



Lytic Polysaccharide Monooxygenases (LPMO) Fongiques : Mise en oeuvre en bioraffinerie et découverte de nouvelles enzymes aux propriétés atypiques

Jean-Lou Reyre

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"Lytic Polysaccharide Monooxygenases (LPMO)" Fongiques : Mise en œuvre en bioraffinerie et découverte de nouvelles enzymes aux propriétés atypiques

Fungal Lytic Polysaccharide Monooxygenases (LPMOs): From their implementation in biorefinery to the discovery of new atypical members

Thèse de doctorat de l'Université Paris-Saclay

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Titre : "Lytic Polysaccharide Monoxygenases (LPMO)" Fongiques : Mise en œuvre en bioraffinerie et découverte de nouvelles enzymes aux propriétés atypiques

Mots clés : Biomasse, Bioraffinerie, LPMO, Champignons filamenteux, Cellulose, Métalloenzymes

Résumé : La biomasse végétale représente un réservoir abondant de carbone renouvelable, stocké sous forme de polysaccharides imbriqués dans une matrice complexe constituant la paroi cellulaire végétale. Dans le contexte de la bioéconomie, le concept de bioraffinerie vise à extraire et valoriser ce carbone, notamment via l'utilisation de cocktails enzymatiques d'origine fongique. Cependant, l'efficacité de ces cocktails sur les biomasses les plus récalcitrantes reste encore limitée impactant la viabilité économique des procédés de bioraffinerie. La découverte des « Lytic Polysaccharide MonoOxygenases » (LPMO), des métalloenzymes accélérant la conversion de la biomasse, a permis une avancée majeure dans l'efficacité de ces cocktails. Cependant, leur mécanisme réactionnel complexe ainsi que leur diversité de spécificité de substrats rend le contrôle de leur activité difficile à mettre en œuvre dans des procédés industriels. Des millions d'années d'évolution ayant conféré aux champignons filamenteux une maîtrise quasi inégalée dans la

dégradation enzymatique de la biomasse, apprendre de ces systèmes biologiques représente une approche prometteuse. Les objectifs de cette thèse furent doubles : Dans un premier axe, afin de mieux contrôler l'activité des LPMO et ainsi d'améliorer les rendements de conversion de plusieurs biomasses récalcitrantes d'intérêt industriel, nous avons étudié l'effet de l'addition d'un partenaire biologique des LPMO, la cellobiose déshydrogénase, sur l'efficacité de cocktails enzymatique reconstitués. En parallèle, dans un deuxième axe, afin d'identifier de nouvelles LPMO à fort potentiel industriel, nous avons étudié des LPMO atypiques sécrétés par des champignons dégradeurs de bois, à savoir des Polyporales. De manière imprévue, ce dernier axe a abouti à la découverte de propriétés atypiques jusqu'alors inconnues du domaine des LPMO, questionnant la nature de leur véritable fonction biologique, allant peut-être au-delà de la conversion de biomasse végétale.

Title : Fungal Lytic Polysaccharides Monoxygenases (LPMOs): From their implementation in biorefinery to the discovery of new atypical members

Keywords : Biomass, Biorefinery, LPMOs, Filamentous fungi, Cellulose, Metallo-enzymes

Abstract : Plant biomass represents an abundant reservoir of renewable carbon, stored in the form of polysaccharides embedded in a complex matrix constituting the plant cell wall. In the context of the bio-economy, the biorefinery concept aims to extract and valorize this carbon, notably through the use of enzymatic cocktails of fungal origin. However, the effectiveness of these cocktails on the most recalcitrant biomasses is still limited, impacting the economic viability of biorefinery processes. The discovery of Lytic Polysaccharide Monoxygenases (LPMOs), metallo-enzymes that boost biomass conversion, has led to a major advance in the effectiveness of these cocktails. However, their complex reaction mechanism and diverse substrate specificities hamper the control their activity in industrial processes. With millions of years of evolution, filamentous fungi are

the master of the enzymatic biomass degradation, and learning from these biological systems represents a promising approach. Thus, the objectives of this thesis were multiple. First, to better control LPMO activity and thus improve conversion yields of several recalcitrant biomasses of industrial interest, we investigated the additional effect of cellobiose dehydrogenase, a well-know LPMO biological partner, on the efficiency of reconstituted enzymatic cocktails. Then, to identify new LPMOs with high industrial potential, we studied atypical LPMOs secreted by fungal wood-decayers, namely Polyporales. Unexpectedly, the latter led to the discovery of atypical properties hitherto unknown in the field of LPMOs, questioning the nature of their true biological function, which may go beyond the conversion of plant biomass.

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List of abbreviations

Enzymes/Microorganisms

LPMO : Lytic polysaccharide monooxygenases
AA: Auxiliary activities
CDH: Cellobiose dehydrogenase
HRP: Horseradish peroxidase
EG : *Endo*-glucanase
CBH : Cellobiose hydrolase
BG : β -glucosidase
Pa : *Podospira anserina*
Tr: *Trichoderma reesei*
Cv: *Collariella virescens*
Nc: *Neurospora crassa*
Ls: *Lentinus similis*
Af: *Aspergillus fumigatus*
Mt: *Myceliophora thermophila*
Ch: *Crassiparpon hotsonii*
Tlj: *Trametes ljubarskyi*
Tel: *Trametes elegans*
Pco: *Pycnoporus coccineus*
Cci : *Coprinopsis cinerea*
Ila: *Irpex lacteus*
Sco: *Schizophyllum commune*
TrCC: *Trichoderma reesei* cellulases minimal cocktail

Substrates/products

PASC: Phosphoric acid swollen cellulose
ATP: Adenosine triphosphate
cAMP: Cyclic adenosine monophosphate
TNP-ATP: 2',3'-O-Trinitrophenyl-ATP
NADPH: Nicotinamide adenine dinucleotide phosphate
PCW: Plant cell wall
SBS: Sulfite-pulped spruce
SEB: Steam-exploded birch
YPD: Yeast Extract–Peptone–Dextrose
BMGY: Buffered Glycerol- complex Medium
BMMY: Buffered Methanol- complex Medium

Methods

HPAEC-PAD: High-performance anion-exchange chromatography coupled with pulsed amperometric detection
MS/MS: Tandem mass spectrometry
UHPLC: Ultra High-Performance Liquid Chromatography
PELE: Protein energy landscape exploration
MSA: Multiple sequences alignment
MW: Molecular weight
SE: Steam explosion
AFEX: Ammonia fiber expansion

Other

PTM: Post-translational modification
P-Loop: Phosphate binding loop

List of papers and contributions

Paper I (Chapter I)

Reyre, J.-L.; Lechar, A.; Margeot, A.; Arragain, S.; Berrin, J.-G.; Bissaro, B. "Implementation of CDH in LPMO-containing reconstituted cocktails to improve bioproducts production from lignocellulosic biomass", *ACS Sustainable Chemistry & Engineering*. In press.

I performed most of the experiments (except for protein production), analyzed the data, designed the figures and drafted of the manuscript.

Paper II (Chapter II)

Reyre, J.-L.; Grisel, S.; Haon, M.; Xiang, R.; Gaillard, J.-C.; Armengaud, J.; Guallar, V.; Margeot, A.; Arragain, S.; Berrin, J.-G.; and Bissaro, B. (2023). "Insights into peculiar fungal LPMO family members holding a short C-terminal sequence reminiscent of phosphate binding motifs", *Scientific Reports*. Vol. 13, No. 1: p. 11586.

I performed most of the experiments (except the proteomic analysis and molecular modelling), analyzed the data, designed the figures and drafted the manuscript.

Paper III (Appendix I)

Vandhana, T. M.; Reyre, J.-L.; Sushmaa, D.; Berrin, J.-G.; Bissaro, B.; and Madhuprakash, J. (2022). "On the expansion of biological functions of lytic polysaccharide monooxygenases", *New Phytologist* (Tansley review). Vol. 233, pp. 2380–2396.

I designed figure 1, wrote the first draft of part II. and contributed to the correction of the final manuscript.

Paper IV (Appendix II)

Yao Assiah, R., Reyre, J.-L.; Tamburrini C. K.; Haon, M.; Tranquet, O.; Nalubothula, A.; Mukherjee, S.; Le Gall, S.; Grisel, S.; Longhi, S.; Madhuprakash, J.; Bissaro, B.; and Berrin, J.-G. (2023). "The *Ustilago maydis* AA10 LPMO is active on fungal cell wall chitin", *Applied and Environmental Microbiology*. Vol. 89, e0057323.

I carried out preliminary bioinformatic analysis, performed the phylogenetic analysis, designed figure 1 and contributed to the correction of the final manuscript.

Other publication by the author (Master thesis)

Reyre, J.-L.; Grisel, S.; Haon, M.; Navarro, D.; Ropartz, D.; Le Gall, S.; Record, E.; Sciara, G.; Tranquet, O.; Berrin, J.-G.; and Bissaro, B. (2022). "The Maize Pathogen *Ustilago maydis* Secretes Glycoside Hydrolases and Carbohydrate Oxidases Directed toward Components of the Fungal Cell Wall," *Applied and Environmental Microbiology*. Vol. 88, No. 23: e0158122

I performed the biochemical characterization of the enzymes, I participated to the bioinformatic analysis (structural prediction and phylogenetic analysis), I designed some of the figures and contributed to the writing of the manuscript.

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Introduction

I. Transition toward a bio-based economy

Modern human societies have drastically evolved when they started to exploit carbon-based resources. Indeed, drawing on a raw material resulting from millions of years of accumulation to produce fossil carbon-based fuels has permitted an unprecedented boom in the 20th century industries (e.g. transport, agriculture...). However, as this fossil carbon is neither renewable nor reusable, this linear mode of consumption will ultimately reach its limits. Hence, with the increase of carbon dioxide emissions and the depletion of fossil resources, the dependence on non-renewable, carbon-based fuels has slowly become one of the biggest threats of this century, global warming and its implications being its clearest manifestation.

With these facts in mind, it is now globally admitted that a transition to a circular and sustainable energy production system, often referred to as bio-economy, is needed for the resilience of human societies. In this regard, the scientific community has been working for decades to explore new renewable energy sources comprising plant biomass, solar, hydro, wind, wave, tidal, ocean current, ocean thermal, and geothermal energies. However, because of their current energy and/or economic lower efficiency, all these new energies are not competitive enough to completely substitute the almighty petroleum. Hence, before the quite unlikely advent of an energy source capable of completely supplanting petroleum, the solution will most likely entail the use of various sustainable energies taking into consideration regional resources and technological means. Yet, a brutal and complete shift to a carbon-neutral society seems for now utopian, especially when considering that most of our infrastructures (especially in the transport sector) rely by-design on the use of carbon-based energies. In this regard, a more conceivable approach could be to valorize local and renewable carbon resources in a circular bio-economy context (**Fig. 1**).

From this point of view, plant biomass holds great potential as a renewable and “easy” to access resource, able to fix and metabolize carbon dioxide via photosynthesis. The becoming of this atmospheric carbon mainly consists in the biosynthesis of glucose, which plants store as food/energy source (e.g. starch) or use to build their cells walls (e.g. (hemi)celluloses). Glucose and other simple sugars represent platform molecules that can be transformed into various products, by chemical or biochemical means, and thus valorized in many fields such as food industry, healthcare, biomaterials, and of course biofuels production, so-called biorefinery.

In this regard, one of the first approach in bio-refinery, referred to as “first generation

(1G) biofuels” (Mohr and Raman 2013), was to take advantage of the carbon contained in food crops: (i) from the glucose mainly found under the form of starch (polymer of α -1,4-linked D-glucose units) notably extracted from corn, or from saccharose (a dimer of fructose-glucose) usually extracted from sugar cane or beetroots, then used to produce bioethanol; or (ii) from vegetal oils (e.g. from rapeseeds) to produce biodiesel (**Fig. 1**). However, this strategy has rapidly raised some awareness over a potential “food vs fuel” conflict considering the value of these food crops in human/animal feed and the agricultural lands required for a large-scale biofuel production (Mohr and Raman 2013). Ethical and economical limits of 1G biofuels have led to the development of “second generation” (2G) biofuels, produced from lignocellulosic biomasses such as energy crops (perennial grasses, miscanthus...) and forestry/agricultural co-products (hardwood, softwood, wheat straw, rice straw...). This non-edible biomass, much more abundant than food crops, is yet harder to tackle as sugars are embedded into a complex polymeric network, called lignocellulose, which is recalcitrant to degradation. Being able to achieve high glucose extraction yields from lignocellulose is therefore a major stake for the current field of 2G biorefinery (Zabed et al. 2017; Champreda et al. 2019; Siqueira et al. 2020).

To cope with the recalcitrance of plant cell walls (PCW), and open-up the packed structure of lignocellulose, physicochemical pre-treatments are often needed (Himmel et al. 2007; Zoghlami and Paës 2019). Enzymatic-based degradation processes, mostly involving hydrolytic enzymes, are then used to break glycosidic bonds between two carbohydrates units or between carbohydrate and non-carbohydrate moieties. This approach is inspired from natural processes involving micro-organisms capable of specifically degrading a wide range of plant biomasses. To date, this bio-inspired approach has imposed itself as the most reliable strategy to deconstruct lignocellulose.

Among these micro-organisms, fungi are candidates of utmost interest. Millions of years of co-evolution with plants have provided them with the ability to adapt their lifestyle to a broad range of lignocellulosic substrates, making them true experts of PCW degradation (Hage and Rosso 2021). Many fungal strains have been scrutinized by researchers in the past decades with the aim of developing efficient cellulolytic cocktails. *Trichoderma reesei* is to date one of the most widely used enzyme producer for 2G biofuel production (Ferreira et al. 2014; Berrin et al. 2014).

Numerous genetic improvements and accessory enzymes supplementation have been one of the keys that have allowed the biofuel industry to become more and more competitive, and with the scarcity of oil increasing, the difference between bioethanol and

petroleum barrel price is expected to diminish. However, efforts are still needed to enhance the depolymerization efficiency of the most recalcitrant 2G substrates such as miscanthus/switchgrass and wood. Today, many enzymatic cocktails of fungal origin have been developed (Cellic Ctec2/3®, ACCELLERASE® TRIO™, Dyadic/C1, DSM enzyme preparation...) and some of them are available on the market (Filiatrault-Chastel et al. 2021). Their main differences lie in the organism they are produced from and/or the biomass they target. Yet different, all these cocktails rely on the same core of enzymatic activities, comprising mainly *endo*- and *exo*-acting glycosides hydrolases and, for some of them, oxidoreductases enzymes. Numerous secreted fungal oxidoreductases are capable of targeting PCW degradation products (simple sugars, oligomers, etc.), but the deconstruction of (hemi)-cellulose fibers by oxidative cleavage is mainly orchestrated by lytic polysaccharide Monooxygenase (LPMOs) (Bissaro et al. 2018; Berrin et al. 2017). The LPMO activity was discovered in 2010 (Vaaje-Kolstad et al. 2010) and has been the subject of intense research in the last decade due to its ability to target the recalcitrant part of PCW polymers and thus boosting the saccharification yield of cellulolytic cocktails (Johansen 2016; Chylenski et al. 2019). More recently, new functions have been proposed that deviate to some extent from the original role LPMO were assigned to, i.e. as pure degraders of recalcitrant biomass (Vandhana et al. 2022). Whether it be in an applied biorefinery context or in the fundamental understanding of their new functions, much remains to be done in the field of LPMOs.

The goal of this PhD thesis was to study fungal LPMOs from these two perspectives. First, by coupling them with their well-known enzymatic partner, we tried try to better control LPMOs activity in industrial cellulolytic cocktails to degrade recalcitrant biomass, and secondly, we attempted to gain new insights into their biological roles through structure/function analyses of atypical LPMOs from basidiomycete fungi.

Hereinbelow, I present knowledge available at the beginning of this PhD thesis, divided into four main sections : in section (II), I present the PCW structure; in section (III), I introduce general fungal life-styles and then further detail the arsenal of PCW-degrading fungal enzymes; in section (IV), I focus on LPMOs, drawing up a portrait of our current knowledge on their mechanism and their known enzymatic partners; and in section (V), I introduce 2G biofuel production processes and the model organism *T. reesei*. Following these state-of-the-art sections, I expose the objectives of this PhD thesis project.

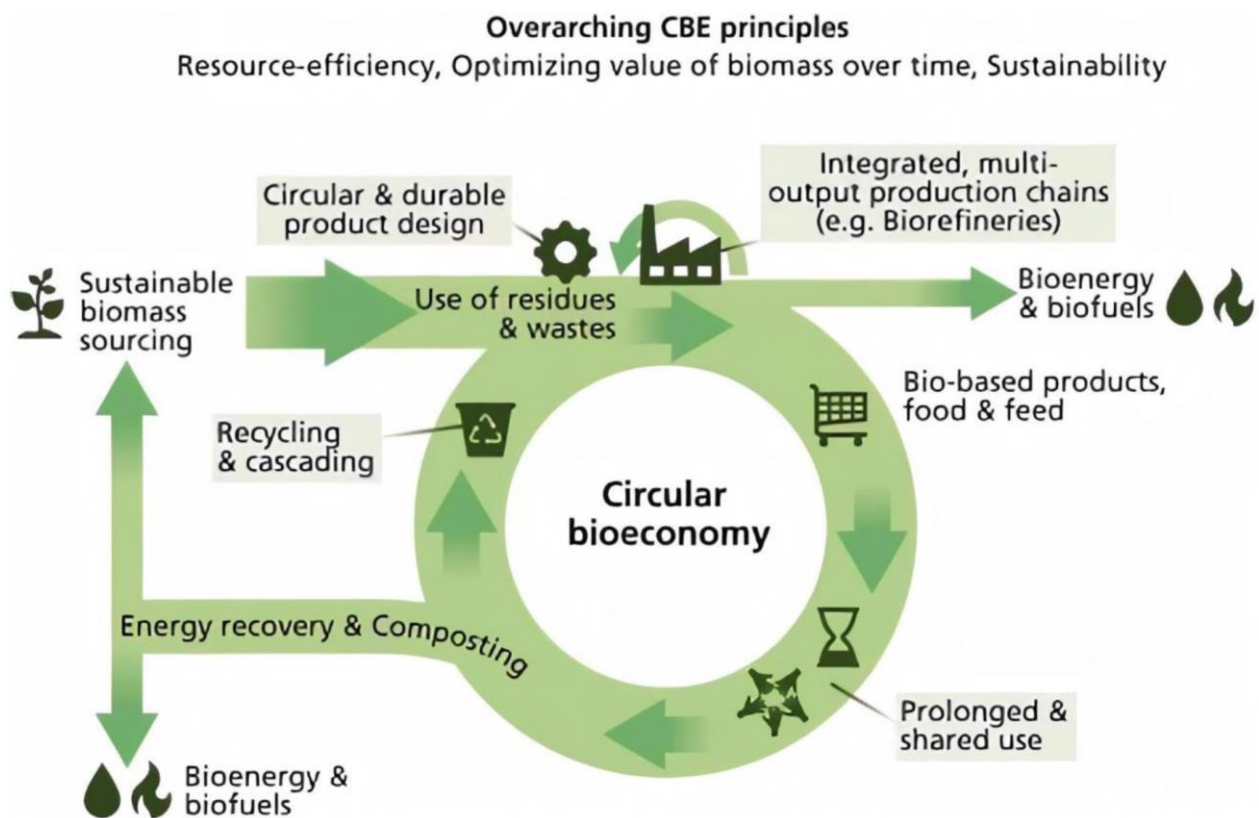


Fig. 1. 2G biofuel production in a circular bio-economy. The figure was reproduced from (Stegmann et al. 2020). CBE, Circular Bio-Economy.

II. The lignocellulose biomass and its diversity

The PCW serves as primary structural framework for plant cells, constituting both a structural support and a protection against biotic (viruses, bacteria, fungi, ...) and abiotic (mechanical, osmotic stress, ...) factors. The PCW exhibits remarkable diversity across plant species, and also varies among different developmental stages and tissues within the same species, sometimes adapted to specific environment (Fangel et al. 2012).

The PCW architecture relies on three main components: cellulose, hemicelluloses, and lignin. Cellulose, which is a linear homopolymer of glucose, forms the scaffold that gives plant cells their mechanical resilience (Somerville et al. 2004). In contrast, hemicelluloses comprise a heterogenous mixture of sugar monomers, often branched, which serve as the matrix connecting cellulose microfibrils (Scheller and Ulvskov 2010). Finally, lignin, a complex and irregular polymer of phenolic moieties, confers rigidity to the PCW and enhances resistance to microbial degradation (Boerjan et al. 2003).

The PCW can be divided into several layers that differ in size and composition. The

first layer to be formed during PCW biosynthesis, so called primary layer, is rich in cellulose, hemicelluloses, and pectin, followed by the middle layer, which is rich in pectin and plays a key role in cell adhesion (Cosgrove 2005). The secondary wall makes up the bulk of PCW and thus represents most of the total mass of the plant. The secondary wall can be divided into three sublayers called S1, S2 and S3, the latter being specific to trees and particularly rich in lignin (Zeng et al. 2017).

An intriguing aspect of PCW lies in its adaptability. Different plant species have evolved unique PCW compositions tailored to their ecological niches. For instance, switchgrass exhibits a cellulose-rich PCW, providing resilience in harsh environment (arid deserts, flood plains, windy coasts...) (Ricketts et al. 2023). On the other hand, softwood from coniferous trees such as pine, features a prominent lignin component, crucial for their growth in temperate climates (Cutter and Guyette 1993; Vanholme et al. 2010). Hardwood, such as poplar, strikes a balance between cellulose and hemicelluloses, enabling versatility in ecological adaptation (Sannigrahi et al. 2010). Understanding this intricate composition is fundamental to unlock the bioenergy potential embedded within these complex natural carbon reservoirs.

The following sections will provide a more extensive description of cellulose, hemicelluloses, and lignin, including their roles and significance in biorefinery processes. Then, the diversity of PCW structure and compositions will be illustrated with a few examples of 2G biorefinery relevant biomasses.

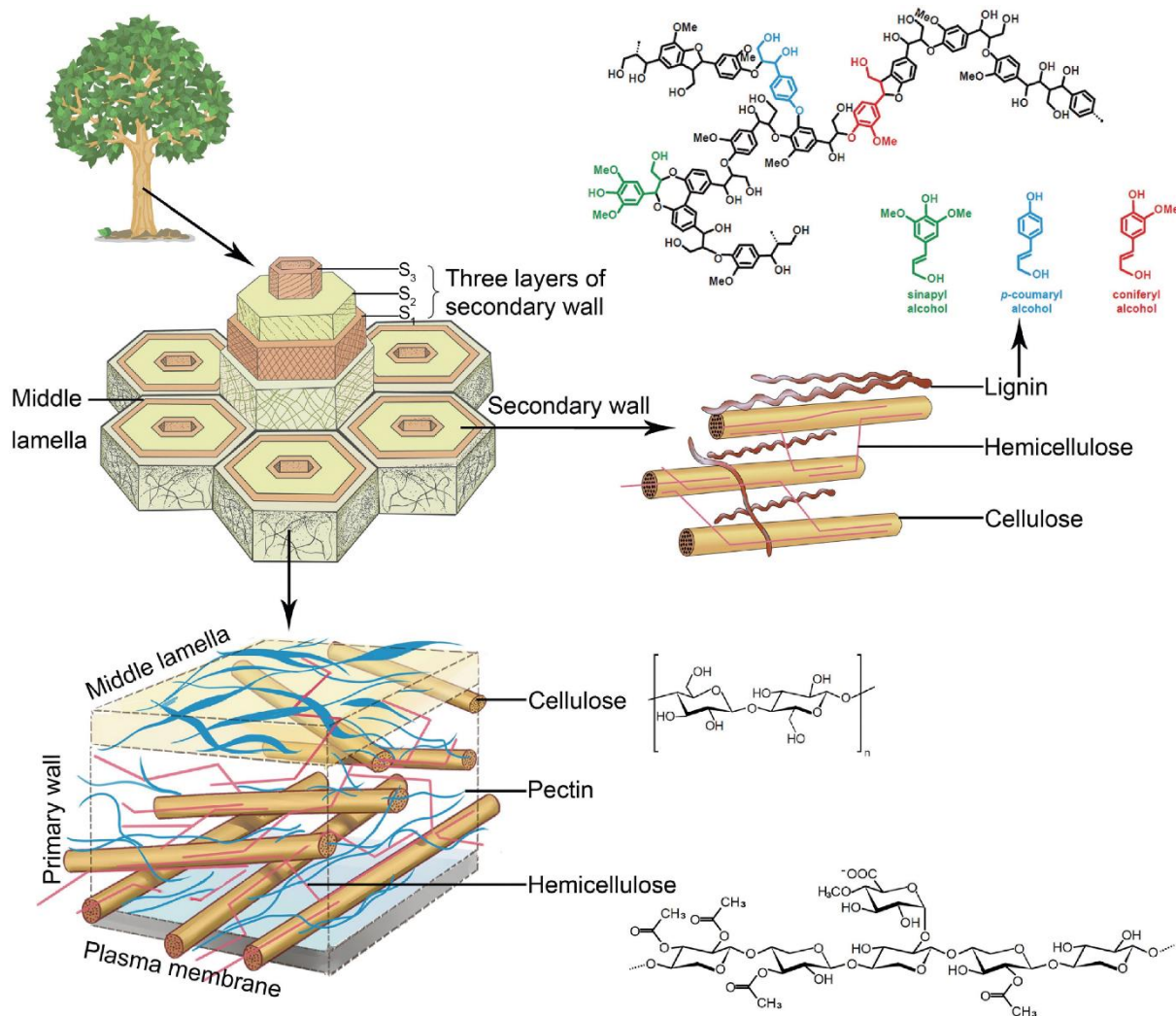


Fig. 2. The structure of the plant cell wall at different scales. The figure was reproduced from (Zhao et al. 2019).

II.1. The main PCW components

II.1.1. Cellulose

Cellulose is the main component found in PCW and is therefore the most abundant biopolymer on earth. This homo-polymer is a linear polysaccharide composed of repeating D glucose units connected by β -1,4-glycosidic bonds (Somerville et al. 2004). In cellulose, each second glucose unit is flipped 180° relative to its neighboring unit, which gives rise to a remarkably recalcitrant linear structure (Brian H. Davison et al. 2013).

As in other polysaccharides, the degree of polymerization (DP) is a crucial structural parameter in cellulose, referring to the number of glucoses units linked together in a linear chain. Cellulose macromolecules are characterized by their exceptionally high DP, typically

ranging from several hundred to several thousand glucose units, depending on the source and isolation method (Baruah et al. 2018). For instance, cotton cellulose can have a DP of around 10 000, while wood cellulose may exhibit a slightly lower DP of approximately 1000 to 2000 (Beck-Candanedo et al. 2005). Each single cellulose chain possesses a reducing end and a non-reducing end.

These individual cellulose chains then align side by side through hydrogen bonding, resulting in the formation of a characteristic linear microfibril structure. Notably, the formation and orientation of cellulose microfibrils can vary between plant species and cell types. Additionally, interactions between cellulose microfibrils and other cell wall components (hemicelluloses, lignin, pectin) occur via hydrogen bonding, van der Waals forces, and electrostatic and hydrophobic interactions (Chami Khazraji and Robert 2013). Cellulose microfibrils structures are not homogeneous as they can adopt two morphologies, including a crystalline and recalcitrant core and more amorphous regions at their surface.

Cellulose exists in three allomorphs, cellulose I, II and III, with slightly different hydrogen bonding patterns and degrees of crystallinity (**Fig. 3**) (Mukarakate et al. 2016). Cellulose I, the native form of cellulose in nature, features parallel cellulose chains held together by both intra- and intermolecular hydrogen bonds resulting in a highly crystalline structure (Nishiyama et al. 2002). Cellulose II and III, on the other hand, are characterized by an altered hydrogen bonding pattern, leading to reduced crystallinity and increased accessibility to enzymatic degradation. Of note, the transition from cellulose I to cellulose II or III is often observed during various cellulose treatments, such as chemical modification (Revol et al. 1992; Mukarakate et al. 2016). For example, to obtain a substrate that is more sensitive to enzymatic degradation, labs usually swollen cellulose I into cellulose II by phosphoric acid pretreatment. We then talk about phosphoric acid swollen cellulose (PASC) (Wood 1988).

The unique properties of cellulose, including its abundance, renewable nature, and the potential to yield glucose through enzymatic hydrolysis, make it of utmost interest in biorefinery processes. To date, the majority of enzymatic cocktails are designed and engineered specifically for the extraction of glucose from cellulose, making it the most coveted PCW compound in biotechnological processes.

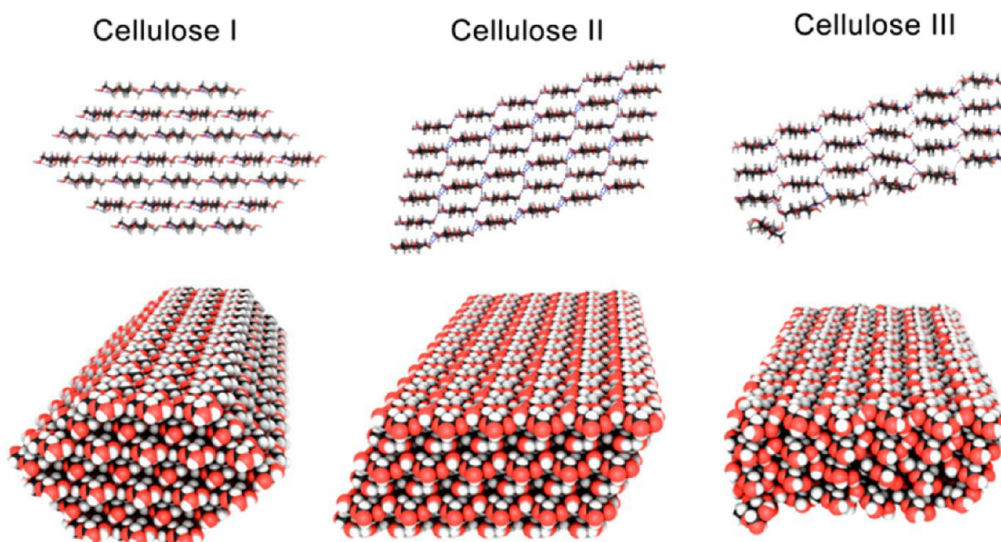


Fig. 3. Molecular models of cellulose allomorphs. Cellulose I is the native form of cellulose in PCW and exhibits two-dimensional intralayer hydrogen-bonding networks. Cellulose II can be generated by ionic liquid treatment and rearranges cellulose chains into antiparallel sheets exhibiting a three-dimensional network of intralayer and interlayer hydrogen bonding. Cellulose III can be generated by treatment with anhydrous ammonia and, like cellulose-II, forms intralayer and interlayer hydrogen bonds, but the chains remain parallel like in cellulose I. Black represents carbon atoms, red represents oxygen, white represents hydrogen atoms, and blue dashed lines represent hydrogen bonds. The figure was reproduced from (Mukarakate et al. 2016).

II.1.2. Hemicelluloses

Hemicelluloses are the second most prominent component of PCW, and are characterized by their complex structure and diverse composition. This heteropolymer is composed of an array of sugar monomers, including pentoses (e.g. xylose, arabinose) and hexoses (e.g. glucose, mannose, and galactose), as well as uronic acids (e.g. glucuronic acid) (Scheller and Ulvskov 2010). Typically, hemicelluloses feature a β -1,4-linked backbone, often branched with various linkage types, further enhancing its complexity (**Fig. 4**). The branching can involve both intra- and intermolecular linkages, resulting in side chains that vary in length and composition. Hemicelluloses can also be linked to other PCW components, notably lignin via ester bonds, contributing to the global recalcitrance of PCW.

The composition and structure of hemicelluloses are highly dependent on the source biomass. Different plant species, and even different tissues within the same plant, can yield hemicelluloses with distinct sugar monomer ratios and branching patterns (**Fig. 4**, see section **II.2.**). This variability contributes to the adaptability and attractiveness of hemicelluloses for various industrial applications (Spiridon and Popa 2008).

Notably, xylose can be fermented into xylitol, a valuable sugar alcohol with

applications in food and pharmaceutical industries (Guirimand et al. 2019; Umai et al. 2022). Beyond biofuels, hemicelluloses can be chemically modified to yield hemicellulosic derivatives with diverse functionalities, such as films, coatings, and hydrogels (Rao et al. 2023). These derivatives have applications in biodegradable packaging materials and pharmaceutical formulations. Moreover, the extraction of valuable hemicellulose-derived compounds, such as arabinoxylans and oligosaccharides, holds promise in the production of functional food ingredients, prebiotics, and dietary fibers (Jana et al. 2021).

However, due to the structural and compositional complexity of hemicelluloses, the enzymatic processes require a more diverse enzymatic arsenal than for cellulose. Conveniently, many hemicelluloses (especially xylan-containing polymers) can be extracted and solubilized from chemical processing such as acid or alkaline pretreatments (Lehuedé et al. 2023). Yet again, harsh chemical conditions may lead to the formation of inhibitors, such as furfural or hydroxymethyl-2-furaldehyde (HMF), impacting subsequent valorization processes.

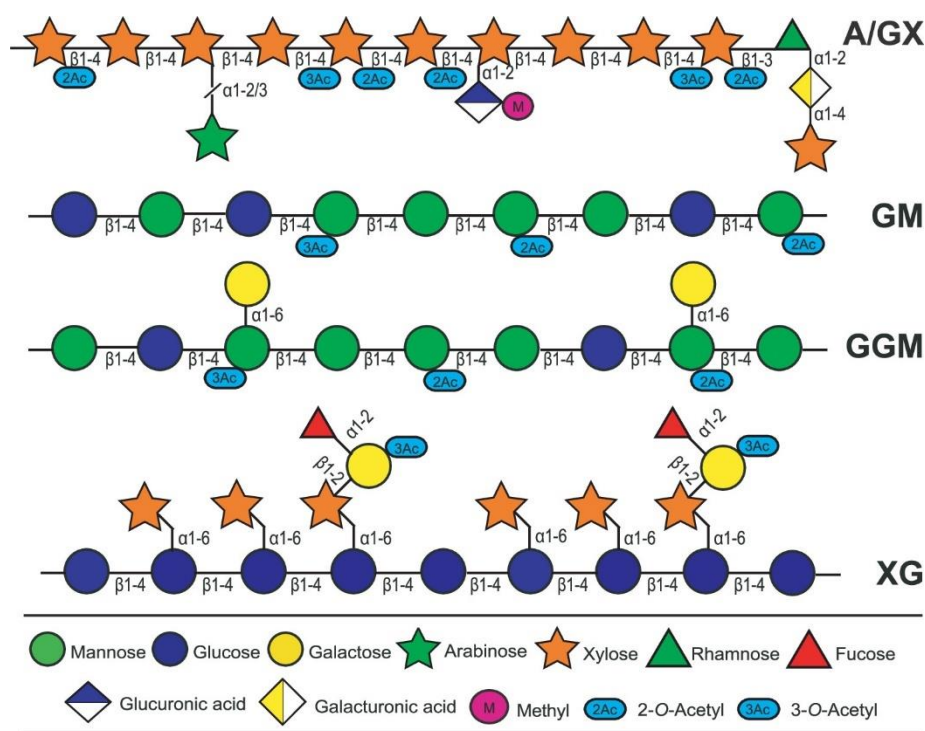


Fig. 4. Structure diversity of hemicelluloses. Arabino/glucuronoxylan (A/GX), glucomannan (GM), galactoglucomannan (GGM) and xyloglucan (XG). α 1-2/3 in A/GX indicates that the arabinose residue may attach to hydroxyl groups at position 2 and/or 3 of the xylose residue. 2-O-Acetyl and 3-O-acetyl indicate that acetylation may occur at the hydroxyl position 2 or 3 of the respective carbohydrate residue. The symbols are sketched according to (Varki et al. 2015). The figure was reproduced from (Abik et al. 2023).

II.1.3. Lignin

Lignin is a complex and irregular polyphenol polymer. It is particularly known to contribute to the PCW rigidity and resistance to microbial degradation. Lignin is primarily composed of three monolignols: G (guaiacyl), S (syringyl) and H (p-hydroxyphenyl) alcohols, which undergo oxidative coupling to form a heterogeneous three-dimensional network (**Fig. 5**, (Boerjan et al. 2003)). In addition to monolignols, caffeyl alcohol and 5-hydroxyconiferyl alcohol can be incorporated into the lignin polymeric structure. As mentioned before, the free hydroxyl groups of lignin can form cross-linkages with hydroxyl/carboxyl groups of hemicellulose components via ester bonds (so-called Lignin-Carbohydrate complexes) (Larsbrink and Lo Leggio 2023). The composition and linkage types of lignin vary among plant species and tissues, leading to diverse structures with unique properties.

It is important to note that one of the unique properties of lignin is its high reactivity with oxygen species. For example, lignin can reduce molecular oxygen to H_2O_2 and reduce H_2O_2 to H_2O (Bissaro et al. 2018). Because it is not a sugar polymer, the enzymes active on lignin differ from those active on cellulose and hemicelluloses. These enzymes comprise mainly oxidoreductases such as laccases and peroxidases that cleave the ester bonds of phenolic compounds through indirect (and possibly direct) oxidative mechanisms (Vrsanska et al. 2016).

Interestingly, the impact of lignin on biomass degradation is discordant. It can for instance have a positive effect by acting as an electron donor for LPMOs (see section **IV.3.1.**), but its derivatives can also act as inhibitors of cellulases, meaning that the presence of lignin affects biomass degradation processes in biorefinery (Fong et al. 2016).

As such, the fate of lignin in biorefinery is traditionally to be combusted to generate heat and power or used as a low-value fuel source. However, increasing efforts are directed toward valorizing lignin into higher-value products (Yu et al. 2023). One approach involves depolymerizing lignin into bio-based chemicals or platform molecules, such as vanillin, syringol, or aromatic monomers, which can be further converted into valuable materials, fuels, or chemicals (Ragauskas et al. 2014). Alternatively, lignin can be used in the synthesis of biodegradable polymers, adhesives, and resins, contributing to the development of sustainable materials (Mili et al. 2022).

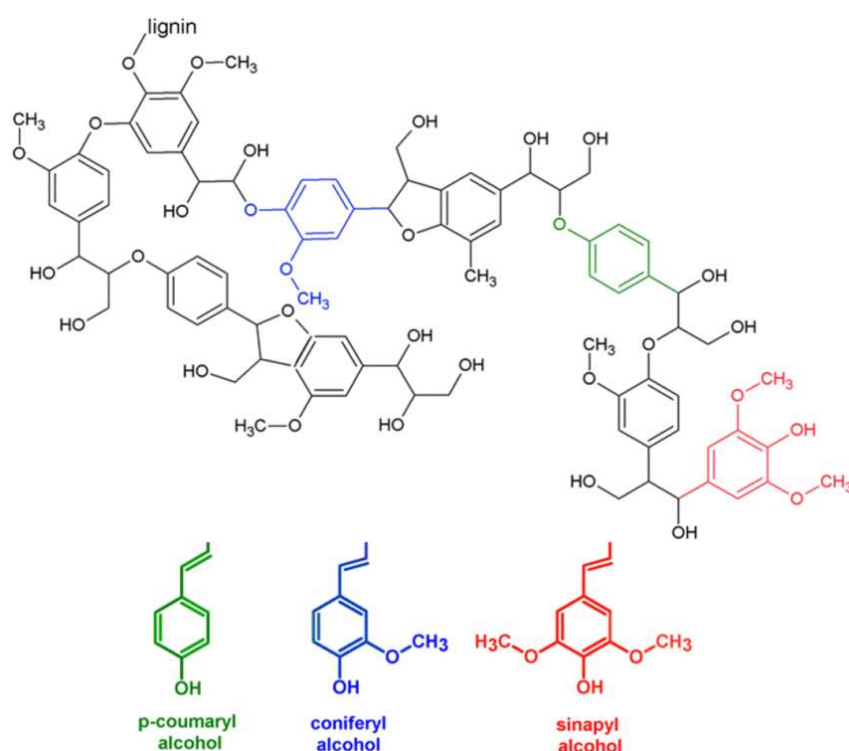


Fig. 5. A representative structural model of lignin. The figure was reproduced from (Serrano et al. 2019).

II.2. Diversity of PCW among plant biomasses

The complexity and wide range of polymers found within PCW give rise to a large possibility of structural arrangements, proportion, and composition. The main differences are (i) the general proportions of cellulose, hemicelluloses, and lignin, (ii) the composition and structure of their hemicelluloses and (ii) the proportion and bonding of lignin units. These differences, which in nature confer special properties to plants in terms of resistance, also give rise to great variability in the performance of cellulolytic cocktails depending on the biomass. Here, we illustrate this versatility of some of the biomasses used in 2G biorefineries.

II.2.1. Softwood

Softwoods, including well-known tree species such as pine, spruce, and Douglas fir are representatives of gymnosperms and are characterized by their coniferous needle-like or scale-like leaves. Softwood trees are abundant in temperate and boreal forests and stand out for their distinct characteristics. They typically possess longer, and thinner cellulose

fibers compared to hardwoods, which contribute to their remarkable tensile strength (Khanjanzadeh et al. 2012). This adaptation allows softwood trees to thrive in regions with harsh environmental conditions, making them valuable resources for various applications.

The PCW of softwood species generally include 33 – 42% cellulose, 22 – 40% hemicelluloses, 27 – 32% lignin, and 2 – 3.5% extractives (Tarasov et al. 2018). As with other plants, the main characteristics that distinguish softwoods PCW from other biomasses lies in their hemicelluloses. In softwood, *O*-acetyl-galactoglucomannan is the principal hemicellulose component, consisting in a β -1,4 backbone made of D-glucose and D-mannose units, the latter being substituted at the C6 position with α -D-galactose units (**Fig. 4**, (Dos Santos et al. 2015)). Noteworthy, the D-mannose units can also sometimes be acetylated at the C2 or C3 position (Berglund et al. 2020). Softwood also contains a specific variation of glucuronoxylan in the form of arabino-4-*O*-methylglucuronoxylan which consist of a backbone of β -1,4-linked D-xylose units with partial substitution by both α -1,2-linked methylated glucuronic acid and α -1,3-linked L-arabinofuranose (Berglund et al. 2020). As to lignin, softwoods are characterized by an abundance of (G) units, a low occurrence of (H) units and the absence of (S) units (Suota et al. 2021).

In addition to cellulose, the specific appeal for softwood in biorefinery lies in its high mannose content (15%) compared to other biomasses such as hardwood and grass (< 2%) (Martínez-Abad et al. 2020). Mannose is a sugar of great interest as it can be valorized in cosmetic, pharmaceutical and food industries. Its moisturizing properties also makes mannose-derived products important ingredients in gels manufacturing in cosmetic (Hu et al. 2016). Moreover, mannose can also be easily chemically reduced to mannitol, a compound that is used in a variety of applications in the pharmaceutical industry (Hu et al. 2016). Challenges regarding softwood valorization in biorefinery include optimizing pretreatment methods to balance delignification and hemicellulose preservation, as well as enhancing the mannolytic power of enzymatic cocktails for the complete extraction of the valuable sugars within softwood PCWs.

II.2.2. Hardwood

Hardwoods encompass a diverse group of tree species, predominantly dicotyledonous angiosperms, renowned for their broad leaves. Examples of hardwood trees include oak, poplar, and birch. As suggested by the name, hardwoods are usually stronger and more hard-wearing than softwoods. Their non-seasonal character and the possibility of growing in rural areas makes them interesting in 2G biorefinery (Del Río et al. 2022).

The PCW components proportions in hardwood are rather similar to that of softwood, including 38 – 51% cellulose, 17 – 38% hemicellulose, 21 – 31% lignin, and 3% extractives (Tarasov et al. 2018). Hemicelluloses in hardwoods are dominated by xylan species, notably in the form of glucuronoxylan. Unlike softwoods, its β -1,4- linked xylan backbone is not decorated with L-arabinose but is instead often esterified with acetyl groups. the branched glucuronic acid can also be esterified with *O*-acetyl groups at their C2 or C3 (Sista Kameshwar and Qin 2018). Noteworthy, mannan can also be found in the form of glucomannan in some hardwood species but in a much lower concentration compared to softwood. Lignin in hardwoods also differs from softwoods, characterized by a higher proportion of (S) units, resulting in an S-rich lignin structure (Boerjan et al. 2003).

The interest of hardwood valorization in biorefinery lies in its high cellulose and xylan content, from which glucose and xylose can be respectively extracted. These sugars are usually co-fermented during bioethanol production processes. Since xylose is not optimally fermented by the yeasts commonly used in 2G biorefinery, it can be dehydrated into furfural, hydrated into xylitol, and oxidized into xylonic acid, which have huge market potential (Del Río et al. 2022). Similar to softwood, pretreatment methods and enzymatic cocktails must be further specifically optimized to make the most of the value-added sugars contained in hardwood.

II.2.3. Grasses

While annual grasses like maize have advantages in terms of rapid growth and established cultivation practices, perennial grasses are gaining prominence for their sustainable and high-yield biomass production, making them increasingly attractive for biorefinery applications. Perennial grasses constitute a diverse group of herbaceous plants primarily belonging to the *Poaceae* family, playing a pivotal role in ecosystems. These grasses, which include species like switchgrass, miscanthus, and reed canary grass, exhibit

distinct characteristics in their PCW.

The PCW components vary greatly among grass species, but there are usually characterized by high level of cellulose (35% – 45%) and hemicellulose (40% - 50%) and lower amount of lignin (20%) compared to trees (Hatfield et al. 2016a). Like hardwood, xylan is the major component of perennial grass hemicelluloses, but display specific features that distinguish it from woody biomass. It is mainly found in the form of arabinoglucuronoxylan but with non-methylated glucuronic acid in contrast to softwood. Additionally, arabinoxylan can be specifically found in grass cell wall consisting in a β -1,4- linked xylan backbone substituted with arabinofuranose linked in α -1,2 and/or α -1,3 as well as ester-linked hydroxycinnamic acids such as *p*-coumaric acid and ferulic acid (Vogel 2008; Coomey et al. 2020). The latter are attached by ester linkages to the C-5 carbon of L-arabinofuranosyl residues, and can be cross-linked to lignin, adding to the complexity of grass PCW structure (Hatfield et al. 2016b). Grass lignin is composed of a balanced proportion of (H), (G) and (S) units, but generally contain more (H) than hardwood or softwood (Boerjan et al. 2003).

The main advantage of grasses over other biomasses in biorefineries lies in their high level of fermentable sugars (glucose and xylose) coupled with a lower lignin content than wood, facilitating thereby their pretreatment. Perennial grasses also grow faster than softwood and hardwood, making them a target of choice for biofuels production.

III. Fungi as biomass degrading models

III.1. General portrait of fungi: classification and lifestyle

The fungal kingdom stands as one of the most diverse within the eukaryotic realm (Choi and Kim 2017), with an estimated diversity ranging between 2.2 and 3.8 million species (Hawksworth and Lücking 2017). Fungi are versatile organisms that can adopt various forms, ranging from the edible fungus *Boletus edulis* to the single-cell yeast *Saccharomyces cerevisiae*. These organisms have succeeded in colonizing a plethora of environments (soil, forest, sea...) thanks to their versatility and remarkable adaptability.

This great diversity of environments is paralleled with a wide array of strategies for carbon assimilation to ensure survival and reproduction, which can vary considerably from one species to another. Like plants, fungi are "non-motile" organisms but unlike plants, they cannot synthesize carbohydrates from light and CO₂ and have to rely on external sources for carbon and energy. As a result, mycologists have devised a fundamental classification based on the fungi's breeding mode and relationship with their carbon source providers (Wainwright and Grayston 1988).

III.1.1. Mode of reproduction

The life cycle of fungi is closely related to their type of spores, the reproductive cells that give rise to a new individual by mitosis or meiosis, in the case of asexual or sexual reproduction, respectively. Consequently, fungi are primarily categorized into two major divisions based on the type of spores they produce: ascomycetes and basidiomycetes (Fig. 6).

- **Ascomycetes** represent the largest phylum in the fungal kingdom (Bennett and Turgeon 2016) and are characterized by both sexual and asexual reproduction. The life cycle of ascomycetes involves two distinct phases: the haploid (n) and diploid (2n) stages. During sexual reproduction, specialized structures called asci (origins of the "asco" prefix), typically contained within fruiting bodies known as ascocarps, produce sexual spores called ascospores through a process called meiosis. Ascospores are typically produced in large numbers and are released into the environment. When two compatible mating types of ascomycetes come into contact, they can undergo sexual reproduction. The fusion of specialized sexual cells called results in the formation of a diploid zygote. This zygote then undergoes meiosis to produce new haploid spores, which can eventually germinate and

form new mycelia. In addition to sexual reproduction, ascomycetes are prolific asexual reproducers. They produce asexual spores called conidia through structures known as conidiophores. Conidia are formed by a process called mitosis and are disseminated into the environment, where they can germinate and give rise to new fungal colonies.

- **Basidiomycetes** encompass the majority of macro-fungi, and represented not only by numerous edible fungi but also by notorious plant pathogens like rusts and smuts. Their life cycle is characterized by a prominent dikaryotic stage and complex fruiting bodies known as basidiocarps (Coelho et al. 2017). Basidiomycetes sexual reproduction begins with the fusion of two compatible hyphae, resulting in a dikaryotic mycelium containing two different but compatible nuclei ($n + n$). This dikaryotic mycelium is a hallmark of basidiomycetes and can persist for extended periods. Under suitable conditions, dikaryotic mycelia can produce basidiocarps, which are often the familiar mushroom structures. Within the basidiocarps, specialized cells called basidia (origins of the “basidio” prefix) produce sexual spores called basidiospores via meiosis. These basidiospores are forcibly discharged into the environment from the gills or pores of the mushroom. When basidiospores germinate, they form a new mycelium, and the cycle continues. Basidiomycetes also have asexual reproductive structures known as conidia, but these are less common than in ascomycetes.

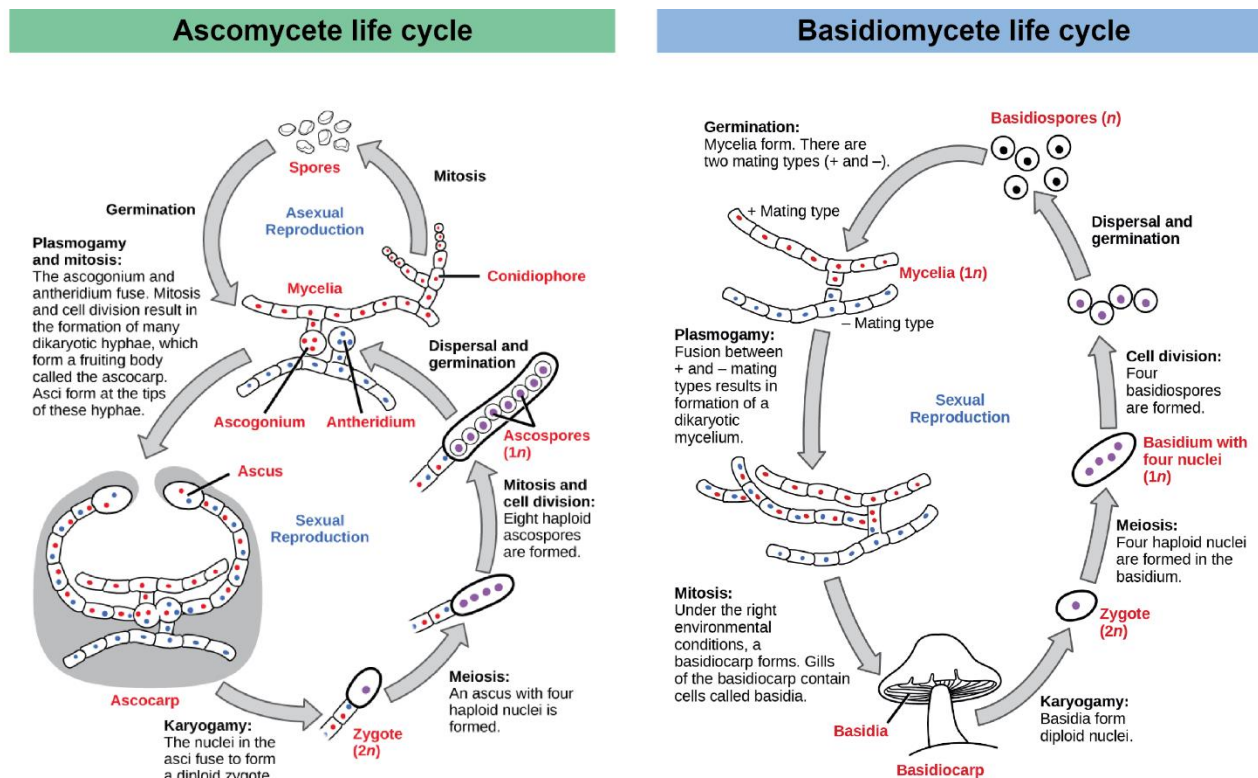


Fig. 6. Ascomycete and basidiomycete life cycle. The figure was adapted from files under the creative common license (<https://creativecommons.org>).

III.1.2. Mode of carbon assimilation

While the precedent section describes the main reproduction mode of fungi, a second essential feature discerning fungal species lies in their mode of carbon assimilation, which can be classified in three main categories: parasitic, symbiotic and saprophytic.

- Parasitic fungi** are engaged in a parasitic lifestyle, infecting living hosts, often causing harm during their proliferation and, in some cases, leading to the host's death. These parasitic fungi can be classified based on their host specificity, modes of infection, and disease symptoms. Plant parasitic fungi, like *Ustilago maydis* (known as corn smut) are major threats to agriculture. They typically infect host plants through specialized infection structures, such as haustoria or appressoria, and can cause devastating crop diseases (Mapuranga et al. 2022). Other parasitic fungi can also affect animals like *Candida albicans* (causing candidiasis in humans), or insects like the entomopathogen *Beauveria bassiana*. Examples include *Magnaporthe oryzae* in rice, *U. maydis* in corn, and *Cryptococcus neoformans* in humans. Parasitic fungi can serve as models for studying host-pathogen interactions, contributing for example to the development of disease-resistant crops and to

bio-based insecticide (Siegwart et al. 2015; Sharma et al. 2019).

- **Symbiotic fungi** maintain mutually beneficial relationships with their hosts, particularly plants, to enhance nutrient uptake and provide various benefits to their host partners. These fungi can be classified into different categories based on their interactions with host organisms and the specific benefits they confer. Mycorrhizal fungi, such as *Rhizophagus irregularis* (formerly known as *Glomus intraradices*), form mutualistic associations with the roots of most plants. They facilitate the uptake of essential nutrients, particularly phosphorus and nitrogen, in exchange for carbon compounds from the host plant (Smith & Read, 2008). Some ectomycorrhizal fungi, like *Laccaria bicolor*, are of major ecological importance in forest ecosystems (Kuo et al. 2014). They can confer various benefits to host plants, including enhanced resistance to herbivores and pathogens (Martin and Selosse 2008; Singh et al. 2011). Some of these mycorrhizal fungi can be used in agriculture to provide nutrients to plants and increase crop yields while reducing dependence on chemical fertilizers (Felföldi et al. 2022). In addition, endophytic fungi can enhance crop resistance to pests and pathogens reducing the need for chemical pesticides (Akram et al. 2023).

- **Saprotrophic fungi** are a diverse group of microorganisms that play a crucial role in ecosystem carbon cycling by decomposing dead organic matter. They are known to be among the most efficient organisms for the degradation of plant biomass and generally possess a vast enzymatic arsenal adapted to the specific deconstruction of PCW. For example, species from the genera *Aspergillus*, *Trichoderma* or *Ustilago* are prolific producers of extracellular enzymes that possess wide applications in industries such as biofuel production, textile, paper, and food industry (Herpoël-Gimbert et al. 2008; Couturier et al. 2012; Ferreira et al. 2014; Ferreira et al. 2016). Among others, one could also cite *Penicillium spp.* used in bio-based antibiotics (e.g. *Penicillium spp.*), *Aspergillus niger* for citric acid production, or *Pleurotus ostreatus* (oyster mushroom) and *Pycnoporus spp.* (white-rot fungi), which can break down complex organic compounds into simpler, less toxic forms and is therefore used in bioremediation to degrade environmental pollutants and xenobiotics (Lomascolo et al. 2011; Ratuchne et al. 2023).

III.1.3. Wood-decaying fungi classification

Since saprotrophic wood-decayers are central to this thesis, it is interesting to take a closer look at their specific classification. These fungi can indeed be further classified based on the type of decay they cause. We can notably distinguish white-rot, brown-rot, and soft-rot fungi (Goodell et al. 2008). It should be underscored that while they prove to be convenient, these somewhat “gross” categories do not capture the full scope of wood-decaying fungi as more nuanced phenotypes have also been reported (such as grey rot fungi).

- **White-rot** fungi account for ca. 90% of total wood decaying fungi and display high diversity of species (Dai et al. 2007). They are known for their remarkable ability to degrade lignin, one of the most recalcitrant components of PCW, leaving the wood with a white color (hence their name). White-rot employ an extensive array of extracellular enzymes, including lignin peroxidases, manganese peroxidases, and laccases, which work synergistically to break down lignin into simpler compounds. By efficiently decomposing lignin, white-rot fungi play a vital role in carbon cycling and contribute to the decay and recycling of woody biomass in ecosystems. Notably, white-rot of the Polyporales order (which belong to the Basidiomycete phylum, Agaricomycetes class), are well-known for their large arsenal of wood-decaying enzymatic activities and will be at the core of this thesis (Couturier et al. 2012; Hage and Rosso 2021).

- **Brown-rot** fungi are a group of wood-decaying fungi that exhibit a different mode of wood degradation compared to white-rot fungi. They target and break down cellulose and hemicellulose components of wood, while lignin is oxidized and only partially degraded, resulting in the characteristic brown coloration of degraded wood (J. Worrall et al. 1997). A unique characteristic of brown rot fungi is that the initial stage of their biomass degradation strategy is nonenzymatic, and relies on the use of small molecules (oxalic acid, hydroxyl radicals...) to degrade cellulose and hemicellulose through Fenton reactions (Langer et al. 2000).

- **Soft-rot** fungi, in contrast to white-rot and brown-rot, are only represented in the Ascomycete phylum. They are characterized by chains of cavities they form within the PCW, and are able to decay wood in a manner intermediate between white-rot and brown-rot fungi. They primarily target cellulose and hemicelluloses, and are less efficient when it comes to

degrade biomass with high lignin content (Goodell et al. 2008). Soft-rot fungi often thrive in conditions with higher moisture content and are commonly associated with wood in contact with soil. They owe their name to the fact that they were first identified from soft, decayed wood surfaces in contact with excessive moisture (Goodell et al. 2008).

Noteworthy, this rather old and empirical wood decay classification has been partially revisited, following the sequencing of hundreds of fungal genomes and knowledge acquired on their enzymatic mechanisms of action (Eastwood et al. 2011; Floudas et al. 2012).

III.2. Fungal toolbox and strategies

In the case of fungal saprotrophs, the energy/carbon source mainly comes from carbohydrates found in the PCW (Rytioja et al. 2014). To extract carbon from such recalcitrant structures, fungi have developed through evolution an enzymatic arsenal capable of specifically degrade the multiplicity of polymers that compose PCW (Hage and Rosso 2021).

Most of these enzymes are now termed as Carbohydrate Active enZymes (CAZymes) and classified in the CAZy database (www.cazy.org/) (CAZypedia Consortium 2018; Drula et al. 2022). The aim of this internationally recognized database is to identify and classify all the CAZymes known to date based on their protein sequence similarity. The founding member of each new CAZy family must have a protein sequence divergent from previously discovered families and must have been biochemically characterized. Then, it is possible to predict the affiliation of an enzyme to an existing family based on sequence comparison.

CAZymes are organized into five enzyme classes and one class of associated carbohydrates binding modules (CBMs). Enzyme classes include Glycoside Hydrolases (GH, involved in the hydrolysis and/or rearrangement of glycosidic bonds), Glycosyl Transferases (GT, involved in the formation of glycosidic bonds), Polysaccharide Lyases (PL, involved in the non-hydrolytic cleavage of glycosidic bonds), Carbohydrate Esterases (CEs, involved in the hydrolysis of carbohydrate ester bonds) and Auxiliary Activities (AA, oxidoreductases that act, in general, in synergy with other CAZymes). Of note some CAZy families have been divided into subfamilies (e.g. GH5, GH16) to optimize the prediction of functions.

III.2.1. Fungal glycoside hydrolases: Main actors of plant cell wall deconstruction

III.2.1.a. Cellulases

Although cellulose may appear to be a simple polymer composed exclusively of glucose, its degradation actually requires the implementation of multiple specific and complementary hydrolytic activities. The hydrolytic enzymes involved in cellulose deconstruction, so-called cellulases, can be distinguished by their mode of action and the polymers they target (Couturier and Berrin, 2013): Endoglucanases (EGs) are active on the middle of cellulose chains (*endo*-acting mode of action), while cellobiohydrolases target the reducing (CBHIs) or non-reducing (CBHIIIs) ends of cellulose chains (*exo*-acting mode of action). Finally, β -glucosidases (BGs) cleave the cellobiose released by the combined action of EGs and CBHs.

EGs are found in GH5_5, GH6, GH7, GH12 and GH45 families. Their active site is characterized by a relatively open cleft able to bind long chains of amorphous cellulose. EGs adsorption on cellulose is not mediated by the recognition of specific cellulose pattern and is rather achieved by random on-off mechanism, hydrolyzing internal glycosidic bonds, and releasing small cello-oligomers after each catalytic cycle.

CBHs are found among GH6, GH7 families. Their active site is characterized by a tunnel-like structure that can accommodate a single cellulose chain. Once bound to their substrate, CBHs progressively degrades cellulose by sliding along the chain, releasing cellobiose after each catalytic cycle. It has to be mentioned that CBHs can also display an *endo*- mode of action in their first contact with cellulose, which is referred to as *endo*-mode initiations (Kurasin and Våljamäe 2011).

The last actors of cellulose hydrolytic degradation are β -glucosidases from GH1 and GH3 families, which act on the soluble cellobiose and other cellodextrins released by EGs and CBHs hydrolytic activity, to convert them into the metabolically essential glucose units.

III.2.1.b. Hemicellulases

As discussed above, hemicellulose is a highly heterogeneous polymer whose composition varies widely among plant species. Then, it is no surprise that its degradation requires a wide range of enzyme activities specifically adapted to each biomass. Regarding xylan, its degradation is mediated by the action of β -1,4-xylanases, which are mainly found in GH8, GH10 and GH11 families. As presented above, the xylan backbone is often substituted with various sugars, whose presence is known to hamper the action of xylanases (Kormelink et al. 1993; Amin et al. 2014), necessitating therefore the assistance of various debranching enzymes. The latter include ferulic and *p*-coumaric esterases, α -glucuronidases, acetyl xylan esterases and α -L-arabinofuranosidases (Rytioja et al. 2014; Bissaro et al. 2018). Finally, similarly to cellulose, small xylo-oligomers released by β -1,4-xylanases are hydrolyzed into xylose by β -1,4-xylosidases (Polizeli et al. 2005).

Regarding galactoglucomannan, the main hemicellulose component of softwood, multiple enzymatic activities are also required. Since mannans are the main constituent of glucomannan backbones, internal glycosidic bonds degradation is handled by the action of β -1,4-mannanases (mainly from GH5_7 and GH26 families) (Mafa and Malgas 2023). Debranching of galactose substitutions and acetylations are respectively operated by α -galactosidases and acetyl mannan esterase. The oligomers released from galactoglucomannan hydrolysis are ultimately cleaved into mannose and glucose by β -mannosidases and β -glucosidases.

III.2.2. Carbohydrate binding modules

CAZymes can be multi-modular enzymes displaying, in addition to their catalytic domain, a great structural diversity, either at their N- or C-terminal end, including the presence of Carbohydrates Binding Modules (CBMs), which bind carbohydrate polymers, promoting the proximity of the enzyme to its substrate, thus enhancing its catalytic efficiency (Boraston et al. 2004; Hervé et al. 2010). CBMs are usually linked to the catalytic domain via a linker of varying length (Sammond et al. 2012). These modules have been classified into three types in the CAZy database, based on the nature of the substrate they target: Type A CBMs bind crystalline polymers, Type B aim internal regions of carbohydrate chains whereas Type C CBMs are specific to polysaccharides ends (Boraston et al. 2004). Additionally, CBMs are more precisely classified based on their substrate specificity, for example, CBM6 are specific for xylan, CBM27 for mannan, CBM18 for chitin and CBM20 for starch. When it comes to cellulose the most common and characterized domains in fungi belong to the CBM1 family.

In fungi, CBM1 can be found appended to the C-terminus of many cellulases, and are notably often observed in many CBHs and EGs. The primary function of CBM1 is to enhance binding to cellulose. Since this polymer is insoluble in water, the possible interactions between cellulases and their substrate are limited to the surface of cellulose, which directly correlates the presence of a CBM1 with catalytic efficiency (Ståhlberg et al. 1991; Nidetzky and Claeyssens 1994). Likewise, it has been shown that CBM removal drastically reduced the activity of CBHs on insoluble substrates, while it had little impact on soluble substrates (Kotiranta et al. 1999; Várnai et al. 2013). Interestingly, CBM1 can to some extent disrupt the surface of cellulose fibers, making it more amorphous and contributing thereby to biomass degradation (Chalak et al. 2019). Importantly, the benefit provided by CBMs is known to be dependent on the substrate concentration. While having a CBM is generally favorable at low biomass content, it can lead to non-productive binding and slow enzymes release at higher substrate loading (Várnai et al. 2013).

Finally, it is worth noting that the specificity of CBMs is intriguingly not always in accordance with the substrate specificity of the catalytic domain they are paired with, which can be the case for instance for some LPMO-CBM multimodular proteins (see section **IV.4.**).

III.2.3. Auxiliary activity enzymes: essential partners

Because of the intricate and recalcitrant nature of lignocellulose, hydrolases cannot fully degrade lignocellulose on their own. The hypothetical existence of another proteinaceous system, acting in synergy with hydrolases to offer them better hydrolysis conditions, has been suggested in the 1950s by Reese and colleagues (Reese et al. 1950). This non-hydrolytic system, named C₁ factor, was described as targeting and disrupting crystalline parts of cellulose, rendered more easily hydrolysable by cellulases (so-called the C_x factor). This vision of the PCW enzymatic degradation was reactivated decades later with the discovery of new auxiliary enzymes acting on polysaccharides (Vaaje-Kolstad et al. 2010; Harris et al. 2010), believed to play the role of Reese's C₁ factor. Of note, non-enzymatic proteins called swollenin in fungi are also believed to mechanically disrupt PCW, although we will not further develop this aspect, it is possible that the auxiliary C₁ factor may not just rely on enzymatic activities (Eibinger et al. 2016).

III.2.3.a. The role of oxidoreductases in lignocellulose degradation

Oxygen species take an important place in the conflict between plants and fungi. The hydroxyl radical OH^{*} is one of the most powerful oxidizing agents on earth, which can be derived from H₂O₂ via Fenton chemistry reactions (Gligorovski et al. 2015). Their ability to oxidize many chemical compounds makes these so-called reactive oxygen species (ROS) true molecular weapons. For example, plants use this chemistry to their advantage through "oxidative burst", releasing huge amounts of ROS to kill pathogens in their early stage of infection (Bolwell 2002). However, fungi do also master ROS chemistry. In fact, in 1974, Eriksson and colleagues reported that cellulose degradation by four white rot species was significantly lower under anaerobic conditions (Eriksson et al. 1974), suggesting that oxygen-based reactions were essential for cellulolytic processes in fungal systems. Research conducted over the past 40 years has enlightened these pioneer observations, as recently summarized in a review entitled "Oxidoreductases and Reactive Oxygen Species in Conversion of Lignocellulosic Biomass" (Bissaro et al. 2018).

Indeed, today, fungal oxidoreductases have been characterized and classified in the CAZy database in the Auxiliary Activities (AA) class (Levasseur et al. 2013). Among these, some AAs families are known to play a major role in the fungal enzymatic arsenal by acting - directly or indirectly - in synergy with other enzymes involved in PCW deconstruction (**Fig. 7** (Bissaro et al. 2018)). Of course, synergies between AAs and other enzymes are not only

limited to PCW degradation, but in the latter context, examples of interplay between secreted oxidoreductases and glycoside hydrolases can take different forms.

Several examples of enzymes interplay can be mentioned, which I classify here depending on their nature:

- **Interplay for substrate accessibility:**

Secreted enzymes targeting PCW (mainly consisting in hydrolases) do not act under optimal degradation conditions. Indeed, due to its complex nature, all the components of the PCW may not be directly accessible to the enzymes, thus greatly reducing the hydrolysis efficiency. To alleviate this issue, some plants degrading organisms are using powerful AAs enzymes to enhance the accessibility of PCW components. Perfect representatives of this kind of interplay between oxidoreductases and hydrolases would be the LPMOs, which are powerful redox enzymes classified in several AAs families (see section IV.). Among these, LPMOs from the AA9 family are notably known to disrupt recalcitrant parts of cellulose, creating new entry point for *exo*- acting cellulases and xylanases. Additionally, esterases may also play a role in increasing the accessibility to hydrolases for cellulose and hemicellulose by cleaving ester bonds linking the lignin-carbohydrate complexes (Jeffries 1990).

Interplay between GHs should also be mentioned. Because of their processive nature, CBHs are highly sensible to potential obstacles in cellulose (change in morphology, proteins, other polymers) creating the so-called traffic jam (Igarashi et al. 2011). Interestingly the *endo*-action of EGs has been observed to shorten the cellulose chain, thus releasing CBHs bound in an unproductive manner (Jalak et al. 2012; Hemsworth et al. 2016).

- **“Activation” interplay:**

To allow the biochemical reactions catalyzed by the enzymes to take place, the direct availability of co-substrate is essential. GHs perform hydrolysis in the presence of water, naturally available *in situ*. However redox enzymes may rely on more complex activation mechanisms, involving less abundant co-substrates. To cope with this, interplays between two AAs, one being able to provide the other with its co-substrate(s), have been recently unveiled. A good example of this kind of interplay would be cellobiose dehydrogenase (CDH) from AA3_1-AA8, which in addition to being able to turn LPMOs into their active state via direct electron transfer (Phillips et al. 2011; Kracher and Ludwig 2016), are also capable to

produce small quantities of the LPMO co-substrate, H₂O₂ (Bissaro et al. 2017; Kracher et al. 2020). In the same way, a cello-oligosaccharide dehydrogenase from AA7 family has also recently been unveiled as a LPMO partner, acting in a similar way as CDHs but without any cytochrome domain (Haddad Momeni et al. 2021) (see section **IV.3.2.**).

- **“Inhibition alleviation” interplay:**

In addition to the availability of substrate and co-substrates, other parameters can hinder the action of CAZymes. Indeed, some lignin-derived phenolic components of the PCW are known to have an inhibitory effect on cellulases and hemicellulases during hydrolysis process (Ximenes et al. 2010). Interestingly, studies have shown that oxidation of these lignin-derived products by specific fungal laccases from the AA1 family can reduce the negative impact of phenols on enzymes (Ladeira-Ázar et al. 2019).

Another well-known form of hydrolases inhibition, also applicable to many other enzymes, is the product inhibition. This phenomenon occurs when the product of an enzymatic reaction is too abundant in the medium. In this case, non-productive bindings between the product and the active site of the enzyme can take place, preventing the renewing of the catalytic cycle. For instance, cellobiose, one of the main products accumulating during long runs of cellulose hydrolysis, is known to inhibit CBHs. Oxidation of cellobiose into cellobiono- δ -lactone (then hydrolyzed into cellobionic acid) by CDHs has been shown to counteract this inhibition (Igarashi et al. 1998).

Hence, in the light of our current knowledge, oxidoreductase enzymes could well be an important piece of the C1 factor hypothesized by Reese and colleagues. However, only an exceedingly small proportion of AAs has been characterized and even less assigned to a biological role. We are still at the dawn of our understanding of GH-AA / AA-AA synergies, and lots of work remains to be done to unveil new enzymatic interplay and even more to understand their biological purpose.

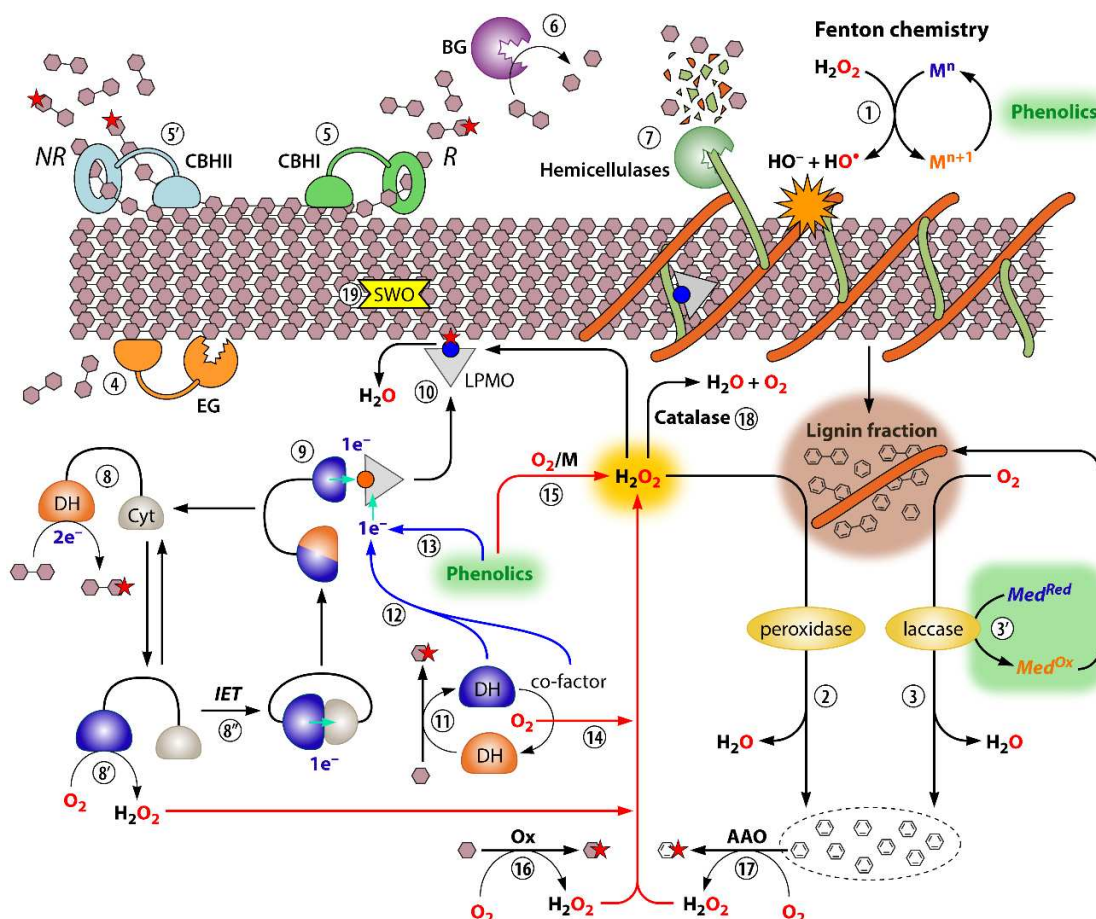


Fig. 7. Enzymatic interplays during PCW degradation. An integrative view on reactions happening during lignocellulolysis. The enzymes presented here do not always all cooccur in genomes or secretomes and may act sequentially, depending on the microorganism or the nature of the biomass. The main nonenzymatic weapon is constituted by Fenton reaction-derived hydroxyl radicals (step 1). The lignin fraction can be modified and to some extent depolymerized via the action of peroxidases (step 2) or laccases (step 3). 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 (step 4), CBHs (steps 5 and 5'), and BGs (step 6). Cellulases will also release minor amounts of products carrying an oxidation at C-1 or C-4 introduced by an LPMO (indicated by a red star). A wide diversity of hemicellulases (Shallom and Shoham 2003; Amore et al. 2013) and possibly pectinases (Willats et al. 2001; Ndeh et al. 2017) 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). LPMOs can oxidize the cellulose (step 10), in the presence of H_2O_2 . LPMOs can also be activated by single-domain dehydrogenases and/or noncovalently bound reduced cofactor (step 11 to 12) or by reduced phenolics (step 13). Single-domain dehydrogenases (step 14) and reduced phenolics (step 15) can also lead to the production of H_2O_2 under aerobic conditions. Several 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). Aryl-alcohol oxidases (AAO) oxidize lignin-derived compounds to generate H_2O_2 (step 17). Catalase can convert excess H_2O_2 into H_2O and O_2 (step 18). Expansins/swollenins (SWO) may contribute to lignocellulolysis by loosening the plant cell wall structure (step 19) (Cosgrove 2015) although their mode of action remains unknown. The figure and caption (slightly adapted) were reproduced from (Bissaro et al. 2018).

II.2.3.b. Discovery of LPMOs, the missing piece of the jigsaw

In 1974, in addition to demonstrating the importance of oxygen in fungal enzymatic systems, Eriksson et al. also found a new oxidizing enzyme of unknown function secreted by the white rot *Sporotrichum pulverulentum* (anamorph of *Phanerochaete chrysosporium*) when grown on cotton cellulose. At that time, they already hypothesized that this enzyme could lead to a swelling of crystalline part of cellulose by the addition of uronic acid moieties (Eriksson et al. 1974) (**Fig. 4**). Later, they isolated a heme-containing enzyme that they called “cellobiose oxidase” (Ayers et al. 1978), which will be later renamed “cellobiose dehydrogenase”, and that they took for responsible of their initial observations (Eriksson 1978).

Later in the 1990s, scientists working on soil bacteria (*Streptomyces* strains) found that the bacteria secreted an unidentified protein when grown on the recalcitrant biopolymer chitin (Schnellmann et al. 1994; Kolbe et al. 1998). Due to its high affinity for chitin, this protein was classified as a Chitin Binding Protein (CBP). Such CBP was then found in *Serratia marcescens* and thus more precisely referred to as CBP21 based on its 21 kDa molecular weight (Suzuki et al. 1998). In the absence of demonstrated catalytic activity, and since one CBP21 was also found attached to a β -1,4 mannanase (Sunna et al. 2000), these proteins were finally defined as a new Carbohydrate-Binding Module family, the CBM33.

In the meantime, another unknown upregulated protein was identified in *T. reesei* during growth on cellulose (Saloheimo et al. 1997). Characterization of this enzyme, named EGIV, revealed a very weak endoglucanase activity, and this enzyme was thus classified as a new GH61 family.

The year 2005 marked a turning point in the understanding of these proteins when Vaaje-Kolstad and colleagues demonstrated that CBP21 protein could disrupt insoluble crystalline chitin and significantly promote its hydrolysis (Vaaje-Kolstad et Horn et al. 2005). The structure of this novel protein was solved the same year and surprisingly did not share any features in common with canonical CBMs (Vaaje-Kolstad et Houston et al. 2005). Instead, this protein displayed a pyramidal structure with highly conserved polar amino acid

on its rather flat surface containing the metal binding site. A few years later, the first structure of a GH61 (from *T. reesei* (Karkehabadi et al. 2008)) revealed that, in addition to sharing no similarity with other GH structures, this enzyme also showed features reminiscent of the newly solved CBM33 structure (Vaaje-Kolstad et al. 2005). With no pocket or tunnel, allowing specific binding to a substrate, and in the absence of any catalytic residues typically found in the active site of most known GHs, the endoglucanase activity of this GH61 could not be related to any known enzymatic mechanism. Interestingly, a conserved metal-binding site could be identified in the structure of both these newly solved structures.

In 2010, alike the boosting effect reported for CBP21 on chitinases activity (Vaaje-Kolstad et al. 2005), a study on a fungal GH61 revealed that its addition to a cellulase mixture (Cel7A and Cel6A) enhanced cellulose hydrolysis, hence again reinforcing the parallel between GH61s and CBM33s (Harris et al. 2010). At that time, although the boost effect of these proteins seemed to be proven, their enzymatic mechanisms remained unknown. The ability of CBP21 to cleave glycosidic bonds of β -chitin through a redox reaction was unveiled in a landmark study by Vaaje-Kolstad and colleagues (Vaaje-Kolstad et al. 2010). Using isotopically labeled dioxygen, the authors demonstrated the introduction of oxygen atom into the carbohydrate chain demonstrating the monooxygenases activity of CBP21 on chitin. In 2011, Quinlan and colleagues clarified the nature of the metal center: a mono-copper atom coordinated by two histidines, therefore called today a 'histidine brace' (Ciano et al. 2018). In 2012, CBM33s and GH61s were collectively termed Lytic Polysaccharide Monooxygenase (LPMO) (Horn et al. 2012). The widely accepted oxidative mechanism (until its revision in 2016/2017; see below) was described as such : the copper Cu(II) atom is first reduced by a reducing agent prior to reacting with O₂, leading in the presence of a polysaccharide substrate (chitin or cellulose) to the monooxygenation of either the C1 or C4 carbons of the scissile glycosidic chain, inducing bond cleavage (Beeson et al. 2012).

After discovering this mechanism, the characterization of other LPMOs on cellulose and chitin followed. These metallo-enzymes were assigned to new AAs families: the formers GH61 active on cellulose were reclassified as AA9 family and the formers CBM33 as the AA10 family (Levasseur et al 2013).

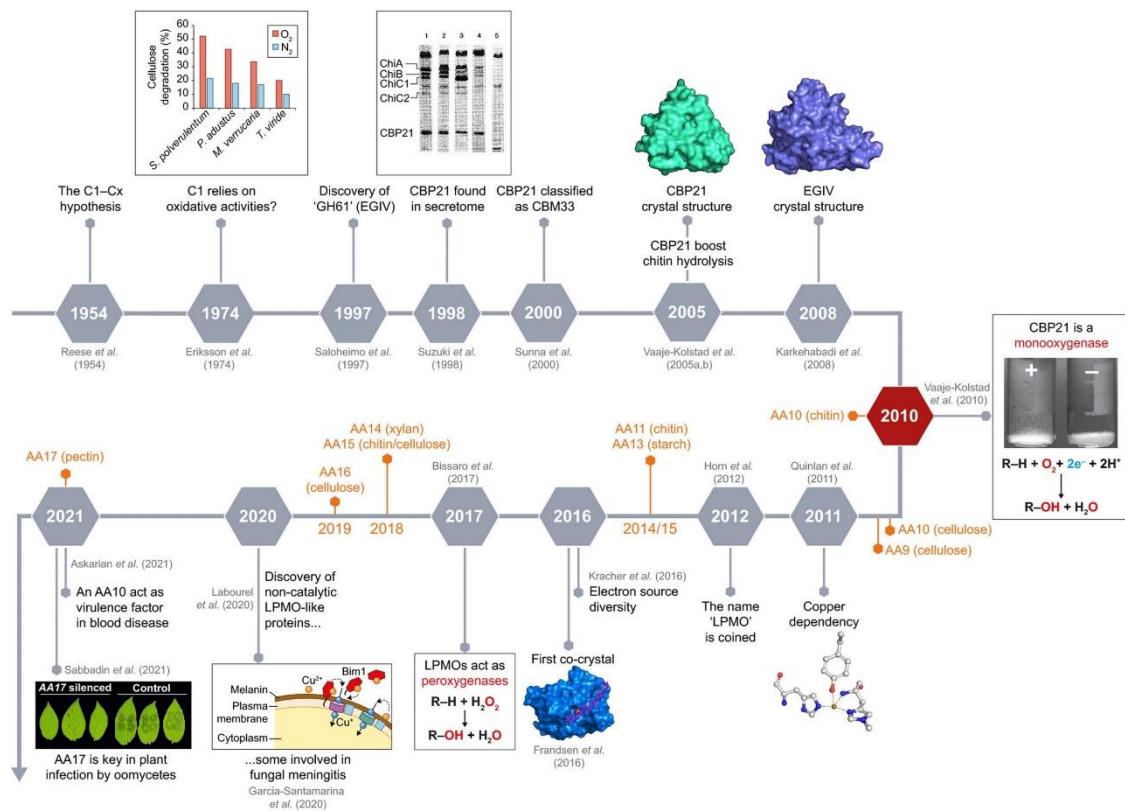


Fig. 8. LPMO research timeline and milestones. The figure was reproduced from (Vandhana et al. 2022).

IV. Overview of LPMOs

IV.1. A diversity of substrate specificities

Since the discovery of the first LPMOs families a decade ago, the potential of these enzymes in both fundamental research and industrial applications have made the characterization of new LPMO sequences growing rapidly. Nowadays, these enzymes are distributed in seven AAs families (AA9, AA10, AA11, AA13, AA14, AA15, AA16 and AA17), based on their sequence similarities:

- **AA9s** are to date only found in fungi. This family encompasses several substrates specificities, mainly targeting glucose-based polymers such as cellulose, cello-oligosaccharides or even hemicelluloses containing glucose backbone (gluco-mannans, xylo-glucans...) (Bennati-Granier et al. 2015; Frommhagen et al. 2015; Simmons et al. 2017; Fanuel et al. 2017).
- **AA10s** are found in various organisms such as bacteria, viruses, archaea and even in some plants (ferns) and pathogenic fungi of the *Ustilaginomycete* class. This LPMO family can target both chitin and/or cellulose polymers (Forsberg et al. 2014; Corrêa et al. 2016). Interestingly, one characterized member of this family from the actinomycete bacteria *Kitasatospora papulosa* seemed to display a broader substrate specificity, cleaving cellulose, chitin and xylan (Corrêa et al. 2019).
- **AA11** family was introduced in 2014, based on a sequence comparison with other uncharacterized AA9s sequences. This rather small family exclusively found in fungi possesses only a few characterized members active on chitin (Hemsworth et al. 2014; Wang et al. 2018).
- **AA13** family, containing only fungal LPMOs, was first reported in 2014. AA13s act on starch substrates (Vu et al. 2014b; Lo Leggio et al. 2015).
- **AA14s** were first identified in 2017 in fungal secretomes (Berrin et al. 2017; Couturier et al. 2015). Two of them from *Pycnoporus coccineus* were then characterized in 2018 and firstly suggested a xylan were specific for xylan, more precisely targeting the heteroxylan that cover cellulose polymers in wood. Their catalytic was recently revisited by Tuveng and colleagues (Tuveng et al. 2023), suggesting that the above described substrate specificity

may not be generalized to all AA14s. Therefore, relevant substrate specificity for AA14s is yet to be revealed (Tuveng et al. 2023).

- **AA15s** stand out from other LPMO families as they are found in various organism including virus (14.5%), and eucaryotes (85.5%) such as algae, oomycetes, and complex animals. The two first members were characterised from the insect *Thermobia domestica* and displayed activity on both cellulose and/or chitin (Sabbadin et al. 2018). More recently, two AA15 LPMOs from the termite *Coptotermes gestroi* (Franco Cairo et al. 2021), one AA15 from the oomycete pathogen *Aphanomyces astaci* ('crayfish plague') (Sabbadin et Henrissat et al. 2021) and one AA15 from the grass moth *Ostrinia furnacalis* (Qu et al. 2022) showed activity on chitin only.

- **AA16s** are mainly found in fungi and some Oomycetes. This family was first identified in the secretome of *Aspergillus japonicus*, and then followed by the characterization of its ortholog in *Aspergillus aculeatus*, revealing activity on cellulose. Further characterization of three other AA16 from *Aspergillus japonicus*, *Aureobasidium pulluans* and *Pestalotiopsis fici* confirmed the specificity for cellulose (Filiatrault-Chastel et al. 2019). Recent studies have also demonstrated a boosting effect of AA16 on cellulose degradation by a cellulase cocktail (Hossain et al. 2020) and implications of this LPMO family in plant pathogenesis in oomycetes have also been suggested (Jagadeeswaran et al. 2021). Even more recently, the crystal structure of *MtAA16A* was solved, and the latter was shown to enhance the activity of another LPMO (*MtLPMO9B*) by providing H₂O₂ in close contact to its active site (Sun et al. 2023).

- **AA17** family is the most recent LPMO family, only found in oomycetes, with a significant expansion of gene copies (up to 65) in hemibiotrophic and necrotrophic plant pathogens (Sabbadin et Urresti et al. 2021). Three AA17s from *Phytophthora infestans* were demonstrated to oxidatively cleave (C4-oxidation) homogalacturonan and oligogalacturonides (DP 10–15), constituents of pectin in plant cell walls (PCW). Prior de-esterification of pectin (by pectin methyl esterases) was required to observe LPMO activity (Sabbadin et Urresti et al. 2021).

IV.2. LPMO structure and reaction mechanisms

IV.2.1. LPMOs structural features

Although some variations across the different families exist, the basal structure of LPMOs is fairly well conserved (Frandsen and Lo Leggio 2016; Vaaje-Kolstad et al. 2017). It consists in an immunoglobulin-like β -sandwich fold, composed of 8 to 10 antiparallel β -strands, linked to each other by α -helix loops (**Fig. 9A**). Regarding their active site, LPMOs stand out as they do not possess the typical tunnel or pocket observed in most other polysaccharide-active enzymes. Instead of that, several loops are forming a flat surface, within which two universally conserved histidines allow the coordination of a copper ion (CuII) (**Fig. 9B**) (Ciano et al. 2018). In addition to this so called “histidine brace”, another conserved tyrosine (or phenylalanine in chitin-active AA10 and AA15) is also essential for the copper stability, and possibly its reactivity (Paradisi et al. 2019; McEvoy et al. 2020). Interestingly, the N-terminal histidine is systematically methylated in LPMOs found in fungi, a feature that we do not observe in LPMO coming from viruses, bacteria or yeasts. The role of this methylation is not clearly elucidated yet, and functional studies of two variants of an AA9 from *Thermascus aurantiacus* (with or without N-terminal methylation) have revealed no differences in substrate affinity or cleavage specificity (Petrović et al. 2018). However, the authors observed that the N-terminal methylated LPMO displayed a higher resistance against oxidative damages and showed better process performance when use in conditions that promote generation of reactive-oxygen species (Petrović et al. 2018). Furthermore, a recent computational study supporting this hypothesis suggests that methylation of the histidine would increase the energy barrier of His oxidation by a reactive intermediate, contributing thereby to prevent autooxidation (Torbjörnsson et al. 2023). Taken together, these observations suggest that this conserved methylation of the N-terminal histidine could be involved in the protection of the active site against oxygen reactive during LPMO catalysis (see below).

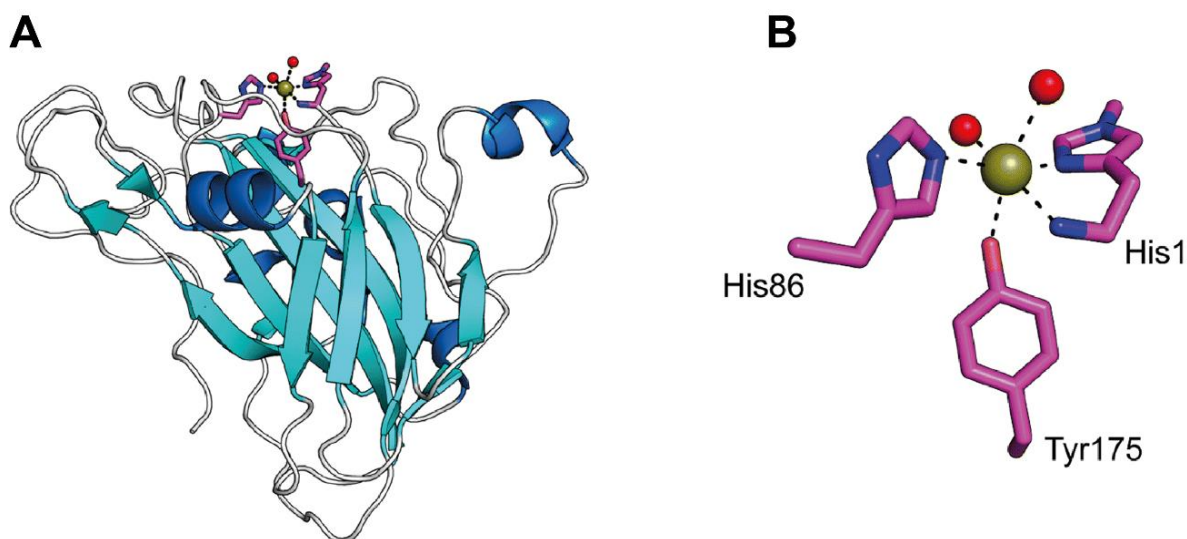


Fig. 9. Typical three-dimensional structure of an LPMO and its active site. (A) crystal structure of TaLPMO9A is displayed in cartoon representation and (B) zoom on the histidine brace. The figure was adapted from (Eijsink et al. 2019).

IV.2.2. LPMO catalytic mechanism

Since its discovery in 2010, the oxidative mechanism of LPMOs has been the subject of heated debate in the LPMO community. Recently, an alternative mechanism is gradually beginning to gain unanimity.

As mentioned earlier in this introduction, the "monooxygenase" terminology attributed to LPMOs was demonstrated by the use of radioactive dioxygen isotope ^{18}O (Vaaje-Kolstad et al. 2010). This experiment showed that LPMOs are able to introduce an oxygen atom from O_2 into a carbohydrate unit at C1 or C4 carbon position. This oxygenation, also called hydroxylation, leads to intramolecular rearrangement inducing glycosidic bond cleavage. A C1 oxidation leads to the formation of a lactone, spontaneously hydrolyzed to aldonic acid; while C4 oxidation yields a ketoaldose, further hydrated into gem-diol (**Fig. 10**).

To achieve this oxygenation reaction, LPMO are relying on the copper ion located at the center of their active site. This copper is initially found in its inactive form Cu(II) and has to first be reduced into Cu(I) (called "priming reduction") prior to entry into the catalytic cycle. the priming reduction can be performed by various electron donors (see **IV.3**).

In its reduced state, Cu(I) is ready to catalyze the oxygenation reaction, relying on an external oxygen donor, the so-called co-substrate. The first described co-substrate for LPMOs was O_2 , which upon reaction with Cu(I) will form a Cu(II)-superoxide intermediate ($\text{Cu(II)-O}_2^{\cdot-}$), which was described as the responsible species for hydrogen atom abstraction

from the target C₁-H or C₄-H bond preceding hydroxylation. Importantly, such monooxygenase reaction requires the delivery of 2 more electrons and 2 hydrogen atoms to complete the catalytic cycle and return to the active Cu(I) state ($\text{R-H} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{R-OH} + \text{H}_2\text{O}$). So far, the delivery mode of this electrons/protons has never been explained (Beeson et al. 2015).

In 2017, this mechanism was challenged by a new scenario in H₂O₂ was proposed to be a catalytically more relevant co-substrate (Bissaro et al. 2017). Bissaro and colleagues showed that LPMO reaction could take place under anaerobic condition by adding small quantities of H₂O₂. Then, they also demonstrated using labeled H₂¹⁸O₂ that H₂O₂ was preferentially used by LPMOs in the presence and in the absence of O₂ in the reaction. In this reaction, the suggested reactive intermediate (copper-oxyl) resulting from the reduction of H₂O₂ would catalyze the oxygenation of the substrate (thus referred to as a peroxygenation reaction) and allow the regeneration of the Cu(II) state at the same time, without the need for extra electrons or protons delivery ($\text{R-H} + \text{H}_2\text{O}_2 \rightarrow \text{R-OH} + \text{H}_2\text{O}$). Various kinetic studies have now demonstrated the much higher catalytic efficiency of the LPMO peroxygenase reaction (Filandr et al. 2020; Kuusk et al. 2019; Kont et al. 2020; Hangasky et al. 2018). Although strongly questioned at the time of its release, this mechanism involving H₂O₂ as a co-substrate of LPMO is starting to be globally accepted as the true mode of action of LPMOs. Yet, we can still qualify this statement by reminding that not all LPMO families have been tested in combination with H₂O₂ and that the nature of the biological co-substrate is not entirely clear. On this note, recent experiments by the R. Ludwig's group have shown that, during wood decay, stable LPMO peroxygenase reactions can occur at low steady-state H₂O₂ concentrations (μM range) and that the LPMO does not consume O₂ (Chang et al. 2022). Further studies aiming at quantifying the actual O₂ and H₂O₂ levels in relevant biological systems would be of great interest. It is also important to note that large amounts of H₂O₂ can cause oxidative damages to catalytically essential amino acid, leading to the inactivation of the LPMO (Bissaro et al. 2017; Hangasky et al. 2018). To prevent such inactivation, small amounts of H₂O₂ must be progressively added or generated *in situ* in the reaction.

LPMOs are also known to catalyze an oxidative side reaction, i.e. the production of H₂O₂ from O₂ (Kittl et al. 2012). This reaction is deleterious as the accumulation of H₂O₂ can lead to auto inactivation of the LPMO. Hopefully, these oxidation events mainly occur in the absence of substrate binding, and it has been shown that the LPMO-substrate complex protects the enzyme from oxidative damages (Bissaro et al. 2017). Recent kinetic studies

estimated the probability of a (methylated) fungal LPMO becoming irreversibly inactivated following a reductant peroxidase reaction (i.e. occurring when the LPMO is both reduced and unbound) to be in the order of 0.01 (Kuusk and Väljamäe 2021; Kuusk et al. 2023). Recently, it has been demonstrated that reduced free copper can produce H_2O_2 in much higher quantities than when it is coordinated in the active site of LPMOs, leading to detrimental effect on LPMO catalysis (Bissaro et al. 2020; Stepnov et al. 2021). This free copper may come from the LPMO copper-loading procedure performed in numerous studies on a routine basis, (including those presented in this thesis) raising the question of whether the measurement of free copper excess should systematically be carried out.

Finally, due to their capacity to react with H_2O_2 , LPMOs can also be forced into a “peroxidase” reaction consisting in the reduction of H_2O_2 into two H_2O molecules ($2 \text{ R-H} + \text{H}_2\text{O}_2 \rightarrow 2 \text{ R}^\bullet + 2 \text{ H}_2\text{O}$). Although this peroxidase reaction is catalytically less efficient than the peroxygenase reaction presented before, this side reaction is used as a standard in LPMOs activity assays using 2,6-dimethoxyphenol as an “artificial” substrate (Breslmayr et al. 2018).

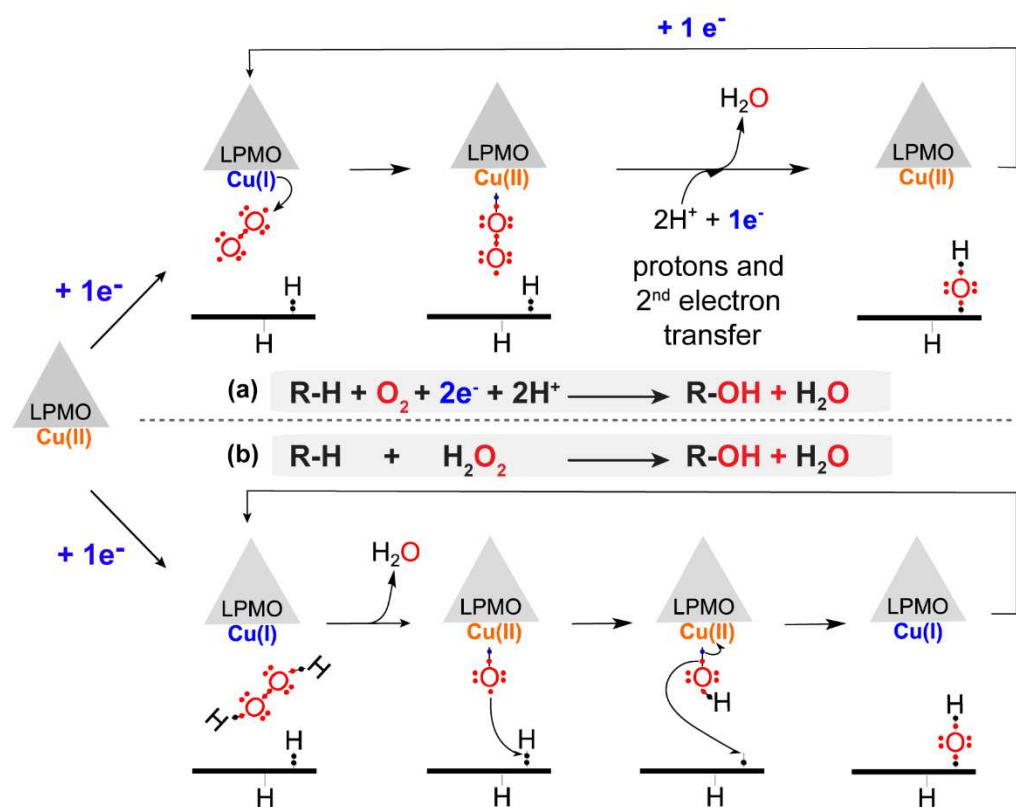
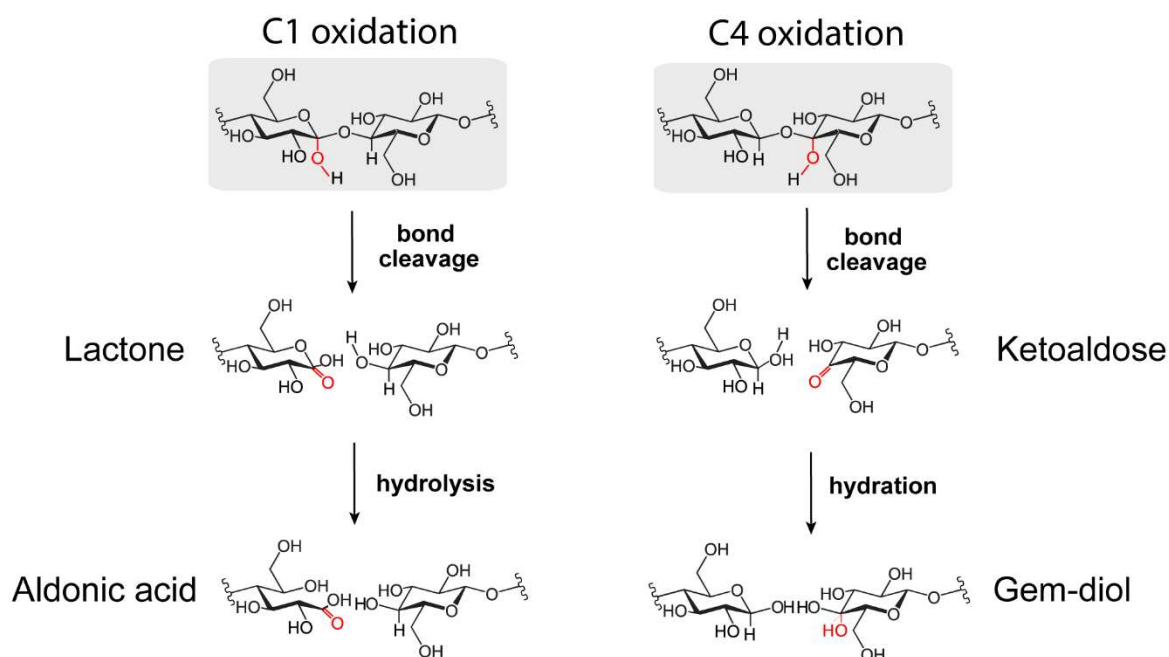
A**B**

Fig. 10: LPMO mechanisms. (A) Comparison of O₂-based (path a) and H₂O₂-based (path b) LPMO reaction pathways. (B) The hydroxylated product (R-OH) can undergo an intramolecular rearrangement leading to formation of a lactone (when C1 oxidized) or ketoaldone (C4) species and bond cleavage. The latter species are further spontaneously hydrolyzed or hydrated, into aldonic acid and geminal-diol, respectively. The figure was adapted from (Bissaro et al. 2018).

IV.3. Electron donor for LPMO reactions

IV.3.1. Small molecules and phenolic compounds

As presented hereabove, LPMOs are enzymes that rely on the exogenous presence of (i) an electron donor and (ii) a co-substrate, H_2O_2 (or O_2 to a lesser extent). To provide the LPMO with these requirements, a wide panel of small molecules can be used as a reductant and H_2O_2 can be artificially added in the reaction. The first and most widely used reductant for LPMOs reactions is ascorbic acid (Vaaje-Kolstad et al. 2010), but other organic compounds such as cysteine, gallic acid, ferulic acid, reduced glutathione have also proven to be efficient LPMOs partners (Quinlan et al. 2011; Kracher et al. 2016; Golten et al. 2023). However, these molecules yet convenient for *in vitro* experiments, are not always present in PCW degradation environments.

In a more biologically relevant context, derived compounds have also been shown to prime the activity of LPMOs. The first evidence that lignin can act as a reductant for LPMOs was provided by Harris and colleagues (Harris et al. 2010) and later confirmed by other studies (Cannella et al. 2012; Dimarogona, Topakas, Olsson et Christakopoulos 2012). Westereng and colleagues also showed that small molecules of lignin could act as shuttles in long-distance electron transfer for LPMOs (Westereng et al. 2015). In a PCW wall degradation context, these lignin-derived compounds can for example come from lignin degradation by laccases, but they can also be generated by pretreatment process in a biorefinery context.

IV.3.2. Enzymatic partners

In a context where a plethora of enzymes are secreted to efficiently deconstruct PCW, one can expect to find interplays between LPMOs and other redox enzymes able to activate and fuel LPMO action.

The first discovered and most studied enzymatic partners of LPMOs are CDHs. They are extracellular hemoflavo-enzymes, member of the glucose–methanol– choline (GMC) oxidoreductase superfamily and are classified in the AA3_1 subfamily in CAZy. CDHs catalyze the oxidation of cellobiose (or other cello-oligosaccharides) to cellobiono-1,5- δ -lactone with concomitant reduction of the FAD to FADH_2 . Electrons coming from the dehydrogenase reaction can then be sequentially transferred to a cytochrome domain

(AA8), connected to the flavo-enzyme via a flexible linker (Kracher et al. 2016; Tan et al. 2015).

The interplay between CDHs and LPMOs in fungal systems has been described in 2011 (Phillips et al. 2011; Langston et al. 2011) and supported by the fact that CDHs and AA9s are often co-expressed in fungi (Navarro et al. 2015; Poidevin et al. 2014). To further support these observations, recent -omics analysis of three white rots of the genus *Pycnoporus* showed that CDHs were systematically co-regulated with at least one AA9 (Miyauchi et al. 2020). In addition to this apparent co-expression, the involvement of CDHs in lignocellulose degradation has also been demonstrated by a mutational study where CDH genes deletion in *Podospira anserina* led to a decrease of biomass degradation (Tangthirasun et al. 2017). Yet, the rather slight decrease observed in this study suggests the existence of mechanisms to compensate for the absence of CDHs.

The mechanism underlying the activation of LPMOs by CDHs is not fully understood yet, but there are clear evidences that it relies on direct (cytochrome-LPMO complex) or indirect (formation of superoxide radical able to reduce the LPMO) electron transfer between the CDH cytochrome domain and the LPMO (Tan et al. 2015; Li et al. 2012). In addition to its “activation” interplay, it is interesting to note that CDHs are also able to produce small amounts of H₂O₂ through an oxidase reaction, providing the LPMO with its co-substrate (**Fig. 11**). The ability of CDHs to fuel LPMO with co-substrate was further emphasized in 2020 by the work of Kracher et al. (Kracher et al. 2020), in which the authors managed to enhance the oxidative power (i.e., the ability to reduce O₂ into H₂O₂) of a CDH from *Crassiacarpon hotsonii* through mutational engineering. The obtained enzyme, so-called CDHox_y+, was able to fuel *Sm*LPMO10-catalyzed reaction 35 times more efficiently than the wild type *Ch*CDH (Kracher et al. 2020).

All hitherto reported studies seem to indicate that CDHs are the perfect LPMOs partners, providing both reducing equivalents for activation and co-substrate. However, some aspects of this interplay must be nuanced. First, the fact that CDHs are totally absent from bacteria raise questions over a potential (non)-enzymatic equivalent of CDHs in these organisms. Of note, even LPMO containing fungi do not all possess genes encoding for a CDH, which should imply that CDH/LPMO interplay is not mandatory, but could be dependent on other factors such as the nature of the degradation environment or the type/number of recruited LPMOs. Furthermore, even if there are clear evidences that CDHs and AA9s are highly co-regulated and co-secreted, such correlation has not been demonstrated for other LPMOs families, although it is worth remembering that CDHs have also been successfully used in pair with some AA10s (Loose et al. 2016).

To cope with the absence of CDHs, other oxidoreductases from the GMC family have been shown to indirectly activate LPMOs by regenerating oxidized phenols back into their reducing state (Kracher et al. 2016). Single-domain flavoenzymes (glucose dehydrogenase and aryl-alcohol dehydrogenase) from the AA3_2 family (Garajova et al. 2016), devoid of any cytochrome, were shown to fuel LPMO reaction. The underlying mechanism remains however unclear.

It is becoming increasingly clear that the CDH/LPMO tandem is not the only system of electron and co-substrate supply for LPMOs. In this regard, a recent study showed that members of a newly discovered oligosaccharide dehydrogenase AA7 subfamily are able to fuel LPMO in the same way as CDH, providing both reducing equivalents for priming reduction and H_2O_2 (Haddad Momeni et al. 2021) (**Fig.11**).

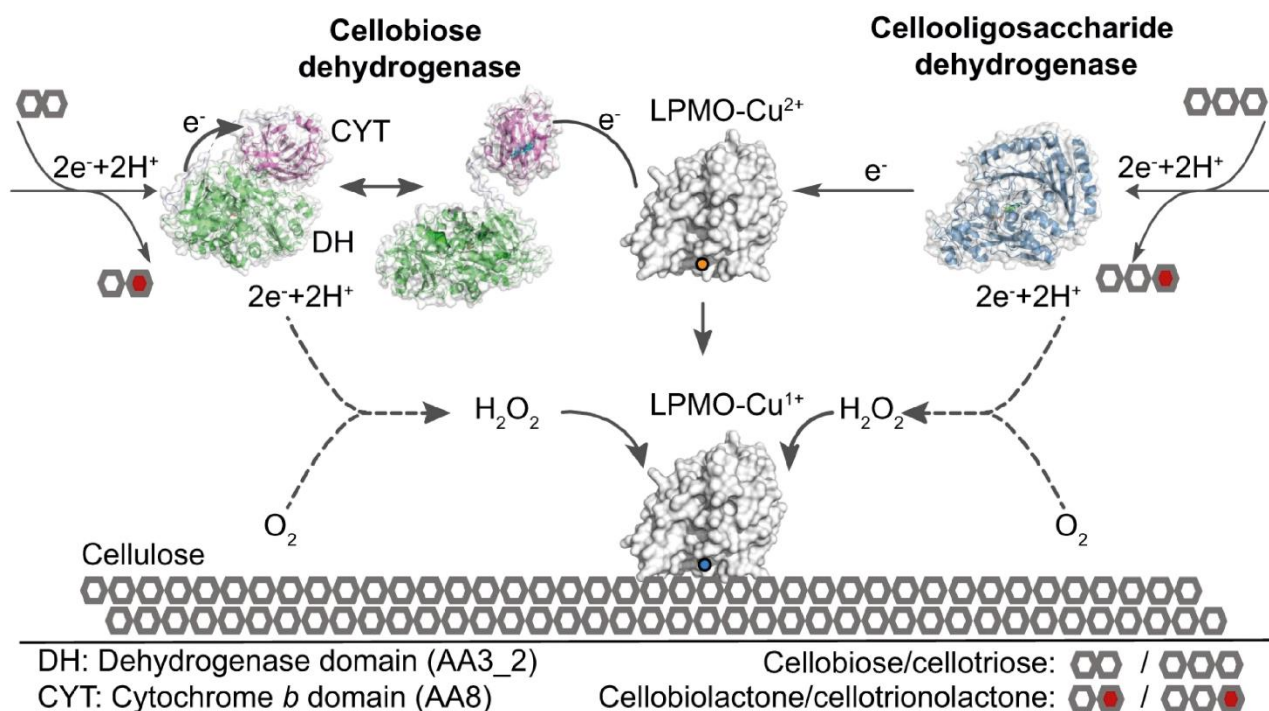


Fig. 11. Schematic model of the interplay of a CDH versus an AA7 cellooligosaccharide dehydrogenase with LPMOs during cellulose degradation. The figure was reproduced from (Haddad Momeni et al. 2021).

IV.3.3. Photo-biocatalysis

As an alternative to the biological partners presented hereinabove, another more artificial, yet industrially interesting, way to activate LPMOs relies on photobiocatalysis. The potential of light to fuel LPMOs reaction was first presented in 2016 by two different proofs of concept: (i) Cannella et al. demonstrated that photo-excited chlorophyllin could efficiently promote AA9 LPMOs reaction (Cannella et al. 2016), (ii) Bissaro et al. reported that the light promoted oxidation of H₂O by V-TiO₂ particles could provide reducing equivalent able to reduce the LPMO (Bissaro et al. 2016). In both examples, the mechanism remained unclear and was further investigated in a later study that demonstrated that LPMO reactions fueled by the light/chlorophyllin and light/V-TiO₂ both relied on the generation of ROS (superoxide and H₂O₂) (Bissaro et al. 2020). Further studies supported these conclusions (Blossom et al. 2020). Recently, it was also shown that light irradiation could drastically enhance the fueling rate of a chitin active LPMO by aromatic compounds, leading to significantly faster chitin solubilization (Kommedal et al. 2022). Even more recently, Monclaro et al. have shown that fungal melanin could also serve as light harvester in light-driven cellulose oxidation by LPMOs (Monclaro et al. 2023). In those last reports, H₂O₂ is believed to play a key role in this light-driven reactions.

IV.4. LPMOs modularity

IV.4.1. CBMs

Just as other enzymes targeting carbohydrate-based substrate, LPMOs can also be linked to CBMs (Horn et al. 2012), attached to the catalytic domain via a linker, which can vary in length, sequence and flexibility (Forsberg and Courtade 2022). Hence, CBMs found attached to LPMOs can come from various families: CBM1 are specific to cellulose (found in AA9, AA10, AA15 and AA16 families), CBM18 and CBM14 are specific to chitin (respectively found in AA9, AA10 and AA15) and CBM20 are specific to starch (found in AA13). Some CBMs usually found associated with AA10s display a broader carbohydrates specificity as it is the case for CBM2 (cellulose, chitin and xylan specific) and CBM5/CBM12 (cellulose and chitin specific).

CBMs are crucial for substrate binding and consequently catalytic activity of LPMO (Forsberg and Courtade 2022). Indeed, various studies have shown that deletion of the CBM leads to a decrease of the LPMO oxidized products (Bennati-Granier et al. 2015; Forsberg

et al. 2016; Crouch et al. 2016; Chalak et al. 2019). For instance, CBM1 containing AA9s from *Podospora anserina* displayed a 3 to 4-fold higher oxidized product generation than the other AA9s from the same organism (Bennati-Granier et al. 2015), which would imply that CBMs are an advantageous feature when it comes to catalytic product generation. To test the specificity of CBM toward their LPMO domain, some CBMs “swap” experiments have also been done by Crouch and colleagues (Crouch et al. 2016). This study revealed that CBMs are most likely substrate- and protein-specific, as introducing a non-natural CBM to *Tc*LPMO9 led to a loss of activity. It is also interesting to note that swapping the endogenous CBM2a of *Cf*LPMO10 or *Tf*LPMO10 (both targeting cellulose) by a CBM3a (cellulose specific module found in cellulases) led to a loss of activity, which would imply that CBMs may guide LPMOs in specific substrate areas (i.e, crystalline, amorphous, complex matrix, end of chains,...) better adapted to their catalytic domain binding and activity (Crouch et al. 2016). Interestingly, CBMs have also been found to play a role in the regioselectivity of LPMOs, as CBM deletion in *Cf*LPMO10 caused a change in the ratio of oxidized over non oxidized products released (Crouch et al. 2016). This observation was confirmed in a recent study where deletion of the CBM of *Pa*AA9H led to a modification of C1/C4 oxidation ratio (Chalak et al. 2019).

Although CBMs seem to be an important tool in PCW targeting LPMO, their presence is not mandatory for LPMOs activity. Indeed, removal of CBM in certain LPMOs does not always lead to a significant loss in activity. For instance, in the case of *Nc*LPMO9C, the deletion of the module even had no effect on the activity of the LPMO on PASC (Borisova et al. 2015).

CBMs are not universally employed by LPMOs, but when appended to an enzyme, they appear to be specifically adapted to their endogenous catalytic domain, allowing to optimize the enzyme reaction by enhancing the amount and driving the profile of oxidized products. Based on these facts it has been hypothesized that CBMs are a feature required for LPMOs that are supposed to target specific/hard to access part of the substrate on which tight bounding may be harder to perform, providing the right positioning of the active site (Crouch et al. 2016). Thus, the fact that the oxidation products of some LPMOs are not that much affected by the deletion of their CBM could imply either (i) that the artificial substrate employed is already in an “optimized” state for the enzymes-substrate ligation, and thus fully available for the LPMO or, (ii) that the presence of the CBM is irrelevant with the substrate tested.

Interestingly, the existence of a AA9-X280-CBM18 modularity (Lenfant et al. 2017) is

rather intriguing in a LPMO family known to target only cellulosic or hemicellulosic substrates and could imply an unreported involvement of some AA9s in chitin/fungal cell wall modification.

IV.4.2. GPI anchors

In some rare cases, LPMOs are found attached to a GlycosylPhosphatidyInositol (GPI) anchors, specific modules that permit the binding to plasma membranes (Ikezawa 2002). The first reported GPI carrying LPMO was from the recently discovered AA16 family (Filiatrault-Chastel et al. 2019). However, the paucity of works on this LPMOs family does not yet allow us to understand the biological function underlying this modularity. One could hypothesize that these AA16-GPI could have a role in the modification of carbohydrates in the vicinity of the cell walls, the purpose of which remains to be unveiled. Although scientific evidence is still lacking to conclude on the biological role for AA16-GPI, it is interesting to draw a parallel with some recent findings on LPMO-like proteins. The latter, formerly referred to as a X325 module of unknown function, were recently demonstrated to be LPMO-like proteins invariably harboring a GPI anchor, and unable to perform polysaccharide oxidative cleavage (Labourel et al. 2020). X325s were found to be essential for copper acquisition during the infection of the pathogen *Cryptococcus neoformans* (responsible for meningitis), providing new insights into the biological functions of GPI-carrying LPMO-like and LPMOs in general (Garcia-Santamarina et al. 2020).

IV.4.3. X modules of unknown function

It is possible to predict and classify modules in CBM families based on the sequence comparison with known module sequences. Nevertheless, when it is impossible to assign a module to a known family, such module is internally referred to as “X module” by CAZy. Today, there are many X families awaiting for biochemical studies, which would eventually lead to new CAZy families.

Regarding the AA9 family, several modules of unknown function have been identified *in silico* (Lenfant et al. 2017), including X278 modules (appended to some AA9 but also GH18 chitinases and chitin-active AA11, suggesting the involvement of this module in chitin recognition), X280 modules (sometime followed by a CBM18, also suggesting a chitin-related function), X281 modules, X282 modules (short module of approximately 30 residues always directly appended to the end of the AA9 domain) and X283 modules (short modules

of 7-8 residues with high concentration of positively charged amino acids) (**Fig. 12**).

The X282 module, only found in AA9 coming from saprotroph agaricomycetes (Hage et al. 2021). Six of these AA9-X282 were found upregulated more than 250 times after three days-growth on Avicel (Hage et al. 2021). At first glance, the upregulation of these AA9-X282s on cellulose made these LPMOs potentially interesting in a PCW degrading context, and they were extensively studied during this thesis (see **Chapter III**).

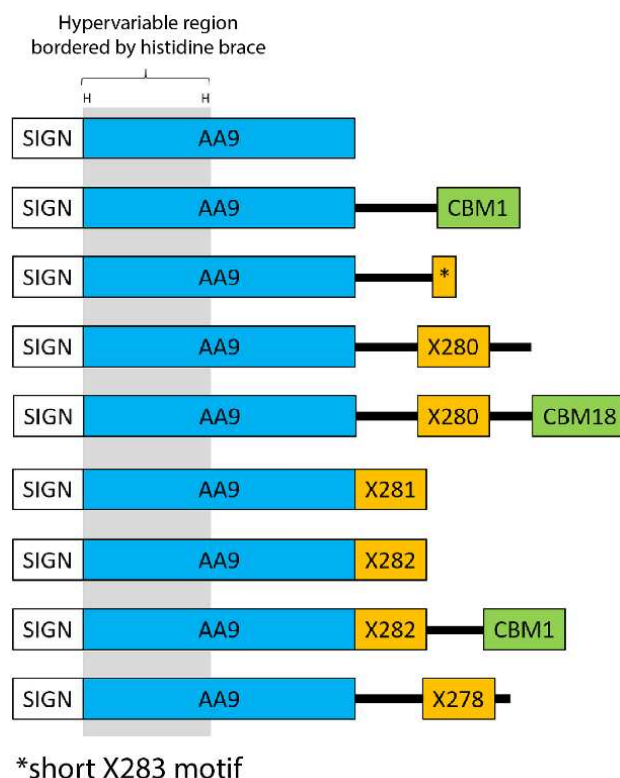


Fig.12. Modular architectures of AA9 LPMOs. The figure was adapted from (Lenfant et al. 2017)

IV.4.4. Disordered regions

Recently, an important and previously unexplored facet of LPMO C-terminal modularity has been investigated for the first time. These are called intrinsically disordered C-terminal regions (dCTRs) and are surprisingly found in over 60% of all LPMOs known to date (Tamburrini et al. 2021). dCTRs are characterized by a lack of stable secondary and/or tertiary structure and seem to be a rather specific feature of LPMOs within glycan-processing enzymes, as it has not been observed yet in other CAZymes. These dCTRs were historically removed to ease recombinant production and have long escaped any form of characterization. Although their function remain unknown, future studies will undoubtedly proceed to unveil their biological role. As observed for dCTRs in other proteins, these functions could possibly be related to stability or interactions with other partners (Tamburrini et al. 2021).

V. Second-generation biorefinery process schemes

The biorefinery differs from the classical petroleum refinery in the sense that it relies on bio-sourced, renewable raw materials, ranging from food crops for 1G biofuels, to lignocellulosic biomass for 2G biofuels. Due to the complex nature of PCW, second generation substrates (2G) are not easy to process. Getting access to the primary compounds of the PCW, embedded in a recalcitrant matrix, is one of the major challenges of the 2G biorefinery. All the components of PCW can be valorized into value-added molecules, but when it comes to bioethanol production, the main targeted molecules are glucose and xylose. Hence, to process this complex matrix and get access to these fermentable sugars, the plant biomass must undergo a succession of treatments, in which the fractions responsible for its recalcitrance are specifically deconstructed. Most bioethanol production processes involve a (physico)chemical pretreatment of the biomass followed by an enzymatic degradation (also referred as to “saccharification”). The extracted sugars from these two steps are then fermented into ethanol or other bioproducts (**Fig. 13**).

V.1. Pretreatment of the biomass

Several approaches have been developed for the pretreatment of biomass but remain similar in their purpose. Biomass pretreatment aims at getting better accessibility to value-added carbohydrates. A universal pretreatment compatible with every biomass does not exist, and therefore it must be adapted based on the biomass composition, downstream transformations, and the desired end products. In this regard, a variety of pretreatments have been developed, characterized by the specific modifications they bring to the PCW components.

Prior to chemical pretreatment, the biomass usually undergoes a first step of mechanical treatment (grinding, milling,...) essential to reduce its size and ensure the efficiency of downstream processes. Noteworthy, some recent technologies also allow the removal of lignin and hemicelluloses before biomass mechanical refining (Kuhn et al. 2020). One of the most commonly used and cost-efficient pretreatments is steam explosion (SE) which consists in the rapid release of high-pressure steam followed by a sudden decompression, leading to biomass explosion. This pretreatment allows the relocation of lignin and modification of its structure, a partial depolymerization and solubilization of hemicellulose and a strong increase of cellulose enzymatic digestibility (Ziegler-Devin et al. 2021). SE efficiency is often further improved with the addition of diluted acid (notably sulfuric acid), but this pretreatment methods leads to the increased formation of enzymes/fermentation inhibitors (Martín et al. 2018; Ho et al. 2019).

Other milder pretreatments such as sulfite-pulping allow the removal of lignin and recovering of varying amount of hemicellulose depending on the pH and temperature used (Zhu et al. 2009). Usually, alkaline pH conditions lead to higher conservation of the hemicellulose fraction (Ho et al. 2019). Accordingly, a pretreatment similar to SE in its principle, but also including ammonia (so-called ammonia fiber explosion or AFEX) has been developed to achieve cellulose decrystallization, better conservation of the hemicellulose and lower production of inhibitors (Balan et al. 2009).

The choice of pretreatment methods is not without consequences, since the resulting changes in the PCW structure and extracted soluble molecules have a direct impact on the efficiency of biomass-degrading enzymes. Therefore, in addition to the type of biomass, it is important to adapt the pretreatments to the composition of the enzymatic cocktails to be used in downstream biorefinery process.

V.2. Enzymatic degradation of the biomass

Enzymatic degradation of the biomass, the keystone of biorefinery process, is based on the implementation of enzymatic activities (notably cellulases and hemicellulases) coming from potent cellulose degrading organisms. To this extent, one of the most studied microorganisms for industrial scale enzyme production is *T. reesei* (teleomorph *Hypocrea jecorina*). This saprotrophic ascomycete was discovered during World War II in the Salomon Islands. At that time, this fungus already attracted scientists' attention for its ability to degrade tent fabrics and uniforms of Americans soldiers, made of cotton. Since then, this first sampled strain, so-called QM6a, has been the object of numerous studies.

Although *T. reesei* is recognized as one of the most efficient organisms for PCW degradation, it possesses a relatively limited set of cellulolytic enzymes. In terms of cellulases, the genome of the wild-type strain QM6a encodes 18 cellulases, including two CBHs (*TrCel7A*; CBHI and *TrCel6A*; CBHII), five EGs (*TrCel7A*, *TrCel5A*, *TrCel5B*, *TrCel12A* and *TrCel45A*) and 11 BGs (*TrCel3A*, *TrCel3B*, *TrCel3E*, *TrCel3F*, *TrCel3H*, *TrCel3J*, *TrCel1A*, *TrCel3C*, *TrCel1B*, *TrCel3G*, and *TrCel3D*), *TrCel3A* remaining the most studied and secreted one (Martinez et al. 2008; Adav et al. 2012). These cellulases are not equally expressed during fungal growth. For instance, the two CBHs represent 80% of *T. reesei* secretome (*TrCel7A* alone representing 60%), while BGs surprisingly account for only 0.5% (Wood 1992; Suominen et al. 1993). Today, most commercially available enzyme cocktails are based on *T. reesei* secretomes, including Celluclast 1.5 L and Cellic CTec2 from Novozymes and Accellerase1500 from DuPont (Filiatrault-Chastel et al. 2021).

To further improve the cellulolytic efficiency of *T. reesei* enzymatic cocktails, several approaches have been conducted. For example, the QM6a strain was subjected to several rounds of random mutagenesis, leading to the development of several hyperproducer strains, e.g. RUT-C30. In addition to strain engineering, improvements have also been made on individual cellulases. However, detailed informations of these improvements (pH, stability, catalytic efficiency...) are not always available in the literature.

A more recent approach to improve the efficiency of cellulolytic cocktails has focused on the addition of external pure enzymes, such as GHs, CEs and AAs (including LPMOs) (Filiatrault-Chastel et al. 2021). Yet, it is worth bearing in mind that this optimization approach is mainly performed at lab scale and not always validated at industrial scale. Typically, the substrate loading used in labs assays are usually ranging from 1 – 10% (w/v) while industrial scale uses much higher concentrations, i.e. > 20%.

Today, part of the challenge for enzymatic cocktails is to adapt them specifically to each type of biomass and pretreatment. This "tailored made" cocktail or "minimal cocktail" approaches has gained increasing interest in recent years, and represent promising strategies in biorefinery to evaluate the impact of a given enzyme. An illustration of this type of approach is presented in **Chapter I**.

V.3. Fermentation step

In the last step of bioethanol production, the sugars (mainly glucose) obtained from upstream biomass degradation are fermented into value-added products (mainly ethanol) (**Fig. 13**) by specialized microorganisms. To this purpose, the most broadly used organism is the yeast *Saccharomyces cerevisiae*, capable of yielding high amount of ethanol. However, as this yeast is not able to metabolize pentoses resulting from the hydrolysis step, modified strains capable of using C5 sugars (i.e. xylose and arabinose) are now implemented during the fermentation process (Caballero and Ramos 2017).

The hydrolysis and fermentation steps are commonly performed separately, a process configuration called SHF (Separate Hydrolysis and Fermentation). Other ways of processing biomass exist, such as SSCF (Simultaneous Saccharification and Co-Fermentation), which allows the sugars resulting from hydrolysis to be simultaneously fermented (Ge et al. 2021). One of the advantages of SSCF is its capacity to mitigate the inhibition of cellulases by sugars during biomass degradation. Fermenting microorganisms in SSCF efficiently consume potential enzymatic inhibitors, such as glucose or cellobiose, thus creating a conducive environment for cellulase activity (Patel and Shah 2021). This phenomenon contributes to improved biomass conversion. However, compatibility between enzymatic hydrolytic systems and requirements for microbial fermentation still poses some challenges. Indeed, the optimal temperature and pH conditions of the fermenting microorganisms (pH 6, 35°C) differ from the one of cellulases (pH 5, 50°C). Additionally, fermentation requires to work in an anaerobic environment, which may not be compatible with the activity of LPMOs, since H₂O₂ production mostly originate from molecular oxygen.

From pretreatment to fermentation, there are several bottlenecks that can negatively impact the cost-effectiveness of biorefinery processes. For instance, these limitations may

be related to the production of inhibitors, low hydrolysis/fermentation yields, or the cost of enzyme production. Hence, by boosting extraction yields from biomasses that are recalcitrant to hydrolysis, and/or by reducing total enzyme loading required to achieve cost effective yields, LPMOs are now the key enzymes in biorefinery processes.

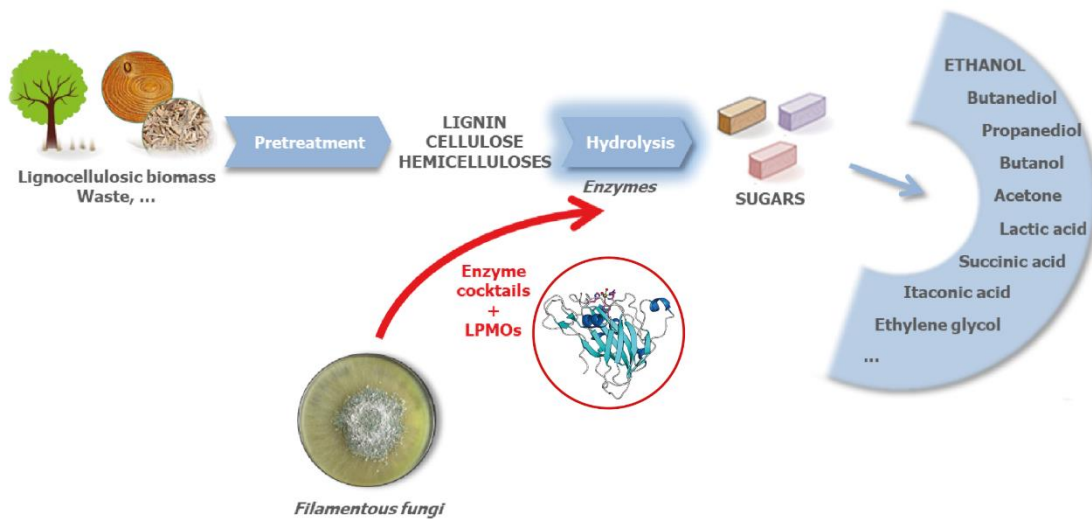


Fig. 13. Biorefinery process and bioproduct production. The figure was adapted from C. Filiatrault-Chastel PhD thesis manuscript (Filiatrault-Chastel 2019)

V.4. LPMOs in a biorefinery context

The LPMO boost effect initially observed (Vaaje-Kolstad et al. 2010; Harris et al. 2010) has been extensively reported in the literature (Harris et al. 2010; Cannella et al. 2012; Lo Leggio et al. 2015) resulting in a median increase in sugar yields of approximately 30% (**Fig. 14**). Conveniently, the LPMOs loadings required to observe this boost generally represent a low fraction of the total enzyme load in cocktails (Hu et al. 2013; Hu et al. 2015), making their implementation in cocktails economically attractive. The use of LPMOs also allowed to consider a wider variety of recalcitrant biomasses (softwood, hardwood, ...) that would otherwise be less attractive as biorefinery substrates because of their low degradability by industrial hydrolytic cocktails.

Besides the extraction of glucose from cellulose, it is also worth reminding that the addition of AA9 has also been shown to increase the extraction of other PCW components such as xylan (Kim et al. 2016). With the emergence of a variety of new LPMO families active on different PCW components (cellulose, xylan, pectin, ...) and a plethora of uncharacterized members, it is certain that the hunt for the best "yield enhancing" LPMOs is not over and that breakthroughs are still possible. However, LPMOs are tricky enzymes as the optimal conditions required for their activity are not easy to control and consist of a fine balance between the amount of reducing power and the availability/amount of H_2O_2 . Therefore, in parallel with the characterization of potentially new promising LPMOs to tackle the still most recalcitrant biomass, it is also essential to address several bottlenecks regarding the control of the LPMOs reaction in enzymatic cocktails.

Interestingly, a first attempt to better control the activity of LPMOs with H_2O_2 in the context of pretreated biomass degradation was made by Muller and colleagues in 2015 (Müller et al. 2015). In this study, H_2O_2 was supplied artificially via a pump system, and the authors successfully managed to fuel the LPMOs present in the commercial cocktail Cellic CTec2 (Novozymes) resulting in a boost in glucan yields during the degradation of steam-exploded birchwood. Although effective, this method of supplementation has limitations: (i) In this set up, H_2O_2 production is not specifically located in the vicinity of the cellulose, where the LPMOs are meant to be active, and thus (ii) the pumped H_2O_2 is more likely to react with other compounds, such as lignin derivatives, resulting in unproductive consumption of a potential LPMO reductant and co-substrate.

Thus, other alternatives that allow for finer control of LPMO activity could be considered, and one promising alternative might be to draw inspiration from the strategies employed by fungi in nature.

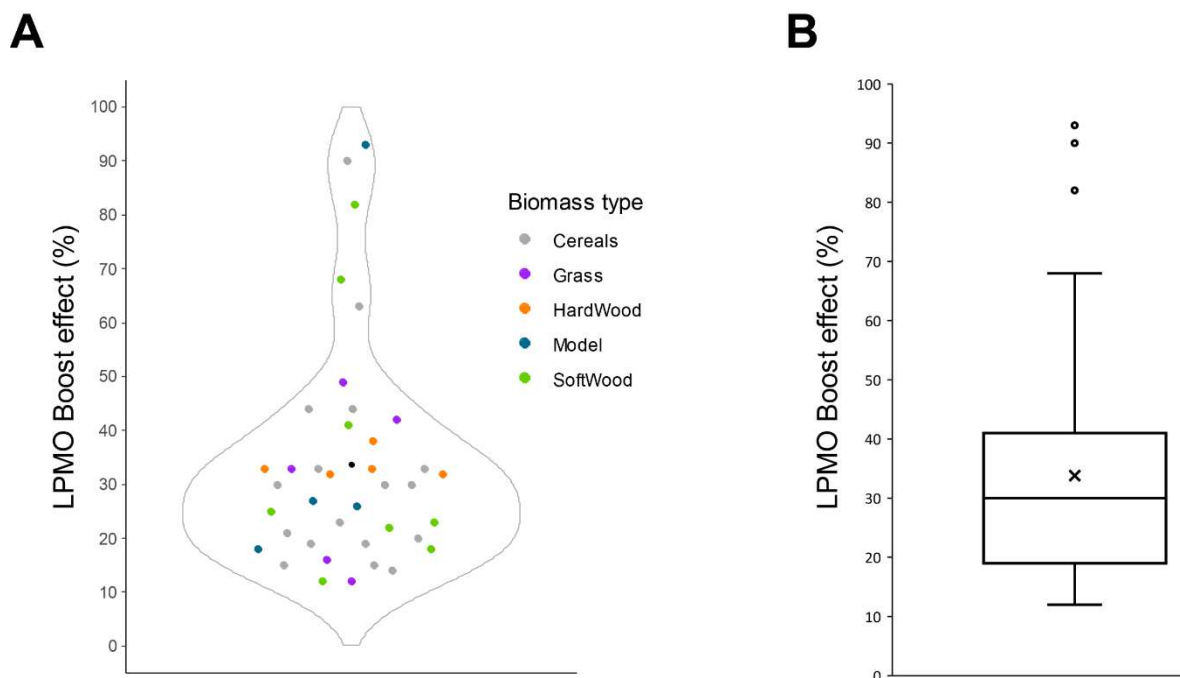


Fig. 14. Review of the best LPMO boost effects reported in the literature. (A) Violin plot showing the distribution of the relative increase in sugar yields upon addition of LPMO in cellulytic cocktails on various types of biomass, so called “LPMO boost effect”. 39 LPMO boost effect values were retrieved from the following studies (Harris et al. 2010; Hu et al. 2015; Müller et al. 2015; Chylenski et al. 2017; Quinlan et al. 2011; Scott et al. 2016; Kim et al. 2017; Du et al. 2018; Abik et al. 2023; Kim et al. 2016; Sanhueza et al. 2018; Dimarogona, Topakas et Christakopoulos 2012; Westereng et al. 2011; Grieco et al. 2020; Chabbert et al. 2017; Couturier et al. 2018; Filiatrault-Chastel et al. 2019; Calderaro et al. 2020; Bernardi et al. 2020; Caputo et al. 2023; Li et al. 2022; Park et al. 2023; Moya et al. 2023) . The type of biomass for which the boost was observed is indicated on the figure. cereals in grey (including wheat straw, corn stover, rice straw and delignified residues), grass in purple (including sugarcane bagasse and miscanthus), hardwood in orange (including poplar and birchwood), model substrates in blue (including microcrystalline cellulose, Avicel and nanofiber cellulose) and softwood in green (including pine, Lodgepole pine, and Norway spruce). (B) Box plot of the above mentioned LPMO boost effects. The central box shows a median boost effect of 30 %, an upper quartile of ~40% and a lower quartile of ~20%, and the whiskers show a minimum value of 12 % and a maximum value of 82 %.

Objectives of the thesis

Since the discovery of their mechanism in 2010, LPMOs have enabled major advances in the field of biomass biorefining. However, the boosting effect generally observed with the application of LPMOs varies greatly depending on the biomass targeted, and the benefit of their addition to cellulolytic cocktails can be relatively low for some plant biomasses. On top of this, the way in which LPMOs are currently incorporated into cellulolytic cocktails does not always take into account all the subtleties of their mechanisms. For example, if present at all, reducing agents are usually added in arbitrary quantities, and the availability of H₂O₂ in reactions is often not controlled.

Therefore, in this PhD thesis, we aimed to answering the following question: “Can we find new LPMOs with great potential in biorefinery, and better control their action in a context of lignocellulosic biomass degradation by cellulolytic cocktails?”. To address this question, we focused on fungal LPMOs from the AA9 family, one of the most studied and promising LPMO family in industrial context and developed two main strategies.

The first approach consisted in the **implementation of some of the best cellulose-active AA9s in combination with one CDH** from the same organism, with the aim to efficiently control their activity (i.e. reduction and H₂O₂ fueling) on challenging 2G biomasses such as softwood, hardwood and grass. The boosting effect of these AA9/CDH couples were evaluated using a reconstituted cocktail of *T. reesei* cellulases. This first axis was initiated at INRAE BBF in Marseille and finalized at IFPEN in Paris where I spent one year.

A second strategy focused on the **search for new AA9s with high potential in a biorefinery context**. To this end, we delved into fungal diversity, focusing on Polyporales, some of nature's most efficient saprotrophic wood degraders. Based on previously published transcriptomic studies, we selected AA9s of interest and proceeded to their in-depth characterization. All the experiments dedicated to this axis were conducted at INRAE BBF in Marseille.

A side objective of my thesis was also to pursue the functional and structural characterization of fungal AA16 LPMOs, notably by solving the crystallographic structure and characterizing new members. Unfortunately, this axis proved unsuccessful at the protein production stage (despite different cloning strategies attempted), and since very little time was allocated to it, the preliminary results I obtained will not be developed in this thesis manuscript.

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Chapter I

Implementation of CDH in LPMO-containing reconstituted cocktails to improve bioproducts production from lignocellulosic biomass

Implementation of CDH in LPMO-containing minimal cellulases cocktails to tune bioproducts production from lignocellulosic biomass

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Abstract

Plant biomass can be converted to bioproducts using fungal enzymatic cocktails that have been improved upon implementation of lytic polysaccharide monooxygenases (LPMOs), known to boost cellulases. However, due to the complexity of their oxidative catalysis, it is still difficult to properly control and sustain LPMOs activity. Here, we investigated whether the implementation of the natural enzymatic partner of LPMOs, i.e. cellobiose dehydrogenase (CDH), could be a promising solution. To this end, we reconstituted a minimal cocktail using the main cellulases from *Trichoderma reesei*, and evaluated the impact of the addition of two LPMOs and one CDH on the conversion of wheat straw, miscanthus, pine and poplar. Surprisingly, while the addition of LPMOs or LPMO/CDH showed little to no increase in glucose equivalent yields, the sole addition of CDH caused an increase of up to 27% (on miscanthus). Interestingly, CDH supplementation allowed to tune the stream of degradation products towards cellobionic acid while maintaining equivalent or increasing the overall conversion yields, in a biomass-dependent manner. Our study shows that extracting reasonable amounts of high value-added oxidized sugars from industrial biomass is feasible, opening thereby new perspectives for the use of CDH in industry.

Keywords: bioeconomy, *Trichoderma reesei*, cellulose, cellulases, CDH, lytic polysaccharide monooxygenase

Introduction

The production of second generation (2G) biofuels and bioproducts is a promising yet still challenging alternative to fossil-based fuels and chemicals. Plant carbohydrates, as products of photosynthesis, constitute an abundant and appealing renewable source of carbon for the bioindustries. However, the extraction of simple fermentable sugars from the complex plant cell wall (PCW) matrix remains a major hurdle, known as “biomass recalcitrance”, which has to be studied for each type of biomass (Himmel et al. 2007). Since cellulose represents the most important source of glucose in PCW, various enzymatic cocktails have been designed in recent years to efficiently degrade this recalcitrant biopolymer (Lopes et al. 2018; Houfani et al. 2020; Contreras et al. 2020; King et al. 2011; Filiatrault-Chastel et al. 2021). Due to biomass-dependent co-polymers content and structure (e.g. lignin, hemicelluloses), and pre-treatment strategies, it becomes increasingly clear that the one-for-all cocktails (such as Cellic CTec® series) need to evolve towards tailor-made cocktails adapted to different types of lignocellulosic biomass.

Today, a striking observation is that most cellulolytic cocktails used in industry are of fungal origin (Filiatrault-Chastel et al. 2021). This is in great part related to the well-documented efficiency of (some) filamentous fungi, notably wood decayers, to deconstruct PCW in nature. To do so, filamentous fungi secrete a wide array of carbohydrate active enzymes (CAZymes ; (Drula et al. 2022)). Notably, in addition to the long-studied glycoside hydrolases (GH), the last decade has seen the emergence of redox-active CAZymes as key players in biomass degradation (Bissaro et al. 2018), forming altogether what has been referred to as “enzyme interactome” (Monclaro et al. 2022). Among these, the discovery of lytic polysaccharide monooxygenases (LPMOs) has marked a turning point (Vaaje-Kolstad et al. 2010; Quinlan et al. 2011; Phillips et al. 2011). LPMOs are monocopper oxidoreductases that perform the oxidative cleavage of glycosidic bonds at the surface of cellulose, thereby creating new chain ends accessible to cellulases and also reducing the cellulose crystallinity by modifying its structure, leading to an overall boost in the biomass depolymerization (Eibinger et al. 2014; Villares et al. 2017). In addition, recent studies have also highlighted that the action of LPMOs increases the hydration of cellulose fibers, further reinforcing their partnership with hydrolases (Uchiyama et al. 2022; Cannella et al. 2023). Because of these peculiar capacities, the boosting effect resulting from the addition of LPMOs in cellulolytic cocktails for biomass conversion purposes, first reported in the early 2010's (Quinlan et al. 2011; Phillips et al. 2011; Harris et al. 2010; Cannella and Jørgensen 2014), has been extensively studied since then (Chylenski et al. 2019; Bernardi et al. 2020).

To perform their reaction, LPMOs need first to be reduced from the Cu(II) resting state to the Cu(I) active state. Then, upon reaction with H₂O₂ as co-substrate (Bissaro et al. 2017), LPMOs will oxidize the C1 and/or C4 carbon of scissile glycosidic bonds. The impacts of the nature, abundance, and mode of action of the reductant and co-substrate on LPMO catalysis are notoriously difficult to control (Eijsink et al. 2019). Furthermore, despite several reports on various redox enzymes competing or partnering with LPMOs (Phillips et al. 2011; Bennati-Granier et al. 2015; Kont et al. 2020; Haddad Momeni et al. 2021; Sun et al. 2023; Kracher et al. 2016), only a few studies have evaluated the effect of such partners in the perspective of designing new cocktails (Scott et al. 2016; Brenelli et al. 2018).

Remarkably, the effect of cellobiose dehydrogenase (CDH), described as a natural redox partner of LPMOs during wood decay by filamentous fungi (Phillips et al. 2011; Bey et al. 2011; Bey et al. 2013; Poidevin et al. 2014; Navarro et al. 2014; Bennati-Granier et al. 2015; Couturier et al. 2015; Tan et al. 2015; Harada et al. 2017; Miyauchi et al. 2017; Felice et al. 2021), has been little studied from such an application perspective (Barbosa et al. 2020; Wang and Lu 2016). These bimodular flavodehydrogenases are able to oxidize cellobiose into its corresponding lactone and sequentially transfer the two electrons resulting from this reaction to their heme-containing cytochrome (Igarashi et al. 2002). One of these electrons can then be further transferred to the LPMO Cu(II) atom to form the active, LPMO-Cu(I) state (Tan et al. 2015). Although less efficient than genuine oxidases, CDHs are also capable to use molecular oxygen as electron acceptor (Bao et al. 1993). Conveniently, when paired with LPMOs, it has been demonstrated that CDH could fulfill both functions of copper reduction (Langston et al. 2011; Kracher and Ludwig 2016) and H₂O₂ supply (Kracher et al. 2020).

In this study, we evaluated the effect of a CDH and two cellulose-active LPMOs from the auxiliary activity family 9 (AA9) on the biomass conversion efficiency of a tailor-made cellulases cocktail from *Trichoderma reesei*. To this end, we used oxidases from the coprophilous ascomycete *Podospora anserina*, viz. *PaCDHB*, the C1-oxidizing *PaAA9E* and the C1/C4-oxidizing *PaAA9H*. Using a design of experiment approach, in which neutral and oxidized products solubilized by different enzyme proportions were quantified, we evaluated the effect of the CDH on the release of soluble products from wheat straw and other recalcitrant lignocellulosic biomass of industrial interest: miscanthus, pine and poplar.

Results

AA9/CDH couples show negative to moderately positive effects on glucose release from wheat straw by a minimal *T. reesei* cellulases cocktail

The first step of this study was to design a minimal *T. reesei* cellulases cocktail. Inspired by data reported in the literature on the use of *T. reesei* enzymes for the deconstruction of various (ligno)cellulosic substrates (**Table S1**), we designed a cocktail including: two cellobiohydrolases (40% w/w of CBHI (also referred to as *TrCel7A*) and 40% w/w of CBHII (*TrCel6A*)), two endoglucanases (5% w/w of EGI (*TrCel7B*) and 5% w/w of EGII (*TrCel5A*)), and one β -glucosidase (10% w/w of *TrBGL1* (*TrCel3A*)). After purifying these enzymes from a secretome of *T. reesei* RUT-C30 strain, we assessed the efficiency of this cellulases minimal cocktail (henceforth called “*TrCC*”) on steam exploded wheat straw. In good agreement with previous results (Billard et al. 2012; Chylenski et al. 2017) (**Table S1**), a glucose yield of 80% was obtained after 48 h of hydrolysis with an enzyme loading of 20 mg/g of dry matter (DM) (**Fig. S1A**). In order to supplement *TrCC* with an oxidative system, we selected two highly active cellulolytic AA9 LPMOs, viz. *PaAA9E* and *PaAA9H* (which both harbors a CBM1 domain) (Bennati-Granier et al. 2015), and the CDHB from the same fungus, *PaCDHB*, previously demonstrated to act in synergy with the selected LPMOs (Bennati-Granier et al. 2015). After heterologous production of each of these enzymes in *Pichia pastoris*, the activity of both LPMO/CDH couples was validated on phosphoric acid swollen cellulose (PASC) by identifying the typical C1 or C1/C4 soluble oxidized products, respectively released by the action of *PaAA9E* or *PaAA9H*. (**Fig. S1B**).

With both the hydrolytic and oxidative systems being separately operational (**Fig. S1**), the next goal was to assess their combined activity on lignocellulosic substrates. Using a design of experiment approach (MODDE software v12.0), we determined the best enzymes proportions to obtain the highest glucose yield from steam exploded wheat straw. To this end, the concentration of *PaCDHB*, AA9 LPMOs (*PaAA9E* or *PaAA9H*) and *TrCC* were set as variables, resulting in a total of 30 combinations (see **Table S2** for more details on the model). Figure 1 shows the resulting contour plot with the glucose yields obtained after 48 h of reaction. Maximum glucose yields of 34 %, 61 % and 82 % were obtained for *TrCC* loadings of 2, 11 and 20 mg/g of DM, respectively. Regardless of the assayed condition, both the C1-oxidizing *PaAA9E* and the C1/C4-oxidizing *PaAA9H* did not appear to have any significant beneficial effect on glucose release. Surprisingly, in all tested conditions, the

presence of *PaCDHB* clearly revealed to be detrimental: the higher the CDH content, the lower the glucose yield (**Fig. 1**). Since *TrBGL1* and *PaCDHB* both share the same substrate (*e.g.* cellobiose), and while noting that the CDH is poorly active on glucose, we think that potential substrates competition between these two enzymes may reduce the overall glucose production coming from the hydrolysis of cellobiose by *TrBGL1*, in favor of cellobiose oxidation by *PaCDHB*. Thus, when aiming at increasing glucose yields, this screening on wheat straw suggested that, if included at all, *PaCDHB* should be implemented at low loading levels in a *TrCC* cocktail complemented with redox-active CAZymes. We then decided to check whether similar results would still be observed on other industrially relevant steam exploded lignocellulosic biomass, *i.e.* miscanthus, pine and poplar (see **Table S3** for biomass pretreatment details and **Table S4** for biomass compositions).

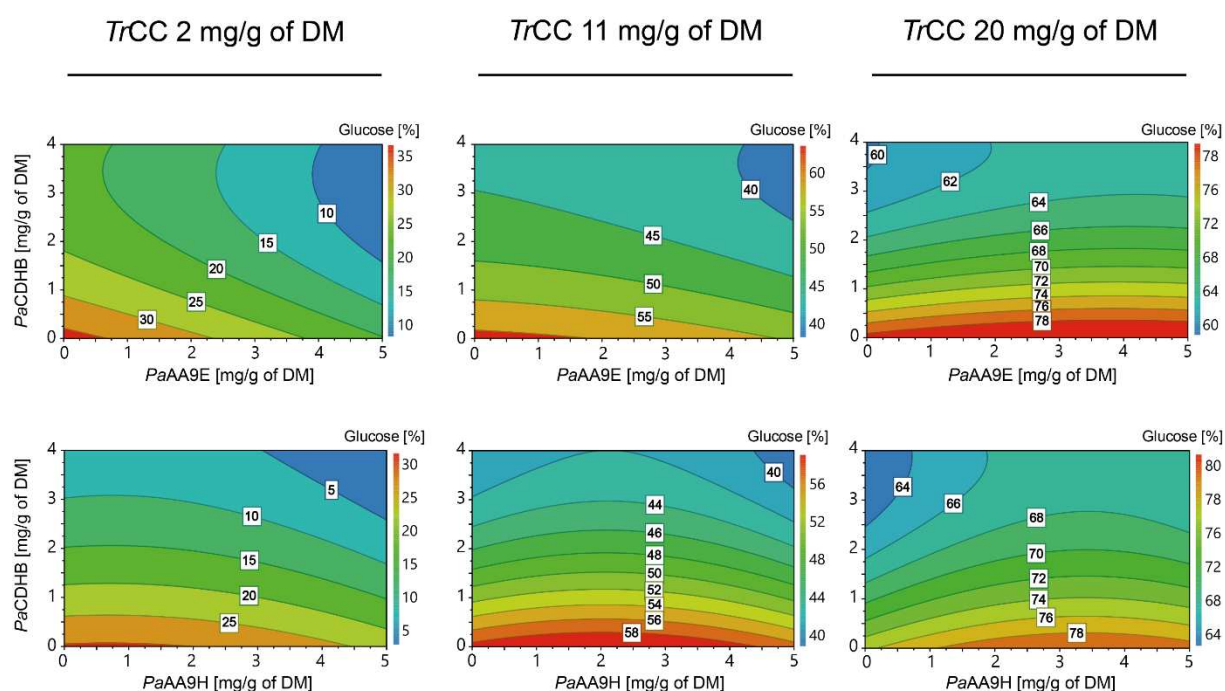


Fig.1. Contour plot of glucose yields obtained on steam-exploded wheat straw degraded by *TrCC* as a function of *PaAA9s* and *PaCDHB* loadings. Based on the design of experiment output (see **Table S2** for more details), we tested three *TrCC* loadings (2, 11 and 20 mg/g of DM, from left to right hand side) mixed with *PaCDHB* (0 to 4 mg/g of DM) and two different AA9s (0 to 5 mg/g of DM), namely *PaAAE* (top row) or *PaAA9H* (bottom row). The glucose yields obtained after 48 h are associated with a color gradient from blue (lowest yields) to red (highest yields) (note that the color scale is specific to each panel). All reactions were carried out with steam-exploded wheat straw (1% w/v) in sodium acetate buffer (50 mM, pH 5.2), at 50°C under stirring (850 rpm).

Conversion yields on miscanthus, pine and poplar

To evaluate the potential impact of the AA9/CDH couples on the soluble products released from miscanthus, pine and poplar, we set the enzymes loading of *TrCC* cocktail at 10 mg/g of DM, in the range of other previously reported cocktails (**Table S1**). As for the oxidative system, LPMO concentration was set at 3 mg/g of DM, and since the results obtained on wheat straw suggested a potential competition between cellobiose hydrolysis and oxidation (**Fig. 1**), we first added *PaCDHB* at a low concentration of 0.25 mg/g of DM. The released products were analyzed after 4, 24 and 48 h of reaction using a high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) (**Fig. 2**). The analysis revealed the presence of four main products identified as glucose, cellobiose, and cellobionic acid (**Fig. S2**). Of note, when detected, gluconic acid was found in negligible amounts (< 0.1% glucose equivalents yields) and was therefore not considered in the following quantifications. Unsurprisingly, glucose stood out as the main released product in all conditions, with yields ranging between 24-27%, 15-19% and 7-8% on pine, miscanthus and poplar, respectively. However, supplementation of *TrCC* with oxidative enzymes did not yield any major improvement in glucose yields, regardless of the biomass (**Fig. 2**). Interestingly when looking at the release of soluble cellobionic acid, some substrate-specific observations could be made. While glucose yields appeared higher on pine (27%) than on miscanthus (19%), the opposite trend was observed when considering cellobionic acid (up to 0.6% on pine and 1.6% on miscanthus). On poplar, no significant release of cellobionic acid could be detected. While the exact reasons for such differences in products yields are complicated to determine, one could speculate on the presence of potential biomass-dependent enzyme inhibitors (such as xylotriose, 3,5-dimethoxy-4-hydroxybenzaldehyde or 4-hydroxy-3-methoxy-cinnamaldehyde) (Momeni et al. 2015; Dondelinger et al. 2016; Ramtanon et al. 2023) and/or different accessibility of the cellulose chains. In general, these assays suggest that (i) *PaCDHB* efficiency seems to be biomass-dependent, (ii) the release of cellobionic acid appears to be LPMO-independent (see **Fig. 2** insets) and mainly due to the action of *PaCDHB*.

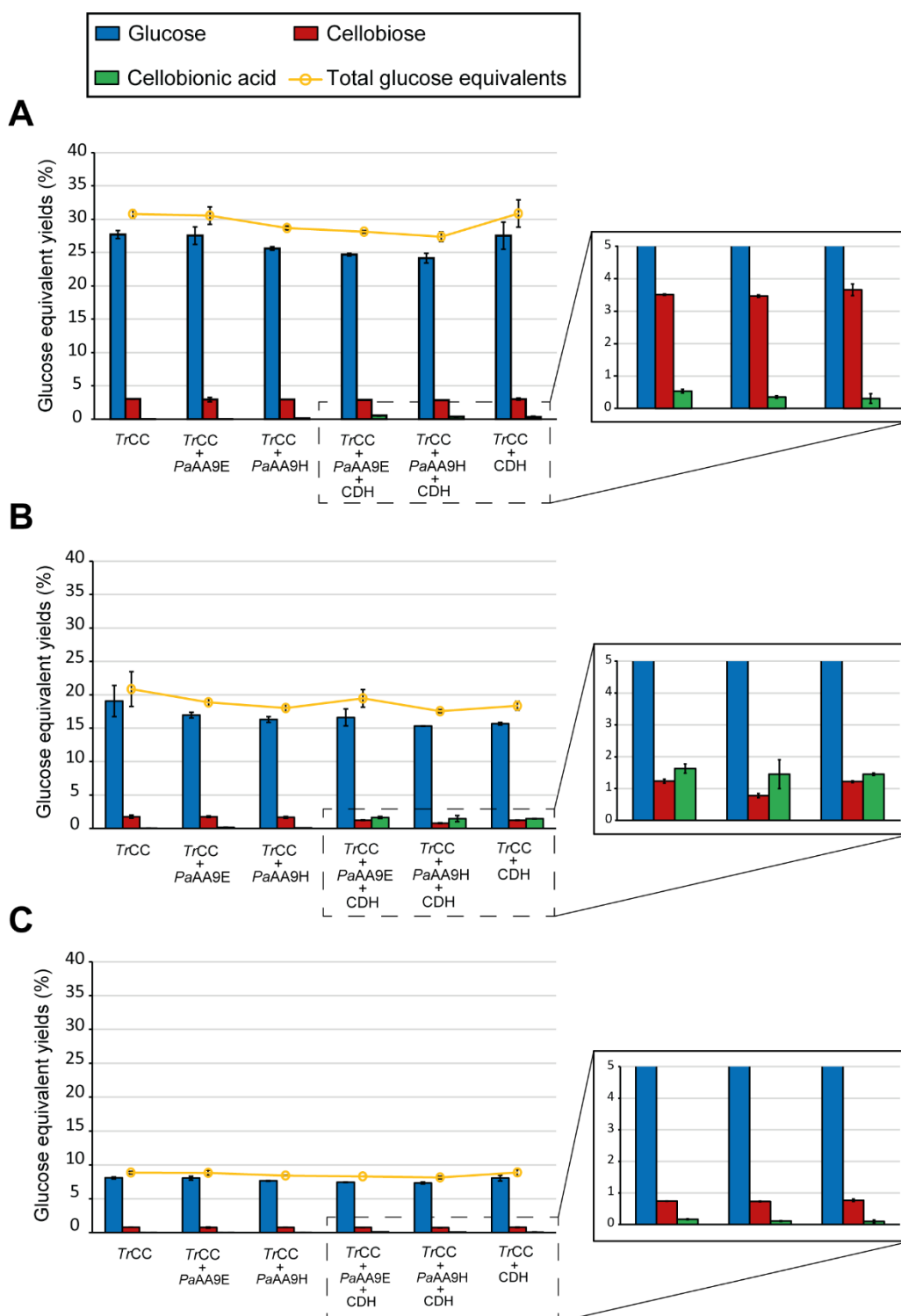


Fig. 2. Total soluble products released from (A) pine, (B) miscanthus and (C) poplar after 48 h of reaction in the presence of *TrCC* supplemented with oxidative enzymes. The graphics show the released soluble products and the corresponding total glucose equivalent yields (one cellobiose or cellobionic acid molecules account for two glucose equivalents) released from pine (A) miscanthus (B) and poplar (C) by *TrCC* (10 mg/g of DM) upon the addition of *PaAA9E* or *PaAA9H* (3 mg/g of DM) and/or *PaCDHB* (0.25 mg/g of DM). All reactions were carried out with pretreated biomass (2% w/v) in sodium acetate buffer (50 mM, pH 5.2), at 50°C under stirring (850 rpm). Bars show average values and error bars represent the standard deviation (n = 3 independent biological replicates).

Impact of *Pa*CDHB on pine and miscanthus cellulose depolymerization

As shown above (**Fig. 2**), C1-oxidized cellobiosaccharides released during biomass degradation by *Tr*CC cocktail spiked with oxidases appeared to originate mainly from *Pa*CDHB action. Therefore, we decided to further investigate the impact of the latter on cellulose depolymerization testing a range of *Pa*CDHB concentrations (from 0.5 to 6 mg/g of DM). In general, glucose yields remained slightly higher on pine (17%, **Fig. 3A**) than on miscanthus (maximum 14%, **Fig. 3B**) (note that poplar was left aside because of very low glucose yields and quasi-null CDH activity, **Fig. 2**). Strikingly, in the *Pa*CDHB concentration range of 0.5 to 3 mg/g of DM, the activity of *Pa*CDHB did not seem impacted by the complex biomass composition as the more CDH the more cellobionic acid was detected, resulting in a quasi linear dose-response ($R^2 = 0.98$) (**Fig. 3C**). Of note, the absolute amount of cellobionic acid appeared always higher (up to 12% glucose equivalent yield) in the reactions performed on miscanthus than on pine. Consistently, a time-course experiment showed that *Pa*CDHB operates at constant speed in reactions done on miscanthus, as shown by the very linear time-dependent release of cellobionic acid until the last analyzed time point (48 h), in contrast to pine on which the CDH reaction nearly stopped at 24 h (**Fig. S3**). As for the total glucose equivalent yields, while no clear differences between the tested conditions could be observed on pine, significant boosts were obtained on miscanthus upon addition of *Pa*CDHB at the two highest tested concentrations (3 and 6 mg of *Pa*CDHB/g of DM), allowing to obtain the best overall yields from this series of experiments (19%). This boost represented a relative increase in total glucose equivalent yields of 27 % when compared to the *Tr*CC alone condition (**Fig. 3D**). We speculate that this boosting effect may originate from the alleviation of cellulases inhibition by cellobiose upon oxidation of the latter by CDH as previously described (Igarashi et al. 1998; Wang and Lu 2016).

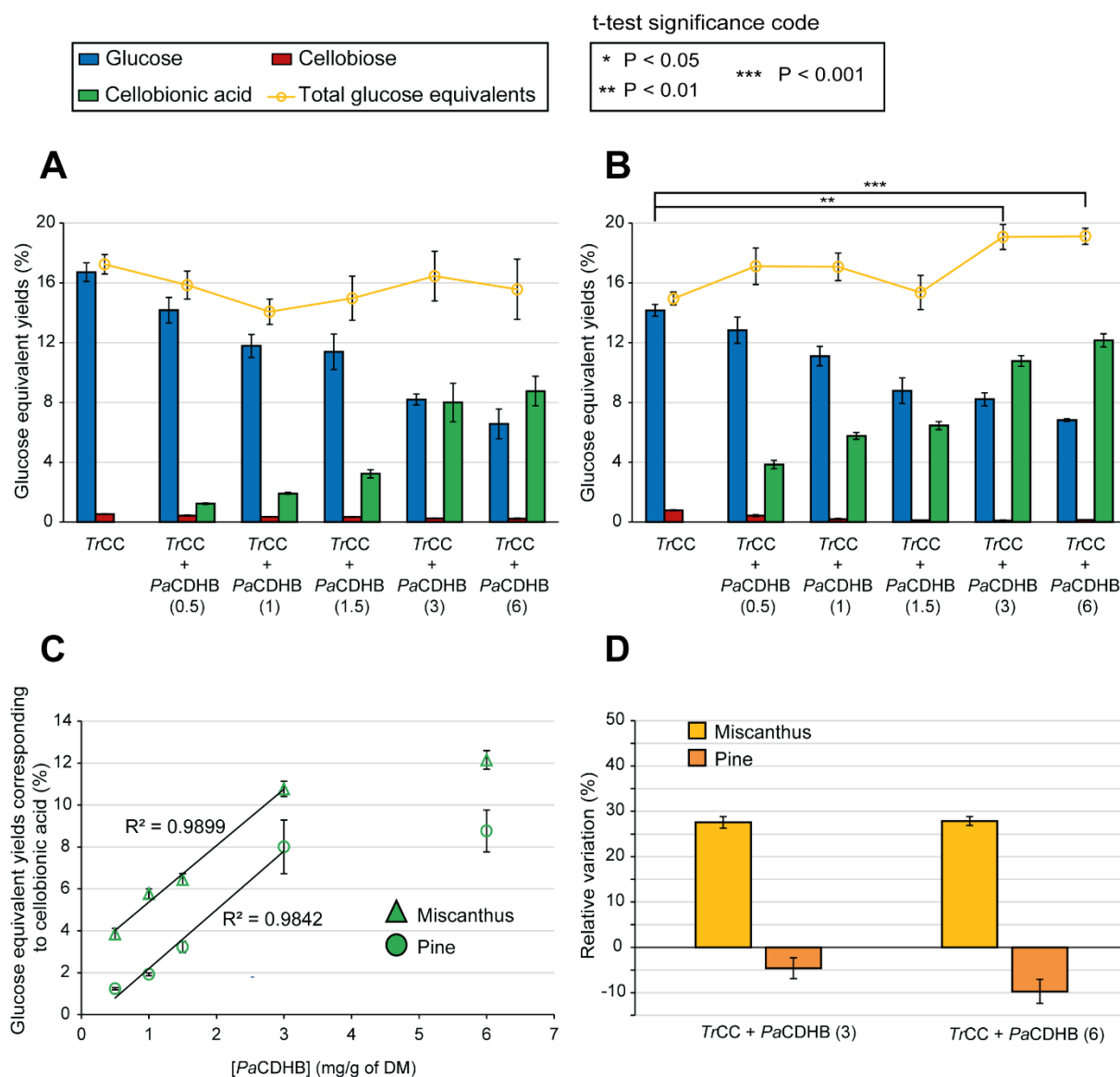


Fig. 3. Effect of *PaCDHB* on total products released by *TrCC* from pine (A) and miscanthus (B) after 48 h. The graphs show soluble products and the corresponding total glucose equivalent yields (one cellobiose or cellobionic acid molecules account for two glucose equivalents) released from pine (A) and miscanthus (B) by *TrCC* (10 mg/g of DM) with varying concentrations of *PaCDHB* (0 to 6 mg/g of DM). All reactions were carried out with pretreated biomass (2% w/v) in sodium acetate buffer (50 mM, pH 5.2), at 50°C under stirring (850 rpm). (C) Glucose equivalent yields corresponding to cellobionic acid as a function of *PaCDHB* concentration in reactions on pine (circles) or miscanthus (triangles). Trend curves and their R^2 are shown for conditions involving 0.5 to 3 mg of *PaCDHB*/g of DM. (D) Relative variation in glucose equivalent yields (vs *TrCC* condition) in the conditions (i.e. *TrCC* + *PaCDHB* at 3 or 6 mg/g of DM) where a significant boost was observed on miscanthus. The corresponding t-test is displayed in panel B (see significance code at the top of the figure). In all panels, bars show average values and error bars represent the standard deviation ($n = 4$ independent biological replicates).

Discussion

In the present study, our goal was to assess the impact of two AA9/CDH couples from the coprophilous ascomycete *P. anserina* on the overall depolymerizing power of a reconstituted minimal cocktail of *T. reesei* cellulases (*TrCC*) on four different lignocellulosic biomass of industrial relevance. Throughout the entire set of experiments presented in this study, the main products extracted from the biomass were glucose, cellobiose and cellobionic acid. While the presence of gluconic acid could have been expected from the hydrolysis of cellobionic acid by *TrBGL*, no evidence of such activity could be detected here, in line with the previously demonstrated very poor BGL activity of commercial cellulolytic cocktails on cellobionic acid (Cannella et al. 2012) or from a purified BGL from *Phanerochaete chrysosporium* (*PcBGL*) (Igarashi et al. 2003). This poor activity may be attributed to an inhibitory effect of cellobionolactone on BGL, as suggested by Igarashi and colleagues, who have shown that the activity of *PcBGL* on cellobiose was reduced in the presence of cellobionic acid (Igarashi et al. 2003). Although the preferred substrate of *PcBGL* is not cellobiose but laminaribiose, a similar negative effect of cellobionic acid on *TrBGL* is plausible. Thus, in addition to a potential substrate competition between *TrBGL* and *PaCDHB*, the inhibitory effect of cellobionic acid itself on *TrBGL* activity could contribute to the observed decrease in glucose production concomitantly to the increase in cellobionic acid production.

By setting up reaction conditions where the LPMO would act in concert with a CDH partner, we expected to better control the overall activity of this oxidative system and potentially boost the yields of extracted sugars (**Fig. 4**). Surprisingly, on all the tested biomass, the addition of either *PaAA9E* or *PaAA9H* to the enzymatic reactions did not yield any significant improvement. The reasons for such lack of “boosting” effect can be manifold. Very recent studies have shown that decrystallization of the cellulose surface by LPMOs can have a positive effect on the cellulose surface water retention (Cannella et al. 2023) and productive binding of CBHs (Angeltveit et al. 2024), improving in turn the overall hydrolysis. It is possible that, under the biomass loadings used in our study ($\leq 2\%$ w/v), CBHs have sufficient productive binding sites, and therefore do not need the action of LPMOs. A non-mutually exclusive hypothesis to explain the lack of boosting effect could be related to the accessibility and nature of the substrate (that depends on the biomass and their pretreatment) or the production and fate of H_2O_2 in reactions. Notably, the lignin remaining after the pretreatment may contribute to redox side reactions that consume or produce H_2O_2 , and may also prevent LPMOs from accessing optimally to cellulose. Indeed, it has been

shown that the hydrophobic properties of CBM1 (which is present in both *PaAA9E* and *PaAA9H*) contribute to the non-productive binding to lignin (Fong et al. 2016; Lee et al. 2022). The biomass used in this study indeed contain high concentrations of remaining lignins (**Table S4**), which could explain the lack of boosting effect coming from the addition of the tested LPMOs. Therefore, although *PaAA9E* and *PaAA9H* are efficient cellulolytic AA9s (Bennati-Granier et al. 2015), it appears clear that observing a high activity on somehow simple cellulosic substrates (such as PASC or Avicel) cannot be taken as a token of efficiency once mixed with cellulases on more complex biomass. Nevertheless, in a recent study Cai and colleagues have showed that an AA9 from *P. anserina* (enzyme ID not reported) could enhance the absolute glucose extraction yield on corncobs by up to 24% (using 8 mg/g of the industrial Cellic CTec3 cocktail and 4 mg/g of LPMO) (Cai et al. 2023). Although it is possible that the boost observed in this study directly originate from the action of the tested *PaAA9*, it is also likely that this LPMO could act as a H₂O₂ supplier for the AA9s potentially present in the Cellic CTec3 cocktail (3 AA9s are encoded in the genome of *T. reesei*). Noteworthy, a similar LPMO-LPMO interplay mediated by H₂O₂-producing activity has already been proposed between two LPMOs from the fungus *Myceliophthora thermophila*, viz. *MtAA16A* and *MtLPMO9s* (Sun et al. 2023). Ultimately, our results suggest that LPMO reduction, co-substrate feeding but also substrate availability should be finely regulated in industrial cellulolytic cocktails and adapted from one biomass to another to ensure optimal boosting effect conditions.

Primarily intended as a partner to fuel LPMOs reactions, the impact of *PaCDHB* alone on pine and miscanthus degradation appeared unexpectedly significant. With a maximum relative boosting effect on the total glucose equivalents yields of 27% on miscanthus (when applied at a concentration threshold ≥ 3 mg/g of DM), *PaCDHB* revealed itself as the only “boosting” oxidative enzyme tested. As a comparison, we carried out a meta-analysis of literature *positive* reports on LPMO boosting effects on glucose yields, measured on a broad range of types of biomass, which revealed a median boost value of 30% (**Fig. S4**). In our case, the boost observed in the soluble glucose equivalents yields mainly relied on the production of cellobionic acid (up to 12% on miscanthus).

On a side note, while the CDH-LPMO interplay has been clearly evidenced in several case studies, we underscore that such association is not systematic. For instance, secretomic analysis of the basidiomycete *Laetisaria arvalis* grown on wheat straw showed that, while AA9s and CDHs are co-secreted at early stage of growth (day 3), the secretion level of AA9s completely drops at day 7 while CDHs continue to be increasingly co-secreted

with cellobiohydrolases (Navarro et al. 2014). On the basis of these observations, we speculate that the role of CDHs in PCW degradation may not be limited to its partnership with LPMOs.

In industry, oxidized products can be considered as platform sugars. For instance, cellobionic acid can be used as additive in the cosmetics and food industry (Bieringer et al. 2023). Additionally, considering its low inhibitory effect on cellulases (as compared to cellobiose) (Hildebrand et al. 2016; Igarashi et al. 1998), it was also investigated as potential substrate in biorefinery for the production of isobutanol (Desai et al. 2015). Gluconic acid, which could be produced from cellobionic acid, is also widely used in the food industry (Sumitra Ramachandran et al. 2006; Cañete-Rodríguez et al. 2016) or as a building block for chemical synthesis (Stottmeister et al. 2005). Hence, by modulating the concentration of BGL and CDH in the cellulolytic cocktails, it may be possible to tune the proportion of neutral and oxidized products depending on the desired bioprocessing route.

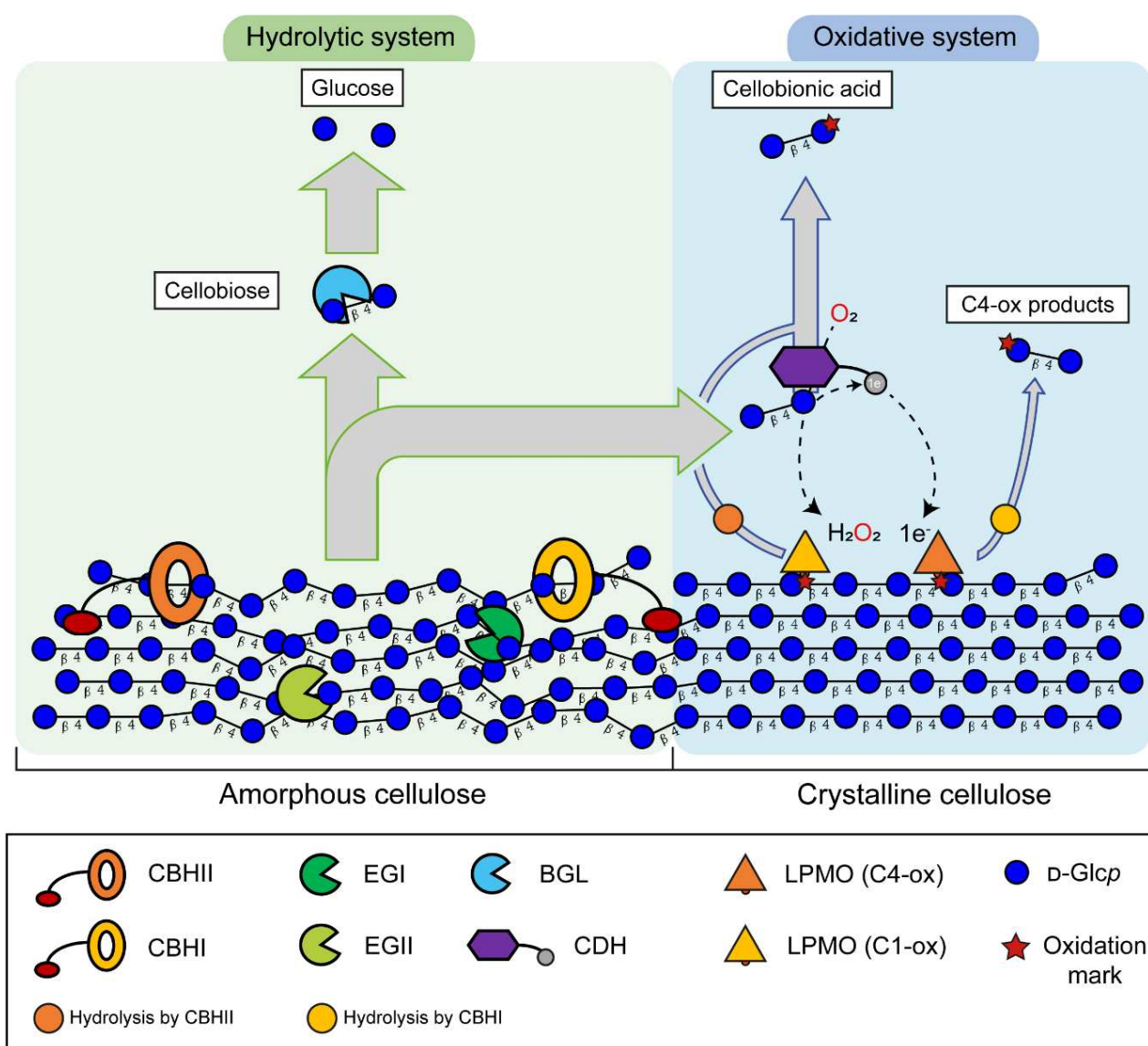


Fig. 4. Theoretical interplay between hydrolases and oxidative enzymes during cellulose degradation. The final products resulting from cellulose degradation by both the hydrolytic and oxidative systems are annotated on the figure. The possible hydrolytic and oxidative paths yielding final products are indicated by green or blue arrows, respectively. Some hydrolytic enzymes such as CBHs may also interact with oxidized products and are therefore also represented on the oxidative system panel. We underscore that, for the sake of clarity, this simplified scheme does not represent the potential action of LPMOs on amorphous regions of cellulose or of that of hydrolases on crystalline ones. BGL, β -glucosidase; CBH, cellobiohydrolase; CDH, cellobiose dehydrogenase; EG, endoglucanase; LPMO, lytic polysaccharide monooxygenase; D-Glcp, D-glucopyranose.

Conclusions

With the emergence of LPMOs as a must-have component of the new best performing enzymatic cocktails, biomass degradation products will tend to become more diversified, including neutral and oxidized sugars. Today, these oxidized soluble oligosaccharides are often overlooked in favour of the hydrolytic system products (namely glucose and cellobiose), used as substrates of various bioprocesses (**Fig. 4**). In the present study, the tested LPMOs did not prove efficient at boosting the conversion of the selected biomass, underpinning the complexity of implementing efficiently these enzymes in biorefinery processes and the need for further research. Nevertheless, our results show that the addition of CDH into industrial enzymatic cocktails could be a promising path to generate high value-added products from industrial lignocellulosic biomass, expanding thereby valorization routes to contribute to the success of the emerging bioeconomy.

Materials and methods

Materials

(Oligo)saccharide substrates were purchased from Megazyme (Wicklow, Ireland), ascorbic acid was purchased from Sigma-Aldrich (Saint-Louis, Missouri, United-states) and PASC was prepared as described in Wood (Wood 1988).

Lignocellulosic substrates

Wheat straw, miscanthus, poplar and pine (*Pinus pinaster*) were obtained from IFP Energies Nouvelles (Rueil-Malmaison, France). Wheat straw, miscanthus and poplar were pre-treated as described in (Paës et al. 2019). In short, the lignocellulosic biomass was steam exploded under acidic conditions, washed in hot water to remove free products, and then dried at 55°C. After one week at room temperature, the biomass was shredded and sieved to a 0.8-mm maximal size. Pine was pre-treated differently. It was first dried at 90°C then shredded and sieved to 30 mm maximal size, further affined to 5 mm. Pine was finally steam exploded under acidic conditions and washed in hot water to remove free products. **Table S2** provides more details on the steam explosion process for each type of biomass. Compositional analysis of pretreated biomass was carried out following standardized procedures developed by the National Renewable Energy Laboratory (NREL/TP-510-42618) (See **Table S3** for composition of the biomass).

Production and purification of *P. anserina* AA9 LPMOs and CDH

PaAA9H (GenBank ID: CAP61476), *PaAA9E* (GenBank ID: CAP67740) and *PaCDHB* (GenBank ID: CAP61651) were recombinantly produced and purified as previously described (Haon et al. 2015; Bennati-Granier et al. 2015). Recombinant *P. pastoris* clones (strain X33) expressing *PaAA9E*, *PaAA9H* and *PaCDHB* were grown in 2 L of Buffered Glycerol- complex Medium (BMGY) media in flasks at 30°C in an orbital shaker (200 rpm) for 16 h to an OD600 of 2 to 6. Expression was induced by transferring cells into 400 mL of Buffered Methanol- complex Medium (BMMY) media at 20°C in an orbital shaker (200 rpm) for another 3 days. Each day, the medium was supplemented with 3% (v/v) methanol. The cells were harvested by centrifugation, and just before purification the pH of the supernatant was adjusted to 7.8 by addition of Tris-HCl buffer (1 M, pH 8) and was filtered on a 0.45 µm membrane (Millipore, Burlington, Massachusetts, USA). The filtered supernatants were then loaded onto a His-Trap Resin (GE Healthcare, Buc, France) column (1.6 × 2.5 cm or 1.60 × 10 cm) equilibrated with buffer A, (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 10 mM), using an Äkta purifier 100 (GE Healthcare). (His)₆-tagged recombinant enzymes were eluted

with buffer B (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 500 mM). Fractions containing recombinant enzymes were pooled, concentrated, and dialyzed against sodium acetate buffer (50 mM, pH 5.2).

The *T. reesei* cellulases were produced, as described in Poggi-Parodi et al. (Poggi-Parodi et al. 2014), in DASGIP bioreactors. *T. reesei* strain CL847 cultures were grown on plates of Potato Dextrose Agar (Difco Laboratories, USA) at 30°C. The cellulase production was performed in two steps. In the first step, a growth phase was conducted at 27°C in 400 mL of starting medium containing 35 g/L of glucose as carbon source during 30 h. The pH was regulated at 4.4 with 5.5 M ammonia. The air flow was adjusted at 0.5 vvm and initial stirring was set at 500 rpm. This parameter was gradually increased to maintain pO₂ above 40% oxygen saturation. The second step was done at 25°C during 170 h and when the glucose was depleted in the media, a fed-batch solution containing 250 g/L lactose was added at a rate of 1 g/h. At the end of the fermentation, the musts were filtered and sodium benzoate at 0.35% w/v were added to the recovered filtrates before storing them in a bottle at 4°C.

T. reesei cellulases were then purified as described in Billard et al. (Billard et al. 2012). Culture supernatants were salted out using a HiTrap desalting column (Biorad, Marnes-la-coquette, France) and equilibrated with 25 mM imidazole-HCl buffer (pH 7.5). Chromatofocusing was performed on an ÄKTA® FPLC (GE Healthcare, Chalfont St Giles, UK) using a Mono Q 5/50 GL column (GE Healthcare, Chalfont St Giles, UK) equilibrated with the initial buffer. Proteins bound (20 mg) under the initial conditions were eluted by a pH gradient (from 7.4 to 3.9) using PB74 Polybuffer (GE Healthcare) at a constant flow rate of 0.7 ml/min. Recovered fractions were analyzed by one dimensional gel electrophoresis indicating a purity of >95% with Image Lab Software.

LPMOs copper saturation and desalting

Prior to enzymatic assays, *PaAA9E* and *PaAA9H* were saturated with Cu(II)SO₄. To this end, the enzymes were incubated in sodium acetate buffer (50 mM, pH 5.2) with a 3-fold molar excess of Cu(II)SO₄ for 30 min at 4°C and excess copper was removed by passing the enzyme solution through a PD MidiTrap G-25 (GE Healthcare) desalting column pre-equilibrated with sodium acetate buffer (50 mM, pH 5.2), at room temperature using gravity flow. Proteins concentration were determined by measuring the absorbance at 280 nm using a Nanodrop NB-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The proteins samples were stored at 4°C until further use.

Design of experiment assays on wheat straw

The MODDE Design of Experiments software version 12.0 (MKS Data Analytics Solutions, Umeå, Sweden) was used for experimental design and data analysis. A quadratic design with three mixture components was used in all experiments including *T. reesei* cellulases cocktail (2 to 20 mg/g of DM loading), *PaCDHB* (0 to 4 mg/g of DM loading) and *PaAA9E/PaAA9H* (0 to 5 mg/g of DM loading). The best subset of experiments from a candidate set was generated with MODDE by Full Fac (3 levels) design (the full list of factors is presented in **Table S1**). An optimal subset consisting of 30 individual experiments were thus obtained and performed for each AA9/*PaCDHB* couples.

Enzymatic reactions

Enzymatic hydrolysis reactions were carried out in 2 mL deep 96-wells plates (ThermoFischer, Waltham, USA), with 1% (w/v; i.e. 10 mg/mL) (for wheat straw) or 2% (for miscanthus, pine and poplar) of DM in sodium acetate buffer (50 mM, pH 5.2), with a total reaction volume of 500 μ L. The reactions were incubated in an incubator (Infors, Massy, France) at 50°C with orbital shaking (850 rpm). For each time point, 5 μ L of the supernatant was sampled and diluted 20 times in milliQ water before heating 5 minutes at 100°C to stop the reactions. The diluted supernatants were then immediately filtered on 96-wells 0.2 μ m micro filter plates (Pall Life Sciences, New-York, USA) and stored at -20°C for further analysis.

For the AA9/*PaCDHB* reaction on PASC, reactions were performed at a final substrate loading of 0.2% in sodium acetate buffer (50 mM, pH 5.2) and incubated in a Thermomixer (30°C, 850 rpm). After 24 h of reactions, reactions were stopped by heating 5 minutes at 100°C and the soluble fractions were analyzed by high-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD). The system is equipped with a CarboPac-PA1 guard column (2 $\times$ 50 mm) and a CarboPac-PA1 column (2 $\times$ 250 mm) kept at 30 °C. Elution was carried out at a flow rate of 0.25 mL.min⁻¹ and 25 μ L of sample was injected. The solvents used were 100 mM NaOH (eluent A) and NaAc (1 M) in 100 mM NaOH (eluent B), and the following gradient was applied: 0 to 10 min, 0 to 10% B; 10 to 35 min, 10 to 35% B (linear gradient); 35 to 40 min, 30 to 100% B (curve 6); 40 to 41 min, 100 to 0% B; 41 to 50 min, 100% A. Integration was performed using the Chromeleon 7.2.10 chromatography data software.

Reaction products analysis

For the reactions on wheat straw, glucose concentration was measured as in (Filiatrault-

Chastel et al. 2019). To perform the assay, 200 µL of the glucose GOD-PAP reagent (Biolabo, Maizy, France) were added to 10 µL of diluted reactions (2 times for the 4 and 8 h time points, 10 times for the 24 and 48 h time points) in 96-well microplates. The microplates were then left at ambient temperature for 30 min before reading the absorbance at 540 nm using a microplate spectrophotometer. Soluble glucose concentrations were evaluated using a standard curve, and yields were computed based on the initial amount of potential cellulosic glucose in each substrate, as follows: glucose yields (%) = extracted soluble glucose x 100 / theoretical total glucose content.

For the reactions on miscanthus, pine and poplar, the diluted supernatants were further analyzed by HPAEC-PAD, using glucose, gluconic acid and cellobiose as standards (Megazyme). Cellobionic acid standard were obtained by incubating 1 to 100 µM of cellobiose (Megazyme) with 1 µM of *PaCDHB* for 24 h in a thermomixer (30°C, 850 RPM).

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Author contributions

J.-L.R. carried out most experiments. S.A. and A.L. performed the expression and purification of *T. reesei* enzymes. S.G. carried part of the HPAEC-PAD analysis. J.-L.R., A.M., S.A., J.-G.B. and B.B. designed the experiments and interpreted the data. A.M, S.A, J.-G.B. and B.B. conceptualized the study. A.M., S.A., J.-G.B. and B.B. supervised the work. All authors contributed to the writing of the manuscript, reviewed, and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

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SUPPORTING INFORMATION FOR

Implementation of CDH in LPMO-containing minimal cellulases cocktails to tune bioproducts production from lignocellulosic biomass

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This file includes:

1. Supplementary Figures 1 to 4 and Supplementary table 1 to 4
2. Full abbreviations list
3. Supplementary references list

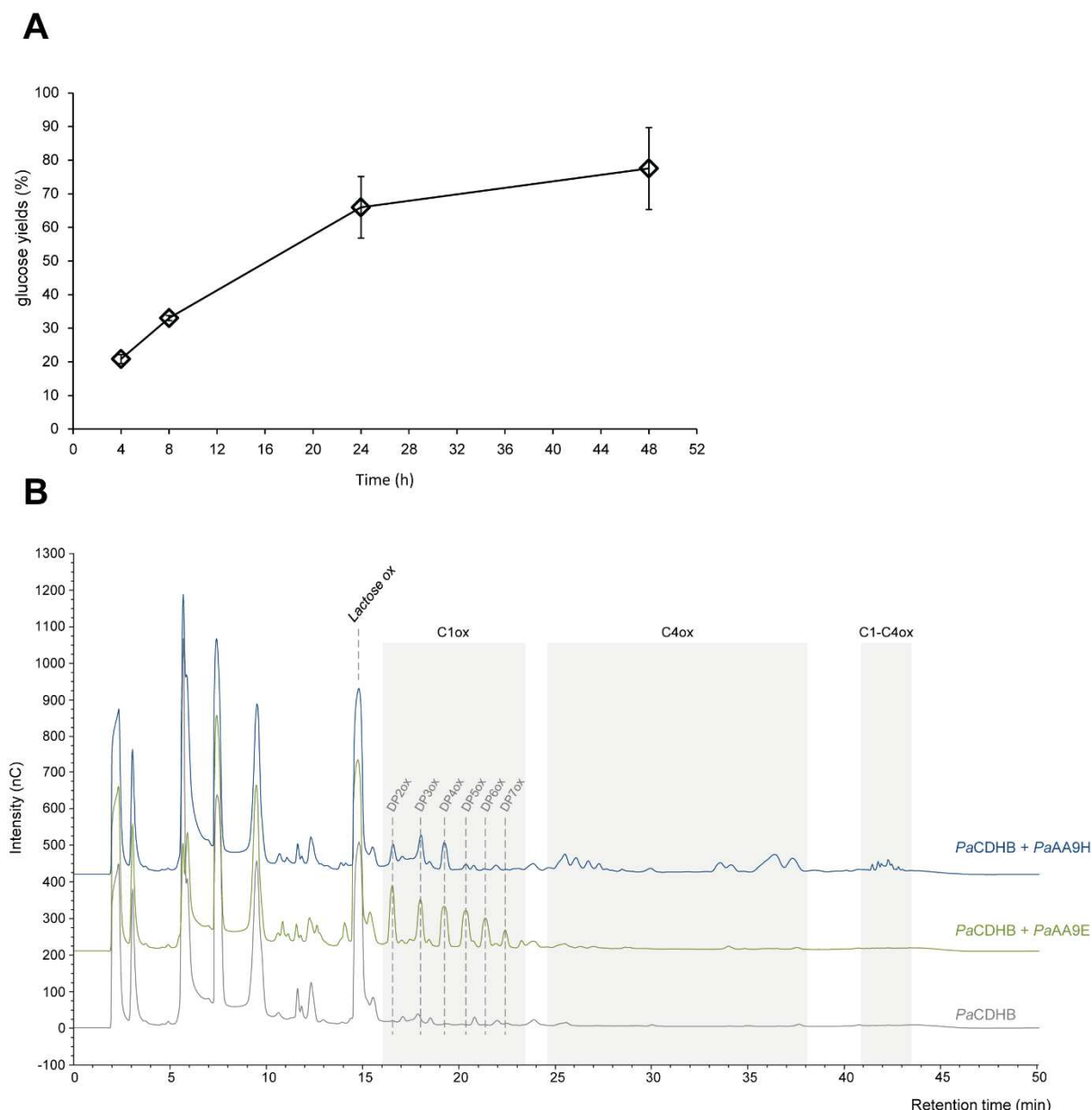


Fig. S1. *TrCC* cocktail and *P. anserina* AA9s/CDH activity on wheat straw and cellulose. (A) Time course of the glucose release (expressed as conversion yield of theoretical glucose content) from wheat straw (1%) by *TrCC* (20 mg/g of DM). Reactions were carried out in sodium acetate buffer (50 mM, pH 5.2) and incubated in a Thermomixer (850 rpm, 30°C). Data points show average values and error bars show standard deviations ($n = 3$ independent biological replicates). **(B)** Oxidized cello-oligosaccharides (DP2-DP6ox) released by *PaAA9E* or *PaAA9H* (1 μ M) from PASC (0.2% w/v) after 24 h of reaction, in the presence of *PaCDHB* (100 nM). Reactions were carried out in sodium acetate buffer (50 mM, pH 5.2) and incubated in a Thermomixer (850 rpm, 30°C). “Lactose ox” (shown on the graph) refers to the C1-oxidized lactose generated upon oxidation of lactose by *PaCDHB*. Products with retention times between 5 and 10 min are neutral oligosaccharides (lactose at 9.5 min; glucose and galactose at 6 and 7.5 min, respectively, which are products of degradation of lactose generated upon boiling when stopping the reaction), while products below 5 min are buffer components.

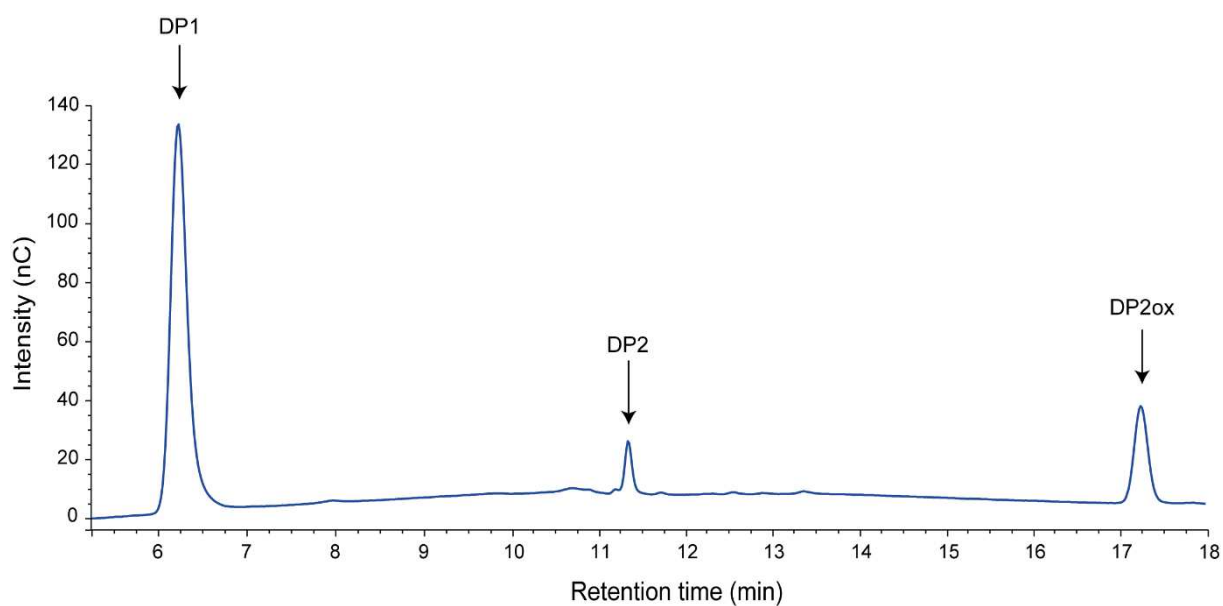


Fig. S2. HPAEC-PAD chromatogram showing the main soluble products released from miscanthus after 48 h reaction. The chromatogram corresponds to one of the replicates of the condition involving *TrCC* + *PaCDHB* at 1 mg/g of DM (see **Fig.3** in main text). Abbreviations: DP1, glucose; DP2, cellobiose; DP2ox, cellobionic acid.

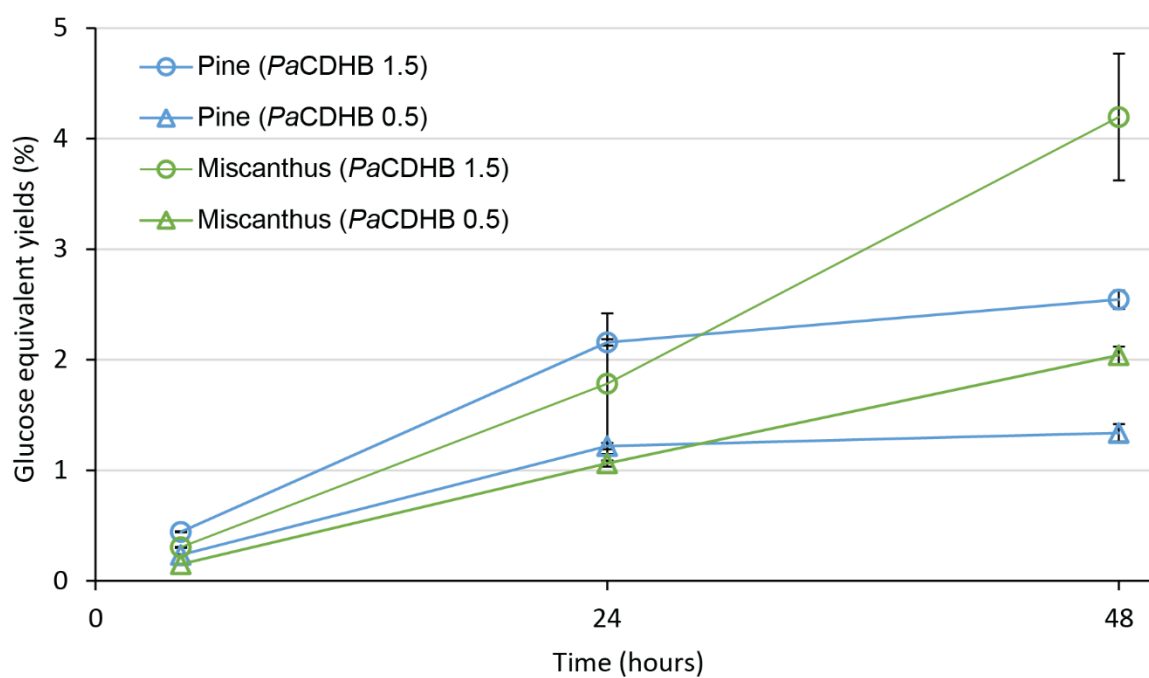


Fig. S3. Time course release of cellobionic acid from miscanthus and pine. The curves correspond to the cellobionic acid production by the *TrCC* in the presence of 0.5 (triangles) or 1.5 (circles) mg of *PaCDHB*/g of DM. Reactions on pine and miscanthus are shown in blue and green, respectively. Data points show average values and error bars show standard deviations ($n = 3$ independent biological replicates).

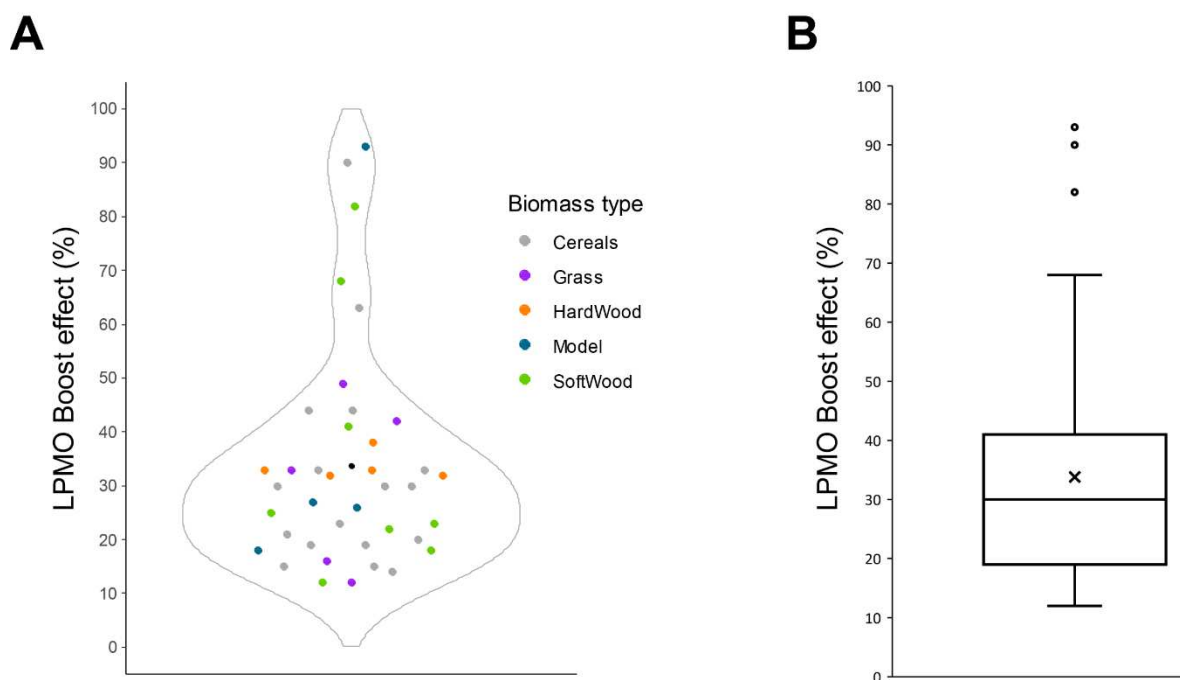


Fig. S4. Review of the best LPMO boost effects reported in the literature. (A) Violin plot showing the distribution of the relative increase in sugar yields upon addition of LPMO in cellulolytic cocktails on various types of biomass, so called “LPMO boost effect”. 39 LPMO boost effect values were retrieved from the following studies (Harris et al. 2010; Hu et al. 2015; Müller et al. 2015; Chylenski et al. 2017; Quinlan et al. 2011; Scott et al. 2016; Kim et al. 2017; Du et al. 2018; Abik et al. 2023; Kim et al. 2016; Sanhueza et al. 2018; Dimarogona et al. 2012; Westereng et al. 2011; Grieco et al. 2020; Chabbert et al. 2017; Couturier et al. 2018; Filiatrault-Chastel et al. 2019; Calderaro et al. 2020; Bernardi et al. 2020; Caputo et al. 2023; Li et al. 2022; Park et al. 2023; Moya et al. 2023) . The type of biomass for which the boost was observed is indicated on the figure. cereals in grey (including wheat straw, corn stover, rice straw and delignified residues), grass in purple (including sugarcane bagasse and miscanthus), hardwood in orange (including poplar and birchwood), model substrates in blue (including microcrystalline cellulose, Avicel and nanofiber cellulose) and softwood in green (including pine, Lodgepole pine, and Norway spruce). (B) Box plot of the above mentioned LPMO boost effects. The central box shows a median boost effect of 30 %, an upper quartile of ~40% and a lower quartile of ~20%, and the whiskers show a minimum value of 12 % and a maximum value of 82 %.

Table S1. Enzymes proportions in previously reported *Trichoderma reesei* minimal cellulases cocktails

References	CBHI (TrCel7a)	CBHII (TrCel6a)	EGI (TrCel7b)	EGII (TrCel5a)	TrBG (TrCel3a or TrCel2a)	Other enzymes	Total enzymes loading	Substrates	Glucose yields (%)
(Rosgaard et al. 2007)	27%	46%	27%	-	-	-	11 mg/g of dry matter	Acid impregnated + steam exploded straw	56
	17%	45%	38%	-	-	-		Water impregnated + steam exploded barley straw	34
	20%	43%	37%	-	-	-		Hot water extracted barley straw	67
(Harris et al. 2010)	50.5%	28%	11.5%	-	-	10%	2.5 mg/g of cellulose	Acid-pretreated corn stover	66
(Gao et al. 2010)*	27 - 30%	17 - 20%	29 - 35%	-	-	11 - 31%	7.5, 15 or 30 mg/g of glucan	AFEX corn stover	52.6 - 80
(Banerjee et al. 2010)**	35 - 43%	4%	26 - 30%	-	5 - 12%	8 - 23%	15 mg/g of glucan	Corn stover	40.7 - 58.2
	16 - 28%	4%	23 - 34%	-	20 - 43%	8 - 24%		Dried distillers' grains plus solubles	22.6 - 29.8
	36 - 48%	4%	32 - 46%	-	4%	8 - 11%		Miscanthus	17.5 - 32.1
	44 - 47%	4%	36 - 37%	-	4%	8%		Poplar	9.8 - 13.8
	31 - 47%	4%	19 - 30%	-	4 - 12%	8 - 39%		Switchgrass	24.4 - 39.1
(Tejirian and Xu 2011)	60%	15%	5%	6%	-	24%	10,8 mg/g of substrate	Avicel	77
							9.8 mg/g of cellulose	Acid-pretreated corn stover	85
(Billard et al. 2012)	41.4%	27.4%	15.9%	6.3%	2.5 mg/g of enzyme mixture	9%	2.5 mg/g of substrate	Steam exploded wheat straw	65.8
(Chylenski et al. 2017)	28%	30%	30%	-	-	13%	8 mg/g of substrate	Sulfite-pulped sugarcane Bagasse	80.5
	28%	17%	48%	-	-	7%		Sulfite-pulped Norway spruce	82.3
(Borisova et al. 2022)	58.4%	19.5%	-	9,80%	-	12.3%	10 mg/g of substrate	Avicel	30

Abbreviations: AFEX, ammonia fiber expansion; BG, β -glucosidase; CBH, cellobiose hydrolase; EG, endoglucanase.

* Glucose yields and enzymes proportion reported in Gao et al. vary depending on the total enzymes loading.

** Glucose yields and enzymes proportions reported in Banerjee et al. vary depending on the pretreatment of the biomass (AFEX, 0.25% NaOH or alkaline peroxide).

Table S2. *TrCC* enzymes proportions and model validity of the design of experiment for wheat straw hydrolysis assays

<i>TrCC</i> (mg/g of DM)	<i>PaCDHB</i> (mg/g of DM)	<i>PaAA9E</i> or <i>PaAA9H</i> (mg/g of DM)	Glucose yields with <i>PaAA9H</i> (%)	Model fit R2*	Predictability Q2**	Glucose yields with <i>PaAA9E</i> (%)	Model fit R2*	Predictability Q2**
2	0	0	34			33.9		
11	0	0	60.4			60.3		
20	0	0	70			81		
2	2	0	9.9			24.1		
11	2	0	36.3			48.4		
20	2	0	69.4			65.2		
2	4	0	7.3			21.1		
11	4	0	43.9			43.1		
20	4	0	68.7			62.8		
2	0	2.5	33.3			34.3		
11	0	2.5	60.2			60.6		
20	0	2.5	77.3			79.2		
2	2	2.5	16.3			23.4		
11	2	2.5	47.5			45.3		
20	2	2.5	69.4	0.98	0.96	64.7	0.98	0.95
2	4	2.5	5.7			18.8		
11	4	2.5	41.4			39.3		
20	4	2.5	63.2			60.9		
2	0	5	15.5			14.6		
11	0	5	60.9			60.1		
20	0	5	81.6			78.5		
2	2	5	6			6		
11	2	5	45.1			44.7		
20	2	5	69.9			68.3		
2	4	5	4.3			3.8		
11	4	5	39.7			41		
20	4	5	62.4			63.4		
11	2	2.5	47.4			42.3		
11	2	2.5	47.7			44.7		
11	2	2.5	48.4			44.1		

*Represents how well the model fits the experimental data (1 = perfect fitting)

**Represents how well a model predicts new data (According to MODDE a model is considered good if Q2 > 0.5)

Table S3. Biomass pretreatment conditions

Pretreatment	Wheat straw	Miscanthus	Poplar	Pine
Temperature (°C)	170	190	190	175
Time (min)	7.5	7.5	7.5	11
[H ₂ SO ₄] (g/100 g of DM)	0.35	0.4	0.7	0.6

Table S4. Pre-treated biomass compositions

	Components (% of DM)						
	Glucose	Xylose	Mannose	Arabinose	Rhamnose	Galactose	Lignin ^a
Wheat straw	53.92	1.58	0.16	0.52	0.15	0	30.3
Miscanthus	59.43	2.09	0	0.39	0.44	0	34.1
Poplar	53.28	0.63	0.18	0.11	0.27	0	45.8
Pine^b	44.8	3.5	5.2	0.8	0	1.9	48.5

^aLignin content was determined by Paës et al. (24) for wheat straw, miscanthus and poplar, and by Auxenfans et al. (25) for pine.

^bPre-treated pine contains 10.7% (w/w) of free glucose monomer.

Full abbreviations list

Enzymes/Microorganisms

AA: Auxiliary activities

CDH: Cellobiose dehydrogenase

Pa : *Podospora anserina*

Tr : *Trichoderma reesei*

TrCC: *Trichoderma reesei* cellulases minimal cocktail

Substrates/products

PASC: Phosphoric acid swollen cellulose

Methods

HPAEC-PAD: High-performance anion-exchange chromatography coupled with pulsed amperometric detection

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Chapter II

Insights into peculiar fungal LPMO family members holding a short C-terminal sequence reminiscent of phosphate binding motifs



OPEN

Insights into peculiar fungal LPMO family members holding a short C-terminal sequence reminiscent of phosphate binding motifs

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Lytic polysaccharide monooxygenases (LPMOs) are taxonomically widespread copper-enzymes boosting biopolymers conversion (e.g. cellulose, chitin) in Nature. White-rot Polyporales, which are major fungal wood decayers, may possess up to 60 LPMO-encoding genes belonging to the auxiliary activities family 9 (AA9). Yet, the functional relevance of such multiplicity remains to be uncovered. Previous comparative transcriptomic studies of six Polyporales fungi grown on cellulosic substrates had shown the overexpression of numerous AA9-encoding genes, including some holding a C-terminal domain of unknown function ("X282"). Here, after carrying out structural predictions and phylogenetic analyses, we selected and characterized six AA9-X282s with different C-term modularities and atypical features hitherto unreported. Unexpectedly, after screening a large array of conditions, these AA9-X282s showed only weak binding properties to cellulose, and low to no cellulolytic oxidative activity. Strikingly, proteomic analysis revealed the presence of multiple phosphorylated residues at the surface of these AA9-X282s, including a conserved residue next to the copper site. Further analyses focusing on a 9 residues glycine-rich C-term extension suggested that it could hold phosphate-binding properties. Our results question the involvement of these AA9 proteins in the degradation of plant cell wall and open new avenues as to the divergence of function of some AA9 members.

White-rot (WR) fungi are pivotal decomposers of dead organic matter in forest ecosystems that have developed various strategies to metabolize the plant biomass substratum, contributing thereby to the global carbon cycle¹. To tackle the complexity of plant cell walls (PCW), a complex matrix composed of cellulose, hemicelluloses and lignin, a common strategy in the fungal kingdom consists in the secretion of a variety of enzymatic activities, notably carbohydrate-active enzymes (CAZymes) including hydrolases and oxidoreductases^{2–4}. A clear picture of the scope of enzymatic activities involved and how these are interconnected and orchestrated remain a major gap in our understanding of biomass decay. A major step forward has been achieved in the past decade with the discovery of lytic polysaccharide monooxygenases (LPMOs)⁵, which has profoundly changed our vision of biomass conversion processes, at both fundamental and industrial levels^{6–8}. Of note, in addition to these canonical activities on biomass, recent works have demonstrated the implication of some LPMOs in new divergent functions, such as microbial pathogenicity, cellular development or copper homeostasis⁹.

LPMOs are copper-dependent oxygenases catalyzing the oxidative cleavage of glycosidic bonds in various recalcitrant polysaccharides, including cellulose and chitin^{5,10–13}. By doing so, LPMOs facilitate the depolymerization of polysaccharides by hydrolases, and thereby boost the overall conversion turnover. Today, LPMOs

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are classified as “auxiliary activities” (AA) within the CAZy database¹⁴, and further distributed into 8 families (AA9-AA11 and AA13-AA17). Among them, the AA9 family has been extensively studied for its ability to target and degrade cellulose, but also cello-oligosaccharides or hemicelluloses with glucan backbone^{15–18}. To catalyze their reaction, LPMOs need a source of electron and an oxygenated substrate (O_2 or H_2O_2). When fueled with H_2O_2 , LPMOs catalyze an efficient peroxygenase reaction¹⁹, which entails the control of a powerful, and yet potentially harmful, Fenton-type chemistry²⁰.

Remarkably, recent genome sequencing campaigns have highlighted the wide range of genes coding for LPMOs of the same family (especially AA9 enzymes, henceforth called AA9s), with up to > 60 different genes in some filamentous fungi²¹. The functional relevance of this redundancy is still not fully understood and the physiological role of most of these LPMOs remains unknown. Such LPMO-rich, white-rot filamentous fungi are notably found within the Polyporales order, belonging to the Agaricomycetes class and Basidiomycota division. Recently, Hage and colleagues assessed the functional adaptation of Polyporales to wood decay activities²² by performing a comparative transcriptional analysis of six Polyporales species (*Artolenzites elegans*, *Leiotrametes* sp., *Pycnoporus cinnabarinus*, *Pycnoporus coccineus*, *Trametes lujbarskyi* and *Irpex lacteus*) grown on wheat-straw, pine, aspen and crystalline cellulose. When focusing on the transcriptional response of genes coding for AA9s, some LPMOs displaying an atypical, conserved C-terminal extension (called X282)²³, and henceforth called AA9-X282, stood out. Interestingly, these AA9-X282s were all predicted as secreted and found among the most up-regulated LPMOs after 3 days of growth on cellulose²².

In this study, we investigated the role(s) of several of these atypical AA9-X282s. To this end, we produced six AA9-X282s in *Pichia pastoris*, displaying different modularities and carried out in-depth characterization using a combination of in silico and biochemical approaches, including enzymatic assays and substrate binding studies.

Results

AA9-X282s form a distinct clade among other AA9s. In the study by Hage et al., the AA9-X282s were found among the most up-regulated LPMOs after 3 days of growth of the six different Polyporales on cellulose (28–500-fold change in transcript read counts compared to the maltose control condition), and all clustered with cellulose-responsive genes (Fig. S1)²². Interestingly, only a single AA9-X282-encoding gene was identified for most Polyporales. Seeking for hints as to the potential activity of these AA9-X282s, we first performed a phylogenetic analysis to determine their evolutionary distance to other characterized AA9s (Fig. 1). To this end, we gathered the AA9 domain only of all the AA9s from the six Polyporales analyzed in Hage et al.²² (104 sequences) to which we added the 39 sequences of all the hitherto experimentally-characterized AA9s available in the CAZy database, and 13 other AA9-X282 sequences selected to fully cover the 3 different C-terminal modularities (see clade X1 in Fig. 1A), for a total of 156 sequences. Of note, X282-containing LPMOs are exclusively found in Basidiomycetes (and more precisely Agaricomycetes)²².

We show that AA9-X282s all cluster within a same clade, so-called clade X1 (Fig. 1A), indicating that the AA9 domain appended with a X282 extension has diverged from other LPMOs (average sequence identity of the AA9 domain of 44% with the closest “non-X282” AA9, Fig. S2). Noteworthy, this clade comprises only one characterized member, the AA9B from the Polyporales *Ceriporiopsis subvermispora* (syn. *Gelatoporia subvermispora*) (CsLPMO9B), which harbors a X282 domain and a carbohydrate binding module from family 1 (CBM1) and has been shown to oxidize amorphous cellulose (i.e. phosphoric acid swollen cellulose; PASC) with strict C1-regioselectivity²⁴. We underscore that in that study the reported oxidative activity (in terms of solubilized oxidized cello-oligosaccharides) seems rather low and that the presence of the X282 extension was not noted, and thus not specifically investigated.

Interestingly, the sister clade X2, which is devoid of X282 extension (Fig. 1A) but phylogenetically close to the clade X1 (percentage of identity ranging from 48 to 66%; Fig. S2) contains only AA9s from Ascomycetes fungi (in contrast to the Basidiomycete-specific clade X1). Three of these AA9s belonging to clade X2 were previously characterized: LPMO9B from *Podospora anserina* (PaLPMO9B), C4-oxidizing on cellulose²⁵; LPMO9J from *Neurospora crassa* (NcLPMO9J), C1-oxidizing on cellulose²⁶; and LPMO9E from *N. crassa* (NcLPMO9E), C1-oxidizing on cellulose²⁷. Despite the absence of X282 extension, the catalytic sites of these AA9s share some common features with AA9-X282s (see below, Fig. S3).

On a more general matter, the phylogenetic analysis also shows that the six Polyporales harboring an AA9-X282 also possess other AA9s, which tend to cluster in distinct clades (colored in yellow in Fig. 1A). These observations suggest that these white-rot fungi have evolved a set of distinct and conserved LPMOs that may fulfill different functions. We also underscore that AA9s from Ascomycetes do not cluster in a unique clade but are rather scattered across the tree (Fig. 1A), further supporting the idea that sequence divergence among AA9s would rather be due to neofunctionalization than to speciation.

The X282 extension is found in three distinct modular architectures. After investigating the phylogenetic relationship of AA9-X282s to other AA9 family members, we carried out a deeper analysis of AA9s containing the X282 extension (174 sequences in the CAZy database). We identified three distinct C-term architectures based on the primary sequences: (i) AA9 harboring only a C-terminal X282 extension [AA9-X282]; (ii) AA9 possessing a CBM1 after the X282 extension [AA9-X282-Linker-CBM1]; and (iii) AA9 holding a conserved motif of 9 residues of unknown function (henceforth called “9res”), directly following the X282 extension [AA9-X282-9res] (Fig. 1A). Structural predictions (AlphaFold2)³⁰ of a representative of each modularity are shown in Fig. 1A.

A phylogenetic analysis of the catalytic domain of the 174 AA9-X282 sequences available in the CAZy database allowed to identify five clades (Fig. 1B and Fig. S4). This analysis revealed that the three AA9-X282 modularities can be found in every clade and that AA9-X282-CBM1 populates mainly clade A and E and is the most

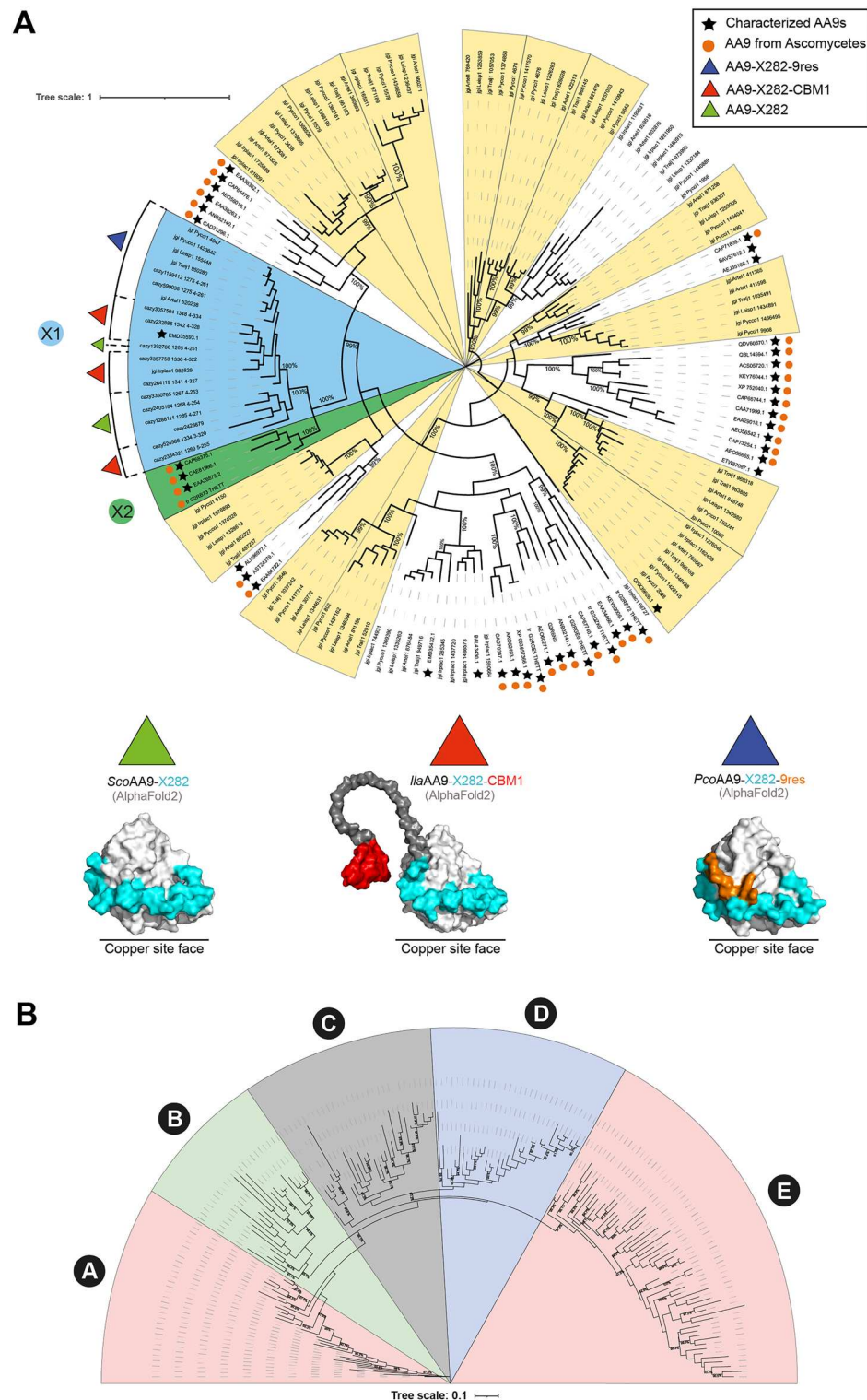


Figure 1. AA9-X282s within the phylogenetic tree of AA9s. **(A)** The tree was built with the catalytic domain only (i.e. without C-term extension, including the X282 extension) of 156 AA9 sequences including (i) 39 sequences of all the biochemically and/or structurally characterized AA9s available in the CAZy database (marked by a black star), (ii) 13 AA9-X282 sequences covering the diversity of their C-terminal modularity and (iii) 104 AA9s sequences regrouping all the AA9s from the six *Trametes* species studied in Hage et al.²². The tree was inferred using IQTree²⁸ (1000 bootstraps; value displayed on tree as percentages) and visualized with Interactive Tree Of Life (ITOL) software²⁹. Clades are colored in yellow when populated with AA9s from at least 5 out of the 6 *Trametes* species studied in Hage et al.²². Clade X1 contains AA9-X282s from Basidiomycetes and clade X2 contains AA9 orthologs (devoid of X282) found in Ascomycetes. The sequences from Ascomycetes are marked with an orange circle. The repartition of the AA9-X282 modularity is shown with colored triangles (see corresponding homology models of the three types of AA9-X282s at the bottom of panel A). In the structure predictions (AlphaFold2³⁰), shown as surface, the catalytic domain is colored in light grey, the X282 extension in cyan, the 9res motif in orange, the linker in dark grey and the CBM1 in red. **(B)** Phylogenetic analysis of 174 AA9-X282 catalytic domains, showing 5 clades (A to E). Each clade is colored according to its most prevalent modularity (> 50% of sequences) using the same color code as in panel A (see Fig. S4 for more details). The tree was generated with IQ-Tree (1000 Bootstraps).

represented modularity (56% of sequences), followed by the AA9-X282-9res (23%) and AA9-X282 (21%) (see Fig. S4 for more details). The presence and frequent occurrence of the CBM1 modularity is indicative of activity on PCW polysaccharides. The AA9-X282-9res and AA9-X282 modularities mainly cluster in clades B and D, accounting respectively for 77% and 66% of the sequences. The fact that a predominant modularity stands out for each of the clades could suggest that the AA9 domain has to some extent evolved in a modularity-dependent manner. It is also worth noting that the presence of the 9res motif is strongly associated with the *Trametes* genus.

The X282 extension is highly conserved and displays a predicted belt-like structure. The X282 extension is a highly conserved stretch of approximately 30 amino acids (Fig. 2A). Its sequence is mostly made up of threonine/serine which could potentially serve as *O*-glycosylation or phosphorylation sites, while five conserved prolines could confer rigidity (Fig. 2A). Interestingly, two conserved aromatic amino acids (Trp/Tyr) are also present (marked by orange stars in Fig. 2). In CAZymes, such surface-exposed conserved aromatic residues are usually involved in substrate binding via CH- π stacking interactions³¹. When present in short modules they can be seen as hallmark of CBM modules³². Interestingly, all molecular models we generated for AA9-X282s rather predict π -stacking interactions with two highly conserved aromatic residues from the AA9 catalytic domain itself (Fig. 2B and Fig. S5). It is tempting to speculate that these interactions drive the X282 extension along the surface of the AA9 domain, hence forcing it to adopt a belt-like structure, the function of which is unknown.

Of note, we also observed another conserved feature in the AA9s from both X1 and X2 clades, namely a negatively charged patch (i.e. conserved acidic residues at the surface of the AA9 structure) next to the active

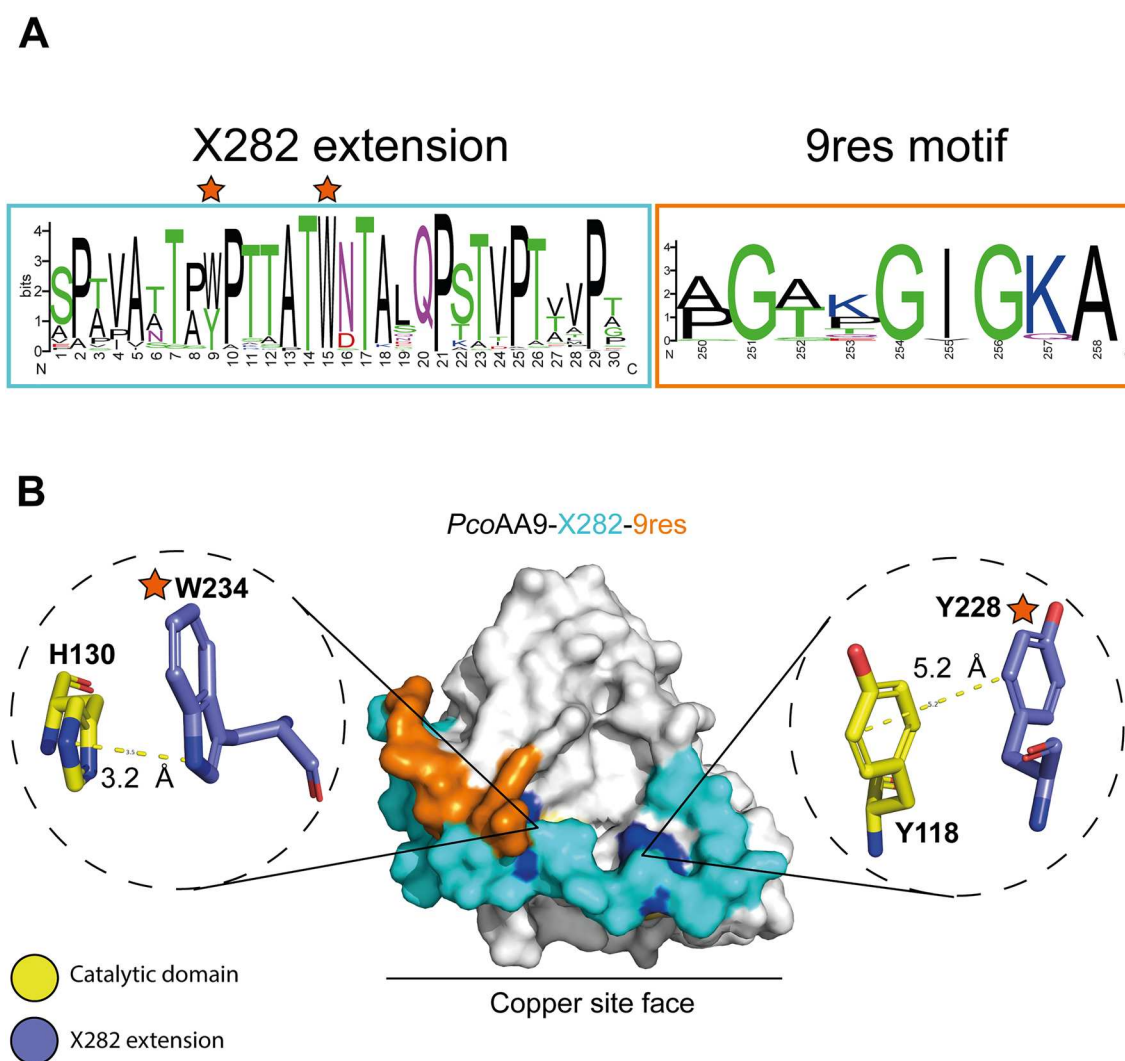


Figure 2. Structural analysis and amino acid conservation of the X282 extension. **(A)** WebLogo of the X282 extension and the 9res motif based on 174 sequences provided by the CAZy database. Conserved aromatic amino acids are marked with an orange star. **(B)** Homology model (AlphaFold2)³⁰ of PcoAA9-X282-9res shown as surface. The X282 extension and 9res motif are colored in cyan and orange, respectively. Zoom-in views (in dotted circles) show the X282 aromatic amino acids (shown as blue sticks) potentially involved in π -stacking interactions with amino acids from the AA9 domain (shown as yellow sticks).

site, just beneath the X282 extension (Fig. S3A). Laurent et al. demonstrated that phylogenetic clustering of AA9s is based on five extended catalytic site segments, annotated from Seg1 to Seg5³³. We note that the sequence of this charged patch is part of segments 2 and 3 and diverge from other AA9s (Fig. S3B). Noteworthy, homology models suggest that these charged-patch sequences contain two strictly conserved cysteines facing each other, seemingly involved in a disulfide bond³³ (Fig. S3B). The potential implication of these conserved features in a biological function also remains to be investigated.

Biochemical characterization of the three AA9-X282 modularities. We heterologously produced in the yeast *Pichia pastoris* six recombinant AA9-X282 representatives of the three different modularities: (i) the AA9-X282-9res from *Pycnoporus coccineus* (syn. *Trametes coccinea*) (*PcoAA9-X282-9res*), *Trametes elegans* (*TelAA9-X282-9res*) and *Trametes ljubarskyi* (*TljAA9-X282-9res*); (ii) the AA9-X282-CBM1 from *Irpex lacteus* (*IlaAA9-X282-CBM1*) and *Coprinopsis cinerea* (*CciAA9-X282-CBM1*); and (iii) the AA9-X282 from *Schizophyllum commune* (*ScoAA9-X282*) (Fig. 3A). To assess the substrate specificity of these AA9-X282, we first

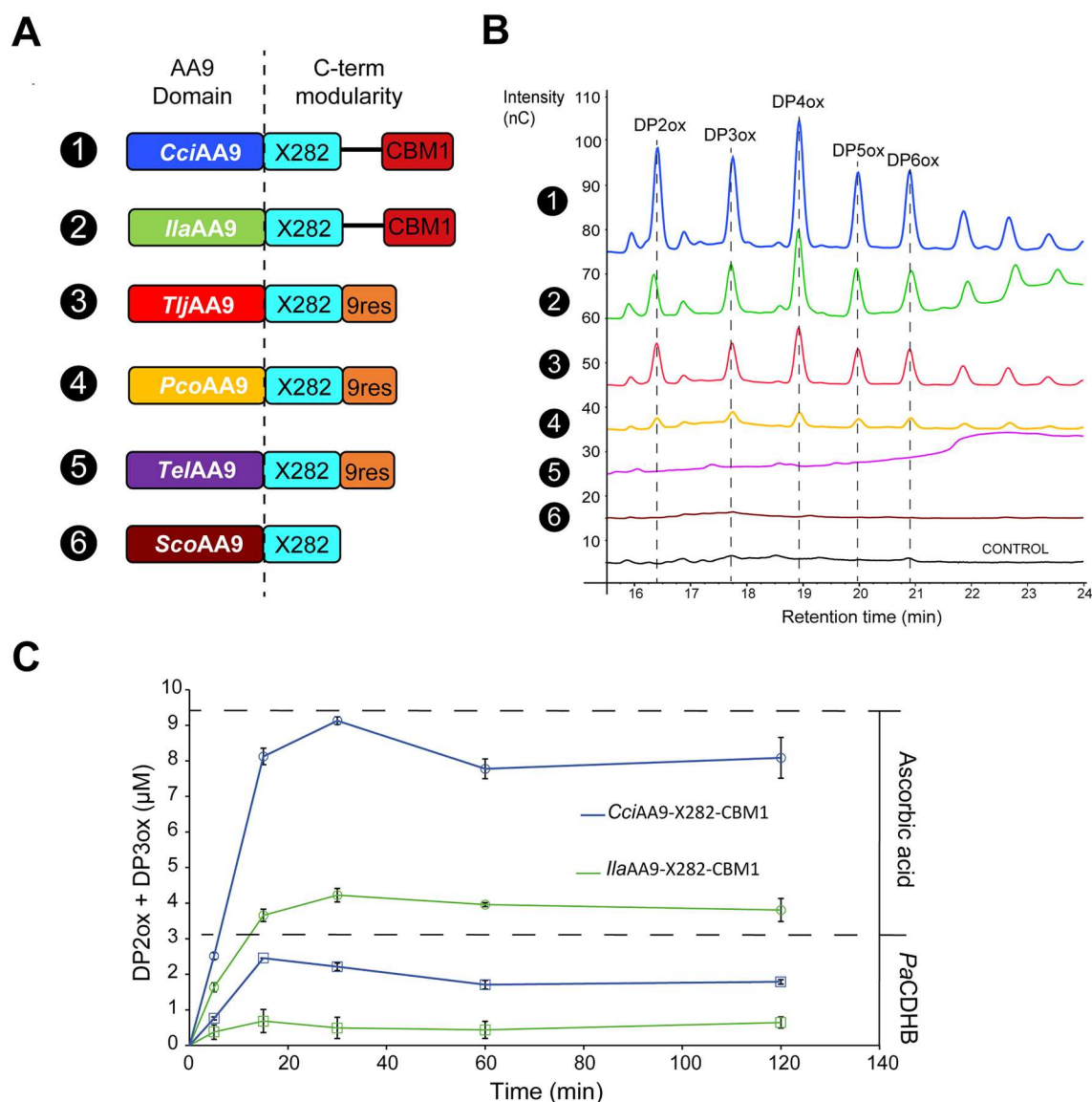


Figure 3. Kinetics and oxidative profile of the produced AA9-X282 on PASC. **(A)** Modularity of the studied AA9-X282s. Enzymes were named based on their organisms of origin: *Coprinopsis cinerea* (*Cci*), *Irpex lacteus* (*Ila*), *Trametes ljubarskyi* (*Tlj*), *Pycnoporus coccineus* (*Pco*), *Trametes elegans* (*Tel*), *Schizophyllum commune* (*Sco*). Each enzyme is associated with a color, consistently used in all panels. **(B)** HPAEC-PAD chromatogram of oxidized products released by the AA9-X282 (1 μM) from PASC (0.2%) in the presence of Asca (1 mM) after 24 h. In the control reaction the AA9 was replaced by CuSO₄ (1 μM). **(C)** Time-course release of oxidized products (DP2ox + DP3ox) by CciAA9-X282-CBM1 and IlaAA9-X282-CBM1 (1 μM each) from PASC (0.2%) using ascorbic acid (1 mM) or PaCDHB (1 μM) as reductant. All reactions were incubated in sodium acetate buffer (50 mM, pH 5.2), in a thermomixer (850 rpm, 40 °C). Data points show average values and error bars show standard deviations (n = 3 independent biological replicates).

assayed them on amorphous cellulose (PASC) (Fig. 3B). For some of the AA9-X282 LPMOs, we detected soluble C1-oxidized cello-oligosaccharides with degrees of polymerization (DP) between 2 and 6 (Fig. 3B). The most active ones harbored a CBM1. The intensity of the peaks observed for the AA9-X282-CBM1 (i.e. *CciAA9-X282-CBM1* and *IlAA9-X282-CBM1*) are in good agreement with the ones observed for *CsLPMO9B*, which also harbors a CBM1 and belongs to clade X1²⁴. However, while keeping in mind potential variations due to equipment and buffers between different labs, we underscore that such intensities are very low when compared to other cellulose-active C1-oxidizing AA9-CBM1 LPMOs^{17,34,35} (Fig. S6A). Strikingly, most of the other AA9-X282 showed low (for *PcoAA9-X282-9res*) or no activity (*TelAA9-X282-9res* and *ScoAA9-X282*) on cellulose. Therefore, we further screened a large range of potential PCW-derived substrates (including diverse xylans, mannans, pectins, cello-oligosaccharides and xylo-oligosaccharides; see Table S1), but no activity could be detected. To get deeper insights into the behavior of the two most active AA9-X282-CBM1, we carried out a time-course analysis (Fig. 3C). We observed a fast initial release of oxidized products from PASC, followed by early reaction ending, which is indicative of possible protein inactivation. Intriguingly, no synergy could be observed when using a CDH (*PaCDHB*) as a redox partner, which is, to our knowledge, an unprecedented observation for AA9s (Fig. 3C). A control experiment using *PaAA9E* LPMO illustrates how AA9s usually behave in the presence of CDH as redox partner and the expected amounts of products (Fig. S6). Intrigued by these observations, we evaluated whether the activity of the AA9-X282 was limited by other, usually key elements of an LPMO reaction, by notably testing the effect of (i) a broad range of reductants at two different pH (Fig. S7A, Table S1) and (ii) the addition of H₂O₂ as co-substrate (Fig. S7B). None of the tested conditions improved the activity of AA9-X282. Furthermore, we measured a rather low binding to PASC for the top 3 most active AA9-X282 (Fig. S7C), which is in agreement with the observed fast ending kinetic (Fig. 3C) since substrate binding has been shown to have a protective effect^{19,36} and this binding is improved by the presence of CBMs^{37–39}. Despite poor activity on cellulose, all AA9-X282 LPMOs displayed an oxidase (i.e. H₂O₂ producing) activity ($4\text{--}6 \times 10^{-3} \text{ s}^{-1}$) similar to that reported for other AA9s ($10^{-4}\text{--}10^{-2} \text{ s}^{-1}$)² (Fig. S7D).

In light of these results, and while bearing in mind their transcriptional upregulation and specific presence in wood-degrading fungi, we questioned the genuine biological role of these enzymes during PCW degradation. In search of new hints, we turned our attention towards the unusual structural features of these poorly active AA9-X282.

The surface of *CciAA9-X282-CBM1*, *TljAA9-X282-9res* and *PcoAA9-X282-9res* displays several phosphorylation sites. When exploring the conservation of the residues constituting the surface of the AA9-X282 catalytic site, we surprisingly noted a high amount of conserved serine and threonine residues. More precisely, throughout our pool of 174 AA9-X282 sequences, 17 serines and 21 threonines display > 50% conservation (Fig. S8A). Among these residues, 2 serines and 7 threonines are part of the X282 extension. Such occurrence of serines and threonines on the surface of AA9-X282 is not inconsequential, as these residues are known to be potential sites of post-translational modifications, which may alter the enzyme activity/properties. In a recent study, a phosphorylated serine was identified in the crystal structure of an atypical AA9 from the Polyporales fungus *Lentinus similis*. This AA9 (*LsAA9B*) is an LPMO-like protein that lacks the conserved histidine brace motif found in LPMOs and displays protein phosphorylation near the N-terminal residue⁴⁰. Interestingly, structural alignment of *PcoAA9-X282-9res* with *LsAA9B* (Fig. S8B) allowed us to identify a conserved serine (Ser24, 93% conservation) at the same position than the phosphorylated *LsAA9B*-Ser25. On the basis of these observations, we decided to probe by high-resolution tandem mass spectrometry the phosphorylation state of three AA9-X282, namely *CciAA9-X282-CBM1*, *TljAA9-X282-9res* and *PcoAA9-X282-9res* (Fig. 4 and Fig. S8A).

Strikingly, while none of the positions corresponding to Ser25 in *LsAA9B* were observed phosphorylated, several other AA9-X282 Ser and Thr residues (two threonines and one serine for *CciAA9-X282-CBM1*; one threonine and two serines for *TljAA9-X282-9res*; two threonines and four serines for *PcoAA9-X282-9res*), all located at the surface of the proteins, were confidently proved to be phosphorylatable (Fig. 4). Intriguingly, the most documented site, with 20% to 26% of peptides covering this site being phosphorylated, was the threonine residue at position 3 (Thr3), which is conserved in almost all AA9-X282 sequences (99%) (Fig. S8A). We underscore that such observation at this position, replicated on several enzymes, is not fortuitous and highlights a possible regulation of the global charge of this patch of residues by kinases/phosphatases interplay. Considering the vicinity of the Thr3 with the N-term His1, it was tempting to speculate that this phosphorylation could affect the structural/redox properties of the histidine brace, which could in turn explain the low activity of these enzymes. To probe this hypothesis, we expressed in *Pichia pastoris* a mutated version (T3V) of the gene coding for *PcoAA9-X282-9res*, and after purification, tested its activity on PASC. The T3V mutation did not allow to restore any significant LPMO activity on cellulose (Fig. S9).

Altogether, these unexpected observations suggest the involvement of the AA9-X282 in a biological function potentially diverging from PCW degradation. Along this line, we focused our attention on the short C-terminal, conserved motif “9res” which displays unusual features.

The 9-residues motif of AA9-X282s is reminiscent of canonical phosphate-binding motifs. The 9res motif is predominantly found in clades C and D in the tree of AA9-X282 proteins and is rather specific of the *Trametes* genus (Fig. S4). Indeed, we underscore that each *Trametes* species possess only one type of AA9-X282, which is the AA9-X282-9res, the only exception being *Trametes punicea*, that possesses both one AA9-X282-9res and one AA9-X282-CBM1. Looking more closely into this motif, we noted the presence of five strictly conserved residues (3 Gly, 1 Ile, and 1 Ala) and one highly conserved lysine (sometime replaced by a glutamine) (Fig. 2). The consensus sequence of this motif “GXXGIGKA” (where “X” can be any amino acids), together with

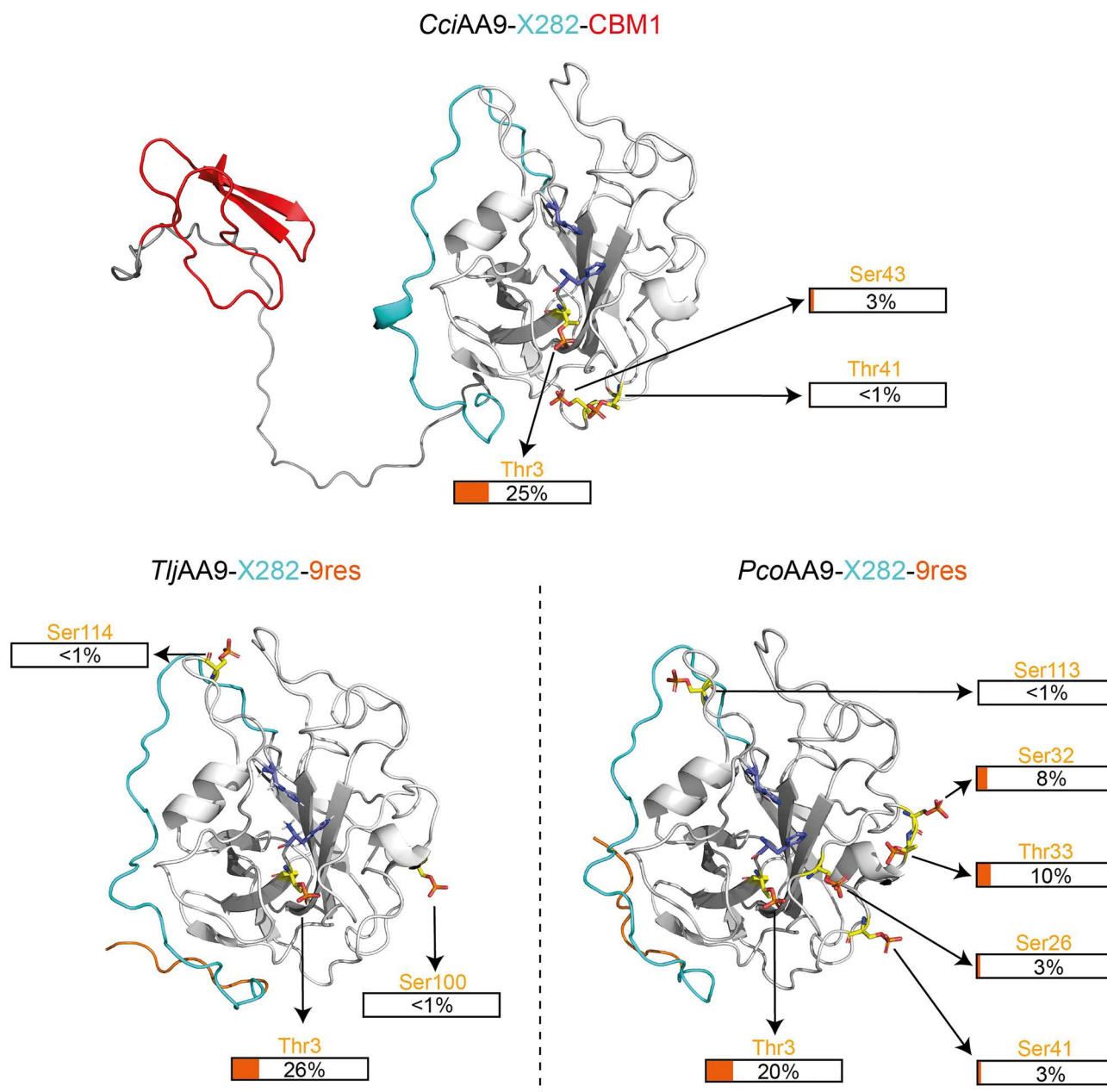


Figure 4. Mapping of phosphorylated residues on three AA9-X282 structure predictions. The phosphorylation (provided in the figure) of *Cci*AA9-X282-CBM1, *Tlj*AA9-X282-9res and *Pco*AA9-X282-9res were evidenced by tandem mass spectrometry. The identified phosphorylated residues are shown as yellow sticks. On the predicted protein structures (AlphaFold²⁰), shown as cartoon, the AA9 domain is coloured in white, the CBM1 in red, the X282 extension in cyan and the 9res motif in orange. The two catalytic histidines are shown as sticks and coloured in blue. Phosphate groups (shown as red sticks) have been added in PyMol using the PyTMs plugin. The percentages of peptides found phosphorylated compared to peptides found not phosphorylated is indicated for each position (we underscore that phosphorylated sites do not necessarily occur on a same protein molecule). The list of all peptides detected is provided in Table S2 (see <https://doi.org/10.6084/m9.figshare.22709599>).

the striking presence of phosphorylated residues in the protein, drew our attention due to its similarity with canonical and widespread phosphate binding loops (P-Loop) found in Walker A and Rossmann fold motifs^{41,42} (Fig. 5). The Walker A motif is a key feature in the AAA + (“ATPases associated with various cellular activities”) proteins superfamily for its ability to bind ATP (Fig. 5A)⁴³. The Rossmann fold refers to a tertiary protein fold composed of a succession of β -strands connected by α -helices⁴⁴ and that can accommodate dinucleotide substrates such as FAD, NAD or NADP⁴⁵. One of the motifs specific to NAD(P)H, have been described as “GXXX-GIG”⁴⁶ (Fig. 5A).

To evaluate the potential of the 9res motif to bind ATP (Fig. S10) or NADPH (Fig. S11), we used both fluorometric and in silico approaches. Of note, to avoid any biases coming from C-term modifications, we cloned,

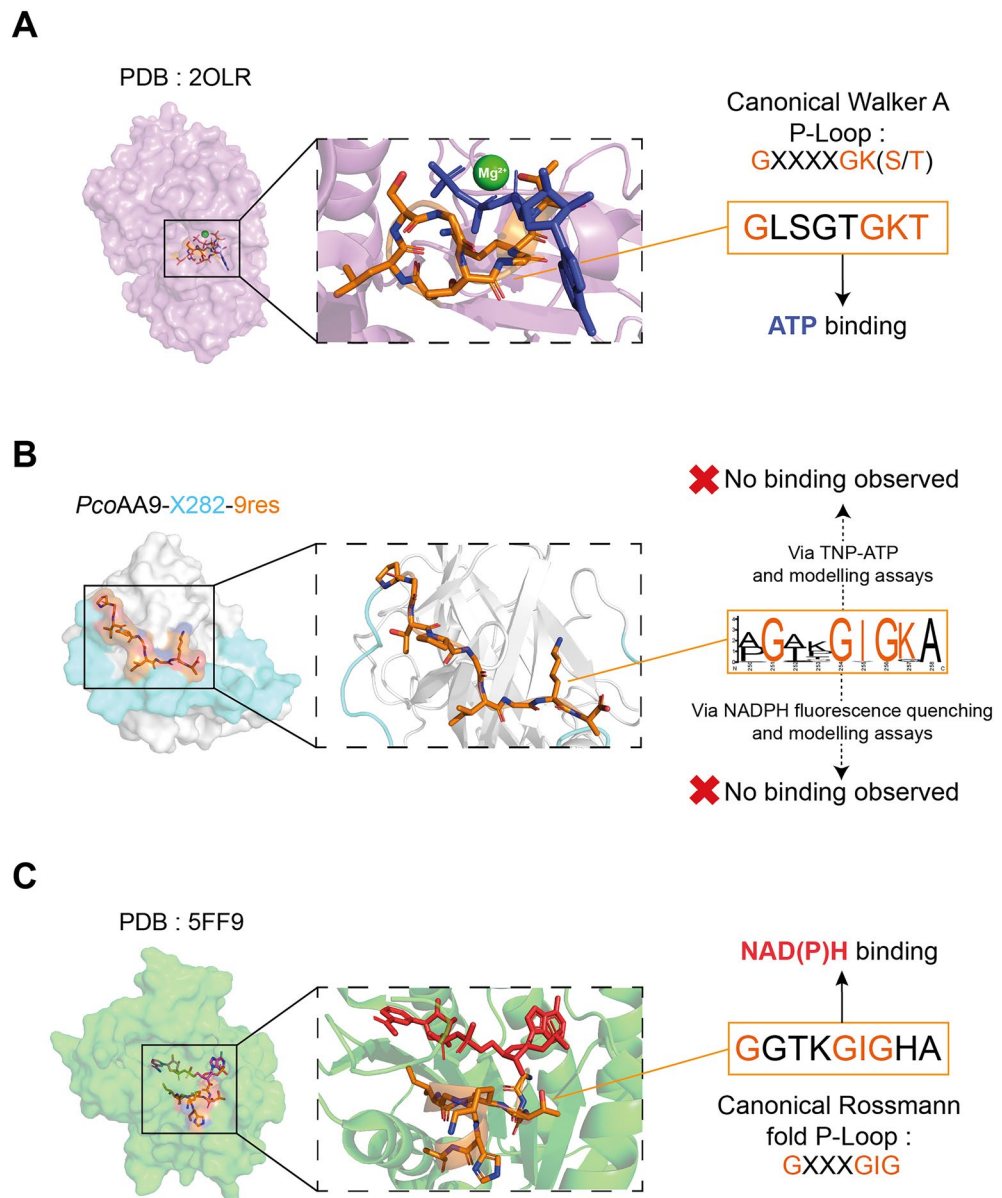


Figure 5. Structural comparison of the “9res” motif with canonical phosphate binding motifs. (A) ATP-dependent enzyme (PDB id 2OLR)⁴⁷. In the canonical sequence “GXXXXGK(S/T)”, the lysine residue is crucial for the coordination of the nucleotide β - and γ -phosphates while the serine/threonine residue ensure the coordination a Mg^{2+} -ATP complex required for its hydrolysis^{41,48,49}. ATP is colored in blue and the Mg^{2+} cation is colored in green. (B) *PcoAA9-X282-9res* structure prediction (AlphaFold2) and zoom-in view of the 9res motif, colored in orange. (C) Zoom-in of the NADPH binding site of a canonical NADPH oxidase crystal structure (PDB id 5FF9)⁵⁰. Key amino acids from the P-loop involved in phosphate binding are colored in orange. NADPH is colored in red.

produced, and purified a non-tagged version of *PcoAA9-X282-9res* devoid of C-terminal His-tag (referred to as “*PcoAA9-X282-9res* NT”). Nevertheless, both the non-tagged and His-tagged variants were evaluated. In the tested conditions, in vitro assays did not allow to show any significant ATP binding (Fig. S10A) nor ATP hydrolysis (Fig. S10B), and no significant binding to NADPH (Fig. S11A,B). In agreement, PELE site finder simulations did not identify the 9res motif as a suitable binding site for ATP (Fig. S10C) or NADPH (Fig. S11C), as the energetic minima, indicative of the location for putative binding sites, are far from the lysine of the “GXXGIGKA” consensus sequence for both ligands. This contrasts with the site finder simulations in a reference ATPase model (PDB id 1XU4), where, as expected, we observed a clear minimum in proximity to the lysine of the Walker A motif when simulated with ATP (Fig. S10C). However, it may be worth noting that, in the case of NADPH, albeit interaction energies remain low, a cluster with a relatively low distance between *PcoAA9-X282-9res* and NADPH was observed (Fig. S11C). It is plausible that the variations observed in the 9res motif of *PcoAA9-X282-9res*, compared to canonical Walker A and Rossmann fold, may alter hypothetical binding to substrates and, in such

alternative, related substrates remain to be found and tested. The conservation and similarity between the 9res motif and canonical phosphate binding motifs remain remarkable and would deserve further study in the future.

Discussion

Our initial objective was to identify and characterize efficient LPMOs for plant biomass degradation. Building on the published observation that some atypical AA9-encoding genes were co-regulated with cellulases during biomass decay, we set off to characterize several of these AA9s displaying a module of unknown function (i.e., X282). However, despite our efforts, we only observed very low LPMO-type activity (on cellulose), and even more striking was the absence of synergy with CDH. In that regard, we hypothesize that the X282 belt may hamper protein–protein interactions. Overall, this poor activity, regardless of the reaction conditions, seems to distance AA9-X282 from a straightforward polymer degradation context. We even wonder whether these AA9s should be referred to as LPMOs, although they display several hallmarks of genuine LPMOs such as a functional histidine brace (as shown by normal H_2O_2 producing activity) and the occurrence of a CBM1 in some members. Nevertheless, these results and observations forced us to take a step back while investigating further the atypical features of these AA9s.

At first glance, we had suspected that the X282 extension could correspond to a new CBM but bioinformatic analyses and structures predictions rather suggest that the X282 extension would cap the AA9 domain as a belt by means of two conserved π -stacking interactions involving strictly conserved aromatic residues. In this configuration, the X282 extension is found relatively close to the copper face, just on top of another conserved negatively charged patch. Noteworthy, the fact that the X282 extension can be found alone at the C-terminus or followed by two distinct modularities (i.e., the 9res motif or a linker-CBM1), suggests that the function the X282 extension and the other motifs are not specifically correlated. Interestingly, peptidic belts have been reported to increase thermostability in fungal glucoamylases⁵¹, and one may wonder if the X282 could fulfill a similar protective function in AA9-X282s.

The second conserved pattern found in some of the AA9-X282s, notably in those produced by fungi belonging to the *Trametes* genus, is a stretch of 9 residues referred to as “9res” motif. We observed that its conserved sequence appears surprisingly reminiscent of canonical P-loops found in ATP and NAD(P)⁺/NAD(P)H dependent enzymes. The latter proteins are described as involved in intracellular mechanisms and, to the best of our knowledge, such P-loop-like motif has never been reported for secreted enzymes. Nonetheless, the presence of extracellular ATP (eATP) has already been demonstrated to be involved in different types of interactions, such as in plant response to biotic and abiotic stresses⁵², or in fungi–bacteria interactions⁵³. The exact role of eATP in these interactions is still poorly understood, and, although no significant binding to ATP could be observed in our conditions, its potential involvement in AA9-X282 functionality should not be definitely ruled out.

The third intriguing observation on AA9-X282 was brought by proteomic analyses, viz. the presence of multiple phosphorylated sites at the surface of the proteins. The presence of these phosphorylated residues could locally impact the charge of the protein and thus enable/prevent interactions with a substrate or protein. Assessing the global occupancy rate of phosphorylated sites per proteins, could allow to estimate the actual localization and occurrence of these potential domains of interaction.

We underscore that the present study stands as the second observation of a phosphorylated AA9 originating from a Polyporales fungus, as a single phosphorylated site has already been observed in the crystal structure of another AA9 from the polypore *Lentinus similis* (LsAA9B)⁴⁰. It is worth noting that so far, no correlation can be established between the phosphorylation of these AA9s and the absence/presence of LPMO activity. Indeed, LsAA9B is devoid of any copper coordination site, and in the case of AA9-X282, the non-phosphorylated T3V mutant did not permit to restore any activity on cellulose. Phosphorylation of extracellular proteins has already been observed in the LPMO field: the virulence factor CbpD from the human pathogen *Pseudomonas aeruginosa* was shown to display 11 phosphorylated residues at its surface when secreted by the bacterium⁵⁴, two of these residues being found on the chitin-binding surface⁵⁵. While CbpD recombinantly produced in *E. coli* was demonstrated to be an AA10 LPMO with chitin-oxidizing activity⁵⁶, the in vivo functional impact of phosphorylations remains to be studied. Given the well-documented importance of protein phosphorylation in various signaling/regulation processes^{57,58}, our study, as well as the few other reports cited above, collectively prompt a thorough examination of the post-translational modifications occurring in LPMOs and an investigation of their biological importance.

Conclusions

Overall, we have shown that the AA9-X282 proteins display several unique features that have never been reported in the LPMO field. We believe that the co-occurrence of structural features reminiscent of phosphate binding loops, together with surface-exposed phosphorylation sites are not the fruit of fortuitous evolution and may be pieces of a larger puzzle. Recent work suggests that the functionality of LPMOs goes beyond their usually ascribed function of recalcitrant polysaccharide oxidizers^{9,59}, such as in some pathogenicity mechanisms^{56,59}, or in allorecognition upon fungal cell–cell contact⁶⁰ where the LPMO catalytic function is not always decisive. Given the wide expansion of AA9 genes in the genomes of some white rot fungi (sometime over 60 genes), it is tempting to hypothesize that, during millions of years of evolution, a certain extent of functional divergence has occurred. Importantly, we wish to stress that the majority of studies have focused on the N-terminal part of LPMOs (mainly on catalytic domain appended or not with a CBM). However, a large proportion of LPMOs contain C-terminal disordered regions⁶¹, modules of unknown functions or small extensions, which are usually removed to ease recombinant production and characterization. Alike the module-walking strategy that has allowed to identify new LPMO families by looking for the presence of well-known CBMs, we believe that further

examination of the C-terminal regions and their unusual properties may be one of the keys to unveil new functions of these moonlighting and enigmatic proteins.

Material and methods

Materials. Most chemicals, including Avicel, lignin-derived compounds and horseradish peroxidase were purchased from Sigma-Aldrich (Saint-Louis, Missouri, United-states). TNP-ATP was purchased from Jena Bioscience (Jena, Germany). Oligo- and polysaccharides substrates were purchased from Megazyme (Wicklow, Ireland). Both α - and β -chitin were purchased from Mahtani Chitosan (Gujarat, India). PASC was prepared as described in Wood⁶².

Enzymes cloning and production. The proteins were produced using the in-house 3PE platform (*Pichia pastoris* Protein Express; www.platform3pe.com) as described in Haon et al.⁶³. The nucleotide sequence of the 6 AA9-X282 from *Coprinopsis cinerea* (CciAA9-X282-CBM1, Uniprot ID: A8NCG7), *Irpex lacteus* (IlaAA9-X282-CBM1; GenBank: KAI0763186.1), *Pycnoporus coccineus* (PcoAA9-X282-9res; GenBank: OSD05161.1), *Trametes ljubarskyi* (TljAA9-X282-9res; GenBank: KAI0367364.1), *Trametes elegans* (TelAA9-X282-9res; GenBank: KAI0761258.1) and *Schizophyllum commune* (ScoAA9-X282; GenBank: KAI4528925.1.) were codon optimized for expression in *Pichia pastoris*. Gene synthesis was performed by Genewiz (South Plainfield, New-Jersey, USA), the region corresponding to the native signal sequence was kept and the genes were inserted into the expression vector pPICZaA (Invitrogen, Cergy-Pontoise, France) in frame with a C-terminal poly-histidine tag, except for PcoAA9-X282-9res NT where the native C-terminal sequence remained unmodified. PmeI-linearized pPICZaA recombinant plasmids were used to transform competent *P. pastoris* cells by electroporation. Zeocin-resistant transformants were then screened for protein production. The SuperMan5 strain was used to produce ScoAA9-X282 and TelAA9-X282-9res while the X33 strain was used for IlaAA9-X282-CBM1, CciAA9-X282-CBM1, TljAA9-X282-9res, PcoAA9-X282-9res, PcoAA9-X282-9res NT and PcoAA9-X282-9res T3V. The best-producing transformants were grown in 2 L of BMGY media in flasks at 30 °C in an orbital shaker (200 rpm) for 16 h to an OD₆₀₀ of 2 to 6. Expression was induced by transferring cells into 400 mL of BMMY media at 20 °C in an orbital shaker (200 rpm) for another 3 days. Each day, the medium was supplemented with 3% (v/v) methanol. The cells were harvested by centrifugation, and just before purification the pH of the supernatant was adjusted to 7.8 by addition of Tris-HCl buffer (1 M, pH 8) and was filtered on a 0.45 μ m membrane (Millipore, Burlington, Massachusetts, USA).

Protein purification. For the His-tagged proteins, the filtered culture supernatant was loaded onto a 5 mL HisTrap excel column (GE Healthcare, Bus, France) equilibrated with buffer A (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 10 mM) that was connected to an Äkta purifier 100 (GE Healthcare). His-tagged recombinant proteins were eluted with 50% of buffer B (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 500 mM). For PcoAA9-X282-9res produced without His-tag (so called “PcoAA9-X282-9res NT”), the protein was purified using a monoQ™ 10/100 GL anion exchange column (Cytiva; Freiburg im Breisgau, Germany) and eluted using a gradient of Tris-HCl buffer (20 mM, pH 8.0) containing 1 M NaCl. The peaks obtained after elution were loaded onto 10% Tris-glycine precast SDS-PAGE gels (BioRad), stained with Coomassie Blue. The fractions containing pure recombinant proteins were pooled together, concentrated and dialyzed against Tris-HCl buffer (20 mM, pH 8.0). The protein concentrations were determined by absorption at 280 nm using a Nanodrop NB-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The protein parameters were the following: CciAA9-X282-CBM1 (MW = 35,346.57 g.mol⁻¹, ϵ_{280} = 63,995 M⁻¹.cm⁻¹); IlaAA9-X282-CBM1 (MW = 33,739.25 g.mol⁻¹, ϵ_{280} = 58,495 M⁻¹.cm⁻¹); PcoAA9-X282-9res (MW = 27,694.72 g.mol⁻¹, ϵ_{280} = 40,255 M⁻¹.cm⁻¹); PcoAA9-X282-9res-T3V (MW = 27,692.75 g.mol⁻¹, ϵ_{280} = 40,255 M⁻¹.cm⁻¹); PcoAA9-X282-9res NT (MW = 26,871.88 g.mol⁻¹, ϵ_{280} = 40,255 M⁻¹.cm⁻¹); TljAA9-X282-9res (MW = 27,732.86 g.mol⁻¹, ϵ_{280} = 37,275 M⁻¹.cm⁻¹); TelAA9-X282-9res (MW = 27,638.74 g.mol⁻¹, ϵ_{280} = 38,765 M⁻¹.cm⁻¹); ScoAA9-X282 (MW = 26,829.63 g.mol⁻¹, ϵ_{280} = 45,755 M⁻¹.cm⁻¹).

Phylogenetic analysis. To build the phylogenetic tree of AA9s, we used 156 AA9 sequences including 39 sequences of all the biochemically and/or structurally characterized AA9s available in the CAZy database at the time of this study, 13 AA9-X282 sequences covering the diversity of their C-terminal modularity and 104 AA9s sequences regrouping all the AA9s from the six Polyporales studied by Hage et al.²². Of note, the variable C-terminal regions of all AA9 sequences were cut using BioEdit⁶⁴ in order to keep the catalytic domain only. The 156 AA9 sequences batches were aligned using MAFFT-DASH (L-INS-i method)⁶⁵, which include structural data input. The resulting multiple sequence alignments were used to infer the phylogenetic trees via the IQ-tree webserver. To infer the tree, the Whelan and Goldman (WAG) amino acid substitution model was selected with a maximum Likelihood optimized (ML-optimized) state frequency. Branch support was calculated by 1,000 bootstraps alignments using the ultrafast bootstrap analysis method. To build the phylogenetic tree of AA9-X282 proteins only, 174 sequences available in the CAZy database were retrieved and the same procedure as above was followed.

LPMO activity assays. Substrates screening of AA9-X282s were performed on 16 substrates (Table S1). All substrates were tested at a concentration of 5 mM (if soluble in water) or 5 mg.mL⁻¹ (if insoluble in water). Reaction on PASC were performed at a concentration of 2 mg.mL⁻¹ (eq. 0.2% w/v), and PASC/Xylan (from beechwood) coupling were tested in different proportions (0.4% PASC / 0.4 or 0.3 or 0.2 or 0.1 or 0.04% xylan; % in w/v). All reactions were performed in sodium acetate buffer (50 mM, pH 5.2) in a final volume of 500 μ L and incubated for 24 h, unless stated otherwise (30 °C, 850 rpm). Reactions were stopped by heating at 100 °C for

10 min and then centrifuged before analysis of the soluble fraction using a high-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) (DIONEX ICS6000 system, Thermo Fisher Scientific). The system is equipped with a CarboPac-PA1 guard column (2 × 50 mm) and a CarboPac-PA1 column (2 × 250 mm) kept at 30 °C. Elution was carried out at a flow rate of 0.25 mL·min⁻¹ and 25 µL of sample was injected. The solvents used were 100 mM NaOH (eluent A) and NaAc (1 M) in 100 mM NaOH (eluent B), and the following gradient was applied: 0 to 10 min, 0 to 10% B; 10 to 35 min, 10 to 35% B (linear gradient); 35 to 40 min, 30 to 100% B (curve 6); 40 to 41 min, 100 to 0% B; 41 to 50 min, 100% A. Integration was performed using the Chromeleon 7.2.10 chromatography data software.

AA9-X282s reductants screening. 14 lignin-derived compounds were tested as reductants for *IlaAA9-X282*. Reaction on PASC (0.2%) were performed as described above at pH 5.2 (50 mM sodium acetate buffer) or pH 8.0 (50 mM Tris-HCl buffer) using 1 mM of reductant and stopped by heating 10 min at 100 °C. After cooling down, the pH 8 reactions were brought back to pH 5–6 by adding 25 µL of acetic acid (1 M). A cellulytic cocktail from the Ascomycete *T. reesei* containing two cellobiose hydrolases, two *endo*-glucanases and one β-glucosidase was then added to all reaction broths at a total concentration of 100 mg/g of substrate and complete hydrolysis of PASC was ensured by incubating the reactions for 24 h in a Thermomixer (50 °C, 850 rpm). The complete fractions (soluble and insoluble) of oxidized products (DP2ox and DP3ox) were then quantified by HPAEC-PAD, using a standard curve.

AA9-X282s oxidase activity assays. Oxidase activity of the six AA9-X282s was measured according to the protocol described by Kittl et al.²⁶. The reaction mixture contained Amplex Red reagent (200 µM; Thermo Fisher Scientific), Horseradish peroxidase (HRP; 0.1 mg·mL⁻¹ final) and AA9-X282s (1 µM). The reactions were performed in 96-well microplates in sodium phosphate buffer (50 mM, pH 7.0) and started by adding 50 µM of ascorbic acid. The H₂O₂ production rate is reflected by the production rate of resorufin, i.e. the product of Amplex Red oxidation by HRP and H₂O₂, which was monitored at 575 nm over a 30 min period. The initial slope of the curve, normalized with the enzyme concentration, was used to calculate the H₂O₂ production rate.

AA9-X282 cellulose binding assays. The binding affinity of AA9-X282s (1 µM) for PASC (0.2%) and Avicel (5 mg·mL⁻¹) was monitored by measuring the concentration of the protein in the supernatant after 2 or 24 h of incubation in a Thermomixer (30 °C, 850 rpm). For each condition, a control reaction in the absence of substrate was carried out. The protein concentration was measured at 280 nm using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A binding of 0% corresponds to the enzyme absorbance in the absence of substrate. All the reactions were performed at pH 5.2 (50 mM sodium acetate buffer) or pH 7.0 (50 mM sodium phosphate buffer). Only the results at 2 h are shown since no increase in binding could be observed after 24 h of incubation.

Tandem mass spectrometry analysis of AA9-X282s. Protein samples (20 µg) were diluted into 200 µL of trifluoroacetic acid (0.1%) and desalted with C18 spin columns (Harvard Bioscience). After their elution, polypeptides were dried and dissolved into 20 µL of 100 mM NH₄HCO₃. Four volumes of 5 µL corresponding to 5 µg of protein were treated for 10 min with 5 mM dithiothreitol at 56 °C under gentle agitation, subjected to 5 mM iodoacetamide for 10 min at room temperature in the dark, and then proteolyzed for 2 h by either trypsin, AspN, trypsin and AspN, trypsin and GluC proteases prepared at 0.1 µg per µL and used at 2% of protein biomass. After proteolysis, trifluoroacetic acid was added (0.1% final volume). The resulting peptides (200 ng) were resolved by reverse phase chromatography on a Vanquish Neo UHPLC (Thermo) coupled to an Exploris 480 tandem mass spectrometer (Thermo). This instrument was operated in data-dependent acquisition mode as previously described⁶⁶. The gradient applied on the reverse phase column was developed over 30 min from 4 to 40% of acetonitrile in 0.1% of formic acid. MS full scan spectra and MS/MS spectra after fragmentation were acquired at resolution 120,000 and 60,000, respectively. Only ions with 2 or 3 positive charges were selected for fragmentation with a 10-s dynamic exclusion window. MS/MS spectra analysis was performed as previously described⁶⁷ with carboxyamidomethylation of cysteine residues as fixed modification, oxidation of methionine, deamidation of asparagine and glutamine, and phosphorylation of serine and threonine as variable modifications. For each residue, the phosphorylation rates were estimated by dividing the number of MS/MS spectra assigned to peptides containing a phosphorylation by the total number of MS/MS spectra attributed to peptides encompassing the phosphorylatable residue. We underscore that such percentage does not represent the true proportion of phosphorylated proteins in each sample as phosphorylated and unphosphorylated peptides may ionized differently in our analytical conditions.

ATP binding assays. Fluorescent emission scanning spectra of TNP-ATP were obtained using a Biotek Synergy HT plate-reader (Bio-Tek Instruments, Winooski, VT, USA), equipped with emission and excitation filters of 540 (± 20) nm or 410 (± 20) nm respectively. Binding assays were performed by mixing 10 µM of enzyme with 20 µM of TNP-ATP in Tris-HCl buffer (50 mM, pH 7.5) and measuring the change in TNP-ATP fluorescence emission at 540 nm upon excitation at 410 nm. All measurements were carried out in 96-well, black, flat-bottom plates (Corning) at 25 °C, in 100 µL reaction volumes.

NADPH binding assays. The binding of *PcoAA9-X282-9res* and *PcoAA9-X282-9res NT* to NADPH was monitored based on a method described in Fjeld et al.⁶⁸. Proteins were excited at 285 nm and fluorescence emission was measured at 325 nm (± 20 nm). When exciting the mixture of NADPH and AA9-X282s at

285 nm, a conserved tryptophan (Trp234) present at the surface of AA9-X282s in the vicinity of the 9res motif is expected to transfer its emitted fluorescence at 325 nm to a potentially bound NADPH via a FRET mechanism. The resulting loss in fluorescence at 325 nm was monitored in a spectrofluorometer (Biotek Synergy HT plate-reader). To prevent any redox interactions between NADPH and the AA9-X282s that could modify the intrinsic fluorescence of the proteins as described elsewhere⁶⁹, *apo*-AA9-X282s were prepared as described by Askarian et al.⁵⁶. Briefly, *PcoAA9-X282-9res* and *PcoAA9-X282-9res* NT solutions (10 μ M final) were incubated overnight with EDTA (2 mM) in Tris–HCl buffer (20 mM, pH 7.5) at 4 °C, followed by addition of MgCl₂ (5 mM), further incubated during 15 min at room temperature, and finally desalted on a PD MidiTrap G-25 column. All fluorescence measurements were performed in 96-well, black, flat-bottom plates at 25 °C, in 100 μ L reaction volumes.

Modelling. PELE (Protein Energy Landscape Exploration) is a Monte Carlo-based simulation algorithm consisting of two stages. In the first stage, the ligand is randomly translated and/or rotated, and the protein is perturbed using a normal mode method based on an anisotropic network model (ANM). In the second stage, the side chains around the ligand are optimized using a rotamer library followed by an overall structural minimization. The new conformation is then accepted or rejected based on the Metropolis criterion⁷⁰. Many protocols have been implemented using this algorithm to tackle different problems of biological interest. One of these protocols is Site Finder^{71,72} which performs a global exploration around the entire surface of the protein to find a suitable binding site for a given ligand. This is achieved by creating N copies of the structure, as many as the number of processors, where the ligand is randomly placed around the protein and allowed to run for a predefined number of PELE steps. The conformations with the lowest interaction energies are then refined with PELE by constraining the ligand to a local exploration in order to generate the most optimal binding mode. The models for the simulation were generated with AlphaFold2 or downloaded from the PDB databank and prepared with the Protein Preparation Wizard, Schrödinger, LLC, New York⁷³. The preparation adds the missing hydrogens, corrects the protonation states, fills the missing side chains and loops as well as the disulfide bonds. Then the structures were manually checked for possible inconsistencies and were set up for the site finder protocol using 100 processors, 12 PELE steps and 50 iterations in a total of 60,000 Monte Carlos steps. Note that to make things equal, the Mg²⁺ ion was removed from the PDB structure of the ATPase which reduced the interaction energy with ATP. The simulation results with the Mg²⁺ is similar but the energy minimum close to the lysine reaches below – 100 kcal/mol.

Data availability

The datasets used and/or analysed during the current study are available in the FigShare repository, <https://doi.org/10.6084/m9.figshare.22709599>, and available from the corresponding author on reasonable request.

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Author contributions

J.-L.R. carried out most experiments. M.H. and S.G. contributed to protein expression and purification and ionic chromatography. J.-C.G. and J.A. carried out the tandem mass spectrometry analysis. R.X. and V.G. carried out the simulations. J.-L.R., A.M., S.A., J.-G.B. and B.B. designed the experiments and interpreted the data. J.-G.B. and B.B. conceptualized the study. A.M., S.A., J.-G.B. and B.B. supervised the work. All authors contributed to the writing of the manuscript, reviewed, and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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SUPPORTING INFORMATION FOR

Insights into peculiar fungal LPMO family members holding a short C-terminal sequence reminiscent of phosphate binding motifs

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This file includes:

1. Supplementary Figure 1 to 11 and Supplementary table 1
2. Full abbreviations list
3. Supplementary references list

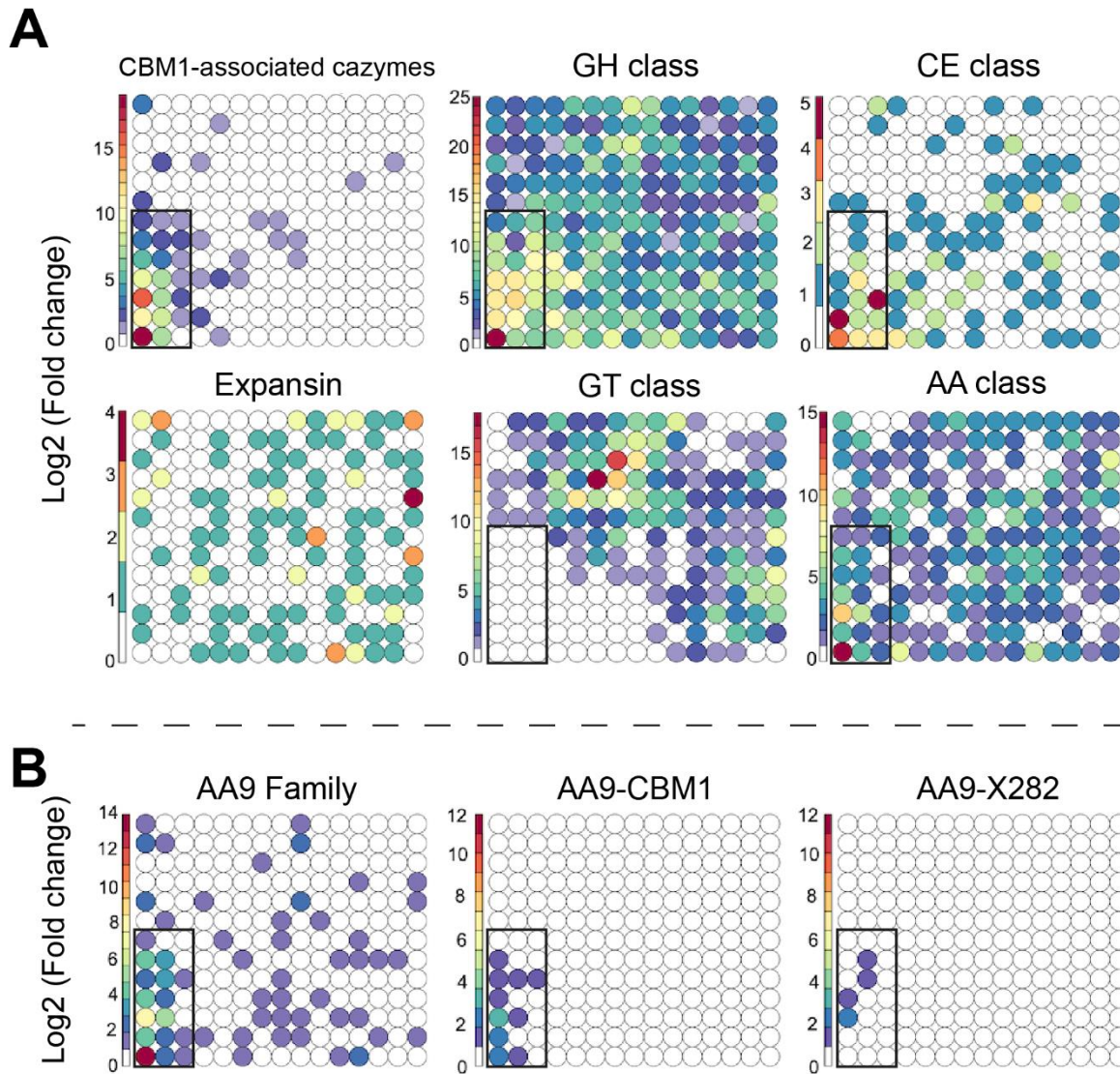


Fig. S1. Overview of the regulation of genes coding for predicted secreted proteins (including CAZymes) from six Polyporales species. The figure is adapted from Hage et al. 2021¹. 5536 genes from six Polyporales species (*Artolenzites elegans*, *Leiotrametes sp.*, *Pycnoporus cinnabarinus*, *Pycnoporus coccineus*, *Trametes lujbarskyi* and *Irpex lacteus*) were grouped on the basis of their regulation similarity using Self-Organizing Map² and the resulting clusters were mapped into nodes (here 195), which locate in the vicinity of each other when sharing similar regulations patterns. The Tatami maps show, for each node, the mean log2 differential transcription (Blue: moderately transcribed, Yellow: over-transcribed, Red: highly transcribed; the log-color scale is provided in the figure) of genes at day 3 on cellulose (Avi), aspen (Asp), pine (Pin), and wheat straw (Whs) (merged data for the 4 lignocellulosic substrates) in comparison to maltose (reference condition). **(A)** Tatami maps showing for each node the counts of commonly up-regulated genes in response to cellulose, aspen, pine and wheat straw corresponding to: CBM1-associated CAZymes and glycoside hydrolase (GH), carbohydrate esterase (CE), expansin, glycosyl transferase (GT) and Auxiliary Activities (AA) classes. **(B)** Same maps as in panel A focusing on AA9, AA9-CBM1 and AA9-X282 genes. The cluster encompassing most of the cellulose-responsive genes are squared in black.

A

		Clade X1	Clade X2	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Clade 9	Clade 10	Clade 11	Clade 12	Clade 13
Clade X1	Mean	76	57	44	38	36	32	35	37	34	37	36	33	39	43	43
Clade X2	Mean	57	78	43	36	38	31	30	34	32	29	33	29	38	43	43

B

		Clade X1	Clade X2	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Clade 9	Clade 10	Clade 11	Clade 12	Clade 13
Clade X1	Min	57	48	38	31	28	28	31	33	29	32	30	29	33	37	36
	Max	100	66	49	42	41	39	42	41	40	41	41	38	43	47	48
Clade X2	Min	48	69	41	34	34	28	26	32	27	25	27	25	34	40	40
	Max	66	100	47	38	40	35	34	40	35	34	39	34	43	48	47

Fig. S2. Condensed percentage identity matrix. Percentage identity matrix of the sequences used to infer the phylogenetic analysis displayed in **Fig.1**. The clades are annotated from 1 to 13, the first one starting right after clade X2 in counterclockwise direction. Minimum, maximum, and average sequences percentage identity are displayed for each clade-to-clade comparison.

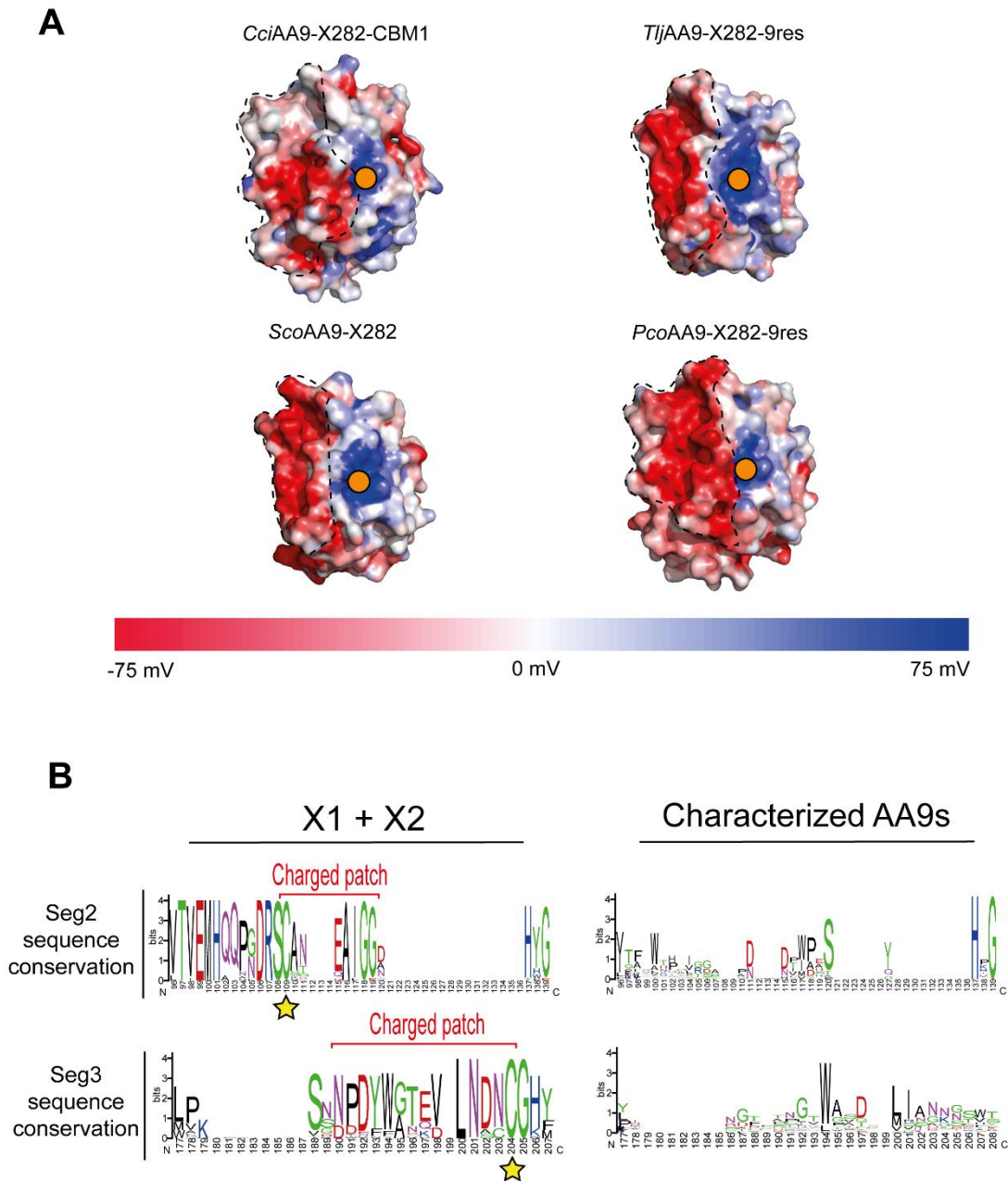


Fig. S3. Surface distribution of electrostatic potential (A) and presence of a conserved charged patch (B) in AA9-X282s. (A) Catalytic site view of four AA9-X282s (structural model predicted with AlphaFold2). Charge distributions were predicted using the ProtSol server³ and represented as color gradient on the structure. The conserved negatively charged patch is circled with a black dotted line and the histidine-brace position is marked by an orange dot. **(B)** Residues conservation comparison of segments seg2 and seg3 (as defined by Laurent et al.⁴) between clades X1 (20 sequences) and X2 (4 sequences) *versus* the characterized AA9s (39 sequences). Residues of the charged patch are framed in red bracket on the Weblogos and the two strictly conserved cysteines from the charged patch are marked by a yellow star.

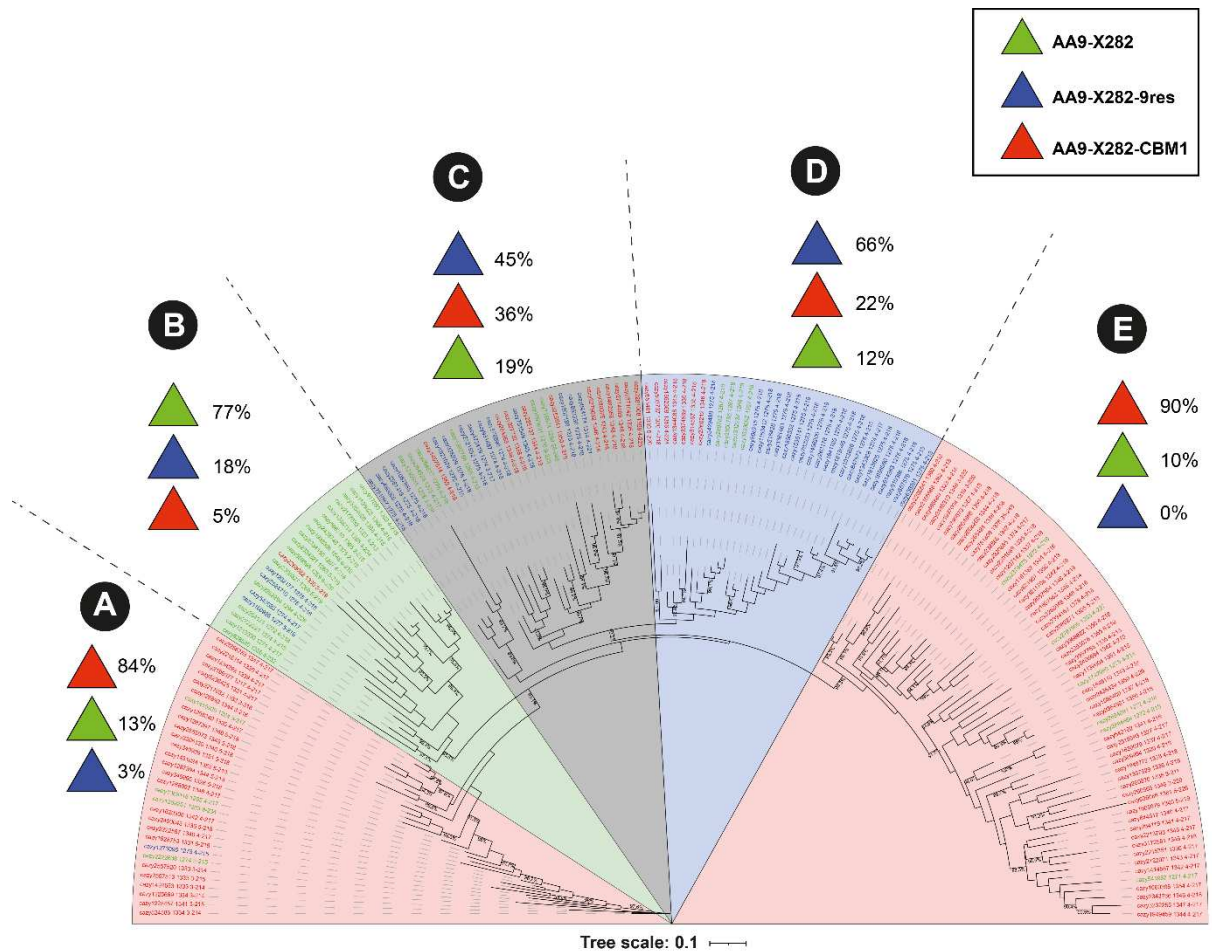


Fig. S4. Phylogeny and structural modularity of AA9-X282. Phylogenetic analysis of 174 AA9-X282 catalytic domains, showing 5 clades (A to E). For each clade, the associated proportions of the three AA9-X282 modularities are shown on the tree. Color code: AA9-X282 in green, AA9-X282-CBM1 in red and AA9-X282-9res in blue. The tree was generated with IQ-Tree (1000 Bootstraps). The protein IDs are CAZy IDs and the corresponding sequences are available upon request.

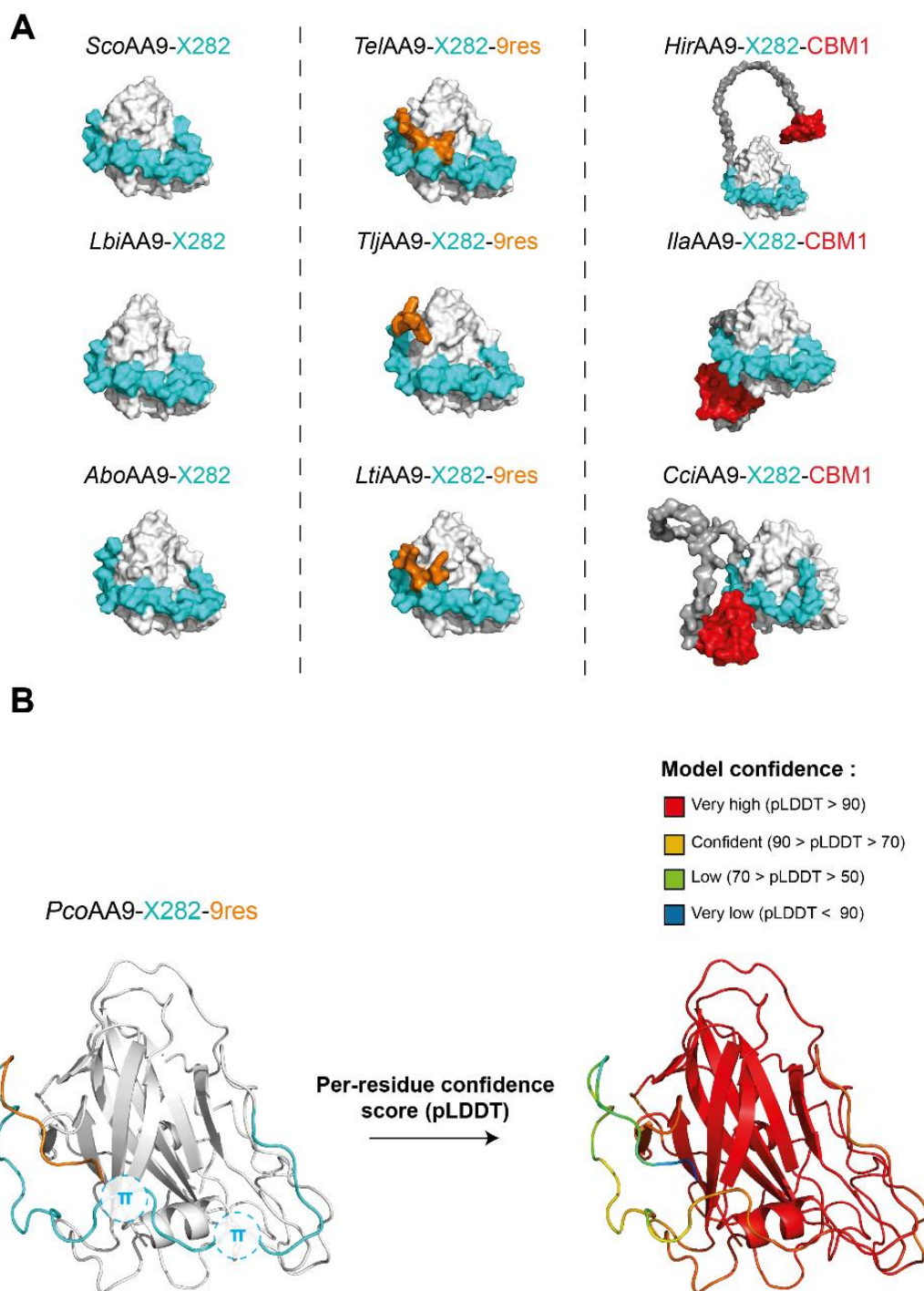


Fig. S5. Structure predictions of 10 AA9-X282s. (A) Structures, predicted with AlphaFold2⁵, for AA9-X282s from *Schizophyllum commune* (*Sco*), *Laccaria bicolor* (*Lbi*), *Armillaria boreal* (*Abo*); AA9-X282-9res from *Trametes elegans* (*Tel*), *Trametes ljubarskyi* (*Tlj*), *Lentinus tigrinus* (*Lti*); AA9-X282-CBM1 from *Heterobasidion irregulare* (*Hir*), *Irpex lacteus* (*Ila*) and *Coprinopsis cinerea* (*Cci*). The X282 (in cyan), the 9res motif (in orange), the linker (in dark grey) and the CBM1 (in red) are colored on the structures shown as surface. (B) Per-residue confidence score (pLDDT) of *Pco*AA9-X282-9res structure prediction provided by AlphaFold2⁵. The color code corresponding to the model confidence is displayed on the figure and the position of the two potential π -stacking interactions are indicated on *Pco*AA9-X282-9res structure prediction.

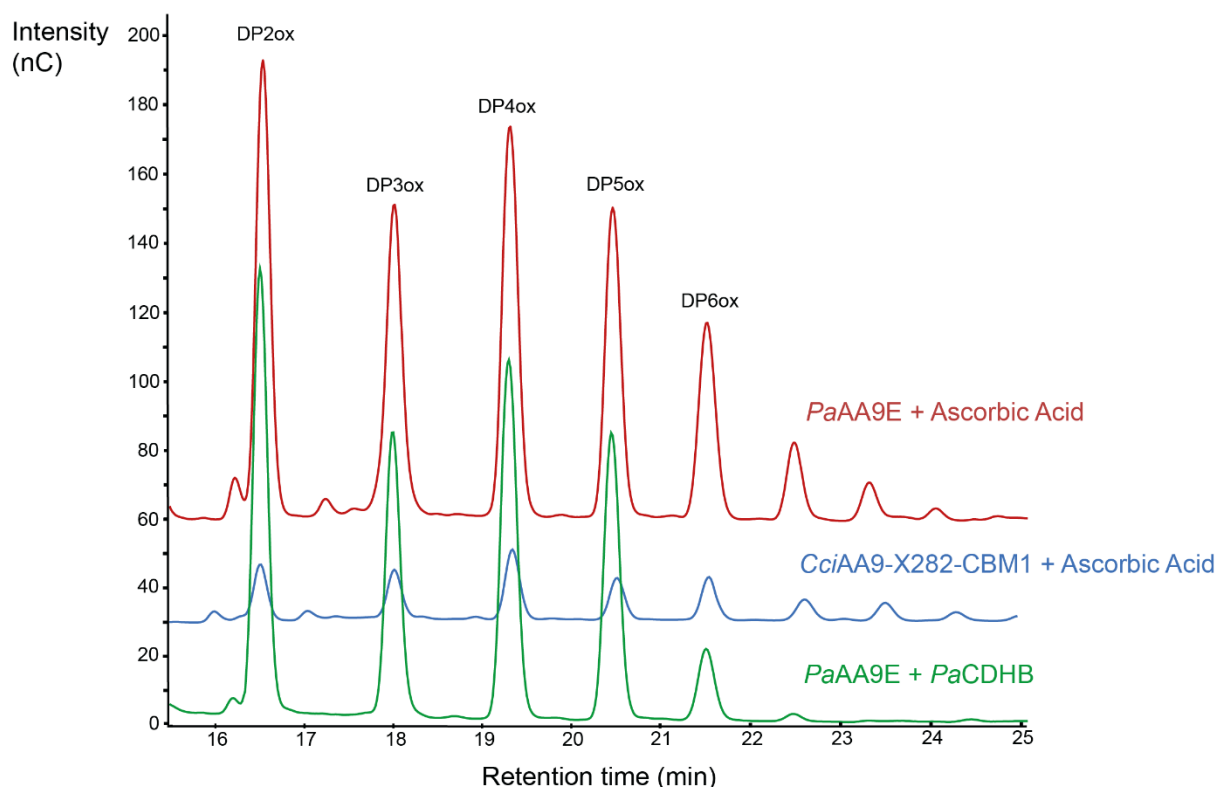
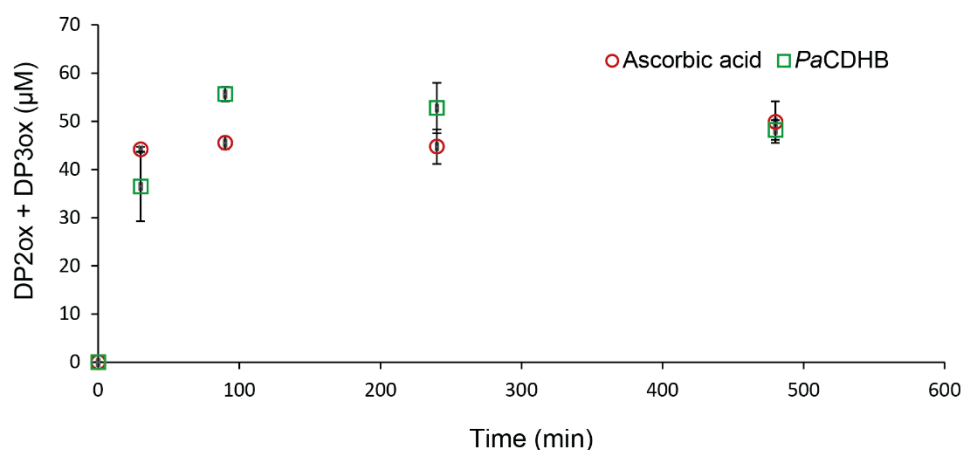
A**B**

Fig. S6. Comparative kinetic of *PaAA9E* reactions on PASC in the presence of *PaCDHB* or ascorbic acid. (A) Oxidized cello-oligosaccharides (DP2-DP6ox) released by *PaAA9E* (1 μM) from PASC (0.2%) after 90 min of reaction, in the presence of ascorbic acid (1 mM) or *PaCDHB* (1 μM). The oxidized products released by *CciAA9-X282-CBM1* (1 μM) after 24 h reaction in the presence ascorbic acid (1 mM) is also displayed for comparison. Reactions were carried out in sodium acetate buffer (50 mM, pH 5.2) and incubated in a Thermomixer (850 rpm, 30°C). **(B)** Time course kinetic of the released C1-oxidized soluble products (DP2ox and DP3ox) from the *PaAA9E* reactions described in (A). Data points show average values and error bars show standard deviations (n = 3 independent biological replicates).

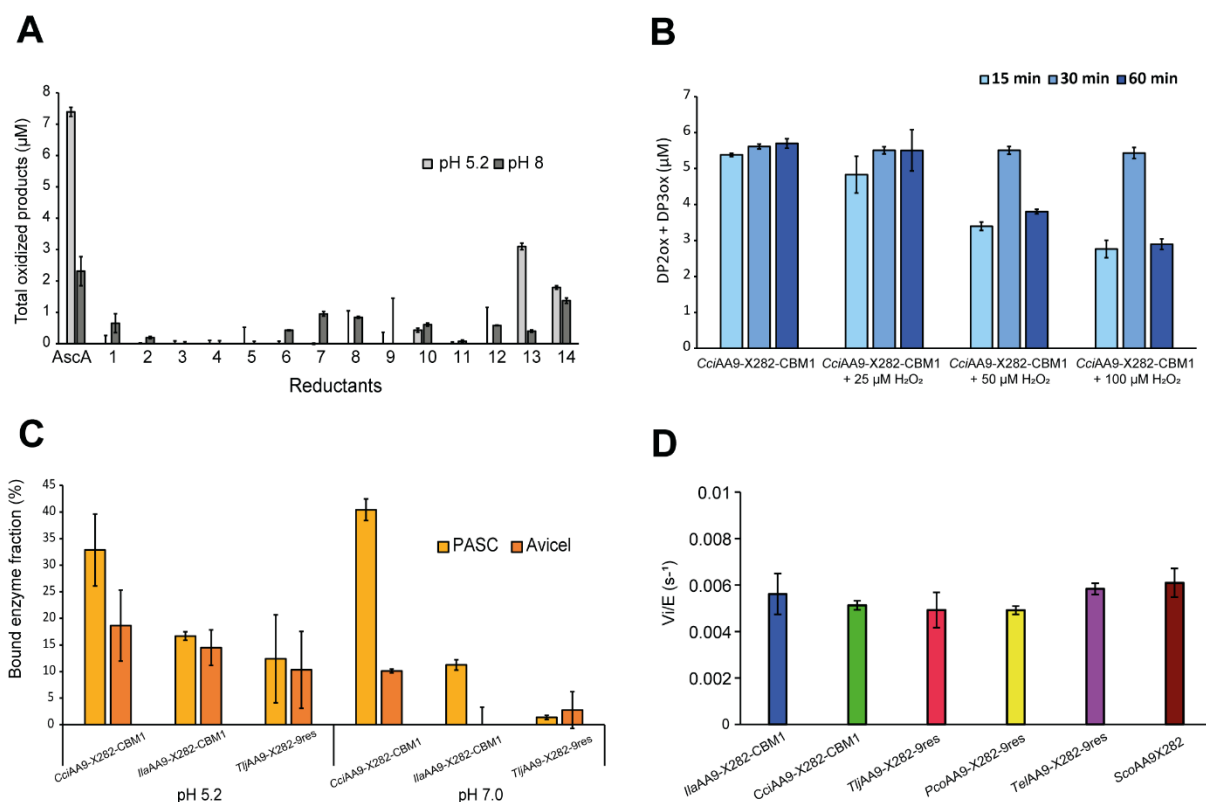


Fig. S7. Biochemical and binding assays of AA9-X282s. (A) C1-oxidized cello-oligosaccharides released from PASC by *llaAA9-X282-CBM1* (1 μM) in the presence of different lignin-derived compounds as reductants (see list with full names corresponding to numerical code in **Table S1**). (B) Effect of H₂O₂ complementation on the time-course release of oxidized products by *CciAA9-X282-CBM1* (1 μM) from PASC (0.2%), in the presence of AscA (1 mM). (C) Binding assay on PASC (0.2%) and Avicel (5 mg.mL⁻¹) for *CciAA9-X282-CBM1*, *llaAA9-X282-CBM1* and *TljAA9-X282-9res* (at 10 μM each). (D) Oxidase activity of the six AA9-X282s (1 μM) assayed with Amplex-red. Histograms show average values and error bars show standard deviations (n = 3 independent biological replicates).

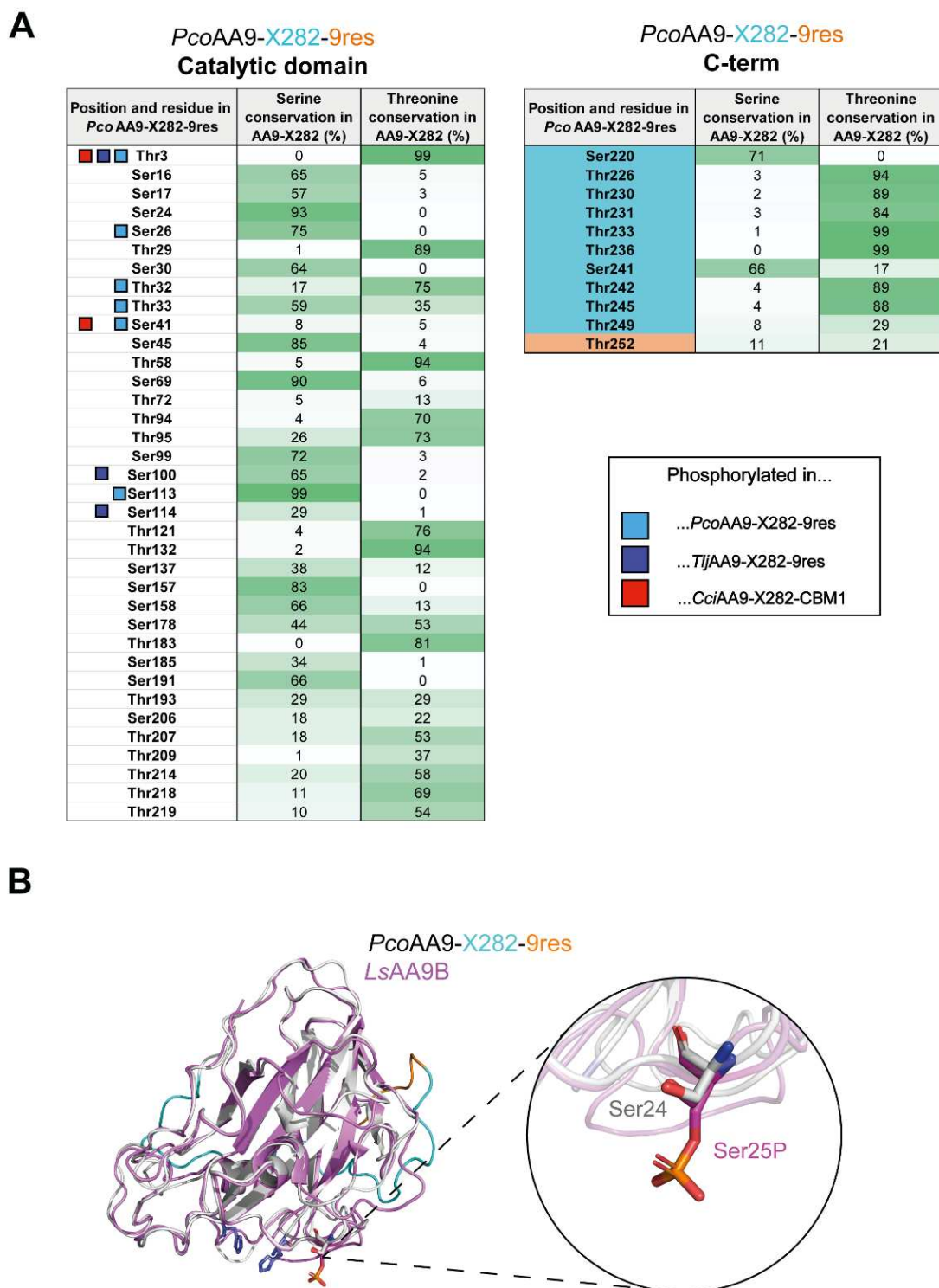


Figure S8. Conservation of surface exposed serines and threonines in AA9-X282. (A) *PcoAA9-X282-9res* serine and threonine conservation rate throughout 174 AA9-X282 sequences. Phosphorylated residues in *PcoAA9-X282-9res* and their structural equivalents in *TljAA9-X282-9res* or *CciAA9-X282-CBM1* are annotated by colored squares (note that the Ser41 phosphorylated in *CciAA9-X282-CBM1* had no Ser/Thr equivalent in *PcoAA9-X282-9res* and is thus not represented). **(B)** Structural alignment of *PcoAA9-X282-9res* (in white) with *LsAA9B* (in purple). A zoom-in on the protruding phosphorylated Ser25 in *LsAA9B* and its equivalent in *PcoAA9-X282-9res* is circled in black. The two catalytic histidines are colored in blue.

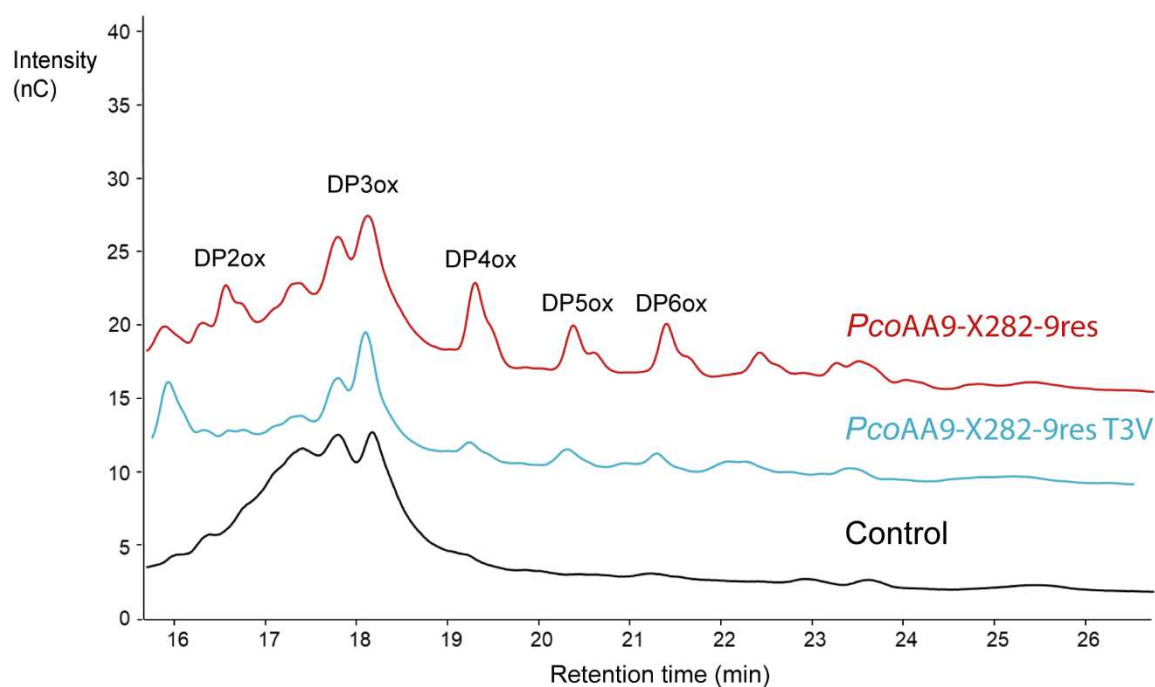


Fig. S9. Soluble oxidized products profile of *PcoAA9-X282-9res* and T3V variant. HPAEC-PAD chromatogram of soluble oxidized products released from PASC (0.2%) by *PcoAA9-X282-9res* (1 μ M) bearing either the original Thr at position 3 (*PcoAA9-X282-9res*, in red) or the T3V mutation (*PcoAA9-X282-9res T3V*, in blue), in the presence of ascorbic acid (1 mM). Reactions were incubated in sodium acetate buffer (50 mM, pH 5.2), during 8 h, under stirring (850 rpm) at 30°C. The control reaction was made by replacing the AA9 by CuSO₄ (1 μ M).

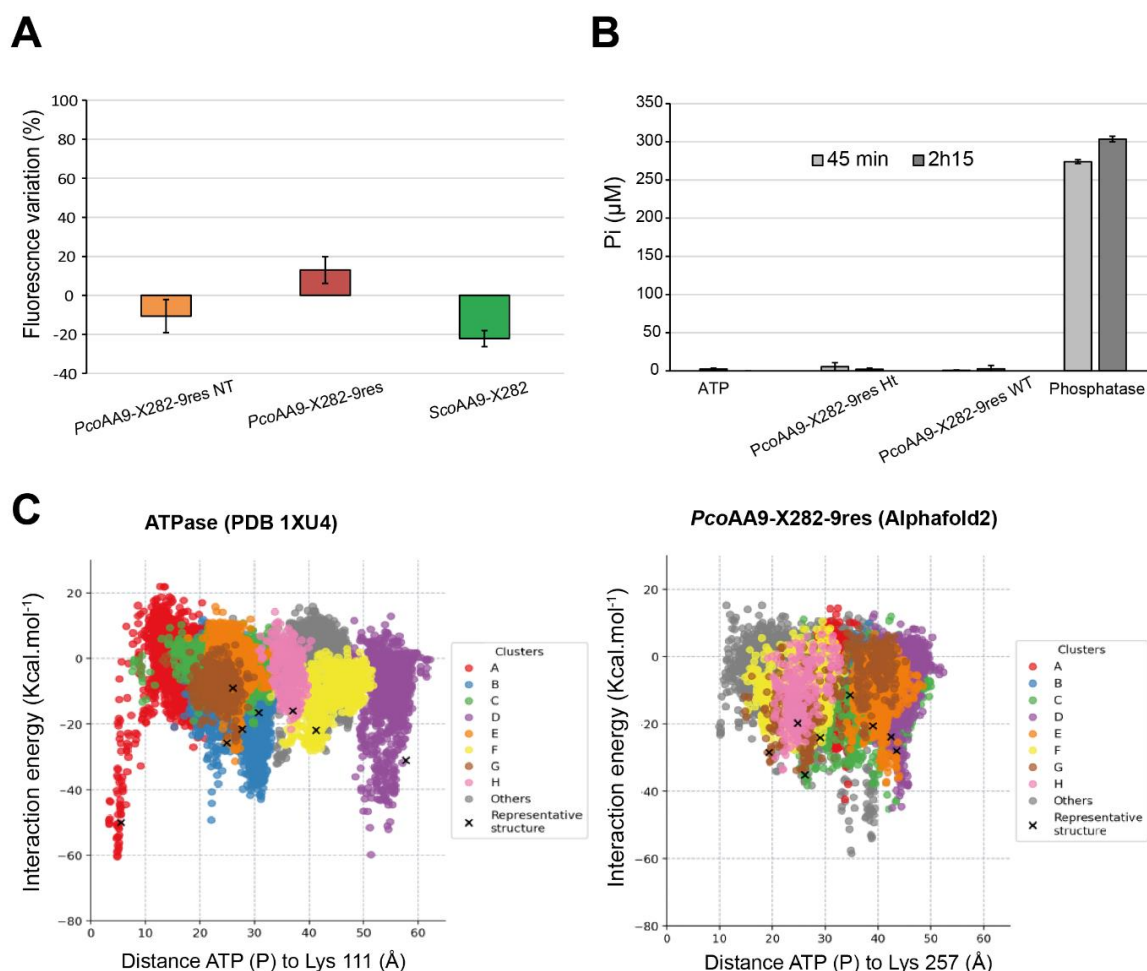


Fig. S10. ATP binding assays. (A) TNP-ATP assay: TNP-ATP (20 μM) was mixed with *PcoAA9-X282-9res* NT (in orange), *PcoAA9-X282-9res* (in red) or *ScoAA9-X282* (in green) (10 μM for each protein). After 5 min, the fluorescence emission was read at 540 nm upon excitation at 410 nm. Control experiments consisted in recording the fluorescence emission background signal of TNP-ATP and AA9 proteins alone. All binding assays were performed in Tris-HCl buffer (50 mM, pH 7.5). When a genuine ATP-binding protein binds to TNP-ATP, the fluorescence of the latter is expected to increase significantly, usually >2 -fold⁶. The variation in fluorescence (F) shown on the y-axis corresponds to the variation of $F(\text{protein} + \text{TNP-ATP})$ relative to the sum of $F(\text{protein})$ and $F(\text{TNP-ATP})$. $y = 0\%$ means that addition of protein to TNP-ATP has no effect on the fluorescence of the latter, reflecting thereby the absence of binding. (B) ATP hydrolysis assay: Released inorganic phosphate (Pi) in complex with the malachite green reagent was monitored at 640 nm. Reactions were performed by mixing AA9-X282 (1 μM) or commercial acidic phosphatase (100 nM) with ATP (200 μM). Reactions were performed in sodium acetate buffer (50 mM, pH 5.2) in the presence of MgCl_2 (100 μM). (C) Site finder simulation. The simulation was performed using an AlphaFold2 model of the *PcoAA9-X282-9res* to detect possible binding sites for ATP, and the results were compared to an ATPase (PDB: 1XU4) as the control. Each point is a different conformation of the binary complex between the ATP and the receptor. The distance between the γ -phosphate and the lysine of the "GXXGIGKA" consensus sequence, in the case of the *PcoAA9*, or the Walker A motif, in the case of the ATPase, is plotted against the interaction energies between the ligand and the protein. The colors show different clusters grouped by ligand RMSD as reported by PELE.

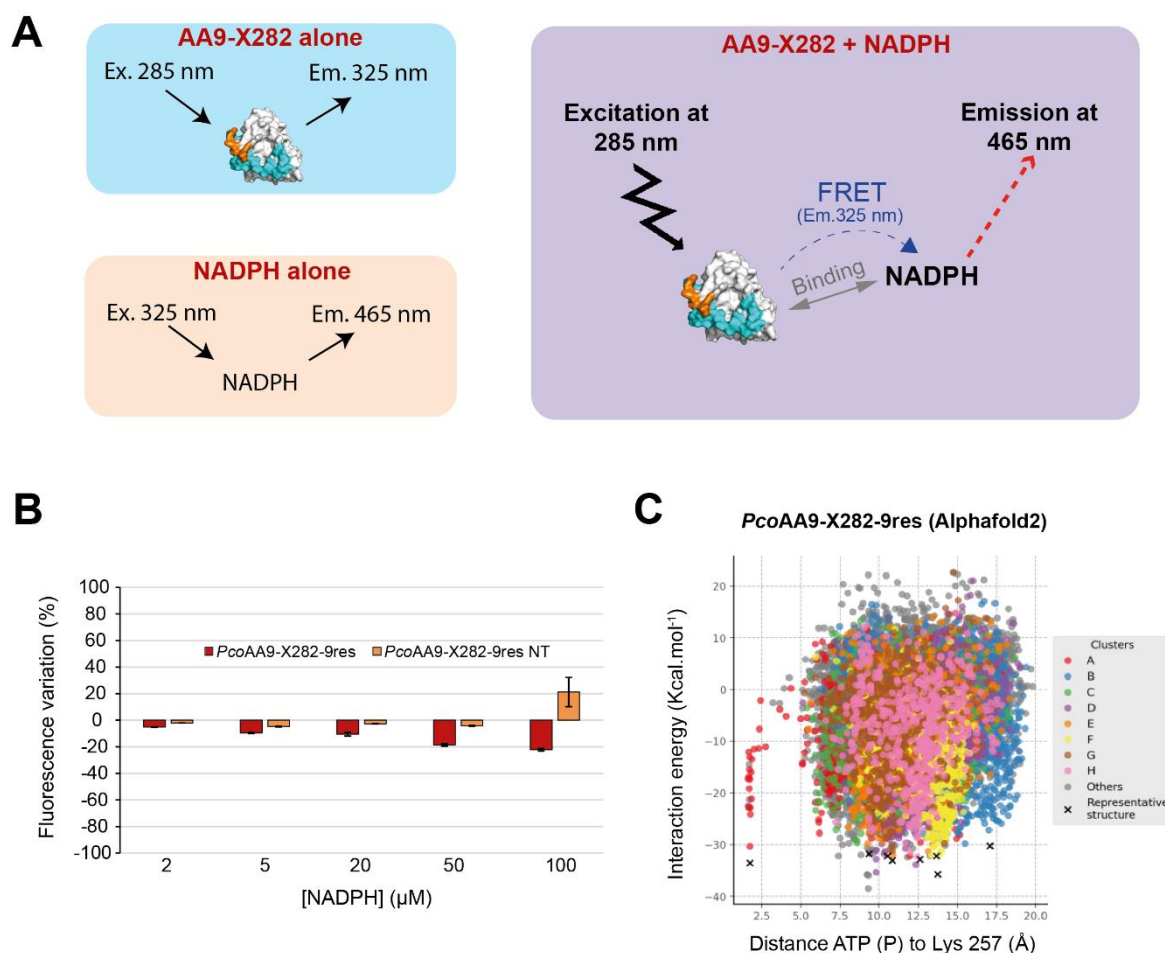


Fig. S11. NADPH binding assays. **(A)** Summary scheme of the method. The NADPH binding assay is based on the method described in Fjeld et al.⁷. When the protein is excited at 285 nm, its emission at 325 nm will be quenched by NADPH via a FRET mechanism if the NADPH is bound to the protein, leading to a specific final emission at 465 nm. **(B)** Evolution of the fluorescence emission (at 325 nm) upon excitation at 285 nm of the *apo*-PcoAA9-X282-9res (5 μM), with and without (“NT”) His-tag, in the presence of different concentrations of NADPH. The variation in fluorescence (F) shown on the y-axis corresponds to the variation of F(protein + NADPH) relative to the F(protein). **(C)** Site finder simulations. The Protein-NADPH interaction energy was plotted as a function of the distance between the protein (the Gly256, next to the Lys in the “GXXGIGKA” consensus sequence) and NADPH (one of the oxygens in the phosphodiester bond). The coloured clusters indicate different conformations of NADPH group by RMSD. Each plot point corresponds to a different binary complex obtained during the PELE site finder simulation. We note that for canonical NAD(P)H binding proteins, one may expect interaction energies in the range -100 to -140 kcal/mol).

Table S1. List of the screened substrates and reductants

Tested reductants	Number in Fig. S6A
Ascorbic acid	/
4-hydrobenzoic acid	1
<i>p</i> -coumaric acid	2
Vanillic acid	3
Vanillin	4
Isovanillin	5
Eugenol	6
4-ethyl-guaiacol	7
Ferulic acid	8
Homovanillyl alcohol	9
2,6-dimethoxyphenol	10
Syringaldehyde	11
Sinapic acid	12
2-methoxyhydroquinone	13
Caffeic acid	14

Tested substrates	Detected activity
PASC	Low
Avicel	Very low
Cello-oligosaccharides (DP2-DP6)	No activity
PASC/Xylan coupling	
Xyloglucan	
Xylan (from beechwood)	
Xylo-oligosaccharides	
Glucorono xylan	
Arabinoxylan	
Arabinan	
Glucomannan	
Galactomannan	
Pectic galactan	
Rhamnogalacturonan	
α -Chitin	
β -Chitin	

Full abbreviations list

Enzymes/Microorganisms

AA: Auxiliary activities
HRP: Horseradish peroxidase
Pco: *Pycnoporus cinnabarinus*
Tlj: *Trametes ljubarskyi*
Tel: *Trametes elegans*
Cci: *Coprinopsis cinerea*
Ila: *Irpepex lacteus*
Sco: *Schizophyllum commune*

Substrates/products

PASC: Phosphoric acid swollen cellulose
TNP-ATP: 2',3'-O-Trinitrophenyl-ATP
PCW: Plant cell wall
YPD: Yeast Extract–Peptone–Dextrose
BMGY: Buffered Glycerol- complex Medium
BMMY: Buffered Methanol- complex Medium

Methods

HPAEC-PAD: High-performance anion-exchange chromatography coupled with pulsed amperometric detection
MS/MS: Tandem mass spectrometry
UHPLC: Ultra High-Performance Liquid Chromatography
PELE: Protein energy landscape exploration
MSA: Multiple sequences alignment
MW: Molecular weight

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General discussion

I. LPMOs in a biorefinery context

I.1. Bottlenecks

I.1.1. In search of powerful fungal enzymes: our strategies and their limits

To develop increasingly effective enzyme cocktails, the choice of enzymes is obviously essential. In the case of cellulases, they have been the subject of numerous optimization studies. Discovered in 1950, the now well-known cellulases producer *T. reesei* has undergone 70 years of genetic engineering to further enhance the hydrolytic performance of its secretomes (Druzhinina and Kubicek 2017; Ferreira et al. 2014; Berrin et al. 2014). At the same time, our improved understanding of biomass degradation processes has allowed to identify key enzymes (such as Cel3A, Cel7A, Cel6B, ...) and engineer their performance through mutagenesis approaches (Berrin et al. 2014; Chokhawala et al. 2015). In comparison, the discovery of LPMOs and their implementation in industrial cocktails is very recent, and the approaches by which LPMOs are implemented in cocktails differs greatly from that of cellulases. So far, there is no such major LPMO-producing model strain, but rather a variety of wood-degrading organisms (especially saprotrophic fungi) with an extremely diverse number of genes encoding LPMOs. For example, the model ascomycete *T. reesei* only possesses 3 AA9s encoding genes, while in other saprotrophic basidiomycetes, like *Coprinellus angulatus*, one can find more than 60!

Among this diversity of enzymes, what strategies can we apply to find the most effective LPMOs in terms of boosting cellulases activity? In bacteria, the recognition, degradation, and transport of polysaccharides is often mediated by highly specific, co-localized protein systems. In some bacterial genomes, the genes encoding CAZymes can be organized as polysaccharide utilization loci (PULs) and are strictly co-regulated (Larsbrink et al. 2014; Terrapon et al. 2015; Grondin et al. 2017). Thus, it is possible to confidently find new substrate-specific CAZymes by analyzing their genomic environments. However, since fungi lack such PULs and considering that most of the industrially-attractive LPMOs (namely AA9) are found only in these organisms, other strategies have been developed to identify enzymes of interest.

A first strategy consists in selecting new LPMOs whose (i) sequence identity is close to other characterized enzymes, or (ii) directly select characterized LPMOs highly active on model substrates. This method has proved successful in numerous studies, but our results for *PaAA9s*, highly active on cellulose (Bennati-Granier et al. 2015), showed that high

activity on model substrates is not necessarily a guarantee of a strong boost effect when added to cellulolytic cocktails (**Chapter I**). The structural and/or catalytic features responsible for these differences in behavior on complex biomass and simple substrates needs to be studied in greater details to define key factors that could be used as benchmarks in the selection of future LPMOs with high industrial potential.

Another commonly used strategy is based on comparative -omics analysis. By selecting LPMOs specifically upregulated and/or secreted on cellulosic substrates and/or a particular biomass, numerous studies have characterized new enzymes capable of enhancing the activity of cellulolytic cocktails. However, this approach also has its limitations, as it proved unsuccessful in predicting the substrate specificity of AA9-X282, which were found upregulated on cellulose but appeared only poorly active on the latter.

Finally, I would like to mention that the advent of new powerful structure prediction tools such as AlphaFold2 (Jumper et al. 2021) and RoseTTAfold (Baek et al. 2021) has been instrumental in advancing my thesis (especially in **Chapter II**). I believe that while these tools will never completely replace crystallographic structures (which, for example, allow the detection of unpredictable modifications), they will become a driving force in our understanding of the structure/function of LPMOs. In addition, even more ambitious tools based on artificial intelligence, such as RFdiffusion (recently developed by the David Baker Lab (Watson et al. 2023)), have been developed to produce *de novo* proteins capable of binding a given substrate, and could well transform many areas of enzymatic biotechnology in future years.

For now, a most promising strategy could be to combine -omics structural and biochemical analyses to unravel the characteristics underlying the "boost" performance of previously/forthcoming characterized LPMOs. I believe that the efficiency of this "combination of methods" approach will only increase with years, nourished by the fundamental knowledge produced by the teams working on LPMOs structure/function, and the development of news bioinformatics tools. In all cases, even if one manages to identify an LPMO with strong industrial potential, the next challenge is to correctly control its activity in the biomass degradation conditions used in biorefinery.

I.1.2. LPMO priming in cellulytic cocktails

The LPMOs reaction lies on a fine balance between the availability of reducing agent, substrate, and co-substrate. As already developed in the introduction, the fate of H_2O_2 in the context of biomass degradation is uncertain, as it can be produced and consumed by numerous components present in reaction, mostly derived from lignin. In this part, I would like to discuss further the fate of LPMOs reduction in the context of biomass degradation by cellulytic cocktails.

The widely accepted starting point of any LPMO reactions consists in the reduction of the resting form, Cu(II), into its active form, Cu(I). As mentioned in the introduction, many electrons sources can serve as reductant LPMOs: organic compounds, reduced phenols, enzymatic partners or photo-catalysts (Cannella et al. 2012; Westereng et al. 2015; Kracher et al. 2016; Cannella et al. 2016; Bissaro et al. 2016; Kommedal et al. 2022; Monclaro et al. 2023). However, as shown in **Chapter I** for *PaAA9H* and in other studies, the addition of reducing agents is not always necessary to observe the boosting effect of LPMOs during biomass degradation (**Table 1** and **Fig. 3, Chapter I**). Hence, one may wonder how copper reduction is really achieved in the LPMO-containing enzymatic cocktails used in biorefinery.

First, at laboratory scale, the way reducing agents are added in such cocktails reactions depends largely on the nature of the substrate under study. While on model substrates (Avicel, PASC, ...) the addition of reducing agents is almost systematic, there is great variability in the literature when it comes to assessing the impact of LPMOs on biomass hydrolysis (**Table 1**). The most commonly used reductant is ascorbic acid, usually added in excess (1 or 2 mM), regardless of the reaction conditions (pH, type of substrate, substrate loading...) (**Table 1**). However, the relevance of using this reductant in such manner to activate the LPMO is questionable, since beyond its reducing role, ascorbic acid (and other reductants) also plays a significant role in the production of LPMO co-substrate in the form of H_2O_2 . Indeed, due to its high reactivity with O_2 and/or free metals (such as copper), ascorbic acid can rapidly produce significant quantities of H_2O_2 , leading to a high initial kinetic rate but also to rapid enzyme inactivation (Kuusk et al. 2019; Bissaro et al. 2017). Conveniently, it has been recently shown that gallic acid, in addition to being poorly reactive to free copper, can prime the LPMO activity more sustainably under pH conditions compatible with cellulases activity, making it a potentially better candidate than ascorbic acid when it comes to activating LPMOs in the enzymatic cocktails (Stepnov et al. 2021).

Interestingly, In these cases, the copper reduction could come from phenolic compounds (probably derived from lignin) already present in the biomass (Westereng et al.

2015; Frommhagen et al. 2017; Muraleedharan et al. 2018). Accordingly, it has already been shown that a positive linear correlation could be drawn between the biomass lignin content and the boosting effect of the LPMO from *Sporotrichum thermophile*, called StCel61a (Dimarogona et al. 2012). In the same trend, a similar correlation between the metal reducing activity of the soluble compounds derived from biomasses and cellulose hydrolysis was demonstrated by Hu et al. (Hu et al. 2014). The true nature of these phenolic compounds remains to be determined, but since they are derived from lignin, their nature and abundance should vary from one biomass to another.

Noteworthy, it has been shown that the production of oxidized lignin-derived phenolic compounds by a tyrosinase could enhance the cellulose oxidative cleavage performed by MtLPMO9B (Frommhagen et al. 2017). It is conceivable that similar oxidized phenols could be produced during the acidic pretreatment of the biomass in industrial processes. Nevertheless, the extraction level of this lignin derivatives is highly dependent on the pretreatment condition/type of biomass, and it is thus difficult to determine the amount of remaining phenolic compounds and their reducing power in cellulolytic cocktails reactions. To top it all off, these lignin derivatives are also known to act as cellulase inhibitors, and it is therefore reasonable to assume that their beneficial effect in enzymatic cocktails could only be achieved through a balance between a low cellulase inhibition and a sufficient LPMO activation.

In all cases, considering the conditions under which industrial hydrolysis is carried out (huge volumes and high enzymes/substrate loading), the external addition of reductant with the sole aim to prime LPMO activity would probably be unprofitable (depending on the benefit provided by the LPMO). Then, relying on the reducing power already present in the biomass remains an interesting alternative.

Table 1. Reducing agents used to prime LPMO activity in cellulolytic cocktails

“-” corresponds to a lack of exogenous reducing agent.

Exogenous reducing agent	pH	Substrate	Hydrolase(s)	Ref.
-	5	Avicel	Cellulase	(Kim et al. 2015)
Reduced glutathione (1.7 mM) or ascorbic acid (1 mM)	6.6	Avicel	Celluclast 1.5 L	(Westereng et al. 2011)
-	5	Acid-pretreated corn stover	Cellulases cocktail	(Harris et al. 2010)
Tris(2-carboxyethyl) phosphine or lignocellulosics methanol extract	5	Steam exploded wheat straw or alkaline treated spruce	Celluclast 1.5 L	(Dimarogona et al. 2012)
Gallic acid (10 mM)	4.8	Steam pretreated and organosolv pretreated corn stover, poplar and pine	Cellulases cocktail	(Hu et al. 2014)
-	5	Pretreated oak and kenaf	Xylanase or cellulase	(Jung et al. 2015)
L-cysteine (4 mM)	6	Retrograded corn starch	β -amylase	(Brander et al. 2020)
Ascorbic acid (1 mM)	5	Sorghum stover	Cellulases mix	(Jagadeeswaran et al. 2016)
Sodium azide (0.02%, w/v)	5	Steam-exploded sugarcane bagasse	Celluclast 1.5 L	(Gouvêa et al. 2019)
-	6	Steam-exploded sugarcane bagasse, corncob and rice straw	Celluclast 1.5 L	(Bernardi et al. 2020)
Ascorbic acid (1 mM)	4.8	Chlorite-treated miscanthus	Celluclast 1.5 L	(Chabbert et al. 2017)
-	5	Alkaline-pretreated wheat straw	Celluclast 1.5 L	(Sanhueza et al. 2018)
Ascorbic acid (1 mM)	5	Alkali and acid-pretreated rice straw and sugarcane bagasse	CellicCTec2	(Basotra et al. 2019)
-	4	Nitric acid-pretreated wheat straw	<i>Penicillium funiculosum</i> secretome	(Ogunyewo et al. 2020)
Ascorbic acid (2 mM)	6	Avicel	Cellulases cocktail	(Cheng et al. 2020)
Ascorbic acid (1 mM)	5	Alkaline-pretreated rice straw, corn stalk or sugarcane bagasse	Commercial cellulase	(Zhou et al. 2020)
Ascorbic acid (1 mM)	7.5	Steam-pretreated corn straw, ethylenediamine-pretreated Caragana korshinskii or rice straw	Commercial cellulase	(Guo et al. 2020)
Ascorbic acid (1 mM)	6	Alkaline-pretreated corn straw	Cellulase	(Li et al. 2022)
Ascorbic acid (2 mM)	5	Steam-pretreated spruce	Celluclast or Novozyme 188	(Caputo et al. 2023)

I.2. Enzymatic partners to fuel industrial LPMO reactions

As mentioned above and in the section of the introduction, the main issues related to the efficient implementation of LPMOs in cellulolytic cocktails stem directly from the control of H_2O_2 and reductant presence in industrial contexts. H_2O_2 can be created/consumed by various components present during biomass degradation, including potential reductants, whose initial amounts in reactions is hard to determine and highly dependent and the pretreatment method and type of biomass.

To address most of the above-mentioned problems associated with reducing agents and H_2O_2 fueling in LPMO-containing cocktails, the promising strategy set out in my thesis relied on using CDHs as a partner for LPMO. In addition to eliminating the need to add reductants, which are potential H_2O_2 producers and consumers, CDH can also supply H_2O_2 on its own at a relatively low rate (which is ideal for LPMOs) (Kracher et al. 2020; Bissaro et al. 2017).

Although we did not manage to observe any clear synergy between *PaCDHB* and *PaAA9s* in terms of improving the degradation yields of several biomasses (wheat straw, pine, miscanthus, poplar), the addition of *PaCDHB* alone surprisingly revealed itself beneficial. In a recent study, Barbosa and colleagues evaluated the hydrolytic power of an enzymatic cocktail similar to the one presented in **Chapter I**, using (i) *NcPMO-02916* or *TrCel61a* (both AA9 LPMOs) coupled with a CDH from *Neurospora crassa* (*NcCDHIIa*) as the oxidative system and (ii) an optimized endoglucanases cocktail as the hydrolytic system (Barbosa et al. 2020). They demonstrated that while the implementation of *NcPMO-02916* had no impact on the hydrolysis of pretreated sugarcane straw, the addition of the *TrCel61a/NcCDHIIa* couple allowed a 1.8-2.7-fold increase in the release of cello-oligosaccharides (i.e., from 22 to 60 mg/g of substrate at best). Noteworthy, the implementation of *NcCDHIIa* was mandatory to witness this boosting effect, but the exact contribution of the CDH and the AA9 has not been determined. To overcome this problem, an alternative already applied in other studies (Bennati-Granier et al. 2015; Østby et al. 2022) could be the use of a strictly C4-active LPMO to discriminate and quantify products originating from the CDH (C1ox) and the LPMO (C4ox). The reason for the absence of boosting effect in the *PaAA9s/PaCDHB* is unclear, but this observation, coupled with the fact that the *TrCel61a/NcCDHIIa* pair was the best boosting condition in Barbosa et al. (Barbosa et al. 2020), suggests that CDH/AA9 synergism could be enzyme dependent, and not necessarily optimal when originating from the same organism. Indeed, our intuitive approach of using a CDH/AA9 couple from the same organism was surprisingly, and

somehow disappointingly, not fruitful at least in our conditions.

One of the most interesting result presented in **Chapter I** remains the beneficial effect observed with the addition of *PaCDHB* alone, especially for the degradation of miscanthus. As already mentioned, the origin of this effect could be attributed to an alleviation of CBHI inhibition by cellobiose oxidation. However, contradictory effects have also been described in the literature, where the presence of C1-ox products was found to have an impeding effect on the kinetics of several CBHIs (Keller et al. 2021). This inhibition phenomenon seems to be enzyme specific and it is reasonable to assume that the proportions of enzymes in the cocktail can also play a key role.

Finally, it is worth noting that other enzymes have also proved to efficiently serve as H₂O₂ producers for LPMO, as shown by Østby and colleagues that managed to fuel the LPMO activity of Cellic CTec2 cocktail during the degradation of sulfite-pulped spruce (SPS) and steam-exploded birch (SEB) using a glucose oxidase from *Aspergillus niger* (*AnGOx*) as H₂O₂ supplier (Østby et al. 2022).

II. LPMOs: from biochemical characterization to biological function

II.1. AA9: a family more diverse than it seems

II.1.1. Diversity in AA9 LPMO substrate specificities

The first reports of cellulolytic activity for AA9 LPMOs (formerly classified as GH61) were made through synergy assays with other cellulases (Harris et al. 2010; Langston et al. 2011), and the first demonstration of cellulose oxidative cleavage through a metal-dependent mechanism was evidenced in mid-2011 (Quinlan et al. 2011). Since then, numerous studies have set out to characterize new AA9s, questioning the straightforward specificity of these enzymes for cellulose. While most AA9s are active on cellulose (amorphous and/or crystalline), the reported cellulose-active AA9 behaviour is not uniform as some members release oxidized products over relatively long kinetics (Bennati-Granier et al. 2015) while others display little or no activity, such as, among others, the six AA9-X282 characterized during this PhD thesis (**Chapter II**; (Reyre et al. 2023)). AA9s behaviour also differs in their substrate specificity, for example, NcLPMO9C is active on soluble cello-oligosaccharides (Isaksen et al. 2014; Bennati-Granier et al. 2015), while *PaAA9H*, *LsAA9A* or *CvAA9A* are capable of oxidizing hemicellulose components (xyloglucan and xylo-oligosaccharides) (Fanuel et al. 2017; Frandsen et al. 2021), these last two being even active on glycosidic bonds adjacent to mannosyl residues (Simmons et al. 2017).

No longer limited to crystalline cellulose, AA9 LPMOs are now known to target a wide range of PCW components. Thus, a first guess as to the reason for their multiplicity in the genomes of some fungal saprotrophs was that it could be related to their multiple substrates preferences, as it is the case for some GHs (e.g. GH5). Nonetheless, as shown in **Table 2** and **Fig. 1**, the 16 AA9s from *P. coccineus* (*PcoAA9s*) are not recruited in the same way during growth on cellulosic substrates (wheat straw, pine and aspen), some of them being hardly transcribed in any of the conditions, questioning their involvement in PCW degradation. Also, some AA9 members do not display any LPMO activity. Indeed, *LsAA9B* is devoid of any genuine LPMO catalytic site, having its first catalytic histidine substituted by an arginine (Frandsen et al. 2019) (see **Fig. 1**). Interestingly, this peculiar feature can also be found in many other AA9s (see cluster 63 in Lenfant et al. (Lenfant et al. 2017)), and seems to be a somewhat specific feature conserved within the *Agaricomycetes* fungal class (Frandsen et al. 2019)(**Fig. 1**).

II.1.2. Diversity in the active sites

AA9s possess some very well conserved features. Like all the other LPMO families reported so far, their core structure consists of a β -sandwich of two β -sheets (Tandrup et al. 2018). AA9s active site also includes several highly distinctive features, including a strictly conserved tyrosine and glutamine, key in the copper coordination sphere, and aromatic residues (tyrosines or phenylalanines) oriented parallel to the substrate binding site, known to be involved in π -stacking interactions with the carbohydrate polymers (Li et al. 2012; Frommhagen et al. 2016). However, despite these conserved features, the overall charge and topology of the AA9 active sites show a much broader diversity (Frandsen and Lo Leggio 2016; Vaaje-Kolstad et al. 2017). It is known that AA9 active site is shaped by variable loops (L2, L3, LS and LC), the L2 Loop showing the highest structural variability (Frandsen et al. 2016). As mentioned above, this diversity in the AA9 active site loops is known to be determinant in the AA9 regioselectivity and was also proposed to be correlated to substrate specificity adaptation (Zhou et al. 2019). In the case of AA9-X282, the L2 loop (also referred to as Seg1 in Laurent et al. (Laurent et al. 2019)) shows a very distinctive structure, which seems to be one of the determinants of their phylogenetic divergence from other AA9s (Laurent et al. 2019). Based on sequence homology, a similar Seg1 sequence and structure was also found in other uncharacterized orthologs in ascomycetes (not having the X282 module) with two notable conserved features whose functions remain to be unveiled: a disulfide bond involving two conserved cysteines and a negatively charged patch (**Fig. S3, Chapter II**).

The charged patches are key when it comes to associate enzymes with a substrate. When it comes to AA9s, one can observe significant differences in charge distribution at the surface of the substrate binding site. **Figure 1** provides a clear example of such diversity among predicted structures of AA9s from a single organism, namely *P. coccineus*. Previous studies have also reported the diversity of charged patches among AA9 and AA10 active sites, and when it comes to cellulose-active members, their substrate binding sites usually consist of a strip of positively charged (usually involving the histidine brace) or neutral residues (Zhou et al. 2019; Book AJ 2014). Such strip can indeed be generally observed in *PcoAA9s*, but their size and orientation seem to vary from one enzyme to another. The AA9 substrate-binding site is also generally surrounded by negatively charged patches ((Zhou et al. 2019) and **Fig. 1**), but the biological function behind the size and positioning of these patches is not yet well understood; these patches could be involved in the recognition of oligosaccharides, proteinaceous partner and/or substrates carrying positive charges.

Noteworthy, the presence of positive charges in PCWs is often associated with that of membrane proteins (Shomer et al. 2003). In the case of AA9-X282 their conserved negatively charged patch covers half of the enzymes face containing the copper site (see **Fig. S3, Chapter II**, (Reyre et al. 2023) and **Fig. 1**) which could partly explain their low binding/activity on cellulose and possibly their lack of synergy with CDH as its cytochrome is also negatively charged. Hence, searching for charge-compatible partners may be a promising strategy to discover potential partners for AA9-X282. Interestingly, one AA9 (ID 1368522) which is predicted to hold a positively charged module of unknown function appeared regulated in a very similar way to *PcoAA9-X282-9res* in Hage et al. and Couturier et al. studies (Couturier et al. 2015; Hage et al. 2021) (see top right-hand side of **Fig. 1** and **Table 2**). Although direct protein-protein interactions are not well documented for LPMOs, we can speculate that these intriguing AA9s could serve as a partner for *PcoAA9-X282-9res*.

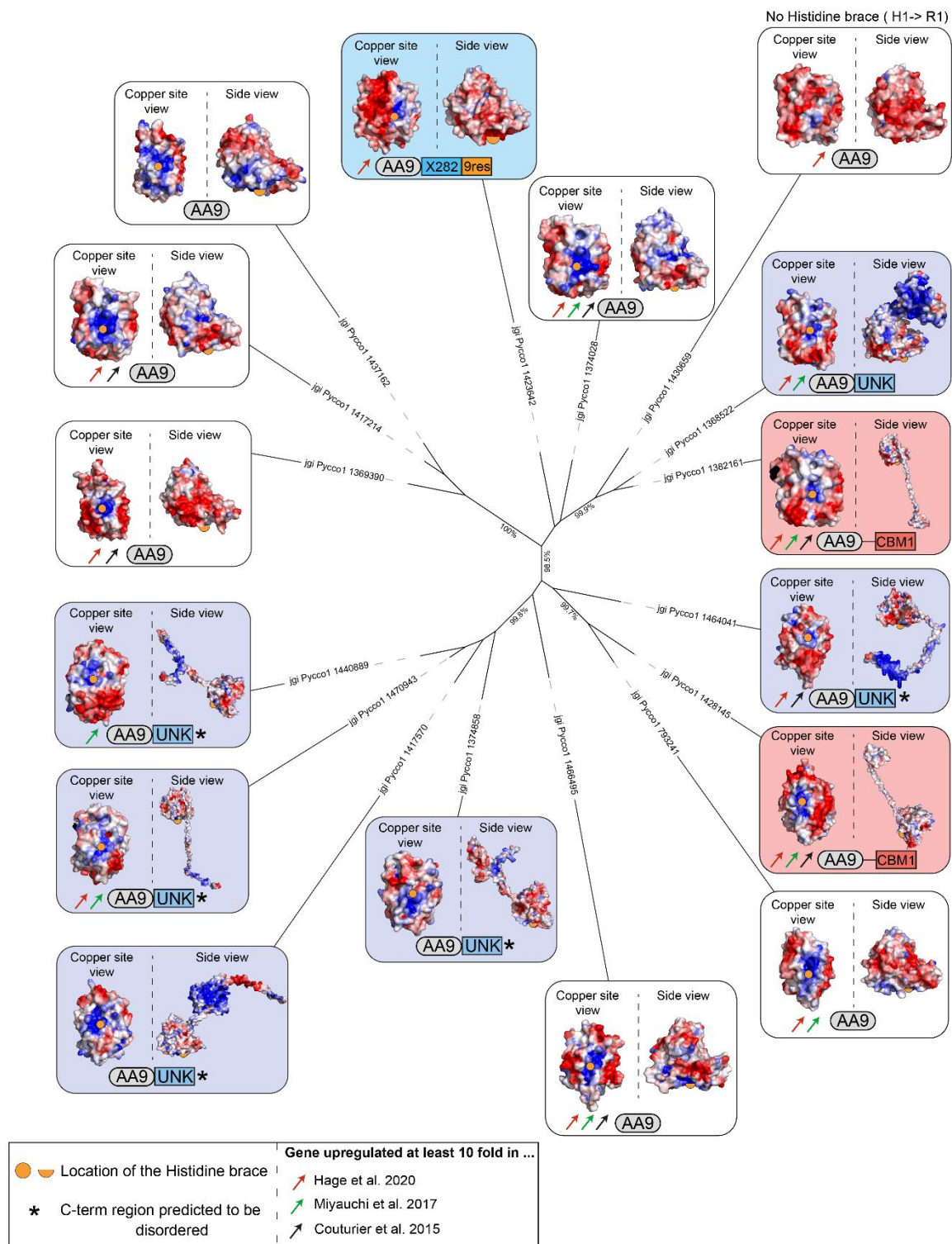


Fig. 1. Phylogenetic tree of all *PcoAA9*s associated with their predicted structure and surface charges. The tree was built with the catalytic domain only (i.e. without additional modules or C-term extension) of the 16 AA9 sequences of *P. coccineus*. The tree was inferred using IQTree (Nguyen et al. 2015)(1000 bootstraps; value displayed on tree as percentages) and visualized with Interactive Tree Of Life (ITOL) software (Letunic and Bork 2019). All AA9 branches are associated with their corresponding structure prediction (RoseTTAFold). The charges of surface of the models were predicted using ProtSol online server (Hebditch et al. 2017) and are represented by a colour gradient from red (negative charge) to blue (positive charge) on the proteins structure. The AA9s are associated with a background colour based on their modularity: unknown (blue), CBM1 (red), X282 (cyan) or no modularity (white). All the proteins models are oriented in the same way based on structure superimposition using PyMOL software. The orange dot shows the copper site.

II.1.3. Diversity in modularity

As mentioned in the introduction, AA9s harbor a wide variety of C-terminal modularities, most of which are annotated as modules of unknown function (referred as “X” domains in the CAZy database) (see **Fig. 12, Introduction**). The existence of these modules of unknown function is intriguing and could represent a significant potential for new discoveries in the field of LPMOs. For example, while the structure of X325 proteins revealed a fold very similar to that of LPMOs, they did not display any LPMO-type activity (Labourel et al. 2020).

In our case, the X282 domain display a short sequence of 30 residues and although it was initially suspected to be a new CBM, it seems to behave more like an extension rather than a folded module, surrounding the catalytic domain as a belt (**Fig. 2, Chapter II**). Despite nearly three years of research efforts, the function of this extension remains unclear, and the fact that it can be followed by different modularities (the “9res” extension or a linker terminated by a CBM1) makes things even more puzzling. Noteworthy, all the structural predictions confidently positioned the X282 extension above the conserved negatively charged patch mentioned above. Of note, the part of the X282 extension covering the charged patch is also predicted as negatively charged. Thus, one hypothesis as to the role of the X282 module could be to expand the negatively charged patch of this peculiar AA9, whose function still needs to be elucidated.

In addition to CBMs and “X” modules of unknown function, the C-terminus of AA9s can also contains uncharacterized domains without any annotations Here, to get further insights into this C-term diversity, I analyzed their phylogenetic distribution in all *PcoAA9s* (**Fig. 1**). Besides *PcoAA9-X282-res*, six other modularities of unknown function (not annotated as “X” module) are found among *PcoAA9s*, accounting for 44% of *PcoAA9s*. A blast search against the PFAM database failed to associate them with any known domains, and, among them, five were predicted to be intrinsically disordered C-terminal regions (dCTRs) (**Fig. 1**). dCTRs are characterized by their lack of tertiary structure, and the multiplicity of dCTRs in LPMOs has recently been highlighted by K. Tamburrini and colleagues (Tamburrini et al. 2021). The function of these dCTRs in AA9s and other LPMO families, and their potential implications in biomass degradation processes, remain to be determined. Of note, the C-terminal domain of one *PcoAA9* (ID:1368522) is not predicted to be disordered, and does not correspond to any known domain (**Fig. 1**).

I wish to underscore that the phylogenetic tree of *PcoAA9s* shown in **Fig. 1** is based on the catalytic domains of AA9s. This representation shows that the catalytic domains do

not necessarily cluster based on the C-terminal classification proposed here (CBM1, unknown, or nothing). An exception is the clade in the lower left corner of the tree, which seems to group only uncharacterized C-terminal modularities, all of which are predicted to be disordered.

To provide new insights as to the potential involvement of *PcoAA9s* with such uncharacterized C-terminal regions during biomass degradation, I also parsed transcriptomics data of *P. coccineus* grown on three different biomasses (wheat straw, pine and aspen) reported in three independent studies (Couturier et al. 2015; Miyauchi et al. 2017; Hage and Rosso 2021). Very interestingly, the top upregulated *PcoAA9s* on all biomass are generally the ones holding a CBM1 or just the AA9 domain. This is notably very clear after 3 days of growth for studies done by Couturier et al. (Couturier et al. 2015) and Hage et al. (Hage et al. 2021), which show very similar transcription rates. While at day 3, some discrepancies in transcription levels should be noted between the study of Miyauchi et al. and the two other ones), it appears clear that at day 7, all *PcoAA9s* holding a modularity of unknown function are less upregulated in all growth conditions compared to their transcriptional values at day 3 (**Table 2**). Interestingly, the *PcoAA9* homolog to *LsAA9B* (ID: 1430659), also lacking the histidine brace, appeared to be significantly upregulated after 7 days of growth.

Finally, I would like to point out that this meta-analysis of transcriptomics data, performed after the publication of **Chapter II**, provides a new vision of the regulation levels of AA9-X282s. Indeed, the initial selection of AA9-X282s was made based on their up-regulation on cellulosic substrate and co-regulation with cellulases reported by Hage et al, highlighting them as potentially interesting in an industrial context. However, when looking at the broader set of data from the 3 studies mentioned above, it appears that the upregulation of *PcoAA9-X282-9res*, no longer appears that clear and is actually significantly lower than many other *PcoAA9s*.

Table. 2. Summary of three *P. coccineus* transcriptomic analyses. Data were retrieved from studies conducted by Couturier et al. (black “C” (Couturier et al. 2015)), Miyauchi et al. (green “M” (Miyauchi et al. 2017)), and Hage et al. (red “H” (Hage et al. 2021)). The background of protein ID is colored based on their modularity: unknown (blue), CBM1 (red), X282 (cyan) or no modularity (white).

JGI protein ID (<i>Pycnoporus coccineus</i> CIRM-BRFM 310 v1.0)	Study	Foldchange vs maltose after 3 days of growth			Foldchange vs maltose after 7 days of growth		
		Wheat straw	Pine	Aspen	Wheat straw	Pine	Aspen
1430659 (H1->R1)	C		n.d	n.d			
	M	0	0	3	410	66	209
	H	5	7	56			
1440889	C		2	1			
	M	67	107	54	1	1	1
	H	1	2	1			
1417570	C		3	1			
	M	0	0	0	2	0	2
	H	6	3	2			
1374858	C		3	1			
	M	1	1	0	1	1	1
	H	3	2	2			
1464041	C		22	7			
	M	3	7	2	8	14	7
	H	7	18	7			
1368522	C		1	5			
	M	25	3	7	28	0	8
	H	17	2	5			
1470943	C		6	3			
	M	42	16	11	1	1	1
	H	10	4	3			
1423642 (X282)	C		1	7			
	M	1	0	0	3	1	2
	H	15	2	8			
1437162	C		1	7			
	M	0	0	0	0	0	0
	H	2	2	2			
793241	C		9	1			
	M	11	8	5	284	8	35
	H	16	12	9			
1374028	C		11	16			
	M	148	32	47	565	33	131
	H	83	16	29			
1369390	C		25	35			
	M	1	1	1	88	14	10
	H	14	25	42			
1466495	C		133	25			
	M	24	6	1	202	26	56
	H	1342	308	55			
1417214	C		12	53			
	M	8	1	3	547	7	132
	H	125	16	90			
1382161	C		16	39			
	M	7	5	11	446	23	85
	H	22	16	48			
1428145	C		12	36			
	M	149	59	134	181	18	31
	H	30	12	42			

II.2. The role of post-translational modifications in LPMOs

II.2.1. Methylation and glycosylation

Post-translational modifications (PTMs) are covalent processing events that modify the properties of a protein by adding a moiety, such as acetyl, phosphoryl, glycosyl and methyl group, to one or more amino acids (Ramazi and Zahiri 2021). PTMs play a key role in numerous biological processes by significantly affecting the function, structure, dynamics and/or recognition of proteins (Audagnotto and Dal Peraro 2017). For LPMOs, the best described PTMs are (i) the methylation of the N-terminal histidine and (ii) glycosylations, which consist of the covalent attachment of carbohydrate chains to the protein surface and/or linker.

In nature, the methylation of the N-terminal catalytic histidine LPMOs is known to occur only for fungal LPMOs and LPMO-like proteins (Navarro et al. 2014; Filiatrault-Chastel et al. 2019; Labourel et al. 2020; Batth et al. 2023). In 2018, Petrovic and colleagues investigated the role of this PTM on various aspect of the catalysis of the AA9A from *Thermoascus aurantiacus* (*TaAA9A*) and revealed that it had no impact on (i) the redox potential of the histidine brace, (ii) the ability to activate molecular oxygen and (iii) the substrate/cleavage specificity (Petrović et al. 2018). The only difference displayed by the methylated form of *TaAA9A* was its improved redox stability, possibly protecting the catalytic copper from oxidative damage caused by excess of H_2O_2 . A recent computational study supporting this hypothesis suggested that methylation of the histidine would increase the energy barrier of His oxidation by a reactive intermediate, contributing thereby to prevent autooxidation (Torbjörnsson et al. 2023). To date, most LPMOs have been characterized without a methylated N-terminal histidine due to their heterologous production in the yeast *P. pastoris*, and since H_2O_2 reactivity is a key factor in LPMO kinetics, it would be interesting to evaluate to what extent the moderate impact of this PTM on *TaAA9A* catalytic properties can be generalized to other LPMOs.

As to glycosylations, two main types can be found in LPMOs: N-glycosylation, linked to the enzyme via nitrogen groups of asparagine residues, and O-glycosylations, linked to the hydroxyl groups of serine, threonine or tyrosine residues (Spiro 2002). In nature or *in vitro*, proteins secreted by fungal hosts (including *Pichia pastoris*) are usually glycosylated in variable proportions, and LPMOs are no exception. These glycosylations are known to play an important role in protein secretion, stability and folding, and in the case of LPMOs,

it has also been suggested that certain *N*-glycosylations close to the active site may enhance substrate binding (Li et al. 2012; Taylor et al. 2012). As for *O*-glycosylations, they are mainly found in linkers, which are often rich in serine/threonine and are known to play a protective role against proteolysis (Payne et al. 2013; Amore et al. 2017; Gaber et al. 2020).

II.2.2. Discussing the role of phosphorylation in AA9 LPMOs

The last PTMs reported in LPMOs are phosphorylations, which are not only the least documented, but arguably the most intriguing. Phosphorylation of proteins are usually mediated by protein kinases, which transfer a phosphoryl group from ATP onto the serine/threonine or tyrosine side chains of a target protein (Adams 2001). These modifications were confidently observed by proteomic analyses in *PcoAA9*-X282-9res, *TljAA9*-X282-9res and *CciAA9*-X282-CBM1 to varying degrees (**Chapter II** (Reyre et al. 2023)). Noteworthy, the peptides containing the X282 sequence resulting from AA9-X282 proteolysis could not be analyzed as they were too large for the method used (nano LC-MS/MS). Given the high amount of serine and threonine in the X282 extension, it could also carry several undetected phosphorylated residues. To answer these questions, the next step in the characterization of AA9-X282 would be to obtain a crystallographic structure to see whether or not the extension is phosphorylated. An AA9 structure harboring a phosphorylated residue has already been obtained for *LsAA9B*, the above-mentioned AA9 from the Polyporale *Lentinus similis*, which lacks the histidine brace (Frandsen et al. 2019). Since *LsAA9B* and AA9-X282s could display divergent function, one may wonder the importance of phosphorylations in these atypical AA9s.

Looking at phosphorylated residues is not part of the standard LPMO characterization process, and it is possible that some of the hitherto characterized AA9s could also carry this unusual PTM. **Figure 2** reports the number of serines and threonines found at the surface of all *PcoAA9*s, as well as four characterized AA9s (*CvAA9A*, *LsAA9A*, *NcLPMO9C* and *AfAA9A*) for which significant activity on cellulose has been reported (Borisova et al. 2015; Frandsen et al. 2016; Simmons et al. 2017; Velasco et al. 2021). Interestingly, although the position of serines and threonines in the sequence alignments is not clearly conserved, it appears that almost all of these residues are always located at the surface of the AA9 domain (**Fig. 2**). Therefore, it would be interesting to analyze other AA9s to verify whether such phosphorylations are present and better understand their potential function (see below).

With the question "is it phosphorylated?" comes the questions "who phosphorylates?", "how?", and "where?". In nature, phosphorylation phenomena are mostly known to be mediated by kinases in an intracellular environment. However, there are strong indications that AA9-X282s (like all other LPMOs) are secreted, such as (i) the presence and conservation of a confidently predicted signal peptide, and (ii) the reported presence of certain AA9-X282 (such as *PcoAA9-X282-9res*) in previously published secretomic studies (Miyachi et al. 2017).

If the phosphorylation of secreted proteins is poorly described in the literature, so are their related mechanisms. Some kinases are known to be located in the Golgi apparatus and could therefore be potential candidates for the phosphorylation of AA9-X282 during their secretion process (Capasso et al. 1989). On the other hand, secretion of kinases has also been reported, such as for the human kinase Fam20C found to be active in the extracellular environment in the presence of sufficient amount of ATP (Tagliabracci et al. 2015). However, evidence for such extracellularly active kinase in fungi is still lacking. It is currently difficult to determine the exact enzymatic and spatial origin of the phosphorylations found in *LsAA9B* and AA9-X282s. Nevertheless, a promising strategy to answer this question could be to search for kinases co-expressed with these AA9s in previously published -omics data and then, unless the protein is phosphorylated intracellularly, refine the search by looking for potential peptide signals in the identified fungal kinases.

In these last, very speculative sections, I would like to discuss the potential biological role of phosphorylation in AA9s. Firstly, it is important to remember that the most documented role of phosphorylation in biology is the regulation of intracellular metabolic pathways. For example, a broad range of transporters can be reversibly activated/inactivated via kinase-mediated phosphorylation (Hanson and Whiteheart 2005). Drawing a parallel of this function in the context of secreted AA9, one may hypothesize that these phosphorylations could serve as a mean to reversibly control their activity by altering their affinity for the substrate and/or copper. However, based on our observations, mutation of the phosphorylated Thr3, the closest residue to the catalytic site of *PcoAA9-X282-9res*, into a non-phosphorylatable residue (T3V) appeared to have no effect on LPMO catalysis (**Fig. S9, Chapter II**).

From an extracellular point of view, the role of phosphorylation is little known and has only been documented in contexts far from the biological context of my PhD project. For

example, the most established role of extracellular phosphorylation relies on the formation of mineralized tissues such as teeth and bones (Redegeld et al. 1999). Hence, much remains to be done to elucidate the function of phosphorylations in AA9s. Since the extracellular context of PCW degradation can be hostile to enzymes (high levels of ROS, proteases...), one could also hypothesize a protective/stability function for this PTM, in the same way as for N-terminal methylation or glycosylations. Finally, the possibility that phosphorylation could allow interactions with other phosphate-interacting proteins for signaling purposes cannot be ruled out either.

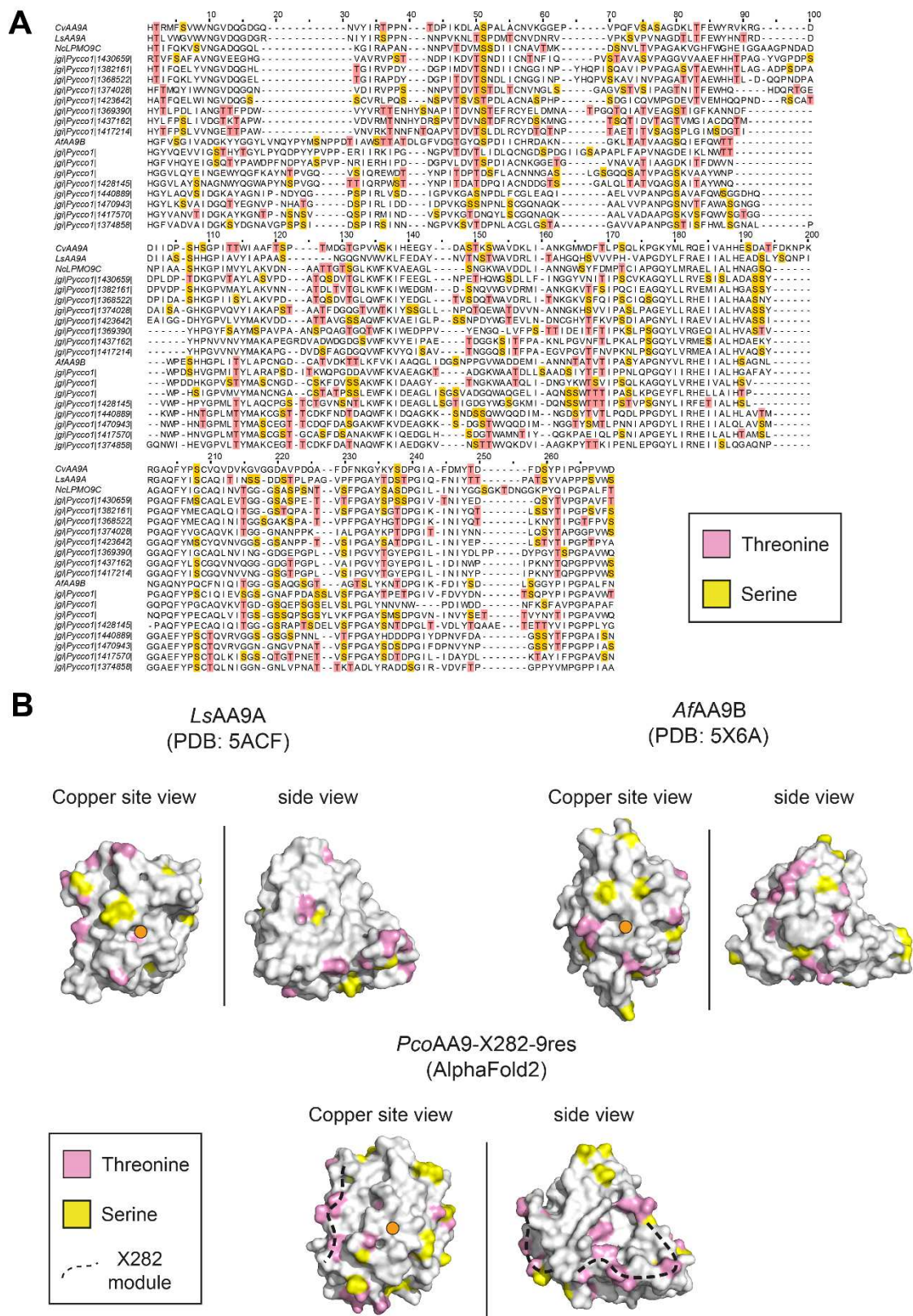


Fig. 2. Illustration of the serines and threonines found at the surface of AA9s. (A) Multiple sequence alignment of 21 AA9 LPMO (All *PcoAA9*s, *NcLPMO9C*, *LsAA9A*, *CvAA9A*, *AfAA9B*). Threonine and serine residues are colored if found at the surface of the protein. (B) Structural analysis of *LsAA9A*, *AfAA9A* and *PcoAA9-X282-9res*. Residues are colored if found at the surface of the protein. The orange dot shows the copper site.

II.2.3. Further insights into potential phosphate containing partner/substrates for AA9-X282

In addition to the unexpected presence of phosphorylations, the occurrence of "P-loop-like" motifs (also referred to as "9res extension" in **Chapter II**, (Reyre et al. 2023)) in the C-terminus of some AA9-X282s was intriguing. Many questions and hypotheses could be raised based on this observation, and I would like to discuss some of them in this, once again, very speculative section.

As a reminder, the 9res extension is very similar to the Walker A motif, well-known feature of ATP binding proteins, whose consensus sequence is GxxxxGK(S/T) (where "x" is any amino acid). Noteworthy, while Walker A is essential for ATP binding, its hydrolysis requires another key motif: the Walker B motif, the consensus sequence of which is "hhhhDE" (where "h" is a hydrophobic residue). In short, several hydrophobic residues form contacts with the nucleotide, the aspartate residue coordinates a Mg^{2+} ion, which is also required for ATP hydrolysis, and the glutamate residue is thought to activate a water molecule for the hydrolysis reaction (Hanson et Whiteheart 2005). Interestingly, a motif that closely resembles a Walker B is found conserved on the surface of AA9-X282 and is predicted to face the Walker A motif in the structure predictions (**Fig. 3**). The presence of this Walker B-like motif seems to be highly correlated to that of the 9res extension (i.e. walker A-like motif) (**Fig. 3**). While the presence of a Walker A-like motif facing a Walker B motif suggested that AA9-X282 could bind and possibly hydrolyze ATP, we did not observe neither binding nor hydrolysis of ATP by *Pco*AA9-X282-9res under our experimental conditions. This could be explained by the fact that: (i) the 9res extension is located at the C-terminus of the protein and does not seem very structurally constrained (as suggested by the low prediction confidence score provided by Alphafold2 for the 9res extension, see **Fig. S5**, **Chapter II**), which is usually the case in classical ATPases to ensure the proper binding to ATP, and/or (ii) the 9res extension lack the key Serine/Threonine residue (replaced by a conserved alanine) and therefore cannot correctly coordinate the catalytic Mg^{2+} as well as the β and γ phosphates of ATP.

Based on this observation, we can speculate that the P-loop-like motifs of AA9-X282 may recognize alternative substrates, such as other phosphate-bearing molecules or shorter versions of ATP, such as cAMP. The latter molecule plays a key role in various signaling pathways and many molecular processes in plants, including sensing and response to biotic and abiotic environmental stresses (Bhadra et al. 2021). Interestingly, the secretion of cAMP in extracellular vesicles have been observed in eucaryotes and bacteria, although its

biological role remains unclear and has not been observed in fungi yet. On the other hand, there are increasing evidences as to the function of extracellular ATP, eATP, showing its involvement in plant response to biotic and abiotic stresses and its recognition by plant immune receptors (Cho et al. 2022; Sowders and Tanaka 2023). If we speculate that fungal AA9-X282s could be able to hydrolyze or bind to eATP, or compounds thereof, it could be conceivable that the 9res extension would compete/interact with plant immune receptors, therefore affecting downstream signaling. Along this line, some nucleotide-binding domain leucine-rich repeat (NLR) proteins (which play a central role in the innate immune systems of plants) of *Arabidopsis lyrata* were reported to possess a P-loop-like motif identical to the one of AA9-X282s, with the sequence of GxxxxGKA (Bonardi et al. 2012). Interestingly, the authors hypothesized that these proteins could act as a scaffold, interacting with other partners featuring functional canonical P-loops. With this set of clues, I think that future studies should attempt to elucidate the role of these puzzling AA9-X282-9res, which might be related to the plant-fungus dialog and could broaden our horizon of the place of phosphate in extracellular environment as well as the biological functions of LPMOs. Ultimately, it would also be interesting to see whether the presence of these motifs could also be observed in other CAZymes.

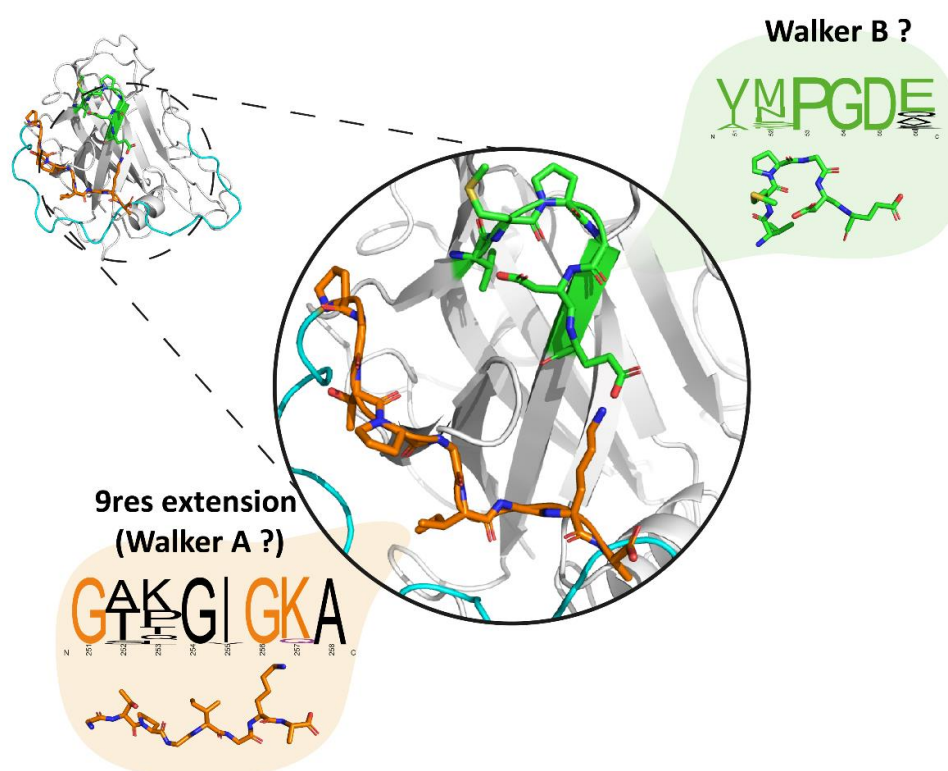


Fig. 3. Zoom-in view of the Walker A and B-like motifs found at the surface of *PcoAA9-X282-9res*. The 9res residue extension and the potential Walker B-like motif are colored in orange and green respectively.

General conclusion and outlook

In a world where the depletion of our natural resources is at the root of the greatest environmental crisis our planet has ever known, it is vital to develop sustainable solutions. The biorefinery concept is a promising alternative, often based on the transformation of carbohydrates, essential building blocks used in many applications (bioenergy, pharmaceuticals, cosmetics, ...). Since plant biomass is mainly composed of sugars, it represents a goldmine for scientists aiming at developing biotechnological concepts. However, compared to fungi, which have been able to thrive on this resource for millions of years, we are still very poor miners! Fortunately, with the discovery of LPMOs, our panel of tools has widened, opening the way to more efficient processes. To become better miners, we now need to use these tools correctly, taking into account a few considerations: (i) understand the structure and composition of the targeted substrates, (ii) choose the right tools to degrade it and (iii) use them efficiently to (iv) fully valorize the end products. In other words, we need to better adapt the process conditions to the LPMOs we choose to implement in our enzymatic cocktails. It is well known that even within a single family, such as the AA9s, one can find different substrate specificities, reductants preference, reactivity with H_2O_2 , sensibility to self-oxidation... Even if we still do not fully understand how fungi deal with these subtleties in nature, part of the answer could rely on the recruitment of specific LPMOs, in combination with well-suited partners, such as CDHs. However, the perfect match in a biological context will not necessarily remain optimal in industrial conditions. To efficiently set up this strategy, I believe it is essential to (i) better understand the compositions of the pretreated biomass, by identifying and quantifying potential inhibitors/LPMO reducing agents, (ii) identify the best performing LPMOs and their redox partners for each substrate and (iii) engineer this LPMO/redox partner pair to make them as efficient as possible under industrial constraints.

Since the driving force behind these innovations mostly lies in the fundamental understanding of LPMOs mode of action, it is not impossible that our quest for “super biorefining LPMOs” will lead to the discovery of hitherto unknown biological functions. In fact, with the recent elucidation of multiple LPMO structures, the rapid emergence of new families, the multiplicity of LPMO-encoding genes in some organisms and the presence of LPMOs in non-cellulolytic/chitinolytic organisms, the community is progressively questioning the universality of the “LPMO dogma”, i.e. LPMOs being merely recalcitrant polysaccharides oxidizers. Therefore, many recent observations, outside the biorefinery context, motivated us to review the literature (see **Appendix I** (Vandhana et al. 2022)) that highlights the

emerging biological functions of LPMOs in different biological contexts, ranging from pathogenicity in numerous organisms (fungi, viruses, oomycetes), to the symbiosis between plants and fungi, not forgetting the remodeling of the cell wall (**Fig. 4, Appendix I** (Vandhana et al. 2022)).

This thesis also turned out to be unexpectedly enriching regarding the biological role of LPMOs, with the discovery of AA9-X282s that can carry an extension reminiscent of phosphate binding motifs. Even if we still don't have the definite answer as to the role of these atypical AA9s, this doctoral thesis opens a new track in the growing landscape of potentially new biological roles played by LPMOs.

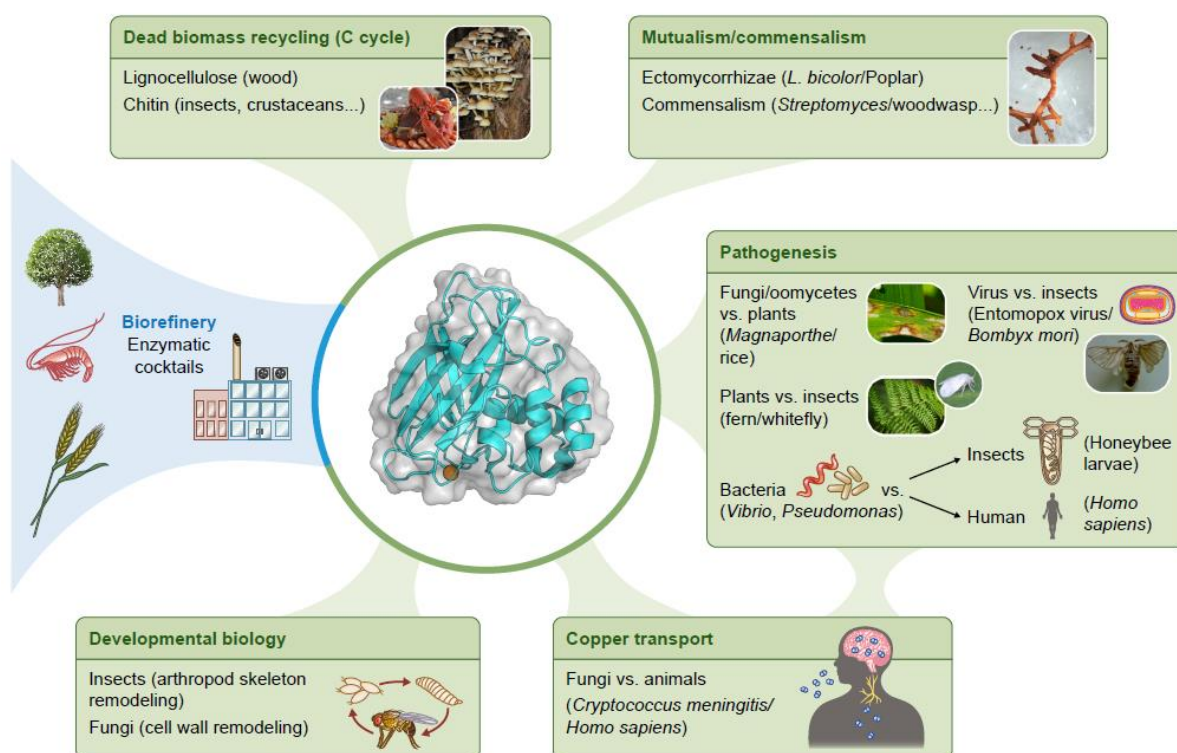


Fig. 4. Overview of the diversity of lytic polysaccharide monooxygenase (LPMO) biological functions. The figure was reproduced from (Vandhana et al. 2022).

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Appendix I

On the expansion of biological functions
of lytic polysaccharide monooxygenases



Tansley review

On the expansion of biological functions of lytic polysaccharide monooxygenases

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Summary

Lytic polysaccharide monooxygenases (LPMOs) constitute an enigmatic class of enzymes, the discovery of which has opened up a new arena of riveting research. LPMOs can oxidatively cleave the glycosidic bonds found in carbohydrate polymers enabling the depolymerisation of recalcitrant biomasses, such as cellulose or chitin. While most studies have so far mainly explored the role of LPMOs in a (plant) biomass conversion context, alternative roles and paradigms begin to emerge. In the present review, we propose a historical perspective of LPMO research providing a succinct overview of the major achievements of LPMO research over the past decade. This journey through LPMOs landscape leads us to dive into the emerging biological functions of LPMOs and LPMO-like proteins. We notably highlight roles in fungal and oomycete plant pathogenesis (e.g. potato late blight), but also in mutualistic/commensalism symbiosis (e.g. ectomycorrhizae). We further present the potential importance of LPMOs in other microbial pathogenesis including diseases caused by bacteria (e.g. pneumonia), fungi (e.g. human meningitis), oomycetes and viruses (e.g. entomopox), as well as in (micro)organism development (including several plant pests). Our assessment of the literature leads to the formulation of outstanding questions, promising for the coming years exciting research and discoveries on these moonlighting proteins.

I. Introduction

The breakdown of carbohydrates is essential for the global carbon cycle. On Earth, the most abundant sources of carbohydrates are found in the form of cellulose and chitin, used as structural biopolymers in the plant and animal/fungal kingdoms, respectively. These polysaccharides are mainly acted upon by enzymes

collectively termed as carbohydrate-active enzymes (CAZymes), which help in their build-up, modification as well as breakdown (Cantarel *et al.*, 2009) (www.cazy.org). In the context of cellulose and chitin conversion, glycoside hydrolases (GHs) act as main players by catalysing the hydrolytic cleavage of polysaccharide chains into smaller oligosaccharides and monosugar building blocks, which can in turn be used as carbon/energy source by

microorganisms. However, polysaccharide chains packed into crystalline lattices are hardly accessible to most canonical enzymes. To circumvent this geometrical and thermodynamic hurdle, Nature has evolved *ad hoc* biocatalytic tools, *viz.* the lytic polysaccharide monooxygenases (LPMOs) (Horn *et al.*, 2012).

Lytic polysaccharide monooxygenases activity was recently discovered in 2010, and they are represented by monocopper enzymes with the unique ability to oxidise the surface of crystalline polysaccharides (Vaaje-Kolstad *et al.*, 2010; Forsberg *et al.*, 2011; Phillips *et al.*, 2011; Quinlan *et al.*, 2011), disrupting thereby the surface topology (Eibinger *et al.*, 2014, 2017; Villares *et al.*, 2017) and creating chain ends tractable by canonical GHs for further depolymerisation. LPMO substrate specificity was later extended to noncrystalline carbohydrate substrates, hinting at a more diverse role and possibly alternative functions of LPMOs (described later). Beyond their biological importance, LPMOs have rapidly proved to be industrially relevant enzymes to boost the efficiency of commercial enzymatic cocktails for plant biomass degradation processes and yield fermentable sugars at competitive cost (e.g. for biofuel production) (Johansen, 2016; Chylenski *et al.*, 2019).

Over the past 10 yr, a significant number of reviews have covered diverse aspects of the LPMO field. We direct the reader to recent comprehensive reviews dealing with mechanistic considerations (Meier *et al.*, 2017; Bissaro *et al.*, 2018b; Wang *et al.*, 2020), applied enzymology (Johansen, 2016; Chylenski *et al.*, 2019), structural aspects (Vaaje-Kolstad *et al.*, 2017; Tandrup *et al.*, 2018) and enzyme secretomes complexity (Berrin *et al.*, 2017; Bissaro *et al.*, 2018b). Recent developments have suggested that LPMOs scope of action would not be restricted to the mere oxidation of structural biopolymers. Here, we aim at drawing a portrait of this expansion of LPMO-related functions. To this end, after succinctly presenting the LPMO field and debated dogmas, we review the emerging biological functions of LPMOs, with a particular emphasis on plant–microorganisms interactions. We then discuss unanswered questions and potential directions that the field may take in the coming years.

II. The bumpy road of LPMO research

Several decades of research have paved the way towards the discovery of LPMOs and the birth of an entire field and community. In Box 1, we provide a historical perspective of this scientific journey.

1. 2010–2020: the race for ‘firsts’

The year 2010 marked a turning point in the field of glycan-processing enzymes. First, like the boosting effect reported for CBP21 on chitinase activity (Vaaje-Kolstad *et al.*, 2005a), a study on a fungal GH61 revealed that its addition to a cellulases mixture enhanced cellulose hydrolysis, reinforcing the parallel between GH61s and CBM33s (Harris *et al.*, 2010). At that time, although the boost effect was clear, their enzymatic mechanisms still remained unknown. This same year, Vaaje-Kolstad and colleagues brought a major piece of the jigsaw, the assembly of which started back in the 1950s (Box 1): they demonstrated that the hitherto

Box 1 A bit of history on pre-LPMOs times

In 1954, in a study entitled *The swelling factor in cellulose hydrolysis*, Reese and Gilligan hypothesised the existence of a nonhydrolytic proteinaceous system (‘C₁ factor’), described as targeting and disrupting crystalline parts of cellulose, rendering them more tractable by cellulases (Reese & Gilligan, 1954).

In 1974, Eriksson *et al.* showed the importance of oxygen and oxidative processes in cotton cellulose conversion by the white-rot fungus *Sporotrichum pulverulentum* (anamorph *Phanerochaete chrysosporium*) (Fig. 1) (Eriksson *et al.*, 1974). The authors highlighted the existence of an unknown oxidative activity that could induce swelling of crystalline cellulose.

In the late 1990s, scientists working on soil bacteria (*Streptomyces*) observed the secretion of a mysterious protein with high affinity for chitin (Schnellmann *et al.*, 1994; Kolbe *et al.*, 1998), classified as a chitin-binding protein (CBP). A 21-kDa CBP orthologue (called CBP21) was then found in *Serratia marcescens* (Suzuki *et al.*, 1998). However, due to the lack of experiments substantiating the catalytic activity, these proteins were defined as a new carbohydrate-binding module family, the CBM33 (Sunna *et al.*, 2000). Meanwhile, the characterisation of another unknown protein (later named EGIV), revealed a very weak endoglucanase activity, and became the founding member of the GH61 family (Saloheimo *et al.*, 1997).

In 2005, Vaaje-Kolstad *et al.* (2005a) demonstrated that CBP21 significantly boosted chitin hydrolysis by chitinases. Structural insights revealed that this protein shared little commonalities with canonical CBMs (Vaaje-Kolstad *et al.*, 2005b) (Fig. 1). A few years later, the first GH61 structure (Karkehabadi *et al.*, 2008) revealed features reminiscent of CBP21 while sharing no similarity with other GH structures. Devoid of a conserved active site of most known GHs, no rational molecular mechanism could be proposed to explain the endoglucanase activity of GH61s (Harris *et al.*, 2010). Interestingly, a conserved metal-binding site could be identified in both CBP21 and GH61.

noncatalytic protein CBP21 was able to cleave glycosidic bonds in β -chitin through a redox reaction (Vaaje-Kolstad *et al.*, 2010). Using isotopically labeled dioxygen, the authors demonstrated the introduction of an oxygen atom into the chitin carbohydrate chain in the presence of a reducing agent. This was the rationale to define CBP21, and its subsequently reported orthologues, as monooxygenases. In 2011, several studies reported an analogous oxidative activity on cellulose (Forsberg *et al.*, 2011; Phillips *et al.*, 2011; Quinlan *et al.*, 2011). Quinlan and colleagues clarified the nature of the metal centre: a mono-copper atom coordinated by two histidines, therefore called today a ‘histidine brace’ (Ciano *et al.*, 2018). In 2012, CBM33s and GH61s were collectively termed ‘LPMO’ (Horn *et al.*, 2012). These pioneer discoveries spurred intense research efforts, first notably in European laboratories and today worldwide (Fig. 1). LPMOs were rapidly assigned to new auxiliary activity (AA) families in the CAZy database (Levasseur *et al.*, 2013): GH61s were reclassified as AA9s and CBM33s as AA10s. A scientific race was engaged to discover new substrate specificities, structures and mechanistic features. Today, LPMOs are distributed in eight AA families: AA9–AA11 and AA13–AA17 (see later). The substrate specificity of LPMOs varies among these AA families, but is mostly towards cellulose, chitin, xylan, starch or

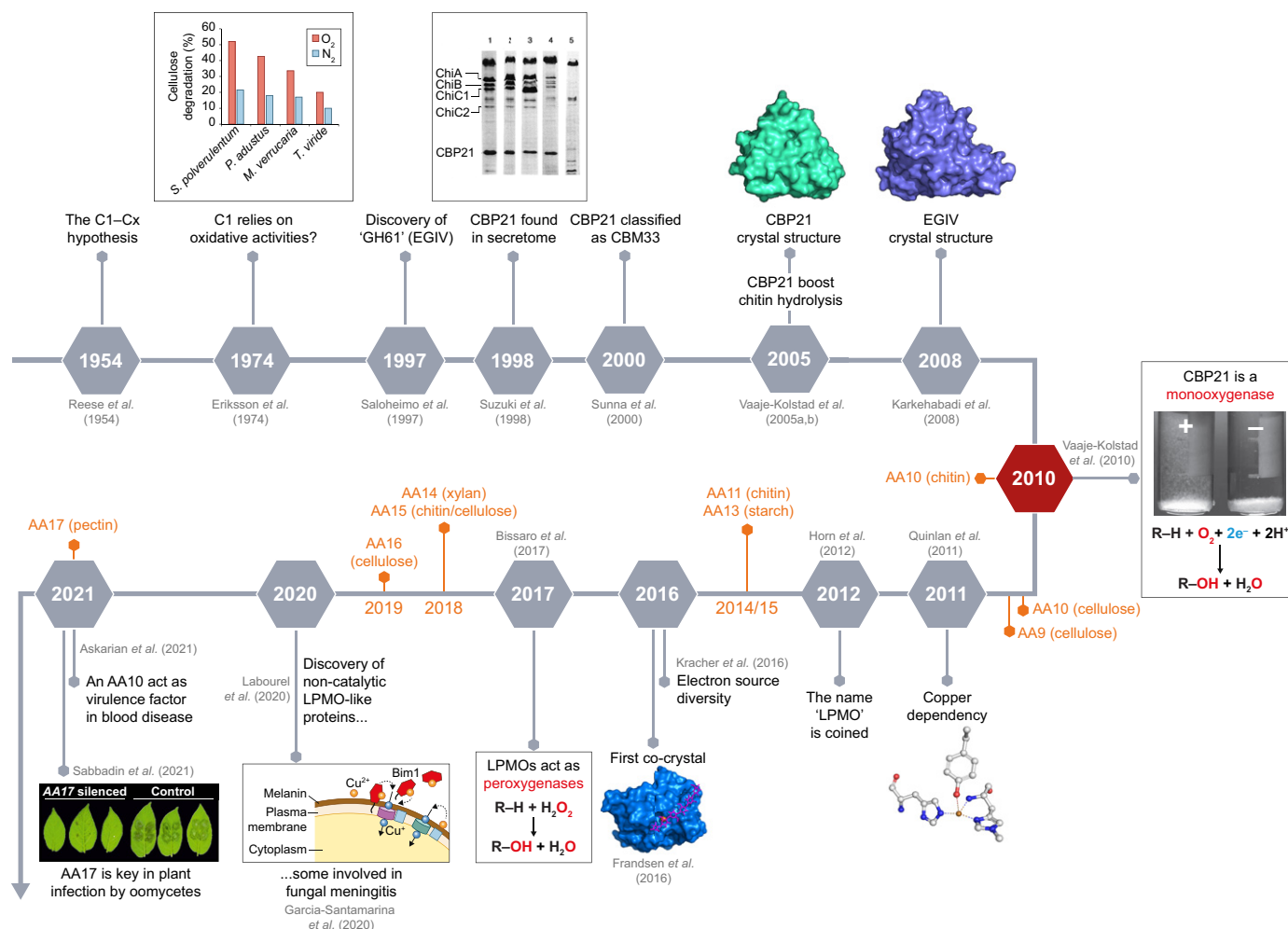


Fig. 1 Lytic polysaccharide monooxygenase (LPMO) research timeline and milestones. Major milestones are associated with the following references, by chronological order: 1954 (Reese & Gilligan, 1954), 1974 (Eriksson *et al.*, 1974), 1997 (Saloheimo *et al.*, 1997), 1998 (Suzuki *et al.*, 1998), 2000 (Sunna *et al.*, 2000), 2005 (Vaaje-Kolstad *et al.*, 2005a,b), 2008 (Karkehabadi *et al.*, 2008), 2010 (Vaaje-Kolstad *et al.*, 2010), 2011 (Quinlan *et al.*, 2011), 2012 (Horn *et al.*, 2012), 2016 (Frandsen *et al.*, 2016; Kracher *et al.*, 2016), 2017 (Bissaro *et al.*, 2017), 2020 (Garcia-Santamarina *et al.*, 2020; Labourel *et al.*, 2021), 2021 (Askarian *et al.*, 2021; Sabbadin *et al.*, 2021b). The discovery of new LPMO families (along with substrate specificity) is displayed in red. The picture on the right-hand side (year 2010) shows the disruptive effect (swelling) of CBP21 on chitin in the presence of oxygen and reductant (vial labelled '+') compared with a reaction devoid of electron source ('-'). The picture on the left-hand side (year 2021) shows the loss of oomycete pathogenicity upon silencing of AA17-encoding gene. AA, auxiliary activity; CBP, chitin-binding protein; EG, endoglucanase.

pectin. They are abundantly present in bacterial and fungal communities but, have also been found in viruses, plants and insects.

(1) The AA9 family contains sequences of fungal origin only, and encompasses several substrate specificities, mainly targeting glucose-based polymers such as cellulose, cello-oligosaccharides or even hemicelluloses with a β -1,4-linked glucose backbone (gluco-mannans, xylo-glucans, mixed linked glucans, etc.) (Agger *et al.*, 2014; Bennati-Granier *et al.*, 2015; Frommhagen *et al.*, 2015; Simmons *et al.*, 2017). Of note, the AA9 family also contains nonactive proteins lacking the conserved His-brace motif (Frandsen *et al.*, 2019).

(2) The AA10 family is probably the most diverse family in terms of taxonomic coverage, inasmuch as it contains sequences originating from bacteria (97%), viruses (2.9%), archaea (0.1%) and even from some pathogenic fungi of the *Ustilaginomycetes* class

(0.1%). Members of this LPMO family can target either chitin or cellulose, or both. Interestingly, one characterised member of this family from the actinomycete bacteria *Kitasatospora papulosa* seemed to display a broader substrate specificity, cleaving cellulose, chitin and xylan (Corrêa *et al.*, 2019), albeit this result needs to be confirmed.

(3) The AA11 family, introduced in 2014, is a rather small, fungal-only family (preponderant in ascomycetes fungi), with only a few characterised members, all active on chitin (Hemsworth *et al.*, 2014; Wang *et al.*, 2018).

(4) The AA13 family, first reported in 2014 (Vu *et al.*, 2014), contains fungal-only LPMOs acting on starch substrates (Vu *et al.*, 2014; Leggio *et al.*, 2015; Momeni *et al.*, 2020).

(5) The AA14 family, published in 2018, unveiled with the characterisation of two homologues from the white-rot fungus *Pycnoporus coccineus*, an oxidative activity on heteroxylan covering

cellulose fibrils in wood (Couturier *et al.*, 2018). While AA14 LPMOs did not show activity on soluble xylan, synergism with GH30 xylanases was observed on wood fibres (Zerva *et al.*, 2020).

(6) The AA15 family LPMOs are found in various organisms including viruses (14.5%) and eukaryotes (85.5%) such as algae, oomycetes and complex animals. The two first members were characterised from the insect *Thermobia domestica* and displayed activity on both cellulose and/or chitin (Sabbadin *et al.*, 2018). More recently, two AA15 LPMOs from the termite *Coptotermes gestroi* (Cairo *et al.*, 2020) and one AA15 from the oomycete pathogen *Aphanomyces astaci* ('crayfish plague') (Sabbadin *et al.*, 2021a) showed activity on chitin only.

(7) The AA16 family contains members mainly found in fungi and some oomycetes (Filiatrault-Chastel *et al.*, 2019). This family was first identified in the secretome of *Aspergillus japonicus*. Characterisation of several orthologues revealed activity on cellulose (Filiatrault-Chastel *et al.*, 2019). Recent studies have also demonstrated a boosting effect of AA16s on cellulose degradation by a cellulase cocktail and suggested implications of this LPMO family in plant pathogenesis in oomycetes (Jagadeeswaran *et al.*, 2020) (see Section III).

(8) The AA17 family is the most recent LPMO family, only found in oomycetes, with a significant expansion of gene copies (up to 65) in hemibiotrophic and necrotrophic pathogens (Sabbadin *et al.*, 2021b). Three AA17s from *Phytophthora infestans* were demonstrated to oxidatively cleave (C4-oxidation) homogalacturonan and oligogalacturonides (degree of polymerisation 10–15), constituents of pectin in plant cell walls (PCW). Prior de-esterification of pectin (by pectin methyl esterases) was required to observe LPMO activity (Sabbadin *et al.*, 2021b).

2. Pitfalls in a recent but established dogma

From the early 2010s, LPMOs have been described as catalysing, via a monooxygenase reaction, the disruption of crystalline regions of polysaccharides (e.g. in chitin or cellulose), otherwise hardly accessible to canonical GHs. Benefiting from the hindsight of a decade of research, it becomes increasingly clear that this dogma is

slowly shattering in several respects, and therefore merits to be discussed and nuanced. Before doing so, some facts should be presented.

The peculiar function of LPMOs is intimately related to their 3D structure (Fig. 2). Although some variations across the different families exist, the basal structure of LPMOs is fairly well conserved (Frandsen *et al.*, 2016; Vaaje-Kolstad *et al.*, 2017), consisting of an immunoglobulin-like β -sandwich fold, composed of 8–10 antiparallel β -strands, linked to each other by α -helix loops. At the active site, located on a usually rather flat, solvent-exposed surface (Fig. 2), two universally conserved histidines coordinate a Cu(II) ion through three nitrogen atoms (Ciano *et al.*, 2018). In addition, a conserved tyrosine (or a phenylalanine in chitin-active AA10 and AA15) is also essential for catalysis (Fig. 2). We underscore that in-depth LPMO–substrate interaction studies are only available for a handful of AA9 (Courtade *et al.*, 2016; Frandsen *et al.*, 2016; Simmons *et al.*, 2017) and AA10 (Aachmann *et al.*, 2012; Bissaro *et al.*, 2018a) LPMOs, due to the fact that working with insoluble substrates (e.g. cellulose or chitin) is a true technical challenge.

A first aspect of the LPMO dogma that needs to be discussed is the nature of the substrate: we, and others, have observed that AA9 LPMOs are often more active on amorphous cellulose than on microcrystalline cellulose (Bennati-Granier *et al.*, 2015; Villares *et al.*, 2017). Intriguingly, some LPMOs are also active on oligosaccharides (Isaksen *et al.*, 2014; Bennati-Granier *et al.*, 2015; Frandsen *et al.*, 2017, 2019), and such activity is unlikely to be related to the direct boosting of biomass depolymerisation. Such LPMOs could, for instance, be involved in alleviating the inhibition of cellulases by cello-oligosaccharides upon oxidation of the latter, a role also suggested for cellobiose dehydrogenases (Igarashi *et al.*, 1998). This remains, however, to be demonstrated. Other LPMOs, such as AA13s and AA14s, with more groovy active sites, would better be described as acting on glycosidic bonds encountered in recalcitrant environments – for example in starch (for AA13s; Leggio *et al.*, 2015; Vu *et al.*, 2019), or on xylan coating cellulose fibrils (for AA14s; Couturier *et al.*, 2018; Zerva *et al.*, 2020) – rather than on crystalline substrates. Lately, the crystal structure of an AA17, active on homogalacturonan, has revealed a

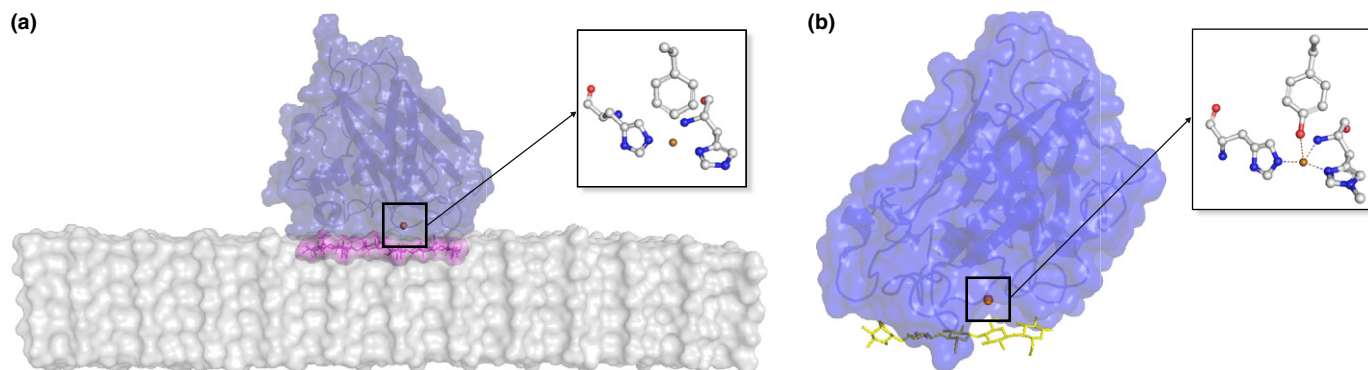


Fig. 2 Lytic polysaccharide monooxygenase (LPMO)–substrate interaction. (a) A model of SmAA10A bound to β -chitin (the chitin crystal is shown in grey and the chitohexaose fragment interacting with the LPMO is highlighted in magenta) (Bissaro *et al.*, 2018a) and (b) LsAA9A–cellopentaose complex (Protein Data Bank (PDB) 5NLS; (Frandsen *et al.*, 2016)). The model in panel (a) was obtained from experiment-guided molecular dynamics (MD) simulations (Bissaro *et al.*, 2018a) and the structure shown in panel (b) was obtained from crystals soaked with oligosaccharides before X-ray diffraction (Frandsen *et al.*, 2016). Inserts show the histidine brace (copper shown as orange spheres).

rather grooved and charged active site, consistent with an adaptation to interact with charged polysaccharides (Sabbadin *et al.*, 2021b).

A second aspect of the LPMO dogma pertains to their catalytic mechanism. Like many other oxidoreductase classes, LPMOs have not been exempt from scientific debates and evolutions when it comes to their mode of action. It is now agreed upon that LPMOs require a priming reduction of the resting Cu(II) state into the active Cu(I) state. It should, however, be underscored that the identity of the reducing agent remains to be clarified for several of the biological contexts involving LPMOs (described later). *In vitro* studies have shown that reduction can occur via reaction with a variety of molecules (Kracher *et al.*, 2016; Frommhagen *et al.*, 2018), including small organic reductants (Vaaje-Kolstad *et al.*, 2010), redox enzyme partners (Phillips *et al.*, 2011; Garajova *et al.*, 2016; Kracher *et al.*, 2016; Várnai *et al.*, 2018; Momeni *et al.*, 2021), lignin and their compounds (Westereng *et al.*, 2015), or even photoactivated molecules (Bissaro *et al.*, 2016, 2020a; Cannella *et al.*, 2016). A more controversial aspect pertains to the nature of the biological cosubstrate bringing the oxygen atom necessary for hydroxylation of either the C1 or C4 carbon of the scissile glycosidic bond (Vaaje-Kolstad *et al.*, 2010; Phillips *et al.*, 2011), and inducing cleavage of the polysaccharide chain (Beeson *et al.*, 2012; Wang *et al.*, 2018). From 2010 onwards, the proposed oxidative mechanism (until its revision in 2016/2017, described later) was described as a monooxygenase reaction, in which O₂ is proposed to serve as an oxygen atom donor. However, in a preprint in 2016 (eventually published in 2017 (Bissaro *et al.*, 2017)), Bissaro and colleagues demonstrated the occurrence of an efficient peroxygenase reaction (Bissaro *et al.*, 2017), that is using H₂O₂ rather than O₂. At this time, multiple kinetics studies have shown that both apparent affinity and catalytic efficiency are several orders of magnitude higher for H₂O₂ (Hangasky *et al.*, 2018; Kuusk *et al.*, 2018, 2019; Bissaro *et al.*, 2020a; Hedison *et al.*, 2020; Jones *et al.*, 2020; Kont *et al.*, 2020; Momeni *et al.*, 2021). This conclusion is further supported by synergistic studies with H₂O₂-producing redox partners (Bissaro *et al.*, 2017; Hegnar *et al.*, 2019; Kracher *et al.*, 2020; Momeni *et al.*, 2021). However, there is the possibility that the actual mechanistic path depends on the biological context and substrate/cosubstrate availability. Furthermore, it should be highlighted that mechanistic studies have mainly been conducted on AA9 and AA10 LPMOs and much remains to be done with the other LPMO families. Clarifying these mechanistic aspects is of utmost importance to then interpret, as correctly as possible, the putative connection between partner enzymes in omics studies, to design relevant *in vivo* localisation studies, and more generally understand the molecular basis underlying the interactions between LPMO-producing (micro)organisms and their host/partner.

Overall, the portrait we draw of the current LPMO field shows that, while there has been dazzling progress within a rather narrow time window, the complexity and subtleties of the biological function(s) of LPMOs clearly escape our understanding. In the next section, we aim at providing an overview of such functions, whether they be supported by sound evidence, or sometimes still at a hypothetical stage.

III. Emerging biological functions

LPMOs are more than mere biomass-degrading enzymes. They have been observed to possess varied biological functions from enabling developmental processes in insects to acting as virulence factors in pathogenic bacteria, oomycetes and fungi, all the while moonlighting as degraders of recalcitrant polysaccharides. This avenue widens the scope of LPMO research while teaching us to look beyond the obvious.

Before diving into specific examples, we carried out a global survey of the number of research articles (over the period 2008–2021) on host–pathogen interactions reporting the expression of an LPMO-encoding gene (studied at transcriptomic level) or the production of an LPMO (at proteomic level, or biochemically characterised) by so-called emitters, whether they be bacteria, oomycetes, fungi, plants or viruses (Fig. 3a). This analysis showed an increasing number of reports, yet modest and mainly focused on bacteria and fungi. Strikingly, this same set of data, represented as an emitter–target map shows that some regions remain empty (Fig. 3b), which could pinpoint territories yet to be explored (e.g. viruses vs animals). To better illustrate the *potential* diversity of LPMOs involved, we have also made an inventory of LPMO and LPMO-like encoding genes found in a selection of bacterial and fungal pathogens discussed here below (Fig. 3c). This inventory clearly illustrates the dichotomy between bacterial (harbouring only a few AA10-encoding genes) and fungal pathogens, which possess mainly AA9s, but also for some plant pathogens – such as *Magnaporthe oryzae* (causing rice blast) or *Leptosphaeria maculans* (causative agent of blackleg disease in Brassica crops, mainly affecting *B. napus*) – a significant number of AA11s and AA16s.

We also underscore that while there are several lines of evidence for the involvement of these LPMOs in pathogenesis processes, crystal-clear proof remains to be provided for many of them (see details later).

1. Role of LPMOs in fungal pathogenesis

The rice blast fungus, *Magnaporthe oryzae*, ranks among the top 10 most devastating plant pathogens worldwide (Dean *et al.*, 2012), causing serious crop yield losses and endangering global food security. This fungus exhibits a hemibiotrophic lifestyle and invades the plant by means of specialised appressoria cells, making use of high turgor pressure to puncture the plant surface, together with various effectors (Wilson & Talbot, 2009). *MoAa91*, an AA9 LPMO of *M. oryzae* was identified in the plant apoplast and acted as an inducer of appressorium formation on artificial inductive surfaces (Li *et al.*, 2020). Of note, upon *MoAa91* gene deletion the formation of appressoria cells was delayed. It is even interesting to note that *MoAa91*, in association with *MoSlp1*, an extracellular LysM domain protein anchoring to chitin, helped the fungus in evading the host immune response by preventing fungal-derived sugars (i.e. fungal cell wall carbohydrates) to be recognised by pattern triggered immunity that relied on the host chitin elicitor-binding protein precursor (CEBiP) (Li *et al.*, 2020). However, we must also underscore that *MoAa91* is a multimodular protein composed of an N-terminal AA9 domain and a C-terminal chitin-

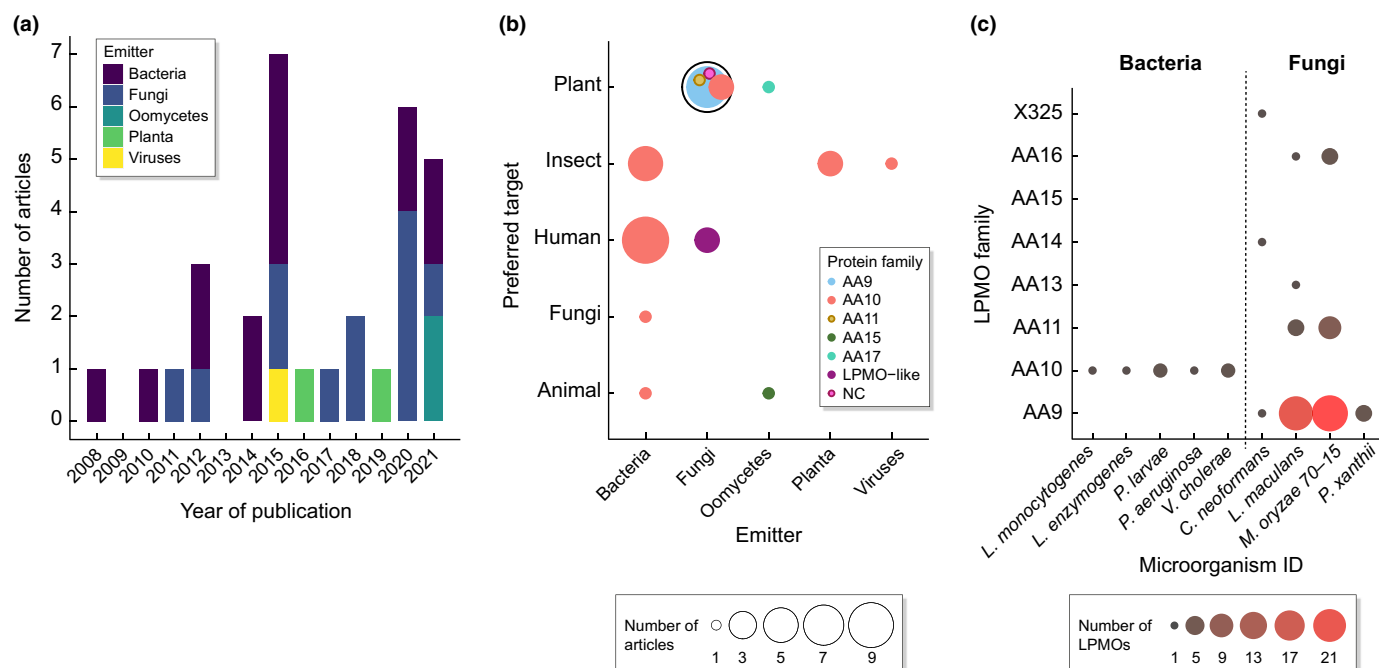


Fig. 3 Literature analysis of the occurrence of lytic polysaccharide monooxygenases (LPMOs) found in various pathosystems. (a, b) Number of research articles reporting an LPMO (or LPMO-like) shown to be relevant by either transcriptomic, proteomic and/or enzyme characterisation in different emitters (Kawada *et al.*, 2008; Chaudhuri *et al.*, 2010; Valente *et al.*, 2011; Stauder *et al.*, 2012; Wong *et al.*, 2012; Yakovlev *et al.*, 2012; Garcia-Gonzalez *et al.*, 2014; Loose *et al.*, 2014; Chiu *et al.*, 2015; Hamre *et al.*, 2015; Huang *et al.*, 2015; Paspaliari *et al.*, 2015; Zhang *et al.*, 2015; Mekasha *et al.*, 2016; Shukla *et al.*, 2016; Liu *et al.*, 2017, 2018, 2020; Hegnar *et al.*, 2019; Yadav *et al.*, 2019; Garcia-Santamarina *et al.*, 2020; Labourel *et al.*, 2020; Li *et al.*, 2020; Munzone *et al.*, 2020; Askarian *et al.*, 2021; Polonio *et al.*, 2021; Sabbadin *et al.*, 2021a,b; Yue *et al.*, 2021), and displayed as (a) a function of the year of publication per emitter and (b) an emitter–target map, in which the circle size indicates the number of articles and the colour indicates the protein family. Note that the ‘preferred target’ shows the potential target of the emitter, and not necessarily the target of the LPMO, which in several cases remains to be fully demonstrated (see main text). The category ‘Animal’ refers to nonhuman animals. (c) Analysis of the number of LPMO and LPMO-like encoding genes found in the genomes of several selected microbial pathogens discussed in the present review, including the bacteria *Vibrio cholerae* N16961, *Paenibacillus larvae* ATCC 9545, *Listeria monocytogenes* EGD, *Pseudomonas aeruginosa* UCBPP-PA14, *Lysobacter enzymogenes* CX03; and the fungi *Magnaporthe oryzae* 70-15, *Cryptococcus neoformans* var. *grubii* H99, *Leptosphaeria maculans* JN3 and *Podosphaera xanthii*. NC, not classified (i.e. PxLPMO1).

binding domain and that inactivation of the former did not alter strain pathogenicity, therefore supporting a mechanism involving chito-oligosaccharide binding rather than oxidative activity (Li *et al.*, 2020).

Adding to the varied functions of LPMOs, the following discussion is about an LPMO-like protein from *Cryptococcus neoformans* (Garcia-Santamarina *et al.*, 2020), the causative agent of lethal meningitis in humans (Srikanta *et al.*, 2014). This LPMO-like protein is encoded by the gene *BIM1*, and is expressed in large quantities by *C. neoformans* during Cu limitation (Garcia-Santamarina *et al.*, 2020). During pathogenesis and brain colonisation, *C. neoformans* shifts from a Cu detox mode to an actively Cu-acquiring state in Cu-limited conditions. Bim1 binds Cu²⁺ and plays a crucial role in Cu acquisition and successively in cryptococcal meningitis. Garcia-Santamarina and colleagues demonstrated that *BIM1*-knockout mutants are Cu deficient (Garcia-Santamarina *et al.*, 2020). The role of Bim1 in Cu uptake and accumulation depends on Cu(II) coordination and cell-surface association via a glycosylphosphatidylinositol (GPI) anchor. Overall, this LPMO-like protein is a critical factor for Cu acquisition in fungal meningitis (Garcia-Santamarina *et al.*, 2020). It is worth noting that *C. neoformans* also expresses other LPMOs belonging to AA9, AA11 and AA14 families, the activities of which are yet to be

determined (Garcia-Santamarina *et al.*, 2020). Importantly, Labourel and coworkers have characterised orthologues of BIM1, called X325, found in yeast and filamentous fungi with different lifestyles, including the ectomycorrhizal fungus *Laccaria bicolor* (Labourel *et al.*, 2020) (see Section III.5 on symbiotic interactions for more details). Of note, the authors have shown that these LPMO-like proteins do not display LPMO activity on any of a wide range of tested polysaccharides. Interestingly, while the 3D structure of one fungal X325 member revealed a conserved LPMO fold, it also showed an atypical His brace with an additional Asp ligand to Cu(II). Interestingly, a parallel can be drawn between Bim1/X325 and mammalian ceruloplasmin or bacterial CopC, which are also involved in binding extracellular Cu but are not redox active (Brander *et al.*, 2020). Whether all X325 proteins act as copper transporters, or if some can catalyse hitherto undetected reactions, remains to be unveiled.

Podosphaera xanthii is the causative organism of cucurbit powdery mildew disease. A novel LPMO, called PxLPMO1, was discovered and characterised by Polonio and colleagues in 2021 (Polonio *et al.*, 2021). PxLPMO1 was seen to possess a predicted protein fold characteristic of LPMOs but showed low sequence similarity with the same. PxLPMO1 displayed a putative chitin-binding domain-3 and was demonstrated to catalyse the oxidative

cleavage of chitin and chito-oligosaccharides. Similarly to what was observed for *MoAa91* (mentioned earlier), RNA silencing of *PxLPMO1* activated chitin-triggered host immunity (chitin receptors) and oxidative burst. Furthermore, *PxLPMO1* was found to be coexpressed along with host (melon) endochitinases, at early stages during infection, supporting the hypothesis that *PxLPMO1* would modify chito-oligosaccharides released from the fungal cell wall by plant enzymes, therefore contributing to evade the plant immune system. Several orthologues of this protein have been observed in other fungal pathogens (such as *Rhynchosporium commune*, *Cadophora* sp. and *Phialophora americana*) demonstrating more avenues to look into for LPMO roles in fungal

pathogenesis (Polonio *et al.*, 2021). Strikingly, our analysis of the *PxLPMO1* sequence (and predicted structure using RoseTTAFold tool (Baek *et al.*, 2021)) indicated that it is an orthologue of BIM1/X325, but belongs to a phylogenetic clade in which the additional copper-coordinating residue specific to X325 (mainly aspartate or histidine) (Labourel *et al.*, 2020) (described earlier), is replaced by an arginine. This major difference may explain why *PxLPMO1* displayed oxidative activity while X325 did not, and also suggests that *PxLPMO1* may be the founding member of a new LPMO family containing active LPMO and nonactive LPMO-like members (Fig. 4).

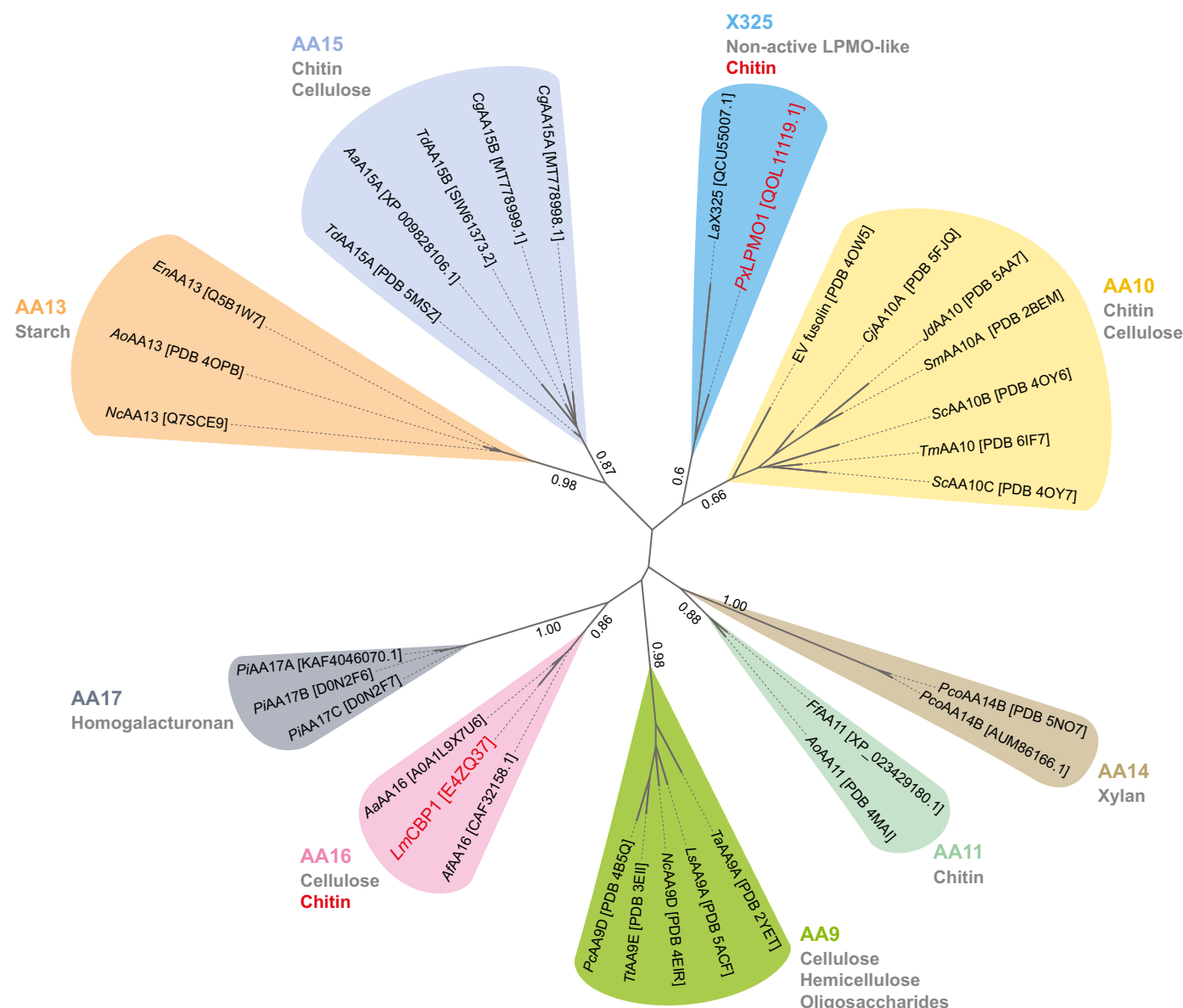


Fig. 4 Phylogenetic tree of lytic polysaccharide monooxygenase (LPMO) families. Sequences of characterised proteins only (accession number or Protein Data Bank (PDB) code provided in the figure) were aligned using T-COFFEE EXPRESSO (Armougom *et al.*, 2006). Phylogeny was inferred using PHYML software (Guindon *et al.*, 2010) and the Whelan and Goldman (WAG) amino acid substitution model (Whelan & Goldman, 2001). Branch support was calculated by 100 bootstrap repetitions (value displayed on tree). The tree was visualised with Interactive Tree Of Life (iTOL) software (Letunic & Bork, 2021). Substrate specificities of each LPMO family are shown in grey (or in red for a few members (printed in red in the tree), in AA16 and X325 families).

Leptosphaeria maculans is a filamentous fungus and the causative agent of blackleg disease in *Brassicaceae* (cabbage, mustard plants). This organism secretes a protein *LmCBP1*, found by the authors as having an AA10 LPMO domain (our expert analysis contradicts this; described later). *LmCBP1* was secreted at high levels during the infection process of *Brassica napus* (Liu *et al.*, 2020), suggesting a role in virulence. Biochemical assays showed that *LmCBP1* could bind chitin. Unfortunately, chitinolytic activity was not properly assessed as the reductant was omitted and only chitinase activity was monitored. Our analysis of *LmCBP1* catalytic domain sequence indicated that it shares only between 13% and 24% sequence identity with most well characterised AA10 LPMOs. However, structural predictions indicated that it displays a typical fibronectin LPMO fold and possesses the conserved histidine brace. Therefore, there is the possibility that, if cautiously produced and characterised (Eijsink *et al.*, 2019), *LmCBP1* could be a functional, chitin-active new LPMO belonging to the AA16 family (see Fig. 4). Additionally, a gene knockout mutant of *LmCBP1* demonstrated a decrease in virulence in the host, causing lesser cell death. Furthermore, the LPMO-knockout mutant was more sensitive to H₂O₂ (usually produced by the attacked plant during the so-called oxidative burst) compared with the wild-type strain. This is an interesting observation considering that chitinolytic AA10 LPMOs have been shown to display efficient peroxygenase activity (Bissaro *et al.*, 2017, 2020b; Kuusk *et al.*, 2018). The authors comment that *LmCBP1* could be utilised as a means to gain tolerance against H₂O₂, and therefore aid the infection in *Brassicaceae* crops. These results collectively demonstrate LPMOs as agents involved in pathogenesis and, in addition, as potential H₂O₂ scavengers.

In a somehow similar context of pathogen–plant warfare, a recent study by Zarattini *et al.* (2021) used LPMO reaction products to trigger the innate immune response in the model plant *Arabidopsis thaliana*. Precisely, they used the fungal AA9 LPMO from *Thermothielavioides terrestris* to generate a pool of native and (C1/C4) oxidised cello-oligosaccharides (collectively referred to as AA9_COS), which were used to treat plants. Following the treatment with AA9_COS, as well as with cellobionic acid, the plants displayed an increased resistance when exposed to the necrotrophic fungus *Botrytis cinerea*, and were observed to undergo a deep transcriptional reprogramming. In more detail, STRESS INDUCED FACTOR 2 and 4 were seen to play a crucial role and signalled the production of camalexin, an antimicrobial phytoalexin. It was also noticed that the levels of ethylene, jasmonic acid and salicylic acid hormones were increased, along with deposition of callose in the cell wall. This study is unique and displays the possible application of LPMO products (AA9_COS) in triggering the damage triggered immunity (DTI) response in plants (Zarattini *et al.*, 2021). However, more research is needed to pinpoint the exact molecular effector and its related recognition mechanism, including the contribution of the oxidised state of the oligosaccharide (i.e. native vs oxidised oligosaccharides), including its degree of polymerisation and its oxidation site (i.e. C1 vs C4), to making a potent elicitor, all of which currently remain unclear. Furthermore, these results are *seemingly* in contradiction with previously cited studies and recent results on the AA17 LPMO (Sabbadin *et al.*, 2021b) (described later) indicating that LPMO's

action would be aimed at getting through the plant defence barriers. In the Concluding remarks section, we discuss several scenarios that could be envisioned to reconcile these observations. These results allow us to draw parallels to previously described studies demonstrating (native) chitin oligomers as agents that induce plant immune response and to their detailed mechanisms. Also, this study makes us wonder about the evolutionary relationship, if any, between LPMO-derived oxidised oligosaccharides and plant cellular receptors (Liu *et al.*, 2012; Shinya *et al.*, 2015). Interestingly, we underscore that the secretion of chemicals by the plant could also be used as a means to regulate fungal LPMO activity, as a very recent report has shown that some plant extracts (containing cinnamtannin B1) acted as a potent LPMO inhibitor (Tokin *et al.*, 2021).

2. Role of LPMOs in pathogenic oomycetes

Oomycetes are a wide group of filamentous, fungal-like microorganisms phylogenetically related to diatoms and brown algae, and causing various severe diseases in agriculture, aquaculture, as well as in natural ecosystems (Derevnina *et al.*, 2016). Oomycete genomes encode three LPMO families (AA15, AA16 and AA17). Sabbadin and colleagues have shown that the array of LPMOs depends on the host preference (Sabbadin *et al.*, 2021a): animal pathogens preponderantly harbour AA15s and AA17s, while plant pathogens mostly have AA16s and AA17s. In 2021, Sabbadin and colleagues reported on the role of an AA15 from the crayfish pathogen *Aphanomyces astaci*. Through the analysis of publicly available transcriptomic data, they showed that one of its AA15 LPMO (called *AaAA15A*)-encoding genes was differentially expressed in both sporulating and growing mycelia, compared with the zoospore stage (Sabbadin *et al.*, 2021a). *In vitro* biochemical analyses proved the chitinolytic activity of this enzyme. Complete demonstration of *in vivo* function (and substrate) remains, however, to be made. In a second study, still by Sabbadin and coworkers, on plant infection by oomycetes, gene silencing of an AA17 from *P. infestans* (*PiAA17C*) yielded a significant decrease in lesion sizes on infected potato leaves (Sabbadin *et al.*, 2021b). Combination of biochemical, structural, transcriptomic and *in vivo* experiments unambiguously supported the role of this AA17 in early stages of the *P. infestans* pathogenic cycle, with two putative, nonexclusive functions: (1) contribution to PCW penetration through the oxidation of de-esterified pectin; and/or (2) by evading plant immune system recognition by oxidising pectin-derived oligosaccharides, otherwise recognised as pathogen associated molecular patterns (Benedetti *et al.*, 2018).

3. Viral LPMO as enhancer for microbial insecticide action

Insect pests have been the cause of damage in agriculture since the beginning of the latter, calling therefore for protection measures, including the use of environmentally friendly insecticides. Microbial insecticides, despite being deemed safe for vertebrates, constitute only a small fraction of the total insecticide market (offering mainly chemical products), due to the higher associated costs, narrow spectrum of targeted insects per product, possible

inactivation in the field due to high temperatures and UV radiation and very selective and focused timely application needed for their use (Mitsuhashi, 2018). A suggested mechanism to increase the use of microbial insecticides is the use of synergistic agents that can enhance the infectivity of microbes therefore also reducing the quantity of insecticide used per square area (Mitsuhashi, 2018). Fusolins, produced by insect viruses (entomopox viruses (EVs) and baculoviruses) have been studied for this purpose (a synergistic agent) and have been known to enhance the insecticidal activity of the bacterium *Bacillus thuringiensis* (Bt) (Liu *et al.*, 2011). Spindles are crystallised EV fusolins seen in host cells, and are spindle-shaped microparticles (inclusion bodies) that are extremely stable. Fusolins and spindles both are seen to enhance EV infectivity (Mitsuhashi & Sato, 2000; Mitsuhashi *et al.*, 2007; Takemoto *et al.*, 2008). Strikingly, the globular domain found in fusolin possesses all the features of chitin-active AA10 LPMOs (Chiu *et al.*, 2015), albeit LPMO activity remains to be properly demonstrated by biochemical assays. The mechanism by which these LPMO-containing fusolins enhance microbial infectivity is proposed to be as follows: orally taken fusolins bind to the peritrophic matrix (PM, a chitin rich acellular membrane lining the gut of insects), oxidise its chitin fibrils and alter the PM conformation (Mitsuhashi, 2018). Next, these conformational changes permit the digestive proteases of the gut to further act and digest the PM, which was previously inaccessible. The PM disruption is accelerated jointly by the fusolins and proteases, and this makes any microbes or microbial toxins (such as Bt toxins) easy to pass through the PM and reach the midgut cells. Therefore, fusolins and spindles may be utilised as synergistic agents with microbial insecticide formulations, therefore reducing the use of microbial insecticides sprayed per unit area. Coexpressing fusolins with Bt toxin in crops is another way to increase insect resistance in crops (Liu *et al.*, 2011; Mitsuhashi *et al.*, 2014).

4. Insecticidal activity by a fern LPMO

It has long been noted that ferns and mosses are rarely affected by pathogenic pests or insects compared with flowering plants. Studies using extracts from the ferns *Christella parasitica*, *Pteridium aquilinum* (Eagle fern) and *Hemionitis arifolia* (Heart fern) have demonstrated that, when sprayed on *Arachis hypogaea* (commonly known as peanut plant) they can confer resistance against pests such as the moths *Helicoverpa armigera* (Old World bollworm) and *Spodoptera litura* (tobacco cutworm) (Hendrix, 1980; Sahayaraj & Selvaraj, 2013). Drawing inspiration from this, Shukla *et al.* (2016) screened several species of ferns and identified the protein Tma12 from the edible fern *Tectaria macrodonta*, which displayed insecticidal activity against whitefly, *Bemisia tabaci*. Tma12, when cloned and expressed in transgenic cotton plants (*Gossypium hirsutum*) showed 99% resistance against whitefly. Following this, a later study relying on sequence analysis, X-ray crystallography and H₂O₂ production by Tma12 showed that Tma12 possessed all the hallmarks of an AA10 LPMO (although chitinolytic activity was not assessed), which would make it the first, and so far unique, plant LPMO (Yadav *et al.*, 2019). In a separate study trying to delineate the evolution of land plants and their symbiotic relationships with

cyanobacteria using fern genomes, the authors have observed Tma12 homologues in 1000 other fern transcriptomes and phylogenetic analysis showed Tma12 to be closely related to chitin-binding proteins from bacteria. This raises the question whether these sequences were obtained by horizontal gene transfer (HGT) from bacterial species at some point during evolution as a mechanism to survive pest attack. Although this cannot be denied completely and functional HGT has been observed in other eukaryotes, there is no direct evidence for the same (Li *et al.*, 2018). These studies echo the general consensus that LPMOs could possess a wide range of functions – insecticidal, in this case – in addition to biomass conversion and an avenue to study if any HGT of LPMO genes from bacteria does exist.

5. LPMOs in mutualistic and commensalistic symbiosis

In the previous paragraphs, we have presented several cases of parasitic symbiosis. There are also situations in which the organisms at play can establish a biological interaction with mutual interest, so-called mutualistic symbiosis. The association between fungi and tree roots falls into this lifestyle. While the fungus obtains sugars from the plant, the latter receives trace elements, such as nitrogen and phosphorus, from the former that weaves a web of hyphae in the soil. Of note, most land plants on Earth rely on such mutualistic symbiosis (Martin *et al.*, 2017). In an article by Veneault-Fourrey *et al.* (2014), the authors studied the transcriptome of *Laccaria bicolor*, an ectomycorrhizal fungus – which does not penetrate the plant cells themselves, in contrast with endomycorrhiza – during symbiosis with the California poplar *Populus trichocarpa*. The study revealed that three of the genes coding for AA9 LPMOs were expressed during the period of first contact and in the final period, that is during the formation of the Hartig network. A soft remodelling of the PCW is likely to occur via the concerted ‘loosening action’ of AA9 LPMOs, GH5 and GH12 endoglucanases (Zhang *et al.*, 2018), together with the modification of pectin in the middle lamella by other CAZymes. Intriguingly, LPMO-like X325 proteins presented above were shown (by immunolocalisation) to be exposed at the interface between fungal hyphae and tree rootlet cells (Labourel *et al.*, 2020). The copper transport function of some X325 orthologues (BIM1 story, described earlier), and the importance of copper trafficking in mycorrhizae, to notably prevent transition metal toxicity, could pinpoint at a role of X325 in establishing a safe symbiotic interface. In agreement with this hypothesis, SymB, an X325 protein from the endophytic fungus *Epichloë festucae* was shown to be a key element of a signalling network impacting hyphal cell fusion and involved in the maintenance of symbiotic interaction between *E. festucae* and the perennial ryegrass *Lolium perenne* (Green *et al.*, 2017).

LPMOs have also been proposed to be involved in a third type of symbiosis, called commensalism, during which (usually) only the host gains benefit from the interaction. For instance, the woodwasp *Sirex noctilio*, a pathogen of pine trees in North America, was reported to be assisted by bacteria belonging to the genus *Streptomyces* that provides the wasp with *ad hoc* tools, such as cellulases, to feed on the tree (Adams *et al.*, 2011). Later, Takasuka

et al. (2013) studied the *Streptomyces* sp. SirexAA-E (ActE) strain associated with the wasp, and found the presence of an AA10 LPMO (called CBM33 in the article) responsible for the degradation of cellulose and hemicellulose. Of note, while the exact mechanism of the symbiosis between *Streptomyces* and *Sirex noctilio* is not explained, the author implied that it may be widespread among insects. Another interesting example is the symbiotic relationship between the bacterium *Teredinibacter turnerae* and the marine caterpillar *Lyrodus pedicellatus*. Like *Streptomyces*, *T. turnerae* produces an AA10 LPMO that is active on cellulose, providing *L. pedicellatus* with wood digestion capacity (Fowler *et al.*, 2019).

6. Lytic polysaccharide monooxygenases in bacterial pathogenesis

Host–pathogen warfare is usually depicted as an arms race in which each antagonist attempts to be smarter than the other. Whether the pathogen plays the false friend card (e.g. biotrophic pathogen) or clear enemy card (e.g. necrotrophic), a very common strategy deployed during early events of bacterial pathogenesis consists of impairing the host defence mechanisms via a series of (enzymatic) reactions (Medie *et al.*, 2012).

Studies on LPMOs from bacteria such as *Vibrio cholerae*, *Paenibacillus larvae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Lysobacter enzymogenes* have shown this class of enzymes to be involved in pathogenesis and virulence roles (Garcia-Gonzalez *et al.*, 2014; Loose *et al.*, 2014; Paspaliari *et al.*, 2015; Askarian *et al.*, 2021; Yue *et al.*, 2021). As virulence factors, LPMOs may be studied as potential targets for synthesis of inhibitors against the pathogen.

The causative agent of cholera, *V. cholerae*, has been studied widely and it is known that its survival in the intestine is largely dependent on its ability to adhere to and colonise the intestinal epithelial cell surfaces (Yoon & Waters, 2019). *Vibrio cholerae* secretes, via a type-2 secretion system, a tetra modular protein called GlcNAc binding protein A (GbpA), composed of an N-terminal AA10 domain, two domains involved in bacterial cell-surface binding, thereby enabling microcolony formation, and a C-terminal CBM5/12 domain capable of binding to crystalline chitin that permits the bacteria to attach to and colonise a wide range of crustaceans (Wong *et al.*, 2012; Yoon & Waters, 2019). In addition to this, GbpA was also found to oxidatively degrade various forms of chitin. GbpA-like proteins were also seen to occur in several other pathogenic bacteria, such as *Listeria monocytogenes*, *Bacillus cereus* and *Yersinia pestis*, further supporting their role in virulence (Loose *et al.*, 2014).

Following this, another interesting study was performed in *Paenibacillus larvae*, the causative agent of the epizootic American Foulbrood affecting honeybees, with a worldwide dramatic ecological impact (Genersch, 2008). This infection that kills honey bee larvae has been widely investigated and Garcia-Gonzalez and colleagues have presented evidence of an AA10 LPMO domain-containing protein, *PICBP49*, to be a key virulence factor of *P. larvae* (Garcia-Gonzalez *et al.*, 2014). To invade the host, the bacterium needs to weaken or degrade the PM, therefore enabling the direct access of bacterial toxins to the gut epithelial cells of the

honeybee larvae. *PICBP49* was demonstrated to have chitinolytic activity on crystalline chitin and colloidal chitin and was inhibited by copper chelators (e.g. EDTA or cyanide). Using Δ *PICBP49* mutants, the authors could show that the bacterial virulence was significantly reduced (95% survival rate of larvae), and the PM was virtually unaltered, therefore cementing *PICBP49* as a key virulence factor of *P. larvae* (Garcia-Gonzalez & Genersch, 2013; Garcia-Gonzalez *et al.*, 2014).

Listeria monocytogenes, causing the deadly foodborne disease listeriosis, is the next bacterial pathogen under discussion. This organism possesses a complete chitinolytic machinery comprising two GH18 chitinases, ChiA and ChiB, and a multimodular *LmLPMO10* (Paspaliari *et al.*, 2015). *LmLPMO10* is a ‘Gbp’ type protein with the ability to bind to α - and β -chitin and cellulose, but active only on α - and β -chitin yielding C1-oxidised products. Unlike chitinases, *LmLPMO10* secretion does not increase in a chitin-containing medium, suggesting that its role is more than biomass degradation, most likely in the realm of pathogenesis and virulence, by mainly enabling bacterial adhesion to the host cell (DeRoy *et al.*, 2006; Kawada *et al.*, 2008; Tran *et al.*, 2011; Frederiksen *et al.*, 2013). Single gene deletion mutants of ChiA, ChiB and *LmLPMO10* of *L. monocytogenes* showed significant reduction in their ability to colonise murine liver and spleen compared with the wild-type strain (Chaudhuri *et al.*, 2010). This further supports the role of these enzymes as virulence factors. Experimental data unravelling the detailed mechanism of action remains to be uncovered.

In a very recent study conducted by Askarian and colleagues on *Pseudomonas aeruginosa*, a multidrug-resistant pathogen causing various infections (e.g. pneumonia or blood infection), the chitinolytic AA10 LPMO, CbpD, was found to promote the survival of the pathogen in host blood, acting therefore as a virulence factor. Deletion mutants of CbpD rendered the pathogen less lethal. Also, Δ *CbpD* mutants were more prone to cell lysis by the host ‘membrane attack complex’ (MAC) and it has been proposed that bacterial CbpD would impair the assembly of a functional host MAC. While the exact molecular mechanism of this phenomenon is yet to be established, these data once again extend the functions of LPMOs from biomass-degrading catalysts to key pathogenicity players (Askarian *et al.*, 2021).

Lysobacter enzymogenes is an interesting bacterium with the ability to produce bioactive metabolites against a wide spectrum of fungal pathogens of plants and humans. A chitinolytic AA10 LPMO secreted by *L. enzymogenes* (*LmLPMO10A*) was seen to induce the production of antimicrobial compounds called ‘heat stable anti-fungal factor’ and its analogues (Yue *et al.*, 2021). The authors further suggested a mechanism of action, yet to be fully demonstrated, for *LmLPMO10A*: the LPMO would cleave the recalcitrant chitin present in the fungal pathogen cell wall, enabling thereby the delivery of the antifungal factors via outer membrane vesicles.

7. Role of LPMOs in development and cell wall remodelling

Insects mainly feed on plant substances and the efficient digestion of the latter is therefore critical for their development. This

degradation process is achieved by their intestinal enzymes and/or the symbiotic microbes therein.

The first characterised LPMO from insects was from *Thermobia domestica* (firebrat) and became the founding member of the CAZy AA15 family (Sabbadin *et al.*, 2018). Members of this family were also observed in the genomes of several protists, crustaceans, mollusks, chelicerates, algae and oomycetes (Sabbadin *et al.*, 2018). Expressed as transcripts per kilobase million (TPM) at medium ($10 < \text{TPM} < 100$), high ($100 < \text{TPM} < 1000$) or very high ($\text{TPM} > 1000$) levels, these LPMO-encoding genes were seen to be mostly present in the midgut region. As *T. domestica* is devoid of any gut microbiome with polysaccharide degrading capacities, AA15s were suggested to play a crucial role in this process. Biochemical characterisation of two paralogues, *TdAA15A* and *TdAA15B*, showed that the former could oxidise both microcrystalline cellulose and β -chitin, while the latter was active on β -chitin only. Furthermore, *TdAA15A*, the most abundant (expression-wise) AA15 LPMO from *T. domestica*, was seen to synergise with cellulases (GH1, GH6, GH7, GH9) and chitinases (GH18), increasing therefore the yield of oligosaccharides released from cellulose or chitin, respectively (Sabbadin *et al.*, 2018).

The AA15 class of LPMOs is conserved from protists to oomycetes to insects, and were suggested to have a pre-Cambrian origin and therefore were proposed to furnish a possible explanation for the thriving of insects during the Carboniferous period (heavily colonised by plants) with the help of their gut endogenous lignocellulolytic enzymes (Sabbadin *et al.*, 2018). In addition to shotgun proteomics analysis, the analysis of public transcriptomic databases revealed that AA15s from *Drosophila melanogaster*, *DmAA15A* and *DmAA15B*, were expressed at high levels during different stages of development and during metamorphosis, and were seen to be coexpressed with genes encoding chitinolytic activities, indicating their involvement in chitin remodelling within the respiratory and digestive systems. Significantly, important phenotypic changes were observed in several *Drosophila* when AA15-encoding genes were silenced or knocked down, including defects in respiration, changes in adult morphology and lethality during pupation (Mummery-Widmer *et al.*, 2009; Hosono *et al.*, 2015). Sabbadin and colleagues proposed that the chitinolytic activities of the early insects of the order *Zygentoma*, initially used for developmental reasons, were later evolved towards cellulolytic functions (Sabbadin *et al.*, 2018; Cairo *et al.*, 2020). Here, one may draw an intriguing parallel with AA10s, in which strict chitinolytic (Vaaje-Kolstad *et al.*, 2010), strict cellulolytic (Forsberg *et al.*, 2011) and hybrid activities have been reported (Forsberg *et al.*, 2014). Indeed, an *in silico* analysis of mutation rate within the AA10 family suggested that chitinolytic AA10s would have preceded the others, which would be in the process of evolving towards different substrate specificities (Book *et al.*, 2014). The biological role of AA10s is, however, still not entirely clear, and may be context dependent.

Cell wall remodelling is a crucial aspect of microorganism life cycle, notably of fungi, which can form different cell types (spores, yeast cells and hyphae) to thrive and explore their environment. Studying the fungus *Neurospora crassa*, Gonçalves and colleagues have shown that a gene, called *cwr-1* (for cell wall remodelling),

together with another gene (*cwr-2*), was involved in a so-called cell fusion checkpoint upon cell-cell contact: that is, regulation of cell wall dissolution, blocking cell fusion upon contact between cells with nonidentical genetic backgrounds. Such regulation is important for reducing the risks of transmission of mycoviruses, senescence plasmids or defective organelles (Gonçalves *et al.*, 2019). *In vivo* inactivation of *cwr-1* altered this regulation and allowed cell fusion between formerly incompatible strains. Strikingly, sequence analysis of *cwr-1* indicated that it was most likely to be an LPMO, belonging to the chitin-active AA11 family (Gonçalves *et al.*, 2019) as it shared 44% sequence identity with its founding member, the AA11 from *Aspergillus oryzae* (Hemsworth *et al.*, 2014).

IV. Concluding remarks and future perspectives

Through this review, we have attempted to highlight how diverse can be the biological functions of LPMOs (Fig. 5). It is truly fascinating how such small, and rather structurally simple proteins have been co-opted by a wide array of living organisms (and viruses) to target nearly all major carbohydrate polymers on Earth. From the C1–Cx factors of Reese & Gilligan (1954) to the discovery of several LPMO families, the research in this area has come a long way. Whereas a decade of LPMO-dedicated research has been necessary to unveil the actual biotechnological potential, biological scope and mode of action of these copper-enzymes secreted by saprotrophic microorganisms (plant biomass-decaying and chitinivorous fungi and bacteria), new facets of LPMO research, including symbiotic relationships (parasitism, mutualism and commensalism), viral infections and developmental biology start to unfold. Excitingly, in regard to this plethora of arenas, much remains to be investigated and understood. While the LPMOs from viruses, bacteria and fungi showcase themselves to be crucial virulence factors, the LPMOs acting as insecticidal agents and those aiding insect development present them to be dextrous proteins with wide-ranging abilities.

A major challenge, and still a lacuna, in LPMO research is the paucity of work on substrates in their natural state (which often remain to be clearly identified). In general, the LPMO community usually assesses LPMOs on a range of (commercially) available substrates, which in many cases may fail to catch the right LPMO–substrate couple. Also, the recent X325/Bim1 story (García-Santamarina *et al.*, 2020; Labourel *et al.*, 2020) teaches us that we should look beyond the only prism of oxidative activity, as other functions, such as in this case copper transport, may have evolved.

Among several outstanding questions (Box 2), an intriguing observation is the high number of LPMO gene copies in fungal and oomycetes genomes. While it could suggest a crucial importance of these proteins for these microorganisms, the exact biological role/significance of this gene multiplicity is still not clear. By contrast, the seemingly virtual absence of LPMOs in plants (excepted in ferns) is another area that has not been tapped into. This question leads us also to reflect on the warfare between plants and pathogens (fungi, oomycetes or insects), and the role of LPMOs in the latter. To breach the PCW, pathogens will secrete PCW polymer-active LPMOs and hydrolases, releasing thereby (oxidised) oligosaccharides. Depending on the evolutionarily advancement in the arms race

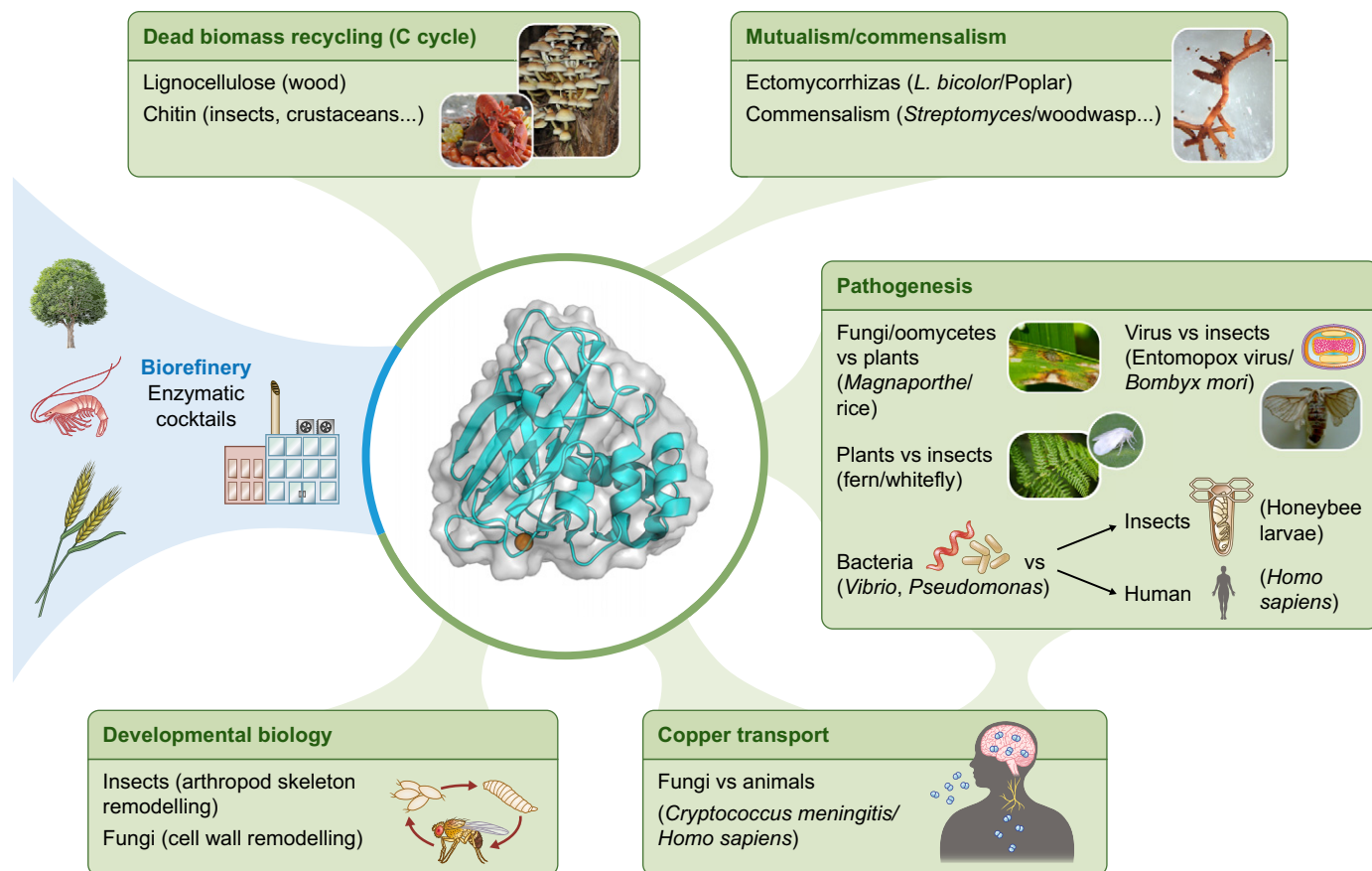


Fig. 5 Overview of the diversity of lytic polysaccharide monooxygenase (LPMO) biological functions. Historically, LPMOs have mainly been harnessed for their boost effect in biorefinery processes (blue zone) and have therefore primarily been studied in natural systems related to biomass degradation and carbon recycling. Lytic polysaccharide monooxygenases have more recently been observed to play crucial roles in pathogenesis in plants, insects and humans, while also enabling some mutualistic and commensalistic behaviours. Another emerging role is their suspected contribution cell wall remodelling during the developmental stages of arthropods and fungi.

Box 2 Outstanding questions

- Why do some microorganisms, especially plant pathogens (e.g. fungi, oomycetes), have dozens of LPMO gene copies?
- What about plant LPMOs? Why are (canonical) LPMOs absent from most plant genomes? Why, like ferns, did other plants not acquire chitin-active LPMOs to defend from fungi/insects? Did they evolve more efficient strategies?
- Does the LPMO oxidative regioselectivity (C1 vs C4) play an important role in the plant–microorganism interaction? Does it have any other biological relevance?
- On substrate specificity of LPMOs: have we already discovered all substrate specificities? What is the evolutionary relationship between *and* within the different families? As proposed for AA10s, could chitinolytic LPMOs be the overall ancestral members?
- On cosubstrate specificity: do LPMOs use H_2O_2 or O_2 in a biological context? What is their respective bioavailability? Do they use it to the same extent? How critical is the cosubstrate nature in the plant–pathogen warfare (oxidative burst)?

(e.g. if the plant is equipped with the right receptors, etc.), these molecular clues of pathogen action can be recognised by the plant, triggering therefore its immune response. Oxidised cello-oligosaccharides have been proposed to induce such response (Zarattini *et al.*, 2021) but it appears rather puzzling, in as much as LPMOs have also been proposed to be used by pathogens to hide their presence by oxidising oligosaccharides. To reconcile these seemingly contradictory observations, we hypothesise that LPMO oxidative regioselectivity (C1 vs C4) and substrate specificity (oligosaccharides vs polysaccharides) needs to be carefully considered. We underscore that C4-oxidising LPMOs are often active on oligosaccharides. Of note, the converse statement is not true as, in a very recent report, Rieder and coworkers showed that an LPMO from *Aspergillus fumigatus* (AfAA11B) was active on chito-oligosaccharides (GlcNAc)₄ (and hardly active on crystalline chitin substrates), while displaying a C1 oxidative regiospecificity (Rieder *et al.*, 2021). Nonetheless, we think that, in the vast majority of cases, C1-oxidised products can be seen as the mere consequence of the combined action of fungal polymer-active LPMOs and hydrolases, possibly used as hints to inform the plant that its cell wall is under

attack. By contrast, the oligosaccharide C4-oxidising LPMOs may be instrumental as *ad hoc* tools to 'mask' oligosaccharides derived from the action of fungal hydrolases on PCW. This hypothesis would require that C1- and C4-oxidised LPMO products are not recognised in a similar way by the plant and hints at possible new biological implications of LPMOs beyond the oxidation of polysaccharide substrates. This remains, however, to be evaluated.

Another crucial aspect pertains to the role of H₂O₂, a double-edged sword, yet central molecule. Conversely, it has been shown that H₂O₂ can efficiently fuel LPMO oxidative activity, while it can also inactivate them if used at too high a dosage (Bissaro *et al.*, 2017). In addition, H₂O₂ and other ROS are known to be secreted in abundance (oxidative burst) by plants when attacked, to an extent that depends on the host–pathogen couple (Gupta *et al.*, 2021). We believe the study of ROS fluxes, and associated redox enzymes, in the plant–microorganism interaction is indubitably a research topic of utmost importance.

Beyond their instrumental role in dead biomass decomposition, it is also suggested that LPMOs are involved at the other end of the carbon cycle, *viz.* in the establishment of the dialogue between living trees and mycorrhizal fungi, possibly in copper homeostasis and/or cell wall remodelling (Veneault-Fourrey *et al.*, 2014; Labourel *et al.*, 2020; Lebreton *et al.*, 2021). We anticipate that more research should be carried out in this direction to better seize the actual scope of action of LPMOs in carbon cycling in terrestrial ecosystems.

Overall, we have seen that trans-disciplinarity is the key to understanding the function of these different LPMO families and to obtain insights into their (co)substrate specificities, biological relevance and, in the future, unveil their biotechnological power. Following what has been achieved in the field of biorefinery, in which LPMOs have been harnessed to boost saccharification processes, we hope that our review will contribute to assessing the promising biotechnological potential that LPMOs still have to offer, notably to address some major issues in the health and agriculture sectors.

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Appendix II

The *Ustilago maydis* AA10 LPMO is
active on fungal cell wall chitin

The *Ustilago maydis* AA10 LPMO is active on fungal cell wall chitin

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ABSTRACT Lytic polysaccharide monooxygenases (LPMOs) can perform oxidative cleavage of glycosidic bonds in carbohydrate polymers (e.g., cellulose, chitin), making them more accessible to hydrolytic enzymes. While most studies have so far mainly explored the role of LPMOs in a (plant) biomass conversion context, alternative roles and paradigms begin to emerge. The AA10 LPMOs are active on chitin and/or cellulose and mostly found in bacteria and in some viruses and archaea. Interestingly, AA10-encoding genes are also encountered in some pathogenic fungi of the Ustilaginomycetes class, such as *Ustilago maydis*, responsible for corn smut disease. Transcriptomic studies have shown the overexpression of the AA10 gene during the infectious cycle of *U. maydis*. In fact, *U. maydis* has a unique AA10 gene that codes for a catalytic domain appended with a C-terminal disordered region. To date, there is no public report on fungal AA10 LPMOs. In this study, we successfully produced the catalytic domain of this LPMO (*UmAA10_cd*) in *Pichia pastoris* and carried out its biochemical characterization. Our results show that *UmAA10_cd* oxidatively cleaves α - and β -chitin with C1 regioselectivity and boosts chitin hydrolysis by a GH18 chitinase from *U. maydis* (*UmGH18A*). Using a biologically relevant substrate, we show that *UmAA10_cd* exhibits enzymatic activity on *U. maydis* fungal cell wall chitin and promotes its hydrolysis by *UmGH18A*. These results represent an important step toward the understanding of the role of LPMOs in the fungal cell wall remodeling process during the fungal life cycle.

IMPORTANCE Lytic polysaccharide monooxygenases (LPMOs) have been mainly studied in a biotechnological context for the efficient degradation of recalcitrant polysaccharides. Only recently, alternative roles and paradigms begin to emerge. In this study, we provide evidence that the AA10 LPMO from the phytopathogen *Ustilago maydis* is active against fungal cell wall chitin. Given that chitin-active LPMOs are commonly found in microbes, it is important to consider fungal cell wall as a potential target for this enigmatic class of enzymes.

KEYWORDS filamentous fungi, fungal cell wall, lytic polysaccharide monooxygenase, chitinase, *Ustilago maydis*, plant pathogen, remodeling

Lytic polysaccharide monooxygenases (LPMOs) are monocopper enzymes that catalyze the oxidative cleavage of glycosidic bonds in carbohydrate polymers. Their discovery has been a major breakthrough in the understanding of the microbial enzymatic mechanisms involved in the degradation of natural recalcitrant polymers, including cellulose and chitin (1–3). LPMOs can act at the surface of polysaccharides, in synergy with other oxidoreductases (4) and glycoside hydrolases (GHs), to overcome polymers recalcitrance factors (such as crystallinity), thereby boosting bioconversion yields (5–7). The unique catalytic properties of LPMOs make them of utmost interest for different types of applications, such as the valorization of lignocellulosic biomass for

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the production of bioproducts (e.g., biofuels) (8, 9) or bio-based materials (10–12). So far, most studies have explored the role of LPMOs from a biotechnology perspective. Excitingly, studies reported over the past few years have shown that LPMOs are present across nearly all kingdoms of life, encompassing thus a wide range of biological contexts, and pointing at the emergence of new roles and paradigms (13).

LPMOs are classified into eight auxiliary activity (AA) families in the carbohydrate-active enzymes (CAZy) database: AA9–AA11 and AA13–AA17 (13, 14). Although their substrate specificity varies among AA families, they are mostly active on cellulose, chitin, xylan, starch, or pectin. The AA10 family is the most taxonomically diverse family with sequences originating from bacteria (15–23), viruses (24), and archaea (25). Interestingly, the presence of AA10s goes beyond the prokaryotic domain of life, as they have recently been shown to be present in some plants (ferns) (26, 27) and in some pathogenic fungi. All AA10 LPMOs hitherto characterized have been found to be active on chitin (C1-oxidizers) and/or cellulose (C1- and C1/C4-oxidizers) (1, 3, 16). Of note, AA10s are often tested on model substrates such as shrimp/crab chitin or pretreated cellulose, and very little is known on their actual biological substrate. The identification of the latter is crucial to get insight into the biological function of a given LPMO but such approaches are challenging and require integration of the physiology of the living organism. A few attempts have been made along this line. Recent studies have notably shown the role of AA10 LPMOs in the chitin oxidative metabolism of a marine bacterium (28), in the virulence of pathogens (29–31), or even in the remodeling of bacterial cell wall (32).

Ustilago maydis is a biotrophic parasite (smut fungus) that depends on living tissue for proliferation and development in maize. This ubiquitous pathogen is also a well-established model organism for the study of plant-microbe interactions (33, 34). Compared to fungal saprotrophs and some plant pathogens, the *U. maydis* genome contains a small set of CAZymes (35) with 107 GHs and 23 AAs. While studying CAZymes from *U. maydis* (36, 37), we noted the unusual presence of a unique AA10-encoding gene in its genome (*UmAA10*), the function of which remains unknown. Interestingly, digging into transcriptomic data collected during *U. maydis* plant infection cycle (38), we noted that this *UmAA10*-encoding gene is overexpressed. Here, we managed to produce *UmAA10* in a heterologous system and characterized it with model substrates and a more biologically relevant substrate prepared from *U. maydis* mycelium. We also probed the concerted action of *UmAA10* together with a GH18 chitinase from *U. maydis* (*UmGH18A*). Our results, analyzed in light of previously published data in the context of *in planta* infection, provide hints on the biological function of *UmAA10*.

RESULTS

Bioinformatics analysis

To get insight into the putative function of *UmAA10*, we built a phylogenetic tree using 197 AA10 amino acids' sequences from bacteria, eukaryotes (fungi and plant), and viruses (Fig. 1). All fungal AA10 LPMOs (including *UmAA10*) cluster together within a larger clade of bacterial AA10s, some of which have been biochemically characterized as chitin-active with C1 regioselectivity. This observation could indicate horizontal gene transfer (HGT) events between bacteria and fungi (see Discussion). Another interesting observation is the presence of a predicted intrinsically disordered C-terminal region (dCTR) in *UmAA10*. dCTRs are regions of unknown function encountered in most LPMO families, and which occur in ~8% of AA10 sequences (39). Interestingly, while most Ustilaginomycetes AA10s display a dCTR, they are distantly related to bacterial AA10-dCTRs. More detailed analysis of these dCTR tails could help in finding common features among fungal AA10s or among AA10s from different species. To the best of our knowledge, none of these microbial AA10s bearing a dCTR have been characterized to date.

The *UmAA10* sequence is composed of 326 amino acids, with a signal peptide (1–32), an AA10 LPMO catalytic domain (cd, 33–203), and a dCTR (204–326). The 3D structure of *UmAA10* (Fig. 2A), predicted using AlphaFold2 (40, 41), shows that the catalytic domain

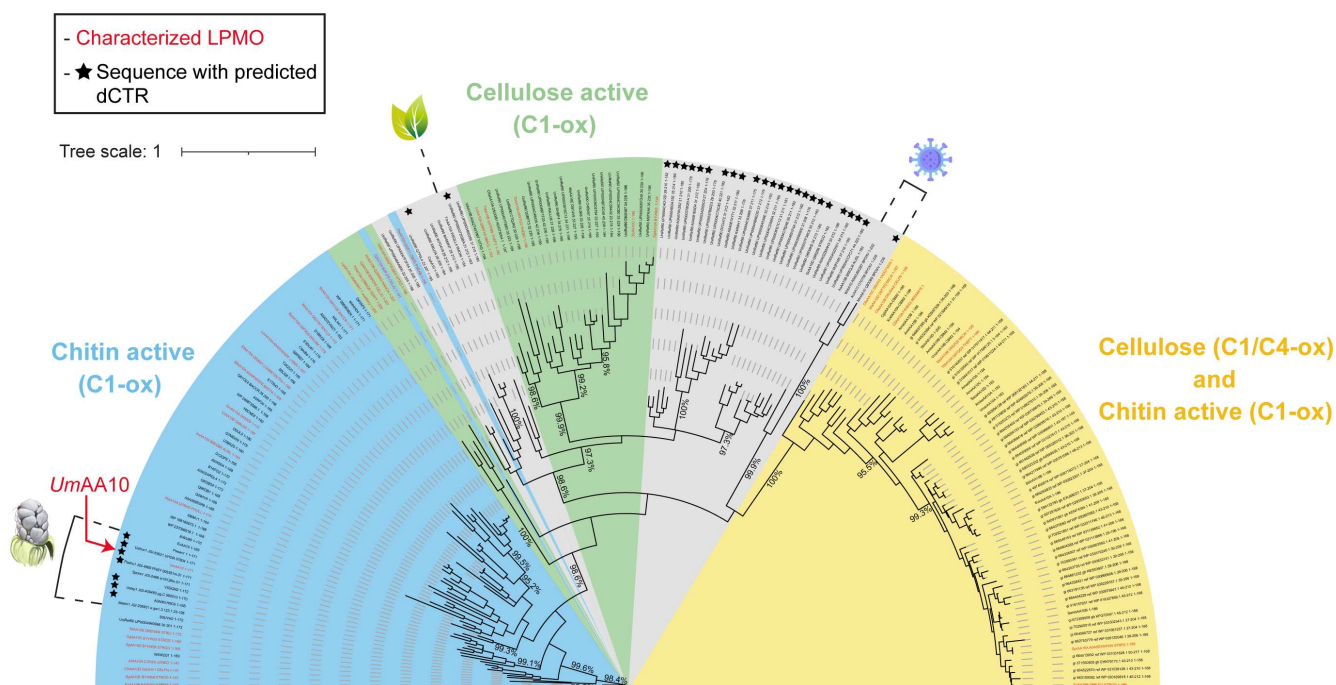


FIG 1 Phylogenetic tree of AA10s. The phylogenetic tree was generated based on a multiple alignment of 197 sequences (AA10 catalytic domain only). The predicted substrate specificity is based on the presence of characterized AA10 LPMOs (in red) within each clade. The substrate specificity of clades highlighted in gray has not been determined yet. AA10 sequences with a predicted dCTR are highlighted with a black star.

displays an immunoglobulin-like β -sandwich fold, typical of LPMOs, with a planar surface exposing the active site formed by two His residues (H33 and H118) coordinating the copper atom. Residues involved in chitin binding and LPMO activity in the archetypal AA10 LPMO from *Serratia marcescens*, SmAA10A [PDB 2BEM (15)], also known as CBP21, have been previously identified using nuclear magnetic resonance (NMR) (42), site-directed mutagenesis (15) and *in silico* quantum mechanics and molecular dynamics (43). Some of these SmAA10A key residues are also conserved in UmAA10, i.e., Gln58, Tyr59, Glu60, Gln62, Ser63, Thr115, Ala116, His118, Asn189, and Asn192 (Fig. 2B). Moreover, the "TAXH" motif (Thr115, Ala116, and His118), conserved in all chitin-active AA10 LPMOs (44), is also found in UmAA10, where X = Gln117. This Gln residue could indicate a preference of UmAA10 for α -chitin. Indeed, it was previously shown that the equivalent residue in SmAA10A (Arg113) is important for its binding preference for β -chitin (44). Altogether, these bioinformatic observations suggest that UmAA10 could target chitin.

As mentioned above, UmAA10 displays a long region (120 residues) predicted to be intrinsically disordered by three different predictors, i.e., MobiDB-lite integrated in InterPro, IUPred2A, and AlphaFold pLDDT (Fig. S1) (41, 45, 46). The length of this dCTR is close to that of the other dCTRs identified in AA10s (median value = 123 residues; Fig. S1). The dCTR of UmAA10, enriched in Ser (35%), Gly (11%), Ala (10%), Arg (8%), and Thr (8%), is overall hydrophilic with a positively charged patch (Arg-rich) at the C-terminus. Charged residues and distribution of residues of opposite charge along the sequence are one of the main determinants of the conformational properties of intrinsically disordered regions (47–49). In addition, they could be involved in protein-protein or protein-membrane electrostatic interactions. Interestingly, the last 47 residues of the UmAA10-dCTR are also predicted by ANCHOR2 (46) to be a disordered binding site. The alignment of the last 50 residues of dCTRs from 30 AA10s revealed some degree of conservation and the presence of a patch of highly positive charged residues at the end of the sequence [see Fig. S2 and reference (50)], which might suggest a common, yet unelucidated, role across fungal AA10s.

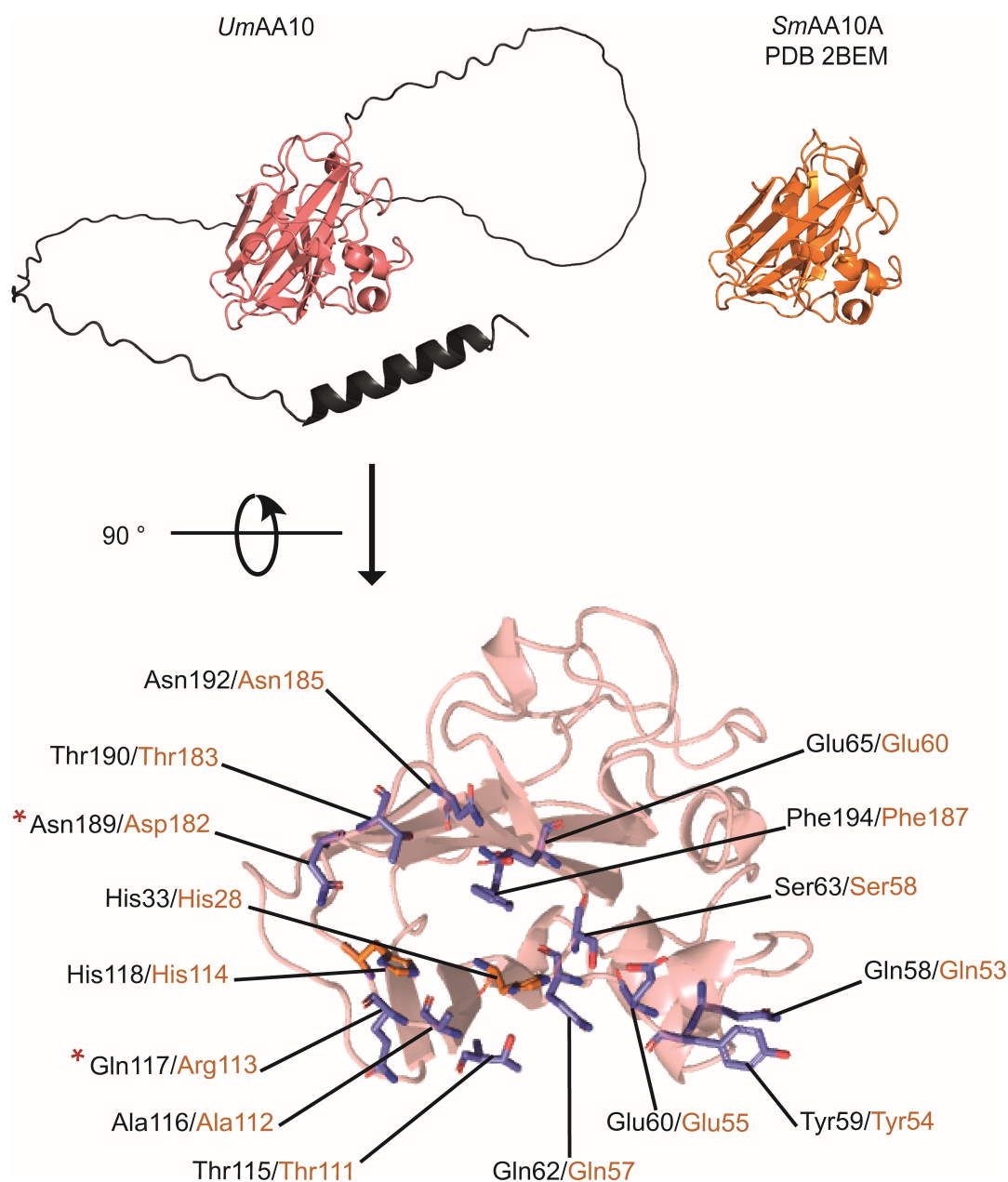


FIG 2 Structural analysis of *UumAA10*. (A) Overview of the predicted 3D structure (AlphaFold2) of *UumAA10* and of the crystal structure of *SmAA10A* [PDB 2BEM; see reference (15)]. The catalytic domain of the predicted *UumAA10* structure is shown in salmon and the C-terminal extension in black. (B) *UumAA10* predicted substrate-binding residues (shown as sticks) based on those identified in *SmAA10A*. *UumAA10* residues (labeled in black) differing from the corresponding residues occurring in *SmAA10A* (labeled in orange) are marked by a red star.

Recombinant production of *UumAA10*

To investigate the biochemical properties of *UumAA10*, we attempted its heterologous production in the yeast *Pichia pastoris*. Unfortunately, even after several attempts, the recombinant production of *UumAA10* (in its full-length form) failed, probably due to the disordered nature of the long C-term extension. Therefore, we decided to produce only the catalytic domain (hereafter referred to as *UumAA10_cd*). Interestingly, we observed upon *UumAA10_cd* recombinant production a striking difference in cells' sedimentation and cell shape/size (Fig. S3). Addition of ethylenediaminetetraacetic acid (EDTA) to the culture (to potentially inactivate *UumAA10_cd* by copper chelation) restored a "normal" sedimentation behavior of *P. pastoris* cells and partially restored cell shape/size (Fig.

S3). However, the recombinant production yield was not further improved (~0.35 mg of pure protein per liter of culture). After a two-step purification by nickel-affinity and size-exclusion chromatography, *UmAA10_cd* was purified to homogeneity. It displayed an apparent molecular weight of ~20 kDa on SDS-PAGE (Fig. S4) in good agreement with the theoretical one (19.5 kDa). After copper loading, *UmAA10_cd* contains ~1.4 copper atom per protein molecule, as determined by inductively coupled plasma mass spectrometry (ICP-MS).

Substrate specificity of *UmAA10_cd*

Before assessing the substrate specificity of *UmAA10_cd*, we first tested its ability to reduce O_2 to H_2O_2 in the presence of an appropriate reducing agent (51). Using ascorbic acid (AscA) as a reductant, we found that *UmAA10_cd* produces H_2O_2 at a rate of $3.8 \times 10^{-4} s^{-1}$, which reflects a relatively average oxidase activity, compared to other LPMOs (4) (Fig. S5). To investigate the substrate specificity of *UmAA10_cd*, we tested several relevant model substrates of AA10 LPMOs, i.e., α -chitin, β -chitin, crystalline cellulose (Avicel), and phosphoric acid swollen cellulose (PASC), with AscA being consistently used as a reducing agent. We observed the release of several products corresponding to C1-oxidized chito-oligosaccharides (DP2–DP4) from α - and β -chitin (Fig. 3A and B). No activity was detected on cellulose (Fig. S6). These results are in line

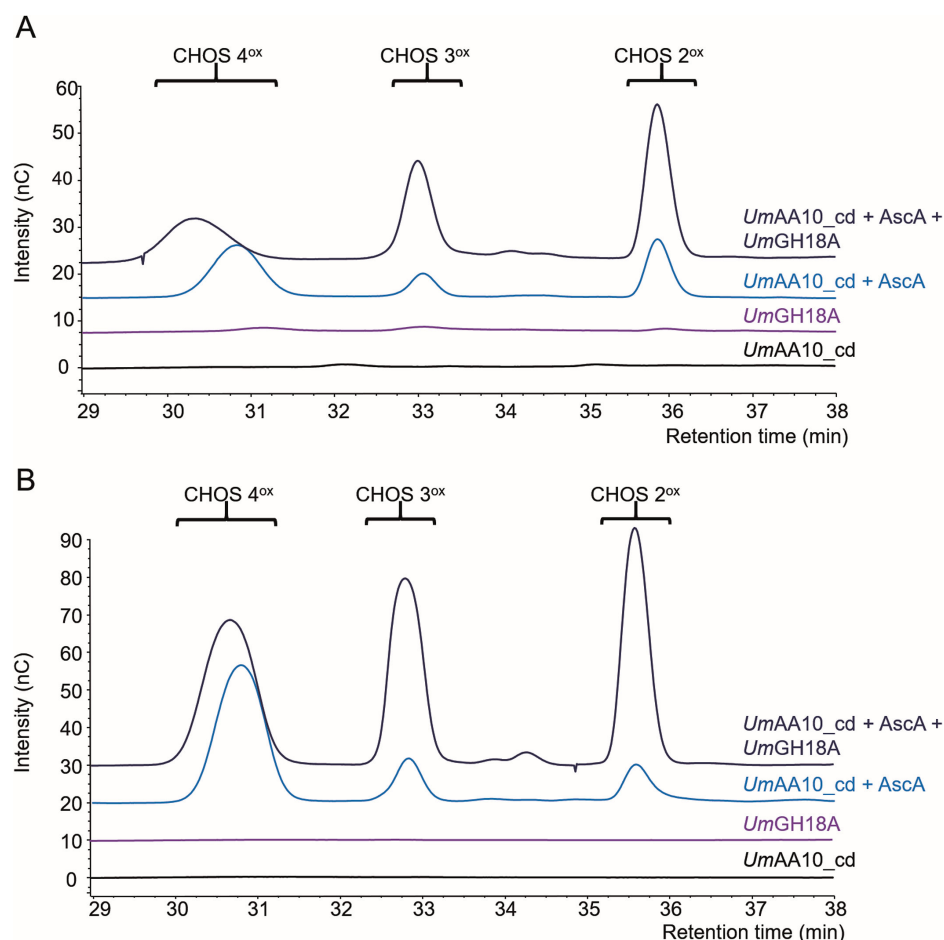


FIG 3 Activity of *UmAA10_cd* on chitin. High-performance anion-exchange chromatography-pulsed amperometric detection chromatograms of oxidized chito-oligosaccharides (CHOS) released from α -chitin (A) and β -chitin (B) by either *UmGH18A* (0.1 μ M) or *UmAA10_cd* (1 μ M), alone or combined. Reactions were carried out in sodium phosphate buffer (50 mM, pH 6.0) under stirring (1,000 rpm) at 30°C for 24 h. For each condition, three independent biological replicates were carried out and, for the sake of clarity, only one replicate is shown.

with our bioinformatic predictions (Fig. 1). To deepen our knowledge on *UmAA10_cd*, we further tested its activity in synergy with a GH18 chitinase. Indeed, many studies have demonstrated the synergistic action of LPMOs and GHs in the degradation of plant polysaccharides (52, 53), chitin (1), and other glycans such as peptidoglycan (32). In the context of our study, we hypothesized that *UmAA10_cd* could work in synergy with one of the GH18 from *U. maydis* for the degradation of chitin. The genome of *U. maydis* contains three genes coding for GH18s: *UMAG_02758* (*UmGH18A*), *UMAG_10419* (*UmGH18B*), and *UMAG_06190* (*UmGH18C*) [respectively, *cts2*, *cts1*, and *cts3* in reference (54)]. While *UmGH18A* and *UmGH18C* harbor a signal peptide, *UmGH18B* is predicted to be secreted by an unconventional pathway (55). In this study, we focused our attention on *UmGH18A*, which was previously shown to be active during yeast growth (54) and upregulated during maize infection (38). After successful production in *P. pastoris* and purification to homogeneity (Fig. S4), we evaluated its activity toward chitin. *UmGH18A* displayed optimal activity at 40°C and pH between 4 and 5 (Fig. S7), and exhibited

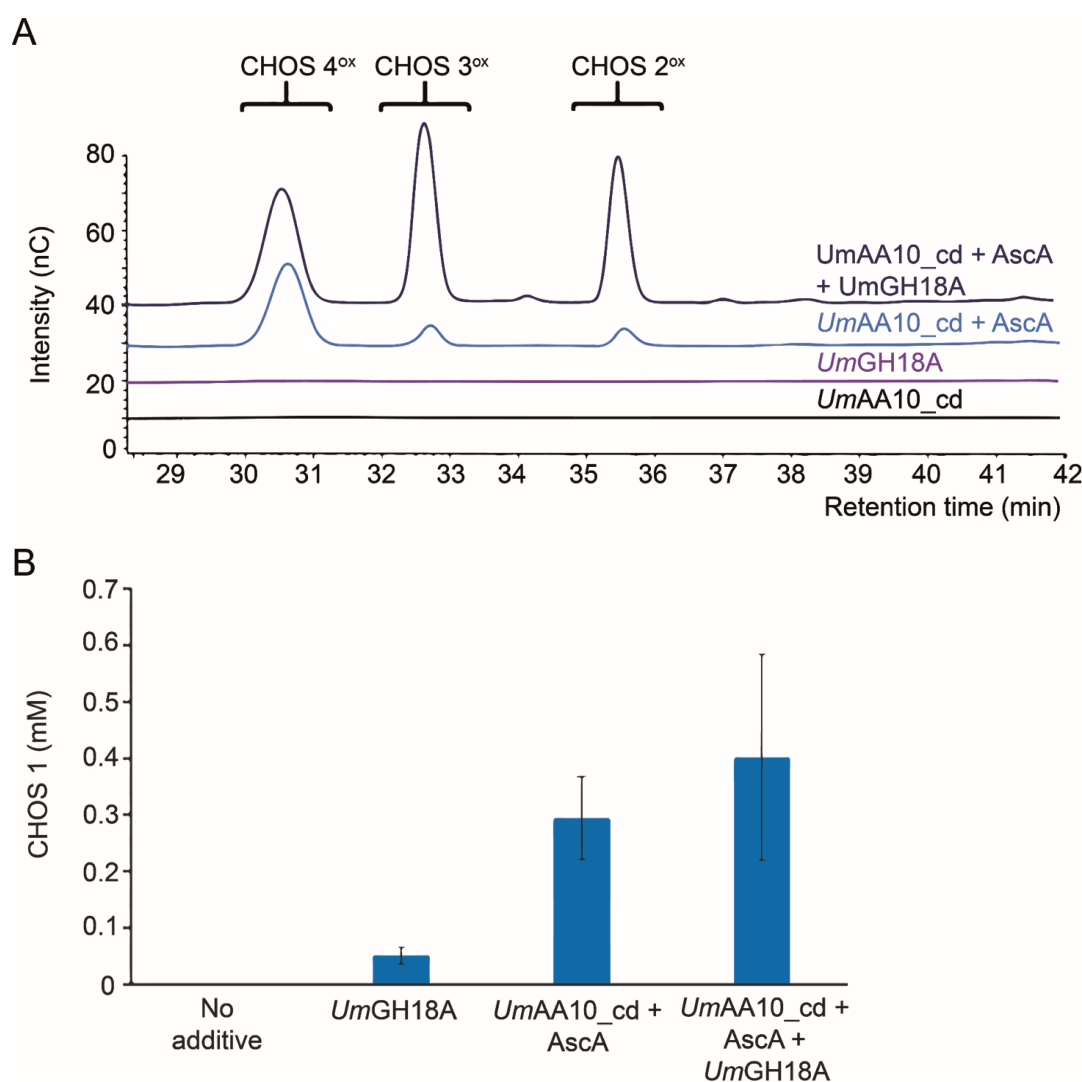


FIG 4 Activity of *UmAA10_cd* on *U. maydis* fungal cell wall chitin. (A) The graph shows high-performance anion-exchange chromatography-pulsed amperometric detection chromatograms of oxidized CHOS released from *UmFCW* (10 g/L) by either *UmGH18A* (0.1 μ M) or *UmAA10_cd* (1 μ M) and AscA (1 mM), alone or combined. (B) Quantification of total soluble products (from the reaction described in panel A). A GH20 chitinase from *Serratia marcescens* (*SmGH20*) was used to degrade the mixtures of CHOS to GlcNAc (CHOS 1) and GlcNAc-GlcNAc1A (CHOS 2^{ox}), as described by Loose et al. (17). All reactions were carried out in sodium phosphate buffer (50 mM, pH 6.0) under stirring (1,000 rpm) at 30°C for 24 h. Data points show average values and error bars correspond to standard deviations from three independent biological replicates.

exo-acting hydrolytic activity on α - and β -chitin, releasing chitobiose (CHOS 2) as a major product (Fig. S8). Interestingly, *UmGH18A* seems to be more active on β -chitin.

It is now clearly established that the preferred co-substrate of LPMOs during the oxidative cleavage of polysaccharides is H_2O_2 (56, 57). LPMOs have also been reported to exhibit a peroxidase activity in the presence of specific organic reductants and H_2O_2 (58). However, using reaction conditions described by Bissaro et al. (44) and Breslmayr et al. (58) (see Materials and Methods for further details), we could not detect any significant peroxygenase activity (on α -chitin) or peroxidase activity (on 2,6-dimethoxyphenol [DMP]) (data not shown). This result is possibly due to an extra-sensitivity of *UmAA10_cd* to H_2O_2 as it has been shown that LPMOs are subject to oxidative inactivation (56, 59, 60), a phenomenon that depends on various reaction conditions.

Enzymatic assays on a biologically relevant substrate

To go further into the biochemical characterization *UmAA10_cd*, we aimed at testing it on a more biologically relevant substrate. As plant cell wall is devoid of chitin, the most obvious source of chitin in the environment of *U. maydis* is its own cell wall, which is remodeled during pathogenesis. We therefore prepared a fraction containing chitin from *U. maydis* mycelium produced at the filamentous stage (see Materials and Methods). The presence of chitin in the alkali-insoluble fraction was confirmed by compositional analysis and by fluorescence microscopy using the labeled wheat germ agglutinin (WGA) lectin (Fig. S9), a reagent commonly used to study chitin and chitin-like molecules (61). Taking advantage of this *U. maydis* fungal cell wall fraction (*UmFCW*), we were able to assay the activity of *UmAA10_cd* on a more natural form of chitin. As previously shown on model chitin, *UmAA10_cd* was found to exhibit enzymatic activity on *UmFCW* with the release of C1-oxidized CHOS. We also provide experimental evidence that *UmAA10_cd* acts in concert with *UmGH18A* on *UmFCW* increasing the overall release of soluble CHOS (Fig. 4).

DISCUSSION

In this study, we demonstrate, using complementary approaches, that *UmAA10* is an LPMO that oxidatively cleaves *U. maydis* FCW extracts containing chitin, and that acts together with a GH18 chitinase from *U. maydis* (*UmGH18A*). We would like to emphasize that this study reports the first characterization of a fungal LPMO belonging to the AA10 family and the first biochemical evidence of LPMO activity toward FCW chitin. The appearance of AA10 LPMOs in the fungal kingdom may be ascribed to HGT. In fact, HGT between bacteria and eukaryotes has been documented for cell wall degrading enzymes (62, 63) and it is well known that HGT can provide a new function or replace a functional loss in the recipient organism, allowing adaptation to its environment (64). It would therefore be interesting to further investigate these events by focusing on the fungal members of the AA10 family.

On the basis of transcriptomic data from Lanver et al. (38), we know that the gene encoding *UmAA10* is overexpressed during advanced stages of maize infection, i.e., between 4 and 12 days post-infection (dpi) (Fig. 5A), when we observe major morphological changes that entail a fast and massive rearrangement of *U. maydis* cell wall with modification of FCW polysaccharides, including chitin. The FCW is a key adaptable scaffold for the survival of the fungus notably during host infection (65). The structural and mechanical properties of the FCW allow the fungus to resist the turgor pressure necessary for polar growth (66) and to withstand environmental stresses (67, 65, 68). While the mechanisms involved in this cell wall biochemical changes are still poorly known, some CAZymes from *U. maydis*, such as chitinases (54) and chitin deacetylases (69), have been shown to be involved in FCW modification and virulence. However, to date the role of oxidative enzymes in FCW modification has not been deeply investigated in a chitinolytic context. Of note, we recently characterized two CAZymes from *U. maydis* that act in synergy on β -glucans, namely a glucanase (*UmGH16_1A*) active on β -1,3-glucans branched with short β -1,6 substitutions and a dehydrogenase (*UmAA3_2A*)

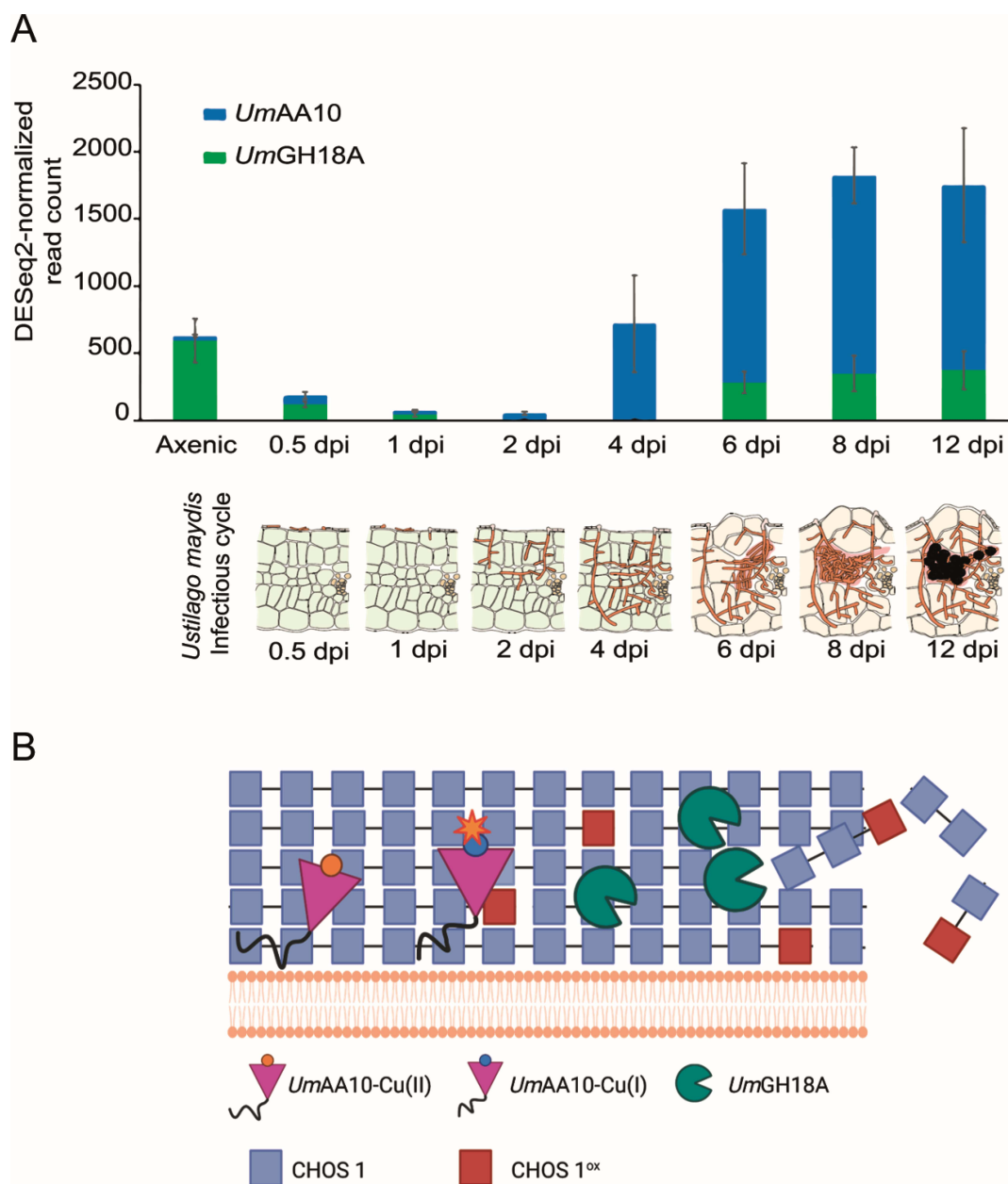


FIG 5 Putative function of *UmAA10* *in vivo*. (A) Normalized expression of genes coding for *UmAA10* and *UmGH18A* during corn infection, illustrated with data adapted from Lanver et al. (38). Under each bar graph, is presented the corresponding *U. maydis* infection cycle stage. dpi: day post-infection. (B) Schematic view of the proposed cooperation between *UmGH18A* and *UmAA10* *in vivo*. The figure was created using BioRender.com.

active on β -1,3 and β -1,6-gluco-oligosaccharides released by the former (37). As these substrates are also FCW components, we believe these enzymes could play a role in FCW remodeling.

In a different context, it was recently discovered that a chitin-active AA11 LPMO from *Neurospora crassa* (CWR-1) is involved in cell-cell allorecognition and cell wall dissolution during cell fusion (70). Additionally, another fungal LPMO from the AA9 family (CnCEL1), which harbors a dCTR and is localized at the cell surface, has been suggested to be involved in cell wall integrity and cell cycle of the human pathogen *Cryptococcus neoformans* (71). Beyond the fungal kingdom, a recent study similarly revealed the involvement of an AA10 LPMO from *Streptomyces coelicolor* (named as LpmP by the authors) in cell wall remodeling (32). This LPMO is retained in the bacterial cell wall and facilitates peptidoglycan degradation by lysozyme. Together with our findings, it

suggests that the remodeling of microbial cell wall polysaccharides by LPMOs may be widespread in nature. Considering the robustness of FCW chitin (72) and while *U. maydis* displays no AA11 LPMO and a unique chitin-active AA10 LPMO, we can hypothesize that *UmAA10*, in addition to *U. maydis* chitinases, could be involved in the modification of chitin upon cell wall remodeling and morphological changes during maize infection (Fig. 5). Despite the need for functional *in cellulose* validation in *U. maydis*, this proposed biological role is supported by the presence of a dCTR that could anchor *UmAA10* in the FCW and by the phenotype observed during recombinant production of *UmAA10_cd* that suggests modification of the yeast cell wall by LPMO.

Conclusion

In the present study, we identified and characterized the catalytic domain of the first fungal LPMO belonging to the AA10 family. This LPMO, which is unique in the genome of *Ustilago maydis*, was found to be active on model chitin but also on chitin-containing *UmFCW* extracts. Importantly, we showed that the LPMO activity contributes to chitin hydrolysis by an endogenous GH18 chitinase (*UmGH18A*). We believe that the acquisition by the plant pathogen *U. maydis* of such unique oxidative activity, possibly by HGT, is not fortuitous and plays an important role in the fungus biology.

MATERIALS AND METHODS

Materials

Most chemicals, including Avicel and horseradish peroxidase (HRP), were purchased from Sigma-Aldrich. Chito-oligosaccharides were purchased from Megazyme (Wicklow, Ireland). Both α - and β -chitin were purchased from Mahtani Chitosan (Gujarat, India). PASC was prepared as described in reference (73).

Bioinformatic analyses

To build up the phylogenetic tree, 197 AA10 amino acids' sequences (catalytic domain only), including nine sequences from fungi (Ustilaginomycetes) and 46 sequences of characterized AA10 LPMOs, were selected. Multiple sequence alignment was performed using the MAFFT-L-INS-i methods via the MAFFT online platform (74). The phylogenetic tree was generated with IQ-TREE online platform (75), with the maximum likelihood method. The Whelan and Goldman amino acid substitution model was selected (76). Branch support was calculated by 1,000 Bootstrap repetitions (values displayed in percent on the tree). The tree was visualized in iTOL (77) and edited in Illustrator CC 2017. The C-terminal regions of unknown function were extracted and analyzed with the disorder predictor IUPred2A (46). The program returns a score between 0 and 1, which represents the probability for each residue of being part of a disordered region (score ≥ 0.5). *UmAA10* C-terminal region was further analyzed with MobidB-lite 3.0 using default parameters and the AlphaFold pLDDT score [values ≤ 68.8 ; see reference (78)]. The properties of the dCTRs were derived from their primary sequence through the toolkit localCIDER (79).

Cloning and production of recombinant enzymes

The proteins were produced using the in-house 3PE platform (*Pichia pastoris* protein express; www.platform3pe.com/) as described in reference (80). The nucleotide sequence coding for the AA10 of *Ustilago maydis* (Genbank ID [XP_011391789.1](https://www.ncbi.nlm.nih.gov/nuclot/XP_011391789.1)) was codon optimized and synthesized (GenScript, Piscataway, USA) for expression in *Pichia pastoris* (syn. *Komagataella phaffii*). The region corresponding to the native signal sequence was kept, and upon production of the catalytic domain only (*UmAA10_cd*), the C-terminal extension region was removed (at position corresponding to between Gly203 and Gly204). The genes encoding *Serratia marcescens* GH20 (*SmGH20*) (Genbank

ID [Q54468.1](#)) and *U. maydis* GH18A (*UmGH18A*) (Genbank ID [XP_011389118.1](#)) were synthesized after codon optimization for *P. pastoris* (GenScript) using the full-length native sequence. Each gene was inserted into the expression vector pPICZaA (Invitrogen, Cergy-Pontoise, France) with a C-terminal (His)₆-tag. PmeI-linearized pPICZaA recombinant plasmids were used to transform by electroporation competent *P. pastoris* cells (SuperMan5 for *UmAA10_cd* and *SmGH20*, and X33 for *UmGH18A*). Zeocin-resistant transformants were then screened for protein production. The best-producing transformants were grown in 2 L of buffered glycerol-complex medium (BMGY) (10 g/L yeast extract, 20 g/L peptone, 3.4 g/L yeast nitrogen base without amino acids, 10 g/L ammonium sulfate, 10 g/L glycerol, potassium phosphate buffer pH 6.0, biotin 0.4 mg/L) in flasks at 30°C in an orbital shaker (200 rpm) for 16 h to an optical density (OD_{600}) of 2–6. Expression was induced by transferring cells into 400 mL of buffered methanol-complex medium (BMMY) (10 g/L yeast extract, 20 g/L peptone, 3.4 g/L yeast nitrogen base without amino acids, 10 g/L ammonium sulfate, potassium phosphate buffer pH 6.0, biotin 0.4 mg/L). The medium was supplemented (initially and everyday) with 1% (vol/vol) methanol at 16°C for *UmAA10_cd* and 3% (vol/vol) methanol at 20°C for *SmGH20* and *UmGH18A* in an orbital shaker (200 rpm) for another 3 days. The cells were harvested by centrifugation (5,000 rpm, 10 min at 4°C), and just before purification, the pH of the supernatant was adjusted to 7.8 with sodium hydroxide and was filtered at 0.45 μ m (Millipore, Burlington, Massachusetts, USA).

Proteins purification

Filtered and pH-adjusted culture supernatants were loaded onto a 5 mL HisTrap HP column (GE Healthcare, Bus, France) equilibrated with buffer A (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 10 mM) that was connected to an Äkta purifier 100 (GE Healthcare). (His)₆-tagged recombinant proteins were eluted with buffer B (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 500 mM). Fractions containing recombinant *UmGH18A* or *SmGH20* enzymes were pooled, concentrated, and dialyzed against Tris-HCl buffer 50 mM, pH 8.0. The concentrated sample of *UmAA10_cd* was further purified by size exclusion chromatography, using a HiLoad 16/600 Superdex 75 pg column (Cytiva, Illkirch, France) operated at 1 mL/min with sodium acetate buffer 50 mM, pH 5.2. The protein concentrations were determined by absorption at 280 nm using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Illkirch, France) and extinction coefficient (*SmGH20*: 152,555 M⁻¹/cm; *UmAA10_cd*: 32,680 M⁻¹/cm; and *UmGH18A*: 84,465 M⁻¹/cm) was determined with ProtParam (ExPASy). Protein purity was checked by analysis onto a 10% Tris-Glycine precast SDS-PAGE (BioRad, Gemenos, France), stained with Blue.

ICP-MS analysis

Copper content was analyzed using ICP-MS as described in reference (81). The samples were mineralized, then diluted in ultrapure water, and analyzed by an ICAP Q apparatus (Thermo Electron, Les Ulis, France). The copper concentration was determined using Plasmalab (Thermo Electron) software, at $m/z = 63$ with an accuracy of $\pm 5\%$.

H₂O₂ consumption and production assay

The ability of *UmAA10_cd* to produce H₂O₂ (oxidase assay) was determined by absorbance measurement, using the Amplex red assay (Thermo Fisher Scientific) and HRP, as described by Kittl et al. (51). The reaction mixture (100 μ L in 96-well microplate) contained sodium phosphate buffer (pH 7.0, 50 mM), HRP (0.1 mg/mL), Amplex red (100 μ M), and *UmAA10_cd* (1 μ M). The reaction, incubated at 23°C, was initiated by adding ascorbic acid (50 μ M) and the release of resorufin was monitored at 575 nm during 40 min. Two control reactions were carried out in the same condition: one without *UmAA10_cd* and another without ascorbic acid. The stoichiometry of the resorufin release in regard to the H₂O₂ produced is 2:1. A standard curve of H₂O₂ was performed under the same conditions.

H₂O₂ consumption was measured using two different assays. The first one was carried out following the protocol described by Breslmayr et al. (58). This assay is based on the oxidation of 2,6-DMP to coerulignone by the enzyme in the presence of H₂O₂ (peroxidase reaction). The stoichiometry of the reaction is 2 H₂O₂ consumed or 2 molecules of 2,6-DMP oxidized per coerulignone produced. Reactions were run in a 100 µL mixture, containing borate buffer (50 mM, pH 7.5), *UmAA10_cd* (1 µM), 2,6-DMP (500 µM), and H₂O₂ (100 µM). The release of coerulignone was monitored at 469 nm in a plate reader ($\epsilon_{469} = 53,200 \text{ M}^{-1}/\text{cm}$). The standard curve obtained was used to determine H₂O₂ consumption kinetic parameters. In the second reaction, H₂O₂ consumption was measured in the presence of the polysaccharide as described by Bissaro et al. (56), with a monitoring by the method described by Kittl et al. (51). Reactions were conducted in a 600 µL mixture containing sodium phosphate buffer (50 mM, pH 6.0), α -chitin as substrate (10 g/L), EDTA (50 µM), and *UmAA10_cd* (50 nM). After 10 min of incubation (30°C, 1,000 rpm), H₂O₂ (100 µM) was added to the mixture and the reaction was initiated by adding ascorbic acid (20 µM). Seventy-five microliters of the samples was taken from the mixture at 0, 5, 10, 15, 20, 30, and 60 min of reaction, filtered using a 96-well filtration plate (0.22 µm filters, Merck Millipore, Ireland) and 25 µL of each filtered sample was added to 75 µL of oxidase reagent as described above. A H₂O₂ standard curve was realized in the condition of the oxidase assay.

Enzymatic characterization of *UmGH18A*

Temperature and pH optima for *UmGH18A* were determined as described previously (82) with slight modifications. The optimal pH was determined using various buffers (50 mM), within the pH range of 3–9. The buffers used were sodium citrate (pH 3, 4, 5, and 6), sodium acetate (pH 4, 5, and 5.5), sodium phosphate (pH 6, 7, and 8), and Tris-HCl (pH 7.2, 8, and 9). While, for determining optimum temperature, the chitinase activity assay was performed under the optimum pH, within a temperature range of 20°C–70°C. In both experiments, the reaction mixture contained colloidal chitin (10 g/L) as the substrate and purified *UmGH18A* (1 µM), incubated at the respective temperature under stirring at 800rpm for 1 h. Chitinase activity was determined by analyzing the presence of reducing sugars using Schales' assay as described previously (82).

Time course degradation of crystalline chitin substrates was performed by incubating *UmGH18A* (1 µM) with α - or β -chitin (10 g/L) under the optimal conditions and shaking at 1,000 rpm. Aliquots were collected at different time points between 1 and 24 h and filtered using a 96-well filter plate (0.45 µm filters; Merck Millipore, USA) operated by a Millipore vacuum manifold. The filtered samples were then mixed with an equal volume of 70% acetonitrile and analyzed using high-performance liquid chromatography (HPLC). The products obtained were separated on Shim-pack GIST NH2 column (5 µm, 4.6 × 250 mm, Shimadzu, Japan), through isocratic elution using 70% acetonitrile, with a 0.7 mL/min flow rate. Throughout the analysis, the column oven temperature was maintained at 45°C, and the products were detected at 210 nm. Quantification of the CHOS was performed essentially as described earlier (83).

Enzymatic characterization of *UmAA10_cd*

To assess the enzymatic activity of *UmAA10_cd*, reactions were performed on model substrates (α - and β -chitin, Avicel, and PASC) and *UmFCW* extract. Activity on model chitin and *UmFCW* was carried out in 300 µL reaction mixture containing *UmAA10_cd* (1 µM), sodium phosphate buffer (50 mM, pH 6.0), and substrate (α - or β -chitin, or *UmFCW*; 10 g/L), which were incubated for 30 min (30°C, 1,000 rpm) before adding ascorbic acid (1 mM) and *UmGH18A* (0.1 µM). Reactions were incubated at 30°C (1,000 rpm) during 24 h and stopped by heat at 100°C during 10 min and then filtered at 0.22 µm using a 96-well filtration plate (0.22 µm filters, Merck Millipore, Ireland). To quantify released chito-oligosaccharides, *SmGH20* (1 µM) was added to the filtrate reaction and the reaction was run for 5 h (30°C, 1,000 rpm) and stopped by heat (100°C, 10 min). Standard C1-oxidized CHOS was prepared as described in reference (84). Activity

tests on cellulose (Avicel or PASC) were carried out in 300 μ L reaction mixture containing *UmAA10_cd* (1 μ M), sodium phosphate buffer (50 mM, pH 6.0), and Avicel (10 g/L) or PASC (0.1%), which were incubated for 30 min (30°C, 1,000 rpm) before adding ascorbic acid (1 mM). Reactions were incubated for 24 h (30°C, 1,000 rpm), stopped by heat (100°C, 10 min), and then filtered using a 96-well filtration plate (0.22 μ m filters, Merck Millipore, Ireland). All reactions were carried out in triplicate. Enzymatic reactions were diluted 10 times, and analyzed using a high-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) (Dionex ICS6000 system, Thermo Fisher Scientific, Waltham, MA, USA). The system is equipped with a CarboPac-PA1 guard column (2 $\times$ 50 mm) and a CarboPac-PA1 column (2 $\times$ 250 mm) kept at 30°C. Elution was carried out at a flow rate of 0.1 mL/min and 25 μ L of the sample was injected. The solvents used were NaOH (100 mM; eluent A) and NaOAc (1 M) in NaOH (100 mM; eluent B). The column was preconditioned with 1.4% eluent B for 24 h, and then the following gradient was applied: 0–10 min, 1.4% B; 10–32 min, 1.4%–14% B; 32–46 min, 1.4% B. Integration was performed using the Chromeleon 7.2.10 chromatography data software.

***Ustilago maydis* growth conditions and cell wall preparation**

The *U. maydis* strain [521/FGSC 9021; see reference (33)], which was provided by the CIRM-CF collection (strain CIRM-BRFM1093) (85) was grown in 100 mL of yeast extract peptone dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) for 48 h at 28°C in 250-mL baffled Erlenmeyer flasks under orbital agitation (150 rpm). Cells were then harvested, washed once in H₂O by centrifugation (1,500g, 10 min), and stored at –80°C in 20% glycerol at 10⁷ cells/mL. To produce material for sequential extraction (Fig. S9), eight Roux flasks containing 180 mL of YPD medium were inoculated at 10⁵ cells/mL and incubated for 17 days at 28°C. The resulting mycelium was then harvested and washed three times with H₂O by filtration on Miracloth and lyophilized. Five grams of this material was resuspended in 500 mL H₂O, homogenized using Ultra-Turrax (13,500 rpm, 2 min), and boiled for 3 h. After centrifugation (6,000g, 10 min), the supernatant was discarded and the pellet was resuspended in 500 mL of NaOH (1.25 M) for 4 h at 60°C. After another centrifugation step, the alkali insoluble residue was washed three times using centrifugation (8,000g, 20 min) in 1 L of H₂O. The pellet referred to as *UmFCW*, which contains chitin, was lyophilized, weighted (967 mg), resuspended in H₂O in 1:1 ratio (wt/vol), and stored at 4°C until further use.

Compositional analysis of fungal cell wall polysaccharides

Identification and quantification of polysaccharide neutral sugars were performed by gas-liquid chromatography (GC) after sulfuric acid degradation as described in reference (86). Briefly, 5 mg of dried alkali insoluble fraction was dispersed in 2 N trifluoroacetic acid and then hydrolyzed 90 min at 121°C. Neutral monosaccharides were converted to alditol acetates and analyzed on a TG-225 GC Column (30 $\times$ 0.32 mm ID) using TRACE Ultra Gas Chromatograph (Thermo Scientific; temperature 205°C, carrier gas H₂). Standard sugars' solution and inositol as internal standard were used for calibration.

Glucosamine residues were quantified after acid hydrolysis [adapted from reference (87)] and HPAEC-PAD analyses [adapted from reference (88)]. Briefly, 5 mg of dried alkali insoluble fraction was dispersed in acetic acid 1% and hydrolyzed in concentrated HCl 10 M at 105°C during 6 h. After dilution in milli-Q water, glucosamine residues were quantified by HPAEC-PAD (ICS-6000, Thermo Scientific) using a CarboPac PA20 column (2 $\times$ 250 mm, Thermo Scientific), thermostated at 30°C. An isocratic elution of 1.7 mM sodium acetate (NaOAc) in 1 mM NaOH was used at a 0.25 mL/min flow rate. Standard glucosamine solutions were used for calibration.

Labeling of fungal cell wall chitin

Fungal cell wall chitin was labeled using the lectin WGA-AF488 (wheat germ agglutinin conjugated to Alexa Fluor 488; Thermo Fisher Scientific). The *U. maydis* mycelium

(~ 1 cm²) was disrupted in 1 mL of H₂O using a FastPrep homogenizer (MP Biomedicals, Illkirch-Graffenstaden, France). Fifty microliters of *U. maydis* mycelium, α-chitin, or UmFCW-AI (all at 20 g/L) was diluted 10-fold in phosphate-buffered saline (PBS) solution with bovine serum albumin (BSA) 0.1% (wt/vol) with or without WGA-AF488 at 1 μg/mL. After 1 h of incubation, samples were washed three times in PBS-BSA 0.1% and resuspended in 500 μL of PBS before imaging using an Olympus microscope BH2 with fluorescence (Rungis, France) at 500× magnification. Images were captured during 1 s using the Archimed software (v5.6.0, Microvision Instruments, Evry, France).

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ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental figures (AEM00573-23-S0001.docx). Figures S1 to S9 and additional references.

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SUPPORTING INFORMATION FOR

The *Ustilago maydis* AA10 LPMO is active on fungal cell wall chitin

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Running title: A fungal AA10 active on fungal cell wall

This file includes:

1. Supplementary Figures S1 to S9.
2. Additional References.

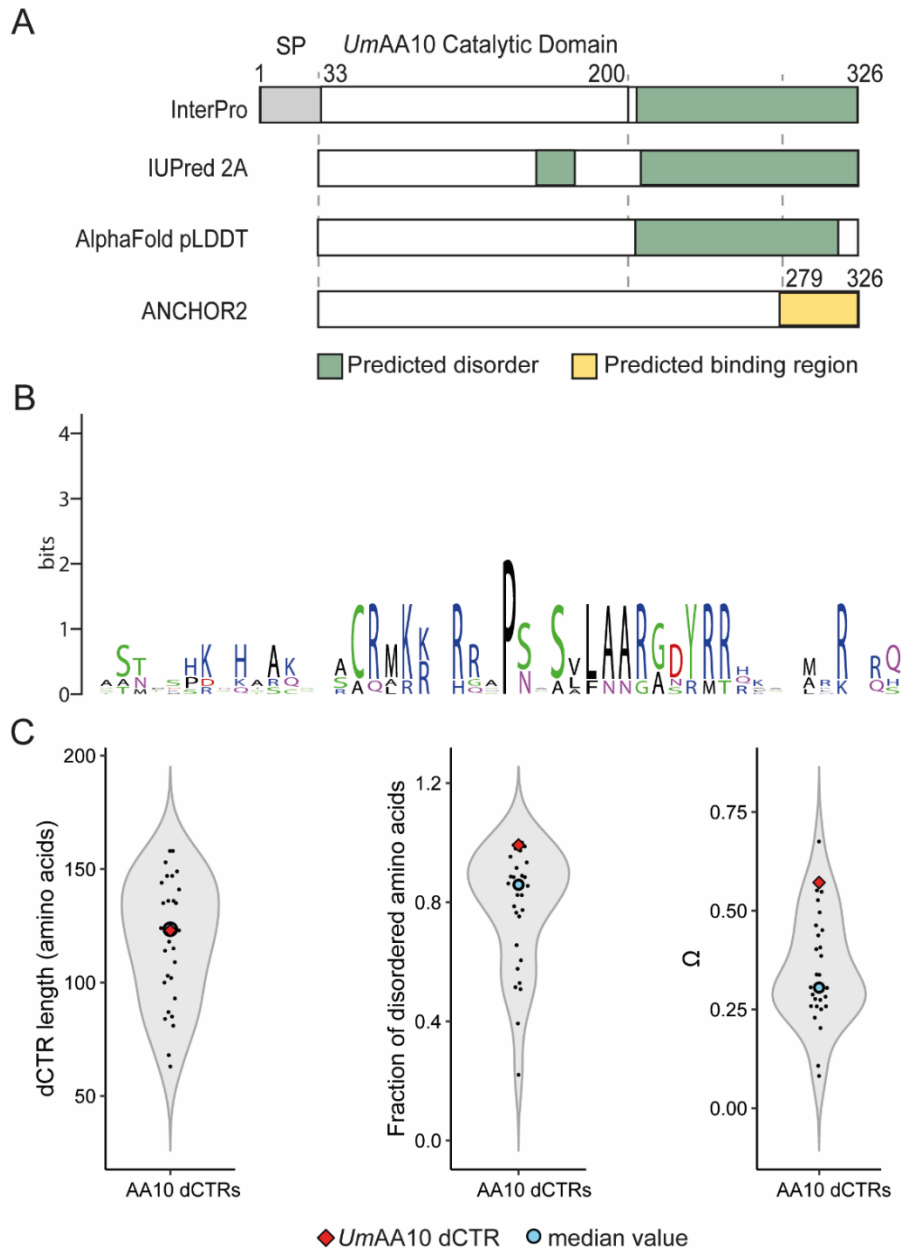


Figure S1. Disorder prediction. **(A)** Prediction of disorder and of disordered binding sites (DiBSs) as derived from Interpro, IUPred 2A (1), AlphaFold (2) and ANCHOR2 (1) Disordered regions are shown in green and disordered binding sites (DiBSs) in gold. **(B)** Amino-acid conservation of the last 50 residues across fungal AA10 LPMO-dCTR visualized using WebLogo (<https://weblogo.berkeley.edu/logo.cgi>) (3)). The x-axis indicates the position of the amino acid and the height of each letter represents the sequence conservation at the corresponding position. **(C)** On the left-hand side, violin plot showing the distribution of the length of dCTRs across AA10 LPMO dCTRs. In the middle, violin plot showing the fraction of disordered residues within dCTRs, as predicted by IUPred2A. On the right-hand side, violin plot showing the distribution of the patterning parameter Ω values of dCTRs (4). This parameter computes the distribution of Pro, Lys, Asp, Arg, Glu across the amino acid sequence: low Ω values indicate that these residues are well dispersed across the sequence, while high Ω values design their clustering. The blue dot in each violin plot represents the median length value.

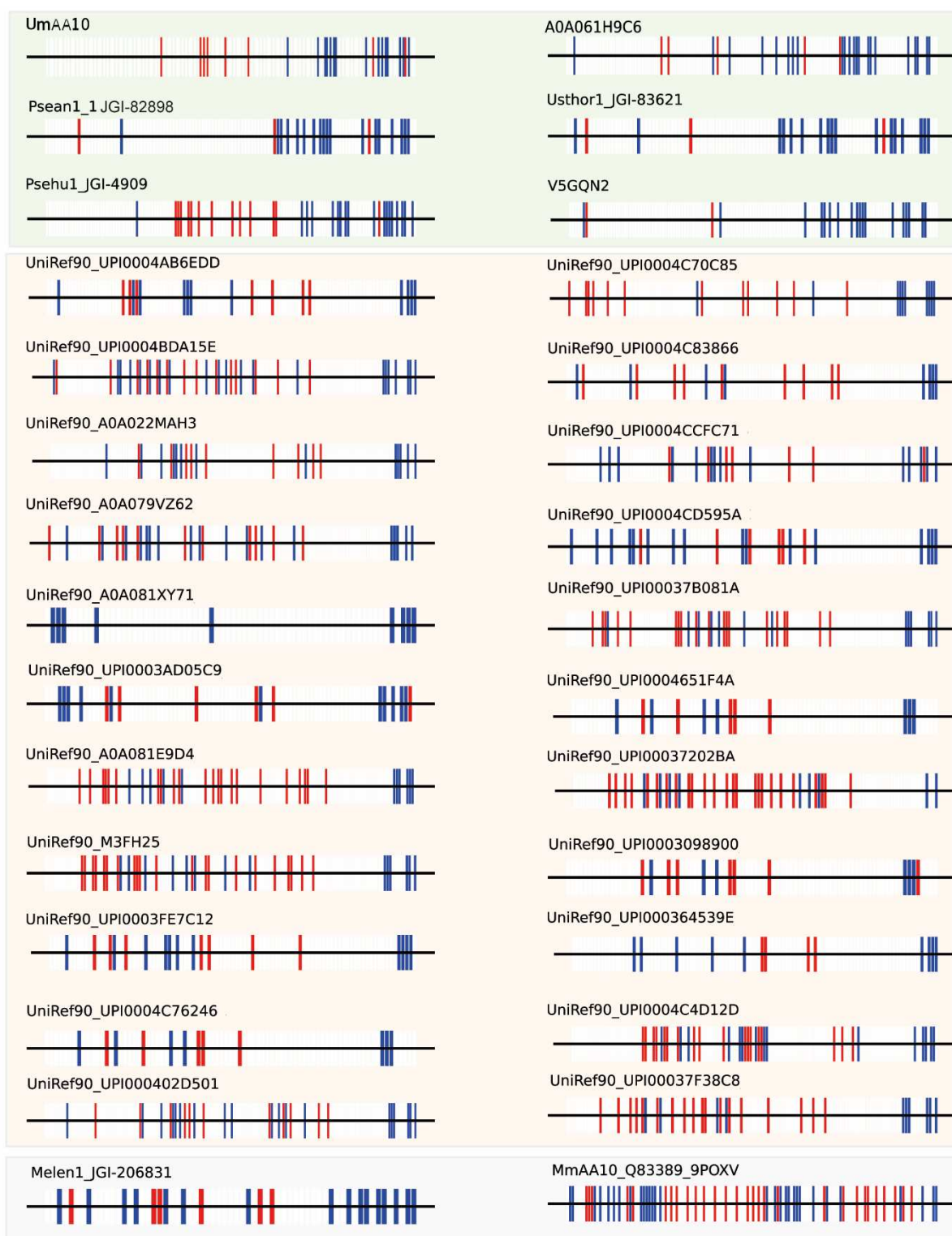


Figure S2. Distribution of positive, negative and neutral residues in the dCTRs of AA10 LPMOs. Asp and Glu residues are colored in red, Arg, Lys and His residues are colored in blue, and other residues are colored in white. Sequences shown with green and orange backgrounds correspond to dCTRs of fungal and bacterial AA10 LPMOs respectively, while the sequences shown with a grey background correspond to AA10 LPMOs from other organisms.

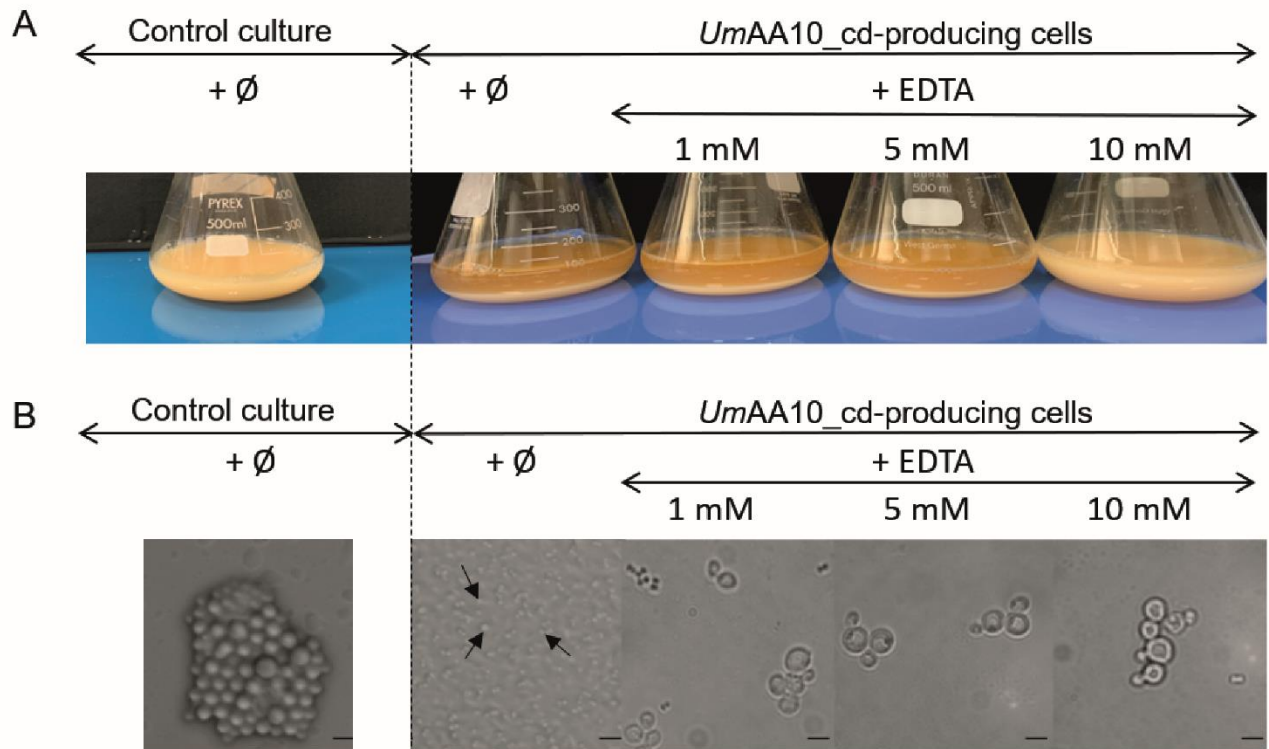


Figure S3. *UmAA10_cd* production in the *Pichia pastoris* system. (A) Cells sedimentation in culture medium at the end of the recombinant protein production (methanol media). From the left to the right: usual aspect of *P. pastoris* in culture medium (production of another recombinant protein) and *UmAA10_cd*-producing *P. pastoris* cells in similar culture conditions, where 0, 1, 5 or 10 mM of the copper-chelating agent EDTA was added. **(B)** *P. pastoris* cell morphology under “normal” conditions (left) and during *UmAA10_cd* production with increasing concentrations of EDTA. Black arrows show yeast cells. Scale bar: 20 μm .

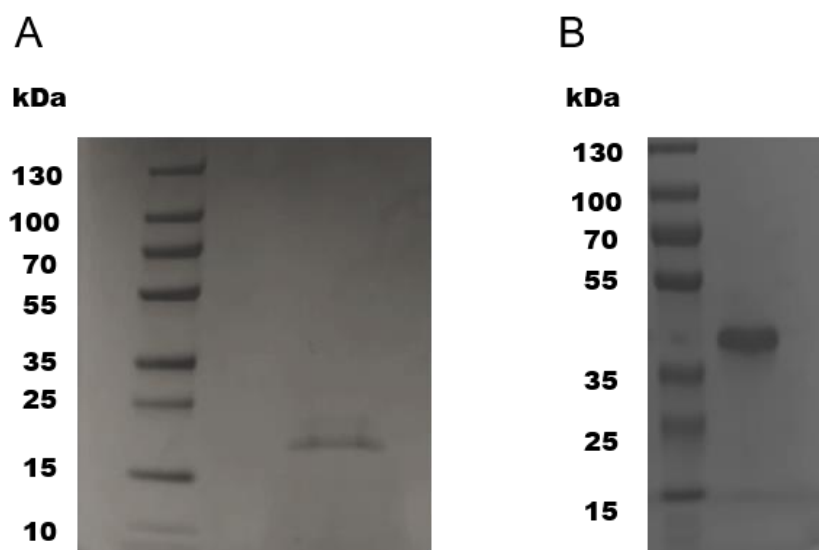


Figure S4. SDS-PAGE analysis of (A) *UmAA10_cd* and (B) *UmGH18A* after purification. The theoretical molecular mass of *UmAA10_cd* and *UmGH18A* are 19.5 kDa and 39.8 kDa, respectively.

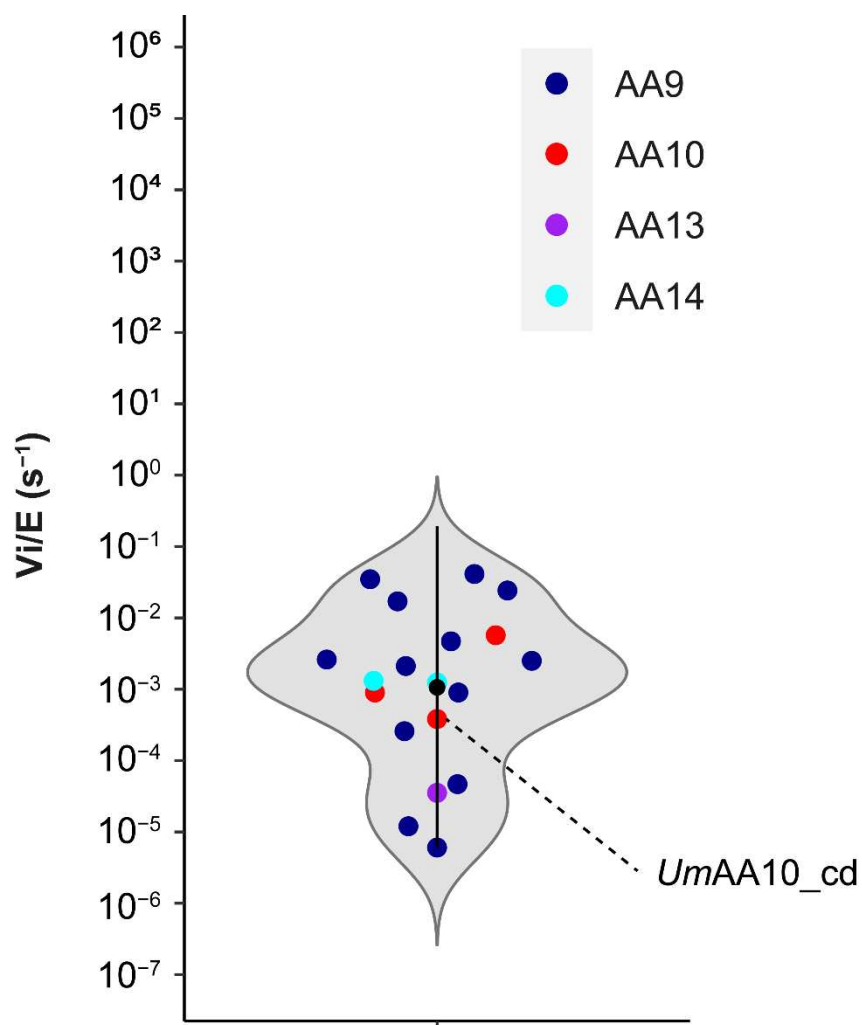


Figure S5. Violin plot of oxidase rates of LPMOs. The graph shows (log-scale) the H_2O_2 production rates (using mainly AscA as reductant) of 19 different LPMOs belonging to AA9, AA10, AA13 and AA14 families (the color code is provided in the figure). Data were taken from Bissaro et al. (5) and completed with the data obtained for *UmAA10_cd* in the present study. The black dot shows the mean value and the black line the double standard deviation.

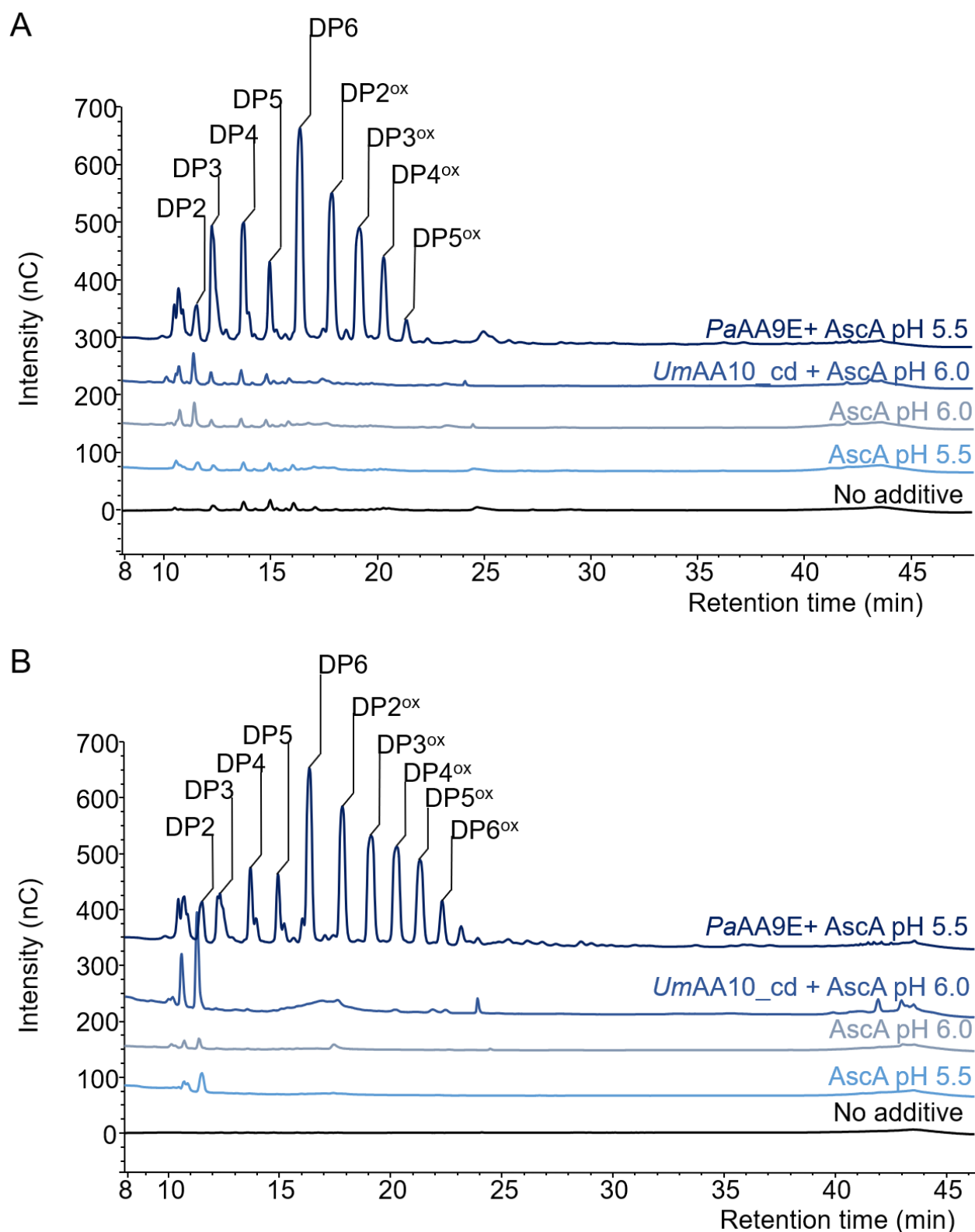


Figure S6. Enzyme assays of *UmAA10_cd* on crystalline (A) and amorphous (B) cellulose. The graphs show HPAEC-PAD chromatograms of products released from (A) Avicel (10 g.L⁻¹) and (B) PASC (0.1%). Reactions were carried out under stirring (1000 rpm) at 30°C for 24 h in sodium acetate buffer (50 mM, pH 5.5) for those containing *PaAA9E* (1 μM) or in sodium phosphate buffer (50 mM, pH 6.0) for those containing *UmAA10_cd* (1 μM). The *PaAA9E* LPMO from *Podospira anserina* (6) was used as a control enzyme. Main products are cello-oligosaccharides of degree of polymerization (DP) from 2 to 6, either non-modified (DP1 to DP6) or C1-oxidized (DP1^{ox}-DP6^{ox}).

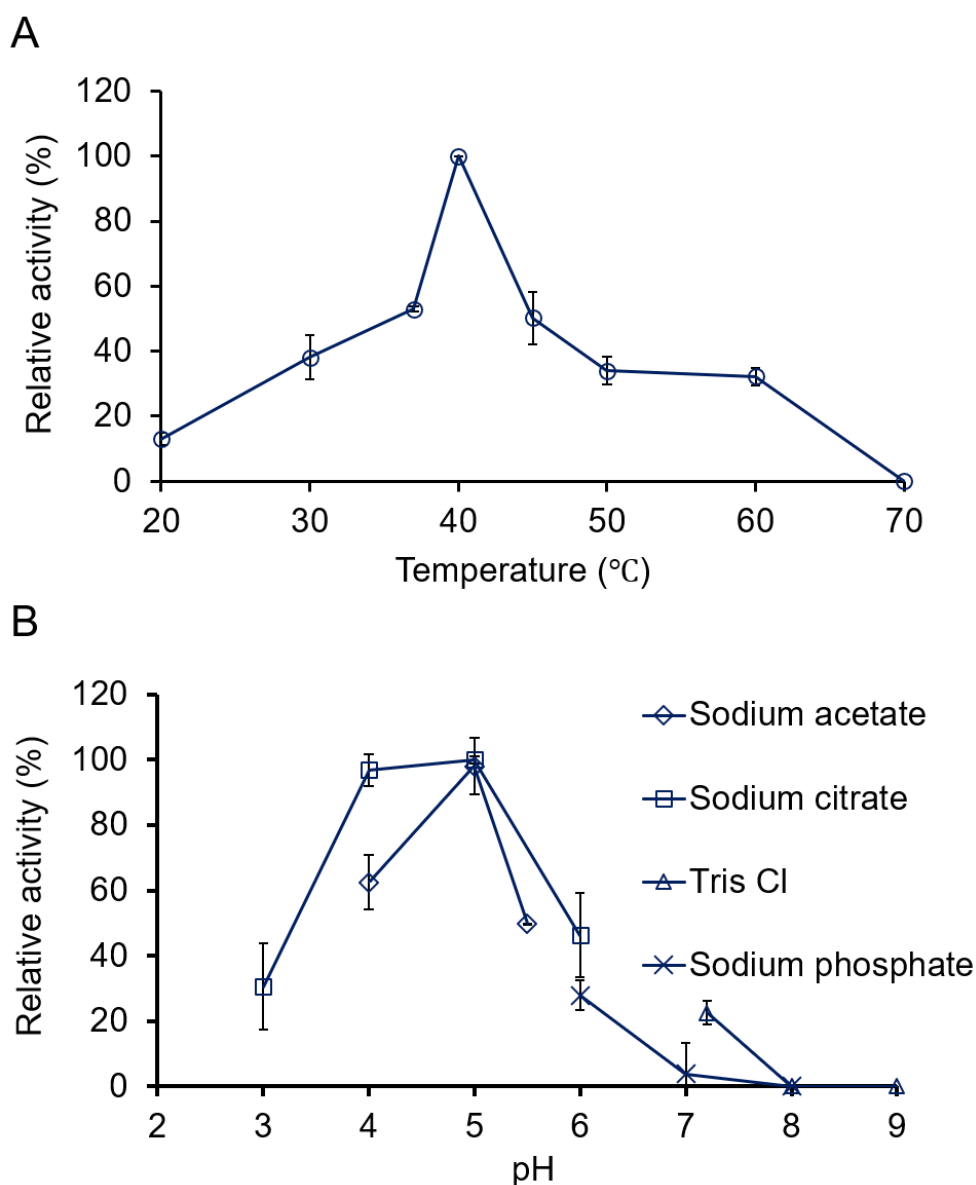


Figure S7. Determination of optimum temperature and pH of *UmGH18A*. (A) Optimum temperature for *UmGH18A* was determined by incubating *UmGH18A* (1 μ M) with 10 g.L⁻¹ colloidal chitin at various temperatures (20–70°C) for 1 h, and the relative activities were plotted against the temperature. (B) The optimum pH was measured in different buffers (50 mM strength) at various pH values. The enzyme load, substrate concentration and time of incubation were same as the temperature optima experiment and the relative activities were plotted against the pH. Data points show average values and the error bars indicate standard deviations from three independent replicates.

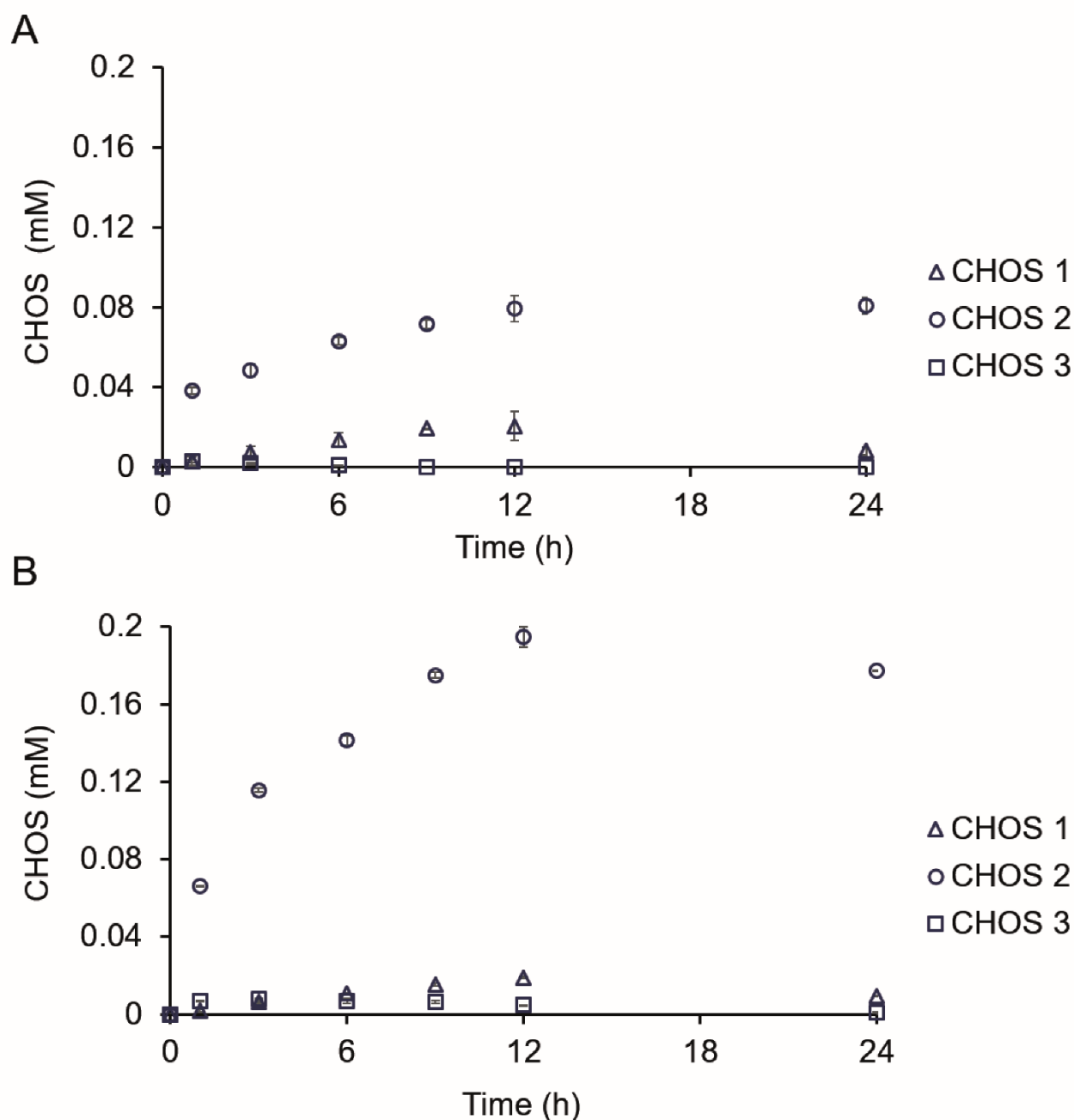


Figure S8. Time-course analysis of the chito-oligosaccharides (CHOS) released by *UmGH18A* from α -chitin (A) and β -chitin (B). All reactions were carried out in sodium citrate buffer (50 mM, pH 5.0), under stirring (1000 rpm), at 40°C using substrate (10 g.L⁻¹) and *UmGH18A* (1 μ M). Data points show average values and the error bars indicate standard deviations from three independent replicates.

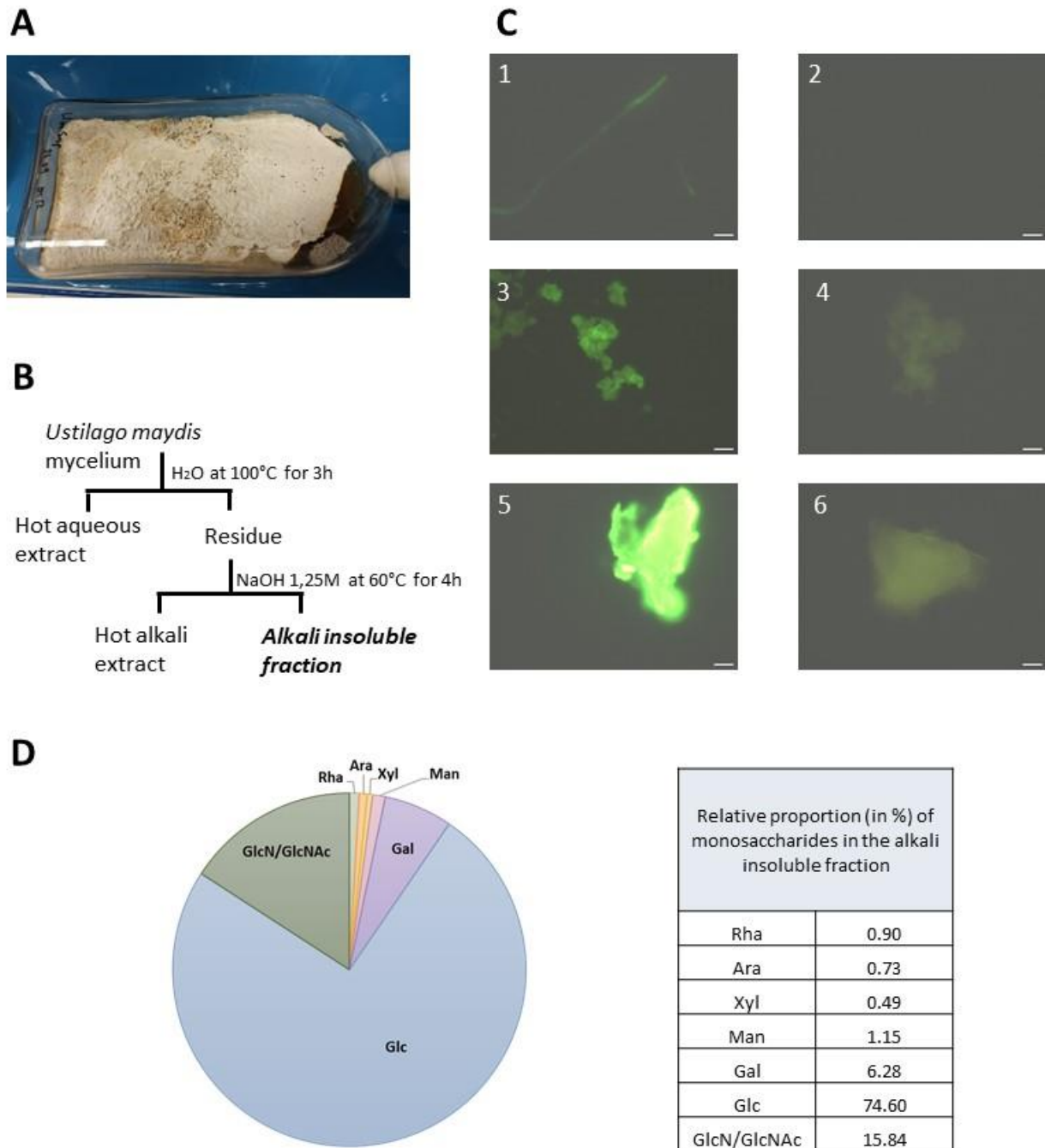


Figure S9. Preparation and analysis of the *Ustilago maydis* chitin-containing fraction. (A) Mycelium of *U. maydis* in Roux flask after 17 days of growth. (B) Outline of the protocol developed to prepare a chitin-containing fraction from *U. maydis* mycelium using sequential water and alkali extraction. (C) Fluorescence microscopy images (500X magnification, scale bar: 10µm) of *U. maydis* intact mycelium (1-top), alkali insoluble *UmFCW* fraction (3-middle) or α -chitin (5-bottom) stained with the fluorescent chitin-specific lectin WGA-AF488, and their corresponding unstained controls (Images 2, 4 and 6 of the right-hand side). (D) Monosaccharide (neutral sugars and glucosamine (GlcN/GlcNAc)) composition of the alkali insoluble fraction after acid hydrolysis.

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