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CHAPTER

1 Chemical aspects of the composite structure of wood and its recalcitrance to enzymatic hydrolysis

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1.1 Introduction

Woody biomass as a renewable resource for chemicals, materials, and energy has garnered significant attention to replace fossil-carbon sources. However, in carbohydrate-based biorefineries, the recalcitrance of wood warrants a pretreatment prior to further processing by enzymatic hydrolysis (EH) and fermentation. Various processes or a combination thereof, including but not limited to, low pH (mild and strong acid pretreatments), neutral pH (autohydrolysis includes hydrothermal and steam explosion pretreatments), high pH (mild and strong alkali pretreatments), organic solvents, ionic liquids, deep-eutectic solvents, biological methods, electron beam and microwave irradiation, and mechanical methods (ultrasound and grinding), are currently under investigation as pretreatment methods to facilitate the subsequent EH. Wood is a natural composite, comprised of structural constituents: cellulose, lignin, and hemicelluloses; and minor, nonstructural compounds: extractives. The recalcitrance of wood is a multifaceted phenomenon inextricably linked to the structure of individual structural polymers, as well as their close interactions. In this chapter, we describe the formation of the wood matrix, the individual constituents (chemical composition), and their contribution to the recalcitrance of wood. In-depth understanding of these concepts is required to pave the way to better tailor processes for the complete valorization of wood as a sustainable feedstock.

1.2 Trees as a renewable resource for biorefineries

During the Ordovician period (~450 million years ago), early vascular plants were able to adapt from the marine environment to terrestrial life based on new developing

[a] Biljana Bujanovic part of contribution is in the Public Domain.

metabolic pathways (Terashima et al., 1993), such as the phenylpropanoid pathway. This provided plants with the necessary defense mechanisms, such as resistance to gravitational forces, ultraviolet radiation, and desiccation stress. The phenylpropanoid biosynthetic pathways further evolved to include lignification as the last phase in the formation of cell walls, incorporating lignin, thereby providing mechanical reinforcement, hydrophobicity for the transport of water and solutes, and protection against pathogens. These features have rendered land plants a major source of biomass on earth, representing >80% of the total biomass estimated at ~550 billion tons, while annually accumulating ~56 billion tons of carbon through photosynthesis (Zhong et al., 2019). As a major carbon sink that stores approximately half of this amount (Weng and Chapple, 2010; Zhong et al., 2019), trees contribute toward reducing the warming effects associated with carbon dioxide emissions. They are a viable source to replace fossil-carbon resources for the production of fuels, materials, and chemicals via wood-based biorefineries, thereby contributing to decarbonization and a sustainable bioeconomy (Devaney and Iles, 2019). The ultimate goal of such a biorefinery would be to design a "zero-waste process" (Zeng et al., 2014), where all the wood constituents, that is, the newly sequestered carbon, would be converted to products that can substitute fossil-based carbon. However, to unleash the full potential of trees for creating novel renewable products, it is necessary to elucidate the chemical structure of the structural polymers and their three-dimensional assembly and interactions that confer recalcitrance toward biological, chemical, and thermal processes required for the complete valorization of wood. Additionally, tremendous progress has been made in deciphering the biosynthetic pathways involved in building the cell wall (Meents et al., 2018; Zhong et al., 2019). This has opened avenues for tailoring the intrinsic features of trees via genetic modifications, thereby facilitating conversion processes to meet the needs of specific applications (Himmel et al., 2007).

1.3 Formation of the wood cell wall matrix and effects on EH

1.3.1 Cell diversity and cell development (xylogenesis) in wood

The cell types and shapes of trees are incredibly diverse, and the differences in the chemical composition and ultrastructure of the cell walls yield variations in the properties of wood among species, genotypes within species, and morphological areas within the same trees. Wood is a product of the vascular cambium (Fig. 1.2), a **lateral meristem,** which consists of meristematic cells organized in radial files that produce the secondary xylem (toward the inside of the growing stem, water- and minerals-conducting tissue) and phloem (toward the outside of the growing stem, bark, food-conducting tissue) facilitating growth in the thickness dimension. The **apical meristem** located at the tips of the stems and roots enables longitudinal growth. The activity of the meristematic cells serves as an important indicator of the growth rate; therefore, factors such as the number of initials and duration of the cell cycle should be considered in efforts directed toward maximizing the growth of trees (Mellerowicz et al., 2001).

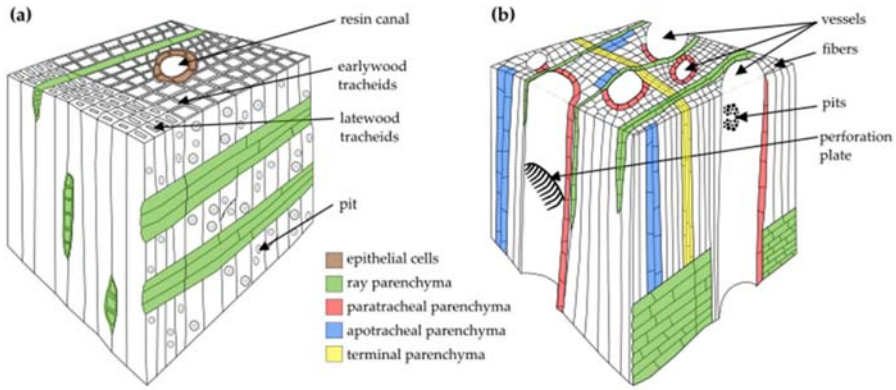

FIGURE 1.1

Schematic representation of (a) softwood and (b) hardwood. Only selected cell types are marked. Cell proportions may not be accurate. A color print is available in the electronic version.

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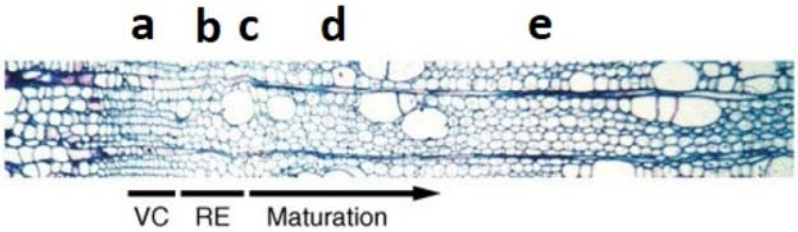

FIGURE 1.2

Wood development in *Populus*. (a) Vascular cambium (VC), (b) = radial expansion (RE), (c) = transition between RE and maturation, (d) = secondary wall formation, (e) = late maturation.

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Xylogenesis describes the formation and development of cells as an ordered process that is spatially and temporally controlled by hormonal signals. This process yields various categories of cells, including supporting cells, conducting cells, and storage cells, specialized to perform specific functions that are vital for trees. Gymnosperms (softwoods (SWs)) contain tracheids that were developed ∼430 million years ago in more primitive plants to provide water transport and mechanical support. The differentiation of the tracheids is induced by two primary phytohormones, namely, auxin, the young leaf signal, and gibberellin, the mature leaf signal. Angiosperms (hardwoods (HWs)) contain vessels and fibers. Both vessels and fibers were developed ∼140 million years ago from tracheids with hormonal specialization, by which auxin and gibberellin induce the differentiation of vessels and fibers, respectively (Aloni, 2015). The thin-walled vessels are joined end-to-end to form long

tubes and serve as water-transporting channels, while fibers provide the support function. In addition, the parenchyma cells in rays are used for storage in both SWs and HWs. The different types of cells in SWs and HWs are depicted in Fig. 1.1. As such, HWs are evolutionarily more advanced and characterized by a more complex structure than SWs.

Xylogenesis (Fig. 1.2) involves the following stages: (1) **Cell division**, where the daughter cells are created from the cambial initials/stem cells. (2) **Cell expansion** in the radial and longitudinal directions establishes the cell diameter and length, respectively. The radial is the symplastic growth, where the neighboring cells grow together, while the longitudinal is the intrusive growth, that is, elongation, where the neighboring cells move past one another. Additionally, the primary wall (P-wall, ~0.1 μm thick) (Fig. 1.3) forms in the cell expansion stage (Mellerowicz and Sundberg, 2008; Plomion et al., 2001). (3) **Secondary (S)-wall deposition** (Fig. 1.3), where the cell wall material consisting mostly of polysaccharides is formed and deposited. (4) **Lignification**, during which the cell wall is stiffened and waterproofed. The S-wall consists of the sequentially arranged S_1, S_2, and S_3 layers with thicknesses of 0.1−0.35, 1−10, and 0.5−1.10 μm, respectively. The S-wall is assembled between the plasma membrane and P-wall, and accounts for the majority of the total thickness of the cell wall; therefore, it governs the properties of wood (Plomion et al., 2001). (5) The last phase of xylogenesis, **programmed cell death** (PCD), described as "cell-autonomous, active, ordered suicide" (Roberts and McCann, 2000) occurs in all cells. Most of the cells undergo PCD soon after the completion of lignification but earlier than the radial and longitudinal parenchyma

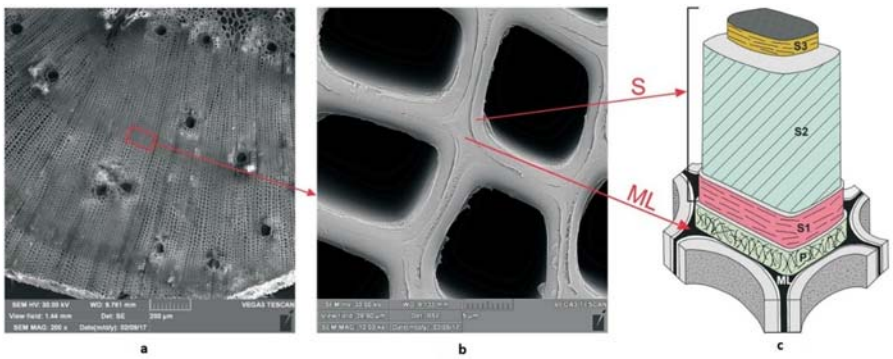


FIGURE 1.3

Scanning electron microscopy (SEM) images of the transverse section of xylem tissue of *Pinus sylvestris*. (a) Early and the latewood tracheids. (b) SEM images of the indicated area of panels (a), and (c) woody cell walls layers; ML, middle lamella; P, primary wall; S, secondary wall; S layers: S1, outer layer; S2, middle layer; S3, innermost layer. A color print is available in the electronic version.

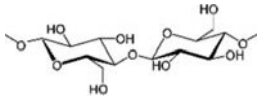
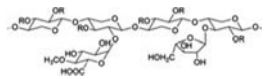
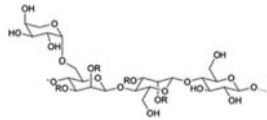
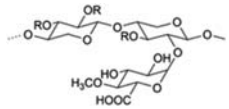
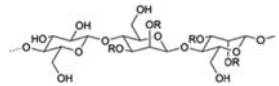
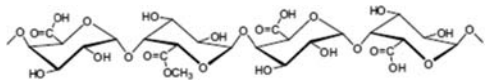
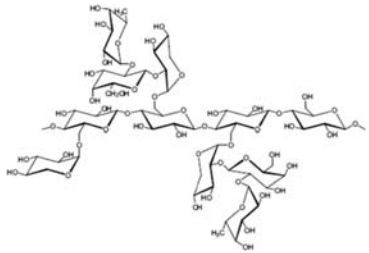
Reprinted with permission from Janusz et al. (2017); (c) has been slightly modified to clearly differentiate between the ML, P, and three S-wall layers: S_1, S_2, and S_3.

cells. PCD occurs via the action of specific proteases, which degrade key cellular proteins while leaving the cell wall intact. Post-PCD, the cells lose their metabolic functions and appear as elongated, stiff, hollow water-conducting tubes (Fig. 1.3). The tracheids, vessels, and fibers are connected to each other by the middle lamella (ML; 0.5−1.5 μm thick), which is composed mostly of lignin. The longitudinal and radial parenchyma cells remain active longer and form the sapwood, the external zone of a mature tree, along with the much more abundant dead cells. The parenchyma cells eventually lose their function, while their contents are also hydrolyzed. The death of the parenchyma cells indicates the transition of sapwood to heartwood, which is located closer to the center of the tree and consists of dead cells only.

Notably, cells within the same category vary in size, structure, and chemical composition depending on various exogenous (e.g., season/temperature; terrain/slope) and endogenous (phytohormones) factors. This variation can be described by the many examples of contrasting features in the various morphological areas and tissues. For example, two distinct regions of juvenile and mature wood may be observed from the stem center outwards, that is, wood formed by a young tree in the area adjacent to the pith produces cells of variable properties, whereas wood formed later by an aged tree produces more uniform cells. Furthermore, the demarcation between these two zones is based on different criteria, including the density, cell length, and microfibril angle (MFA: the angle between the cell long axis and cellulose microfibrils (MFs)). In addition, owing to the seasonal variability, annual rings are formed and contain spring- or early-wood and summer- or late-wood. Further, reaction wood differs from normal wood; it is formed in response to mechanical stresses in the growth environment, such that it assists in restoring the vertical position of an inclined stem. The reaction wood in HWs and SWs is referred to as tension wood and compression wood, respectively (Mellerowicz et al., 2001; Mellerowicz and Sundberg, 2008; Plomion et al., 2001; Sjöstrom, 1993). In that respect, it should be noted that fibers in tension wood terminate toward the lumen by a thick gelatinous layer (G-layer), which is high in cellulose content. Further, the cellulose in the G-layer exhibits a high degree of crystallization and a low MFA. In contrast to tension wood, compression wood has a lower content of cellulose and a higher content of lignin than normal wood in SWs (Mellerowicz and Sundberg, 2008; Plomion et al., 2001; Sjöstrom, 1993).

Concurrently with cell division, a cell plate is formed between 2 cells, along with the pectin-rich ML, where the pectins help adhere the cells. Homogalacturonan (HG), representing ∼65% of all pectins, has a backbone of α-(1 → 4) linked-galacturonic acid chains that are partially methylesterified at the C_6 carboxyl group and acetylated at O_2 and O_3. The extent of methylesterification of HGs changes through the different phases of cell wall formation, stimulating (esterified form) or inhibiting the growth (de-esterified form) (Mellerowicz and Sundberg, 2008). During cell expansion, the P-wall is simultaneously formed mainly from the deposition of pectins, xyloglucan (XG), and cellulose (Table 1.1). XG is a β-(1 → 4) glucan backbone with α-(1 → 6) xylopyranoside residues distributed at different intervals along the chain and can be further decorated by galactose, fucose, and

Table 1.1 Structure of polysaccharides in softwoods (SWs) and hardwoods (HWs); R═H, C(═O)─CH_3.

	Polysaccharides		
Wood	**Cellulose**	**Xylans**	**Galactoglucomannans**
SW		Arabinoglucuronoxylan 	Galactoglucomannans 
HW		Glucuronoxylan 	Glucomannan 
SW & HW		Pectin—homogalacturonan  Xyloglucan 	

glucuronic acid. It interacts with the cellulose MFs forming hydrogen bond-based cross-links and a strong but extensible XG–cellulose network that serves as the main load-bearing component of the P-wall. Moreover, XG may be associated with pectins through chemical bonds (Pauly et al., 2013), playing a key role in the regulation of cell wall extensibility (Mellerowicz et al., 2001). The cell expansion step of xylogenesis is characterized by high expression of genes that encode numerous enzymes, including expansins, pectinases, pectin methyl esterases, glycosyl transferases, xyloglucan endotransglycosylases, and endoglucanases. Recently, genetic modifications, aimed to tailor the biosynthesis of pectins and XG to improve biomass growth yields, loosen the cell wall structure and improve the release of sugars during EH, have been attempted (Biswal et al., 2018; Damm et al., 2016; Pauly and Keegstra, 2008). The pectin- and XG-containing ML and P-wall are designated as compound ML (CML), which is mainly composed of lignin. By the end of cell wall formation, CML accounts for <20% of the total thickness of the cell wall; therefore, the contribution of XG and pectin toward wood recalcitrance has received limited attention as compared to that of hemicelluloses and lignin.

Following the cessation of cell expansion, the deposition of the S-wall sequentially continues with the biosynthesis of cellulose and hemicelluloses in the three layers, S_1, S_2, and S_3 (simplified structure in Fig. 1.3). The three layers are sandwiched by the P-wall and plasma membrane, that is, lumen after the PCD, and can be differentiated with respect to the inherent orientation of the cellulose MFs, that is, MFA, and the degree of lignification/lignin content following lignin deposition. Since the S-wall contains >80% of the total biomass in wood, it is the primary contributor to wood recalcitrance (Meents et al., 2018; Sjöstrom, 1993).

1.4 Cellulose

1.4.1 Structure and function

Cellulose is the most abundant natural polymer on earth, synthesized by different organisms, including plants, marine animals (e.g., tunicates), algae, and bacteria, with an estimated annual production of 10^{10}–10^{11} tons (Meents et al., 2018). Cellulose content in wood can range from 40% to 50% (Plomion et al., 2001; Sjöstrom, 1993; Zhong et al., 2019). Its extensive use as a feedstock for the pulping and energy industries has prompted thorough research to understand its structure, biochemistry, and synthesis of MFs. Cellulose is a homopolysaccharide composed of β-D-glucopyranose units bound together with a β-(1 → 4) glycosidic bond (Table 1.1). The glucopyranose units polymerize via an enzymatically controlled condensation reaction, where water is expelled and the glycosidic bond is formed. The enzymes employed in cellulose synthesis are glycosyltransferases (GT) from the cellulose synthase type 1 GT2 family (CAZy - GT2, n.d.; Lombard et al., 2014). The degree of polymerization (DP) of cellulose in situ can vary from 10,000 to 15,000 (Moon

et al., 2011). Cellulose has two ends: the reducing end (RedE), which is a chemically reducing functionality (C_1−OH group), and a nonreducing end (NRedE, C_4−OH group). The cellulose chains are reinforced by intrachain and interchain hydrogen bonds (HBs), which coalesce into MFs that are packed together in aggregates/macrofibrils to form cellulose fibrils as the major load-bearing elements (McKendry, 2002; Robak and Balcerek, 2018).

Comprehensive studies of the cellulose structure by X-ray diffraction (XRD), nuclear magnetic resonance (NMR), and Raman spectroscopy (Gardner and Blackwell, 1974; Peter, 2021; Sjöstrom, 1993) were further expanded by Gross and Chu (2010), who characterized the intrachain, interchain, and intersheet interactions (Fig. 1.4) of cellulose via all-atom molecular dynamics simulations. Intrachain interactions are the specific HBs, (OH)−O, between neighboring residues of the same chain, whereas interchain interactions are those between residues of neighboring chains. Intrachain HBs limit the rotation of glucose units about the glycosidic linkages. Intersheet interactions are the (CH)−O bonds and van der Waals interactions connecting residues on neighboring sheets and are the most robust. Higher intensities of infrared (IR) signals and dynamic Fourier transform infrared (FTIR) spectra indicate that the (OH)3−(O)5 intrachain HB is the dominant form, has a higher Young's modulus, and predominantly responds to applied stress. The inter- and intrachain HBs are an important feature of cellulose that determine its overall strength and yield the four different crystalline forms of cellulose (I−IV). Cellulose I is the native or naturally occurring and the most unstable form found in plants and

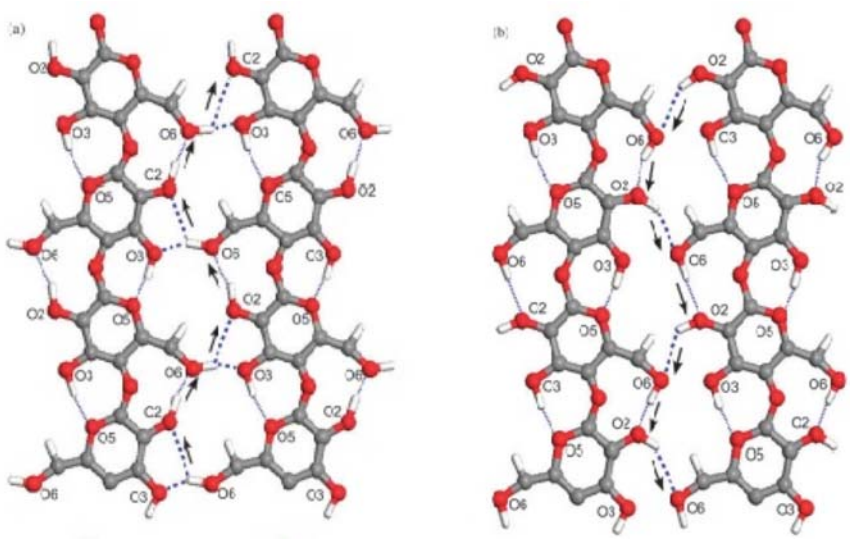


FIGURE 1.4

Cellulose hydrogen bonds. A color print is available in the electronic version.

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some bacteria and algae. It has parallel chains packed together to form MFs, with two intrachain bonds at (OH)3−(O)5 and (OH)2−(O)6, and an interchain bond at (O)6−(O)3. Physical and chemical deformations of cellulose I yield the more inherently stable isoforms of cellulose II−IV. Cellulose II has antiparallel chains, with an intrachain bond at (OH)3−(O)5, two interchain bonds at (OH)6−(O)2 and (OH)6−(O)3, and additional intersheet bonds at (OH)2 (center chains) and (O)2 (corner chains). The higher number of inter- and intrachain HBs render it more thermodynamically stable (O'Sullivan, 1997). It has been suggested that despite cellulose II being more stable than cellulose I and cellulose II provides greater enzyme access and enhances EH (Wada et al., 2010).

1.4.2 Cellulose biosynthesis

Cellulose biosynthesis is conducted by the action of the membrane-bound cellulose synthase complex (CSC). It is an essential process in higher plants that affects cell wall formation, including the formation of P-wall and S-wall, creating a highly ordered fibrous scaffold for the deposition of hemicelluloses and lignin in the S-wall (Fig. 1.3). The CSC uses UDP-glucose as a substrate for cellulose biosynthesis. CSCs are thought to be assembled in the Golgi apparatus and delivered to the plasma membrane by Golgi vesicles guided by cortical microtubules. It has been shown that CSCs consist of a group of cellulose synthase proteins, CesAs (inverting enzymes of the Type 1 GT2 family), which remain attached to the growing cellulose chains. Their number and arrangement in the CSC are dependent on the organism producing cellulose (Guerriero et al., 2010; Malcolm Brown et al., 1996).

Each CesA protein synthesizes one cellulose chain at the plasma membrane, unlike other polysaccharides, which are synthesized in the Golgi.

The arrangement of the CSC and its role in guiding the cellulose biosynthesis in vascular plants have been the subject of much debate. The CSC has a six-lobed structure (Brown, 2004; Malcolm Brown et al., 1996; Nixon et al., 2016), often referred to as the rosette; its extracellular face has a diameter of approximately 25−30 nm (Saxena and Brown, 2005), whereas a larger globular structure emerges on the cytosolic face with a diameter of ∼40 nm (Meents et al., 2018). Initially, the CSC rosette was presumed to synthesize an MF comprised of 36 (6 6) cellulose chains, which is now considered less likely. New evidence suggests that one rosette CSC synthesizes an 18-chain (6 3) MF, that is, three chains per lobe. Recent spectroscopic analysis of cell walls from various plants has verified MFs with 18−24 chains (Fernandes et al., 2011; Jarvis, 2018; Newman et al., 2013; Thomas et al., 2014; Turner and Kumar, 2018; Wang et al., 2015; Wang and Hong, 2016) (Fig. 1.5). MFs are 3−4 nm wide as demonstrated by atomic force microscopy, small-angle neutron scattering (SANS), and wide angle X-ray scattering (Fernandes et al., 2011; Song et al., 2020; Zhang et al., 2016). Closely associated MFs form larger cellulose macrofibrils aided by branched heteropolysaccharides, hemicelluloses, and surrounded by lignin to create a strong, recalcitrant composite cell wall structure through adhesion or dehydration (Donaldson, 2007). The macrofibrils

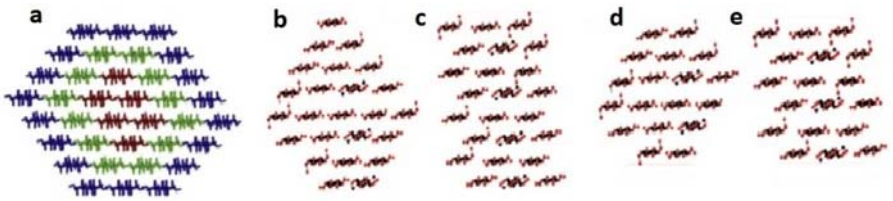


FIGURE 1.5

Potential cross-sectional shapes of cellulose microfibrils and impact on interactions with other wall components (a) 36-chain (hexagonal shape; the colors represent chain mobility, with internal residues (red) more rigid than surface residues (blue); the 36-chain model is now considered unlikely), (b) 24-chain (diamond shape), (c) 24-chain (hexagonal shape), (d) 18-chain (diamond shape), and (e) 18-chain (hexagonal). A color print is available in the electronic version.

Adapted from Cosgrove (2014) with permission.

can span up to 50 nm in width in S-walls (Anderson et al., 2010; Fernandes et al., 2011; Song et al., 2020; Zhang et al., 2016). Furthermore, CSCs move bidirectionally at an average speed of ∼350 nm/min (range 150−500 nm/min) in wood (Gu et al., 2010; Paredez et al., 2006). Therefore, with the step-wise addition of glucopyranose units, cellulose chains in wood are extended by an average of ∼700 GlcP residues per minute (300−1000), which is approximately one third of the rates of cellulose formation predicted for algal cellulose (Gu et al., 2010; Paredez et al., 2006) and only approximately one 10th of the rate of in vitro formation of bacterial cellulose (90 GlcP residues per second) (Haigler and Roberts, 2019). This production rate creates 7 μm-long cellulose chains in 20 min, while they serve as load-bearing elements in trees with a life expectancy exceeding 1000 years.

The polymerization of the cellulose chain occurs because the glucose molecule is directly transferred from the UDP-glucose onto the NRedE of the growing chain, releasing the UDP. Alternating glucopyranose molecules along chains are rotated by 180 degrees (Table 1.1, Fig. 1.4). Cellulose contains regions that are highly ordered (crystalline) and disordered (amorphous) (Horn et al., 2012; Moon et al., 2011). Regions of amorphous cellulose have been reported to be embedded within the highly crystalline microfibrils in spruce, evidenced by SANS (Fernandes et al., 2011) and transmission electron microscopy (Haigler et al., 2014). Additionally, almost entirely pure crystalline cellulose occurs in tension wood, most likely because of high tensile stress (Foston et al., 2011).

Intra- and interchain HBs stabilize the glucan chains in the MF, while hydrophobic van der Waals forces have been observed in aqueous environments (Cousins and Brown, 1997), indicating their higher prevalence in the P-wall, which has a high water content. In the S-wall, intrachain HBs are more dominant and result in a rigid, semi-crystalline polymer that provides strong structural support to the cell.

The DP is an important parameter regulating the cell wall function and ultrastructure and differs between the primary and secondary wall (DP: P-wall < S-

wall). Although cellulose elongation is partly controlled by the spatial constraints of the cell wall, the specific mechanism regulating the end of polymerization has not been defined. Furthermore, the factors controlling the length of cellulose chains may include the speed and lifetime of the CSC at the plasma membrane (Meents et al., 2018).

1.4.3 Effects of cellulose on wood recalcitrance

The cellulose MFs are embedded into the matrix of hemicelluloses and lignin to create a resilient structure that effectively resists biological attacks from enzymes and microorganisms—wood recalcitrance (Foston and Ragauskas, 2012). The poor hydrolyzability is also attributed to the higher number of HBs in long cellulose chains, whereas shorter cellulose chains contain a weaker hydrogen-bonding system, promoting enzyme accessibility (Hallac and Ragauskas, 2011; Meng et al., 2017). However, a positive correlation has been observed between cellulose content and glucose release (Yoo et al., 2017).

The DP of cellulose is considered a major factor influencing wood recalcitrance, as changes in DP alter structural parameters such as crystallinity and porosity; however, its exact role remains unclear (Zoghlami and Paës, 2019). Further, a decrease in DP has been correlated with an increase in EH efficiency (Lu et al., 2019; Puri, 1984). A lower DP indicates an increase in the number of REs and NREs available for the action of cellobiohydrolase I (CBHI) and cellobiohydrolase II (CBHII), respectively, thereby promoting EH (Hallac and Ragauskas, 2011). In particular, with a large amount of CBHI present among cellulase enzymes (~65%), it is expected that a lower DP leads to an increase in EH rates and yields (Hallac and Ragauskas, 2011). However, limited information is available on the effect of DP on cellulase adsorption. It has been shown that the endo—exo and exo—exo synergisms and the processivity of cellulases depend on the DP. Studies by Hallac and Ragauskas (2011) also suggest that the role of the various pretreatment methods on the reduction of DP varies, such that low-pH pretreatments cause a greater decrease in DP than alkali pretreatments. An increase in cellulose crystallinity has been reported to decrease the adsorption of cellulases, including a decrease in the cellulose-binding module, which in turn decreases the EH efficiency (Hall et al., 2010; Kong et al., 1992; Yang et al., 2011). Li et al. demonstrated that an increase in the crystallinity index (CrI) from 0.43 to 0.72 and 0.81 (CrI calculated from XRD patterns) led to a decrease in cellulose hydrolyzability from 80% to 68% and 57%, respectively (Li et al., 2018). Accordingly, the EH of amorphous cellulose has been noticed to proceed more rapidly (Yang et al., 2011). However, EH performed on different cellulose substrates produced the highest conversion yield of 90.7% on cellulose characterized by a relatively high degree of crystallinity of 63.3% (estimated from CP/MAS ^{13}C-NMR measurements) (Peciulyte et al., 2015). Similarly, there is evidence of tension wood exhibiting higher EH yields compared to normal wood despite a greater crystallinity of cellulose in its G-layer (Foston et al., 2011). Different cellulases exhibit different adsorption capacities and enzyme activities

for cellulose. Therefore, although there are reports indicating that the processivity of CBHI improves as cellulose crystallinity reduces, the relationship between cellulase processivity and cellulose crystallinity must be further clarified (Yang et al., 2011).

There are attempts to increase biomass production via genetic modifications to improve biomass yield. The overexpression of the cellulose synthase CesA2 gene isolated from *Pinus massoniana* in the poplar hybrid clone yielded a thicker S-wall and increased the content of cellulose and lignin, thereby causing an improvement in biomass production (Maleki et al., 2020). Further, transgenic studies on Arabidopsis have demonstrated that changes in MF can be induced and cellulose crystallinity reduced to improve EH (Harris et al., 2012).

1.5 Hemicelluloses

Hemicelluloses are not generally considered to be resistant to chemical, thermal, or biological decomposition. However, their association with cellulose MFs and covalent linkages with lignin and lignin–carbohydrate bonds (LC bonds) in lignin–carbohydrate complexes (LCCs) make them an important contributor to biomass recalcitrance (Martínez-Abad et al., 2018).

1.5.1 Structure, function, and content

Hemicelluloses are an intermediate wood constituent, which, based on the substitution pattern, change from high-energy regions to low-energy regions to interact with cellulose and lignin, respectively. The structure of hemicelluloses enables them to act as a bridge between lignin and cellulose through covalent bonds with lignin (LC bonds and LCC) and HBs with cellulose. In the wood cell wall, hemicelluloses behave somewhat like surfactants, facilitating the association between cellulose and lignin. Cellulose (Table 1.1, and Figs. 1.4 and 1.5) and lignin (Fig. 1.9) are characterized by different chemical structures and surface energies, which would be chemically incompatible in the absence of hemicelluloses (Hansen and Björkman, 1998).

The solubility parameter (i.e., cohesion energies) is a guiding principle in understanding the role of hemicelluloses in facilitating the network between lignin and cellulose (Hansen and Björkman, 1998). Tailored substitution of the hemicelluloses' main chain facilitates a dual role: segments with acetylated hydroxyl groups provide a decrease in solubility parameter/cohesive energy increasing interactions with (lower-energy) lignin, while segments featuring nonacetylated hydroxyl groups are more compatible with (higher-energy) cellulose through their hydrophilic surfaces. Thus, hemicelluloses are parallelly oriented to cellulose MFs in the regions with hydroxyl groups. This phenomenon was referred to as "segmental interactions" by Hansen and Björkman (Hansen and Björkman, 1998). The nature and substitution pattern of hemicelluloses have been shown to have significant effects on the mechanical properties of cellulose/hemicellulose hydrogels designed to mimic the S-wall prior to lignification (Berglund et al., 2020). Molecular modeling has also been

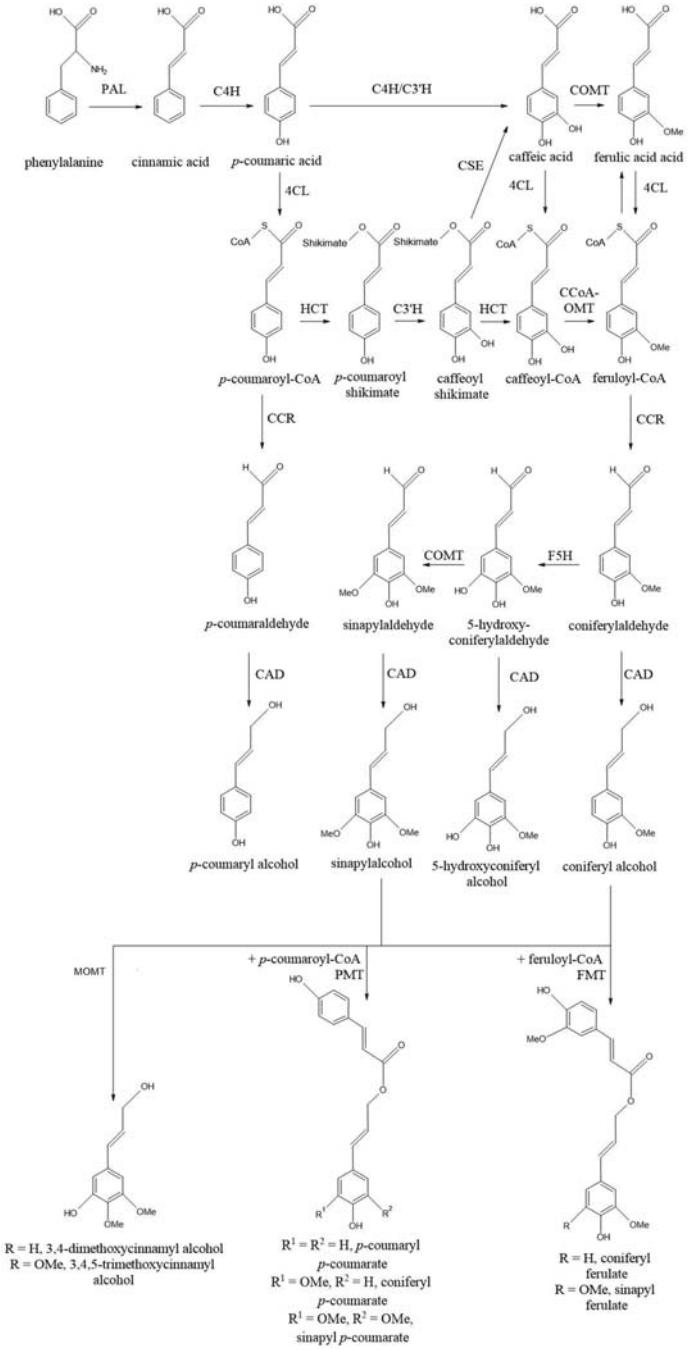


FIGURE 1.6

Lignin biosynthetic pathway. 4*CL*, 4-coumarate:CoA ligase; *C_3'H*, *p*-coumaroyl quinate/shikimate 3′-hydroxylase; *C_4H*, cinnamate 4-hydroxylase; *CAD*, cinnamyl alcohol dehydrogenase; *CCoAOMT*, caffeoyl-CoA O-methyltransferase; *CCR*, cinnamoyl-CoA reductase; *COMT*, caffeic acid O-methyltransferase; *CSE*, caffeoyl shikimate esterase; *F5H*, ferulates 5-hydroxylase/Cald5H, coniferaldehyde 5-hydroxylase; *FMT*, feruloyl-CoA monolignol transferase; *HCT*, *p*-hydroxycinnamoyl-CoA:quinate/shikimate-*p*-hydroxycinnamoyltransferase; *PAL*, phenylalanine ammonia-lyase; *PMT*, *p*-coumaroyl-CoA monolignol transferase.

Adapted from Chanoca et al. (2019) open access.

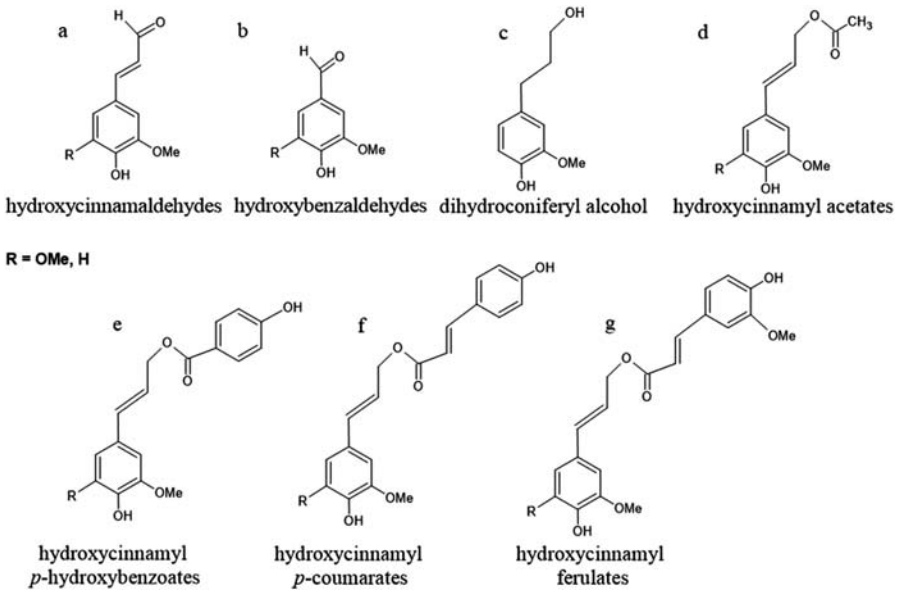


FIGURE 1.7

Lignin monomers.

Adapted from Vanholme et al. (2008).

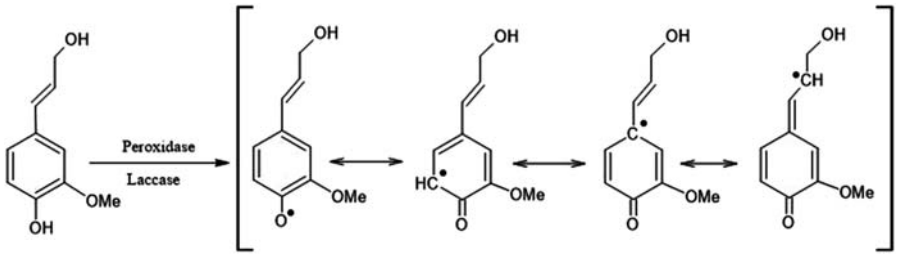


FIGURE 1.8

Resonance-stabilized monolignol phenoxy radicals (Sjöstrom, 1993).

employed to elucidate the role of substitution on cellulose–hemicellulose interactions and cell wall structural development (Pereira et al., 2017).

Through their capacity to interact/orient favorably with both lignin and cellulose, hemicelluloses vitally contribute to the stabilization of the composite cell wall structure. The substitution pattern as a guiding principle for interactions between hemicelluloses and lignin on one side, and cellulose on the other side has been underscored in recent studies (Berglund et al., 2020; Busse-Wicher et al., 2016; Wierzbicki et al., 2019). Additionally, the possibility of direct interactions between lignin and cellulose, especially through the hydrophobic area of the cellulose

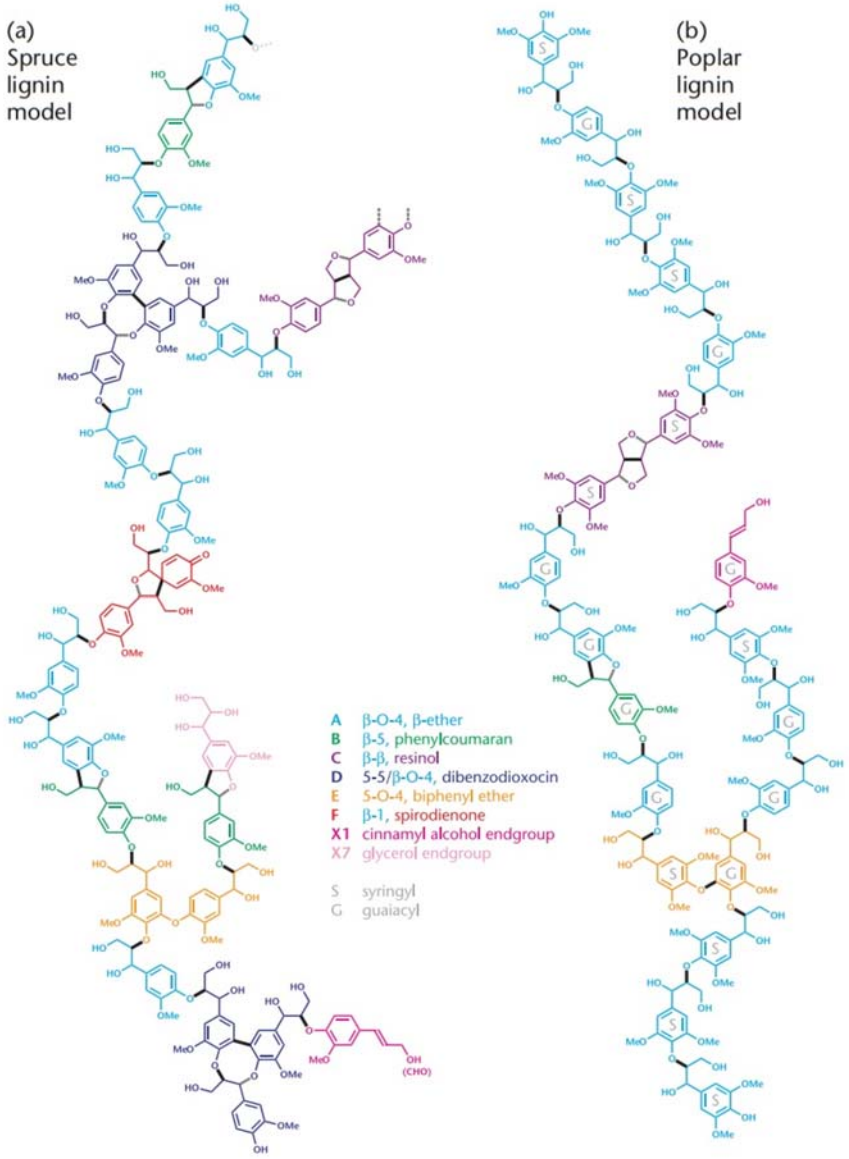


FIGURE 1.9

Representative structures of lignin (a) a softwood (spruce) lignin and (b) a hardwood (poplar) lignin. A color print is available in the electronic version.

Reprinted with permission from Ralph et al. (2007).

surface, has been suggested based on molecular simulation studies (Vermaas et al., 2019). These interactions are also discussed in consideration of lignin playing an inhibiting role in EH (Lindner et al., 2013).

1.5.2 Xylans

1.5.2.1 Glucoronoxylans in HWs

Xylans are the most abundant hemicelluloses in HWs, which also contain glucomannans in lesser amounts. The content of O-acetyl-4-O-methyl-α-D-glucurono-β-D-xylans (glucuronoxylans (GXs); Table 1.1) varies between 15% and 30%. GXs represented ∼60% of the noncellulosic sugar units in all, but one of 11 industrially relevant HW species surveyed; these 11 species were: four poplar species (*Populus* sp.; *P. tremula, P. tremuloides, P. grandidentata*, and *P. deltoides x nigra*), two eucalyptus species (*Eucalyptus* sp.; *E. globulus* and *E. dunnii*), common oak (*Quercus robur*), silver birch (*Betula pendula*), European beech (*Fagus sylvatica*), and two wattle species (*Acacia* sp.; *A. crassicarpa* and *A. mangium*). Noncellulosic glucose represented up to 10% of sugar units in most HWs in the same study (Willför et al., 2005). GXs are acetylated at C_2 or C_3 of the xylopyranose (XylP) residues (on average ∼7 Ac/10 XylP) and have a DP of ∼200 (Koshijima et al., 1965; Rowell, 2012). 4-O-methyl-α-D-glucuronic acid residues are linked at C_2 of the XylP residues (on average ∼1 MeGlcA/10 XylP) (Sjöstrom, 1993). The tetrasaccharide sequence of 1,4-β-D-XylP-1,3-α-L-RhaP-1,2-α-D-GalA-1,4-D-XylP has been observed at the RedE of GXs in HWs. Moreover, the substitution degree and pattern of xylan decorations depend on the wood species and specific tissue and stage of development. For example, GXs of the *Acacia* species have been shown to contain a relatively higher content of acidic MeGlcA groups. *Eucalyptus globulus* and *Populus deltoides* both contain more noncellulosic sugar units in the sapwood than in the heartwood, whereas other species contain relatively similar amounts in both tissues (Willför et al., 2005).

The complex nature of xylan decorations (and that of hemicelluloses in general) provides for the adoption of secondary/tertiary structures that promote interactions with other cell wall structural constituents such as cellulose and lignin. These decorations likely play an important role in rendering xylans relatively resistant to biological attack in nature and limiting xylan solubility in the lab (Busse-Wicher et al., 2016). A number of molecular modeling studies have shed light on the impact of the substitution pattern on the hemicellulose−cellulose interactions in the S-wall (Bromley et al., 2013; Busse-Wicher et al., 2016; Pereira et al., 2017). Two distinct domains have been observed in xylans: a "major" domain with more evenly spaced and less frequent modifications providing for a two-fold helical screw conformation and a "minor" domain with more frequent and irregular modifications providing for a three-fold helical screw conformation. These two domains have been hypothesized to provide for differential interactions with lignin and cellulose by the same xylan chain (Bromley et al., 2013). The two domains were found to be inseparable, suggesting that their tight association plays a critical role in building the composite, recalcitrant wood structure.

Acetylation in GXs in HWs plays a key role in modulating this secondary structure formation and the resulting interactions with cellulose and lignin. Random and frequent acetylation hampers the formation of consistent hydrophilic surfaces for interaction with cellulose MF faces and, where present, may promote interaction with lignin. Furthermore, acetylation has been shown to impede xylanase activity (Busse-Wicher et al., 2016). The pattern of acetylation and glucuronation of GX has also been shown to affect xylan−lignin interactions, including LCCs (Martínez-Abad et al., 2018).

As such, structural features of xylans extensively contribute to the composite cell wall structure, resulting in the resistance to chemical/enzymatic decomposition. Clarification of the biosynthetic machinery resulting in intrinsic substitution patterns characterizing the two xylan domains would be beneficial for overcoming recalcitrance and improving wood processability (Martínez-Abad et al., 2018; Silveira et al., 2013; Wierzbicki et al., 2019).

1.5.2.2 Arabinoglucuronoxylans in SWs

In SWs, galactoglucomannans (GGMs) are the major hemicelluloses (∼20% (Sjöstrom, 1993)) and considered the most important cellulose-binding polysaccharide (Busse-Wicher et al., 2016). However, a considerable quantity of xylans are present (5%−10% (Sjöstrom, 1993)). In contrast to GXs in HWs, SW xylans are not acetylated and contain arabinose decorations; arabinoglucuronoxylans (GAX) (Table 1.1) (Busse-Wicher et al., 2016). SW GAX feature arabinofuranose (AraF) and MeGlcA decorations on C_3 and C_2 positions of the xylan chain, respectively, and have a DP of ∼100 (Rowell, 2005). Similarly to HWs, it is believed that compatible interactions between hydrophilic surfaces of xylan and cellulose through even patterning of MeGlcA and AraF attached to the main xylan backbone provide for a clear differentiation between one hydrophilic unsubstituted surface and the other more hydrophobic substituted surface (Busse-Wicher et al., 2016).

Decoration of GAXs with MeGlcA is consistent among conifers with every sixth XylP unit featuring a MeGlcA unit. Additionally, AraF-linked units are located two XylP residues apart from MeGlcA units. Other gymnosperm lineages (Cycads, Gingko, and Gnetophyta) only show minimal variation in the distribution of linked units (Busse-Wicher et al., 2016). This consistent substitution pattern has been elucidated by molecular modeling to allow for two-fold helical winding of SW GAXs such that multiple xylan chains can bind to hydrophilic cellulose microfibril faces, thereby stabilizing the S-wall similar to HW xylans (Busse-Wicher et al., 2016; Pereira et al., 2017).

1.5.3 (Galacto)glucomannans

1.5.3.1 Glucomannans in HWs

Glucomannans (GMs) are generally cited as being the second most abundant hemicellulose in HWs (after GXs). However, it has been reported that polysaccharides and pectins (polygalacturonans, for example, HGs and rhamnogalacturonans are

not considered hemicelluloses) may be more plentiful in certain HWs (Willför et al., 2005). GMs (3%−5% (Rowell, 2005; Sjöstrom, 1993; Teleman, 2009)) are linear polymers of β-(1 → 4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl units with a DP of ∼200 (Table 1.1). ManP and GlcP units are present in the main chain at a ratio of 1−2:1 (Sjöstrom, 1993). GMs in aspen and birch have been found acetylated at the C_2 and C_3 positions of ManP residues with the degree of acetylation (DS_{Ac}) of 0.3, similar to that reported for SW GGMs (∼0.33 (Teleman et al., 2003)). NMR analysis of GMs extracted from aspen and birch indicated random decoration of acetyl groups, which agrees with reports on acetyl group distribution in SW GGMs (Teleman et al., 2003).

1.5.3.2 Galactoglucomannans in SWs

SW hemicelluloses are dominated (∼20% (Sjöstrom, 1993)) by two classes of GGMs differing in the content of α-D-galactopyranose (Table 1.1). GalP are (1 → 6) linked to ManP units as a single-unit side chain (Sjöstrom, 1993). GGMs may serve as the main cellulose-binding hemicelluloses in SWs (Busse-Wicher et al., 2016). They are partially acetylated at the C_2 and C_3 positions of ManP residues, with a DS_{Ac} range of 0.17−0.36 (Teleman, 2009). It has recently been suggested that GalP substituents significantly contribute toward the stability of spruce GGMs through noncovalent association with the main chain (Berglund et al., 2020).

Decoration of GGMs is generally believed to be random. However, the pattern of GalP decorations on GGMs in the seed pod cell walls of Carob and Guar (members of the legume family) was reportedly highly improbable assuming the substitution pattern was statistically random. This result implies the presence of biosynthesis-derived ordered arrangements of blocks of mannans with distinct galactosyl branches (in legumes, at least) (Buckeridge and de Souza, 2014; McCleary et al., 1985). The function, if any, of this ordered branching is not clear, but it can be hypothesized to play a role in the interactions of GGMs with other cell wall constituents, similar to those described for xylans. More recent work has provided further support for a biosynthetic origin of patterning of GGM substitution and its importance in enabling/moderating interactions with cellulose fibrils (non-wood-derived GGMs) (Yu et al., 2018). Further work is warranted to determine if a similar effect occurs with GGM in wood xylem.

1.5.4 Effects of hemicelluloses on wood recalcitrance

EH of cellulose substrates with and without added hemicelluloses has provided evidence that precipitation of hemicelluloses onto cellulose after high-temperature pretreatments likely contributes to reduced hydrolyzability (Kumar et al., 2018). Hemicelluloses that remain after delignification of native biomass were less inhibitory toward cellulases than exogenous hemicelluloses, even at relatively higher concentrations (Kumar et al., 2018). The addition of xylanases to the enzyme cocktail was effective at enhancing cellulose conversion by cellulases. Many commercial enzyme cocktails that are marketed for hydrolysis of pretreated lignocellulosic biomass include both cellulases and hemicellulases.

1.5.5 Genetic manipulation of hemicelluloses to reduce recalcitrance

Biosynthesis of hemicelluloses, including xylans, is believed to occur in the Golgi apparatus through a two-step process. The backbone is first constructed by a polysaccharide synthase, followed by glycosyltransferase-catalyzed decoration of the backbone polymer with side-chain residues (Plomion et al., 2001). Studies indicate that xylan deposition is closely associated with cellulose biosynthesis with xylan spontaneously binding/coating newly formed cellulose MFs in the S-wall (Mellerowicz et al., 2001). It has been suggested that xylan is not uniformly present in all secondary cell wall layers, with the S_2 layer containing relatively more xylan (Mellerowicz et al., 2001; Mellerowicz and Sundberg, 2008). Studies of xylan biosynthesis have demonstrated the feasibility of employing genetic engineering to modify xylan in woody plants (Mellerowicz and Sundberg, 2008).

Different approaches to genetic manipulation of hemicelluloses aimed at reducing recalcitrance have been explored with promising results. Strategies include reducing total content and manipulating various aspects of side-chain decorations, such as removal of acetyl groups (Kong et al., 1992) and MeGlcA (Lyczakowski et al., 2017). These efforts generally have a goal of increasing biomass susceptibility to pretreatments by limiting cell wall stabilization achieved by hemicelluloses and minimizing LC bonds, that is, covalent association between hemicelluloses and lignin (Marriott et al., 2016).

A two-fold increase in hydrolyzability of poplar was demonstrated via the overexpression of genes associated with xylanase or xyloglucanase biosynthesis (Kaida et al., 2009). Additionally, a number of studies have demonstrated a significant enhancement of cellulose EH by reducing the GX content. Lee et al. applied RNA interference inhibition of poplar glycosyltransferase to achieve up to a 50% increase in EH efficiency via reduced GX content (Lee et al., 2009). In separate studies, genes that are associated with xylan biosynthesis in *Arabidopsis* were disrupted, resulting in dramatic increases in glucose yields during cellulose EH (Brown et al., 2011; Petersen et al., 2012). In addition to containing half of the xylan content of the wildtype, xylan in the mutant samples featured MeGlcA residues in place of GlcA side chains (Brown et al., 2011).

Acetylation of hemicelluloses is considered as a contributing factor to lignocellulose recalcitrance (Hu and Ragauskas, 2012). Deacetylation of xylan has been shown to result in significant increases in the efficiency of cellulose EH, although this effect may be reduced beyond 75% removal of acetyl groups (Yang et al., 2011). EH of cellulose has been improved by the controlled removal of acetyl groups from the GXs in aspen, with higher sugar yields and an increased deacetylation degree (Kong et al., 1992).

The downregulation of reduced wall acetylation gene families produced enhanced xylose and glucose yields during EH of hybrid aspen wood (Pawar et al., 2017). Recently, improved EH of transgenic *Arabidopsis* engineered to contain reduced DS_{Ac} was observed, emphasizing the importance of xylan acetylation (Rastogi et al., 2022).

In contrast, a minor impact of xylan acetylation on EH of poplar wood has been reported (Chang and Holtzapple, 2000). Additionally, DS_{Ac} of GXs in birch did not show a noticeable effect on enzyme access (Martínez-Abad et al., 2018), warranting more studies on GXs in HWs to clarify the importance of xylan acetylation in HW recalcitrance.

Members of the glucuronosyltransferase and arabinosyltransferase enzymes families have been implicated in the construction of the xylan backbone and side-chain addition during xylan biosynthesis. Modification of genes related to the production of such enzymes has been shown to enhance biomass saccharification (Oliveira et al., 2019). Lyczakowski et al. (2017) identified enzymes related to the addition of glucuronic acid side chains to xylans in SWs and demonstrated up to 30% and 700% more glucose and xylose release, respectively, during saccharification of Arabidopsis mutants. The observed reduced recalcitrance in these studies may be due to a reduced or modified presence of side chains capable of covalent cross-linking with lignin (such as AraF, ferulic acid in monocotyledons, and GlcA side chains) resulting in a reduced frequency of LC bonds.

Studies showing the impact of xylan modification/downregulation on the enhancement of EH, primarily on glucose yields, demonstrate the important role that xylans play in stabilizing the plant cell wall, protecting cellulose MFs, and bolstering the lignocellulosic biomass/wood recalcitrance (Marriott et al., 2016).

1.6 Lignin

1.6.1 Structure, function, and biosynthesis

The third structural constituent of wood is lignin, the most abundant natural aromatic polymer. Its incorporation aids in the stiffening of plants and provides resistance against the gravitational force, thereby playing a vital role in the survival of land plants (Ralph et al., 2007). Lignin accounts for 12%−33% of lignocellulosic biomass, while trees contain the largest amount of lignin 18%−33%, mainly composed of three phenylpropanoid C_9 units. Extensive research on different plants, cell types, and morphological areas has suggested that the lignin polymer is a product of diverse precursors and monomers (or monolignols) derived from the general phenylpropanoid pathway (Fig. 1.6). In addition to the canonical (Mottiar et al., 2016), or the most prevalent hydroxycinnamyl alcohols, lignin monomers belong to 10 other different categories of phenolic compounds, including hydroxybenzaldehydes, hydroxybenzoic acids, hydroxycinnamaldehydes, hydroxycinnamic acids, and hydroxycinnamyl esters (Fig. 1.7) (Vanholme et al., 2019). They are linked by randomly distributed ether and C−C bonds, where the ether bonds, especially β-aryl ether bonds, are predominant. In the last step of plant cell growth, the lignin monomers, that is, the three hydroxycinnamyl alcohols (Fig. 1.6): *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which differ in the degree of methoxylation on the aromatic ring, undergo dehydrogenative polymerization (DHP). This

is the combinatorial radical coupling process, producing *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively (Boerjan and Ralph, 2019; Ralph et al., 2007, 2008; Rinaldi et al., 2016).

The lignin random assembly model or the "combinatorial" coupling hypothesis remains the widely accepted governing mechanism for lignin biosynthesis (Ralph et al., 2008). This is based on the overwhelming evidence regarding the last step of lignin biosynthesis, where the polymer is produced from the supplied monomers via a two-step oxidative polymerization, DHP. DHP starts with an enzymatically controlled oxidation of lignin monomers, assisted by phenoloxidases, namely peroxidases and laccases, yielding resonance-stabilized phenoxy radicals (Fig. 1.8). Subsequently, chemically controlled combinatorial coupling reactions involving free radicals occur, influenced by factors such as pH, temperature, ionic strength, and H_2O_2 concentration. Lignin polymerization in situ is carried out in a gradual "end-wise" fashion, which favors the coupling of monomer radicals onto the free-phenolic ends of the growing polymer, particularly dominated by the formation of β-O-4 bonds. This is in contrast to the rapid "bulk" polymerization that may be conducted in vitro, which promotes the coupling of the monomer radicals with each other instead of cross-coupling the monomers with dimers and higher oligomers; this process is dominated by the formation of C−C bonds and dehydrodimerization reactions. Consequently, differences in the chemical structure between native (end-wise polymers) and synthetic (bulk polymers) lignins have been observed (Ralph et al., 2004). Note that lignin polymerization/deposition is spatially governed by the scaffold of the previously deposited polysaccharides as lignification occurs in the last phase of wood cell wall formation (Mellerowicz et al., 2001; Plomion et al., 2001; Terashima et al., 1993).

SW and HW lignins result from DHP of different amounts of hydroxycinnamyl alcohols; therefore, they vary in the content of the H-, G-, and S-units (C_9 units). SWs predominantly consist of G-lignin with <5% H-units, and HWs consist of SG-lignin with trace amounts of H-units (Ralph et al., 2007). During DHP, *p*-coumaryl alcohol and coniferyl alcohol undergo coupling at the C_3,C_5 available positions and form condensed structures; the C_9 units are linked to others via the C_3/C_5 ring positions, whereas sinapyl alcohol cannot couple through these positions and is predominantly incorporated into lignin via the β-O-4 bonds (Fig. 1.9). Therefore, SW G-lignin is more condensed than HW SG-lignin, although most recent studies have proposed that in general, native lignins contain a relatively low content of condensed units (Rinaldi et al., 2016). Further, lignin monomers may be acylated at the γ-carbon and participate in lignin biosynthesis as acetates or *p*-hydroxybenzoates, coumarates, and ferulates (Fig. 1.7d−g). In wood, they are predominantly incorporated into lignin as acetates and *p*-hydroxybenzoates (especially in Salicaceae). Furthermore, pendant groups, such as C_γ ester-linked *p*-hydroxybenzoates, have been observed, which are almost exclusively connected to S-units owing to the ability of *p*-hydroxybenzoates to facilitate DHP of lignin via a more efficient oxidation of sinapyl alcohol after C_γ-acylation. This also leads to a higher content of β-O-4 bonds in lignin (Goacher et al., 2021; Regner et al., 2018; Stewart et al.,

2009). Other "uncommon" lignin monomers, such as hydroxycinnamaldehydes and hydroxybenzaldehydes (Fig. 1.7a and b), have also been detected in wood lignin; hydroxycinnamaldehydes are more likely to be incorporated in HW lignin than in SW lignin (Boerjan et al., 2003; Ralph et al., 2007). Therefore, although wood lignin has a versatile structure, G- and S-units, followed by H-units predominantly govern its structure, properties, and reactivity. The monolignol distribution widely varies in the lignifying cell wall types: *p*-coumaryl alcohol is mainly present in the ML and coniferyl alcohol in the secondary wall of the xylem elements, while sinapyl alcohol is distributed across regions in the fiber-forming cell walls. The monolignols polymerize and endow the cell walls with distinct biophysical properties, which is apparent in the corresponding wall properties of the fiber and xylem elements (Fergus and Goring, 1970; Fukushima and Terashima, 1991; Terashima et al., 1986; Terashima and Fukushima, 1988; Whiting and Goring, 1982).

1.6.2 Effects of lignin on wood recalcitrance

Lignin is one of the most significant contributors to lignocellulosic biomass/wood recalcitrance, requiring pretreatment processes and a higher enzyme amount, thereby increasing the processing cost (Mosier et al., 2005; Pu et al., 2011). Thus, the lignin content of biomass significantly influences its hydrolyzability, decreasing the lignin content in plants results in higher sugar yields from EH (Chen and Dixon, 2007; Mahon and Mansfield, 2019; Saleme et al., 2017). The role of caffeoyl shikimate esterase (CSE) in lignin biosynthesis is of particular interest (Saleme et al., 2017); CSE catalyzes the conversion of caffeoyl shikimate into caffeic acid (Fig. 1.6), and its downregulation leads to the accumulation of its substrate caffeoyl shikimate and increased levels of H-units in the lignin polymer. A 25% reduced lignin deposition, an increased level of H-units in the lignin polymer, and a relatively higher cellulose content because of downregulating the expression of this enzyme in hybrid poplar was observed. Thus, up to 62% more glucose was released per plant without pretreatment and 86% and 91% with acid and alkaline pretreatments, respectively, during EH.

There are two proposed mechanisms by which lignin inhibits the EH of biomass: (i) formation of a physical barrier and/or LCCs that restrict the accessibility of enzymes to polysaccharides and (ii) nonproductive binding with enzymes, that is, lignin adsorbs onto cellulases, restricting enzymatic access to cellulose and resulting in steric hindrance (Li et al., 2016, 2018). Although steric hindrance is often considered less critical in obstructing EH, some studies have documented a higher cellulase adsorption capacity for lignin than cellulose (Li and Zheng, 2017). Moreover, lignin may nonproductively and irreversibly adsorb onto cellulases via hydrogen bonding, where free phenolic hydroxyl (PhOH) groups play a critical role, and hydrophobic and electrostatic interactions (highly pH-dependent carboxyl and hydroxyl amino acid residues on lignin and enzymes, respectively) (Li and Zheng, 2017; Zheng et al., 2021). Further, the adsorption depends on the nature of cellulase enzymes and on the origin/structure of lignin, for example, endoglucanase II (EGII) was

more likely to bind to lignin than CBHI owing to the more open active sites available in EGII than in the tunnel-shaped CBHI (Li and Zheng, 2017).

Furthermore, lignin undergoes structural changes during pretreatments such as steam explosion, which is suggested to promote nonproductive enzyme adsorption (Rahikainen et al., 2013). This is attributed to the lignin−lignin condensation reactions due to the electrophilic benzylium cations (carbocations) formed in lignin under acid conditions, even under mild acid conditions of autohydrolysis. It is possible to reduce lignin repolymerization by adding nucleophiles, such as the highly nucleophilic 2-naphthol, which readily scavenges these cations and is incorporated into lignin. Hydrothermal pretreatment (HTP) of SWs spruce (*Picea abies*) and pine (*Pinus sylvestris*) in the presence of 2-naphthol revealed a significant increase in hydrolyzability, demonstrating the major contribution of lignin repolymerization toward recalcitrance (Pielhop et al., 2017). Other agents that help suppress lignin repolymerization include dimethylphloroglucinol, which is slightly more potent than 2-napthol. Its high efficiency has been attributed to the structure activated by three PhOH groups with a single reactive site (Pielhop et al., 2016).

Hydrophobicity, which is considered a major factor in lignin−enzyme interactions, varies with the content of hydroxyl and carboxylic groups. Generally, a higher content of PhOH groups and lower content of aliphatic OH content render it less hydrophobic. High PhOH and low aliphatic OH amounts in lignin have been associated with higher cellulases adsorption affinity and binding strength (Guo et al., 2014; Rahikainen et al., 2013; Yu et al., 2014); however, the effects vary with the pretreatment and origin of lignin. Milder pretreatments promote access to cellulose while minimizing lignin hydrophobicity through minimizing recondensation (Li et al., 2016; Rahikainen et al., 2013). Further, lignin modification by hydroxypropylation, carboxylation, and sulfonation to block PhOH groups significantly reduced the inhibitory effect of lignin on enzyme activity (Yang and Pan, 2016). The presence of carboxylic groups in lignin alter the physicochemical effects of lignin by (i) increasing hydrophilicity and (ii) creating electrostatic charge, both of which reduce nonproductive enzyme binding (Nakagame, 2010). Notably, the surface of SW lignin is more hydrophobic than that of HW lignin. The former also has fewer available negative surface charges that can promote the electrical repulsion between lignin and enzymes; thus, SW lignin tends to be more inhibitory than HW lignin during EH (Li and Zheng, 2017).

The composition of lignin, expressed as the H/G/S ratio, also influences biomass digestibility, and the contribution of individual units toward recalcitrance has been discussed (Li et al., 2016; Marriott et al., 2016). *Populus* natural variants (Studer et al., 2011) that were subjected to a high throughput pretreatment and EH (Studer et al., 2010) showed enhanced hydrolyzability with higher S/G ratios (2) and a strong negative correlation between lignin content and EH efficiency for samples with an S/G ratio <2.0. Similarly, saccharification improved with a higher S/G ratio for HT-pretreated Arabidopsis (Li et al., 2010), poplar subjected to steam explosion pretreatment (Mansfield et al., 2012), and HW species subjected to green liquor and kraft pulping (Min et al., 2013; Santos et al., 2011). This suggests that lignin that is

modified during pretreatment facilitates hydrolysis. It can be postulated that lignin with a low S/G ratio is more likely to contain fewer β-O-4 linkages and more condensed units; therefore, a more developed 3D structure is generated. This structure is bulkier, creating more steric hindrance and reducing enzyme accessibility (Foston and Ragauskas, 2012). For untreated biomass, some have reported no influence of the S/G ratio on its hydrolyzability, such as transgenic poplar lines with a high S content (S/G ratios of 6.7 and 13.2) (Mansfield et al., 2012) and Arabidopsis with high G (S/G = 0.05) and S (S/G ratio = 10.1) contents (Li et al., 2010), while others reported a negative correlation (Min et al., 2013; Papa et al., 2012; Zhang et al., 2011). Based on a proposed molecular model (Besombes and Mazeau, 2005), the negative correlation was attributed to the extended shape of S-enriched lignin, which offers more efficient coverage of the cellulose fibrils, compared to the relatively more branched G-lignin. SG-lignins are characterized by a less condensed structure that is more easily depolymerized than G-lignins. Furthermore, SG-lignins with more S-units have higher concentrations of the more labile β-O-4 bonds (bond dissociation energy (BDE) = 54−72 kcal/mol) and lower concentrations of the more recalcitrant β-5 and 5-5 C−C (BD > 110 kcal/mol) bonds (Parthasarathi et al., 2011). SG-lignins also contain more β-β bonds (SW: 2%−6% and HW: 3%−12% (Rinaldi et al., 2016)), yielding a lower molecular weight, which enables lignin migration and removal (Li et al., 2016). This was also observed by Trajano et al. (2013) during HTP, confirming that a higher S/G ratio reduces recalcitrance.

Additionally, based on molecular simulation studies, the possibility of direct interactions between lignin and cellulose has been suggested (Vermaas et al., 2019). Based on model studies, these interactions have also been discussed with respect to the inhibiting role of lignin. It has been proposed that the crystalline regions of cellulose have a greater tendency to associate with lignin than the more hydrophilic amorphous regions. In this context, pretreatment techniques that reduce the crystallinity degree of cellulose have a dual-positive effect on increasing the efficacy of EH; such a pretreatment would result in more access to less-crystalline cellulose, which is less likely to be blocked by lignin adsorption (Lindner et al., 2013).

1.6.3 Lignin genetic modifications to reduce recalcitrance

To reduce lignocellulosic/wood recalcitrance, various approaches in genetic modification of lignin are being pursued, including studies on reducing the lignin content and altering its composition by upregulating or downregulating the lignin biosynthetic pathway. A comprehensive list of the effects of the modified expression of lignin biosynthesis genes on the lignin content and composition that ultimately affect efficiencies of EH and delignification processes has been provided by Chanoca et al. (2019).

Designer lignins have been proposed that can contribute toward reducing recalcitrance with the following characteristics (Mottiar et al., 2016):

(1) Lignins with a lower DP, as they can be easily extracted. This can be achieved by incorporating monomers capable of only single coupling reactions that either initiate or terminate the growth of the polymer, thereby reducing the average size of the lignin polymers. The gene encoding monolignol 4-O-methyltransferase (MOMT4), which leads to the formation of 4-O-methylated coniferyl and sinapyl alcohols (Fig. 1.6), was activated in poplar. Since these cannot be incorporated into the growing lignin polymer owing to the lack of aromatic hydroxyl group, lignin polymerization is halted, yielding trees with lower lignin content and size, and higher EH efficiency (Bhuiya and Liu, 2010; Cai et al., 2016). Alternatively, S-units were successfully incorporated into lignin in pine by introducing genes that induce enzymes needed for biosynthesis of sinapyl alcohol (F5H, COMT, and CAD; Fig. 1.6) (Edmunds et al., 2017; Wagner et al., 2015), as lignin with incorporated S-units results in improved EH efficiency (Li et al., 2016; Marriott et al., 2016). In addition to the formation of SG-lignin, overexpressing F5H yielded lignin with a lower average DP of approximately 10 (Stewart et al., 2009). Nongenetically modified–based approaches to yield lignin of lower DP include the incorporation of dihydroconiferyl alcohol (Fig. 1.7d), found in gymnosperms lignin (Sederoff et al., 1999), hydroxyphenylglycerols and hydroxybenzenoids, detected in a variety of plants, which serve as lignin initiators (Eudes et al., 2012; Ralph et al., 2004). If these initiators are present in higher amounts during active polymerization, it is expected that more lignin moieties of smaller size will be formed.

(2) More hydrophilic lignins can be expected to be more readily removed during pretreatment processes. They would also lead to fewer hydrophobic interactions in the cell wall, enhancing EH by decreasing unproductive enzyme adsorption. Alternative strategies, including monomers with additional hydroxyl groups or accessory hydrophilic moieties, such as carbohydrates, have also been proposed (Grabber et al., 2012). Notably, molecular dynamic simulations predicted that increasing hydrophobicity reduced the noncovalent interactions between lignin and hemicelluloses (Carmona et al., 2015), rendering the cell wall polysaccharides more accessible.

(3) Lignins with fewer bonds to structural carbohydrates. The interactions between lignin and carbohydrates (LCCs) also significantly contribute to the recalcitrance in wood (discussed in detail in Section 1.7). When a monolignol combines with another monomer or with the growing polymer via its β position, a quinone methide intermediate (Fig. 1.10), is produced, which can be quenched and rearomatized via a nucleophilic attack from water (Mottiar et al., 2016) and from hydroxyl and carboxyl groups of carbohydrates, giving rise to benzyl ether or benzyl ester bonds, respectively (Table 1.2). Less common units containing *o*-diphenol groups, such as those arising from caffeoyl alcohol or 5-hydroxyconiferyl alcohol (Fig. 1.6), result in quinone methide intermediates that can potentially be internally trapped as benzodioxane structures prior to the possibility of an external nucleophilic attack.

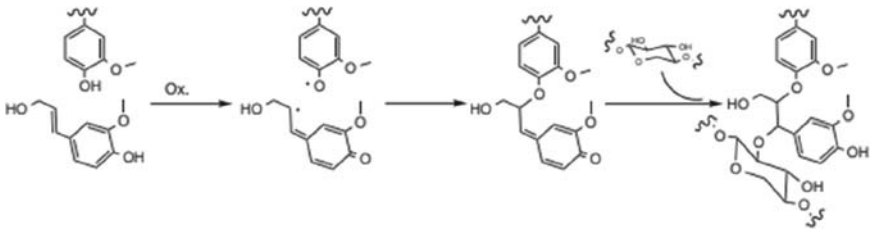
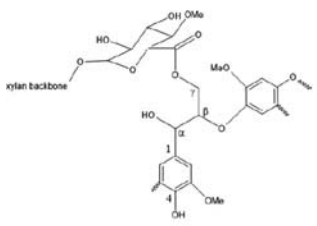
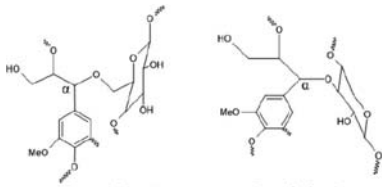
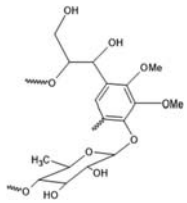
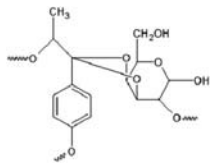


FIGURE 1.10

Formation of lignin–carbohydrate benzyl–ether two linkage via nucleophilic attack of carbohydrates on the quinone methide intermediate during dehydrogenative polymerization.

Reprinted with permission from Oinonen et al. (2015).

Table 1.2 Types of lignin–carbohydrate (LC) linkages.

LC-linkage	Structure
γ-ester, γE	 Ester bond between lignin and MeGlcpA on xylan
Benzyl ether, BE	 Benzyl ether 1 Benzyl ether 2
Phenyl–glycosidic, PG	
Acetal	

Adapted from Giummarella et al. (2019).

(4) Lignins containing chemically labile bonds. Another strategy is to introduce ester linkages into the lignin backbone. This enables alkaline extractability and in turn, EH. Coniferyl ferulate esters were introduced into poplar lignin by activating the feruloyl-CoA:monolignol transferase gene, FMT (Fig. 1.6), which resulted in improved saccharification efficiency following various pretreatments (Bhalla et al., 2018; Kim et al., 2017; Wilkerson et al., 2014), in addition to an enhanced kraft pulping efficiency (Zhou et al., 2017).

1.7 Lignin–carbohydrate complexes

1.7.1 Structure and formation

The complex chemical moieties formed through the covalent linkages between lignin and hemicelluloses; LC linkages, in native wood are termed LCCs (Westerberg et al., 2012). LCCs have been reported to have distinct structural, physicochemical, and biological properties (Sakagami, 2014; Sakagami et al., 2010; Zhao et al., 2020), arising from a variety of functional groups, molecular weights, and LC linkages present among them (Zhao et al., 2020). LCCs have also been thought to contribute to the greater thermal stability and resistance to thermal degradation, relative to lignin-free carbohydrates (Zhao et al., 2020). The LC linkages are mainly of three types (Table 1.2): (1) γ-ester (γE) linkages between acidic side chains, for example, MeGlcA in xylans, and C_γ-hydroxymethyl group on lignin (Nylander et al., 2016), (2) benzyl ether (BE) linkages: BE1 ether linkages formed between the C_α in lignin and primary CH_2OH-groups of carbohydrates (C_6 for GlcP, GalP, and ManP mainly in GGMs and C_5 for Araf in AGXs) and BE2 ether linkages between the C_α in lignin and secondary −CHOH groups of carbohydrates, (mainly C_2 and C_3 for XylP for AGX and GX) (Wen et al., 2013), and (3) the least common LC bonds are phenyl−glycosidic (PG) linkages between the C_1, anomeric carbon; RedE of hemicelluloses backbone and lignin (Cornu et al., 1994). Ester linkages are alkali-labile, while BE linkages are relatively stable in alkaline conditions (Toledano et al., 2010). γE linkages are thought to be more common in HWs because of the frequent acetylation of C_2 and C_3 of XylP in GXs that hinders the formation of BE linkages (Zhao et al., 2020). In addition, acetal linkages have been less commonly reported as the fourth type (Zhao et al., 2020).

Owing to the relatively hydrophobic nature of lignin and partial hydrophobic–hydrophilic nature of xylans, a core–corona structure has been proposed for LCCs in solutions, where a hydrophobic lignin core is surrounded by xylans that have their hydrophilic side chains oriented toward the surface (Sedlmeyer, 2011). A similar molecular structure has been hypothesized for the organization of LCCs in native wood (Oinonen et al., 2015).

The γE- and BE-type LC linkages are hypothesized to form in native lignin through the nucleophilic addition reaction between the hydroxyl group on carbohydrates or uronic acids and the electrophilic sites on the quinone methide (QM) intermediate (Fig. 1.10) formed by coupling of phenoxy radicals (Fig. 1.8) during DHP

(Giummarella et al., 2016; Zhao et al., 2020). The mechanism of the formation of the PG bonds is not yet fully understood. However, the existence of LC linkages in native lignin remains a topic of debate, as they may form during biomass processing because of the relatively harsh methods generally employed in their isolation from lignocellulosic biomass (Lawoko et al., 2005).

1.7.2 Effect of pretreatment on LC linkages

Currently used methods for LCC isolation are ball milling (Zhao et al., 2020; Giummarella et al., 2019), enzymatic isolation (Giummarella et al., 2019; Zhao et al., 2020), acid hydrolysis (Giummarella et al., 2019), alkaline hydrolysis (Giummarella et al., 2019; Hu and Ragauskas, 2012; Zhao et al., 2020), sequential fractionation (Giummarella et al., 2019), and hydrothermal treatment (Giummarella et al., 2019). Ball milling is especially useful for isolation of native structure of LCCs, while the rest of the methods differentially break the LC linkages to release the resulting fragments. For example, alkali treatment results in the cleavage of γE bonds through saponification (Hu and Ragauskas, 2012).

Enzyme cocktails such as lignin peroxidases, laccases, and esterases can also cause the cleavage of γE bonds, but the process is relatively slower (Zhao et al., 2020). BE linkages are broken down during acid treatments (Giummarella et al., 2019). Although alkali treatments have been reported to cleave BE linkages, they are also thought to favor the reformation of BE linkages through additional reactions on the QM intermediates produced during strong alkali treatments, such as kraft pulping (Giummarella et al., 2019). PG bonds have been reported to be susceptible to both acid and alkali treatments (Zhao et al., 2020). Organosolv treatment, which tends to be mildly acidic when run at an elevated temperature, can also result in the cleavage of BE, as well as γE bonds (Zhao et al., 2020). The effect of HTP on LC linkages has been explored in detail. Different types of LC linkages have been documented to be differentially affected, depending on the intensity of the HTP as discussed in Chapter 9 of this book.

1.7.3 Effects of LCCs on wood recalcitrance

LCCs have been reported to contribute to the recalcitrance of woody biomass to chemical and biological processing of lignocellulosic biomass and delignification (Boukari et al., 2009; Giummarella and Lawoko, 2017; Košíková and Ebringerová, 1994; Martínez-Abad et al., 2018; Yang et al., 2011; Zhao et al., 2020). They reduce the accessibility of chemicals and/or enzymes to cellulose and prevent selective solubilization of lignin and hemicelluloses (Hu and Ragauskas, 2012). This can create bottlenecks affecting the economical sustainability of a biorefinery (Zhao et al., 2020). Hence, the cleavage of LC bonds, along with the removal of hemicelluloses, is recommended to improve efficiency of pretreatments and ultimately reduce recalcitrance, that is, improve hydrolyzability (Hu and Ragauskas, 2012). More studies are needed to understand the effects of the initial LCC concentration on the rate-

limiting steps in various pretreatments (Zhao et al., 2020). LCCs and lignin associated with hemicelluloses can also create steric hindrance and nonproductive binding with cellulases and hemicellulases, thereby decreasing EH yield (Wang et al., 2017; Zhao et al., 2020). In addition to hindering the access of enzymes to polysaccharides, LCCs also affect the valorization opportunities for the individual wood constituents by negatively affecting downstream separation and purification of lignin and hemicelluloses (Zhao et al., 2020).

1.7.4 Valorization of LCCs in a modern biorefinery

Possible valorization opportunities for LCCs have been suggested in the literature based on their antioxidant and bioactive properties and biocompatibility (Sakagami, 2014; Sakagami et al., 2010; Zhao et al., 2017, 2020). The wide range of bioactive properties of LCCs has been proposed to stem from their heterogenous nature, where the phenylpropanoid rings contribute to antiviral properties and the carbohydrate moieties contribute to immunoactivity (Zhao et al., 2020). Examples of valorization of LCCs are discussed elsewhere in the book (Chapter 9). Additional structural and chemical exploration of LCCs can lead to a deeper understanding of the lignocellulosic matrix and increase the utility of commercial biorefineries.

1.8 Conclusion

Wood, as the most promising alternative to fossil-carbon resources, is inherently characterized by resistance to deconstruction; recalcitrance. Recalcitrance is embedded into the wood composite structure during the spatially and temporally regulated biosynthesis of its entangled structural constituents. Decreasing the recalcitrance of wood for efficient EH is a necessary step to realize a successful biorefinery. Some major factors that contribute toward recalcitrance include cellulose crystallinity, cellulose−hemicelluloses interactions, content, distribution and structure of lignin, and lignin−carbohydrate complexes. Improvements in standard analytical protocols for the characterization of the chemical composition and ultrastructure of wood and in the analysis of wood polymers in situ and in biorefinery streams are of utmost importance in support of these efforts. Recent advances in strategies to tailor wood formation and structure through genetic modifications and improve pretreatment methods and enzyme cocktails offer a bright outlook for wood-based biorefineries. It is evident that substantial progress has been made in developing model pretreatments capable of simultaneously inhibiting lignin condensation pathways and promoting EH. However, the commercialization of these technologies requires overcoming the barriers of poor economic feasibility and environmental sustainability. It is becoming abundantly clear that lignin valorization is an essential step to make the biorefinery economically viable. Thus, the integrated wood-based biorefinery must be designed to optimize the production of sugars and lignin as raw materials for high-value products.

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