Valorization of deinking sludge as a substrate for lignocellulolytic enzymes production by *Pleurotus ostreatus*

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Abstract

Disposal of waste sludges produced in large amounts in the pulp and paper industry imposes significant environmental and economical problems. One strategy to address these issues involves revalorization of paper mill sludges by their application as substrates for microbial production of biotechnologically relevant enzymes. The application of lignocellulolytic enzymes in paper, textile and bioenergy industries is encouraged in order to decrease chemicals and energy consumptions. In the following work, deinking sludge was assessed as a substrate for production of lignocellulases. Based on the results of growth and activity screenings, *Pleurotus ostreatus* PLAB was chosen as the most promising candidate among 30 tested strains and its secretome was further studied by quantitative enzyme assays and mass spectrometry. While endoglucanase and xylanase activities detected in *P. ostreatus* secretome produced on deinking sludge were similar to activities of cultures grown on other lignocellulosic substrates, average laccase activity was significantly higher (46 000 U / kg $_{DIS}$). Mass spectrometry identification of the most prominent proteins in the secretome of the target strain confirmed that significant amounts of different lignin-modifying oxidases were produced on this substrate despite its low lignin content, indicating the presence of other inducible compounds. The findings of this study suggest deinking sludge may represent a good substrate for fungal production of the aforementioned enzymes with broad biotechnological applications, including bioremediation, paper and bioenergy industries.

Key words: paper mill waste / deinking sludge / fungi / lignocellulolytic enzymes / solid-state fermentation

Abbreviations: DIS – deinking sludge, SF – submerged fermentation, SSF – solid state fermentation, TOC – total organic carbon, TON – total organic nitrogen, WRF – white rot fungi, PDA – potato dextrose agar, SEEs – secreted enzyme extracts, CMC – carboxymethyl cellulose, BX – birchwood xylan, EG – endoglucanase, EX – endoxylanase, ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), PDA – potato dextrose agar, SDS PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis, TCA – trichloroacetic acid, LC MS/MS – liquid chromatography coupled to tandem mass spectrometry

1. Introduction

Eleven million tonnes of waste are produced yearly by the European pulp and paper industry (Monte et al., 2009). Approximately 70% of these originate from manufacturing tissue paper from recovered fibre, leading to the generation of considerable amounts of deinking sludge (150 kg dry solids/t paper manufactured), which must be properly managed to avoid negative effects on the environment (Deviatkin et al., 2016). Deinking sludge (DIS) exists as a mixture of short cellulosic fibers and inorganic fillers, such as calcium carbonate and china clay, and residual chemicals dissolved in water (Likon and Saarela, 2012). DIS originating from printed recycle mills is high in ash content compared to sludge originating from corrugated recycle mills and virgin pulp mills (Boshoff et al., 2016).

Traditional methods of DIS management include landfilling, landspreading, composting, incineration and pyrolysis, utilisation as construction material and landfill capping material (Likon and Trebše, 2012). However, due to high moisture content some of these recovery methods, such as incineration and pyrolysis, are expensive for large amounts of sludge while the environmental impact of others is questionable due to the possibility of hazardous substances leaking into the environment. As a result, numerous possibilities for biological valorisation of paper sludge waste, including its fermentation and anaerobic digestion are currently being explored (Gottomukkala et al., 2016). These aim for efficient microbial transformation of cellulose waste into bioethanol (Boshoff et al., 2016), biomethane (Mohan et al., 2016a), biohydrogen or other value added chemicals (Liguori and Faraco, 2016).

The main advantage of exploiting paper sludges as sources of cellulose-derived energy and chemicals in comparison to other lignocellulose substrates is their amenability, which is associated with an extensive pulping process that removes the majority of the lignin and exposes cellulose fibers to enzymes. This results in substantial cost savings on energy for substrate pretreatment in comparison to other lignocellulose fuel production technologies

(Gottumukkala et al., 2016). Studies on direct production of bioethanol from paper sludge have shown that tissue printed recycle sludge (a type of DIS) resulted in significantly lower ethanol yields when compared to corrugated recycle and virgin pulp mill sludges (Williams 2017), so other possible ways for valorization of this type of substrate need to be explored.

One of the major limiting factors for bioconversion of cellulosic waste such as paper sludge to valuable products is the cost of the enzymes, as they are commercially produced using high cost feedstocks. Reducing the enzyme dosage per gram cellulose/feedstock has been a major research area for the past few years (Robus et al., 2016). Efficient transformation of cellulosic feedstock to value added products requires a mix of synergistically acting enzymes (CAZYmes) that are able to work at low dosages. White-rot fungi (WRF) are known to produce significant amounts of powerful extracellular oxidative and hydrolytic enzymes that degrade lignin and cellulose biopolymers (Manavalan et al., 2015). Major functional groups of glycoside hydrolases (cellulases and hemicellulases) produced by WRF involve endoglucanases (EG; EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91, EC 3.2.1.176), beta-glucosidases (3.2.1.21), endoxylanases (EX; EC 3.2.1.8), beta-xylosidases (EC 3.2.1.37) and alpha-glucuronidases (E.C. 3.2.1.131). On the other hand, lignin degradation enzyme systems are based on oxidative enzymes, such as lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), versatile peroxidases (EC1.11.1.16), and laccases (EC 1.10.3.2) (Manavalan et al., 2015). Recently, oxidative enzymes, namely lytic polysaccharide monooxygenases, have also been shown to play an important role in the degradation of cellulose (Garajova et al., 2016). The composition of the enzyme mixtures produced on different substrates (ratios between different types of enzymes) reflects substrate composition (Elisashvili et al., 2008).

The applications of fungal enzymes in paper industry involve biobleaching of pulp, pulp de-inking, degradation of dissolved and suspended organic compounds in concentrated effluents

of mills and enhanced fibrillation. Enzymes usage is encouraged in paper industry in order to reduce the use of chemicals and energy consumption as well as to improve quality of the products (Bajpai, 2014). A desirable side-effect of processing paper mill waste by WRF enzymes is non-specific degradation of organic environmental pollutants, commonly present in these substrates (Kűes, 2015).

Most industrial enzymes currently produced by fungi are products of submerged fermentation (SF) which uses significant amounts of water and generates large quantities of liquid waste stream during the filtration process. Major cost contributor in commercial cellulase production is cost of the feedstock which is mostly glucose and accounts for 50% of the total process cost (Humbird et al., 2011). However, solid state fermentation (SSF) has emerged as an economically attractive alternative for the *in situ* production of lignocellulolytic enzymes due to lower energy consumption, direct use of low-cost lignocellulosic wastes as substrates, reduction in cost of dewatering in downstream processing, higher concentrations of enzymes and lower demand for the sterility of the equipments (Yoon et al., 2014). At present, the production of lignocellulolytic enzymes by SSF on several agricultural wastes has been reported, such as coffee pulp (Velazquez-Cedeno et al., 2002), spend brewery grains (Gregori et al., 2008), straw and fruit peels (Kurt and Buyukalaca, 2010), rice straw (Khalil et al., 2011) and tomato pomace (Iandolo and Piscitelli, 2011). Conversely, there are few reports on enzyme production from paper sludge and no report specifically on fungal enzymes production from deinking paper sludge.

The aim of the following work was to untap the potential of DIS as a low-cost substrate for fungal production of lignocellulolytic enzymes via SSF. Fungal strains with the ability to transform the unpretreated DIS to a mixture of industrially relevant lignocellulases were identified. Furthermore, the composition, production dynamics and performance of the extracted enzymes were studied in detail in the most promising of the tested strains (*P.*

ostreatus PLAB). The advantages of lignocellulolytic enzymes production on DIS and their applications in different industries is also discussed from a perspective of cleaner production.

2. Materials and methods

2.1. Substrate characterization

DIS was obtained from a paper mill in Vipap, Krško, Slovenia. After the substrate was sieved through a mesh with 0.9 cm openings, its pH and moisture content were measured (Kern MLS-D). For the determination of cellulose, hemicellulose and lignin content of the substrate, 10 g of air-dried DIS was first mixed with 200 ml of 6 M HCl and than incubated for 1h to dissolve the inorganic fraction. Following filtration and drying, the cellulose content of DIS was determined according to the method described in Kurschner and Hoffer (1993), hemicellulose content as described in Wise and Karl (1962) and lignin content according to the method as described in Fukushima et al., 2015. Dry content was measured according to European standard SIST EN 14346:2007, total organic carbon (TOC) according to standard SIST EN 13137:2002, total organic nitrogen (TON) according to persulfate digestion method 10071 (Test'N Tube, Hach Lange). Heavy metals were determined according to the following standards: DIN 38406-E7-2:1991 (Cu), SIST ISO 8288:1996 (Zn), DIN 38406-E11-2:1991 (Ni), SIST EN ISO 5961:1996 (Cd), SIST ISO 5666:2000 (Hg), SIST ISO 9174:1999 (Cr), DIN 38406-E6-2:1998 (Pb).

2.2. Screening of fungal strains based on growth efficiency on DIS

Thirty fungal strains of different origin obtained from culture collection Mycomedica d.o.o. (Suppl. 1) were first screened for their growth on agar plates incorporating the target substrate (DIS agar). The strains were stored as slant test tubes on Potato Dextrose Agar (PDA, Difco, USA) at 3 °C. Before each experiment, mycelium from each fungal strain was transferred to PDA agar and incubated in the darkness at 23 °C until overgrowing the entire surface. A 9 mm diameter disc was cut from the resultant mycelium and used to inoculate a DIS agar plate. DIS agar, prepared by mixing bacteriological agar (Sigma), deinking sludge and distilled water in ratio 1:10:100, was autoclaved for 20 min at 121 °C, 1.1 bar. Additional sets of media were prepared similarly except without autoclaving in order to test the growth on non-pretreated substrate (energetically favourable option). All strains were inoculated in triplicates and their growth was observed for 50 days and classified in different categories according to its efficiency. Ten strains with the highest average growth rate on DIS agar were chosen for further enzyme activity screenings.

2.3. Enzyme activity screening

Based on the highest growth rates achieved on DIS agar, 10 strains were selected for additional semi-quantitative screening for endoglucanase, xylanase and laccase activities. Secreted enzyme extracts (SEEs) were prepared by first homogenizing the overgrown DIS agar by Ultraturax disperser (Ika) at 1000 rpm for 5 min. Aliquotes (20 ml) of homogenate were mixed with equal amounts of 0.5 M Na-phosphate buffer pH 7 and shaken on ice at 150 rpm for 3 h. Following centrifugation at 18500 x g, the clear supernatants (representing SEEs) were transferred to sterile Falcon tube and stored at -20 °C until further use. Aliquots of 10 µL of SEEs from different fungal strains were applied in the hollow areas of equal size (0.5 mm diameter) wells cut into 0.6% (w/v) agarose plates incorporating target substrates. The following substrates were used: 0.2% (w/v) carboxymethyl cellulose (CMC) when screening

for endoglucanase activity, 0.4% (w/v) birchwood xylan (BX), when screening for xylanase activity and 0.4% (v/v) guaiacol when screening for laccase activity. SEEs denatured by heating at 100 °C for 15 min were used as negative controls. The plates were incubated at 30 °C for 4 h. Guaiacol plates were assessed directly by measuring radius of brown areas that developed around wells with applied SEEs due to laccase activity. Plates incorporating CMC and BX were stained by Kongo red for 30 min and subsequently washed by 1 M NaCl (Teather and Wood, 1982). The radii of clear zones indicating substrate degradation were measured and compared in order to assay xylanase and endoglucanase activities in SEEs from different fungal strains. Based on growth and enzyme screening, *P. ostraetus* PLAB was selected for further investigation.

2.4. Enzymes production and extraction from deinking paper sludge (DIS)

P. ostreatus PLAB mycelia were first inoculated to PDA media in petri dishes as described in the section 2.2. After suspending in distilled water, 5 ml of mycelium was used to inoculate 200 g of each sterilized DIS medium (prepared by sieving DIS trough the mesh and adjusting its moisture content to 65% (w/w) in 1 L glass jars. Uninoculated DIS medium was used as a negative control. Jars were incubated at 23 °C until mycelial growth was observed. For enzyme activity quantification, cultures were incubated for 51 days and collected in 7 day intervals (first sampling took place after mycelium started to grow in all of the samples). Four cultures were collected at each time point and frozen for further analysis. Following homogenization, selected buffers were added to each homogenate at the ratio 1:1. Based on literature and preliminary experiments, 0.5 M acetate buffer pH 4 was used for laccase, 0.5 M acetate buffer pH 5 for endoglucanase (EG) and 0.5 M citrate buffer pH 6 for xylanase extraction. Extraction was performed by incubating the samples on ice for 3 h with intermittent mixing by Vortex (every 30 min for 30 s).

Following centrifugation at 18500 x g, clear supernatant (representing SEEs) was separated, aliquoted and stored at -20 °C until further processing.

2.5. Spectrophotometric assays for quantitative determination of total protein, endoglucanase and xylanase activities in SEEs

Cellulase (endoglucanase) and xylanase activities in SEEs were measured via quantitative determination of released reducing sugars by para-hydroxybenzoic acid (PAHBAH) assay (Lever, 1977). Suspensions of 1% beechwood xylan (BX) in 0.5 M citrate buffer pH 6 (for xylanase activity) or 1% carboxymethyl cellulose (CMC) in 0.5 M acetate buffer pH 5 (endoglucanase activity) were used as substrates. Twenty µl of enzyme extracts were added to 230 µl of substrate suspensions and incubated in microcentrifuge tubes at 30 °C for 150 min. Reactions were stopped by adding 30 µl of 15% trichloroacetic acid (TCA). In control samples, enzyme extracts were added after the incubation, just before enzyme inactivation. 100 µl of each incubated sample was added to 5 ml of reaction mixture in test tubes and heated in boiling water for 10 min. Absorbance of the mixture was measured at 420 nm. Total protein concentrations in SEEs were quantified by Lowry assay, using bovine serum albumin (BSA) as a standard (Lowry et al., 1951). Two technical replicates were performed for each sample in order to assess method variability.

2.6. Spectrophotometric assay for quantitative determination of laccase activity in SEEs

A modified assay described in Childs and Bardsley (1975) was used to measure laccase activities in SEEs. Reaction mixtures (prepared in duplicate) consisted of 100 µl 5 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Calbiochem), 895 µl 0.1 M acetate buffer pH 4.5 and 5 µl enzyme extract. Cooled ABTS was added to the sample after heating reaction mixture to 30 °C and absorbance was measured at 420 nm continuously.

Enzyme extracts previously inactivated by heating at 100 °C for 20 min were used as negative controls.

2.7. SDS-PAGE and mass spectrometry

Four 30-days old cultures were homogenized and SEEs were prepared by extraction of proteins from 20 g of each homogenate in equal amounts of phosphate buffer (pH 8) with the addition of protease inhibitors (cOmplete ULTRA Tablets, Sigma-Aldrich) and freeze-dried for transport at ambient temperature. Before the analysis, the samples were dissolved in 750 µl deionized water and precipitated with the same volume of 20% TCA at 4 °C for 1 h. The precipitate was collected by centrifugation (16400 x g, 10 min, 4 °C), subsequently washed twice with 2.25 ml of acetone (15 min, -20 °C) and 2.25 ml of ethanol (15 min, -20 °C). After air-drying the final precipitates were dissolved in 200 µl of urea buffer. The protein concentration determination was carried out with Amidoblack (Schweikl et al., 1989) using BSA as standard. SDS-PAGE was performed according to Laemmli (1970). After separation, the bands were cut from the gel and digested with trypsin as described previously by Heyer et al. (2013). The peptides were extracted, dried and resuspended in 11 µL of chromatographic mobile phase A (98% LC-MS water, 2% acetonitrile, 0.05% trifluoroacetic acid). Five µL of sample were injected and separated by UltiMate® 3000 nano splitted reversed phase nano HPLC (Thermo Fisher Scientific, Dreieich, Germany) equipped with a reversed phase trap column (nano trap cartridge, 300 µm i.d. x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100 Å, nanoViper, Bremen, Germany) and a reversed phase separation column (Acclaim PepMap RSLC, C18, 2 µm, 75 µm, 15 cm, Bremen, Germany).
The gradient was 4% to 55% mobile phase B (80% acetonitrile, 20% LC-MS water, 0.1% formic acid) over 120 min at a flow rate of 0.3 µL min^{-1}. The amaZon^{TM} ETD Ion Trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was equipped with a Captive

Spray (using acetonitrile) nanoBooster™ Module and operated in positive ion mode. MS/MS data acquisition was performed using collision induced dissociation (CID) with 5 precursor ions per measurement and an active exclusion of the same precursor after 2 spectra for 9 seconds. DataAnalysis 2.0 (Bruker Daltonik) and ProteinScape 3.1.3 461 software (Bruker Daltonik) were used for automatic deconvolution and analysis as well as database search using Mascot™ 2.5 (Matrix Science, London, UK) in NCBINr database (version 2014/01/22). One missed cleavage was allowed. Other parameters were chosen as follows: monoisotopic mass, variable carbamidomethyl (C) and oxidation (M) modification, 0.3 Da precursor and MS/MS tolerance, one ^{13}C and peptide charge of +2/+3.

2.8. Zymograms

Separated active glycoside hydrolases in SEEs were detected by modified SDS-PAGE (Laemmli, 1970). Separation gels (10%) were modified by adding 0.2% (w/v) CMC (for detection of endoglucanases) or 0.4% (w/v) water soluble fraction of 0.4% (w/v) BX (for detection of xylanases). Enzyme extracts were mixed with equal amounts of loading buffer, heated for 6 min at 85 °C and applied on 4% (w/v) stacking gel. For detection of enzymes with laccase activity, native samples were applied on the gels. Glycoside hydrolases were renaturated and stained as described previously (Vodovnik and Marinšek Logar, 2012). Separated enzymes exhibiting laccase activity were detected by applying ABTS solution (Calbiochem, 194430) directly over the gel after its equilibration in 0.1 acetate buffer pH 4 as described by Castanera et al. (2012). The properties of the detected enzymes were compared to the information on putative *P. ostreatus* enzymes available in the NCBI protein (Wheeler et al., 2002) and BRENDA databases (Schomburg et al., 2004). Where absent, putative conserved domains were detected by Interproscan (Quevillon et al., 2005) while other

properties of interest (Mw and pI) were calculated using ProtParam tool (Gasteiger et al., 2005).

2.9. Data processing and statistical analysis

Results are presented as the mean ± standard deviation (SD) of at least 3 replicates. The data presentation and analysis were carried out using R version 3.1.3 and Microsoft Excell 2013. Statistically significant differences in enzyme activities between different fungi were evaluated by one-way ANOVA, while growth-point-related activities analysis was done by repeated measures ANOVA (paired samples Student's t-test). A significant difference was considered if $p<0.05$ applying Tukey multiple-comparisons HSD.

3. Results and discussion

3.1. Substrate characterization

Commercial cellulases are produced using recombinant fungal strains to yield enzyme titres of close to 100 g/L with superior activity. Inducible carbon sources such as purified cellulose, lactose and sophorose are used for high-level cellulase production. Feedstock cost is an important consideration for commericail enzyme production as this contributes more than 50% of the total cost of the final enzyme (Ellila et al, 2017). Other than cost factor, there are also environmental concerns that impact commercial enzyme production as bioprocesses using genetically modified organisms, excessive amounts of nutrients used for process intensification and use of large quantities of fresh water can adversely affect regulatory costs and carbon emissions (Junker, 2010). Utilisation of waste streams rich in cellulose such as paper sludge as feedstocks for enzyme production will not only reduce the feedstock cost for enzyme production but also support cleaner production by reducing the amounts of waste

disposal in comparison to high amounts of nutrient rich liquid and solid waste generated by the bioprocess industry that mainly uses pure substrate and nutrient source.

Deinking paper mill sludge, a major waste stream from the pulp and paper industry, was selected for this study, as it is mainly composed of short cellulose fibers. It also includes fines, inks and other additives that can influence the growth of the microoganisms and enzyme production. It has been reported that even among mills using similar processes and feedstocks, the sludge composition may vary due to different operating conditions (Williams, 2017). So DIS used in our study was characterized for its chemical composition.

DIS used in our study has a moisture content of 39% and cellulose content of 34.2%. Hemicellulose and lignin contents are 6.2% and 3.7% respectively, which were lower than stated in previous reports (Gottomukkala et al., 2016). The remaining is mostly ash and heavy metals (Table 1). Cellulose is the major carbon source contributor for fungal growth and also an inducer of cellulases. Hemicellulose and lignin fractions on the other hand play an important role in production of plant biomass-degrading enzyme cocktail including xylanases and ligninases (Fernández-Fueyo et al., 2016).

The high moisture content of DIS is due to the water holding capacity of cellulose fibres in the sludge which makes it unsuitable feedstock for thermochemical conversion process. Breaking down of cellulose fibres in DIS by enzymes has been shown to not only add value to the feedstock, but also significantly reduce the water content and bulk of the sludge that has to be discarded (Steffen et al. 2017a). Williams (2017) has studied three different types of paper sludge for ethanol production and has reported that usage of enzymes and break down of cellulose has significantly reduced the water holding capacity and increased the bulk density of all the three types of paper sludge. This clearly indicates the advantage of using DIS as feedstock for enzyme production as cellulase producing fungi are able to use cellulose present in the DIS as a substrate and reduce the bulk of sludge to be disposed.

Other important parameters anticipated to influence the growth and enzyme production (activity) of microorganisms on DIS include pH, C/N ratio as well as concentrations of bioavailable nutrients, ash and heavy metals (Table 1). The DIS used in this study has a dry weight of 61.35% with 34.3% total organic carbon (TOC) and 1.48% nitrogen, indicating C/N ratio of 23.3, which falls within the optimal range for maintaining optimal fungal growth and cellulose utilization (Wan and Li, 2012). This indicates that there is no additional requirement for a nitrogen source for fungal enzyme production using DIS as a substrate. This is advantageous in comparison to other cellulose-rich substrates, where nitrogen content and availability are among the main limitations affecting microbial degradation and enzyme production (Huang et al. 2017). Excess amounts of salts and nutrients used for process intensification and to maintain high cell mass no only increase the cost of the process, but can also be left unutilised or accumulated in the cells and are of environmental concern (Junker 2010).

Low concentrations of certain heavy metals are essential for transcription activation and activity of certain oxidative enzymes, but excessive amounts may have inhibitory or toxic effects on some production strains (Lorenzo et al., 2006; Bhattacharya et al., 2013). The concentrations of heavy metals in DIS (Table 1) were lower than reported for some other paper mill sludges (Beauchamp et al., 2002; Abdullah et al., 2015) and did not exceed the concentrations typically found in non-contaminated soils (Tóth et al., 2016) which indicates that the risk of heavy metal inhibition on enzymes production is negligible with DIS as a substrate.

Appreciable amounts of cellulose, low concentration of heavy metals, desirable C/N ratio (thus negating the need for supplementing additional nutrient requirements) indicate that DIS is a suitable feedstock for lignocellulolytic enzymes production. Enzyme production with DIS

as a substrate can be further improved by reducing the ash concentration and increasing the cellulose concentration in the substrate. Robus et al. (2016) has shown that washing the paper sludge can reduce the ash content in the paper sludge significantly. On the other hand, washing utilises large quantities of fresh water and hence is not considered suitable for sustainable and cleaner production. So, the fungal strains capable of growing under high pH, high ash conditions and minimum nutrients should be targeted for enzymes production.

3.2. Screening growth of selected strains on DIS

Results from our inititial screening, conducted to select fungal strains exhibiting the best growth on DIS, are presented in Fig. 2. Conceptually, these strains should be those producing enzyme mixtures that are most effective at breaking down the non-pretreated DIS with no or minimal supplementation of nutrients. Moreover, the strains exhibiting the best growth on DIS should be resistant to high pH of the substrate, chlorinated organic compounds or inks that are commonly found in this type of substrate (Monte et al., 2009). The growth of the 30 selected fungal strains was monitored for 50 days and classified in different categories according to its ability / efficiency of growth on autoclaved (A-DIS) and non-pretreated (NA-DIS) medium (Fig. 2). While the autoclaved substrate supported the growth of 15 fungal strains, only 8 of these strains (all white rots), were able to grow on NA-DIS. However, all except *P. eryngii* grew significantly better on the autoclaved substrate. This may be due to partial breakdown of cellulose fibres in DIS during hot steam treatment that may increase the bioavailability of nutrients, supporting larger spectrum of the strains (Zheng et al., 2014). In addition, the indigenous microbial community colonizing the NA-DIS may have exerted a competitive exclusion effect that precluded growth of some of the less competitive fungal strains. Under such conditions, it is reasonable to conclude that the 8 strains able to grow on

the NA-DIS were able to successfully compete against the indigenous comunity for essential nutrients and resist the conditions established in the substrate.

Pretreatment is considered as one of the major energy and cost intensive unit operation in lignocellulose biomass conversion and pressure vessels for sterilisation are one of the major capital investment cost in bioprocess industries (Brodeur et al., 2011). Steam sterilisation requires fresh water to generate steam and to cool the waste steam. The ability of the fungal strains to outgrow indigenous microbes colonizing DIS and to utilise untreated DIS is therefore a significant advantage in terms of energy and water usage. The majority of the strains that were able to grow on NA-DIS belonged to the genus *Pleurotus* which has previously been reported to have high tolerance to different types of environmental pollutants, such as heavy metals and polycylic aromatic compounds (Kapahi and Sachdeva, 2017). This has been associated with the ability to accumulate and immobilize (chelate) high levels of heavy metals (Yang et al., 2017). In addition, *P. ostreatus* and some white-rots producing ligninolytic enzymes have been shown to mineralize several persistent organic pollutants (POPs) to CO_2 (Bezalel et al. 1996) and have therefore already been used for bioremediation of contaminated solid organic wastes (such as olive mill wastes) and soils (Kůes, 2015). The potential and capacity of the strains selected in this work for bioremediation of DIS from different paper mills and pollutant loadings should further be studied.

3.3. Screening enzyme activities of selected strains on DIS

Extracted secretomes of 10 strains exhibiting the most growth on A-DIS and ability to grow on NA-DIS were screened for target enzyme activities by plate assays. The latter reflected the inducibility of the genes encoding (ligno)cellulolytic enzymes by the elements in the substrate, their translational efficiency as well as the stability and activity of the extract mixtures in given conditions (Lee, 2012). The SEEs of all the strains exhibited similar

xylanolytic activity, while endoglucanase activity was significantly higher in the four *Pleurotus* strains (PLAB, PP3, PLO4, PLOZ), *C. purpereum* (CHP4), *H. ulmarius* (HYUL1), *P. ciliates* (PC1) and *G. lucidum* (GAL5) than in the remaining two tested strains (Fig. 3). Laccase activity was only detected in seven of the tested strains and was most pronounced in two *Pleurotus* strains (PLAB, PP3) and *H. ulmarius* (HYUL1).

As extracellular enzymes were separated from mycelia before testing, the enzymes performance must reflect their intrinsic stability (not associated with mycelial defense mechanisms, such as accumulation of toxic compounds or buffering) in the applied conditions, such as high pH and potentially inhibitory compounds.

Enzyme performance at high pH values (8.6 in case of our substrate), typically associated with high amounts of mineral fillers present in DIS, is advantageous for many applications in the paper industry, as most of the current commercial cellulases perform best in weakly acidic conditions (pH 5-7)(Steffen et al, 2017a). Other factors that may affect stability and activity of enzymes produced on DIS are remaining organic pollutants or heavy metals (originating from inks). However, lignin-degrading oxidoreductases from different white rots have demonstrated not only resistance, but also the ability to degrade or transform broad range of organic pollutants (including pharmaceuticals) to less toxic species (Naghdi et al., 2018). Consequently, they have already been used to purify contaminated water or solid materials prior to release into an environment, as well as for bioremediation within different environments (Kűes, 2015).

On the other hand, variable effects of different heavy metals on lignocellulolytic enzyme activities and stability were reported. While Fe^{2+} and Cu^{2+} mainly exerted inhibitory effects on fungal endoglucanases, exoglucanases, and β-glucosidases, the effects of other divalent ions seems to be inconsistent among the enzymes secreted by different microorganisms (de Cassia Pereira et al., 2017). In contrary, most of the studies report positive effects of some heavy

metals (in particular Cu^{2+} and Cd^{2+}) on oxidative enzymes, especially laccases (Yin et al., 2017).

3.4. Major proteins identified in *P. ostreatus* DIS-associated secretome

During their evolution, microorganisms have optimized the expression of genes encoding lignocellulolytic enzymes by sensing and responding to the composition of the available substrate in most efficient ways (Fernández-Fueyo et al., 2016). Studying the composition of enzyme consortia secreted on different target substrates is therefore useful for understanding the mechanisms of their degradation and for the successful design and production of enzyme cocktails optimized for specific industrial processes (Vodovnik and Marinšek Logar, 2010).

Our study revealed six most abundant proteins in the secretome of *P. ostreatus* PLAB likely involved in the degradation of DIS (Table 2, Suppl. 4, 5). Among these were two laccase isoenzymes with the theoretical Mw of 57.4 and 56.7. Two enzymes with ABTS-oxidasing activity were also confirmed by zymogram approach (Suppl. 6C) – however, their activity was only detected following native separation conditions and was irreversibly lost if the samples were pretreated by SDS. SDS-associated inhibition of isolated *Pleurotus sp*. laccase has also been observed by More et al. (2011). Despite their sensitivity to certain compounds, laccases have huge biotechnological potential in the pulp and textile industry as well as for beverage processing, organic synthesis, the manufacture of biodevices and the detoxification of pollutants (Cañas and Camarero, 2010).

Feruloyl esterase, a hydrolase catalyzing the cleavage of bonds between plant cell wall polysaccharide and phenolic acid, and bilirubin oxidase, an oxidoreductase from cupredoxin family, were also identified among the secreted proteins. Both of these enzymes are

considered promising tools for different biotechnological applications. While feruloyl esterases may be used for bio-refining of lignocellulosic material for the recovery and purification of ferulic acid and related hydroxycinnamic acids ubiquitously found in the plant cell wall (Gopalan et al., 2015), bilirubin oxidases have promising applications in dye effluent decolorization (Liu et al., 2009) and in biofuel cells (Durand, 2012).

A cellobiohydrolase (enzyme releasing cellobiose from the non-reducing ends of cellulose chains) was also identified among the major *P.ostreatus* proteins produced on DIS (Table 2). In addition, six other cellulases were also detected in zymograms (Suppl. 6A). The Mw range of the three enzymes with the most pronounced activites correlated with four theoretically predicted Mw of *P. ostreatus* cellulases listed in the NCBI Protein database (Suppl. 3A). In addition, three bands revealing enzymes with xylanolytic activity correlating with *P. ostreatus* putative xylanases KDQ24406, KDQ24407 and KDQ33930 were visualized (Suppl. 3B). Another fainter degradation band with a Mw between 45 and 50 kDa did not correlate with any of the predicted gene products indicating it may be a post-translationaly modified enzyme. Interestingly, glycoside hydrolases detected in the zymograms proved resistant to rigorous pre-separation treatment (heating in SDS-buffer), as their activities were recovered after the renaturation steps. Similar stability has previously been reported with some other extracellular glycoside hydrolases, i.e. from anaerobic cellulolytic bacteria (Vodovnik and Marinšek Logar, 2012; Hatefi et al., 2017).

In addition to proteins traditionally involved in lignocellulose degradation, peptidase 1 was also detected among proteins of *P. ostreatus* PLAB produced on DIS. Fungal proteases typically act as virulence factors (Monod et al., 2002), but the results of current research indicate that they may also play a role in debranching polysaccharide linkages within plant cell wall proteins and thus providing access to proteins as sources of carbon and nitrogen. The low content of available nitrogen that is typical for the majority of lignocellulosic substrates

implies that the expression of specific proteases could be advantageous for releasing nitrogen from organic matter such as that contained in plant material or associated microbial biomass (Sato et al., 2007). High expression of proteases and peptidases was also detected in *Aspergillus fumigatus* (Adav et al., 2015), *Trichoderma reesei* (Borin et al., 2015), *Pycnoporus coccineus* (Couturier et al., 2015) and *Pleurotus ostreatus* PC9 (Fernández-Fueyo et al., 2016) secretomes produced on different lignocellulosic biomasses. The enzyme identified in our study belongs to family S8 peptidases (subtilase clan), which are typically thermostable alkaline peptidases, that may be suitable for different biotechnological applications (especially detergent industry) (Apolinar-Hernandéz et al., 2016). Implementation of enzyme technologies in paper, textile and biofuel industry is a promising means toward cleaner industrial production. The results of several studies show that implementing enzymatic processes in place of conventional processes generally results in a reduced contribution to global warming and also a reduced contribution to acidification, eutrophication, photochemical ozone formation and energy use (Jeganathan and Nielsen, 2013).

3.5. Enzymes production from DIS

Due to its efficient growth on pretreated as well as non-pretreated DIS and its highest screened enzyme activities, *P. ostreatus* PLAB was chosen as the most promising candidate for further quantitative analysis of enzyme production on target substrate. Target enzyme activities detected in enzyme extracts at different fermentation times are shown Fig. 4. The endoglucanase activities recovered from overgrown substrate consistently remained between 1.5 – 3.0 µmol/min/g_{DIS} throughout all the growth stages. This is higher than previously reported for *P. ostreatus* during SSF on coffee pulp and similar as in cultures grown on a mixture of supplemented wheat and rice straw as a substrate (Kurt and Buyukalaca, 2010). On

the other hand, Khalil et al. (2011) reported higher endoglucanase activity after 12 days of SSF on rice straw (Suppl. 2A).

Xylanase activities in the extracts varied from 1.5 - 4.0 µmol/min/g. These activities are lower than detected in extracts from mycelia grown on corn and wheat bran, tomato waste or a mixture of *Fagus sylvatica* leaves and wheat straw as substrates (Suppl. 2B). This may be associated with the lack of inducing compounds originating from the substrate, since natural (ligno)cellulosic substrates usually contain a substantial portion of hemicelluloses (20-30%), while its amounts in paper sludge remain very low due to their removal during the pulping process, with only 3.7% hemicellulose remaining in the DIS used in our study. The mechanisms of regulation and substrate-associated expression patterns of genes encoding hemicellulases in *P. ostreatus* are not yet well understood. However, research on related fungi confirmed their coordinated regulation is associated with molecular signals reflecting substrate composition (Cai et al., 2017).

The changes in endoglucanase activity and xylanase activity did not correlate with total protein concentration time-wise, since enzyme production apparently peaked in earlier fermentation (growth) phases (maximal endoglucanase and xylanase activity was detected at 23 days of growth), which is in accordance with the fact that the production of these enzymes is an essential predisposition for nutrient acquirement from the substrate (Fig. 4A).

In contrast to endoglucanase and xylanase, laccase activity in *P. ostreatus* DIS-induced secretome strongly correlated with total proteins (reflecting growth of fungal mycelia) and peaked between 30 and 44 days of incubation, achieving its highest average activity of 49 ± 5 µmol $ABTS^{\cdot+}$/min/g_{DIS} after 37 days and declining thereafter (Fig. 4B). Laccase activity detected in enzyme extracts from *P. ostreatus* on DIS were significantly (10-30 times) higher than reported for other lignocellulose substrates, such as lyophilized wheat straw, wheat bran

and spent brewery grains (Suppl. 2C). Since DIS contains lower amounts of lignin than reported for the aforementioned substrates (6.2% in our study), high laccase activity in *P. ostreatus* PLAB secretome may be associated with other inducing compounds present in this substrate. In addition to nitrogen source and concentration, heavy metal ions and certain phenolic and (poly)aromatic compounds with structural similarities to lignin have previously been shown to positively regulate expression of laccases (Zhuo et al., 2017) and other ligninolytic enzymes (Zhang et al., 2016). The deinking sludge used in our study contained significant amounts of copper (64.99 mg/kg dry content), which has been reported to act as a strong transcriptional inducer and activity promotor of laccases (Lorenzo et al., 2006) and lignin-modifying peroxidases with several white rots (Zheng et al., 2017).

Due to possible effects of substrate composition on enzyme production, it is important that the substrates from different paper milly be characterized so as to support optimization of amounts and types of enzymes produced. If the production of specific type of enzymes would be an objective, the substrate composition should be adjusted by adding relevant inducers (i.e. xylan for increasing xylanase production or specific heavy metals to increase ligninase production).

3.6. Advantages of enzyme production from DIS via SSF

The advantage of SSF processes for enzyme production is lower water consumption and hence relatively less energy is required to concentrate the target enzymes (Yoon et al., 2014). DIS produced in current practice is a waste stream with zero value, substantial disposal costs and no proper environmentaly friendly disposal method, therefore repurposing it as a feedstock not only reduces the waste to be disposed from tissue printed recycle mills but also adds revenue to the industry (Gottomukkala, 2016). Laccases are enzymes with tremendous application potential in paper and pulp industry for delignification and brightening of pulp, as

well as in bioremediation of toxic compounds released from the industry (Virk et al., 2012). Endoglucanases and small quantities of xylanases produced may be used for conversion of cellulosic rich feedstock to value added chemicals and hence can be used within the paper industry to convert paper sludge or undigested residues of enzyme fermentation to fuel and chemicals (Gottomukkala et al., 2016). This study has proven that DIS is a potential feedstock for production of lignocellulytic enzymes and induces laccase production at high concentrations. Moreover, this study has shown that DIS as a feedstock needs no additional supplementation of nutrients which thus reduces the burden of waste on the environment. Well known bioprocess like direct ethanol production from paper sludge requires high solids loading to achieve threshold ethanol concentrations of 40 g/L (Robus et al., 2016). Viscosity of paper sludge at such high solids loading is a major limitation and high ash content of the sludge significantly influences the final ethanol concentration (Gottumukkala et al., 2016). Hence, the suggested technology, to use *Pleurotus ostreatus* for enzymes production with DIS by SSF without any supplementation, pretreatment and sterilization requirement, may therefore be a suitable cost-effective alternative for paper sludge valorisation to ethanol. However, to increase the enzyme concentration and yields further experiments testing co-fermentation with additional nitrogen rich waste streams need to be explored in the future. This approach would help in valorisation and treatement of two different waste streams. In addition, the effects of the variations in the DIS composition on the ratios of the different types of the produced enzymes should be explored. The residues from the SSF of DIS should be investigated and potentially used for biogas production (Steffen et al., 2017b). Further studies are warranted, to optimize the production, economic feasibility and environmental benefits of this technology.

4. Conclusions

Enzymes production for lignocellulose degradation is currently a cost intensive process and utilises fresh water and nutrients which in turn results in additional waste stream generation. If combined with efficient and robust microbial enzyme producers, lignocellulosic wastes have an enormous potential for developing a sustainable chemical and energy industry as the most inexpensive feedstock among all the renewable resources. The results of our study have shown that the deinking paper sludge represents a suitable feedstock for the production of lignocelulolytic enzymes by selected *Pleurotus* strains. The selected strains exhibited outstanding ability to transform untreated and unsterilized DIS to a mixture of alkali-stable enzymes (mainly with endoglucanase, xyalanase and particulary high laccase activities). Production of lignocellulases from unsterilized DIS by SSF should therefore be considered at an industrial level to add value to the major waste stream of paper industry by reducing waste disposal, costs associated with the disposal of wastes to landfill sites and by yielding high value enzymes suitable for application in biorefinery, paper and pulp, detergent and textile industries. Further research should be done to analyse (anticipated positive) effects of the process on the levels and bioavailability of environmental pollutants potentially present in DIS. In addition, the enzymes production should be further optimized and process conditions fine-tuned to increase the yield and productivity of individual enzymes and the cocktail. Major advantages of this process such as less water usage, less energy usage and reduced waste disposal should be studied in detail for its energy, economic and environment benefits.

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FIGURE CAPTIONS

Fig. 1. Schematic representation of experimental set-up.

Fig. 2. A heatmap representing the ability/efficiency of selected fungal strains to grow on autoclaved (A-DIS) and non-autoclaved (NA-DIS) deinking paper sludge. 0 – no growth observed in any of the inoculated samples, 1 – mycelium observed in less than half of the samples, 2- mycelium overgrew more than 50% of the samples, 3- mycelium overgrew more than 75% of the samples (N=4).

Fig. 3. Degradation/oxidation zone diameters around secreted enzyme extracts (SEEs) from different fungal strains applied on screening plates indicating endoglucanase (red), xylanase (grey) and laccase (blue) activities. Results represent an average values ± standard deviations from 3 biological replicates. Different capital letters above solid bars denote significant ($P < 0.05$) differences in endoglucanase activity, while different lowercase letters above hollow bars denote significant ($P < 0.05$) differences in laccase activity.

Fig. 4. Activities of target lignocellulolytic enzymes (EA) in *P. ostreatus* PLAB at different stages of its growth. A: glycoside hydrolase (endoglucanases, xylanases), B: laccase (ABTS-oxidases). Results represent an average values ± standard deviations from 3 biological replicates.

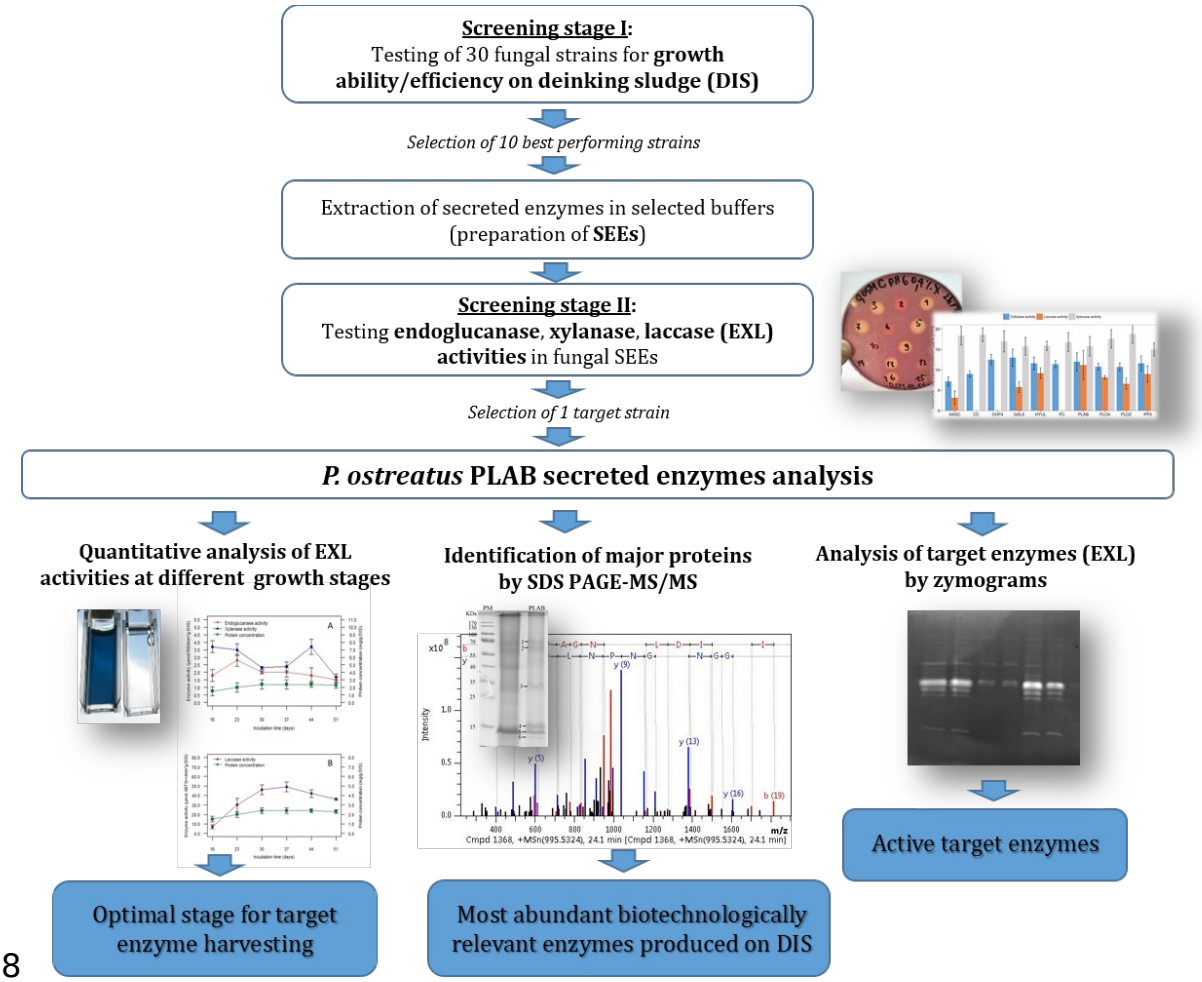
Table 1. Properties (composition) of deinking sludge (DIS) used as a substrate for selected fungal strains.

Parameter	Value
pH	8.6
Dry content (DC)	61.35 %
Total organic carbon (TOC)	34.53 % (w/w_{DC})
Total nitrogen (TON)	1.48 % (w/w_{DC})
Cellulose content	34.2 % (w/w_{DC})
Lignin content	6.2 % (w/w_{DC})
Hemicellulose content	3.7 % (w/w_{DC})
Copper (Co)	64.99 mg/kg DC
Zinc (Zn)	84.90 mg/kg DC
Cadmium (Cd)	0.40 mg/kg DC
Chromium (Cr)	11.60 mg/kg DC
Nickel (Ni)	2.20 mg/kg DC
Lead (Pb)	17.04 mg/kg DC
Mercury (Hg)	0.09 mg/kg DC

Table 2. Major fungal proteins likely involved in the degradation of deinking sludge (DIS) identified in the secretome extracts of *P. ostreatus* PLAB grown on A-DIS.

Protein hits (fungal enzymes)	Accession No. (NCBInr)	Mw [kDa]	pI	Detected in band(s)	Matching Peptides #	Peptide scores
Laccase	CAC69853	57.4	5.7	1, 2, 3	8	308.2
Laccase 2	Q12739	56.7	4.7	1, 2	1	132.7
Cellobiohydrolase	AAA50608	46.1	4.8	2	1	90.0
Bilirubin oxidase	BAA85185	56.8	4.6	1, 2	3	158.8
Feruloyl esterase	CDI44666	59.3	6.9	1	2	159.6
Peptidase 1	ACR25273	38.7	7.9	3	3	84.5

Fig. 1



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946**Fig. 2**

Species	Strain accesion code	A-DIS	NA-DIS
Agaricus bisporus	AGB1	1	0
Agrocybe aegerita	AASC	2	1
Auricularia auricula	AAP3	1	0
Auricularia sambucina	AS	0	0
Chondrostereum purpureum	CHP4	2	0
Coprinus comatus	CC	2	0
Cordyceps militaris	CM2	0	0
Fomes fomentarius	FF366	1	0
Ganoderma lucidum	GAL5	2	1
Gleophyllum trabeum	GT	0	0
Grifola frondosa	GF5	0	0
Grifola frondosa	GF2	0	0
Hypsizygus ulmarius	HYUL1	2	1
Inonotus obliquus	IO	1	0
Lentinula edodes	LE3770	0	0
Lentinula edodes	LE7401	0	0
Meripilus giganteus	MG2	1	0
Monascus ruber	M2	0	0
Pleurotus eryngii	POE1	1	1
Pleurotus ostreatus	PLO4	2	1
Pleurotus ostreatus	PLAB	3	1
Pleurotus ostreatus	H35	2	1
Pleurotus pulmunarius	PP3	2	0
Pleurotus sp.	PLOZ	2	1
Polyporus umbellatus	PLUI	0	0
Polyporus ciliatus	PC	2	0
Poria vaillantii	PV2	0	0
Psilocybe cubensis	PSCUII.	0	0
Trametes versicolor	TVER	1	0
Wolfiporia cocos	DSM1216	0	0

Fig.3

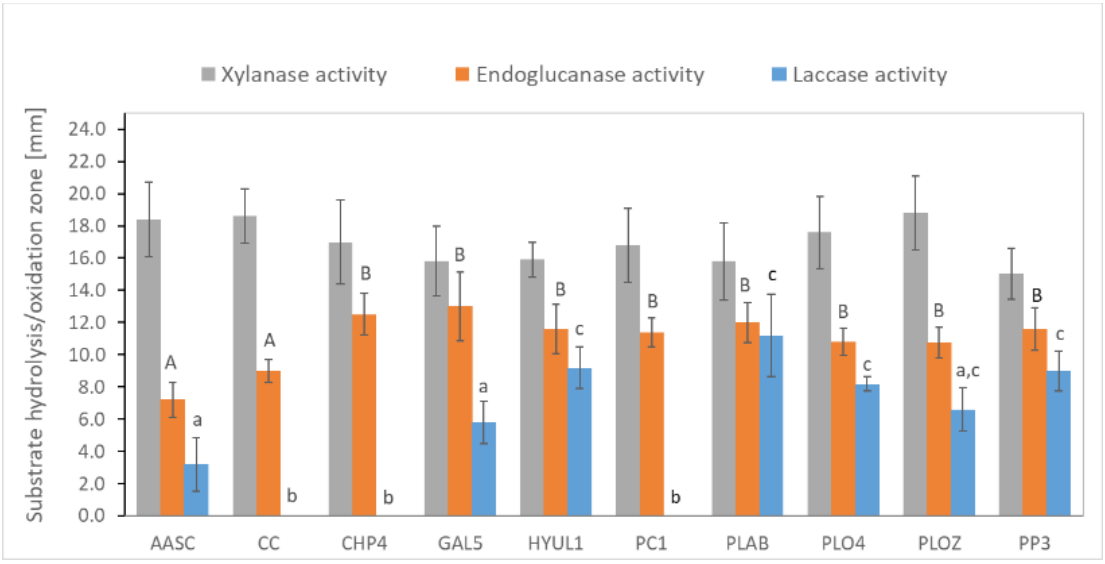
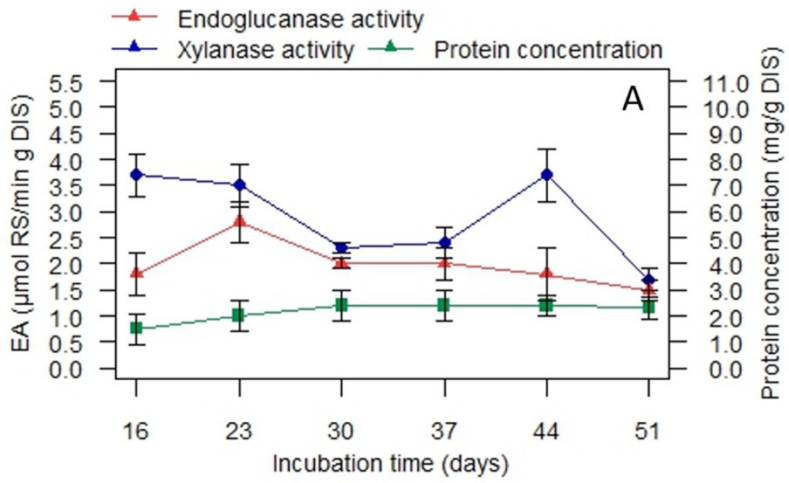
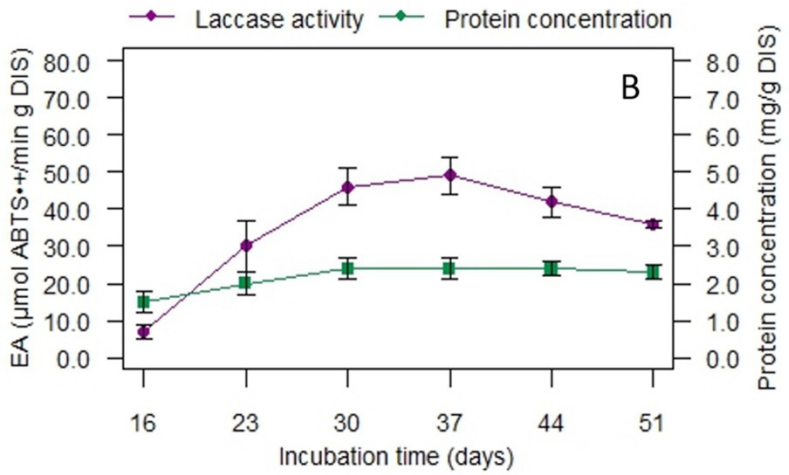

Xylanase activity
Endoglucanase activity
Laccase activity
Substrate hydrolysis/oxidation zone [mm]
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
A
a
A
b
B
b
B
a
B
c
B
b
B
c
B
c
B
a,c
B
c
AASC
CC
CHP4
GAL5
HYUL1
PC1
PLAB
PLO4
PLOZ
PP3

Fig. 4


Endoglucanase activity
Xylanase activity
Protein concentration
A
EA (µmol RS/min g DIS)
Protein concentration (mg/g DIS)
Incubation time (days)


Laccase activity
Protein concentration
B
EA (µmol ABTS•+/min g DIS)
Protein concentration (mg/g DIS)
Incubation time (days)

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