

Biofuels: Analytical and Imaging Technologies for Studying Lignocellulosic Material Degradation

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Effects of Pretreatments onto Lignocellulosic Materials as Studied by Raman Microscopy and Mass Spectroscopy

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Project Goals: The project is to develop correlated optical (Raman and SH-OCT) and mass spectrometric (SIMS and MALDI MS) imaging approaches for spatial and temporal characterization of lignocellulosic materials at specific processing stages.

The ability to efficiently use lignocellulosic materials (LCMs) to feed the biorefinery of the future depends on high-efficiency pre-enzymatic processing to render lignin separable from cellulose/hemicelluloses. The complex 3-D network structure and chemical characteristics of LCMs pose daunting challenges for imaging and molecular characterization: (1) they are opaque and highly scattering; (2) their chemical composition is a spatially variegated mixture of heteropolymers; (3) the nature of the matrix evolves in time during processing. Presently, there are few *in situ* characterization tools that can be applied to materials with these characteristics, especially *during* processing—yet acquiring this information is of paramount importance. Here we present a combination of Raman microscopy and mass spectrometric imaging (secondary ion mass spectrometry, SIMS, and laser desorption ionization mass spectrometry, LDI MS) to visualize the structural and chemical changes of LCMs upon various treatments, such as H₂SO₄, NaClO₂, NaOH, etc. *Miscanthus x giganteus* as a model LCM was sectioned into 50 μm thick, and was investigated in this work. Raman and SIMS imaging results indicated that lignin and cellulose are collocated in the cell wall of raw *miscanthus*. A globular structure, composed predominantly of hemicellulose and lignin, is associated with the interior cell wall. Pretreatment of *Miscanthus* using NaOH or NaClO₂ solutions results in the removal of lignin at long processing time. Interestingly, Raman experiments reveal that the H₂SO₄ treated *Miscanthus* exhibits a higher autofluorescence, which might be due to the formation of highly conjugated hydrocarbon species during H₂SO₄ treatment. We are currently correlating these Raman results with mass spectrometric imaging to identify the unknown

species, thereby realizing the full power of correlated optical-mass spectrometric imaging. Together these studies promise to provide a much more complete picture of the effects of various treatments on LCMs.

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Dynamic Visualization of Lignocellulose: A Biofuels Scientific Focus Area

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Project Goals: Lignocellulosic biomass is recalcitrant to deconstruction and saccharification due to its fundamental molecular architecture and multicomponent laminate composition. A fundamental understanding of the structural changes and associations that occur at the molecular level during biosynthesis, deconstruction, and hydrolysis of biomass is essential for improving processing and conversion methods for lignocellulose-based fuels production. The objective of this research is to develop and demonstrate a combined neutron scattering and computer simulation technology for multiple-length scale, real-time imaging of biomass during pretreatment and enzymatic hydrolysis. Integration and application of the combined capabilities of the Spallation Neutron Source (SNS), the High Flux Isotope Reactor (HFIR) and the National Center for Computational Science (NCCS) at ORNL will provide new information on lignocellulosic degradation at an unprecedented level of detail.

The Oak Ridge National Laboratory (ORNL) Scientific Focus Area (SFA) Biofuels program will provide fundamental information about the structure and deconstruction of plant cell walls that is needed to drive improvements in the conversion of renewable lignocellulosic biomass to biofuels. This program integrates neutron scattering with computational simulation and molecular dynamics to understand the physicochemical processes taking place across multiple length scales during deconstruction of lignocellulosic biomass. These molecular-level methods are assisted and complemented by technical expertise in lignocellulose characterization at the Institute of Paper Science and Technology (Georgia Tech), and chemical force microscopy methods for surface characterization. A multipurpose neutron imaging chamber will be designed and used for in situ, dynamic observation of biomass processing. Deuteration of the biomass crop switch grass and other cellulose sources is being carried out to enable higher contrast between the components of lignocellulosic biomass for neutron scattering that will enable the examination of surface accessibility. These novel technological capabilities are being applied to

specific problems in the pretreatment and enzymatic hydrolysis of biomass to produce the fundamental understanding of plant cell architecture that is needed to develop the next generation of cost-effective cellulosic ethanol production. The interrelated research is organized as three principal tasks: (1) sample preparation and characterization; (2) neutron scattering and diffraction; and (3) computer simulation and modeling.

This overview poster will present the project goals as well as results in sample preparation and characterization. Two companion posters will specifically target the results from neutron scattering and from computer modeling.

The Biofuels Science Focus Area is supported by the Office of Biological and Environmental Research (BER) of the Office of Science (SC), U.S. Department of Energy (DOE), Genomic Science research program.

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SANS Study of Dilute Acid Pretreatment of Switchgrass

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Small-angle neutron scattering (SANS) and wide-angle X-ray diffraction (WAXD) were used to obtain a better understanding of the morphology of the cellulose/lignin composite to aid in understanding and ultimately selecting biomass pretreatment methods that are required to prepare lignocellulosic biomass for conversion to ethanol.

The structural changes that occur during acid pretreatment of switchgrass were investigated by a series of SANS and WAXD experiments. Samples of switchgrass and its component biopolymers were prepared using two different chemical processes: (a) the dilute acid pretreatment method used to break down lignocellulosic biomass and (b) the

extraction treatment for removing one component at a time from the biomass without disrupting its overall structure. The pretreatment, extractions, and compositional analysis of the samples were carried out at the Institute of Paper Science and Technology (Georgia Tech). SANS experiments were carried out with the BIO-SANS instrument at the High Flux Isotope Reactor (ORNL). Dilute acid pretreatment (1) increases the small-scale structure which can be related either to the crystalline core cross-section or pores in the fibrils; (2) decreases in the interconnectivity of the fibrils and forms additional distinct structures at length scales of 100-150 Å that are due to formation of lignin aggregates; and (3) at length scales larger than 1000 Å, does not change the smooth domain boundaries. In contrast, the extraction treatment: (1) produced a smaller increase in the small-scale structure; and (2) did not create an additional structure assigned to the re-precipitation of lignin.

This Biofuels Science Focus Area is supported by the Office of Biological and Environmental Research (BER) of the Office of Science (SC), U.S. Department of Energy (DOE), Genomic Science research program.

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Examination of Lignin Aggregation by Computer Simulation Integrated with Neutron Experiments: Molecular Dynamics Studies of Lignin

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Computer simulation can provide the integration of structural information obtained from multiple imaging and analysis methods that is needed to visualize the molecular structure of lignocellulose and its changes during pretreatment and hydrolysis. The objective is the development and demonstration of computer simulation technology that will provide multi-length scale, real-time

imaging of biomass during pretreatment and enzymatic hydrolysis based on experimental data obtained through neutron science, surface force microscopy, and advanced NMR methods. The integration and application of these combined capabilities will provide new information on lignocellulosic degradation at an unprecedented level of detail.

Computer simulation can complement experimental techniques in gaining an atomic- and molecular-level understanding of the structure and dynamics of lignocellulose. The power of the high-performance computation facilities of the National Center for Computational Sciences (ORNL) enables the development of such dynamic, atomistic models of the very large, heterogeneous molecules that compose lignocellulosic biomass. Here we present how simulation is integrated with Small Angle Neutron Scattering (SANS) to examine the morphology of lignin aggregates in solution. A very brief description of the experimental input that was used to construct the simulation models as well as the derivation of a force field for lignin [1] will be followed by a presentation of the results of our Molecular Dynamics (MD) studies of lignin in solution. The models were built using information on composition, distribution, and location of covalent bonds in lignin from specific sources as determined by state-of-the-art techniques of chemical analysis and ¹³C- and ¹H/²H- NMR combined with chemical modification methods that were carried out at the Institute of Paper Science and Technology (Georgia Tech). The surface morphology and compactness of the lignin aggregates are examined and the results are discussed in the context of SANS experiments using the analogous samples that were carried out with the BIOSANS instrument at the High Flux Isotope reactor (ORNL). Simulation of more complex models that include cellulose and hemicellulose is incredibly computationally demanding because of the sheer size of these models. For this reason, a new strategy [2] is discussed that allows efficient simulation of such large systems on petaflop supercomputers, such as the JaguarXT5 at ORNL. These advances extend the length- and time-scales that can be probed using simulation and as a result microsecond time scale MD of multimillion-atom lignocellulose systems appear now within reach.

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Student Presentation

Understanding and Engineering Outer Membrane Protein Export in Gram-Negative Bacteria

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Project Goals: Extracellular secretion of cellulases by engineered bacteria

Escherichia coli K12 is a common host for preparative protein production, however it is limited in its ability to deliver proteins to the extracellular environment. The lack of robust outer membrane (OM) protein translocation machinery presents a bottleneck in the development of bioenergy as cellulosic biomass is not readily transported into the bacterial cell. A recent study identified a protein in *E. coli* called YebF that can efficiently carry recombinant proteins into the culture medium. Here, we identify a novel OM protein translocation mechanism mediated by the *yebEFG* gene cluster that is responsible for delivering YebF and its fusion partners across the OM. We also describe the development of multiple extracellular secretion assays that provide a rapid means to study and engineer the *yebEFG* translocon and type II secretion of gram-negative bacteria.

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Systems Biology of Cellulose Fermentation to Ethanol: From Domestication of New Organisms to Understanding of Microbial Synergies

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Project Goals: Present communication is based on preliminary results of research supported by DOE (Grant Number: ER64507, "To Bioethanol through Genomics of Microbial Synergies"). The strategic goal of this project is to facilitate development of cellulosic ethanol technology by achieving better understanding of lignocellulose degradation in nature: in anaerobic biocompost, anoxic soils and sediments. We believe that industrial consolidated bioprocessing of fuel from various feedstocks cannot be realized by a single 'superbug', even after profound metabolic engineering. More feasible solution would be to construct a set of specialized microbial consortia each one adapted to particular feedstock.

Emerging bioethanol technology uses narrow range of fermenting organisms from genera *Clostridium* and *Caldicellulosiruptor*, the *C. thermocellum* being the most carefully studied (Lynd et al., 2002). New organisms will likely come from the pool of as yet uncultivated species accounting for up to 99% of the natural microbial diversity. We have recently shown that such species could be recovered by using in situ incubation devices (Kaeberlein et al., 2002). We have demonstrated also that 'uncultivable' species can be grown in standard media in the presence of microbial helpers, deliberately added organisms which provide signaling metabolites, siderophores or other extracellular stimulatory factors to a 'difficult' microbe (Nichols et al., 2008). Here we report on further development of this approach combined with culture-independent techniques and dynamic mathematical simulations as applied to particular case of cellulose and lignocellulose digestion/fermentation.

Methods. Anaerobic cellulose degradation and fermentation were followed in situ as well as in laboratory incubation experiments with cellulose-amended soil and biocompost samples incubated under constant environmental conditions (temperature, moisture, gas flow). To improve recovery of natural cellulolytic organisms, we used cellulose-traps followed by standard serial dilution optimized for recovery of strict anaerobes. The isolated consortia and pure cultures were tested for their degrading activity by using high throughput screening system based on continuous off-gas analysis by IR- and mass-spectrometry. 16S rRNA survey of the isolates, consortia and natural communities (after cloning) provided taxonomic identification and assessment of degree of uncertainty: how many species (OTU's) involved in cellulose degradation remained uncultivable. Finally, the active strains were grown on suspended cellulose or pretreated wood under full fermentation control (pH, mixing intensity, temperature, red-ox conditions) and computer-aided instrumental monitoring of residual substrate, cell mass, fermentation products and base titration rate (Panikov and Lynd, 2010)

Results: Stable cellulose-degrading consortia were obtained in most tested soils and biocomposts. The typical fermentation products in enrichments and stabilized consortia were CO₂, H₂, CH₄, acetate, ethanol and lactate. In some cases, acetate was replaced with formate. At high sulfate concentration (some soils and sediments), cellulose degradation was suppressed, probably because of toxic by-products formed by sulfate-reducing bacteria. The methanogenic communities grew slow (specific growth rate 0.05–0.1 h⁻¹ at 55°C) with sustained oscillations of fermentation rate (the effect of reversible product inhibition).

More than 15 pure cultures of novel organisms related to *C. clariflavum*, *C. straminisolvens* and *C. thermocellum* have been isolated. *C. clariflavum* was able to ferment cellulose, xylan and their mixture as well as pretreated wood into ethanol, formate, CO₂ and H₂. Other isolates degraded only cellulose with acetate as end product. New strains varied in respect to growth and maintenance rates, enzymes localization (free and cell-bound), sensitivity to product inhibition as well as to starvation and O₂-stress (see illustration below).

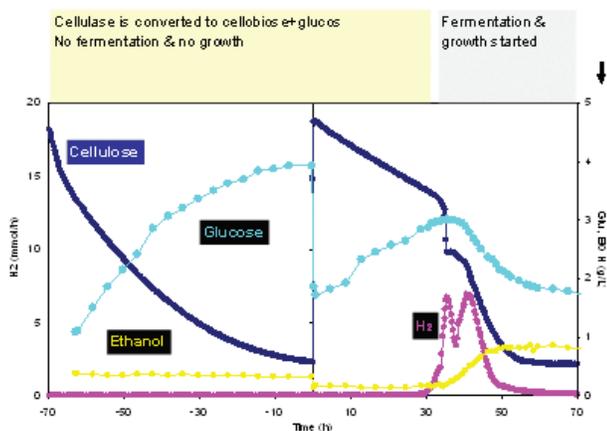


Fig 1. Example of starvation stress. Culture of cellulolytic bacteria *C. thermocellum* enters non-growing state after short-term starvation. First addition of cellulose-mineral medium (time -70 h) does not result in a normal cell growth: extracellular cellulases remaining from the past growing period decompose cellulose into glucose and cellobiose, but cells are unable to consume released sugars. At time zero (\downarrow), the second addition of fresh medium induces growth after another 30 h of latent phase. The growth and fermentation start abruptly and proceed until depletion of cellulose (but not glucose).

Mathematical simulation of community dynamics.

Growth of communities and consortia as well as pure cultures was simulated by structured dynamic model based on high-order set of non-linear ordinary differential equations (Panikov, 1995; 2008). Model takes into account differential gene expression in delayed response to concentration of limiting substrate orchestrated by transcription factors. The simulation of community was possible with aggregated model containing linear approximation of the vector of intracellular polymeric constituents. We are testing the validity of two basic ecological concepts on the nature of microbial cellulolytic community. The first concept identifies a community as a super-organism with firm internal interactions between individual populations stemming from the metabolic stoichiometry of decomposition network and regulatory effects of signaling metabolites. The second, continuum paradigm allows relative freedom for members to enter or leave community dependent upon their success in acquiring limiting nutrient resources. The competitive advantage of each population depends on inherited growth characteristics (growth rate, colonization potential, yield, affinity of transporters, maintenance, stress-tolerance).

Conclusion: Preventing starvation stress in industrial strains seems to be as important as the level of enzymatic activity. We discuss the ways to improve productivity and robustness of fermenting *Clostridia* by selection and metabolic engineering.

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A New Solution-State NMR Approach to Elucidate Fungal and Enzyme/Mediator Delignification Pathways

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Project Goals: New methods for solution-state NMR spectroscopy of lignocellulose are being used, in conjunction with enzymology and molecular biology approaches, to determine how filamentous fungi cleave lignin and what agents they employ to accomplish this chemistry.

Brown rot basidiomycetes remove cellulose from wood efficiently, even though this sugar polymer is initially shielded by a biochemically recalcitrant barrier of lignin. During this process, the lignin appears to remain in situ, which raises the question of how the polysaccharide-degrading systems of brown rot fungi circumvent the lignin to access their substrates. New results based on solution-state NMR analysis of ball-milled, dissolved, brown-rotted wood are now available to clarify this picture. ¹H-¹³C HMBC spectra of aspen degraded by the brown-rotter *Postia placenta* showed that the lignin sidechains had been cleaved between C_α and C_β, yielding new benzoic acid and benzaldehyde residues in the polymer. In addition, arylglycerol-β-aryl ether linkages had been cleaved in the lignin to generate new phenylglycerol residues, as shown by three-dimensional ¹H-¹³C HSQC-TOCSY spectra. The HSQC results, in conjunction with quantitative ¹³C NMR spectroscopy, indicated that roughly 6% of the monomeric units in the residual lignin were cleaved structures. Our results show that *P. placenta* is ligni-

nolytic, contrary to the prevailing view of brown rot. Since this fungus lacks ligninolytic peroxidases, it is also clear that some other mechanism is responsible for its ability to cleave lignin. Results to date suggest that reactive oxygen species generated via extracellular oxidation of a fungal metabolite may be the responsible oxidants: (a) The wood colonized by the fungus contained a laccase that is encoded in the *P. placenta* genome. (b) The biodegrading wood contained a fungal metabolite, 2,5-dimethoxyhydroquinone, and also Fe^{3+} as its oxalate complex. (c) Heterologously expressed *P. placenta* laccase oxidized 2,5-dimethoxyhydroquinone with concomitant production of perhydroxyl radicals, which are known initiators of hydroxyl radical production in the presence of Fe^{3+} complexes.

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Real-Time Chemical Imaging of *Clostridium cellulolyticum* Actions on *Miscanthus*

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Project Goals: Our purpose is to perform technology research and development, and to apply the technology to develop a comprehensive picture of actions of cellulolytic microorganisms on natural plant biomass, which will help elucidating the important processes of different temporal and spatial scales underlying the microbial destruction of plant biomass for a cost-effective production of biofuel.

In nature, microorganisms are important agents in the cycling of elements. Those that can hydrolyze cellulose rapidly may come to play an important role in carbon cycling and breaking the barriers to cost-competitive production of cellulosic ethanol. Microbe-induced cellulose hydrolysis is generally a slow and incomplete process. However, many microorganisms among *Clostridia* species have been linked to elevated rates of cellulose hydrolysis in compost and landfills. Cellulolytic action by *Clostridia* sp. is facilitated primarily at the surface of cellulosic materials. *C. cellulolyticum* is a mesophilic anaerobic bacterium. The wealth of information on cellulosomes, genomics and carbon flux in *C. cellulolyticum* made it a prime model system for understanding microbial strategies in biofuels processing. We have developed synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy which has enabled us to non-invasively make molecular measurement and images of *C. cellulolyticum* interactions with cellulose substrates, as well as plant materials. Our SR-FTIR results show that even in the simple cellulose system, the surface chemistry is quite variable spatially at scales that range from a fraction of a micron to hundreds of microns, with concentrated features locally. However, nearly all the kinetic studies within the last two decades were conducted in batch cultures, or in continuous cultures, or in chemostat cultures. These results point to the importance of physiochemical parameters at a microscopic level under relatively uniform and dilute conditions.

We are extending these observations with an improved SR-FTIR approach, to explore *C. cellulolyticum* actions on *Miscanthus*, a natural perennial plant that grows as tall as 13 feet with little to no fertilizer, and can be conveniently stored for an almost indefinitely time period. *Miscanthus* shows promises for more efficient biofuel production. Therefore, we use *Miscanthus* as the lignin-cellulose substrates in this study. The destruction of *Miscanthus* will be followed in real time by SR-FTIR chemical imaging. The controls will include known enzymes on various carbohydrate polymers. Our aim is to develop a comprehensive picture of actions of cellulolytic microorganisms on natural plant biomass, which will help elucidating the important processes of different temporal and spatial scales underlying the microbial destruction of plant biomass for a cost-effective production of biofuel.

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Multi-Mode Real-Time Chemical Imaging as a Systems Biology Approach to Decipher Microbial Depolymerization of Lignocellulose

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Project Goals: We are developing multi-mode real-time imaging methods to probe at the chemical level plant biomass depolymerization by living cellulolytic microbes. This integrated imaging effort will provide unique insights into the highly complex physical and chemical transformations that occur during the depolymerization problem. The integrated system should also prove useful for studying the fundamental chemical processes of other energy conversion technologies, such as next-generation solar energy conversion devices and fuel cells.

We are developing multi-mode real-time imaging methods to probe at the chemical level plant biomass depolymerization by living cellulolytic microbes. In nature, some microbes use suites of enzymes to break down the highly heterogeneous solid substrates present in plant biomass; others convert them to biofuels like ethanol. The mechanisms of these actions require surface chemistry, since the plant biomass substrates are solids. It is imperative, therefore, to study not only the enzymes, but also the properties of the substrates as they are degraded. Furthermore, the production of enzymes is affected by the physiological states of the microorganisms, which can be altered by the metabolites and end products including ethanol because of their inherent toxicity. To understand this dynamic system of biomass depolymerization for cellulosic ethanol production, we need to approach it at a systems biology level. Due to the highly complex nature of the substrates and enzyme mixtures, we think that a systems biology approach should be considered

in broader terms. It should include multi-mode chemical imaging methods.

To this end, a “grand challenge” is the acquisition of integrated knowledge on multiple time and length scales. We develop and use both single-molecule imaging of enzyme dynamics and Fourier transform infrared spectroscopy of solid substrates with living cellulolytic bacteria to probe plant cell wall depolymerization as a function of space and time. For example, using our newly developed real-time 3D single-particle tracking (RT-3DSPT) spectromicroscopies for single-molecule spectroscopy and imaging, we will examine cellulase and cellulosome processivity and cooperativity in the degradation of lignocellulose. These experiments will exploit the genetic tools that we are developing to incorporate fluorescent tags into the enzyme and enzyme complexes to enable tracking. We will also exploit the high temporal and spatial resolution of synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy to follow changes in the chemical composition of the cell wall substrates as they are depolymerized (see our second poster, Holman et. al.). To obtain a truly quantitative understanding, we found that the heterogeneity inherent in biomass depolymerization ultimately cannot be tackled with separate measurements that occur at different times or on different, albeit similar, samples. This integrated imaging effort will provide unique insights into the highly complex physical and chemical transformations that occur during the depolymerization problem. The integrated system should also prove useful for studying the fundamental chemical processes of other energy conversion technologies, such as next-generation solar energy conversion devices and fuel cells.

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In Vivo Mapping of ROS Gradients Produced by Wood Decay Fungi during Early Colonization

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<http://www.bact.wisc.edu/faculty/hammel/index.php>

Project Goals: In this project we have developed a modular system to image and quantify ROS (or other metabolite) from fungi in their natural substrate with minimal disturbance. We are using this knowledge of ROS concentrations to understand the mechanisms at work during incipient decay.

Background

Wood decay fungi are successful in removing carbohydrate from wood, despite the presence of lignin. We hope to improve prospects for woody biomass saccharification by understanding the mechanisms of fungal wood decay.

To make lignocellulosics permeable to enzymes, filamentous fungi use a variety of small, diffusible reactive oxygen species (ROS) such as hydroxyl radicals, peroxy radicals, and possibly phenoxy radicals. These radicals diffuse into the cell walls and initiate biodegradative radical reactions. When lignin is the target, radical attack results in various extents of oxidation and depolymerization.

These small diffusible oxidative species are important tools used by filamentous fungi to make the cell wall accessible to enzymes. Despite this, we have a poor knowledge of how these oxidants are spatially distributed in biodegrading lignocellulose relative to the fungal hyphae that produce them. The goal of this project is to remedy this deficit through fluorescence microscopy of newly designed sensors that will serve as in situ reporters of biodegradative radical production. We will use these sensors to produce oxidative maps that will help us to understand how fungi generate ROS and how they use these ROS to make cell walls more accessible to enzymes.

Method

We are placing fluorescent beads in wood at the start of fungal decay, and then imaging the beads after the fungus has colonized the wood a few days later. Our images can tell us the local concentration of oxidants as well as an overlay with the location of hyphae.

The strategy of covalently attaching fluorescent dyes to silica beads has many advantages. We design the bead to emit two fluorescent signals, so that the ratio of the two signal intensities provides quantitative information. Immobilized dyes are prevented from moving after reaction, so partitioning is impossible. In addition, they cannot be ingested, and the fluorescence from the dye is clearly distinguishable from background.

Our first bead has BODIPY 581/591® on a 3µm porous HPLC bead. This dye’s emission changes irreversibly from red to green upon oxidation by ROS. The ratio of red to green emission provides a quantitative measure of the cumulative oxidation at that point in space. Dyes with reactivity to specific ROS, pH, or other metabolites of interest are envisioned.

Results

We showed that our oxidant detection system is tied to wood decay by comparing bead oxidation in with the wood decay fungus *Phanerochaete chrysosporium* to the oxidation from a wood inhabiting fungus that does not degrade wood, *Ophiostoma piliferum*. The decay fungus cause much more oxidation, and also ate holes completely through the wood sections if left to incubate for a month.

By observing the oxidation of beads around a hyphal tip, we have concluded that oxidation occurs gradually over time. The extracellular enzymes to create ROS are typically excreted from the hyphal tip, but the enzymes do not immediately oxidize the wood. The enzymes make low molecular weight diffusible ROS which attack wood. These enzymes continue to operate over hours and days, continually creating ROS which gradually degrades cell walls.

While the current generation of beads is not sensitive enough to observe oxidation in the first few hours after the passing of a hyphal tip, three day old cultures show oxidation gradients around almost every hypha we investigated (see below). We expect these gradients to tell us about the relative rates of reactivity and diffusion for the oxidative species in the culture.

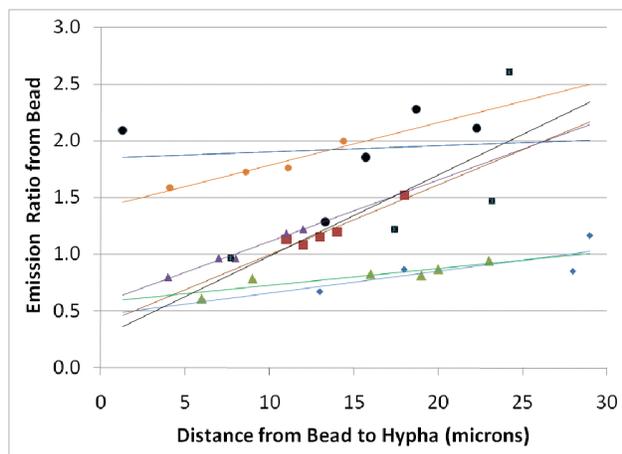


Figure 1: Fluorescent emission from individual beads as a function of distance from hypha. Lower Y axis values indicate more oxidation.

Calibration of the beads was done by measuring the oxidation of the beads after incubation with different concentrations of a free radical initiator in cultures. From this calibration, we can estimate the number of oxidant molecules produced per time for given conditions.

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Integrated Nondestructive Spatial and Chemical Analysis of Lignocellulosic Materials during Pretreatment and Bioconversion to Ethanol

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Project Goals: Our *long-term goal* is to develop a quantitative structural model for changes that occur in the organization and chemical composition of plant biomass during pretreatment, enzymatic degradation and bioconversion to ethanol or other products. The *objectives* of this proposed work are to 1) use advanced high resolution magnetic resonance microscopy (MRM), micro and nano x-ray computed tomography (x-ray CT), electron microscopy and imaging mass spectrometry (IMS) to quantify

changes in the architecture, porosity, permeability, surface area, pore size, interconnectivity, chemical organization and composition of bagasse and particularly *Populus* and pine wood chips during pretreatment and enzymatic degradation, and 2) integrate the results from these different quantitative imaging methods into a model for disassembly of the plant cell wall during pretreatment and bioconversion. We are developing methods for imaging biomass with MRM, x-ray CT and IMS.

IMS with a MALDI linear ion trap + MS: The full-scan MS of biomass shows intense ions at every mass-to-charge ratio (m/z), making the analysis very complex. The use of the linear ion trap (LIT) and MSⁿ fragmentation are required to interpret the complex spectra and map the distribution of cellulose, lignin and hemicelluloses within the biomass during pretreatment and hydrolysis. Because so many ions are present, we analyzed standard compounds that are normally present in wood. Full-scan and MSⁿ spectra were obtained for beta 1,4-glucan, 4-O-methylglucouronoxylan, β -glucan, starch and microcrystalline cellulose. The complex carbohydrates typically present in lignocellulosic biomass yield oligomeric fragments of characteristic ionization patterns. Interestingly, starch fragmented and ionized very differently than microcrystalline cellulose. Figure 1 shows typical spectra from microcrystalline cellulose (top) and birch beta 1,4-glucan, 4-O-methylglucouronoxylan (bottom).

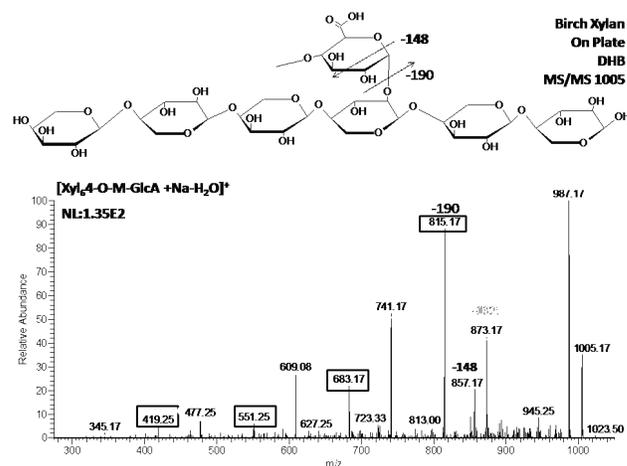
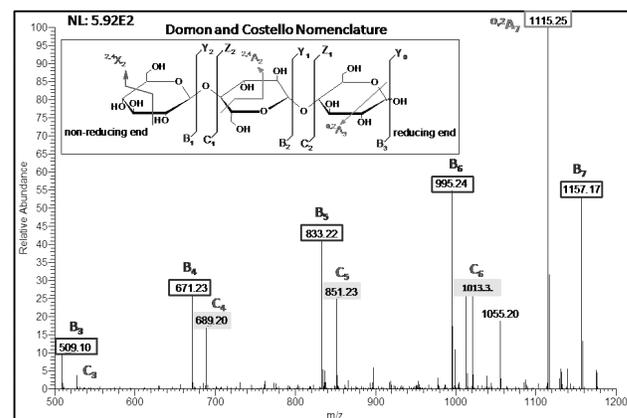


Figure 1

IMS images with radial sections of poplar wood pretreated with dilute acid at 145°C created by extracting the m/z 1175 derived from cellulose shows inconsistent patterns suggesting microheterogeneity in release of the ion.

Magnetic Resonance Microscopy: Excellent image quality is obtained from *Populus* wood and bagasse samples using T2 and diffusion weighted modes. In T2 images, vascular bundles appear dark consistent with the knowledge that lignified cells contain limited free water. A chemical shift has been found in some samples and the cause for this is under investigation. Image quality is quite comparable to optical microscopy. T2 weighted MRM images acquired at 39 μM resolution of *Populus* wood chips treated with mild acid and 145°C, conditions considered more typical of pretreatments considered commercially viable, were obtained. Small differences were observed comparing untreated with pretreated wood, even though chemical analyses show that the xylan was quantitatively removed and the wood clearly becomes more brittle and is substantially softer in the 2 and 3% acid treatments at 145°C as expected. We hypothesize that the resolution of the instrument using larger rf coils is too low to quantify the relatively subtle differences in structure induced by pretreatment. New images were collected with state of the art rf microcoils at 8 μM resolution. Figure 2 shows the improved signal to noise ratios provide much better images.

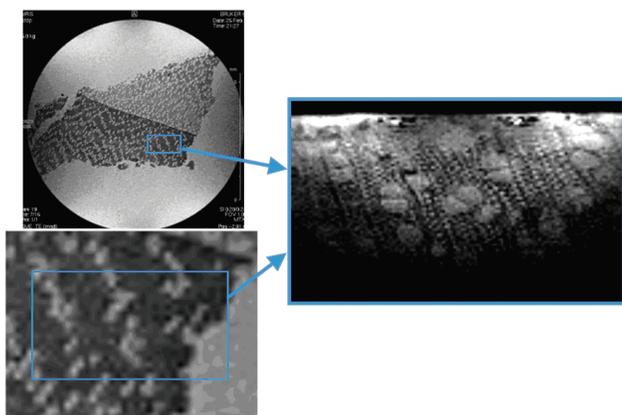
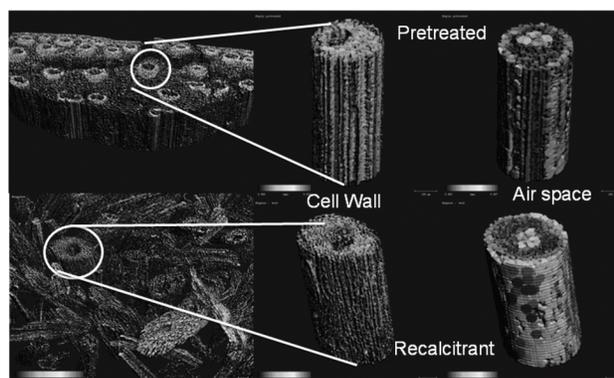


Figure 2. Illustration of the microcoil SNR improvement. On the left is a conventional 39 micron resolution image (top) with an expanded view (bottom). Using a microcoil an 8 micron image (right) with an equivalent field of view (indicated by the boxes) is shown – the clear improvement in the ability to see the wood microstructure is evident.

X-ray micro CT: Excellent images have been obtained at high resolution from *Populus*, pine, and bagasse samples. In addition to the basic density, images are readily segmented and the material and airspace sizes can be quantified. Small changes in surface area and surface area to volume ratios were observed after dilute acid pretreatment. Analysis of the recalcitrant material left after steam gun pretreatment and simultaneous saccharification and fermentation from our pilot facility shows that the recalcitrant material was mainly the lignified and dense vascular bundles as expected. Micro CT imaging of the recalcitrant material shows thinner cell

walls and some degree of degradation on the periphery compared with the internal regions of the bundles.



One constraint of CT imaging is that for quantification samples need to be dried and the wood shrinks by ~10% in the radial and tangential planes, thus dried wood measurements underestimate those in wet wood. To overcome this limitation we are exploring the use of nanoparticle contrast agents designed for CT imaging.

95 Cell Wall Assembly and Deconstruction Revealed through Multi-Platform Imaging in the *Zinnia elegans* Model System

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Project Goals: To improve our knowledge of the native architecture of the plant cell wall and assess applications for efficient deconstruction, using a combination of imaging approaches, including atomic force microscopy (AFM), fluorescence microscopy, and synchrotron radiation Fourier-transform infrared spectroscopy (SR-FTIR).

With the ambition of manufacturing alternative fuels, the biotechnology industry has turned to the plant cell wall, a source of fermentable sugars, which can become the starting material for biofuel production. However, detailed changes in plant cell walls in response to chemical, enzymatic and microbial treatments have not been monitored at high resolution. Our project seeks to understand more about the structural organization of the cell wall and how it can be efficiently deconstructed. For this purpose, we are imaging single cells from *Zinnia elegans* that have been induced in culture to develop into tracheary elements (TEs), individual

components of xylem tissue. Mature TEs develop large secondary cell wall thickenings that are deposited underneath the primary cell wall and are rich in lignocellulose. We have imaged TEs using a variety of platforms, including atomic force microscopy (AFM), fluorescence microscopy and synchrotron radiation based Fourier-transform infrared spectroscopy (SR-FTIR). Our approach of imaging the ultrastructure of the cell wall at nanometer scale, coupled with the capability to reveal the corresponding chemical composition, can profoundly improve the fundamental understanding of the native architecture and mechanisms of deconstruction of the plant cell wall.

To probe the cell wall for the presence of specific polysaccharides, we used fluorescently-tagged carbohydrate binding modules (CBMs) from *Clostridium thermocellum*. After treating TEs with oxidative chemicals to remove lignin, we observed a dramatic increase in fluorescence using CBM3, a family 3 CBM that binds to crystalline cellulose. This increase in fluorescence suggested that that cellulose was more accessible or likely to bind to CBM3 following chemical treatments.

When we imaged the surface of *Zinnia* TEs by AFM, we observed that these were covered with pronounced granular structures, ranging in size from approximately 20 to 100 nm. After oxidative treatment, we found that this surface granular material was absent and that the underlying meshwork of cellulose fibrils (ranging in width from 10 to 20 nm) from the primary cell wall had become exposed. This result corroborated the increased physical accessibility of cellulose in the cell wall after oxidative treatment. When pre-treated TEs were examined by SR-FTIR, we found that their chemical composition changed significantly.

To examine secondary cell wall ultrastructure, we found that physical disruption of TEs using mild sonication was sufficient to produce cell fragments that were conducive to AFM imaging. We focused on discrete ring-like secondary wall structures, which revealed cellulose fibrils decorated with particles and arranged in parallel bundles. Chemical treatments generally removed particles from these cellulose bundles.

We are currently developing experimental techniques to structurally and chemically probe the dynamic response of *Zinnia* TEs to enzymatic and microbial degradation of lignocellulose. We anticipate that our imaging-based studies will help elucidate mechanisms of cell wall degradation and improve models of the organization and composition of the plant cell wall.

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Label-Free, Real Time Monitoring of Biomass Processing with Stimulated Raman Scattering Microscopy

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Project Goals: (a) To develop novel optical imaging technology based on coherent Raman scattering that is capable of real time, label-free chemical imaging and (b) to apply these techniques to image the process of biomass conversion to biofuels in real time in three dimensions in intact plant tissue. The new information available from these techniques will offer insight into this complex series of chemical reactions and help to better understand and optimize their efficiency.

The conversion of plant biomass into “cellulosic” ethanol is an alternative energy technology that has attracted significant research interest over the past decades and requires new tools to understand and optimize the conversion process. We demonstrate that stimulated Raman scattering (SRS) microscopy can be used to selectively map plant cell wall polymers, such as lignin and cellulose, simultaneously, at sub-micron spatial resolution and with linear concentration dependence and high speed. We then follow the acid chlorite delignification process, to further demonstrate the real-time imaging of lignin bleaching with a time resolution of a few seconds. SRS microscopy is a high sensitivity, label-free chemical imaging technique, and provides a new tool to improve our understanding of biomass conversion processes.

submitted post-press

New Imaging Tools for Biofuel Research: Correlated Soft X-ray Tomography and Visible Light Cryo-Microscopy

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Project Goal: Develop correlated imaging technologies required to advanced bioenergy research.

In this poster we will present the recent results from our work developing instruments and methods for carrying out correlated soft x-ray tomography and high numerical aperture immersion light microscopy on cryogenic specimens^{1,2}. These new imaging modalities have enormous potential as precision structural phenotyping tools for bioenergy research. The novel use of a cryogenic immersion fluid in the cryolight microscope minimizes the refractive index mismatch between the specimen and lens, leading to a more efficient coupling of the light from the sample to the image forming system³. The instrument can be used for correlating detailed spectral imaging with a high fidelity x-ray tomographic map of any microorganism. We will show results of correlated imaging on yeast, and also show results of using soft x-ray tomography to phenotype algae for biofuel production.

For more information on the National Center for X-ray Tomography: <http://ncxt.lbl.gov>

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