Chapter 14
Linking Plant Biology and Pretreatment: Understanding the Structure and Organization of the Plant Cell Wall and Interactions with Cellulosic Biofuel Production

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Abstract In order to more economically process cellulosic feedstocks using a biochemical pathway for fuel production, it is necessary to develop a detailed understanding of plant cell wall characteristics, pretreatment reaction chemistry, and their complex interactions. However given the large number of thermochemical pretreatment methods that are currently being researched and the extreme diversity of plant cell wall structure and composition, this prospect is extremely challenging. Here we present the current state of research at the interface between plant biology and pretreatment chemistry. The first two sections discuss the chemistry of the secondary plant cell wall and how different pretreatment methods alter the overall cell wall structure. The third section addresses how the characteristics of the cell wall and pretreatment efficacy are impacted by different factors such as

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plant maturity, classification, and plant fraction. The fourth section summarizes current directions in the development of novel plant materials for improved biochemical conversion. And the final section discusses the use of chemical pretreatments as a screening and analysis tool for rapid identification of amenable plant materials, and for expansion of the fundamental understanding of plant cell walls.

Keywords Enzymatic digestibility • Lignocellulose • Plant breeding and transgenesis • Plant cell wall • Pretreatment chemistry • Screening tools

Abbreviations

AFEX™	Ammonia fiber expansion
BMIMCl	1-butyl-3-methylimidazolium chloride
CBM	Carbohydrate binding module
EMIMAc	1-ethyl-3-methylimidazolium acetate
EMIMCl	1-ethyl-3-methylimidazolium chloride
G	Guaiacyl
GAX	Glucuronoarabinoxylan
H	*p*-hydroxyphenyl
IL	Ionic liquid
S	Syringyl
TAGs	Triacylglycerols

14.1 Introduction

Lignocellulosic materials are a promising source of biofuels because of their abundance and availability. One potential conversion pathway is the biochemical route, through enzymatic hydrolysis and fermentation of cell wall carbohydrates. The difficulty is that although plant cell walls are permeable to small molecules, such as water, carbon dioxide, sugars, and amino acids (Ivakov and Persson 2012), while enzymes, with a diameter of around 51Å (Ishizawa et al. 2007), are too large to penetrate. Therefore to obtain access to polysaccharides embedded within the cell wall in an industrially relevant time scale, some form of chemical or physical pretreatment is needed to disrupt the cell wall structure. A large number of pretreatments are currently being researched (da Costa Sousa et al. 2009; Zhao et al. 2012), corresponding to a wide range of chemistries and modes of action. In addition there is enormous diversity of plant cell walls in terms of their structure and organization (Cosgrove 2005). The chemical and physical interactions between variables related to the feedstock (Fig. 14.1a), and pretreatment (Fig. 14.1b), determines the specific types and magnitudes of effects on cell wall structure (Fig. 14.1c), and ultimately the extent of enzymatic deconstruction.

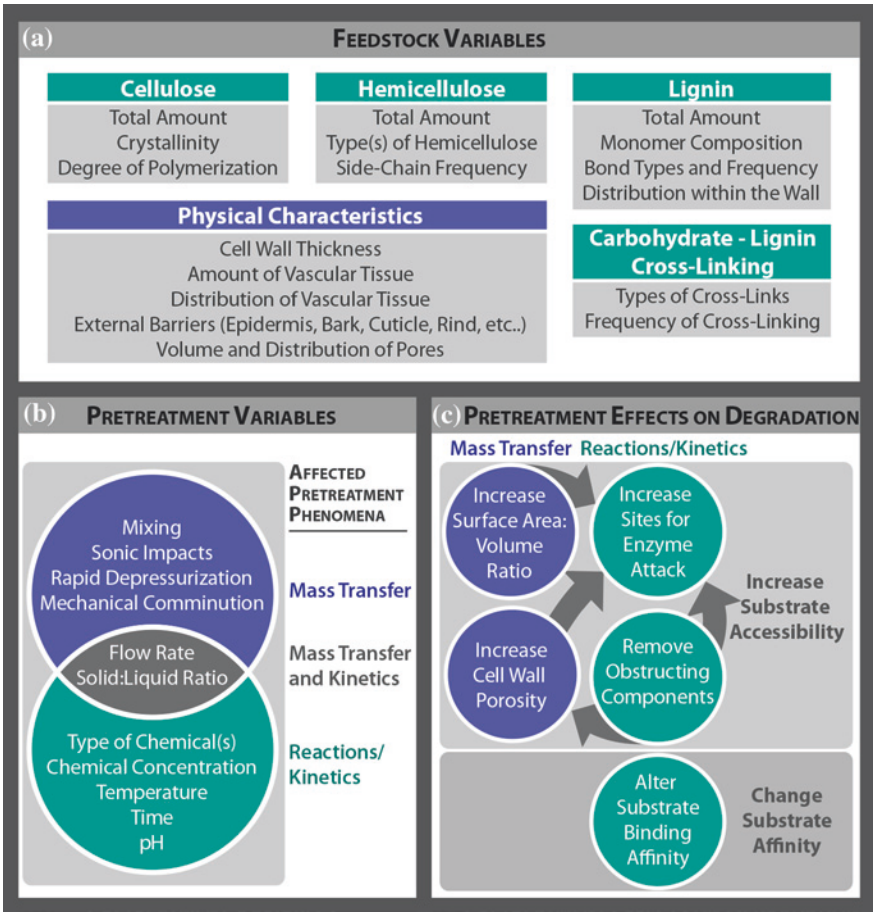

Fig. 14.1 Feedstock variables (**a**), pretreatment variables (**b**), and resulting modes of action (**c**) for improved enzymatic degradation of plant cell wall carbohydrates. Gray arrows in part C represent the potential for a direct impact of one mode of action on another

14.2 Secondary Cell Wall Chemistry

Higher plants have two main cell wall types, with different functions and compositions. Primary walls are laid down during cell growth and elongation, and secondary walls are laid down after cessation of cell growth (Cosgrove 2005; Ivakov and Persson 2012). The middle lamella is located between adjacent cells and binds them together (Cosgrove 2005). After growth stops, lignin deposition begins in the middle lamella and cell corners and progresses to the primary and secondary walls (Ralph et al. 2007; Ivakov and Persson 2012). In woody plants, the primary wall is degraded before secondary wall deposition (Jarvis 2012), however for herbaceous plants the secondary wall is deposited directly inside the primary wall (Wilson and Hatfield

1997; Engels and Jung 1998). Not all types of cells have secondary cell walls, mainly those requiring greater strength or rigidity (Cosgrove 2005), and some secondary walls never lignify (Engels and Jung 1998). But because of their greater thickness, secondary walls make up the bulk of lignocellulosic biomass and cell volume, especially in woody materials (Wilson and Hatfield 1997; Ivakov and Persson 2012). Cellulose, hemicelluloses, and lignin are the major components of the secondary cell wall. Cellulose forms the scaffolding of the cell wall and comprises β-(1 → 4)-linked glucan chains arranged in crystalline microfibrils. The hemicelluloses are a diverse class of amorphous carbohydrates that cross-link cellulose microfibrils and lignin within the cell wall. All hemicelluloses have β-(1 → 4)-linked backbones of glucose (glucans), mannose (mannans), glucose and mannose (glucomannans), or xylose (xylans), and may be substituted with sugars, uronic acids, and acetyl groups. Lignin is an amorphous phenylpropanoid polymer that fills in most of the remaining space and is comprised of three different subunits that are differentiated by the number of methoxyl groups on the phenyl ring: syringyls (S) have 2; guaiacyls (G) have 1; and *p*-hydroxyphenyls (H) have 0. Pectins are another class of carbohydrate and represent a major portion of the dicot and gymnosperm primary wall, however they are comparatively easy to extract from the cell wall or degrade (Willför et al. 2005a, b; DeMartini et al. 2011a). For more detailed explanations on cell wall composition and structure of the polymers please refer to a number of reviews (Carpita and Gibeaut 1993; Ralph et al. 2007; Scheller and Ulvskov 2010; Ivakov and Persson 2012).

14.2.1 Variation in Chemistry Due to Classification, Cell Type, and Location

Bioenergy plants are grouped in three classes based on their cell wall composition: grass-like (commelinid monocots), dicot-like (non-commelinid monocots, herbaceous dicots, and hardwoods), and gymnosperm (softwoods). Grass-like secondary cell walls contain glucuronoarabinoxylan (GAX) as the main hemicellulose substituted with arabinose and some glucuronic acid, and lignin comprised of S and G subunits with low levels of H subunits; dicot-like secondary cell walls predominantly contain glucuronoxylan substituted with 4-*O*-methyl-glucuronic acid and infrequently with arabinose, and lignin comprised of similar levels of S and G subunits and trace H subunits; and gymnosperm secondary walls contain slightly more galactoglucomannan than glucuronoarabinoxylan, and lignin comprised mostly of G subunits and low levels of H subunits (Ralph et al. 2007; Scheller and Ulvskov 2010). The type and distribution of the polymers varies within the cell and between cell types. For all plant classifications, the cell corners and middle lamella generally have the highest lignin content compared to the primary and secondary wall (Singh et al. 2009; Siqueira et al. 2011; Sun et al. 2011). For corn stover, cell types can be arranged in order of decreasing lignin and cellulose content: sclerenchyma and tracheids > epidermis > bundle sheath > parenchyma (Sun et al. 2011). Sugarcane follows a similar trend with lignin concentrated in the vessels followed by fiber and parenchyma cells (Siqueira et al. 2011). Lignin in herbaceous dicots is concentrated

in the vascular ring (Wilson and Hatfield 1997; Engels and Jung 1998), but pith parenchyma cells, though thin, are also lignified (Engels and Jung 1998).

14.2.2 Covalent Linkages

The S/G ratio determines the types of inter-unit cross-linking that occur within the lignin matrix. The β-O-4 (β-aryl ether) linkage is the most frequent linkage and one of the most easily cleaved chemically (Ralph et al. 2007) and is more common in lignin containing more S subunits (Kishimoto et al. 2009). 4-*O*-5 linkages are more common with a 1:1 S/G ratio, and the β-β linkage is more common with a greater proportion of S subunits (Kishimoto et al. 2009; Rencoret et al. 2011). Lignins with a greater proportion of G subunits tend to be more branched, and also contain more chemically and thermally resistant structures (β-5 and 5-5) (Ralph et al. 2007; Kishimoto et al. 2009; Rencoret et al. 2011). As a result, hardwood lignin is easier to degrade and has a lower glass transition temperature compared to softwood lignin, which contains no syringyl subunits (Lundquist and Lundgren 1972; Lundquist 1973; Awal and Sain 2011).

Lignin is also covalently linked to hydroxycinnamic acids, with *p*-coumaric acids forming ester-linked terminal residues. Ferulic acids, which are also able to form oligomers, are ether-linked to lignin and ester-linked to carbohydrates, either pectins in certain dicots, or GAX arabinose side-chains in grass and dicot secondary walls (Iiyama et al. 1990; Harris and Trethewey 2010), though the frequency is lower for dicots due to significantly lower arabinose substitution (Scheller and Ulvskov 2010; Chiniquy et al. 2012). Ferulate cross-links limit enzymatic degradation (Grabber et al. 1998), but the ester link with hemicellulose is easily cleaved by most pretreatments. In addition to covalent cross-linking through hydroxycinnamic bridges, a variety of direct cross-links have also been proposed between lignin subunits and cell wall carbohydrates (Imamura et al. 1994; Karlsson et al. 2004; Lawoko et al. 2006).

14.2.3 Non-Covalent Interactions

A great deal of interaction between cell wall polymers is in the form of hydrogen bonding and van der Waals forces. In higher plants the glucan chains in the cellulose microfibril are present predominantly in the I_β crystal conformation (Atalla and Vanderhart 1984; Stone 2005). The microfibrils may interact with each other and other cell wall polysaccharides through non-covalent interactions (Altaner and Jarvis 2008; Ivakov and Persson 2012) and through these form aggregate- or bundle-like structures (Donaldson 2007; Abe and Yano 2009). Glucomannans bind more strongly to cellulose and are more resistant to extraction compared to glucuronoxylans (Clayton and Phelps 1965; Åkerholm and Salmén 2001, 2004; Zhang et al. 2011a). Strength of hemicellulose binding is likely related to

interactions between the specific sugars in the hemicellulose backbone and cellulose, and a recent modeling study showed fewer hydrogen bonds but greater bond strength between cellulose and glucomannan compared to between cellulose and xylan (Zhang et al. 2011a). However stronger binding of glucomannan may also be related to lower side-chain substitution compared to xylan (Clayton and Phelps 1965). For the same class of hemicellulose, those with lower substitution bind more strongly to cellulose (Whitney et al. 1998; Kabel et al. 2007; Dammström et al. 2009), and the pattern of substitution also appears to have an impact (de Lima and Buckeridge 2001). In addition to the sugar side-chains, most mannans and xylans are acetylated (Scheller and Ulvskov 2010), which likely reduces binding affinity towards cellulose (Altaner and Jarvis 2008). It has also been hypothesized that hemicelluloses may be covalently linked to or embedded within cellulose microfibrils (Cosgrove 2005).

14.3 Pretreatment Chemistry

Thermochemical pretreatments alter the cell wall through chemical reactions that cleave covalent bonds and/or disrupt non-covalent interactions between cell wall polymers (Fig. 14.2) as well as through thermal softening and solubilization of biomass components. These chemical changes in combination with the physical removal and/or relocalization of cell wall components cause structural changes that improve enzymatic digestibility. Most pretreatments can be grouped based on their general effect on cell wall structure: those that remove lignin (alkaline/oxidative), those that remove hemicellulose and relocalize lignin (acidic), and those that fractionate cell wall components (ionic liquid, organosolv, and phosphoric acid) (Fig. 14.2). Most pretreatments, except for biological pretreatments and ionic liquids (ILs), can also be arranged in a continuum based on the nucleophilicity/electrophilicity of their main reactants (Fig. 14.3). Though less precise, the continuum can also be thought of in terms of pH (Pedersen and Meyer 2010; Garlock et al. 2011). Almost all of these pretreatments cleave some fraction of acetyl groups from the hemicellulose backbone (Maloney et al. 1985; Kumar et al. 2009; Shi et al. 2011) and use conditions that break α-ether linkages in lignin (Saake and Lehnen 2007).

The main mode of action for alkaline and oxidative pretreatments is through nucleophilic substitution and/or oxidation of esters and β-ethers within lignin and between cell wall polymers (Tarkow and Feist 1969; Iiyama et al. 1990; Sewalt et al. 1996). At very high alkali concentrations, carbohydrate monomers can be removed via peeling reactions and converted to acids (e.g. lactic acid) (Knill and Kennedy 2003). As reactant concentration and temperature decrease, peeling reactions become less likely to occur and fewer β-aryl-ether bonds are broken. Ammonia, a weaker nucleophile, does not cleave β-ethers but is known to cleave ester-linkages between hemicellulose and hydroxycinnamic acids (Wang et al. 1964; Azarpira et al. 2011). In contrast, acidic pretreatments mainly act through electrophilic hydrolysis of ester cross-links, β-ether bonds, and glycosidic

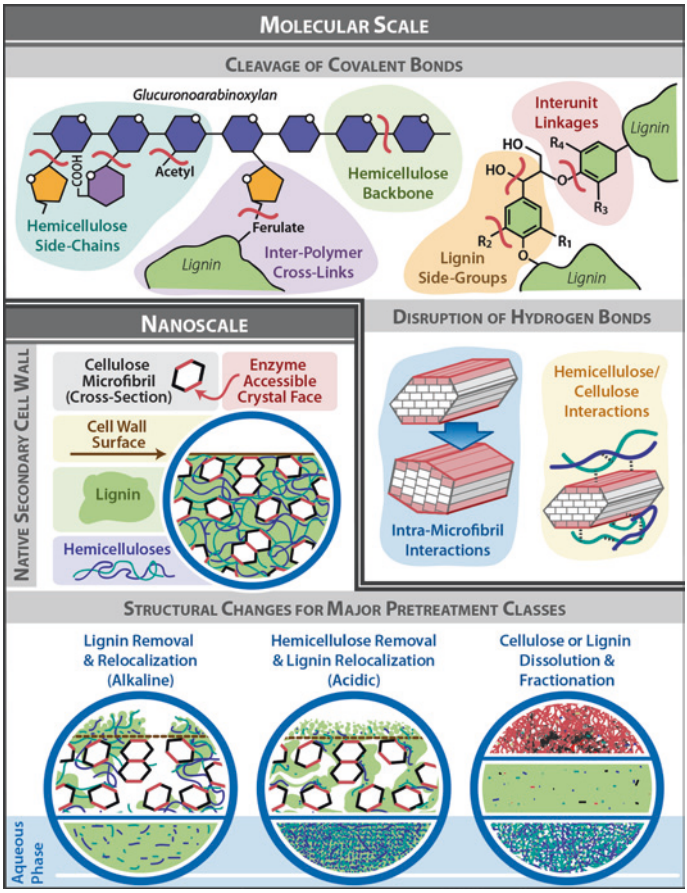

Fig. 14.2 Main molecular scale (chemical) impacts to plant cell wall components by thermochemical pretreatments and, in conjunction with mass transfer of biomass components, the resulting nanoscale (structural) changes for the three main classes of pretreatment

linkages, and they can also catalyze the dehydration of monomeric sugars. Room temperature acid treatment is able to break ether linkages between hydroxycinnamic acids and lignin/hemicelluloses (Wallace et al. 1995); while higher temperatures are needed to hydrolyze esters (Sannigrahi et al. 2009). Though β-ether bonds can be broken by strong acidic pretreatments, they are more readily hydrolyzed by alkali (Saake and Lehnen 2007). The key feature of acidic pretreatments is the hydrolysis of glycosyl linkages that allows for extraction of hemicellulose-derived oligomers and monomers. Xylans are more easily hydrolyzed than mannans (McGee and April 1982; Tunc and van Heiningen 2008; Várnai et al. 2010), and for side-chains, arabinose is more easily removed than galactose, and galactose than 4-*O*-methyl-glucuronic acid (McGee and April 1982; Sun and Cheng 2005). During hydrothermal pretreatments, hydronium ion concentration is

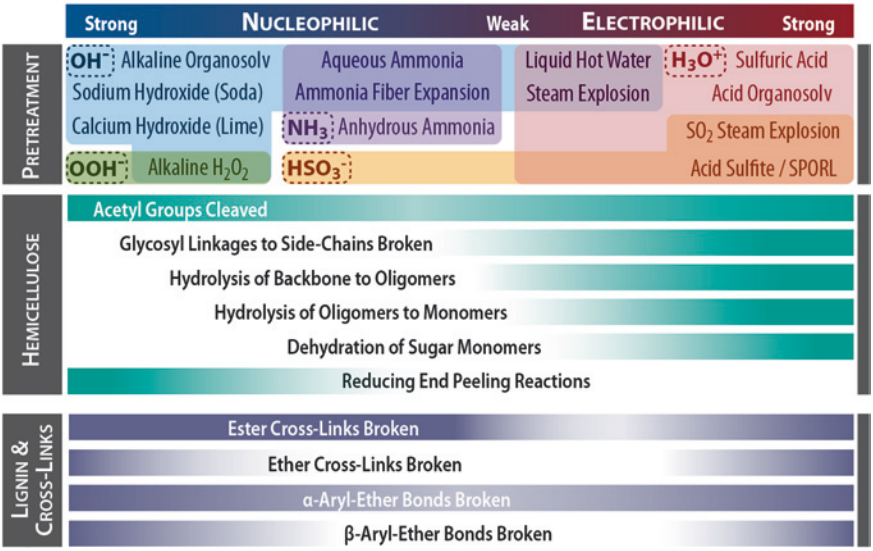

Fig. 14.3 Thermochemical pretreatments arranged in order of reactant nucleophilicity and effect on cell wall covalent linkages

initially governed by water autoionization and later by release of weak, biomass-derived acids (Garrote et al. 1999), and for these weakly acidic pretreatments, most hemicellulose is released as oligomers, only the most amenable side-chains are cleaved, and most lignin inter-unit linkages remain intact (Garlock et al. 2011).

A number of pretreatments (AFEX™[1], liquid hot water, dilute acid, and acid-catalyzed organosolv) have also been shown to deposit lignin-rich globules on the surface of the cell wall (Donohoe et al. 2008; Chundawat et al. 2011; Donohoe et al. 2011; Koo et al. 2012). For acidic pretreatments, particularly those catalyzed by sulfuric acid, lignin can condense and form new bonds (Xiao et al. 2013; Lundquist 1973; Karlsson et al. 1988). Most pretreatments also generate degradation compounds that influence downstream processes, and the specific compounds that are formed are determined by the interaction of plant cell wall chemistry (grass, hardwood, or softwood) with pretreatment chemistry (Chundawat et al. 2010; Du et al. 2010).

In addition to cleavage of covalent bonds, some pretreatments (sodium hydroxide, liquid ammonia, phosphoric acid, and ILs) disrupt hydrogen bonding within cellulose microfibrils and generate more digestible forms of cellulose (amorphous > II, III > I). The main mode of action for ionic liquids is the disruption of hydrogen bonding and decrystallization of cellulose to the extent that fractionation and lignin removal may not be necessary for high enzymatic conversions (Wu et al. 2011). There are indications that IL reactivity is related to both the ability of the

[1] AFEX™ is a registered trademark of MBI International, Lansing, MI.

anion to accept hydrogen bonds (Tadesse and Luque 2011; Gericke et al. 2012; King et al. 2012) and the length of the alkyl substituent chain on the cation, with shorter chain lengths leading to more effective cellulose dissolution (Zhang et al. 2005). Combined, the anion and cation form an electron donor–acceptor matrix with the cellulose hydroxyl groups that facilitates dissolution (Tadesse and Luque 2011; Xu et al. 2012). In ILs, pH is a measure of dissociation between the anion and cation (MacFarlane et al. 2006) and anions and cations can be classified as acidic, basic, or neutral. For example, the imidazolium ring has acidic properties that are believed to result in acid catalytic effects (MacFarlane et al. 2006). An IL with an acidic cation and basic anion like 1-ethyl-3-methylimidazolium acetate (EMIMAc) has a larger degree of dissociation (~pH 11) (Singh et al. 2009; Muhammad et al. 2012), which is likely related to its ability to both decrystallize cellulose and dissolve lignin. Imidiazolium ILs with a weakly basic anion like 1-butyl-3-methylimidizolium chloride (BMIMCl) and 1-ethyl-3-methylimidizolium chloride (EMIMCl) (~pH 6) are more selective for dissolving cellulose (Zhang et al. 2013b). The IL anion may also enhance catalytic reactions, and ILs with anions that are less basic than water (e.g. Cl^-) turn strong acids into weaker acids, however ILs with anions that are more basic than water (e.g. acetate) turn weak acids (like water and acetic acid) into stronger acids (MacFarlane et al. 2006). This may be one reason for the beneficial effect of water observed in EMIMAc, though this may also be related to reductions in viscosity (Fu and Mazza 2011). IL viscosity, which is much higher than conventional solvents, impacts cellulose dissolution through mass transfer and this can be difficult to separate from kinetic impacts (Gericke et al. 2012). The effectiveness of an IL is also dependent on temperature. Pure cellulose dissolves in imidazolium ILs between 80 and 100 °C (Zhang et al. 2005; Vitz et al. 2009), but pretreatment of whole biomass requires higher temperatures (~130 °C) for significant decrystallization of undissolved fractions (Kimon et al. 2011), which might be related to the glass transition temperature of lignin (Keskar et al. 2011; Li et al. 2011).

14.4 Impacts of Plant Characteristics on Cell Wall Degradation

14.4.1 Plant Classification

Different pretreatments process certain classifications of plants more effectively than others (Wyman et al. 2013), however, for the same pretreatment method, plant materials can almost always be arranged in the following order, either with regard to digestibility for the same conditions, or severity of conditions required for equivalent digestibility: grasses > herbaceous dicots > hardwoods > softwoods (Arantes and Saddler 2011; DeMartini and Wyman 2011a; Garlock et al. 2012b). This order is largely due to four factors that increasingly hinder pretreatment reaction kinetics and mass transfer: (1) increase in proportion of recalcitrant covalent linkages within the cell wall (esters - > ethers - > carbon–carbon bonds); (2) increase in strength of

hydrogen-bonding of major hemicellulose sugars to cellulose; (3) increase in average cell wall thickness and proportion of the cell volume occupied by cell wall; and (4) increase in the proportion of lignin versus cellulose, although if cellulose accessibility is sufficiently increased, the actual presence of lignin during hydrolysis is not a major issue (Jeoh et al. 2007; Chundawat et al. 2011; Rollin et al. 2011; Wiman et al. 2012).

14.4.2 Plant Varieties

A handful of studies have looked at differences in digestibility and yields for cultivars within the same species. Upland and lowland switchgrass when harvested around the same time in the same location had similar sugar yields for most pretreatment methods (Kim et al. 2011) and similar optimal pretreatment conditions and enzyme loading (Garlock et al. 2012a). Results for wheat straw were varied, with one study that indicated sugar yields (g/g dry biomass) from hydrothermally pretreated wheat straw were not influenced by cultivar (Larsen et al. 2012), while two other studies found a significant variation in sugar yields across all cultivars (Lindedam et al. 2010; Lindedam et al. 2012).

14.4.3 Plant Cell Types and Tissues

Herbaceous feedstocks can show major differences in conversion between different portions of the plant or different cell types that may influence practical considerations such as harvesting methods and fractionation prior to pretreatment. In general, pith tends to be more digestible than the vascular bundles and the rind/epidermis. One study found that sugar yields follow the same pattern of digestibility for both hydrothermally pretreated and untreated materials (pith > leaves > rind) (Zeng et al. 2012). Pith cells of sugar cane were highly digestible by enzymes even without pretreatment and following chlorite treatment the rind cells became significantly more digestible (Siqueira et al. 2011).

For herbaceous botanical fractions, the general trend is that stems are easier to digest than leaves. For AFEX™-pretreatment, corn fractions were more digestible in order of decreasing lignin content (husk > leaf > stem > cob) (Garlock et al. 2009). For sodium hydroxide pretreatment, corn stover fractions released the most glucan in order of husks, cob, and leaves > upper stem > lower stem (Duguid et al. 2009) and corn stover and wheat straw fractions that contained more lignin showed a greater improvement with a higher catalyst loading (Duguid et al. 2007, 2009). Hydrothermally pretreated grasses and legume stems had lower percent sugar conversions than leaves, but higher total sugars released (DeMartini and Wyman 2011a). Miscanthus fractions showed decreasing cellulose conversion with: leaves > sheath > stem (Le Ngoc Huyen et al. 2010).

14.4.4 Harvest Date and Maturity

For herbaceous crops that have annual growth cycles, harvest date significantly impacts composition and biomass yields. As the plant approaches full maturity and senescence, the relative proportion of lignin and structural carbohydrates increase with a simultaneous decrease in soluble sugars, protein, and minerals (Dien et al. 2006). Harvest during the growing season can result in a highly digestible material, but one that also has significant nitrogen and ash content (Bals et al. 2010), which can impact farm economics, sustainability, and conversions. Some studies have shown little impact on total sugars released due to maturity (Dien et al. 2006; Garlock et al. 2009). However, there is a consistent decrease in digestibility and biomass yields when harvest is delayed from fall to winter or spring, largely due to loss of leaves and other fragile, digestible portions of the plant (Pordesimo et al. 2005; Adler et al. 2006; Le Ngoc Huyen et al. 2010; Kim et al. 2011). With regard to woody materials, one paper examined sugar yields from different growth rings and found no significant variation between mature wood and juvenile wood, despite an increase in lignin content with age of the ring (DeMartini and Wyman 2011b).

14.4.5 Composition

The most common trend reported for the effect of biomass composition on hydrolysis yields, is that glucan digestibility is negatively correlated to total lignin content (Davison et al. 2006; Dien et al. 2006; Rock et al. 2009; Garlock et al. 2012b). Lignin monomer composition may also be important, as a decrease in the S/G ratio leads to more recalcitrant linkages, and pretreatments that can break them would be expected to show a higher digestibility compared to those that do not. However, based on a number of studies S/G ratio may or may not be correlated to improved digestibility, depending on other plant cell wall properties and whether and how the plant was pretreated (Chen et al. 2002; Mechin et al. 2005; Davison et al. 2006; Li et al. 2010; Studer et al. 2011b; Zhang et al. 2011b).

14.5 Designing Improved Feedstocks

A number of strategies for developing “plants designed for deconstruction” have been reviewed in recent years (Carpita 2012; Jung et al. 2012; Abramson et al. 2013). These strategies can be grouped broadly as altering lignin (content, monolignol composition, and degree of polymerization), increasing and/or altering polysaccharides (content, composition, or crystallinity), expressing cell wall-degrading or modifying enzymes *in planta,* or producing oils in vegetative tissues.

14.5.1 Alterations to Lignin

Initial studies on plants with altered lignin contents began with the "brown midrib" mutations (Barrière et al. 2004) for improved ruminant digestibility. Plant lines have subsequently been engineered with decreased and altered lignin content by changing the expression of monolignol biosynthetic enzymes. Decreasing expression of one or more of the monolignol synthesis enzymes has been shown to decrease total lignin content and improve the enzymatic digestibility of alfalfa following hot water pretreatment (Chen and Dixon 2007). However, decreasing the total lignin content of the cell wall also impairs the overall fitness of the plant and can lead to dwarfed plants and failure to accumulate biomass (Casler et al. 2002; Voelker et al. 2011). As a consequence of this, more recent strategies have been focused on altering the ratio of monolignols, and increasing the S/G ratio in hybrid poplar has been shown to improve alkaline delignification (Stewart et al. 2009) and digestibility following alkaline and dilute acid pretreatment, though there was no significant difference following AFEX™ treatment (Ong 2011). Increasing S/G in *Arabidopsis* was shown to improve the enzymatic release of glucose following hot water pretreatment (Li et al. 2010). A recent study found that decreasing total lignin content concurrently with decreasing S/G in switchgrass improved the enzymatic glucose yield following dilute acid pretreatment, as well as decreasing pretreatment severity and cellulase loadings, and increasing ethanol yield (Fu et al. 2011).

Another strategy has been to introduce novel monolignols or proteins that make the cell wall more amenable to chemical deconstruction without impacting total lignin content or plant fitness. These approaches, all of which have been shown to increase digestibility and/or lignin removal to some extent include adding monolignols that shorten the degree of polymerization (*p*- hydroxybenzyaldehydes) (Eudes et al. 2012), monolignols that incorporate alkali-labile ester linkages within the lignin matrix, e.g. novel ester-based di-lignols as lignin precursors (Grabber et al. 2008; Simmons et al. 2010), and glycoproteins that participate in cross-couplings with lignin, such as tyrosine-rich hydroxyproline-rich glycoprotein (Liang et al. 2008).

14.5.2 Alterations to Polysaccharides

Altering cell wall polysaccharides is another method to reduce cell wall recalcitrance or increase the amount of substrate. One strategy is to decrease cellulose crystallinity by overexpressing cellulose synthases with impaired functionality (Harris et al. 2009) or by overexpressing a membrane-bound endoglucanase, KORRIGAN (Maloney and Mansfield 2010). Another strategy is to increase the carbohydrate content of the plant cell wall. Cellulose content and crystallinity increased in poplar by over-expressing a sucrose synthase gene (Coleman et al. 2006) and various amorphous polysaccharides have been targeted for accumulation, including starch (Chuck et al. 2011 and mixed-linkage β-glucans (Pauly et al. 2011). In contrast, reductions in glucuronoxylan content in poplar showed an

increase in digestibility by enzymes alone (Lee et al. 2009). In rice, loss of activity for a xylosyltransferase thought be responsible for arabinosyl substitution of the xylan backbone resulted in a slight increase in arabinose substitution and decrease in hydroxycinnamic acid content, resulting in increased extractability of xylan and enzymatic digestibility (Chiniquy et al. 2012). Alteration of *O*-acetylation of hemicelluloses may also lead to a decrease in acetate content for reduced inhibition of fermentation or altered capacity of hemicelluloses to hydrogen bond with other cell wall polymers (Gille and Pauly 2012). Other work has demonstrated improved enzymatic digestibility of plant cell walls by preventing de-methyl esterification in the pectin homogalacturonan (Lionetti et al. 2010), which limits the ability to form Ca^{2+}-mediated cross-links, increasing primary cell wall porosity and decreasing rigidity and cell-to-cell adhesion in primary cell walls.

14.5.3 Transcription Factors for Secondary Cell Wall Formation

Regulatory networks have recently been identified comprising several transcription factors that act as "master switches" responsible for controlling the temporal and spatial regulation of collections of genes involved in the secondary cell wall synthesis, assembly, and thickening (Shen et al. 2012). One study ectopically overexpressed a MYB transcription factor in switchgrass to down-regulate the genes associated with monolignol biosynthetic pathways and identified phenotypic outcomes of reduced lignin and reduced *p*-coumarate to ferulate ratios that resulted in a tripling of enzymatic sugar release (Shen et al. 2012). Other work identified a mutation in WRKY transcription factors to be responsible for secondary cell wall thickening and significantly increased cellulose, hemicellulose, and lignin deposition in the pith cells of model dicots, increasing the overall plant density, and potentially providing a route for increasing accumulation of fermentable sugars in plant cell walls (Verma et al. 2010).

14.5.4 Expression of Cell Wall Degrading Enzymes in Planta

The high cost and doses of enzymes required for cellulosic biofuels are critical economic barriers for commercialization. Expression of thermophilic cellulases in the apoplast (Sticklen 2006) or mesophilic cellulases in chloroplasts (Verma et al. 2010) are one possible route for generating some of the cellulolytic enzymes in situ. Cellulolytic enzymes can be generated *in planta* to supplement other enzymes during hydrolysis, however even mild pretreatment of the biomass can significantly lower their activity (Teymouri et al. 2004). Expression of feruloyl esterases in grasses which cleave ferulate ester cross-links has been found to improve both enzymatic and in vitro ruminant digestibilities (Buanafina et al. 2008). Expression of plant cellulolytic enzymes that are active under plant physiological conditions (Hartati et al. 2008) or

cellulose binding modules (CBMs) (Shoseyov et al. 2006) in the apoplast have been found to increase growth and biomass accumulation, presumably by increased cell wall loosening, but with the potential disadvantage of impaired plant fitness.

14.5.5 Production of Oils in Vegetative Tissues

One way to increase the energy content of lignocellulosic biomass is to modify plants to produce oils, fatty acids, or triacylglycerols (TAGs) in vegetative tissues (Durrett et al. 2008). In one study triacylglycerols were accumulated in senescing *Arabidopsis* leaves by either blocking fatty acid breakdown, or by ectopically expressing the LEC2 seed development transcription factor in leaves (Slocombe et al. 2009). Another study successfully shifted the carbon flux in *Arabidopsis* leaves from starch biosynthesis to the production and accumulation of triacylglycerols by simultaneously reducing the expression of a catalytic subunit of ADP-glucose pyrophosphorylase and ectopically expressing the WRINKLED1 transcription factor that is involved in seed oil biosynthesis (Sanjaya et al. 2011).

14.6 Pretreatment as a Screening and Analysis Tool: Expanding Our Understanding of the Plant Cell Wall

Re-engineering plants to provide phenotypic traits desirable of an ideal biofuel energy crop is an area of intense research, as highlighted previously. However, it is vitally important to evaluate processing capabilities of new materials as they are being generated, as biomass recalcitrance may not favorably correlate with the traits selected for during transgenesis or breeding. With recent advances in high-throughput analytical techniques, it is now feasible to quickly screen for desirable traits from very large libraries of biomass phenotypes, while requiring only small sample quantities for detailed analyses. In addition to screening, high-throughput techniques are also helping to further understanding of the relationship between biomass conversion and plant cell wall characteristics. For example, high throughput composition analysis techniques allowed for screening of thousands of poplar samples for lignin content and S/G ratios, and from this a fairly large subset was further tested using a high-throughput pretreatment and enzymatic hydrolysis method in order to determine the relative impacts of lignin and S/G ratio on sugar yields (Studer et al. 2011b).

As in the example above, high-throughput pretreatments can now be carried out in custom-designed microplate-based reactors that have been developed for both acidic and alkaline pretreatments (Santoro et al. 2010; Selig et al. 2010; Studer et al. 2011a). Rapid, small-scale compositional analysis methods are able to determine cell wall composition, both before and after pretreatment (DeMartini et al. 2011b; Selig et al. 2011). These techniques can be coupled to medium/high-throughput analyses using LC–MS/MS and 2D-NMR for more detailed elucidation of changes in cell wall structure, composition, and degradation (Chundawat et al. 2008; Kim and Ralph 2010;

Morreel et al. 2010). Semi-automated (medium/low throughput) electron micrography and immunolabeling based techniques have also been used in recent years to characterize the complex interplay of pretreatment severity and cell wall ultra-structural modifications (Donohoe et al. 2009; Pattathil et al. 2010; Chundawat et al. 2011; Zhang et al. 2013a). To this end a bio-analytic toolkit was developed, comprising more than 200 glycan-directed monoclonal antibodies that recognize distinct epitopes present on various categories of plant cell wall polysaccharides (Pattathil et al. 2010). This microplate-based, quantitative assay has provided insights into the relationship between pretreatment severity and cell wall polysaccharide accessibility and extraction, and the molecular architecture of the plant cell wall (Alonso-Simón et al. 2010; DeMartini et al. 2011a). As indicated by Moller et al. (2007), monoclonal antibodies directed against cell wall glycans provides complementary compositional data that could be used to optimize pretreatment conditions and enzyme cocktails necessary for more efficient degradation of lignocellulose.

The effectiveness of pretreatments on bioconversion has been evaluated using micro-scale based rapid enzymatic hydrolysis (Chundawat et al. 2008; Banerjee et al. 2010; Gomez et al. 2010; Jäger et al. 2011; Riedlberger and Weuster-Botz 2012) and microbial fermentation based assays (Funke et al. 2010; Riedlberger and Weuster-Botz 2012). These assays can be coupled with microplate-based pretreatments to facilitate rapid screening of several hundred biomass specimens (Studer et al. 2010). Additionally, with developments in micro-scale cell-free protein expression systems it is possible to selectively optimize enzyme combinations necessary for different pretreatments and biomass types (Chandrasekaran et al. 2010).

14.7 Conclusions

In recent years understanding of the chemistry and structure of the plant cell wall has progressed rapidly. Pretreatment research has contributed to understanding of the distribution and composition of various cell wall polysaccharides within the many different classes of cell walls. Future work will continue to delve more deeply into the complex relationships between cell wall and pretreatment chemistry to improve and develop novel conversion methods for release of cell wall sugars and to improve biomass characteristics for conversion to biofuels. High-throughput analytical techniques and tools that allow for rapid analysis of small quantities of samples will allow for more efficient comparisons in the development of new feedstocks and processing methods, and improved understanding of the fundamental relationships between cell wall chemistry and structure and pretreatment chemistry.

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