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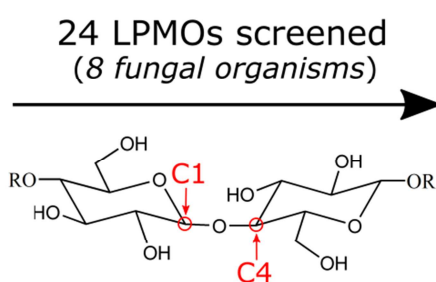
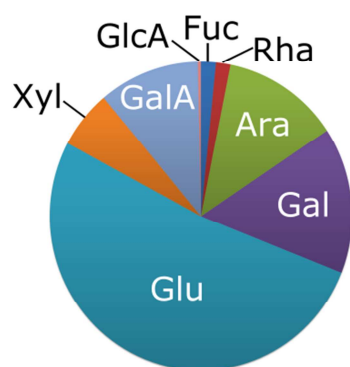
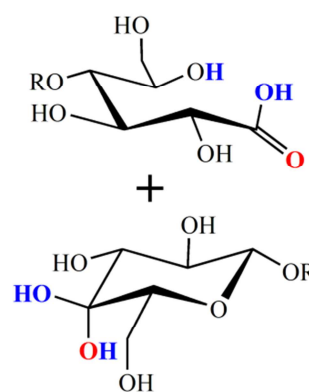
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Pretreated Soybean Polysaccharides**7 Active**

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Title

A comparative study on the activity of fungal lytic polysaccharide monoxygenases for the depolymerization of cellulose in soybean spent flakes

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1 Abstract

2 Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes capable of the
3 oxidative breakdown of polysaccharides. They are of industrial interest due to their ability to
4 enhance the enzymatic depolymerization of recalcitrant substrates by glycoside hydrolases. In this
5 paper, twenty-four lytic polysaccharide monooxygenases (LPMOs) expressed in *Trichoderma*
6 *reesei* were evaluated for their ability to oxidize the complex polysaccharides in soybean spent
7 flakes, an abundant and industrially relevant substrate. *TrCel61A*, a soy-polysaccharide-active AA9
8 LPMO from *T. reesei*, was used as a benchmark in this evaluation. In total, seven LPMOs
9 demonstrated activity on pretreated soy spent flakes, with the products from enzymatic
10 treatments evaluated using mass spectrometry and high performance anion exchange
11 chromatography. The hydrolytic boosting effect of the top-performing enzymes was evaluated in
12 combination with endoglucanase and beta-glucosidase. Two enzymes (*TrCel61A* and *Aspte6*)
13 showed the ability to release more than 36% of the pretreated soy spent flake glucose – a greater
14 than 75% increase over the same treatment without LPMO addition.

15 Keywords

16 lytic polysaccharide monooxygenase; soy spent flake; soy polysaccharides; cellulose hydrolysis;
17 *Trichoderma reesei*; *Chaetosartorya cremea*; *Aspergillus fumigatus*; *Aspergillus terreus*; *Penicillium*
18 *citrinum*; *Malbranchea cinamomea*; *Myceliophthora thermophila*; *Talaromyces leycettanus*

19 Abbreviations

20 LPMO, lytic polysaccharide monooxygenase; HPAEC, high performance anion exchange
21 chromatography; PASC, phosphoric acid swollen cellulose; DP, degree of polymerization

22

23 1. Introduction

24 Soybean polysaccharides are produced in large quantities globally as an industrial by-product of
25 soy protein, soy milk, and tofu production. When resulting from tofu or soy milk production they
26 are generally referred to as okara, and when generated as a by-product of soy protein isolate
27 production they are known as spent flakes¹. These materials are predominantly composed of high
28 molar mass, water-insoluble cell wall polysaccharides including type I arabinogalactan, cellulose,
29 arabinoxylan, rhamnogalacturonan, arabinan, xyloglucan, and homogalacturonan². Each year,
30 approximately 3.91×10^6 tons of soybean polysaccharides are produced through Chinese,
31 Japanese and Korean tofu production, with the vast majority of this material disposed of as waste
32 or burned³⁻⁵. This disposal not only adds significant costs to the production process, but also raises
33 environmental sustainability concerns. It is therefore of significant interest to develop alternate
34 applications for improved utilization of this abundant industrial by-product.

35 Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes capable of
36 polysaccharide depolymerization through oxidation of sugar residues⁶. They have been classified
37 as auxiliary activity (AA) enzymes in the Carbohydrate-Active enZyme database (CAZy;
38 <http://www.cazy.org>)⁷, and are currently categorized into four families: AA9 (formerly GH61),
39 AA10 (formerly CBM33), AA11, and AA13. AA9 comprises fungal LPMOs active on lignocellulose,
40 AA10 predominantly comprises LPMOs of bacterial and viral origin cleaving cellulose and chitin,
41 AA11 comprises fungal LPMOs that cleave chitin, and AA13 comprises fungal LPMOs that cleave
42 starch⁸. The importance of this oxidative mechanism in cellulose degradation was first suggested
43 in 1974 by Eriksson et al.⁹. In 2010, Vaaje-Kolstad et al. described the oxidative mode of action of
44 LPMOs and demonstrated their capacity to boost the enzymatic degradation of chitin¹⁰. There has

45 been a significant amount of research into these enzymes, with LPMO activity being demonstrated
46 for a variety of substrates including cellulose, xylan, xyloglucan, glucomannan, lichenan, starch, β -
47 glucan, and soluble cello-oligosaccharides¹¹⁻¹⁶. The precise oxidative mode of action of LPMOs is
48 still under debate, but has been postulated to involve internal electron channels¹⁷ or, more
49 recently, Fenton-type H_2O_2 chemistry¹⁸. The majority of LPMO oxidations have been observed at
50 the C1 or C4 positions of glucose^{6, 19}, however oxidation at the C6 position has also been
51 suggested²⁰. A key feature of all structurally characterized LPMOs is the presence of a histidine
52 brace at the active site. This site coordinates to the catalytic copper ion and consists of the
53 imidazole and amino group of an N-terminal histidine, as well as the imidazole of an additional
54 histidine²¹. LPMOs which have been expressed in fungi routinely display a characteristic N ϵ
55 methylation of the N-terminal histidine imidazole nitrogen. While the role of this modification is
56 still unclear and under debate, it has been proposed that the presence of an alkyl group on the
57 imidazole side chain of the N-terminal histidine could enhance the electrostatic interaction
58 between the enzyme and the substrate²². It has also recently been suggested that this methylation
59 may play a role in protecting fungal LPMOs from oxidative self-destruction¹⁸.

60 One reason for the recent interest in LPMOs is their ability to boost the activity of cellulolytic
61 enzymes in the degradation of biomass²³⁻²⁵. Our previous work demonstrated the ability of the
62 AA9 LPMO *TrCel61A* from *Trichoderma reesei* to oxidize the cellulosic polysaccharides of NaOH
63 pretreated soybean spent flakes at both the C1 and C4 positions²⁶. In addition, *TrCel61A*
64 treatment of pretreated soybean spent flakes was shown to result in a significant increase in the
65 glucose release observed from endoglucanase (EG) treatment of this substrate. The aim of the
66 current study is to explore the suitability of other LPMOs to degrade this complex substrate. For
67 this purpose, twenty-three additional fungal LPMOs from seven host organisms were expressed in

68 a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for select cellulase genes and beta-
69 glucosidase²⁷. Following expression, the N-terminal protein sequence was assessed in order to
70 verify the N-terminal histidine and evaluate the presence of the N-terminal histidine Nε
71 methylation. The activities of each LPMO were investigated against a variety of substrates
72 (phosphoric acid swollen cellulose (PASC), native soy spent flakes, NaOH pretreated soy spent
73 flakes, and Avicel® PH-101 microcrystalline cellulose) and oxidation products of these treatments
74 were analyzed with ESI mass spectrometry and high performance anion exchange
75 chromatography (HPAEC). For the LPMOs displaying activity on soy spent flakes, the effect of
76 LPMO addition on substrate glucose release through endoglucanase (EG) and beta-glucosidase (BG)
77 treatment was also investigated.

78 2. Results and Discussion

79 2.1 Protein characterization

80 Table 1 provides a list of the twenty-four LPMOs utilized in this study. To date, the majority of
81 these proteins have not yet been reported to have LPMO activity, with only four of them having an
82 exact amino acid sequence match in the National Center for Biotechnology Information (NCBI)
83 BLAST® non-redundant protein sequence database^{28, 29}. The closest matches for all twenty-four
84 LPMOs from a BLAST® search are provided in Supplementary Information Section 2. Note that ten
85 of the LPMOs showed high levels of sequence homology with proteins listed in the sequence
86 database, giving 100% sequence coverage and matching scores of at least 99% identity, 99%
87 positives, and 0 gaps. Table 1 also contains the results of an InterPro protein sequence domain
88 analysis indicating whether a cellulose binding domain (CBD) is predicted based on the sequences
89 of the LPMOs analyzed³⁰. Supplementary Information Figure S1 shows the SDS-PAGE gels obtained

90 for each LPMO used in this study. The presence of band spreading and increased molecular
91 weights over those expected from the amino acid sequence demonstrate the presence of
92 glycosylation, a common post-translational event which occurs during protein expression in fungi³¹.

93 Protease digestion in combination with MS/MS-MS analysis was used to identify the N-terminal
94 sequences of the twenty-four expressed LPMOs. A summary of the results from this analysis are
95 presented in Supplementary Information Section 3. All twenty-four proteins were identified to
96 primarily have N-terminal histidine residues. Chacr2 (G), Aspfu3 (G), Aspfu5 (G), Aspte1 (G),
97 Aspte3 (G), Aspte4 (G), Aspte5 (A), Aspte6 (A), Penci1 (G), Penci2 (G), and Mycth3 (A) showed the
98 presence of some proteins containing one additional N-terminal amino acid, indicated in
99 parenthesis, resulting in the lack of an N-terminal histidine. While one LPMO (Penci2) showed a
100 significant amount of expression with the amino acid addition, the relative abundance of these
101 observed peptides for the other LPMOs were several orders of magnitude lower than peptides
102 containing the N-terminal histidine. It is interesting to note that the majority of these amino acid
103 additions occur with the presence of glycine immediately preceding the N-terminal histidine. In
104 fact, only two protein sequences featuring a glycine prior to the expected terminal histidine lacked
105 any observed N-terminal glycine addition (Malcil1 and *TrCel61A*). Aspfu1 (G), Aspfu2 (G), Aspfu3
106 (G), Aspfu4 (G), Aspfu5 (G), Aspte5 (G), Aspte6 (G), Aspte7 (Y), Aspte8 (Y), Mycth1 (Y), Mycth3 (T),
107 and Talle1 (G) showed the presence of a one amino acid truncation, with the new N-terminal
108 amino acid indicated in parenthesis. Significant levels of the truncated sequence were observed
109 for Aspfu4, Aspte6, Aspte7, Aspte8, and Mycth1; however, in all cases the relative abundances of
110 these observed peptides were significantly lower than the peptides containing the N-terminal
111 histidine.

112 The presence of N-terminal histidine methylation was also evaluated due to its expected presence
113 following expression in *T. reesei*²³. The Nε methylation of the N-terminal histidine was observed
114 for all twenty-four LPMOs in this study. It is interesting to observe that the methylation from *T.*
115 *reesei* expression is observed even though leader peptides that are not endogenous to this
116 organism were used. For the majority of proteins, the methylated N-terminal histidine was
117 identified as the most prominent N-terminal fragment. Ten of the proteins (Aspte1, Aspte3,
118 Aspte4, Penc1, Aspte7, Aspte8, Malci1, Malci2, Mycth2, and TrCel61A) were observed to have
119 methylation present on all or the vast majority of their N-terminal histidine residues. However, a
120 non-methylated N-terminal histidine was identified to varying degrees in the remaining fourteen
121 proteins (Chacr1, Chacr2, Aspfu1, Aspfu2, Aspfu3, Aspfu4, Aspfu5, Aspte2, Aspte5, Aspte6, Penc1,
122 Mycth1, Mycth3, and Talle1). For the vast majority of these proteins, peptides corresponding to
123 the methylated N-terminal histidine were significantly more abundant than those of its non-
124 methylated counterpart. However, five of the proteins (Chacr1, Aspfu1, Aspfu2, Aspfu4, and Talle1)
125 showed a higher abundance of the non-methylated protein.

126 2.2 Evaluation of LPMO Activity

127 The twenty-four LPMOs listed in Table 1 were evaluated for their ability to oxidatively cleave the
128 polysaccharides in four substrates: PASC, native soy spent flakes, NaOH pretreated soy spent
129 flakes, and Avicel®. The NaOH pretreatment of soy spent flakes serves to convert the cellulose I
130 allomorph into cellulose II, in addition to reducing the cellulose crystallinity³². As previously
131 reported, this NaOH pretreatment results in a reduction of substrate hemicellulose and a
132 corresponding enrichment in cellulosic polysaccharides. The molar percentages of galactose,
133 arabinose, glucose, and xylose for the native spent flakes are 34.65%, 20.67%, 19.16%, and 8.37%,

134 respectively. Following NaOH pretreatment, these levels change to 15.30%, 12.56%, 51.77%, and
135 6.08%, respectively²⁶. This reduction in substrate hemicellulose may also lead to the additional
136 benefit of increased substrate accessibility during enzymatic treatment.

137 Table 2 shows the results of this substrate screening, with the lack of activity (-) or presence and
138 degree of activity (+, ++, or +++) indicated for each enzyme. The degree of activity was assessed
139 using ESI-MS to determine the presence of DP 2-6 oxidized oligomers following the 24 hr enzyme
140 treatment. For a given neutral oligomer of mass M, samples were evaluated for signals
141 characteristic of LPMO oxidation such as a [M-2] signal indicating C4-ketone oxidation and a
142 [M+16] signal characteristic of either C1-aldehydic acid oxidation and/or C4-gemdiol oxidation. The
143 results for this full in-depth analysis are provided in Supplementary Information Section 4, but
144 have been condensed to a single activity level per enzyme for a given substrate in Table 2. Twenty-
145 one of the LPMOs investigated in this study demonstrated some degree of oxidative activity on
146 PASC after 24 hrs. The lack of observed activity on PASC for Aspte1, Mycth1, and Mycth3 may
147 indicate that the enzyme treatment conditions utilized in this study were not sufficient for these
148 enzymes to produce enough detectable product. While all LPMO treatments presented in this
149 study were standardized at equivalent protein levels, the relative amount of LPMO protein in each
150 enzyme sample may have differed due to the lack of extensive purification. For this reason, it is
151 possible that the relative levels of LPMO protein for these three enzyme treatments were too low
152 for activity to be observed. It can be observed from Table 1 that these three LPMOs lack a
153 predicted cellulose binding domain (CBD) at the C-terminus of the protein; however, there does
154 not appear to be a clear correlation between the presence of a predicted CBD and activity for the
155 LPMOs investigated in this study. It is also possible that these three proteins simply do not have
156 LPMO activity on the substrates investigated in this study. The lack of activity cannot be directly

157 linked to the N-terminal characterization data presented in 2.1 and Supplementary Information
158 Section 3, as all three enzymes displayed the required N-terminal histidine following expression.

159 No oxidative activity was observed for any of the LPMOs on the native soy spent flakes; however,
160 seven of the LPMOs investigated (*TrCel61A*, *Chacr2*, *Aspfu1*, *Aspfu4*, *Aspfu5*, *Aspte6*, and *Talle1*)
161 did show activity on the NaOH pretreated spent flakes. This finding matches our previously
162 reported results concerning the activity of *TrCel61A* on soy polysaccharides, and also indicates
163 that the complex polysaccharides of soybean are highly resistant to oxidative degradation by
164 LPMOs using the conditions employed in this study²⁶. While the exact structural relationship
165 between the cellulosic and hemicellulosic polysaccharides in soybean has not yet been fully
166 characterized, it can be hypothesized from these findings that the hemicellulose is capable of
167 shielding the cellulose from enzymatic attack by LPMOs. It is interesting to note from Table 2 that
168 all LPMOs which showed activity on the NaOH pretreated soy spent flakes also showed strong
169 activity on PASC and some degree of activity on the crystalline cellulose in Avicel®. This
170 commonality between Avicel® activity and NaOH pretreated soy spent flake activity suggests a
171 degree of similarity between these two substrates, and may be an indication of remaining
172 cellulose crystallinity in the spent flakes despite the pretreatment employed in this study. While
173 this trend is almost universal for the LPMOs considered in this study, it was observed that *Aspte2*
174 showed no detectable activity on NaOH pretreated spent flakes despite its observed activity on
175 both PASC and Avicel®.

176 From the seven LPMOs showing activity on the pretreated spent flakes, three were selected as the
177 top-performing enzymes (*TrCel61A*, *Aspte6*, and *Talle1*) for further screening based on the
178 abundance of oxidized products detected with ESI MS. Figure 1 shows the ESI MS-MS CID

179 fragmentation spectra of the sodiated and oxidized $[M+16-Na]^+$ ion (m/z 381.10) following the
180 LPMO treatment of PASC with these three LPMOs. The fragments have been labeled with the
181 nomenclature of Domon and Costello³³, with Figure 1D illustrating the possible fragmentation
182 products for both the C4 oxidized gemdiol, Glc4gemGlc (blue), and the C1 oxidized aldonic acid,
183 GlcGlc1A (red) as described by Isaksen et al.¹⁶. As was previously reported²⁶, *TrCel61A*
184 demonstrates characteristics of both C1 and C4 oxidation following treatment with PASC (Figure
185 1A). Characteristic C4 fragmentations include the loss of two water molecules (m/z 363.09 and
186 345.08); the presence of $^{0,2}A_2-H_2O$ (m/z 303.07) and $^{3,5}A_2$ (m/z 275.07); the prominent Y_1 peak (m/z
187 203.05); and loss of water in the B_1 fragment (m/z 183.02). Characteristic C1 fragmentations
188 include the characteristic loss of a carboxyl group ($^{(0),1}A_2$; m/z 335.09) and the presence of a large
189 Y_1 fragment (m/z 219.05). While this latter signal could also correspond to the C_1 fragment of C4
190 oxidation, this fragment is expected to be in low abundance after undergoing rapid dehydration
191 and has therefore been marked in parenthesis to indicate this effect.

192 Comparing Figure 1A to the other LPMO treatments, we continue to observe the characteristic C4
193 oxidation fragments; however, fragmentations characteristic of C1 oxidation ($^{(0),1}A_2$; Y_1) are no
194 longer present. This is evidenced by the lack of observed signals corresponding exclusively to C1
195 oxidation (red). In particular, the low signals observed for the (C_1)/ Y_1 fragment (m/z 219.05) for
196 the non-*TrCel61A* LPMO treatments indicate a lack of C1 oxidation. *Aspte6* and *Talle1* show an
197 additional C4 oxidation fragment, $^{3,5}X_2$, not observed in the *TrCel61A* sample; however, they also
198 lack the C1/C4 oxidation fragment $^{2,4}X_1/^{(0),2}A_2$, which is observed in the *TrCel61A* sample. In order
199 to further explore the observation that all LPMOs showing activity on NaOH pretreated spent
200 flakes demonstrate C4 oxidation, HPAEC-PAD profiles were run to analyze the oxidation products.

201 Figure 2 shows the HPAEC-PAD chromatograms of the products produced by these three enzymes
202 on NaOH pretreated soy spent flakes both with (black) and without (red) ascorbic acid as a
203 reductant. In addition, the elution pattern of the neutral cello-oligomers from DP 2-6 is presented
204 for reference (Figure 2D). In the presence of ascorbic acid, signals are observed in the
205 characteristic neutral (~5-22 min), C1 oxidized (~20-30 min), C4 oxidized (~30-40 min), and C1-C4
206 oxidized (~40-46 min) regions of this chromatogram as described by Westereng et al.³⁴. The
207 strongest signals are observed for *TrCel61A* and *Aspte6*, corresponding well to the strength of
208 LPMO activity summarized in Table 2. Both of these enzymes show evidence of C1 and C4
209 oxidation (Figure 2A and 2B), while *Talle1* shows the majority of its signals in the C4 oxidation
210 region (Figure 2C). While signals are observed in the late-eluting C1-C4 oxidized region, the
211 presence of additional signals in this region both with and without ascorbic acid makes it difficult
212 to unambiguously confirm the presence of these doubly oxidized products.

213 To further investigate the observed correlation between C4 oxidation and activity on the
214 pretreated spent flakes, additional HPAEC-PAD experiments were conducted where the soluble
215 fractions following LPMO treatment of PASC were further treated with BG. Due to the non-
216 reducing end activity of BG, this treatment will result in degradation of neutral and C1 oxidized
217 soluble oligomers, but will leave the C4 oxidized compounds intact due to their modified non-
218 reducing end. Evaluating the HPAEC-PAD chromatograms of all BG treated LPMO samples for
219 retained signals in the C4 oxidized region (~30-40 min), we observe that seven enzymes (*TrCel61A*,
220 *Chacr2*, *Aspfu3*, *Aspfu4*, *Aspfu5*, *Aspte6*, and *Talle1*) continue to show strong evidence of C4
221 oxidation and four enzymes (*Aspfu1*, *Aspte2*, *Aspte3*, and *Aspte4*) show weak evidence of C4
222 oxidation (results not shown). All other LPMOs show no signals in this region of the chromatogram,
223 indicating that no C4 oxidation took place. Comparing these results to the activity data presented

224 in Table 2, we observe that all LPMOs demonstrating activity on the NaOH pretreated spent flakes
225 also show confirmed C4 oxidation. In addition, the three top-performing enzymes (*TrCel61A*,
226 *Aspte6*, and *Talle1*) selected above show strong C4 oxidation, confirming the MS-MS results
227 presented in Figure 1. It is interesting to note that four LPMOs (*Aspfu3*, *Aspte2*, *Aspte3*, and
228 *Aspte4*) showed evidence of C4 oxidation, but no activity on the pretreated spent flakes. Lastly, it
229 should be noted that all oxidation effects described above were absent in control samples treated
230 with each LPMO in the absence of ascorbic acid (results not shown).

231 2.3 Hydrolytic boosting effect between LPMO, endoglucanase, and beta-glucosidase on soy spent
232 flakes

233 In our previous work, we demonstrated the hydrolytic boosting capability of *TrCel61A* when dosed
234 in combination with EG for the enzymatic degradation of NaOH pretreated spent flakes²⁶. In order
235 to further explore this effect, the top-performing LPMOs described in Section 2.2 were evaluated
236 for their hydrolytic boosting ability. Figure 3 shows the HPAEC measured glucose release from
237 NaOH pretreated soy spent flakes following LPMO treatment in combination with EG and BG for 0-
238 72 hrs. We observe that all three of the LPMOs evaluated (*TrCel61A*, *Aspte6*, and *Talle1*) show a
239 significant hydrolytic boosting effect when compared to samples treated without LPMO. Statistical
240 evaluation by the Tukey-Kramer HSD method was conducted and the results are presented in
241 Figure 3. Additional statistical evaluation with two-way analysis of variance (ANOVA) describes the
242 significant correlation between glucose release and both reaction time and LPMO treatment
243 (Supplementary Information Section 5). This analysis confirms that the glucose release depends on
244 the interaction of these factors, as evidenced by the data presented in Figure 3. In addition to the
245 Tukey-Kramer analysis presented in Figure 3, additional Tukey pairwise comparisons were

246 conducted on the complete dataset in Figure 3 (irrespective of time point) and are provided in
247 Supplementary Information Section 5. These results show that the LPMO treatments from 8-72
248 hrs result in statistically significant increases in glucose release over the controls lacking LPMO. In
249 addition, differences between the boosting effects of the three enzyme treatments are not
250 statistically significant at each of these time points. The addition of *TrCel61A*, *Aspte6*, and *Talle1*
251 result in glucose yield increases of 88%, 76%, and 55%, respectively, when compared to enzymatic
252 treatment with EG and BG alone. *TrCel61A* and *Aspte6* are capable of ~36% substrate glucose
253 release after 72 hrs of reaction, the highest yields obtained in this study and a comparable result
254 to those obtained in previous work with *TrCel61A* ($34.48 \pm 4.84\%$)²⁶. It is also interesting to note
255 the rate of reaction observed in Figure 3. Significant glucose release is obtained within the first 2
256 hrs of reaction (10-15%), however no significant additional glucose release is observed from 2-4
257 hrs. After 24 hrs of reaction, the samples treated with LPMOs capable of hydrolytic boosting begin
258 to significantly differentiate themselves from controls. While samples containing *Talle1* showed
259 the highest glucose release after 24 hrs, the reaction appears to have been completed in this
260 timeframe as no additional glucose release was observed beyond this point.

261 In addition to evaluating the hydrolytic boosting activity of each LPMO alone, two-enzyme
262 combinations of the top-performing LPMOs were also assessed. Figure 4 shows the glucose
263 release from NaOH pretreated spent flakes after 72 hr incubation with these enzyme
264 combinations. A one-way analysis of variance showed no statistically significant differences
265 between these six treatments, indicating that the LPMO combinations tested are unable to
266 increase the glucose release when compared with the individual LPMO treatments. Lastly, it
267 should be noted that all hydrolytic boosting effects observed in Figures 3 and 4 were not present
268 in the absence of ascorbic acid (results not shown).

269 The data presented in Figures 1 and 2 indicate that the three top-performing LPMOs from this
270 study (*TrCel61A*, *Aspte6*, and *Talle1*) show similar oxidation on NaOH pretreated spent flakes. As
271 described above, the hydrolytic boosting effect demonstrated in Figure 3 is consistent across these
272 three enzymes as well, with Figure 4 showing that 50% of the LPMO dosage can be replaced with
273 another similarly acting LPMO to result in comparable hydrolytic boosting effects. These results
274 indicate that this is a more generalized effect, and not necessarily tied to the specific activity of an
275 individual LPMO. However, the results presented above do show commonality between the
276 LPMOs displaying activity on the pretreated spent flakes. *TrCel61A*, *Aspte6*, and *Talle1* all show
277 significant C4 oxidizing activity (Figures 1 and 2) as well as activity on Avicel[®], suggesting a link
278 between these features and activity on pretreated spent flakes.

279 3. Conclusions

280 In this study, we have characterized the LPMO activity of twenty-four enzymes against four
281 substrates: PASC, native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel[®] PH-101
282 microcrystalline cellulose. Following expression in *T. reesei*, the enzymes were evaluated for the
283 presence of N-terminal histidine and methylation of this histidine, with all enzymes showing these
284 features. Twenty-one enzymes displayed oxidative activity on PASC, seven showed activity on
285 Avicel[®], seven showed activity on NaOH pretreated soy spent flakes, and none showed activity on
286 native soy spent flakes. Of the seven LPMOs showing activity on pretreated soy spent flakes
287 (*TrCel61A*, *Chacr2*, *Aspfu1*, *Aspfu4*, *Aspfu5*, *Aspte6*, and *Talle1*), three were selected as the top-
288 performing enzymes (*TrCel61A*, *Aspte6*, and *Talle1*). ESI MS-MS fragmentation analysis
289 demonstrated predominantly C4 oxidizing activity in these enzymes, and the presence of C4
290 oxidized compounds was confirmed with HPAEC analysis. These results show a link between an

291 LPMO's C4 oxidizing ability, activity on the microcrystalline Avicel® substrate, and oxidative activity
292 on pretreated soy spent flakes. This observed correlation between C4 oxidizing activity and activity
293 on NaOH pretreated soy spent flakes demonstrates a link between substrate structure and LPMO
294 oxidative regioselectivity. This observation may offer some insight into the reasons for the
295 evolution of regioselectivity in LPMOs, and highlights the continued need for research in this area
296 to more fully understand the reasons behind oxidative regioselectivity. In addition to these
297 observations, the hydrolytic boosting effect of these enzymes towards the enzymatic degradation
298 of pretreated spent flakes by EG and BG was evaluated. Two of the enzymes (*TrCel61A* and *Aspt66*)
299 showed the most significant hydrolytic boosting ability, with release of 36% substrate glucose
300 after 72 hours compared with the 20% released in the absence of LPMO. Investigations into the
301 glucose release with LPMO combinations showed no direct evidence of increased hydrolytic
302 boosting when compared to treatment with individual LPMOs. The current results further expand
303 our understanding of the ability of LPMOs to degrade this complex substrate, and open new and
304 exciting opportunities for the application of these enzymes in the enzymatic degradation of soy
305 polysaccharides and other industrially-relevant substrates in the future.

306 4. Materials and Methods

307 4.1 Materials

308 Soy spent flakes were obtained from DuPont™ Nutrition & Health – Protein Solutions (St. Louis,
309 MO) as a by-product of the soy protein isolate production process. The material was lyophilized
310 and ground to pass a USA-Standard ASTM 40-mesh screen. Avicel® PH-101 microcrystalline
311 cellulose, L-fucose (Fuc), L-rhamnose (Rha), D-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-
312 xylose (Xyl), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), D-(+)-cellobiose, D-(+)-cellotriose,

313 cellotetraose, cellopentaose, and cellohexaose were obtained from Sigma-Aldrich (St. Louis, MO).
314 GE Healthcare PD MidiTrap G-25 columns were purchased from VWR (Radnor, PA). PASC was
315 obtained from DuPont™ Industrial Biosciences (Palo Alto, CA) where it was prepared as described
316 by Wood³⁵. Invitrogen NuPAGE 4-12% Bis-Tris Gels, SeeBlue® Plus2 Pre-Stained Standard, and
317 SimplyBlue™ SafeStain were obtained from Thermo Scientific (Waltham, MA).

318 4.2 Chemicals

319 Concentrated sodium hydroxide (50%; NaOH), 96% glacial acetic acid, copper(II) sulfate
320 pentahydrate, methanol, acetonitrile, urea, ammonium bicarbonate, DL-dithiothreitol (DTT),
321 iodoacetamide, formic acid, and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich
322 (St. Louis, MO). L(+)-ascorbic acid AnalaR NORMAPUR® was obtained from VWR (Radnor, PA).
323 Sodium azide (NaN₃) was purchased from Merck Performance Materials (Darmstadt, Germany).
324 The Bio-Rad Protein Assay Kit II (dye reagent and bovine serum albumin (BSA) standard) was
325 obtained from Bio-Rad (Hercules, CA).

326 4.3 Enzymes

327 Twenty-four LPMOs (Table 1) were obtained from DuPont™ (Shanghai, China) following expression
328 in a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for the major cellulase genes and beta-
329 glucosidase as described in PCT Pat. Appl. Publ. No. WO2009/048488²⁷. Expression was conducted
330 with the native leader peptide for each protein. The amino acid sequences, including the leader
331 peptide sequences, are provided in FASTA format in Supplementary Information Section 1. Cell
332 debris was removed by centrifugation and the supernatant was concentrated using a VivaFlow50
333 PES ultrafiltration membrane (MWCO 10 kDa) from Sartorius (Göttingen, Germany), with final
334 protein concentrations (Table 1) determined as described in 4.5.3. The purity of the enzymes

335 were assessed using SDS-PAGE gels (Supplementary Information Figure S1) as described in 4.5.4,
336 and N-terminal characterization was performed as described in 4.5.5 to verify the expected
337 presence of an N-terminal histidine with methylation of the imidazole¹¹. Endo-1,4- β -D-glucanase
338 from *Bacillus amyloliquefaciens* (EC 3.2.1.4; GH5) with 3500U activity (17.07 mg/mL protein) and
339 beta-glucosidase from *Agrobacterium sp.* (EC 3.2.1.21; GH1) with 600U activity (2.48 mg/mL
340 protein) were obtained from Megazyme International (County Wicklow, Ireland). Trypsin from
341 bovine pancreas (EC 3.4.21.4), α -Chymotrypsin from bovine pancreas (EC 3.4.21.1), and
342 endoproteinase Asp-N from *Pseudomonas fragi* (EC 3.4.24.33) were obtained from Sigma-Aldrich
343 (St. Louis, MO).

344 4.4 Soy Spent Flake NaOH Pretreatment

345 NaOH pretreated soy spent flakes were produced as described by Mittal, Katahira, Himmel &
346 Johnson³². In brief, dry soy spent flakes were combined with 16.5% w/w NaOH in an amount of
347 8.57 mg per mL of 16.5% w/w NaOH. This mixture was stirred under nitrogen at 25 °C for 2 hrs.
348 Upon completion, the material was separated by centrifugation and the soluble fraction was
349 decanted off with the remaining insoluble fraction re-diluted in MilliQ water to the original
350 reaction volume and vortexed. The centrifugation, separation and dilution described above were
351 repeated until the pH of the soluble fraction was neutral. The final, rinsed insoluble material was
352 lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen.

353 4.5 Material Characterization

354 4.5.1 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
355 (HPAEC-PAD)

356 The free monosaccharides in each sample were quantified by HPAEC-PAD using a Dionex ICS-3000
357 Ion Chromatography system with a CarboPac® PA100 guard column (50 x 2 mm) followed by a
358 CarboPac® PA100 analytical column (250 x 2 mm) and a PAD, all from Dionex Corporation
359 (Sunnyvale, CA). The flow rate used was 0.25 mL/min under the following elution profile, with
360 MilliQ water as eluent A and 600 mM NaOH as eluent B: 0-18 min, 1.5% B; 18-19 min, 1.5-75% B;
361 19-29 min, 75% B; 29-29.1 min, 75-1.5% B; 29.1-43 min, 1.5% B. Monosaccharides were
362 quantified using both external calibration standards containing a mixture of Fuc, Rha, Ara, Gal, Glc,
363 Xyl, GlcA, and GalA, as well as variable internal calibration with Fuc added to each sample in the
364 range of 6-13 ppm. The standard curves for each sugar were fit with a linear calibration curve
365 without offset, except for Fuc where a second order polynomial calibration curve without offset
366 was used.

367 HPAEC-PAD was also used for the analysis of the oxidation products of LPMO reactions using the
368 method of Westereng et al.³⁴. This involved the use of a Dionex ICS-3000 Ion Chromatography
369 system with a CarboPac® PA1 analytical column (250 x 2 mm) and a PAD, all from Dionex
370 Corporation (Sunnyvale, CA). Note that no guard column was used in these experiments. The flow
371 rate used was 0.25 mL/min under the following elution profile, with 0.1 M NaOH as eluent A and 1
372 M NaOAc in 0.1M NaOH as eluent B: 0-10min, 0-10% B; 10-35 min, 10-30% B; 35-40 min, 30-100%
373 B (Dionex curve 6 - exponential); 40-41 min, 100-0% B; 41-50 min, 0% B.

374 4.5.2 Direct Infusion Electrospray Ionization Mass Spectrometry (ESI-MS)

375 Mass spectrometry analysis by ESI-MS was performed on a linear ion trap LTQ Orbitrap Fusion
376 from Thermo Scientific (Waltham, MA) coupled to a TriVersa NanoMate® chip-based electrospray
377 device from Advion (Ithaca, NY). The analyses were done by direct infusion to the MS without

378 chromatographic separation. Samples were prepared in a 96-well microtiter plate with a
379 methanol:sample ratio of 1:2. The electrospray was controlled with Advion ChipSoft Manager
380 version 8.3.1 and operated in positive mode with a gas pressure (N₂) of 0.6 psi, voltage of 1.8 kV, 5
381 µL tip collection, and sample temperature of 7 °C utilizing plastic tips (part no. 1004763) and a 5
382 µm ESI Chip[®] (part no. 1003446). Fluoranthene was used as an internal calibrant and the ion
383 transfer tube temperature was set to 200 °C. The acquisition time was set to 2.0 min, with
384 approximately 40 scans per minute for MS and MS-MS. MS full scans were performed in the m/z
385 300–1,200 mass range, utilizing an Orbitrap detector at 120K resolution and 2 microscans. MS-MS
386 scans were performed on Na-adducts with CID (collision-induced dissociation), using He as the
387 colliding gas in the m/z 105–400 scan range with quadrupole isolation, Orbitrap detection at 15K
388 resolution, an isolation window of 2, 50% collision energy, and 3 microscans. MS Data from ESI-MS
389 was processed in Xcalibur 3.0.63 from Thermo Scientific (Waltham, MA).

390 4.5.3 Measurement of total protein concentration

391 Total protein concentration was measured using the Bio-Rad Colorimetric Protein Assay based on
392 the Bradford method³⁶. In brief, 50 µL of enzyme sample was combined with 2.5 mL of 4x diluted
393 Protein Assay Dye Reagent Concentrate and incubated at room temperature for 5 mins.
394 Absorbance was measured at 595 nm and compared to the absorbance measured against known
395 concentrations of BSA from 0.1-0.9 mg/mL. All measurements were performed in duplicate.

396 4.5.4 SDS-PAGE

397 SDS-PAGE gels were run using an Invitrogen XCell SureLock™ Mini-Cell Electrophoresis system,
398 Invitrogen NuPAGE 4-12% Bis-Tris Gels, and SeeBlue[®] Plus2 Pre-Stained Standard from Thermo
399 Scientific (Waltham, MA). Proteins were incubated at 90 °C for 5 mins with 1.0 M sodium-

400 phosphate buffer, pH 6 and sample buffer containing DTT and 1% bromophenol blue. Following
401 electrophoresis, gels were stained with SimplyBlue™ SafeStain from Thermo Scientific (Waltham,
402 MA).

403 4.5.5 N-terminal Characterization with Liquid Chromatography-Mass Spectrometry (LC-MS)

404 Proteomic analysis was performed using a modified spin filter protease digestion as previously
405 described by Manza et al. and Wiśniewski et al.^{37,38}. Samples were reduced with DTT and alkylated
406 with iodoacetamide prior to protease digestion. Amino acid sequences were used to select an
407 optimal protease for N-terminal characterization: Chymotrypsin (Chacr1, Aspfu1, Aspfu2, Aspte2,
408 Aspte5, Talle1, and TrCel61A), Trypsin (Aspfu3, Aspfu4, Aspfu5, Aspte6, Penc12, Aspte7, Aspte8,
409 Malci2, Mycth1, and Mycth3), and Asp-N (Chacr2, Aspte1, Aspte3, Aspte4, Penc11, Malci1, and
410 Mycth2). Following protease digestion, samples were dried in a Thermo Scientific (Waltham, MA)
411 Savant™ SpeedVac™ at 45°C for 8 hrs, and dissolved in 100 µL of 0.1% TFA for LC-MS analysis.

412 LC separation was performed using an UltiMate 3000 Nano LC system from Dionex Corporation
413 (Sunnyvale, CA), a 5 µm Acclaim PepMap C18 guard column (20 mm x 100 µm) from Thermo
414 Scientific (Waltham, MA), and a 3 µm ReproSil-Pur C18-AQ column (100 mm x 75 µm) from Dr.
415 Maisch GmbH (Ammerbuch-Entringen, Germany). The flow rate used was 300 nL/min under the
416 following elution profile, with H₂O/formic acid (100:0.1) as eluent A and acetonitrile/formic acid
417 (100:0.1) as eluent B: 0-3 min, 2% B; 3-21 min, 2-41% B; 21-23 min, 41-95% B; 23-29 min, 95% B;
418 29-31 min, 95-2% B; 31-44 min, 2% B. MS and MS-MS analysis by ESI-MS was performed in
419 positive mode on a linear ion trap LTQ Orbitrap Fusion with a capillary temperature of 200°C, 2.1
420 kV ESI voltage, and quadrupole isolation. For MS-MS analysis, both CID (collision-induced
421 dissociation) and HCD (higher-energy collisional dissociation) fragmentation were performed. MS

422 data was processed in Xcalibur 3.0.63 from Thermo Scientific (Waltham, MA) and Mascot Server
423 from Matrix Science Inc. (Boston, MA). A summary of the results from this analysis is provided in
424 Supplementary Information Section 3.

425 4.6 Enzymatic Reactions

426 4.6.1 Primary LPMO Screening

427 Reaction mixtures of 200 μ L total volume contained 4 mg/mL substrate cellulose, 0.02% sodium
428 azide, and 1 mM ascorbic acid in 10 mM Na-acetate, pH 6.0. All LPMOs were Cu(II) saturated by
429 incubating 0.3 mg/mL protein in 10 mM Na-acetate, pH 6.0 with a 3-fold molar excess of Cu(II)SO₄
430 for 30 minutes at room temperature. Excess copper was removed by PD MidiTrap G-25 filtration,
431 as described by Loose, Forsberg, Fraaije, Eijsink, & Vaaje-Kolstad³⁹ with 10 mM Na-acetate, pH 6.0
432 used in place of the 20 mM Tris-HCl, pH 8.0. LPMOs were dosed based on protein content at 0.2
433 mg/mL. Samples were incubated at 40 °C with 600 rpm mixing for 24 hrs. Following the reaction,
434 all enzyme samples were heat-inactivated through incubation at 95 °C for 5 min and centrifuged at
435 16,300 x g for 20 mins to isolate the supernatants from the pellets.

436 4.6.2 Glucose release with LPMO, endoglucanase, and beta-glucosidase

437 All LPMOs were Cu(II) saturated as described in 4.6.1, however a protein concentration of 0.2
438 mg/mL was used during copper saturation. LPMOs were dosed based on protein content at 0.05
439 mg/mL with samples incubated at 40 °C with 600 rpm mixing for varying times from 0-72 hrs. For
440 reactions utilizing a combination of LPMOs, the total LPMO protein dosage was divided equally,
441 with the total maintained at 0.05 mg/mL. All reactions also contained endo-1,4- β -D-glucanase (EG)
442 and beta-glucosidase (BG) dosed at 0.05 and 0.125 mg/mL, respectively. Following the reaction, all

443 enzyme samples were heat-inactivated through incubation at 95 °C for 5 min and centrifuged at
444 16,300 x g for 20 mins to isolate the supernatants from the pellets. Glucose release was quantified
445 by HPAEC-PAD as described in 4.5.1 and sample data was converted into substrate glucose
446 released (%) by dividing the amount of glucose released in the enzyme treatment by the total
447 amount present in the original reaction substrate and multiplying by 100. Glucose release was
448 compared in Minitab® 17 from Minitab Inc. (State College, PA) using two-way analysis of variance
449 and means comparisons by the Tukey pairwise comparison method.

450 4.6.3 Beta-glucosidase treatment of LPMO reaction products

451 Following LPMO enzymatic treatment, supernatants were treated with beta-glucosidase from
452 *Agrobacterium sp.* to degrade non-C4 oxidized reaction products for analysis with HPAEC-PAD as
453 described in 4.5.1. Beta-glucosidase was dosed based on protein at 0.05 mg/mL in 100 µL
454 reactions containing 40% sample from the primary enzymatic treatment in 10 mM Na-acetate
455 buffer, pH 6.0. Samples were incubated at 40 °C with 600 rpm shaking for 20 hrs. Following the
456 reaction, beta-glucosidase was heat-inactivated by incubating the samples at 95 °C for 5 min and
457 samples were analyzed by HPAEC-PAD using the method of Westereng et al.³⁴.

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469 The author contributions are as follows: B. C. P. contributed to the study design, analysis and
470 interpretation of the results as well as the writing of the manuscript. J. W. A. contributed to the
471 study design, data interpretation, and manuscript revision. Z. Z. conducted the enzyme expression
472 and production for the samples utilized in this study. J. W. and A. S. M. contributed to the study
473 design, data interpretation, manuscript revision, and project supervision.

474 **Figure Captions**

475 **Figure 1** – ESI CID MS-MS fragmentation of the DP2 oxidized product (m/z 381.10) from LPMO
476 treated PASC after 24 hrs. Masses are labeled based on expected fragmentation from the C4
477 oxidized product Glc4gemGlc (blue) and/or the C1 oxidized product GlcGlc1A (red). A: *TrCel61A*; B:
478 *Aspte6*; C: *Talle1*; D: Expected fragmentation products from Glc4gemGlc and GlcGlc1A.
479 Parenthesis indicate an unlikely product.

480 **Figure 2** – HPAEC-PAD profile of LPMO treated NaOH pretreated soy spent flakes both with (black,
481 1 nC offset) and without (red) ascorbic acid as per Westereng et al.³⁴. A: *TrCel61A*; B: *Aspte6*; C:
482 *Talle1*; D: DP 2-6 cello-oligosaccharides.

483 **Figure 3** – Glucose release from NaOH pretreated soy spent flakes following treatment with LPMO
484 (0.05 mg/mL protein), EG (0.05 mg/mL protein), and BG (0.125 mg/mL protein) in the presence of

485 1 mM ascorbic acid, as determined by HPAEC-PAD analysis. Data are shown as averages of
486 duplicate analyses with standard deviations given by error bars. Tukey-Kramer HSD comparisons
487 are provided for each time point with data points marked by differing letters at a given timepoint
488 signifying statistically different means.

489 **Figure 4** – Glucose release from NaOH pretreated soy spent flakes following 72 hr treatment with
490 LPMO(s) (0.05 mg/mL protein), EG (0.05 mg/mL protein), and BG (0.125 mg/mL protein) in the
491 presence of 1 mM ascorbic acid, as determined by HPAEC-PAD analysis. Samples contain either a
492 single LPMO (red) or two LPMOs (blue). Data are shown as averages of duplicate analyses with
493 standard deviations given by error bars. One-way analysis of variance showed no statistically
494 significant differences ($p > 0.05$) between the six treatments presented.

495 **Table Captions**

496 **Table 1** – LPMOs utilized in this study listed with their reference ID, native organism, NCBI
497 reference ID²⁹, presence of an InterPro predicted C-terminal cellulose binding domain (CBD)³⁰, and
498 measured protein concentration.

499 **Table 2** – LPMO activity on PASC, native soy spent flakes, NaOH pretreated soy spent flakes, and
500 Avicel®. Legend: - = no activity observed; + = weak activity; ++ = moderate activity; +++ = strong
501 activity.

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569 3440.

Table 1:

LP MO ID	Native Organism	NCBI Reference ID	InterPro Predicted CBD	[Protein] (mg/mL)
TrCel61A	<i>Trichoderma Reesei</i>	UniProt:O14405	Yes	38.30
Chacr1	<i>Chaetosartorya cremea</i>	-	Yes	0.37
Chacr2	<i>Chaetosartorya cremea</i>	-	-	0.46
Aspfu1	<i>Aspergillus fumigatus</i>	-	Yes	0.38
Aspfu2	<i>Aspergillus fumigatus</i>	-	Yes	0.40
Aspfu3	<i>Aspergillus fumigatus</i>	GenBank: GAQ10694.1	-	0.65
Aspfu4	<i>Aspergillus fumigatus</i>	-	Yes	0.48
Aspfu5	<i>Aspergillus fumigatus</i>	GenBank: GAQ07946.1	-	0.55
Aspte1	<i>Aspergillus terreus</i>	-	-	0.65
Aspte2	<i>Aspergillus terreus</i>	-	Yes	0.74
Aspte3	<i>Aspergillus terreus</i>	-	-	0.56
Aspte4	<i>Aspergillus terreus</i>	-	-	0.66
Aspte5	<i>Aspergillus terreus</i>	-	Yes	0.67
Aspte6	<i>Aspergillus terreus</i>	-	Yes	0.59
Penci1	<i>Penicillium citrinum</i>	-	-	0.46
Penci2	<i>Penicillium citrinum</i>	-	-	0.25
Aspte7	<i>Aspergillus terreus</i>	GenBank: GAQ40595.1	Yes	0.35
Aspte8	<i>Aspergillus terreus</i>	-	-	0.23
Malci1	<i>Malbranchea cinamomea</i>	-	-	0.21
Malci2	<i>Malbranchea cinamomea</i>	-	-	0.36
Mycth1	<i>Myceliophthora thermophila</i>	-	-	0.16
Mycth2	<i>Myceliophthora thermophila</i>	-	Yes	0.28
Mycth3	<i>Myceliophthora thermophila</i>	-	-	0.31
Talle1	<i>Talaromyces leycettanus</i>	-	Yes	0.56

Table 2:

LPMO	PASC	Native Soy	NaOH Soy	Avicel®
<i>TrCel61A</i>	+++	-	+++	-
Chacr1	+	-	-	-
Chacr2	++	-	+	++
Aspfu1	++	-	+	+
Aspfu2	++	-	-	-
Aspfu3	+++	-	-	-
Aspfu4	+++	-	+	+
Aspfu5	+++	-	+	+
Aspte1	-	-	-	-
Aspte2	+++	-	-	+
Aspte3	++	-	-	-
Aspte4	++	-	-	-
Aspte5	++	-	-	-
Aspte6	+++	-	+++	+++
Penci1	++	-	-	-
Penci2	++	-	-	-
Aspte7	+	-	-	-
Aspte8	+	-	-	-
Malci1	+	-	-	-
Malci2	++	-	-	-
Mycth1	-	-	-	-
Mycth2	+	-	-	-
Mycth3	-	-	-	-
Talle1	+++	-	++	+++

Figure 1:

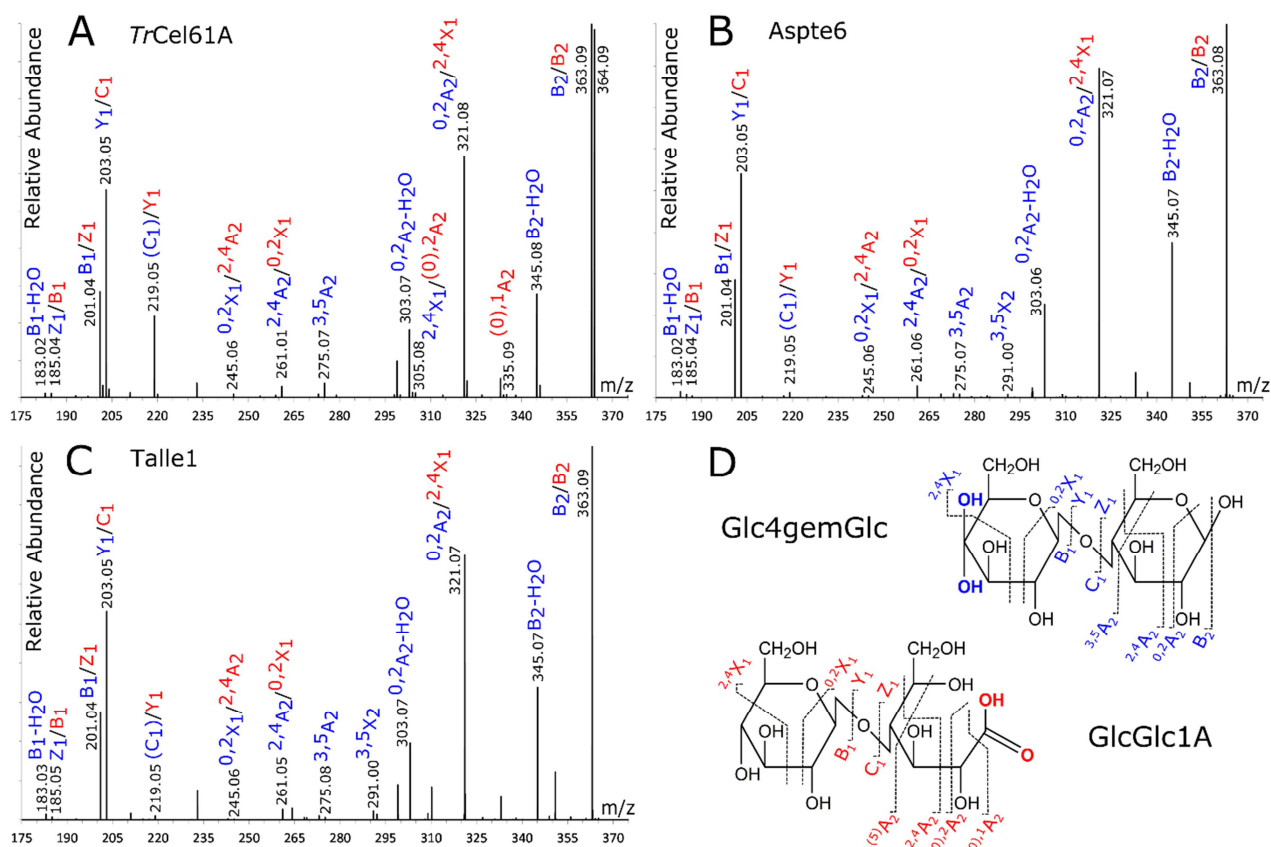
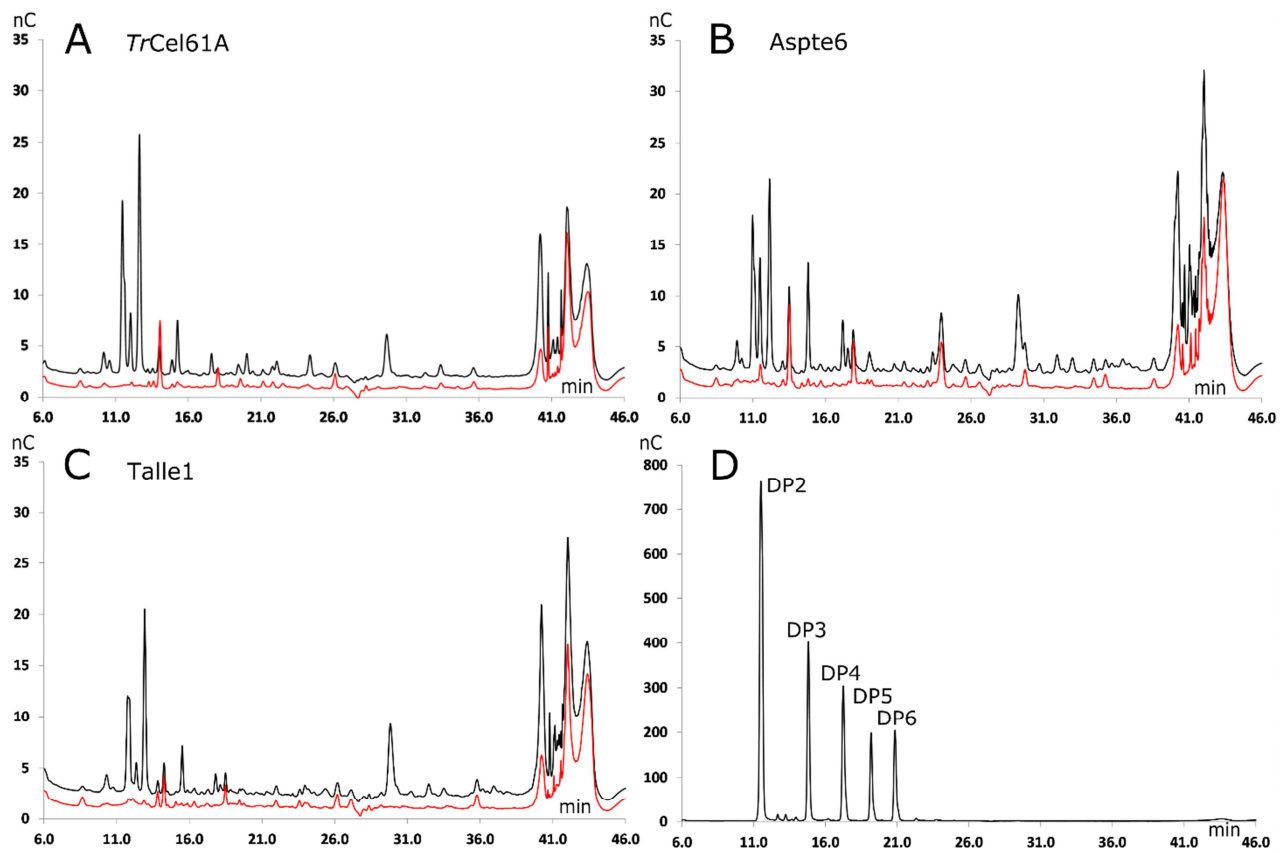


Figure 2:



ACCEPTED

Figure 3:

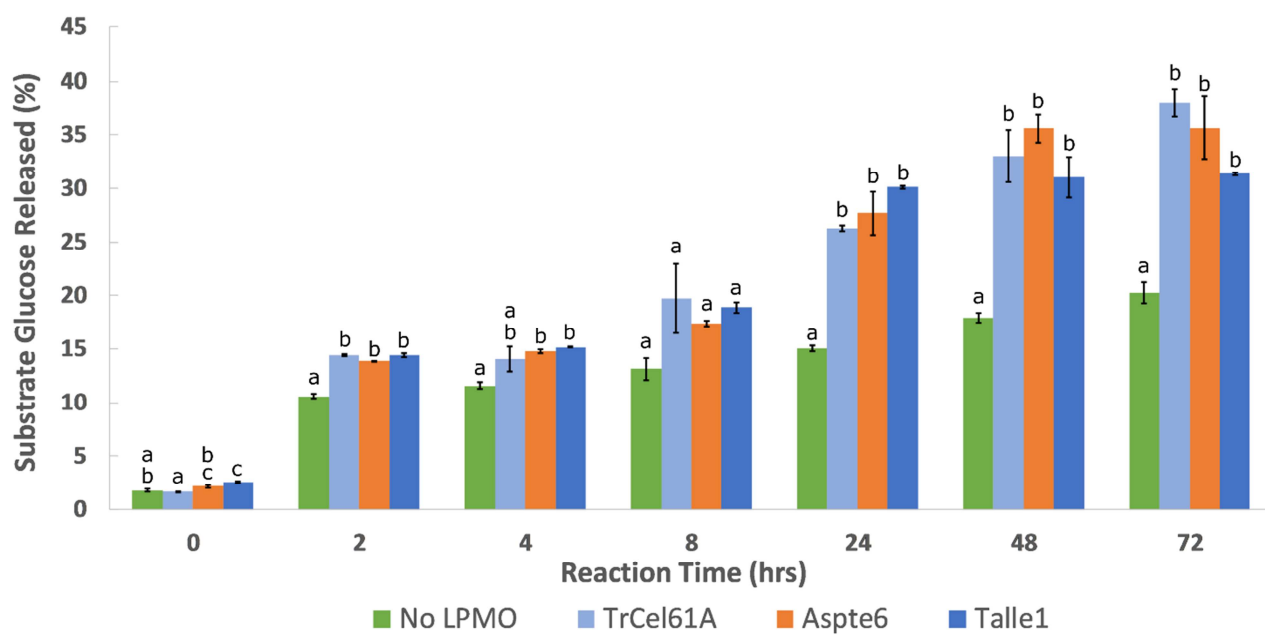
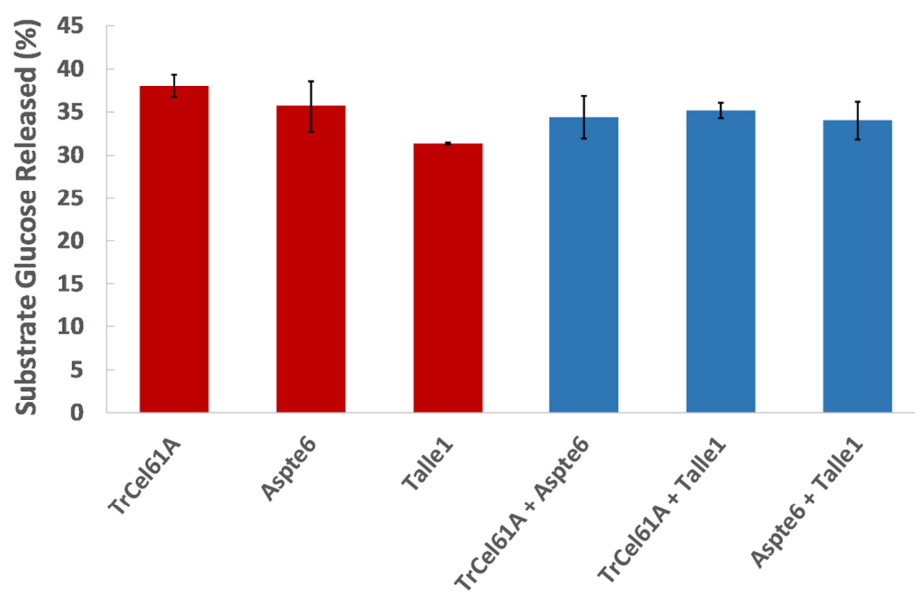


Figure 4:



Highlights

- Twenty-four LPMOs were investigated for activity on NaOH pretreated soy spent flake.
- Seven LPMOs showed activity on pretreated soy spent flake.
- Oxidative activity (C1, C4, and C1-C4) was determined with ESI-MS and HPAEC-PAD.
- Two LPMOs (*TrCel61A* and *Aspte6*) showed significant hydrolytic boosting.
- LPMOs boost glucose release from soy spent flakes by EG and BG by more than 75%.