1 **Cultivation of *Podospora anserina* on soybean hulls results in an efficient enzyme**
2 **cocktail for plant biomass hydrolysis**
3

4 **Miia R. Mäkelä[a,b*], Ourdia Bouzid[a,c*], Diogo Robl[a,d], Harm Post[e,f], Mao Peng[a], Albert**
5 **Heck[e,f], Maarten Altelaar[e,f] and Ronald P. de Vries[a#]**
6
7 [a]Fungal Physiology, CBS-KNAW Fungal Biodiversity Centre & Fungal Molecular
8 Physiology, Utrecht University, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands
9 [b]Department of Food and Environmental Sciences, Division of Microbiology and
10 Biotechnology, P.O. Box 56, Viikinkaari 9, University of Helsinki, Helsinki, Finland
11 [c]Microbiology, Utrecht University, Padualaan 8, 3584 Ch Utrecht, The Netherlands
12 [d]Brazilian Laboratory of Science and Technology of Bioethanol, Giuseppe Maximo Scolfaro
13 10.000, Campinas, Brazil.
14 [e]Biomolecular Mass Spectrometry and Proteomics Bijvoet. Center for Biomolecules
15 Research and Utrecht Institute for Pharmaceutical Sciences, Padualaan 8, 3584 CH Utrecht,
16 The Netherlands.
17 [f]Netherlands Proteomics Center, Padualaan 8, 3584 CH Utrecht, The Netherlands.
18
19 [*]Equal contribution
20
21 [#]corresponding author: email: r.devries@cbs.knaw.nl, tel: + 31 (0)30 2122600, fax: + 31
22 (0)30 2512097
23
24
25

26 **Abstract**

27 The coprophilic ascomycete fungus *Podospora anserina* was cultivated on three different
28 plant biomasses, i.e. cotton seed hulls (CSH), soybean hulls (SBH) and acid-pretreated wheat
29 straw (WS) for four days, and the potential of the produced enzyme mixtures was compared
30 in the enzymatic saccharification of the corresponding lignocellulose feedstocks. The enzyme
31 cocktail *P. anserina* produced after three days of growth on SBH showed superior capacity to
32 release reducing sugars from all tested plant biomass feedstocks compared to the enzyme
33 mixtures from CSH and WS cultures. Detailed proteomics analysis of the culture
34 supernatants revealed that SBH contained the most diverse set of enzymes targeted on plant
35 cell wall polymers and was particularly abundant in xylan, mannan and pectin acting
36 enzymes. The importance of lytic polysaccharide monooxygenases (LPMOs) in plant
37 biomass deconstruction was supported by identification of 20 out of 33 AA9 LPMOs in the
38 SBH cultures. The results highlight the suitability of *P. anserina* as a source of plant cell wall
39 degrading enzymes for biotechnological applications and the importance of selecting the
40 most optimal substrate for the production of enzyme mixtures.

41

42 **Keywords**

43 Proteomics; saccharification; plant biomass; *Podospora anserina*; carbohydrate active
44 enzymes; auxiliary activities

45

46 **Highlights**

47 **-** *P. anserina* produced an efficient enzyme set for saccharification in SBH cultures
48 **-** The most diverse set of CAZymes was detected in SBH secretomes
49 **-** The enzyme cocktail from SHB was superior in hydrolysis of different plant biomasses
50 - The secretome from SBH was rich in xylan, mannan and pectin acting enzymes

51 **Abbreviations**

52 ABF, α-arabinofuranosidase; ABX, exoarabinanase; AE, acetyl esterase; AGD, α-
53 glucosidase; AGL, α-1,4-D-galactosidase; AGU, α-glucuronidase; AMY, amylase; APO,
54 aromatic peroxygenase; AXH, arabinoxylan arabinofuranohydrolase; BGL, β-1,4-
55 glucosidase; BXL, β-xylosidase; CAZy, carbohydrate active enzyme; CBH,
56 cellobiohydrolase; CDH, cellobiose dehydrogenase; CMCase, carboxymethyl cellulase; CSH,
57 cotton seed hull; DNS, dinitrosalicylic acid; EGL, endoglucanase; FAE, feruloyl esterase;
58 FDR, false discovery rate; GAL, endogalactanase; GE, glucuronoyl esterase; GMC, glucose-
59 methanol-choline oxidoreductase; GLA, glucoamylase; LAC, β-1,4-D-galactosidase; LPMO,
60 lytic polysaccharide monooxygenase; MEA, malt extract agar; MM, minimal medium; MAN,
61 endo-β-1,4-mannanase; MND, β-1,4-mannosidase; MCO, multicopper oxidase; PCA,
62 principal component analysis; PL, pectin lyase; PME, pectin methyl esterase; *p*PN, *p*-
63 nitrophenol; PSM, peptide spectrum match; RGAE, rhamnogalacturonan acetyl esterase;
64 RHA, α-rhamnosidase; SBH, soybean hulls; WS, wheat straw; XLN, endoxylanase.

65

66 **1 Introduction**

67 *Podospora anserina* is a dung inhabiting ascomycete fungus that has traditionally
68 been studied as a model species for several biological questions, such as ageing, prion
69 mechanisms and sexual development [1–4]. The genome sequence of *P. anserina* revealed
70 that this species possesses a large and highly specialized gene repertoire encoding hydrolytic
71 and oxidative carbohydrate active enzymes (CAZymes; [5]) that match with the carbon
72 sources present in the natural biotope of the fungus [6]. Therefore, more recently the
73 characterization of the lignocellulose acting enzyme machinery of *P. anserina* has gained
74 increasing attention and led e.g. to recombinant production and biochemical studies of several
75 of its CAZymes [7].

76 As a late coloniser of herbivorous dung [8], *P. anserina* has adapted to grow on
77 lignocellulosic substrates from which easily degradable carbon sources have already been
78 largely consumed by primary colonising species. This is reflected in its genome content
79 comprising a large repertoire of CAZymes putatively targeted to (crystalline) cellulose
80 degradation and including both hydrolytic and oxidative activities. These include e.g. several
81 glycoside hydrolases from CAZy families GH6, GH7, GH45 and GH131, and, even more
82 noticeably, 33 putative lytic polysaccharide monooxygenases (LPMOs) from family AA9 [6].
83 In addition, *P. anserina* possesses an abundant xylan degrading machinery with several
84 candidate enzymes from GH10, GH11, GH3 and GH43 families. Also, the relatively high
85 number of putative esterases possibly indicates the ability of *P. anserina* to detach xylan from
86 lignin and utilise this more recalcitrant fraction of lignocellulose as a carbon source.
87 Moreover, compared to other plant biomass degrading ascomycete species, the *P. anserina*
88 genome encodes a large number of oxidative auxiliary activities connected to lignin
89 modification, including several genes for putative AA1_3 laccase-like multicopper oxidases
90 (MCOs) and AA3_2 H_2O_2-producing enzymes. It also possesses three cellobiose
91 dehydrogenases (CDHs, AA1_3 and AA8), which may function in the degradation of all
92 main lignocellulose polymers [9,10].
93 The ability of *P. anserina* to degrade recalcitrant components of plant biomass makes
94 it a highly interesting species as a source of novel enzymes for plant biomass pre-treatment in
95 biotechnological applications. To further explore this ability, we cultivated *P. anserina* on
96 three lignocellulosic biomass substrates: cotton seed hulls (CSH), soybean hulls (SBH) and
97 pre-treated wheat straw (WS). The choice for CSH and SBH was based on their potential as a
98 biorefinery feedstock as they are abundant agricultural waste streams. In addition, we
99 included acid-pre-treated WS, as it is a currently used feedstock in biorefineries and therefore
100 presents a real case to test the potential of this fungus. We used a combination of enzyme and

101 saccharification assays with proteomics to obtain an in depth insight into the abilities of *P.*
102 *anserina*.
103

104 2 Materials and methods

105 2.1 Fungal cultivations

106 *Podospora anserina* S mat$^+$ was kindly provided by Dr. Fabienne Malagnac (Institut de
107 Génétique et Microbiologie, UMR 8621, Université Paris Sud-CNRS, France). The strain
108 was maintained on 2% (w/v) malt extract agar (MEA) plates at 4°C. For precultures, medium
109 that contained 1% of fructose and *P. anserina* modified minimal medium (MM, 0.25 g/l
110 KH_2PO_4; 0.3 g/l K_2HPO_4; 0.25 g/l $MgSO_4 \cdot 7H_2O$; 0.05 mg/l thiamine 0.25 µg/l biotin; 25 µg/l
111 boric acid; 2.5 mg/l $ZnSO_4$; $CuSO_4$ 0.5 mg/l; $MnSO_4$ 125 µg/l and vishniac solution [11] at
112 pH 7.0) was prepared. The inoculum was obtained by mixing a quarter of mycelium-covered
113 2% MEA agar plate with 100 ml of MM, and 20 ml of this mixture was used to inoculate 400
114 ml MM medium supplemented with 1% fructose. After 3 days of shaken (200 rpm)
115 incubation at 27°C, the fungal mycelium was collected by filtration and washed with MM. 2
116 g wet biomass was used to inoculate 100 ml MM supplemented with 3% of powdered cotton
117 seed hulls (CSH), soybean hulls (SBH) and wheat straw (WS) as carbon source. WS treated
118 with 20% HCl for 24 h and then washed and dried, was provided by Green Sugar GmbH,
119 Germany. Biological duplicate cultures were incubated for 4 days at 27°C and shaken (200
120 rpm).
121 Culture supernatants from each cultivation were collected from day 1 to day 4 for further
122 analyses.
123

124 2.2 Enzyme activity assays

125 Cell free samples were used for measurement of extracellular CAZyme activities. All
126 measurements were performed as three technical replicates, and the averages and standard
127 deviations were calculated using two biological replicate cultures and three technical
128 replicate measurements.

129 α-Arabinofuranosidase (ABF), cellobiohydrolase (CBH), α-1,4-D-galactosidase (AGL), β-
130 1,4-D-galactosidase (LAC), glucoamylase (GLA), β-1,4-glucosidase (BGL), β-1,4-
131 mannosidase (MND) and α-rhamnosidase (RHA) activities were determined by using *p*-
132 nitrophenol (*p*PN) -linked substrates (Sigma-Aldrich) as described previously [12]. Reaction
133 mixtures were incubated at 30°C for 4 h after which the reactions were terminated by adding
134 100 μl 0.5 M sodium carbonate. The amount of the released *p*NP was measured
135 spectrophotometrically (FLUOstar OPTIMA, BMG Labtech) at 405 nm. Activities are
136 expressed as nmol *p*NP/ml of sample/min.

137 Endoxylanase (XLN) and carboxymethyl cellulase (CMCase) activities were determined
138 using beechwood xylan (Sigma-Aldrich) and carboxymethyl cellulose (CMC, Sigma-
139 Aldrich) as substrates, respectively. Reaction mixtures containing 5 μl of culture supernatant
140 and 45 μl of 1% substrate in 50 mM sodium acetate, pH 5.0, were incubated at 50°C for 30
141 min. Dinitrosalicylic acid (DNS) assay was used to measure the amount of released sugars
142 [13]. Reaction was stopped by 75 μl DNS solution and boiling for 30 min. The absorbance
143 was measured at 550 nm. Activities are expressed as nmol of glucose released/ml of
144 sample/min.

145

146 **2.2 SDS-PAGE and protein concentration measurement**

147 For protein profiling, 30 μL of culture supernatant from each sample was loaded on
148 12% SDS-PAGE gel and stained by silver staining. SigmaMarker wide range (Sigma-
149 Aldrich) was used as a molecular mass marker.

150 The protein content of the culture supernatants was estimated by using BioRad protein
151 kit (BioRad) following the instructions of the manufacturer. Bovine serum albumin (Sigma)
152 was used as standard and the results are expressed in mg/l.
153
154 **2.3 Composition analysis of the used substrates**
155 Neutral carbohydrate composition of CSH, SBH and WS was analysed according to Englyst
156 and Cummings, 1984 [14], using inositol as an internal standard. Samples were treated with
157 72% (w/w) H_2SO_4 (1 h, 30°C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100°C and
158 the constituent sugars released were derivatised and analysed as their alditol acetates using
159 gas chromatography.
160
161 **2.4 Saccharification**
162 Culture supernatants obtained from the CSH, SBH and WS cultures of *P. anserina*
163 after three days of cultivation were used for saccharification of CSH, SBH and acid-pre-
164 treated WS. The 500 µl reactions were conducted with 3% (w/v) solid substrate in 50 mM
165 Na-phosphate buffer, pH 5.0, in the presence of 0.02% sodium azide. Total protein loading in
166 each reaction was 621 µg. The reactions were incubated for 24 h, 300 rpm, at 50°C, after
167 which the amount of reducing sugars was determined using DNS method. The experiments
168 were performed in technical triplicates.
169
170 **2.5 Proteomics analysis**
171 **2.5.1 Protein separation and digestion**
172 Samples were ran on a 12% Bis-Tris 1D SDS-PAGE gel (BioRad) for 2-3 cm and
173 stained with colloidal coomassie dye G-250 (Gel Code Blue Stain Reagent, Thermo
174 Scientific). The lane was cut into three bands, which were treated with 6.5 mM dithiothreitol

175 (DTT) for 1 h at 60°C for reduction and 54 mM iodoacetamide for 30 min for alkylation. The
176 proteins were digested overnight with trypsin (Promega) at 37°C. The peptides were
177 extracted with 100% acetonitrile and dried in a vacuum concentrator.
178
179 **2.5.2 Mass spectrometry: RP-nanoLC-MS/MS**
180 Samples were resuspended in 10% formic acid / 5% DMSO and 30% of the sample
181 was analyzed using a Proxeon Easy-nLC100 (Thermo Scientific) connected to an Orbitrap Q-
182 Exactive mass spectrometer. Samples were first trapped (Dr Maisch Reprosil C18, 3 μm, 2
183 cm x 100 μm) before being separated on an analytical column (Agilent Poroshell EC-C18,
184 2.7 μm, 40 cm x 50 μm), using a gradient of 60 min at a column flow of 150 nl/min. Trapping
185 was performed at 8 μL/min for 10 min in solvent A (0.1 M acetic acid in water) and the
186 gradient was as follows 7- 30% solvent B (0.1 M acetic acid in acetonitrile) in 31 min, 30-
187 100% in 3 min, 100% solvent B for 5 min, and 7% solvent B for 13 min. Nanospray was
188 performed at 1.7 kV using a fused silica capillary that was pulled in-house and coated with
189 gold (o.d. 360 μm; i.d. 20 μm; tip i.d. 10 μm). The mass spectrometers were used in a data-
190 dependent mode, which automatically switched between MS and MS/MS. Full scan MS
191 spectra from *m/z* 350 – 1500 were acquired at a resolution of 35,000 at *m/z* 400 after the
192 accumulation to a target value of 3E6. Up to ten most intense precursor ions were selected for
193 fragmentation. HCD fragmentation was performed at normalised collision energy of 25%
194 after the accumulation to a target value of 5e4. MS2 was acquired at a resolution of 17,500
195 and dynamic exclusion was enabled (exclusion size list 500, exclusion duration 10 s).
196
197 **2.5.3 Data analysis**
198 Raw files were processed using Proteome Discoverer 1.4 (version 1.4.1.14, Thermo
199 Scientific, Bremen, Germany). Database search was performed against the *P. anserina*

200 database (JGI Mycocosm, http://genome.jgi.doe.gov/Podan2/Podan2.home.html) using
201 Mascot (version 2.5, Matrix Science, UK) as the search engine. Carbamidomethylation of
202 cysteines was set as a fixed modification and oxidation of methionine was set as a variable
203 modification. Trypsin was specified as enzyme and up to two miss cleavages were allowed.
204 Data filtering was performed using percolator [15], resulting in 1% false discovery rate
205 (FDR). Additional filters were; search engine rank 1 peptides and ion score >20. Raw files
206 corresponding to one sample were merged into one result file. The MS proteomics data have
207 been deposited to the ProteomeXchange Consortium via the PRIDE [16] partner repository
208 with the dataset identifier PXD005170 and 10.6019/PXD005170. The number of peptide
209 spectrum matches (PSMs) for each protein in each sample were merged into one matrix file.
210 Principal component analysis (PCA) was performed on the resulting matrix table with the
211 FactoMineR package, a package written in R dedicated to multivariate exploration data
212 analysis [17].
213
214 **3 Results**
215 **3.1 Extracellular enzyme activities and protein profiles in *P. anserina* cultures**
216 Enzyme activities of selected hydrolytic CAZymes were determined from cultures
217 where powdered CSH, SBH or WS were used as sole carbon sources during 4 days of
218 cultivation (Fig. 1). The highest activities were detected from the cultures with SBH as a
219 carbon source, which most probably was due to the higher protein production produced on
220 this substrate (Supplementary Table 1). While CBH, BGL and XLN activities were detected
221 in all studied media, LAC, ABF, AGL and CMCase activities were only observed in SBH
222 supernatants (Fig. 1B). The highest CBH activity was determined at the third day of growth
223 in all three cultures. Activities measured in CSH and WS supplemented media were at similar
224 level (2.1 and 1.5 nmol of *p*NP released/min/ml, respectively; Fig. 1A, C), while in SBH

225 medium, 6- and 9-fold higher CBH activity was detected (Fig. 1B). The highest BGL (3.5
226 nmol of *p*NP released/min/ml) and XLN (4.1 nmol of glucose released/min/ml) activities
227 were also detected from the SBH cultures at the fourth and third day of growth, respectively
228 (Fig. 1B). Compared to CSH and WS cultures, 3- and 5-fold higher BGL activity, and 8- and
229 3-fold higher XLN activity was produced in the SBH cultures of *P. anserina* (Fig. 1B). No
230 MND, GLA and RHA activity were detected in the culture supernatants.
231
232 [Figure 1]
233
234 SDS-PAGE analysis revealed that *P. anserina* produced variable sets of extracellular
235 proteins over time in different plant biomass containing cultures (Supplementary Fig. 1), thus
236 indicating adaptation to each carbon source. In line with the detected enzyme activities,
237 higher amount of extracellular proteins was produced in SBH cultures compared to CSH and
238 WS cultures (Supplementary Table 1). As expected, the protein amount increased in the
239 cultures during the cultivation resulting in 171, 376 and 173 mg/l in CSH, SBH and WS,
240 respectively, at the third day (Supplementary Table 1E), which was selected for
241 saccharification experiments (see 3.3).
242
243 **3.2 Carbohydrate composition of the plant biomass substrates**
244 The plant biomass substrates used in this work consisted of one monocot, i.e. WS, and
245 two dicots, i.e. CSH and SBH, which substantially differ in their chemical composition
246 (Supplementary Table 2). The glucose content of CSH is approximately 10 mol% lower
247 compared to SBH and WS. In contrast, the xylan content of CSH is significantly higher than
248 that of the two other substrates. Furthermore, SBH contains mannan and distinguishes from

249 the others with its high pectin content shown by the presence of galactose, arabinose and
250 uronic acids (Supplementary Table 2).
251
252 **3.3 Saccharification of lignocellulosic substrates by the enzyme mixtures produced by *P.*
253 *anserina***
254 Since the highest enzyme activities were detected after 3 days of *P. anserina* growth
255 on CSH, SBH and WS cultures, these extracellular enzyme mixtures were selected for
256 saccharification assays of the lignocellulosic feedstocks by measuring the amount of released
257 reducing sugars (Fig. 2). The enzyme cocktail originating from SBH cultures hydrolysed all
258 tested plant biomass substrates resulting in release of 0.7, 1.2 and 0.3 g/l reducing sugars from
259 CSH, SBH and acid-pre-treated WS, respectively. In contrast, the enzyme set from CSH was
260 able to saccharify only SBH, whereas enzymes from WS hydrolysed CSH and SBH.
261 Moreover, the superiority of the enzyme mixture produced on SBH was seen in that it
262 released more than 2-fold higher amount of reducing sugars from CSH compared to enzymes
263 produced on WS, and 4- and 2-fold higher amount from SBH compared to enzymes produced
264 on CSH and WS, respectively (Fig. 2). In line with the chemical composition of the
265 biomasses, acid-pre-treated WS was the most recalcitrant substrate lacking the easily
266 hydrolysable sugars compared to the non-pre-treated WS (Supplementary Table 2), and only
267 the enzyme mixture from SBH cultures accomplished to release reducing sugars from it.
268 Surprisingly, in the studied conditions, the enzymes from CSH and WS cultures did not
269 hydrolyse the substrates on which they were produced.
270
271 [Figure 2]
272
273

274 **3.4 Proteomics of *P. anserina* cultures**

275 In order to study *P. anserina* enzyme mixtures more thoroughly, the culture liquids
276 from the first and third day cultures were selected for proteomics analysis, based on the
277 detected protein profiles and enzyme activities. The PCA analysis showed that the duplicate
278 samples were very similar to each other (Supplementary Fig. 2). *P. anserina* produced the
279 highest number of selected plant cell wall degradation related enzymes on SBH at both
280 studied time points. Their number was slightly higher at the early time point of SBH (114)
281 compared to WS (99) and substantially higher compared to CSH with 47 detected proteins
282 (Fig. 3, Supplementary Table 1). Of these proteins, 39 (83%), 106 (93%) and 88 (89%) from
283 CSH, SBH and WS, respectively, have predicted secretion signal peptides (Supplementary
284 Table 1C). While the number of the plant cell wall degradation related enzymes stayed
285 almost the same after 3-day cultivation in SBH (116) and WS (98), it notably increased in
286 CSH (103). Putative secretion signals were present in 89 (86%), 104 (90%) and 89 (91%) of
287 the selected enzymes detected from CSH, SBH and WS cultures, respectively. The actual
288 number of secreted proteins may be higher than is suggested by the *P. anserina* genome
289 annotation, since half of the genes without a secretion signal sequence that were selected for
290 the analysis (Supplementary Table 1C) were revealed to contain a putative secretion signal
291 after manual correction of the gene model (Supplementary Table 1F).

292 The number of enzymes that were unique to SBH was high (22) compared to CSH (1)
293 and WS (11) after 1-day cultivation. The number of enzymes common to all three cultures
294 increased from 40 to 69, while the number of enzymes shared between SBH and WS notably
295 decreased (from 47 to 14) during the cultivation (Fig. 3). Significant increase in the number
296 of unique proteins in CSH cultures (from 1 to 17) was detected after 3 days of cultivation,
297 while it did not change in SBH (22) and slightly diminished in WS (from 11 to 9).

298

299 [Figure 3]

300

301 With respect to cellulose degradation, several CBH, endoglucanase (EGL) and BGL
302 enzymes were produced by *P. anserina* (Supplementary Table 1). GH6 and GH7 CBHs were
303 highly produced in WS cultures at both time points. Furthermore, one GH6 and one GH1
304 BGL were among the 15 most highly produced, and one GH7 CBH was the most abundant
305 CAZyme in WS supernatants at both time points (Supplementary Table 1C). GH5 EGLs were
306 present at the highest level in SBH cultures. In accordance with the highest release of
307 reducing sugars from all plant biomass substrates used in this work, the highest CAZyme
308 activities were detected from the SBH cultures of *P. anserina*. However, the measured
309 enzyme activities did not correlate in all cases with the proteomics analysis. For example,
310 CMCase activity was measured only from the supernatants obtained from SBH. This was
311 most probably due to inactivation or instability of the enzymes in the other samples. In
312 addition, it is possible that not all enzymes produced by *P. anserina* are active on the *p*NP-
313 linked substrates. No notable differences between the cultures were detected in the abundance
314 of BGLs.

315 Proteomics analysis revealed that majority (20 out of 33) of the AA9 LPMOs were
316 present in *P. anserina* SBH cultures (Supplementary Table 1). Most of these (18) were
317 produced after 3 days of growth, while 15 were identified at the early time point. Also, a
318 large variety of LPMOs were detected in WS cultures with 15 isoenzymes present in both
319 time points, and two of them were among the 15 most highly detected CAZymes
320 (Supplementary Table 1C). A narrower selection of 6 LPMOs was produced in CSH medium
321 after 1 day of cultivation, but 15 representatives were recognised on day 3. In addition,
322 LPMOs were generally produced in higher amounts in the later time point of SBH than of
323 CSH and WS containing cultures. The number of unique LPMOs was also highest in SBH

324 with 3 and 4 representatives in day 1 and 3, respectively (Fig. 3). In line with the high
325 production of LPMOs, all three *P. anserina* putative AA8-AA3_1 CDHs were also detected
326 in the studied cultures. One CDH, Pa_7_2650, was among the 15 most abundantly produced
327 plant cell wall acting proteins at both time points of all cultures, and another CDH, Pa_0_280
328 also in the 3-day CSH and WS supernatants (Supplementary Table 1C).

329 The ample ability of *P. anserina* to degrade hemicelluloses was seen when the
330 proportions of the selected plant cell wall acting enzymes were compared with their putative
331 substrates (Fig. 4). Accordingly, galactomannan degrading GH5 and GH26 endo-β-1,4-
332 mannanases (MANs) and GH27 AGLs were abundant in SBH cultures compared to CSH and
333 WS cultures. In addition, one GH26 MAN, one GH5_5 MAN and one GH2 MND belonged
334 to the 15 CAZymes that were most highly produced in SBH cultures (Supplementary Table
335 1C). However, AGL activity was only detected from 4-day culture liquids from SBH.
336 Especially, GH5 endo-β-1,4-mannanases were almost exclusively detected from SBH
337 supernatants, resulting also with GH5 proteins detected uniquely from SBH (Fig. 3). This is
338 in accordance with the chemical composition of SBH containing 7.1 and 4.0 mol% of
339 mannose and galactose, respectively (Supplementary Table 2, [18]). GH2 mannosidases were
340 notably produced at the later time point in SBH and CSH, although no MND activity was
341 detected from any of the studied culture supernatants.

342

343 [Figure 4]

344

345 Xylan degrading activities were also highly present in *P. anserina* secretomes (Fig.
346 4). XLNs from GH10 were highly produced after 3 days of growth in CSH and SBH media,
347 while those from GH11 were abundant in WS and SBH (Supplementary Table 1). GH11
348 XLN (Pa_1_1010) was the most abundant plant cell wall degrading enzyme in SBH in both

349 time points (Supplementary Table 1C). From putative β-xylosidases (BXLs), the enzymes
350 classified to GH43 were slightly more abundant in SBH than in the two other substrates.
351 GH62 arabinoxylan arabinofuranohydrolase (AXH) was highest on SBH, and GH115 and
352 GH67 α-glucuronidases (AGUs) on CSH. The high xylan degrading potential of the 3-day
353 SBH supernatant used for the saccharification was also evidenced by a GH10 XLN, GH62
354 AXH and GH43 BXL that were among the 15 most highly detected proteins. From putative
355 xyloglucan depolymerising enzymes, GH12 Pa_4_6020 was most abundant in WS, whereas
356 GH74 Pa_4_7820 was highest in SBH. The same was true for two CE15 glucuronoyl
357 esterases (GEs, Pa_0_910 and Pa_5_11620) and a putative CE16 acetyl esterase (AE,
358 Pa_5_11290), which were more abundant in the *P. anserina* proteome from 3-day SBH
359 cultivations compared to CSH and WS. In addition, CE15 Pa_5_11620 was unique for SBH
360 secretomes (Fig. 3). However, putative CE1 feruloyl (FAEs) and AEs were detected in all
361 samples. GH2 LACs, and GH43 and GH51 ABFs, which may act on several polysaccharides,
362 were almost exclusively produced in SBH. This is in line with the detection of LAC and ABF
363 activity only from SBH cultures. Interestingly, highly variable GH43 isoenzymes were
364 produced on different substrates on day 3 (Fig. 3).
365 Pectin acting enzymes were prevalent on SBH, while they were absent or lowly
366 produced on CSH and WS (Fig. 4, Supplementary Table 1). These included CE12
367 rhamnogalacturonan acetyl esterase (RGAE, Pa_1_22170), CE8 pectin methyl esterase
368 (PME, Pa_4_7580) GH53 endogalactanase (GAL, Pa_1_16660), and PL1, 3 and 4 pectin
369 lyases (PLs). This corresponds with the high pectin content of SBH (Supplementary Table 1).
370 The only exception was GH93 exoarabinanase (ABX, Pa_5_950) being most abundant in WS
371 cultures. *P. anserina* genome harbours one putative GH78 RHA, but this enzyme was not
372 identified from the studied secretomes and the corresponding activity was not detected from
373 the culture liquids. Enzymes targeted for starch cleavage, including GH13 amylases (AMYs),

374 GH15 GLAs and GH31 α-glucosidases (AGDs), were also most abundant in SBH enzyme
375 mixtures (Fig. 4, Supplementary Table 1). However, no GLA activity was detected from the
376 *P. anserina* culture supernatants, which may be due to the corresponding enzymes not being
377 active on the synthetic *p*NP-linked substrate. Most notable was the high production of GH31
378 AGDs.

379 From the large array of putative lignin acting oxidative enzymes encoded by the
380 genome of *P. anserina*, the relatively highest portion of enzymes were detected in CSH
381 cultures (Fig. 4). Three out of 10 putative AA1_3 laccase-like MCOs were identified in the
382 cultures. Of these, Pa_1_15470 showed the highest production especially in both studied time
383 points on SBH. In addition, several H_2O_2-producing enzymes were detected in the cultures,
384 namely 11 AA3_2 glucose-methanol-choline oxidoreductases (GMCs) and an AA5_1
385 glyoxal oxidase (GLX). They were abundant in the 3-day CSH culture supernatant, in which
386 two of the GMCs and the GLX were among the 15 highest produced plant cell wall acting
387 proteins. Notable was that one laccase and five GMCs were unique for 3-day CSH
388 secretomes (Fig. 3). While H_2O_2 is an essential factor in efficient breakdown of
389 lignocellulose, it is a harmful compound to fungi of which the concentration must be tightly
390 regulated. In accordance with the detection of H_2O_2-producing enzymes, catalase CATB
391 (Pa_7_1610) was detected from all studied *P. anserina* cultures. Interestingly, a putative
392 aromatic peroxygenase (APO, Pa_0_370) was produced in all studied substrates and was
393 among the 15 most abundant plant cell wall degrading enzymes in all samples in the later
394 time point (Supplementary Table 1).

395

396 **4 Discussion**

397 As a late coloniser of herbivore dung, the ascomycete fungus *P. anserina* is able to
398 use recalcitrant parts of plant cell walls as its carbon source. This feature together with the

399 exceptionally large repertoire of CAZymes encoded by its genome, makes studying the *P.*
400 *anserina* plant cell wall degrading enzyme machinery attractive. With the current interest for
401 efficient plant biomass converting enzyme cocktails, the aim of this study was to find culture
402 conditions in which *P. anserina* produces enzyme sets that are able to hydrolyse various plant
403 biomass feedstocks.

404 Our results show that compared to enzyme mixtures from CSH and WS containing
405 cultures of *P. anserina*, the enzyme cocktail produced on SBH, a major by-product from
406 soybean processing industry [19], was outstanding in its ability to release reduced sugars
407 from CSH, SBH and acid-pre-treated WS. Results obtained with e.g. the enzyme mixtures
408 produced by *Aspergillus niger, Penicillium subrubescens* and *Trichoderma reesei* have
409 indicated that saccharification of plant biomass is more effective when the enzyme mixtures
410 are produced on the corresponding feedstocks [20,21]. Also, enzyme supernatants produced
411 by *Trichoderma atroviride* TUB F-1663 mutant on steam-pretreated spruce, WS and
412 sugarcane bagasse yielded comparable amounts of glucose in the hydrolysis of the same
413 feedstocks [22], but none of them was distinguished by superior performance. In contrast to
414 this, the results of our study suggest that SBH, with the most variable chemical composition
415 of the studied substrates, induces a wider array of plant cell wall degrading enzymes of *P.*
416 *anserina* with robust ability to hydrolyse different plant biomass feedstocks.

417 Several hydrolytic cellulases from GH1, GH3, GH5, GH6 and GH7 families were
418 present in all cultures showing their importance in the degradation of cellulose. This is in
419 accordance with the results reported from the *P. anserina* secretomes produced in Avicel and
420 sugar beet pulp [23]. Putative secretion signal sequences could not be determined for all the
421 gene models selected for the study, including one BGL that was highly produced in CSH and
422 WS, thus indicating intracellular location for these proteins. However, part of the proteins
423 lacking a secretion signal may also be secreted non-classically as suggested earlier for

424 *Aspergillus nidulans* [24]. In addition, several putative cellulose oxidising AA9 LPMOs were
425 present in all *P. anserina* enzyme mixtures used for the saccharification, but in general they
426 were detected in higher amounts in the later time point of SBH than of CSH and WS
427 containing cultures. Also the number of unique LPMOs was highest in SBH supernatants.
428 LPMOs are copper-dependent enzymes that oxidatively cleave glycosidic bonds in various
429 polysaccharides after activation by an electron donor [25–27]. They are ubiquitous in fungi
430 among which the *P. anserina* genome is exceptionally rich with 33 genes encoding putative
431 AA9 LPMOs, thus resembling genomes of plant cell wall degrading basidiomycetes [28]. In
432 a previous study, no peptides corresponding to AA9 LPMOs were detected from the Avicel
433 cultures of *P. anserina* and only seven of these proteins were present in sugar beet pulp
434 cultures [23]. All these proteins were produced in our study.

435 In line with the high production of LPMOs, all three *P. anserina* putative AA8-
436 AA3_1 CDHs were also detected in the studied cultures. CDHs act as potential electron
437 donors for LPMO oxidative catalysis, and a striking feature of *P. anserina* is that its genome
438 encodes three CDHs, which most often are present as one to two copies in fungal genomes
439 [28,29]. Two of these, Pa_0_280 and a CBM1 containing Pa_7_2650, have been
440 heterologously expressed and biochemically studied [30,31]. The influence of Pa_7_2650
441 CDH in saccharification of wheat straw has also been investigated as a supplement to
442 industrial *T. reesei* enzyme cocktail [30]. In our study, this CDH was the among the 15 most
443 abundant plant cell wall acting enzymes in all studied conditions highlighting their function
444 in the lignocellulose degradation. Four *P. anserina* LPMOs have been shown to function
445 together with CDH on cellulose regiospecifically and release a different range of oxidised
446 products [31,32]. All of these characterised LPMOs that carry a CBM1 (Pa_1_16300,
447 *Pa*LPMO9E; Pa_2_6530, *Pa*LPMO9A; Pa_4_1020, *Pa*LPMO9H and Pa_7_3160,
448 *Pa*LPMO9B) were produced in all three plant biomass substrates used in the current study. In

449 addition, three of the characterized *P. anserina* LPMOs have been shown to target different
450 components of plant cell wall, i.e. cellulose, soluble oligosaccharides and hemicellulose [31],
451 thus further supporting their role in the plant biomass degradation by *P. anserina*.
452 One feature, which distinguished SBH enzyme mixtures from those produced on CSH
453 and WS, was the abundance of the enzymes putatively acting on hemicelluloses. These
454 included one GH5_7 (Pa_6_490) and one GH26 (Pa_5_1950 with a CBM35) mannanases,
455 which were highly produced in SBH. These enzymes (*Pa*Man5a and *Pa*Man26A,
456 respectively) have been shown to improve the saccharification of WS and spruce softwood
457 when added to the enzyme cocktail of the industrial *Trichoderma reesei* CL847 strain [33].
458 Especially, *Pa*Man5a (Pa_6_490) showed up to 28% improvement of spruce hydrolysis [33].
459 The same isoenzyme was unique in the SBH enzyme mixture that resulted in superior
460 saccharification in this work, thus supporting the importance of this mannanase in the
461 pretreatment of different lignocellulosic substrates. In addition, crystal structures of these
462 mannanases have been solved, and they have been suggested to act synergistically in
463 hydrolysis of mannan polymers [34], which is supported by the results of our study.
464 Several xylanolytic enzymes were abundant in SBH cultures, while their production
465 was more variable in CSH and WS cultures. In contrast, low amount of xylan acting enzymes
466 has been reported from the *P. anserina* secretomes from Avicel and sugar beet pulp (Poidevin
467 et al. 2014). In addition, GH2 LACs, and GH43 and GH51 ABFs, which can hydrolyse
468 several polysaccharides, i.e. xylan, xyloglucan and pectin, were scarce in CSH and WS
469 proteomes. However, at the later time point, different GH43 enzymes were detected in the
470 substrates, which highlights the interesting features of that CAZy family, which has recently
471 been reorganized into subfamilies [35]. Two *P. anserina* ABFs (GH51 Pa_5_11670 and
472 GH62 Pa_0_1370) and one XLN (GH11 Pa_2_13730 with a C-terminal CBM1) have been
473 recombinantly produced in *Pichia pastoris* and biochemically characterized [33]. However,

474 these enzymes were not among the highest representatives from their respective CAZy
475 families in our study.
476 *P. anserina* genome contains several genes encoding candidate esterases assigned to
477 families CE1, CE15 and CE16. In addition to that, *P. anserina* genome encodes 14 putative
478 FAEs [36]. In the current work, putative CE1 FAEs and AEs were evenly present in all
479 studied samples and did not exhibit high level production, while two CE15 GEs (Pa_0_910
480 and Pa_5_11620) were more abundant in the *P. anserina* proteome from 3-day SBH
481 cultivations compared to CSH and WS. GEs are suggested to release xylan that is connected
482 to aromatic alcohols of lignin by ester bonds [37], and recombinant Pa_0_910 has been
483 demonstrated to cleave the ester bond between synthetic lignin alcohols and 4-*O*-methyl-D-
484 glucuronic acid [38]. These glucuronic acid units have been shown to be present in SBH [19].
485 A representative from family CE16 containing a CBM1 (Pa_5_11290) was also more highly
486 present in proteomes from SBH than from CSH and WS, and interestingly, this enzyme has
487 been shown to possess versatile activities towards polymeric xylan [39].
488 Although the genome content of *P. anserina* indicates a weak potential for the
489 degradation of pectin, e.g. by absence of GH28 pectin hydrolases and a lower number of
490 pectin lyases compared to *A. niger* [6], the secretome analysis from SBH cultures showed
491 abundant production of different pectin acting enzymes on this pectin rich substrate. In
492 accordance with this result, good growth of *P. anserina* has been shown on soy pectin,
493 although poor growth on other pectins and pectin structural elements was reported [40]. This
494 suggests that the (partial) removal of pectin is essential for efficient degradation of SBH.
495 Also sugar beet pulp, another plant biomass with high pectin content, has been shown to
496 induce high pectinolytic activity and production of pectin acting enzymes in *P. anserina* [23].
497 The high amount of GH93 ABX produced in WS cultures could be due to the possible
498 activity of this enzyme towards arabino-oligomeric side chains of xylan, although its main

499 role appears to be the degradation of rhamnogalacturnonan I [40]. Enzymes targeted for
500 starch cleavage, including GH13 AMYs, GH15 GLAs and GH31 AGDs, were also most
501 abundant in SBH enzyme mixtures. Most notable was the high production of GH31 α-
502 glucosidases. *P. anserina* has been shown to produce GH31 AGD Pa_2_60 in soluble starch
503 [41], but this enzyme was not detected in our study.

504 From the laccase-like MCOs of *P. anserina*, Pa_1_15470 was the most highly
505 produced protein especially in both studied time points on SBH. In a laccase gene deletion
506 study, this enzyme was shown to have an effect on the optimal growth of *P. anserina* on
507 *Guibourtia demeusei* wood shavings, thus suggesting a role in lignin depolymerisation or
508 tolerance to lignin-derived compounds (Xia et al. 2014). In a proteomic study of *P. anserina*
509 secretomes, this protein was induced in microcrystalline cellulose (Avicel) containing
510 cultures, but was not detected in sugar beet pulp cultures of *P. anserina* (Poidevin et al.
511 2014).

512 Several AA3_2 GMCs and an AA5_1 GLX were produced in the studied *P. anserina*
513 cultures. It has been suggested that, in addition to assisting lignin-modifying peroxidase
514 activity or participating in Fenton-chemistry based lignocellulose degradation, H_2O_2-
515 producing enzymes may participate in the electron transfer mechanism for LPMOs with
516 several target polymers in lignocellulose [42,43]. Accordingly, several putative AA9 LPMOs
517 were present in all *P. anserina* secretomes. Although the enzyme mixtures from CSH
518 contained the highest proportion of putatively lignin oxidising activities, especially GMCs,
519 they did not assist in the saccharification of the different plant biomass feedstocks as CSH
520 supernatant was only able to hydrolyse SBH.

521 Also, a catalase (Pa_7_1610) was detected from all studied cultures, thus implying
522 participation in the regulation of the concentration of extracellular H_2O_2. In fact, a gene
523 deletion study of *P. anserina* catalases suggested that CATB (Pa_7_1610) is a major

524 contributor in protecting fungal cells from reactive oxygen species [44]. Interestingly, *P.*
525 *anserina* showed abundant production of a putative APO especially in the later time point of
526 all studied substrates. *P. anserina* possesses three putative APO encoding genes [6,45], and
527 although the biological relevance of these enzymes is not known [46], it may suggest a role in
528 detoxification of lignocellulose-derived aromatic compounds.
529

530 **5 Conclusions**

531 Our study shows that SBH as a carbon source triggered *P. anserina* to secrete an
532 enzyme mixture that was superior in saccharification of the tested plant biomasses, i.e. CSH,
533 SBH and acid pre-treated WS, when compared to the enzymes from CSH and WS cultures.
534 Interestingly, SBH was hydrolysed by all enzyme mixtures produced by *P. anserina*. The
535 detailed proteomics analysis showed that the main differences in the enzyme sets were
536 detected in xylan, mannan and pectin acting enzymes, which were relatively abundant in the
537 supernatants originating from SBH cultures. These results support the potential of *P. anserina*
538 as a versatile of source plant cell wall degrading enzyme activities for plant biomass
539 pretreatment for biorefinery applications.
540

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550

551 **Supplementary data**

552 **Supplementary Figure 1** Extracellular proteins from liquid cultures of *P. anserina*. The
553 proteins from the cultures supplemented with (1) 3% CSH, (2) 3% WS and (3) 3% SBH after
554 1 to 4 days of growth were separated by SDS-PAGE. M, molecular mass standard.
555

556 **Supplementary Figure 2** Variable factor map based on the PCA result from 1,487 proteins
557 abundance (approximated with peptide spectra counts, PSMs) across different samples and
558 their duplicates. CSH, cotton seed hulls; WS, wheat straw; SH, soybean hulls.
559

560 **Supplementary Table 1** Details on the analysis of the proteomics data.
561

562 **Supplementary Table 2** Monosaccharide composition (mol%) of the plant biomasses used
563 in this work.
564

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704

705 **Figure captions**

706 **Figure 1** Extracellular CAZyme activities detected from the culture supernatants of *P.*
707 *anserina* during 4-day cultivation in liquid cultures with A) 3% cotton seed hulls (CSH), B)
708 3% soybean hulls (SBH) and C) 3% wheat straw (WS) as sole carbon sources.
709 Cellobiohydrolase (CBH), β-glucosidase (BGL), β-1,4-D-galactosidase (LAC), α-
710 arabinofuranosidase (ABF) and α-1,4-D-galactosidase (AGL) activities were determined by
711 using *p*-nitrophenol (*p*NP) linked substrates, and carboxymethyl cellulase (CMCase) and
712 endoxylanase (XLN) activities by measuring the amount of released reducing sugars as
713 glucose equivalents. The vertical bars show the standard deviation of two biological replicate
714 cultures and three technical replicate reactions.
715

716 **Figure 2** The amount of released reducing sugars from 3% cotton seed hulls (CSH), 3%
717 soybean hulls (SBH) and 3% wheat straw (WS) incubated with the extracellular enzyme
718 mixtures produced by *P. anserina* after 3 days of growth in 3% CSH, 3% SBH and 3% WS
719 supplemented cultures. The vertical bars show the standard deviation of two biological
720 replicate cultures and three technical replicate reactions.
721

722 **Figure 3** Distribution of the plant cell wall degradation related extracellular enzymes
723 produced by *P. anserina* over the different plant cell wall compounds they putatively act on.
724 The proteins were identified from cotton seed hull (CSH), soybean hull (SBH) and wheat
725 straw (WS) containing cultures after 1 and 3 days of growth. The size of the pie chart reflects
726 the amount of total extracellular protein in the culture liquid. The details are given in
727 Supplementary Table 1E.
728
729 **Figure 4** Unique and common enzymes related to plant cell wall degradation detected from
730 the different cultures of *P. anserina*. The unique proteins in each sample are listed next to the
731 Venn graph. The font colour of the protein corresponds to the plant cell wall polymer it
732 putatively acts on according to the legend of Fig. 3. Cotton seed hulls, CSH; soybean hulls,
733 SBH; wheat straw, WS. To construct the Venn graphs, all proteins with a lower PSM value
734 than 2 were excluded from the analysis. The details are given in Supplementary Table 1D.
735 ABF, α-arabinofuranosidase; AE, acetyl esterase; AGD, α-glucosidase; AGL, α-1,4-D-
736 galactosidase; AGU, α-glucuronidase; AMY, amylase; BGL, β-1,4-glucosidase; BXL, β-
737 xylosidase; CBH, cellobiohydrolase; EGL, endoglucanase; FAE, feruloyl esterase; GAL,
738 endogalactanase; GE, glucuronoyl esterase; GMC, glucose-methanol-choline oxidoreductase;
739 GUS, β-glucuronidase; LAC, β-1,4-D-galactosidase; LPMO, lytic polysaccharide
740 monooxygenase; MAN, endo-β-1,4-mannanase; MCO, laccase-like multicopper oxidase;
741 PLY, pectin lyase; PME, pectin methyl esterase;
742