Investigation of *Abortiporus biennis* lignocellulolytic toolbox, and the role of laccases in polystyrene degradation

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Abstract

White-rot basidiomycetes are the only microorganisms able to produce both hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) enzymes for degrading all lignocellulose constituents. Their enzymatic machinery makes them ideal for the discovery of novel enzymes with desirable properties. In the present work, *Abortiporus biennis*, a white-rot fungus, was studied in regard to its lignocellulolytic potential. Secretomics and biochemical analyses were employed to study the strain's enzymatic arsenal, after growth in corn stover cultures and xylose-based defined media. The results revealed the presence of all the necessary enzymatic activities for complete breakdown of biomass, while the prominent

role of oxidative enzymes in the lignocellulolytic strategy of the strain became evident. Two novel laccases, *Abi*Lac1 and *Abi*Lac2, were isolated from the culture supernatant with ion-exchange chromatography. Characterization of purified laccases revealed their ability to oxidize a wide variety of phenolic and non-phenolic substrates. *Abi*Lac1 was found to oxidize polystyrene powder, showing high depolymerization potential, based on radical chain scission mechanism as evidenced by molecular weight decrease. The results of the present study demonstrate the biotechnological potential of the unexplored enzymatic machinery of white-rot basidiomycetes, including the design of improved lignocellulolytic cocktails, as well as the degradation and/or valorization of plastic waste materials.

Keywords: *Abortiporus biennis*, laccase, secretomes, lignocellulosics degradation, plastics degradation

1. Introduction

Lignocellulosic biomass (LCB) is the most abundant natural resource on earth, and it is currently the most promising candidate for the transition from petroleum-based refineries to sustainable biorefineries. The complete depolymerization of lignocellulose can be facilitated by exploiting the enzymatic arsenal produced by wood-decomposing microorganisms. All the enzymes acting on LCB are classified in CAZy database (http://www.cazy.org/; (Lombard et al., 2014)). The most important enzyme activities in this respect are cellulases, xylanases and oxidative enzymes. However, many more accessory enzymatic activities are required for complete LCB depolymerization, such as esterases and enzymes acting on side chains.

White-rot basidiomycetes are especially good decomposers, due to their ability to completely degrade all LCB components. Despite the diversity of wood-rotting fungi in natural ecosystems, only a few have been studied in detail regarding their secretomic data during

growth in LCB substrates, such as *Irpex lacteus* (Salvachúa et al., 2013), *Pycnoporus coccineus* (Couturier et al., 2015), *Pleurotus ostreatus* (Xiao et al., 2017), *Ceriporiopsis subvermispora* (Hori et al., 2014), *Laetiporus sulphureus* (de Figueiredo et al., 2021), *Laetisaria arvalis* (Navarro et al., 2014) and a few others (Sethupathy et al., 2021). Given the need for discovery of novel LCB-degrading enzymes, it is essential to gather data regarding the secretomes of LCB-degrading fungi from a variety of strains/species from diverse habitats, in order to explore differentiating mechanisms of microbial LCB attack, but also guide future efforts for the design of enzyme cocktails.

The ligninolytic enzyme system of white-rot fungi has been studied in detail. Laccases (EC 1.10.3.2, benzenediol-oxygen oxidoreductases) are some of the most important lignin-acting enzymes, catalyzing the four-electron oxidation of a substrate and the concomitant two-electron reduction of molecular oxygen to water (Bassanini et al., 2020; Leynaud Kieffer Curran et al., 2022). Their substrate spectrum is very wide, including various phenolic compounds and amines, but it can be further broadened to include non-phenolic compounds by the use of redox mediators, facilitating oxidation of recalcitrant substrates (Bassanini et al., 2020). Laccases have been used for the biodegradation and detoxification of various environmental contaminants, such as pesticides (Chen et al., 2019), synthetic dyes (Mishra et al., 2019), pharmaceuticals (Alharbi et al., 2019), and many others (Bilal et al., 2019). Recently, laccases have also been used for synthetic polymer degradation, such as polyethylene (PE), polyurethane (PU) and polystyrene (PS), revealing a promising scope of these enzymes (Nikolaivits et al., 2021). However, synthetic polymer degradation is often accomplished using mediators, such as 1-hydroxybenzotriazole (HBT), which allow the degradation of a wider range of substrates by improving laccase's oxidation potential (Fujisawa et al., 2001).

Abortiporus biennis (Basidiomycota, Polyporales) is a white-rot fungus, which grows well in phenol-containing media, such as olive oil mill effluents and waste (Aggelis et al., 2002; Hong et al., 2016; Koutrotsios and Zervakis, 2014; Zerva et al., 2021b), mostly due to its ability to produce laccases and ligninolytic peroxidases (Koutrotsios and Zervakis, 2014; Yin et al., 2017; Zhang et al., 2011). All the necessary enzyme activities for LCB breakdown are present in its genome, while previous transcriptomic studies confirmed the expression of multiple carbohydrate-acting enzymes (Grąz et al., 2017). Therefore, this strain was selected to further study its secretome during growth on LCB material, as a potential new source for novel enzymes with superior properties. Two new laccases were isolated from the culture supernatants, and they were fully characterized. One of them, *Abi*Lac1, was applied on the depolymerization of PS powder, which underwent accelerated degradation, revealing that the enzyme can be considered as a promising tool towards plastic waste valorization.

2. Materials and methods

2.1 Microorganisms and culture procedures

The *A. biennis* LGAM 436 strain used in this work was obtained from the culture collection of the Laboratory of General and Agricultural Microbiology (Agricultural University of Athens), and was maintained in Potato Dextrose Agar plates (PDA- Applichem, Germany) at 4 °C. The strain was grown on 100 mL precultures, in 250 mL Erlenmeyer flasks, with the following medium composition: D-xylose 5.7 % (w/v), 30 g L^{-1} yeast extract, 1 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, and the pH was adjusted to 6 with HCl. After autoclaving, the cultures were inoculated with three 6-mm agar plugs with fully grown mycelium, and filter-sterilized ampicillin was added at a final concentration of 0.1 mg mL^{-1}. The cultures were incubated at 27 ºC and 100 rpm for 24 days. Then, the biomass was aseptically filtered,

washed and inoculated in fresh medium with 4 % (w/v) corn stover, 1 g L^{-1} NH_4NO_3, 0.8 g L^{-1} KH_2PO_4, 0.2 g L^{-1} Na_2HPO_4, 0.5 g L^{-1} $MgSO_4.7H_2O$ and 2 g L^{-1} $(NH_4)_2C_4H_4O_6$, while the pH was adjusted to 6 with HCl. The cultures were incubated for further 14 days in 27 ºC and 100 rpm. Samples were taken at selected intervals, centrifuged, and the supernatant was used for the analyses.

2.2 Enzyme activities

In the culture supernatants of *A. biennis*, the following enzymatic activities were determined: cellulase, endoxylanase, total esterase, laccase, acetyl xylan esterase, α-arabinofuranosidase, feruloyl esterase, glucuronoyl esterase, aryl alcohol oxidase and versatile peroxidase, following previously established protocols (Camarero et al., 1999; Zerva et al., 2019, 2014), as described in Supplementary Material (Text S1). The hydrolytic potential of the secreted enzymes from *A. biennis* against LCB was determined as described in Supplementary Material (Text S1).

2.3 Proteomic analysis

2.3.1 Sample preparation and LC-MS/MS analysis

For the secretome analysis, *A. biennis* was cultured as described in Section 2.1. After 14 days of growth the mycelia were removed by filtration, and the supernatant was ultrafiltrated and freeze-dried. Protein extracts were reduced, alkylated trypsin – digested and purified as described in Supplementary Material (Text S2). The mass spectrometer (Ultimate 3000 RSLC nano- LC, Thermo Fisher Scientific, Bremen, Germany) in-line connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific)) was operated in data-dependent, positive ionization mode (Text S2). QCloud was used to control instrument longitudinal performance during the project (Chiva et al., 2018).

2.3.2 Data analysis

The LC-MS/MS runs of all samples were searched together using MaxQuant v2.0.3.0 (Tyanova et al., 2016a) against the proteome of *A. biennis* CIRM-BRFM 1778 (v1.0, release date 2021-06-21, 11,767 sequences) (Hage et al., 2021) downloaded from MycoCosm (Grigoriev et al., 2014) and the MaxQuant common contaminant database (Text S2). Downstream processing was performed with Perseus v1.6.15.0 (Tyanova et al., 2016b) and the pandas Python package (https://zenodo.org/record/3715232#.YjGciDyxWrx and 10.25080/Majora-92bf1922-00a) (Text S2). The functional annotation, and the statistical comparison were performed as described in Text S2.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD032691 and 10.6019/PXD032691".

2.4 Purification and protein analysis of AbiLac1 and AbiLac2

The supernatant of 500 mL cultures was concentrated 10-fold with ultrafiltration (10 kDa cutoff membrane), and dialyzed against 20 mM Bis-Tris-HCl buffer pH 7.5. Then, the crude enzyme sample was loaded on a Q-Sepharose™ High Performance column (100 mL, GE Healthcare, Uppsala, Sweden) equilibrated in the same buffer, using an Econo Gradient peristaltic pump (Biorad, U.S.A.). The proteins were eluted with the following gradient (Solvent B is 1 M NaCl in 20 mM Bis-Tris-HCl buffer pH 7.5): 0-60 min isocratic 90 % A:10 % B (3 CV), 60-140 min gradient step 10-25 % B (4 CV), 140-160 min gradient step 25-50 % B (1 CV), 160-180 min 100 % B isocratic step (1 CV), with a flow of 5 mL min^{-1}. The proteins eluting from the column were monitored with a UV detector Econo UV Monitor (Biorad, U.S.A.). Fractions (10 mL) displaying activity on catechol were pooled and concentrated. The purity of the isolated enzymes was confirmed with SDS-PAGE electrophoresis (Laemmli, 1970). Zymograms for the detection of catechol oxidase activity were performed in non-denaturing conditions, and

the gels were activity-stained with 2 mM catechol in 50 mM phosphate-citrate-buffer pH 4.5, in 40 °C. For the identification of the isolated proteins, the bands were cut from the gels, and an in-gel digestion protocol was applied, as described in Text S3.

Peptide LC-MS/MS analysis was performed with MaxQuant, as described in Supplementary material (Text S2).

2.5 Biochemical characterization of AbiLac1 and AbiLac2

For the determination of optimum activity conditions of purified laccases, 5 mM catechol was used as substrate (ε_{450}=928 $M^{-1}cm^{-1}$). Optimum pH was determined by measuring the residual activity in pH values 3 to 9, with respective buffers citrate- potassium phosphate, potassium phosphate, Tris-HCl, all in 100 mM concentration, in 35 °C. The temperature dependence of the enzyme activity was determined by measuring the residual activity at temperatures 30-70 °C.

The activity screening in different substrates was performed by incubating each purified enzyme with 2 mM of the respective substrate in the optimum pH for each laccase. After incubation for 0, 6, and 24 h in 35 °C, a UV/Vis spectrum was taken for each reaction, together with the respective blanks. For the determination of substrate specificity for each purified laccase, the enzymes were incubated for 10 min with the following substrates: ABTS (ε_{420}=36000 $M^{-1}cm^{-1}$), catechol (ε_{450}=928 $M^{-1}cm^{-1}$), guaiacol (ε_{456}=12100 $M^{-1}cm^{-1}$), 2,6-dimethoxyphenol (ε_{469}=27500 $M^{-1}cm^{-1}$) and pyrogallol (ε_{420}=3724 $M^{-1}cm^{-1}$). Michaelis-Menten kinetic parameters were calculated for ABTS and catechol, from the respective nonlinear regression plots, with the software GraphPad Prism 8. Thermostability of *Abi*Lac1 and *Abi*Lac2 was determined by incubating each enzyme at various temperatures from 30 to 70 °C and measuring the residual activity, with ABTS as the substrate, at the indicated time points. Protein quantification was performed with the Bradford assay (Bradford, 1976).

2.6 Enzymatic degradation of polystyrene

PS in the form of pellets (PS Crystal 154) was purchased from Total Petrochemicals and cryomilled into powder (particle diameter < 500 μm) in a Pulverisette 14 (Fritsch Corp., Idar-Oberstein, Germany). Enzymatic reactions containing 10 mg of PS powder were performed in 1 mL of 0.1 M phosphate-citrate buffer (pH 5.5). Reactions were initiated after the addition of 16 U of enzyme (activity determined with ABTS) and incubated at 30 °C under agitation (1200 rpm) in an Eppendorf Thermomixer Comfort (Eppendorf, Germany) for four days. In the control reactions, an equal amount of 0.1 M phosphate-citrate buffer was added, instead of enzyme. After four days, the residual material was isolated by centrifugation, washed with ultra-pure water twice, freeze-dried and its weight was measured in order to quantify any mass loss (%) after enzymatic treatment.

Average molecular weights (number-average and weight-average molecular weights, ($\overline{M_n}$, $\overline{M_w}$, g mol^{-1}) and polydispersity index (PDI) of PS powder was determined by gel permeation chromatography (GPC), which was carried out with the Agilent 1260 Infinity II instrument (Agilent Technologies, Germany). Molecule separation was performed using two PLgel MIXED-D 5 μm columns (300 x 7.5 mm) equipped with a guard column (PLgel 5 μm) and elution was carried out with tetrahydrofuran (THF >=99.9% purity, Macron Fine Chemicals, Poland) at a flow rate of 1 ml min^{-1}. The temperature of the analysis was maintained at 40^{o}C using the Agilent 1290 Infinity II Multicolumn Thermostat (G7116B), while the injection volume was set at 100 μL. The analysis was performed using an Agilent 1260 Infinity II refractive index detector (RID) (G7162A). The calibration of the instrument was carried out with PS standards of molecular weight from 162 to 500.000 g mol^{-1} (EasiVial PS-M 2 ml, Great Britain).

Fourier-Transform Infrared (FT-IR) spectroscopy was used in order to detect any changes in PS molecular fingerprint. FT-IR spectrum was recorded by the Bruker Alpha II FTIR spectrometer using ATR method with a diamond crystal. Software OPUS 8.5 was used to analyze the recorded spectrum, which was received as 16 scans in the 400–4000 cm^{-1} range. Identification of PS characteristic peaks was performed based on vibrational bands and comparing IR spectra against standards in software's spectral library.

3. Results and discussion

3.1 Enzymatic activities in the culture supernatants of A. biennis grown on corn stover

The first step towards our attempt to understand the enzymatic potential of *A. biennis* in lignocellulose deconstruction was to monitor selected enzyme activities during its growth on corn stover, a common agroindustrial byproduct. The selected enzymatic activities were endoxylanase, esterase and laccase activity, and their time course profile is shown in Fig. 1a. *A. biennis*, similarly to most white-rot Basidiomycetes, secretes high titers of laccase activity, indicating the strain's potential in lignin degradation. As shown in Fig. 1a, the time profile of laccase production showed two peaks, which could possibly indicate the differential production of laccase enzymes in the course of the culture. Significant endoxylanase production was measured, indicating that *A. biennis* can simultaneously degrade more than one lignocellulose component. Also, low total esterase activity was measured throughout the course of the culture, consistent with the auxiliary nature of esterases in biomass deconstruction. Several other enzyme activities were measured only the last day of the culture (14^{th} day), such as acetyl xylan esterases (8.8 ± 0.4 U L^{-1}), cellulases (212 ± 43 U L^{-1}) and aryl alcohol oxidases (3.5 ± 0.5 U L^{-1}), while versatile peroxidase, ferulic acid esterase, α-arabinofuranosidase and glucuronoyl esterase activities were not detected.

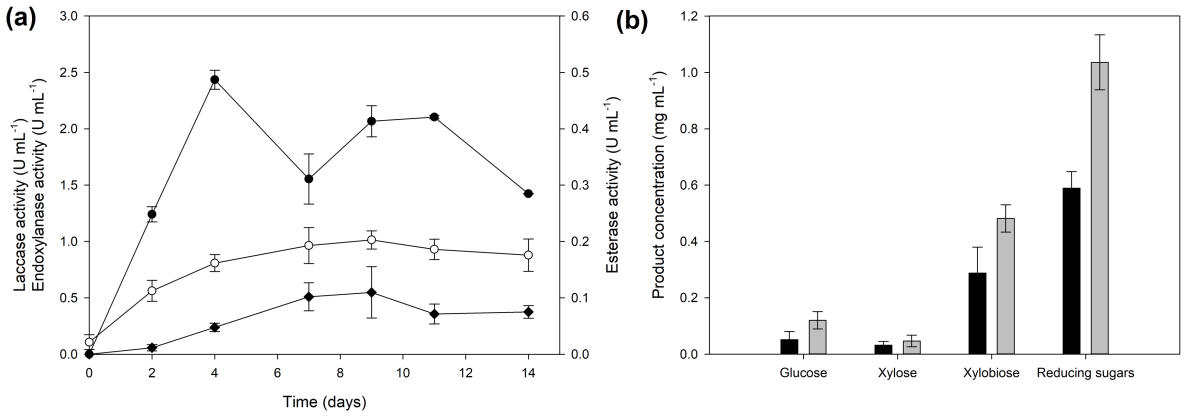

Fig. 1. (a) Time course profile of the enzyme activity during the growth of *A. biennis* in corn stover. *Black circles*: laccase, *white circles*: xylanase, *black diamonds*: total esterase, measured with pNPhA. (b) Hydrolysis of pretreated substrates by the secreted enzymes of *A. biennis* after growth in corn stover. *Black bars*: pretreated corn stover, *grey bars*: pretreated beechwood.

After the end of the cultures, the supernatants were concentrated and applied to the hydrolysis of pretreated lignocellulosic samples. The results, in terms of the release of reducing sugars, xylose, xylobiose and glucose, are shown in Fig. 1b. Consistently with the high titers of polysaccharide-degrading enzymes, the secretomes of *A.biennis* were able to release reducing sugars both from cellulose and xylan of pretreated biomass substrates. The xylobiose measured was significantly higher than the monomer xylose, which could indicate the lack of β-xylosidase activity in the supernatant. This is not surprising, since β-xylosidases are commonly found associated to the cell wall. Moreover, the released sugars were higher in the case of beechwood substrate than corn stover, which is rather expected, due to the variety of decorations in corn xylan, contributing to its high recalcitrance. Nonetheless, the secretome of *A. biennis* was shown to contain all the necessary enzymes for complete decomposition of all polysaccharides from biomass. This property is shown for the secretomes of many LCB-

degrading fungi (de Figueiredo et al., 2021; Machado et al., 2020; Paës et al., 2019; Yang et al., 2018), especially when they are used to supplement commercial cellulolytic cocktails. In the case of *Penicillium chrysogenum* P33, the addition of its secretome alone to delignified corn stover resulted in 2.4 mg mL^{-1} reducing sugars release, which is comparable with the results of the present study (Yang et al., 2018). Similarly, the secretomes of *P. chrysosporium* and *T. versicolor* after growth in cellulose led to 30-60 % glucan conversion from sugar cane substrates (Machado et al., 2020). This effect has also been shown for the secretomes of *L. sulphureus* after growth in sugarcane bagasse, acting on sugarcane straw and pine substrates (de Figueiredo et al., 2021). It is therefore evident that, in accordance with previous data, LCB-degrading fungi are an untapped source of LCB-acting enzymes and hold significant potential for the improvement of existing lignocellulolytic cocktails.

3.2 Proteomic analysis

LC-MS/MS was used to identify the secretome of *A. biennis* during growth on LCB material, namely corn stover and xylose. This analysis identified 525 proteins in corn stover and 326 in xylose samples. Of the identified proteins, many were likely involved in intracellular processes, such as ribosomal proteins and histones (accounting for around 10.2% of the identified proteins), possibly due to cell lysis during sample preparation (Bengtsson et al., 2016). After filtering for secreted and extracellular proteins, 235 proteins were detected in corn stover, and 185 of them were also found in xylose secretomes. The abundance of the detected proteins was in agreement with previous studies from other white-rot fungi during growth in LCB materials (Fernández-Fueyo et al., 2016; Hori et al., 2014; Xie et al., 2021). Xylose was chosen as a simple carbon source for control cultures, aiming to avoid endoplasmic reticulum stress-related issues, caused by high glucose concentrations (Heimel,

2015). However, the high number of shared proteins among the two conditions implies that xylose might act as an inducer of many LCB-degrading enzymes. The ten most abundant proteins in corn stover secretomes include two laccases, two CBM-containing cellobiohydrolases, two CBM-containing GH10 xylanases, one unknown protein, one AA3-AA8 cellobiose dehydrogenase, and one CBM-containing amylase (supplementary excel file 1). This reflects a rather balanced production of the main enzymes for the degradation of each LCB component, namely lignin, cellulose and xylan, together with starch, and it could serve as an indication that *A. biennis* does not resemble preferential lignin degraders, such as some *Pleurotus* species (Fernández-Fueyo et al., 2016; Valadares et al., 2019), but rather white-rot fungi attacking all LCB components simultaneously, such as *I. lacteus* (Salvachúa et al., 2013) and *P. chrysosporium* (Arntzen et al., 2020). *A. biennis* was previously shown to strongly express xylanases, cellulases and β-glucosidases in response to lignocellulosic substrates (McGregor et al., 2022). Based on KEGG classification (Fig. S1a) the metabolic pathways expressed in corn stover were mainly associated with carbohydrates and to a lesser extent with complex lipids.

Protein intensities of shared proteins were compared with Students t-test, resulting in 35 upregulated and 32 downregulated proteins found in corn stover cultures (Fig. S1b). Among the ten proteins with the highest fold change are a FAD oxidase, four glycoside hydrolases (GHs; two GH10, one GH115, one GH74), an aldose/glucose epimerase (EC. 5.1.3.3), a cellobiose dehydrogenase containing an iron reductase domain (AA3_1+AA8), and a protein of unknown function that lacks any Interpro domains. The 235 proteins detected in corn stover constitute 19.95 % of the total predicted secretome of *A. biennis* and 50 % of its CAZy secretome, while 49.4 % of the detected proteins were CAZymes (Fig. S2). These numbers reflect the complexity of corn stover as carbon source, requiring multiple enzymatic activities, acting in concert, for its effective decomposition.

The enzymatic activities measured in the culture supernatants of *A. biennis* grown on corn stover and the proteins detected by LC-MS/MS were in good agreement. GH was the most frequent CAZy class, constituting 70 % of the detected CAZymes (Fig. 2a), which supports the hypothesis that the response of *A. biennis* to lignocellulosic substrates might deviate significantly from typical white-rot preferential lignin degraders. In Fig. 2b, proteins were classified based on the plant-cell wall component they act on and their function. Cellulase activity is corresponding to as many as 23 proteins, including cellobiohydrolases and endo-β-1,4-glucanases (belonging to CAZy families GH5_9, GH5_5, GH6, GH7 and GH131), as shown in Fig.2a. Mannanases were represented by nine sequences in the secretome, belonging to CAZy families GH5_7, 47, 92 and 125, although mannanase activity was not determined.

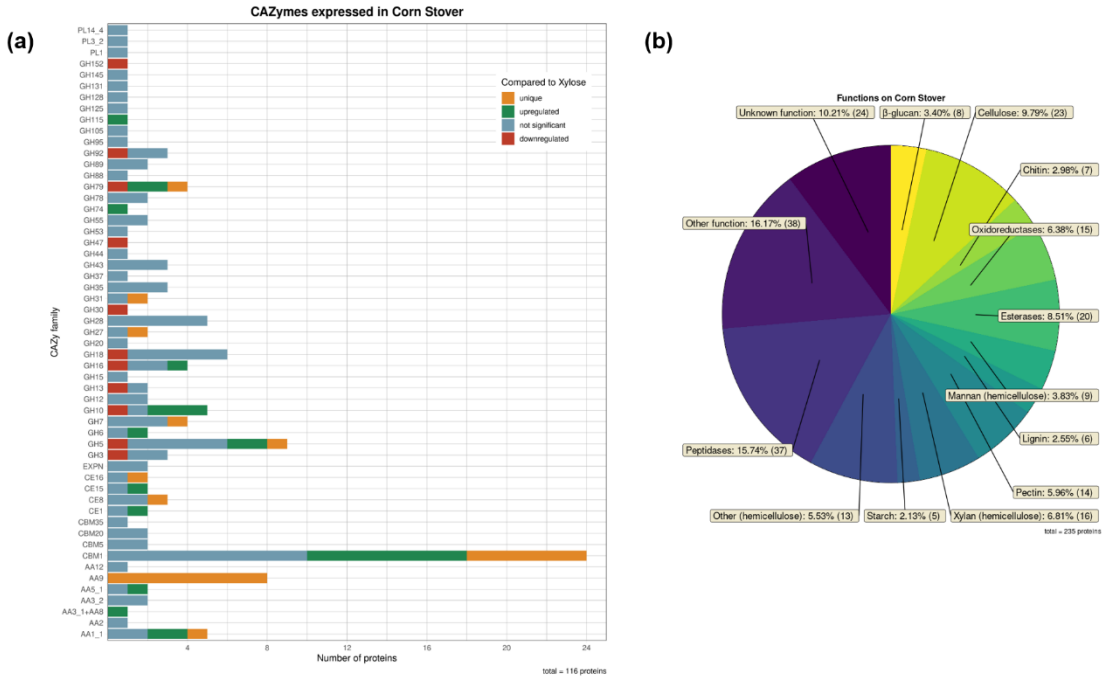

Fig. 2. (a) CAZymes detected in the *A. biennis* secretomes, after growth in corn stover. Their intensities are compared with their intensities after growth in xylose. CAZy classes: GH: glycoside hydrolases, CBM: carbohydrate-binding module, CE: carbohydrate esterase, PL:

polysaccharide lyase and AA: auxiliary activities (b) Relative abundance of protein functions detected in *A. biennis* secretome after growth in corn stover.

Significant endoxylanase activity can be attributed to five GH10 endoxylanases, some of which showed the highest upregulation in corn stover cultures, while GH11 and GH30 counterparts were absent from the secretome. Regarding xylan-active accessory hydrolases, three sequences were found from the GH43 family, two of them corresponding to α-L-arabinofuranosidases, despite the fact that α-L-arabinofuranosidase activity was not detected, and one to a β-galactosidase. Moreover, one β-glucuronidase of the family GH79 and one of the family GH115 were found, which was also one of the most upregulated enzymes in corn stover cultures, while those of the family GH67 were absent.

Twenty esterases were detected in the corn stover secretome, 9 of which act on carbohydrates. Two glucuronoyl esterases of the CE15 family were identified, despite the fact that their activity was not detected under the chosen assay conditions. Acetyl xylan esterase activity is reflected in the presence of two CE1 and two CE16 enzymes, and pectinesterases of the family CE8 were represented by three sequences.

CBM1 was the most frequently represented carbohydrate-binding module (CBM) and was carried by enzymes acting on cellulose, xylan and mannan. Of the twenty-nine CAZymes found bearing CBMs, three were AA9 LPMOs, four were carbohydrate esterases (1 CE16, 1 CE15 and 2 CE1) and twenty-three were GHs, belonging to families GH5-7, GH10, GH13, GH15, GH18, GH43, GH74, and GH131.

LPMOs of the AA9 CAZy family were expressed uniquely in corn stover. AA9 LPMOs are one of the most abundant families present in the secretomes, with eight sequences detected, from a total number of 17 sequences present in the genome (Fig. S2). This is consistent with previous studies (Arntzen et al., 2020; Couturier et al., 2015; Machado et al., 2020; Miyauchi

et al., 2017; Peña et al., 2021), and also with the preference of white-rot fungi for oxidoreductive mechanisms of LCB breakdown (Miyauchi et al., 2020; Schilling et al., 2020; Zerva et al., 2021b). The pivotal role of AA9 LPMOs for LCB breakdown by *A. biennis* is highlighted by the simultaneous presence of their redox partners: an upregulated cellobiose dehydrogenase AA3_1-AA8 (530992) and two AA3_2 (Fig. 2a). The co-secretion of CDHs and AA9 LPMOs has been evidenced in several fungi grown on cellulose and plant biomass (Machado et al., 2020; Miyauchi et al., 2017), while various studies report the co-regulation and co-secretion of AA3_2 with AA9 LPMOs (Berrin et al., 2017; Miyauchi et al., 2020), acting as electron donors (Bey et al., 2013), or through H_2O_2 production (Miyauchi et al., 2020). These results are consistent with the AAO activity measurements. Moreover, one upregulated glyoxal oxidase AA5_1 was detected, which can also act as an H_2O_2 provider for LPMOs (Miyauchi et al., 2020). Interestingly, despite the variety of hemicellulose-acting main and accessory enzymes present in *A. biennis* secretomes, the two xylan-acting AA14 LPMOs coded in the genome were not detected in the culture supernatants under the tested conditions (Fig. S2).

The high titers of laccase activity are justified by the five laccases detected, two of which are among the three most abundant proteins in the corn stover samples (supplementary excel file 1). The production of multiple laccase enzymes is a common property of white-rot fungi (Pezzella et al., 2013; Zerva et al., 2021a). Surprisingly, lignin-acting heme peroxidases (AA2 CAZy family) were represented by only one, moderately expressed, sequence in the secretomes (Fig. 2a), despite the presence of 14 sequences in the genome (Fig. S2). In our previous study, moderate peroxidase activity was demonstrated for this strain (~ 20 U L^{-1}) when grown in LCB- or phenol-containing substrate (Zerva et al., 2021b).

Feruloyl esterases (FAEs) are not included in the classification of CAZy database, with the exception of some enzymes belonging to CE1. In order to detect putative FAEs, Blast searches were performed against the proteome of *A. biennis*, using characterized fungal FAEs from different subfamilies as templates (Dilokpimol et al., 2016). This resulted in the identification of three putative FAEs (279665, 711062, and 716080). The best hit for 279665 was a xylanase-related FAE from *Aspergillus terreus* (*At*FAE-2, EAU39455.1, 48.99% identity) (Kumar et al., 2013), and for the other two a cinnamoyl esterase from *Talaromyces funiculosus* (FaeB, CAC14144; 46.2 % identity with 711062 and 47.0 % with 716080) (Kroon et al., 2000). All three proteins harbored a CBM1, were classified as CAZymes (279665 was classified as GH10, while 711062, and 716080 were annotated as CE1) and were present in both xylose and corn stover secretomes, while 279665 and 711062 were upregulated in corn stover. This highlights the need for improvement of the current annotation tools to include reliable FAE identification.

A. biennis was also found to secrete many CAZymes active on pectin (Fig. 2b), revealing the strain's adaptability to all plant biomass components. For pectin decomposition, hydrolases belonging to families GH28, GH78, GH88 and GH105 were found and complemented with methylesterases of the CE8 family and lyases of the families PL1 and PL3. The enzymatic removal of pectin is considered a necessary enzymatic 'pretreatment' of LCB, permitting the access of lignocellulolytic enzymes to their target polymers (Presley et al., 2018).

In Fig. 2b, sequences belonging to CAZy families with activity on β-1,3 or β-1,6 glucans were grouped together, since they most probably participate on β-glucans formation, which is a known constituent of the fungal cell wall (Ruiz-Herrera and Ortiz-Castellanos, 2019). Some strains are also reported to secrete β-glucans to their extracellular medium (Zerva et al.,

2017). Similarly, seven chitin-acting enzymes were grouped together due to their possible role in cell wall formation (Adams, 2004).

Hemicellulose-acting enzymes were the most abundant group, followed closely by peptidases (Fig. 2b). Induction of secreted peptidases has been previously reported for fungi grown on LCB material but their role in lignocellulose degradation is not clear (Adav et al., 2012; Arntzen et al., 2020). In addition to the above mentioned, annotated proteins, there are also many hypothetical proteins of unknown function, of which four are upregulated and seven are uniquely expressed in corn stover. These data are not uncommon for white-rot fungal secretomes: most previous studies report the existence of upregulated proteins with unknown function (Alfaro et al., 2020; Arntzen et al., 2020; Fernández-Fueyo et al., 2016; Poidevin et al., 2014), which could contribute to LCB degradation in unknown ways, and thus, constitute targets for further study. Therefore, the untapped potential of white-rot degraders for the discovery of novel enzymes with unique activities becomes evident. One such example is the recent study by Oates et al., (Oates et al., 2021), describing the omic-driven approach to the discovery of a novel enzyme with β-ether-cleaving oxidase activity.

From the above results, it can be concluded that all the necessary main and auxiliary activities for complete degradation of glucuronoarabinoxylan-containing substrates are present in *A. biennis* secretomes, revealing an impressive enzymatic machinery tailored to the breakdown of lignocellulosics.

3.3 Purification of AbiLac1 and AbiLac2, identification and protein analysis

The supernatant from the culture of *A. biennis* in corn stover was loaded in a Q- Sepharose column, in order to isolate new enzymes with laccase activity. From the fractionation of the culture supernatant, two separate peaks were visible, and the corresponding fragments were pooled and handled separately. SDS-PAGE electrophoresis of the two enzyme preparations

revealed that they correspond to two different proteins, which, in addition, were electrophoretically pure (Fig. 3a). Zymograms revealed that the enzymes present in both these bands were able to oxidize catechol (Fig. 3a). The purification yields were quite low (Table S1), indicating that the crude laccase activity measured probably corresponds to multiple laccase enzymes, which is in accordance with the results of the secretome analysis, where five proteins were found, corresponding to AA1_1 subfamily. According to SDS-PAGE gels, one of the bands corresponded to 63 kDa, which was named *Abi*Lac1 and the other to 70 kDa, which was named *Abi*Lac2 (Fig. 3a). Both these bands were cut from the gel, in-gel digested and the peptides were analyzed by LC-MS/MS in order to identify the sequences. The proteomic identification revealed that *Abi*Lac1 corresponds to the JGI Protein ID 805259 (unique sequence coverage 48.4 %), while *Abi*Lac2 corresponds to 811425 (unique sequence coverage 27.4 %) (supplementary excel file 2). Both proteins are among the three most abundant proteins in corn stover supernatants (supplementary excel file 1).

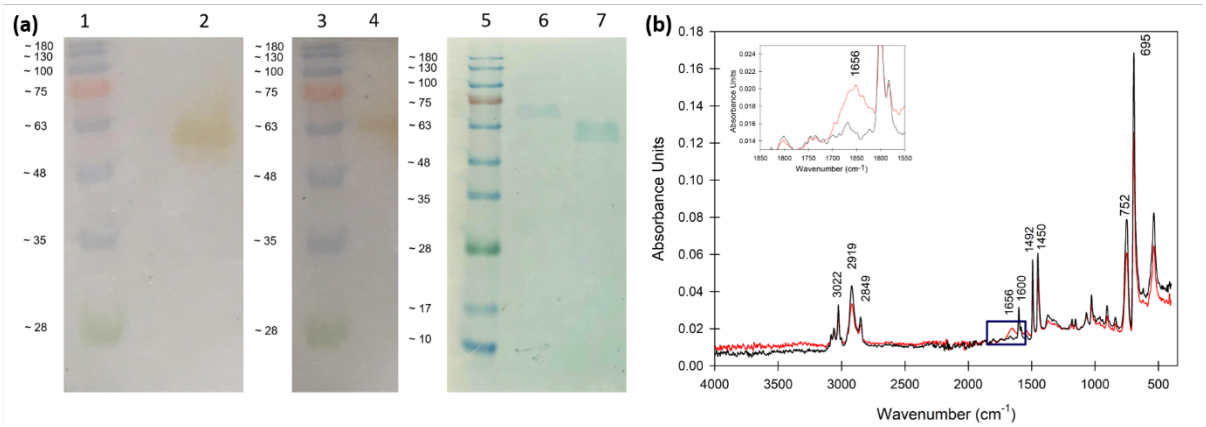

Fig. 3. (a) Zymograms and SDS-PAGE gels of the purified *Abi*Lac1 and *Abi*Lac2. Lanes 1, 3, 5: Protein marker, Lanes 2, 7: Purified *Abi*Lac1, Lanes 4, 6: Purified *Abi*Lac2. (b) FT-IR spectrum of control PS sample (*black line*) and enzymatically treated PS sample (*red line*)

Both laccases correspond to a theoretical MW of around 55 kDa (55.5 kDa for *Abi*Lac1 and 55.6 kDa for *Abi*Lac2), according to MaxQuant software (supplementary excel file 2), although their molecular weights were quite different on the SDS-PAGE gels. This could be due to different glycosylation patterns, which are rather common for fungal secreted enzymes. Indeed the existence of three *N*-glycosylation positions in both enzymes was predicted with the Net*N*Glyc 1.0 tool (Blom et al., 2004), but only five *O*-glycosylation positions in *Abi*Lac2 compared to eight positions in *Abi*Lac1 were calculated with the Net*O*Glyc 4.0 tool (Steentoft et al., 2013). Nonetheless, their sequence identity is 65.8 %; therefore, they probably do not correspond to isoforms of the same enzyme. Both contain signal peptide for secretion, and they have similar amino acid count, 517 for *Abi*Lac1 and 518 for *Abi*Lac2.

The sequences of both *Abi*Lac1 and *Abi*Lac2 are very similar to other white-rot fungal laccases with high redox potentials: Both show more than 60 % sequence identity with laccases from *Phlebia radiata* (Q01679, (Saloheimo et al., 1991)), *T. versicolor* (Lcc2, Q12718, (Piontek et al., 2002)), *Trametes villosa* (Lcc1, Q99044, (Yaver et al., 1996)) and *P. ostreatus* (POX2, Q12739, (Giardina et al., 1996)).

3.4 Characterization of AbiLac1 and AbiLac2

The maximal activity parameters were investigated for both *Abi*Lac1 and *Abi*Lac2. The maximum activity was observed in pH values 5.5 and 4.5 for *Abi*Lac1 and *Abi*Lac2, respectively, among the values tested (Fig. S3), while the activity of both laccases drops significantly above pH 7. The acidic pH maxima displayed by both laccases is a common trait of white-rot fungal laccases (Zerva et al., 2021a). The maximum activity was found in temperatures 50 ºC for *Abi*Lac1 and 60-65 ºC for *Abi*Lac2. At 30 ºC, *Abi*Lac1 retains almost 60 % of the maximum activity, while in the same temperature *Abi*Lac2 only retains about 20

% (Fig. S3). Both laccases show satisfactory thermostability in temperatures up to 40 ºC (Fig. S4); however, at 50 ºC *Abi*Lac1 lost quickly the activity, in contrast with *Abi*Lac2, which retained over 60 % of the initial activity after 8 h. This is in agreement with other basidiomycete laccases (Ai et al., 2015; Zerva et al., 2021a), displaying moderate thermostability properties.

Table S2 shows the results of the substrate screening performed for both *Abi*Lac1 and *Abi*Lac2. Both laccases display a remarkably broad substrate specificity, including the ability to oxidize hydroxybenzoles, methoxyphenols, amines, flavonoids, hydroxycinnamic and hydroxybenzoic acids, and cresols. Most importantly, both laccases were able to oxidize high redox potential compounds, such as vanillin and 3,4-dimethoxybenzyl alcohol, without the use of mediator. This property is uncommon, even for high redox potential laccases, such as the *T. versicolor* laccase (Bourbonnais and Paice, 1990). The substrate specificity of both *Abi*Lac1 and *Abi*Lac2 was tested in more detail for standard laccase substrates, and the results are shown in Table S3. *Abi*Lac1 shows significantly higher specific activity for all the tested substrates, compared with *Abi*Lac2. Both enzymes showed a preference for catechol over pyrogallol, which is also not common for laccases since pyrogallol contains an extra electron-donating -OH group, and therefore, is more prone to oxidation. Kinetic parameters were determined for both enzymes in ABTS and catechol, and the results are shown in Table S4. The kinetic parameters are similar for both laccases, which show a slight preference for ABTS over catechol. The kinetic constants calculated are comparable to other high-redox potential laccases from white-rot fungi, such as those from *T. versicolor* and *T. hirsuta* (Frasconi et al., 2010), *P. radiata* (Kaneko et al., 2009) and *P. ostreatus* (Palmieri et al., 1997).

3.5 Application of AbiLac1 in polystyrene degradation

Based on the remarkable biodegradation potential of *Abi*Lac1 on the tested pollutants, the enzyme was further applied on polystyrene powder, since lignocellulose -degrading enzymes, including laccases, manganese peroxidases and lignin peroxidases, have been reported to be involved in the radical-based degradation of synthetic polymers, especially polyolefins (Wei and Zimmermann, 2017). Polystyrene in particular, is mainly reported to degrade slowly via a mixture of scission and oxidation reactions under UV radiation stimuli (Billingham, 2002). Therefore, oxidoreductases producing free radicals, such as laccases, might be suitable for its depolymerization. Regarding styrene microbial assimilation, many microorganisms have been reported to convert styrene into phenylacetate, with the latter entering the TCA cycle. The predominant catabolic pathway includes the secretion of oxidoreductive enzymes, which oxidize the vinyl or, more rarely, the aromatic ring (Mooney et al., 2006). Supposing that polystyrene degradation follows the same catalytic mechanism, oxidoreductases producing free radicals, such as laccases, might be ideal for its controlled depolymerization.

The results of the enzymatic treatment are shown in Fig. S5 and Table S5. More specifically, the treatment of polystyrene powder with purified *Abi*Lac1, resulted in 6.4 ± 0.7 % mass loss of the polymer sample, while the number-average molecular weight ($\overline{M_n}$) and weight-average molecular weight ($\overline{M_w}$) decreased by 19.7 ± 2.9 and 7.7 ± 1.5 %, respectively, compared to the control. The simultaneous mass loss with average MW decrease reveal random chain scission reactions, along with end scission ones. Furthermore, the higher decrease % in $\overline{M_n}$ compared to $\overline{M_w}$, combined with the polydispersity index (PDI) increase, imply that *Abi*Lac1 activity affects the higher molecular weight chains at a lower extent, of which $\overline{M_w}$ is the representative value.

Apart from polymer average molecular weight decrease, IR spectrum of control and treated samples was also recorded (Fig. 3b). Spectrum peaks recorded were typical for polystyrene.

In more detail, characteristic peaks at 695 cm^{-1} and 752 cm^{-1} correspond to aromatic C-H bond, while peaks located at 1450, 1492 and 1600 cm^{-1}, indicate aromatic C=C bond stretching vibrations. Moreover, peaks at 2849 and 2919 cm^{-1} correspond to aliphatic C-H bond, while aromatic C-H stretching vibration peak appeared at 3022 cm^{-1} (Nayanathara et al., 2018).

Interestingly, almost all the peaks recorded in the enzymatically treated sample were less intensive than those of the control sample. Especially, the peak recorded at 695 cm^{-1} (aromatic C-H bond) decreased by 26 %, indicating that probably the enzyme oxidized carbon atoms located in benzene rings. Moreover, a new peak appeared at 1656 cm^{-1}, which could correspond to the insertion of carbonyl groups to the polymer, due to laccase oxidation (Nayanathara et al., 2018). Interestingly, despite the fact that fungi, such as basidiomycetes and ascomycetes, possess and secrete oxidative enzymes involved in lignocellulose degradation, none of them is known to substantially degrade polyolefins, including solid polystyrene, probably because of the substrate recalcitrance and the low redox potential of the secreted enzymes (Krueger et al., 2017; Tian et al., 2017).

Regarding enzyme-driven oxidative plastic degradation, only one previous study is available, employing the commercial laccase from *T. versicolor*, acting on water soluble PS sulfonate (PSS; (Krueger et al., 2015b)). In contrast to the present study, the action of *T. versicolor* laccase alone had no effect on the $\overline{M_n}$ of PSS, while the use of different mediators resulted in PSS depolymerization up to 25 %, based on the $\overline{M_n}$ determination (Krueger et al., 2015b). It should be mentioned that the redox potential of LCB degrading enzymes plays a key role in polymer degradation. Many of these enzymes can successfully attack lignin, which is more hydrophilic because of its functional groups. However, the high-energy bonds of synthetic polymers, makes them considerably more recalcitrant to the attack of LCB-degrading

enzymes (Krueger et al., 2015a). Considering all of the above, the superiority of *AbiLac*1 is apparent, as a potent, high redox potential laccase, and can be a promising candidate for synthetic polymer degradation, with highly inert chemical structure.

In conclusion, the present work demonstrates the potential of the white-rot fungus *A. biennis* for the production of biotechnologically relevant enzymes. As shown by secretomic analysis, the strain secretes a complete enzymatic arsenal for the depolymerization of all lignocellulose constituents. Moreover, the purification and characterization of two laccases from the cultures of *A. biennis* revealed their broad substrate specificity. Most importantly, *Abi*Lac1 was shown to be able to degrade PS, a very recalcitrant plastic polymer, without the use of mediators or pretreatment of the material.

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Declaration of competing interests

The authors declare no competing interests.

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Figure legends

Fig. 1. (a) Time course profile of the enzyme activity during the growth of *A. biennis* in corn stover. *Black circles*: laccase, *white circles*: xylanase, *black diamonds*: total esterase, measured with pNPhA. (b) Hydrolysis of pretreated substrates by the secreted enzymes of *A. biennis* after growth in corn stover. *Black bars*: pretreated corn stover, *grey bars*: pretreated beechwood.

Fig. 2. (a) CAZymes detected in the *A. biennis* secretomes, after growth in corn stover. Their intensities are compared with their intensities after growth in xylose. CAZy classes: GH: glycoside hydrolases, CBM: carbohydrate-binding module, CE: carbohydrate esterase, PL: polysaccharide lyase and AA: auxiliary activities (b) Relative abundance of protein functions detected in *A. biennis* secretome after growth in corn stover.

Fig. 3. (a) Zymograms and SDS-PAGE gels of the purified *Abi*Lac1 and *Abi*Lac2. Lanes 1, 3, 5: Protein marker, Lanes 2, 7: Purified *Abi*Lac1, Lanes 4, 6: Purified *Abi*Lac2. (b) FT-IR spectrum of control PS sample (*black line*) and enzymatically treated PS sample (*red line*)