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1 **Activity-Based Protein Profiling Reveals Dynamic Substrate-**
2 **Specific Cellulase Secretion by Saprotrophic Basidiomycetes**

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5 **Keywords:** activity-based probe, cellulase, filamentous fungi, secretome, enzyme secretion, kinetics,
6 fluorescence, cyclophellitol

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14

15 **Abstract**

16 **Background.** Fungal saccharification of lignocellulosic biomass occurs concurrently with the secretion of
17 a diverse collection of proteins, together functioning as a catalytic system to liberate soluble sugars from
18 insoluble composite biomaterials. How different fungi respond to different substrates is of fundamental
19 interest to the developing biomass saccharification industry. Among the cornerstones of fungal enzyme
20 systems are the highly expressed cellulases (*endo*- β -glucanases and cellobiohydrolases). Recently, a
21 cyclophellitol-derived activity-based probe (ABP-Cel) was shown to be a highly sensitive tool for the
22 detection and identification of cellulases.

23 **Results.** Here we show that ABP-Cel enables *endo*- β -glucanase profiling in diverse fungal secretomes. In
24 combination with established ABPs for β -xylanases and β -D-glucosidases, we collected multiplexed in-gel
25 fluorescence activity-based protein profiles of 240 secretomes collected over ten days from biological
26 replicates of ten different basidiomycete fungi grown on maltose, wheat straw, or aspen pulp. Our
27 results reveal the remarkable dynamics and unique enzyme fingerprints associated with each species-
28 substrate combination. Chemical proteomic analysis identifies significant arsenals of cellulases secreted
29 by each fungal species during growth on lignocellulosic biomass. Recombinant production and
30 characterization of a collection of probe-reactive enzymes from GH5, GH10, and GH12 confirms that
31 ABP-Cel shows broad selectivity towards enzymes with *endo*- β -glucanase activity.

32 **Conclusion.** Using small-volume samples with minimal sample preparation, the results presented here
33 demonstrate the ready accessibility of sensitive direct evidence for fungal enzyme secretion during early
34 stages of growth on complex lignocellulosic substrates.

35 **Keywords:** cellulase, glycoside hydrolase, activity-based protein profiling, cyclophellitol, basidiomycete,
36 biomass, secretome, fluorescence, enzyme identification, *Pichia pastoris*.

37 **Introduction**

38 The diversity of biomass sources, containing different composition of various polysaccharides
39 such as hemicelluloses (1) and pectins (2), presents a challenge to saprotrophs. The organism must
40 possess the right combination of enzyme systems and molecular logic to efficiently sense and degrade
41 the various linkages holding the material together. Identifying the right saprotrophic organism(s) to
42 degrade industrially available biomass presents a match-making challenge in bioprocess development. It
43 is clear that no single biomass-degrading organism is proficient at digesting all types of biomass, and
44 that a variety of species will be needed to facilitate the utilisation of the various agricultural biomass
45 streams that are available today (3,4). Tools to rapidly screen different fungi for their ability to recognise
46 and grow on distinct complex carbohydrate-based substrates, particularly broadly accessible tools
47 amenable to efficient small-scale enzyme detection and identification, are needed to enhance enzyme
48 discovery and species characterisation.

49 Lignocellulosic biomass is a highly variable complex composite material assembled from non-
50 carbohydrate and carbohydrate polymers, including cellulose, hemicelluloses (primarily β -xylans, β -
51 mannans, and non-cellulosic β -glucans), pectins, and lignin (1,5–7). The carbohydrate components of
52 this biomass represent the bulk of the chemical potential energy available to saprotrophic organisms.
53 Thus, saprotrophs produce large arsenals of carbohydrate-degrading enzymes when growing on such
54 substrates (8–10). These arsenals typically include polysaccharide lyases, carbohydrate esterases, lytic
55 polysaccharide monooxygenases (LPMOs), and glycoside hydrolases (GHs) (11). Of these, GHs and
56 LPMOs form the enzymatic vanguard, responsible for generating soluble fragments that can be
57 efficiently absorbed and broken down further (12).

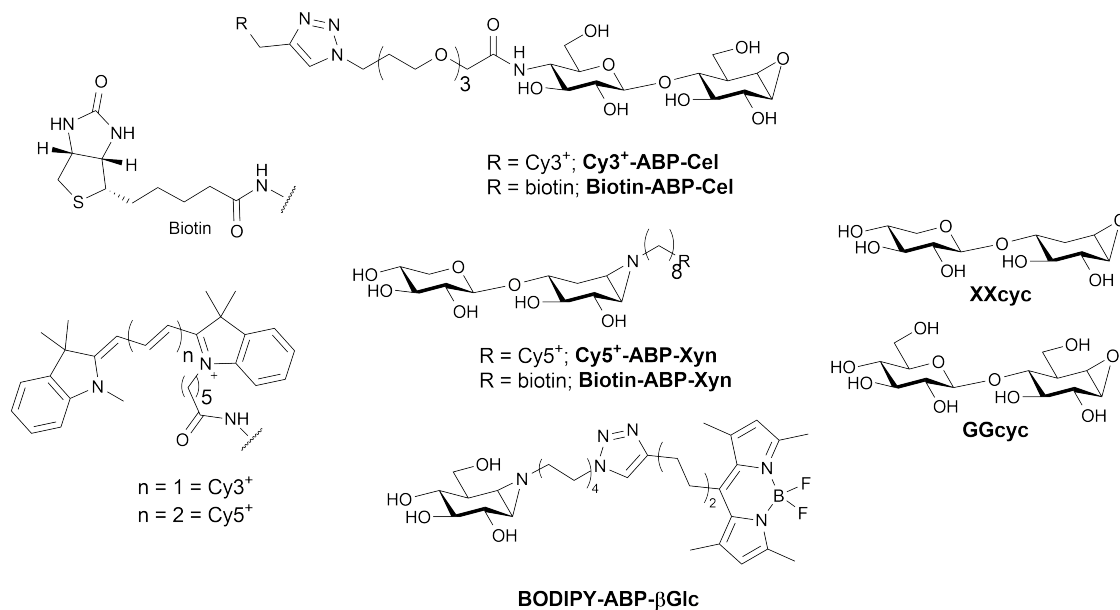
58 The identification, usually *via* bioinformatic analysis of comparative transcriptomic or proteomic
59 data, of carbohydrate-active enzymes (CAZymes) that are expressed in response to specific biomass

60 substrates is an essential step in dissecting biomass-degrading systems. Due to the underlying molecular
61 logic of these fungal systems, detection of carbohydrate-degrading enzymes is a useful indicator that
62 biomass-degrading machinery has been engaged (9). Such expression behaviour can be hard to
63 anticipate and methods of interrogation generally have low throughput and long turn-around times.
64 Indeed, laborious scrutiny of model fungi has consistently shown complex differential responses to
65 varied substrates (13–15). Much of this complexity still remains obscure, presenting a hurdle in
66 saccharification process development (16). In particular, while many ascomycetes, particularly those
67 that can be cultured readily at variable scales, have been investigated in detail (17,18), only a handful of
68 model organisms from the diverse basidiomycetes have been studied, with a focus on oxidase enzymes
69 (19,20).

70 Made possible by the recent sequencing of various basidiomycete genomes (21,22), activity-
71 based protein profiling (ABPP) offers a rapid, small-scale method for the detection and identification of
72 specific enzymes within the context of fungal secretomes (23,24). ABPP revolves around the use activity-
73 based probes (ABPs) to detect and identify specific probe-reactive enzymes within a mixture (25). ABPs
74 are covalent small-molecule inhibitors that contain a well-placed reactive warhead functional group, a
75 recognition motif, and a detection handle (26). Cyclophellitol-derived ABPs for glycoside hydrolases
76 (GHs) use a cyclitol ring recognition motif configured to match the stereochemistry of an enzyme's
77 cognate glycone (27,28). They can be equipped with epoxide (29), aziridine (30), or cyclic sulfate (31,32)
78 electrophilic warheads, which all undergo acid-catalyzed ring-opening addition within the active site.(33)
79 Detection tags have been successfully appended to the cyclitol ring (29) or to the (N-alkyl)aziridine, (34)
80 giving highly specific ABPs. The recent glycosylation of cyclophellitol derivatives has extended such ABPs
81 to targeting retaining *endo*-glycanases, opening new chemical space. ABPs for *endo*- α -amylases, *endo*- β -
82 xylanases and cellulases (encompassing both *endo*- β -glucanases and cellobiohydrolases) have been

83 developed (35–37). Initial results with these probes have demonstrated that their sensitivity and
84 selectivity is sufficient for glycoside hydrolase profiling within complex samples.

85 To profile fungal enzymatic signatures, we sought to combine multiple probes that target
86 broadly distributed biomass-degrading enzymes (Figure 1). Cellulases and β -glucosidases are known to
87 be some of the most broadly distributed and most highly expressed components of enzymatic plant
88 biomass-degrading systems (11,38). Among the hemicellulose-degrading enzymes, GH10 xylanases are
89 broadly distributed, being found in every kingdom of life (5,39). Using validated probes targeting
90 cellulases, xylanases, and β -glucosidases, we report here the results from a rapid, small-scale multiplex
91 in-gel fluorescence-based ABPP assay. We demonstrate the ability of this assay to detect and identify
92 diverse enzymes that are secreted by a collection of 10 different basidiomycete fungi over time under
93 different growth conditions. Recombinant production of a collection of detected GH family
94 representatives shows correlation between probe reactivity and enzyme activity.



95
96 Figure 1: Structures and given names (bold) of probes and inhibitors used in this study.

97

98 **Results and Discussion**

99 *Preparation of basidiomycete secretomes*

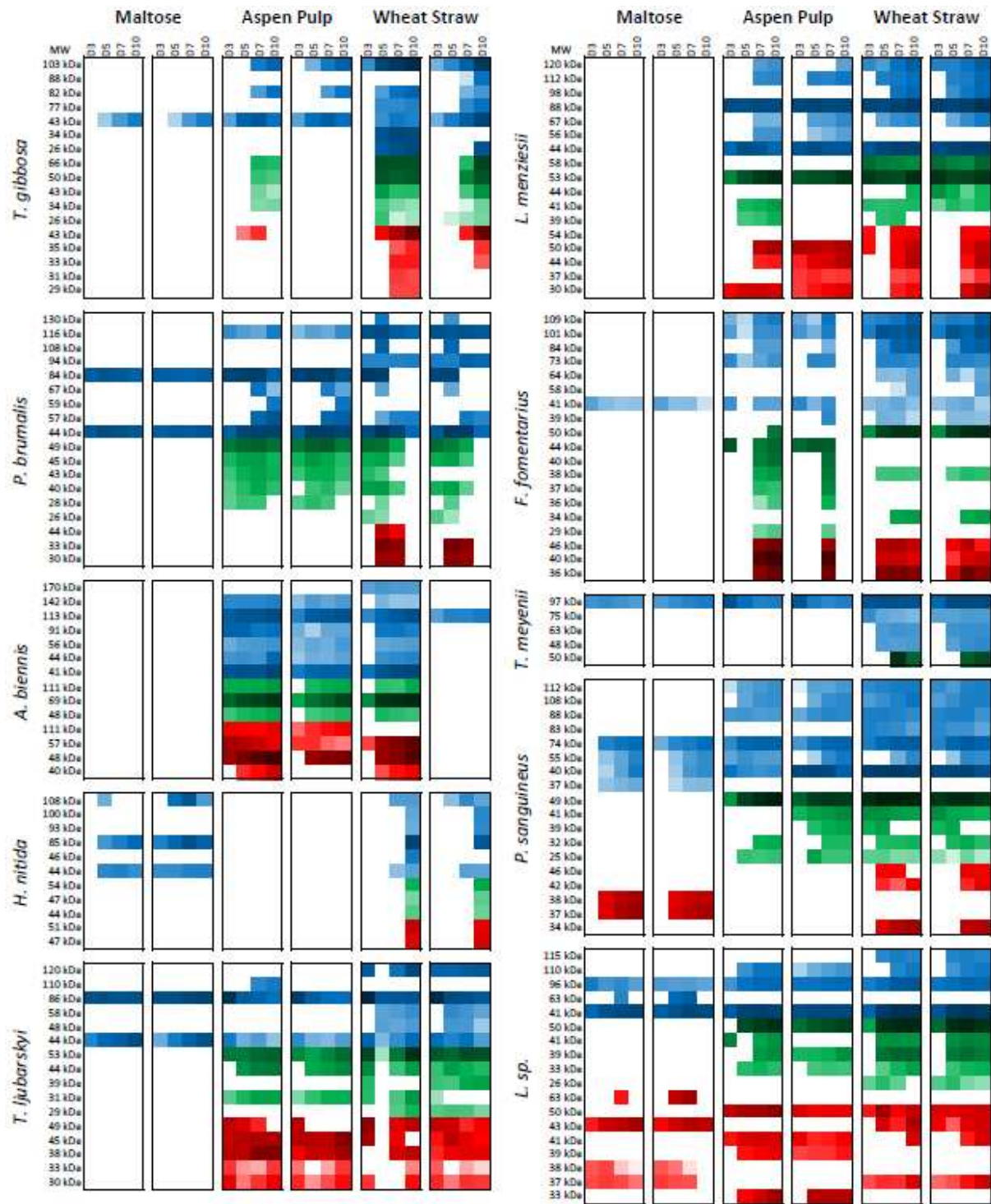
100 Ten fungi were selected from the CIRM (“Centre International des Ressources Microbiennes”)
101 collection for profiling on the basis that are all known basidiomycete saprotrophs with sequenced
102 genomes (supplementary table 1). These included *Abortiporus biennis* (40), *Fomes fomentarius* (41),
103 *Hexagonia nitida*, *Leiotrametes menziesii* (42), *Polyporus brumalis* (43), *Trametes ljubarskyi* (44),
104 *Trametes gibbosa* (45), *Pycnoporus sanguineus* (46), *Leiotrametes* sp. 1048 (47), and *Trametes meyenii*
105 (47). Annotated genomes for each of these are available publicly through JGI Mycocosm (22).

106 Each fungus was cultured in a general minimal medium (see methods) supplemented with either
107 wheat straw (an abundant monocot lignocellulosic substrate rich in arabinoxylan) (48), aspen pulp (a
108 woody dicot biomass rich in glucuronoxylans and mannans) (49,50), or maltose (a control substrate
109 which does not induce biomass-degrading enzyme production (21)). The use of wheat straw and aspen
110 pulp facilitates comparison to previous integrative omics studies of basidiomycetes (46,51). Duplicate
111 time-course cultures were grown from individual mycelial starter cultures for 10 days to give ample time
112 for substrate recognition and digestion. The use of small, baffled flasks shaking at 120 rpm minimized,
113 but likely did not eliminate, mechanical cell lysis while promoting aeration. Secretomes collected at days
114 3, 5, 7, and 10 from maltose and aspen-grown cultures developed minimal colour over time, varying
115 from clear to light yellow. Wheat straw cultures developed strong yellow-to-brown colour over the
116 course of culturing, generally giving a denser, more aggregated mycelium.

117 *Fluorescence-based secretome profiling*

118 The inclusion of maltose in the complex substrate cultures allows rapid early expansion of
119 biomass, typically being consumed over the course of the first two days of culture (21). Thus, it was
120 expected that day 3 secretomes would be dominated by early oxidative enzymes as observed previously

121 (8,52) and that cellulose- and hemicellulose-degrading enzymes would be detected at later time points,
122 with increasing signal over time. Incubation of each of our 240 secretome samples (centrifuged and
123 filtered) with the triplex probe mixture for 1 hour followed by SDS-PAGE separation and fluorescence
124 imaging yielded a collection of visual species-specific enzyme profiles (Figures S1-10). Qualitative
125 inspection of these images reveals clear signatures of biomass recognition in most cases, with
126 differential glycoside hydrolase expression between each substrate and significant variation over time.
127 Surprisingly, the gel images clearly show the presence of low levels of cellulase secretion following only
128 three days of culturing in many cases, particularly *A. biennis*, *P. brumalis*, and *L. menziesii*. Background
129 interference can be seen in the Cy5⁺ channel in many of the wheat straw secretomes. This interference
130 correlates with the darkness of secretome colour, visible as a tan-coloured streak in the gel following
131 separation of some of the most darkly coloured, notably *P. brumalis*, wheat straw-grown secretomes.
132 We were not able to remove this material via selective precipitation or adsorption (e.g. using PVPP)
133 without losing proteins of interest, so xylanase detection was partially obscured in some cases. To
134 quantify relative enzyme levels and provide good estimates of enzyme molecular weight, fluorescent
135 lane profiles were determined for each channel and peaks were integrated with subtraction of a rolling
136 ball baseline. Integrated peak intensities were then plotted over time on a log scale to show enzyme
137 concentration variation for each detected band across ~3.5 orders of magnitude (Figure 2).



138

139 Figure 2: Quantified ABP fluorescence of bands detected following SDS-PAGE of basidiomycete

140 secretomes stained with BODIPY-ABP-βGlc (blue), Cy3⁺-ABP-Cel (green), and Cy5⁺-ABP-Xyn (red). The

141 intensity of the colour of each square represents the integrated fluorescence for the observed bands on

142 a log scale from white (<100,000 counts) to full colour (at ~4,000,000 counts) to black (>250,000,000
143 counts). The apparent molecular weight of the observed band is given to the left of each row of squares.
144 Data are organized by species (abbreviated to the left of each collection of squares) and by substrate
145 (top). Two sets of four time points (D3, D5, D7, and D10, noted above each column of squares) represent
146 two biological replicates measured for each substrate-species combination.

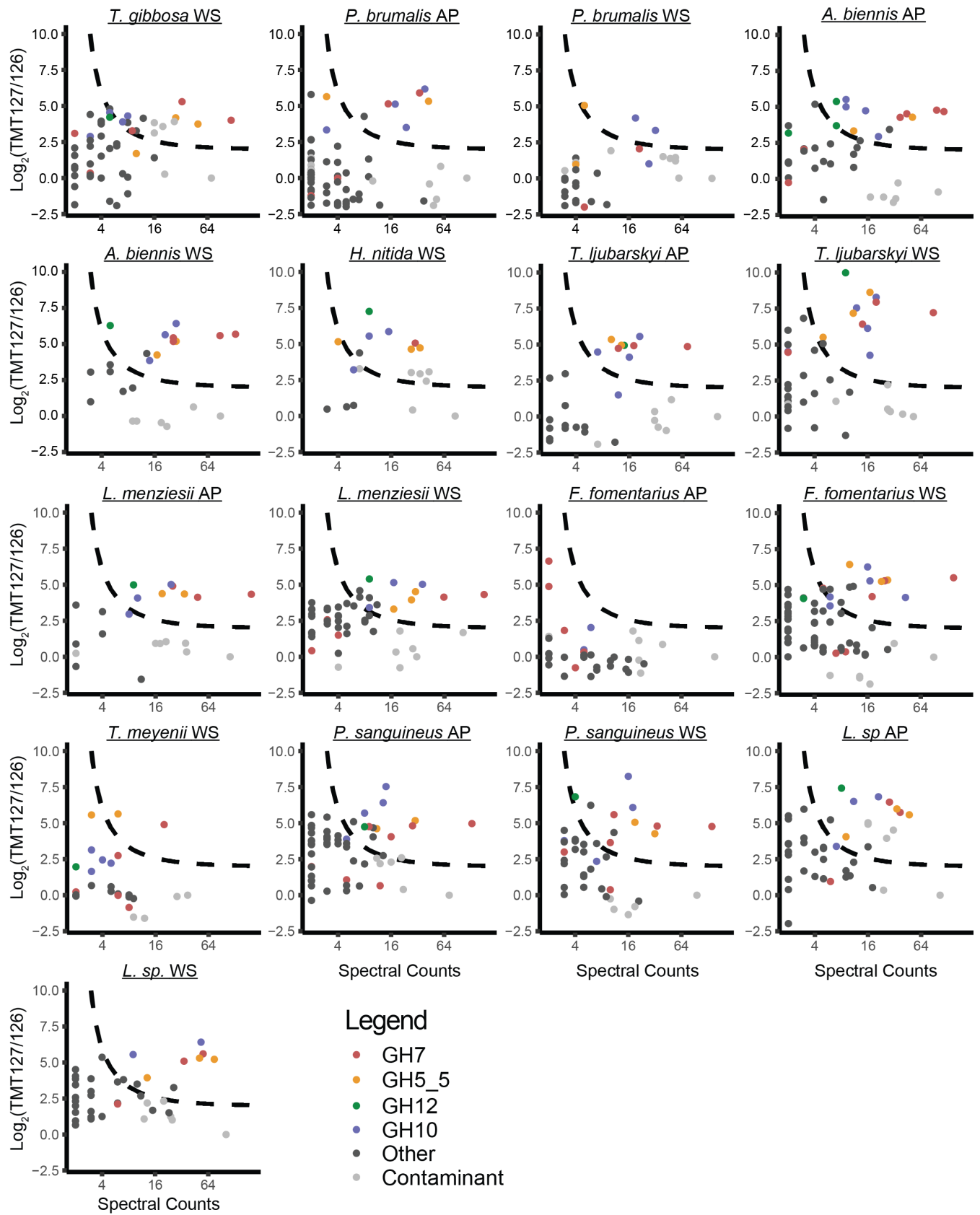
147
148 Each species showed a distinct pattern of behaviour. *T. gibbosa* took 5-7 days to initiate enzyme
149 secretion. Following this extended lag phase, it showed a strong response to wheat straw, producing an
150 array of apparent cellulases, glucosidases, and xylanases. Its response to aspen was much more muted,
151 with exceptionally weak cellulase expression in one replicate and weak glucosidase expression in both.
152 *P. brumalis* recognized both substrates rapidly, showing significant cellulase expression at 3 days.
153 Interestingly, cellulase and glucosidase levels peaked at day 5-7 in all cultures, with xylanases only
154 detected in the wheat straw culture. Strikingly, the *P. brumalis* secretome decayed rapidly following its
155 day 5-7 peak. *A. biennis* showed clear strong recognition of both substrates after 3-5 days, secreting
156 xylanases, cellulases, and glucosidases. A major xylanase band at ~57 kDa was lost over time in the
157 aspen culture but increased over time in the wheat straw culture. An apparent xylanase band at 111 kDa
158 may be a β -xylosidase, given the high molecular weight of GH3 xylosidases and the known tendency of
159 this probe to cross-react (35). *H. nitida* did not appear to strongly recognize any of the substrates,
160 though a mixture of enzymatic signatures could be detected in the wheat straw cultures at the 10 day
161 mark, suggesting that longer culturing is needed for the full development of *H. nitida* under these
162 conditions. *T. ljubarskyi* showed remarkably complex behaviour. When grown on aspen pulp, it rapidly
163 produced an array of xylanases, some of which grew over time while others decayed. Cellulase levels
164 were low, but consistently rose. When grown on wheat straw, it rapidly produced a high level of

165 cellulases and xylanases. This was then followed by a rapid loss of most of these enzymes, correlated
166 with a notable increase in background fluorescence in the Cy5⁺ channel. Slow background decay and
167 restoration of most of these hydrolases followed, with the two replicates showing different enzyme
168 levels. We speculate that this is indicative of variable growth behaviour, oscillating between oxidative
169 and hydrolytic catabolism. *L. menziesii* showed rapid wheat straw recognition and slower aspen
170 recognition, characterized by low levels of xylanase, and high levels of cellulase and glucosidase
171 production. Interestingly, the higher molecular weight cellulase band was only observed during growth
172 on wheat straw. *F. fomentarius* recognized substrate rapidly, producing detectable cellulase and
173 glucosidase at day 3. Like *T. ljubarskyi*, it showed the remarkable ability to temporarily eliminate its
174 diverse complement of secreted glycoside hydrolases, particularly evident in the aspen cultures at day 5
175 in the first replicate and day 10 in the second replicate. The wheat straw cultures showed more
176 consistent behaviour, with a steady increase in xylanase, cellulase, and glucosidase levels over time. *T.*
177 *meyenii* did not appear to recognize the aspen pulp, but did recognize the wheat straw after 7 days,
178 expressing a high level of a singular cellulase and a small host of apparent glucosidases. *P. sanguineus*
179 produced the most diverse complement of enzymes, producing high levels of cellulase, particularly after
180 5 days. Diverse glucosidases and xylanases were also detected, particularly in the wheat straw
181 secretome. *P. sanguineus* was the only organism that produced an apparent xylanase in the maltose
182 culture, though this was a different molecular weight from those detected during growth on biomass.
183 Similarly, *Leiotrametes sp.* 1048 produced consistently high levels of cellulase and a diverse collection of
184 xylanases and glucosidases following 5 days of growth on either wheat straw or aspen pulp substrates.
185 Together, these results show the diversity of fungal strategies for biomass degradation and highlights
186 the challenge of identifying apparently productive fungus-substrate interactions. Taking rising cellulase
187 and xylanase titres as an indicator of a productive interactions between fungus and substrate, we can

188 see clear preferences of *T. gibbosa*, *L. menziesii*, *Leiotrametes sp.* 1048, and *P. sanguineus* for wheat
189 straw, while *T. ljubarskyi* and *A. biennis* showed an apparent preference for aspen pulp.

190 *Chemical proteomic identification of putative cellulases*

191 Interested in the identities of the apparent cellulases in the basidiomycete secretomes and the
192 identification of novel *endo*- β -glucanases, we used the biotinylated derivative of ABP-Cel (Biotin-ABP-
193 Cel) to label the cellulases found in the day 10 secretomes. Labelled enzymes (and a negative control
194 treated with vehicle) were pulled down from 2 mL of secretome using streptavidin beads and peptides
195 were generated *via* on-bead digestion using trypsin. To assist in the filtration of background signals,
196 while facilitating the throughput needed to analyze 17 samples using the relatively small sample volume
197 available, we labelled negative control samples with TMT²-126 and probe-treated samples with TMT²-
198 127. These were mixed 1:1 prior to separation and analysis. Thus, orthogonal signals of spectral counts
199 (indicative of overall abundance in the pulldown) and TMT ratios (indicative of selective enrichment in
200 the pulldown) were collected for each identified protein in a single 1 hour run. This enabled the
201 identification of both major and minor probe-reactive secretome components (figure 2, supplemental
202 files 1-10). Contaminating proteins common to both probe-treated and negative control samples (i.e.
203 trypsin, streptavidin) were generally found to have TMT ratios close to 1, indicating that a TMT 127/126
204 ratio close to 1 is a robust basis on which to exclude background signals.



206 Figure 3: CAZymes identified in the pulldown from the day 10 secretomes using biotinylated ABP-Cel.
207 Each plot shows a point for each protein detected (minimum 2 peptides at 1% FDR) in the day 10
208 secretome listed above the plot (AP = aspen pulp, WS = wheat straw). The x axis is the number of
209 spectra collected for peptides assigned to each protein (\log_2 scale) and the y-axis is the \log_2 TMT127/126
210 ratio (127 = labelled, 126 = vehicle control) calculated by Scaffold for the protein, normalised using the
211 TMT ratio of streptavidin. Points corresponding to putative retaining *endo*- β -glucanases/xylanases are
212 coloured according to glycoside hydrolase family, other proteins are coloured dark grey. Detected
213 contaminants not derived from the fungi under study (e.g. streptavidin, trypsin, keratins) are coloured
214 light grey. A hyperbolic hit cut-off line is shown as a black dashed line with lower limits at 2 spectral
215 counts and a 127/126 ratio of 4. Points found above this line are both well-detected in the pulldown
216 sample and depleted in the vehicle control. Source data (Excel format) can be found in the
217 supplementary information. Plots were prepared using ggplot2.

218

219 In all cases, the strongest hits from ABP-Cel were putative cellulases or xylanases from families
220 GH7, GH5_5, GH10, and GH12. The detected enzymes represent a majority of the total predicted GH5_5
221 (85% of the annotated genes across all 10 fungi), and GH7 (83% of annotated genes) cellulases
222 annotated in the genomes of each fungus (table 1), indicating that this method is suitable for the
223 broadly specific detection of core cellulases. Similarly, our method achieved reasonably comprehensive
224 detection of annotated GH10 enzymes, identifying 66% of the annotated genes. GH12 enzymes,
225 however, gave a significantly lower detection rate (35% of annotated genes). It remains unclear if this is
226 a result of a low levels of GH12 expression, a general lack of reactivity, reduced detection efficiency due
227 to low molecular weights, specificity for certain GH12 enzymes or a combination of these factors. All of
228 the GH7 enzymes detected are close homologues of known, and well-characterized, cellobiohydrolases

229 (53,54). Similarly, the GH5_5 enzymes that were detected are homologues of well-known *endo*- β (1,4)-
 230 glucanases that show specificity towards linear glucans such as carboxymethylcellulose (CMC, an
 231 artificial soluble cellulose derivative) or mixed-linkage β -glucan (bMLG) (55,56). GH10 enzymes are only
 232 known to be *endo*- β (1,4)-xylanases, though weak *endo*- β (1,4)-glucanases activity has been reported in
 233 the family (57). GH12 enzymes have been reported to have variable specificities, recognizing linear or
 234 branched (i.e. xyloglucan) β (1,4)-glucans (58,59). This divergent substrate specificity within GH12 may
 235 explain the low number of detected GH12 enzymes, though low levels of GH12 expression during
 236 growth on wheat straw and aspen pulp, reduced detection efficiency due to their low molecular weight,
 237 or generally poor reactivity of the probe with GH12 enzymes may also contribute.

238

Enzyme Family	<i>Trametes gibbosa</i>	<i>Polyporus brumalis</i>	<i>Abortiporus biennis</i>	<i>Hexagonia nitida</i>	<i>Trametes I jubarskyi</i>	<i>Leiotrametes menziesii</i>	<i>Fomes fomentarius</i>	<i>Trametes Meyenii</i>	<i>Pycnoporus sanguineus</i>	<i>Leiotrametes sp 1048</i>
GH5_5	3/3	2/3	2/3	3/3	3/3	3/3	3/3	2/4	3/4	3/3
GH7	3/4	2/3	4/4	1/4	3/3	3/3	3/4	4/4	3/3	3/3
GH10	4/6	4/6	4/6	3/5	5/6	4/6	5/7	4/7	4/6	3/6
GH12	1/5	0/3	3/3	1/4	1/3	1/3	2/7	1/3	2/3	1/3

239 **Table 1.** Detected hits from pulldown experiments compared to the total number of GH family members
 240 in each fungal genome. Each cell contains (the number of detected GH family members)/(the number of
 241 annotated GH family members in the genome).

242

243 Several unexpected proteins also gave significant hits. The most abundant and consistently
 244 detected of these were members of GH5_7 (11), a well-characterized subfamily of *endo*- β -mannanases.
 245 Other less frequent marginal detections included a handful of enzymes from GH families 6 (invertin), 28
 246 (invertin), 74 (invertin), and 152 (thaumatin-like), as well as a glutamic protease (eqolisin-like). These
 247 detection events may point to a weak broader non-specific reactivity with enzymes containing activated

248 glutamate residues. However, such non-specific reactivity is not in-line with general epoxide reactivity,
249 which favours cysteine residues (60). Larger datasets are needed to explore the significance and
250 consistency of the marginal detections observed in pulldown experiments using ABP-Cel.

251 Comparing the predicted molecular weights (MWs) of proteomic hits with observed bands on
252 SDS-PAGE presents a challenge due to the known tendency of fungi to glycosylate or proteolyze
253 secreted protein and the complexity of the band patterns on each gel. However, we attempted some
254 inference considering both expected correlations between band intensity and spectral count (SC), and
255 between theoretical and apparent MWs. Considering the case of the *P. sanguineus* wheat straw
256 secretome, we observed minor bands at 25, 32 and 41 kDa and a strong broad band at 49 kDa. The only
257 hit close to 25 kDa, is a GH12 weak hit (4 SCs) with a predicted MW of 26 kDa. No hit could be readily
258 matched to the observed 32 kDa band, perhaps indicating that it was either undetected or a result of
259 proteolysis. The dominant 49 kDa band matches the theoretical MW of a GH7 cellobiohydrolase, which
260 gave the single strongest signal observed in the proteomic data (142 SCs). However, considering the
261 remainder of the observed hits, most of these are not apparently resolved on SDS-PAGE. We conclude
262 from this that analysis of in-gel fluorescence bands is generally not sufficient to assess the diversity of
263 the often microheterogeneous *endo*- β (1,4)-glucanase components of basidiomycete secretomes,
264 necessitating routine chemical proteomic analysis for the assessment of molecular diversity. Alternative
265 separation techniques (e.g. liquid chromatography, capillary electrophoresis) may offer the resolution
266 needed to better distinguish enzymes with such similar apparent molecular weights.

267 *Testing enzyme specificity via recombinant production*

268 To assess the specificity of ABP-Cel for cellulases, we sought to determine the true substrate
269 specificities of representatives of the detected enzyme clades. Towards this end, pure enzyme samples
270 were needed. Thus, we selected a GH5_5 enzyme (LsGH5_5A; 27 spectral counts (SCs), TMT ratio

271 (127/126) = 52), a GH10 enzyme (LsGH10A; 20 SCs, 127/126 = 93), a GH12 enzyme (TIGH12A; 10 SCs,
272 127/126 = 31), and a GH5_7 enzyme (LsGH5_7A; 3 SCs, 127/126 = 52) for recombinant production.
273 Homologues of all of these were detected as components above the cut-off in pulldowns from multiple
274 fungal species. Each sequence was codon-optimized for *P. pastoris*, synthesized and cloned into pPICZ α
275 with a C-terminal 6xHis tag, and native signal peptide replaced with the α -factor secretion tag. They
276 were transformed into *Pichia pastoris* X-33 and produced under methanol-induction in shake flasks,
277 giving high yields of electrophoretically pure enzymes (supplemental figure 11).

278 To establish a basis for an inhibition assay we measured hydrolytic activity towards 4-
279 methylumbelliferyl cellobioside (4MU-GG). LsGH5_5A, LsGH10A, and TIGH12A all showed detectable
280 hydrolytic activity towards 4MU-GG (supplemental table 2, supplemental figure 12), while LsGH5_7A did
281 not. As an initial test of specificity, we compared activity towards 4MU-GG and 4-methylumbelliferyl
282 xylobioside (4MU-Xyl2), finding no detectable activity towards 4MU-Xyl2 among LsGH5_5A and
283 TIGH12A, and a strong preferential activity towards 4MU-Xyl2 for LsGH10A (supplemental table 2). Using
284 4MU-GG as substrate, we measured inhibition of LsGH5_5A, LsGH10A and TIGH12A over time by
285 glucosyl- β (1,4)-cyclophellitol (36) (GGcyc) at inhibitor concentrations as high as 50 μ M under optimal
286 buffer conditions (see supplemental figures 13 and 14 for effects of buffer and pH on enzyme activity).
287 This revealed clear time-dependent inhibition of LsGH5_5A, TIGH12A, and LsGH10A by GGcyc
288 (supplemental figures 15-17) with similar performance constants (k_i/K_i , supplemental table 3), providing
289 an explanation for the comparable detections of GH5, GH10, and GH12 enzymes in the pulldown.
290 Comparison to inhibition with xylosyl- β (1,4)-xylocyclophellitol (35) (XXcyc) provided further evidence the
291 LsAA5_5A and TIGH12A are specific *endo*- β -glucanases while LsAA10A is a specific *endo*- β -xylanase
292 (supplemental table 3). The move from GGcyc to ABP-Cel somewhat reduced potency towards TIGH12A
293 compared to GGcyc and had no apparent impact on reactivity with LsGH5_5A. In contrast, Biotin-ABP-

294 Xyn bound to LsGH10A non-covalently with 21 nM affinity, but no covalent inhibition was discernable
 295 after 1 hour, similar to previously reported behavior among GH10 xylanases (35). Thus, the addition of
 296 Biotin-ABP-Xyn to a secretome-labelling reaction can serve as a way to “block” GH10 active sites, but
 297 does not efficiently label xylanases on the time-scales used in this assay, preventing pulldown and
 298 identification of xylanases using Biotin-ABP-Xyn.

299

<i>Enzyme</i>	bMLG	CMC	tXyG	wAX	cGM
<i>LsGH5_5A</i>	19±2	11±1	<0.01	<0.01	<0.01
<i>LsGH5_7A</i>	0.06±0.01	0.04±0.01	<0.01	<0.01	14±2
<i>LsGH10A</i>	<0.01	0.05±0.01	<0.01	8±1	<0.01
<i>TIGH12A</i>	20±2	13±1	0.04±0.01	<0.01	<0.01

300 **Table 2.** Enzyme specificity. Specific activity values (μmol/min/mg) measured for LsGH5A, LsGH5B,
 301 LsGH10A, and TIGH12A acting on 1 mg/mL barley mixed-linkage glucan (bMLG), carboxymethylcellulose
 302 (CMC), tamarind xyloglucan (tXyG), wheat arabinoxylan (wAX), or carob galactomannan (cGM)

303

304 To assess enzyme polysaccharide specificity, reducing end-based activity assays were performed
 305 with a panel of β-glucan, β-xylan, and β-mannan substrates (Table 2). TIGH12A showed strong activity
 306 towards CMC and bMLG with only weak xyloglucanase activity, suggesting that this is indeed a cellulase-
 307 type GH12. LsGH10A showed strong activity towards wheat arabinoxylan (wAX), with weak activity
 308 towards bMLG and CMC, confirming that it does have cellulase activity, though it is primarily a xylanase.
 309 LsGH5_7A showed dominant activity towards carob galactomannan (cGM), in line with previous
 310 observation that GH5_7 enzymes are β(1,4)-mannanases (61). LsGH5_7A also displayed weak activity
 311 against CMC and bMLG, a previously unreported phenomenon possibly rationalizing the observed weak
 312 hit in the pulldown. Finally, LsGH5_5A showed dominant activity towards CMC and bMLG with no

313 detectable xyloglucanase activity, confirming that it is a cellulase. Thus, we conclude that ABP-Cel is
314 selective towards enzymes that recognize glucans, allowing the identification of a list of probable
315 cellulases. However, detectable reactivity with ABP-Cel should not be taken as sufficient evidence to
316 assign enzyme specificity, as detected enzymes may be either *endo*-glucanases or *endo*-xylanases.

317 **Conclusions**

318 Here we have presented an ABPP-based method for the rapid detection of multiple cellulose-
319 and xylan-degrading glycoside hydrolases in fungal secretomes. This method enables time-resolved
320 studies of fungal enzyme secretion in response to lignocellulosic substrates using small-volume samples.
321 Applying this method to basidiomycete secretomes, we have shown that most of the fungi in this study
322 produce significant complements of cellulases, glucosidases, and xylanases in response to different
323 sources of lignocellulosic biomass. Furthermore, we have shown that the secreted enzyme complements
324 can vary significantly over time, being completely degraded and restored on the timescale of days. Using
325 chemical proteomic methods, we have identified a collection of putative cellulases and shown, through
326 recombinant production and characterization, that they do, in fact, possess *endo*-glucanase activity.
327 Despite this, we find that the major detected enzymes may either be *endo*-glucanases or *endo*-
328 xylanases. Thus, the function of enzymes identified using ABP-Cel should be assigned with consideration
329 of the functions of characterised homologues or supplemental functional assays of purified enzymes.
330 We expect that the development of improved ABPs for other *endo*-glycanases built on the ABP-Cel
331 architecture will enable ABPP-based specificity determination.

332

333 **Experimental**

334 All chemicals were purchased from Sigma unless otherwise specified.

335 *Design and synthesis of cyclophellitol-derived probes*

336 For multiplex fluorescent ABPP, three probes, each bearing a different fluorophore and a
337 different combination of recognition motif and reactive warhead, were used. JJB376, an established N-
338 alkyl aziridine probe bearing a BODIPY-FL (62) tag was used to label β -glucosidases (34). ABP-Xyn, an
339 established N-alkyl aziridine probe bearing a Cy5⁺ tag was used to label *endo*- β -xylanases (35). *Endo*- β -
340 glucanase probe CB644 was prepared through click modification of ABP-Cel with Cy3⁺ alkyne in place of
341 previously reported Cy5⁺ alkyne (36).

342 *Basidiomycete culture preparation and secretome collection*

343 The strains *Abortiporus biennis* BRFM 1215 (*A. biennis*), *Fomes fomentarius* BRFM 1323 (*F.*
344 *fomentarius*), *Hexagonia nitida* BRFM 1328 (*H. nitida*), *Leiotrametes menziesii* BRFM 1557 (*L. menziesii*),
345 *Polyporus brumalis* BRFM 985 (*P. brumalis*), *Trametes ljubarskyi* BRFM 957 (*T. ljubarskyi*), *Trametes*
346 *gibbosa* BRFM 952 (*T. gibbosa*), *Pycnoporus sanguineus* BRFM 902 (*P. sanguineus*), *Leiotrametes sp.*
347 BRFM 1048 (*L. sp.*) and *Trametes meyenii* BRFM 1361 (*T. meyenii*) were obtained from the CIRM-CF
348 collection (International Centre of Microbial Resources dedicated to Filamentous Fungi, INRA, Marseille,
349 France). All strains were identified by morphological and molecular analysis of ITS (Internal Transcribed
350 Spacer) sequences. The strains were maintained on malt agar slants at 4°C.

351 Five discs (5 mm each) of fungal mycelium grown on malt agar plates were used to inoculate
352 Roux flasks containing 100 ml of medium (glucose 10 g/L ; bactopectone 20 g/L ; yeast extract 1 g/L).
353 After incubation during 15 days at 30°C without shaking, the fungal mycelium was ground (ultraturax
354 10000 rpm, 60 s) in 50 ml of purified water (MilliQ, Millipore). Five mL of this suspension were used for
355 the inoculation of each 250-ml baffled Erlenmeyer flasks containing 100 mL medium with 2.5 g L⁻¹ of

356 maltose as a starter (except for the maltose control condition; 20 g L⁻¹), 1.842 g L⁻¹ of diammonium
357 tartrate as a nitrogen source, 0.5 g L⁻¹ yeast extract, 0.2 g L⁻¹ KH₂PO₄, 0.0132 g L⁻¹ CaCl₂/2H₂O and 0.5 g
358 L⁻¹ MgSO₄/7H₂O, and as a main carbon source, 15 g L⁻¹ (dry weight) of ball-milled wheat straw (*Triticum*
359 *aestivum*) or Wiley-milled aspen (*Populus grandidentata*). Cultures were incubated in the dark at 30°C
360 with shaking at 120 rpm. 5 mL of each culture was sampled at 3, 5, 7, and 10 days after inoculation and
361 the culture broths (secretomes) were centrifuged, filtered using 0.2 µm polyethersulfone membrane
362 (Millipore) and then stored at -20°C until used.

363 *In-gel fluorescence ABPP assay*

364 Each probe (samples available from Prof. Herman Overkleeft upon request) was dissolved in
365 DMSO at 5 mM and then mixed and diluted with ultrapure water. We prepared a 6x mixture of probes
366 containing 60 µM each of BODIPY-ABP-βGlc, Cy3⁺-ABP-Cel, and Cy5⁺-ABP-Xyn (see supplemental figure
367 18 for probe and inhibitor structures used in this study). Secretome samples were buffered with 0.1
368 volumes of 1 M NH₄OAc pH 5.5 to ensure consistent labelling conditions. 25 µL samples of buffered
369 secretome were mixed with 5 µL of 6x probe stock and incubated at 30°C for 1 hour with a heated lid to
370 prevent evaporation. Samples were diluted with 10 µL of 4x SDS-PAGE loading dye, heated to 95°C for 2
371 minutes, and 15 µL of this was separated through 4-15% Criterion gels in an actively cooled Dodeca cell
372 at 200 V for 55 minutes. Gels were then imaged using the Cy2, Cy3, and Cy5 filter/laser sets in the
373 Typhoon 5 laser scanner. Bands were identified and integrated using ImageQuant (GE Healthcare) with
374 molecular weight estimation based on a Pageruler 10-180 kDa ladder (ThermoFisher), using the bands
375 from 25-180 kDa for calibration.

376 *Pulldown of endo-β-glucanases using ABP-Cel*

377 1.8 mL of buffered day 10 secretomes that showed detectable ABP-Cel signal via fluorescence
378 (17 samples total) were supplemented with 10 µL of 1 mM Biotin-ABP-Cel in DMSO and incubated for 2

379 hours at 30°C. A separate set of samples treated with 10 μ L of DMSO were prepared as negative control.
380 200 μ L of 10x denaturing buffer (40 mM DTT, 2% SDS) was added and the samples were heated to 80°C
381 for 5 minutes in a water bath, then cooled to RT. 100 μ L of 0.5 M IAA was then added. Following 30
382 minutes of incubation at RT in the dark, 9 mL of acetone was added to each sample and they were
383 incubated at -20°C overnight. Precipitate (varying in colour from tan to dark orange) was collected by
384 centrifugation at 4000xg for 15 minutes. Supernatant was decanted and the pellets were air dried for ~1
385 hour to remove residual acetone. Pellets were dissolved in 40 μ L of 10 M urea at RT, transferred to a 0.5
386 mL lo-bind tube (Eppendorf), then diluted with 360 μ L of 0.05% SDS in 50 mM pH 7.4 NaP_i buffer. 20 μ L
387 of strep mag sepharose suspension was added to each tube and they were shaken at 25°C for 1 hour.
388 Beads were collected using a magnetic rack and the supernatant was discarded. Beads were washed
389 (resuspended, shaken for 5 minutes, then collected and supernatant discarded) with 500 μ L of 2% SDS
390 at 40°C twice, then 500 μ L of 2 M urea at rt once, then 500 μ L of water at rt twice. Beads were finally
391 resuspended in 20 μ L of 0.05 M TEAB (Thermo) and supplemented with 0.5 μ L of 0.5 μ g/ μ L Trypsin Gold
392 (Promega V5280). Digests were incubated with vigorous shaking overnight at 37°C. Tubes were then
393 spun down to ensure consistent volume, beads were collected, and the supernatant was supplemented
394 with 2 μ L of 20 mg/mL TMT² reagent in absolute ethanol (126 added to negative control and 127 added
395 to probe-treated samples). Labelling reactions were incubated for 1 hour at rt, then excess labelling
396 reagent was quenched by addition of 1 μ L of 5% hydroxylamine (~65 mM final) and incubation for 15
397 minutes at rt. 10 μ L of TMT²-126-labelled negative control and 10 μ L of TMT²-127-labelled sample
398 peptide solutions were then mixed together and 6 μ L was analysed.

399 *LC-MS analysis of peptides*

400 Peptides from each sample were collected on a 180 μ m x 20 mm 5 μ m Symmetry C18 trap
401 column (Waters) flowing at 2500 nL/min and subsequently separated over a 75 μ m x 250 mm 1.7 μ m

402 Peptide CSH C18 (C18) flowing at 300 nL/min using a nanoAcquity M-Class LC system (Waters). The
403 column was maintained at 60°C. Solution A was 0.1% formic acid in LC-MS grade water and solution B
404 was 0.1% formic acid in LC-MS grade acetonitrile. The separation gradient was 3 minutes of isocratic
405 2.5% B followed by a 7 minute gradient to 8% B, then a 30 minute gradient to 30% B, a 5 minute
406 gradient to 80% B, a 4 minute gradient to 95% B, a 1 minute gradient to 2.5% B, and 15 minutes of
407 isocratic 2.5% B. All samples were analysed on an Orbitrap Fusion Tribrid mass spectrometer. TMT-
408 labelled samples were analysed using synchronous precursor selection MS³ analysis (63). MS/MS peaks
409 were picked using Compass. MS²/MS³ spectra were paired using MASCOT. Searches were performed
410 against the predicted proteome of each fungal species supplemented with common contaminants using
411 Mascot with a mass tolerance of 5 ppm and a false discovery rate of 1%. Variable modifications including
412 cysteine carbamidomethylation, methionine oxidation, and cysteine or glutamate modification with 1
413 were included in the search. TMT ratios were determined using SCAFFOLD. For quantitative analysis,
414 protein hits were filtered for >2 quantifiable peptide matches at 95% confidence.

415 *Production and purification of recombinant enzymes in Pichia pastoris*

416 Amino acid sequences were selected for recombinant production at random from collections of
417 homologous sequences detected across multiple pulldowns. These included LsGH5_5A, LsGH5_7A,
418 LsGH10A, and TIGH12A (Sequences found in supplemental table 4. Genes, with signal peptides removed
419 (64), were synthesized and cloned into pPICZαA between the EcoRI and Sall restriction sites by Genscript
420 (Netherlands) to generate sequences with α-factor secretion signals and C-terminal 6xHistidine
421 purification tags. Plasmids were propagated in *E. coli* Stellar cultured in low-salt LB with 25 µg/mL
422 zeocin. For transformation, ~1 µg of plasmid DNA was digested with SacI and purified using a PCR
423 purification kit. ~100 ng of the resulting linearized DNA was electroporated into *Pichia pastoris* X-33
424 prepared following the method of Wu *et al* (65). From each transformation, a selection of 3-8 colonies

425 that grew on YPD supplemented with 100 µg/mL of zeocin were streaked for purity. A single colony was
426 taken from each streak plate and grown overnight in 5 mL of BMGY, then induced with two additions of
427 50 µL (1% final) methanol over two days. Culture supernatants were checked for protein of interest *via*
428 SDS-PAGE and staining with Coomassie dye. The best-producing colony was used for scale-up to 500 mL
429 cultures in 2.5 L baffled flasks, induced in the same manner. Supernatant was collected following
430 centrifugation. The pH was adjusted to 7.5 with NaOH, the cultures were 0.45 µm-filtered, and protein
431 was collected on a 5 mL Histrap FF crude column (GE Healthcare). Following a 10 CV wash with 20 mM
432 imidazole, 300 mM NaCl, 20 mM NaP_i, pH 7.5, bound protein was eluted with a gradient from 20-500
433 mM imidazole in the same buffer. Protein-bearing elution fractions were pooled, concentrated using a
434 10 kDa MWCO centrifugal filter, then purified into 20 mM sodium acetate pH 6, 100 mM NaCl using XK
435 16/60 columns containing Superdex 75 (TIGH12A) or Superdex 200 (LsGH5_5A, LsGH5_7A, LsGH10A)
436 medium. Protein-bearing fractions were pooled and concentrated to 10-50 mg/mL using a 10 kDa
437 centrifugal filter and stored at -80°C. Two LsGH10A elution peaks were observed from Superdex 200;
438 only the later-eluting peak was used, though both showed activity and ran indistinguishably on SDS-
439 PAGE. The total protein yields were 54 mg/L (6xHis tag intact) for LsGH5_5A, 38 mg/L (6xHis tag intact)
440 for LsGH5_7A, 26 mg/L for LsGH10A, and 135 mg/L for TIGH12A. Notably, LsGH5_5A and LsGH5_7A
441 produced extremely well (>200 mg/L based on SDS-PAGE), but the majority of the protein did not bind
442 to a Histrap column, suggesting proteolytic trimming of the C-terminal tag from these enzymes.

443 *Hydrolysis of substrates by recombinant enzymes*

444 Polysaccharide hydrolysis was measured through the detection of reducing ends using the BCA
445 assay. Briefly, enzyme (<10 µg/mL) was mixed with substrate in 50 mM pH 4.0 NaOAc buffer with 100
446 mM NaCl and incubated at 30°C for 15 minutes. The reaction was stopped by the addition of freshly
447 mixed BCA reagent (250 mM Na₂CO₃, 140 mM NaHCO₃, 2.5 mM bicinchoninic acid, 1.25 mM CuSO₄, 2.5

448 mM L-serine), then colour was developed by incubation at 80°C for 10 minutes before measuring A₅₆₃.
449 Reducing ends were determined relative to a glucose calibration series from 10-200 µM. A substrate
450 blank was measured and subtracted from each sample measurement. Minor activities were quantified
451 by the same method using 50 µg/mL enzyme with a boiled enzyme control (95°C, 15 minutes) added to
452 substrate for background subtraction.

453 The pH optimum of each enzyme was measured using 1 mg/mL cGM (LsGH5_7A), wAX
454 (LsGH10A) or bMLG (LsGH5_5A, TIGH12A) in a collection of buffers (citrate, acetate, formate, MES,
455 HEPES, phosphate) at different pH values (see supplemental figure 15) at 30°C. The temperature-activity
456 profile of each enzyme was measured from 32-83°C using the same substrates in 50 mM pH 4.0 NaOAc
457 buffer. Enzyme was incubated at temperature for 5 minutes, then substrate was added and reducing
458 ends were quantified relative to a substrate blank following 15 minutes of incubation with substrate
459 (see supplemental figure 16).

460 Hydrolysis of 4-methylumbelliferyl cellobioside (4MU-GG) and 4-methylumbelliferyl xylobioside
461 (4MU-Xyl2) were quantified at 25°C in 50 mM pH 4.0 NaOAc buffer using excitation at 360 nm and
462 detection at 450 nm. 4MU fluorescence was calibrated using a dilution series from 100-0.8 µM 4MU in
463 the same buffer.

464 *Inhibition kinetics of recombinant enzymes*

465 Inhibition kinetics were monitored using a continuous assay as described previously (32). Briefly,
466 enzyme in 100 mM pH 4.0 NaOAc buffer was mixed 1:1, to a final concentration selected to hydrolyse
467 ~5% of the substrate over 2 hours, with inhibitor and 0.4 mM substrate (diluted from 100 mM in DMSO)
468 in water. Inhibitor concentrations from 0 to 50 µM or 0 to 25 µM were monitored for fluorescence
469 continuously for up to 2 hours. To test enzyme recognition specificity, inhibition was measured with
470 glucosyl-β(1,4)-cyclophellitol (GGcyc) (36) or xylosyl-β(1,4)-xylocyclophellitol (XXcyc) (35). To test the

471 impact of the different linker chemistries, inhibition kinetics were also measured using Biotin-ABP-Xyn
472 (35) and Biotin-ABP-Cel (36).

473 **Declarations**

474 *Ethics approval*

475 Not applicable.

476 *Consent for publication*

477 Not applicable

478 *Availability of data and materials*

479 *Pichia pastoris* strains and samples of recombinant proteins may be available from Gideon Davies
480 (Gideon.davies@york.ac.uk). Samples of ABP-Cel, ABP-Xyl, and ABP-Glc may be available from Herman
481 Overkleeft (h.s.overkleeft@lic.leidenuniv.nl). Basidiomycete fungi are available from the fungal culture
482 collection of the International Centre of Microbial Resources (CIRM-CF) at the French National Institute
483 for Agricultural research (INRA; Marseille, France). Genome sequences for each of the fungi used in this
484 study are available from MycoCosm (<https://mycoCosm.jgi.doe.gov/mycoCosm/home>) (DOE Joint
485 Genome Institute, Walnut Creek, California). Other datasets used and/or analysed during the current
486 study are available from the corresponding author on reasonable request.

487 *Competing interests*

488 The authors declare no competing interests.

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499 *Authors contributions*

500 NM, GJD, HSO, and JGB designed and planned the research. MH prepared fungal cultures. CB and SS
501 prepared activity-based probes used in this study. NM collected secretome samples and performed
502 activity-based protein profiling experiments. NM collected and analyzed proteomic data. DN performed
503 bioinformatic analysis. NM and MS prepared *P. pastoris* strains, produced and purified recombinant
504 enzymes, and performed activity assays. NM wrote the manuscript with input from all authors.

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507 aspen (*Populus grandidentata*).

508 **Abbreviations**

509 ABP: Activity-based probe
510 ABPP: Activity-based protein profiling
511 BCA: bicinchoninic acid
512 bMLG: barley mixed-linkage glucan
513 CAZyme: carbohydrate-active enzyme
514 cGM: carob galactomannan
515 CMC: carboxymethyl cellulose
516 DMSO: dimethylsulfoxide

517 DTT: dithiothreitol
518 GH: glycoside hydrolases
519 IAA: iodoacetamide
520 LPMO: lytic polysaccharide monooxygenase
521 MW: molecular weight
522 PVPP: Polyvinylpyrrolidone
523 SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
524 TEAB: tetraethylammonium bicarbonate
525 TMT: tandem mass tag
526 wAX: wheat arabinoxylan

527

528 **References**

- 529 1. Scheller HV, Ulvskov P. Hemicelluloses. *Annu Rev Plant Biol.* 2010 Jun 2;61:263–89.
- 530 2. Luis AS, Briggs J, Zhang X, Farnell B, Ndeh D, Labourel A, et al. Dietary pectic glycans are degraded
531 by coordinated enzyme pathways in human colonic Bacteroides. *Nat Microbiol.* 2018 Feb
532 18;3(2):210–9.
- 533 3. Celińska E, Nicaud JM, Białas W. Hydrolytic secretome engineering in *Yarrowia lipolytica* for
534 consolidated bioprocessing on polysaccharide resources: review on starch, cellulose, xylan, and
535 inulin. *Appl Microbiol Biotechnol.* 2021 Feb 1;105(3):975–89.
- 536 4. Schlembach I, Hosseinpour Tehrani H, Blank LM, Büchs J, Wierckx N, Regestein L, et al.
537 Consolidated bioprocessing of cellulose to itaconic acid by a co-culture of *Trichoderma reesei* and
538 *Ustilago maydis*. *Biotechnol Biofuels.* 2020 Dec 1;13(1):207.
- 539 5. Smith PJ, Wang HT, York WS, Peña MJ, Urbanowicz BR. Designer biomass for next-generation

- 540 biorefineries: Leveraging recent insights into xylan structure and biosynthesis. *Biotechnology for*
541 *Biofuels*. 2017.
- 542 6. Doblin MS, Pettolino F, Bacic A. Evans Review: Plant cell walls: The skeleton of the plant world.
543 *Funct Plant Biol*. 2010 May 21;37(5):357–81.
- 544 7. Lodish H, Berk A, Zipursky S. Section 22.5 The Dynamic Plant Cell Wall. *Mol Cell Biol* 4th Ed.
545 2000;1–5.
- 546 8. Miyauchi S, Navarro D, Grisel S, Chevret D, Berrin JG, Rosso MN. The integrative omics of white-
547 rot fungus *Pycnoporus coccineus* reveals co-regulated CAZymes for orchestrated lignocellulose
548 breakdown. Cullen D, editor. *PLoS One*. 2017 Apr 10;12(4):e0175528.
- 549 9. Henske JK, Wilken SE, Solomon K V., Smallwood CR, Shutthanandan V, Evans JE, et al. Metabolic
550 characterization of anaerobic fungi provides a path forward for bioprocessing of crude
551 lignocellulose. *Biotechnol Bioeng*. 2018 Apr 8;115(4):874–84.
- 552 10. Solomon K V., Haitjema CH, Henske JK, Gilmore SP, Borges-Rivera D, Lipzen A, et al. Early-
553 branching gut fungi possess large, comprehensive array of biomass-degrading enzymes. *Science*.
554 2016 Mar 11;351(6278):1192–5.
- 555 11. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active
556 enzymes database (CAZy) in 2013. *Nucleic Acids Res*. 2014 Jan 1;42(D1):D490–5.
- 557 12. Østby H, Hansen LD, Horn SJ, Eijsink VGH, Várnai A. Enzymatic processing of lignocellulosic
558 biomass: principles, recent advances and perspectives. *J Ind Microbiol Biotechnol*. 2020 Oct
559 1;47(9–10):623–57.
- 560 13. MacDonald J, Doering M, Canam T, Gong Y, Guttman DS, Campbell MM, et al. Transcriptomic
561 responses of the softwood-degrading white-rot fungus *Phanerochaete carnosae* during growth on
562 coniferous and deciduous wood. *Appl Environ Microbiol*. 2011 May 15;77(10):3211–8.

- 563 14. Diaz AB, Blandino A, Webb C, Caro I. Modelling of different enzyme productions by solid-state
564 fermentation on several agro-industrial residues. *Appl Microbiol Biotechnol.* 2016 Nov
565 1;100(22):9555–66.
- 566 15. Navarro D, Couturier M, da Silva G, Berrin J-G, Rouau X, Asther M, et al. Automated assay for
567 screening the enzymatic release of reducing sugars from micronized biomass. *Microb Cell Fact.*
568 2010 Jul 16;9(1):58.
- 569 16. Posch AE, Herwig C, Spadiut O. Science-based bioprocess design for filamentous fungi. Vol. 31,
570 Trends in Biotechnology. Elsevier Current Trends; 2013. p. 37–44.
- 571 17. Amore A, Giacobbe S, Faraco V. Regulation of Cellulase and Hemicellulase Gene Expression in
572 Fungi.
- 573 18. Kjærboelling I, Vesth T, Frisvad JC, Nybo JL, Theobald S, Kildgaard S, et al. A comparative genomics
574 study of 23 *Aspergillus* species from section *Flavi*. *Nat Commun.* 2020 Dec 1;11(1):1–12.
- 575 19. Fernández-Fueyo E, Ruiz-Dueñas FJ, López-Lucendo MF, Pérez-Boada M, Rencoret J, Gutiérrez A,
576 et al. A secretomic view of woody and nonwoody lignocellulose degradation by *Pleurotus*
577 *ostreatus*. *Biotechnol Biofuels.* 2016 Feb 29;9(1):1–18.
- 578 20. Syed K, Shale K, Pagadala NS, Tuszynski J. Systematic Identification and Evolutionary Analysis of
579 Catalytically Versatile Cytochrome P450 Monooxygenase Families Enriched in Model
580 Basidiomycete Fungi. Yu J-H, editor. *PLoS One.* 2014 Jan 22;9(1):e86683.
- 581 21. Miyauchi S, Hage H, Drula E, Lesage-Meessen L, Berrin J-G, Navarro D, et al. Conserved white-rot
582 enzymatic mechanism for wood decay in the Basidiomycota genus *Pycnoporus*. *DNA Res.* 2020
583 Apr 1;27(2):1–14.
- 584 22. Hage H, Miyauchi S, Virágh M, Drula E, Min B, Chaduli D, et al. Gene family expansions and
585 transcriptome signatures uncover fungal adaptations to wood decay. *Environ Microbiol.* 2021;

- 586 23. Wu L, Armstrong Z, Schröder SP, de Boer C, Artola M, Aerts JM, et al. An overview of activity-
587 based probes for glycosidases. *Curr Opin Chem Biol.* 2019 Dec 1;53:25–36.
- 588 24. Chauvigné-Hines LM, Anderson LN, Weaver HM, Brown JN, Koech PK, Nicora CD, et al. Suite of
589 activity-based probes for cellulose-degrading enzymes. *J Am Chem Soc.* 2012 Dec
590 19;134(50):20521–32.
- 591 25. Cravatt BF, Wright AT, Kozarich JW. Activity-Based Protein Profiling: From Enzyme Chemistry to
592 Proteomic Chemistry. *Annu Rev Biochem.* 2008 Jun 2;77(1):383–414.
- 593 26. Fang H, Peng B, Ong SY, Wu Q, Li L, Yao SQ. Recent advances in activity-based probes (ABPs) and
594 affinity-based probes (AfBPs) for profiling of enzymes. *Chem Sci.* 2021;
- 595 27. Willems LI, Beenakker TJM, Murray B, Gagestein B, Van Den Elst H, Van Rijssel ER, et al. Synthesis
596 of α - And β -galactopyranose-configured isomers of cyclophellitol and cyclophellitol aziridine.
597 *European J Org Chem.* 2014 Sep;2014(27):6044–56.
- 598 28. Kuo CL, van Meel E, Kytidou K, Kallemeijn WW, Witte M, Overkleeft HS, et al. Activity-Based
599 Probes for Glycosidases: Profiling and Other Applications. *Methods Enzymol.* 2018 Jan
600 1;598:217–35.
- 601 29. Witte MD, Kallemeijn WW, Aten J, Li KY, Strijland A, Donker-Koopman WE, et al. Ultrasensitive in
602 situ visualization of active glucocerebrosidase molecules. *Nat Chem Biol.* 2010 Dec 31;6(12):907–
603 13.
- 604 30. Kallemeijn WW, Li KY, Witte MD, Marques ARA, Aten J, Scheij S, et al. Novel activity-based probes
605 for broad-spectrum profiling of retaining β -exoglycosidases in situ and in vivo. *Angew Chemie -*
606 *Int Ed.* 2012 Dec 7;51(50):12529–33.
- 607 31. Artola M, Wu L, Ferraz MJ, Kuo CL, Raich L, Breen IZ, et al. 1,6-Cyclophellitol Cyclosulfates: A New
608 Class of Irreversible Glycosidase Inhibitor. *ACS Cent Sci.* 2017 Jul 26;3(7):784–93.

- 609 32. McGregor NGS, Artola M, Nin-Hill A, Linzel D, Haon M, Reijngoud J, et al. Rational Design of
610 Mechanism-Based Inhibitors and Activity-Based Probes for the Identification of Retaining α -I-
611 Arabinofuranosidases. *J Am Chem Soc.* 2020 Mar 11;142(10):4648–62.
- 612 33. Gloster TM, Madsen R, Davies GJ. Structural basis for cyclophellitol inhibition of a β -glucosidase.
613 *Org Biomol Chem.* 2007 Jan 25;5(3):444–6.
- 614 34. Jiang J, Beenakker TJM, Kallemeijn WW, Van Dermarel GA, Van Den Elst H, Codée JDC, et al.
615 Comparing Cyclophellitol N-Alkyl and N-Acyl Cyclophellitol Aziridines as Activity-Based
616 Glycosidase Probes. *Chem - A Eur J.* 2015 Jul 20;21(30):10861–9.
- 617 35. Schröder SP, De Boer C, McGregor NGS, Rowland RJ, Moroz O, Blagova E, et al. Dynamic and
618 Functional Profiling of Xylan-Degrading Enzymes in *Aspergillus Secretomes* Using Activity-Based
619 Probes. *ACS Cent Sci.* 2019 May 26;5(6):1067–78.
- 620 36. de Boer C, McGregor NGS, Peterse E, Schröder SP, Florea BI, Jiang J, et al. Glycosylated
621 cyclophellitol-derived activity-based probes and inhibitors for cellulases. *RSC Chem Biol.*
622 2020;1(3):148–55.
- 623 37. Chen Y, Armstrong Z, Artola M, Florea BI, Kuo C-L, de Boer C, et al. Activity-Based Protein Profiling
624 of Retaining α -Amylases in Complex Biological Samples. *J Am Chem Soc.* 2021 Feb
625 10;143(5):2423–32.
- 626 38. De Eugenio LI, Méndez-Líter JA, Nieto-Domínguez M, Alonso L, Gil-Muñoz J, Barriuso J, et al.
627 Differential β -glucosidase expression as a function of carbon source availability in *Talaromyces*
628 *amestolkiae*: A genomic and proteomic approach. *Biotechnol Biofuels.* 2017 Jun 23;10(1):161.
- 629 39. Collins T, Gerday C, Feller G. Xylanases, xylanase families and extremophilic xylanases. *FEMS*
630 *Microbiol Rev.* 2005 Jan 1;29(1):3–23.
- 631 40. Jaszek M, Grzywnowicz K, Malarczyk E, Leonowicz A. Enhanced extracellular laccase activity as a

- 632 part of the response system of white rot fungi: *Trametes versicolor* and *Abortiporus biennis* to
633 paraquat-caused oxidative stress conditions. *Pestic Biochem Physiol.* 2006 Jul 1;85(3):147–54.
- 634 41. Ishikawa H, Schubert WJ, Nord FF. Investigations on lignins and lignification. XXVIII. The
635 degradation by *Polyporus versicolor* and *Fomes fomentarius* of aromatic compounds structurally
636 related to softwood lignin. *Arch Biochem Biophys.* 1963 Jan 1;100(1):140–9.
- 637 42. Alexandropoulou M, Antonopoulou G, Fragkou E, Ntaikou I, Lyberatos G. Fungal pretreatment of
638 willow sawdust and its combination with alkaline treatment for enhancing biogas production. *J*
639 *Environ Manage.* 2017 Dec 1;203:704–13.
- 640 43. Rouches E, Zhou S, Sergent M, Raouche S, Carrere H. Influence of white-rot fungus *Polyporus*
641 *brumalis* BRFM 985 culture conditions on the pretreatment efficiency for anaerobic digestion of
642 wheat straw. *Biomass and Bioenergy.* 2018 Mar 1;110:75–9.
- 643 44. Paës G, Navarro D, Benoit Y, Blanquet S, Chabbert B, Chaussepied B, et al. Tracking of enzymatic
644 biomass deconstruction by fungal secretomes highlights markers of lignocellulose recalcitrance.
645 *Biotechnol Biofuels.* 2019 Dec 1;12(1):76.
- 646 45. Berrin JG, Navarro D, Couturier M, Olivé C, Grisel S, Haon M, et al. Exploring the natural fungal
647 biodiversity of tropical and temperate forests toward improvement of biomass conversion. *Appl*
648 *Environ Microbiol.* 2012 Sep;78(18):6483–90.
- 649 46. Miyauchi S, Navarro D, Grigoriev I V., Lipzen A, Riley R, Chevret D, et al. Visual comparative omics
650 of fungi for plant biomass deconstruction. *Front Microbiol.* 2016 Aug 24;7(AUG):1335.
- 651 47. Pretreatment of Lignocellulosic Biomasses with Filamentous Fungi for the Production of
652 Bioenergy [Internet]. France: Fr. Pat.; FR1460472, 2015.
- 653 48. Sun R, Lawther JM, Banks WB. Fractional and structural characterization of wheat straw
654 hemicelluloses. *Carbohydr Polym.* 1996 Apr 1;29(4):325–31.

- 655 49. Gabriellii I, Gatenholm P, Glasser WG, Jain RK, Kenne L. Separation, characterization and
656 hydrogel-formation of hemicellulose from aspen wood. *Carbohydr Polym.* 2000 Dec 1;43(4):367–
657 74.
- 658 50. Jun A, Tschirner UW, Tauer Z. Hemicellulose extraction from aspen chips prior to kraft pulping
659 utilizing kraft white liquor. *Biomass and Bioenergy.* 2012 Feb 1;37:229–36.
- 660 51. Miyauchi S, Rancon A, Drula E, Hage H, Chaduli D, Favel A, et al. Integrative visual omics of the
661 white-rot fungus *Polyporus brumalis* exposes the biotechnological potential of its oxidative
662 enzymes for delignifying raw plant biomass. *Biotechnol Biofuels.* 2018 Dec 23;11(1):201.
- 663 52. Berrin JG, Rosso MN, Abou Hachem M. Fungal secretomics to probe the biological functions of
664 lytic polysaccharide monooxygenases. *Carbohydr Res.* 2017 Aug 7;448:155–60.
- 665 53. Henrissat B, Driguez H, Viet C, Schülein M. Synergism of cellulases from *trichoderma reesei* in the
666 degradation of cellulose. *Bio/Technology.* 1985;3(8):722–6.
- 667 54. Ubhayasekera W, Muñoz IG, Vasella A, Ståhlberg J, Mowbray SL. Structures of *Phanerochaete*
668 *chrysosporium* Cel7D in complex with product and inhibitors. *FEBS J.* 2005 Apr;272(8):1952–64.
- 669 55. Lo Leggio L, Larsen S. The 1.62 Å structure of *Thermoascus aurantiacus* endoglucanase:
670 Completing the structural picture of subfamilies in glycoside hydrolase family 5. *FEBS Lett.* 2002
671 Jul 17;523(1–3):103–8.
- 672 56. Liu G, Li Q, Shang N, Huang JW, Ko TP, Liu W, et al. Functional and structural analyses of a 1,4-β-
673 endoglucanase from *Ganoderma lucidum*. *Enzyme Microb Technol.* 2016 May 1;86:67–74.
- 674 57. Wang K, Cao R, Wang M, Lin Q, Zhan R, Xu H, et al. A novel thermostable GH10 xylanase with
675 activities on a wide variety of cellulosic substrates from a xylanolytic *Bacillus* strain exhibiting
676 significant synergy with commercial Celluclast 1.5 L in pretreated corn stover hydrolysis.
677 *Biotechnol Biofuels.* 2019 Mar 9;12(1):48.

- 678 58. Gloster TM, Ibatullin FM, Macauley K, Eklöf JM, Roberts S, Turkenburg JP, et al. Characterization
679 and three-dimensional structures of two distinct bacterial xyloglucanases from families GH5 and
680 GH12. *J Biol Chem*. 2007 Jun 29;282(26):19177–89.
- 681 59. McGregor N, Morar M, Fenger TH, Stogios P, Lenfant N, Yin V, et al. Structure-function analysis of
682 a mixed-linkage β -glucanase/xyloglucanase from the key ruminal bacteroidetes *Prevotella*
683 *bryantii* B14. *J Biol Chem*. 2016 Jan 15;291(3):1175–97.
- 684 60. Zanon PRA, Yu F, Musacchio P, Lewald L, Zollo M, Krauskopf K, et al. Profiling the Proteome-Wide
685 Selectivity of Diverse Electrophiles. 2021 Mar 10;
- 686 61. Couturier M, Roussel A, Rosengren A, Leone P, Stålbbrand H, Berrin JG. Structural and biochemical
687 analyses of glycoside hydrolase families 5 and 26 β -(1,4)-mannanases from *Podospira anserina*
688 reveal differences upon manno-oligosaccharide catalysis. *J Biol Chem*. 2013 May
689 17;288(20):14624–35.
- 690 62. Verdoes M, Hillaert U, Florea BI, Sae-Heng M, Risseuw MDP, Filippov D V., et al. Acetylene
691 functionalized BODIPY dyes and their application in the synthesis of activity based proteasome
692 probes. *Bioorganic Med Chem Lett*. 2007 Nov 15;17(22):6169–71.
- 693 63. McAlister GC, Nusinow DP, Jedrychowski MP, Wühr M, Huttlin EL, Erickson BK, et al. MultiNotch
694 MS3 enables accurate, sensitive, and multiplexed detection of differential expression across
695 cancer cell line proteomes. *Anal Chem*. 2014 Jul 15;86(14):7150–8.
- 696 64. Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, et al.
697 SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat Biotechnol*. 2019
698 Apr 1;37(4):420–3.
- 699 65. Wu S, Letchworth GJ. High efficiency transformation by electroporation of *Pichia pastoris*
700 pretreated with lithium acetate and dithiothreitol. *Biotechniques*. 2004 Jan 6;36(1):152–4.

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