6 Degradation of Plant Cell Wall Polymers by Fungi

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I. Introduction

Plants are the main producers of primary biomass through carbon fixation. By photosynthesis, they convert light energy into chemical energy, thereby transforming carbon dioxide to carbohydrates. Fungi as heterotrophs depend on these organic compounds as energy and nutrient source. They are today the most important and widespread group of organisms responsible for the recycling of plant material into the ecosystem and are therefore essential components of the global carbon cycle. Saprotrophic or saprobic fungi grow on dead material and are particularly important for the enzymatic breakdown of various polymers in the cell wall of plants. This lignocellulosic plant matter is the most abundant natural material on earth and is mainly composed of the three polysaccharides cellulose, hemicellulose, and pectin and the polymer lignin. Fungi are also adapted to grow as parasites or pathogens on living plants, with biotrophic parasites feeding from living cells and necrotrophic parasites killing the host organism before they feed on the dead cells. In addition, a number of economically important plants are attacked by fungi, leading to significant crop losses either by plant decomposition or by the production of toxic substances. Hyphal fungi are not only the most efficient degraders of plant biomass but are also the main source of enzymes for commercial lignocellulose degradation. The polysaccharides of the plant cell walls have attracted the biotech industry as a source of fermentable sugars, not only for bio-ethanol production but also as raw material in the production of a wide range of other biorefinery products (Carroll and Somerville 2009; Pauly and Keegstra 2008; Youngs and Somerville 2012; Somerville et al. 2010).

The importance of fungi in global carbon cycling and the applications of these organisms and their enzymes in industry have heavily promoted research over the past decades. Applications are found amongst others in the food, animal feeding, textile, and pulp and paper industries. The fungi best studied with respect to -biopolymer-degrading enzyme formation are *Aspergillus* sp., *Neurospora crassa*, *Penicillium* sp., and *Trichoderma reesei* or—as an example of lignin degradation—the white-rot fungus *Phanerochaete chrysogenum*. In nature, most of these fungi produce considerable amounts of extracellular enzymes to get access to metabolizable sugars entrapped in the different biopolymers. Strain improvement and specific

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selection programs have resulted in industrial strains of *Aspergillus niger* and *T. reesei* which can yield more than 100 g/l of amylases or cellulases (Durand et al. 1988; Berka et al. 1991; Cherry and Fidantsef 2003), therefore making them also versatile production hosts for other enzymes and recombinant proteins. Another advantage of these fungi is that they are easy and inexpensive to grow in large bioreactors and are suitable for genetic recombination technologies. Indeed, it was not surprising that these biotechnological applied fungi were among the first filamentous fungi for which a genome sequence became available (Martinez et al. 2008; Pel et al. 2007). The recent advances in the sequencing and annotation of fungal genomes give an impression of the hidden enzymatic potential in these organisms, and transcript and proteome analysis has accelerated our current understanding of the different genes expressed on various natural substrates. However, despite the advanced technologies used today, the inability to efficiently convert the crystalline and insoluble cellulose to fermentable sugars still poses a major barrier for the commercialization of biofuel production (Himmel and Bayer 2009).

This chapter aims to summarize our current knowledge on plant cell wall degradation, giving the reader an overview about the structures of the substrates and the enzymatic strategies for their degradation. As an example for the complex deconstruction of lignocellulose, the biopolymer degradation strategy of brown- and white-rot fungi is highlighted in more detail. A complete listing of all genes and their encoded enzymes involved in the breakdown of plant cell walls is beyond the scope of this chapter. Today, a number of web-based databases are available which accomplish these tasks. One example is the Carbohydrate-Active Enzymes Database (www.CAZy.org) which is a manually curated list of enzyme classes and families known to act on carbohydrates (Cantarel et al. 2009; Levasseur et al. 2013; Lombard et al. 2014). As a complement to CAZy, the CAZypedia (www.cazypedia.org) describes the different enzyme classes and families in more detail. Initially focusing on the glycoside hydrolase (GH) families, other CAZyme classes were introduced to the CAZy database within the last years, including polysaccharide lyases (PL), glycosyltransferases, carbohydrate esterases (CE), as well as auxiliary redox enzymes and non-catalytic carbohydrate-binding modules (CBMs). A database specialized on the biochemical properties of lignocellulose-active proteins is *myco*CLAP (https://mycoclap.fungalgenomics.ca/mycoCLAP/ Murphy et al. 2011). Biochemical properties and functional annotations described in *myco*CLAP are manually curated, and the database covers fungal GHs, CEs, and PLs and enzymes with auxiliary activities.

II. Structure and Composition of Plant Cell Walls

Plant cells are enclosed by cell walls which provide rigidity to the cell for structural and mechanical support, maintain and determine cell shape, counterbalance osmotic pressure, direct growth, and, ultimately, determine the architecture and form of the plant. In addition, the plant cell wall protects against environmental factors or pathogens. The plant cell walls consist mainly of polysaccharides such as cellulose, hemicelluloses, pectins, and the aromatic polymer lignin. Together, they form a complex and rigid structure termed lignocellulose.

Typically, three regions are distinguished in plant cell walls, which are the middle lamella, the primary, and the secondary cell wall. The middle lamella is the outermost layer, composed primarily of pectin and its function is to cement the cell walls of adjacent cells together. Primary walls are synthesized during growth, whereas secondary walls are thickened structures containing lignin and surrounding specialized cells such as vessel elements or fiber cells. All differentiated cells contain walls with distinct compositions, resulting in a spectrum of specialized cell walls with primary and secondary walls as two extremes (Keegstra 2010). Primary walls are comprised of 15–40 % cellulose, 30–50 % pectins, and 20–30 % xyloglucans and minor amounts of arabinoxylans and proteins. The primary cell wall is expanded inside the middle lamella and consists of several interconnected matrices. In these matrices, cellulose microfibrils are aligned at all angles and cross-linked via hemicellulosic tethers to form the cellulose–hemicellulose network (e.g., with xyloglucan and galactoglucomannan). This network is embedded in the gelatinous pectin matrix composed of homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (O'Neill and York 2003). The pectins are particularly important for wall hydration. Two main types of primary cell walls are distinguished (Carpita and Gibeaut 1993): type I is found in all dicotyledons, non-graminaceous monocotyledons, and gymnosperms

and typically contains xyloglucan and/or glucomannan and 20–35 % pectin. Type II is found in grasses, where arabinoxylans constitute most of the matrix, and mixed-linkage glucans transiently comprise 10–20 % of wall mass (Carpita et al. 2001; Gibeaut et al. 2005). An exception are celery and sugar beet parenchyma which are rich in cellulose and pectin, but have little hemicelluloses (Thimm et al. 2002; Zykwinska et al. 2007).

In addition to primary walls, many plants synthesize a secondary wall, which is assembled between the plant cell and the primary wall. These secondary walls are found in plant tissues that have ceased growing. They provide strength and rigidity and are comprised primarily of cellulose and lignin. In addition, the hemicelluloses xylan, and glucomannan replace xyloglucan and pectins. Secondary cell walls are also less hydrated than primary cell walls. The cellulose microfibrils are generally aligned in the same direction but, with each additional layer, their orientation changes slightly. The secondary wall is altered during development by successive encrustation and deposition of cellulose fibrils and other components. Nonstructural components of the secondary wall represent generally less than 5 % of the dry weight of wood and include compounds such as phenols, tannins, fats, sterols, proteins, and ashes.

The structure of the polysaccharides found in the cell wall is highly diverse and comprises a spectrum ranging from simple linear polymers composed of a single type of glycosyl residue (e.g., cellulose is composed of 1,4-linked β-glucosyl residues), over polymers with a regular branching pattern (e.g., xyloglucan and rhamnogalacturonan II), to rhamnogalacturonan I, a polymer substituted with a diverse range of arabinosyl and galactosyl-containing oligosaccharide side chains. Understanding the structures of these polymers and determining their primary structures remain a major challenge, especially because their biosynthesis is not template driven (O'Neill and York 2003).

III. Degradation of the Plant Cell Wall

Due to the overall structural and chemical complexity of plant cell walls, a complete breakdown of the different components is brought about only by a wide range of organisms acting in a consortium. This degradation follows a characteristic decomposition sequence, which starts with the colonization of living plants and ends with the production of highly persistent soil humus. The fungi found in these decomposition sequences live in complex and diverse communities and are often specialized to degrade only certain types of polymers, reflecting their genomic capabilities. The efficient breakdown of the plant cell wall by fungi is linked, on the one hand, to their hyphal growth, which provides penetrating power, and, on the other hand, to their highly specialized extracellular plant cell wall-degrading enzyme systems. The enzymatic decomposition of plant cell walls is usually synergistic: individual, highly specialized enzymes operate as components of multi-enzyme systems to efficiently degrade the polymers. The synthesis of these enzymes is governed by a sophisticated signal transduction and gene regulation system, and a highly productive secretory machinery is available for their cellular export. These characteristics enable fungi to successfully compete with other microorganisms in their environment, and they are today the main agents of decomposition in terrestrial and aquatic ecosystems.

Degradation occurs extracellularly, since the substrates are usually large polymers which are insoluble or even crystalline. Two principal types of extracellular enzymatic systems for the degradation of the polymeric fraction have been developed: the hydrolytic system, which degrades the polysaccharides mainly by glycoside hydrolases, and a unique oxidative ligninolytic system, which depolymerizes lignin. But even for the complete degradation of the chemically simple polysaccharide cellulose, several enzymes are necessary and the number of enzymes is further increased when the polysaccharides are substituted. Historically, three classes of enzymes can be distinguished although recent advancements have revealed overlapping roles of some of the enzymes originally classified into one of these classes. These classes include (1) exo-acting enzymes, which release mainly mono- and dimers of the ends of the polymeric chain, (2) endo-acting enzymes, which cleave in the middle of the sugar chain, and (3) enzymes (often exo-acting) which are specialized in cleaving the resulting oligosaccharides into their monomers. The resulting low molecular break down

products are then readily taken up into the cell and further degraded by a wide range of specialized catabolic pathways. In contrast to polysaccharides, the complex heteropolymer lignin lacks a stereochemical regularity and is therefore degraded by a nonspecific and non-stereoselective mechanism.

GHs are the main actors in polysaccharide degradation and are represented by 135 GH families in the CAZy database. Additional families involved in plant cell wall degradation are found in the group of the polysaccharide lyases which employ β-elimination reaction mechanisms to cleave pectins and carbohydrate esterases which catalyze the de-O or de-N-acylation of substituted polysaccharides. To date, polysaccharide lyases form 23 and carbohydrate esterases 16 characterized families. "Auxiliary activities" or AAs cover redox enzymes that are currently represented by 13 families, with the majority active in lignin degradation and with four families active on polysaccharides, namely the lytic polysaccharide monooxygenases (LPMOs). The catalytic modules or functional domains of the different enzymes are often associated with other functions to improve their action on lignocellulose. The most prominent additional domain are carbohydrate-binding modules (CBMs), which are represented by 71 families in the CAZy database.

A. Cellulose Degradation

Cellulose is the most abundant biopolymer on earth, with approximately 10^{10} metric tons of CO_2 and H_2O getting transformed into this polymer every year (Field et al. 1998). It consists of a linear chain of several 100 up to 12,000 β-1,4-linked D-glucopyranose units. The glucose hydroxyl groups from one chain form hydrogen bonds with the oxygen atoms on the same, or on a neighboring chain, which leads to the formation of cellulosic microfibrils. These microfibrils provide high tensile strength in cell walls and prevent the cell wall from stretching laterally. Cellulose crystallizes shortly after its biosynthesis, but less-ordered amorphous regions can occur. A large number of fungi are able to grow on amorphous cellulose and water-soluble derivates, but relatively few are able to produce a complete enzyme system necessary to hydrolyze crystalline cellulose. For the complete hydrolysis of cellulose (Fig. 6.1) to D-glucose, a number of synergistically acting enzymes are necessary. These include cellobiohydrolases (EC 3.2.1.91; 1,4-β-D-glucan cellobiohydrolases), which attach to the cellulose chains and act processively from their ends to generate mainly the glucose disaccharide cellobiose. The cellobiohydrolases are classified in two GH families with GH6 enzymes characterized by an inverting mechanism and cleavage from the nonreducing cellulose end. In contrast, GH7 family members cleave cellobiose from the reducing end by a retaining mechanism. Endoglucanases (EC 3.2.1.4; 1,4-β-D-glucan 4-glucanohydrolase) are capable of cleaving within the amorphous regions inside the cellulose chain, thereby generating additional sites for the attack of the cellobiohydrolases. β-Glucosidases (EC 3.2.1.21) degrade the accumulating cellodextrines and cellobiose to D-glucose (Beguin 1990; Teeri 1997). Within the last years, another main player in cellulose degradation was discovered with the lytic polysaccharide monooxygenases (LPMOs). These copper dependent oxidases originally classified as fungal GH61 enzymes attack the highly crystalline regions of cellulose by an oxidative cleavage. Studies on the *T. reesei* cellulase CEL61A revealed that its addition to commercial enzyme mixes can significantly boost degradation (Harris et al. 2010). These LPMOs act on crystalline cellulose by generating both oxidized and non-oxidized chain ends. One group oxidizes the C_1 of D-glucose, thereby releasing lactones that are hydrolyzed to aldonic acids (Beeson et al. 2011). Other LPMOs act on the nonreducing end, producing ketoaldoses, or a combination thereof (Beeson et al. 2012). Therefore, these CEL61 enzymes were reclassified in auxiliary activity family 9 (AA9), which exclusively contains eukaryotic LPMOs. Originally, these LPMOS were detected in the degradation of chitin (Hemsworth et al. 2014; Vaaje-Kolstad et al. 2010) before their action on cellulose or starch was discovered (Vu et al. 2014; Levasseur et al. 2013; Lo Leggio et al. 2015; Horn et al. 2012).

By disrupting the crystalline structure of cellulose, they facilitate the enzymatic cleavage of the classical cellulases and allow other enzymes like cellobiohydrolases to attack the polymer at otherwise inaccessible sites

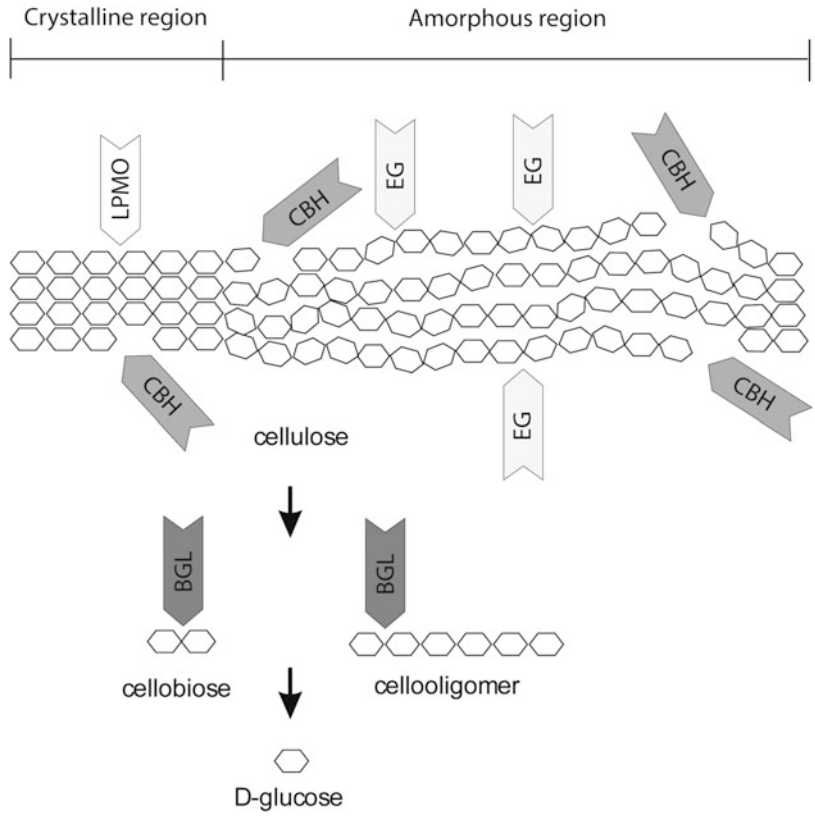

Fig. 6.1 Enzymatic degradation of cellulose. Cellobiohydrolases (CBH) act on either the reducing or non-reducing end of the glucose chain. Endoglucanases (EG) hydrolyze internal glycosidic bonds, thereby providing additional sites for the CBHs. Finally, smaller oligomers and the dimer cellobiose are cleaved by β-glucosidases (BGL) into D-glucose. The lytic polysaccharide monooxygenases (LPMO) cleave within the highly crystalline regions of cellulose in an oxidative manner

(Horn et al. 2012). In this respect, LPMOs may be the true C_1 enzyme in the C_1-C_x model proposed by Elwyn T. Reese to explain cellulose degradation (Mandels and Reese 1964; Reese et al. 1950). In this model, the non-hydrolytic C_1 enzyme is required for the initial attack on crystalline cellulose and the C_x enzymes for the hydrolysis of soluble cellulose. Later, in the 1990s, the classical endo/exo model of cellulase degradation was proposed (Wood and McCrae 1972) which consisted of EGs attacking amorphous cellulose regions of the microfibrils, thereby generating new sites for exoglucanases. The exoglucanases attack the free cellulose ends. Finally, β-glucosidases convert cellodextrines and cellobiose to glucose. Here, the dominant EG act only on amorphous cellulose and functions as the C_x activity whereas the C_1 is represented by the exoglucanases, i.e., cellobiohydrolases. But Reese suggested that C_1 was a decrystallizing protein factor which possesses the ability to swell or disrupt cellulose; the LPMOs might act like the C_1 enzyme proposed by Reese.

A structural comparison of the different cellulases shows that these proteins comprise besides the catalytic domain carbohydrate-binding modules. These modules (Várnai et al. 2014) can be found N- or C-terminally and are about 40 aa in size. They were originally described as cellulose-binding domains due to their presence in cellobiohydrolases but were later also found in other carbohydrate-degrading enzymes and therefore renamed to carbohydrate-binding modules (CBMs). Removal of this domain leads to enzymes which are still able to cleave glycosyl linkages

from smaller oligosaccharides, but their binding to cellulose and, therefore, the action on crystalline cellulose is impaired. CBMs are structurally similar, and their carbohydrate-binding capacity can be attributed to several amino acids constituting the hydrophobic surface. The CBM domain is usually spatially separated from the catalytic core domain by a flexible linker region (Sammond et al. 2012). This linker region is rich in prolines, serines, and threonines, and the latter two amino acids are highly O-glycosylated to impair proteolytic degradation (Langsford et al. 1987; Srisodsuk et al. 1993). Recent studies on this connector have shown that their length and sequences are optimized towards the type of GH and CBM domain, which indicates that also the linker is of importance for the activity of the enzyme (Sammond et al. 2012; Payne et al. 2013).

The catalytic domain of the cellobiohydrolase II (CEL6A) of *T. reesei* was the first cellulase crystal structure resolved at the atomic level (Rouvinen et al. 1990). Its structure demonstrates why the cellobiohydrolases are only able to attack cellulose chain ends. The crystal structure shows a tunnel-shaped active site which is so tight that it can incorporate only one cellulose chain. Despite a similar overall structure, endoglucanases possess a more open active site which allows the enzyme to attack the cellulose chains in the middle. The active site topology of these polymer-degrading enzymes shows that these enzymes have extended active sites which provide binding places for a number of sugar units. These subsites position the substrate tightly and correctly with respect to the catalytic amino acids.

In addition to the classical glycoside hydrolases and the new LPMOs, other types of proteins have been described to be involved in cellulose degradation. One of them is the *T. reesei* swollenin SWO1 which shows similarity to plant expansins (Saloheimo et al. 2002; Sampedro and Cosgrove 2005). Expansins induce the extension of isolated cell walls by a non-hydrolytic activity on different cell wall polymers, e.g., pectins and xyloglucans, which are tightly bound to the cellulose microfibrills (McQueen-Mason and Cosgrove 1995). A proposed model is, that swollenins are able to disrupt the cellulose microfibrils without any hydrolytic activity and thereby they make the cellulose fibers more accessible for the cellulases to act upon (Saloheimo et al. 2002). Although SWO1 has cellulose disrupting activity (Andberg et al. 2015; Jäger et al. 2011), its actual role seems to be in aiding degradation of hemicelluloses, as synergistic effects with endo-xylanases rather than endo-cellulases or cellobiohydrolases have been reported (Gourlay et al. 2013).

Numerous genes encoding cellulases have been isolated and the respective enzymes were studied in great detail. They are found in GH families 5, 6, 7, 12, and 45. One of the best studied cellulolytic fungi is *T. reesei*, which was discovered during World War II on the Solomon Islands as a severe degrader of cellulosic material of the US Army (Reese 1976) and is today the most commonly used organism for commercial production of cellulases (Gupta et al. 2014). Detailed analysis of the enzymes and gene regulation is available for, e.g., *A. niger*, *T. reesei*, and *P. chrysogenum* (Aro et al. 2005; de Vries 2003; Louise Glass et al. 2013; Kubicek et al. 2009).

B. Hemicellulose Degradation

Hemicelluloses are the second most abundant polysaccharide in plant cell walls and represent a heterogeneous group of polymers with a backbone of 1,4-linked β-D-pyranosyl residues, with the exception of arabinogalactan (O'Neill and York 2003; O'Neill and Selvendran 1985). The backbone can consist of xylosyl-, glucosyl-, galactosyl-, arabinosyl-, or mannosyl residues and, depending on the dominant sugar, they are, e.g., named xylans or arabinogalactans, if both sugars occur in near-equal amounts. This chemical diversity of hemicellulosic structures requires a larger set of enzymes which attack the main chain or side chains. The main chain is cleaved by (mainly) endo-acting enzymes whereas exo-acting enzymes liberate the respective monomers. Only a few exo-acting enzymes are known which attack the main chain (Tenkanen et al. 2013). Accessory enzymes are activities necessary to cleave off the side chains, leading to the release of various

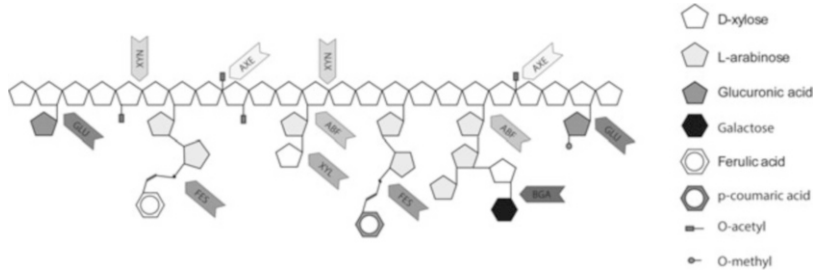

Fig. 6.2 Enzymatic degradation of hemicelluloses. The main chain of xylan is degraded by endo-1,4-β-xylanases (XYN). Accessory enzymes necessary for side group removal are β-xylosidase (XYL), α-glucuronidase (GLU), feruloyl esterase (FES), acetyl xylan esterase (AXE), 1,4-galactosidases (BGA), and α-L-arabinofuranosidase (ABF)

mono- and disaccharides. In this way, the main chain also becomes more accessible for the other group of enzymes. Most extensively studied is the enzymatic degradation of xylan, which involves exoxylanases, endoxylanases, β-xylosidases, and accessory enzymes for the side chains (Fig. 6.2).

1. Xylans

Xylans are characterized by a β-1,4-linked β-D-xylosepyranose backbone substituted by different side chains. They are the major hemicellulose in the cell walls of cereals and in hardwood and represent a minor component of the walls of dicotyledons and non-graminaceous monocotyledons.

Xylans found in cereals are highly substituted with single residues or short side chains of α-1,2- or α-1,3-linked L-arabinofuranose residues and are therefore referred to as arabinoxylans. Glucuronoxylans are typical hardwood xylans and contain large amounts of α-1,2- and α-1,3-linked 4-*O*-methyl-α-D-glucuronic acid and acetyl groups at *O*-2 or *O*-3. Glucuronoarabinoxylans are found in softwood and are substituted with a higher content of α-1,2-linked 4-*O*-methyl-α-D-glucuronic acid compared to hardwood and, in addition, contain α-L-arabinosefuranose but no acetyl groups. The L-arabinose residues may be esterified at *O*-5 with feruloyl or *p*-coumaroyl residues, and a number of other minor residues have been detected, too (Darvill et al. 1980; Ebringerova and Hienze 2000; Izydorczyk and Biliaderis 1995).

The hydrolysis of the xylan backbone involves endo-1,4-β-xylanases (endo-1,4-β-D-xylan xylanohydrolases; EC 3.2.1.8) and β-xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). Exo-1,4-β-xylanase which cleaves xylan from the ends has received less attention but, e.g., XYN4 from *T. reesei* shows both exo- and endo-xylanase activity (Tenkanen et al. 2013). Endoxylanases cleave the main sugar chain depending on the type of xylan, the degree of branching, and the presence of different substituents (Polizeli et al. 2005). The main hydrolysis products are substituted or non-substituted oligomers which are further converted by β-xylosidases into tri-, di-, and monomers. Endoxylanases can be classified according to their end product into debranching and non-debranching enzymes, based on their ability to release L-arabinose from arabinoxylan (Wong et al. 1988). Some enzymes cut randomly between unsubstituted D-xylose residues whereas the cleavage site of some endoxylanases is dependent on the neighboring substituents of the side chains. β-Xylosidases can be classified according to their relative affinities for xylobiose or larger xylooligosaccharides and release β-D-xylopyranose by a retaining mechanism from the nonreducing end. β-Xylosidases are in general highly specific for small unsubstituted D-xylose oligosaccharides, and the activity decreases with increasing polymerization of the substrates. Accumulation of the short oligosaccharides would inhibit the action of the endoxylanases, but the hydrolysis of these products by β-xylosidases removes this possible cause of inhibition, thereby increasing the efficiency of xylan hydrolysis (Andrade et al. 2004). Similar to cellulases, most of the genes encod-

ing endoxylanases and β-xylosidases have been characterized in different *Aspergillus* spp., *T. reesei*, and *Penicillium* spp. as well as in *Agaricus bisporus* and *Magnaporthe grisea*.

2. Xyloglucan

Xyloglucan is the predominant hemicellulosic polysaccharide of dicotyledons and non-graminaceous monocotyledons, constituting up to 20 % of the plant cell wall.

Xyloglucans cross link cellulose microfibrills and support in this way the structural integrity of the cell wall (Hayashi and Kaida 2011). The backbone is composed of 1,4-linked β-D-glucopyranose residues which are substituted at *O*-6 by D-xylopyranose via an α-1,6-linkage. Depending on the number of D-glucopyranose residues attached to the main chain, they are classified as XXXG or XXGG type. In the XXXG type, three consecutive D-glucopyranose residues are substituted with D-xylopyranose followed by a fourth unbranched D-glucopyranose residue. Additional sugars found substituted to the D-xylopyranose include α-1,2-L-fucopyranose, β-1,2-D-galactopyranose, α-1,2-L-galactopyranose, or α-1,2-L-arabinose residues. Some of these residues can also contain O-linked acetyl groups (O'Neill and York 2003). XXXG-type glucans are present in numerous plants whereas the XXGG type occurs in solanaceous plants.

Some of the endoglucanases active against cellulose are also active on xyloglucans; in addition, xyloglucan hydrolases (EC 3.2.1.151) which are specific for xyloglucan have been reported (Pauly et al. 1999; Hasper et al. 2002; Grishutin et al. 2004; Baumann et al. 2007). Its degradation is not limited to glycoside hydrolases, as NcLPMO9C, a LPMO from *N. crassa*, was shown to degrade various hemicelluloses, in particular xyloglucan. It generates oxidized products and primarily acts on the xyloglucan backbone, accepting various substitutions (xylose, galactose) and helps in this way to depolymerize recalcitrant cellulose xyloglucan structures (Agger et al. 2014).

3. (Galacto-)glucomannan

Mannan consists of a β-1,4-linked β-D-mannopyranose backbone whereas in the glucomannan backbone randomly distributed β-1,4-linked β-D-glucopyranose and β-D-mannopyranose are found. Further substituents include α-1,6-linked α-D-galactopyranose residues which can be substituted further by α-1,2-linked α-D-galactopyranose. These polysaccharides are usually referred to as galactomannans and galactoglucomannans (Brett and Waldren 1996) which are the major hemicellulose structures of softwoods whereas glucomannan dominates in hardwood (Stephen 1982; Aspinall 1980). The D-glucose or D-mannose residues are partially substituted with acetyl residues linked to *O*-2 or *O*-3. The backbone is degraded by endo-1,4-β-mannanases (Mannan endo-1,4-β-mannosidase, EC 3.2.1.78) and β-mannosidases (EC 3.2.1.25). The ability of the endo-1,4-β-mannanases to degrade these polymers depends on the number and position of the side chain substituents. The enzymes releasing glucose (β-glucosidase, EC 2.1.21) and galactose (α-galactosidase, EC 3.2.1.22) residues act in synergism with endo-1,4-β-mannanases and β-mannosidases. β-Mannosidases split off the β-D-mannose residue from the nonreducing end of the manno-oligosaccharides and are characterized by a retaining mechanism.

C. Degradation of Pectins

Pectins are the most complex and heterogeneous group of polysaccharides in plant cell walls and are characterized by a significant content of α-1,4-linked D-galacturonic acids. They are found mainly in the middle lamella and in the primary cell wall where, their proportion ranges from 5–10 % in grasses to 30 % in dicotyledons. The carbohydrate composition and, hence, the structure vary depending on the species and cell type. Pectin is made up of several distinct domains which, depending on the presence of side chains, are called either "smooth" or "hairy" regions (Ridley et al. 2001; Pérez et al. 2000).

The "smooth" region or homogalacturonan (HG) is the most abundant pectin structure and consists of

linear chains of α-1,4-linked D-galacturonic acid residues which can carry methyl esters at the terminal carboxyl group and acetyl esters at the *O*-2 or *O*-3 position. Homogalacturonan with a high degree of methyl esterification is referred to as pectin whereas pectic acid (pectate) has a low degree of esterification. The esterification of the uronic acid group results in the elimination of the negative charge, which is of great significance for the gelling process of pectin, since the complexes between the carboxyl groups and Ca^{2+} ions are involved in this (Vincken et al. 2003). Additionally, the number of methyl- and acetyl esters has a strong influence on the susceptibility to cleavage by the different pectinases. Rhamnogalacturonan I+II and xylogalacturonan (XGA) are responsible for the "hairy" regions of the pectin due to their abundant and often branched side chains. The backbone of rhamnogalacturonan I consists of D-galacturonic acid and L-rhamnose in a $[1\rightarrow2)$-α-L-Rha-$(1\rightarrow4)$-α-D-GalA-$(1\rightarrow]_n$ linkage. Whereas D-galacturonic acid can be substituted with either methyl- or acetyl esters similar to those in HG, 20–80 % of the L-rhamnose residues are substituted at the *O*-4 position by L-arabinose and D-galactose with varying size from monomers up to branched, heterogeneous oligomers. They can be terminated with α-L-fucose and (4-*O*-methyl)-β-D-glucuronic acid. Arabinan chains are formed of α-1,5-linked L-arabinoses which can be further substituted with α-1,3-linked L-arabinose residues. In addition, two types of arabinogalactan side chains are present: Type I consists of a chain of β-1,4-linked D-galactopyranose whereas type II contains a backbone of β-1,3-linked D-galactopyranose residues which can be substituted with β-1,6-linked D-galactopyranose residues. Both are occasionally substituted with L-arabinose at *O*-3. In addition, ferulic acid and *p*-coumaric acids have been identified in the pectic hairy regions attached to *O*-2 of L-arabinose and *O*-6 of D-galactose. Rhamnogalacturonan II consists of a short backbone of α-1,4-linked D-galacturonic acid which is substituted either at the *O*-2 or *O*-3 position (Vidal et al. 2000). Its side chains have been found to be either dimers or branched oligomers and to contain rare sugars such as D-apiose and L-fucose in addition to L-arabinose, D-galactose, and L-rhamnose. The backbone of another substructure found in hairy regions, the xylogalacturonans, is similar to that of the HGs, but a major part of the D-galacturonic residues carry β-D-xylose substituents at the *O*-3 position. It has been found in reproductive tissues, including soybean seed, apple fruit, and pine pollen (Schols et al. 1995). Although the composition and structure of the individual subunits are well established, the manner in which they make up the pectin polymer is still under investigation. For a long time, pectin was thought to consist of linear chains of homogalacturonan interspersed with hairy regions. In another model (Vincken et al. 2003), rhamnogalacturonan I forms the backbone, substituted with homogalacturonan and the abovementioned arabinan and galactan side chains.

To efficiently degrade this complex pectin structure, fungi have developed a broad spectrum of pectinolytic enzymes. Enzymatic depolymerization of pectin weakens the cell wall and exposes the other cell wall polymers to degradation by other plant cell wall-degrading enzymes. Therefore, pectin degradation is important for plant pathogenic fungi (Kubicek et al. 2014), while other often fungi show a reduced set of these enzymes.

Pectinases can be classified in terms of their reaction mechanism into hydrolases or lyases and further according to their substrate specificity into, e.g., polygalacturonases or rhamnogalacturonases. Beside glycoside hydrolases, different lyases such as pectin- (EC 4.2.2.10), pectate- (EC 4.2.2.2), and rhamnogalacturonan lyases (EC 4.2.2.-) cleave polysaccharide chains via a β-elimination mechanism resulting in the formation of a Δ-4,5-unsaturated bond at the newly formed nonreducing end.

Most pectinases are found in GH family 28 as they share a common conserved structure which is in contrast to the cellulases and hemicellulases, which are characterized by a high diversity of protein structures. Although the overall sequence similarity is low, pectinases share a central core consisting of parallel β-strands forming a large, right-handed helix defined as parallel β-helix (Jenkins and Pickersgill 2001). Even though hydrolases and lyases differ in their catalytic mechanism, the substrate-binding sites are all found in a similar location within a cleft formed on the exterior of the parallel β-helix. This structure facilitates the binding and cleaving of the buried pectin polymers in the undamaged cell wall. The parallel β-helix fold confers the stability needed by these pectinases for efficient aggressive action in a variety of hostile extracellular environments. An exception to this rule is the rhamnogalacturonan lyase from *A. aculeatus*, which displays a unique arrangement of three distinct modular domains (McDonough et al. 2004).

The pectinolytic system has been studied in great detail in *Aspergillus* spp. including *A. niger* (de Vries and Visser 2001). About 60 genes of *A. niger* are predicted to encode pectinases for which the expression of 46 was detected (Martens-Uzunova and Schaap 2009)

and a high number was identified by mass spectrometry (Tsang et al. 2009).

Polygalacturonases (PGAs) are the most extensively studied class of pectinases and include endopolygalacturonases (1,4-α-D-galacturonan glycanohydrolase; EC 3.2.1.15) which catalyze the hydrolytic cleavage of α-1,4 D-galacturonic bonds within the chain and exopolygalacturonases (galacturan 1,4-α-galacturonidase; EC 3.2.1.67) which cleave from the nonreducing end. Both endo- and exoPGAs belong to glycoside hydrolase family 28 and have similar reaction mechanisms and substrate specificities, but their level of sequence identity is surprisingly low (Biely et al. 1996; Markovic and Janecek 2001; Henrissat and Bairoch 1993). Among the endoPGAs, some enzymes cleave only once per chain (single attack or non-processive) whereas others attack multiple times (processive behavior). Single-attack PGAs generally produce longer fragments, which are only gradually degraded into dimers, trimers, or short oligomers, providing possible sites for exoPGAs.

A factor which significantly influences the activity of PGAs is the number (and distribution) of methyl- and acetyl ester groups on the substrate. In general, most endo- and exoPGAs prefer substrates with a low degree of esterification, although some exceptions exist (Parenicova et al. 2000). In most cases, the activity of a methyl/acetyl esterase is required to prepare the pectin molecule for PGA digestion. Pectin-degrading fungi often produce multiple isozymes with a wide range of enzymatic properties, substrate specificities, and pH optima, which may reflect the complexity of the pectin molecule in plant cell walls and the need for enzymes capable of cleaving the homogalacturonan backbone in a variety of structural contexts. Another structural feature which may determine the functional diversification of these enzymes is the presence or absence and type of N-terminal extension, which has been suggested to influence their substrate specificity and to play a role in their interaction with particular regions of the pectin polymer (Gotesson et al. 2002; Parenicova et al. 2000). The degree of esterification is also important for the functional classification of lyases. Pectate lyases prefer substrates with a low degree of methyl esterification, which therefore have a more acidic character, and are strictly dependent on Ca^{2+} for catalysis. Pectin lyases, on the other hand, favor highly methyl-esterified substrates and do not require Ca^{2+} ions (Jurnak et al. 1996).

The pectinolytic enzyme system of *A. niger* serves as an example of how a microorganism can degrade a pectin molecule. *A. niger* produces seven PGAs, two of them (PgaA and PgaB) constitutively. These two are most active on pectins containing 22 % methyl esters (Parenicova et al. 2000), thus making them suitable for an initial attack on the native substrate. Enzymes subsequently induced at this early stage during growth on pectin are pectin methyl esterase PmeA, an exopolygalacturonase PgxA, and pectin lyases (PelA and PelD; (de Vries et al. 2002). The action of the methyl esterase renders the substrate accessible to PGAs, which are expressed at a later stage after removal of the methyl esters, while the pectin lyases contribute to the breakdown of the still esterified polymer. The exoPGAs cleave D-galacturonic acid monomers from the homogalacturonan poly- and oligomers, which may serve as inducers for the other pectinases. Similar to homogalacturonans, the degradation of the rhamnogalacturonan I backbone is catalyzed by hydrolases and lyases (Fig. 6.3). Endorhamnogalacturonases have been isolated from *A. aculeatus* and *A. niger* (de Vries and Visser 2001) and hydrolyze the α-1,4 glycosidic bonds in saponified hairy regions. The resulting fragments were tetra- and hexamers of the backbone, which partly were still substituted with D-galactose. This suggests that—similar to some exoPGAs ability to cleave XGA (van den Broek et al. 1996)—endorhamnogalacturonases are tolerant towards monomeric substituents. Depending on the monosaccharide cleaved from the nonreducing end of the rhamnogalacturonan, two kinds of exorhamnogalacturonases have been described: rhamnogalacturonan α-D-galactosyluron-hydrolase and rhamnogalacturonan α-L-rhamnohydrolase. All rhamnogalacturonan hydrolases were classified as members of GH family 28. Rhamnogalacturonan lyases cleave the rhamnogalacturonan backbone via β-elimination. Unlike the hydrolases, they act on the Rha-(1→4)-α-D-GalA bond, resulting in the formation of Δ4,5-unsaturated D-galacturonic acid residues at the nonreducing end.

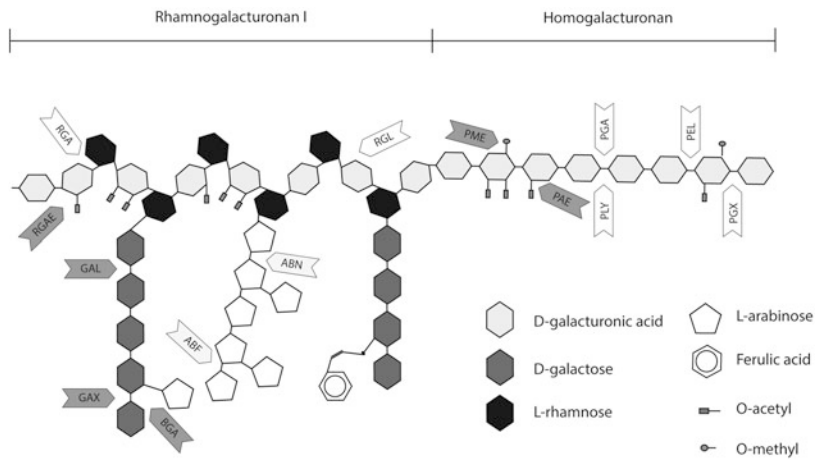

Fig. 6.3 Enzymatic degradation of rhamnogalacturonan I and homogalacturonan. The main chain of rhamonogalacturonan I is degraded by rhamnogalacturonan hydrolase (RGA) and rhamnogalacturonan lyase (RGL). The side chains are degraded by rhamnogalacturonan acetyl esterase (RGAE), endoarabinase (ABN), endo-β-1,6-galactanases (GAL), and exogalactanases (GAX). Terminal monosaccharides are removed by α-L-arabinofuranosidases (ABF) and β-galactosidases (BGA). The main chain of homogalacturonan is degraded by endopolygalacturonases (PGA), exopolygalacturonases (PGX), pectin lyases (PLY), and pectate lyases (PEL). Pectin methyl esterase (PME) and pectin acetyl esterase (PAE) act on the side groups

D. Accessory Enzymes for Plant Cell Wall Degradation

Most plant cell wall-degrading enzymes described above act on the backbone of the respective polysaccharide, but often their activity is impaired by monomeric substituents or larger side chains present in hemicelluloses and pectins. To ensure an efficient and complete breakdown of such polysaccharides, these substituents have to be removed and degraded by accessory enzymes. These accessory enzymes work in synergism with the enzymes attacking the main chain and often depend on each other for an efficient breakdown of the whole substrate to monomeric sugars. The substrate specificity of these enzymes varies; some of the enzymes can hydrolyze the intact polymer whereas others show maximum activity only in the presence of shorter breakdown products (Puls and Schuseil 1993; Tenkanen and Siika-aho 2000). A detailed list of all the enzymes involved in the degradation of hemicellulose and pectinase side chains and their mode of action has been reviewed for e.g. *Aspergillus* spp. by de Vries and Visser (2001), and only some of the major enzymes are listed here (Figs. 6.2 and 6.3).

Side groups in xylans are generally small (mono-, di-, and trimers) but can consist of several different sugars and acids (e.g., acetic acid, L-arabinose, ferulic acid, D-galactose, D-glucuronic acid), and, consequently, multiple enzymes are required to make the backbone fully accessible for the xylanases. α-L-Arabinofuranosidases (EC 3.2.1.55) remove terminal L-arabinose residues but differences in the specificity towards α-1,2-, α-1,3-, or α-1,5-arabinosidic bounds and towards the substrates themselves have been observed. Whereas several representatives are also able to release L-arabinose from pectins and xylans, arabinoxylan arabinofuranohydrolases (EC 3.2.1.99) are strictly specific for L-arabinose bound to xylan. In addition, some α-L-arabinofuranosidases are inhibited by the presence of D-glucuronic acid residues adjacent to the targeted L-arabinose. α-L-arabinofuranosidases contain also CBMs which support their binding to cellulose, xylan, or arabinofuranose side chain. The D-glucuronic acid and its 4-*O*-methyl ethers are removed by α-glucuronidases (EC

3.2.1.139) and by xylan-α-1,2-glucuronosidases (EC 3.2.1.131). In addition to these carbohydrate substituents, esters are found in xylans. Several types of feruloyl esterases (EC 3.1.1.73) have been described, their activity varying with the presence of additional methoxy or hydroxyl substituents on the ferulic acid's aromatic ring, and with the type of linkage (*O*-2, *O*-5, or *O*-6) to the carbohydrate chain. Acetyl xylan esterases (EC 3.1.1.72) participate in the breakdown of the xylan backbone by removing acetyl ester groups from *O*-2 and *O*-3 of the D-xylose chain, thereby facilitating the action of the endoxylanases (de Vries et al. 2000; Kormelink et al. 1993; Tenkanen 1998). So far, only two types of substituents have been described in galacto(gluco)mannans: D-galactose mono- and dimers and acetyl esters. The former are removed from the backbone by α-galactosidases (EC 3.2.1.22) whereas the latter are hydrolyzed by acetylglucomannan esterases (EC 3.1.1.-). Both reactions result in an increased activity of the endomannases and β-mannosidases (Tenkanen 1998; de Vries et al. 2000). For the removal of α-1-6-linked D-xylose side groups of the xyloglucan, specific α-D-xylosidases (EC 3.2.1.177) are required.

Pectin contains acetyl esters at the *O*-2 or *O*-3 and methyl esters at the carboxy group bound to the D-galacturonic acid residues in the smooth regions. Pectin methyl esterases (EC 3.1.1.11) and pectin acetyl esterases (EC 3.1.1.-) have been isolated from different fungal species, and they show a prominent synergism with polygalacturonases (de Vries et al. 2000). Similar results were achieved with rhamnogalacturonan acetyl esterases (EC 3.1.1.-) and rhamnogalacturonase or rhamnogalacturonan lyase (de Vries et al. 2000). Polymeric side chains in pectin consist mainly of L-arabinose and D-galactose. The D-galactose or L-arabinose side chains are often substituted with several other carbohydrates or acids. These chains are cleaved by endoarabinases (EC 3.2.1.99), exoarabinases (EC 3.2.1.-), endo-β-1,4-galactanases (EC 3.2.1.89), endo-β-1,6-galactanases (EC 3.2.1.-), and exogalactanases (EC 3.2.1.23). Terminal monosaccharides are removed by α-L-arabinofuranosidases (EC 3.2.1.55) and α-galactosidases (EC 3.2.1.23), but additional enzymes (e.g., α-L-fucosidases, α-glucuronidases) are required to completely degrade the side chains. α-L-rhamosidases (EC 3.2.1.40) are another group involved in pectin degradation.

IV. Degradation of Lignin

Next to cellulose, lignin is the most abundant polymer in nature and accounts for 15–36 % of the lignocellulosic material. It forms an extensive cross-linked network within the cell wall and confers structural support and decreases water permeability. It protects the other, more easily degradable, cell wall components and is therefore the main obstacle for an efficient saccharification of cellulose and hemicelluloses in bioethanol production.

The aromatic polymer is synthesized from the three substituted phenylpropanoid alcohols coniferyl (guaiacyl propanol), synapyl (syringyl propanol), and *p*-coumaryl (*p*-hydroxyphenyl propanol). The softwood of the gymnosperms contains mainly coniferyl alcohols, some *p*-coumaryl, but no sinapyl alcohol, whereas, in the hardwood of the angiosperms, coniferyl and sinapyl alcohols are found in equal amounts (46 %), with a minor proportion of *p*-coumaryl (8 %). The lignin polymer is synthesized by the generation of free phenoxy radicals, which is initiated by plant peroxidases-mediated dehydrogenation of the three precursor alcohols. The result of this polymerization is a highly insoluble, complex-branched, and amorphous heteropolymer joined together by different types of linkages such as carbon–carbon and different ether bonds. The chemical complexity and structural variability of the lignin polymer make it resistant to breakdown by conventional enzymatic hydrolysis and therefore the initial attack is oxidative, nonspecific, non-hydrolytic, and extracellular (Hatakka 1994; Higuchi 1990; Kirk and Farrell 1987).

Fungi are the most efficient group of organisms able to decompose the wood structure, and they use different mechanisms to make cellulose and heimcellulose accessible. White-rot basidiomycetes act on both hard and softwood and are the major group of wood rots. It is the only group which can completely degrade lignin into CO_2 and H_2O. They can overcome difficulties in wood decay, including the low nitrogen content of wood (a C:N ratio of about 500:1) and the presence of toxic and antibiotic

compounds. Besides these fungi, brown-rot fungi are also able to degrade wood extensively. Analysis of the genomes of different Basidiomycota suggests that the categorization into white rot or brown rot is an oversimplification because of gradations both in the expression of metabolites and the resulting patterns of decay (Riley et al. 2014; Arantes et al. 2012). Some ascomycetes also colonize wood in contact with soil but alter the lignin component only slightly. Their action leads to decrease in the mechanical properties of wood, giving rise to so-called soft rot, a process which often involves bacteria. Soft-rot fungi can degrade wood under extreme environmental conditions (extreme wetness or frequent dryness) which prohibit the activity of other wood-degrading fungi. They are relatively unspecialized (hemi-) cellulolytic ascomycetes in the genera *Chaetomium*, *Ceratocystis,* and *Phialophora*, and some basidiomycetes can also cause a soft rot-type of decay pattern. In soft rot, decay by fungi is closely associated with penetration by the fungal hyphae, because the enzymes cannot cross the plant cell wall. Two distinct types of soft rot are currently recognized. Type 1 is characterized by longitudinal cavities formed within the secondary wall of wood cells and type 2 by an erosion of the entire secondary wall (Martinez et al. 2005). Although many white rots and brown rots secrete oxidative and hydrolytic enzymes, it is generally recognized that their enzymes are unable to diffuse through healthy wood and that smaller, non-proteinaceous molecules are involved in the initiation of decay.

A. Brown-Rot Fungi

Brown-rot fungi degrade mainly cellulose and hemicellulose of coniferous softwoods and partially modify the lignin mainly by demethylation but not by oxidation (Eriksson et al. 1990). Brown-rot fungi attack cellulose in wood, which promotes rapid loss of mechanical strength leaving modified lignin behind. The term "brown rot" refers to the characteristics of this decayed wood: a reddish-brown material consisting of oxidized lignin, which cracks into characteristic brick-like pieces. Representatives of brown-rot basidiomycetes comprise *Schizophyllum commune*, *Fomes fomentarius*, *Serpula lacrimans*, *Postia placenta*, *Piptoporus betulinus*, and *Gloeophyllum trabeum*. They are also the major cause of decay of woods in commercial use and have an important role in coniferous ecosystems through their contribution to humus formation. These fungi grow mainly in the cell lumen of the woody cells, and the degradation is not localized to the fungal hyphae but found at greater distances from these. The extracellular enzymes formed are too large to penetrate healthy cell walls and therefore—as noted above—degradation of cellulose by brown-rot fungi must involve diffusible low-molecular agents. They employ small molecule reactive species to depolymerize lignin, cleave propyl side chain, and also demethoxylate the ring structures before repolymerizing the material elsewhere as a means of freeing the cellulosic components and generating greater access for deconstruction (Arantes and Goodell 2014). Brown rot fungi have evolved multiple times from the predecessors of current white rot fungi. During this evolution, the typical lignolytic enzyme systems of white rots and crucial types of cellulases have been lost (Floudas et al. 2012).

Although some brown rots possess cellobiohydrolases, they generally lack the ability to hydrolyze crystalline cellulose enzymatically. Nonenzymatic deconstruction of cellulose uses iron-dependent Fenton chemistries (Arantes and Goodell 2014). Crystalline cellulose can efficiently be degraded by a combination of classical endoglucanases and an oxidative degradation system such as extracellular reactive oxygen species (ROS). These include hydroxyl radicals (•OH) and the less reactive peroxyl (ROO•) and hydroperoxyl (•OOH) radicals (Hammel et al. 2002). There is a well-established pathway for the generation of these radicals via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \bullet OH$). In order not to destroy the fungal hyphae and to act in the lignified parts of the secondary cell wall, the •OH production has to occur at a distance

from the hyphae, and the fungal reductants should be stable enough to diffuse before they react to reduce Fe^{3+} and O_2 to Fe^{+2} and H_2O_2. The production of •OH radicals can take place in several ways including secreted hydroquinones, cellobiose dehydrogenases, low molecular-weight glycopetides, and phenolate chelators. A chelator-mediated Fenton (CMF) system has evolved in different brown rot fungi (Gloeophyllales, Polyporales, and Boletales), providing an efficient mechanism for depolymerization and modification of lignocellulolytic biomass (Arantes and Goodell 2014; Eastwood et al. 2011). The CMF system is unique as it is based on oxygen radical chemistry that permits nonenzymatic deconstruction at a considerable distance from the organism. The efficiency of the CMF system is thought to provide brown rot fungi advantages in exploiting ecological niches, and, for example, these fungi have displaced white rot predecessors in the degradation of conifer wood.

The principle of the quinone redox cycling for •OH production is that the fungus reduces the quinone extracellularly to its hydroquinone which then reacts with Fe^{3+} to Fe^{2+} and a semiquinone radical. The semiquinone reduces O_2 to •OOH, which is a source for H_2O_2, and is in this way recycled to quinone. *Gloeophyllum trabeum* produces extracellular quinones including 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone which can reduce Fe^{3+} and O_2 rapidly under physiological conditions, thereby generating both Fe^{2+} and H_2O_2. Moreover, the fungus was shown to reduce the resulting dimethoxyquinones back to hydroquinones, possibly by the action of an intracellular quinone reductase. Another nonenzymatic system includes phenolate or catechole chelators (Goodell 2003). They have a high affinity for the binding of iron and have the ability to reduce Fe^{3+} to Fe^{2+}. The two compounds 4,5-dimethoxy-1,2-benzenediol and 2,5-dimethoxy-1,4-benzenediol were identified in the Gt chelator fraction and also their oxidized benzoquinone forms (see above). •OH radicals can also be produced by the extracellular flavohaemoprotein cellobiose dehydrogenase (CDH). CDH production has been reported for all types of wood-rotting fungi (Zamocky et al. 2006), and CDH can act as cellobiose oxidase by reducing O_2 to H_2O_2. However, Fe^{3+} is a better electron acceptor than O_2 and, thus, CDHs are actually Fe^{3+} reductases. Glycopeptides, implicated in wood degradation, have been isolated from *G. trabeum* and *Tyromyces palustris*. They reduce Fe^{3+} to Fe^{2+} and bind Fe^{2+} (Goodell 2003; Enoki et al. 2003). In the presence of H_2O_2, the glycopeptide generates one-electron oxidation and possesses the ability to oxidize NADH in the presence of oxygen and thereby produces H_2O_2.

Most brown rots secrete oxalic acid, which is a strong chelator of Fe^{3+} and Fe^{2+} but also reduces the pH. The pH of wood itself is generally in the range 3–6 and is lowered to pH values between 2.5 and 1.7. The reduction of pH is important for the function of the extracellular enzymes and has been identified as a key factor in several hypotheses related to molecular weight degradation systems, as discussed in the reviews listed above.

B. White-Rot Fungi

White rots are the most frequently found wood-rotting organisms and are mainly basidiomycetes, but also some ascomycetes (*Diatrypaceae* and *Xylariaceae*) are able to cause white rot. They are characterized by their ability to completely degrade lignin, hemicelluloses, and cellulose, thereby giving rise to the name-giving cellulose-enriched white colored wood material. A typical white rot degradation is primarily enzymatic, and the attack of the wood cell wall proceeds only from lignocellulose surfaces because degradative enzymes are too large to penetrate the intact cell wall. The enzymes employed by the white rot fungi include a complete suite of cellulases, with some fungi possessing a large number of LPMO encoding genes (Busk and Lange 2015) and enzymes that can oxidize lignin components, including lignin, manganese, and versatile peroxidase or laccases (Pollegioni et al. 2015). Two different white-rot patterns have been described which are simultaneous and selective delignification. Simultaneous or nonselective delignification acts mainly on hardwood and degrades cellulose, lignin, and hemicellulose simultaneously. The cell wall is attacked progressively from the cell lumen towards the middle lamella. Degradation is associated with the fungal hyphae and substantial amounts of undecayed wood remains. Basidiomycetes (e.g., *Trametes versicolor*, *Irpex lacteus*, *P. chrysosporium*, *Heterobasidion annosum*, and *Phlebia radiata*) and some ascomycetes (e.g., *Xylaria hypoxylon*) perform this type of degradation. Selective delignification, or sequential decay, is found in hardwood and softwood. The initial attack is selective for lignin and hemicellulose, and the cellulose is attacked later. Lignin is degraded in

the middle lamella and in the secondary wall. This type of degradation is performed exclusively by basidiomycetes (e.g., *Ganoderma australe*, *Phlebia tremellosa*, *C. subvermispora*, *Pleurotus* spp., and *Phellinus pini*). Many white-rot fungi cause both types of rot, and the amount of simultaneous or selective decayed wood depends on the substrate and varies even among different strains of the same species (Martinez et al. 2005; Eriksson et al. 1990). To date, *P. chrysosporium* is the most intensively studied white-rot fungus (Martinez et al. 2004; Cullen and Kersten 2004).

White-rot fungi degrade lignin via an oxidative process involving peroxidases and laccases (phenol oxidases) which act nonspecifically by generating highly reactive, nonspecific free radicals that attack lignin causing spontaneous cleavage reactions (Hammel and Cullen 2008).

Heme peroxidases, such as the lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13), and the versatile peroxidase (VP; EC 1.11.1.16), have been described as true ligninases due to their high redox potential which enables them to oxidize non-phenolic aromatic substrates constituting up to 90 % of the lignin structure. Usually, a number of isoenzymes are produced which is either due to posttranslational modifications or the presence of multiple genes in the genome. Peroxidases require the presence of H_2O_2 as oxidizing substrate and oxidize phenolic and non-phenolic compounds (Hatakka 1994; Kersten 1990; Hammel et al. 1986; Eggert et al. 1997). LiP and MnPs were firstly described for *P. chrysosporium* (Glenn and Gold 1985; Kirk and Farrell 1987; Paszczyński et al. 1985).

The catalytic, oxidative cycle of LiP is similar to those of other peroxidases. A heme group (Fe^{3+}) in the active center oxidizes H_2O_2 while forming an intermediate compound I known as LiP oxyferryl. Compound I is reduced by veratryl alcohol via one-electron transfer forming compound II before it is reduced to its peroxidase resting state via a second one-electron transfer (Hofrichter et al. 2010; Liu et al. 2003). Veratryl alcohol is oxidized to short-lived cation radicals which oxidize lignin directly or pass on the charge to other, more stable carriers which can act as diffusible mediators. Since enzymes such as LiPs are too large to enter the plant cell, direct degradation is carried out only in exposed regions of the cell lumen (simultaneous delignification). But microscopic studies of selective lignin biodegradation revealed that white-rot fungi remove the polymer from inside the cell wall, which can be performed only by indirect oxidation mediated by low molecular-weight diffusible compounds capable of penetrating the cell wall.

MnPs are closely related to LiPs and have an iron protoporphyrin IX (heme) prosthetic group. They have the same catalytic cycle involving a two-electron oxidation of the heme by H_2O_2, followed by two subsequent one-electron reductions. But instead of veratryl alcohol, MnPs oxidize Mn^{+2} to Mn^{+3}, which is stabilized by organic acids such as oxalate, fumarate, and malate.

Chelated Mn^{+3} ions can act as diffusible oxidizer on phenolic substrates and oxidize non-phenolic substrates via lipid peroxidation reactions (Martinez et al. 2005; Hofrichter et al. 2010). The reaction is similar to LiPs and initiated by the enzyme and H_2O_2 to form MnP compound I, a Fe^{4+}-oxo-porphyrin-radical complex. A monochelated Mn^{+2} ion transfers one electron to the porphyrin intermediate to form compound II and is oxidized to Mn^{3+} before the resting state is reached by one electron transfer to form Mn^{+3} by an electron transfer from Mn^{+2}. The more recently discovered VP combines the enzymatic properties of both LiP and MnP and oxidizes Mn^{+2} and veratryl alcohol (Hofrichter et al. 2010). A VP was firstly described for *Pleurotus eryngii* (Martinez et al. 1996). The catalytic cycle of VP combines both LiP and MnP, but this cycle differs from the classical MnPs by catalyzing the Mn^{+2}-independent oxidation of simple amines and phenolic monomers (Perez-Boada et al. 2005). The catalytic versatility of VP permits its application in Mn^{+3}-mediated or Mn-independent reactions in both low- and high-redox potential aromatic substrates. Although VP from *P. eryngii* catalyzes the oxidation of Mn^{+2} to Mn^{+3} with H_2O_2, it differs from classical MnPs in its manganese-independent activity, thereby enabling it to oxidize substituted phenols and the veratryl alcohol (Camarero et al. 1999).

The crystal structures have been resolved for both LiP and MnP (Piontek et al. 1993; Poulos et al. 1993; Sundaramoorthy et al. 1994). The prosthetic group (iron protoporphyrin IX) of LiPs is accessible only through a narrow pore (Piontek et al. 2001). Although its catalytic cycle is common to peroxidases, it is noted that the position of the iron-binding histidine residue in ligninolytic peroxidases is located further away from the heme iron which increases the redox potential. Also the existence of specific binding sites for substrate oxidation are unique (Martinez 2002). The substrate-

binding sites have been identified for LiP, MnP, and VP, and explain the dual catalytic properties of VP. Mn^{2+} oxidation occurs at a binding site near the cofactor which enables direct electron transfer. By contrast, veratryl alcohol is oxidized at the surface of the protein by a long-range electron transfer mechanism. The rationale of the existence of this electron transfer mechanism is related to the fact that many of the aromatic substrates cannot penetrate inside the LiP/VP and, therefore, these substrata are oxidized at the enzyme surface, and electrons are transferred to the heme (Doyle et al. 1998; Gold et al. 2000; Sundaramoorthy et al. 1997).

Laccases (EC 1.10.3.2) are multicopper phenoloxidases and generally larger than peroxidases. They directly oxidize phenols and aromatic amines and catalyze a one-electron oxidation combined with a four electron reduction of O_2 to H_2O. Laccase catalysis requires four copper atoms, which are held in place at the catalytic center by four histidine-rich, copper-binding regions (Claus 2004). The phenolic nucleus is oxidized by removal of one electron, generating phenoxy-free-radical products, which can lead to polymer cleavage. Due to their low redox potential, non-phenolic substrates have to be oxidized by other mediators. Metabolites such as 3-hydroxyanthranilate can mediate oxidation in *Pycnoporus cinnabarinus* (Eggert et al. 1997), and also lignin degradation products can act as redox charge transfer molecules (ten Have and Teunissen 2001).

H_2O_2-generating enzymes are essential for the function of the peroxidases and include glyoxal oxidase, glucose 1-oxidase, methanol-oxidase, aryl-alcohol oxidase, and oxalate decarboxylase oxidases which reduce O_2 to H_2O_2 (Zhao and Janse 1996). Flavin is often used as cofactor, with the exception of, e.g., copper-containing glyoxal oxidase from *P. chrysosporium*. Cellobiose dehydrogenase oxidizes soluble cello- and mannodextrine and uses a wide spectrum of electronacceptors (Fe^{3+}, Cu^{2+}, quinone, and phenoxy radicals). Proposed roles of CDH in the ligninolytic system comprise (1) the reduction of aromatic radicals formed by ligninolytic enzymes, thereby preventing repolymerization and supporting lignin degradation, (2) the production of •OH radicals via a Fenton-type reaction to modify cellulose hemicellulose and lignin, and (3) a cooperation with the manganese peroxidases to make the abundant non-phenolic components of lignin accessible for MnP and laccases. The role of ROS in the initial attack of lignin has also been discussed (Hammel et al. 2002) and was reviewed already in the brown-rot section.

V. Conclusions

The recycling of polymers by fungi is an essential process for life on earth which is today exploited in the biotech industry to produce fermentable sugars for different biorefinery products including bioethanol. Fungi produce a plethora of plant cell wall-degrading enzymes and efficiently degrade this highly complex carbon source. Although most of these enzymes are known for decades, the recent discovery of a new group, the lytic polysaccharide monooxygenases, as main actors in polysaccharide degradation shows that our understanding of polysaccharide and lignocellulose deconstruction is still limited. Comparative genomics, transcriptomics, and proteomics have provided us over the past years with a number of potentially interesting enzymes and accessory proteins, which will reveal new insights into how fungi use plant cell walls for their metabolism. Still, a major challenge for future research is to understand the complex multienzyme process of lignocellulose decomposition and to transfer this knowledge for the development of novel enzyme formulations to increase the efficiency of plant cell wall saccharification in the bio-based economy.

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