Understanding and Enhancing Alkaline and Oxidative Chemical Pretreatments for the Production of Biofuels through Improved Characterization

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Plant Tissues are Diverse and Highly Heterogeneous

Storage Tissues: Food Crops

- Starch Granules in Corn Endosperm
  Source: USDA/ARS/ERRC

- Arabidopsis Oleosomes
  Source: John Sedbrook, U. Illinois

- Corn Stem
  Source: USDA/ARS/ERRC

Structural Tissues: Plant Cell Walls

- Softwood secondary xylem (tracheids)
  Source: USDA/ARS/ERRC
  [http://www.cb.uu.se/](http://www.cb.uu.se/)

- Hardwood secondary xylem (vessel elements and fibers)
  Source: USDA/ARS/ERRC

- Arabidopsis stem
  Source: USDA/ARS/ERRC
  PNAS. 107(51): 22338–22343
## Diversity of Cell Wall Polymers

<table>
<thead>
<tr>
<th></th>
<th>Primary Cell Wall</th>
<th>Secondary Cell Wall</th>
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</thead>
<tbody>
<tr>
<td>Commelinoid</td>
<td>pp GAX β-Glucans</td>
<td>GAX GM</td>
</tr>
<tr>
<td>(Grasses)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-commelinoid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herbaceous</td>
<td>PP XyG GX</td>
<td>GX GM</td>
</tr>
<tr>
<td>Dicot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Woody Dicot</td>
<td>PP XyG GX</td>
<td>GM GX</td>
</tr>
<tr>
<td>(Hardwood)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Softwood</td>
<td></td>
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</tr>
</tbody>
</table>

- **GAX**: glucuronoarabinoxylans
- **GX**: glucuronoxylans
- **XyG**: xyloglucans
- **PP**: pectic polysaccharides
- **GMs**: glucomannans
- **G**: Guaiacyl
- **S**: Syringyl
- **H**: p-hydroxyphenyl
- **FA**: Ferulates

**Stem cross-sections**
Monocot Lignins

- Differences in composition and structural organization relative to herbaceous and woody dicots or gymnosperm lignins
  - Ferulates and p-coumarate can comprise a significant fraction of grass lignins
  - Ester crosslinks
  - Highly condensed (~85%)
  - High phenolic hydroxyl content
    - High alkali solubility
Barriers to the (Biological) Utilization of Plant Cell Wall Components

- Ultrastructure of plant cell walls needs to be broken down
- Lignin prevents access to carbohydrate fraction
- Crystalline cellulose resists depolymerization, blocks access to internally packed polymers

How pretreatments address this problem

- At the molecular level:
  - Chemical modifications
  - Depolymerization
  - Solubilization

- At the structural level:
  - Physical redistribution of components

Targets/problems

Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production

Michael E. Himmel,1* Shi-You Ding,2 David K. Johnson,1 William S. Adney,1 Mark R. Nimlos,3 John W. Brady,4 Thomas D. Foust5

Science 315, 804 (2007)
Biochemical Conversion of Plant Cell Wall Polysaccharides to Biofuels

Lignocellulose Feedstock
(Plant Cell Walls)

Chemical Pretreatment

Enzymatic Depolymerization of Polysaccharides

Hemicellulose

Cellulose

Microbial Metabolites
Ethanol, Butanol, Carboxylic Acids, Alkanes, Isoprenoids, ...

Biological Conversion of Monosaccharides

(A) Hexoses
β-D-Galactose
β-D-Glucose
β-D-Mannose

(B) Pentoses
β-D-Xylose
β-L-Arabinose
Oxidative Deconstruction of Lignocellulose

Biological Mechanism 1: Secreted set of cellulases
- Individual proteins with CBM, flexible linker, and catalytic domain (e.g. *Trichoderma reesei*)

Biological Mechanism 2: Brown rot fungi
- Deconstruction of cellulose and lignin using biologically-generated ·OH

Biological Mechanism 3: White rot fungi
- Peroxidases and oxidases for lignin degradation

Images source: [http://chemistry.umeche.maine.edu/CHY431/Wood4.html](http://chemistry.umeche.maine.edu/CHY431/Wood4.html)
Alkaline Hydrogen Peroxide Pretreatment

- Based on existing alkaline hydrogen peroxide pulp bleaching stages in the paper industry
- Alkaline-oxidative pretreatments as either standalone pretreatments OR delignifying “finishing” post-pretreatment step
- Unique advantages
  - Well-suited for grasses
- Current Challenges:
  - Process integration
  - Economics
  - Water use/recycle

Alkaline hydrogen peroxide bleaching tower >1000 tpd capacity at Smurfit-Kappa Kraftliner, Piteå, Sweden (photo courtesy: Outokumpu Oy)
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Agricultural Residues:
- Corn Stover/Corn Fiber
- Wheat Straw
- Sugar Cane Bagasse
- Sorghum Bagasse
- Rice Straw

Bioenergy Grasses:
- Switchgrass
- Miscanthus sp.
- Reed Canary Grass
Alkaline Hydrogen Peroxide Pretreatment

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Improved Processes? Catalytic approaches?

![Graph showing Pretreatment Chemical Cost vs. Gal EtOH](image-url)

- Switchgrass
- Corn Stover

Upper Theoretical Limit

Pretreatment Chemical Cost / Gal EtOH

Current Challenges:

- Process integration
- Economics
- Water use/recycle
Alkaline Hydrogen Peroxide Pretreatment

**Pretreatment**

**Liquefaction/Saccharification**

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Integration of AHP Pretreatment, Hydrolysis, and Fermentation

Xylose-fermenting *Saccharomyces*

1.67 g/L YNB w/o ammonium sulfate and 2.27 g/L of urea

Filter Sterilize (0.22 μm)

Liu et al. (in preparation).

Pretreatment, 0 h

Pretreatment, 48 h

Hydrolysis, 0 hr

Hydrolysis, 24 hr
Relating Cell Wall Properties to Recalcitrance

- Understanding cell wall properties impacting recalcitrance and relationship of these properties to enzymatic digestibility
  - Using pretreatment of plant cell walls with diverse phenotypes to generate samples with diverse recalcitrance properties

<table>
<thead>
<tr>
<th>Property Impacting Recalcitrance</th>
<th>Analytical Tool</th>
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</thead>
<tbody>
<tr>
<td>Cell wall hydrophilicity</td>
<td>Water adsorption/retention</td>
</tr>
<tr>
<td>Cellulose oxidation</td>
<td>Potentiometric titration</td>
</tr>
<tr>
<td>Polysaccharide accessibility</td>
<td>Glycome Profiling</td>
</tr>
<tr>
<td>Lignin content</td>
<td>Klason Lignin</td>
</tr>
<tr>
<td>Ferulate content</td>
<td>HPLC</td>
</tr>
<tr>
<td>S/G ratio</td>
<td>Pyrolysis-GC/MS</td>
</tr>
<tr>
<td>Lignin condensation</td>
<td>Thioacidolysis-GC/MS</td>
</tr>
<tr>
<td></td>
<td>HSQC NMR</td>
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</tbody>
</table>
Diverse Cell Wall Properties: Biomass

**Corn Stovers** *(Zea mays)*
- Pioneer hybrid 36H56
- Inbred brown midrib bm1
- Inbred brown midrib bm3

**Switchgrass** *(Panicum virgatum cv. Cave-in-Rock)*

**Miscanthus** *(Miscanthus x giganteus)*

**Hybrid poplar** *(Populus nigra var. Charkoviensis x Caudina)*

**Goldenrod** *(Solidago sp.)*
Diverse Cell Wall Properties: Pretreatments

- AHP
- Soda pulping
- Liquid Hot Water + AHP post-treatment
- Cu(bpy)-catalyzed AHP pretreatment

Liquid Hot Water (LHW)

AHP Pretreatment

Hydrolysis

0%, 6%, 12.5%, 50% H\textsubscript{2}O\textsubscript{2} (g/g biomass)
15% (w/v) Solids
9% w/w solids
30mg/g Gluc Enzymes
62% Accelerase
24% Multifect Xyl
14% Multifect Pect

pH 8–12

pH > 12
Cell Wall – Water Interactions Objectives

How can cell wall–water interactions impact deconstruction

- Water Retention Value
- Particle Settling
- Water Activity

**Quantifiable metrics**

**Property: Cell Wall Composition**
- Polysaccharide Content
- Lignin Content

**Property: Cell Wall Hydrophilicity**
- Accessible vs. Amorphous
- Inaccessible vs. Crystalline

**Property: Cell Wall Porosity**

**Outcomes:**
- Water Penetration
- Enzyme Penetration
- Polysaccharide Hydrolysis

15% Solids
Corn Stover
Switchgrass
Introduction of Carboxyl Groups with Oxidative Pretreatments?
Interactions Between Water and Cell Wall

Water retention value (WRV)

Step 1: Filter wash
Filter wash using 200mesh screen to ~ 80% moisture

Step 2: Centrifuge
"Cookie Cutter" pad onto 200 mesh disk from filter funnel; Centrifuge at 900xg for 15min

Step 3: Weighing
Determine Moisture Content of Pad

WRV = \( \frac{M_{\text{water}}}{M_{\text{drypad}}} \)

Weigh wet pad; Dry in oven at 105°C for ~2hrs then weigh dry pad
Interactions Between Water and Cell Wall

- Both water retention value (WRV) and swelling volume are increased with pretreatment
  - More water confined in cell wall
- WRV is a very good predictor of digestibility

Settling height (ht)

Total height (Ht)

LHW = Liquid Hot Water pretreated

Williams et al. (in preparation).
Interactions Between Water and the Cell Wall

- Pretreated corn stover can adsorb more water in the cell wall
  - Quantifiable by decreased water activity at a moisture content
  - *i.e.* more water is present in a constrained environment

Water Activity ($a_w$) = Relative Humidity ($h_r$) of vapor

*Williams et al. (in preparation).*
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Williams et al. (in preparation).
Hydrolysis Results for AHP Pretreatment of Diverse Cell Wall Phenotypes

- **Grasses**: Increasing response to AHP pretreatment
- **Herbaceous Dicot**: Conversion saturates
- **Cell wall properties?**

Li et al. (in preparation)
Correlating Digestibility to Plant Cell Wall Properties: Lignin Removal

- Digestibility and lignin content correlated
  - Extracted with alkali or AHP pretreated
  - Negative relationship with "threshold" value for lignin removal
  - Critical level for cell wall hydrophobicity?
- Properties associated with lignin removal?
Lignin Analytical Methods: Pyrolysis GC-MS

- High-throughput destructive characterization of complete cell walls
- Based on characterization of volatilized pyrolysis products
  - Pyrolysis for 10 s at 600°C, heated transfer line to GC/MS at 300°C
Correlating aromatic products to monomers in the lignin polymer

- Only represents a fraction of original lignin
  - Poor yield on condensed lignins
- Unique products for each lignin monomer?
- pCA and FA yield clearly identifiable markers
  - 4-vinylphenol and 4-vinylguaiacol
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5 pools of lignin monomers identifiable

Can be used to:

- Estimate S/G
- Characterize changes to lignin during pretreatment
Correlating Digestibility to Plant Cell Wall Properties

- Solubilized ferulate is correlated to glucan digestibility
- Pyrolytic 4-vinylguaiacol (Ferulate/Lignin):
  - Correlated to lignin content and glucan digestibility
**Lignin Methods: Thioacidolysis GC/MS**

- Determine relative quantities of G, S, H lignins
  - Foster et al., 2010. J Vis Exps, 37

- Cleavage of alkyl-aryl ethers in lignin
  - Quantification of derivatized fragments by GC-MS
  - Problematic in grasses?
    - Highly condensed lignins

- 

\[
\text{EtSH, BF}_3 \quad \rightarrow \quad \text{BSTF} \quad \rightarrow \quad \text{CPG-SM} \quad \rightarrow \quad \beta-O-4 \text{ linkage}
\]

- \[ \text{Li et al. (2012). Biotechnol Biofuels. 5(1)38.} \]
Quantitative Thioacidolysis

- Determines the fraction of lignin involved in only ether linkages
  - Thioacidolysis yield decreased from 5-12% w/w to 1% w/w after pretreatment
  - AHP pretreatment solubilizing 50% lignin
  - Fraction of condensed lignin increased from 88-95% w/w to 99% w/w

Cu(bpy)-Catalyzed AHP

Catalyst significantly improves subsequent enzymatic conversion of cellulose

Li et al. (accepted 2012). Biotechnol Bioeng.
Oxidative Depolymerization of Cellulose

- AHP depolymerizes cellulose (filter paper)
- Introduces -COOH

Li et al. (accepted 2012). Biotechnol Bioeng.
Summary

Water adsorption and retention can be correlated to enzymatic conversion

Correlating digestibility to plant cell wall properties
- Negative relationship between Klason lignin and digestibility across diverse cell wall types
- Ferulate content quantified by Py-GC/MS in residual cell wall and LC/MS in hydrolysates can be correlated to lignin removal and digestibility

Oxidative cellulose depolymerization and –COOH introduction demonstrated
- Impact on digestibility improvement?
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Questions?

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