Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Effects of supplementation of sea buckthorn press cake on mycelium growth and polysaccharides of *Inonotus obliquus* in submerged cultivation

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Keywords
exopolysaccharide, *Inonotus obliquus*, intracellular polysaccharide, mycelium, sea buckthorn, submerged cultivation.

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2021/2430: received 12 November 2020, revised 26 January 2021 and accepted 3 February 2021

doi:10.1111/jam.15028

Abstract

Aims: Investigation of the influence of cultivation time and sea buckthorn press cake (*Hippophaë rhamnoides*) dosage on mycelium yield of *Inonotus obliquus* in submerged cultivation and on the yield, monomer composition, and macromolecular properties of the exopolysaccharides (EPS) from culture media and intracellular polysaccharides (IPS) extracted from mycelia.
Methods and Results: Supplementation at 5 g l^{-1} combined with cultivation time of 250 h granted highest yield increase in mycelia (by 122%). The supplementation reduced extraction yield and decreased the molecular weight of the main IPS population. The supplementation increased production and molecular weight of EPS. The relative content of arabinose and rhamnose in EPS positively correlated with dosage of the press cake. The press cake supplementation increased the content of galacturonic acid in IPS, but not in EPS.
Conclusion: Sea buckthorn press cake is a food industry fibrous side stream with high oil content. It increases the cultivation yield of *Inonotus obliquus* mycelium and influences the produced polysaccharides.
Significance and Impact of the Study: Mycelium is a resource of bioactive polysaccharides, attracting the interest of nutraceutical companies. Sea buckthorn press cake is a promising supplement for increasing mycelium production. The utilization of this agricultural side stream would therefore favour circular economy.

Introduction

Inonotus obliquus (Fr.) Pilát is a basidiomycete of the family Hymenochaetaceae. It is an obligate parasite of birch trees, less commonly of beech, and it is classified as a white-rot fungus. It is distributed in the northern hemisphere, mainly above the 40th parallel. Its infection causes the formation of a charcoal black, cracked-shape conk on the birch stem, consisting of sclerotial mycelium and wood. Such conk, commonly called Chaga, is a renowned folk medicine in many regions such as the Baltic countries, Russia and China. Traditionally, hot water extracts, such as tea and decoction, prepared from Chaga have been used for the treatment of different health conditions, including digestive disorder, tuberculosis and cancer (Lee *et al.* 2008). Numerous studies on the hot water extracts of Chaga performed in recent years have suggested multiple biological activities of the extracted polysaccharides, in particular immunomodulating function (Lau and Abdullah, 2016). The interest in the exploitation of Chaga has been increasing rapidly among the food supplement and healthcare industry. However, an economically feasible and environmental sustainable harvesting of Chaga is hampered by logistical difficulties connected to its natural location and, most importantly, by its slow growth (Zheng *et al.* 2010).

The mycelium of basidiomycetes, obtained after submerged cultivation (SbmC), has been considered an alternative source of bioactive polysaccharides of *I. obliquus* (Zheng *et al.* 2010). Such methodology allows the use of supplements and stimulants to increase the cultivation yield. Multiple reports regarding the cultivation of *I. obliquus* and the production of polysaccharides are available. Research has focused also on the simultaneous collection of exopolysaccharides (EPS), secreted by the mycelium, which has shown immunomodulation activity (Xu *et al.* 2014b; Lee *et al.* 2017). Previous research has shown that the supplementation of fibrous material is able to increase the cultivation yield of *I. obliquus* (Xu *et al.* 2014a). In addition, the supplementation of fatty acids and plant oils to the submerged cultivation medium of basidiomycetes has also showed positive effects in promoting mycelium growth (Xu *et al.* 2015; Berovic and Podgornik, 2016).

It could then be hypothesized that the simultaneous supplementation of fibres and oil would have a positive effect on the mycelium yield of the submerged cultivation of *I. obliquus*. However, no research has been reported using simultaneous addition of fibres and lipids in submerged cultivation of *I. obliquus*.

Sea buckthorn (*Hippophaë rhamnoides* L.) is a deciduous shrub distributed across Eurasia. Its berries contain a vast array of hydrophilic bioactive compounds such as ascorbic acid and flavonoids. Sea buckthorn berries are rich in lipophilic compounds, including carotenoids, tocopherols, triacylglycerols, phospholipids, plant sterols and waxes (Bal *et al.* 2011). In particular, the oil content of the dry pulp varies in the range of 4–34% (Yang and Kallio, 2002). Sea buckthorn berries are used as raw materials for food and nutraceuticals. Juice pressing is a common way of industrial processing of sea buckthorn berries, producing berry press cakes as side stream. Sea buckthorn press cakes consist of pulp/peels and seeds of the berries rich in pulp oils, fibres, and phenolic compounds. After pressing, moreover, the extractability of pectin from the berry is enhanced (Hilz *et al.* 2006).

In the present work, our aim was to investigate the impact of supplementation of sea buckthorn press cake to the culture medium on the mycelium growth yield and polysaccharide production of *I. obliquus* under submerged cultivation conditions. The sea buckthorn press cakes were supplemented at different dosages, and two cultivation times were applied. The influence of these parameters was investigated in terms of mycelium yield and monomer composition of carbohydrates in the mycelium. Intracellular polysaccharides (IPS) were extracted from the mycelium of *I. obliquus* to study the possible influences of the press cake to their yield and macromolecular properties.

Furthermore, EPS fractions were isolated from the cultivation medium and their monomer composition and molecular weight were determined, to investigate the impact of different cultivation parameters.

Materials and methods

Submerged cultivation of *I. obliquus*

Dried sea buckthorn press cake was obtained from Polarforma Oy (Tornio, Finland). The total lipid and phenolic contents of the press cake were $0{\cdot}40\ g\ g^{-1}$ and $0{\cdot}43\ mg\ g^{-1}$ respectively (Damerau *et al.* 2020). The content of acid-insoluble matters of the press cake was $0{\cdot}47\ g\ g^{-1}$, which was estimated as difference between the content of defatted press cake and the content of hemicellulose ($0{\cdot}13\ g\ g^{-1}$) measured with methanolysis. The press cake was ground and passed through a 30-mesh sieve. Particles with size below 30 mesh were collected and used as supplement in submerged cultivation. For comparison with mycelium polysaccharides, press cake polysaccharides were extracted with 200 ml of deionized water in autoclave for 30 min at 120°C and, and after filtration to remove press cake residue, the sea buckthorn polysaccharides were precipitated with 3 volumes of technical ethanol.

The mycelium of *I. obliquus* was isolated from wild sclerotia collected from the birch forest in Yichun (Heilongjiang province, China) and maintained on potato dextrose agar slants. The strain was deposited at the Institute of Microbiology of the Heilongjiang Academy of Sciences. Slants were inoculated, incubated at 30°C for 7 days and thereafter stored at 4°C until further use. The mycelium was subcultured every 3 months. About 1 cm length of agar slant, free from aerial hyphae, was cut, smashed and transferred to a 500 ml Erlenmeyer flask containing 200 ml of aqueous cultivation medium, previously autoclaved at 120°C for 30 min. The cultivation medium contained ($g\ l^{-1}$): glucose 15; maltose 15; peptone 2; beef extract 1·3; $MgSO_4{\cdot}7\ H_2O$ 1·5; KH_2PO_4 3; vitamin B_1 0·01. The inoculated flasks were incubated in a rotary shaker at 27°C, with a rotation speed of 140 rev min^{-1}. Parallel to the control flask, the medium of the treatment flasks was supplemented with the different dosages of sea buckthorn press cake ($g\ l^{-1}$): 2·5; 5; 10; 30. For each dosage, two cultivation times were investigated: 200 and 250 h. Each cultivation was performed in triplicate.

EPS and mycelium isolation

At the end of the cultivation time, the medium was filtered with a 30-mesh sieve. Since ground press cake was screened with the same sieve, there was no retention of remainder particles of press cake. Moreover, the methanolysis proved the absence of press cake residues in the filtrated mycelium. After extensive washing with distilled water, the collected mycelium (free from press cake particles) was oven dried at 80°C for 1 h and the yield was measured gravimetrically. The obtained cultivation liquid was further filtrated, concentrated to 50 ml, and 3 volumes of technical ethanol were added to precipitate the EPS. After overnight storage at 4°C, the precipitates were collected with ultracentrifugation (9000***g*** for 20 min at 4°C). To further remove sugars, the precipitates were recovered with fresh technical ethanol and ultracentrifuged again. Thereafter, the precipitates were then redissolved in deionized water. Insoluble material was removed from EPS with freeze-thawing cycle and soluble EPSs were freeze-dried. Finally, the yield of EPS was measured gravimetrically.

Methanolysis of mycelium and press cake fractions

The monomer composition of dried mycelium, ground press cake, autoclaved press cake extract and autoclaved press cake residue were analysed with methanolysis (Sundberg *et al.* 1996). Briefly, about 10 mg of dried mycelium sample was mixed with 2 ml HCl 2 mol l^{-1} in MeOH and hydrolysed at 105°C for 5 h. Each methanolysis was performed in duplicate. After cooling down, neutralization with pyridine, addition of internal standard (resorcinol in MeOH), and sedimentation, an aliquot of 1·5 ml of clear phase was transferred into a test tube and dried with nitrogen flow. After the recovery of the hydrolysate with pyridine, the samples were silylated overnight with 150 μl of HMDS and 70 μl of TMSC. The clear phases containing silylated sugars and uronic acids were transferred into autosampler vials and analysed with GC-FID. Arabinose, fucose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, rhamnose and xylose were used as standard and derivatized in the same way before GC-FID analysis.

Extraction of polysaccharides from *I. obliquus* mycelium

Polysaccharides were extracted from the mycelia obtained with different cultivation parameters using extraction protocol previously reported (Beltrame *et al.* 2019). Mycelia obtained without supplementation of the press cake and with supplementation at the dosage of 2·5 g l^{-1} were pooled and studied separately. Mycelia obtained with the supplementation of press cake at higher dosages (5 and 10 g l^{-1}) were pooled, and polysaccharides were extracted and analysed. For comparison, polysaccharides were extracted from mycelium cultivated without the supplementation of sea buckthorn. Briefly, after removal of phenolics with technical EtOH, mycelium was extracted sequentially with hot water and aqueous 2% KOH. The alkali extract was neutralized with acetic acid. The hot water- and alkali-extracted polysaccharides were precipitated with the addition 3 volumes of technical EtOH, recovered with ultra-centrifugation and deproteinized with the Sevag method (Shi, 2016). Thereafter, the polysaccharides were then dialysed (cut off 12–14 kDa), recovered after freeze-thawing cycles and finally freeze-dried. The final yields of polysaccharides were measured gravimetrically. In the case of mycelium cultivated without supplement, only the hot water polysaccharides fraction was produced. The intracellular polysaccharide fractions (IPS) produced were labelled IPSsb5-10 HW, IPSsb5-10 2% and IPSsb0 HW respectively.

Quantification and macromolecular properties of *I. obliquus* polysaccharides

The sugar contents of polysaccharide fractions (EPS and IPS) were measured with the phenol-sulphuric acid method adapted for microplate reader (Masuko *et al.* 2005). The protein content of IPS fractions was measured with the Lowry method (Markwell *et al.* 1978).

The monomer composition was analysed after TFA hydrolysis. About 10 mg of samples were mixed with an aliquot of TFA 2 mol l^{-1} solution to bring the sample concentration to 1 mg ml^{-1}. The solutions were heated for 6 h at 100°C. Then, they were filtered with 0·45 μm RC filters, added with internal standard (myo-inositol) and dried with nitrogen flow. The samples were silylated with 500 μl of TriSil at 70°C, and 1 μl of clear phase TriSil solution was injected into a GC-MS system for identification and GC-FID system for quantification. Temperature programs and GC-FID parameters were as reported previously (Beltrame *et al.* 2019). The mass spectrometer (EI positive ion mode) had transfer line and ion source temperatures of 280°C and 260°C, respectively, and ionization voltage of 70 eV. The same sugar standards reported above were used. The correction factor of galactose was used for the quantification of 3-O-Me-galactose. Monomer composition was expressed as relative molar percentage.

The molecular weight was analysed with size exclusion chromatography as described previously (Beltrame *et al.* 2019). The analytical system was calibrated with pullulan standards (Polymer Standard Service, Mainz, Germany). Weight-average (M_w) and number-average (M_n) molecular masses and polydispersity index (PDI) of the EPS fractions were calculated from RI signal, using the software Origin 2016 (OriginLab Corp., Northampton, MA, USA). On the other hand, the molecular weight of the mycelium polysaccharides was calculated from the pullulan calibration curve and reported as peak molecular weight (M_p).

Statistical analysis

Statistical analysis was performed with RStudio (RStudio, 2020). Shapiro–Wilk test was used to assess normality distribution of the data. One-way ANOVA with Levene test was used to analyse the variance among samples. Tukey-HSD and Games-Howell were used as *post hoc* tests. Correlation among variables was assessed with Pearson or Spearman methods. Significance was assigned at $P < 0{\cdot}05$. Second-order polynome and asymptotic regressions of the data were performed with the software Origin 16 (OriginLab Corp., Northampton, MA, USA).

Results

Effect of sea buckthorn addition and incubation time on mycelium yield

The supplementation of sea buckthorn press cake had a remarkable positive effect on the growth of *I. obliquus*. The cultivation yields, expressed as dry weight of mycelium per litre of medium (g l^{-1}), are reported in Fig. 1. When sea buckthorn was added to the culture medium, both the dosage of sea buckthorn addition and the cultivation time showed a significant impact on the yield of mycelium. At cultivation time of 200 h only the addition of 5 g l^{-1} of supplement led to a significant ($P < 1 \times 10^{-4}$) increase in yield by 71% compared to the control. Press cake addition at higher or lower dosages did not produce any significant impact on the mycelium yield compared to the control. Conversely, the addition of press cake had more noticeable positive effect on yield obtained after 250 h (Fig. 1). At this cultivation time, significant ($P < 1 \times 10^{-4}$) and the highest yield increase was obtained with the addition of press cake at 5 g l^{-1} or 10 g l^{-1} (increase by 122% from the control). Also, addition of the press cake at 2·5 g l^{-1} resulted in significant increase (94%).

The increase in cultivation time from 200 to 250 h had no significant effect on the mycelium growth in absence of sea buckthorn press cake. The influence of the cultivation time was most evident at the addition of 10 and 2·5 g l^{-1} of press cake, where the increase in cultivation time resulted in yield increase by 72% and 69% respectively. However, addition of the press cake at 30 g l^{-1} had no significant effect on the cultivation yield, at either of the cultivation times.

Monomer composition of cultivated mycelium of *I. obliquus*

Cultivated mycelium of *I. obliquus* was subjected to methanolysis, in order to investigate the possible impact of supplementation of sea buckthorn press cake and the cultivation time on the content and monomer composition of mycelium polysaccharides. The monomer concentrations (mg g^{-1} of mycelium) are reported in Fig. 2. The monomer composition of sea buckthorn press cake was analysed with methanolysis before and after autoclaving for comparison with the monomer composition of mycelium, and the results are reported in Fig. S1. While the press cake polysaccharides were mainly composed of galacturonic acid (35%) and xylose (20%) before autoclaving, the polymers extracted by the medium during autoclaving were mainly pectin (50·2% of total sugars were galacturonic acid). On the other hand, xylose represented 39·8% of total sugars the polysaccharides retained by the press cake after autoclaving, indicating hemicelluloses being the major component. As shown in Fig. 2, glucose was the main monomer released by the mycelium during methanolysis, with a concentration between 263 and 475 mg g^{-1} mycelia (75–93% w/w of the total sugars), being at least one magnitude higher than all the other monomers. The most abundant monomers after glucose were galactose and mannose, with ranges of relative abundance of 3–16% and 1–9% w/w, respectively, of the total sugars. The monomer composition showed no significant differences between the concentrations of glucose and xylose in the mycelia. On the other hand, there was a significant increase in arabinose, rhamnose and galacturonic acid in mycelia cultivated with higher dosage of sea buckthorn press cake (dosage 10 and 30 g l^{-1} medium). Spearman correlation test showed strong positive correlation between arabinose, rhamnose and galacturonic acid and press cake dosage ($0{\cdot}8 < \rho < 0{\cdot}9$, $P < 1 \times 10^{-4}$) (Table S1). On the other hand, a strong negative correlation between press cake dosage and glucuronic acid and mannose was highlighted ($\rho = -0{\cdot}58$, $P = 0{\cdot}008$ and $\rho = -0{\cdot}71$, $P < 1 \times 10^{-4}$, respectively), while a weak negative correlations was found between press cake dosage and galactose. The lack of significant correlation between fucose and press cake concentration indicates that this monomer was produced by the mycelium irrespectively of the cultivation conditions. Finally, cultivation time showed no significant correlation with other variables.

Polysaccharides extracted from *I. obliquus* mycelium

Polysaccharides were extracted from *I. obliquus* mycelium with a sequential method and their macromolecular properties determined. The results of the analyses are

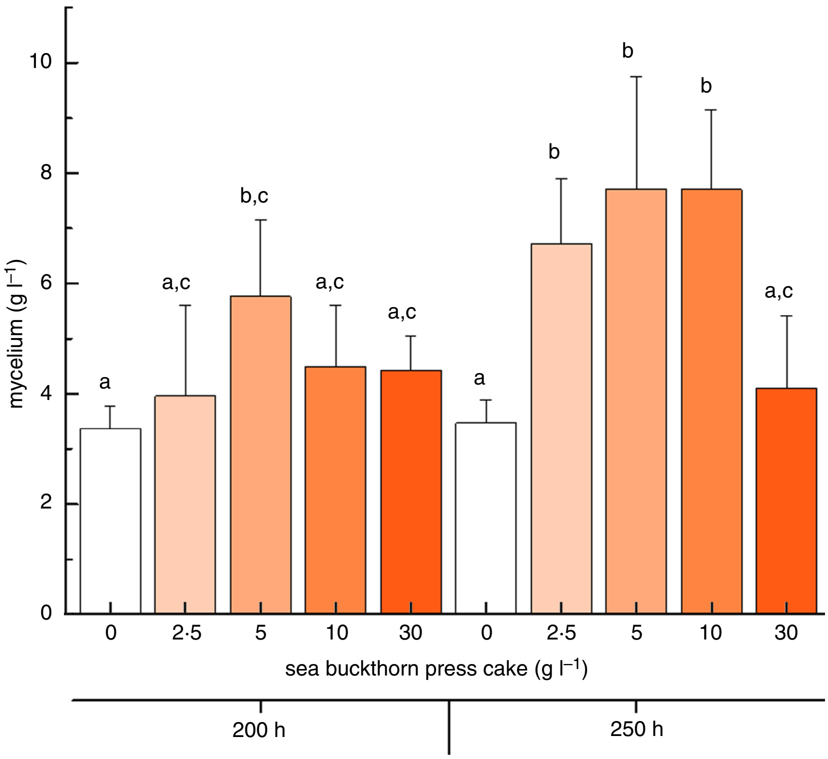

Figure 1 Dry mycelium yield of the submerged cultivation of *Inonotus obliquus*. Results of different press cake dosages (0, 2·5, 5, 10 and 30 g l^{-1}) are reported as average and grouped by cultivation time (200 and 250 h). Different letters mark significant difference ($p < 0{\cdot}05$)

reported in Table 1. The aim of alkali extraction was to disrupt the fungal cell wall and to facilitate the extraction of polysaccharides from the inner layers of the cell wall (Komura *et al.* 2014; Beltrame *et al.* 2019). In this study, all extractions resulted in yield close to 1% (w/w dry weight) of the starting mycelium. The sugar content decreased from 85% w/w in IPSsb0 HW to 59% w/w in IPSsb5-10 2%. The observed decrease in production of polysaccharides by the mycelium might be connected to the presence of press cake. This was in agreement with the methanolysis results, which showed negative Spearman correlations between press cake content and hydrolysis yield ($\rho = -0{\cdot}33$, Table S1). A clearer negative correlation was observed with the hydrolysis of the mycelium obtained after 200 h of cultivation ($\rho = -0{\cdot}54$). Conversely, the use of alkali resulted in an increase in the protein content of the fractions, from 23% to 34% w/w.

Glucose was the main sugar (65%) of the polysaccharides extracted from the mycelium of *I. obliquus* with hot water, followed by mannose (18%) and galactose (11%). The content of mannose was slightly higher in IPSsb0 HW than respective fraction obtained from supplemented mycelium. The use of alkali decreased the relative molar percentage of glucose to 50%, and increased the relative content of other monomers, in particular mannose (from 17% to 27% of the molar composition). Galactose, on the other hand, decreased only slightly (from 12% to 11%) by the alkali extraction. The monomer composition analysis showed the presence of galacturonic acid in the extracts, however, in low abundance (2% and 1% for hot water and alkali extracts respectively). The amount was lower in IPSsb0 (0·5%). The increase in this monomer can be attributed to the sea buckthorn in the cultivation medium, as shown by the monomer composition of the mycelium. Based on the results of the mycelium methanolysis (Fig. 2), it can be estimated that IPSsb5-10 HW and IPSsb5-10 2% extracted from the pooled mycelium contained in total around 5% of the galacturonic acid.

The molecular weight of IPS polysaccharides is reported in Table 1. The HPSEC chromatograms are reported in Fig. S3. No relevant peaks were observed at the penetration limit of the column. Almost 80% of the polymers extracted with hot water had M_p of 65 kDa, while the 70% of the polymers extracted with alkali had M_p of 15 kDa. The decrease in molecular weight of the main population can be attributed to the hydrolysing effect of the alkali solution. The HPSEC chromatograms

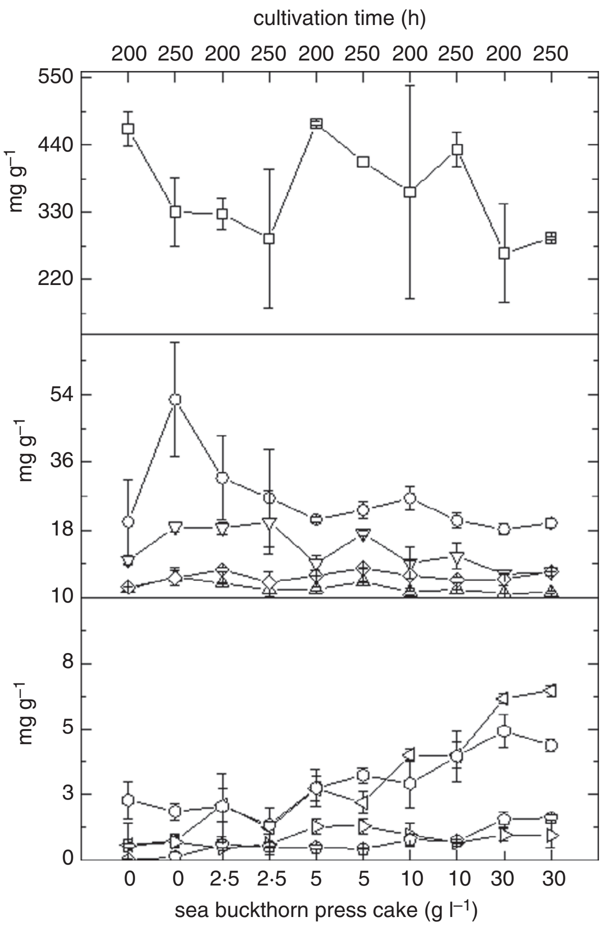

Figure 2 Monomer composition of the cultivated mycelium reported as concentration of monomers (mg g^{-1} of mycelium). Samples are identified by the combination of press cake dosage (lower *x*-axis) and cultivation time (upper *x*-axis). Symbols: (□) glucose, (○) galactose, (Δ) glucuronic acid, (∇) mannose, (◇) xylose, (◁) arabinose, (▷) fucose, (□) galacturonic acid, (□) rhamnose.

showed also that both IPSsb5-10 fractions contained polymers with a M_p of $2{\cdot}0 \times 10^2$ kDa at lower percentages (18·5% and 21·4% for hot water and alkali fractions respectively). This population was present also in IPSsb0, however, it was the most abundant (58% of total area). Nevertheless, IPSsb5-10 2% contained (about 9% of the total area) a polysaccharide population of high molecular weight ($1{\cdot}4 \times 10^3$ kDa) that was not observed in the IPSsb5-10 HW chromatogram.

Content, monomer composition and macromolecular properties of EPS

In this study, we investigated the influence of sea buckthorn press cake supplementation and cultivation time on macromolecular properties of the EPS isolated from the growth medium of *I. obliquus*. The concentrations of EPS in the culture medium and sugar content of the EPS fractions are reported in Table 2, expressed as grams of EPS per litre of medium and weight % respectively. The production of exo-polysaccharide, expressed as mg g^{-1} mycelium, was calculated from these values. Highest EPS concentration in the culture medium (2 g l^{-1}) and sugar contents in EPS fraction (17 w/w %) were obtained with sea buckthorn press cake at the highest dosage (30 g l^{-1}), after 250 and 200 h of cultivation respectively. EPS content in the medium showed, with both cultivation times, a negative trend with a turning point followed by an increase, when the dosage of press cake was increased. The minimum concentration (0·33 g l^{-1} of EPS) was obtained with supplementation at dosages of 5 and 2·5 g l^{-1}, respectively, after 200 and 250 h of cultivation. The observed trends fitted a second-order polynomial regression function, as reported in Equations 1 and 2:

$$\mathrm{EPS_{200h}} = 0\cdot61658 - 0\cdot06393 \times [\mathrm{SB}] + 0\cdot00283 \times [\mathrm{SB}]^2 \quad R^2 = 0\cdot774, \tag{1}$$

$$\mathrm{EPS_{250h}} = 0\cdot37278 - 0\cdot00721 \times [\mathrm{SB}] + 0\cdot00243 \times [\mathrm{SB}]^2 \quad R^2 = 0\cdot854, \tag{2}$$

where $\mathrm{EPS_{200h}}$ and $\mathrm{EPS_{250h}}$ represent the EPS concentration (g l^{-1}) after 200 and 250 h of cultivation, respectively, and [SB] represents the dosage of sea buckthorn press cake (g l^{-1}) in the cultivation medium. On the other hand, the sugar content of the EPS positively correlated with the dosage of sea buckthorn press cake ($r = 0{\cdot}48$, $P = 0{\cdot}006$). The production of exo-polysaccharides (mg g^{-1} mycelium) had a clear quadratic relationship with the supplementation of sea buckthorn press cake, as reported in Equations 3 and 4:

$$\mathrm{P_{200h}} = 9\cdot91238 + 0\cdot05939 \times [\mathrm{SB}] + 0\cdot02896 \times [\mathrm{SB}]^2 \quad R^2 = 0\cdot983, \tag{3}$$

$$\mathrm{P_{250h}} = 7\cdot71521 - 0\cdot72105 \times [\mathrm{SB}] + 0\cdot08179 \times [\mathrm{SB}]^2 \quad R^2 = 0\cdot995, \tag{4}$$

where $\mathrm{P_{200h}}$ and $\mathrm{P_{250h}}$ represent the production of exo-polysaccharide and [SB] represents the dosage of sea buckthorn press cake (g l^{-1}) in the cultivation medium.

The monomer composition of the EPS is reported in Fig. 3. The most abundant monomer was glucose (23–46 mol%), followed by mannose (14–28% mol%) and galacturonic acid (7–26% mol%). Significant differences in relative molar percentages between different cultivation conditions were found mainly for arabinose, rhamnose, 3-O-Me-galactose and the galactose/3-O-Me-galactose ratio. Pearson correlation values between monomer composition, cultivation conditions, molecular weight and polydispersity index (PDI), are showed in Fig. 4. A summary of Pearson correlation values between the same variables, distinguishing between samples

Table 1 Sugar and protein contents, monomer composition and molecular weight of the polysaccharides extracted from *Inonotus obliquus* mycelium

	Sugar content (w/w %)	Protein content (w/w %)	Monomer composition (relative mol %)										Mp (area) kDa (%)		
			Xyl	Gal	Rha	Glc	Man	Ara	Fuc	GalA	3-O-MeGal	Gal/3-O-MeGal (mol mol^{-1} %)	Population 1	Population 2	Population 3
IPSsb0 HW	85·3 ± 5·3	15·6 ± 1·1	3·42 ± 0·14	4·98 ± 0·24	0·74 ± 0·09	66·02 ± 0·66	20·13 ± 0·24	2·34 ± 0·07	1·26 ± 0·11	0·56 ± 0·10	0·56 ± 0·10	9 ± 1	$3{\cdot}8 \times 10^2$ (57·8)	4·8 (14·2)	2·4 (28·0)*
IPSsb5-10 HW	74·3 ± 2·6	22·8 ± 0·8	0·84 ± 0·02	11·49 ± 0·14	0·35 ± 0·08	64·07 ± 0·13	17·59 ± 0·14	1·21 ± 0·01	1·02 ± 0·02	1·96 ± 0·07	1·43 ± 0·04	8 ± 0	$2{\cdot}0 \times 10^2$ (18·5)	65 (79·5)	6·7 (2·0)
IPSsb5-10 2%	58·9 ± 2·2	34·4 ± 0·9	6·66 ± 0·06	10·73 ± 0·24	0·52 ± 0·02	49·16 ± 0·26	26·57 ± 0·37	1·95 ± 0·04	1·54 ± 0·02	1·17 ± 0·06	1·61 ± 0·01	7 ± 0	$1{\cdot}40 \times 10^3$ (8·6)	$2{\cdot}0 \times 10^2$ (21·4)	15 (70·0)

*Two peaks combined.

obtained after 200h and 250 h of cultivation, can be found in Table S2. In particular, the content of arabinose and rhamnose correlated with press cake dosage ($r = 0{\cdot}55$, $P = 0{\cdot}002$). On the other hand, the relative amount of galacturonic had no significant correlation with supplement dosage. This was in contrast to the findings in IPS extracted from mycelia, where the proportion of galacturonic acid clearly increased after supplementation. The concentration of press cake, while unaffecting the relative amounts of galactose, had a negative effect on the amount of 3-O-Me-galactose ($r = -0{\cdot}40$, $P = 0{\cdot}03$), which explained the significance in the increase of the galactose/3-O-Me-galactose ratio at higher dosages ($r = 0{\cdot}51$, $P = 0{\cdot}004$) (Fig. 4).

The molecular weight analysis focused on the major peak found in the chromatograms. The size-exclusion chromatograms are reported in Fig. S2. The weight-average molecular mass (M_w) and polydispersity index (PDI) of the main EPS population for the different cultivation conditions are reported in Table 2. Overall, the average M_w was $4{\cdot}20 \pm 0{\cdot}10$ kDa and the average PDI was $1{\cdot}28 \pm 0{\cdot}04$. As showed in Fig. 4, a significant negative correlation was found between M_w and the relative amounts of galactose ($r = -0{\cdot}56$, $P = 0{\cdot}001$) and 3-O-Me-galactose ($r = -0{\cdot}58$, $P = 0{\cdot}001$). The relationship was more marked at 250 h of cultivation (about $r = -0{\cdot}82$ for both monomers, $P < 1 \times 10^{-4}$). It suggested an association between galactan and low molecular weight polymers. On the other hand, a positive correlation between M_w and arabinose and rhamnose was found ($r = 0{\cdot}53$ and $r = 0{\cdot}52$, respectively, $P = 0{\cdot}003$) and between M_w and press cake dosage ($r = 0{\cdot}73$, $P < 1 \times 10^{-4}$) (Fig. 4). For the latter relationship, experimental data suggested an asymptotic exponential trend, as reported in Equations 5 and 6:

$$M_{w(200h)} = 4348 \cdot 14 - 275 \cdot 05 \times 0 \cdot 91^{[SB]} \quad R^2 = 0 \cdot 566, \quad (5)$$

$$M_{w(250h)} = 4292 \cdot 17 - 212 \cdot 22 \times 0 \cdot 84^{[SB]} \quad R^2 = 0 \cdot 648, \quad (6)$$

where $M_{w(200h)}$ and $M_{w(200h)}$ represent the M_w (Da) of the EPS population and [SB] the dosage of sea buckthorn press cake (g l^{-1}) in the medium. In the comparison among the different cultivation conditions, cultivation time had no clear influence on M_w. Finally, our results showed no clear trend in the PDI of the analysed EPS.

Discussion

Effect of sea buckthorn addition and incubation time on mycelium yield

The use of sea buckthorn press cake as supplement for the submerged cultivation of the mycelium of *I. obliquus*

Table 2 Exopolysaccharides (EPS) yield, sugar content, exo-polysaccharide production, weight-average molecular mass (M_w) and polydispersity index (PDI) of the isolated EPS fractions

Sea buckthorn (g l^{-1})	Cultivation time (h)	EPS concentration* (g l^{-1} ± SD‡)	Polysaccharide content† (w/w % ± SD‡)	Production of polysaccharide (mg g^{-1} mycelium)	M_w (kDa ± SD‡)	PDI (M_w/M_n ± SD‡)
0	200	0·63 ± 0·07[a]	5·47 ± 3·09[a]	9·99	4·06 ± 0·03[a,b]	1·28 ± 0·02[a,b]
0	250	0·55 ± 0·19[a]	7·40 ± 5·38[a]	9·65	4·07 ± 0·10[a,b]	1·37 ± 0·01[a,b]
2·5	200	0·46 ± 0·37[a]	12·37 ± 4·51[a,b]	11·69	4·15 ± 0·10[a,b]	1·30 ± 0·06[a,b]
2·5	250	0·33 ± 0·21[a]	8·47 ± 2·49[a,b]	3·78	4·19 ± 0·06[a]	1·25 ± 0·00[a]
5	200	0·33 ± 0·07[a]	14·97 ± 2·06[a,b]	8·36	4·20 ± 0·01[a]	1·31 ± 0·00[b]
5	250	0·40 ± 0·00[a]	11·39 ± 1·00[a,b]	5·87	4·19 ± 0·05[a,b]	1·25 ± 0·00[a]
10	200	0·67 ± 0·28[a]	10·27 ± 3·18[a,b]	14·52	4·22 ± 0·10[a]	1·32 ± 0·00[b]
10	250	0·60 ± 0·10[a]	12·72 ± 1·82[a,b]	9·77	4·24 ± 0·01[b]	1·25 ± 0·00[a]
30	200	1·10 ± 0·67[a,b]	17·08 ± 4·74[b]	37·70	4·34 ± 0·10[b,c]	1·26 ± 0·02[a,b]
30	250	2·04 ± 0·85[b]	12·30 ± 1·32[a,b]	59·60	4·30 ± 0·03[c]	1·25 ± 0·01[a]

Different letters mark significant difference ($p < 0·05$).
*Concentration of EPS in culture medium measured gravimetrically.
†Measured as total sugar content in the EPS fractions.
‡Average of cultivations in triplicate.

is here reported for the first time. The increase in the cultivation time granted a significant increase in yield, while the increase in supplement dosage granted yields statistically equal to each other. On the other hand, the highest amount of press cake (30 g l^{-1}) had no effect on the cultivation yield. At this dosage, the supplement might have formed physical barrier limiting the mycelium from access to oxygen. A difference in particle size of the supplemented material could explain the contrast with the results obtained by Xu (Xu *et al.* 2014a), where the addition of 30 g l^{-1} of wheat straw to the medium resulted in a significant increases in cultivation yield at 216 and 240 h (about 15% and 10% respectively) of *I. obliquus* mycelium.

The supplementation of sea buckthorn press cake provided, at the tested experimental conditions, greater yield enhancements than those obtained with the supplementation of fatty acids or oils. Xu (Xu *et al.* 2015) reported a yield increase for *I. obliquus* mycelium of 27% and approximately 15% after the addition (0·1% v/v) of stearic and oleic acid respectively. Yang (Yang *et al.* 2000) reported, for *Ganoderma lucidum*, an increase in cultivation yield of 62% and 52% after the supplementation (1%) of olive and corn oil respectively. Huang (Huang *et al.* 2009) reported a yield increase of about 100% after a 2% supplementation of corn oil to *G. lucidum* cultivation medium, which is close to the increase observed in the current study with sea buckthorn press cake. The fatty acid composition of sea buckthorn press cake, dominated by palmitic and palmitoleic acids (Damerau *et al.* 2020), was different from all the aforementioned supplements. The absence of clear correlation between fatty acid chain length of the supplement material and cultivation yield of mycelia has already been reported (Berovic and Podgornik, 2016).

Fibrous biomass and lipids used separately have been proven to act as efficient growth enhancers (Huang *et al.* 2009; Wolters *et al.* 2016). Therefore, their simultaneous presence in the sea buckthorn press cake could explain the obtained results. Previously, it was reported (Krupodorova and Barshteyn, 2015) that supplementations of press cakes of oilseeds, such as camelina or rapeseed, failed to enhance the mycelium growth of *I. obliquus*. Similar finding was observed in the cultivation of other white-rot basidiomycetes, such as *Trametes versicolor* (Krupodorova and Barshteyn, 2015). Moreover, the supplementation of olive mill wastewater (containing sugars, free fatty acids, phenolic compounds and lipids) to mycelium liquid cultivation medium of *G. lucidum* granted a yield increase of 72%, while failed to enhance the yield of multiple *Pleurotus* strains (Ntougias *et al.* 2013; Zerva *et al.* 2017). On the other hand, the effects of the supplementation of pectin to the medium of mycelium submerged cultivation have received little attention. Also, the pectinolytic activity of *I. obliquus* has never been subject of investigation. However, strong pectinase activity has been measured from the culture of *Inonotus rickii* (Xavier-Santos *et al.* 2004). Moreover, Kruporodova and Barshteyn incremented the cultivation yield of *I. obliquus* by 55% (Krupodorova and Barshteyn, 2015) with the supplementation of rose hip fruit, which contains about 1% w/w pectin (Ognyanov *et al.* 2016). Hence, the pectin present in sea buckthorn press cake could have been used as carbon source by *I. obliquus* and stimulated its

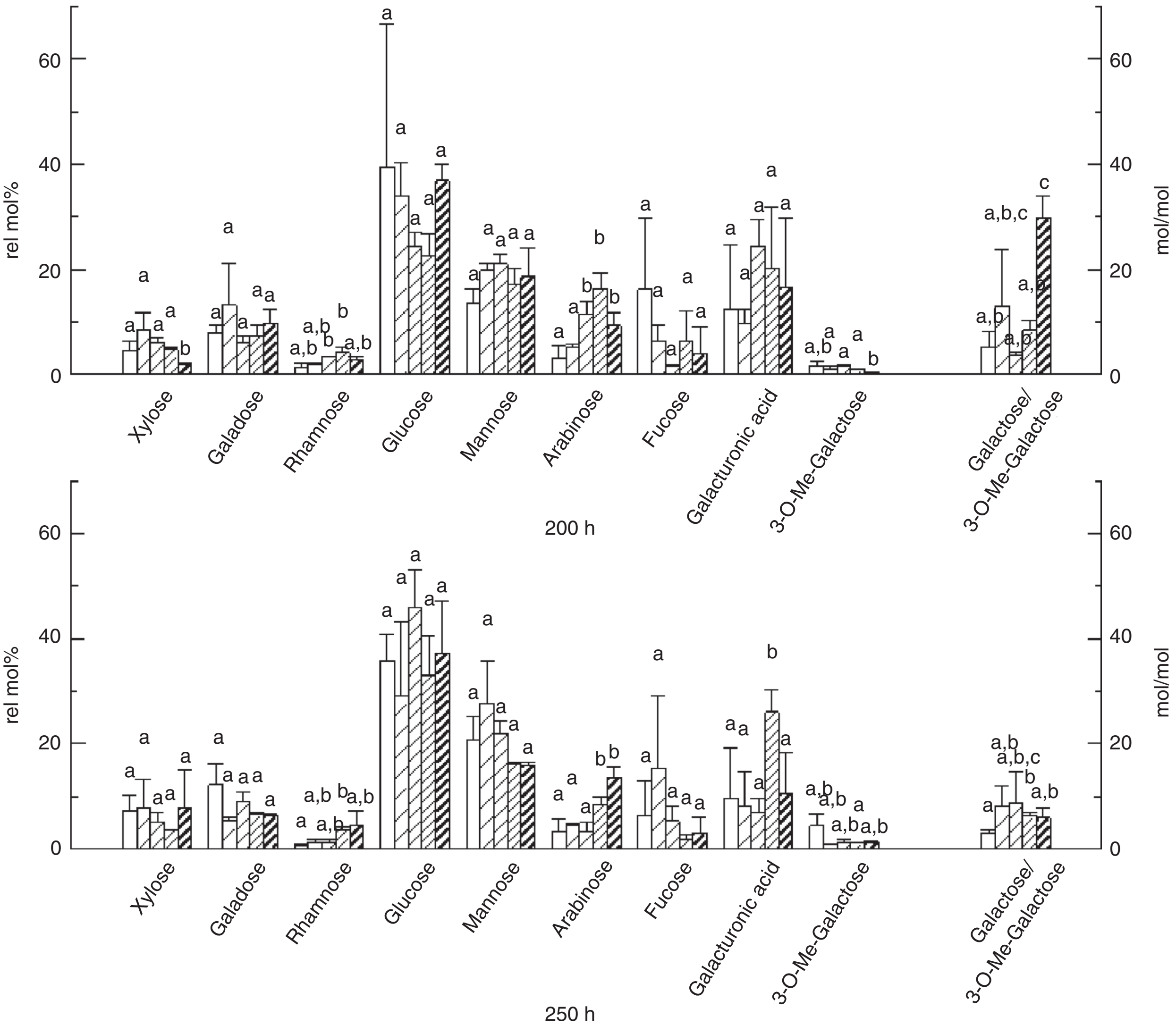

Figure 3 Monomer composition, expressed as relative molar percentage and molar ratio, of the exopolysaccharides (EPS) fractions isolated from the cultivation medium of *Inonotus obliquus*. Increase in pattern density indicates increase in supplement dosage (0, 2·5, 5, 10 and 30 g l^{-1}). Results are grouped by monomers and separated by the cultivation time. Different letters mark significant difference ($p < 0{\cdot}05$).

mycelial growth. However, further studies are required to verify the pectinolytic activity and the effect of supplementation of pectins on the submerged cultivation yield of *I. obliquus*. In addition, some phenolic compounds, while toxic for most fungal species, are known to be detoxified by white-rot fungi and act as mild growth stimulants (Mäkelä *et al.* 2015). Sea buckthorn press cake is rich in phenolic compounds, mainly flavonols and phenolic acids (Damerau *et al.* 2020), which might have contributed to the growth promoting effects of the sea buckthorn press cake at 5 and 10 g l^{-1}. At higher dosages such as 30 g l^{-1}, the antimicrobial activities of phenolic compounds in sea buckthorn might have dominated over the growth stimulating effect.

Monomer composition of cultivated mycelium of *I. obliquus*

The results of the hydrolysis of the *I. obliquus* mycelium in the present study were in partial agreement with the monomer composition of cultivated mycelium of *Pleurotus pulmonarius* (Smiderle *et al.* 2012). While the latter had mannose as second most abundant monomer, our *I. obliquus* hydrolysates showed a higher amount of

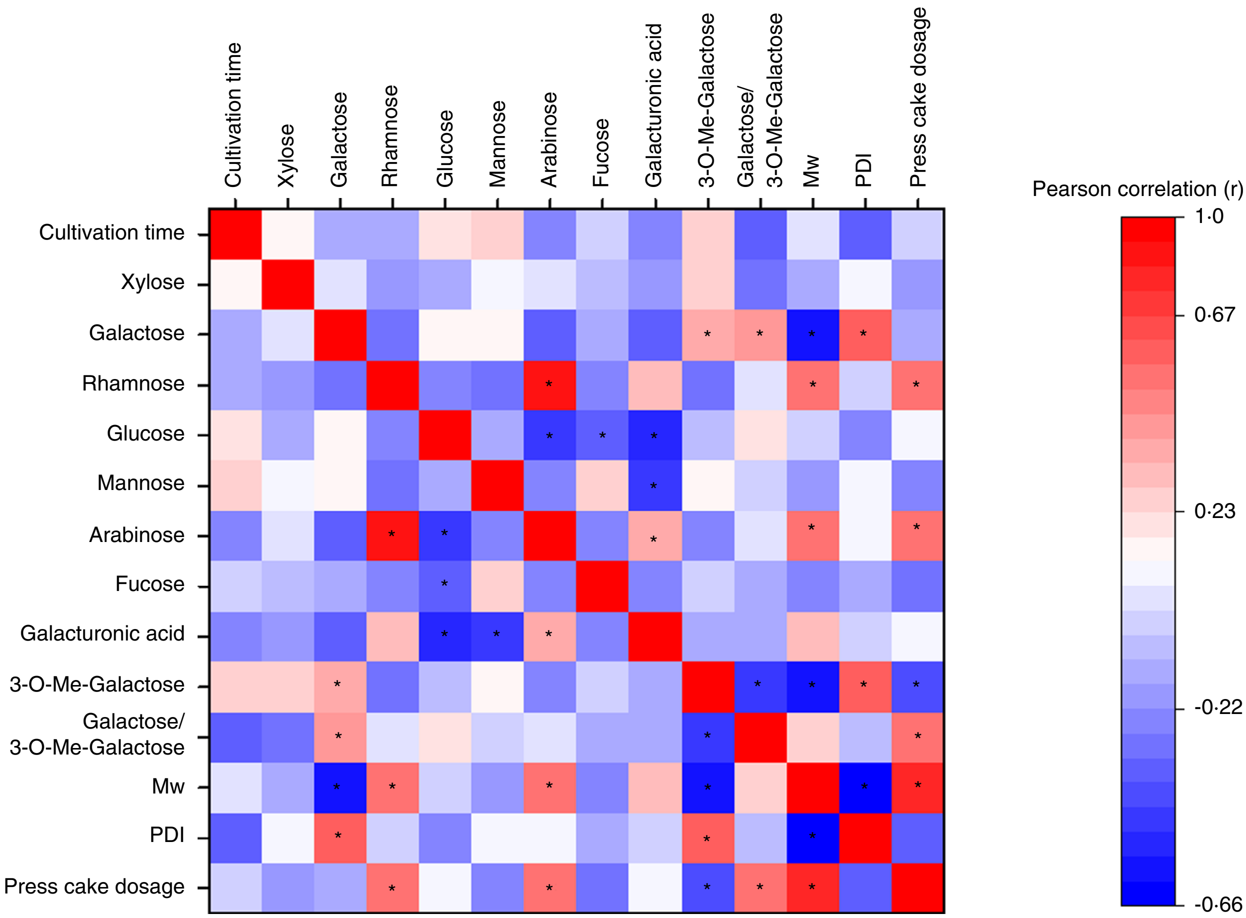

Figure 4 Heatmap of Pearson correlation coefficients (*r*) of exopolysaccharides (EPS) monomers, Mw, PDI, and cultivation conditions. Symbol marks statistical significance ($P < 0{\cdot}05$).

galactose. These two monomers constitute polysaccharides which have already been reported as mycelium cell wall components (Ruthes *et al.* 2016). In the study on *P. pulmonarius*, the carbon source had a significant effect on the relative amount of glucose. Conversely, and in agreement with our study, supplementation had no effect on the mycelial glucose of *G. lucidum* measured after hydrolysis (Zerva *et al.* 2017). However, this study reported biomass content of glucose of 5%, while the w/w content of glucose of *I. obliquus* was in the range 21-49%. This difference could be attributed to the species examined or to the difference in hydrolysis method. The strong correlations of arabinose, rhamnose and galacturonic acid with the press cake dosage suggested a partial retention of pectin by the mycelium. Interestingly, the increment of arabinose was more pronounced than galacturonic acid, despite the higher amount of the latter in the medium. The results of Xu and coworkers (Xu *et al.* 2014a, 2019) show a correlation between amount of arabinose in *I. obliquus* polysaccharides and arabinose-containing supplement, further indicating the increase in arabinose was due to press cake pectin.

Polysaccharides extracted from *I. obliquus* mycelium

Our analysis of the polysaccharides extracted from the cultivated mycelium of *I. obliquus* have shown a decrease in the sugar content of the fractions obtained after supplementation. The negative trend in mycelial polysaccharide production is in agreement with Xu (Xu *et al.* 2015), who has reported a negative effect on the extraction yield of IPS from *I. obliquus* after the supplementation of fatty acids. Moreover, the supplementation of different lignocellulose materials to *I. obliquus* had little or negative effects on the IPS production of the mycelium (Xu *et al.* 2014a). However, supplementation of plant oils had positive effect on the IPS production of *Antrodia cinnamomea* (Shih *et al.* 2006). Besides species and strains, differences in mycelial IPS content at the variation in concentration of nutrients and medium components have been attributed to changes in mycelium metabolism and morphology (Cui *et al.* 2016; Tao *et al.* 2018). The prevalence of glucose on the other monomers of the extracted IPS was in agreement with previous results on *I. obliquus*, which also have shown scant influence of lignocellulose supplementation on the monomer composition (Xu *et al.* 2014a). The M_p of the polysaccharides extracted from *I. obliquus* mycelium was similar to molecular weights of IPS obtained from the same mycelium, although with different population abundances (Xu *et al.* 2014b). Basing on the increase in the relative amount of mannose, the $1{\cdot}4 \times 10^3$ kDa population of IPSsb5-10 2% could be attributed to the heterosaccharides that are present in the lower layers of the fungal cell wall (Latgé, 2007; Bernabé *et al.* 2011). The population of 65 kDa of IPSsb5-10 HW was absent from IPSsb0 HW and from previously reported results. The differences might be attributed to the presence of the press cake. Oil supplementation has been proven already to influence the expression of mycelial polysaccharide biosynthesis enzymes (Reverberi *et al.* 2004).

Content, monomer composition and macromolecular properties of EPS

Our results have shown the influence of supplementation on the production of EPS isolated from *I. obliquus* cultivation medium. The EPS concentration in the medium and the production of polysaccharide were quadratically influenced by the dosage of sea buckthorn press cake. This observation could be explained by the findings of Huang and colleagues (Huang *et al.* 2009), who reported a quadratic relationship between mycelial EPS concentration in the medium and carbon source consumption. Interestingly, the reported regression equation for relationship between EPS yield and oil consumption differed from Equation 2 mainly in the linear coefficient. The monomer composition analysis has shown absence of correlation between the galacturonic acid content and the amount of supplement. The presence of galacturonic acid in EPS even in absence of press cake indicated that this monomer was present in the polysaccharides secreted into the medium by *I. obliquus* mycelium. Polysaccharides containing galacturonic acid have been already isolated from the submerged cultivation medium of Basidiomycetes (Li *et al.* 2019). While the observed weak positive correlation between galacturonic acid and arabinose ($r = 0{\cdot}38$, $p = 0{\cdot}036$) could hint to the retention of pectin in the EPS, the ratio between the two monomers resulted unaffected by the concentration of press cake ($r = -0{\cdot}29$, $p = 0{\cdot}121$) and was, however, lower than the ratio observed after methanolysis of the supplement (Fig. S1). This evidence suggested that *I. obliquus* is able to depolymerize pectin and use galacturonic acid as carbon source, which was as well suggested by the results of the mycelium methanolysis. Differences in carbon source dosages have as well resulted in little differences in EPS monomer composition for *G. lucidum* (Fraga *et al.* 2014), while differences in lignocellulose supplement dosage had significant effects on the monomer composition of EPS isolated from *I. obliquus* (Xu *et al.* 2014a). Polymers with M_w of the same magnitude of the EPS main population have been already isolated from the cultivation medium of some Basidiomycetes, although they are more common for Ascomycetes (Osińska-Jaroszuk *et al.* 2015). Our results showed a statistically significant influence of the sea buckthorn press cake dosage on the M_w of the EPS and the results fitted an asymptotic exponential trend. A similar trend was found by Shu and Lung, relating log M_n of fungal EPS and culture pH (Shu and Lung, 2004). However, due to the high complexity of the supplement, further experiments would be required to verify whether pH of the medium alone was responsible of the observed trend.

In conclusion, the present work showed that the mycelium yield of the submerged cultivation of *I. obliquus* can be significantly increased with the supplementation of sea buckthorn press cake. Methanolysis of the mycelium highlighted little retention of pectin after cultivation. The amount of press cake quadratically influenced the production of EPS in the cultivation medium but reduced the production of IPS extractable from the mycelium. While the relative amounts of only few EPS monomer were influenced by the supplement dosage, the molecular weight of EPS resulted exponentially increased. IPS, on the other hand, showed little amount of berry pectin but their molecular weight resulted affected by the press cake.

Acknowledgements

The present work was supported by Business Finland (formerly Tekes – the Finnish Innovation Funding Agency), Niemi Foundation, Turku University Foundation, Magnus Ehrnrooth Foundation and by the Graduate School of the University of Turku. The technical help of Andrew Pranada (Institut Pertanian Bogor University) and Kirushaanki Shadakopan (University of Turku) is acknowledged.

Author contributions

G.B., Z.H. and B.Y. designed the research. G.B., J.H. and H.Y. performed the experiments. G.B. analysed the data and wrote the manuscript. All the present authors have read and approved the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Monomer composition, expressed as relative molar %, of sea buckthorn press cake.

Figure S2 HPSEC-RID chromatograms of EPS fractions obtained from cultivation media of *I. obliquus*.

Figure S3 HPSEC-RID chromatograms of IPS fractions obtained from the mycelium of *I. obliquus*.

Table S1 Spearman correlation (ρ) values of mycelium monomers and cultivation conditions.

Table S2 Summary of Person correlation values between variables of EPS obtained after 200 and 250 h of cultivation.