Exploring and Exploiting
The Fundamental Chemistry of Laccase

Art J. Ragauskas
Professor of Wood Chemistry
Institute of Paper Science and Technology
Exploring and Exploiting The Fundamental Chemistry of Laccase

- Lignocellulosic Research Opportunities
- Overview of Lignin Chemistry
- Laccase Bio-bleaching Studies
- Laccase Bio-grafting Studies
- Future Opportunities
Research Opportunities: Carbohydrate Economy

David Morris - early 1980s

• Industrial raw materials consist of renewable carbohydrates not hydrocarbons.

– Agricultural Risk Protection Act of 2000 (PL 106-224)
  *Title III: Biomass R&D Act of 2000, established the Biomass Research and Development Board.*
Research Opportunities

Carbohydrate Economy

- Environmental, manufacturing, and sustainability costs are pulling a variety of industrial processes toward carbohydrate feedstocks: agro/wood
  - Pulp/paper
  - Fuel and energy
  - Plastics – biochemicals/biopolymers
- Many of these processes dependent are on lignin/carbohydrate chemistries and separation technologies
Chemical Pulp/Paper Manufacturing

Industrial Goal:

Conversion of wood into high-value products by degrading lignin while retaining cellulose and hemicellulose polymers

- Lignin is a major component in wood: HW ~20%  SW:~ 28%

Lignin Biosynthesis

Enzymatic Radical Coupling

Coniferyl Alcohol Synapyl Alcohol p-Coumaryl Alcohol

SW/HW HW trace
Native Wood Lignin—an overview

<table>
<thead>
<tr>
<th></th>
<th>%</th>
<th>SW</th>
<th>HW</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: $\beta$-O$_4$</td>
<td>50</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>B: $\alpha$-O$_4$</td>
<td>8</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>C:</td>
<td>15</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>D: $\beta$-5</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>E: 5-5</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>F: 5-O$_4$</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>G: $\beta$-1</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>H: $\beta$- $\beta$</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>
Chemical pulping-kraft process

• **Kraft pulping chemistry**
  – Degrade lignin, retain carbohydrates in a fiber structure
  – NaOH/Na₂S solution
  – 2 hours at 170° C
  – Removes ≈ 95% lignin
  – Macro-molecule lignin is degraded into lower MW fragments with hydrophilic units
Typically Pulping Reactions

Fragmentation of β-Aryl Ether from non-phenolic lignin units

Lower MW and generation of new alkaline soluble phenolic centers
Residual Lignin After Kraft Pulping

Alkaline Stable Structures

- Lignin carbohydrate complexes
- Free phenolic groups
- Condensed units
Pulp Bleaching

• Purpose of bleaching is 2-fold:
  – to remove the residual lignin
  – to brighten the pulp

• Current bleaching technologies consist of:
  – O₂, ClO₂, H₂O₂, & O₃
  – Capital expensive, environmental & selectivity issues

  – New bleaching agents needed!
LACCASE-MEDIATED BIOBLEACHING
Laccase: Overview

Laccase

- Oxidoreductase enzyme
- Reduces O$_2$ to H$_2$O$_2$
- Concomitantly oxidizes
- MW varies 65,000-140,000.
- Carbohydrate content $\sim$10-45 (% wt).
- Catalysis occurs due to 4 copper atoms/active site
- Active sites near surface

$\text{Cu}^{1+}$

$\text{HisN}$

$\text{Cu}^{1+}$

$\text{SCys}$

$\text{Cu}^{1+}$

$\text{HisN}$

$\text{Cu}^{2+}$

$\text{HisN}$

$\text{SCys}$

$\text{Cu}^{2+}$

$\text{OH}$

$\text{O}_2$

$\text{Fully Reduced}$

$\text{Fully Oxidized}$

E.I. Solomon et al

Active sites on surface
Laccase: Overview

- Proposed to be involved in lignin biosynthesis
- Oxidize a wide array of phenolic substrates

Higuchi, Wood Sci. Techn., 24, 22(1990)
Laccase Biobleaching

Low MW Co-factor - Mediator

O₂

Pulp Fiber

Cellulose

Lignin

Hemicellulose
Laccase Mediators-1\textsuperscript{st} generation

ABTS : 2-2’azinobis(3-ethylbenzthiazoline-6-sulfonate)
Paice, ~89-90

- First mediator used to demonstrate that a laccase-mediator system (LMS) can delignify kraft pulps.
- Not ideal $>>$ low delignification levels and cost prohibitive
Laccase Biobleaching

LMS-Process

Laccase Biobleaching

O₂ → LACCASE → LACCASE等一系列反应

H₂O → LACCASEoxide → MEDIATOR → MEDIATORoxide → LIGNIN → LIGNIN oxide
Laccase Biobleaching

Conditions LMS(E)

• LMS: 45°C, 1-2 h, 5-20% csc, pH 4 - 6, 1-4% mediator, +40 psi
• E: 70°C, 1-3 h, 1-2% NaOH

Highly selective for lignin, little degradation of pulp carbohydrates

Laccase Biobleaching

Research Goals:

• Identify chemical mechanisms contributing efficiency of RRN-OH mediators

• Establish how LMS treatments degrade lignin

• Establish relationship between LMS treatments and pulp properties
Chemistry of RRN-OH
Laccase Mediators
LMS Biobleaching: Mediator Chemistry

Nitrogen Incorporation

Fate of mediator during LMS?
LMS Biobleaching: Mediator Chemistry

Fate of HBT in LMS

1. $^1$H NMR spectrum of N-hydroxybenzotriazole in $D_2O$

2. $^1$H NMR spectra of the reaction between laccase (5.2 U) and N-hydroxybenzotriazole for 4 hours at 23°C.
LMS Biobleaching: Mediator Chemistry


LMS Biobleaching: Mediator Chemistry

Among these mediators, violuric acid has been found to be most effective at delignification of pulps.

Efficacy is not well predicted by a single term:
- Oxidation potential
- $K_m/K_{cat}$

Calculations have been performed to evaluate the electronic and energetic differences that might account for mediator behavior.
Mediators chemistry has not been modeled.
LMS Biobleaching: Computational Studies

- Computational methods
  - Reactions of mediator radicals with β-O-4 lignin model compound
  - 3-21G* ab initio calculations
  - Geometry optimizations
  - UHF (unrestricted Hartee-Fock) calculations for radicals
  - Gaussian 98
LMS Biobleaching: Computational Studies

• The reduction of violuric acid is exothermic

• The reductions of HBT and NHA are endothermic
LMS Biobleaching: Computational Studies

Spin density

HBT

NHA

VA
Spin density (unpaired electron density) is greatest on the oxygen of violuric acid (0.98), followed by NHA (0.92) and HBT (0.91).

This trend is consistent with the reduction in kappa numbers.

Mulliken charges at oxygen:
- Violuric acid = -0.097
- HBT = -0.146
- NHA = -0.158

The violuric acid may be less repellant toward the addition of an electron than the others.
LMS Biobleaching: Computational Studies

- Identified fundamental properties that may contribute to the improved performance of VA
  - Spin density
  - Mulliken charges
  - Electrostatic repulsion
- Insight provides new opportunities

*Accepted: Progress in Biotechnology: Chandra, R.; Allison, L.; Kim, D.; Elder, T.; Ragauskas, A.*
Lignin Chemistry of LMS Treatments
LMS Biobleaching: Lignin Chemistry

Research Goal:
Determine the functional groups in lignin that are oxidized during an LMS(E) treatment.

Experimental Procedure:
• Analyze lignin structure in starting pulp
• Characterize lignin in post LMS(E) fibers and in effluents
**LMS\textsubscript{VA} Delignification**

**LMS Conditions**
- $5.4 \times 10^5$ U laccase/gram dry pulp
- $45^\circ C$, 2h, 120 psi $O_2$, 9% csc, med. = VA
- $E$
- $80^\circ C$, 1.5 h, 10% csc, 2.5% NaOH
- 0.50% $H_2O_2$

<table>
<thead>
<tr>
<th>Pulp</th>
<th>% Delign.</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW High-lignin</td>
<td>43</td>
<td>99.9</td>
</tr>
<tr>
<td>SW Low-lignin</td>
<td>62</td>
<td>100.0</td>
</tr>
</tbody>
</table>
LMS Biobleaching: Lignin Isolation

Pulp
reflux for 2hr (4% csy)
in 0.1N HCl 9:1
dioxane: water

Filter (coarse)
Filter (fine)
Neutralize

Remove dioxane
under reduced
pressure

Precipitate lignin
wash lignin x3
Freeze dry

NMR

Diagram of chemical structures:

Lignin
H₃CO
HO
OH

Xylan
O
HO
OH

O
OH

O
HO
OH

O
HO
OH
LMS Biobleaching: NMR Analysis Lignin

Substituted Aromatic C’s

Cβ in β-O-4

Cγ in β-O-4

Chemical Structures:

1. CH₃O
2. O
3. O
4. OCH₃

5. CH₃O
6. O
7. O
8. OCH₃

9. HO
10. CH₃O
11. O
12. OCH₃
13. O
14. OCH₃
LMS Biobleaching: $^{13}$C NMR Analysis Lignin

![Bar chart showing the comparison of SW(50.0 K)-Acid, SW(27.5 K)-Acid, SW(50.0 K)-OMe, and SW(27.5 K)-OMe between Start and LMS(EP).]
LMS Biobleaching: $^{13}$C NMR Analysis Lignin

NB: differs from model compound studies
LMS Biobleaching: $^{31}$P NMR Analysis Lignin

LMS Biobleaching: $^{31}$P NMR Analysis Lignin

mmol/g lignin

<table>
<thead>
<tr>
<th>SW(50.0 K)</th>
<th>SW(27.5 K)</th>
<th>SW(50.0 K)</th>
<th>SW(27.5 K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Start}$</td>
<td>$\text{LMS(EP)}$</td>
<td>$\text{Start}$</td>
<td>$\text{LMS(EP)}$</td>
</tr>
</tbody>
</table>

Start and LMS(EP) values for different temperatures.
LMS Biobleaching: $^{31}$P NMR Analysis Lignin

mmol/g lignin

<table>
<thead>
<tr>
<th></th>
<th>SW(50.0 K)</th>
<th>SW(27.5 K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>LMS(EP)</td>
<td>0.08</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Chemical structure of lignin.
LMS Biobleaching: $^{31}$P NMR Analysis Lignin

![Graph showing mmol/g lignin](image)

- SW(50.0 K)-ROH
- SW(27.5 K)-ROH

**mmol/g lignin**

**Legend:**
- Green: Start
- Blue: LMS(EP)
LMS Biobleaching

Where Are the Quinones?
Trimethylphosphite Chemistry: Reaction with Ortho-Quinone Structures

Ramirez et al., Sidky et al., & Medvecz

Ramirez et al., Sidky et al., & Medvecz
Trimethylphosphite Chemistry: Reaction with Para-Quinone Structures
$^{31}$P NMR Quinone Lignin Analysis

Quinone Adduct

Internal Standard

31P NMR Quinone Lignin Analysis for LMS Treated Pulps

- First direct evidence for quinone formation in lignin post LMS treatment with NHA, VA, & HBT.

- Data indicates a decrease after E stage.

Quinone Lignin Chemistry During Alkaline Extraction
LMS Biobleaching: Conclusions

- Stability of mediator is key component in determining performance of LMS
- Radical energetics of the mediator influences effectiveness of an LMS-stage
- LMS delignification of lignin in pulp differs from model compound studies (or homogenous lignin solution studies)
- In pulp LMS$_{VA}$ oxidizes phenolics
  - C5- noncondensed preferred
  - Minor oxidation of C5- condensed and p-hydroxyl phenyl (NB: nature of mediator is important VA>> HBT or NHA)
- LMS$_{VA}$ treatments result in slight de-methoxylation
- LMS treatments do not degrade $\beta$-O-aryl ethers in pulp
- LMS generated quinones are limitation for some bleaching applications
- Type of lignin in fiber influences LMS delignification
Laccase Future Directions

• Design of new mediators for LMS pulp bleaching/paper dyes
• Biografting of natural macropolymers/fibers

Biografting/crosslinking ligno-cellulosic fibers has several applications:

• Composite Wood Products > replace formaldehyde in OSB, MDF, PB
• New plastic-wood materials, new paper materials
Laccase Biografting: Experimental Approach

Laccase/O₂

pH 4.5, 45°C

After treatment fibers exhaustively washed

Preliminary solution work reported by
Laccase Biografting: Conductometric Acid Determination

- Laccase + 4-hydroxy benzoic acid approx. doubled the bulk acid groups
- Control experiments support coupling hypothesis
Laccase and 4-hydroxybenzoic acid exhibited largest % increase of surface acid groups.
Laccase Biografting: Conclusions/Future

• Biografting in a heterogenous system is possible

• Biografting impacts physical properties

• Chemistry of the process has been investigated employing model systems

• Assorted reaction parameters need further investigation

• Research opens a new frontier in topochemistry modification of lignocellulosic materials and controlling physical properties
Acknowledgements

T. Elder/Auburn U., N. Franks/Novozymes
J. Sealey, K. Haynes, D. H. Kim, L. Allison, R. Chandra
A. Suurnaki/VTT, F. Wolfaardt/ U. Orange Free State
M. Lund/The Royal Veterinary & Agricultural U.

U.S. Department of Energy
Member Companies of IPST
Potlatch Corporation
Ph.D. Student Research

• L. Vander Wielen
  – Corona Discharge Initiated Grafting to High-Lignin Content Pulp Fibers
• A. Puckett
  – Topochemistry of D - Z bleaching
• T. Dyer
  – Fundamentals of color formation during kraft pulping
• K. Knutson
  – Biobleaching MOW
M.S. Student Research

- K. Nelson
  - Chemical crosslinking of lignocellulosic pulp fibers
- Jason Montegna
  - Chemical mechanisms involved in curing wood composites
- Shanna VanTassel
  - Peroxidase biobleaching of azo-dyes
External Funded Research Activities

• DOE: High Selectivity Oxygen Delignification
• DOE: Curing of Composite Wood Products
• USDA: Lignocellulosic Photostabilization
• Industrial Consortium:
  Fiber Modification/Fiber-Fiber Bonding