



Oxidoreductases and Reactive Oxygen Species in Conversion of Lignocellulosic Biomass

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SUMMARY Biomass constitutes an appealing alternative to fossil resources for the production of materials and energy. The abundance and attractiveness of vegetal biomass come along with challenges pertaining to the intricacy of its structure, evolved during billions of years to face and resist abiotic and biotic attacks. To achieve the daunting goal of plant cell wall decomposition, microorganisms have developed many (enzymatic) strategies, from which we seek inspiration to develop biotechnological processes. A major breakthrough in the field has been the discovery of enzymes today known as lytic polysaccharide monoxygenases (LPMOs), which, by catalyzing the oxidative cleavage of recalcitrant polysaccharides, allow canonical hydrolytic enzymes to depolymerize the biomass more efficiently. Very recently, it has been shown that LPMOs are not classical monoxygenases in that they can also use hydrogen peroxide (H_2O_2) as an oxidant. This discovery calls for a revision of our understanding of how lignocellulolytic enzymes are connected since H_2O_2 is produced and used by several of them. The first part of this review is dedicated to the LPMO paradigm, describing knowns, unknowns, and uncertainties. We then present different lignocellulolytic redox systems, enzymatic or not, that depend on fluxes of reactive oxygen species (ROS). Based on an assessment of these putatively interconnected systems, we suggest that fine-tuning of H_2O_2 levels and proximity between sites of H_2O_2 production and consumption are important for fungal biomass conversion. In the last part of this review, we discuss how our evolving understanding of redox processes involved in biomass depolymerization may translate into industrial applications.

KEYWORDS fungi, LPMO, peroxidase, catalase, redox enzymes, hydrogen peroxide, Fenton reaction, lignocellulose

INTRODUCTION

One of the main pillars of the Earth's carbon cycle is the depolymerization of complex plant biomass (1), and a complete understanding of this process is of utmost interest for fundamental biology and crucial for the emerging bioeconomy (2, 3). The structural intricacy of this raw material, primarily composed of cellulose, various hemicelluloses, and lignin, is mirrored by the complexity of the network of enzymatic and chemical reactions developed by microorganisms to decompose it. This network is far from fully understood. Until recently, degradation of the recalcitrant polysaccharides in plant biomass was thought to be mainly achieved by an arsenal of hydrolytic enzymes called glycoside hydrolases (GHs) (4). In some ecosystems, the enzymatic decomposition process is thought to be supported by Fenton chemistry, i.e., transition metal-driven *in situ* generation of H_2O_2 -derived hydroxyl radicals, which are among the most powerful oxidizing species found on Earth (5) and are able to unspecifically oxidize both polysaccharides and lignin in plant biomass (6).

Several decades ago, Elwyn Reese and colleagues proposed that nonhydrolytic proteins were involved in cellulose decomposition. This proposal is known as the C_1 - C_x postulate in which the C_1 factor (nonhydrolytic proteins) acts as an enhancing protein for the C_x factor (hydrolytic enzymes) (7). In 1974, Eriksson and colleagues noticed that cellulose degradation by culture filtrates of white-rot fungi was more efficient in the presence of O_2 , leading to the hypothesis that oxidative processes contribute to cellulose conversion (8). In 2005, studying enzymatic chitin degradation, Vaaje-Kolstad et al. showed that a 21-kDa protein named chitin-binding protein, or CBP21, by Suzuki et al. (9), drastically enhanced the efficiency of classical (hydrolytic) chitinases (10). Five years later, in 2010, i.e., 60 years after Reese's postulate, it was shown that CBP21 represents a new class of enzymes that carry out oxidative cleavage of polysaccharides (11). These enzymes, today known as lytic polysaccharide monoxygenases (LPMOs) (12), are mono-copper redox enzymes (13, 14) that hydroxylate the C-1 or C-4 carbons of scissile glycosidic bonds (11, 15–18) in an O_2 - and reductant-dependent manner (11).

As illustrated by the many different names they have been given over the years, LPMOs still retain many secrets. After having been considered sluggish fungal glycoside

hydrolases belonging to family GH61 (19, 20) or noncatalytic bacterial carbohydrate binding modules (CBMs) belonging to family CBM33, the 2010 discovery led to gathering these proteins of diverse origins under the common acronym LPMO (12) or, alternatively, polysaccharide monooxygenase (PMO) (14). Today, LPMOs are classified as auxiliary activities (AA) (21) in the database of carbohydrate-active enzymes (CAZy) (22, 23), where they form families AA9, -10, -11, -13, -14, and -15. LPMOs are today seen as key frontline weapons in the warfare between attackers (e.g., fungi and bacteria) and defenders (e.g., plants) (24).

LPMOs are unique in the sense that they are able to attack polysaccharides that are organized in recalcitrant structures (e.g., crystalline cellulose or chitin; hemicellulose-cellulose complexes) (25). While canonical glycoside hydrolases (GHs) interact with single polysaccharide chains, meaning that a decrystallization penalty needs to be paid (26), LPMOs act on surfaces; that is, they cleave a polysaccharide chain while this chain is in a crystalline context (11). By doing so, LPMOs render a relatively inaccessible substrate tractable to further depolymerization by GHs (27–30). In this connection, real-time atomic force microscopy studies have shown that LPMOs are relatively immobile on the cellulose surface and that cellulase-catalyzed substrate turnover is higher after LPMO treatment (31). In a very interesting study using *in situ* imaging, it has also been shown that the progression of hydrolases and the boosting effect of LPMOs are dependent on the type of plant tissue (30).

A recent milestone in the field concerns the discovery that H_2O_2 can drive LPMO reactions in the absence of O_2 (32, 33). In fact, it has been claimed that H_2O_2 is the preferred, and perhaps even the only, cosubstrate of LPMOs (32, 34), which contrasts with established paradigms and raises questions as to whether LPMOs should be classified as monooxygenases. Importantly, H_2O_2 is also a reaction product or substrate in several other enzyme-catalyzed lignocellulolytic reactions, in particular, in lignin conversion (35). Thus, H_2O_2 may play a central role in the reaction networks of biomass conversion. Interestingly, H_2O_2 and reactive oxygen species (ROS) in general are increasingly considered metabolites with a variety of possible (regulatory) functions beyond simply being oxidants (36, 37). It is thus worth considering whether H_2O_2 could be a central regulatory metabolite in biomass conversion, the levels of which are temporally and spatially regulated by the actions of substrate-specialized (and thus localized) H_2O_2 -producing and -consuming enzymes.

In this context, we wish to first introduce the concept of LPMO catalysis by presenting the monooxygenase (MO; O_2 -based) and peroxygenase (PO; H_2O_2 -based) reaction paradigms and discuss the knowns and unknowns. Then, a critical retrospect on LPMO literature is carried out with the aim of shedding new light on previously reported results. The second part of this review describes other H_2O_2 -producing or -consuming systems encountered during lignocellulose conversion and discusses potential and proven interconnections in the light of available biochemical and multi-omics data. The last part of this review focuses on how our improving understanding of natural biomass conversion translates into the design of better industrial biorefining processes, today and tomorrow.

RETROSPECT ON LPMO RESEARCH

Introduction to LPMO Catalysis: Knowns and Unknowns

The present review does not aim to summarize all aspects of LPMOs, such as structural aspects and putative mechanistic routes, since these have been comprehensively covered in other recent reviews (12, 17, 18, 24, 38–42). Nevertheless, the recent discovery of the peroxygenase activity of LPMOs (32, 34, 43, 44) shatters a widely accepted paradigm for LPMO catalysis that laid the foundations for previous discussions and analyses. Therefore, a clarification of what has been explicitly proven and what remains hypothetical is required.

A conserved catalytic core embedded in an evolutionarily divergent binding surface. The uniqueness of LPMOs comes in part from the fact that they can carry out the oxidative cleavage of a glycosidic chain embedded in a crystalline lattice, an unprec-

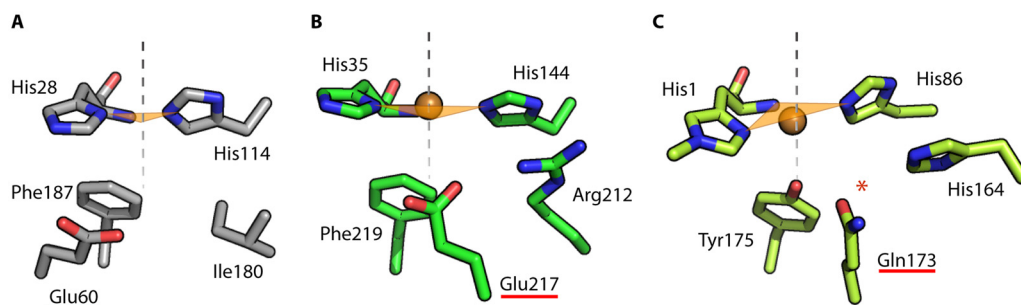


FIG 1 LPMO active sites. The figure shows a close-up view of the catalytic center of *SmAA10A* (also known as CBP21; PDB accession number **2BEM**) (A), *SCAA10C* (also known as CelS2; PDB accession number **4OY7**) (B), and *TAA9A* (also known as *TaGH61A*; PDB accession number **2YET**) (C), which are representatives of bacterial chitin-active, bacterial cellulose-active, and fungal cellulose-active LPMOs, respectively. The gray dotted line shows the axis defined as axial, and the orange triangle represents the equatorial plane defined by the three copper-coordinating nitrogens in the histidine brace (best visible in panel C). The red star indicates the location of an oxygen species observed in the neutron structural studies by O'Dell et al. (69), and the Glu/Gln potentially interacting with this oxygen species is underlined. Note that Phe187, Phe219, and Tyr175 are in equivalent positions in their respective proteins, namely, the proximal axial coordination position. The distal axial position is solvent exposed and will be occupied by substrate upon binding (52).

edented ability in the world of CAZymes. To do so, LPMOs have to bring their active site close to the crystalline surface at the right location (45). Importantly, there is growing evidence that LPMOs also act on noncrystalline substrates (46–48) or copolymeric structures (25, 49–51), indicating that substrate crystallinity is not a *sine qua non* condition for LPMO catalysis to occur. Numerous crystallographic structures (39, 52) show that LPMOs, irrespective of their phylogenetic origins and substrate specificities, display rather flat, solvent-exposed substrate-binding surfaces that include two conserved histidines coordinating a single copper atom, also known as a histidine brace (13). The histidine brace is the only totally conserved feature across the LPMO superfamily (Fig. 1). Other surface-exposed residues involved in substrate binding (45, 52–58) and/or the second coordination shell of the active-site copper are often relatively conserved within phenotypic subgroups and therefore probably dictate complementarity with the target substrate (39). Despite a few studies aimed at unraveling the determinants of oxidative regioselectivity (56, 59, 60) and substrate specificity (61), these determinants remain largely unknown.

Some cellulose-active LPMOs are regiospecific (i.e., exclusively oxidizing the C-1 or C-4 carbon), while others display a lack of specificity, oxidizing both C-1 and C-4 carbons. Notably, data on enzyme-substrate complexes (45, 52) show that the C-1 and C-4 carbons are both close to the copper site, meaning that minor variation in substrate positioning could lead to a change in oxidative regioselectivity, a notion that is supported by various studies (56, 60). It is intriguing that some LPMOs seemingly are somewhat ambiguous when it comes to substrate binding and positioning, while accurate assembly of the catalytic complex is crucial to control the very powerful redox chemistry and to prevent off-pathway reactions, as explained in “Oxidative self-inactivation of LPMOs,” below.

The O₂ reaction mechanism(s): oxidase and monooxygenase activities. The seminal study by Vaaje-Kolstad et al. unraveled the oxidative activity of the chitin-active CBP21, AA10A, from the bacterium *Serratia marcescens* (*SmAA10A*) by showing that aldonic acids were released from chitin under aerobic conditions and in the presence of reductant. Using mass spectrometry and labeled oxygen (¹⁸O₂), it was shown that the introduced oxygen atom was derived from O₂ (11), and this was later also shown for a cellulose-active fungal LPMO, AA9E, from *Neurospora crassa* (*NcAA9E*-CBM1, also known as *NcPMO*-08760) (16). The combined use of mass spectrometry and labeled water (H₂¹⁸O) showed that the detected aldonic acids result from spontaneous hydrolysis of a lactone form (11). The lactone form has been proposed to arise from a spontaneous elimination reaction that happens upon hydroxylation of the C-1 carbon

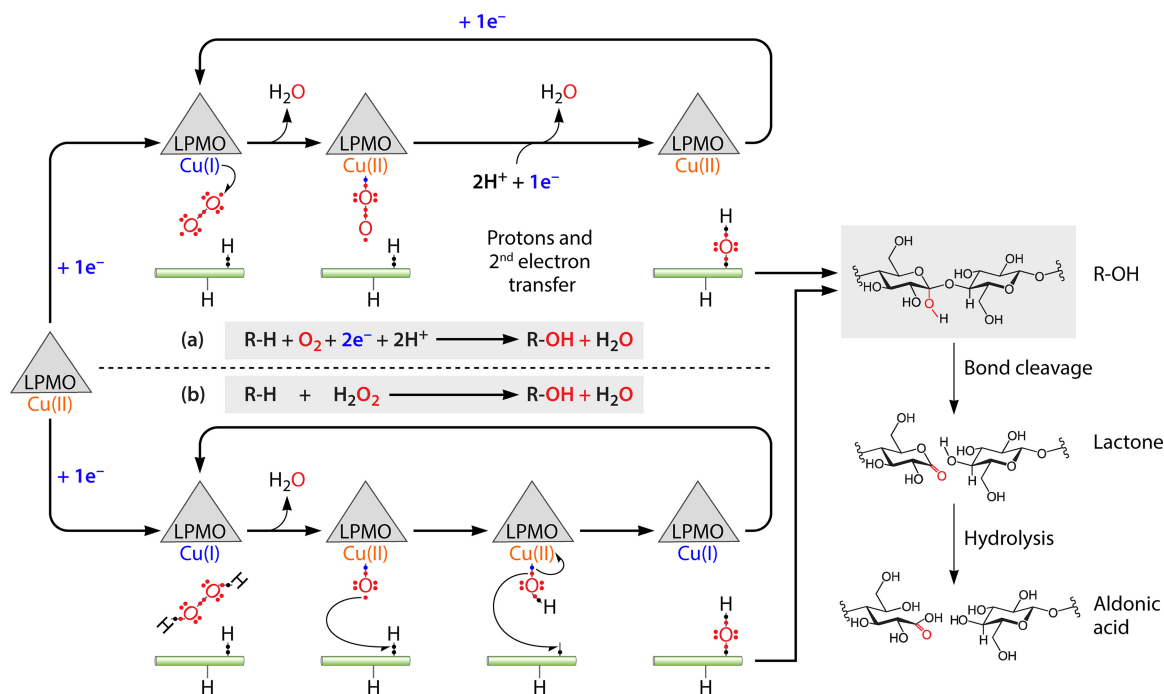


FIG 2 Comparison of O_2 -based (a) and H_2O_2 -based (b) reaction pathways. To enter both pathways, a reduction is necessary to reduce Cu(II) to Cu(I). In pathway a, transfer of a second electron and of 2 protons is necessary to complete the catalytic cycle. In pathway b, H_2O_2 alone is sufficient to complete a reaction cycle. Detailed potential catalytic pathways involving O_2 (17, 18) or H_2O_2 (32) as a cosubstrate have been described elsewhere. Both pathways are thought to generate a hydroxylated end product (either at C-1 or C-4). The right-hand side of the figure shows a C-1-hydroxylated product undergoing a spontaneous elimination reaction that leads to cleavage of the glycosidic bond and formation of a lactone, which is hydrolyzed to become an aldonic acid.

(16) (Fig. 2, right side). A similar mechanism is envisaged for C-4-oxidizing LPMOs, but in this case the product is a ketoaldehyde, which is hydrated to a gem-diol (14, 62).

LPMOs were assigned as monooxygenases based on (i) the monooxygenated nature of the reaction product, (ii) the apparent dependency of the reaction on O_2 and reducing equivalents, and (iii) similarities with other copper-dependent enzymes known as monooxygenases, in particular, methane monooxygenase (13, 14). This logical reasoning has been widely accepted by the scientific community. However, it has recently been pointed out that while the apparent O_2 dependency shows that O_2 can be the source of the incorporated oxygen, this dependency is not a *proof stricto sensu* that O_2 is the cosubstrate of LPMOs since, in a reducing environment, O_2 -derived intermediates such as superoxide or H_2O_2 will be generated (32).

Among the knowns, it has unambiguously been established that LPMOs are mono-copper enzymes (13, 14) which can catalyze the monooxygenation of several carbohydrate substrates (11, 13, 14, 25, 46, 47, 62–65). It is also well known that the LPMO-Cu(II) state is the resting inactive state and that reduction of the copper precedes catalytic action. It is also established that in the absence of substrate and presence of reductant, LPMOs can act as oxidases, i.e., that they are able to carry out the reduction of O_2 (15), leading to H_2O_2 formation (62, 66). Importantly, this implies that even in reactions with substrate, non-substrate-bound LPMOs that become reduced may generate H_2O_2 , which could fuel H_2O_2 -driven reactions catalyzed by substrate-bound LPMOs (see below for more details).

While the ability of LPMOs to produce H_2O_2 is well established, it is not clearly established whether the superoxide species resulting from O_2 single-electron reduction [by LPMO-Cu(I)] is released in the reaction mixture before undergoing spontaneous disproportionation, as suggested by some density functional theory (DFT) calculations (15), or if H_2O_2 is produced in the LPMO active site. The latter scenario was recently suggested by Span et al., who noted that addition of superoxide dismutase (SOD) to an

AA9 LPMO reaction mixture had no effect on H_2O_2 production (67). This would suggest that a second electron and two protons have to be delivered to the LPMO active site to complete the two-electron reduction of O_2 to H_2O_2 . Span et al. further showed that a second-shell glutamine (Fig. 1) likely contributes to keeping the superoxide bound to the active site. In support of the latter observations, analysis of LPMO structural diversity shows that a conserved second-shell glutamate or glutamine is pointing toward the active site in all LPMOs, suggesting a key role in LPMO catalysis (Fig. 1) (39). Such a role of this glutamine residue is also supported by recent quantum mechanics/molecular mechanics (QM/MM) studies (Fig. 1C) (68). Accordingly, recent neutron structures of *NcAA9D* from *Neurospora crassa* (also known as *NcPMO-01050*) indicate that His157 and Gln166 (equivalent to His164 and Gln173 shown in Fig. 1C) interact with an equatorially bound oxygen species (69). Similar equatorial binding of an oxygen species has also been observed in a neutron structure of a bacterial AA10 LPMO (70). Mutational studies have shown that Glu60 in *SmAA10A* (54) (Fig. 1A) and Gln151 in *Thielavia terrestris* AA9E (*TtAA9E*) (19) (equivalent to Gln173 in *Thermoascus aurantiacus* AA9A [*TaAA9A*]) (Fig. 1C) are important for activity on chitin and cellulose degradation, respectively. While this indicates that the studies on oxygen activation discussed above are relevant for understanding LPMO catalysis, generally, great caution is needed when observations made under substrate-free conditions are transposed to productive conditions (i.e., in the presence of substrate; see below). Of note, the acid/base chemistry that may be exerted by second-sphere amino acid residues such as Glu60 in *SmAA10A* or His164 in *TaAA9A* (Fig. 1A) during O_2 reduction and/or catalysis is not understood, and no pK_a values for these residues have been reported.

One of the major challenges in understanding LPMO catalysis pertains to the insoluble nature of natural substrates that constitutes a hurdle for the use of most standard experimental approaches. In this respect, a landmark study has reported the crystallographic resolution of the first AA9 LPMO-oligosaccharide complex revealing interactions between the enzyme and a soluble substrate (52). This study showed that substrate binding, expectedly, shields the copper ion from the solvent and that the catalytic oxygen species must bind in the equatorial position. The latter was supported by the presence of a chloride ion in the equatorial copper coordination position in the enzyme-substrate complex, which could mimic a reactive oxygen species. Frandsen et al. also confirmed previous observations by Borisova et al. (71) that substrate binding leads to changes in copper coordination, as was also reported, more recently, for the chitin-active *SmAA10A* (45), leading to the suggestion that substrate binding increases the catalytic competence of the enzyme. This would make sense since it would help the enzyme to unleash its oxidative power only if substrate is present. Despite claims indicating the opposite, still very little is known about polysaccharide cleavage by LPMOs. A key point to be taken from the work by Frandsen et al. and others is that the active-site environment of an LPMO in solution is different from that of an LPMO bound to a crystalline polysaccharide surface. Notably, confinement of the active site upon crystalline polysaccharide binding seems to restrict access to reactants, as indicated by simulations suggesting formation of a gated tunnel connecting the active-site cavity to the bulk solvent (45).

There is clear and growing evidence (crystallographic, biochemical, and computational) for activation of O_2 in the absence of substrate, but there is no strong experimental proof for such activation in the presence of a bound substrate (see reference 44 for a very recent discussion). One particularly intriguing issue concerns the fact that the monooxygenase reaction paradigm ($\text{R-H} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{R-OH} + \text{H}_2\text{O}$) requires that two electrons and two protons are recruited during catalysis. In the case of an LPMO, the first electron can be stored in the form of Cu(I), but the second electron either has to be stored by the enzyme or timely supplied when required. In the mono-copper and otherwise cofactor-free LPMO, a second electron could be derived from transient residue-derived radicals, such as radicals observed for the modified tyrosine in galactose oxidase (72, 73). A tyrosine, located in the axial direction (Fig. 1C), is conserved across fungal LPMOs and may play such a role, whereas a phenylalanine

is usually found at the equivalent position in bacterial LPMOs. Alternatively, some researchers have proposed an electron transport chain or channel that would allow delivery of an electron to the substrate-bound LPMO (17, 18) (Fig. 2, option a). The existence of such an electron channel has so far not been established, and there are no conserved sequence features among LPMOs that could support the existence of such a channel.

It is worth noting that the monooxygenase reaction also requires the proper supply of two protons and that nothing is known about how this could happen. Knowledge about second-sphere residues' acid/base chemistry and proton networks (67) will be useful to elucidate this.

The H₂O₂ reaction mechanism(s): peroxygenase and peroxidase activities. Recently, it has been proposed that the monooxygenase paradigm applied to LPMOs may need to be revised based on experiments showing that H₂O₂ can efficiently drive LPMO reactions. Importantly, inhibition of LPMO catalysis by a H₂O₂-scavenging enzyme (horseradish peroxidase [HRP]) under standard reaction conditions (i.e., excess of O₂ and reductant) suggested that O₂ is a precursor molecule for the true cosubstrate, H₂O₂ (32, 33). Accordingly, it was also shown that H₂O₂-driven reactions are much faster than O₂-driven reactions and that the enzymes prefer H₂O₂ over O₂ in competition experiments. The ability of H₂O₂ to promote fast AA9 LPMO catalysis has recently been confirmed in independent experiments by Hangasky et al. (44). Bissaro et al. (32, 33) concluded that H₂O₂ is not just an alternative to O₂ but that it is the catalytically relevant cosubstrate of the polysaccharide oxidation by different LPMOs, which would thus act as peroxygenases (R—H + H₂O₂ → R—OH + H₂O). This conclusion has been debated in the field (44, 74) and still needs more proof, but the ability of H₂O₂ to drive fast AA9 and AA10 LPMO reactions is now well proven.

The proposed H₂O₂-based mechanism entails an initial reduction from LPMO-Cu(II) to Cu(I), termed the priming reduction. The reduced enzyme reacts in a controlled and substrate-associated manner with H₂O₂ to unleash the intrinsic oxidative power of the latter. This leads to hydroxylation of the substrate, concomitant release of a water molecule, and regeneration of the LPMO-Cu(I) state, which can enter a new catalytic cycle (Fig. 2, option b). It is important to note that the redox state of the LPMO has not been experimentally monitored along the reaction. The concept of the priming reduction has mainly been deduced from the observation that supra-stoichiometric amounts of oxidized products (relative to the reductant) were generated when the AA10 LPMO was supplied with H₂O₂, indicating that a reduced LPMO catalyzes multiple turnovers (32). Of note, such substoichiometric consumption of reductant is not compatible with the originally proposed O₂-dependent reaction mechanism (Fig. 2).

The nature of the most likely reactive oxygen species emerging during catalysis remains a matter of discussion for both the O₂ and the H₂O₂ mechanisms (32, 42, 44, 74). One route proposed for the H₂O₂ reaction mechanism (32) involves a [CuO⁺] core intermediate as the species catalyzing the hydrogen atom abstraction from the glycosyl unit (Fig. 2). Earlier QM/MM calculations, in the working frame of an O₂ reaction mechanism, also suggested that the [CuO⁺] intermediate would be the relevant catalytic species (68, 75, 76). Still, other mechanisms, such as a mechanism involving the formation of a H₂O₂-derived hydroxyl radical as oxidant, cannot be excluded (32, 44). While mechanistic details remain to be elucidated, it is worth noting that the H₂O₂ mechanism solves the conundrum of second-electron delivery discussed above. H₂O₂ carries both the protons and reducing equivalents necessary for LPMO catalysis to occur.

Prompted by the discovery of H₂O₂-driven LPMO catalysis in 2016 (32, 33), several follow-up studies have recently appeared. A detailed kinetic study of the peroxygenation of chitin by SmAA10A yielded a catalytic constant of 6.7 s⁻¹ and a K_m of 2.8 μM, suggesting high affinity for H₂O₂ (34). The resulting catalytic efficiency, in the range of 10⁶ M⁻¹ · s⁻¹, is similar to catalytic efficiencies typically reported for peroxygenases (77). In another study, Breslmayr et al. used 2,6-dimethoxyphenol as chromogenic substrate and H₂O₂ as a cosubstrate to assess the peroxidase activity of several AA9s,

yielding catalytic rates varying from 0.9 to 18.6 s⁻¹ (43). Two different QM/MM studies have shown that the peroxygenase reaction is plausible from a theoretical point of view, with low overall energy barriers and involving a [CuO⁺] intermediate as oxidant (74, 78).

Using a fungal AA9 active on cellohexaose, Hangasky et al. found much higher reactivity with H₂O₂ as a cosubstrate (4.75 to 15.25 s⁻¹) than with O₂ (0.28 s⁻¹) (44), thus confirming the findings by Bissaro et al. Interestingly, despite a difference of four orders of magnitude in the catalytic efficiencies observed with H₂O₂ (10⁶ M⁻¹ · s⁻¹) (34) and O₂ (10² M⁻¹ · s⁻¹) (44), based on additional experiments, these authors concluded that O₂ may still be the most relevant and natural cosubstrate.

Regarding the stoichiometry of the reaction, data obtained for H₂O₂-driven LPMO reactions support a 1:1 molar ratio between consumed H₂O₂ and produced oxidized sugars for both AA9 and AA10 cellulose-active (32, 44) and AA10 chitin-active (34) LPMOs. No equivalent data are available for O₂-driven reactions although one may expect a similar 1:1 stoichiometry in a system where nonproductive events are minimized (e.g., reduction of O₂ to water). All in all, accumulating experimental evidence suggests that, in the presence of substrate, AA9 and AA10 LPMOs react much more efficiently with H₂O₂ than with O₂. The question of whether LPMOs at all use O₂ directly, that is, without prior reduction to H₂O₂, remains to be settled. Importantly, as described in the following sections, there are plenty of sources of H₂O₂ in lignocellulolytic environments.

The carbohydrate intermediate species. Little is known about the carbohydrate intermediates potentially occurring along the reaction pathway since the substrate radical resulting from hydrogen abstraction and the subsequent hydroxylated product have never been experimentally observed. It is not known either whether the molecular rearrangement induced by the destabilizing hydroxylation, which leads to glycosidic bond cleavage and lactone formation, occurs spontaneously in the reaction mixture or in an enzyme-assisted manner. Recent calculations, however, suggest that hydroxylation-induced glycosidic bond cleavage can occur in the absence of enzyme (74). This mechanistic ambiguity is at the origin of the debate on the relevance of the term “lytic” in the LPMO acronym and explains why some choose to call these enzymes PMOs (18). The term lytic is meant to indicate the fact that bond cleavage occurs, which contrasts with most other monooxygenases, which tend to catalyze oxy-functionalization but not cleavage of their substrates.

Oxidative self-inactivation of LPMOs. A key aspect of LPMO catalysis pertains to operational stability, a parameter that is of high importance in industrial, biological, and chemical contexts. When analyzing the literature, one can observe that a correlation can be established between poor substrate binding and rapid enzyme inactivation (56, 79–81). We know today that this enzyme inactivation is due to oxidative self-inactivation of the LPMO and that oxidative damage of the enzyme is confined to the active site, notably the copper-coordinating histidines (32). These suicide reactions can be prevented by productive substrate binding (32, 34, 44). In studies with H₂O₂ as a cosubstrate, Kuusk et al. showed that the rate of inactivation of *SmAA10A* in the absence of substrate was about 1,000 times lower than the rate of substrate cleavage in reactions with substrate (34).

In general, it is thus extremely important to have full control of the reduction state of the LPMO during its handling (i.e., protein extraction, purification, storage, and reaction setup) since accidental reduction of the copper center in the absence of the proper substrate and in the presence of O₂ or H₂O₂ will lead to enzyme oxidative self-inactivation and thus to nonfunctional protein. It must be noted that an oxidatively damaged LPMO will still look normal on an SDS-PAGE gel and that the damage thus may remain undetected and lead to false conclusions as to the activity of the enzyme in question. To cope with such issues, several precautions could be envisaged, such as using of metal chelators (e.g., EDTA) to remove the copper ion while the enzyme is not used, working under anaerobic conditions, or avoiding reducing conditions. Research pertaining to the control of these inactivation processes will likely be a topic of

investigation in the near future, notably by seeking inspiration from the fields of peroxidases and peroxygenases that face similar problems (82).

What is in the name? As mentioned in the introduction, over time, LPMOs have had several names. Today, the term LPMO is widely accepted, and most researchers name their LPMOs as $XxLPMOnX$ or $XxAAnX$, where Xx indicates the source microbe (e.g., *Nc* for *Neurospora crassa*), n indicates the LPMO family according to CAZy (currently 9, 10, 11, 13, 14, or 15), and X is a capital letter that is assigned to multiple LPMOs from a certain organism, often by the order of their functional characterization or the order of their gene numbers in the genome. Considering the recent discoveries on the role of H_2O_2 , the term LPMO may need revision. While including peroxygenase (PO) in the name (e.g., LPPO) may appear premature, the existing term monooxygenase (MO), which by definition implies O_2 as a cosubstrate, seems incorrect. Informal discussions among scholars have led to the suggestion to use the more general term oxidase to refer to LPMOs. The term LPO, for lytic polysaccharide oxidase, entails a simple change relative to LPMO, does not assume the nature of the cosubstrate, and includes the indisputable ability of LPMOs to generate H_2O_2 (which is an oxidase reaction). On the other hand, the term oxidase does not reflect the fact that an oxygen atom is incorporated into the final product, and in that sense “oxygenase” appears more appropriate. Discussions, debates, and scientific progress should allow sorting out this issue in the near future.

In the following sections, we revisit previously published studies on LP(M)Os to pinpoint overlooked incoherencies or unexplained phenomena that find sense in the frame of the peroxygenase paradigm for LPMO action.

A Wide Diversity of Reductants

LPMO reductants and associated catalytic rates. It has been demonstrated that LPMOs can be activated by a wide diversity of reductants (Table 1). These include organic compounds such as ascorbic acid (AscA) (11), cysteine (48, 65), reduced glutathione (11), and a wide range of plant- and fungus-derived phenols (65, 83), as well as lignin and fragments thereof (84–86). Functional reductants also include enzymatic systems such as cellobiose dehydrogenase (CDH) (see subsection “The CDH Case: a Multifunctional Redox Partner,” below) or photocatalytic systems (87, 88) (see subsection “Stimulation of LPMO Activity by Photocatalytic Systems,” below). The ability of CDH to drive fungal AA9 LPMO reactions was detected early in the development of the field (14, 64). It should be noted that an equivalent natural enzymatic redox partner has not yet been found in bacteria.

The highly surface-exposed active sites of LPMOs are unusual and may explain the apparent absence of reductant specificity. Nevertheless, taking into account the fact that the reductants listed above display very different sizes and topologies, as well as electrostatic or hydrophobicity properties, it appears intriguing that they can all directly reduce the LPMO copper center. Of note, redox partner diversity has also been observed for cytochrome P450 monooxygenases (89). Both for P450 monooxygenases (90) and LPMOs (83) a correlation between the reduction potential of the redox partner and the reduction rate of the enzyme has been established. Also, it has been reported that an increase in pH led to a decrease in reduction potential of the reductant and to an increase in AA9 LPMO initial rates (91).

Importantly, no correlation has ever been established between the rate of reduction of LPMOs (per millisecond range) and the apparent catalytic rate (per minute range), which is orders of magnitude lower. Assuming the O_2 -based mechanism, this remarkable discrepancy in rates indicates that transfer of the second electron, the rate of which cannot be measured directly, is rate limiting and affected by the reductant type. Alternatively, in the H_2O_2 -based mechanism, priming reduction of the copper may not be rate limiting, and the dependency of the LPMO catalytic rate on the reductant may reflect different potentials of each reducing system to generate and/or accumulate H_2O_2 . Table 1 shows an overview of available kinetic data for a large diversity of LPMO substrates and reductants. Importantly, with a few exceptions, the apparent enzyme

TABLE 1 Diversity of reductants promoting LPMO activity and associated apparent catalytic rates^a

Enzyme (concn) ^b	Electron-supplying system (concn) ^f	Reaction conditions			T (°C)	Mixing (rpm) ^j	Quantification method (fraction) ^j	Observed oxidative rate (min ⁻¹) ^j	Reference
		Substrate(s) (concn) ^g	Buffer ^h						
<i>SmAA10A</i> (1 μM) ^c	Reduced glutathione (1 mM)	β-Chitin (0.45 g/liter)	Tris-HCl (20 mM, pH 8.0)	37	Thermomixer (1,000)	UHPLC-UV (total)	1.28	11	
<i>ScAA10C-CBM2</i> (1 μM) ^d	AscA (2 mM)	PASC (2 g/liter)	Am-Ac (20 mM, pH 6.0)	50	Thermomixer (900)	HPAEC-PAD (soluble)	0.22*	351	
<i>LsAA9A</i> (1 μM)	AscA (5 mM)	FRET substrate (10–100 μM) ^d	BT-HCl (20 mM, pH 7.0)	37	96-Well MP	FRET (total)	6.6	52	
<i>NcAA9C-CBM1</i> (1.47 μM)	AscA (1 mM)	XG ₁₄ (0.2 mM)	Am-Ac (25 mM, pH 8.0)	40	Thermomixer (600)	HPAEC-PAD (total)	3.6	46	
	AscA (1 mM)	Cellopentaose (0.2 mM)	Am-Ac (25 mM, pH 8.0)	40	Thermomixer (600)	HPAEC-PAD (total)	1.8	46	
	AscA (1 mM)	Tamarind XG (5 g/liter)	Na-P (5 mM, pH 8.0)	50	Thermomixer (1,000)	DNS	6.6	46	
	AscA (1 mM)	PASC (5 g/liter)	Na-P (5 mM, pH 8.0)	50	Thermomixer (1,000)	DNS	6.6	46	
<i>NcAA9C-CBM1</i> (4 μM)	AscA (2 mM)	Tamarind XG (5 g/liter)	Na-P (40 mM, pH 6.5)	50	Thermomixer (1,000)	DNS	6.0	71	
	AscA (2 mM)	PASC (5 g/liter)	Na-P (40 mM, pH 6.5)	50	Thermomixer (1,000)	DNS	2.4	71	
<i>VcAA10B-X-Y-CBM73</i> (2 μM) ^e	AscA (1 mM)	β-Chitin nanofibers (5 g/liter)	BTm-HCl (50 mM, pH 6.8)	37	Thermomixer (800)	UHPLC-UV (soluble)	2.7	429	
<i>SmAA10A</i> (1 μM)	AscA (0.5 mM)	β-Chitin (10 g/liter)	BTm-HCl (50 mM, pH 6.0)	40	Thermomixer (1,000)	UHPLC-UV (soluble)	4.17*	102	
	AscA (1 mM)	β-Chitin (10 g/liter)	BTm-HCl (50 mM, pH 6.0)	40	Thermomixer (1,000)	UHPLC-UV (soluble)	6.6*	102	
	AscA (2 mM)	β-Chitin (10 g/liter)	BTm-HCl (50 mM, pH 6.0)	40	Thermomixer (1,000)	UHPLC-UV (soluble)	9.72*	102	
	AscA (5 mM)	β-Chitin (10 g/liter)	BTm-HCl (50 mM, pH 6.0)	40	Thermomixer (1,000)	UHPLC-UV (soluble)	13.2*	102	
<i>CfAA10-CBM2</i> (1 μM)	AscA (1 mM)	PASC (0.3 g/liter)	Na-P (50 mM, pH 6.0)	37	Thermomixer (150)	HPAEC-PAD (soluble)	0.49	80	
	AscA (1 mM)	Avicel (0.3 g/liter)	Na-P (50 mM, pH 6.0)	37	Thermomixer (150)	HPAEC-PAD (soluble)	0.35	80	
	AscA (1 mM)	BMCC (0.3 g/liter)	Na-P (50 mM, pH 6.0)	37	Thermomixer (150)	HPAEC-PAD (soluble)	0.78	80	
<i>TbAA10-CBM2</i> (1 μM)	AscA (1 mM)	PASC (0.3 g/liter)	Na-P (50 mM, pH 6.0)	37	Thermomixer (150)	HPAEC-PAD (soluble)	0.23	80	
	AscA (1 mM)	Avicel (0.3 g/liter)	Na-P (50 mM, pH 6.0)	37	Thermomixer (150)	HPAEC-PAD (soluble)	0.10	80	
	AscA (1 mM)	BMCC (0.3 g/liter)	Na-P (50 mM, pH 6.0)	37	Thermomixer (150)	HPAEC-PAD (soluble)	0.32	80	
<i>CjAA10A-CBM5-CBM73</i> (0.5 μM)	AscA (1 mM)	α-Chitin (10 g/liter)	BTp-HCl (20 mM, pH 7.2)	37	Thermomixer (1,000)	UHPLC (soluble)	1.43*	79	
<i>TrAA9A-CBM1</i> (4 μM)	AscA (0.4 mM)	PASC (4 g/liter)	Na-Ac (10 mM, pH 5.0)	37	Mixing in miniplate well	Oxygen consumption ^k	1.26	104	
<i>TtAA9E</i> (4 μM)	AscA (0.4 mM)	PASC (4 g/liter)	Na-Ac (10 mM, pH 5.0)	37	Mixing in miniplate well	Oxygen consumption	0.88	104	
<i>ThtAA9A</i> (4 μM)	AscA (0.4 mM)	PASC (4 g/liter)	Na-Ac (10 mM, pH 5.0)	37	Mixing in miniplate well	Oxygen consumption	0.93	104	
<i>ThtAA9B-CBM1</i> (0.27 μM)	AscA (1 mM)	RAC (2.8 g/liter)	Am-Ac (50 mM, pH 5.0)	50	HOTSR (20)	HPAEC-PAD (total)	0.28 ^{nm}	91	
<i>ScAA10C-CBM2</i> (0.5 μM)	AscA (1 mM)	Avicel (10 g/liter), H ₂ O ₂ (200 μM)	Na-P (50 mM, pH 7.0)	40	Magnetic stirring	HPAEC-PAD (soluble)	82.4 ^{no}	32	
	AscA (1 mM)	Avicel (10 g/liter)	Na-P (50 mM, pH 7.0)	40	Magnetic stirring	HPAEC-PAD (soluble)	3.2 ^{no}	32	
<i>PcAA9D</i> (0.5 μM)	AscA (1 mM)	Avicel (10 g/liter), H ₂ O ₂ (100 μM)	Na-P (50 mM, pH 7.0)	40	Magnetic stirring	HPAEC-PAD (soluble)	15.6 ^{no}	32	
	AscA (1 mM)	Avicel (10 g/liter)	Na-P (50 mM, pH 7.0)	40	Magnetic stirring	HPAEC-PAD (soluble)	2.1 ^{no}	32	
<i>SmAA10A</i> (50 nM)	AscA (100 μM)	CNW (sat), H ₂ O ₂	Na-Ac (50 mM, pH 6.1)	25	Static	¹⁴ C radioactivity	402	34	
<i>ThtAA9E</i> (50 nM)	AscA (2 mM)	Cellohexaose (1 mM), H ₂ O ₂ (100 μM)	MES/MOPS (100 mM, pH 6.5)	40	NR	HPAEC-PAD (total)	916 ^p	44	
<i>ThtAA9E</i> (1 μM)	AscA (2 mM)	Cellohexaose (sat)	MES/MOPS (100 mM, pH 6.5)	40	NR	HPAEC-PAD (total)	10 ^q	373	
<i>SmAA10A</i> (1 μM)	Lactose (3 mM)/ <i>MtCDH</i> (1.5 μM)	β-Chitin (10 g/liter)	BTm-HCl (25 mM, pH 6.0)	40	Thermomixer (1,000)	UHPLC-UV (total)	3.3	102	
<i>TtAA9E</i> (2.22 μM)	Chl (1.6 mM)/visible light + AscA (2 mM)	PASC (7.5 g/liter)	Cit-P (100 mM, pH 6.3)	50	Thermomixer (1,000)	HPAEC-PAD (total)	33	87	
<i>ScAA10C-CBM2</i> (0.5 μM)	Chl (0.5 mM)/visible light + AscA (1 mM)	Avicel (10 g/liter)	Na-P (50 mM, pH 7.0)	40	Magnetic stirring	HPAEC-PAD (soluble)	96 ^{no}	33	
<i>ScAA10C-CBM2</i> (1 μM)	H ₂ O ₂ /V-TiO ₂ (5 g/liter)/visible light	Avicel (10 g/liter)	Na-P (50 mM, pH 6.0)	40	Magnetic stirring	HPAEC-PAD (soluble)	0.28*	88	

(Continued on next page)

TABLE 1 (Continued)

Enzyme (concn) ^b	Electron-supplying system (concn) ^f	Reaction conditions			T (°C)	Mixing (rpm) ⁱ	Quantification method (fraction) ^j	Observed oxidative rate (min ⁻¹) ^k	Reference
		Substrate(s) (concn) ^g	Buffer ^h						
	H ₂ O + MeOH/V-TiO ₂ (5 g/liter)/visible light	Avicel (10 g/liter)	Na-P (50 mM, pH 6.0)		40	Magnetic stirring	HPAEC-PAD (soluble)	0.78 [*]	88
NcAA9C-CBM1 (1.25 μM)	Fungal- and plant-derived phenols (1 mM)	MCC (25 g/liter)	K-P (50 mM, pH 6.0)		30	Thermomixer (800)	HPLC-ED40 (soluble)	NR ^r	83
<i>ThtAA9A</i> , <i>ThtAA9B</i> -CBM1, and <i>ThtAA9C</i> (2.5, 5, and 2.5 mg/g substrate)	Plant-derived phenols (1 mM)	RAC (1.5 g/liter)	Am-Ac (50 mM, pH 5.0)		50	HOTSR (20)	HPAEC-PAD (soluble)	NR ^r	65
<i>TtAA9E</i> (1 μM)	Lignin (1 mg of HMW + 2 mM LMW)	PASC (7.5 g/liter)	Cit-P (20 mM, pH 5.9)		50	Thermomixer (1,000)	HPAEC (soluble)	NR ^s	86

^aThe table contains apparent rates for only full-length enzymes. Note that this table should not be used to draw conclusions concerning the substrate specificities of LPMOs since assays with varying substrates were carried out under highly varying conditions and since the listed rates are apparent and not true kinetic parameters. If one assumes that the H₂O₂-mechanism is valid, production of H₂O₂ was likely the rate-limiting step in many of the reported experiments with solid substrates, but not, for example, in the experiments with cellobiose carried out by Hangasky et al. (44, 373). See the text for details.

^b*Cf*, *Cellulomonas fimi*; *Cj*, *Cellvibrio japonicus*; *Ls*, *Lentinus similis*; *Tht*, *Thermothelomyces thermophila* (previously *Myceliophthora thermophila*); *Nc*, *Neurospora crassa*; *Pc*, *Phanerochaete chrysosporium*; *Sc*, *Streptomyces coelicolor*; *Sm*, *Serratia marcescens*; *Ta*, *Thermoascus aurantiacus*; *Tb*, *Thermobispora bispora*; *Tr*, *Trichoderma reesei*; *Tt*, *Thielavia terrestris*.

^cAlso known as CBP21.

^dAlso known as CelS2.

^eAlso known as *Vibrio cholerae* colonization factor, GbpA (GlcNAc binding protein A). GbpA is a four-domain protein where X and Y denote unknown domains related to the flagellin protein p5 and pilus-binding chaperone FimC, respectively (430).

^fComplex electron supplying systems include light-driven oxidation of water, catalyzed by vanadium-doped titanium dioxide (V-TiO₂), light-excited chlorophyllin (Chl) in the presence of ascorbic acid (AscA), lactose oxidation catalyzed by cellobiose dehydrogenase from *Myriococcum thermophilum* (MtCDH), and mixture of high-molecular-weight (HMW) and low-molecular-weight (LMW) lignins.

^gCNW, chitin nanowhiskers; FRET, Förster resonance energy transfer; (B)MCC, (bacterial) microcrystalline cellulose; PASC, phosphoric acid-swollen cellulose; RAC, regenerated amorphous cellulose; sat, saturating concentration; XG, xyloglucan; XG₁₄, a 14-mer xyloglucan.

^hAm-Ac, ammonium acetate buffer; BT, Bis-Tris; BTm, Bis-Tris-methane; BTp, Bis-Tris-propane; Cit-P, citrate phosphate buffer; K-P, potassium phosphate buffer; MES, morpholineethanesulfonic acid; MOPS, morpholinepropanesulfonic acid; Na-P, sodium phosphate buffer.

ⁱHOTSR, head-over-tail Stuart rotator; MP, microplate; NR, not reported.

^jDNS, dinitrosalicylic acid assay for concentration of reducing ends; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPLC-ED40, high-performance liquid chromatograph equipped with a Dionex ED40 electrochemical detector; UHPLC, ultra-high-performance liquid chromatography.

^kValue independent of the extent of solubilization of oxidized products and reflects thus total LPMO activity.

^lValues marked with an asterisk were calculated by us on the basis of progress curves reported in the original article.

^mAn approximate molecular weight of 31 kDa, which does not account for glycosylations, was considered to convert the rate from micromolars per minute to per minute.

ⁿEstimated based on product quantity released after a 2-min reaction.

^oEstimated based on product quantity released after a 3-min reaction.

^pApparent rate values 285, 444, and 647 min⁻¹ were also determined as with [H₂O₂] = 12.5, 25, and 50 μM, respectively (44).

^qDetermined at ambient O₂. Note that Hangasky et al. have determined a full set of kinetic parameters for *ThtAA9E* at different O₂ and cellobiose concentrations (373).

^rIn a 24-h reaction.

^sIn a 12-h reaction.

rates are low and fall in a relatively narrow window roughly between 1 and 10 min⁻¹. Much higher LPMO rates (in the range of 1 to 10 per second) have been obtained in two settings: (i) when a photocatalytic system is used (87) (see also subsection "Stimulation of LPMO Activity by Photocatalytic Systems," below) and (ii) when H₂O₂ is used to drive the reaction (32, 34, 44). Determination of LPMO rates is generally complicated because of the inactivation processes discussed above. In reactions with added H₂O₂, the LPMO catalytic rate depends on the H₂O₂ supply rate, but saturation kinetics may not be reached before inactivation phenomena occur, which is a common problem encountered in the field of H₂O₂-using enzymes (82). Of note, when available kinetic data for LPMOs are evaluated (Table 1), it is important to consider that in most studies only the carbohydrate substrate concentration was controlled, whereas neither the identity nor the quantity of the oxygen-containing cosubstrate was known or controlled.

Almost all rates listed in Table 1 are apparent rates and not true kinetic parameters. As a consequence, the listed rates for various substrates cannot be used to draw any conclusions as to the substrate specificity of LPMOs. In fact, whereas LPMOs are thought to have evolved primarily to attack crystalline substrates (11, 29), the true substrate preferences of LPMOs, e.g., in terms of k_{cat}/K_m values, remain unknown.

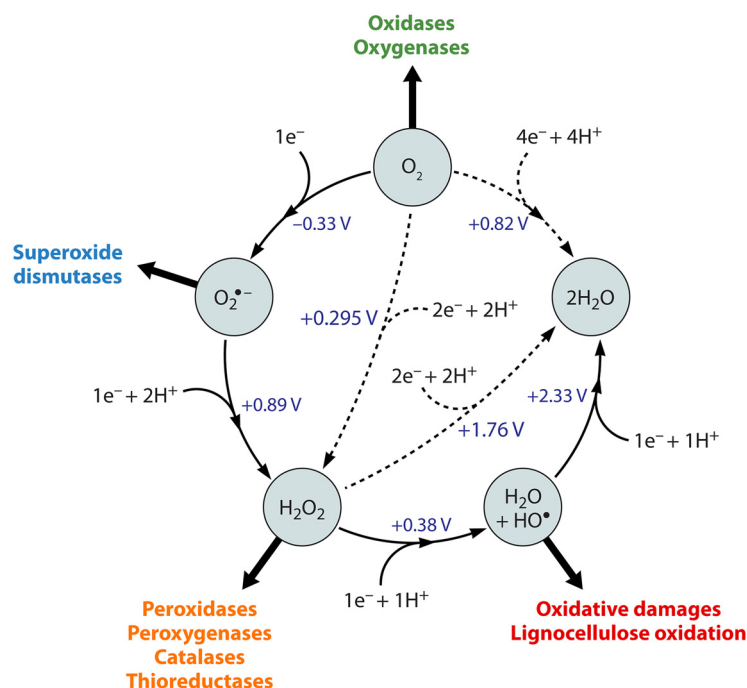


FIG 3 The reduction cycle of reactive oxygen species (ROS) from molecular oxygen (O_2) to water. O_2 can undergo a single-electron reduction leading to the formation of superoxide ($O_2^{\cdot-}$), which can be further reduced to H_2O_2 , either spontaneously, enzymatically, or by small organic reductants. H_2O_2 can also be generated via a two-electron reduction of O_2 . H_2O_2 can enter pathways leading either to the formation of a hydroxyl radical after a single-electron reduction [e.g., by Fe(II) or by Cu(I)], i.e., Fenton reactions] or to the production of two H_2O molecules via a two-electron reduction. H_2O molecules can also be obtained by a direct four-electron reduction of O_2 . Reduction potentials are indicated in the figure (at pH 7 versus SHE) (99, 100). Each ROS can also be the substrate of other chemical or enzymatic reactions, as indicated by the large filled arrows.

ROS as reductants. It has been shown that superoxide ($O_2^{\cdot-}$), the product of O_2 single-electron reduction (Fig. 3), can reduce and thus activate AA10 LPMOs, while H_2O_2 cannot (32). Therefore, superoxide constitutes a possible electron shuttle between a reductant and the LPMO active site, as has been shown for myeloperoxidases (92). Many photosystems display the appropriate reduction potential to catalyze production of superoxide (93). Also, $O_2^{\cdot-}$ can be produced by a wide range of oxidases (94) and can emerge in reactions involving semiquinones (95), which are intermediates between hydroquinones and quinones, all abundant in biomass-degrading ecosystems (96). Notably, $O_2^{\cdot-}$ will spontaneously disproportionate to H_2O_2 in protic solvents such as H_2O , decreasing its lifetime as an LPMO reductant but generating an LPMO cosubstrate. This disproportionation process can be accelerated by ascorbic acid (97, 98) or by phenolics present in biomass (see subsection "Nonenzymatic Production and Use of H_2O_2 ," below). Notably, in the H_2O_2 -dependent mechanism, only a priming reduction is needed, meaning that small amounts of $O_2^{\cdot-}$ could be sufficient to activate LPMOs.

To complicate things, it is known that reduced LPMOs can catalyze the single-electron reduction of O_2 into $O_2^{\cdot-}$ (15), which eventually results in H_2O_2 . In light of this, it is worth noting the single electron reduction potentials of H_2O_2 ($E_0 = +0.38$ V) and O_2 (-0.33 V) (Fig. 3) (99, 100), which suggests that single-electron transfer from LPMO-Cu(I) would be more thermodynamically favorable for H_2O_2 than for O_2 . Indeed, the ^{18}O competition experiments alluded to above (32) clearly showed that AA10 LPMOs prefer to react with H_2O_2 rather than with O_2 when presented with both. Having this in mind, in a biological context, prerduction of O_2 to H_2O_2 via an energetically easier two-electron reduction process (+0.295 V) catalyzed by enzymes evolved in nature for this purpose (e.g., flavin adenine dinucleotide [FAD]-dependent oxidases) (99, 101) (Fig. 4) represents an appealing and efficient strategy to provide H_2O_2 and fuel

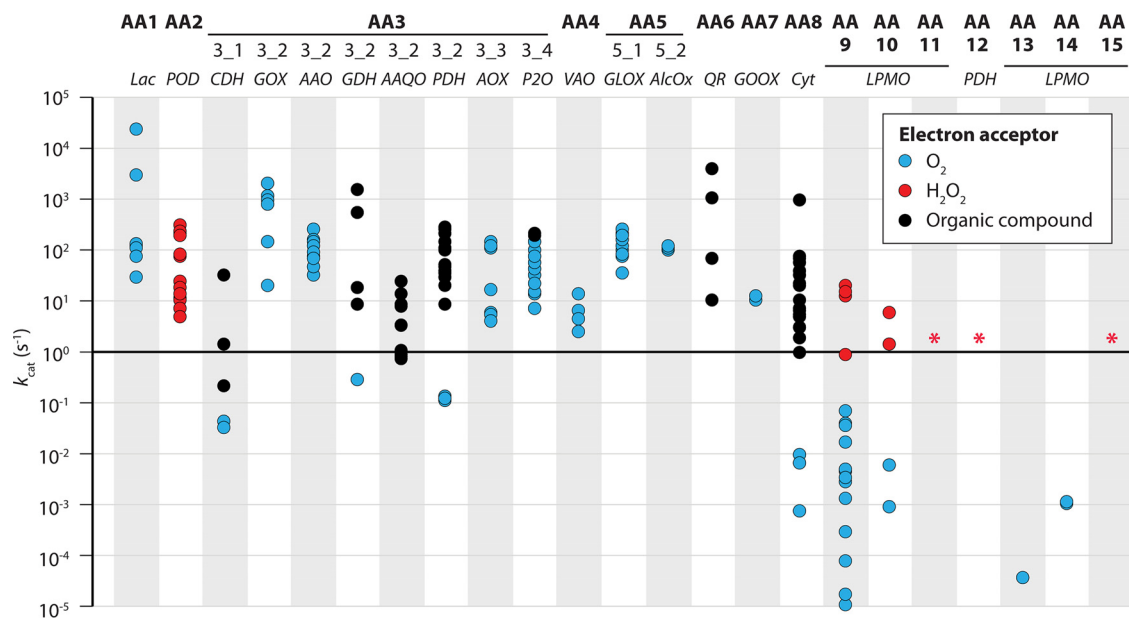


FIG 4 Catalytic constants of auxiliary activities. Auxiliary activities comprise redox enzymes involved in biomass conversion and include the LPMOs (21). For each AA family, apparent catalytic constants collected from the literature are indicated by dots that are colored depending on whether the final electron acceptor is O_2 , H_2O_2 , or an organic compound/protein, as indicated. Details on the sources of the displayed data, including a reference to the correct publication for each data point, are provided in Table S1 in the supplemental material. Red stars indicate the absence of kinetic data. Abbreviations (and associated references for corresponding data) are as follows: Lac, laccase (380–383); POD, peroxidase (380, 384–388); CDH, cellobiose dehydrogenase (102, 128–130, 389–392); GOX, glucose oxidase (167, 393–398); AAO, aryl alcohol oxidase (166, 399, 400); GDH, glucose dehydrogenase (401, 402); AAQO, aryl alcohol quinone oxidoreductase (403); PDH, pyranose dehydrogenase (404–407); AOX, alcohol oxidase (177, 408–410); P2O, pyranose 2-oxidase (181, 411–413); VAO, vanillyl alcohol oxidase (186, 187); GLOX, glyoxal oxidase (170, 414); AlcOx, alcohol oxidase (172); QR, quinone reductase (415–418); GOOX, gluco-oligosaccharide oxidase (173, 174, 419, 420); Cyt, cytochrome *b* (83, 102, 124, 130, 353, 389, 391, 421); LPMO, lytic polysaccharide monoxygenase (AA9, (43, 44, 66, 67, 103, 422, 423); AA10, (32, 34, 56); AA13, (424); AA14 (25); PDH, pyranose dehydrogenase.

LPMO reactions. Indeed, such enzymes are found together with LPMOs in biomass-degrading ecosystems (see Insights into the Network of Lignocellulolytic Redox Reactions).

How much reducing power is needed to fuel LPMOs? An intriguing aspect of LPMO biochemistry pertains to observed dose-response relationships for the reductant (102). In most experiments published so far under standard conditions (aerobic, reductant, and no added H_2O_2) molar enzyme/reductant ratios were in the 1:10,000 range (Table 1). One may wonder why variation in such a large excess of reductant influences the catalytic rate of the LPMO, knowing that LPMO reduction is a fast process (83). One possible answer is that the reductant, in addition to reducing the LPMO, is involved in the generation of the H_2O_2 whose availability is rate limiting for the reaction. Alternatively, in an O_2 -based mechanism, the reductant concentration could affect the delivery rate for the second electron (see above).

H_2O_2 may be generated from O_2 by reduced LPMOs in solution (62, 66, 103) or by reactions involving O_2 and reductant. As to the latter option, Gusakov et al. have shown that O_2 consumption in the absence of LPMO increased when the concentration of Asca increased (104). It is important to note that generated H_2O_2 may engage in all kinds of redox reactions, for example, those involving free metals (105) or phenolic compounds present in the lignocellulosic substrate. In this respect, LPMO experimentalists have certainly noticed variability and reproducibility issues when they compared different batches of substrate or used different batches of reductant.

If one wishes to control LPMO reactions, it is of utmost importance to control the levels of reductant and oxygen species, which, considering all the possible reactions, is a major challenge. One way of reaching such control is to run reactions anaerobically, with small amounts of reductant and a steady, slow supply of H_2O_2 (32). When reactions

are run with O_2 , current data indicate that, instead of using small-molecule reductants, it is better to use a source of reducing equivalents that is less prone to uncontrollable auto-oxidation, such as a dehydrogenase and its substrate (102) or photocatalytic systems (33, 87, 88). Such systems are discussed below.

Why LPMOs Do Not Seem To Produce H_2O_2 in the Presence of Substrate

The binding of an LPMO to its target substrate is thought to be controlled by structural properties of the enzyme surface (45, 52) and, in the case of multimodular LPMOs, by appended CBMs (12, 39). Substrate binding has also been suggested to be influenced by other players of the LPMO reaction since cyanide and chloride, both mimics of superoxide, increase binding (52, 55). Current data suggest that binding is strengthened by formation of a ternary complex with substrate and an oxygen species.

Available data show that the extent of LPMO binding varies a lot. For AA10 chitin-active LPMOs, the bound fraction has been reported to lie between 80% or more (54, 81) and down to 40% (106) or 19% (107). Regarding cellulose-active LPMOs, one can find qualitative estimations for bound fractions spanning from ca. 100% bound (for a full-length enzyme with CBM) (80) to 40% (108). Thus, in most cases, a significant fraction of the LPMO is not bound to the substrate and is free in solution.

As noted above, H_2O_2 accumulates in LPMO reactions that lack substrate (62, 66, 103). This is usually considered a futile reaction, also known as an uncoupling reaction. In the presence of the appropriate substrate, such LPMO-mediated H_2O_2 production is not observed, and this is usually attributed to the fact that the catalytically competent LPMO acts on the substrate rather than being engaged into the uncoupling reaction. However, one may wonder why the unbound fractions of LPMOs apparently do not produce H_2O_2 . For instance, in the case of the fungal NcAA9C-CBM1, no H_2O_2 was detected in reaction mixtures containing 5 mM $Gluc_6$ (62) although 14% of the enzymes were probably free in solution, given a K_d (dissociation constant) value for this substrate of 0.81 mM (71). One possible explanation is that reduced LPMOs are never free in solution because they bind much more strongly to the substrate than suggested by the K_d value, which was determined in the absence of reductant. This explanation is supported by a recent report showing that LPMO-Cu(I) binds cellulose more strongly than LPMO-Cu(II) (109), but also in this case, binding did not seem complete. In any case, strong and even 100% binding of the reduced LPMO cannot account for the complete absence of H_2O_2 since, under the conditions used, H_2O_2 will also be produced by non-LPMO-catalyzed reactions involving the reductant, O_2 , and transition metals in solution.

An obvious alternative explanation for these observations follows from the H_2O_2 mechanism, which dictates that H_2O_2 produced by non-LPMO-catalyzed reactions or by unbound LPMOs is readily consumed by bound LPMOs carrying out H_2O_2 -driven catalysis on the substrate. In this scenario, the very low H_2O_2 concentrations observed in LPMO reaction mixtures containing substrate are hiding production and consumption fluxes (Fig. 5).

The CDH Case: a Multifunctional Redox Partner?

It is now well established that fungal CDHs, which are more common in white-rot fungi than brown-rot fungi (110), can promote AA9 LPMO activity (14, 64). Genomic cooccurrence and coexpression of CDH along with AA9 LPMOs is often observed (18, 83). Knocking out the *cdh* gene leads to lower efficiency of the cellulolytic secretome (14) and has also been shown to promote putatively compensatory mechanisms by the fungus, such as the secretion of additional β -glucosidases as well as AA3_2 flavo-oxidases (111). A plethora of roles have been proposed for CDHs throughout the last decades, one of them being reduction of transition metals [e.g., Fe(III)] and generation of H_2O_2 to drive hydroxyl radical-generating Fenton reactions (112–116) (see subsection "Nonenzymatic Production and Use of H_2O_2 ," below). Of note, the H_2O_2 production ability varies between CDHs and is pH and substrate dependent (117, 118) but is relatively low (119–122) compared to that of classical oxidases (Fig. 4; see also Table S1

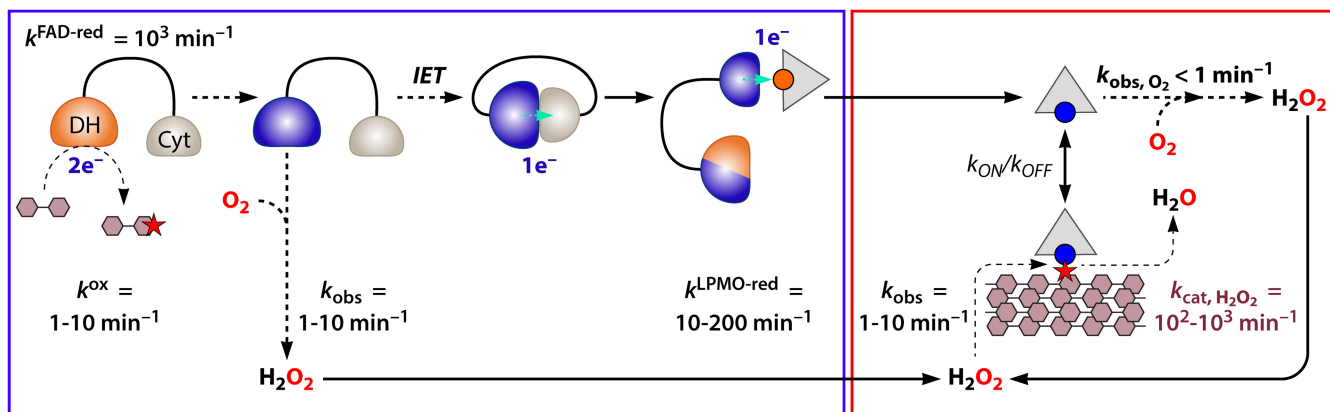


FIG 5 On the disappearance of H_2O_2 during LPMO catalysis, illustrated using CDH as an LPMO-independent H_2O_2 -generating system. Dotted arrows indicate chemical reactions, whereas solid arrows indicate diffusion. IET stands for internal electron transfer; blue and orange indicate reduced and oxidized states, respectively. The blue frame shows a system generating reducing equivalents that both produce H_2O_2 and serve to reduce the LPMO. The reduced LPMO (red frame) is in equilibrium between the bound and unbound forms and will either generate H_2O_2 (uncoupling reaction, $k_{\text{obs}, \text{O}_2}$) or oxidize a polysaccharide. Here, the blue frame depicts a CDH system (see subsection “The CDH Case: a Multifunctional Redox Partner” for a detailed explanation), but this system could be replaced by several alternative reducing systems. In the figure, from left to right, rates are shown as follows: for cellobiose oxidation with O_2 being the electron acceptor (102); for the reduction ($k^{\text{FAD-red}}$) of the flavin (FAD)-containing DH domain (83, 124, 391); for LPMO reduction (83, 102, 124); for H_2O_2 generation by CDH (102, 117, 119, 129–131). The apparent rates of LPMO-catalyzed reactions are given in Tables 1 and S1. Note that LPMO reduction ($k^{\text{LPMO-red}}$) by CDH-Cyt obeys pseudo-first-order kinetics and depends on the LPMO concentration (102). Thus, for comparative purposes, the $k^{\text{LPMO-red}}$ range is given for 1 μM LPMO (a common concentration in most published assays). Note that the catalytic constant for H_2O_2 -driven polysaccharide oxidation by LPMOs ($k_{\text{cat}, \text{H}_2\text{O}_2}$) is much higher than the apparent rate constant, derived from standard reactions without added H_2O_2 (k_{obs}). In the latter reactions H_2O_2 generation is rate limiting for LPMO action.

in the supplemental material). This low rate led scholars to adopt the name cellobiose dehydrogenase (CDH) instead of the initial cellobiose oxidase (CBO) (123).

Today, there is strong evidence that CDH constitutes a natural redox partner for AA9 LPMOs (55, 124, 125) although the exact mode of interplay still needs to be fully elucidated, including the second-electron conundrum in the O_2 -based LPMO mechanism. Considering the recent doubts concerning this mechanism, it is worthwhile to revisit some of the available kinetic data for CDHs and their interplay with LPMOs.

CDHs are bi-modular redox enzymes belonging to the superfamily of glucose-methanol-choline (GMC) oxidoreductase (EC 1.1.99.18) containing a flavin adenine dinucleotide (FAD)-dependent dehydrogenase domain (DH; AA3_1 subfamily) and a cytochrome domain (Cyt; family AA8) connected by a flexible linker allowing mobility between the two domains. The DH domain constitutes the catalytic part of the enzyme where a two-electron oxidation of the substrate (cellobiose and several other oligosaccharides) reduces the flavin cofactor ($\text{FAD} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{FADH}_2$). Reoxidation of the flavin may happen by reduction of a two-electron acceptor (e.g., dichlorophenolindophenol [DCPIP], benzoquinone, or O_2) or by sequential single-electron transfer to the Cyt domain (126). It is known that the reduced Cyt domain can transfer electrons to AA9 and AA10 LPMOs (102, 124). The existence of a CDH binding site on a fungal AA9 LPMO has been suggested (127), but theoretical considerations (42) and lack of sequence conservation in the proposed docking site (125), as well as studies of interactions between CDH and an AA9 LPMO by computational modeling (124) or nuclear magnetic resonance (NMR) (55), rather support direct electron transfer at the copper site.

The reoxidation of the reduced DH by direct (from the FAD) (Fig. 5) and indirect (via Cyt) (not shown in Fig. 5) reduction of O_2 is slow, with observed rates typically being in the order of 10^{-1} to 10^{-2} and 10^{-2} to 10^{-3} s^{-1} , respectively (128–130) (Fig. 4). It has been shown that the reduced Cyt domain reacts much faster with the LPMO (AA9) than with atmospheric O_2 (47, 83, 124). This is logical when the reduction potential of Cyt ($E_0 = +93$ to 163 mV versus standard hydrogen electrode [SHE]) (131) is compared with the potential for the thermodynamically challenging single-electron reduction of O_2 ($E_0 \approx -330$ mV versus SHE) and the reduction potential of LPMO-Cu(II), which is much

more prone to reduction ($E_0 \approx +250$ mV versus SHE). Note that the total spin is conserved for the reduced cytochrome reaction with both LPMO-Cu(II) and O_2 . Under standard conditions (i.e., $1 \mu\text{M}$ LPMO) the LPMO reduction rate by reduced Cyt lies in the range of 10 to 200 min^{-1} (83, 102, 124), which is one to two orders of magnitude faster than reported LPMO catalytic rates (Fig. 5 and Table 1). Therefore, the initial reduction step probably does not constitute a rate-limiting step in CDH-driven LPMO catalysis. However, CDH does play a rate-limiting role under certain conditions, as it has been shown that increasing the amount of CDH activity in a reaction increases AA10 LPMO activity (102).

Taken together, the existing data on the interactions between CDH and AA9 and AA10 LPMOs may seem to suggest that delivery of the second electron is rate limiting. However, this reasoning requires reconsideration in light of a possible role of H_2O_2 in LPMO catalysis. Incubation of CDH with a substrate (e.g., lactose) in the absence of an electron acceptor will lead to the production of H_2O_2 , whereas such production seemingly does not happen when the reaction mixture also contains an LPMO and its substrate. The common explanation for the disappearance of H_2O_2 when the LPMO is present requires that the reducing equivalents acquired by CDH upon lactose oxidation are preferentially transferred to the LPMO rather than to O_2 . Such a conclusion, however, is questionable given the abundance of O_2 (ca. $250 \mu\text{M}$) versus that of LPMO ($1 \mu\text{M}$), suggesting that the balance between both processes may be less tilted in favor of LPMO reduction than usually thought. Moreover, it has been shown that the rate of H_2O_2 production by the lactose/CDH system in the absence of LPMO plus substrate (1 to 10 min^{-1}) (see Table S1 in the supplemental material) is remarkably similar to the LPMO (AA10) oxidative rate when the latter is fueled by the lactose/CDH system (Table 1) (102, 128). This observation may, of course, be due to coincidence but does suggest that the rate-limiting step in CDH-driven LPMO catalysis is the formation of H_2O_2 , which does not accumulate because it is consumed by the LPMO.

Comparing the high reduction rate of the FAD domain (by electrons derived from oligosaccharide oxidation) with all other rates of subsequent reactions in the CDH system (Fig. 5) shows that the rate of reoxidation of CDH is determining the overall turnover rate and that this rate is driven by the nature of the electron acceptor and its ability to accept electrons from CDH (Table S1) (125). In the CDH-LPMO systems, there is a mismatch between the (relatively high) electron-donating capacity of CDH and the (relatively low) electron consumption by the LPMO. Thus, electrons will inevitably be routed from the reduced dehydrogenase domain toward O_2 , leading to slow H_2O_2 generation. The similar rates of H_2O_2 generation by the CDH and of CDH-driven LPMO catalysis strongly suggest that generation of H_2O_2 is the rate-limiting step in CDH-LPMO systems. The observation that CDH-fueled LPMO systems are inhibited by a peroxidase competing for H_2O_2 fully supports this view (32, 44). With hindsight, it is interesting that the poor oxidase activity (i.e., slow H_2O_2 generation) and the more efficient dehydrogenase activity (i.e., reducing equivalent generation) of CDHs may both be biologically relevant.

Stimulation of LPMO Activity by Photocatalytic Systems

In 2016, two photocatalytic systems were reported to promote LPMO activity. First, Cannella et al. showed that AA9 LPMOs can be fueled by the combined use of a pigment (e.g., chlorophyllin) and a reductant (e.g., AscA) when exposed to (low-intensity) visible light (87). This approach resulted in an impressive boost in LPMO activity, reaching rates that were 10- to 100-fold faster than those of reference experiments under standard conditions. The authors of this study proposed that high redox potential electrons, generated by the photoexcited pigment, would be at the origin of LPMO activation and the rate enhancement. In this system, the reductant would merely serve to regenerate the electron pool of the pigment.

It has been proposed (33) that the generation of ROS could be the underlying reason for the activity boost observed by Cannella et al. (87). Several photosystems encountered in nature perform the single-electron reduction of molecular oxygen to super-

oxide, which requires a low reduction potential, i.e., a high reducing strength ($O_2 + 1e^- \rightarrow O_2^{\cdot-}$; $E_0 = -0.33$ V) (Fig. 3). Bissaro et al. showed that superoxide is formed when chlorophyllin is exposed to (high-intensity) light (in the absence of reductant) and that superoxide can activate an AA10 LPMO (33). When a reductant (AscA) is added to the system, a dramatic boost in LPMO activity was observed (33), thus confirming the original work by Cannella et al. (87). However, based on several experiments, Bissaro et al. claimed that this boost was correlated to the fact that AscA accelerated the conversion of superoxide into H_2O_2 (33), which will speed up LPMO catalysis (32, 34, 44). In a follow-up study, the idea of ROS being involved in the photocatalytically promoted LPMO activity was dismissed, mainly on the basis of the absence of effects of the addition of catalase on AA9 LPMO activity (132). It could be argued that the lack of a catalase effect could be due to the micromolar affinity for H_2O_2 of LPMOs (32, 34), which likely enables these enzymes to compete with catalases that have apparent K_m values in the millimolar range (see “The housekeeping role of catalases,” below). The issue remains controversial, and a direct comparison of the two studies is complicated, primarily due to the use of different light intensities (33, 87). More work is required to decipher the underlying mechanism of light/pigment-driven LPMO catalysis.

In the same year, 2016, it was also shown that light-driven oxidation of water, catalyzed by vanadium-doped titanium dioxide (V-TiO₂), can provide the reducing equivalents that LPMOs need to oxidize polysaccharides, thus alleviating the need for externally added electron donors (88). This proof of concept yielded much lower LPMO rates than the chlorophyllin system described above (tested for both AA9 and AA10) (Table 1). With hindsight, it is likely that the light-driven LPMO activity observed in this study reflected light-driven production of H_2O_2 , which is a known ability of photoexcited TiO₂-based photocatalysts (133–136). Indeed, the ability of TiO₂ to catalyze light-driven peroxygenase reactions has recently been demonstrated using the unspecific peroxygenase from *Agrocybe aegerita* as a model enzyme (137).

INSIGHTS INTO THE NETWORK OF LIGNOCELLULOLYTIC REDOX REACTIONS

Our understanding of biomass conversion in natural environments, notably by fungi (4), is constantly challenged and improved, as illustrated by the relatively recent discovery of LPMOs (10, 11). As reviewed by Berrin et al. (138) and others (139), several studies conducted during the past few years have reported biomass-dependent up-regulation of LPMO expression or secretion by many fungi: *Hypocrea jecorina* (140), *Myceliophthora thermophila* (141), *Schizophyllum commune*, *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* (142), *Aspergillus nidulans* (143), *Phanerochaete carnosa* (144, 145), *Postia placenta* (146), *Ceriporiopsis subvermispota* (147), *Pycnoporus coccineus* (148), *Phlebia radiata* (149), *Podospora anserina* (111), and *Neurospora crassa* (150). Beyond an array of well-known hydrolases, fungi tend to coexpress/cosecrete a plethora of other oxidoreductases along with LPMOs, and many of these generate or consume H_2O_2 . The ability of H_2O_2 to efficiently drive LPMO catalysis sheds new light on the potential interplay between the different enzymatic and nonenzymatic elements of lignocellulolytic enzyme systems. In the next paragraphs, we describe different redox enzyme systems thought to be involved in depolymerization of lignocellulose, followed by a discussion of their spatial and temporal interconnections (Fig. 6).

The Function of Lignocellulolytic Oxidoreductases

From an evolutionary perspective. Our current understanding of the role of different oxidoreductases involved in lignocellulose conversion is essentially derived from studies on two kinds of living laboratories, namely, wood-decaying basidiomycetes commonly classified as white-rot or brown-rot fungi. The difference in appearance of the rotted wood is a direct consequence of the depolymerization strategy adopted by either fungus, which reflects the enzymatic arsenal that the fungi deploy (Fig. 7). Notably, fungi with hybrid phenotypes, sometimes called gray rot, have been described previously (151). With the exception of hemicellulases, which are equally abundant in the genomes of both types of fungi, the two fungal

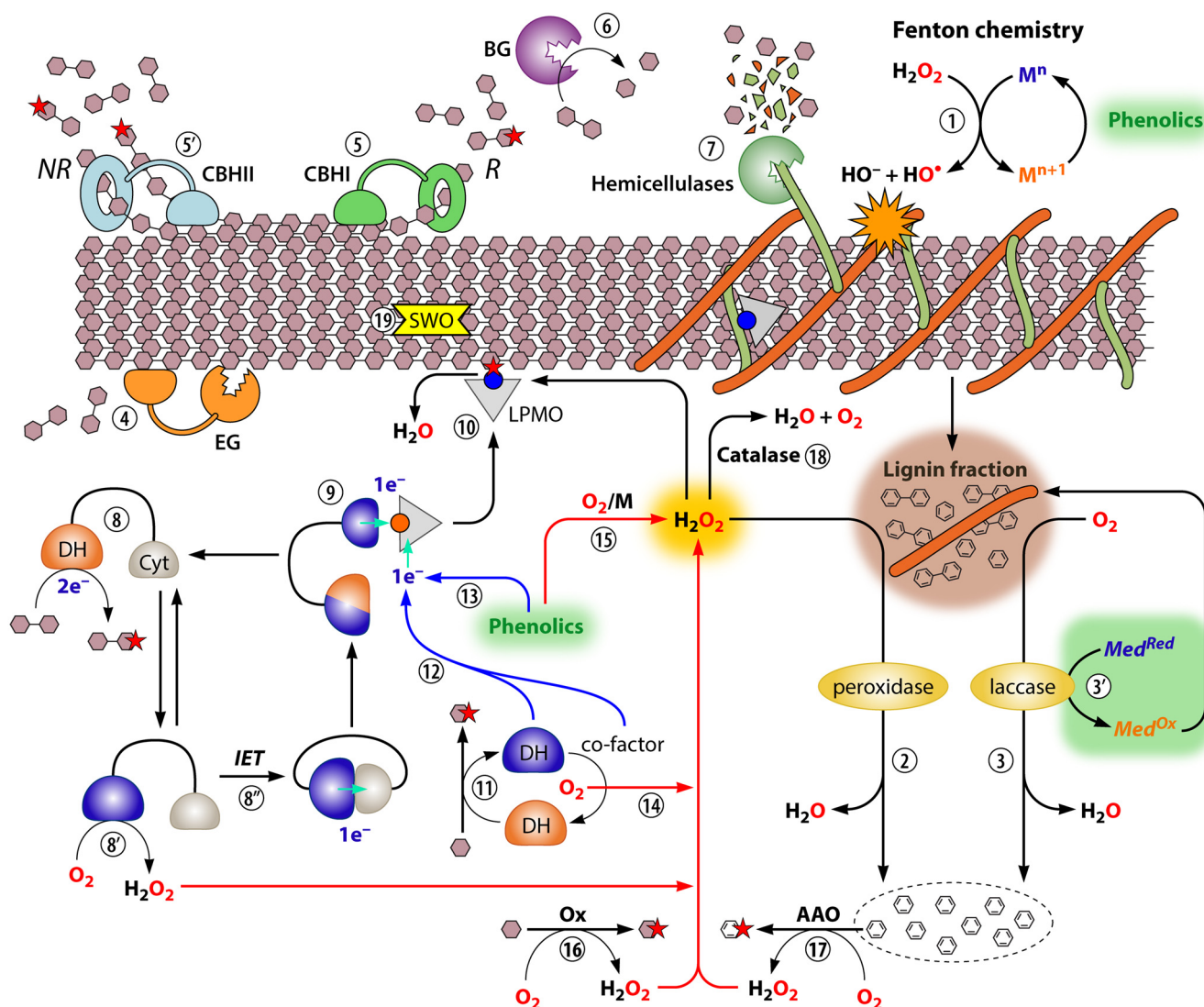


FIG 6 An integrative view on reactions happening during lignocellulolysis. The enzymes presented here do not always all cooccur in genomes or secretomes and may act sequentially, depending on the microorganism or the nature of the biomass. The main nonenzymatic weapon is constituted by Fenton reaction-derived hydroxyl radicals (step 1). The lignin fraction, which constitutes a physical barrier, can be modified and to some extent depolymerized via the action of enzymes such as peroxidases (step 2) or laccases (step 3). Note that the peroxidases and laccases are also involved in repolymerization of lignin (not shown). These enzymes can also catalyze the oxidation of mediators that may be involved in lignin oxidation (step 3'; shown only for laccases). The main cellulases are endoglucanases (EGs) (e.g., Cel7B) acting internally (step 4), cellobiohydrolases CBHI (e.g., Cel7A) and CBHII (e.g., Cel6A) acting, respectively, from reducing (R) and nonreducing (NR) chain ends and primarily releasing cellobiose (steps 5 and 5'), which is further hydrolyzed to glucose by β -glucosidases (BG) (step 6). The various cellulases will also release minor amounts of products carrying an oxidation at C-1 or C-4 that was introduced by an LPMO (the presence of such oxidations is indicated by a red star). A wide diversity of hemicellulases (261, 425) and possibly pectinases (426, 427) acts on the hemicellulosic and pectin fractions, respectively (step 7). CDH oxidizes cello-oligosaccharides (step 8), and acquired reducing equivalents can be used to generate H_2O_2 (step 8') or be transferred to the cytochrome (Cyt) domain (step 8'), which then reduces LPMOs (step 9). Once reduced, LPMOs can oxidize the cellulose (step 10), provided that the cosubstrate H_2O_2 (or O_2) is present. (As noted elsewhere in this review, the question of whether O_2 can act as a cosubstrate without prior reduction to H_2O_2 is still under debate [44].) LPMOs can also be activated by single-domain dehydrogenases and/or noncovalently bound reduced cofactor (steps 11 to 12) or by reduced phenolics (step 13). Single-domain dehydrogenases (step 14) and reduced phenolics (especially in the presence of transition metals [M]) (step 15) can also lead to the production of H_2O_2 under aerobic conditions. Several oxidases (Ox) such as methanol oxidase, glyoxal oxidase, copper radical oxidase, or diverse oligosaccharide oxidases can generate H_2O_2 (step 16) to fuel the different H_2O_2 -consuming systems (here, a secreted pyranose 2-oxidase is shown) (see subsection "The Function of Lignocellulolytic Oxidoreductases"). Aryl-alcohol oxidases (AAO) oxidize lignin-derived compounds to generate H_2O_2 (step 17). Catalase acts as a safety belt by converting excess H_2O_2 and O_2 (step 18). Expansins/swollenins (SWO) may contribute to lignocellulolysis by loosening the plant cell wall structure, also called amorphogenesis (step 19) (282) although their mode of action remains unknown. Note that for the sake of simplicity the stoichiometry of reactions is not taken into account. See Table S1 in the supplemental material and Fig. 7 for an overview of known lignocellulolytic redox enzyme activities.

types have quite different enzyme arsenals. In particular, white-rot fungi have more laccases, cellulases, and lignin-active peroxidases than brown-rot fungi. It has been proposed that the first wood-rotting fungi appeared through acquisition of ligninolytic peroxidases (110) by ancestral basidiomycetes, whereas the later transition

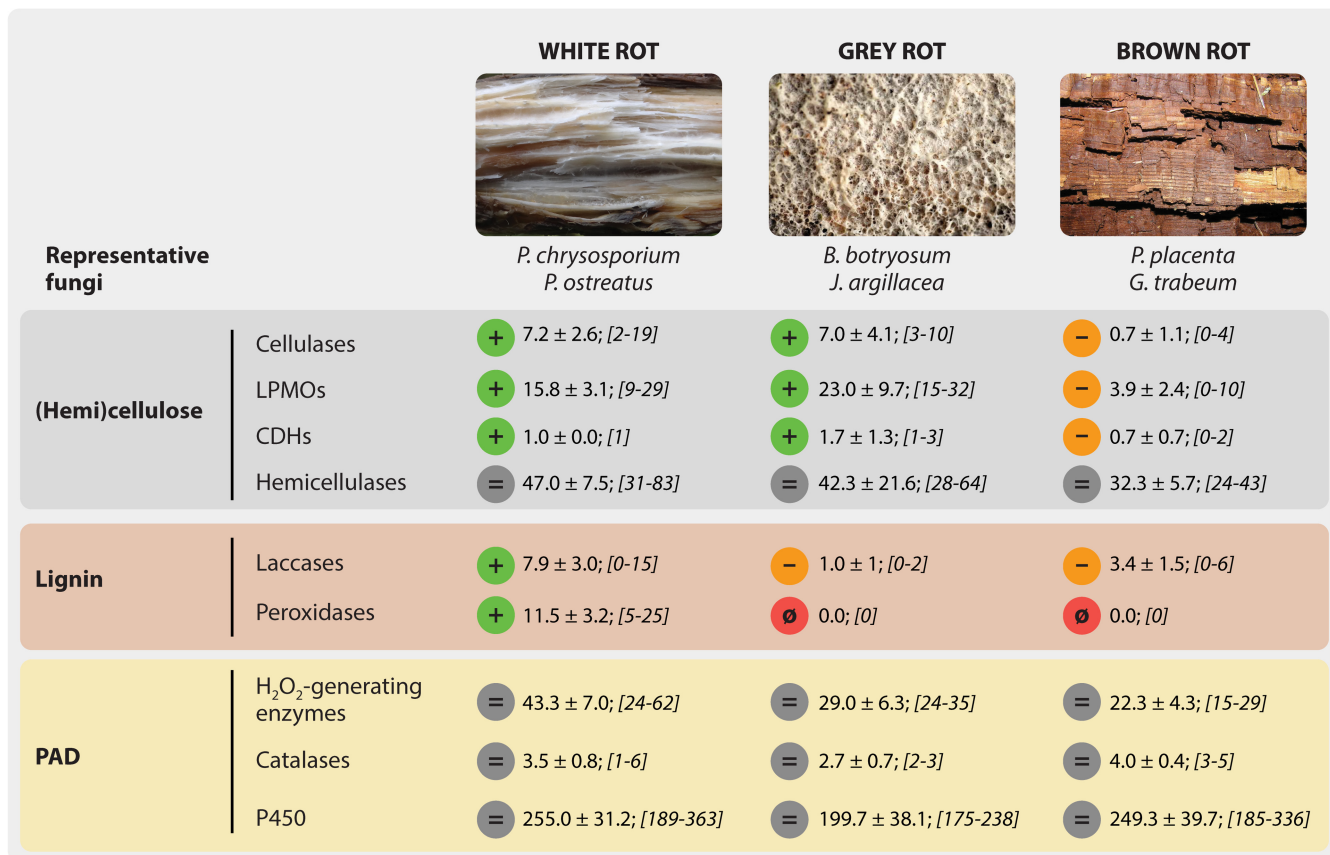


FIG 7 Enzymatic features of wood-decaying basidiomycetes. The phylum of *Basidiomycota* contains 32% of all described fungi and contains mostly saprotrophic fungi, including most wood-decaying fungi. Brown rots represent approximately only 6% of the latter but dominate in boreal forests, where they are associated with conifer wood. Here, we report average numbers of genes for different classes of enzymes in white-rot, brown-rot, and gray-rot fungi. For each group of fungi, the plus and minus symbols indicate whether the number of genes is higher or lower than that of at least one of the other two groups. An equal sign indicates that the numbers are similar in all three groups, whereas the null symbol (\emptyset) indicates the absence of any gene encoding a given type of (known) oxidoreductase. For each species, the number of genes encoding the major cellulases (GH6 and GH7), hemicellulases (GH10, -11, -16, -51, -62, and -74), CDH (AA3_1), laccases (AA1_1), peroxidases (AA2), and main secreted H₂O₂-generating CAZymes (AA3_2, AA3_3 and AA3_4, AA5_1, and AA7) were retrieved from Riley et al. (151). The list of individual fungi is provided in the legend of Fig. 8. The numbers of putative catalases and P450s (secreted and cytosolic) were obtained from the MycoCosm online database (258). For each species, the total number of genes corresponding to each enzyme category was calculated, and then an average value \pm 95% confidence interval was calculated; the interval of minimum to maximum values is provided in brackets for each phenotypic subgroup. PAD, prooxidant, antioxidant, and detoxifying enzymes (which notably include GMC oxidoreductases). (The rotting-wood pictures were obtained from Wikimedia Commons. The white-rot photo, by Jerzy Opiola, is licensed under the Creative Commons Attribution-Share Alike 4.0 International license [<https://creativecommons.org/licenses/by-sa/4.0/legalcode>]; the gray-rot photo, by James K. Lindsey, is licensed under the Creative Commons Attribution-Share Alike 2.5 Generic license [<https://creativecommons.org/licenses/by-sa/2.5/deed.en>]; and the brown-rot photo is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license [<https://creativecommons.org/licenses/by-sa/3.0/deed.en>].)

from white-rot to brown-rot decay among fungi involved the loss of lignin peroxidases but maintenance of oxidases (152–154).

The oxidases that are maintained in all wood-decaying fungi are well-known H₂O₂ producers and belong to the superfamilies of glucose-methanol-choline (GMC) oxidoreductases (AA3) or copper radical oxidases (CRO; AA5). Among AA3s, aryl-alcohol oxidases (AAO), glucose oxidases (GOX), and alcohol oxidases (AOX) are phylogenetically the most related enzymes, followed by pyranose 2-oxidase (P2O) and CDH, which share the oldest ancestor with other GMC oxidoreductases (155). In white-rot fungi, an obvious role for these oxidases is to fuel lignin peroxidases with H₂O₂. In brown-rot fungi, which lack lignin peroxidases, H₂O₂ may be used to drive the Fenton systems that are unique for these fungi. Importantly, Fenton systems may not be the only H₂O₂ sink since there is a strong cooccurrence of AA9 LPMOs and AA3-encoding genes in brown-rot fungi (83), and we know now that LPMOs efficiently use H₂O₂ to catalyze the oxidative cleavage of polysaccharides. Interestingly, cooccurrence of *lpmo* and *cdh* genes is more scarce in brown-rot fungi than the strong correlation found in white-rot

fungi (83), suggesting that alternative LPMO activation systems exist in brown-rot fungi, as detailed below.

Laccases. Along with peroxidases (discussed below), laccases are major contributors of ligninolysis in white-rot fungi (156, 157). Also known as benzenediol:O₂ oxidoreductases (EC 1.10.3.2; AA1 CAZy family) belonging to the multicopper oxidase superfamily (73), they use O₂ as a cosubstrate and can directly oxidize a wide range of phenolic substrates (156). The resulting reduced form of the laccase catalyzes the reduction of O₂ to H₂O (158) while the oxidized (poly)phenols get involved in depolymerization, cross-linking, or internal reactions such as ring cleavage or quinone formation. The reduction potential of laccases, ca. +0.8 V (compared to >+1 V for lignin-active peroxidases), does not allow activity on nonphenolic moieties ($E_0 > +1.3$ V). This limitation is overcome by the so-called laccase-mediator systems (159, 160). In short, the mediator (e.g., a phytophenolic) is oxidized by the laccase and acts as an electron shuttle by diffusing out of the laccase active site to further oxidize substrates, such as nonphenolic lignin subunits, otherwise not directly tractable by the laccase itself (161). The interplay between laccases and LPMOs is not clear yet. It is clear that soluble lignin fragments emerging from laccase action, which still contain phenolic groups and reducing power, can activate AA9 LPMOs (86, 162). Under certain conditions, the competition of laccases for oxygen may be relevant.

Lignin-active peroxidases. Peroxidases are so far the only known type of ligninolytic enzymes relying on the use of H₂O₂. Peroxidases belong to four independently evolved superfamilies (163), spanning all kingdoms of life. The largest family, the peroxidase-catalase superfamily, contains three families (called families I to III) (163) (PeroxiBase database [peroxibase.toulouse.inra.fr]). Three types of lignin-modifying peroxidases, all belonging to family II (164), have been identified thus far, namely, the lignin (LiPs), manganese (MnPs), and versatile (VPs) peroxidases. All of these enzymes belong to family AA2 in CAZy.

LiPs, MnPs, and VPs all use H₂O₂ as an oxidant but employ very different strategies to act on lignin (165). While LiP, with a high redox potential ($E_0' \approx +1.2$ V versus SHE at pH 3) can directly oxidize nonphenolic aromatic compounds, MnP uses an indirect pathway relying on the oxidation of Mn(II) to Mn(III), which is released from the enzyme. Mn(III) is then chelated by organic compounds (e.g., oxalate or malate) and can act as a diffusible oxidizing agent on phenolic (but not on nonphenolic) substrates. VP, with a very high redox potential ($E_0' > 1.4$ V versus SHE), shows features that are common to LiP and MnP and can oxidize both nonphenolic and phenolic compounds. It is worth noting that despite, or because of, its indirect mode of action, MnP is often the most abundant lignin-active peroxidase found in white-rot fungal secretomes.

Peroxidases compete with LPMOs for H₂O₂ and may thus inhibit LPMO activity under certain conditions, as has been shown by several authors (32, 44).

A diversity of enzymatic H₂O₂ producers. In lignocellulolytic systems, H₂O₂ can be produced by several extracellular enzymes, most of which belong to the GMC oxidoreductase superfamily: cellobiose dehydrogenases (AA3_1; see above); aryl alcohol oxidase (AAO) (AA3_2; EC 1.1.3.7) (166), glucose oxidase (GOX) (AA3_2; EC 1.1.3.4) (167), methanol oxidase (MOX) (AA3_3; EC 1.1.3.13) (168), and pyranose 2-oxidase (P2O) (AA3_4; EC 1.1.3.10) (169). H₂O₂ may also be generated by extracellular copper radical oxidases (CRO), which include glyoxal oxidase (GLOX) (AA5_1; EC 1.2.3.15) (170, 171) and alcohol oxidase (AlcOx) (AA5_2; EC 1.1.3.13) (172), and by gluco-oligosaccharide oxidases (GOOX) (AA7, EC 1.1.3.—) (173, 174) (Fig. 8). H₂O₂-generating enzymes are widely distributed in both white-rot and brown-rot fungi (Fig. 7) (155, 175), indicating that H₂O₂ will be generated during biomass conversion, regardless of the decomposition strategy.

These H₂O₂-producing enzymes act on many different compounds derived from lignocellulose (see Table S1 in the supplemental material). MOX, which seems equally abundant in white- and brown-rot fungi (155), oxidizes methanol, which is a product of the demethoxylation of lignin-derived phenolics (176) and has been suggested to be the main H₂O₂ supplier of Fenton chemistry during brown-rot

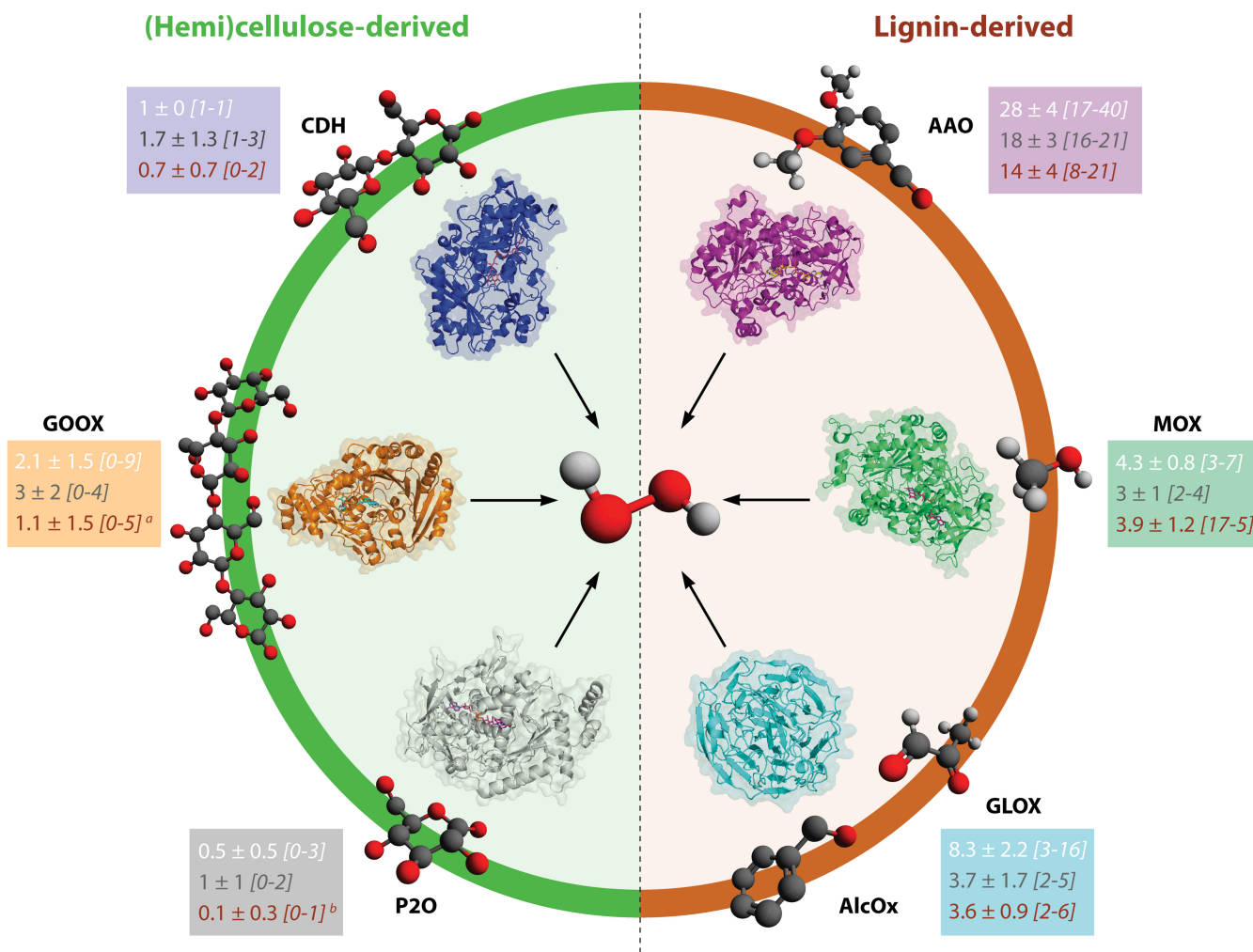


FIG 8 Extracellular H₂O₂ producers encountered during fungal wood decay. The figure shows enzyme structures and associated representative substrates, derived from (hemi)cellulose or lignin fractions, for the CDH (AA3_1) from *Phanerochaete chrysosporium* (PDB accession number 1KDG; dark blue), an AAO (AA3_2) from *Pleurotus eryngii* (PDB accession number 5OC1; magenta), an MOX (AA3_3) from *Pichia pastoris* (PDB accession number 5HSA; green), a P2O (AA3_4) from *Phanerochaete chrysosporium* (PDB accession number 4MIF; gray), an AlcOx (AA5_2) from *Colletotrichum graminicola* (PDB accession number 5C86; light blue), and a GOOX (AA7) from *Sarocladium strictum* (PDB accession number 1ZR6; orange). Note that there is no structure available for GLOX (AA5_1). The average numbers of genes ($\pm 95\%$ confidence interval) found in the genomes of white-, gray-, and brown-rot fungi are indicated in the colored boxes (from top to bottom, respectively) and were retrieved from Riley et al. (151). The interval of minimum to maximum values of actual (varying) gene numbers per genome is indicated in brackets. The white-rot species include the following: *Auricularia subglabra*, *Ceriporiopsis subvermisporea*, *Dichomitus squalens*, *Fomitiporia mediterranea*, *Galerina marginata*, *Heterobasidion annosum*, *Phanerochaete carnosa*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Punctularia strigosozonata*, *Stereum hirsutum*, and *Trametes versicolor*. The gray-rot species are *Botryobasidium botryosum*, *Jaapia argillacea*, and *Schizophyllum commune*; the brown-rot species are *Coniophora puteana*, *Dacryopinax* sp., *Fomitopsis pinicola*, *Gloeophyllum trabeum*, *Postia placenta*, *Serpula lacrymans*, and *Wolfiporia cocos*. ^a*P. placenta* and *Dacryopinax* sp. are the only brown-rot fungi containing GOOX-encoding genes (3 and 5 genes, respectively). ^b*G. trabeum* is the only brown-rot fungus containing a P2O gene (1 gene). It should be noted that the dichotomy between lignin and (hemi)cellulose-derived substrates is not always clear-cut since substrates such as veratryl alcohol can be synthesized as a secondary metabolite *de novo* from glucose (428).

decay (177). AAO, one of the most frequent GMC oxidases in white-rot fungi (155) (Fig. 8), is an extracellular enzyme acting preferentially on fungal metabolites such as 4-methoxylated benzoyl alcohols (166). These aromatic alcohols are the products of lignin-derived aldehydes or acids that have been reduced by aryl-alcohol dehydrogenases (EC 1.1.1.90) and aryl aldehyde dehydrogenases (EC 1.2.1.30) (178). GOXs, which are structurally and sequentially closely related to AAO and found both intra- and extracellularly, are not widespread in wood decayers, and their role in lignocellulolysis is not clear. P2O is found at the hyphal periplasmic space or in the secretome (putatively upon cell lysis) and has a broader substrate specificity than GOX as it can catalyze the oxidation of several aldo-pyranoses at the C-2 position (179, 180). Although efficient at reducing O₂, P2O has also been shown to reduce

quinones and complexed metals (Fig. 4 and Table S1) (181, 182). The involvement of P2O in lignocellulose conversion is supported by immunocytochemical and substrate-dependent gene regulation studies (169, 183–185).

Vanillyl-alcohol oxidases (AA4) are intracellular FAD-dependent enzymes that act on activated aromatic alcohols such as 4-hydroxybenzyl alcohols, leading to the concomitant production of H₂O₂ (186, 187). As intracellular enzymes, AA4s are not thought to be directly involved in lignocellulolysis but in the metabolism of lignin-derived compounds (188, 189).

GLOX (AA5_1) is widely distributed among wood decayers. The enzyme acts mainly on aldehydes that are released during lignin and carbohydrate processing (190) and is considered to be physiologically coupled to lignin-active peroxidases (149, 170, 175, 190, 191). The substrate specificity of the AA5 family has recently been expanded by the characterization of two alcohol oxidases (constituting a subclade of AA5_2) active on aliphatic primary alcohols (e.g., butan-1-ol, benzyl, and cinnamyl alcohol) (172). The authors of the study speculated that these AA5_2 enzymes may have a role in plant cell wall depolymerization even though the identity of the natural substrate has not yet been determined.

Glucosyl-oligosaccharide oxidase (GOOX) activity was reported more than 25 years ago (192). GOOX enzymes, classified as AA7s, have hitherto received less attention than other H₂O₂ suppliers in fungal secretomes. GOOXs are secreted enzymes, sharing some substrate specificity with CDH, P2O, and GOX (173). They are primarily active on glucosyl-oligosaccharides, but activity on xylo-oligosaccharides has also been described (174). Very little is known about their biological role. Some researchers have proposed that GOOX may fuel lignin-active peroxidases, which is the usual proposal when it comes to identifying a H₂O₂ sink in lignocellulolytic enzyme systems. Today, LPMOs provide an alternative H₂O₂ sink. Interestingly, considering the substrate specificities of GOOX and LPMOs, the two enzymes could be acting in close proximity, which could lead to better reaction control. AA7s have been detected together with AA9s in the secretome of *S. commune*, a gray-rot fungus, when they colonize artichoke stalk (142).

Several members of the GMC oxidoreductase superfamily have now been shown to activate LPMOs (83, 193). As described above (see subsection “The CDH Case: a Multifunctional Redox Partner”), CDH is a known redox partner that, by means of its two-domain structure, plays the dual role of reducing the LPMO and supplying H₂O₂. Interestingly, the Cyt domain is not absolutely required for GMC oxidoreductases to drive LPMO reactions as single-domain DHs, such as the glucose dehydrogenase (GDH) or aryl-alcohol quinone oxidoreductases (AAQO) (subfamily AA3_2), can also drive AA9 LPMO activity (193). On the other hand, single-domain strict oxidases such as the GOX or AAO are not able to reduce or activate the LPMO (32, 193). The way in which GDH or AAQO reduces the LPMO remains unclear and may involve mediators, such as superoxide or cofactors. Interestingly, some fungal secretomes contain many H₂O₂-generating oxidases as well as LPMOs while lacking both CDH and any usual H₂O₂ consumer (e.g., peroxidase) (83, 143). In light of the recent insights into LPMO functionality, these oxidases can be viewed as H₂O₂-generating partners of LPMOs although they may be fulfilling other roles that remain to be discovered. In this respect, it is worth noting that knocking out *cdh* genes in *Podospira anserina* did not alter its growth on lignocellulose but led to increased production of (H₂O₂-generating) flavo-oxidases (AA3_2) and CRO (AA5_1) along with β -glucosidases (111). It is possible that LPMOs are reduced by other factors, such as redox mediators, and that the oxidases then fuel the reaction by delivering H₂O₂. Indeed, it has been shown that glucose oxidases, which alone cannot drive LPMO reaction, do so very well upon reduction of the LPMO by a reductant (32). Notably, the natural environment of wood-decaying fungi is rich in phenolic compounds, which can reduce LPMOs (83).

As shown in Fig. 4, H₂O₂-producing oxidases (AA3_2, _3, _4, AA4, AA5, and AA7) display, in general, catalytic constants spanning two orders of magnitude, from 10 to 300 s⁻¹, approximately (see Table S1 in the supplemental material for details). On the other hand, the H₂O₂-producing ability of CDH is much lower and lies in the range of

10^{-2} to 10^{-1} s^{-1} . LPMOs present the lowest capacity to reduce O_2 into H_2O_2 , with reported catalytic rates ranging between 10^{-4} and 10^{-2} s^{-1} .

Whether the relatively low H_2O_2 production rate displayed by CDH would be a way to control LPMO action during white-rot decay is an interesting possibility. The necessity of a match between H_2O_2 production and H_2O_2 consumption in lignocellulolytic enzyme systems has been argued in the past by Philip Kersten, who noticed that the H_2O_2 generator GLOX drives the lignin peroxidase action and that the much higher catalytic efficiency of GLOX (than that of the peroxidase) may explain why it is yet a minor component of the lignocellulolytic broth (170). It is also worth noting that H_2O_2 has been proposed as a limiting factor in lignocellulolysis by *P. chrysosporium* (194, 195) or limiting the action of MnP during compost lignin conversion by *Agaricus bisporus* (196). Overall, it is tempting to think that, in order to match and control H_2O_2 fluxes between emitters and receptors, nature has coevolved catalytic efficiencies and enzyme secretion levels. However, such correlations remain to be clearly demonstrated.

The housekeeping role of catalases. As highlighted above, H_2O_2 appears to be a central molecule produced and used by several enzymatic systems involved in lignocellulolysis. H_2O_2 is *per se* a rather stable molecule (197) but can be destabilized. Indeed, in a complex environment, such as biomass-decomposing litter, free reduced metals are likely to be found, and these may react with H_2O_2 , leading to the generation of hydroxyl radicals by Fenton chemistry. Beyond metal-catalyzed reduction of H_2O_2 , direct reduction by reductants is also a possibility (Fig. 3) although it will occur at much lower rates. For instance, the second-order rate constant for the reaction of H_2O_2 with the strong reductant ascorbate is only $1.03 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.0 (198).

The generation of hydroxyl radicals and other ROS exposes the system's components to irreversible and destructive oxidative damage. Housekeeping enzymatic activities referred to as catalase activities have evolved to avoid such damage (199–201). Catalase activity, i.e., the enzymatic disproportionation of H_2O_2 into O_2 and H_2O ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$), is found in all kingdoms of life and can be carried out by three groups of proteins, namely, by typical (also known as monofunctional) catalases (EC 1.11.1.6), by some members of the peroxidase-catalase family (EC 1.11.1.6/7), and by nonheme manganese catalases (a minor group) (EC 1.11.1.6). Typical catalases, which constitute the predominant group (199), are heme iron-dependent enzymes with high k_{cat} (10^4 to 10^5 s^{-1}) but rather poor apparent K_m values for H_2O_2 (3 to 10^3 mM range) (200, 201). Similar values apply to the bifunctional peroxidase-catalases (10^3 to 10^4 s^{-1} and 3.7 to 8 mM for the catalase activity) (202). These values differ strongly from values for peroxidases, i.e., enzymes that use H_2O_2 as oxidant, which show lower catalytic rates (10^{-1} to 10^3 s^{-1}) but also much lower K_m values (10^1 to $10^3 \mu\text{M}$) (203–206). Typical catalases display a deeply buried active site, to which H_2O_2 molecules gain access via a long channel, the properties of which (shape, size, and composition) are optimized to increase the ratio of H_2O_2 to H_2O and, thus, reactivity (207, 208). Variations in channel features, impacting inlet and outlet fluxes, may explain the wide range of observed catalytic efficiencies (199, 202).

The kinetic properties of catalases, with their high, noncompetitive K_m values, seem adapted to keeping the level of H_2O_2 low enough to avoid oxidative damage without inhibiting H_2O_2 -dependent enzymes with their much more competitive K_m values. High catalytic efficiencies displayed by catalases may, on the other hand, be related to the necessity of a fast response in the case of H_2O_2 accumulation. This scenario would obviously benefit lignocellulosic redox enzyme systems. It has been shown that catalases are secreted simultaneously with H_2O_2 -consuming enzymes, such as peroxidases, during the biodegradation of spruce wood by the white-rot fungus *Phanerochaete chrysosporium* (209). The brown-rot fungus *Postia placenta* secretes catalases along with GHs in a later phase of wood degradation, possibly as a means to protect the GHs from residual Fenton reagents used in the earlier phases of the brown-rot process (146) (see subsection "Enzyme Production during Lignocellulose Depolymerization," below). The role of catalases in H_2O_2 regulation may translate into antagonistic effects, as observed with knockout of catalase genes in *Podospora anserina* (210, 211). Bourdais et al. (210)

showed that the five *P. anserina* catalases play different roles and that there may be a trade-off between the need for protection and the need for H₂O₂ to increase the efficiency of lignocellulose depolymerization efficiency. The multiplicity of catalase genes in microbial genomes suggests a need for flexibility and underpins a need for further research on the roles of these enzymes during biomass conversion.

The dynamic aspect of H₂O₂ fluxes renders the determination of real H₂O₂ levels encountered in biological systems rather challenging. Impressively, values reported in the literature span approximately seven orders of magnitude. Intracellular levels are thought to be extremely low to protect from lethal ROS-induced damage, i.e., in the low-nanomolar range (212, 213). Conversely, extracellular levels are higher and may be bacteriostatic (micromolar) or even bactericidal (millimolar) (214). Some pathogenic microorganisms, such as some *Streptococcus* species (215) or the rice blast fungus *Magnaporthe oryzae* (216), can resist millimolar concentrations of H₂O₂. In the 1 to 100 μM range, effects with different amplitudes can be observed on fungal growth depending on the region considered, with the hyphae being for instance more resistant than the conidia (217–219). Plants can tolerate up to several millimolars of H₂O₂ (100 μM to 200 mM) (220, 221). Importantly, H₂O₂ produced intracellularly is likely to be used intracellularly while H₂O₂ produced extracellularly is consumed extracellularly, suggesting that both systems are partly disjoint. Indeed, it has been shown that H₂O₂ transmembrane transport in *Saccharomyces cerevisiae* and bacteria is very limited (222), and this is consistent with the (potentially) enormous difference in the concentrations encountered on both sides of the cellular membrane.

It is known that plants trigger a defense mechanism called oxidative burst when they are under threat (223), which implies production of ROS and, notably, H₂O₂ (224, 225). Interestingly, for hitherto unexplained reasons, necrotrophic fungal pathogens such as *Botrytis cinerea* or *Colletotrichum graminicola* take advantage of this response to proliferate (226, 227). These microorganisms can express multiple LPMOs and peroxidases (24, 228), and one may wonder if the pathogens harness plant-generated H₂O₂ to fuel their degradative redox enzyme machinery. The ambivalent role of H₂O₂ is also highlighted by the fact that some plants use catalases as defense against pathogenic fungi such as *Aspergillus flavus* (229). Another indication for a role of H₂O₂ and H₂O₂-producing enzymes in pathogenicity is provided by two independent studies showing that *Botrytis cinerea* became less virulent upon deletion of genes encoding either superoxide dismutase (SOD) (230) or GLOX (231). Membrane-bound GLOX has also been implicated in pathogenesis for crop pest *Fusarium* species (232).

Nonenzymatic Production and Use of H₂O₂

In ecological niches where biomass decomposition occurs, several nonenzymatic reactions can generate or consume H₂O₂. This additional layer of complexity needs to be taken into account in order to understand lignocellulose conversion.

Nonenzymatic sources of H₂O₂. The reduction of O₂ into H₂O₂, possibly via superoxide, O₂^{•-}, as an intermediate (Fig. 3) requires compounds with low enough reduction potentials. Many plant-derived phenols, also called phytophenolics, have this capacity and are abundant in decomposing matter (233). Phenolics are also known metabolites of brown-rot fungi (234), and it has been shown that the activity of some microbial extracellular enzymes is modulated by these compounds (235). Like many reducing agents with antioxidant properties, phenolics can also act as prooxidants, e.g., by reducing transition metals that subsequently engage in damaging oxidative processes such as Fenton chemistry (236). What happens in a biological system depends on the concentration of the chemicals in question, the presence of transition metals (Fe and Cu) and of O₂, and the reduction potentials of the derived radical(s). Phytophenolics have different structures, which affect their anti- and prooxidant properties (237). Importantly, the H₂O₂ production potential of phenolics is also pH dependent (238). As outlined herein, several phytophenolics promote LPMO activity with different efficiencies (65, 83), and one may question if their role is only to reduce the copper site or also

to provide H_2O_2 . The role of phytophenolics during lignocellulolysis remains rather unclear, but their potential importance is well documented.

Fenton-type chemistry: a sink for H_2O_2 . Regarding nonenzymatic H_2O_2 sinks, the chelator-mediated Fenton (CMF) reaction (6, 239), notably used by brown-rot and certain LiP-deficient white-rot fungi, is considered central. Fenton reactions, i.e., the reaction of H_2O_2 with a reduced transition metal, generate hydroxyl radicals, which are thought to be involved in an initial, nonenzymatic pretreatment of lignocellulose. The CMF system finds biological relevance when one considers that enzymes are usually too big (20 to 100 Å in diameter assuming a globular shape [240]) to penetrate wood pores (10 to 40 Å in diameter; up to 100 Å in some cases [239, 241, 242]). On the other hand, such a chemical strategy is rather dangerous for the microorganism, and, therefore, the hydroxyl radicals have to be generated at a safe distance. Due to the diffusion-limited and nonspecific character of their reactivity, with a half-life of nanoseconds (239), hydroxyl radicals have to be generated *in situ*, i.e., in wood pores, to be both efficient and not harmful for the fungus. To perform such spatial control, it has been proposed that the pH dependency of metal reduction and concentration gradients of metal chelators such as oxalic acid or 3,4-dihydroxybenzoic acid (DHBA) play a major role (243), as has been reviewed elsewhere (234).

In the gradient scenario, low pH (≈ 2) and a high concentration of oxalate (a fungal metabolite) at close proximity of the fungal hyphae keep Fe(III) in a stable chelated form that can diffuse into wood pores. Once within the wood, the combined effect of higher pH (≈ 5.5 to 6) and lower oxalate concentration leads to release of Fe(III), which can then be reduced to Fe(II) by wood-penetrating redox compounds such as catechol or hydroxamic acids (244), oxalate (245), or, potentially, by low-molecular-weight peptides (234, 246). Redox cycling involving low-molecular-weight metabolites such as hydroquinones (247) or involutin (248) has also been proposed to be involved in iron reduction. The reduced transition metal can then react with H_2O_2 (249, 250) via the well-known Fenton reaction reported in 1894 (251), which produces a hydroxyl radical and a hydroxide ion (Fig. 6). CDH has often been linked to Fenton chemistry, both as a producer of H_2O_2 and because of its ability to reduce iron (112–116); such a role, however, seems questionable because CDH likely cannot penetrate wood pores. Of note, superoxide can also play the role of reductant via the Haber-Weiss reaction (252).

The control of H_2O_2 levels, which directly impact the efficiency of the CMF reaction, is a key parameter to consider. In brown-rot fungi the CMF system is thought to be fueled with H_2O_2 by the action of GMC oxidoreductases and/or copper radical oxidases (155) (see subsection “The Function of Lignocellulolytic Oxidoreductases,” above). Little is known about the actual H_2O_2 concentrations needed. *In vitro* studies of Fenton reactions often use high (millimolar) H_2O_2 concentrations and time scales that do not seem compatible with *in vivo* conditions.

While Fenton chemistry, with its seemingly clear biological relevance, is almost by default mentioned as an H_2O_2 sink in discussions of fungal biomass-related enzymology, it is important to note that the CMF system is just one way of exploiting the oxidative power of combining H_2O_2 with a reductant. Reactions between the reductant and H_2O_2 may take place in many locations, in a controlled or noncontrolled manner, and be beneficial or detrimental. For example, it has been suggested that such chemistry occurs in the termite gut, which seemingly lacks LPMOs and peroxidases but contains many H_2O_2 -generating and iron-reducing enzymes (253). Similarly, Fenton-type reactions were shown to occur in the midgut of a leaf-feeding caterpillar (254). It is also worth noting that the use of a Fenton-like system during biomass decomposition has recently been described for a bacterium (255).

Enzyme Production during Lignocellulose Depolymerization

Connections between two enzymes are likely biologically relevant if (i) the product (a compound or reducing equivalents) of one enzyme is used by another and (ii) the enzymes are cooccurring in a common environment. The availability of next-generation sequencing methods and developments in adjacent omics technologies now offer

TABLE 2 Current overview of plant cell wall-degrading enzymes^a

Substrate and enzyme activity(ies) ^b	Common abbreviation(s) ^c	CAZy family(ies)
Lignin		
Laccases	LAC or Lcc	AA1
Versatile, lignin, and manganese peroxidases	VP, LiP, and MnP	AA2
Aryl-alcohol oxidases	AAO	AA3_2
Methanol oxidase	MOX	AA3_3
Glyoxal oxidase	GLOX	AA5_1
1,4-Benzoquinone reductase	QR	AA6
Hemicellulose		
β -Mannosidase	MANB	GH2
β -1,4-Endo-mannanases	MAN	GH5_7, GH5_26
β -1,4-Galactosidase	AGA, AGL	GH27, GH36
β -1,4-Galactosidase	LAC, BGA, GAL	GH2, GH35
Galactomannan acetyl esterase	GMAE	
β -Xylosidases	XYL	GH3, GH39, GH43, GH52
β -1,4-Endo-xylanases	XYN, XLN	GH10, GH11
Arabinoxylan arabinofuranohydrolase	AXH	GH62
α -L-Arabinofuranosidases	ABF	GH43, GH51, GH54, GH62
α -Glucuronidases	AGU	GH67, GH115
Feruloyl esterases	FAE	CE1
Acetyl esterases	AE	CE1–CE7, CE12, CE16
4-O-Methyl glucuronoyl methylesterases	GE/GCE	CE15
Xyloglucan transferase/hydrolases	XTH	GH12, GH16, GH74
α -Xylosidases	AXL/XYL	GH31
Galactose 6-oxidase	GalOx	AA5_2
Lytic xylan oxidase (LPMO)	LPMO14	AA14
Cellulose		
β -Glucosidases	β -Glu, BG, BGL	GH1, 3
Endoglucanases	EG	GH5_5, GH5_7, GH5_9, GH5_12, GH5_45, GH5_48, GH5_74, GH5_131, GH5_148
Cellobiohydrolases	Cel, CBH	GH6, GH7
Cellobiose dehydrogenase	CDH	AA8-AA3_1-(CBM1)
Glucose 1-oxidase	GOx	AA3_2
Pyranose 2-oxidase	P2O	AA3_4
Gluco-oligosaccharide oxidases	GOOX	AA7
Fungal LPMOs	LPMO9	AA9
Bacterial LPMOs	LPMO10	AA10
PQQ-dependent pyranose dehydrogenase ^d	PDH	AA8-AA12-CBM1

^aThe table summarizes the main enzymatic activities that are thought to be involved in lignocellulose conversion. They have been ordered according to the class of substrate they act on, namely, lignin, hemicelluloses, pectin or cellulose. For each type of enzyme we provide the most common abbreviation(s) and the CAZy (sub)family (21). Catalases, cytochrome P450s and other non-CAZymes are not included.

^bSee Rytioja et al. (263) for a detailed list of pectin-active CAZymes (GH2, -3, -28, -35, -43, -51, -53, -54, -62, -78, -88, -93 and -105; PL1, -3, -4, -9, and -11; and CE1, -8, and -12), which should be updated with several recently discovered pectin-active CAZyme families (GH137 to GH143 [427], GH145 [431], and GH146 and GH147 [432]).

^cFor historical reasons (evolving terminology and enzyme reclassification), several abbreviations may be found for the same activity. Early names are often kept by habit but also for optimal tracking of the literature. A more standardized approach consists in using the CAZy (sub)family preceded by initials, in italics, of the microorganism of origin. For example, *Sm*AA10A would be the first characterized AA10 from *Serratia marcescens* (historically known as CBP21). Alternatively, the two-letter code indicating the CAZy class may be replaced by a three- or four-letter abbreviation that indicates what the enzyme does. For example, the GH5 family contains both cellulases and mannanases; hence, one may find *Tr*Cel5A and *Tr*Man5A, which indicate a GH5 endo-cellulase and a GH5 endo-mannanase from *Trichoderma reesei*, respectively.

^dPQQ, pyrroloquinoline quinone.

possibilities to get insight into enzyme systems involved in lignocellulose degradation. The first white-rot fungus genome (from *P. chrysosporium*) was published in 2004 (256). Since the takeoff in fungal genomics in 2009 (257), more than 1,000 fungal genomes have been hitherto sequenced, mostly under the flag of the 1,000 Fungal Genomes Project (258) led by the U.S. Department of Energy's Joint Genome Institute. Lignocellulose conversion involves many players (Fig. 6 and Table 2), and genomes alone are not sufficient to elucidate how the orchestra of lignocellulose-active enzymes is regulated in time and space. With the emergence of multi-omics data, the reconstruction of biomolecular reaction networks involved in plant biomass decomposition becomes a possibility (259, 260). Based on accumulating omics data, we briefly discuss below (i) the orchestration of enzymatic machineries in simultaneous or sequential depolymer-

ization of lignin and (hemi)cellulose and (ii) the interplay between Fenton chemistry and enzymes in brown-rot decay. The regulation and properties of cellulases and hemicellulases have been extensively covered by other investigators in comprehensive reviews (261–264).

Insights from recent multi-omics studies. It is known that some microorganisms have evolved enzymatic arsenals that are specifically adapted to a given habitat (265–267) while others have kept a broad array of activities (110, 263, 268, 269), ensuring adapted responses to diverse environments (270). In 2017, a large-scale study analyzed 22 transcriptomics data sets from 10 species of plant-decaying basidiomycetes displaying a white-, gray-, or brown-rot phenotype (271). Among 328 consistently differentially expressed genes, the authors could define a core set of 50 CAZymes involved in the degradation of plant polysaccharides. Among these, five enzyme types were found upregulated in at least 18 of the data sets: β -glucosidase, β -xylosidase, endo-xylanase, endo-mannanase, and α -glucuronidase. This result highlights the point that enzymatic depolymerization of hemicellulose is common among all fungal types. According to this same meta-analysis, the most prevalent additional common enzyme types were cellulases, hemicellulases, and pectinases (271). Oxidoreductases were not present in these sets of commonly employed enzymes, and this is logical since different wood-decaying fungi employ different oxidative strategies (Fig. 7).

With this global, but static, enzymatic picture being drawn, we need to consider the temporal fate of the lignin, hemicellulose, and cellulose fractions in order to understand which enzymes may or may not be physiologically linked. Owing to the high recalcitrance of lignin, it has been thought for decades to be the last biopolymer to be degraded, eventually leading to the production of humic substances. However, we know today that several scenarios can take place, depending on both the fungus and the type of substrate. In contrast to most white-rot fungi, which generally (but not always) carry out simultaneous degradation of lignin and cellulose (110, 272–274), fungi such as *Phanerochaete carnosa* (144, 145) and *Ceriporiopsis subvermispota* first attack lignin and then cellulose (275). For instance, when grown on aspen wood, *C. subvermispota* employs a strategy whereby oxidative lignin-active enzymes are first produced (AAO and MnP) and (hemi)cellulolytic enzymes are secreted later (GH5_5, GH12, and GH45 endoglucanases [EGs], GH6 cellobiohydrolase II [CBHII], GH7 CBHI, EG, GH10 xylanase, CE1 acetyl/feruloyl esterase and CE15 glucuronoyl esterases, LPMOs, and CDH) (147). During the ligninolytic phase, genes involved in lipid metabolism (e.g., encoding desaturases) are upregulated along with genes directly involved in lignin modification (MnP) (276), in agreement with the hypothesis that a connection exists between lipid peroxidation products and oxidation of nonphenolic structures present in lignin (277–279).

When the white-rot *P. chrysosporium* is grown on spruce wood, expression of genes encoding ligninolytic enzymes (MOX and GLOX) is upregulated, whereas cellulase genes are expressed in a more constitutive manner (209). Earlier reports have shown that CDH expression is also induced early, particularly by products of cellulase action (280). In a recent comprehensive study, Miyauchi et al. used an integrative omics approach to show that the saprophytic white-rot fungus *Pycnoporus coccineus* simultaneously secretes GHs, esterases, and oxidoreductases when grown on various lignocellulosic substrates (148). In this study, peroxidases (AA2), CDH (AA3_1-AA8), AAO (AA3_2), GLOX (AA5_1), and LPMOs (AA9) were highly upregulated, highlighting that H₂O₂-dependent peroxidases, H₂O₂-producing enzymes, and LPMOs cooccur during lignocellulose breakdown.

Studies of the transcriptomes of *Aspergillus niger* and *Trichoderma reesei*, which are used in the industry for the production of enzymatic cocktails, during growth on steam-exploded sugarcane bagasse revealed large temporal differences between the two fungi and showed the importance of redox enzymes in both (281). *A. niger* showed fast upregulation of CAZyme genes and a relatively early decay in expression levels, whereas the situation was the opposite for *T. reesei*. A total of 53% and 39%, respectively, of all AA-encoding genes were induced by the biomass for *A. niger* and *T. reesei*

(out of a total of 68 and 33, respectively). Several extracellular H₂O₂-generating enzymes (AA3_2 and AA3_3) were highly upregulated in both fungi, as well as several AA9s, highlighting the importance of these enzymes. Interestingly, next to catalases and glutathione S-transferases, with putative housekeeping functions, and AA9 LPMOs, only a single known H₂O₂-consuming enzyme, a putative peroxidase, was transcribed. Of note, the *T. reesei* genome does not contain any *cdh* gene whereas the *A. niger* genome contains only one that was not induced (281).

The results of comparative studies of the secretome of the white-rot fungus *Dichomitus squalens* grown on woody (aspen and spruce) and nonwoody (wheat bran and cotton seed hulls) biomasses further support the notion that one main role of H₂O₂-generating enzymes could be to fuel other enzymes (LPMOs) than peroxidases. In this study, H₂O₂-consuming peroxidases were expressed at much lower levels on nonwoody (lignin-poor) substrates whereas the secretome pattern of H₂O₂-supplying enzymes (AA3_3 and AA5_1) was not affected by the nature of the substrate (270). AA9 LPMOs were secreted on all substrates, and their expression patterns were found to cluster with different sets of CAZymes, suggesting that LPMOs target different structures.

Several other enzymes or proteins with putative roles in biomass conversion appear in studied secretomes and transcriptomes. One example is the family of expansins, which, by changing the cell wall structure through a hitherto unknown mechanism, may affect the wall's susceptibility to enzymatic degradation (282, 283). Another class of enzymes worth mentioning are the P450 monooxygenases, which are abundant and largely nonsecreted but have been detected in the secretomes of white-rot fungi (110, 152, 175, 276, 284) and in the hyphal front of brown-rot fungi (146). P450s are ubiquitous heme enzymes, catalyzing a wide range of redox reactions, and their potential role in biomass conversion remains obscure. Most interestingly, in 2018, Reisky et al. described P450s catalyzing the oxidative demethylation of carbohydrates present in algal polysaccharides, likely decreasing the recalcitrance of this marine biomass (285). A role of P450s in lignocellulose conversion is plausible since they are more abundant in wood-decaying basidiomycetes than in non-wood-decaying relatives (286).

How do brown-rot fungi deal with ROS? The simultaneous use of potentially harmful ROS (generated in the Fenton reaction) and enzymes by brown-rot fungi is intriguing. As discussed herein, the feasibility of this system is usually ascribed to a spatial partition between the generation of nonselective ROS and GHs (see subsection "Nonenzymatic Production and Use of H₂O₂," above). Recent studies suggest that the brown-rot fungi *Postia placenta*, *Serpula lacrymans*, and *Gloeophyllum trabeum* (also) use separation in time (146, 287). Expression data indicate that these fungi use a two-step mechanism where oxidative and hydrolytic actions are temporally separated by differential gene expression (146). Zhang et al. showed that oxidative processes (i.e., H₂O₂ and Fe²⁺-generating enzymatic systems) involved in Fenton reactions are first triggered at the hyphal front, constituting thus a sort of pretreatment, which is followed by expression of (hemi)cellulases at a later stage. It is worth noting that expansins and pectinases (GH28) were also expressed during this early phase, in agreement with their potential action in initial loosening of the plant cell wall. Importantly, AAO and superoxide dismutase, which are H₂O₂ suppliers, were also expressed during the later stage of the process along with hydrolytic enzymes. The authors of this study noted that H₂O₂ produced by AAO must be directed toward a pathway that remains to be identified (the usual recipients, lignin peroxidases, are absent in brown rots). Interestingly, a single AA9 LPMO was also expressed during the later stage of biodegradation, and this enzyme may use the AAO-generated H₂O₂.

In general, the simultaneous secretion of enzymes with complementary (H₂O₂ producers versus H₂O₂ consumers) or competitive (different H₂O₂ consumers) impacts on H₂O₂ fluxes highlights that fungi possess regulatory systems and/or have evolved well-balanced enzymatic arsenals that allow the maximizing of enzyme efficiency while minimizing inhibition or oxidative damage. One particularly intriguing question is

whether fungi have systems for generating proximity between redox partners. For example, proximity between the sites of production and consumption of H_2O_2 could be one way to minimize damage caused by side reactions involving free H_2O_2 . This is discussed further below.

On abundance and importance. When omics data are assessed, an important point of discussion concerns the implications of the numbers. Why are there so many LPMO- and GMC oxidoreductase-encoding genes? Why are there so few CDH genes? Why are there so few catalase genes? These questions underpin the complexity of analyzing genomic, transcriptomics, or secretomics data since the number of genes and proteins does not necessarily reflect their importance. While abundance is likely a sign of importance, importance is not necessarily reflected in abundance. This is why quantities (of genes, of transcripts, and of secreted proteins) may need to be weighted by the catalytic efficiency of a given biocatalyst, something that is rarely done.

The abundance of LPMO genes may reflect the need to face several different recalcitrant structures on copolymeric plant cell walls (288, 289), as beautifully illustrated by the recent discovery of an LPMO that specifically acts on copolymeric cellulose-xylan structures (25). On the other hand, white-rot fungi harbor only a single, but conserved, CDH gene. While it could be envisioned that a single CDH enzyme could serve as a common reductant and H_2O_2 generator for any kind of LPMOs, the question of proximity between these two enzymes with potentially different targets is more cumbersome. The diversity of H_2O_2 -generating enzymes, beyond CDH, may reflect a need to produce this central ROS at different specific locations during plant material decomposition.

IMPLEMENTATION OF REDOX STRATEGIES INTO BIOREFINING PROCESSES

The first two sections of this review highlighted the observation that oxidative processes form an essential part of biomass degradation in nature. In this section, we will illustrate how biorefining processes can leverage our current knowledge on these oxidative processes. Microorganisms do achieve complete biomass conversion, but these biological processes occur on relatively long timescales (weeks and months) due to physiological and environmental constraints. From a biotechnological standpoint, one of the main challenges consists of not only mimicking but also speeding up the microbial strategies at reasonable cost (290). Here, we provide a nonexhaustive overview of the (possible) impact of the most recent advances in our understanding of the role of LPMOs and other oxidoreductases on industrial biomass conversion. We discuss oxidative pretreatments of biomass, consider the composition of lignocellulolytic enzyme cocktails and the contribution of oxidoreductases, and end by addressing the implementation in bioprocess design.

Oxidative Pretreatments

In the lignocellulose-based biorefinery concept, the biomass is first rendered more accessible to enzymes by pretreatment methods (291, 292) and then enzymatically depolymerized to platform molecules (e.g., mono- and oligosaccharides) (293), which can, in turn, be converted into value-added products by microorganisms. As several comprehensive reviews on pretreatment technologies are available, here we address only pretreatment methods utilizing oxidative processes. Pretreatment technologies using oxidative processes can be classified into biological (294), biochemical, and (physico)chemical processes: biological pretreatment methods use a biomass-degrading microorganism (295), biochemical pretreatment methods use oxidative enzymes, and (physico)chemical pretreatment methods utilize oxidizing agents for the generation of reactive oxygen species (ROS).

Biological pretreatment. The most common biological pretreatments include treating the biomass with lignin-degrading bacteria, white-rot fungi, or brown-rot fungi (294). Such treatments primarily target lignin, which is generally considered the most important hindrance for efficient depolymerization of the polysaccharides. One promising strategy is the use of microbes that can depolymerize lignin and take up the

resulting products, thus acting as microbial sinks. Uptake of lignin-derived compounds prevents repolymerization reactions and thereby improves overall biomass delignification (296, 297). Some white-rot fungal species preferentially target lignin during the onset of biomass conversion, which renders them a suitable choice for selective biological delignification. For instance, pretreatment (during 18 days) of several biomass feedstocks by *Ceriporiopsis subvermispota* resulted in a 2- to 3-fold increase in saccharification efficiency compared to the level with untreated raw material (298). Similarly, pretreatment of bamboo culms with the white-rot basidiomycete *Punctularia* sp. improved the subsequent enzymatic release of total sugars by 60% (299). A very recent study showed impressive improvements in cellulose hydrolysis yields from corn stover when it was pretreated (for 28 days) with the white-rot fungus *Physisporinus vitreus*: saccharification of the pretreated material with a commercial cellulase cocktail from *Trichoderma longibrachiatum* gave an overall hydrolysis yield of cellulose of 92%, in contrast to 26% without pretreatment. The extent of the beneficial effect was proportional to the duration of the pretreatment, and the obtained maximum yields were similar to what could be obtained after thermochemical pretreatment (300). In order to reduce long biological pretreatment times, often associated with sugar loss, Brethauer et al. proposed an alternative approach that consists of simultaneous saccharification and fermentation combined with *in situ* pretreatment by a white-rot fungus (*Irpex lacteus*) (301).

Biochemical pretreatment. While biological pretreatment has produced some success, it usually requires long incubation times (ca. 20 to 90 days) and, depending on the species used, reduces overall yields through sugar consumption by the microorganism (294). Sugar yield loss may be circumvented by using biochemical approaches inspired by the oxidative strategies employed by these organisms. Lignin-active enzymes, such as peroxidases and oxidases, from white-rot fungi may be used to generate free radicals that nonspecifically attack lignin. For instance, Asgher et al. have shown that pretreatment of sugarcane bagasse with a secretome from *Pleurotus ostreatus* led to saccharification yields (ca. 70% glucose yields) similar to those of chemical pretreatment with 4% NaOH (302). In principle, enzymatic delignification is advantageous because one uses mild conditions that require low energy input and may give high yields. However, so far, this approach still suffers from long reaction times (several weeks) compared to those with classical physico-chemical treatments (303). The cost of the enzymes and their cofactors is also a limiting factor.

Mimicking brown-rot fungi by using Fenton (304) and chelator-mediated Fenton (CMF) (243) treatments to generate lignin-destroying hydroxyl radicals from H₂O₂ close to the biomass is an attractive scenario (305). Indeed, Fenton-type reactions have been successfully used to pretreat cotton fibers (306), garden biomass (307), rice straw (308), steam-exploded poplar (309), *Miscanthus* (310), switchgrass (310), corn stover (310), or wheat straw (310), allowing up to 5-fold improvements in saccharification yields (310). However, these Fenton reaction-based treatments require large amounts of chemicals (typically in the 0.1 to 10 M range for H₂O₂) and may lead to considerable mass loss, while special care must be taken to ensure innocuousness of the reaction mixture prior to proceeding with downstream steps.

(Physico)chemical oxidative pretreatment. In addition to biological and biochemical pretreatment strategies that are inspired by natural processes, some known (physico)chemical pretreatment methods employ reactive oxygen species. The most common of these methods are ozonolysis (using ozone at ambient temperature and pressure for lignin removal) and wet oxidation (using air/oxygen with water or H₂O₂ at elevated temperatures for lignin and hemicellulose removal) (291). Although these methods are time-efficient and give good subsequent saccharification yields, they require high energy input (e.g., high pressure and temperature) and/or harsh conditions (e.g., high concentrations of chemicals such as hydrogen peroxide and acid) and usually lead to the generation of inhibitory compounds.

One may wonder if a future, deeper understanding of microbial strategies will allow

development of natural-process-inspired biochemical/biological pretreatments that are cost- and time-efficient as well as environmentally friendly.

Design of Enzymatic Cocktails: the Effect of Oxidoreductases

Although the costs of enzymes used for lignocellulose saccharification have decreased nearly 20-fold during the past two decades (41), enzymes are still a major cost factor in biorefining (311–313), especially for the more recalcitrant of substrates. Today, the most common commercial products for saccharification are enzyme cocktails produced by heavily modified versions of the filamentous ascomycetes *Trichoderma reesei* (also known as *Hypocrea jecorina*) (314), *Aspergillus niger*, and *Myceliophthora thermophila*. In recent years, it has become more apparent that enzyme mixtures need to be customized for each target substrate (depending on the biomass type and the pretreatment method it has been subjected to), just as in nature (see Insights into the Network of Lignocellulolytic Redox Reactions, above).

Enzymes may be produced on site (312) or purchased from commercial enzyme producers (315, 316). In this respect, one has to keep in mind that the composition of hitherto available commercial enzyme blends has been optimized under conditions which may be suboptimal for the given application. As an example, Novozymes launched a hemicellulase preparation (Cellic HTec series) to complement their commercial cellulase preparation (Cellic CTec series) to be used on substrates rich in xylan.

In the past years, the rise in awareness of the key role played by oxidative enzymes in biomass conversion has prompted industrialists and scholars to investigate their synergy with canonical (hemi)cellulases. In the following paragraphs, we review the reported effects of LPMOs, lignin-active oxidoreductases, detoxifying enzymes, and CDHs on the conversion of industrially relevant biomasses.

The effect of LPMOs. The performance of cellulolytic enzyme cocktails produced by Novozymes experienced a 10-fold improvement between 2000 and 2012, which in part is due to the inclusion of LPMOs (41, 317). The effect of LPMOs on the efficiency of cellulolytic cocktails has been assessed in several studies. In an early study, before the oxidative nature of LPMO activity was described, Harris et al. had demonstrated that the secretome of an engineered *T. reesei* strain expressing the GH61A from *Thermoascus aurantiacus* (today known as *TaAA9A*) reduced the enzyme loading needed to reach 90% conversion of pretreated corn stover 2-fold compared to the level with a natural *T. reesei* secretome (19). Also, a relative increase of 30% in hydrolysis yield (from 69% to 89%) was measured. Interestingly, this positive effect was not observed on pure cellulose (19), which, with hindsight, can be explained by the lack of reducing power to drive the LPMO reaction (in pretreated corn stover lignin fulfills this role). The results obtained by Harris et al. were similar to the results obtained with CBP21 in 2005, when LPMO activity was first observed in studies of a chitinolytic enzyme system (10, 318).

Subsequent to the discovery of the LPMO activity, including the need for reducing power and oxygen (11), several studies assessed the effects of supplementing enzyme cocktails with LPMOs. In an early study, Cannella et al. noted a 25% difference in the saccharification efficiency of pretreated wheat straw in a comparison of LPMO-deficient Celluclast and LPMO-containing Cellic CTec2 (84). In 2014, Hu et al. showed that spiking of Celluclast with *TaAA9A* increased hydrolysis yields for corn stover, poplar, or lodgepole pine by up to 25%, depending on the pretreatment method employed (319). In 2015, Müller et al. showed that supplementation of a mixture of Celluclast and Novozym 188 (a β -glucosidase) with *TaAA9A* improved glucose yields by up to 32% (i.e., from 64% to 85% of maximum theoretical yield) for steam-exploded birchwood (320), whereas a similar study later showed a 22% relative increase (from 63% to 77%) for sulfite-pulped Norway spruce (321). Importantly, these beneficial effects were not observed under anaerobic conditions or in the absence of sufficient reducing power (320, 321). In 2016, Scott et al. also showed the beneficial effect of *TaAA9A* addition to a mixture of Celluclast and β -glucosidase on the conversion of steam-exploded wheat straw, leading to a 43% relative increase in saccharification yield (from 39% to 56%) (322). Another study has shown that addition of an AA9 from *Penicillium oxalicum* to a

commercial cellulase cocktail from the same fungus increased cellulose conversion by 33% (from 67% to 89% yield) and 31% (from 63% to 82%) for alkali-pretreated wheat straw and corn stover, respectively (323). The effect of the LPMO was less when washed substrates were used, which is likely due to a lack of reducing power (323).

LPMOs are estimated to make up ca. 15% (wt/wt) of the composition of Cellic CTec2 from Novozymes (320). Interestingly, Hu et al. have shown that the amount of LPMO (AA9) required to reach optimal hydrolysis depends on the biomass concentration, with high-solid loading requiring less LPMO (324). This important finding likely relates to the most recent discoveries on LPMOs that, among other things, show that activated LPMOs must bind to substrate in order to avoid self-destructive off-pathway processes (32, 34, 44). From our own unpublished work, we have strong indications that, due to unfavorable process conditions, only a fraction of the LPMOs in commercial cellulase cocktails is actually used, whereas a large fraction is subject to oxidative self-inactivation (see below).

Although the beneficial effect of hemicellulases on biomass conversion has been studied for decades, the potential synergy between hemicellulases and oxidative enzymes such as LPMOs is, for obvious reasons, a fairly recent matter of investigation. As alluded to above, LPMO activity on various hemicelluloses (xyloglucan, glucomannan, and xylan) has been detected. The impact of these hemicellulolytic activities on biomass processing and the extent of direct synergies between hemicellulases and hemicellulose-active LPMOs in hemicellulose conversion remain largely unknown. These effects are difficult to study because of multiple enzyme activities and synergistic effects that are hard to deconvolute unless one uses an extensive analytical toolbox (for example, simple measuring of reducing sugars does not show which reactions are actually happening). Another complication lies in the fact that complex formation between cellulose and hemicelluloses may affect the activity of LPMOs on each of the components. For example, the first xylan-active LPMO ever described showed this activity only for xylan that was grafted onto cellulose (49). Studies with mixtures of phosphoric acid-swollen cellulose (PASC) and hemicelluloses have shown that hemicelluloses restrict enzyme access to the cellulose and that LPMO activity on these hemicelluloses increases cellulose conversion (50, 51).

It has been shown that coinubation of an LPMO, AA9, from *Chaetomium globosum* (CgAA9) with a xylanase led to a 30% increase in reducing sugar yield from an insoluble xylan preparation compared to level with the LPMO-free reference reaction (325). The same authors also showed that addition of CgAA9 to Celluclast led to a 10% to 20% increase in solubilized reducing sugar obtained from pretreated rice straw. Another recent study showed that a mixture of a xylanase (GH10A) and an LPMO (AA9A) from the fungus *Gloeophyllum trabeum* (GtGH10A and GtAA9A, respectively) improves the performance of Celluclast (final ratio of Celluclast/GtGH10A/GtAA9A of 60:20:20, wt/wt/wt) in the saccharification of wheat straw (326). Interestingly, the presence of the GH10 was necessary to unlock the full potential of the AA9. Notably, these interesting studies remain somewhat inconclusive since LPMO products were not analyzed, and it is thus not clear whether the observed effects are due, at least in part, to oxidative cleavage of xylan.

A potentially major breakthrough came in 2018 when Couturier et al. described the first member of a new LPMO family, AA14 (25). This AA14 acts specifically on copolymeric assemblies of cellulose and xylan, cleaving xylan chains that glue cellulose fibrils together and likely promoting the dissociation of hemicellulose from cellulose. While the studied enzyme was only a minor component of the fungal secretome when the fungus was grown on pine and poplar, it played a significant role in the overall biomass conversion efficiency. It will be exciting to see if additional LPMOs specifically acting on certain copolymeric structures in plant cell walls will be discovered and to what extent these enzymes can improve the efficiency of cellulase cocktails.

The effect of lignin-active oxidoreductases. Lignin constitutes a hurdle for efficient biomass conversion into fuels and chemicals, and, in spite of recent progress (327), biorefineries have thus far overcome this issue mainly by using harsh thermochemical

pretreatments. As discussed above, redox enzymes such as lignin peroxidases (AA2) or laccases (AA1) could be part of oxidative pretreatment strategies, and such lignin-active oxidoreductases may be beneficial when added to commercial enzymatic cocktails (328). To the best of our knowledge, the potential of such redox enzymes has not yet been harnessed in industrial settings, which is likely due in part to the inherent complexity of redox enzyme systems, which require cofactors and are subject to damaging off-pathway redox processes.

A few recent studies have addressed the possible contribution of such redox enzymes at lab scale. Although laccases have been studied for decades (mainly on model substrates), their effect on real biomass decomposition is still poorly understood. Laccases have been used as a delignifying pretreatment tool (329) and/or for decreasing the toxicity of lignin-derived compounds, which could hamper enzymatic saccharification and/or subsequent fermentation steps (330–332). Regarding the direct addition of laccases into cellulolytic cocktails, Rytioja et al. found a 1.5-fold increase in reducing sugar yield (from 5.3% to 8%) when a fungal laccase (from *M. thermophila*) was added to a mix of cellulases acting on sugar beet pulp while no effect was observed on lignin-rich wheat bran (333) (Note the low yields, which are due to the absence of pretreatment.) Very recently, Singh et al. studied the effect of a bacterial laccase (from *Amycolatopsis* sp. strain 75iv3) on saccharification of steam-exploded poplar by Celluclast and found an 8% increase in cellulose hydrolysis yield (from 61% to 66%), which they ascribed to the lignin depolymerization action of the laccase and/or to its detoxification effect (334). On the other hand, Brenelli et al. found that addition of laccases from *M. thermophila* (MtL) or *Trametes villosa* (TvL) had a negative effect on the efficiency of the LPMO-containing Cellic CTec2 cocktail during conversion of various biomasses, with a 49% decrease in hydrolysis yield on steam-exploded sugarcane bagasse (162). Interestingly, the addition of laccases led to considerable consumption of O₂, which may inhibit LPMO action and may explain the lower glucose yields. Laccases are indeed relatively efficient O₂ consumers (Fig. 3; see also Table S1 in the supplemental material). It would be interesting to supply laccase-containing reaction mixtures with H₂O₂, which is a cosubstrate for LPMOs but not for laccases.

All in all, while effects of adding laccases to cellulolytic enzyme cocktails have been observed, the nature of these effects remains largely unknown. The effects may vary from detoxification effects to a direct effect on lignin structure.

Lignin peroxidases have mainly been explored as oxidative pretreatment tool (see subsection “Oxidative Pretreatments,” above). Studies assessing the potential synergy between peroxidases and cellulolytic cocktails are scarce. A patent from Novozymes (335) describes the beneficial effects of peroxidases that are ascribed to the removal of excess H₂O₂ that may induce oxidative damage. In light of current knowledge, this concept likely needs some revision since peroxidases will compete with LPMOs for H₂O₂ (32, 44). In a rare public report, Salvachúa et al. showed that the addition of a fungal peroxidase (DyP from *Irpex lacteus*) to a cellulase cocktail (Celluclast complemented with commercial β-glucosidase, β-xylosidase, and xylanases) improved saccharification of wheat straw to an extent (between 16% and 41% relative increase) that was dependent on whether the biomass had undergone biological pretreatment and/or alkali washing (336). Another study showed that combining a commercial cellulase cocktail from *T. longibrachiatum* with the versatile peroxidase from *P. vitreus*, supplemented with a glucose oxidase as H₂O₂ generator, led to a 14% increase in the yield of released glucose from raw corn stover (from 85% to 97%) (300). In light of recent findings on LPMOs, this effect could also be due to promotion of LPMO activity by the generated H₂O₂.

The effect of prooxidant, antioxidant, and detoxifying enzymes. Prooxidant, antioxidant, and detoxifying (PAD) enzymes include SOD, catalase, and aldo-keto reductase (AKR). These enzymes are thought to be involved in securing the stability of redox systems and, in some cases, in converting potential lignin-derived inhibitory compounds. The (potential) roles of these enzymes in biomass processing are intriguing and complex and have not yet been investigated in sufficient depth.

A study published in 2017 has addressed the role of an AKR from the termite *Coptotermes gestroi* (CgAKR-1) on the conversion efficiency of sugarcane bagasse (337). Beyond a role in the detoxification of yeast fermentation inhibitors (e.g., furfural and 5-hydroxymethylfurfural [HMF]), it was shown that supplementation of Celluclast with CgAKR-1 led to a 33% increase (after 24 h) in reducing sugar yields during saccharification of phosphoric acid-pretreated sugarcane bagasse. Interestingly, CgAKR-1 catalyzed the NADPH-dependent reduction of O₂ to H₂O₂, and the improvement in saccharification was correlated with H₂O₂ generation. The authors of this study proposed that H₂O₂ was involved in oxidation of the lignin fraction, which could improve substrate access for cellulolytic enzymes (337). An effect on LPMO activity could also be envisaged although LPMO concentrations in Celluclast are low (320).

Scott et al. have demonstrated that the addition of a catalase to the Cellic CTec3 cocktail decreases the rate of enzyme inactivation during conversion of pretreated wheat straw with, however, only modest effects (ca. 10% relative increase) on final glucose yields (322). They found a larger catalase effect when working with a cocktail of Celluclast (80%), TaAA9A (10%), and β -glucosidase (10%); in this case, catalase addition increased the final glucose yield from ca. 56% to 68% of the theoretical maximum (i.e., a 20% relative increase) (322). Based on these and additional observations for reaction mixtures with added H₂O₂, the authors proposed that the catalase protects the cellulolytic enzymes from getting inactivated by H₂O₂-derived species (e.g., hydroxyl radicals). H₂O₂ was proposed to originate from biotic and abiotic reactions, and its production/accumulation was proposed to depend on parameters such as O₂ availability, temperature, pH, dry matter, and metal content (for further discussion, see below and "The housekeeping role of catalases," above). Of note, such a beneficial effect of catalase addition had been observed as early as 1992 in a study of the synergism between *P. chrysosporium* CDH (PcCDH) and a cellulase mixture (338). In this study, the inhibitory effects of high concentrations of CDH, attributed to high H₂O₂ production rates leading to oxidative inactivation of cellulases, were alleviated by the addition of a catalase, which led to a 25% increase in the hydrolysis yield of microcrystalline cellulose.

Superoxide dismutases (SODs) have received massive attention in the past decades since they convert superoxide, which is toxic for cells, to H₂O₂ that is further reduced by H₂O₂-scavenging enzymes. SODs have been implemented in many health- and cosmetics-related applications (339) but have not made a breakthrough in the field of biomass conversion. However, a few patents (340, 341) mention the use of SOD in enzymatic mixtures for biomass conversion. Although SODs are usually intracellular enzymes, Rashid et al. have recently reported the existence of two extracellular SODs from the bacterium *Sphingobacterium* sp. strain T2 that displays ligninolytic activity (342). The mode of action of these SODs is not established, but the authors proposed that the enzymes may be involved in the generation of hydroxyl radicals acting as lignin oxidants. The role of SOD is complex because the enzyme removes one ROS species, superoxide, while generating another, hydrogen peroxide. As alluded to above, H₂O₂ can engage in a wide variety of reactions, varying from beneficial (improved LPMO activity and well-controlled Fenton chemistry) to highly detrimental. Interestingly, it has been shown that SOD can be beneficial for AA10 LPMO activity under conditions that generate a constant flux of superoxide, an effect that was ascribed to the beneficial effect of continuous *in situ* generation of H₂O₂ (33).

The effect of CDHs. In pre-LPMO times, it was shown that addition of PcCDH to a mixture of cellulases from *Trichoderma viride* promoted saccharification of microcrystalline cellulose (338). Nevertheless, the use of CDH to improve industrial lignocellulose conversion has so far been little discussed, and the patent literature shows seemingly conflicting results (343, 344). While an early patent, from 2010, by Sweeney et al. describes a negative effect of CDH (343), in 2013, Sigoillot et al. claimed that spiking an enzyme cocktail from *T. reesei* with a CDH from the fungus *Pycnoporus cinnabarinus* would lead to an increase in total sugar release (344). In a related publication, Bey et al.

showed that the addition of this CDH to the *T. reesei* GC220 enzyme cocktail (Genecor-Danisco) supplemented with a β -glucosidase (Novozym 188) doubled sugar release from wheat straw (345). The authors suggested that differences in reported CDH effects could be due to differences between the enzymes, such as the absence or presence of a CBM (345). With hindsight, variation in the H_2O_2 production ability and the interplay with H_2O_2 -dependent enzymes present in the enzyme mixture could be seen as another explanation for the observations since H_2O_2 can represent both a beneficial and a harmful factor. A recent study showed that addition of a CBM-free CDH from *Volvariella volvacea* to a secretome from *T. reesei* D-86271 leads to a CDH dose-dependent increase in overall hydrolysis yield for both filter paper and delignified wheat straw (346).

The effect of CBMs. The efficiency of carbohydrate-active enzymes is affected by the presence of carbohydrate binding modules (CBMs) (347, 348). The substrate affinity provided by CBMs brings the catalytic domain into close proximity of the substrate, a property that may be particularly beneficial in the case of hardly accessible and nondiffusible substrates such as the copolymeric plant cell wall. Of note, it has been shown that the strong substrate binding enabled by CBMs becomes less beneficial, and even negative, at higher substrate concentrations, which lead to an increased frequency of enzyme-polysaccharide association events (349, 350). These observations are particularly relevant in industrial settings, where high-solid loadings are desirable.

As of August 2018, 83 families of CBMs have been included in the CAZy database, and LPMOs are appended to several of these (12). While there are many natural LPMOs without a CBM, deletion of the CBM from LPMOs with a CBM is deleterious for enzyme efficiency (56, 79, 80, 351, 352), and this deleterious effect may be substrate dependent (71, 80). Today, there are clear indications that, in the absence of substrate, LPMOs provided with electrons and their oxygen-containing cosubstrate suffer from autoxidation of key amino acids in their catalytic center (32). Substrate affinity provided by attached CBMs may postpone or prevent such oxidative self-inactivation.

Interestingly, a recent study of activation of an AA9 LPMO by a pyranose dehydrogenase (PDH) belonging to the AA12 family (353) showed that deletion of the CBM from this three-domain CDH analogue (AA8-AA12-CBM1) reduced the overall efficiency of the PDH-LPMO system (354). This relatively preliminary result is potentially of great importance because it could reflect a proximity effect. The presence of the cellulose-binding CBM could ensure that reduction of the LPMO and/or production of its cosubstrate H_2O_2 , both catalyzed by the PDH, happen close to the LPMO substrate, thus preventing off-pathway reactions in the LPMO.

The effect of GHs. Since LPMO stability is affected by substrate availability, the interplay between GHs and LPMOs needs attention in the designing of enzyme cocktails. GHs could play an indirect role in stabilizing LPMOs by removing obstacles from the polysaccharide surfaces, including chains already oxidized by LPMOs, and thus increasing the available (crystalline) surface area for LPMO binding. Details of the interplay between GHs and LPMOs remain remarkably unexplored. We predict that increasing insights into LPMO functionality, including LPMO stability, will lead to adjustments in the composition of GHs in enzyme cocktails for biomass processing.

The Impact of Oxygen Dependency on Bioprocessing Strategies

As discussed above, LPMOs are major players in the saccharification of several biomasses of industrial relevance. Until recently, LPMO reactions were thought to be driven by O_2 (only), and this O_2 dependency poses several challenges to industrial application. Aeration, i.e., dissolution and homogeneous dispersion of oxygen, at an industrial scale is expensive. Capital and operational costs for aeration are considered major hurdles for the development of large-scale (e.g., 100 to 1,000 m^3) aerobic fermentation processes (355, 356). As recently underlined by Humbird et al., the aeration-related costs are even more critical for low-margin and high-volume production commodities such as biofuels (357). Furthermore, at an industrial scale, high substrate loadings (>10%, wt/wt) are necessary to lower water consumption and

maximize the sugar concentration after saccharification, leading to viscous and heterogeneous slurries and increasing the technological challenges and costs associated with mixing and aeration (358–360). In the case of lignin-poor biomasses, LPMO action requires not only O_2 but also addition of reducing agents (320, 321), which adds costs. In oxygen-driven processes, reductants need to be supplied in equimolar amounts (two electrons per LPMO reaction [11]). Importantly, the use of O_2 can lead to the waste of valuable reducing equivalents via unproductive, and sometimes harmful, oxidative reactions.

The peroxygenase nature of LPMOs may offer a more cost-efficient and process-friendly alternative for LPMO activation at large scale since, in this case, the cosubstrate is a pumpable liquid, and stoichiometric amounts of reductant are not needed. Indeed, efficient, H_2O_2 -fueled saccharification with the LPMO-containing cocktail Cellic CTec2 has been demonstrated for Avicel, sulfite-pulped Norway spruce, and steam-exploded birch wood (361). Importantly, and well known from work with peroxygenases (362), tight regulation of H_2O_2 supply is required to avoid side reactions that deplete reducing power or inactivate the enzymes (361). In other words, conditions should be such that the concentration of H_2O_2 is kept at a minimum (likely in the low-micromolar range) (32, 34). Indeed, Scott et al. have shown that the batch-wise addition of H_2O_2 to a saccharification reactor, leading to temporarily high H_2O_2 concentrations, has negative effects on overall process efficiency (322).

As an alternative to supplementing external H_2O_2 , H_2O_2 may be generated *in situ*, for example, using glucose oxidase (32), a widely used enzyme in industry (363), or via electrochemical or photocatalytic reduction of molecular oxygen (88, 364). These last approaches, however, would not solve the problem of aeration although the demands for aeration could differ due to different kinetics of the various alternatives.

An important aspect of biomass conversion concerns the main overall strategy adopted to optimize potential synergies and minimize the effects of inhibiting compounds generated during the various processing steps. Two main approaches are usually considered which differ in that the processes of biomass saccharification and microbial conversion of sugars into added-value products are run sequentially (separate hydrolysis and fermentation, or SHF) or simultaneously (simultaneous saccharification and fermentation, or SSF). So far, SSF approaches have been considered incompatible with harnessing the enzymatic power of LPMOs since the fermenting microorganisms either compete for the supplied O_2 or require anaerobic conditions (365, 366). Activation of LPMOs with H_2O_2 , at low, nonharmful concentrations, under anaerobic conditions opens up new possibilities for SSF.

While the H_2O_2 -driven saccharification of a relatively clean substrate such as Avicel works well, with seemingly stoichiometric incorporation of the cosubstrate into oxidized sugars (32, 44), the situation becomes more complicated when complex biomass is used. Lignin-containing biomass contains lots of redox-active components that may engage in reactions with H_2O_2 , as clearly shown in a recent study by Müller et al. (361).

Finally, one issue for future studies concerns the temporal orchestration of enzyme additions. Inspired by extensive knowledge of glycoside hydrolases (367) and LPMOs and by increased knowledge of natural strategies (see Insights into the Network of Lignocellulolytic Redox Reactions, above), the impact of simultaneous or sequential and timely addition of various enzyme types at different stages of biomass conversion may be worth (re)investigating. Orchestrating the concert of oxidative and hydrolytic activities will certainly be one of the future challenges on the road toward more efficient and sustainable processes of biomass conversion.

CONCLUDING REMARKS

The controlled decomposition of biomass in general and of lignocellulose in particular involves a wide diversity of enzymatic activities and chemical reactions, which are probably not all identified yet and whose interconnections are far from clear. Here, we have reviewed enzymes, processes, and possible interconnections while focusing on LPMOs and the potentially central role of hydrogen peroxide. Based on the discovery

of the peroxygenase activity of LPMOs (32, 33) and subsequent supporting studies (34, 43, 44, 74), we have revisited established views on the LPMO paradigm and provided alternative H_2O_2 -based interpretations of literature data.

The question as to what extent LPMO activity directly driven by O_2 is relevant remains open. It is worth noting the history of the discovery of GH mechanisms. Initially, GHs were thought to employ one of two main mechanisms (368, 369), but as research progressed, a variety of family-dependent mechanistic features was discovered (370–372). There may be mechanistic variations and not yet discovered alternative mechanisms within the LPMO superfamily. In the case of GHs, variations in mechanism are often related to the nature of the target substrate. The same could very well apply to LPMOs, especially since current (limited) data indicate that binding of the two substrates (i.e., the polysaccharide and the oxygen-delivering cosubstrate) is interdependent (34, 373). Considering the importance of ternary complex formation and the effect of substrate binding on the confinement of the otherwise exposed catalytic center (45), it is possible that substrate-dependent variations in the catalytic mechanism occur, as also recently suggested by Simmons et al. (57).

Regardless of the true mechanism(s) of LPMOs, the efficient use of H_2O_2 by fungal LPMOs (AA9) is compatible with their ecological context. Above, we have stressed the Janus-type role played by H_2O_2 in lignocellulose conversion, being a product of oxidases and of the oxidation of organic compounds, being a substrate of peroxidases, peroxygenases, and catalases or in Fenton-type reactions, but also being a potentially damaging entity, primarily through its reaction with transition metals. Several authors have pointed out the intriguing genomic cooccurrence and coexpression of H_2O_2 -generating enzymes (e.g., strict oxidases) along with GHs and LPMOs in the absence of classical, known H_2O_2 sinks. Considering H_2O_2 -driven catalysis by LPMOs, which are abundantly encoded in genomes and abundantly expressed during growth on biomass, the cooccurrence of these enzymes now has a plausible explanation. It appears reasonable to speculate that, in order to tame ROS, dedicated enzymatic tools have evolved to deal efficiently with the respective chemistries of their reduction, resulting in optimized cooperation between enzymes. While oxidases, often FAD-dependent enzymes, appear equipped to deal with the reduction of O_2 , to provide *in fine* H_2O_2 , LPMOs are clearly much more efficient in reducing H_2O_2 than in reducing O_2 directly.

The role of nonenzymatic entities such as phytophenolics in the redox processes occurring during biomass conversion is undeniable and highly relevant, both biologically and industrially (19, 83, 86, 374, 375). For example, it is clear that lignin and fragments thereof affect LPMO catalysis. Furthermore, it is obvious that ROS such as H_2O_2 will react with some of these nonenzymatic redox entities. Finally, the role of seemingly uncontrollable Fenton chemistry remains intriguing. Integrating these chemical aspects, which do not appear from omics studies, into our understanding of lignocellulolysis constitutes a considerable future challenge.

Another future challenge concerns the need for more insight into spatial and temporal aspects. The impacts of simultaneous versus sequential decomposition and the temporal and spatial regulation of enzyme expression still need more attention (261). Another remaining issue concerns the possible existence of backup mechanisms, as illustrated by the study of a Δcdh strain of *Podospora anserina*, whose growth was not affected due to the activation of alternative strategies (111) (see above for details).

The remarkable diversity of H_2O_2 -generating enzymes encountered during lignocellulose conversion raises interesting questions. As pointed out above, the widely accepted idea that these enzymes are general partners of peroxidases may need revision. Notably, if general H_2O_2 production, rather than substrate oxidation, is the main purpose of these enzymes, why then are there so many different enzymes employed to generate an identical product? Noting the wide variation in substrate specificity among these enzymes, which act on a variety of (hemi)cellulose- or lignin-derived compounds, one may wonder if this wide spectrum of H_2O_2 -producing enzymes has evolved to ensure that H_2O_2 is produced only at specific locations, i.e., locations where the oxidase substrate is present and an enzyme such as an LPMO or a

peroxidase is ready to use the produced H_2O_2 . Such a strategy would reduce hazardous bulk production of H_2O_2 and satisfy the need for proximity between redox partners, minimizing undesired competition between enzymes and off-pathway reactions. Such proximity effects may be crucial for optimizing enzyme system efficiency and may also be achieved by appending CBMs to interacting redox partners, as alluded to above (354).

H_2O_2 is a ubiquitous molecule. It is a relatively stable carrier of reducing equivalents and of oxygen atoms and is sometimes referred to as a master hormone, notably in plant metabolism (220). Compared to atmospheric O_2 , which constitutes a hardly controllable and infinite input in air-exposed environments, H_2O_2 is a liquid compound offering a much wider concentration range that nature can tightly regulate. This is illustrated by the wide diversity of H_2O_2 -using enzymes, with catalytic efficiencies and substrate affinities spanning several orders of magnitude. Interestingly, within the context of biomass conversion, Robert Blanchette pointed out in 1991 that oxygen concentrations are extremely low within decaying tree trunks, leading to the suggestion that “some mechanism must be operative that delivers necessary oxygen for oxidative reactions involved in lignin degradation” (272). In ancient times, before the Great Oxidation Event (GOE; controversially dated around 2,450 and 800 million years ago [376]), H_2O_2 was highly abundant on Earth while O_2 was not (377). This has led to the suggestion that P450 cytochromes were initially peroxygenases and that their monooxygenase activity evolved later (378). LPMOs are ancient enzymes, putatively already in use more than 400 million years ago (153, 379). Given their ability to harness H_2O_2 , one may wonder if LPMOs were already present in pre-GOE times, and, if so, on which substrate they were acting. Cytochrome P450s use H_2O_2 only at high (millimolar) concentrations and with low total turnover numbers, in what is referred to as a shunt reaction that is a putative remainder of ancestral function. In contrast, LPMOs operate in an efficient and stable manner when supplied with low concentrations of H_2O_2 .

The regulation of the network of lignocellulolytic reactions, involving hydrolases, oxidoreductases, other enzymes, and nonenzymatic entities, is essential for the stability and efficiency of microbial systems and, thus, for the global carbon cycle. Next to the discovery and understanding of individual players, there is a clear need to better understand their interconnection in a biological situation, which eventually may inspire the design of improved, substrate-adapted industrial processes. We hope that the present overview of known and potential players in lignocellulolysis will help in targeting future research in this field.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/MMBR.00029-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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