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PERSPECTIVE

Exploring new strategies for cellulosic biofuels production

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A research program has been initiated to formulate new strategies for efficient low-cost lignocellulosic biomass processing technologies for the production of biofuels. This article reviews results from initial research into lignocellulosic biomass structure, recalcitrance, and pretreatment. In addition to contributing towards a comprehensive understanding of lignocellulosic biomass, this work has contributed towards demonstrated optimizations of existing pretreatment methods, and the emergence of new possible pretreatment strategies that remain to be fully developed.

Introduction

Petroleum currently comprises more than 40% of the world's energy and is continuously adding to the planet's atmospheric carbon dioxide burden. World energy demand is projected to double within the next 10 years, and as petroleum and natural gas supplies become less plentiful and more costly, it is imperative to identify and develop renewable, carbon-neutral alternatives.[1] As the world's top consumer of crude oil and the second largest greenhouse gas emitter,[2] the USA has a large stake in this crisis.[3] While stationary energy needs for power generation, manufacturing, and other similar processes can be addressed by renewable energy sources such as nuclear, solar, wind, and geothermal power, sustainable and cost-effective production of liquid transportation fuels remains a paramount and an urgent problem. One strategy to address this problem is to produce biofuels from biomass feed stocks. Several types of biofuels are possible, which are renewable, sustainable, and emit less carbon during consumption.[4–8]

At present, the commercial production of biofuels in the USA is largely based on the use of starch in grains such as maize (95% of grains) to produce ethanol,[9] although sugars, mostly sucrose, in crops such as beet and cane are commonly used in place of starch elsewhere. This is a mature commercial technology with well understood economics and the USA is now the world's largest producer of starch-based ethanol, used mainly (99% of the US supply) as a 10% additive to gasoline (known as E-10 or gasohol).[10] However, starched-based ethanol production on its own and under current conditions has the potential to displace

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Broader context

It is imperative for our future energy and environmental needs to identify and develop renewable, carbon-neutral alternatives to petroleum for the sustainable and cost-effective production of liquid transportation fuels. One strategy is to produce biofuels from biomass. Lignocellulosic biomass is a particularly attractive because it is the cheapest, most abundant, and fastest growing form of biomass, it can be grown on marginal or non-arable lands without displacing food crops, and it can be found in the residues and wastes from agricultural crops. A research program has been initiated to formulate new strategies for lignocellulosic biomass processing to biofuels. In addition to contributing towards a comprehensive understanding of lignocellulosic biomass, early results have contributed towards demonstrated optimizations of existing biomass pretreatment methods, and the emergence of new possible pretreatment strategies that remain to be fully developed. A feature of this work is the use of combinations of several complementary experimental and theoretical methods in order to obtain a more complete understanding of each problem. These multi-technique approaches have the potential to be applied to other research areas of importance to energy and environmental science that require the characterization and understanding complex material systems.

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only a relatively small proportion (10%) of gasoline. This is due to several issues. Converting more cropland to maize, and shifting maize utilization to biofuels and away from other uses, would have severe consequences for other agricultural markets and food prices. Furthermore, although ethanol can adequately serve as a land transportation fuel for the public sector, it simply does not have the energy density required for aviation fuels.[4] Consequently, there is considerable support from the US government through legislation and through funding for industry and National Laboratories to lead the development of alternative or additional biomass feed stocks and more advanced high energy density biofuels.[10–12]

Lignocellulosic biomass, the woody fibrous material derived from plant cell walls, is a particularly attractive feed stock because it is the cheapest, most abundant, and fastest growing form of terrestrial biomass, it can be grown on marginal or non-arable lands without displacing food crops, and it can be found in the residues and wastes from agricultural crops.[10,13,14] It can serve as a source of carbon based feedstock for fuel and chemical production in much the same way as crude oil serves as the carbon feedstock in petrochemical refineries.[15] However, clear strategies for feedstock supply, handling, and processing, and the technologies required for large-scale and economic conversion of lignocellulosic biomass into biofuels and its distribution have not yet been fully developed.[16–19]

A number of different approaches are being explored at present for converting lignocellulosic biomass into biofuels.[7] The technologies required for thermochemical pathways, in particular gasification, are relatively mature.[11] Several alternative approaches to thermochemical pretreatment are based around three distinct stages; 1) pretreatment to disrupt the plant cell wall architecture and expose or separate the sugar containing cellulose and hemicellulose material 2) hydrolysis of those components to simple sugars 3) conversion of sugars to fuel products. Approaches exist for carrying out each of these three stages, mainly derived from the pulp, paper, and ethanol fermentation industries, but the economics of the complete life-cycle for lignocellulosic derived biofuels production remains unclear. Several reviews have been published that describe the technological barriers that limit cost-competitiveness and the advances that are being made to overcome these barriers.[11,17,20,21] Numerous demonstrations and commercialization efforts are ongoing.

This article describes preliminary results from a research program whose primary aim is to formulate new strategies for efficient low-cost lignocellulosic biomass processing technologies. The program builds on major advances already made by other researchers and is designed to be complementary to other ongoing research programs.[11] The initial focus has been on relatively low temperature (compared to thermochemical) biological and chemical technologies. Research began with institutional investment and support from Los Alamos National Laboratory to explore lignocellulosic biomass structure, recalcitrance, and pretreatment and the scope of this article is limited to reviewing results from this early effort. However, research in this program has now broadened to also explore hydrolysis into simple sugars, the conversion of these sugars to ethanol, thermochemical approaches, and the production of more advanced biofuels. These broader activities and also future directions are briefly discussed in the concluding section. It is beyond the scope of this article to review the tremendous progress being made by other research programs in this field.

The work described in this article has been driven by collaborations with the United States Department of Agriculture Forest Products Laboratory and Agricultural Research Service; the Universities of New Mexico, Michigan, Tokyo, and Toledo; the Centre de Recherches sur les Marcomolécules Végétales; and the Great Lakes Bioenergy Research Center. Furthermore, extensive use of central user research facilities throughout the world has been important to this research, in particular the Advanced Photon Source at Argonne National Laboratory, Los Alamos Neutron Science Center at Los Alamos National Laboratory, and the reactor neutron source at the Institute Laue Langevin.

Lignocellulosic biomass structure and recalcitrance

Lignocellulosic biomass is the fibrous material derived from plant cell walls. It is comprised mostly of lignin, hemicelluloses, and cellulose in a highly complex architecture with structure sizes ranging from Ångströms to micrometres. This architecture has evolved for strength, vascular function, and resistance to biological, physical and chemical attack and is a barrier to effective hydrolysis to monosaccharides. Understanding the architecture of biomass is a key to addressing its recalcitrance.[19] In the work described here an array of complementary experimental techniques is being applied to characterize biomass at different length scales. At the same time, different theoretical approaches are being developed and applied to interpret the experimental data at each length scale and to allow transition between them. The long-term goal in combining experiment and theory in this way is to develop what can be called a virtual microscope that can be refocused to build a multi-level understanding of lignocellulosic biomass (Fig. 1).

The initial focus has been on poplar trees as a source of biomass because they have potential to be a highly abundant, low cost, feed stock,[22] although there are other promising feed stocks such as switch grass and corn stover.[23] Furthermore it is relatively easy to handle poplar as a microtomed thin wafer that can be analyzed using a variety of different noninvasive experimental techniques. Poplar species have cellulose, hemicelluloses, and lignin content ranging 42–49%, 16–23%, and 21–29%, respectively.[22] The exact composition can depend on several factors including species, strain, location and physiological function in the tree, harvest time and storage. Despite the variation, the cellulose content of poplar is usually higher than that of other potential feedstock such as switch grass and corn stover.

At the Ångström scale

The major component of poplar biomass, cellulose, is generated at the plasma membrane as a crystalline fibrous material consisting of polymers of glucose sugar monomers (1–4 linkedβ-D glucose) (Fig. 2). These high tensile strength fibers add mechanical strength to the cell wall. In higher plants two different crystal forms of cellulose can occur, I_α and I_β, collectively referred to as cellulose I.[24] The proportions of I_α and I_β, and the size and crystallinity of fibers, vary greatly depending on

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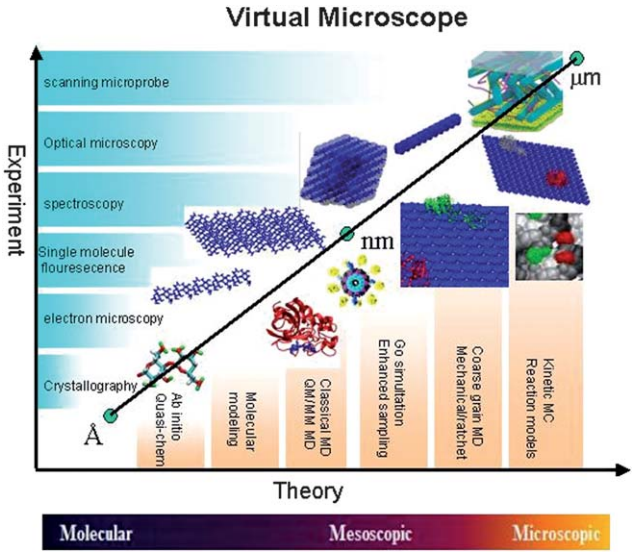

Fig. 1 An array of complementary experimental techniques is being used to characterize biomass at different length scales, including crystallography, small angle scattering, spectroscopy, synthetic chemistry, enzyme and chemical activity assays. At the same time, different theoretical approaches are being developed and applied to interpret the experimental data at each length scale, including atomistic and coarse-grained molecular dynamics, statistical mechanics, quantum chemistry, and kinetic Monte Carlo models. Coarse graining methodologies are being used to integrate individual theoretical models that traverse length scales from the Ångström to the micron. The multi-scale tool that will result can be thought of as a "virtual microscope" that can be refocused to build a multi-level understanding of lignocellulosic biomass and its pretreatment.

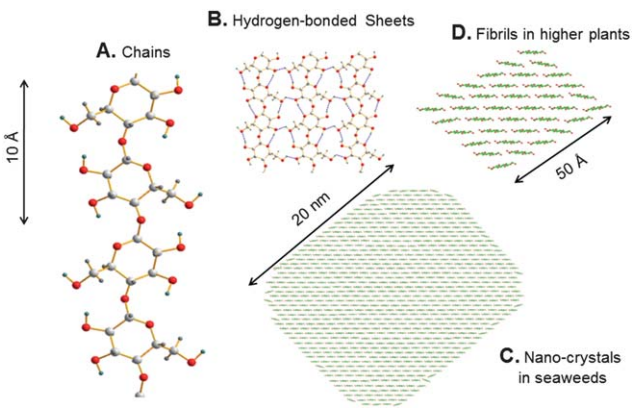

Fig. 2 Cellulose, is generated as a crystalline fibrous material consisting of polymers of hexose glucose sugar monomers (1–4 linked β-D glucose) (**A**). The polymer chains are arranged in sheets that are held together by strong hydrogen bonds (blue dashed lines) between the hydroxyl groups of neighboring chains (**B**). These sheets stack on top of each other through more frequent but weaker stacking interactions, such as van der Waals forces. The thick highly crystalline cellulose fibers (cross section shown in **C**) that can be extracted from seaweeds and marine creatures can be clearly characterized as corresponding to individual nanocrystals. Fibers have crystalline interiors, but less ordered and more dynamic chains at their surfaces. The presence of surface disorder has profound implications for the smaller fibers found in poplar (cross section shown in **D**) because approximately half of their cellulose chains are located at the surface.

the origin of the cellulose and its location within the plant cell wall. In poplar the cellulose fibers are predominantly in the I_β form,[25] with a diameter of ~5 nm and up to several micrometres in length.[26,27] Within these fibers about 63% of the cellulose is crystalline with the remaining cellulose being less well ordered.[25]

The crystal structures of cellulose fibers in the I_α and I_β forms were determined by using high resolution fiber diffraction studies.[28–31] In these studies large (~20nm in diameter) cellulose fibers (sometimes referred to as whiskers or nanocrystals) were used. These can be found in seaweeds and marine creatures and serve as good model systems because they are highly crystalline and therefore provide very accurate atomic resolution structures, and also because they can be found in purely the I_α or I_β forms. X-Rays were used to visualize the arrangement of carbon and oxygen atoms that make up the molecular skeleton of the cellulose chains, but neutrons had to be used to visualize the smaller and more mobile hydrogen atoms involved in hydrogen bonding.[30] In the I_β form the parallel chains are arranged in sheets that are held together by strong hydrogen bonds between the hydroxyl groups of neighboring chains. These sheets stack on top of each other through more frequent but weaker stacking interactions, such as van der Waals forces. Quantum mechanics calculations revealed that these stacking interactions are highly cooperative and contribute just as much to the stability of cellulose I_β as hydrogen bonding.[32] The flexible hydroxymethyl group is oriented so that it can make hydrogen bonds with atoms within the same chain (intra-chain and intra-sheet) and also neighboring chains (inter-chain and intra-sheet).[28] These fundamental insights provide a foundation for experimental and theoretical studies of the smaller cellulose fibers found *in situ* in poplar plant cell walls.

An unexpected result from these studies is that the hydrogen bonding arrangement in naturally occurring cellulose is disordered. This was an important discovery because the exact nature of hydrogen bonding in cellulose has long been thought to play a key role in determining the properties of cellulose fibers, in particular their recalcitrance to hydrolysis.[33] A novel statistical mechanical approach was developed to investigate this disorder further, and it was found that its presence can allow for the hydrogen bonding to rearrange and adapt to different environmental conditions, particularly temperature. The adaptive nature of the hydrogen bonding arrangement, thus, serves to stabilize the fibers and make them more resistant to degradation.[34] Furthermore, a combination of low temperature neutron crystallography, quantum mechanics calculations, and molecular dynamics simulations was applied to discover that most chains (70–80%) in model seaweed fibers have the same hydrogen bonding arrangement, but that disorder exists at defects in the fibers and also at their surfaces.[35]

The presence of surface disorder has profound implications for the smaller fibers found in poplar because approximately half of their cellulose chains are located at the surface. Further molecular dynamics simulations were carried out on cellulose fibers of the size found in poplar, and also in the presence of water. In those simulations, the central core of the fiber maintained an average structure that was essentially the same as the crystallographic structure, but chains at the surfaces had significantly different structures and hydrogen bonding arrangements and were more disordered.[36] Support for differences between interior

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and surface chains has also come from ^{13}C NMR studies carried out by other researchers.[37] The surface chains interact strongly with water, forming a dense layer of water near the surfaces, in agreement with previous molecular dynamics studies by others.[38] This hydration layer will affect the way hydrolyzing enzymes interact with cellulose fibers.

What happens when a cellulose chain is pulled from the surface of a fiber and becomes free in solution was also investigated.[39] In order to accomplish this, the first application of replica exchange molecular dynamics (REMD) to polysaccharides was developed. It was found that cellulose chains in solution have different properties compared to those within fibers. In particular, they have greater dynamic freedom, although the conformational flexibility about each glycosidic linkage becomes more restricted as the chain length is increased. The main reason for this increase in stiffness with chain length is a tendency to form intrachain hydrogen bonds, like those found within crystalline fibers. In solution the hydroxymethyl groups have a preference to adopt a different conformation compared to those within crystalline fibers of cellulose I. The hydroxymethyl groups of the chains at the surfaces of crystalline fibers can also have different conformations from those within. Although the cellulose chains in the more disordered regions of fibers may be bent and twisted in a way that also demonstrates conformational flexibility, in solution flexibility is associated with greater dynamic freedom and is influenced by solvent, which competes for hydrogen bonding and causes the disruption of intra-chain hydrogen bonds.

The second most abundant component of poplar biomass, lignin, is a complex, branched polymer biosynthesized by radical polymerization of three monolignol phenolpropanoid precursors which differ in the degree of methoxylation on their aromatic rings (Fig. 3). Monomeric units are added non-stereoselectively to the growing lignin polymer through free radical coupling reactions, which give rise to three types of racemic, predominantly non-phenolic, structural units commonly referred to as H (*p*-hydroxyphenyl), G (guaiacyl), and S (syringyl). These structural units can be linked by diverse carbon-oxygen and carbon-carbon (condensed) bonds, including arylglycerol-β-aryl ether (β-*O*-4), phenylcoumaran (β-5), resinol (β-β), and spirodienone (β-1) linkages.[40] Theβ-*O*-4 non-condensed linkages predominate (~40–80%) in most lignins.

Whereas the linkages between monomer units in cellulose and hemicelluloses can be hydrolyzed under relatively mild physiological conditions, the linkages between lignin monomers cannot, but rather require oxidative cleavage. Under non-physiological conditions, such as very acidic pH or high temperature, the ether linkages in lignin, and between lignin and hemicellulose, can be cleaved.[41–43] Lignin also has a glass transition temperature in biomass which varies between 100 to 160 °C, depending on monolignol chemical composition and biomass source.[44–46] Above the glass transition temperature it is possible for lignin to melt, resulting in its redistribution in biomass.

Both the quantity and structural nature of lignins vary among plant species, but in poplar mostly the G and S moieties are present. Methoxylation prevents the formation of some types of linkages and therefore monomer composition affects the total number, and the relative proportion, of linkages that are present. This will have a direct effect on the glass transition temperature of lignin. The proportion of non-condensed β-*O*-4 linkages will also increase with the ratio of S/G. In order to study the structure and properties of lignin the approach used in this project has been to synthesize small dimeric and tetrameric lignin model compounds that are representative of the linkages found in poplar and then to investigate these compounds with a combination of chemical, enzymatic and quantum chemistry approaches.[47,48] No X-ray diffraction features from lignin in poplar have been identified in this work, indicating that it is relatively disordered at a molecular level. However, it has been reported by other researchers that linkage patterns affect the structure of lignin, as β-*O*-4-linked structures tend to form elongated chains in which the aromatic rings lie in the same plane with each other and the cellulose fibers.[49]

The third most abundant component in poplar biomass, the hemicelluloses, consists of complex, branched polysaccharides. They are mostly xylans with a main chain formed by pentose sugar monomers (β-1,4 linked D-xylopyranose) and α-1,2 linked 4-*O*-methylglucuronic acid monomers. In poplar there are about 6–7 acetyl groups per 10 xylose units. These acetyl groups can be easily degraded to liberate acetic acid. The pentose sugar arabinose, and the hexose sugars mannose, glucose and galactose, are also present to a lesser degree.[50] Hemicellulose forms covalent bonds with lignin, the most common being an ether linkage of the hydroxyl group at the α-position of lignin with the alcoholic hydroxyl of a hemicellulose sugar monomer.[51] There is also extensive hydrogen bonding between hemicelluloses and other polysaccharide components such as cellulose. Hemicelluloses are therefore tightly associated with both lignin and cellulose in the cell wall and act effectively as a binder with non-branched xylans preferentially associated with cellulose fibers and branched xylans interacting with lignin.[52]

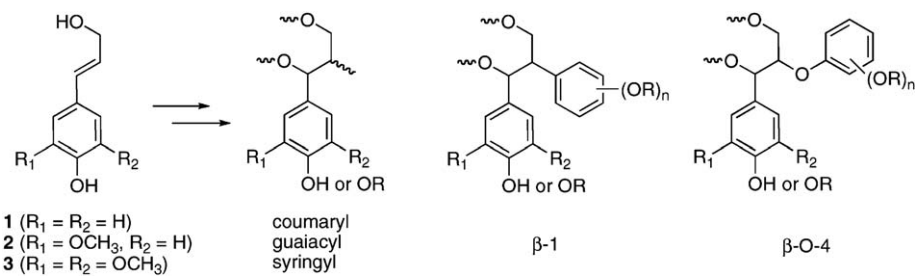

Fig. 3 Structural unit found in lignin. The approached used in this project has been to synthesize small dimeric and tetrameric lignin model compounds that are representative of the linkages found in poplar and then to investigate these compounds with a combination of chemical, enzymatic and quantum chemistry approaches (adapted from Cho *et al.*[47]).

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As with lignin, no diffraction features from hemicelluloses in poplar were identified in this work. However, isolated xylan is known to form crystalline assemblies of three-fold or two-fold helices when pure or acetylated, respectively.[53] Furthermore, composites of cellulose and xylan have been shown to give X-ray diffraction patterns that are identical to those from pure cellulose, with the xylan polymers incorporated within the cellulose fibers without disrupting their crystal structure.[54] Although no evidence was observed for the crystalline structures seen with isolated xylans, it is possible that some hemicellulose chains are adsorbing on to the surface of poplar cellulose fibers in the same manner as cellulose chains and therefore contributing to diffraction features that are identified with cellulose. Hemicelluloses are thought to be more thermally unstable than cellulose and lignin and are more easily hydrolyzed than cellulose.[55]

At the nanometre scale

The thick highly crystalline cellulose fibers extracted from seaweeds and marine creatures can be clearly characterized as individual nanocrystals using electron microscopy and diffraction techniques, with rectangular, square, or parallelogram cross sections depending on their source.[56] However, in the cell walls of higher plants the nature of the supramolecular structure of cellulose is less clear. Higher plant cellulose is known to be synthesized by membrane bound hexagonal arrays of synthase complexes called rosettes or terminal complexes.[57] Each synthase complex is thought to synthesize an elemental fibril consisting of about 36 chains. Depending on the exact cross sectional shape, this would result in a fibril width of ~4 nm which is only slightly smaller than that determined in this work from diffraction studies of poplar (~5 nm). The difference may be due to a diffraction contribution from hemicellulose on the surface of the fibrils, or incorrect assumptions made about fiber shape in the diffraction analysis.[27] From molecular (Å) level studies it can be concluded that these elemental fibrils have crystalline interiors, but less ordered and more dynamic chains at their surfaces. Molecular dynamics simulations of solvated elemental fibrils by other researchers typically show the development of a persistent right-handed twist.[58] Although it has been suggested that twist may be an artifact of the force-field used in these simulations, it is not inconsistent with the diffraction data collected in this work[59] and there is some experimental evidence for its presence *in vivo*.[60] The degree of twisting that can appear in molecular dynamics simulations is found to decrease with increasing fiber thickness and this may explain its complete absence in molecular images of large cellulose nanocrystals from seaweeds and marine creatures.[56]

In addition to the possibility of twisting, there is evidence for periodic disorder along the length of cellulose fibrils from studies using a combination of small angle neutron scattering, acid hydrolysis, gel permeation chromatography, and viscosity studies.[61] Every 300 or so glucose units along the crystalline fibril there appears to be a disordered region of about 4 or 5 glucose units. Periodic disorder is not observed in seaweed or marine creature nanocrystals, suggesting that it is restricted to higher plant cellulose biosynthesis. Interestingly, whereas cellulose produced at the cell wall of *rubusfruticosus* (common name: blackberry) does show periodic disorder, cellulose produced *in vitro* without the constraints of the cell wall does not.[62] This suggests that periodic disorder is associated with the cell wall environment, within which disordered regions may act as anchoring points for hemicelluloses and may also relieve any twist induced strain.

Elemental fibrils are thought to be coated with hemicelluloses, and then assembled into larger bundles that are referred to as macrofibrils, although there is often confusion with use of the terms fibril, fiber, microfibril and macrofibril.[56,57] In order to understand the assembly and properties of these macrofibrils they are currently being studied using coarse-grained molecular dynamics approaches. In this work, which is still at an early stage, new coarse-grained, off-lattice models are being based on the relevant degrees of freedom that have been identified from the analysis of the fully-atomistic molecular dynamics simulations. Off-lattice models have been previously applied to proteins[63] and there have been several applications of other coarse-grain modeling approaches to cellulose.[34, 64–67]

At the micron scale

The cell wall of poplar is organized in several layers that have different structures and compositions; the middle lamella, the primary cell wall, the three sub-layers of the secondary cell wall S1–S3, and the empty lumen.[68,69] The thick secondary cell wall is deposited after the cells have reached their final size, followed by lignification. *In situ* X-ray micro-diffraction (with a beam size of a few μm) recorded from wafers of poplar with the thickness of about a single cell gives patterns that are characteristic of partially crystalline fibers of cellulose I_β.[27] In fact two superimposed patterns are observed with a relative orientation of approximately 25° due to diffraction from both the front and back walls of a cell. Most of the material in poplar biomass is associated with the thick S2 sub-layer. Within S2 the elemental fibrils and macrofibrils are known to have a winding angle, referred to as the microfibril angle (MFA), which is related to wood strength. This winding angle has been previously shown by other researchers to correspond to the cellulose fibers in S2 of wood being in a helical arrangement as they spiral around the central lumen.[70] The observed relative orientations of the two superimposed diffraction patterns correspond to a value for the MFA of 12.5°, which is in about the middle of the wide range observed for different clones and ring positions in poplar trees.[71] Periodic disorder and any twist in the elemental fibrils and macrofibrils may be intimately associated with this winding.

During cell wall deposition, lignification is known to start from the cell corners and middle lamella and to proceed towards the lumen, filling the spaces between the packed cells, but not penetrating the lumen.[72] Auto-fluorescence images can be used to reveal the distribution of lignin in poplar cell walls,[73] and it is found that although most lignin is located in the thick S2 sub-layer, its concentration is greatest in the middle lamella.[27] This agrees with previous studies that have shown that lignin can represent 85% of the total material in the middle lamella.[49] Lignin adds structural rigidity and hydrophobicity to plant cell walls, but it also protects the cellulosic component from hydrolyzing enzymes. Raman microspectra from poplar cell walls show clear bands that can be assigned to not only lignin and cellulose but

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also hemicellulose.[27] These microspectra offer a non-invasive approach to monitoring the overall composition of poplar cell walls.

Towards a multi-scale understanding of the complete cell wall

By developing the application of an array of complementary experimental and theoretical techniques, models of the complex architecture of the cell wall at different length scales have been built. The expectation is that the structure and properties of lignocellulosic biomass and its pretreatment at these different length scales will be highly correlated. Only by integrating results from these different scales can a comprehensive and predictive understanding of the cell wall be achieved. In the multi-scale approach being developed in this work, a key initial requirement is the quantitative integration of the experimental results with the associated simulations and theory at the same length scale.

Upon establishing this correspondence, and after validation, coarse graining methodologies are being used to integrate individual theoretical models that traverse length scales from the Ångström to the micron. Hopefully the resulting multi-scale tool will allow the prediction of various changes in the cell wall structure during pretreatment such as the development of pores and the diffusion of oxidative and hydrolyzing enzymes at the micron scale, the interaction and binding of enzymes to lignin, hemicellulose, and cellulose fibers at the nanometre scale, and the decrystallization and chemical hydrolysis of cellulose or hemicellulose at the molecular and atomic scale.

Lignocellulosic biomass pretreatment

Pretreatment is one of the main economic costs of producing lignocellulosic biofuels. Several approaches are being developed to pretreat lignocellulosic biomass so that it can be more efficiently converted to fermentable sugars, including dilute acid, steam explosion, hydrothermal processes, ionic liquids, organic solvents in aqueous media, biological and enzymatic processes, ammonia fiber explosion (AFEX), strong alkali processes, and highly-concentrated acid treatment.[21,74,75] Major advances continue to be made by researchers across the world to improve and optimize these different pretreatment technologies. Some pretreatments, in particular those using ionic liquids and concentrated acids, are effective because, in addition to increasing accessibility to cellulose, they decrease its crystallinity making it more easily digested by cellulases.[76,77] Other pretreatments, such as dilute acid pretreatment, increase accessibility at the expense of increasing cellulose crystallinity.[78] These different approaches all have their advantages and disadvantages and it is likely that no one approach will be best for all potential feed stocks. Effective optimization of pretreatment will likely require a fundamental understanding of the basic interactions that occur with biomass during its pretreatment.

Ammonia fiber expansion (AFEX)

Conventional AFEX involves treating biomass with ammonia at about 130 °C in a pressure vessel for periods of up to an hour and then releasing the pressure, causing the ammonia to expand rapidly, thereby disrupting the biomass and enhancing cellulose accessibility.[79] In addition to mechanical disruption there is a significant redistribution of lignin and hemicelluloses in the cell wall material, but no major decrystallization of cellulose.[80,36] Although some deacetylation of hemicelluloses can occur, a major advantage of AFEX is that it does not produce large amounts of degradation products that can inhibit downstream processing.[81] Conventional AFEX under hydrous conditions does not change the crystal structure of cellulose. However, when cellulose fibers are treated with anhydrous amines such as ammonia they can be converted from the naturally occurring cellulose I crystal form[28,29] to another form called cellulose III_I.[82] Cellulose III_I has been shown to have enhanced accessibility and chemical and biological reactivity.[83] Furthermore, combinations of enzymes that are typically effective for hydrolysis of cellulose I can be adjusted to greatly improve their effectiveness in hydrolyzing cellulose III_I.[36] There are several different research groups advancing pretreatment methods that are based on the use of ammonia or other amines. The focus of the work in this program has been to contribute to the efforts of research collaborators at Great Lakes Bioenergy Research Center by investigating the molecular aspects of the conversion of cellulose I to cellulose III_I and how AFEX can be altered to maximize cellulose accessibility whilst simultaneously producing a conversion to cellulose III_I.

Anhydrous ammonia converts cellulose I to a transient crystalline complex, called ammonia-cellulose I, and then when the ammonia is evaporated under anhydrous conditions this complex is converted to cellulose III_I (Fig. 4). However, if a significant amount of water is present at certain stages, the complex will revert back to cellulose I. One of the keys to maximizing cellulose III_I formation during AFEX is therefore controlling the amount of water present at different stages of the process. In an effort to characterize the solid-state structural pathway involved in this conversion at a molecular level, a combination of X-ray and neutron crystallography, atomistic and coarse-grained molecular dynamics simulations, and quantum mechanics calculations has been applied in this work.[28,29,32,82,84,85] Investigations have been carried out on how amines penetrate into cellulose fibers at both molecular and fibrillar scales using time-resolved scanning microprobe X-ray diffraction and ^{13}C nuclear magnetic resonance. In these latter studies ethylenediamine (EDA) was used as a substitute for ammonia because it is easier to handle.[86,87] Replica exchange

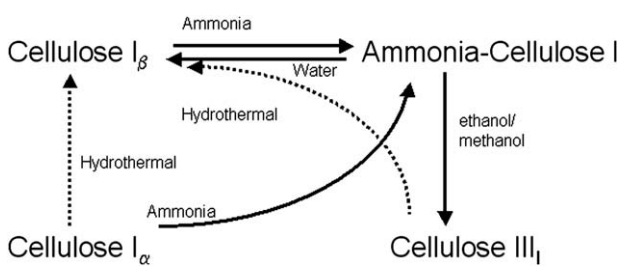

Fig. 4 The solid-state conversion of cellulose between various crystal phases involving ammonia and hydrothermal treatment. A key to maximizing cellulose III_I formation during AFEX is controlling the amount of water present at different stages of the process (adapted from Wada *et al.*[85]).

molecular dynamics is also being used to study amine penetration and the structural conversion of macrofibrils of cellulose I_β to cellulose III_I at the coarse-grained level. The structural transition in these coarse-grained simulations is being analyzed within the framework of the Ginzburg-Landau formalism.

Ammonia preferentially penetrates cellulose fibers from particular crystal faces, and as it penetrates it acts as a strong hydrogen bond acceptor and donor, pulling at the flexible hydroxymethyl groups, causing them to rotate away from their positions in cellulose I and breaking the intra-chain hydrogen bonding. After penetration ammonia sits in distinct sites in the binary complex, tethered by its hydrogen bond with the hydroxymethyl group, but rotating so that it forms transient hydrogen bonds with the hydroxyl groups of other chains. There is a significant amount of entropy associated with the confined rotating ammonia molecules and dynamical disorder associated with the hydrogen bonding in ammonia-cellulose I. The ammonia molecule can be thought of as holding the hydroxymethyl groups in the correct configuration to form an inter-sheet hydrogen bond that stabilizes cellulose III_I during ammonia evaporation.

The conversion decreases intra-sheet hydrogen bonding and introduces inter-sheet hydrogen bonding. Atomistic molecular dynamics simulations show that these rearrangements increase the number of hydrogen bonds that surface cellulose chains can make with water.[36] The surface chains have significantly fewer hydrogen bonds with other chains compared to those within the crystalline core of the fiber. Further, the hydroxymethyl groups of chains at the surface of fibers have different preferred conformations, and are more disordered, compared to those within the fiber core. There are also significant differences between the properties of chains at the surfaces of fibers of cellulose I and cellulose III_I. The chains at the surfaces of cellulose III_I fibers, compared to those at the surfaces of cellulose I fibers, have preferred hydroxymethyl group conformations, hydrogen bond interactions with solvent moelcules, and dynamic disorder more similar to that found in solution and in amorphous material. This might partly explain the greater susceptibility of cellulose $III_I$to enzyme hydrolysis compared to cellulose I. In addition to changing the crystal structure of cellulose to cellulose III_I,optimizing the combination of enzymes applied to different crystal forms can greatly enhance hydrolysis.[36] Optimizing the synergistic activity of enzymes for different crystal forms is an example of co-optimizing catalyst and substrate and has allowed near theoretical glucan conversion at industrially relevant enzyme loading for cellulose III_I (Fig. 5).

These results with pure cellulose have been used to alter AFEX pretreatment in preliminary studies with lignocellulosic biomass. An 80% increase in glucan digestibility was observed within 6 h of enzymatic hydrolysis for cellulose III_Irich AFEX compared to conventional AFEXpretreated corn stover (Fig. 6). Although there are further improvements to be made, and although this is only a small part of a much broader effort to use ammonia for biomass pretreatment that is being investigated by several different researcher groups, it was demonstrated that formation of cellulose III_I within a realistic lignocellulosic biomass can indeed improve overall hydrolysis yields. This conceptually new approach may help to reduce the enzyme loading necessary to achieve cost-effective conversion of lignocellulosic biomass.[36]

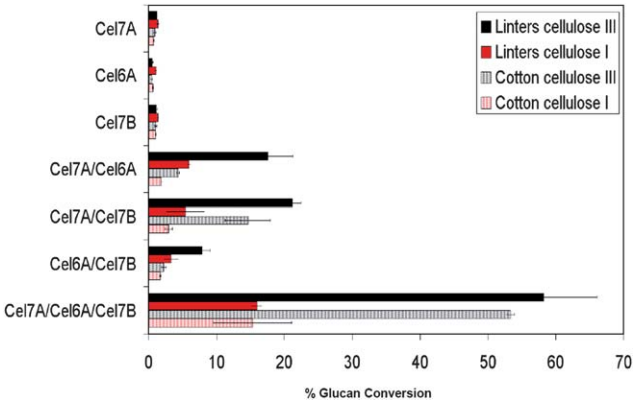

Fig. 5 Enzymatic digestibility of cellulose I and III_I (black bars)for various combinations of *Trichodermareesei basedexo-* (Cel7A, Cel6A) and *endo-* (Cel7B, Cel5A) cellulases. Optimizing the synergistic activity of enzymes for different crystal forms has allowed near theoretical glucan conversion at industrially relevant enzyme loading for cellulose III_I (adapted from Chundawat *et al.*[36]).

Ionic Liquids (ILs)

Ionic Liquids (ILs) are being developed by several research groups as a promising new approach to pretreatment because they are non-derivatizing, they do not produce fermentation inhibitors, and they are amenable to "easy recovery".[21] Pretreatment with ILs not only disrupts the plant cell wall and separates cellulosic, hemicellulosic, and lignin components, but it also disrupts the crystallinity of cellulose making it more rapidly digestible by hydrolyzing enzymes.[89–91] Several ILs have been identified by different research groups as effective in enhancing biomass saccharification. The focus of the work in this program has been on EMIMAC (1-*n*-ethyl-3-methylimidazolium acetate) because it can be readily separated from dissolved cellulose by the addition of an anti-solvent, such as water or ethanol, by a solute displacement mechanism.[90] Imidazolium based ILs with short alkyl chains, such as EMIMAC, have been shown to be less toxic than those with longer alkyl side groups.[91] Furthermore the acetate ion renders the IL less corrosive than ILs with halide anions (*e.g.* Cl) that have been effective in cellulose dissolution.[90]

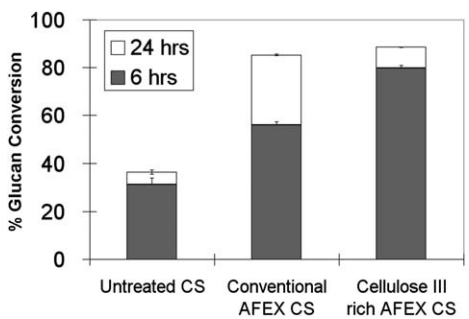

Fig. 6 Enzymatic digestibility (15 FPU Spezyme CP cellulase/g glucan) of untreated cornstover (CS), conventional AFEX treated CS (AFCS) and cellulose III rich AFEX treated CS. No cellulose III was formed during conventional AFEX. Cellulose III in AFEX CS was produced by treating AFCS with anhydrous liquid ammonia at 25 C for 2 h (adapted from Chundawat *et al.*[36]).

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Several studies by other researchers have shown that total dissolution and separation of the cellulosic, hemicellulosic, and lignin components of biomass can be achieved in IL pretreatments at elevated temperatures.[92,93] However, these elevated temperatures add significantly to the cost of lignocellulosic biorefining. The development of a room temperature IL pretreatment process would therefore be of very high value, and is a focus of research collaborators at the University of Toledo.

In this work towards that goal, the pretreatment of poplar biomass at room temperature was studied using a combination of time-resolved scanning microprobe X-ray diffraction, Raman spectroscopy and auto-fluorescence (Fig. 7).[27] It was found that EMIMAC penetrates the cell wall from the lumen, swelling it by about a factor of two in towards the empty lumen. However, the middle lamella remains unchanged, preventing the cell wall from swelling outwards. During swelling, most of the cellulose microfibrils are solubilized but chain migration is restricted and a small percentage of microfibrils persist. When the EMIMAC is expelled, the cellulose recrystallizes as microfibrils of cellulose I. There is little change in the relative chemical composition of the cell wall and the action of EMIMAC would appear to be a reversible swelling of the cell wall and a reversible decrystallization of cellulose I.

Both the disruption of the cell wall and also the conversion of cellulose I into another form called cellulose II[94,95] or into amorphous material are important factors in the effectiveness of high temperature IL pretreatments for improving the efficiency of subsequent enzyme hydrolysis of cellulose. Room temperature treatment with EMIMAC under ambient conditions achieves neither, the main obstacle being the intransigence of the lignin rich middle lamella. However, a room temperature IL pretreatment may be effective following some other pretreatment process that specifically attacks the middle lamella. It was observed that reversible swelling by room temperature IL pretreatment can serve as a general approach to introducing chemicals and materials into cell wall structures (Fig. 8).[96] X-ray fluorescence spectroscopy was used to quantify uptake, and confocal near infrared surface enhanced Raman microscopy was used to verify penetration, of particles up to 4 μm in size into the poplar cell wall. The possibility of using room temperature IL pretreatment to improve the access of enzymes and catalyst particles to the lignocellulosic matrix is currently being further explored. Deeper penetration into the biomass is expected to lower the cost of reducing the biomass to a fine size. It was shown that impregnated samples when subjected to surface enhanced Raman microscopy display a strong surface-enhanced effect. Room temperature IL pretreatment can significantly increase the speed in Raman microscopy image acquisition, as well as the ability to place nanosensors at the cell wall to measure chemical environment at the wall during fungal or biochemical treatments. This technique is generally applicable to biochemical analysis.[97,98]

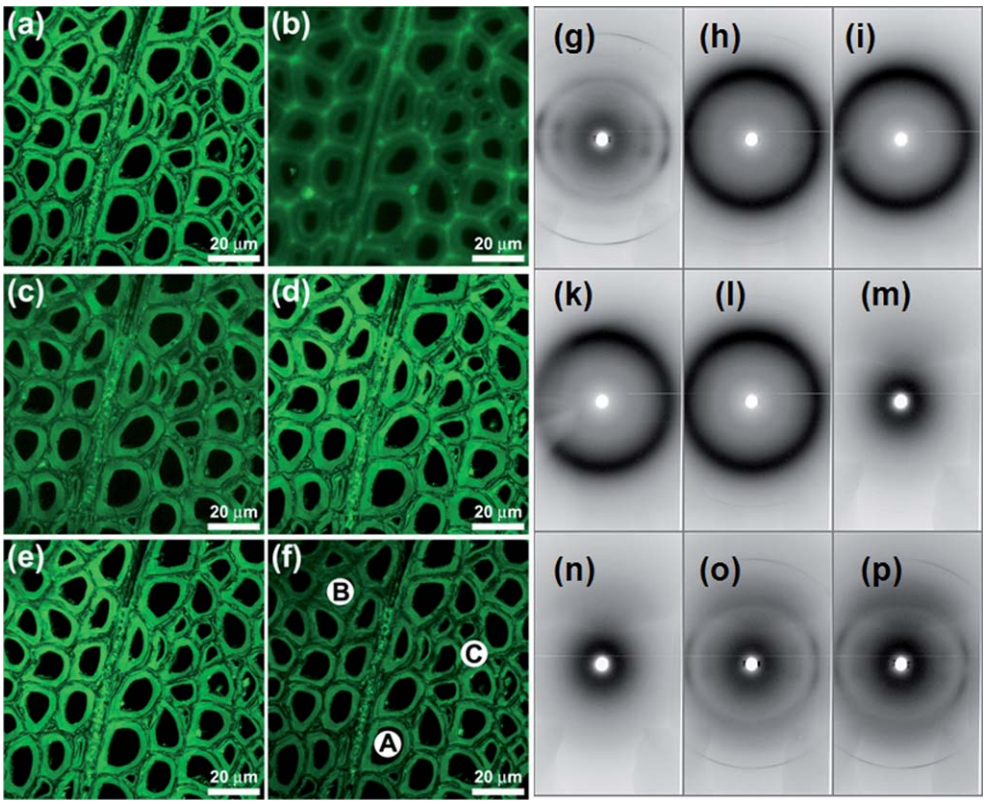

Fig. 7 Time-resolved autofluorescence images (**a** to **f**) and X-ray microdiffraction images (**g** to **p**) of poplar wood cells as they are reversibly swollen by ionic liquid. Auto-fluorescence reveals the distribution of lignin in poplar cell walls, whereas X-ray diffraction reveals the structure of cellulose fibers (adapted from Lucas *et al.*[27]). The ionic liquid penetrates the cell wall from the lumen, swelling the cell wall by about a factor of two. However the middle lamella remains unchanged. During swelling most of the cellulose microfibrils are solubilized but recrystallize as cellulose I on expulsion of the ionic liquid. The action of the ionic liquid is therefore a reversible swelling and a reversible decrystallization of the cell wall.

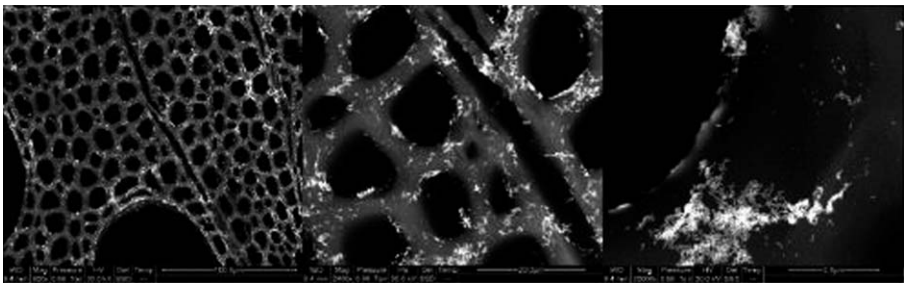

Fig. 8 Poplar wood cells after impregnating with gold nanoparticles during ionic liquid reversible swelling. The bright spots are the nanoparticles. The possibility of using this approach to improve the access of enzymes and catalyst particles to the lignocellulosic matrix is currently being explored.

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Biological approaches

Although many fungi degrade parts of plant cell walls, white rot fungi are unique in their ability to degrade it completely.[99] In particular, they can efficiently degrade lignin to CO_2[100] and this has stimulated interest in their use as a biological pretreatment (sometimes called "seasoning").[21,101] A big advantage of this approach is environmental friendliness. Rather than developing the use of these microorganisms themselves as a pretreatment, in this work a different approach is being taken of trying to understand their key degradation processes, so that new insights can be obtained that might lead to new pretreatment strategies. Although several strains of white rot fungi have been investigated by other researchers for their potential use in biological pretreatments, *Phanerochaete chrysosporium* has been a primary focus because it has already been widely studied and its genome sequence has been available for some time.[102] More recently the sequences of several other lignin degrading fungi have become available. The ability of *P. chrysosporium* to degrade lignin is due to its unique extracellular oxidative ligninolytic system that includes secreted enzymes and small molecule metabolites.[103] Of these enzymes, multiple isozymes of lignin peroxidase (LiP) and manganese peroxidase (MnP) have been identified. LiP and MnP are iron-heme containing enzymes which catalyze oxidation reactions that lead to lignin bond cleavage.[104]

MnPs from *P. chrysosporium* oxidize Mn^{2+} to Mn^{3+}, which acts as a small diffusible mediator for the oxidation of lignin.[99] There is evidence that during diffusion, the bidentate chelator oxalate, an extracellular metabolite of the fungus, stabilizes Mn^{3+} against disproportionation to Mn^{2+} or the insoluble Mn^{4+}.[104] However, these Mn^{3+}/oxalate complexes are considerably weakened oxidants compared to more weakly chelated Mn^{3+} and are not strong enough to oxidize the predominant (90%) non-phenolic units in lignin, attacking only the terminal phenolic units. For that reason they are unlikely candidates for development for biomass pretreatment and attention has been focused on other possible Mn^{3+}/chelator complexes and on studying the stronger oxidative mechanisms of LiPs.

Recently it was discovered that Mn^{3+} produced by MnPs from *P. chrysosporium* can chelate and oxidize certain unsaturated long chain fatty acids and it may be that a downstream product of these fatty acids can act a non-phenolic lignin oxidant.[104] Preliminary evidence suggests that the proximal oxidants in this system may be long-chain unsaturated aldehydes. This exciting discovery may have potential for development towards a lignin degrading pretreatment, although considerable further research is required to define the exact sequence of single electron transfers. It also suggests that if MnP does have a major role in the lignin degradation strategy of *P. chrysosporium*, it may be through subsequent reactions of Mn^{3+} with other mediator oxidants that cleave the non-phenolic lignin structures.

In order to search for other possible mediator oxidants small molecules that are generated during fungal degradation of poplar are being studied using mass spectrometry. Although this is at a preliminary stage, several compounds have been identified, with one fungus secreting a previously unknown metabolite. This metabolite is aromatic, as shown by the finding that it becomes radiolabeled when ^{14}C-labeled phenylalanine is supplied to the wood cultures. In associated work, a transporter protein, located in the cell membrane of *P. chrysosporium*, has been observed which is up-regulated during ligninolytic metabolism and thus may have a role in the secretion of biodegradative metabolites. Also identified during this study was a ligninolytically up-regulated, membrane-associated alcohol oxidase which likely has a role in production of the hydrogen peroxide needed to support the action of lignin-degrading peroxidases during wood deconstruction by *P. chrysosporium*.In addition to searching for natural chelators and mediator oxidants of Mn^{3+}, the possibility of directly designing Mn^{3+}/chelator complexes that incorporate synthetic ligands that can stabilize Mn^{3+} whilst also preserving or enhancing its oxidative power is being explored by other researchers.

Unlike MnPs, LiPs are strong oxidants that have the ability to interact directly with non-phenolic lignin units to cleave them. They are oxidized by H_2O_2 to give a two electron-oxidized intermediate (Compound I) in which the iron is present as Fe(IV) and a free radical resides on the tetrapyrrole ring (or on a nearby amino acid). Compound I then oxidizes a donor lignin substrate by single electron transfer, yielding a lignin radical cation and Compound II, in which the iron is still present as Fe(IV)but no radical is present on the tetrapyrrole. The lignin radical cation then undergoes spontaneous carbon-carbon bond cleavage. Compound II then oxidizes a second donor lignin substrate, giving another lignin radical cation and the resting state of the enzyme. This pathway can occur through direct interaction (binding) of the heme group with small lignin fragments, or longer-range electron transfer between a surface exposed tryptophan residue and more bulky and enzyme-impenetrable lignin polymers.

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An alternative route which would also allow oxidation of bulky lignin polymers has been suggested that involves mediation by diffusible small molecules (*e.g.*, veratryl alcohol) whose cation radicals formed by single electron transfer to LiP are responsible for oxidation of lignin. In mass spectrometry studies of the fungal degradation of poplar in this work, veratryl alcohol and its oxidation product, veratryl aldehyde, are the most common small molecules seen, and it is a metabolite known to be secreted by *P. chrysosporium*. However, veratryl alcohol may have other roles in the oxidative pathway of LiPs including reduction of LiP Compound II to the resting state after lignin oxidation.[104]

Although LiPs are attractive because of their unusually high activity, an efficient expression system that would allow their production on an industrial scale has yet to be developed in this work. However, significant progress has been made in understanding the details of the single electron oxidation reactions which LiPs promote. In initial studies, information was gained about the efficiencies/rates of carbon-carbon bond cleavage of linkages in lignin. For this purpose, single electron transfer, photochemical, Ce(IV), and LiP promoted oxidation reactions were carried out on dimeric lignin model compounds that had been synthesized to represent the condensed β-1 and non-condensedβ-*O*-4 groups that are predominant in the lignin skeleton, and compared to quantum mechanics calculation of bond ionization energies. It was discovered that regardless of the method used for their generation, cation radicals derived by single electron transfer oxidation of β-1 linked dimers undergo carbon-carbon bond cleavage more rapidly than those produced from β-*O*-4 linked dimers.[47]

These studies were then extended to tetrameric lignin compounds that included both the both β-1 and β-*O*-4 linkages. It was found that regardless of how the charge and odd electron in the radical cations are distributed over the two linkages, the β-1 linkage is preferentially cleaved.[48] This is an important finding because it suggests that the genetic design of plants that have greater abundances of the spirodienone(β-1) linkages in lignin may be more efficiently pretreated through oxidative pretreatments. The structural and electronic features of neutral and radical cation lignin model compounds containing β-1 and β-*O*-4 linkages were also investigated using *ab initio* density functional theory (Fig. 9). The calculated bond dissociation energies clearly indicated the selective C_α–C_β bond weakening upon electron delocalization and charge localization upon radical formation. These calculations addressed the reactivity of these model compounds in terms of the possible influence of stereoselectivity and solvent effects.

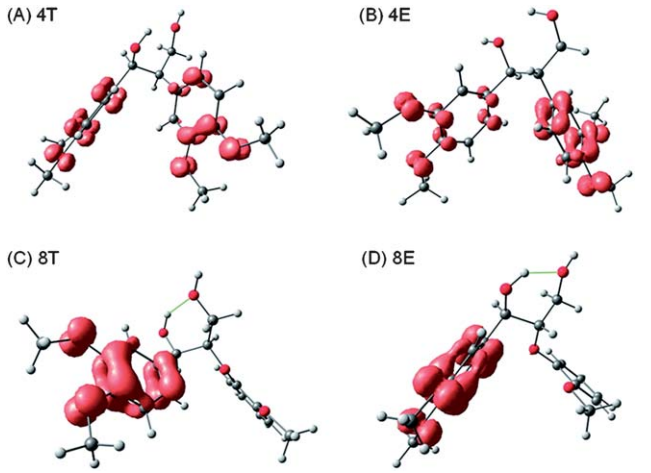

Fig. 9 The structural and electronic features of neutral and radical cation lignin model compounds containing β-1 and β-*O*-4 linkages were calculated using *ab initio* density functional theory. The distribution of positive electron density is shown for different structural units. The calculated bond dissociation energies clearly indicated that oxidation of β-1 linked dimers undergo carbon-carbon bond cleavage more rapidly than those produced from β-*O*-4 linked dimmers, suggesting that the genetic design of plants that have greater abundances of β-1 linkages in lignin may be more efficiently pretreated through oxidative pretreatments (adapted from Cho *et al.*[47]).

Discussion

Initial results from a program researching lignocellulosic biomass structure, recalcitrance, and pretreatment have been reviewed. This program is designed to contribute in a complementary way to the many other research programs in this field, and it builds on major advances already made by other researchers across the world. It was described how by altering conventional AFEX to maximize cellulose accessibility whilst simultaneously producing a cellulose crystal conversion, cost-efficiency can be significantly improved. In related work it was observed that optimizing the combination of enzymes applied to different types of cellulose crystal forms found in pretreated lignocellulosic biomass can improve hydrolysis by enhancing synergistic activity. At room temperatures, and ambient conditions, pretreatment with EMIMAC ionic liquid cannot break down the middle lamella and disrupt the cell wall components of biomass, as occurs during effective pretreatment at higher temperatures. However, the secondary cell wall can be reversibly swollen and this allows access to nanometre sized particles. This insight is being exploited to allow deeper penetration of enzymes and chemical catalysts in order to lower the cost of mechanically reducing lignocellulosic biomass. Biologically inspired approaches to oxidizing lignin as a possible pretreatment strategy were explored. The chelated Mn^{3+} ions that are the immediate oxidation products of the enzyme MnP are unlikely candidates for industrial development, but other downstream oxidation mediators that are more promising were found. Research into the more powerfully oxidizing enzyme LiP has provided new insights into how bonds in lignin are preferentially cleaved, and these results may be helpful for genetic manipulations to lignin synthesis in plants to reduce the costs of their pretreatments. In addition to contributing towards a comprehensive understanding of lignocellulosic biomass, this work has also contributed towards demonstrated optimizations of existing pretreatment methods, and the emergence of completely new pretreatment strategies that remain to be fully developed.

The nascent research program reviewed here has more recently grown into a broader program in lignocellulosic biofuels research involving several collaborations between different technical divisions at Los Alamos National Laboratory, as well as with industry, other national laboratories, and academic institutes. In the other areas of this growing program noteworthy progress is being made. In particular, in addition to investigating

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biologically inspired Mn^{3+}/chelator complexes as catalysts for lignin degradation, other base metal complexes, such as vanadium(v) dipicolinate have been developed that have the potential to provide an earth-abundant (non-precious) metal catalyst and air as an oxidant for lignin.[105–107] It is hoped that selective oxidative cleavage of lignin by this catalyst will result in smaller, more utilizable components, for example, aromatic product streams that could feed into existing industrial markets.

Enzymes represent a significant proportion of the cost of producing lignocellulosic biofuels. Industrially relevant properties such as thermal stability, pH tolerance, activity, substrate specificity and inhibition, of cellulose (cellulases) and hemicellulose (xylanases) hydrolyzing enzymes are being improved using several different bioengineering approaches. A novel protein thermostabilization method has recently been developed,[108] which is being applied to cellulases from the fungus *Trichoderma longibrachiatum*, so that they remain active for longer at the elevated temperatures of some proposed industrial conversions. A second, independent bioengineering project is focusing on the hemicellulose degrading enzyme from the soil bacterium *Bacillus circulans*. A two-pronged approach has been implemented in order to identify the best method for rapid evolution of cellulases from this xylanase based on enzyme function. The first utilizes the power of Rosetta molecular modeling software.[109] The second method leverages the detailed knowledge of the enzyme catalyzed reaction mechanism for construction of an intricate directed evolution approach for evolving cellulase function.[110] In yet another project, the catalytic mechanism of another xylanase from the fungus *Trichoderma longibrachiatum* is being studied by neutron crystallography in order to provide mechanistic insights that can guide rational engineering of the enzyme for improved performance in the alkaline conditions sometimes found in proposed industrial conversion.[111]

The above examples of bioengineering involve enzymes that are secreted by both fungi and soil-derived bacteria for extracellular degradation of biomass. These are just a few examples of industrially important enzymes that are being pressed to perform new and improved functions. However, other examples involve enzymes that are introduced into the microorganism in order to endow their new hosts with new metabolic capabilities. In particular, a major problem for the efficient production of lignocellulosic biofuels is the presence of xylose in biomass hydrolazates. Xylose is a pentose sugar which cannot be fermented by the industrial yeast *S. cerevisiae*. Extensive efforts are being made by several other researcher groups to engineer *S. cerevisiae* with an efficient xylose metabolic pathway to produce xylulose, the fermentable keto isomer of xylose. One pathway is isomerization of xylose to xylulose by expression of *xylA*, the gene found in some anaerobic fungi and bacteria that encodes the enzyme D-xylose Isomeraze. In work being carried out in collaboration with the Great Lakes Bioenergy Research Center, neutron crystallography is being used to elucidate the catalytic mechanism of D-xylose isomerase in detail in order to provide insights that will allow rational design to improve catalytic performance and binding specificity and which are generalizible to a broad range of enzymes with different thermal and pH stabilities.[112–115] In related work, mechanistic insights from neutron crystallography are being used to improve the enzyme carbonic anhydrase for increased carbon fixation during algal biofuels production.[116,117]

Conclusion

This work contributes towards a comprehensive understanding of lignocellulosic biomass. It builds on, and is complementary to, the major advances made by other researchers in this field. Although several different approaches to pretreatment methods are possible, and are being advanced and demonstrated by other researchers, this work has contributed towards the emergence of completely new pretreatment strategies that remain to be fully developed. A feature of the research reviewed here is the use of combinations of several complementary experimental and theoretical methods when necessary, in order to obtain a more complete understanding of each problem. Thus insights that contribute to a basic understanding of the structure and properties of lignocellulosic biomass and its pretreatment have been gained by the application of crystallography, small angle scattering, spectroscopy, synthetic chemistry, enzyme and chemical activity assays, molecular dynamics, statistical mechanics, and quantum chemistry. These new combined theoretical and experimental platforms will continue to be developed and applied to research in lignocellulosic biofuels, but also have potential to be applied in other research areas requiring complex material characterization.

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References

1 R. A. Kerr, Oil resources – the looming oil crisis could arrive uncomfortably soon, *Science*, 2007, **316**, 351–351.
2 EIA. *Petroleum Basic Statistics*, Washington, DC, 2009, available at: http://www.eia.doe.gov/basics/quickoil.html, July 27, 2009.
3 D. Inman, N. Nagle, J. Jacobson, E. Searcy and A. E. Ray, Feedstock handling and processing effects on biochemical conversion to biofuels, *Biofuels, Bioprod. Biorefin.*, 2010, **4**, 562–573.
4 L. R. Lynd, Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy, *Annu. Rev. Energy Environ.*, 1996, **21**, 403–465.
5 D. L. Klass, in *Encyclopedia of Energy*, ed. C. J. Cleveland, Elsevier, London, 2004, vol. 1.

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Published on 10 August 2011 on http://pubs.rsc.org | doi:10.1039/C1EE01268A

6 C. E. Wyman, S. R. Decker, M. E. Himmel, J. W. Brady, C. E. Skopec and L. Viikari, in *Polysaccharides*, ed. S. Dumitriu, Marcel Dekker, New York, 2nd edn, 2005.
7 G. W. Huber, S. Iborra and A. Corma, Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering, *Chem. Rev.*, 2006, **106**, 4044–4098.
8 A. Carroll and C. Somerville, Cellulosic biofuels, *Annu. Rev. Plant Biol.*, 2009, **60**, 165–182.
9 R. Schnepf, *Agriculture-based renewable energy production*, Congressional Research Service Report to Congress, 2006, RL32712.
10 D. Somma, H. Lobkowicz and J. P. Deason, Growing America's fuel: an analysis of corn and cellulosic ethanol feasibility in the United States, *Clean Technol. Environ. Policy*, 2009, **12**, 373–380.
11 R. E. H. Sims, W. Mabee, J. N. Saddler and M. Taylor, An overview of second generation biofuel technologies, *Bioresour. Technol.*, 2010, **101**, 1570–1580.
12 EISA. *Energy Independence and Security Act of 2007*. Public Law 110–140 (2007).
13 R. D. Perlack *et al.* Report TM-2005, under contract DOE/GO-102005-2135, Oak Ridge, TN, 2005.
14 Y. C. Lin and G. W. Huber, The critical role of heterogeneous catalysis in lignocellulosic biomass conversion, *Energy Environ. Sci.*, 2009, **2**, 68–80.
15 A. Weislogel, S. Tyson and D. Johnson, in *Handbook on Bioethanol: Production and Utilization*, ed. C. Wyman, Taylor and francis, 1996, pp. 105–118.
16 J. R. Mielenz, Ethanol production from biomass: technology and commercial status, *Curr. Opin. Microbiol.*, 2001, **4**, 324–329.
17 M. Knauf and M. Moniruzzaman, Lignocellulosic biomass processing: A perspective, *Int. Sugar J.*, 2004, **106**, 147–150.
18 C. Somerville, The billion-ton biofuels vision, *Science*, 2006, **312**, 1277–1277.
19 M. E. Himmel, S. Y. Ding, D. K. Johnson, W. S. Adney, M. R. Nimlos, J. W. Brady and T. D. Foust, Biomass recalcitrance: Engineering plants and enzymes for biofuels production, *Science*, 2007, **315**, 804–807.
20 M. Saraf and A. Hastings, Biofuels, the role of biotechnology to improve their sustainability and profitability, *Biodiversity, Biofuels, Agroforestry and Conservation Agriculture*, 2010, 123–148.
21 P. Alvira, E. Tomas-Pejo, M. Ballesteros and M. J. Negro, Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review, *Bioresour. Technol.*, 2010, **101**, 4851–4861.
22 P. Sannigrahi, A. J. Ragauskas and G. A. Tuskan, Poplar as a feedstock for biofuels: A review of compositional characteristics, *Biofuels, Bioprod. Biorefin.*, 2010, **4**, 209–226.
23 H. S. Moon, J. M. Abercrombie, A. P. Kausch and C. N. Stewart, Jr., Sustainable use of biotechnology for bioenergy feedstocks, *Environ. Manage.*, 2010, **46**, 531–538.
24 R. H. Atalla and D. L. VanderHart, Native cellulose – a composite of 2 distinct crystal forms, *Science*, 1984, **223**, 283–285.
25 M. Foston, C. A. Hubell and M. Davis, Variation in cellulosic ultrastructure of poplar, *BioEnergy Res.*, 2009, **2**, 193–197.
26 K. Leppänen, S. Andersson, M. Torkkeli, M. Knaapila, N. Kotelnikova and R. Serimaa, Structure of cellulose and microcrystalline cellulose from various wood species, cotton and flax studied by X-ray scattering, *Cellulose*, 2009, **16**, 999–1015.
27 M. Lucas, G. Wagner, Y. Nishiyama, L. Hanson, I. P. Samayam, C. A. Schall, P. Langan and K. Rector, Reversible swelling of the cell wall of poplar biomass by ionic liquid at room temperature, *Bioresour. Technol.*, 2011, **102**, 4518–4523.
28 Y. Nishiyama, P. Langan and H. Chanzy, Crystal structure andhydrogen-bonding system in cellulose $I_\beta$from synchrotron X-ray and neutron fiber diffraction, *J. Am. Chem. Soc.*, 2002, **124**, 9074–9082.
29 Y. Nishiyama, J. Sugiyama, H. Chanzy and P. Langan, Crystal structure and hydrogen bonding system in cellulose I_α from synchrotron X-ray and neutron fiber diffraction, *J. Am. Chem. Soc.*, 2003, **125**, 14300–14306.
30 P. Langan, Neutron diffraction from fibers, *Crystallogr. Rev.*, 2005, **11**, 125–147.
31 Y. Nishiyama, P. Langan, M. Wada and V. T. Forsyth, Looking at hydrogen bonds in cellulose, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2010, **66**, 1172–1177.
32 R. Parthasarathi, G. Bellesia, S. Chundawat, B. E. Dale, P. Langan and S. Gnakaran. New Insights into Hydrogen Bonding and Stacking Interactions in Cellulose. *J. Physical Chem*, submitted.
33 G. T. Beckham, J. F. Matthews, B. Peters, Y. J. Bomble, M. E. Himmel and M. F. Crowley, Molecular-level origins of biomass recalcitrance: decrystallization free energies for four common cellulose polymorphs, *J. Phys. Chem. B*, 2011, **115**, 4118–4127.
34 T. Shen and S. Gnanakaran, The stability of cellulose: A statistical perspective from a coarse-grained model of hydrogen bond networks, *Biophys. J.*, 2009, **96**, 3032–3040.
35 Y. Nishiyama, G. P. Johnson, A. D. French, V. T. Forsyth and P. Langan, Neutron crystallography, molecular dynamics, and quantum mechanics studies of the nature of hydrogen bonding in cellulose I_β, *Biomacromolecules*, 2008, **9**, 3133–3140.
36 S. Chundawat, G. Bellesia, N. Uppugundla, L. da Costa Sousa, D. Gao, A. Cheh, U. P. Agarwal, C. M. Bianchetti, G. N. Phillips, Jr, P. Langan, V. Balan, S. Gnanakaran and B. E. Dale, Restructuring crystalline cellulose hydrogen bonding network enhances its depolymerization rate, *J. Am. Chem. Soc.*, 2011, **133** (29), 11163–11174.
37 R. H. Newman, Estimation of the lateral dimensions of cellulose crystals using ^{13}C NMR signal strengths, *Solid State Nucl. Magn. Reson.*, 1999, **15**, 21–29.
38 J. F. Matthews, C. E. Skopec, P. E. Mason, P. Zuccato, R. W. Torget, J. Sugiyama, M. E. Himmel and J. W. Brady, *Carbohydr. Res.*, 2006, **341**, 138–152.
39 T. Shen, P. Langan, A. P. French, G. P. Johnson and S. Gnanakaran, Conformational flexibility of soluble cellulose oligomers: chain length and temperature dependence, *J. Am. Chem. Soc.*, 2009, **131**, 14786–14794.
40 T. K. Kirk and R. L. Farrell, *Annu. Rev. Microbiol.*, 1987, **41**, 465.
41 R. El Hage, N. Brosse, P. Sannigrahi and A. Ragauskas, Effects of process severity on the chemical structure of Miscanthusethanol organosolv lignin, *Polym. Degrad. Stab.*, 2010, **95**(6), 997–1003.
42 B. B. Hallac, Y. Pu and A. J. Ragauskas, Chemical transformations of Buddlejadavidii lignin during ethanol organosolv pretreatment, *Energy Fuels*, 2010, **24**(4), 2723–2732.
43 P. Sannigrahi, A. J. Ragauskas and S. J. Miller, Effects of two-stage dilute acid pretreatment on the structure and composition of lignin and cellulose in loblolly pine, *BioEnergy Res.*, 2008, **1**, 205–214.
44 R. Arora, C. Manisseri, C. Li, M. D. Ong, H. V. Scheller, K. Vogel, B. A. Simmons and S. Singh, Monitoring and analyzing process streams towards understanding ionic liquid pretreatment of switchgrass (Panicumvirgatum L.), *BioEnergy Res.*, 2010, **3**, 134–145.
45 M. J. Selig, S. Viamajala, S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant, Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose, *Biotechnol. Prog.*, 2007, **23**, 1333–1339.
46 T. Hataeyama, K. Nakamura and H. Hatakeyama, Studies on heat capacity of cellulose and lignin by differential scanning calorimetry, *Polymer*, 1982, **23**, 1801–1804.
47 D. W. Cho, R. Parthasarathi, A. S. Pimentel, G. D. Maestas, H. J. Park, U. C. Yoon, D. Dunnaway-Mariano, S. Gnanakaran, P. Langan and P. S. Mariano, Nature and kinetic analysis of carbon-carbon bond fragmentation reactions of cation radicals derived from SET-oxidation of lignin model compounds, *J. Org. Chem.*, 2010, **75**, 6549–6562.
48 D. W. Cho, J. A. Latham, H. J. Park, P. Langan, U. C. Yoon, D. Dunnaway-Mariano and P. S. Mariano, Regioselectivity of enzymatic and photochemical single electron transfer promoted carbon-carbon bond fragmentation reactions of tetrameric lignin model compounds, *J. Org. Chem.*, 2011, **76**, 2840–2852.
49 L. Donaldson, Lignification and lignin topochemistry – and ultrastructural view, *Phytochemistry*, 2001, **57**, 859–873.
50 S. B. Kim and Y. Y. Lee, Kinetics in acid catalyzed hydrolysis of hardwood hemicelluloses, *Biotechnol. Bioeng. Symp.*, 1987, **8**, 75–88.
51 C. Crestini and D. S. Argyropoulos, Structural analysis of wheat straw lignin by quantitative P-31 and 2D NMR spectroscopy.The occurrence of ester bonds and alpha-O-4 substructures, *J. Agric. Food Chem.*, 1997, **45**, 1212–1219.
52 S. Dammström, L. Salmén and P. Gatenholm, On the interactions between cellulose and xylan, a biomimetic simulation of the hardwood cell wall, *Bioresources*, 2009, **4**, 3–14.

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53 S. M. Gabbay, P. R. Sundararajan and R. H. Marchessault, X-ray and stereochemical studies on xylandiacetate, *Biopolymers*, 1972, **11**, 79–94.
54 P. J. Weimer, J. M. Hackney, H.-J. G. Jung and R. D. Hatfield, Fermentation of a bacterial cellulose/xylan composite by mixed ruminalmicroflora: implications for the role of polysaccharide matrix interactions in plant cell wall biodegradability, *J. Agric. Food Chem.*, 2000, **48**, 1727–1733.
55 T. Q. Yuan, F. Xu, J. He and R. C. Sun, Structural and physic-chemical characterization of hemicelluloses from ultrasound-assisted extractions of partially delignified fast-growing poplar wood through organic solvent and alkaline solutions, *Biotechnol. Adv.*, 2010, **28**, 583–593.
56 Y. Nishiyama, Structure and properties of the cellulose microfibril, *J. Wood Sci.*, 2009, **55**, 241–249.
57 S. Y. Ding and M. E. Himmel, The maize primary cell wall microfibril: a new model derived from direct visualization, *J. Agric. Food Chem.*, 2006, **54**, 597–606.
58 J. F. Matthews, C. E. Skopec, P. E. Mason, P. Zuccato, R. W. Torget, J. Sugiyama, M. E. Himmel and J. W. Brady, *Carbohydr. Res.*, 2006, **341**, 138–152.
59 G. Bellesia, A. Asztalos, T. Shen, P. Langan, A. Redondo and S. Gnanakaran, Insilico studies of crystalline cellulose and its degradation by enzymes, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2010, **66**, 1184–1188.
60 R. Atalla, *Celluloses, in Comprehensive natural products chemistry, Carbohydrate*, ed. B. H. Piuto, Elsevier, Cambridge, 1999.
61 Y. Nishiyama, U. J. Kim, D. Y. Kim, K. S. Katsumata, R. P. May and P. Langan, Periodic disorder along ramie cellulose microfibrils, *Biomacromolecules*, 2003, **4**, 1013–1017.
62 J. Lai-Kee-Him, H. Chanzy, M. Muller, J. L. Putaux, T. Imai and V. Bulone, *In vitro versus in vivo* cellulose microfibrils from plant primary wall synthase: Structural differences, *J. Biol. Chem.*, 2002, **277**, 36931–36939.
63 F. H. Stillinger, T. Head-Gordon and C. L. Hirshfield, Toy model for protein folding, *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.*, 1993, **48**, 1469–1477.
64 J. Wohlert and L. A. Berglund, A coarse-grained model for molecular dynamics simulations of native cellulose, *J. Chem. Theory Comput.*, 2011, **7**(3), 753–760.
65 B. Lintao, G. T. Beckham, M. F. Crowely, C. H. Chang, J. F. Matthews, Y. J. Bomble, W. S. Adney, M. E. Himmel and M. R. J. Nimlos, The energy landscape for the interaction of the family 1 carbohydrate binding module and the cellulose surface is altered by hydrolyzed glycosidic bonds, *J. Phys. Chem. B*, 2009, **113**, 10994–11002.
66 B. Lintao, M. E. Himmel and M. R. Nimlos, *Mesoscale modeling of polysaccharides in plant cell walls: an application to translation of CBMs on the cellulose surface, in Computational modeling in lignocellulosic biofuel production*, ch. 5, pp. 99–117, ACS Symposium Series, vol. 1052.
67 V. Molinero and W. A. Goddard III, M3B: A coarse grain force field for molecular simulations of malto-oligosaccharides and their water mixtures, *J. Phys. Chem. B*, 2004, **108**, 1414–1427.
68 J. R. Barnett and V. A. Bonham, Cellulose microfibril angle in the cell wall of wood fibers, *Biol. Rev.*, 2004, **79**, 461–472.
69 W. A. Côté, Jr. and A. C. Day, A contribution to ultrastructure of tension wood fibers, *Wood Sci. Technol.*, 1969, **3**, 257–271.
70 H. Lichtenegger, M. Müller, O. Paris, Ch. Riekel and P. Fratzl, Imaging of the helical arrangement of cellulose in wood by synchrotron X-ray microdiffraction, *J. Appl. Crystallogr.*, 1999, **32**, 1127–1133.
71 S. Fang, W. Yang and Y. Tian, Clonal and within-tree variation in microfibril angle in poplar clones, *New For.*, 2006, **31**, 373–383.
72 N. Terashima, K. Fukushima, S. Tsuchiya and K. Takabe, Hetergeneity in the formation of lignin VII. An autoradiographic study of the formation of guaiacyl and syringyl lignin in poplar, *J. Wood Chem. Technol.*, 1986, **6**, 495–504.
73 V. De Micco and G. Aronne, Combined histochemical and autofluorescence for identifying lignin distribution in cell walls, *Biotech. Histochem.*, 2007, **82**, 209–216.
74 T. Eggeman and R. T. Elander, Process and economic analysis of pretreatment technologies, *Bioresour. Technol.*, 2005, **96**, 2019–2025.
75 B. Yang and C. E. Wyman, Pretreatment: the key to unlocking low-cost cellulosic ethanol, Biofuels, *Biofuels, Bioprod. Biorefin.*, 2008, **2**, 26–40.
76 Y. H. Zhang, J. Cui, L. R. Lynd and L. R. Kuang, A transition from cellulose swelling to cellulose dissolution by o-phophoric acid: Evidence from enzymatic hydrolysis and supramolecular structure, *Biomacromolecules*, 2006, **7**, 644–648.
77 R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, Dissolution of cellulose with ionic liquids, *J. Am. Chem. Soc.*, 2002, **124**, 4974–4975.
78 S. V. Pingali, V. S. Urban, W. T. Heller, J. McGaughy, H. O'Neill, M. Foston, D. A. Myles, A. Ragauskas and B. R. Evans, Breakdown of cell wall nanostructure in dilute acid pretreated biomass, *Biomacromolecules*, 2010, **11**, 2329–2335.
79 M. W. Lau, C. Gunawan and B. E. Dale, The impacts of pretreatment on the fermentability of pretreated lignocellulosic biomass: a comparative evaluation between ammonia fiber expansion and dilute acid pretreatment, *Biotechnol. Biofuels*, 2009, **2**, 30.
80 S. P. S. Chundawat, B. Donohue, L. Sousa, T. Elder, U. Agarwal, F. Lu, J. Ralph, M. Himmel, V. Balan and B. Dale, Multiscale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment, *Energy Environ. Sci.*, 2011, **4**, 973–984.
81 S. P. S. Chundawat, R. Vismeh, L. N. Sharma, J. F. Humpula, L. D. Sousa, C. K. Chambliss, A. D. Jones, V. Balan and B. E. Dale, Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments, *Bioresour. Technol.*, 2010, **101**, 8429–8438.
82 M. Wada, H. Chanzy, Y. Nishiyama and P. Langan, Cellulose III_I Crystal Structure and Hydrogen Bonding by Synchrotron X-ray and Neutron Fiber Diffraction Macromolecules, *Macromolecules*, 2004, **37**, 8548–8555.
83 K. Igarashi, M. Wada and M. Samejima, Activation of crystalline cellulose to cellulose III_I results in efficient hydrolysis by cellobiohydrolase, *FEBS J.*, 2007, **274**, 1785–1792.
84 M. Wada, Y. Nishiyama and P. Langan, X-ray structure of ammonia cellulose I: new insights into the conversion of cellulose I to cellulose III_I, *Macromolecules*, 2006, **39**, 2947–2952.
85 M. Wada, Y. Nishiyama, G. Bellesia, V. T. Forsyth, S. Gnanakaran and P. Langan, Rearrangement of Hydrogen Bonding during the Treatment of Cellulose with Ammonia: Neutron Crystallographic Structure of Ammonia-Cellulose, *Cellulose*, 2011, **18**, 191–206.
86 M. Wada, L. Heux, Y. Nishiyama and P. Langan, X-ray crystallographic and cross-polarized/magic angle spinning ^{13}C nuclear magnetic resonance studies of the structure of the complex of cellulose I with EDA, *Cellulose*, 2009, **16**, 943–957.
87 Y. Nishiyama, M. Wada, B. L. Hanson and P. Langan, Time-resolved X-ray diffraction microprobe studies of the conversion of cellulose I to ethylenediamine-cellulose I, *Cellulose*, 2010, **17**, 735–745.
88 A. P. Dadi, C. A. Schall and S. Varanasi, Mitigation of cellulose recalcitrance to enzymatic hydrolysis by ionic liquid pretreatment, *Appl. Biochem. Biotechnol.*, 2007, **137–140**, 407–422.
89 A. P. Dadi, A. P. Varanasi and C. A. Schall, Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step, *Biotechnol. Bioeng.*, 2006, **95**, 904–910.
90 I. P. Samayam and C. A. Schall, Saccharification of ionic liquid pretreated biomass with commercial enzyme mixtures, *Bioresource tech.*, 101, pp. 3561–3566.
91 A. Romero, A. Santos, J. Tojo and A. Rodriguez, Toxicity and biodegradability of imidazolium ionic liquids, *J. Hazard. Mater.*, 2008, **151**, 268–273.
92 D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna and G. Moyna, Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-n-butyl-3-methylimidazolium chloride, *Green Chem.*, 2007, **9**, 63–69.
93 C. Li, B. Kneirim, C. Manisseri, R. Arora, H. V. Scheller, M. Auer, K. P. Vogel, B. A. Simmons and S. Singh, Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification, *Bioresour. Technol.*, 2010, **101**, 4900–4906.

 This journal is © The Royal Society of Chemistry 2011

94 P. Langan, Y. Nishiyama and H. Chanzy, A revised structure and hydrogen bonding scheme in cellulose II from a neutron fibre diffraction analysis, *J. Am. Chem. Soc.*, 1999, **121**, 9940–9946.
95 P. Langan, Y. Nishiyama and H. Chanzy, X-ray structure of mercerized cellulose II at 1 Å resolution, *Biomacromolecules*, 2001, **2**, 410–416, 2001.
96 M. Lucas, B. Macdonald, G. Wagner, S. Joyce and K. Rector, Ionic liquid pretreatment of poplar wood at room temperature: Swelling and incorporation of nanoparticles, *ACS Appl. Mater. Interfaces*, 2010, **2**, 2198–2205.
97 K. Nowak-Lovato, B. Wilson and K. Rector, SERS nanosensors that report pH of endocytic compartments during FceRI transit, *Anal. Bioanal. Chem.*, 2010, **398**, 2019–2029.
98 K. Nowak-Lovato and K. Rector, Targeted Surface-Enhanced Raman Scattering Nanosensors for Whole-Cell pH Imagery, *Appl. Spectrosc.*, 2009, **63**, 387–395.
99 R. Ten Have and J. M. Teunissen, Oxidative mechanisms involved in lignin degradation by white rot fungi, *Chem. Rev.*, 2001, **20**, 3397–3413.
100 D. Cullen and P. J. Kersten, Enzymology and molecular biology of lignin degradation, red. R. Brambl, G. A. Marzluf, *The Mycota III. Biochemistry and molecular biology*, Berlin-Heidelberg: Springer-Verlag, 2004, pp. 249–273.
101 F. A. Keller, J. E. Hamilton and Q. A. Nguyen, Microbial pretreatment of biomass, *Appl. Biochem. Biotechnol.*, 2003, **105–108**, 27–40.
102 D. Martinez, *et al.*, Genome sequence of the lignocelluloses degrading fungus Phanerocaetechrysosporium strain RP78, *Nat. Biotechnol.*, 2004, **22**, 695–700.
103 P. Kersten and D. Cullen, Extracellular oxidative systems of the lignin-degrading basidiomycetePhanerochaetechrysosporium, *Fungal Genet. Biol.*, 2007, **44**, 77–87.
104 K. E. Hammel and D. Cullen, Role of fungal peroxidases in biological lignolysis, *Curr. Opin. Plant Biol.*, 2008, **11**, 349–355.
105 S. K. Hanson, J. C. Gordon, R. T. Baker, B. L. Scott, A. D. Sutton and D. L. Thorn, Aerobic Oxidation of Polyalcohols by Vanadium (V) Complexes, *J. Am. Chem. Soc.*, 2009, **131**, 428.
106 S. K. Hanson, R. T. Baker, J. C. Gordon, B. L. Scott and D. L. Thorn, Aerobic Oxidation of Lignin Models Using a Base Metal Vanadium Catalyst, *Inorg. Chem.*, 2010, **49**, 5611–5618.
107 S. K. Hanson, R. T. Baker, J. C. Gordon, B. L. Scott, L. A. P. Silks and D. L. Thorn, Mechanism of Alcohol Oxidation by Dipicolinate Vanadium(V): Unexpected Role of Pyridine, *J. Am. Chem. Soc.*, 2010, **132**, 17804–17816.
108 C. Kiss, J. Temoriv, L. Chasteen, G. S. Waldo and A. M. Bradbury, *et al.* Directed evolution of an extremely stable fluorescent protein, *Protein Eng., Des. Sel.*, 2009, **22**, 313–323.
109 Y1. Jiang, *et al.*, De novo computational design of retro-aldol enzymes, *Science*, 2008, **319**, 1387–1391.
110 D. L. Zechel and S. G. Withers, Glycosidase mechanisms: Anatomy of a finely tuned catalyst, *Acc. Chem. Res.*, 2000, **33**, 11–18.
111 A. Y. Kovalevsky, L. B. Hanson, S. Z. isher, M. Mustyakimov and P. Langan, Preliminary Joint X-ray and Neutron Protein Crystallographic Studies of Endoxylanase II from fungus Trichodermalongibrachiatum, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.*, 2011, **67**, 283–286.
112 A. Katz, X. Li, H. L. Carrell, B. L. Hanson, P. Langan, L. Coates, B. P. Schoenborn, J. P. Glusker and G. J. Bunick, Location of the active-site hydrogen atoms enzymes: time-of-flight neutron diffraction, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 8342–8346.
113 A. Y. Kovalevsky, A. K. Katz, H. L. Carrell, L. Hanson, M. Mustyakimov, S. Z. Fisher, L. Coates, B. P. Schoenborn, G. J. Bunick, J. P. Glusker and P. Langan, Hydrogen location in stages of an enzyme-catalyzed reaction: time-of-flight neutron structure of D-xylose isomerase with bound D-xylulose, *Biochemistry*, 2008, **47**, 7595–7597.
114 A. Kovalevsky, B. L. Hanson, S. Z. Fisher, S. A. Mason, V. T. Forsyth, M. Mustyakimov, M. Blakeley, D. Keen, J. Glusker and P. Langan, *Structure*, 2010, **18**, 688–699.
115 J. P. Glusker, A. Y. Kovalevsky, L. Hanson, S. Z. Fisher, M. Mustyakimov, S. Mason, T. Forsyth and P. Langan, Using Neutron Protein Crystallography to Understand Enzyme Mechanisms, *Acta Crystallogr.*, 2010, **D66**, 1257–1261.
116 S. Z. Fisher, A. Y. Kovalevsky, J. Domsic, M. Mustyakimov, D. N. Silverman, R. McKenna and P. Langan, Enzymes for carbon sequestration: Neutron crystallographic studies of carbonic anhydrase, *Acta Crystallogr.*, 2010, **D66**, 1178–1183.
117 "The neutron structure of human carbonic anhydrase II: Implications for proton transfer." S. Z. Fisher, A. Y. Kovalevsky, M. Mustyakimov, R. McKenna, D. N. Silverman and P. Langan, *Biochemistry*, 2010, **49**, 415–421.

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