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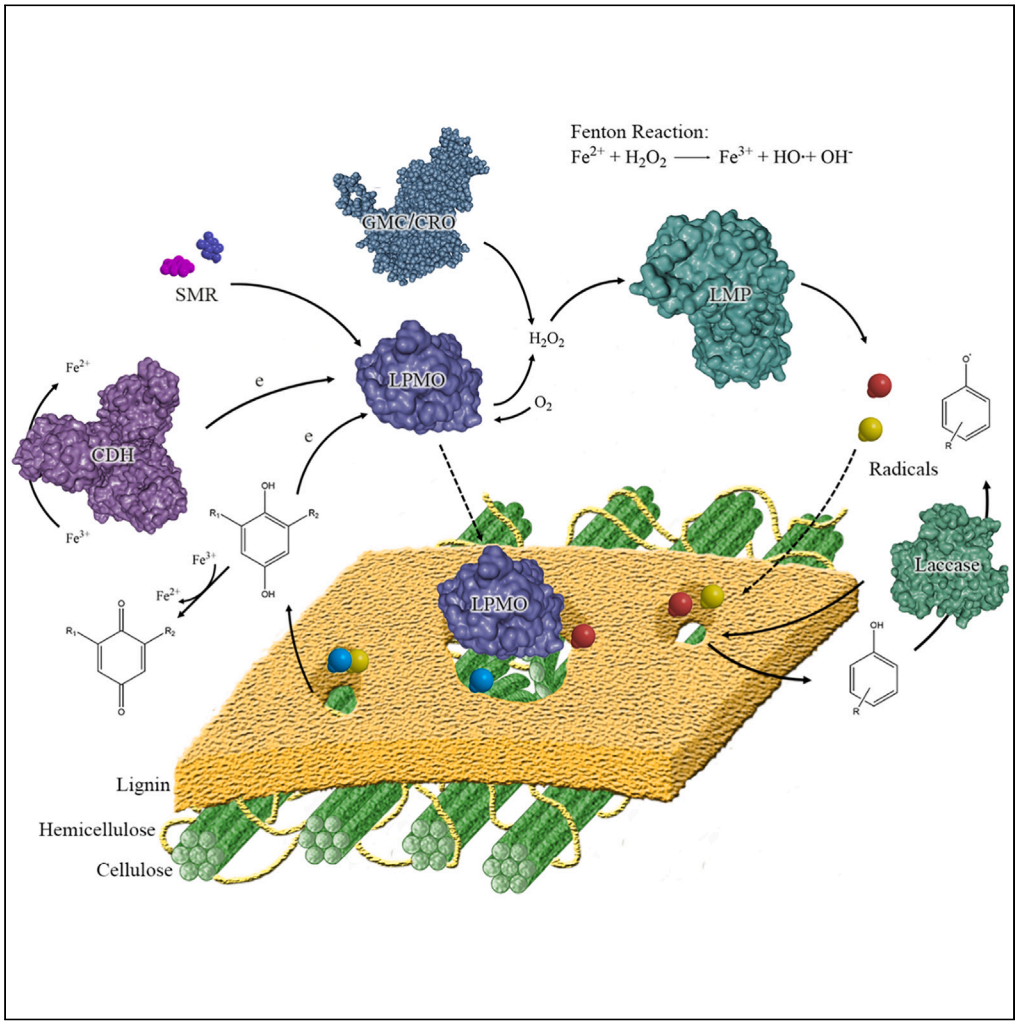

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Highlights

LPMO can directly participate to the process of lignin degradation in white-rot fungi

LPMOs co-regulate with lignin-degrading enzymes during fungal biomass degradation

LPMO drives Fenton reaction and synergizes with MnP for lignin oxidation

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Lytic polysaccharide monooxygenase synergized with lignin-degrading enzymes for efficient lignin degradation

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SUMMARY

Even though the discovery of lytic polysaccharide monooxygenases (LPMOs) has fundamentally shifted our understanding of biomass degradation, most of the current studies focused on their roles in carbohydrate oxidation. However, no study demonstrated if LPMO could directly participate to the process of lignin degradation in lignin-degrading microbes. This study showed that LPMO could synergize with lignin-degrading enzymes for efficient lignin degradation in white-rot fungi. The transcriptomics analysis of fungi *Irpex lacteus* and *Dichomitus squalens* during their lignocellulosic biomass degradation processes surprisingly highlighted that LPMOs co-regulated with lignin-degrading enzymes, indicating their more versatile roles in the redox network. Biochemical analysis further confirmed that the purified LPMO from *I. lacteus* CD2 could use diverse electron donors to produce H_2O_2, drive Fenton reaction, and synergize with manganese peroxidase for lignin oxidation. The results thus indicated that LPMO might uniquely leverage the redox network toward dynamic and efficient degradation of different cell wall components.

INTRODUCTION

The deconstruction of lignocellulosic biomass into its molecular building blocks is essential for global carbon cycle and sustainable production of renewable fuels, chemicals, and materials. Lytic polysaccharide monooxygenases (LPMOs) attracted broad interest for lignocellulose deconstruction due to its unique capacity to oxidize carbohydrates and to synergize with hydrolytic enzymes for biomass conversion to sugars.[1,2] LPMOs were originally characterized as glycoside hydrolase family 61 or carbohydrate-binding module 33.[3] Recent breakthroughs expanded the substrates of LPMO and discovered the oxidative activities of LPMOs on all major polysaccharides, including cellulose,[4,5] hemicellulose,[6–8] chitin,[3,9] and starch.[10] Current studies also suggested that LPMOs oxidized carbohydrate through recruiting oxygen to copper active site[11–13] and accepting electrons from a variety of external electron donors, including cellobiose dehydrogenase (CDH),[14] photosynthetic pigment,[15] and small molecular reductants.[1,3,5,16,17] Despite the progresses, it is still unclear if LPMOs oxidize the substrates beyond the carbohydrates in lignocellulose. Our recent work showed that the purified LPMO could supply H_2O_2 for versatile peroxidase to derive its oxidation activity to lignin oxidation.[18] Meanwhile, we also demonstrated that LPMO participated in the degradation of lignin macromolecular and lignin-carbohydrate linkages in fungus *Pleurotus ostreatus* through enhancing the extracellular hydroquinone-quinone redox cycling.[19] Given the broad range of electron donors for LPMO and the extensive redox network involved, it will be highly interesting to further study the role of LPMO involved in degradation of other components of biomass, like lignin. It is also essential to understand how LPMOs can synergize with other enzymes in the redox network for biomass deconstruction. The fundamental understanding will not only better define the roles of LPMOs in biomass deconstruction but also reveal how the redox network involved in degrading different cell wall components synergistically.

White-rot basidiomycete fungi have evolved with unique capacity to degrade all cell wall components, including the highly recalcitrant lignin, with synergistic enzyme and none-enzyme radical generation systems.[20,21] White-rot fungi provide ideal model systems to study the lignocellulosic degradation mechanisms. More interestingly, our recent study showed that LPMO gene family expansion correlated

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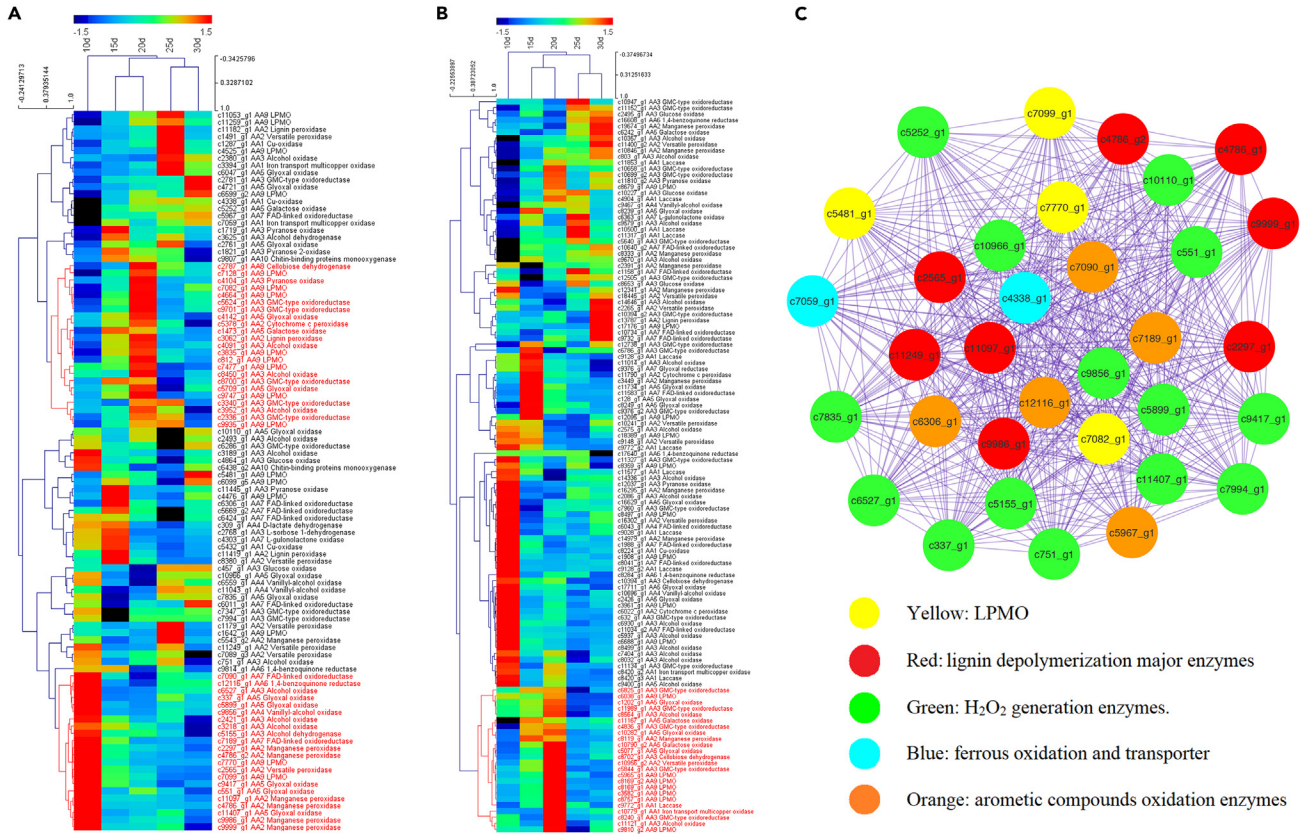

Figure 1. The heatmap cluster analysis and co-regulation network analysis of AA class genes

(A and B) Heatmap cluster of AA class genes in *I. lacteus* CD2 (a) and *D. squalens* DSM 9615 (b). Many LPMO-encoding genes in both strains were clustered into the same group with the other lignin degradation-related enzymes including lignin-depolymerizing major enzymes and H_2O_2-generating enzymes which were highlighted in red color.

(C) Co-regulation network of *I. lacteus* CD2. Only AA class genes were displayed in this co-regulation network from one of the co-regulated modules enriched with oxidation reduction. Each node represented one gene in the module. The line represented the interaction among genes.

with lignin-depolymerizing enzymes in the 43 analyzed biomass-degrading microbes, suggesting that lignin degradation may be driving the evolution of LPMO family.[22] In order to unveil the role of LPMO in biomass-degrading redox network, we carried out comparative systems biology analysis of two white-rot fungus species, *Irpex lacteus* CD2 and *Dichomitus squalens* DSM 9615, with different lignocellulosic degradation patterns and enzymatic systems to study the composition and dynamics of extracellular redox networks.[23,24] With the systems-biological understanding, we further carried out *in vitro* study to approve that the purified LPMOs could drive Fenton reaction and synergize with manganese peroxidase for lignin oxidation. These studies provided a new insight to the role of LPMOs in biomass degradation and to understand the mechanisms of synergistic redox network for lignin and polysaccharide degradation.

RESULTS

Comparative transcriptomics analysis reveals the co-regulation of LPMOs with other lignin degradation enzymes

Two white-rot fungi with different lignocellulosic degradation pattern and enzymatic system were chose in this study. *I. lacteus* CD2 lacks the laccase system, yet has a higher selectivity in lignin degradation than cellulose degradation.[24] *D. squalens* DSM 9615 degrades lignin and cellulose simultaneously (Figure S1).[23] The transcriptomics of the two fungi were analyzed every 5 days from the day 10 to day 30 during biomass degradation. The results highlighted the dynamic expression of genes coding for lignocellulosic-degrading enzymes throughout different stages of biomass deconstruction (Table S1; Figures S2–S6).

The most interesting fact is that most of the LPMOs were clustered and co-regulated with H_2O_2-generating enzymes and lignin-depolymerizing major enzymes (LDMEs) in both fungi (Figures 1A and 1B). To get access to the cellulose, fungi need to overcome the structural recalcitrance of lignin. To this end, the fungi evolved with a comprehensive extracellular redox system that consists of LDMEs, accessary enzymes, and the generation systems for non-enzyme active-radical species. The LDMEs include laccase, manganese peroxidase (MnP), lignin peroxidase, and versatile peroxidase.[21] Fewer LDME-encoding genes were identified in *I. lacteus* CD2. However, most LDMEs in *I. lacteus* CD2 were expressed at a much higher level as compared to those in *D. squalens* DSM 9615 (Figure S7). To supply sufficient H_2O_2 for the fungal

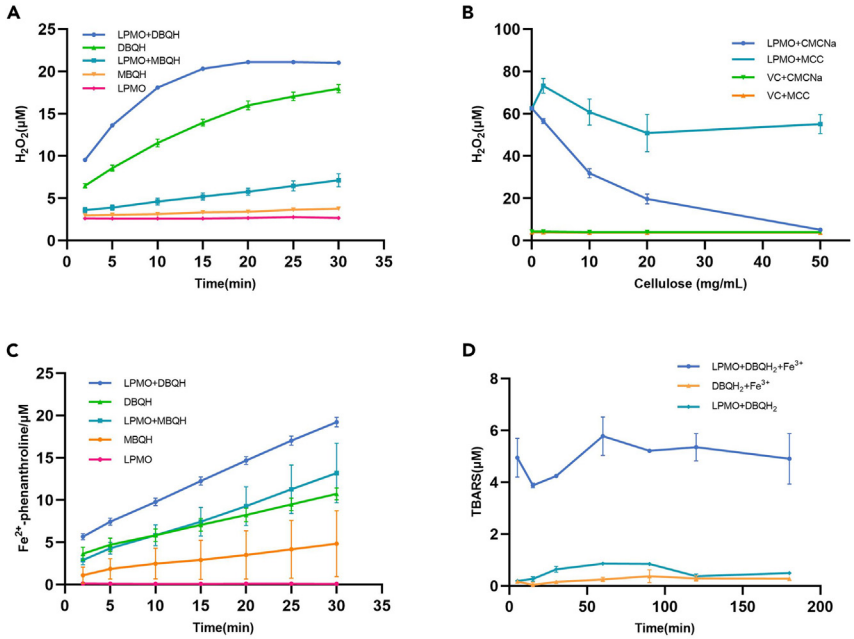

Figure 2. LPMO drives the Fenton reaction

(A) The H_2O_2 producing by *Il*LPMO99 incubated with hydroquinone $MBQH_2$ or $DBQH_2$ as electron donors.

(B) The H_2O_2 producing by *Il*LPMO99 in presence of different type celluloses. Ascorbic acid (VC) was used as electron donor for *Il*LPMO99. CMCNa, carboxymethylcellulose sodium; MCC, microcrystalline cellulose.

(C) The Fe^{3+} reduction by LPMO in presence of hydroquinone.

(D) The hydroxyl radical generation by LPMO-driven Fenton reaction via incubating LPMO with $DBQH_2$ and Fe^{3+}.

All the data are represented as mean ± SD.

class II lignin-modifying peroxidases, both fungi expressed diverse accessary enzymes to generate H_2O_2 from different substrates, including various glucose-methanol-choline oxidoreductases (GMCs) and copper radical oxidases (CROs) (Figures 1A and 1B). Interestingly, the cluster analysis highlighted that most of the LPMOs were clustered with the aforementioned H_2O_2-generating enzymes and LDMEs. The co-regulation network analysis of differentially expressed genes also showed that the LPMOs were co-regulated with LDMEs, H_2O_2-generating enzymes, and aromatic compound catabolism enzymes (Figure 1C). The co-regulation of hydrogen peroxide producing enzymes with LPMOs may also be related to provision of hydrogen peroxide for LPMO catalysis as being demonstrated that H_2O_2 could serve as co-substrate for LPMO.[11] However, it was noted that many LPMOs were upregulated when little cellulose degradation was observed after 20 days in *I. lacteus* CD2 (Figure S6). Meanwhile, it could be noticed that most of LPMO were also upregulated and co-regulated with H_2O_2-generating enzymes and lignin degradation peroxidases during the stage when highest lignin degradation ratio was observed in *D. squalens* DSM 9615 (Figures 1B and S6). Considering the diverse role of LPMO in redox network, the co-regulation of LPMO with LDMEs and entire lignin-degrading system suggested that LPMO may also play a role in H_2O_2 generation and synergize with LDMEs to form the fungal extracellular redox network for lignin oxidation.

The LPMO involves in hydroxyl radicals generating via Fenton reaction

In order to further elucidate the role of LPMO in biopolymer degradation during cell wall deconstruction, we first investigated the role of LPMO in generating hydroxyl radicals. Hydrogen peroxide plays a critical role in lignin oxidation either by generating hydroxyl radicals through Fenton reaction or acting as electron acceptor for fungal class II lignin-modifying peroxidases (LMPs).[21,25] Our previous studies have showed that Fenton reaction plays an important role in lignin depolymerization in fungus *I. lacteus*.[24,26] To investigate if the LPMOs are involved in lignin oxidation by generating hydrogen peroxide, one LPMO (c7099_g1, named *Il*LPMO99) was heterologously expressed in *Pichia pastoris* and purified for *in vitro* studies. The enzyme was overexpressed together with LDMEs in the transcriptomics analysis and could be involved in providing H_2O_2 for lignin degradation. The H_2O_2 concentration was measured when the *Il*LPMO99 was incubated with lignin model compound hydroquinone or ascorbic acid as electron donors. Hydroquinone can be derived from lignin degradation and be reduced by quinone reductase, making it a potential key mediator for redox transfer during lignin degradation. The results showed that the *Il*LPMO99 could produce the H_2O_2 with a suitable electron donor. Even though 2-methoxyhydroquinone ($MBQH_2$) and 2,6-dimethoxyhydroxyquinone ($DBQH_2$) could produce H_2O_2 absent of LPMO at a certain level, the addition of *Il*LPMO99 significantly improved the H_2O_2 generation at the maximum rate of 4.2-folds and 1.9-folds to $MBQH_2$ and $DBQH_2$ in 15 min, respectively ($p < 0.05$) (Figure 2A). When ascorbic acid was used as the electron donor, the H_2O_2 was only produced in the presence of *Il*LPMO99 (Figure 2B). The results highlighted that

*Il*LPMO99 could use lignin, aromatics, and other extracellular reductants as electron donor to produce H_2O_2 for synergizing with LDMEs toward lignin depolymerization.

It has been well established that LPMO can oxidize cellulose substrates to promote carbohydrate degradation. We thus explored the *Il*LPMO99's H_2O_2 generation capacity in presence of cellulose to evaluate the substrate preference of the enzyme. Different types of cellulose were used to investigate their effect on the production of H_2O_2 by *Il*LPMO99. The results showed that microcrystalline cellulose (MCC) could slightly increase the H_2O_2 production at low concentration (2 mg/mL) and slightly decrease the H_2O_2 production at a higher concentration ($\geq$10 mg/mL) (Figure 2B). However, the addition of carboxymethylcellulose sodium (CMCNa) caused a rapid decrease in H_2O_2 production by *Il*LPMO99, with an increase in CMCNa concentration (Figure 2B). The significant decrease of H_2O_2 production could be related to that most of the *Il*LPMO99 bound to the surface of CMCNa. The reduction of hydrogen peroxide production in presence of celluloses would mean that the enzyme was oxidizing cellulose. Having lower effect with crystalline cellulose could be related to its less accessible surface-binding area. These results suggested that the soluble cellulose or oligosaccharides could affect the H_2O_2 production by LPMO, while *Il*LPMO99 could still release H_2O_2 in the presence of crystalline cellulose. The generation of H_2O_2 by *Il*LPMO99 thus depends on the redox environment and substrate availability in the extracellular environment, where the enzyme could generate with H_2O_2 for LDMEs without soluble cellulose presence.

We also investigated the ability of reducing Fe^{3+} into Fe^{2+} by *Il*LPMO99 in the presence of $MBQH_2$ or $DBQH_2$, which will indicate the enzyme's capacity to drive Fenton reaction for lignin degradation. Effective Fe^{3+} reduction by *Il*LPMO99 was observed in presence of $MBQH_2$ and $DBQH_2$ (Figure 2C). The reducing of Fe^{3+} into Fe^{2+} had a maximum increase of 2.8-folds and 1.8-folds by $MBQH_2$ and $DBQH_2$ in the presence of *Il*LPMO99, as compared to those without *Il*LPMO99, respectively (Figure 2B). When the hydroquinone is oxidized to quinone, the active intermediate produced during the reaction could reduce Fe^{3+} into Fe^{2+}.[27] Although the hydroquinone could be autoxidized (Figure S8), LPMO could significantly promote the oxidation of hydroquinone to quinone and thus increase the Fe^{3+} reduction efficiency. The results highlighted more complicated roles of *Il*LPMO99 in redox network for biomass degradation.

By generating the H_2O_2 and reducing the Fe^{3+} into Fe^{2+}, LPMO could drive the Fenton reaction to produce the hydroxyl radicals, which can be used to oxidize the lignin.[28,29] The hydroxyl radicals were consistently produced when the *Il*LPMO99 was incubated with Fe^{3+} and hydroquinone (Figure 2D). In order to verify the role of *Il*LPMO99 in lignin degradation, we further investigated the oxidation of lignin model compounds by LPMO-driven Fenton reaction (Figure 3A). The results highlighted that the oxidation ratios of lignin model compounds, including *p*-coumaric acid, sinapic acid, 3-methoxycinnamic acid, and 3,5-dimethoxycinnamic acid, were increased by 68.1%, 52.7%, 33.0%, and 246% in presence of *Il*LPMO99 as compared to the controls ($p < 0.05$) (Figure 3A). The different promotion effect of the oxidation of lignin model compounds could be related to their different redox potentials, where the higher redox potential of lignin model compounds resulting to the more difficult to be oxidized by the hydroxyl radicals generated by Fenton reaction.[30,31] Overall, the results suggested that the LPMO may involve the generation of hydroxyl radicals, by producing H_2O_2 and reducing Fe^{3+} to drive the Fenton reaction for lignin oxidation. The H_2O_2 generation could also be synergized with LDMEs for lignin depolymerization.

The LPMO synergizes with MnP for lignin degradation

Considering that LPMO could produce H_2O_2 in the presence of an electron donor and was co-regulated with other H_2O_2-generating enzymes and lignin-modifying peroxidases, we hypothesized that LPMO could supply H_2O_2 to the fungal class II LMPs to synergize the lignin degradation. LMPs are hydrogen peroxide-dependent enzymes for lignin degradation. In order to prove this hypothesis, we first investigated the oxidation ability of MnP on lignin model compounds with and without *Il*LPMO99. The results showed that the MnP alone without H_2O_2 or *Il*LPMO99 had no oxidation activity on the lignin model compounds such as *p*-coumaric acid, 3-methoxycinnamic acid, and 3,5-dimethoxycinnamic acid (Figure 3B). When the *Il*LPMO99 and its electron donor, ascorbic acid, were incubated with MnP, the oxidation of lignin model compounds could be observed, where the highest oxidation rate could be over 80% for *p*-coumaric acid (Figure 3B). The results highlighted that the H_2O_2 produced by LPMO could supply redox potential needed for MnP oxidation activity. The biochemical and enzyme expression results strongly suggested the role of LPMO in supplying H_2O_2 for peroxidase and in reducing Fe^{3+} for Fenton reaction during lignin degradation. We further explore the function of LPMO in presence of cellulose to understand the role of the enzyme at different stages of biomass degradation. H_2O_2 generation by LPMO and lignin oxidations synergized with MnP was still slightly inhibited by both MCC and CMCNa (Figure 3B). As aforementioned, the CMCNa (10 mg/mL) caused 50% of inhibition to the H_2O_2 production by LPMO (Figure 2B). In contrast, it only caused 19%–35% of inhibition to the oxidation of lignin model compounds by MnP with LPMO (Figure 3B). These results suggested that either the LPMO could synergize with MnP for lignin model compounds oxidation through enzyme interactions or MnP might have higher affinity to H_2O_2 than LPMO, which could reduce the effects caused by soluble cellulose on H_2O_2 production by LPMO.

In addition to elucidating the role of LPMO in lignin degradation using model compounds, enzymatic hydrolysis lignin was further used to verify the lignin degradation caused by the *Il*LPMO99-driven Fenton reaction and the synergized oxidation reaction by *Il*LPMO99 and MnP. Phosphorus-31 NMR (^{31}P-NMR) was used to characterize the functional structure groups of oxidized lignin from different chemical reactions. ^{31}P-NMR is a well-developed and widely used analytic approach to quantify the major functional groups in native lignin or processed lignin isolates, which could define the cleavage of inter-unit linkages and/or oxidation degradation.[32–34] As shown in Figure 3C, the lignin oxidized by the LPMO-driven Fenton reaction has shown the reduced content of aliphatic hydroxyl group, syringyl hydroxyl group, guaiacyl group, and carboxyl group by 13%, 29%, 17%, and 36% comparing to the control raw lignin, respectively. The results confirmed that the hydroxyl radicals generated by *Il*LPMO99-driven Fenton reaction could oxidize lignin to promote the lignocellulosic biomass degradation. When the lignin was oxidized by combination of MnP and *Il*LPMO99, most of the detected functional structure groups, including aliphatic hydroxyl group, syringyl hydroxyl group, guaiacyl group, *p*-hydroxyphenyl hydroxyl group, and tricin hydroxyl group, were significantly decreased by 27%, 47%, 32%,

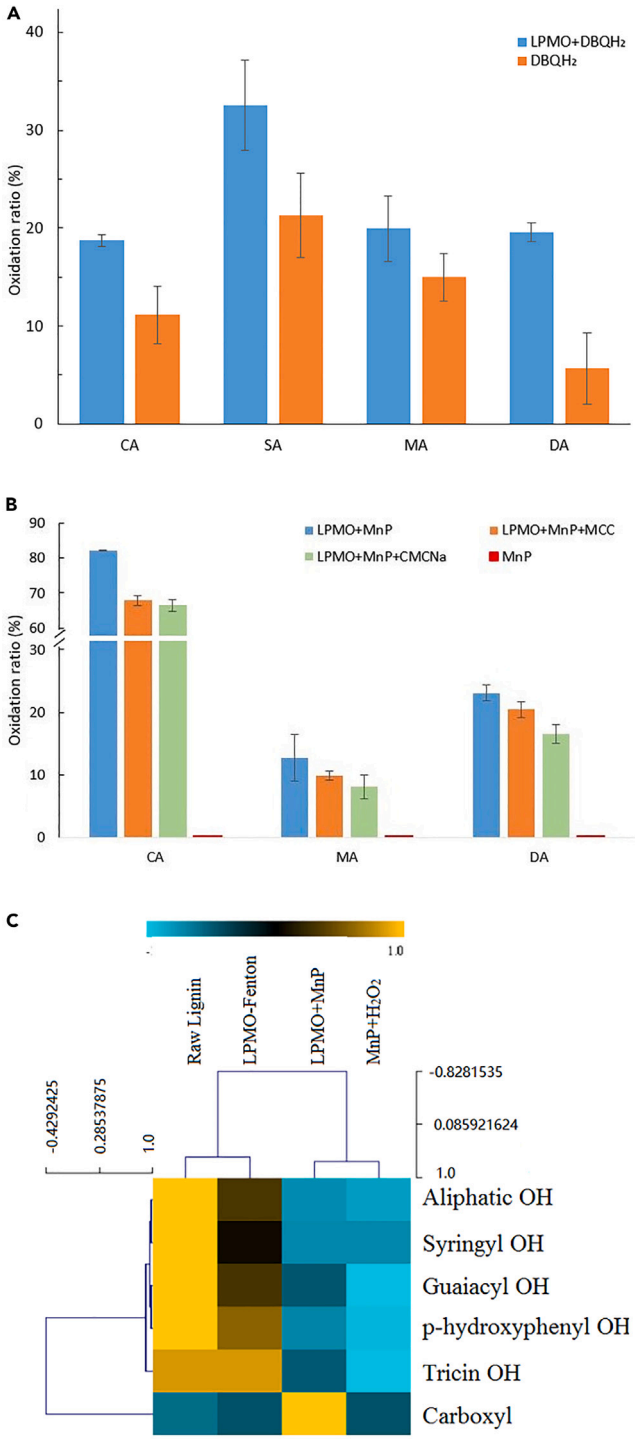

Figure 3. LPMO involves in lignin oxidation

(A) The oxidation of lignin model compounds by *Il*LPMO99-driven Fenton reaction in 24 h. Compounds: CA, *p*-coumaric acid; SA, sinapic acid; MA, 3-methoxycinnamic acid; DA, 3,5-dimethoxycinnamic acid. Data are represented as mean ± SD.

(B) The oxidation of lignin model compounds by *Il*LPMO99 and MnP with or without cellulose (10 mg/mL). CMCNa, carboxymethylcellulose sodium; MCC, microcrystalline cellulose. Data are represented as mean ± SD.

(C) The ^{31}P-NMR characterization of functional structure groups in lignin. LPMO-Fenton: enzymatic hydrolysis lignin was incubated with LPMO, $DBQH_2$, and Fe^{3+}; LPMO+MnP, enzymatic hydrolysis lignin was incubated with MnP, $MnSO_4$, and LPMO; MnP + H_2O_2, enzymatic hydrolysis lignin was incubated with MnP, $MnSO_4$, and H_2O_2.

22%, and 13% compared to the raw lignin, respectively (Figure 3C). These functional group-oxidation efficiencies were very similar to that of lignin oxidized by MnP with H_2O_2. However, the lignin oxidized by MnP with *Il*LPMO99 had a 5.5-folds increase in carboxyl group as compared to the raw lignin, while the lignin oxidized by MnP with H_2O_2 had only 36% increase of carboxyl group (Figure 3C). The significant increase of carboxyl group in oxidized lignin may be due to the ring cleavages or side-chains oxidation.[35] Overall, the results from enzymatic hydrolysis lignin oxidation well verified the hypothesis that LPMO supplied H_2O_2 to MnP for lignin degradation. More than that, the detailed mechanisms for MnP synergy with LPMO were yet to be unveiled, considering that MnP synergized with LPMO more efficiently than that with H_2O_2 and soluble cellulose had effect on the MnP and LPMO synergy.

DISCUSSION

The discovery of LPMO represents a major breakthrough for understanding lignocellulosic biomass degradation mechanisms in nature.[1,3,35,36] LPMO has been found to be able to oxidize diverse carbohydrate substrates in biomass, and the roles of LPMO in redox network for biomass degradation are continuing to expand. Our previous study also revealed that the specific *Il*LPMO99 used in this study also showed traditional polysaccharides oxidation capability.[19] Recently, more and more researches have illustrated the linkages between lignin degradation and cellulose degradation through LPMO.[3,15,16,37,38] However, all these studies only demonstrated that the lignin degradation-derived aromatic compounds could serve as electron transfer for LPMO to enhance the polysaccharide oxidation. In this study, we have further demonstrated that LPMO is also involved in lignin degradation in white-rot fungi. The discovery was established by gene expression analysis and biochemical assay. We first found that LPMOs were expressed throughout the biomass degradation process and co-regulated with the redox enzymes related to lignin degradation in the white-rot fungi *I. lacteus* CD2 and *D. squalens* DSM 9615. Some of the LPMOs were significantly upregulated in *I. lacteus* CD2 at the point of time when lignin was significantly degraded. The gene expression analysis led to the hypothesis that LPMO might be involved in lignin degradation. The hypothesis was well validated in biochemical analysis, where we have established that LPMO could generate H_2O_2 and promote the Fe^{3+} reduction to drive Fenton reaction, supply H_2O_2 to LMPs, and even synergize with MnP for lignin oxidation. The LPMO activities and enzyme synergies were well verified with both model compounds and enzymatic hydrolysis lignin substrates. The biochemical analysis was further substantiated by finding that gene family expansion of LPMO is more driven by the lignin degradation capacity in biomass-degrading fungi as shown in our recent study.[22] More interestingly, the lignin oxidation capacity of LPMO is only slightly affected by insoluble cellulose, which indicated that LPMO may play a more important role in lignin degradation in absence of soluble carbohydrate. The study suggested that LPMO may play a unique role to bridge the degradation of different components of plant cell wall (cellulose, hemicellulose, and lignin), where LPMO may promote lignin degradation in absence of soluble carbohydrate during early stage of wood degradation.

Therefore, the study strongly suggested dual roles of LPMOs for both lignin and polysaccharides degradation depending on the substrate availability, co-expressed enzymes, and redox environment (Figure 4). In other words, LPMO might uniquely harness the extracellular redox network with temporal and spatial precisions to specifically target lignin or carbohydrate at different stages of biomass degradation to maximize the efficiency. In the context of extracellular redox network, LPMO could be activated by direct accepting electrons from CDH[14] and some small molecular reductants like diphenols[1] and ascorbic acid[3] to carry out oxidation reactions. When activating in absence of cellulose, LPMO could produce H_2O_2 by recruiting O_2 and accepting electron from the donors.[39,40] On one side, the H_2O_2 produced by LPMO could be used to generate hydroxyl radicals via Fenton reaction with Fe^{2+} for lignin and cellulose oxidation. Fe^{2+} could be either reduced from Fe^{3+} by CDH or LPMO-hydroquinone. As we demonstrated in this study, LPMO-hydroquinone could significantly promote the production of H_2O_2 and Fe^{2+}, which could in turn generate hydroxyl radicals to oxidize lignin. On the other side, the H_2O_2 produced by LPMO could be used to activate the fungal lignin-modifying peroxidases for lignin degradation as we demonstrated in our recently publication.[18] Unlike the other H_2O_2-generating enzymes in GMC and CRO families, LPMOs can accept electrons from much broader donors to produce H_2O_2, which could make the fungi adaptable to various extracellular environments during lignocellulose degradation. Although a recent study showed that H_2O_2 could be co-substrate of LPMOs, it only happens in the presence of carbohydrate substrates such as cellulose.[11] Our results showed that the H_2O_2 production by LPMO was significantly inhibited by soluble polysaccharides, consistent with previous studies,[39] while the insoluble MCC did not significantly inhibit the H_2O_2 production by LPMO. Considering that the native cellulose microfibrils are embedded in lignin and exist in crystalline structure rather than soluble polysaccharide,[41] many LPMOs could react in an environment with low-soluble polysaccharide. In addition, many LPMOs have no carbohydrate-binding modules, resulting in enzymes with much lower carbohydrate affinity. Therefore, the free LPMOs (unbound to cellulose) might generate H_2O_2 for lignin degradation in the fungal extracellular redox environment and synergize with LMPs for lignin oxidation. Recent studies also suggested that the peroxidases have higher affinity to H_2O_2 than LPMOs, and thus the peroxidases could compete the H_2O_2 produced by LPMOs to affect the LPMOs' activity on cellulose and drive the reaction to H_2O_2 generation, even when the LPMOs are binding to cellulose.[11] Overall, the study has revealed a much more versatile role of LPMOs (Figure 4), where the enzyme might be involved in lignin oxidation via Fenton reaction and synergizing with lignin-modifying peroxidases besides the oxidation of polysaccharides.

To our knowledge, this is the first study to show the role of LPMOs in lignin degradation from different aspects. The discovery indicated that LPMO's role is much more dynamic depending on the redox environment, substrate availability, and co-regulated enzymes. It could be that LPMOs in wood-degrading enzymes acted together with peroxidases and Fenton reaction agents to break down lignin at early stage of biomass degradation, when less-soluble carbohydrate is available. With more crystalline cellulose broke down and more soluble polysaccharides available for hydrolysis, LPMOs synergize with hydrolase to break down the polysaccharides. The scheme makes perfect evolutionary sense in that the enzyme could explore the redox network in a very versatile way to maximize the efficiency of degrading different cell wall

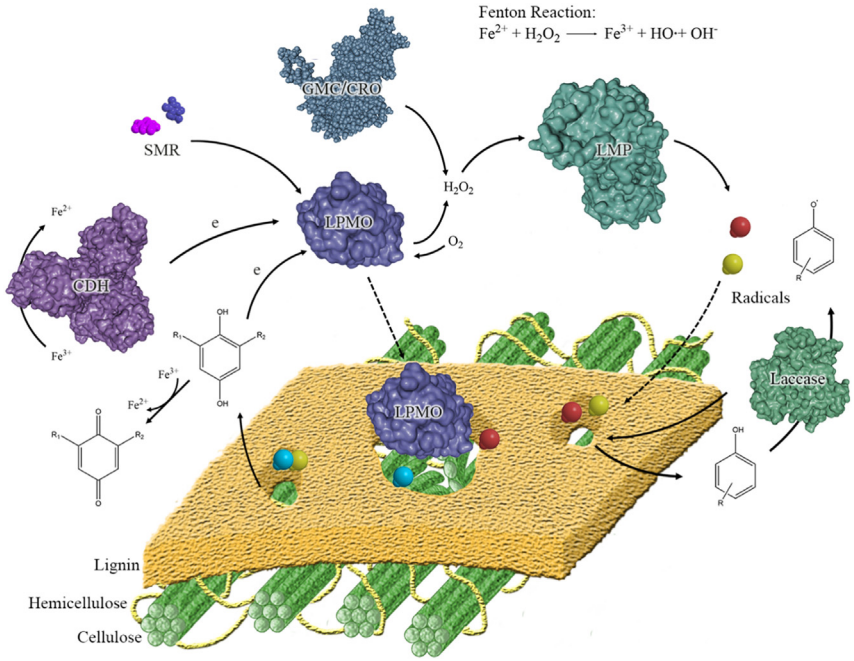

Figure 4. The proposed extracellular redox network for lignin oxidation

The free LPMO (non-binding to cellulose), glucose-methanol-choline oxidoreductases (GMCs) and copper radical oxidases (CROs) produce H_2O_2 for Fenton reaction and lignin-modifying peroxidases (LMPs). The LPMO receives electron from cellobiose dehydrogenases (CDH) and small molecular reductants (SMRs) to initiate its active. The CDH will reduce Fe^{3+} to Fe^{2+} for Fenton reaction when it donates its electron to LPMO. Hydroquinone was oxidized into semiquinone, which was further oxidized by Fe^{3+}-EDTA and lead to the production of quinone. In the process of oxidation of hydroquinone to quinone by LPMO, semiquinone promoted ferric iron reduction, generating in Fe^{2+}. The hydroxyl radicals produced via Fenton reaction and other reactive radicals produced by LMPs and laccase will generate phenol compounds, which could serve as electron donor for LPMO and mediator for laccase.

components. The study thus brought new perspectives of biomass degradation that a broader redox network involving LPMOs needs to be dissected for both lignin and carbohydrate degradation. The dynamics, synergy, and regulation of such network need to be further defined so that novel enzyme mixtures may be developed for holistic degradation of cell wall components including both lignin and cellulose.

Limitations of the study

We analyzed the transcriptome data to provide the genetic background of LPMOs in the role of biomass degradation, and verified the new function of LPMO *in vitro* through biochemical analysis, and how it synergizes with MnP for lignin oxidation through driving Fenton reaction. More research is still needed to identify whether other lignin degradation enzymes besides MnP interact with LPMOs in the process of biomass degradation, and how they could effectively use redox networks to efficiently degrade different cell wall components remains to be further explored. The biological functions of LPMO in lignocellulose still need to be further illustrated through *in vivo* biological experiments. In addition, since our previous studies showed that LPMO only could not oxidize the lignin model compounds, lignin dimers, or lignin polymers,[18] we did not include the LPMO alone as control in this study for its synergistic lignin degradation analysis with MnP.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107870.

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AUTHOR CONTRIBUTIONS

S.X., J.S.Y., H.Y., S.S., and F.L. designed the study; transcriptomics was carried out by S.S.; bioinformatics analysis was conducted by S.X., S.S., W.Z., M.L., and Z.J.; LPMO-driven experiments were carried out by F.L. and H.Z.; lignin oxidation and characterization were carried out by S.X., Y.P., and A.J.R.; the manuscript was drafted by S.X. and S.S., and revised by J.S.Y., S.X., H.Y., X.Z., and S.D.

DECLARATION OF INTERESTS

The authors declare no competing financial interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Irpex lacteus	HUST, China	CD2
Dichomitus squalens	German Collection of Microorganisms and Cell Cultures	DSM 9615
Deposited data		
Raw sequence reads	NCBI	PRJNA451480
Transcriptomics analysis data	NCBI	PRJNA450991

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Shangxian Xie (shangxian_xie@hust.edu.cn).

Materials availability

This study did not generate new materials.

Data and code availability

All the transcriptomics data was accessible from NCBI BioProject with the accession number PRJNA451480 and PRJNA450991. All the other data presented in this study could be requested through the lead contact, Shangxian Xie (shangxian_xie@hust.edu.cn).

METHOD DETAILS

Strains

The white rot fungi *I. lacteus* CD2 was originally isolated from rotted wood in Shennongjia Nature Reserve (Hubei province, China) and maintained on potato dextrose agar (PDA) slant culture. *D. squalens* DSM 9615 was purchased from the German Collection of Microorganisms and Cell Cultures.

The comparative composition analysis of degraded biomass

Four grams of milled corn straw (40-60 mesh) was mixed with 10 mL distilled water in 250-ml Erlenmeyer flask with addition of and sterilized at 121°C for 30 min with liquid autoclave cycle. Two different white rot fungi *I. lacteus* CD2 and *D. squeens* DSM 9615 were inoculated to the straw substrate respectively and incubated at 25°C for 30 days. The biomass samples were collected every 5 day from the day 10 to day 30 during their biomass degradation, and the biomass compositions including structural carbohydrates and lignin in biomass were determined according to the Laboratory Analytical Procedure from the National Renewable Energy Laboratory after the periodical degradation by these two different white rot fungi. The degradation ratio of lignin (or cellulose) in each stage was calculated by the percentage of weight loss of lignin (or cellulose) during the specific biomass degradation period to the original weight of lignin (or cellulose) in the untreated biomass.

Total RNA extraction, sequencing, transcriptomics and bioinformatics analysis

The fungal mycelium for RNA extraction was collected from *I. lacteus* CD2 and *D. squalens* DSM 9615 grown on the straw at different time (10-day, 15-day, 20-day, 25-day and 30-day). RNA was extracted according to the manufacturer's for filamentous fungi in the Rneasy Plant Mini Kit (Qiagen Inc., TX, USA). The isolated RNA concentration and quality were analyzed by bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). cDNAs were synthesized using the mRNA-Seq Sample Prep Kit (Illumina Inc., San Diego, USA) and sequenced by the Illumina Hiseq2500 platform. The obtained high-quality clean reads were *de novo* assembled with Trinity program with default parameters and the Unigenes were predicted with Trinity anlyasis toolkit again Uniprot database.[26,28] All the transcriptomics data was accessible from NCBI BioProject with the accession number PRJNA450991.

Heterologous protein expression and purification

The genes of *Il*LPMO99 and *Il*MnP encoding a mature peptide according to the transcriptomics data were synthesized by Tsingke Biotechnology Co., Ltd. (Wuhan, China) and then inserted into the pPICzαA and pET-28a vector, resulting in recombinant plasmids

pPICzαA-*Il*LPMO99 and pET 28a-*Il*MnP, respectively. For *Il*LPMO99 expression and purification,[18] the pPICzαA-*Il*LPMO99 was linearized with Pme I and transformed into *P. pastoris* X33 cells by electroporation. The transformants were cultivated in BMGY medium with 200 μM $CuSO_4$ and induced with 0.5% methanol. The supernatant was concentrated and loaded onto an anion-exchange column for purification. To avoid the influence of copper ions on the experiment, excess copper was removed through a PD MidiTrap G-25 desalting column.[42] For *Il*MnP expression and purification, the pET 28a-*Il*MnP was transformed into *E. coli* Rosetta (DE3) cells. The recombinant was cultivated in LB medium with 30 μM Hemin and induced with 1 mM IPTG. The culture was collected and lysed using the high-pressure cracker. The inclusion bodies were refolded *in vitro* and purified using a Ni^{2+} His-tag column (GE).

Measurement of hydrogen peroxide produced by LPMO

The H_2O_2 produced by LPMO in presence of small molecular reductants was quantified by using Amplex Red/horseradish peroxidase assay according to a published protocol reported by Bissaro et al. and Kittl et al.[11,43] The reaction mixture was carried out in a total volume of 100 μL. 100 μM $DBQH_2$ (or $MBQH_2$, or 500 μM ascorbic acid) and 20 μg/mL LPMO was first dissolved in 50 μL phosphate buffer (50 mM, pH6.0) and then mixed with 50 μL detection reagent solution containing 50 μM Amplex Red (Invitrogen, USA) and 7.14 U/ml peroxidase. The H_2O_2 concentration was determined by measuring the absorbance change at 560 nm with H_2O_2 solutions as the standard curve. Reactions with only LPMO or small molecular reductants were used as controls. To determine the effect of cellulose on H_2O_2 produced by LPMO, different concentration of MCC and CMCNa were added to aforementioned reaction using ascorbic acid as electron donor. To avoid the effect of the insoluble MCC to the absorbance measurement, the control with insoluble MCC and without LPMO was set and the samples were centrifuged before determination.

Determination of the iron-reducing and hydroxyl radical

The iron-reducing activity by LPMO with hydroquinone was determined by measuring the formation of Fe^{2+} reduced from Fe^{3+}. Typical experiments were carried out by mixing 100 μM Fe^{3+}-EDTA, 100 μM $MBQH_2$ (or $DBQH_2$), 20 μg/mL purified LPMO and 1.5 mM 1,10-phenanthroline (ε = 12,110 $M^{-1}cm^{-1}$) in 50 mM phosphate buffer (pH5.0). The concentration of Fe^{2+} was measured by detecting the formation of its chelate with 1,10-phenanthroline at 510 nm using a UV-visible spectrometer.[40] To determine the hydroxyl radical production, the experiment was performed according to a modified method from a previous publication.[40] The reaction mixture contained 400 μg/mL LPMO, 500 μM hydroquinone, 100 μM Fe^{3+}-EDTA and 2.8 mM 2-deoxyribose in phosphate buffer (pH5.0). The production of HO· was estimated as the conversion of 2-deoxyribose into TBA-reactive substances (TBARS). The absorbance of TBARS was read at 532 nm against blanks without LPMO or hydroquinone.

The oxidation of lignin model compounds

The oxidation efficiency of lignin model compounds by LPMO driven Fenton reaction was determined by measuring the concentration of the compounds before and after the reaction. The experiments were carried out by incubating 200 μM lignin model compound with 2 mM $DBQH_2$, 100 μM Fe^{3+}-EDTA and 0.65 mg/mL LPMO in 100 mM phosphate buffer (pH5) at 28°C for 24 hours.

To determine the oxidation efficiency of lignin model compounds by MnP with LPMO, the experiments were carried out by incubating 500 μM lignin model compound with 1 mM $MnSO_4$, 0.5 mM ascorbic acid, 70 U/L MnP and 0.8 mg/mL LPMO in 100 mM malonate buffer (pH4.5) at 28°C for 72 hours. The structure changes of the lignin model compounds were determined by high-performance liquid chromatography (HPLC) according to a previous publication.[20] The oxidation rate of the compound was calculated as: oxidation rate (%) = (Ci-Ct)/Ci × 100%, where, Ci: initial peak area of the substrate, Ct: peak area of the substrate along the time.

Lignin oxidation and characterization

The lignin oxidation experiments were carried out with similar aforementioned method for the oxidation of model compounds. The lignin, extracted from wheat straw by ball milling and enzymatic digestion,[44] was used in this study. For the oxidation by LPMO-driven Fenton reaction, 20 mg lignin was incubated with 10 mM $DBQH_2$, 1 mM Fe^{3+}-EDTA and 2 mg/mL LPMO in 100 mM phosphate buffer (pH5.0). For the oxidation by MnP, 20 mg lignin was incubated with 1 mM $MnSO_4$, 0.5 mM ascorbic acid, 1000 U/L MnP and 0.8 mg/mL LPMO (or 0.1 mM H_2O_2) in 100 mM malonate buffer (pH4.5). The changes of functional groups in lignin were quantified by phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) according to the method published in our previous studies.[29,34,45] The lignin samples (ca. 20 mg each) were dissolved in a solvent of pyridine/CDCl3 (1.6/1.0 v/v, 500 μL) and derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. The spectrums were acquired using an inverse-gated decoupling pulse sequence (Waltz-16), a 90° pulse, and a 25-s pulse delay. 128 scans were accumulated for each sample. TopSpin 2.1 software (Bruker BioSpin) and MestreNova (Mestre Labs) software packages were used to process the NMR data.

All assays were conducted in triplicate and expressed as means (SD), whereas the graphical and statistical analysis were conducted using ORIGIN 9.0 (Microcal Software, Northampton, MA, USA).