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ORIGINAL PAPER

Valorization of Olive Mill Wastewater for the Production of β-glucans from Selected Basidiomycetes

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Abstract
Purpose The aim of the present study was to investigate the feasibility of polysaccharides production by selected basidiomycetes in submerged culture. Olive mill wastewater (OMWW) was also tested as a potential substrate for polysaccharides production by mushroom strains, focusing on the simultaneous degradation and valorization of the waste material.
Methods The tested strains were grown in two different substrates, and after biomass harvesting, polysaccharides were isolated using two different methods. The extracellular polysaccharides were isolated from the culture broth, with ethanol precipitation. The isolated fractions were partially characterized with FT-IR spectroscopy.
Results All three strains performed well in both substrates. Maximum degradation performance of OMWW was achieved by *Ganoderma lucidum*, achieving 19.4% phenols reduction together with 47.56% decolorization. The extracellular polysaccharides (EPS) produced by all strains were found to be richer in total glucans during growth in semi-synthetic medium, compared to growth in OMWW-based medium. In regard to biomass polysaccharides, *Pleurotus ostreatus* biomass was found to be richer in glucans, reaching 8.68% (w/w) total glucan content when grown in semi-synthetic medium and 7.58% (w/w) when grown in OMWW-based medium. After purification of biomass polysaccharides with two methods, the fraction with the highest glucan content was found to be the one from *G. lucidum* after growth in semi-synthetic medium cultures, with 49.1% (w/w) total glucans. FT-IR spectra of the isolated samples revealed the bands corresponding to α- and β-glucosidic bonds, but also the existence of protein contamination.
Conclusions Purification of biomass polysaccharides with two distinct methods revealed that α-amylase and Sevag treatments failed to remove completely α-glucans and proteins respectively, leading to the suggestion that these two steps could be omitted without significant impact. Moreover, the results imply that the valorization of OMWW might be feasible with the use of mushroom strains, leading to the production of important products, such as glucans.

Keywords β-Glucan · Olive oil mill waste valorization · Basidiomycetes submerged culture · Polysaccharide isolation

Introduction

Glucans are polysaccharides consisting solely of glucose units. The number of different glucosidic bonds with which the glucose monomers can be connected, together with the occurrence of branched chains, results in a variety of different polysaccharide molecules with distinct properties. Among them, β-1,3-D-glucans or β-1,4-D-glucans can be found in the cell walls of higher plants and cereal seeds, while β-1,3-D-glucans and β-1,6-D-glucans are usually found in the fungal cell walls [1], together with α-1,3-D-glucans, frequently in complex with chitin or proteins

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[2]. β-D-glucans are usually branched chains, with varying molecular weight, while α-D-glucans are linear polymers.

Research interest in glucans is steadily growing the last 20 years, due to their important effects in the human health, including antitumor, immunostimulating, (activating phagocytosis) [3], antidiabetic [4], apoptosis-inducing [4], and antioxidant [5] activities. Activity against bacteria, viruses and fungi has also been reported [5]. Moreover, they are found to enhance patient recovery from the toxic effects of chemotherapy, lower blood cholesterol concentration, and participate in blood pressure control [3].

Many fungal strains produce polysaccharides, either as a secreted metabolite, or as a constituent of their cell wall. Among them, the basidiomycetes species belonging to the genera *Pleurotus* and *Agaricus*, with great significance in human nutrition, and some of pharmaceutical importance, such as *Ganoderma*, are the most promising candidates for polysaccharides production, and thus the focus of most research studies [4, 6–10]. There are also some studies reporting the detection of the enzymes responsible for the synthesis of polysaccharides, such as β-D-glucan synthases, and their corresponding genes [5, 11].

Apart from their ability to produce glucans with dietary value, many basidiomycetes are also very efficient lignin degraders, due to their potent oxidative enzyme system, comprising mainly of laccases (Lac, EC 1.10.3.2), manganese peroxidases (MnP, EC 1.11.1.13) and manganese-independent peroxidases, such as lignin peroxidase (LiP, EC 1.11.1.14), together with a variety of other accessory enzymes. Due to the production of this biocatalytic factory, basidiomycetes are able to degrade complex phenolic substrates and use them as a carbon source to sustain their growth. Taking this trait into advantage, suitable basidiomycetes could be grown in lignin-containing wastes, and while degradation of phenols is under way, the secreted polysaccharides could be isolated from the extracellular fluid, and the biomass could be harvested for the isolation of cell wall polysaccharides. This way, a combined bioremediation and valorization of the waste can be achieved [12–14]. A promising phenolic waste for this purpose is olive mill wastewater (OMWW), a common effluent from the olive oil production process. OMWW is highly toxic against plants, soil microorganisms and marine organisms, due to its high organic load and phenolic content [15].

Total annual production of OMWW amounts to 30 million m^3 [16]. The amount of the produced waste is posing a global challenge regarding its managing, disposal and/or treatment. Deposition in landfills has been a popular approach in the Mediterranean basin, but it usually leads to a variety of severe environmental issues, including contamination of underground or surface water reservoirs, and soil acidification [17]. In view to these issues, several treatment methods have been proposed, spanning from chemical or mechanical treatment [18] to various biological treatment approaches, among them the use of OMWW as fermentation substrate for bioethanol production [16], enzymes production [19], or even biopolymers production [20], enzymatic hydrolysis of the waste [21], or even composting approaches [22]. However, the most efficient methods of biological treatment of OMWW up to date implement the use of white-rot basidiomycetes fungi, achieving up to 90% degradation of the phenol content of OMWW [13]. Basidiomycetes are able to grow using the components of OMWW as carbon and energy source, seeing that, apart from phenol compounds that can be oxidized by their ligninolytic enzyme system, OMWW also contains sugars, lipids and organic acids that promote fungal growth [13, 14].

In the present work, we report the production and isolation of β-D-glucans from three wild Greek strains, *Pleurotus ostreatus, Pleurotus citrinopileatus* and *Ganoderma lucidum*. The production of biomass polysaccharides and exo-polysaccharides was tested during growth in two different substrates, a semi-synthetic, sugar based medium, and a complex wastewater medium, containing OMWW. The isolation of biomass polysaccharides was tested with two different methods. The isolated fractions were further analyzed with FT-IR spectroscopy.

Materials and Methods

Olive Oil Mill Wastewater

OMWW was obtained from an olive oil mill with a three-phase decanter in Kalamata (Peloponnese, S.W. Greece), pretreated and maintained at −20 °C. The composition and physicochemical properties of OMWW was previously assessed [23]. Briefly, the main properties of the OMWW used were as follows: pH 5, proteins 10.26 g L^{-1}, total nitrogen 0.9 g L^{-1}, total phenolics 3.88 g L^{-1}, total solids 43.67 g L^{-1}. Prior to use, pH was adjusted to 6 with 3 N NaOH, and the OMWW was centrifuged at 8000 rpm, 4 °C for 20 min and subsequently filtered through Whatman No. 1, to remove any suspended solids.

Microorganisms and Culture Procedures

The *P. citrinopileatus* LGAM 28684, *G. lucidum* LGAM 9720 and *P. ostreatus* LGAM 1123 strains used for this study, were obtained from the culture collection of the Laboratory of General and Agricultural Microbiology (Agricultural University of Athens), and were selected after a screening evaluation including numerous white-rot basidiomycetes [24]. The strains were maintained in Potato Dextrose Agar plates (PDA- Applichem, Germany) at 4 °C.

Liquid cultures were prepared as reported previously [25]. Briefly, the 100 mL liquid medium contained xylose 57 g L^{-1}, corn steep liquor 37 g L^{-1}, K_2HPO_4 1 g L^{-1}, and $MgSO_4(H_2O)_7$ 0.2 g L^{-1}. Alternatively, for the preparation of liquid cultures, OMWW was diluted at a final concentration of 50% (v/v) with the appropriate buffer solution to a final volume of 100 mL in 250 mL Erlenmeyer flasks with cotton stops, supplemented with 30 g L^{-1} yeast extract, and autoclaved at 121 °C for 20 min. Batch fermentations were also carried out in 2.5 L Bio-Flo 310 bioreactors (New Brunswick Scientific, US), using either two-fold diluted OMWW with 0.1 M potassium phosphate buffer pH 6, or the semi-synthetic medium described previously. Agitation speed was set to 100 rpm, and the medium was supplemented with 30 g L^{-1} yeast extract prior to sterilization. The inoculation of 1.5 L of medium was carried out with 200 mL of fully grown precultures, resulting at a starting concentration of 1.5–2.5 g L^{-1} of dry biomass. The fermentation was maintained at 26 °C, 100 rpm, unless otherwise stated. Daily samples were taken, centrifuged (1520 g, 10 min), and the supernatant was used for analysis. At the end of the bioreactor fermentation, biomass was separated from the culture medium by centrifugation at the same conditions. The precipitate was washed twice with deionized water, for the removal of salts and other impurities, freeze-dried and weighed.

Determination of OMWW Total Phenolic Content and Decolorization

Total phenols content was determined, as described by Waterhouse [26]. Phenols concentration was expressed in ppm of gallic acid equivalents, using the appropriate calibration curve. OMWW decolorization was estimated spectrophotometrically by measuring the absorbance at 525 nm, as previously described [27].

Assessment of Glucan Content in Fungal Biomass

The content in total glucans and α-glucans were determined in the freeze-dried mycelium of all tested species, obtained from cultures grown in OMWW-based media, according to the mushroom and yeast β-glucan Assay Procedure K-YBGL 10/2005 (Megazyme International Ireland, Bray, Ireland). The content in β-glucans was calculated as the difference between total glucans and α-glucans.

Isolation and Partial Purification of β-glucans

The purification of β-glucans from the mycelial biomass was tested with two methods: The first was a modified method of Synytsya et al., [9] (Fig. 1, Protocol A), and the second the method described by Wei et al., 2008 [28] (Fig. 2, Protocol B).

Extracellular polysaccharides (EPS) were isolated as follows: The liquid culture supernatant was concentrated 10-fold in a rotary evaporator (Rotavapor Buchi RE 111, Buchi, Switzerland). In the concentrated supernatant, polysaccharides were precipitated with the addition of 4 volumes cold ethanol (96%) and overnight incubation at 4 °C. The mixture was centrifuged and the precipitate was freeze-dried and weighed.

FT-IR Spectroscopy

FT-IR spectra of the dried glucan preparations were recorded with a Nicolet Magna-IR 560 Spectrophotometer (Thermo Fisher Scientific Inc.) in the 4000-400 cm^{-1} range in the form of KBr pellets.

Statistical Analyses

Data analysis was performed with the use of SigmaPlot software (Systat Software, Inc., San Jose, CA, USA). Error values represent the standard deviation of the mean value.

Results

Fungal Growth Under Submerged Fermentation

All three strains showed a satisfactory growth in submerged culture in both media tested. Moreover, the final harvested biomass at the end of the fermentation was higher in the OMWW-based cultures compared to semi-synthetic medium cultures, for all tested strains, as shown in Table 1. More specifically, the harvested biomass from the cultures of *P. ostreatus* after 13 days of growth was almost twofold higher in the case of OMWW-based medium, compared to semi-synthetic medium. The same result was obtained also in the case of *G. lucidum* biomass production, after 6 days of growth. However, this was not the case for *P. citrinopileatus* bioreactor cultures, where the harvested biomass, after 4 days of growth, was only slightly higher in the OMWW-based medium compared to semi-synthetic medium (Table 1).

Regarding the degradation of OMWW, the three tested stains showed a very variable performance. In the case of *P. citrinopileatus* OMWW cultures, significant phenol reduction was not observed, while decolorization reached only 7.1%. However, the degradation efficiency for the other two strains is quite higher: *P. ostreatus*, when grown in OMWW showed 43.6% total phenolics reduction and 11.1% medium decolorization, while the reduction of total phenolics in the

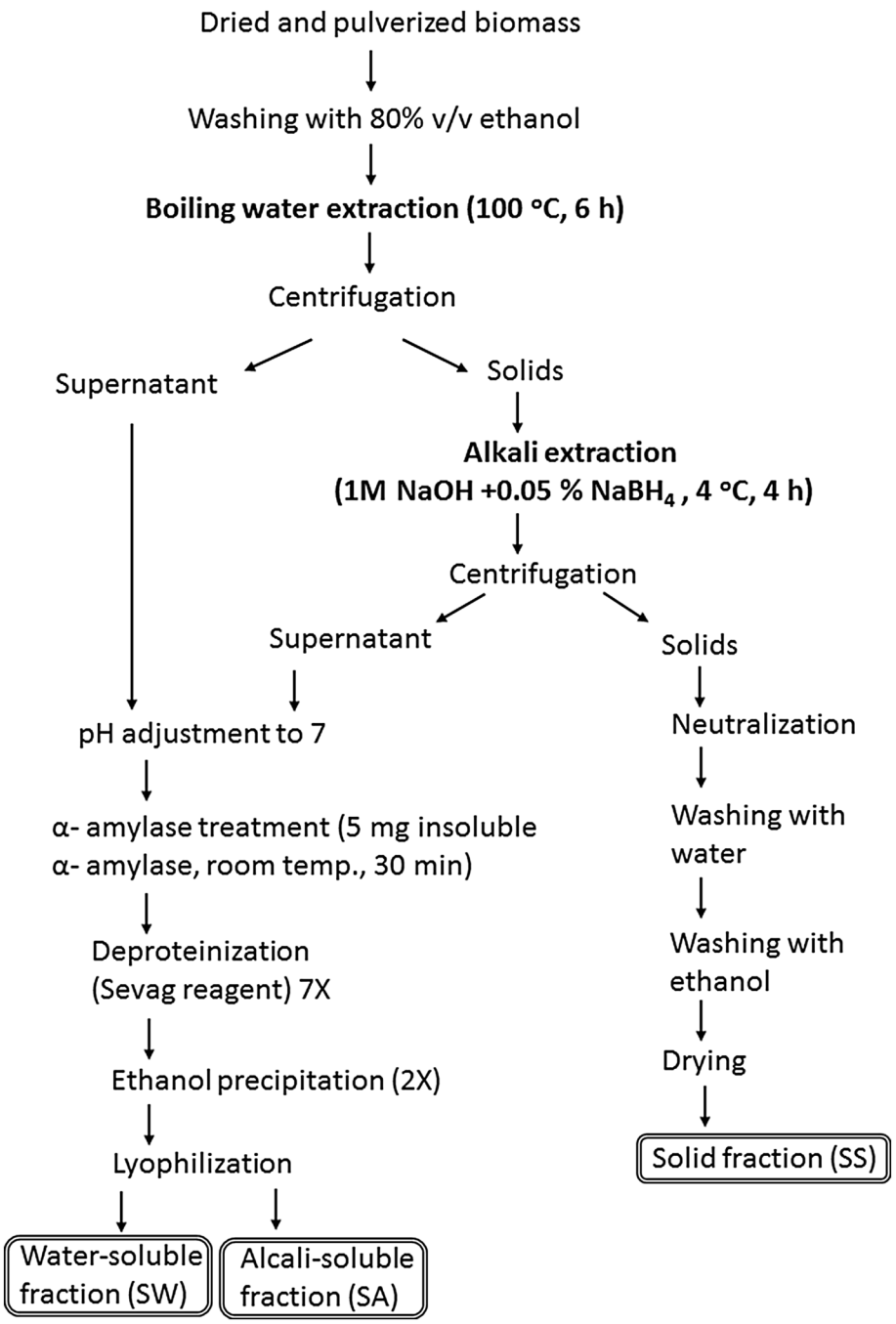

Fig. 1 Outline of polysaccharides purification protocol, as described in Synytsya et al., [9] (Protocol A)

case of *G. lucidum* OMWW cultures reached 19.4%, with concomitant medium decolorization as high as 47.56%.

Production of Extracellular Polysaccharides

The production of extracellular polysaccharides was found to be quite satisfactory from all three strains, in both tested media. All strains seem to produce a higher quantity of extracellular polysaccharide fractions in the case of OMWW-based cultures compared to semi-synthetic medium cultures (Table 2). Another interesting observation was that in all the tested strains and culture conditions, the EPS produced contained β-glucans at a significantly higher percentage than α-glucans. In all cases, α-glucan content was lower than 1% (w/w). Moreover, all the strains seem to produce EPS at similar yields when grown in OMWW-based media. As shown in Table 2, the total EPS isolated from OMWW-grown cultures from all three strains do not present many differences in terms of quantity, ranging from 18.8 to 21.02 mg mL^{-1}, as well as total glucan content, ranging from 1.91 to 2.4% (w/w). β-glucan content is also quite similar, ranging from 1.45 to 1.87% (w/w)

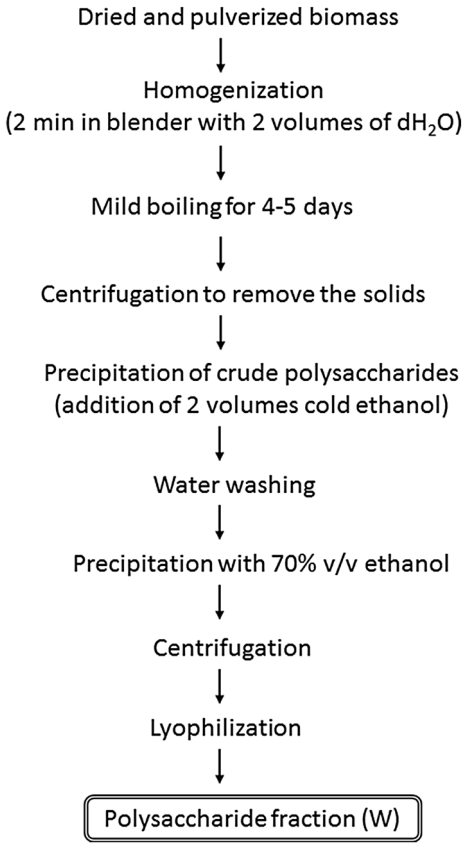

Fig. 2 Outline of polysaccharides purification protocol, as described in Wei et al., (Protocol B) [28]

Table 1 Biomass production in bioreactor cultures from the three tested basidiomycetes strains

Biomass production (mg mL^{-1})	*P. ostreatus*	*P. citrino-pileatus*	*G. lucidum*
Semi-synthetic medium cultures	4.87	5.97	2.65
OMWW cultures	8.38	6.19	4.2

(Table 2). In spite of the higher quantity of EPS isolated in the case of OMWW-grown cultures, these fractions contain significantly fewer glucan molecules than the EPS fractions isolated from semi-synthetic medium cultures. This apparent discontinuity can be explained by the method used for the EPS isolation: In the present work, the isolation of the EPS was performed with a simple ethanol precipitation, without further purification steps. Due to the complexity of the chemical composition of OMWW, it is possible that the precipitates contained many other ethanol-insoluble compounds, yielding an EPS fraction with much more impurities, compared to the EPS fractions obtained after growth in semi-synthetic medium. As shown in Table 2, these EPS fractions might be lower in quantity, but the glucan content was considerably higher, compared to EPS obtained from OMWW-based cultures. Moreover, the most significant differences among the three strains were observed in the case of EPS production in the semi-synthetic medium cultures. The highest glucan content was observed in the EPS of *G. lucidum*, reaching 37.92% (w/w) total glucan content, of which α-glucans account for only a small percentage, under 0.5% (w/w). These results are in accordance with previous studies, making *G. lucidum* a promising candidate for large-scale secreted production of valuable β-glucans.

Among the *Pleurotus* strains, the EPS isolated from *P. citrinopileatus* cultures were found to be slightly richer in total and β-glucan content compared to *P. ostreatus*, but *P. ostreatus* was found to produce a larger amount of exo-polysaccharides, as shown in Table 2.

Production of Mycelial Biomass-Derived Glucans

The mycelial biomass harvested at the end of each fermentation was analyzed for total, α-, and β-glucan content, before the application of any purification step (Tables 3, 4, 5). *P. ostreatus* (Table 3) and *G. lucidum* (Table 5) mycelia were found to be slightly richer in glucans in the case where the mushrooms were grown in semi-synthetic medium, compared to OMWW-based medium. However, this was not the case for the mycelia of *P. citrinopileatus* (Table 4), as in this case the growth in OMWW-based medium was found to enhance at a certain degree the accumulation of biomass polysaccharides. Nonetheless, in most

Table 2 Glucan composition of the EPS fractions from the culture supernatants from the three tested basidiomycetes strains

	P. ostreatus	*P. citrinopileatus*	*G. lucidum*
Semi-synthetic medium cultures	15.9 mg mL^{-1}	1.2 mg mL^{-1}	2.53 mg mL^{-1}
Total glucans (% w/w)	4.57 ± 0.08	6.62 ± 0.16	37.92 ± 2.8
α-glucans (% w/w)	0.27 ± 0.02	0.46 ± 0.01	0.34 ± 0.01
β-glucans (% w/w)	4.3 ± 0.06	6.16 ± 0.15	37.58 ± 2.86
OMWW cultures	18.8 mg mL^{-1}	19.7 mg mL^{-1}	21.02 mg mL^{-1}
Total glucans (% w/w)	1.91 ± 0.2	2.4 ± 0.4	2.01 ± 0.65
α-glucans (% w/w)	0.46 ± 0.03	0.52 ± 0.01	0.48 ± 0.03
β-glucans (% w/w)	1.45 ± 0.17	1.87 ± 0.4	1.52 ± 0.07

Table 3 Glucan composition of the isolated fractions from the *P. ostreatus* mycelia

Semi-synthetic medium cultures	Crude biomass (% w/w)	Water- soluble fraction (% w/w)	Purification (fold)	Yield (%)	Alkali-soluble fraction (% w/w)	Purification (fold)	Yield (%)
Total glucans	8.68 ± 2.1^{a}	19.6 ± 2.1^{a}	2.36 ± 0.81^{a}	20.92 ± 7.1^{a}	15.71 ± 0.93^{a}	1.88 ± 0.56^{a}	1.7 ± 0.5^{a}
	2.59 ± 0.33^{b}	42.87 ± 1.96^{b}	6.7 ± 1.4^{b}	13.28 ± 1.1^{b}	n.d.[b]	n.d.[b]	n.d.[b]
α-glucans	3.36 ± 0.09^{a}	10.59 ± 0.71^{a}	3.15 ± 0.12^{a}	28.03 ± 1.1^{a}	10.57 ± 0.83^{a}	3.15 ± 0.16^{a}	2.85 ± 0.14^{a}
	1.64 ± 0.06^{b}	9.77 ± 0.02^{b}	5.97 ± 0.19^{b}	4.75 ± 0.15^{b}	n.d.[b]	n.d.[b]	n.d.[b]
β-glucans	5.32 ± 0.21^{a}	9.01 ± 1.39^{a}	1.9 ± 0.11^{a}	16.92 ± 0.92^{a}	5.14 ± 0.1^{a}	1.06 ± 0.05^{a}	0.96 ± 0.04^{a}
	0.95 ± 0.27^{b}	33.1 ± 1.9^{b}	36.2 ± 8.46^{b}	28.83 ± 6.7^{b}	n.d.[b]	n.d.[b]	n.d.[b]
OMWW cultures							
Total glucans	7.58 ± 0.45^{a}	33.32 ± 0.9^{a}	4.4 ± 0.14^{a}	27.63 ± 0.9^{a}	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]
	4.65 ± 0.54^{b}	20.82 ± 0.06^{b}	4.51 ± 0.5^{b}	12.72 ± 1.5^{b}			
α-glucans	1.1 ± 0.05^{a}	2.9 ± 0.11^{a}	2.92 ± 0.12^{a}	18.3 ± 0.78^{a}	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]
	0.78 ± 0.0^{b}	2.37 ± 0.14^{b}	3.05 ± 0.19^{b}	8.58 ± 0.52^{b}			
β-glucans	6.48 ± 0.06^{a}	30.42 ± 0.8^{a}	4.7 ± 0.16^{a}	29.49 ± 1.03^{a}	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]
	3.87 ± 0.55^{b}	18.45 ± 0.21^{b}	4.82 ± 0.74^{b}	13.59 ± 2.1^{b}			

The isolation was performed following both protocols. Crude biomass results refer to the mycelia obtained at the end of the cultures, before treatment

[a]Results from purification protocol A, [b]Results from purification protocol B, *n.d.* not detected

Table 4 Glucan composition of the isolated fractions from the *P. citrinopileatus* mycelia

Semi-synthetic medium cultures	Crude biomass (% w/w)	Water- soluble fraction (% w/w)	Purification (fold)	Yield (%)	Alkali-soluble fraction (% w/w)	Purification (fold)	Yield (%)
Total glucans	$6.49 \pm 0.24^{a,b}$	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]
α-glucans	0.56 ± 0.09^{b}	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]
β-glucans	$5.93 \pm 0.34^{a,b}$	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]	n.d.[a,b]
OMWW culture							
Total glucans	$6.13 \pm 0.24^{a,b}$	12.58 ± 1.1^{a}	2.06 ± 0.25^{a}	1.3 ± 0.17^{a}	22.77 ± 2.73^{a}	3.71 ± 0.3^{a}	3.32 ± 0.27^{a}
		31.71 ± 1.37^{b}	2.26 ± 0.05^{b}	2.37 ± 0.05^{b}	n.d.[b]	n.d.[b]	n.d.[b]
α-glucans	$1.36 \pm 0.02^{a,b}$	6.07 ± 0.39^{a}	4.47 ± 0.22^{a}	2.9 ± 0.15^{a}	2.12 ± 0.01^{a}	1.56 ± 0.03^{a}	1.4 ± 0.03^{a}
		15.72 ± 0.59^{b}	5.08 ± 0.21^{b}	5.33 ± 0.22^{b}	n.d.[b]	n.d.[b]	n.d.[b]
β-glucans	$4.77 \pm 0.26^{a,b}$	6.51 ± 0.68^{a}	1.37 ± 0.21^{a}	0.89 ± 0.14^{a}	20.65 ± 2.72^{a}	4.32 ± 0.34^{a}	3.87 ± 0.3^{a}
		15.99 ± 0.72^{b}	1.44 ± 0.06^{b}	1.51 ± 0.06^{b}	n.d.[b]	n.d.[b]	n.d.[b]

The isolation was performed following both Protocols A and B. Crude biomass results refer to the mycelia obtained at the end of the cultures, before treatment

[a]Results from purification protocol A, [b]Results from purification protocol B, *n.d.* not detected

cases, the differences were marginal, accounting for less than 1% (w/w) of total glucans. Moreover, in accordance with the results obtained for extracellular polysaccharides, the β-glucan content in the crude biomass samples was significantly higher than that of α-glucan, in all cases.

The purification of biomass glucans was performed in all cases following the two protocols presented in Figs. 1 and 2. The results obtained for each strain are presented in Tables 3, 4 and 5. The application of Protocol A usually results in two different polysaccharide fractions, according to the solubility of the isolated glucan molecules, a water-soluble glucan fraction, and an alkali-soluble glucan fraction. On the other hand, Protocol B yields only one, water-soluble, polysaccharide fraction.

The results for the isolation of the biomass-bound glucan molecules for strain *P. ostreatus* are presented in Table 3. Protocol A yielded a water-soluble glucan fraction and an alkali-soluble glucan fraction in the case of the biomass grown in semi-synthetic medium, but in the case of biomass grown in OMWW-based medium, alkali-soluble polysaccharide fraction was not obtained. This result indicates that the type of glucan molecules produced from *P. ostreatus* may depend on the growth conditions, at least concerning their physicochemical properties.

Table 5 Glucan composition of the isolated fractions from the *G. lucidum* mycelia

Semi-synthetic medium cultures	Crude biomass (% w/w)	Water-soluble fraction (% w/w)	Purification (fold)	Yield (%)	Alkali-soluble fraction (% w/w)	Purification (fold)	Yield (%)
Total glucans	5.36 ± 0.36^a	n.d.[a]	n.d.[a]	n.d.[a]	5.58 ± 0.36^a	1.05 ± 0.14^a	4.79 ± 0.63^a
	6.2 ± 0.72^b	49.1 ± 5.6^b	7.9 ± 1.8^b	36.61 ± 8.4^b	n.d.[b]	n.d.[b]	n.d.[b]
α-glucans	0.17 ± 0.01^a	n.d.[a]	n.d.[a]	n.d.[a]	0.08 ± 0.004^a	0.44 ± 0.02^a	2.03 ± 0.1^a
	0.83 ± 0.04^b	0.51 ± 0.04^b	0.71 ± 0.03^b	3.23 ± 0.15^b	n.d.[b]	n.d.[b]	n.d.[b]
β-glucans	5.19 ± 0.35^a	n.d.[a]	n.d.[a]	n.d.[a]	5.5 ± 0.4^a	1.07 ± 0.15^a	4.88 ± 0.69^a
	5.39 ± 0.27^b	48.58 ± 5.7^b	9.05 ± 1.5^b	41.4 ± 6.9^b	n.d.[b]	n.d.[b]	n.d.[b]
OMWW cultures							
Total glucans	4.83 ± 0.5^a	n.d.[a]	n.d.[a]	n.d.[a]	4.68 ± 0.6^a	0.98 ± 0.02^a	7.62 ± 0.7^a
	5.56 ± 0.33^b	14.96 ± 1.45^b	2.7 ± 0.42^b	14.34 ± 2.23^b	n.d.[b]	n.d.[b]	n.d.[b]
α-glucans	0.12 ± 0.02^a	n.d.[a]	n.d.[a]	n.d.[a]	0.06 ± 0.003^a	0.5 ± 0.03^a	3.91 ± 0.84^a
	0.13 ± 0.0^b	0.64 ± 0.04^b	5.04 ± 0.31^b	26.72 ± 1.64^b	n.d.[b]	n.d.[b]	n.d.[b]
β-glucans	4.7 ± 0.48^a	n.d.[a]	n.d.[a]	n.d.[a]	4.62 ± 0.57^a	0.99 ± 0.2^a	7.7 ± 0.72^a
	5.43 ± 0.33^b	14.32 ± 1.41^b	2.65 ± 0.42^b	14.05 ± 2.23^b	n.d.[b]	n.d.[b]	n.d.[b]

The isolation was performed following both Protocols A and B. Crude biomass results refer to the mycelia obtained at the end of the cultures, before treatment

[a]Results from purification protocol A, [b]Results from purification protocol B, *n.d.*: not detected

Protocol B purification method yielded a polysaccharide fraction from the semi-synthetic medium grown biomass, containing 42.87% (w/w) total glucans, a surprisingly high content, one of the highest obtained in the present work, indicating a rather successful purification process. However, due to the low quantity of this specific fraction, the polysaccharide yield of Protocol B is ultimately lower than the yield obtained from Protocol A. Indeed, the highest purification yields in the case of *P. ostreatus* were obtained with the application of Protocol A, and this result, at least in the case of the semi-synthetic medium grown mycelia, seems to be due to the higher quantity of the derived products, despite the lower glucan content of these fractions, indicating the existence of more impurities. The polysaccharide fractions obtained from the OMWW-grown biomass seem to be of higher glucan content and higher glucan yield in the case where the isolation was performed with Protocol A. Overall, these results indicate that Protocol A was more efficient in the case of *P. ostreatus*-biomass glucan isolation, despite the existence of residual α-glucans in the final products.

Both purification protocols A and B (Figs. 1, 2) were applied in the crude biomass samples of *P. citrinopileatus*. Interestingly, in this case, neither purification method yielded any polysaccharide fractions in the case where the mushroom was grown in semi-synthetic medium cultures (Table 4). The obtained water-soluble polysaccharide fraction following Protocol B was only 37 mg, and thus the determination of its glucan composition was not possible. Protocol A purification method yielded similar results. These observations indicate a certain inhibition of the polysaccharides isolation in the case where the strain is grown in semi-synthetic medium cultures, possibly due to enzymes or unknown compounds produced by the fungus in these conditions.

The results are quite different in the case of OMWW-grown cultures. Despite the lower glucan content of the starting material, both purification protocols were successful (Table 4). Protocol B seem to be more efficient in this case. As shown in Table 4, Protocol B application yielded polysaccharide fractions with higher glucan content, and better yield, compared to Protocol A. The water-soluble polysaccharide fraction derived from Protocol B purification contained 31.71% (w/w) total glucans, a quite satisfactory result, despite the fact that α- and β-glucan contents are almost equal. On the other hand, the same conclusion can be drawn also from the results of Protocol A application in OMWW-grown biomass: the water-soluble fraction was found to contain almost equal amounts of α- and β-glucans, despite the α-amylase treatment step of Protocol A (Fig. 1). The application of Protocol A also yielded an alkali-soluble polysaccharide fraction that was found to be richer in total glucans than the respective water-soluble fraction (Table 4).

Finally, the two purification protocols tested were applied also in the fungal biomass of *G. lucidum*. The results in this case were of special interest: The application of Protocol A in the crude biomass samples of *G. lucidum* from both culture media yielded only alkali-soluble polysaccharide fractions, while the water-soluble fractions obtained were of negligible quantity (Table 5). On the other hand, the biomass polysaccharides of *G. lucidum* were

isolated in the water-soluble fraction resulting from Protocol B application. These results suggest that the extensive boiling step of Protocol B might be responsible for the solubilization in water even of the glucan molecules that are, in milder conditions, alkali-soluble. Nonetheless, the single alkali-soluble fraction resulting from Protocol A purification, was found to be rather poor in glucan content in both the cases of semi-synthetic medium and OMWW-medium grown biomass (Table 5). Overall, Protocol B was found to be the most effective in the case of biomass polysaccharides isolation from *G. lucidum*. Especially in the case of the polysaccharide fraction obtained from the cultures grown in semi-synthetic medium, its glucan content was the highest achieved in this study, a remarkable 49.1% (w/w), of which only 0.51% (w/w) accounts for α-glucans (Table 5). These results highlight the superiority of Protocol B in this case.

FT-IR analysis of Isolated Polysaccharide Fractions

For the identification of the isolated polysaccharide fractions, FT-IR spectroscopy was employed. All spectra shown in Fig. 3 present a wide peak around 3300–3400 cm^{-1}, corresponding to free –OH groups of a large molecule, indicating the existence of high MW polysaccharide molecules [5]. The peaks in 881 and 893 cm^{-1} present in EPS from cultures grown in both semi-synthetic and OMWW medium for the samples derived from the species *P. citrinopileatus* and *G. lucidum*, corresponding to β-glucosidic bond, and thus indicating the existence of β-glucan molecules [9, 29, 30]. The peaks at 854, 862 and 845 cm^{-1} indicate accordingly, the existence of α-glucan molecules in the samples of *P. ostreatus* and *P. citrinopileatus* [2, 29, 30]. Moreover, the peaks around 520–550 cm^{-1} in the *P. ostreatus* EPS sample, and some of the other samples, also indicate the presence of α-glucans according to several studies [2, 9]. Peaks at 1127, 1153, 1152, 1158, 1150 and 1143 cm^{-1} represent the stretch of the C–O–C bond of the pyranose ring of sugar moieties [9, 30]. The peak at 1650 cm^{-1} found in all the samples, corresponds to the C-N bond of the aminoacids, and thus indicates the presence of protein contamination in the samples [9]. The peaks around 2920–2930 cm^{-1} correspond to $-CH_2$ groups of lipids, as previously reported [9].

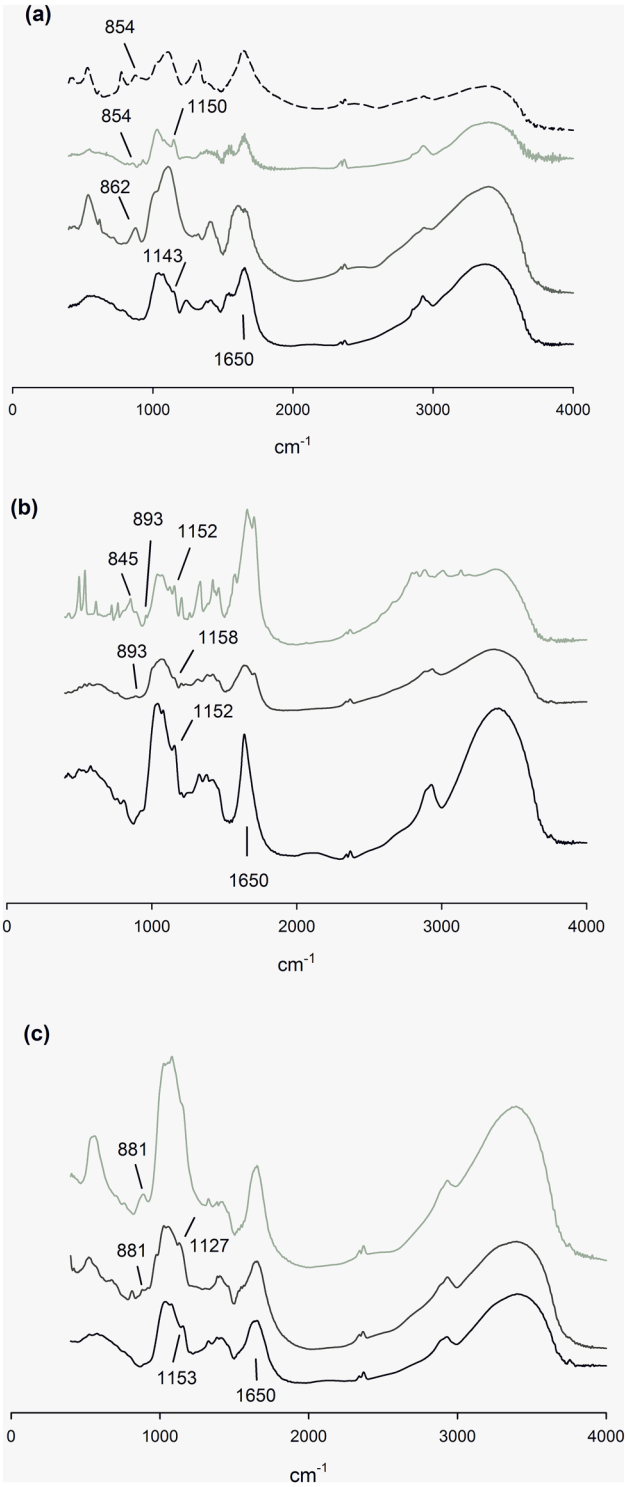

Fig. 3 FT-IR spectra of partially isolated polysaccharide samples derived from *P. ostreatus* (**a**), *G. lucidum* (**b**) and *P. citrinopileatus* (**c**). *Black and dark grey lines* represent the FT-IR spectra of the polysaccharides isolated from mycelial biomass and EPS, respectively, isolated both from cultures grown in semi-synthetic medium. *Light grey and dotted lines* represent the FT-IR spectra of the polysaccharides isolated from mycelial biomass and EPS, respectively, isolated both from cultures grown in OMWW medium

Discussion

In the present work, all the studied basidiomycetes grew well in both media, yielding a satisfactory production of biomass through fermentations in semi-synthetic, as well as in OMWW media. OMWW supplemented with nitrogen source seems to be able to sustain mycelial growth from all strains. However, waste degradation in these cases was not

as high as reported previously for other basidiomycetes [13, 31, 32] indicating that further optimization steps are necessary. However, the results suggest that the submerged fermentation might be more efficient in terms of productivity, due to the fact that biomass growth was considerably faster compared to solid state approaches, where complete fruit body formation might take up to 2 months [33].

P. ostreatus appears to be more productive in terms of EPS compared to the other two strains, at least in the case of the semi-synthetic medium cultures. In this case, a considerable amount of EPS was isolated in both tested media, albeit with a low concentration of glucans. Interestingly, EPS obtained from *G. lucidum* cultures were isolated as a gel-like substance, as previously reported for basidiomycete's EPS [8], consisting from β-glucans at a high percentage. The yields obtained were comparable to those reported previously for other species [34]. Unfortunately, the glucan concentration in the EPS after growth in OMWW-based media was considerably lower. This was also the case for *P. citrinopileatus*, however, in the semi-synthetic medium cultures, the EPS yield exceeded those obtained in the previous work by Wang et al. [10], by the same species, where only 0.56 mg mL^{-1} EPS were isolated.

In the case of *P. citrinopileatus* biomass-derived polysaccharides, the purification yields obtained with the two methods were quite different but comparable to relevant studies [35–37]. In this case, Protocol B seems to result in a significant recovery of partially purified polysaccharides, leading to the conclusion that the laborious and costly steps of Protocol A, mainly the incubation with α-amylase and the Sevag treatment steps, may be omitted without significant loss in the final product. Unfortunately, the results obtained refer only to the OMWW cultures, since in semi-synthetic medium cultures, the isolated fractions following both protocols were of low quantity for glucan composition analysis.

Following Protocol A, *P. ostreatus* biomass glucans were found mainly in the water-soluble fraction. The obtained purification yields were surprisingly high, reaching almost 30% for OMWW-grown mycelium β-glucans. This is quite interesting, taking into account that these yields are considerably higher than others reported in the literature [6, 8, 38]. However, comparable yields have been also obtained previously [39]. Similar results were also obtained using Protocol B, where the β-glucan content in the water-soluble fraction was surprisingly high. These results also support the feasibility of Protocol B, taking into account the simplicity of the process, compared to Protocol A.

On the other hand, regarding the isolation of glucans from *G. lucidum* biomass following Protocol B (Fig. 2) of Wei et al. [28], the obtained yields were very high in this case, exceeding 40% for the β-glucans isolated from the mycelium grown in semi-synthetic medium, as reported previously for other species [39] but quite higher than most relevant studies [6, 8, 38]. Isolation was also performed with protocol A in this case, with very poor results, highlighting the superiority of Protocol B in the case of purification of biomass-related polysaccharides.

FT-IR spectra of the isolated fractions revealed protein contamination, despite multiple treatments with Sevag reagent, as shown in previous studies [9]. Glucans often occur in mushrooms in complex with proteins called proteoglucans and thus complete protein removal from the samples can be challenging [40]. In terms of protein removal, Protocol A does not seem to be superior to Protocol B, despite the Sevag reagent treatments, indicating that this step of the procedure might be omitted without significant changes in the final product.

Moreover, in the case of the glucans isolated following Protocol A, the treatment with α-amylase failed to remove completely α-glucans from the isolated samples. The yield of obtained α- glucans varied from 1.4% in *P. citrinopileatus* alkali-soluble biomass polysaccharide to 28.03% in *P. ostreatus* water-soluble biomass polysaccharide fraction. The corresponding obtained yields in the case of the samples isolated with Protocol B varied from 3.23 to 26.72%, indicating that the use of the commercial α-amylase, a laborious and costly step of the procedure, may be omitted without significant change in the α-glucan yields.

Conclusions

Overall, the results of the present work support that the production and purification of fungal polysaccharides might be feasible in submerged culture and in large scale, providing an alternative method for the valorization of wastewater as media for fungal growth. The three wild Greek basidiomycetes tested in this study performed well during submerged culture in semi-synthetic medium, but also during growth in OMWW. Although production and purification of basidiomycete's glucans can be easier and more cost-effective in solid state cultures and in basidiocarp form, submerged culture could offer an interesting alternative due to the following main advantages; first, the mycelial growth is much more rapid leading to higher productivity, while the use of liquid media required, leads to a greater variety of growth media choices, including liquid wastes, such as OMWW. Basidiomycetes are known to effectively degrade OMWW, and after the necessary optimization of parameters, the two processes of OMWW degradation and polysaccharides production could be combined in a single procedure, leading to the valorization of a toxic waste. Finally, with the submerged culture, the isolation of extracellular glucans can also be achieved, leading to further increase of the total polysaccharide yield of the procedure.

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