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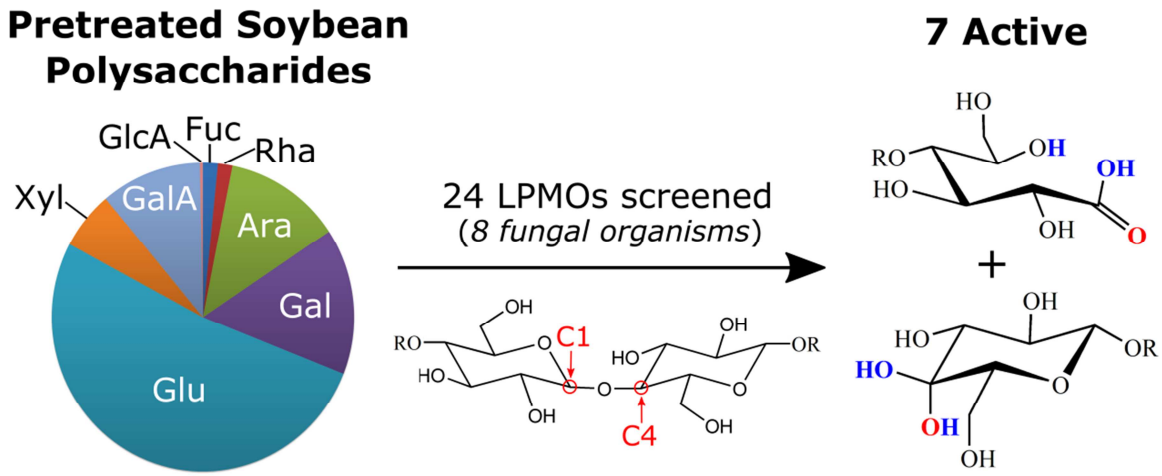
Published in:
CARBOHYDRATE RESEARCH

Link to article, DOI:
10.1016/j.carres.2017.07.004

Publication date:
2017

Document Version
Peer reviewed version



Title

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

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Abstract

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes capable of the oxidative breakdown of polysaccharides. They are of industrial interest due to their ability to enhance the enzymatic depolymerization of recalcitrant substrates by glycoside hydrolases. In this paper, twenty-four lytic polysaccharide monooxygenases (LPMOs) expressed in *Trichoderma reesei* were evaluated for their ability to oxidize the complex polysaccharides in soybean spent flakes, an abundant and industrially relevant substrate. *Tr*Cel61A, a soy-polysaccharide-active AA9 LPMO from *T. reesei*, was used as a benchmark in this evaluation. In total, seven LPMOs demonstrated activity on pretreated soy spent flakes, with the products from enzymatic treatments evaluated using mass spectrometry and high performance anion exchange chromatography. The hydrolytic boosting effect of the top-performing enzymes was evaluated in combination with endoglucanase and beta-glucosidase. Two enzymes (*Tr*Cel61A and Aspte6) showed the ability to release more than 36% of the pretreated soy spent flake glucose – a greater than 75% increase over the same treatment without LPMO addition.

Keywords

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

Abbreviations

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

1. Introduction

Soybean polysaccharides are produced in large quantities globally as an industrial by-product of soy protein, soy milk, and tofu production. When resulting from tofu or soy milk production they are generally referred to as okara, and when generated as a by-product of soy protein isolate production they are known as spent flakes[1]. These materials are predominantly composed of high molar mass, water-insoluble cell wall polysaccharides including type I arabinogalactan, cellulose, arabinoxylan, rhamnogalacturonan, arabinan, xyloglucan, and homogalacturonan[2]. Each year, approximately 3.91 x 10^6 tons of soybean polysaccharides are produced through Chinese, Japanese and Korean tofu production, with the vast majority of this material disposed of as waste or burned[3-5]. This disposal not only adds significant costs to the production process, but also raises environmental sustainability concerns. It is therefore of significant interest to develop alternate applications for improved utilization of this abundant industrial by-product.

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes capable of polysaccharide depolymerization through oxidation of sugar residues[6]. They have been classified as auxiliary activity (AA) enzymes in the Carbohydrate-Active enZyme database (CAZy; http://www.cazy.org)[7], and are currently categorized into four families: AA9 (formerly GH61), AA10 (formerly CBM33), AA11, and AA13. AA9 comprises fungal LPMOs active on lignocellulose, AA10 predominantly comprises LPMOs of bacterial and viral origin cleaving cellulose and chitin, AA11 comprises fungal LPMOs that cleave chitin, and AA13 comprises fungal LPMOs that cleave starch[8]. The importance of this oxidative mechanism in cellulose degradation was first suggested in 1974 by Eriksson et al.[9]. In 2010, Vaaje-Kolstad et al. described the oxidative mode of action of LPMOs and demonstrated their capacity to boost the enzymatic degradation of chitin[10]. 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-oligosaccharides[11-16]. The precise oxidative mode of action of LPMOs is
48 still under debate, but has been postulated to involve internal electron channels[17] or, more
49 recently, Fenton-type H_2O_2 chemistry[18]. 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 suggested[20]. 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 histidine[21]. LPMOs which have been expressed in fungi routinely display a characteristic Nϵ
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 substrate[22]. It has also recently been suggested that this methylation
59 may play a role in protecting fungal LPMOs from oxidative self-destruction[18].

60 One reason for the recent interest in LPMOs is their ability to boost the activity of cellulolytic
61 enzymes in the degradation of biomass[23-25]. Our previous work demonstrated the ability of the
62 AA9 LPMO *Tr*Cel61A from *Trichoderma reesei* to oxidize the cellulosic polysaccharides of NaOH
63 pretreated soybean spent flakes at both the C1 and C4 positions[26]. In addition, *Tr*Cel61A
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

a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for select cellulase genes and beta-glucosidase[27]. Following expression, the N-terminal protein sequence was assessed in order to verify the N-terminal histidine and evaluate the presence of the N-terminal histidine Nϵ methylation. The activities of each LPMO were investigated against a variety of substrates (phosphoric acid swollen cellulose (PASC), native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel® PH-101 microcrystalline cellulose) and oxidation products of these treatments were analyzed with ESI mass spectrometry and high performance anion exchange chromatography (HPAEC). For the LPMOs displaying activity on soy spent flakes, the effect of LPMO addition on substrate glucose release through endoglucanase (EG) and beta-glucosidase (BG) treatment was also investigated.

2. Results and Discussion

2.1 Protein characterization

Table 1 provides a list of the twenty-four LPMOs utilized in this study. To date, the majority of these proteins have not yet been reported to have LPMO activity, with only four of them having an exact amino acid sequence match in the National Center for Biotechnology Information (NCBI) BLAST® non-redundant protein sequence database[28, 29]. The closest matches for all twenty-four LPMOs from a BLAST® search are provided in Supplementary Information Section 2. Note that ten of the LPMOs showed high levels of sequence homology with proteins listed in the sequence database, giving 100% sequence coverage and matching scores of at least 99% identity, 99% positives, and 0 gaps. Table 1 also contains the results of an InterPro protein sequence domain analysis indicating whether a cellulose binding domain (CBD) is predicted based on the sequences of the LPMOs analyzed[30]. Supplementary Information Figure S1 shows the SDS-PAGE gels obtained

for each LPMO used in this study. The presence of band spreading and increased molecular weights over those expected from the amino acid sequence demonstrate the presence of glycosylation, a common post-translational event which occurs during protein expression in fungi[31].

Protease digestion in combination with MS/MS-MS analysis was used to identify the N-terminal sequences of the twenty-four expressed LPMOs. A summary of the results from this analysis are presented in Supplementary Information Section 3. All twenty-four proteins were identified to primarily have N-terminal histidine residues. Chacr2 (G), Aspfu3 (G), Aspfu5 (G), Aspte1 (G), Aspte3 (G), Aspte4 (G), Aspte5 (A), Aspte6 (A), Penci1 (G), Penci2 (G), and Mycth3 (A) showed the presence of some proteins containing one additional N-terminal amino acid, indicated in parenthesis, resulting in the lack of an N-terminal histidine. While one LPMO (Penci2) showed a significant amount of expression with the amino acid addition, the relative abundance of these observed peptides for the other LPMOs were several orders of magnitude lower than peptides containing the N-terminal histidine. It is interesting to note that the majority of these amino acid additions occur with the presence of glycine immediately preceding the N-terminal histidine. In fact, only two protein sequences featuring a glycine prior to the expected terminal histidine lacked any observed N-terminal glycine addition (Malcil1 and *Tr*Cel61A). Aspfu1 (G), Aspfu2 (G), Aspfu3 (G), Aspfu4 (G), Aspfu5 (G), Aspte5 (G), Aspte6 (G), Aspte7 (Y), Aspte8 (Y), Mycth1 (Y), Mycth3 (T), and Talle1 (G) showed the presence of a one amino acid truncation, with the new N-terminal amino acid indicated in parenthesis. Significant levels of the truncated sequence were observed for Aspfu4, Aspte6, Aspte7, Aspte8, and Mycth1; however, in all cases the relative abundances of these observed peptides were significantly lower than the peptides containing the N-terminal histidine.

The presence of N-terminal histidine methylation was also evaluated due to its expected presence following expression in *T. reesei*[23]. The Nε methylation of the N-terminal histidine was observed for all twenty-four LPMOs in this study. It is interesting to observe that the methylation from *T. reesei* expression is observed even though leader peptides that are not endogenous to this organism were used. For the majority of proteins, the methylated N-terminal histidine was identified as the most prominent N-terminal fragment. Ten of the proteins (Aspte1, Aspte3, Aspte4, Penci2, Aspte7, Aspte8, Malci1, Malci2, Mycth2, and *Tr*Cel61A) were observed to have methylation present on all or the vast majority of their N-terminal histidine residues. However, a non-methylated N-terminal histidine was identified to varying degrees in the remaining fourteen proteins (Chacr1, Chacr2, Aspfu1, Aspfu2, Aspfu3, Aspfu4, Aspfu5, Aspte2, Aspte5, Aspte6, Penci1, Mycth1, Mycth3, and Talle1). For the vast majority of these proteins, peptides corresponding to the methylated N-terminal histidine were significantly more abundant than those of its non-methylated counterpart. However, five of the proteins (Chacr1, Aspfu1, Aspfu2, Aspfu4, and Talle1) showed a higher abundance of the non-methylated protein.

2.2 Evaluation of LPMO Activity

The twenty-four LPMOs listed in Table 1 were evaluated for their ability to oxidatively cleave the polysaccharides in four substrates: PASC, native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel®. The NaOH pretreatment of soy spent flakes serves to convert the cellulose I allomorph into cellulose II, in addition to reducing the cellulose crystallinity[32]. As previously reported, this NaOH pretreatment results in a reduction of substrate hemicellulose and a corresponding enrichment in cellulosic polysaccharides. The molar percentages of galactose, 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%, respectively[26]. 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-aldonic 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

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

No oxidative activity was observed for any of the LPMOs on the native soy spent flakes; however, seven of the LPMOs investigated (*Tr*Cel61A, Chacr2, Aspfu1, Aspfu4, Aspfu5, Aspte6, and Talle1) did show activity on the NaOH pretreated spent flakes. This finding matches our previously reported results concerning the activity of *Tr*Cel61A on soy polysaccharides, and also indicates that the complex polysaccharides of soybean are highly resistant to oxidative degradation by LPMOs using the conditions employed in this study[26]. While the exact structural relationship between the cellulosic and hemicellulosic polysaccharides in soybean has not yet been fully characterized, it can be hypothesized from these findings that the hemicellulose is capable of shielding the cellulose from enzymatic attack by LPMOs. It is interesting to note from Table 2 that all LPMOs which showed activity on the NaOH pretreated soy spent flakes also showed strong activity on PASC and some degree of activity on the crystalline cellulose in Avicel®. This commonality between Avicel® activity and NaOH pretreated soy spent flake activity suggests a degree of similarity between these two substrates, and may be an indication of remaining cellulose crystallinity in the spent flakes despite the pretreatment employed in this study. While this trend is almost universal for the LPMOs considered in this study, it was observed that Aspte2 showed no detectable activity on NaOH pretreated spent flakes despite its observed activity on both PASC and Avicel®.

From the seven LPMOs showing activity on the pretreated spent flakes, three were selected as the top-performing enzymes (*Tr*Cel61A, Aspte6, and Talle1) for further screening based on the abundance of oxidized products detected with ESI MS. Figure 1 shows the ESI MS-MS CID

fragmentation spectra of the sodiated and oxidized $[M+16-Na]^+$ ion (*m/z* 381.10) following the LPMO treatment of PASC with these three LPMOs. The fragments have been labeled with the nomenclature of Domon and Costello[33], with Figure 1D illustrating the possible fragmentation products for both the C4 oxidized gemdiol, Glc4gemGlc (blue), and the C1 oxidized aldonic acid, GlcGlc1A (red) as described by Isaksen et al.[16]. As was previously reported[26], *Tr*Cel61A demonstrates characteristics of both C1 and C4 oxidation following treatment with PASC (Figure 1A). Characteristic C4 fragmentations include the loss of two water molecules (*m/z* 363.09 and 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* 203.05); and loss of water in the B_1 fragment (*m/z* 183.02). Characteristic C1 fragmentations include the characteristic loss of a carboxyl group ($^{(0),1}A_2$; *m/z* 335.09) and the presence of a large Y_1 fragment (*m/z* 219.05). While this latter signal could also correspond to the C_1 fragment of C4 oxidation, this fragment is expected to be in low abundance after undergoing rapid dehydration and has therefore been marked in parenthesis to indicate this effect.

Comparing Figure 1A to the other LPMO treatments, we continue to observe the characteristic C4 oxidation fragments; however, fragmentations characteristic of C1 oxidation ($^{(0),1}A_2$; Y_1) are no longer present. This is evidenced by the lack of observed signals corresponding exclusively to C1 oxidation (red). In particular, the low signals observed for the $(C_1)/Y_1$ fragment (*m/z* 219.05) for the non-*Tr*Cel61A LPMO treatments indicate a lack of C1 oxidation. Aspte6 and Talle1 show an additional C4 oxidation fragment, $^{3,5}X_2$, not observed in the *Tr*Cel61A sample; however, they also lack the C1/C4 oxidation fragment $^{2,4}X_1/^{(0),2}A_2$, which is observed in the *Tr*Cel61A sample. In order to further explore the observation that all LPMOs showing activity on NaOH pretreated spent flakes demonstrate C4 oxidation, HPAEC-PAD profiles were run to analyze the oxidation products.

Figure 2 shows the HPAEC-PAD chromatograms of the products produced by these three enzymes on NaOH pretreated soy spent flakes both with (black) and without (red) ascorbic acid as a reductant. In addition, the elution pattern of the neutral cello-oligomers from DP 2-6 is presented for reference (Figure 2D). In the presence of ascorbic acid, signals are observed in the characteristic neutral (~5-22 min), C1 oxidized (~20-30 min), C4 oxidized (~30-40 min), and C1-C4 oxidized (~40-46 min) regions of this chromatogram as described by Westereng et al.[34]. The strongest signals are observed for *Tr*Cel61A and Aspte6, corresponding well to the strength of LPMO activity summarized in Table 2. Both of these enzymes show evidence of C1 and C4 oxidation (Figure 2A and 2B), while Talle1 shows the majority of its signals in the C4 oxidation region (Figure 2C). While signals are observed in the late-eluting C1-C4 oxidized region, the presence of additional signals in this region both with and without ascorbic acid makes it difficult to unambiguously confirm the presence of these doubly oxidized products.

To further investigate the observed correlation between C4 oxidation and activity on the pretreated spent flakes, additional HPAEC-PAD experiments were conducted where the soluble fractions following LPMO treatment of PASC were further treated with BG. Due to the non-reducing end activity of BG, this treatment will result in degradation of neutral and C1 oxidized soluble oligomers, but will leave the C4 oxidized compounds intact due to their modified non-reducing end. Evaluating the HPAEC-PAD chromatograms of all BG treated LPMO samples for retained signals in the C4 oxidized region (~30-40 min), we observe that seven enzymes (*Tr*Cel61A, Chacr2, Aspfu3, Aspfu4, Aspfu5, Aspte6, and Talle1) continue to show strong evidence of C4 oxidation and four enzymes (Aspfu1, Aspte2, Aspte3, and Aspte4) show weak evidence of C4 oxidation (results not shown). All other LPMOs show no signals in this region of the chromatogram, indicating that no C4 oxidation took place. Comparing these results to the activity data presented

in Table 2, we observe that all LPMOs demonstrating activity on the NaOH pretreated spent flakes also show confirmed C4 oxidation. In addition, the three top-performing enzymes (*Tr*Cel61A, Aspte6, and Talle1) selected above show strong C4 oxidation, confirming the MS-MS results presented in Figure 1. It is interesting to note that four LPMOs (Aspfu3, Aspte2, Aspte3, and Aspte4) showed evidence of C4 oxidation, but no activity on the pretreated spent flakes. Lastly, it should be noted that all oxidation effects described above were absent in control samples treated with each LPMO in the absence of ascorbic acid (results not shown).

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

In our previous work, we demonstrated the hydrolytic boosting capability of *Tr*Cel61A when dosed in combination with EG for the enzymatic degradation of NaOH pretreated spent flakes[26]. In order to further explore this effect, the top-performing LPMOs described in Section 2.2 were evaluated for their hydrolytic boosting ability. Figure 3 shows the HPAEC measured glucose release from NaOH pretreated soy spent flakes following LPMO treatment in combination with EG and BG for 0-72 hrs. We observe that all three of the LPMOs evaluated (*Tr*Cel61A, Aspte6, and Talle1) show a significant hydrolytic boosting effect when compared to samples treated without LPMO. Statistical evaluation by the Tukey-Kramer HSD method was conducted and the results are presented in Figure 3. Additional statistical evaluation with two-way analysis of variance (ANOVA) describes the significant correlation between glucose release and both reaction time and LPMO treatment (Supplementary Information Section 5). This analysis confirms that the glucose release depends on the interaction of these factors, as evidenced by the data presented in Figure 3. In addition to the Tukey-Kramer analysis presented in Figure 3, additional Tukey pairwise comparisons were

conducted on the complete dataset in Figure 3 (irrespective of time point) and are provided in Supplementary Information Section 5. These results show that the LPMO treatments from 8-72 hrs result in statistically significant increases in glucose release over the controls lacking LPMO. In addition, differences between the boosting effects of the three enzyme treatments are not statistically significant at each of these time points. The addition of *Tr*Cel61A, Aspte6, and Talle1 result in glucose yield increases of 88%, 76%, and 55%, respectively, when compared to enzymatic treatment with EG and BG alone. *Tr*Cel61A and Aspte6 are capable of ~36% substrate glucose release after 72 hrs of reaction, the highest yields obtained in this study and a comparable result to those obtained in previous work with *Tr*Cel61A (34.48 ± 4.84%)[26]. It is also interesting to note the rate of reaction observed in Figure 3. Significant glucose release is obtained within the first 2 hrs of reaction (10-15%), however no significant additional glucose release is observed from 2-4 hrs. After 24 hrs of reaction, the samples treated with LPMOs capable of hydrolytic boosting begin to significantly differentiate themselves from controls. While samples containing Talle1 showed the highest glucose release after 24 hrs, the reaction appears to have been completed in this timeframe as no additional glucose release was observed beyond this point.

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

The data presented in Figures 1 and 2 indicate that the three top-performing LPMOs from this study (*Tr*Cel61A, Aspte6, and Talle1) show similar oxidation on NaOH pretreated spent flakes. As described above, the hydrolytic boosting effect demonstrated in Figure 3 is consistent across these three enzymes as well, with Figure 4 showing that 50% of the LPMO dosage can be replaced with another similarly acting LPMO to result in comparable hydrolytic boosting effects. These results indicate that this is a more generalized effect, and not necessarily tied to the specific activity of an individual LPMO. However, the results presented above do show commonality between the LPMOs displaying activity on the pretreated spent flakes. *Tr*Cel61A, Aspte6, and Talle1 all show significant C4 oxidizing activity (Figures 1 and 2) as well as activity on Avicel®, suggesting a link between these features and activity on pretreated spent flakes.

3. Conclusions

In this study, we have characterized the LPMO activity of twenty-four enzymes against four substrates: PASC, native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel® PH-101 microcrystalline cellulose. Following expression in *T. reesei*, the enzymes were evaluated for the presence of N-terminal histidine and methylation of this histidine, with all enzymes showing these features. Twenty-one enzymes displayed oxidative activity on PASC, seven showed activity on Avicel®, seven showed activity on NaOH pretreated soy spent flakes, and none showed activity on native soy spent flakes. Of the seven LPMOs showing activity on pretreated soy spent flakes (*Tr*Cel61A, Chacr2, Aspfu1, Aspfu4, Aspfu5, Aspte6, and Talle1), three were selected as the top-performing enzymes (*Tr*Cel61A, Aspte6, and Talle1). ESI MS-MS fragmentation analysis demonstrated predominantly C4 oxidizing activity in these enzymes, and the presence of C4 oxidized compounds was confirmed with HPAEC analysis. These results show a link between an

LPMO's C4 oxidizing ability, activity on the microcrystalline Avicel® substrate, and oxidative activity on pretreated soy spent flakes. This observed correlation between C4 oxidizing activity and activity on NaOH pretreated soy spent flakes demonstrates a link between substrate structure and LPMO oxidative regioselectivity. This observation may offer some insight into the reasons for the evolution of regioselectivity in LPMOs, and highlights the continued need for research in this area to more fully understand the reasons behind oxidative regioselectivity. In addition to these observations, the hydrolytic boosting effect of these enzymes towards the enzymatic degradation of pretreated spent flakes by EG and BG was evaluated. Two of the enzymes (*Tr*Cel61A and Aspte6) showed the most significant hydrolytic boosting ability, with release of 36% substrate glucose after 72 hours compared with the 20% released in the absence of LPMO. Investigations into the glucose release with LPMO combinations showed no direct evidence of increased hydrolytic boosting when compared to treatment with individual LPMOs. The current results further expand our understanding of the ability of LPMOs to degrade this complex substrate, and open new and exciting opportunities for the application of these enzymes in the enzymatic degradation of soy polysaccharides and other industrially-relevant substrates in the future.

4. Materials and Methods

4.1 Materials

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

cellotetraose, cellopentaose, and cellohexaose were obtained from Sigma-Aldrich (St. Louis, MO). GE Healthcare PD MidiTrap G-25 columns were purchased from VWR (Radnor, PA). PASC was obtained from DuPont™ Industrial Biosciences (Palo Alto, CA) where it was prepared as described by Wood[35]. Invitrogen NuPAGE 4-12% Bis-Tris Gels, SeeBlue® Plus2 Pre-Stained Standard, and SimplyBlue™ SafeStain were obtained from Thermo Scientific (Waltham, MA).

4.2 Chemicals

Concentrated sodium hydroxide (50%; NaOH), 96% glacial acetic acid, copper(II) sulfate pentahydrate, methanol, acetonitrile, urea, ammonium bicarbonate, DL-dithiothreitol (DTT), iodoacetamide, formic acid, and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich (St. Louis, MO). L(+)-ascorbic acid AnalaR NORMAPUR® was obtained from VWR (Radnor, PA). Sodium azide (NaN_3) was purchased from Merck Performance Materials (Darmstadt, Germany). The Bio-Rad Protein Assay Kit II (dye reagent and bovine serum albumin (BSA) standard) was obtained from Bio-Rad (Hercules, CA).

4.3 Enzymes

Twenty-four LPMOs (Table 1) were obtained from DuPont™ (Shanghai, China) following expression in a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for the major cellulase genes and beta-glucosidase as described in PCT Pat. Appl. Publ. No. WO2009/048488[27]. Expression was conducted with the native leader peptide for each protein. The amino acid sequences, including the leader peptide sequences, are provided in FASTA format in Supplementary Information Section 1. Cell debris was removed by centrifugation and the supernatant was concentrated using a VivaFlow50 PES ultrafiltration membrane (MWCO 10 kDa) from Sartorius (Göttingen, Germany), with final protein concentrations (Table 1) determined as described in 4.5.3. The purity of the enzymes

were assessed using SDS-PAGE gels (Supplementary Information Figure S1) as described in 4.5.4, and N-terminal characterization was performed as described in 4.5.5 to verify the expected presence of an N-terminal histidine with methylation of the imidazole[11]. Endo-1,4-β-D-glucanase from *Bacillus amyloliquefaciens* (EC 3.2.1.4; GH5) with 3500U activity (17.07 mg/mL protein) and beta-glucosidase from *Agrobacterium sp*. (EC 3.2.1.21; GH1) with 600U activity (2.48 mg/mL protein) were obtained from Megazyme International (County Wicklow, Ireland). Trypsin from bovine pancreas (EC 3.4.21.4), α-Chymotrypsin from bovine pancreas (EC 3.4.21.1), and endoproteinase Asp-N from *Pseudomonas fragi* (EC 3.4.24.33) were obtained from Sigma-Aldrich (St. Louis, MO).

4.4 Soy Spent Flake NaOH Pretreatment

NaOH pretreated soy spent flakes were produced as described by Mittal, Katahira, Himmel & Johnson[32]. In brief, dry soy spent flakes were combined with 16.5% w/w NaOH in an amount of 8.57 mg per mL of 16.5% w/w NaOH. This mixture was stirred under nitrogen at 25 °C for 2 hrs. Upon completion, the material was separated by centrifugation and the soluble fraction was decanted off with the remaining insoluble fraction re-diluted in MilliQ water to the original reaction volume and vortexed. The centrifugation, separation and dilution described above were repeated until the pH of the soluble fraction was neutral. The final, rinsed insoluble material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen.

4.5 Material Characterization

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

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

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

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

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

chromatographic separation. Samples were prepared in a 96-well microtiter plate with a methanol:sample ratio of 1:2. The electrospray was controlled with Advion ChipSoft Manager version 8.3.1 and operated in positive mode with a gas pressure (N_2) of 0.6 psi, voltage of 1.8 kV, 5 µL tip collection, and sample temperature of 7 °C utilizing plastic tips (part no. 1004763) and a 5 µm ESI Chip® (part no. 1003446). Fluoranthene was used as an internal calibrant and the ion transfer tube temperature was set to 200 °C. The acquisition time was set to 2.0 min, with approximately 40 scans per minute for MS and MS-MS. MS full scans were performed in the m/z 300–1,200 mass range, utilizing an Orbitrap detector at 120K resolution and 2 microscans. MS-MS scans were performed on Na-adducts with CID (collision-induced dissociation), using He as the colliding gas in the m/z 105–400 scan range with quadrupole isolation, Orbitrap detection at 15K resolution, an isolation window of 2, 50% collision energy, and 3 microscans. MS Data from ESI-MS was processed in Xcalibur 3.0.63 from Thermo Scientific (Waltham, MA).

4.5.3 Measurement of total protein concentration

Total protein concentration was measured using the Bio-Rad Colorimetric Protein Assay based on the Bradford method[36]. In brief, 50 µL of enzyme sample was combined with 2.5 mL of 4x diluted Protein Assay Dye Reagent Concentrate and incubated at room temperature for 5 mins. Absorbance was measured at 595 nm and compared to the absorbance measured against known concentrations of BSA from 0.1-0.9 mg/mL. All measurements were performed in duplicate.

4.5.4 SDS-PAGE

SDS-PAGE gels were run using an Invitrogen XCell SureLock™ Mini-Cell Electrophoresis system, Invitrogen NuPAGE 4-12% Bis-Tris Gels, and SeeBlue® Plus2 Pre-Stained Standard from Thermo 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 *Tr*Cel61A), Trypsin (Aspfu3, Aspfu4, Aspfu5, Aspte6, Penci2, Aspte7, Aspte8,
409 Malci2, Mycth1, and Mycth3), and Asp-N (Chacr2, Aspte1, Aspte3, Aspte4, Penci1, 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 H_2O/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 µL 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 $Cu(II)SO_4$
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-Kolstad[39] 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].

458 **Acknowledgement**

459 The authors would like to thank Zheyong (Jeff) Yu for his generosity in providing enzymatic
460 samples, Maria Nielsen for her assistance with enzymatic reactions, and Janne Bech Thøgersen for
461 her protein sequencing guidance.

462 This work was supported by DuPont™ and Innovation Fund Denmark [project 1355-00146]. This
463 work was conducted as part of the Danish Industrial PhD Program, as a joint collaboration

between DuPont™, Innovation Fund Denmark, and the Technical University of Denmark (DTU). The project funding sources had no involvement in the study design, writing of the report, the decision to submit the article for publication, or the collection, analysis and interpretation of the data. The authors were solely responsible for these aspects, without any outside influence from their respective organizations.

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

Figure Captions

Figure 1 – ESI CID MS-MS fragmentation of the DP2 oxidized product (*m/z* 381.10) from LPMO treated PASC after 24 hrs. Masses are labeled based on expected fragmentation from the C4 oxidized product Glc4gemGlc (blue) and/or the C1 oxidized product GlcGlc1A (red). A: *Tr*Cel61A; B: Aspte6; C: Talle1; D: Expected fragmentation products from Glc4gemGlc and GlcGlc1A. Parenthesis indicate an unlikely product.

Figure 2 – HPAEC-PAD profile of LPMO treated NaOH pretreated soy spent flakes both with (black, 1 nC offset) and without (red) ascorbic acid as per Westereng et al.[34]. A: *Tr*Cel61A; B: Aspte6; C: Talle1; D: DP 2-6 cello-oligosaccharides.

Figure 3 – Glucose release from NaOH pretreated soy spent flakes following treatment with LPMO (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 ID[29], 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:

LPMO ID	Native Organism	NCBI Reference ID	InterPro Predicted CBD	[Protein] (mg/mL)
*Tr*Cel61A	*Trichoderma Reesei*	UniProt:O14405	Yes	38.30
Chacr1	*Chaetosartorya cremea*	-	Yes	0.37
Chacr2	*Chaetosartorya cremea*	-	-	0.46
Aspfu1	*Aspergillus fumigatus*	-	Yes	0.38
Aspfu2	*Aspergillus fumigatus*	-	Yes	0.40
Aspfu3	*Aspergillus fumigatus*	GenBank: GAQ10694.1	-	0.65
Aspfu4	*Aspergillus fumigatus*	-	Yes	0.48
Aspfu5	*Aspergillus fumigatus*	GenBank: GAQ07946.1	-	0.55
Aspte1	*Aspergillus terreus*	-	-	0.65
Aspte2	*Aspergillus terreus*	-	Yes	0.74
Aspte3	*Aspergillus terreus*	-	-	0.56
Aspte4	*Aspergillus terreus*	-	-	0.66
Aspte5	*Aspergillus terreus*	-	Yes	0.67
Aspte6	*Aspergillus terreus*	-	Yes	0.59
Penci1	*Penicillium citrinum*	-	-	0.46
Penci2	*Penicillium citrinum*	-	-	0.25
Aspte7	*Aspergillus terreus*	GenBank: GAQ40595.1	Yes	0.35
Aspte8	*Aspergillus terreus*	-	-	0.23
Malci1	*Malbranchea cinamomea*	-	-	0.21
Malci2	*Malbranchea cinamomea*	-	-	0.36
Mycth1	*Myceliophthora thermophila*	-	-	0.16
Mycth2	*Myceliophthora thermophila*	-	Yes	0.28
Mycth3	*Myceliophthora thermophila*	-	-	0.31
Talle1	*Talaromyces leycettanus*	-	Yes	0.56

Table 2:

LPMO	PASC	Native Soy	NaOH Soy	Avicel®
*Tr*Cel61A	+++	-	+++	-
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:

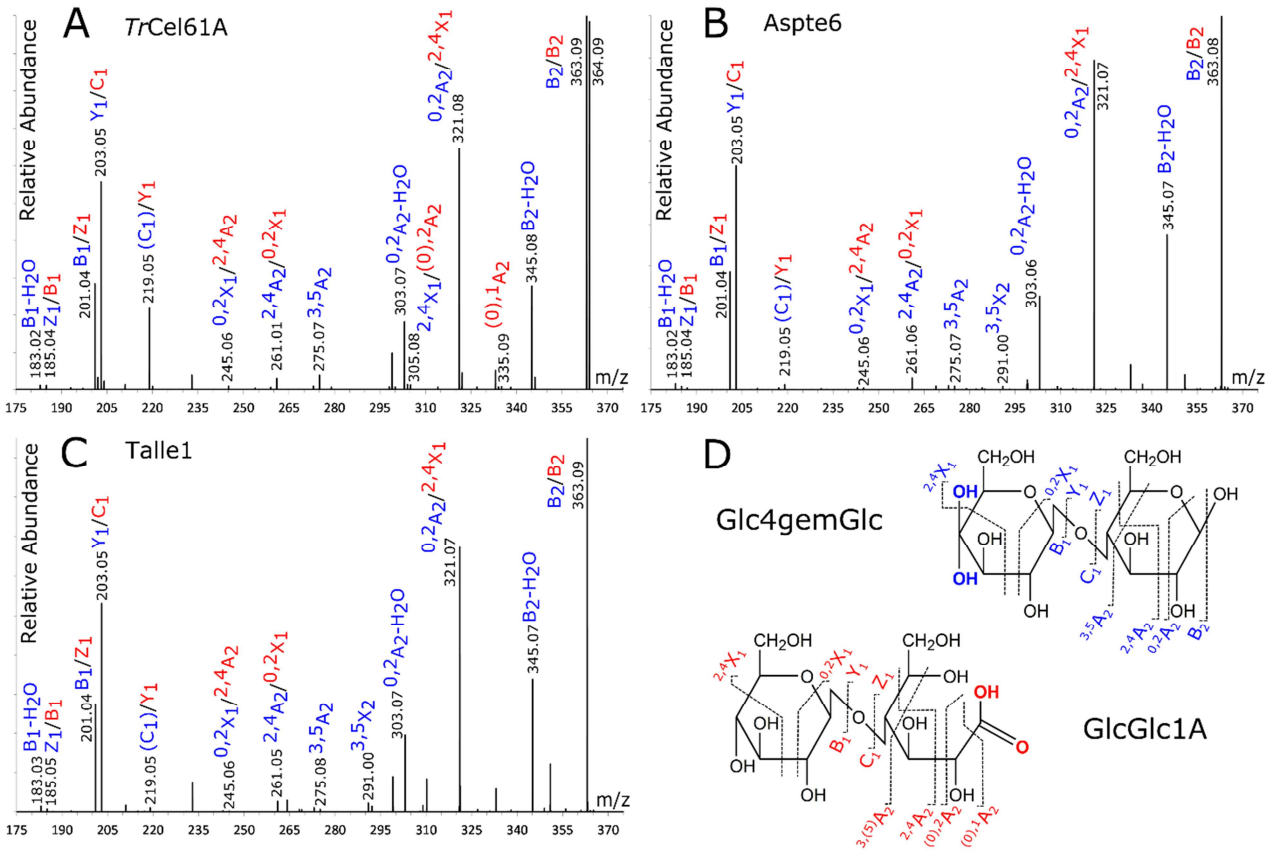


Figure 2:

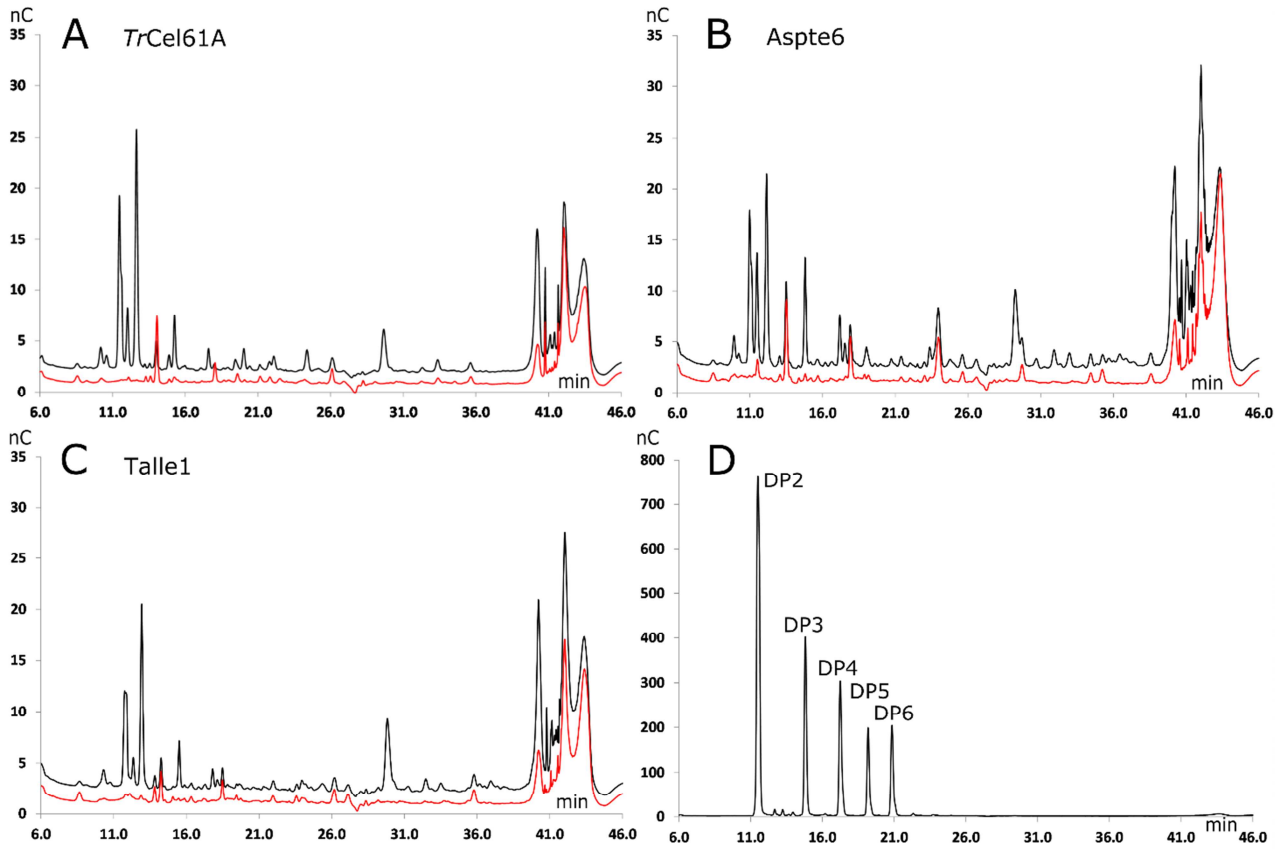


Figure 3:

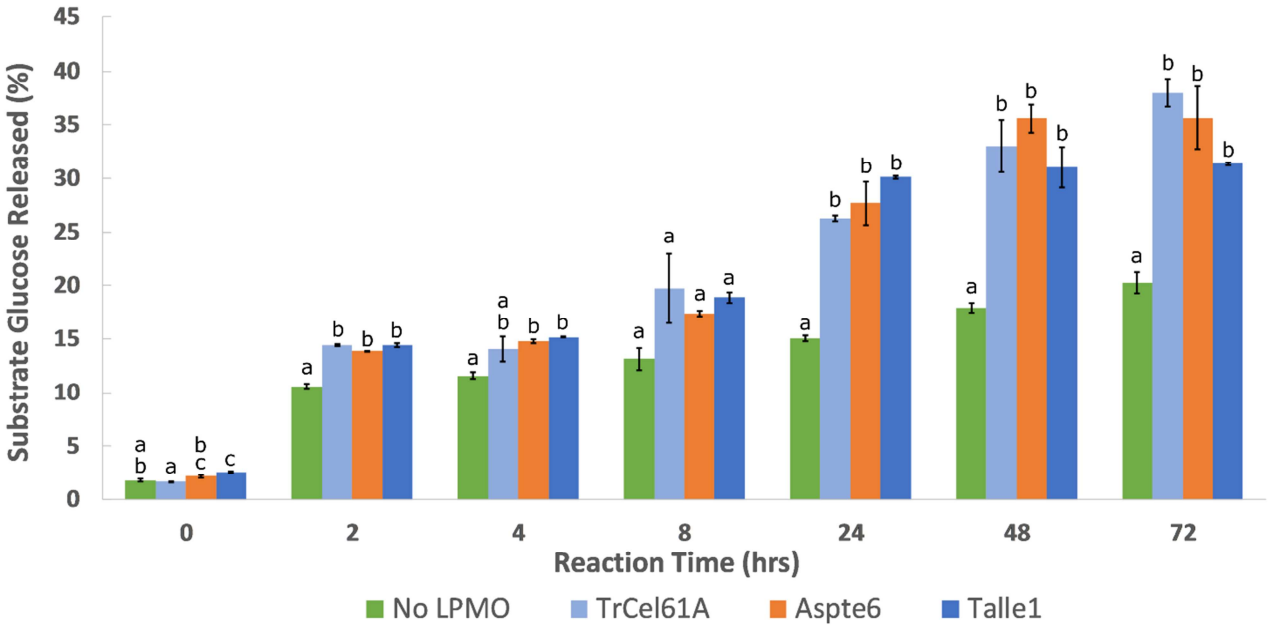
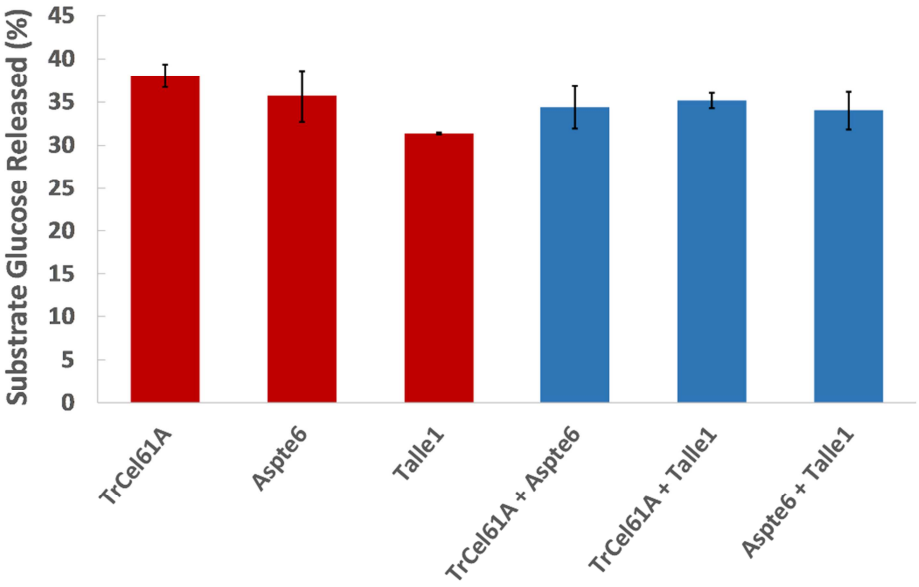


Figure 4:



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 (*Tr*Cel61A and Aspte6) showed significant hydrolytic boosting.
- LPMOs boost glucose release from soy spent flakes by EG and BG by more than 75%.