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Exopolysaccharides, proteins and lipids in *Pleurotus pulmonarius* submerged culture using different carbon sources

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ABSTRACT

For many years mushrooms have been consumed and appreciated by their nutritional value, and medicinal properties. The traditional mushroom cultivation takes too long and the macrofungi biotechnology has not been explored in its full potential yet. The goal of this work was to observe if different carbon sources could improve the yield and diversify fungi nutrient composition in submerged culture.

Pleurotus pulmonarius mycelia and exopolysacharide productions were evaluated using glucose, galactose, xylose and arabinose. The mycelia yield varied depending on the culture medium, and galactose showed to be the best carbon source to produce EPS. Samples that showed the highest protein contents were grown with xylose (19.44%) and arabinose (26.05%). Furthermore, the biomass cultivated with these carbohydrates and with galactose showed five essential amino acids. All cultured biomass showed low lipid contents (~1%), being composed mainly of unsaturated fatty acids. All EPS fractions showed as main structures glucans and mannogalactans.

1. Introduction

Pleurotus spp. are appreciated not only for their sensory characteristics, but also for their nutritional value and functional properties (Alexopoulos, Mims, & Blackwell, 1996). The terpenes, lactones, amino acids, and carbohydrates present determine a range of aromas and flavor characteristics from both their fruiting body and mycelial biomass (Tsai et al., 2009). Apart from their low lipid contents, important characteristics are observed concerning other chemical compounds, for example polysaccharides, which have shown antitumor, antioxidant, and hypoglycemic properties (Jose & Janardhanan, 2000; Wasser, 2002).

Mushrooms or mushroom polysaccharide preparations have been extensively studied as immune modulators and adjuvant agents in cancer treatment. These fungi have been increasingly consumed by the majority of cancer patients, during their treatments, as dietary supplements (Hardy, 2008). Among the mushroom extracts, the most consumed are *Trametes versicolor*, *Grifola umbellatae*, *Ganoderma lucidum*, *Agaricus blazei*, and *Lentinus edodes*. All of them are rich in β-glucans (Hardy, 2008; Wasser, 2002).

The chemical composition of mushrooms varies, according to the species and growth conditions. In the latter sense, submerged culture has the advantage of reduced space for mycelial biomass and exopolysaccharide (EPS) production in bulk, besides more precise control on a range of factors such as pH, moisture, light, and temperature (Lin & Sung, 2006). The composition of the culture medium can also influence the yield and chemical components of basidiomycete mycelium and EPS (El-Dein, El-Fallal, Toson, & Hereher, 2004).

In general, mushrooms have high contents of fiber, protein and some minerals, apart from low level of lipids and calorie content (Furlani & Godoy, 2007; Silva, Costa, & Clemente, 2002). Fungi are an excellent dietary fiber source, because their cell wall is composed mainly of chitin, hemicellulose, mannans and β-glucans, which can assist intestinal motility and increase stool bulk, decreasing absorption of harmful toxic, carcinogenic substances, leading to lower incidence of colorectal cancer (Manzi & Pizzoferrato, 2000).

The fruiting body of *Pleurotus eryngii* contained 0.8% lipids (Manzi, Marconi, Aguzzi, & Pizzoferrato, 2004). Furthermore, the fruiting bodies of 23 mushroom spp. collected in India had 52–87% unsaturated fatty acids, being *cis*-9-*cis*-12-octadecadienoic acid (C18:2 *n*-6) and *cis*-9-octadecaenoic acid (C18:1 *n*-9) the predominant ones (Kavishree, Hemavathy, Lokesh, Shashirekha, & Rajarathnam, 2008). *Pleurotus sajor-caju*, cultured in banana straw, contained 18.4% protein (Bonatti, Karnopp, Soares, & Furlan, 2004). Mdachi, Nkunya, Nyigo, and Urasa (2004) found that 5 of 10 edible mushroom spp. had between 2 and 7 essential amino acids.

Most *Pleurotus* species have good development rates in different substrates, due to their capacity of secreting a wide spectrum of enzymes able to digest a large number of materials (Das &

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Mukherjee, 2007). El-Dein et al. (2004) examined submerged cultured of *P. pulmonarius* using the following carbon sources: arabinose, galactose, glucose, glucuronic acid, sorbitol, glycerol, lactose, mannose, raffinose, sorbose, starch, sucrose and xylose. They concluded that biomass production varied according to the carbohydrate source.

These mushroom species have a commercial importance duo to their appreciation as food. Moreover, their consumption as food supplement nowadays shows the opportunity of an open market for the production of goods enriched with such nutrients. Therefore the submerged culture technique is a biotechnology tool capable of supplying interesting metabolites, on a large scale and in a short period of time, which has been demanded by the food industry. We now evaluate mycelial biomass and EPS production of *Pleurotus pulmonarius* submerged culture using different carbon carbohydrate sources to determine its chemical composition and structure, respectively.

2. Materials and methods

2.1. Fungal material

Pleurotus pulmonarius (Fr.) Quel. was furnished by Renato A. Yamasita, Fazenda Bom Jesus das Araucárias, located in Reserva (24°39′05″ S; 50°50′40″ W), State of Paraná (PR), Brazil. A voucher specimen was deposited in the Museu Botânico Municipal (n° 332755), Curitiba-PR, Brazil.

2.2. Culture conditions

P. pulmonarius was isolated from fruiting bodies fragments and maintained in Petri dishes with potato dextrose agar (PDA) medium.

Pre-inocula were prepared using a PDA culture medium, while potato filtrate plus dextrose (PD) was used for liquid inoculum preparation. *P. pulmonarius* was cultured in C/N 24:1 liquid medium (Wu, Cheung, Wong, & Huang, 2003, 2004) with varying the carbon sources. The medium consisted of yeast extract 4.0 g l^{-1}, K_2HPO_4 1.0 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.6 g l^{-1}, and carbohydrate 30.0 g l^{-1}. The carbon sources used for mushroom culture were glucose as standard, galactose, xylose, and arabinose.

Pre-inocula were grown in 250 ml Erlenmeyer flasks containing 100 ml of PD liquid medium, at 22 °C in the dark, on a rotary shaker at 120 rpm. After 18 days, the pre-inocula were transferred to 2000 ml Erlenmeyer flasks containing 1000 ml of C/N 24:1 liquid medium, as described previously, and incubated for a further 18 days under the same conditions as for pre-inocula. From each carbohydrate-containing culture, the biomass were separated by filtration, dialyzed against tap water, and freeze-dried to obtain mycelia yields. While the culture media have their volumes reduced, followed by addition to excess EtOH, dialysis and freeze-dried to obtain EPS samples.

2.3. Analytical methods

2.3.1. Protein and lipid contents

Total nitrogen content was estimated by the Kjeldahl method and total protein was determined from total nitrogen content, using the correction factor 4.38 (Breene, 1990). Lipids were extracted in a Soxhlet apparatus using petroleum ether. All analyses were carried out in triplicate.

Analyses of protein and lipid components of mycelial fractions were performed, using AOAC procedures (1995).

2.3.2. Gas chromatography–mass spectrometry

Alditol acetates obtained from EPS fractions, were submitted to gas liquid chromatography–mass spectrometry (GC–MS) using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap model 810 R-12 mass spectrometer, with He as carrier gas for carbohydrate analysis. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection and then programmed at 40 °C min^{-1} to 220 °C or 215 °C (constant temperature) was used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates, respectively.

Fatty acid and amino acid derivatives (FAME and aaMAs, respectively) were submitted to GC–MS analysis using a Varian 4000 gas chromatograph, equipped with fused silica capillary columns of CP-Sil-43CB. The injector temperature was maintained at 260 °C, with the oven starting at 50 °C (hold 2 min) to 90 °C (20 °C min^{-1}, then held for 1 min), 280 °C (5 °C min^{-1}, then held for 2 min) and to 310 °C (3 °C min^{-1}, then held for 5 min). Helium was used as carrier gas at a flow rate of 1.0 ml min^{-1}.

2.3.3. ^{13}C NMR spectroscopy

^{13}C NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer, incorporating Fourier transform. Analyses were performed at 70 °C on samples dissolved in D_2O or Me_2SO-d_6. Chemical shifts of water-soluble samples are expressed in δ (ppm) relative to acetone at δ 32.77 (^{13}C) and 2.21 ^{1}H), based on DSS (2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate sodium salt; $\delta = 0.0$ for ^{13}C and ^{1}H), and at δ 39.70 (^{13}C) and 2.40 (^{1}H) for those solubilized in Me_2SO-d_6.

2.4. Experimental procedures

2.4.1. Process of EPS purification

After 18 days of pre-inocula and 18 days of mycelia production, mycelial biomass were separated by filtration, and culture media had their volumes reduced, followed by addition to EtOH (3:1; v/v). This procedure gave rise to a precipitate, which was separated by centrifugation (8000 rpm, 20 min, 5 °C), dialyzed and freeze-dried to obtain EPS samples. Thus, EPS fractions obtained from media with different carbon sources were submitted to successive purification processes.

The crude EPS fractions were submitted to a freeze-thawing process furnishing cold water-soluble (SEPS) and water-insoluble polysaccharides (IEPS), which were separated by centrifugation (8000 rpm, 20 min, 5 °C). The water-soluble fractions were then treated with Fehling solution (Jones & Stoodley, 1965) and the soluble fractions (FSEPS) were isolated from the insoluble Cu^{2+} complexes, provided by centrifugation under the above conditions. The respective fractions were each neutralized with HOAc, dialyzed against tap water and deionized with mixed ion exchange resins and then freeze dried (Fig. 1).

2.4.2. Monosaccharide composition of EPS

Each EPS fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The residues were successively reduced with $NaBH_4$ (1 mg) and acetylated with Ac_2O–pyridine (1:1, v/v; 200 μl) at 100 °C for 30 min following the method described by Sassaki et al. (2008). The resulting alditol acetates were analyzed by chromatography–mass spectrometry (GC–MS) as indicated below (item 2.3.2) and identified by their typical retention times and electron impact profiles.

2.4.3. Fatty acid composition

Aliquots of mycelia (10 mg) were submitted to methanolysis with 600 μl of 1.2 M MeOH–HCl for 2 h. The products were evaporated to dryness and residual fatty acid methyl esters (FAMEs) were qualitatively and quantitatively analyzed by GC–MS. These

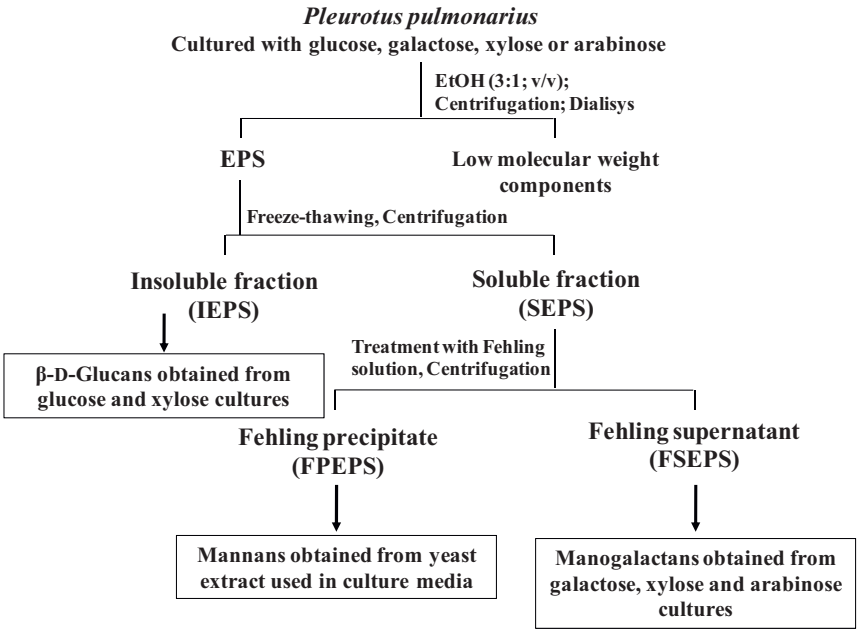

Fig. 1. Scheme of fractionation and purification of EPS fractions obtained from *P. pulmonarius* submerged culture testing different carbon sources.

were identified from their retention times and *m*/*z* of their positive ions, by comparison with standards, the results being expressed as a relative percentage of each component (Sassaki et al., 2008).

2.4.4. Amino acid in mycelial composition

Aliquots (20 mg) of mycelial fractions were hydrolyzed with 6 M HCl at 100 °C for 24 h, with 1% phenol (v/v) to avoid tyrosine decomposition. The solution was then evaporated to dryness and the residue was dissolved in aqueous-MeOH (200 μl; 1.5:8.5, v/v) containing 0.6 M HCl with 0.1% phenol (v/v) and heated at 100 °C for 15 min. The mixture was evaporated giving rise to a residue containing amino acid methyl esters which was treated with pyridine–MeOH–Ac_2O (300 μl, 1:1:4, v/v) at 100 °C for 60 min, generating amino acetate methyl esters (aaMAs). These were analyzed by GC–MS and the resulting ions were identified by comparison with standards. The results are expressed as a relative percentage of each amino acid (Sassaki et al., 2008).

2.4.5. Statistical analysis

Protein and fat content were expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $p < 0.05$, <0.01 and <0.001.

3. Results and discussion

3.1. Mycelial biomass and EPS production

After the growing period for *P. pulmonarius* submerged culture, mycelial biomass were separated by filtration and dialyzed, while culture media had their volumes reduced, followed by addition to EtOH (3:1; v/v) and dialysis. All EPS and mycelia biomass samples were freeze-dried to obtain their yields (Table 1).

Galactose showed to be the best carbon source for EPS production, which varied from 0.3 g l^{-1} to 0.42 g l^{-1} (Table 1). These results were in contrast with other data reported in the literature, where glucose is generally the best monosaccharide source for EPS production, including the species in concern (El-Dein et al., 2004; Lin & Sung, 2006; Pokhrel & Ohga, 2007).

Regarding the mycelial biomass, *P. pulmonarius* submerged culture resulted in great yield variations according to the different carbon sources used. When cultured with arabinose it showed 0.94 g l^{-1} yield and with glucose it reached 9.07 g l^{-1} yield (Table 1). Similarly, the greatest biomass yields for *Lyophyllum decastes* (Pokhrel & Ohga, 2007), *Grifola umbellata* (Huang & Liu, 2008), and *P. pulmonarius* (El-Dein et al., 2004) were obtained when these basidiomycetes were cultured in the presence of glucose monosaccharide. This was preferred by the mycelium instead of other monomers, probably due to its efficient incorporation into the respiratory pathway (Moore-Landecker, 1996).

Despite of microfungi have been used in production of alcoholic beverages, baker and cheese products, and some food condiments and/or additives, the macrofungi biotechnology has not been explored in its full potential yet (Ghorai et al., 2009). The great amount of mycelial biomass obtained from the glucose-based culture in this work showed this method to be a promising biotechnology tool for the food industry. Besides that, the submerged culture procedure requires less time and space than the traditional cultivation of basidiomycetes.

The crude EPS fractions and mycelia biomass were further analyzed according to their monosaccharide content (Table 2). Both sources showed manose, galactose and glucose as major components. EPS fractions cultured in media containing glucose and galactose also presented considerable amounts of 3-*O*-Me-galactose (confirmed by the presence of the fragments *m*/*z* 130 and 190, after hydrolysis, reduction with NaB^2H_4 and acetylation), 17.9% and 23.6%, respectively (Table 2).

Analyzing EPS monosaccharide composition, it is possible to observe that mannose is present in highest levels, mainly for xylose (61.8%) and arabinose (74.8%) samples (Table 2). In contrast, mycelia samples showed glucose as major component when cultured with glucose (84.1%), galactose (79.9%) and xylose (64.1%), while for arabinose-based mycelium; mannose is once more the major monosaccharide, 84.0%. Xylose-based mycelium also showed xylose in considerable amount, 18.9% (Table 2).

Comparing the monosaccharide composition of EPS and mycelium for each carbon source, only for the culture medium using arabinose it was observed similarities, having mannose as the major component in both samples.

Table 1
Yields of EPS, mycelial biomass, protein and lipid contents of *P. pulmonarius* cultured with different carbon sources.

Carbon source	EPS ($g l^{-1}$)	Biomass ($g l^{-1}$)	Protein (% ± SD)	Lipid (% ± SD)
Glucose	0.36	9.07	14.02 ± 0.19	0.80 ± 0.00
Xylose	0.33	1.86	19.44 ± 0.48***	0.97 ± 0.01
Galactose	0.42	1.08	15.50 ± 0.39**	0.91 ± 0.00
Arabinose	0.30	0.94	26.05 ± 0.50***	1.61 ± 0.14***

Results are expressed as percentage of mycelial dry weight (mean ± SD, $n = 3$, with $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. glucose-based mycelium).

3.2. EPS characterization

In order to obtain pure polysaccharides from *P. pulmonarius* submerged culture EPS fractions, crude EPS were recovered by ethanol precipitation from culture media, followed by dialysis and freeze-dried process. The yields obtained from each medium culture are shown in Table 1. Each EPS sample showed as major monosaccharide components manose, galactose and glucose (Table 2), and it was examined by ^{13}C NMR spectroscopy (Fig. 2). Their spectra were very similar, presenting a great number of signals at anomeric region.

Fractionation and purification of EPS were carried out by a freeze-thawing procedure (Gorin & Iacomini, 1984) furnishing cold water-soluble (SEPS) and water-insoluble polysaccharides (IEPS), which were separated by centrifugation (8000 rpm, 20 min, 5 °C) (Fig. 1).

IEPS fractions obtained from glucose- and xylose-based culture media (IEPS-Glc and IEPS-Xyl, respectively) contained only glucose as their monosaccharide component (GC–MS), and they were examined using ^{13}C NMR spectroscopy (Fig. 3). The signals observed in both spectra are characteristic of a fungal β-glucan (Barreto-Bergter & Gorin, 1983; Gorin, 1981), and suggest that this fractions are composed by branched (1 → 3), (1 → 6)-linked β-D-glucans, as found in *P. pulmonarius* fruiting body. All the signals were assigned using literature values for similar polysaccharides (Carbonero et al., 2006; Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996; Santos-Neves et al., 2008; Smiderle et al., 2008a; Tabata, Ito, & Kojima, 1981; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985).

^{13}C NMR spectra contained C1 signals at δ 102.9, corresponding to non-reducing end-units, 3-*O*- and 3,6-di-*O*-substituted Glc*p* residues (Fig. 3). The β-glycosidic configuration was confirmed by C-1 signals at high frequency (Hall & Johnson, 1969). The glycosidic linkage of these glucans were shown by the presence of 3-*O*-substituted signals at δ 86.6, 86.2 and 85.9, and O-substituted-CH2-6 signal at δ 68.4 (Carbonero et al., 2006; Santos-Neves et al., 2008; Smiderle et al., 2008a; Yoshioka et al., 1985) (Fig. 3).

The β-D-glucan found in *P. pulmonarius* fruiting bodies showed a backbone structure consistent with a linear (1 → 3)-linked β-D-Glc*p* chain (Gorin, 1981) after controlled Smith degradation. Therefore it can be suggested that both fractions, IEPS-Glc and IEPS-Xyl also present a glucan with a linear main-chain β-D-(1 → 3)-linked with typical C/H signals at δ 102.9 (C-1); 86.2 (C-3); 76.3 (C-5); 72.6 (C-2); 68.4 (C-4), and 60.8 (C-6) (Gorin, 1981) (Fig. 3).

Surprisingly, these glucans were not isolated from the galactose- and arabinose-based culture media. This polymer is commonly present in mushroom fruiting bodies and also in submerged cultures. Although, the different carbon sources used for the cultivation seemed to interfere in glucan production. Galactose and glucose are isomers which present *trans*-configuration on C3–C4, while xylose is the analogous pentose of glucose (Sobotka & Reiner, 1930). Sobotka and Reiner (1930) observed that considerable differences in the reducing power of hexoses are due mainly to the *cis-trans*-distribution of the hydroxyls on the third and fourth carbon atom. These differences could explain why the two hexoses gave rise to different exopolysaccharides when used as carbon sources. Furthermore, the similar distribution of the hydroxyls between glucose and xylose may be an explanation for the consumption of this pentose by the fungus, and consequently the production of similar glucans obtained when glucose is the carbon source.

The water-soluble fractions were then treated with Fehling solution (Jones & Stoodley, 1965) and the soluble fractions (FSEPS) were isolated from the insoluble Cu^{2+} complexes (FPEPS) by centrifugation under the above conditions to avoid the contamination of yeast mannans arising from yeast extract used in culture media (Komura et al., 2010). The respective fractions were each neutralized with HOAc, dialyzed against tap water and deionized with mixed ion exchange resins and then freeze dried (Fig. 1).

FSEPS were also analyzed by ^{13}C NMR spectroscopy, and for samples derived from galactose, xylose and arabinose-based media similar spectra were obtained (Fig. 4). These fractions showed to be composed of manose, 3-*O*-methyl-galactose (confirmed by the presence of the fragments *m*/*z* 130 and 190, after hydrolysis, reduction with NaB^2H_4 and acetylation) and galactose, suggesting the presence of mannogalactans. Thus, the spectra now obtained were compared with that of *P. pulmonarius* fruiting bodies mannogalactan ^{13}C NMR spectrum.

Table 2
Monosaccharide composition of *P. pulmonarius* submerged culture EPS and mycelia obtained from media using different carbon source.

Source	Carbon source	Monosaccharide composition[a] (%)[b]						
		Fuc	Ara	Xyl	Man	3-*O*-Me-Gal	Gal	Glc
EPS	Glucose	–	–	–	34.4	17.9	35.7	12.0
	Galactose	–	–	–	33.3	23.6	24.1	19.0
	Xylose	–	–	–	61.5	Tr[c]	9.5	28.7
	Arabinose	–	–	–	74.6	Tr	11.1	14.1
Mycelium	Glucose	0.9	–	2.0	9.8	Nd[d]	3.2	84.1
	Galactose	0.9	–	1.4	8.8	Nd	9.0	79.9
	Xylose	0.9	1.3	18.9	9.3	Nd	5.5	64.1
	Arabinose	–	–	–	84.0	Nd	2.7	13.3

[a] Analyzed by GC–MS after total acid hydrolysis (TFA 2 M, 100 °C for 8 h), reduction ($NaBH_4$) and acetylation.
[b] Relative percentage to peaks areas.
[c] Tr – traces.
[d] Nd – not determined.

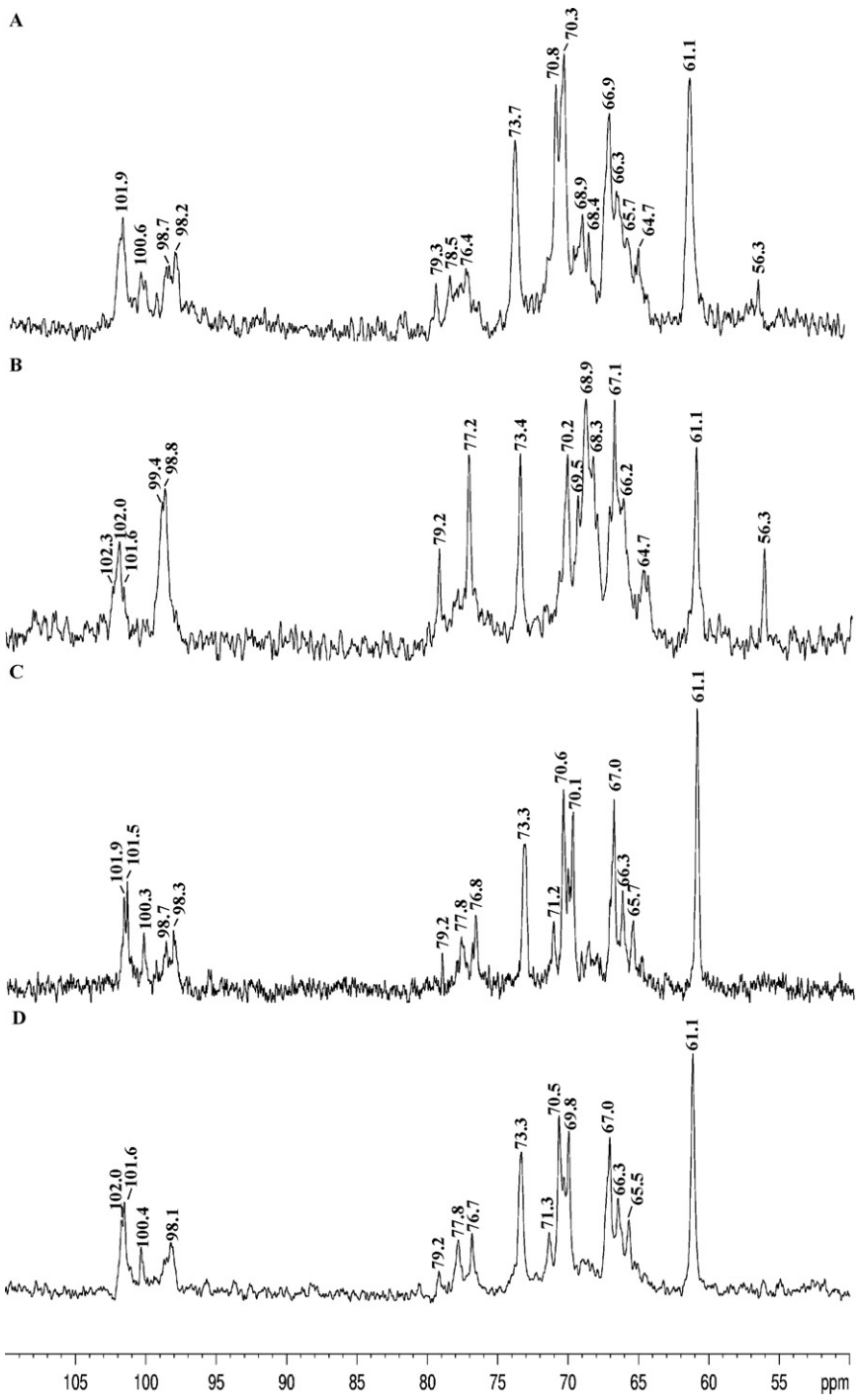

Fig. 2. ^{13}C NMR spectra of EPS fractions obtained from *P. pulmonarius* submerged cultured varying carbon source: glucose (A) galactose (B), xylose (C) and arabinose (D), EPS, in D_2O at 70 °C (chemical shifts are expressed in δ ppm).

By comparison ^{13}C NMR spectra shown in Fig. 4 contain signals in the anomeric region, corresponding to C1 of mannopyranosyl, 2,6-di-*O*- and 6-*O*-Gal*p*-substituted units (δ 103.9, 101.0 and 100.6, respectively).

Glycosidic configurations could be attributed based on literature reports, as β-configuration for Man*p* and α-configuration for Gal*p* and 3-*O*-Me-Gal*p* units, in accordance with the C-1 high-field signals in its ^{13}C NMR spectrum (Fig. 4) (Smiderle et al., 2008b).

The signal at δ 79.4 in the ^{13}C NMR spectra could be attributed to C-2 of 2,6-di-*O*-substituted Gal*p* units, in accordance with *P. pulmonarius* fruiting body mannogalactan (Smiderle et al., 2008b), confirming the O-2 substitution by non-reducing end units of Man*p*. The presence of O-methyl groups is confirmed by signals at δ 58.7, which is more evident in the purified ^{13}C NMR spectra samples than in the crude ^{13}C NMR spectra. In addition, one signal at δ 81.4 arose from C3 of 6-*O*-substituted *O*-Me-Gal*p* units. Signals at δ 72.8, 75.5, 69.4, 78.6 and 63.6 could be assigned to C-2, C-3, C-4, C-5 and C-6 of β-D-Man*p* units (Smiderle et al., 2008b). Thus, based on mannogalactan characterized from *P. pulmonarius* fruiting body, one can assume that FSEPS obtained from galactose, xylose and arabinose-based media polysaccharide has a main chain composed of (1 → 6)-linked α-D-Gal*p* and *O*-Me-α-D-Gal*p*,

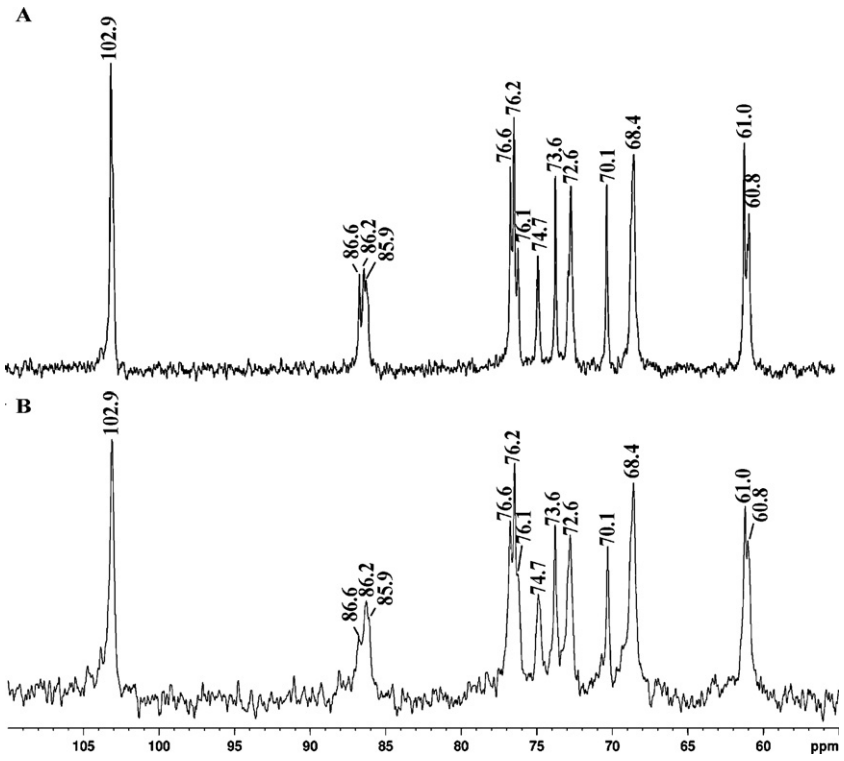

Fig. 3. ^{13}C NMR spectra of fractions IEPS-Glc (A) and IEPS-Xyl (B) obtained from *P. pulmonarius* submerged cultured with glucose and xylose, respectively (in Me_2SO-d_6 at 70 °C, with chemical shifts are expressed in δ ppm).

some of them substituted at O-2 by β-D-Man*p* non-reducing end units.

Similar 3-*O*-methyl mannogalactans have been isolated from fruiting body of *P. ostreatus* (Jakovljevic, Miljkovic-Stojanovic, Radulovic, & Hranisavljevic-Jakovljevic, 1998), and as cited above, also for *P. pulmonarius* (Smiderle et al., 2008b). An *O*-methyl galactan, with the same main chain of the above cited polysaccharide, has been extracted from *P. eryngii* and *P. ostreatoroseus* (Carbonero et al., 2008). These findings indicate that such polysaccharides could be characteristic of the genus *Pleurotus*.

Among the polysaccharides encountered in mushrooms, only glycogen is digestible by human enzymes (Manzi & Pizzoferrato, 2000; Smiderle et al., 2010). Therefore, β-glucans and mannogalactans, which are also isolated from basidiomycetes, can be considered as fibers. By the NMR spectra (Figs. 3 and 4) obtained from *P. pulmonarius* no sign from glycogen was observed, which allow us to suggest that almost all the carbohydrate content of the EPS fractions tested can be considered as fibers.

3.3. Protein and lipid contents

Approximately 60–70% of the total nitrogen of the fungal cell-wall consisted of protein, the remainder being nucleic acids, chitin, phospholipids and vitamins, among others (Moore-Landecker, 1996). The overall protein content of mushrooms is in a constant state of flux, depending on the developmental stage, substrate, harvest period, and species. The crude protein content usually found for basidiomycetes is between 11% and 44% (Botha & Eicker, 1992), although 56.3% was reported for *Agaricus arvensis* (Barros et al., 2007).

The analyzed samples contained high levels of protein (Table 1), with significant difference between the biomass grown with galactose, xylose and arabinose ($p < 0.01$, 0.001 and 0.001, respectively), compared to that one grown with glucose. The biomass cultured using the pentoses, xylose and arabinose, had the highest protein contents (19.44% and 26.05%, respectively).

The medium or substrate composition was shown to influence significantly the quantity and quality of resulting proteins (Bonatti et al., 2004). It was reported that *Morchella hybridia* produced only 10.5% protein, when grown in a synthetic glucose medium, while it was 34.8% when molasses was used as a substitute (Kurbanoglu, Algur, & Zulkadir, 2004). Molasses are composed mainly of sucrose, with glucose, fructose and other reducing substances, among other components (Teclu, Tivchev, Laing, & Wallis, 2009). It is well known that the amino acid composition in the substrate can affect protein quality, but it still remains unclear why the protein content is higher when the mycelium was cultivated using pentoses.

The lipid content was evaluated and it was found in low proportions in the mycelia. It was observed less than 1% for galactose-, xylose-, and glucose-based cultures. The major difference was when comparing the glucose mycelium and that cultivated with arabinose ($p < 0.001$), which showed its highest content of 1.61% (Table 1).

Several studies have suggested that cell wall composition of mycelium can be similar to that of mushroom fruiting body. The *P. pulmonarius* fruiting body contained 14.7% protein and 1.8% lipid (data not shown), which is similar when compared with the mycelial biomass of the fungus. Moreover, the mushroom biomass showed protein contents as high as those of other sources, such as chicken breast (21.5%), beef topside (16.7%), whitefish (21.5%) and chicken egg (13.3%), which showed the potential of these mushrooms as protein sources (NEPA, 2006).

3.4. Fatty acid profile

Mycelial biomass fatty acid compositions are shown in Table 3. Unsaturated fatty acids (UFA) were predominant, and the glucose mycelium contained the greatest quantity of this group (89.01%). *Cis*-9-*cis*-12-octadecadienoic acid (C18:2 *n*-6), or linoleic acid, accounted for most of the fatty acids present in all samples, representing up to 80.82% of total fatty acids when the mycelium was cultured with arabinose. Approximately 33–68% of total fatty acids

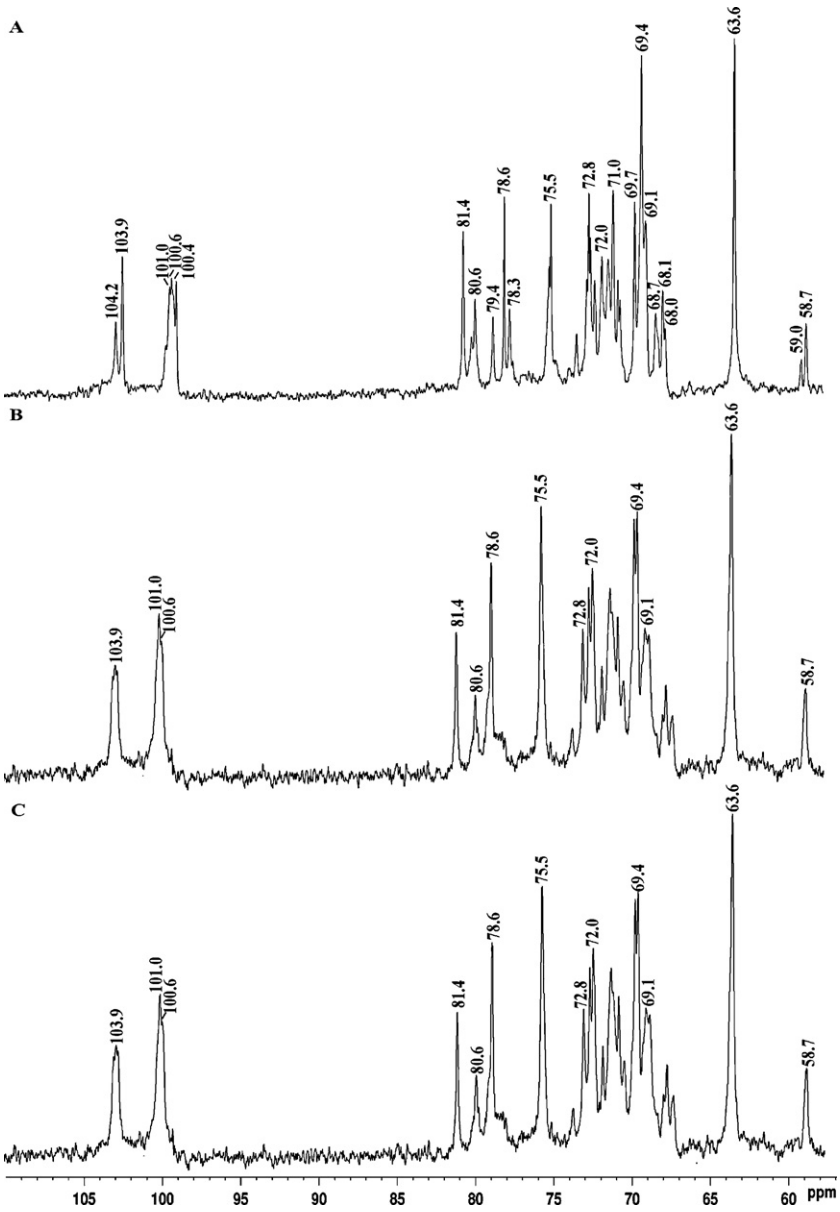

Fig. 4. ^{13}C NMR spectra of mannogalactans obtained from *P. pulmonarius* submerged cultured with galactose (A), xylose (B) and arabinose (C) (in D_2O at 70 °C, with chemical shifts are expressed in δ ppm).

Table 3
Lipid fatty acid profile from *P. pulmonarius* mycelial biomass cultured with different carbon sources.

Fatty acid (% of total fatty acid methyl esters)[a]	% of fatty acid from each carbon source			
	Glucose	Galactose	Xylose	Arabinose
C15:0	2.41	0.35	0.83	2.42
C16:0	8.58	15.14	17.30	12.26
C16:1 *n*-11	Nd[b]	0.19	3.87	Nd
C16:1 *n*-9	5.28	Nd	2.41	Nd
C17:0	Nd	0.03	0.19	Nd
C18:0	Nd	0.58	2.16	Nd
C18:1 *n*-9	9.54	5.37	10.63	4.49
C18:1 *n*-11	Nd	2.79	2.63	Nd
C18:2 *n*-6	74.19	75.55	59.98	80.82
ΣSFA[c]	10.99	16.10	20.48	14.68
ΣMUFA[d]	14.82	8.35	19.54	4.49
ΣPUFA[e]	74.19	75.44	59.98	80.82

[a] GC–MS analysis of FAMEs on a DB-225 capillary column.
[b] Nd – not detected.
[c] ΣSFA – total saturated fat acid content.
[d] ΣMUFA – total monounsaturated fat acid content.
[e] ΣPUFA – total polyunsaturated fat acid content.

Table 4
Retention times and amino acid composition from *P. pulmonarius* mycelial biomass cultured with different carbon sources.

Amino acid (% of total amino acetate Me ester)[a]	R_t (min)[b]	Aa (%)			
		Glucose	Galactose	Xylose	Arabinose
Ala	8.90	–	0.33	0.43	–
Gly	9.60	–	0.51	0.63	3.53
Val*	11.21	–	2.17	0.74	2.09
Ile*	13.32	6.97	9.95	7.47	4.17
Leu*	13.43	1.60	2.25	0.38	0.72
Thr*	15.12	7.17	–	–	5.28
Pro	15.34	12.55	23.70	20.75	13.39
Asp	15.75	19.70	12.98	13.84	10.26
Ser	18.10	1.83	–	–	0.85
Glu	18.58	23.78	27.26	20.64	34.01
Met*	19.15	–	1.05	8.62	–
Gln	19.23	–	–	5.48	–
Phe*	21.33	26.40	19.80	21.01	25.71
ΣEAA[c]	–	42.14	35.22	38.23	32.69
ΣNEAA[d]	–	57.86	64.78	61.77	67.31

[a] GC–MS analysis of aaMAs on a CP-Sil-43CB capillary column.
[b] R_t – retention time.
[c] ΣEAA – total essential amino acids content.
[d] ΣNEAA – total non-essential amino acids.
* Essential amino acids (Kalač, 2009).

observed in *Pleurotus* spp. mycelium were linoleic acid (Dimou, Georgala, Komaitis, & Aggelis, 2002).

Followed by linoleic acid, the saturated fatty acid hexadecanoic (C16:0), or palmitic acid, accounted for 17.3%, 15.14%, and 12.26% of total fatty acids when the mycelium was cultured with xylose, galactose, and arabinose, respectively. In contrast, mycelium cultured with glucose showed a higher content of *cis*-9-octadecaenoic acid (C18:1 *n*-9) or oleic acid (9.54%), when compared with the other tested carbon sources.

According to Moore-Landecker (1996), triglycerides are generally responsible for most of the lipids present in fungi. The fatty acids composed of 16–18 carbons are the most common and unsaturated fatty acids (UFAs), especially oleic and linoleic acids, predominate over saturated fatty acids (SFA).

3.5. Amino acid composition

Table 4 shows the content of amino acid derivatives (aaMAs) present in samples of mycelial biomass. Our results showed that there was a variation in amino acid contents, depending on the carbon source. In the glucose medium biomass only eight amino acids were detected, while in the xylose medium biomass there were eleven different ones.

All samples showed high contents of phenylalanine, aspartate, glutamate and proline, which corresponded to an average of 81.44% of all the amino acids present in the mycelial biomass cultured with different carbon sources (Table 4). The biomass cultivated with xylose, arabinose and galactose presented 5 of 8 essential amino acids, but the glucose-based mycelium showed the highest content of these nutrients, representing 42.14% of total amino acids. Most studies showed that the essential amino acid present in highest quantities in *Pleurotus* spp. is leucine (Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999; Wang, Sakoda, & Suzuki, 2001). However, the essential amino acid found in greatest proportion for this work was phenylalanine, which was observed in high amounts for all analyzed samples. Glutamine was detected only in the mycelium grew with xylose.

Our results showed the significance of the carbon source in the culture medium, concerning the quantity and quality of protein and lipid contents.

4. Conclusions

The yields of mycelial biomass and its chemical composition varied depending on the carbohydrate source presented in *P. pulmonarius* submerged culture. Concerning the growth conditions, glucose gave rise to the greatest mycelial biomass. Although all samples had a high protein content, arabinose-based mycelium showed the highest amount of this nutrient. The production of exopolysaccharides was similar for glucose-, xylose-, and arabinose-based media, but it was higher when galactose was added to the culture. Regarding the EPS composition, mannose, galactose and glucose were found for all the carbon sources, although the fungus cultivated with arabinose presented higher content of mannose, as its mycelium. For the other carbon sources, the mycelia were composed of great amounts of glucose. The content of lipids was low, and it was mainly composed of unsaturated fatty acids and especially linoleic acid. Six essential amino acids were detected in the biomass samples and phenylalanine was the most abundant.

The production of mushrooms is highlighted nowadays due to the great interest in healthy food with low-fat, low-calorie, and cholesterol-free. Considering the increasing consumption of dietary supplements, the high content of protein, and the low levels of lipids as well as the presence of unsaturated fatty acids, the use of submerged culture of mushrooms can be an alternative for vegetarian diets (Ghorai et al., 2009). Processed fungal food represents an important alternative to obtain large amounts of nutrients and bioactive compounds. Mycelial biomass studies are necessary to explain the factors which influence its chemical composition. The data obtained can shed light on the chemical composition of cultivated mycelium as well as on the production components for the food industry.

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