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Purification and Characterization of a Native Lytic Polysaccharide Monooxygenase
from *Thermoascus aurantiacus*

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16 **Abstract**

17 Lytic polysaccharide monooxygenases (LPMOs) have emerged as key proteins for
18 depolymerization of cellulose. These copper-containing enzymes oxidize C-1 and/or C-4
19 bonds in cellulose, promoting increased hydrolysis of the oxidized cellulose chains. The
20 LPMO from *Thermoascus aurantiacus*, a thermophilic ascomycete fungus, has been
21 extensively studied and has served as a model LPMO. A method was developed to purify the
22 LPMO from culture filtrates of *T. aurantiacus* along with its native cellobiohydrolase and
23 endoglucanase. The activity of the purified LPMO was measured with a colorimetric assay
24 that established the T_{opt} of the native LPMO at 60°C. Purification of the components of the *T.*
25 *aurantiacus* cellulase mixture also enabled quantification of the amounts of
26 cellobiohydrolase, endoglucanase and LPMO present in the *T. aurantiacus* culture filtrate,
27 establishing that the LPMO was the most abundant protein in the culture supernatants. The
28 importance of the LPMO to activity of the mixture was demonstrated by saccharifications
29 with Avicel and acid-pretreated corn stover.

30

31 INTRODUCTION

32 The conversion of plant biomass to biofuels and biochemicals is a critical component in
33 implementing the bioeconomy (Scarlat et al 2015). This conversion comprises chemical
34 pretreatment of lignocellulose followed by enzymatic depolymerization of the
35 polysaccharides to sugars for microbial conversion (Blanch et al 2011). Cellulose is the most
36 abundant polysaccharide in plant biomass, and cellulases produced by filamentous fungi have
37 been harnessed for cellulose depolymerization on an industrial scale (Kuhad et al 2016).
38 Fungi produce a variety of hydrolytic enzymes (cellobiohydrolases, endoglucanases and beta-
39 glucosidases) that hydrolyze glycosidic bonds in the cellulose chain and in cellobiose, the
40 hydrolytic product of the cellobiohydrolases (Payne et al 2015). Recently, an enigmatic
41 glycoside hydrolase family, GH61, which was comprised of low activity endoglucanases, was
42 reclassified as lytic polysaccharide monooxygenases (LPMOs) in the auxiliary activity family
43 9 (AA9) (Forsberg et al 2019). LPMOs are monocopper-containing proteins that oxidize C-1
44 and/or C-4 bonds in cellulose, promoting increased hydrolysis of the oxidized cellulose
45 chains and lowering the protein loading requirements for high levels of glucose release from
46 cellulose (Harris et al 2010; Quinlan et al 2011). LPMOs require a reductant for activity and
47 have been shown to use a variety of reductants, including: exogenously added compounds
48 (ascorbate and gallate), aromatics derived from biomass, redox-active proteins (cellobiose
49 dehydrogenase) and photochemistry (Kracher et al 2016). Initial studies identified O₂ as the
50 co-substrate for LPMOs; recent work has amassed evidence for H₂O₂, which LPMOs can
51 produce from O₂, being the primary co-substrate for cellulose oxidation (Bissaro et al 2017)
52 (Müller et al 2018).

53

The LPMO identified in *Thermoascus aurantiacus* has been central to the understanding of the biochemistry of these oxidative enzymes. A boosting effect of the *T. aurantiacus* supernatants on the cellulolytic activity of Cellulcast, a commercial cellulase mixture, was observed and subsequently attributed to the activity of *T. aurantiacus* LPMO by expressing it in a *Trichoderma reesei* strain that produces high levels of the hydrolytic enzymes (Rosgaard et al 2006) (Harris et al 2010). The structure of the *T. aurantiacus* LPMO defined its active site as having a unique N-methylated histidine residue that binds strongly binds Cu^{2+} and spectroscopy of the Cu active site indicated that the Cu(I) form of the active site adopts a three coordinate T-shaped structure (Quinlan et al 2011) (Kjaergaard et al 2014; Petrović et al 2018). Recent spectroscopic work on the *T. aurantiacus* LPMO has identified a Cu(III) oxidation state, possibly stabilized by a tyrosyl radical, that is produced by reaction with H_2O_2 and may be involved in the catalytic cycle (Singh et al 2020).

Previous studies on *T. aurantiacus* enzyme production have shown that the LPMO is produced at high levels when *T. aurantiacus* is grown on xylan, cellulose or concentrations of xylose that do not trigger carbon catabolite repression (McClendon et al 2012) (Schuerg et al 2017a; Schuerg et al 2017b). Under these conditions *T. aurantiacus* produces the LPMO along with glycoside hydrolases (cellobiohydrolase, endoglucanase, xylanase, and beta-glucosidase). Purification of the *T. aurantiacus* LPMO along with the other cellulase enzyme will allow the the LPMO to be characterized and its abundance in comparison with with the other cellulases enzymes quantitatively assessed. Here we describe purification and characterization of the native *T. aurantiacus* LPMO along with its native cellobiohydrolase and endoglucanase.

77 MATERIALS AND METHODS

78 *Protein production by T. aurantiacus*

79 *T. aurantiacus* ATCC 26409 was grown on potato dextrose agar plates for 2 days at 50 °C
80 followed by 4 days at 45°C. Precultures (250 ml) containing McClendon's salts with 2%
81 sucrose and 0.8% soymeal peptone (Amersco, VWR, Radnor, PA, USA) were inoculated
82 with agar plugs from the plates and incubated at 50°C and 200 rpm for 2 days (McClendon et
83 al 2012). The precultures were filtered under sterile conditions and the recovered mycelia
84 were inoculated into a medium with McClendon's salts, 0.8% soy meal peptone and 1%
85 beechwood xylan (Megazyme, Bray, Ireland). After 3 days of incubation, the cultures were
86 filtered through Miracloth (Millipore, Burlington, MA, USA) and the filtrate was centrifuged
87 for 10 min at 4000 rpm and subsequently filtered through a 0.22 µm filter. After filtration,
88 Bradford protein assays indicated a protein concentration of ~1 mg/mL. The filtrate was
89 concentrated to 2 mg/mL at 4°C *in vacuo* with a 10 kDa molecular weight cutoff membrane
90 (Millipore, Burlington, MA, USA).

91

92 *LPMO purification*

93 The culture filtrate (2 mg/ml, 10 mL) was concentrated to 2.5 mL and diluted to 5 mL with
94 loading buffer (30 mM NaOAc pH 3.5). The diluted filtrate was loaded on to a PD-10 column
95 and eluted with loading buffer. The eluted sample was concentrated to 1 mL and filtered
96 through a 0.22 µm filter (Millipore, Burlington, MA, USA). The sample was loaded on to a 1
97 mL SP-Sepharose HP column (GE Healthcare Life Sciences, Chicago, IL, USA) that had
98 been equilibrated with 1 mL of loading buffer and, after loading, was washed with 5 mL of
99 loading buffer. The endoglucanase and cellobiohydrolase were eluted with a 7 mL NaCl
100 gradient (0 to 0.5 M) in loading buffer; the endoglucanase and cellobiohydrolase eluted at

125 mM NaCl. The cation exchange column was re-equilibrated with 4 mL of loading buffer and the LPMO eluted at pH 6 with a 10 mL pH gradient, starting with loading buffer (30 mM NaOAc, pH 3.5) and continuously increased inflow of a potassium phosphate buffer (30 mM) with a finishing pH of 8. The xylanase co-eluted with the LPMO. The salt gradient fractions were rebuffed with elution buffer (potassium phosphate buffer, 30 mM, pH 6), concentrated to 0.5 mL and loaded on to a Superdex Increase 75 10/300 GL size exclusion column (24 mL), which was pre-equilibrated with 30 mM potassium phosphate buffer pH 6 containing 20 mM NaCl (elution buffer). The cellobiohydrolase and endoglucanase were separated by washing of the column with 1.5 volumes of elution buffer. In a separate purification step, the pH gradient sample was concentrated to 0.5 mL and loaded on to the Superdex Increase 75 10/300 GL column. The LPMO and xylanase were separated by elution with 1.5 column volumes. The LPMO was estimated to be >90% pure by SDS-PAGE (Figure 1D).

Enzyme assays

Endoglucanase activity was measured by the DNS method using carboxymethylcellulose as the substrate (Gladden et al 2011). In brief, enzymes and substrates (5 μ l and 75 μ l, respectively) in NaOAc buffer (pH 5, 50 mM) were incubated at 65 °C for 60 min (CMC) followed by addition of 80 μ l DNS and incubation at 90 °C for 5 min. Release of glucose was determined with optical density readings at λ = 540 nm. Cellobiohydrolase activity was measured using the p-nitrophenyl- β -D-cellobioside method (Park et al 2012). Enzyme samples and 10 mM pNPC (final concentration) in 100 mM NaOAc (pH 5.0) were incubated for 15 min at 50 °C. Spectrophotometric measurement of p-nitrophenol release was performed at λ = 415 nm after addition of 2 M Na₂CO₃ to the reaction mixtures.

LPMO activity was measured by the H₂O₂-dependent oxidation of 2,6-dimethoxyphenol to coerulignone as previously described (Breslmayr et al 2018). The temperature dependence of the LPMO activity was measured by pre-equilibrating the reaction mixture at the chosen temperature for 30 minutes and then adding the LPMO. One unit of CMCase, cellobiohydrolase or LPMO activity was defined as nmol of released glucose, p-nitrophenol or coerulignone per min, respectively. Protein concentration assays were performed using the Bradford method as previously described.

Protein quantification on SDS-PAGE gels

Quantification of cellobiohydrolase, endoglucanase and LPMO in the culture filtrate was performed with SDS-PAGE gel analysis using ImageJ (<https://imagej.nih.gov/ij/features.html>). Laemmli buffer (5 µl) and 2-mercaptoethanol (1 µl) were added to a dilution series of purified proteins and culture filtrate. 15 µl samples and 5 µL of Novex™ sharp pre-stained protein standard molecular weight marker (Thermo Fisher Scientific, Waltham, MA, USA) were loaded a 4–12% precast protein gel (Bio-Rad) and run at 140 V for 45 min in Tris/glycine SDS running buffer (Bio-Rad, Hercules, CA, USA). Gels were stained with SimplyBlue™ SafeStain solution (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h and destained according to the manufacturers protocol. The gel was pictured with a chemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA) and uploaded to ImageJ. Rectangular areas of each band were selected and histograms indicating the band intensities were calculated by the software. Numerical intensity values of bands of each dilution series and applied amounts of protein were plotted for standard curve calculations. Protein amounts in the culture filtrate were measured by obtaining the band intensities as described above.

149

150 *LPMO assays with enzymatic mixtures*

151 *T. aurantiacus* culture filtrates produced by controlled feeding of xylose and cellobiose were
152 used in this experiment (Schuerg et al 2017b). The filtrates were produced by preculturing *T.*
153 *aurantiacus* in minimal medium with McClendon's salts and 2% glucose/0.8% soymeal
154 peptone as described above. The pre-cultures were incubated for 48 h at 50 °C in a rotary
155 shaker and filtered *in vacuo* under sterile conditions. The mycelia (1.5 g) were weighed into
156 baffled 250 ml Erlenmeyer culture flasks containing 50 ml of culture medium without a
157 carbon substrate. Solutions of 1% xylose and cellobiose were autoclaved separately and
158 added by a peristaltic pump (BT100-1L Multi-Channel Peristaltic Pump (Langer Instruments
159 Corp., Boonton, NJ, USA)) according to a previously established protocol (Schuerg et al
160 2017b). The filtrates were isolated as described above. Saccharifications were
161 performed in 96-well plates with 2% w/v biomass (Avicel or acid-pretreated corn stover)
162 suspended in 2mL of 50 mM NaOAc pH 5.0 containing 0 or 1 mM sodium ascorbate.
163 Aliquots of *T. aurantiacus* filtrates from xylose or cellobiose-grown cultures were added to
164 provide reaction mixtures with 20 mg of enzymes/g of biomass. The plates were incubated
165 for 24 h at 50 °C and 180 rpm and the mixtures subsequently analyzed for glucose production
166 using a YSI analyzer (YSI, Yellow Springs, Ohio, USA).

167 RESULTS AND DISCUSSION

168 *Protein purification*

169 A purification protocol was developed for the *T. aurantiacus* LPMO using culture
170 supernatants obtained from mycelia grown on sucrose and shifted to induction medium with
171 beechwood xylan as the carbon substrate. This *T. aurantiacus* cultivation method produces
172 high levels of extracellular proteins (>1 mg/mL), and visual inspection of the SDS-PAGE
173 gels of the supernatant indicated that the cellobiohydrolase (58 kDa) and LPMO (23 kDa)
174 were the dominant proteins in the fraction, with the endoglucanase (35 kDa) and xylanase (32
175 kDa) present at lower levels in an overlapping band (McClendon et al 2012). A purification
176 protocol was devised that targeted the cellulases and would separate the LPMO from the
177 hydrolytic proteins. Binding of the supernatant to cation exchange column and elution with a
178 salt gradient separated the cellobiohydrolase and the endoglucanase from the LPMO and
179 xylanase, which were eluted with a pH gradient (Figure 1A). The cellobiohydrolase and
180 endoglucanase were separated by gel filtration chromatography, with the cellobiohydrolase
181 eluting first, as predicted by its higher molecular weight (Figure 1B). In contrast, for gel
182 filtration chromatography of the fraction containing the LPMO and xylanase, the lower
183 molecular weight LPMO eluted first and the xylanase co-eluted with a second fraction of the
184 LPMO (Figure 1C). The specific activity of the endoglucanase and LPMO increased after the
185 gel filtration step; however the specific activity of the cellobiohydrolase decreased (Table 1).
186 Previously, purification of *T. aurantiacus* cellobiohydrolase and endoglucanase was reported
187 from a strain isolated in India and grown with blotting paper as the carbon source (Khandke
188 et al 1989). The reported molecular weights (52 kDa for the CBH and 32 kDa for the EG) are
189 consistent with the molecular weights for the protein isolated here (Figure 1D). The specific

activity for the CBH from the Indian strain, which was measured by hydrolysis of Avicel, also decreased during the purification, suggesting that either other proteins may contribute to the cellobiohydrolase activity in the supernatant or the CBH is unstable during purification (Khandke et al 1989). Interestingly, SDS-PAGE of the supernatant from the Indian *T. aurantiacus* strain lacked a prominent band for an LPMO, indicating that strains of *T. aurantiacus* may respond differently to lignocellulosic substrates or not encode an LPMO.

Temperature dependence of LPMO assay

Previous studies of the *T. aurantiacus* LPMO measured its activity through its ability to enhance the hydrolysis of cellulose by enzymatic mixtures with glycoside hydrolases or by MALDI-TOF detection of cellodextrins upon incubation of phosphoric-acid swollen cellulose with the LPMO and beta-glucosidase (Harris et al 2010; Quinlan et al 2011). These studies were performed at 50°C, which is generally the temperature which saccharifications with commercial enzymatic mixtures are performed. Recently, a colorimetric assay was developed to measure LPMO activity by taking advantage of the ability of the LPMO to act as a peroxidase, coupling oxidation of 2,6-dimethoxyphenol to coeruleinone to reduction of H₂O₂ to water (Breslmayr et al 2018). This colorimetric assay was used to test the temperature dependence of LPMO activity (Figure 2), which reached a maximum at 60°C, which is in the same range (60-70 °C) as the T_{opt} previously measured for the *T. aurantiacus* cellobiohydrolase and endoglucanase (Khandke et al 1989). The observation of a T_{opt} of ≥60°C for the three cellulases from *T. aurantiacus* is consistent with the maintenance of high saccharification rates for *T. aurantiacus* supernatant enzyme mixture at elevated temperatures compared with commercial enzymatic mixtures (Schuerg et al 2017b).

213

214

215 *Quantification of LPMO in T. aurantiaucs supernatant*

216 Though the prominence of the LPMO in the *T. aurantiacus* culture supernatant was evident
217 in SDS-PAGE, quantification of the LPMO in this mixture has not been reported. The
218 simplicity of the enzyme mixture produced by *T. aurantiacus* and availability of purified
219 LPMO, CBH and EG enabled the quantification of the amount of LPMO relative to the total
220 protein in the mixture (Figure 3). Band intensities of culture filtrate samples (lanes 1-3) were
221 compared to dilution series of CBH, EG/xylanase and LPMO (lane 4-7, 8-10, 11-13,
222 respectively). To determine relative quantities of each enzyme in the filtrate, lane 3 was
223 quantitatively analyzed. In total, 2.5 µg of culture filtrate protein was separated and plotting
224 the band intensities to those of the dilution series, 1.8 µg of target enzymes were found in the
225 native culture filtrate. This analysis results in relative quantities of 34%, 31%, 7%, and 28%
226 for LPMO, CBH, EG/Xylanase and other proteins, respectively. Previous studies adding the
227 heterologously produced LPMO from *T. aurnatiacus* to commercial hydrolytic enzyme
228 mixtures (Celluclast) demonstrated that at optimal boosting of activity was achieved with the
229 LPMO set at 15% of the total enzymatic mixture. Therefore, the relative proportion of the
230 LPMO in the native *T. aurnatiacus* mixture is more than double than the optimal loading in
231 enzymatic mixtures (Müller et al 2015; Chylenski et al 2017).

232

233 *LPMO Activity in T. aurantiacus enzyme mixtures*

234 The identification of the LPMO as the most abundant protein produced by *T. aurantiacus*
235 suggested that the influence of the LPMO on the overall activity of the *T. aurantiacus*
236 enzymatic mixture may be larger than in commercial enzymatic mixtures. To test this
237 prediction, the effect of ascorbate on saccharification of Avicel and acid-pretreated corn
238 stover by the *T. aurantiacus* enzyme mixture was performed and compared to CTec2. The
239 saccharification was performed at 50°C since this temperature is optimal for CTec2. Previous
240 results with commercial enzymatic mixtures and purified proteins has demonstrated that a
241 reducing agent such as ascorbate was required to activate the *T. aurantiacus* LPMO in Avicel
242 saccharifications, but native phenolic compounds served as reductants for the LPMO in
243 saccharifications with acid-pretreated corn stover (Quinlan et al 2011). *T. aurantiacus*
244 supernatants from fed-batch incubations with xylose and cellobiose were tested (Schuerg et al
245 2017b). Saccharifications were performed in the presence or absence of 1 mM ascorbate and
246 20 mg/g biomass; CTec2 was used as a control. For Avicel saccharifications, CTec2
247 produced 5.8-8.1x glucose in 24 h compared to the *T. aurantiacus* supernatants in the absence
248 of ascorbate (Figure 4A). In the presence of 1 mM ascorbate, glucose release from Avicel
249 was increased 46% for the CTec2 saccharifications. However, glucose release from
250 saccharifications with the *T. aurantiacus* enzymes was increased 91-151% when ascorbate
251 was present, consistent with the higher level of LPMO in the *T. aurantiacus* supernatant. For
252 acid-pretreated corn stover, CTec2 produced 2.0-2.5x glucose compared to the *T. auranaticus*
253 supernatants, and addition of 1 mM ascorbate had a negligible effect on glucose release
254 (Figure 4B). The comparative results for glucose release of CTec2 and the *T. aurantiacus*
255 supernatants on acid-pretreated corn stover after 24h are comparable to saccharifications
256 reported previously for xylose-grown *T. aurantiacus* cultures Schuerg et al 2017b). Of
257 particular interest in these saccharifications is the divergence between glucose release on

258 Avicel, which strongly favors CTec2, versus corn stover, where the glucose release is
259 comparable. These results may reflect the differences in protein content of each enzymatic
260 mixture. In the commercial cellulase mixtures, cellobiohydrolase is the most abundant protein
261 (Taylor et al 2018), while in the *T. aurantiacus* mixture the LPMO is the most abundant
262 protein. This observation suggests that the LPMO-dominated enzymatic mixture from *T.*
263 *aurantiacus* is relatively poor at depolymerizing cellulose in purified preparations, but
264 performs well on cellulose still embedded in the lignocellulose matrix. The abundance of the
265 *T. aurantiacus* LPMO raises questions about the biological role of the LPMO and the other
266 cellulases in biological biomass deconstruction by *T. aurantiacus*. In particular, how
267 important is the LPMO in *T. aurantiacus* growth on cellulose and will eliminating the LPMO
268 reduce or prevent growth? In addition, are there accessory enzymes that are required for the
269 in vivo activity of the LPMO?

270

271 CONCLUSIONS

272 Purification of the native LPMO from *T. aurantiacus* has enabled a measurement of its
273 temperature dependence and demonstrated that it is the most abundant protein produced by
274 fungus in cultures with xylan as the inducing substrate. The importance of the LPMO to the
275 overall activity of the *T. aurantiacus* enzyme mixture was shown in up to 150% increases in
276 glucose release from Avicel in the presence of ascorbate. The abundance of the LPMO in
277 proteins produced by *T. aurantiacus* suggests that cellulolytic enzymatic mixtures may be
278 designed with LPMOs as the dominant protein, as opposed to CBHI, which is the most
279 abundant protein in most commercial enzymatic mixtures (Taylor et al 2018; Hamre et al
280 2019)..

281

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293

294 **AUTHOR CONTRIBUTIONS**

295 S.W.S. and R.G. designed research; S.F., C.H. J.G and R.G, performed research.

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373 **TABLES**

374

375 Table 1. Purification table of native cellobiohydrolase, endoglucanase and LPMO from *T.*
376 *aurantiacus*

Sample	Volume (mL)	Concentration (mg/mL)	Total activity (U)	specific activity (U/mg)
culture filtrate	1	3.53	720.2 ± 89.3	203.9 ± 25.3
NaCl gradient eluate	1.5	0.99	147.3 ± 8.6	98.4 ± 5.8
gel filtration eluate	1.5	0.46	56.1 ± 1.7	81.3 ± 2.5
culture filtrate load	1	3.53	2.0 ± 0.4	0.6 ± 0.1
NaCl gradient eluate	1.5	0.99	1.9 ± 0.3	1.3 ± 0.2
gel filtrated	1.5	0.17	0.6 ± 0.2	2.43 ± 0.59
culture filtrate load	1	3.53	4.4 ± 0.1	1.3 ± 0.1
pH gradient eluate	5	0.4	2.6 ± 0.5	1.3 ± 0.3
gel filtrated	3.5	0.35	15.9 ± 3.1	13.2 ± 2.6

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378 **FIGURE LEGEND**

379 **Figure 1.** Purification of native cellobiohydrolase, endoglucanase and LPMO from *T.*
380 *aurantiacus*. SDS-PAGE of (a) Cation exchange chromatography. Gel represents loaded
381 culture filtrate (1), column wash fractions (2,3), NaCl-gradient (0-0.5 M) fraction containing
382 CBH and EG, eluted at 125 mM NaCl (4), pH-gradient (pH 3.5-8) fractions containing
383 LPMO and xylanase, eluted at pH 6 (5-8). (b) Size exclusion chromatography of CBH and
384 EG. SDS-PAGE analysis of loaded sample (1) and elution fractions (2-9). CBH was
385 predominantly found in fractions of the first elution peak (4-5), pure EG eluted within a
386 second peak (8-9). (c) Size exclusion chromatography of xylanase and LPMO. Lane 1
387 represents loaded sample, LPMO eluted within the first peak (3-9) followed by co-elution of
388 xylanase co-eluted (10-12). (d) Summary of stepwise protein purification. Comparison of
389 initially loaded culture filtrate (1), NaCl-gradient purified CBH and EG (2), SEC-purified
390 CBH (3) and EG (4), pH-gradient purified Xylanase and LPMO (5) and SEC-purified LPMO.
391 4-12% pre-cast gels were run for 45 min at 120 V in Tris/glycine SDS running buffer and
392 stained with SimplyBlue™ SafeStain, ladder: Novex™ sharp pre-stained protein standards
393 (3.5 to 260 kDa). Cation exchange and size exclusion chromatography were performed on an
394 ÄKTA avant 150 chromatography system with a HiTrap™ SP HP and Superdex Increase 75
395 10/300 GL column, respectively. (d)

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397 **Figure 1.** Temperature-dependent relative activities of LPMO in preincubated buffer. The
398 LPMO was assayed with 1 mM 2,6-DMP and 100 μ M H₂O₂ in 100 mM sodium acetate buffer
399 pH 6. After preincubation of the reaction mixture for 30 min, the assay was started by adding
400 the LPMO. Formation of coerulignone ($\epsilon_{469} = 53,200 \text{ M}^{-1} \text{ cm}^{-1}$) was followed over 10 min by

401 spectrophotometric measurement of absorbance at $\lambda = 469$ nm. Error bars show the standard
402 deviation of two independent protein preparations measured in triplicates. A relative activity
403 of 100% was set at 60°C based on the observed temperature maxima of the two experiments.

404 **Figure 3.** Quantification of LPMO in *T. aurantiacus* culture filtrate using SDS-PAGE
405 imaging. (a) SDS-PAGE gel electrophoresis of dilution series of whole culture filtrate (1-3)
406 and purified cellobiohydrolase (4-7), endoglucanase/xylanase (8-10) and LPMO (11-13).
407 Standard curves were obtained by plotting band intensities and concentration of proteins
408 applied to the gel. (b) ImageJ protein quantification analysis. Graph represents relative
409 quantities based on band intensities of lane 3 (2.5 μ g total protein).

410 **Figure 4.** Saccharification of Avicel and acid-pretreated corn stover by CTec2 and culture
411 filtrates obtained from xylose (Xyl) and cellobiose (Cell)-grown *T. aurantiacus* cultures with/
412 without the reductant ascorbic acid (AscA) added. (a) Saccharification of 2% Avicel; (b)
413 Saccharification of 2% acid-pretreated corn stover. Saccharification reactions were performed
414 at 50°C and 180 rpm for 24 h with an enzyme loading of 20 mg/g biomass. Error bars
415 represent the standard deviation of 4 technical replicates, asterisks represent significant
416 difference among conditions (pval < 0.05).

417

418 **FIGURES**

419 Figure 1

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421 Figure 2

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427 Figure 4

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