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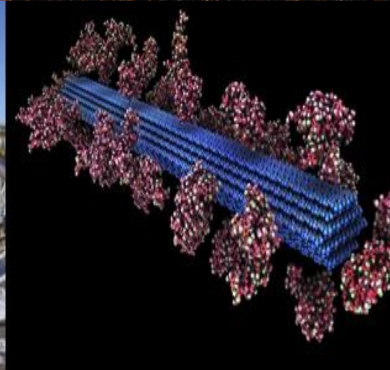


2015 DOE BioEnergy Technologies Office (BETO) Project Peer Review

Date: March 25th, 2015

Technology Review Area: Biochemical Conversion

Biological Lignin Depolymerization (WBS 2.3.2.100)

Principal Investigators:
Gregg Beckham (NREL)
John Gladden (SNL)

Organizations: National Renewable Energy Laboratory and Sandia National Laboratory

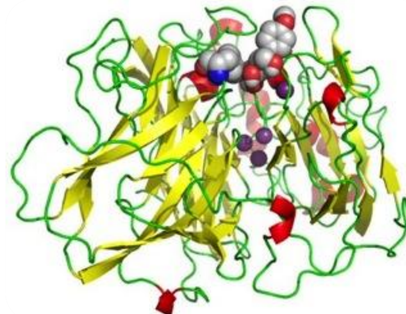
Project Goal

Goal and Outcome: develop a biological approach to depolymerize solid lignin for upgrading of low MW aromatic compounds to co-products

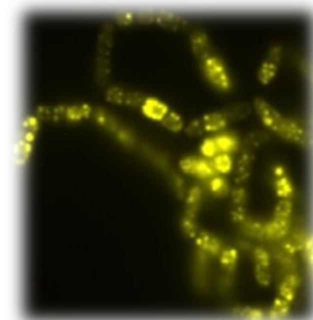
Relevance to BC Platform, BETO, industry: lignin valorization is key for meeting HC fuel cost and sustainability targets in integrated biorefineries

Residual Biorefinery Lignin

Ligninolytic Enzymes



Aromatic-catabolizing bacteria



NREL NATIONAL RENEWABLE ENERGY LABORATORY

Sandia National Laboratories

jbei Joint BioEnergy Institute

Quad Chart

Timeline

- Start date: October 2014
- End date: September 2017
- Percent complete: ~30%

Barriers

- Bt-F Hydrolytic Enzyme Production
- Bt-I Catalyst Efficiency
- Bt-J Biochemical Conversion Process Integration

Budget

	FY14 Costs	Total Planned Funding (FY15-Project End Date)
DOE funded	$222,410	$1,752,590

Funds are split 50/50 between NREL and SNL

Partners and Collaborators

- **BETO Projects**: Pretreatment and Process Hydrolysis, Lignin Utilization, Biochemical Platform Analysis, Pilot Scale Integration, Enzyme Engineering and Optimization
- **BETO-funded National Lab Projects:** Oak Ridge National Laboratory (A. Guss)
- **Office of Science funded efforts:** Joint BioEnergy Institute, Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory (R. Robinson, E. Zink)
- **Academic collaborators:** Swedish University of Agricultural Sciences, University of Portsmouth

Project Overview

Project History:

- Began as a BETO seed project in FY14 between NREL and SNL
- Achieved major milestone at end of FY14
- Complementary effort to other BETO-funded lignin projects

Project Context:

- Lignin valorization is a primary challenge in biochemical conversion processes
- Leverage studies in biological lignin depolymerization with new synthetic biology techniques and new process concepts in biological funneling

High-Level Objective:

- Employ biology for depolymerization and aromatic catabolism of residual lignin

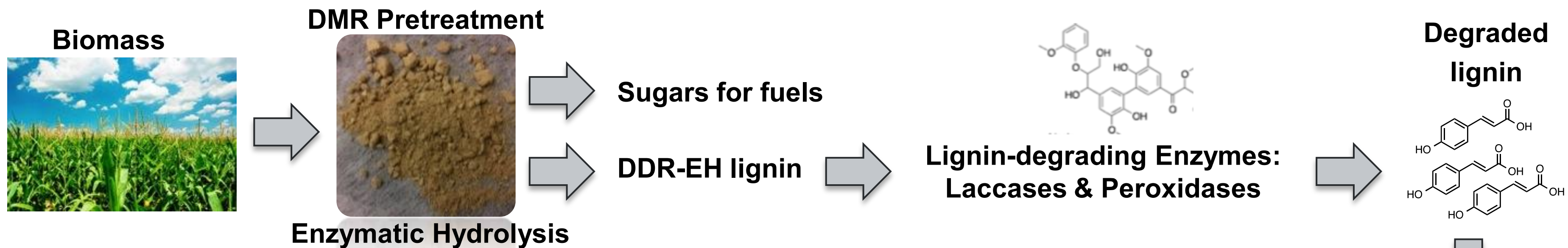
Lignin Depolymerization

- Focus on residual solids from Deacetylation-Mechanical Refining Prt.
- Examine fungal and bacterial ligninolytic enzymes for solid lignin depolymerization
- Develop effective enzyme secretion systems

Lignin Upgrading

- Develop aromatic metabolic map
- Elucidate aromatic transport mechanisms
- Understand rate limitations in aromatic catabolism
- Develop optimized modules for production of value-added products

Technical Approach



Substrate: residual lignin from deacetylation and mechnical-refining pretreatment and enzymatic hydrolysis (DMR-EH)

Approach:

- Characterize enzymatic degradation of DMR-EH lignin
- Identify parameters for optimal biological lignin degradation
- Engineer organisms convert depolymerized lignin into valuable bioproducts

Primary challenges:

- Effective **analytical tools** to quantify lignin depolymerization
- Minimizing **repolymerization** of high MW lignin during depolymerization
- Identifying and overcoming **aromatic transport and catabolic limitations**

Use microbial cell factories to upgrade lignin

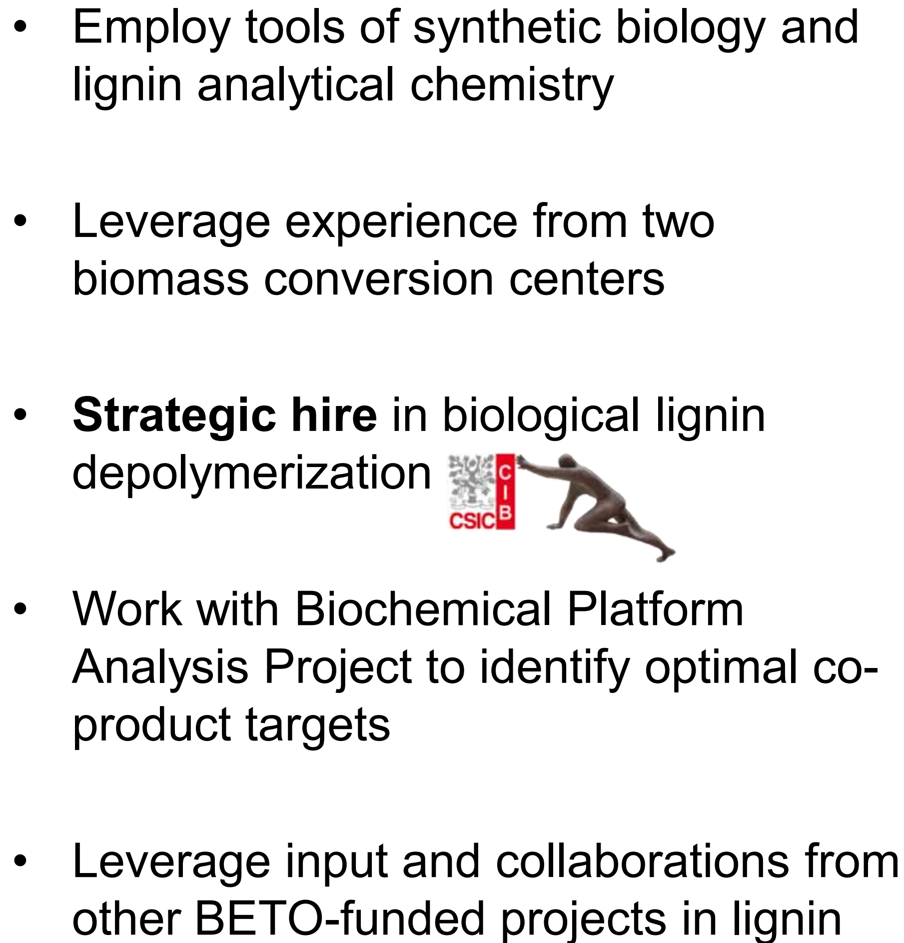
Success factors:

- Achieving high yields of low MW lignin species
- Generating aromatics that can be metabolized by microbes
- Producing products with high market value from lignin

Management Approach

Critical Success Factors:

- Employ tools of synthetic biology and lignin analytical chemistry
- Leverage experience from two biomass conversion centers
- **Strategic hire** in biological lignin depolymerization



- Work with Biochemical Platform Analysis Project to identify optimal co-product targets
- Leverage input and collaborations from other BETO-funded projects in lignin

Management Approach:

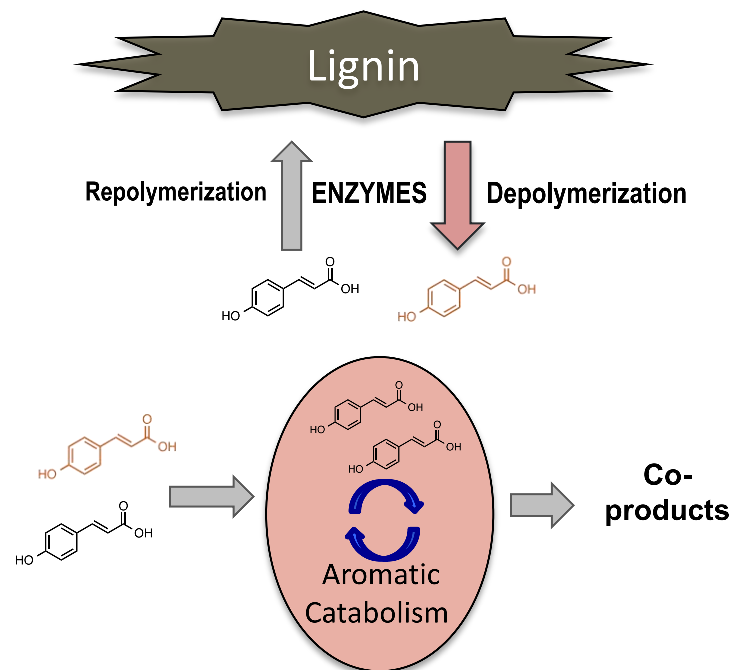
- Use quarterly milestones to track progress and down-select options
- **Milestone priority dictated by depolymerization and overcoming key risks in yields**
- Phone calls every 3 weeks, site visits every 6 months
- Contributions from both partners on lignin analytical chemistry leveraging respective expertise
- Divide research in a manner that leverages each partners strengths

Technical Results – Outline

- Examined **purified enzymes** and fungal-derived **secretomes** on several lignin substrates for ability to depolymerize lignin

- Determined that reaction conditions and enzyme composition are key factors in promoting **lignin depolymerization**

- Found evidence that lignin repolymerization is an issue that must be addressed to achieve maximum lignin depolymerization. Solution: microbial "sink"



- For FY15, we have assembled a list of microbial "sinks" and initiated a detailed characterization of DMR-EH lignin

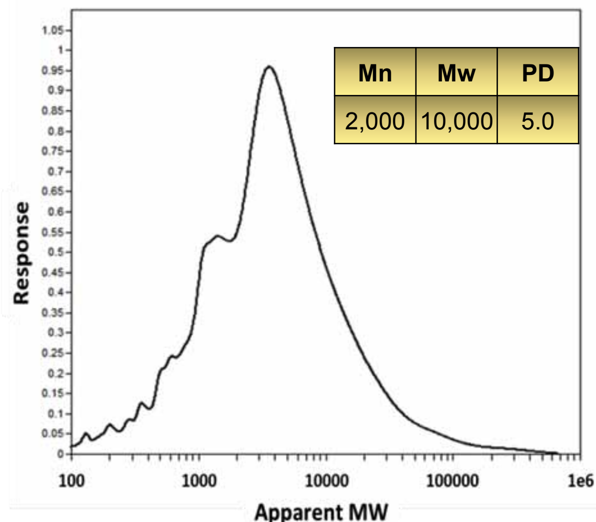
Strain	Consumes Aromatics	Growth on Lignin
Amycolatopsis sp.	Y	Y
Pseudomonas fluorescens	Y	Y
Pseudomonas putida KT2440	Y	Y
Etc…	Y	Y

FY15 Milestone: Characterization of solid DMR-EH

Compositional analysis

Sample ID	Content (%)
Ash	2.18
Lignin	66.0
Glucan	9.24
Xylan	9.36
Galactan	1.04
Arabinan	1.62
Fructan	0.00
Acetate	0.72
Total sugar	21.26
Total	90.2

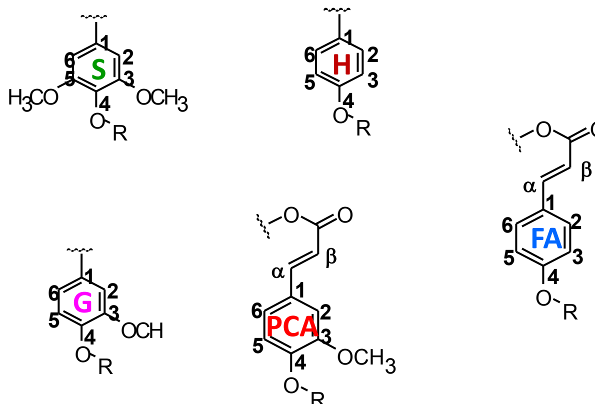
Molecular weight distribution



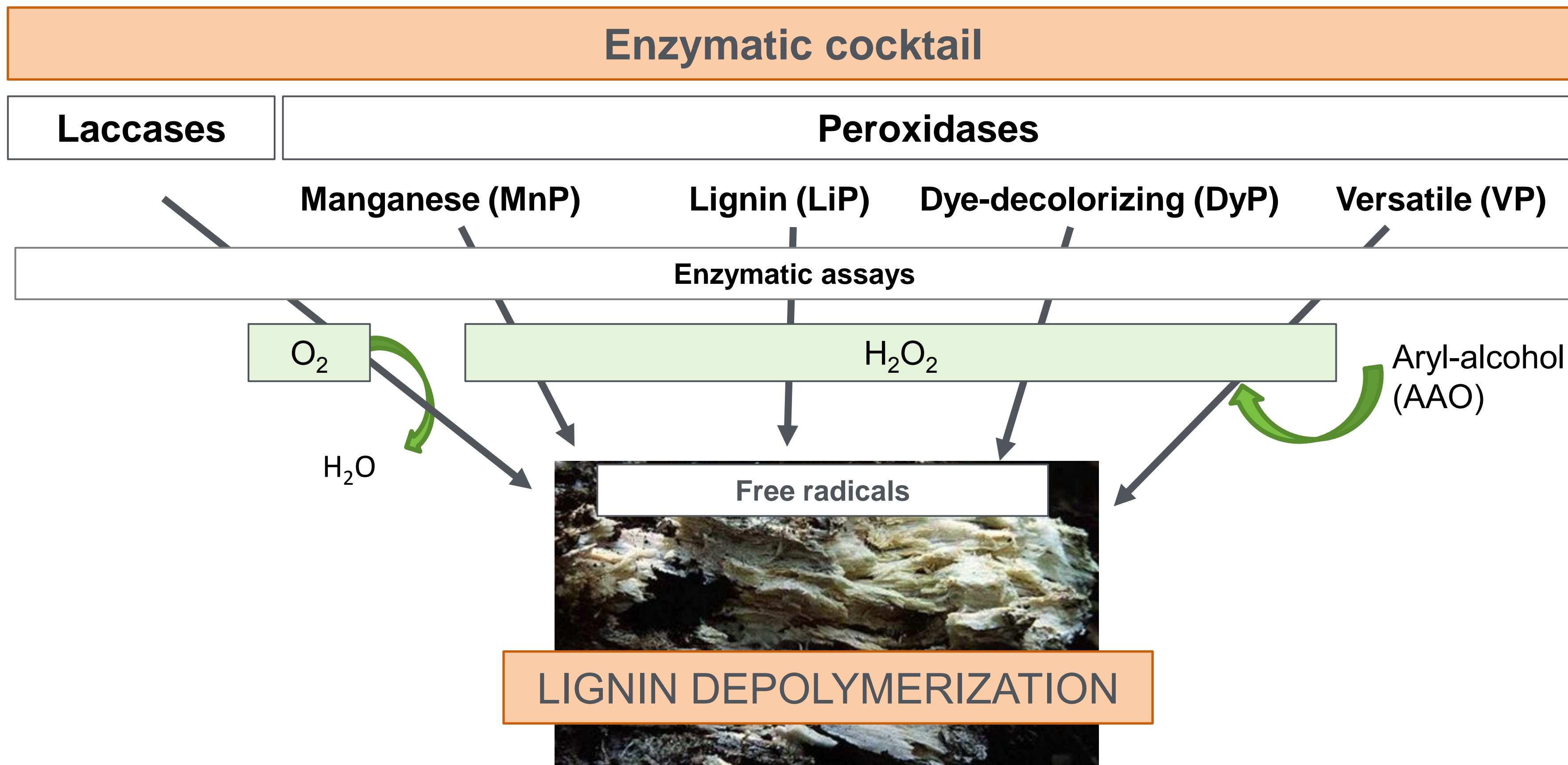
Mn	Mw	PD
2,000	10,000	5.0

2D-NMR for lignin chemical composition (HSQC)

Moiety	Abundance
S	17%
G	45%
H	2%
PCA	27%
FA	9%



Microbial Lignin Depolymerization



FY14 Research: Start with Fungi

- Use **purified fungal enzymes** to optimize conditions for lignin depolymerization
- Examine **ligninolytic enzyme cocktails produced by basidiomycete fungi** to optimize conditions and identify the enzymes present in natural secretomes

Optimizing Enzymatic Lignin Depolymerization

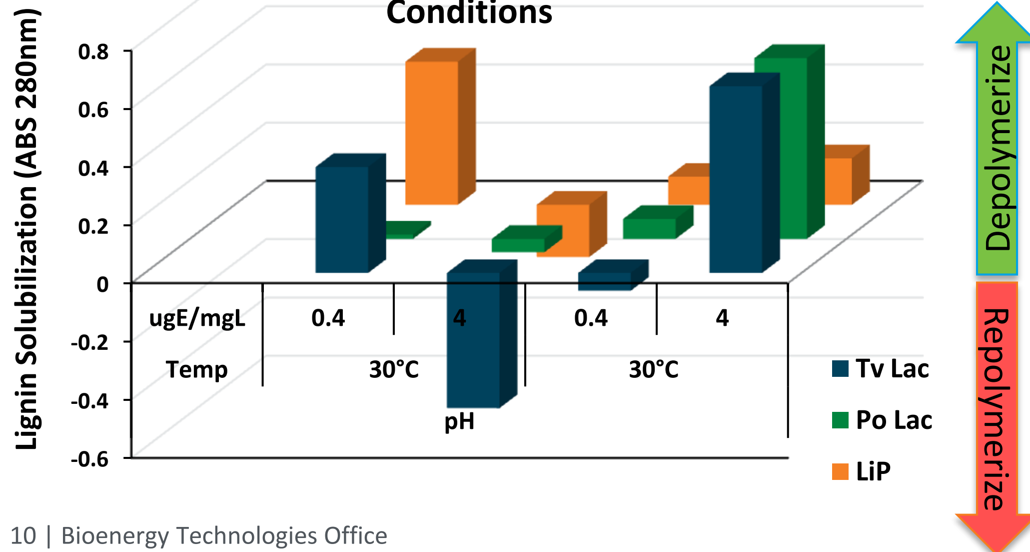
Ligninolytic enzyme activity is a strong function of temperature and pH

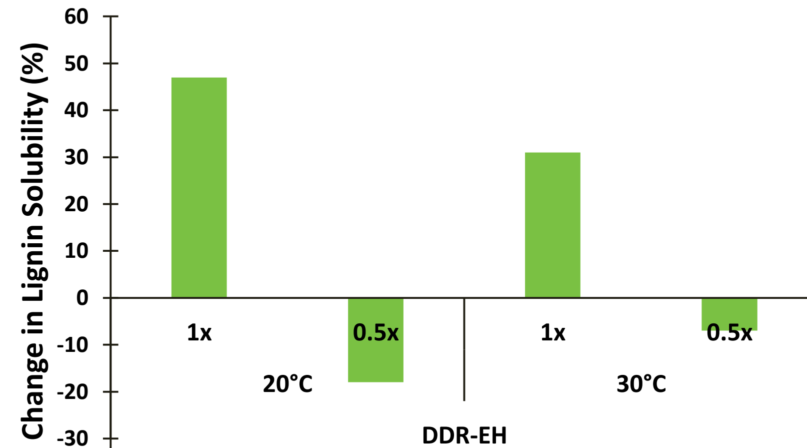
- Screened 9 commercial purified fungal laccases and peroxidases for ability to solubilize lignin
- Used active enzymes to optimize the reaction conditions and create cocktail
- pH and Temp are critical factors for depolymerization
- Optimal enzyme loads depend on pH, T

Top Enzymes

Enzyme Function	Source Organism	Name
Laccase	*Trametes versicolor*	*Tv*
Laccase	*Agaricus bisporus*	*Ab*
Laccase	*Rhus vernicifera*	*Rv*
Laccase	*Pleurotus ostreatus*	*Po*
Lignin Peroxidase	Unknown	LiP

Green enzymes used in ligninolytic cocktails





Enzyme Loadings are also very important!

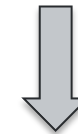
Screening of natural fungal secretomes

Screen with 12 white-rot fungi

- *Pleurotus ostreatus*
- *Irpex lacteus*
- *Panus tigrinus*
- *Bjerkandera adusta*
- *Bjerkandera* sp.
- *Cerioporopsis subvermispora*
- ***Pleurotus eryngii***
- *Phellinus robustus*
- *Polyporus alveolaris*
- *Stereum hirsutum*
- *Trametes versicolor*
- *Phanerochaete chrysosporium*

Ligninolytic Enzyme induction on DDR-EH lignin

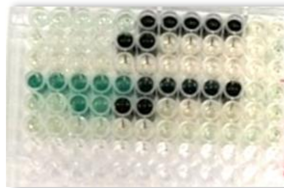
Production of fungal secretomes in the presence of lignocellulose can induce and/or accelerate the production of ligninolytic and cellulolytic enzymes



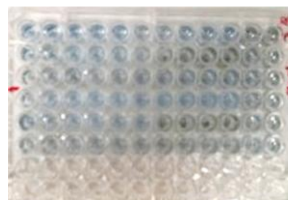
Production of ligninolytic cocktails

Profile ligninolytic enzyme activities

ABTS assay



Reactive Blue 19 assay



Characterization of ligninolytic cocktails

Pleurotus eryngii

(↑↑↑laccase, AAO, VP, ↓↓MnP)

9 days

Laccase x 10

Phellinus robustus

(↑laccase, ↑MnP, DyP, AAO)

7 days

Bjerkandera sp.

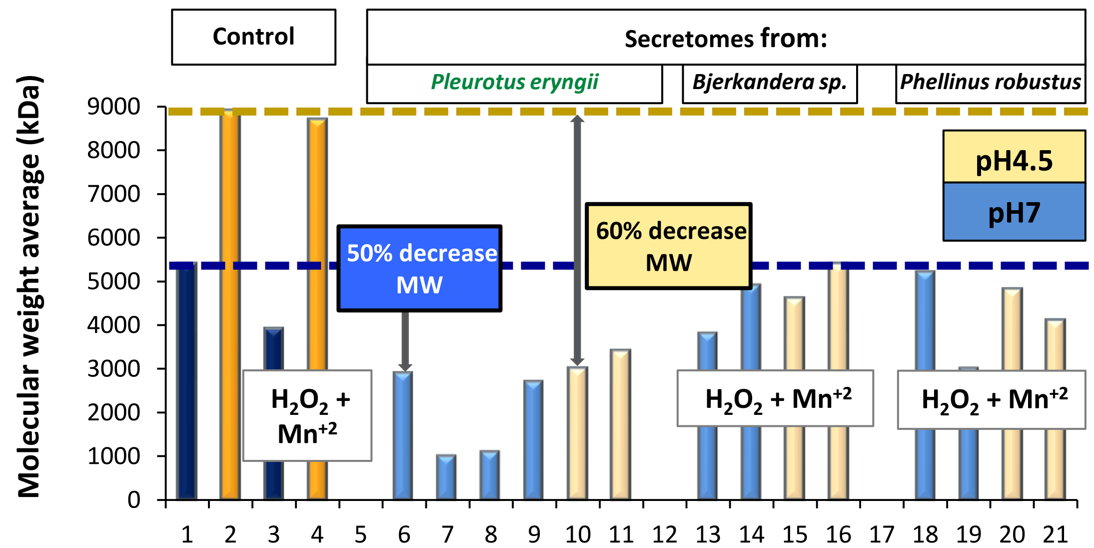
(↑↑MnPs)

9 days

mU/mL

Days

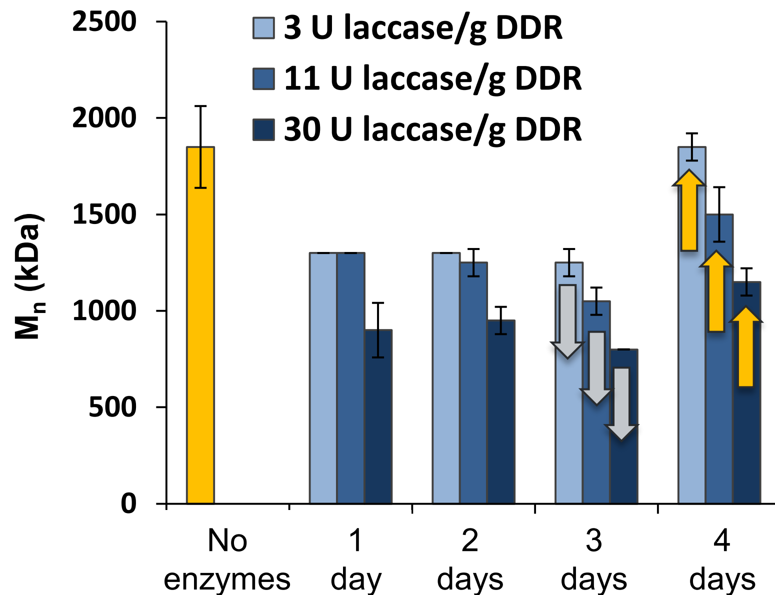
Laccase
Peroxidase
MnP I
MnP II
AAO
LiP
DyP

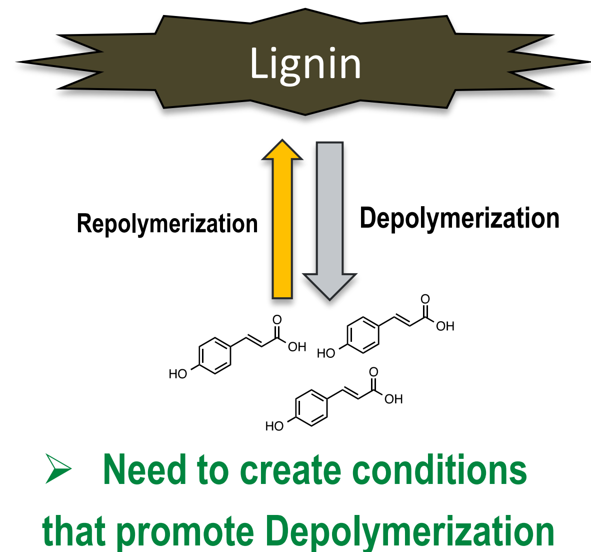


- Identified organisms that produce the most activity of specific ligninolytic enyzmes
- ***P. eryngii*** **shows highest lignin depolymerization, lowest MW pH 7**
- **Surpassed FY14 Go/No-Go**
- pH plays a major role in lignin depolymerization extents

P. eryngii secretome depolymerizes DMR-EH Lignin

Number-averaged Molecular Weight Distribution upon ligninolytic enzyme treatment at pH 7





➢ Need to create conditions that promote Depolymerization

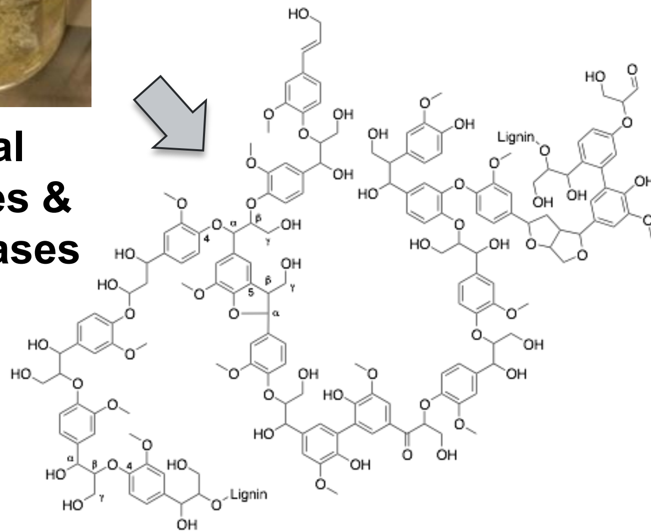
P. eryngii is able to depolymerize DDR-EH lignin to low MW species

- 50% of lignin species are smaller than the control (M_n) in a single day of treatment
- Apparent repolymerization by 4 days of incubation
- Repolymerization may be prevented if we incorporate a low MW aromatic compound “sink”
- Work in *Pseudomonas putida* suggests microbes are an ideal “sink”” as they can be used to convert the lignin degradation products into value-added compounds

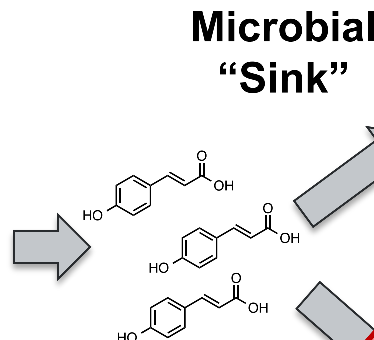
One primary strategy going forward: microbial "sink"

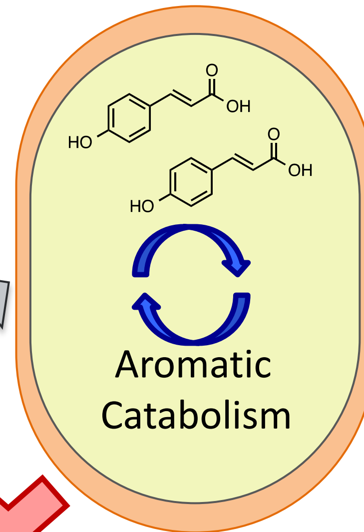
Fungal Laccases & Peroxidases

High MW Lignin



Microbial "Sink"





Co-products

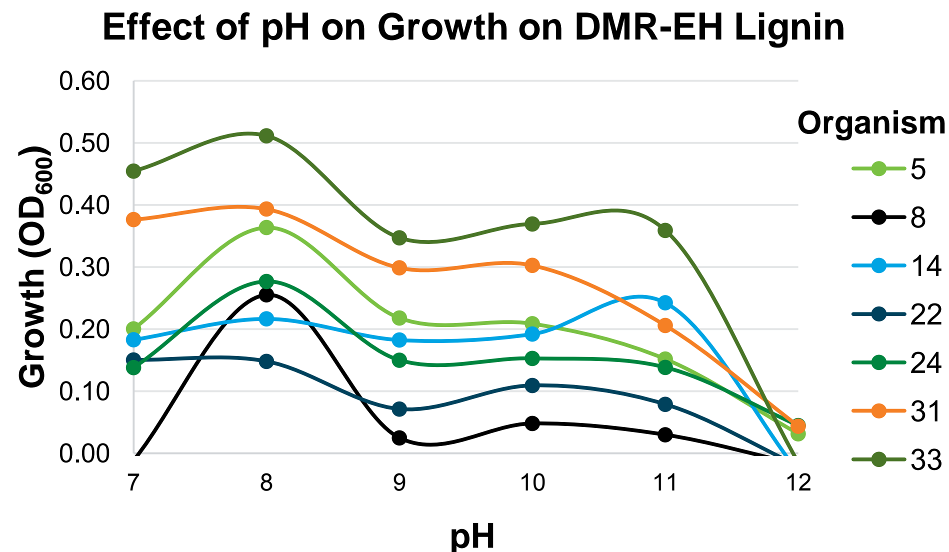
Re-polymerization

Utilize microbial sink to uptake aromatic species upon depolymerization

- Similar to SSF or CBP concepts in polysaccharide valorization approaches
- Requires understanding of aromatic transport and catabolism: "aromatic metabolic map"
- Need a promiscuous microbe with broad substrate specificity, genetic tractability
- Co-product selection will require consideration of separations and other process variables including oxygen demands in solid media

Conditioning DMR-EH lignin for biological treatments

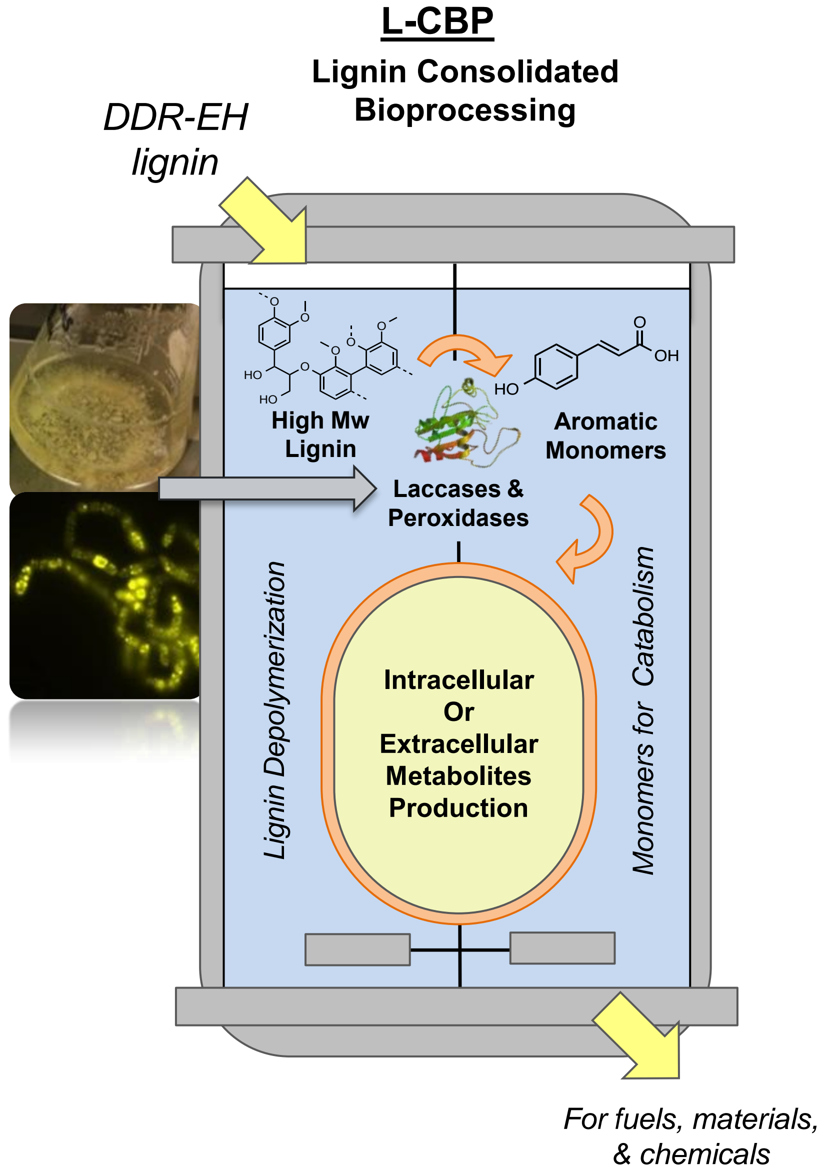
- DMR-EH lignin is a challenging substrate to obtain high conversion yields to monomeric species.
- pH will be crucial for obtaining high lignin solubilization as well as performing enzyme treatments and growing the organisms



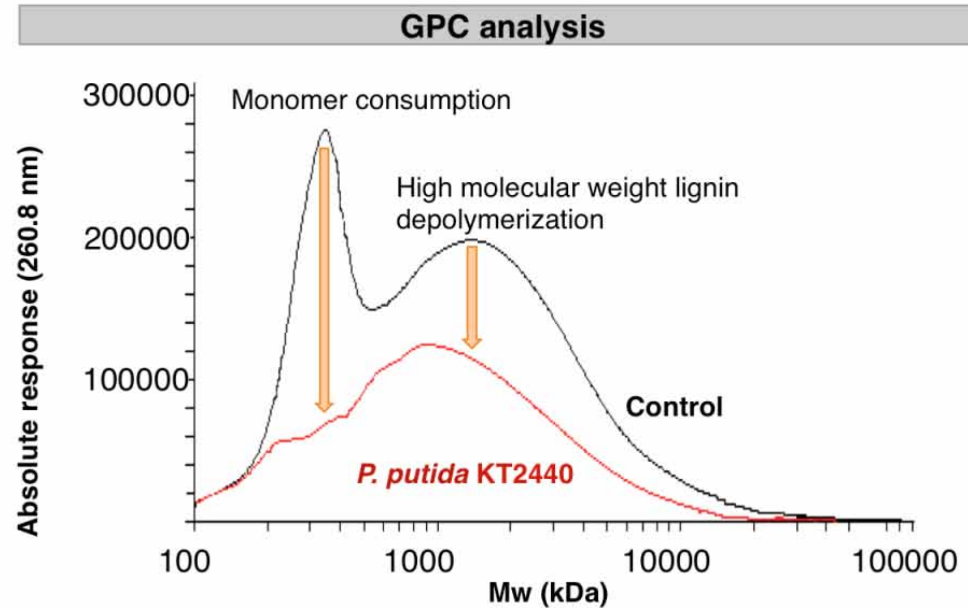
- **FY15 Milestone: Identified 27 organisms (bacteria and fungi) that can metabolize lignin subunits and can grow on lignin**
- Remaining FY15 Milestones: Down-select to top microbial “sinks” and begin detailed characterization of aromatic metabolism

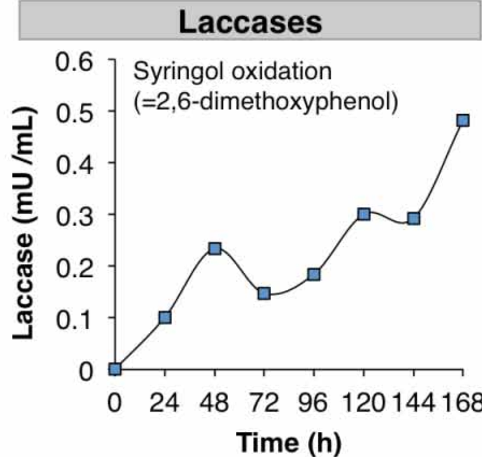
L-CBP: Bacteria also depolymerize lignin

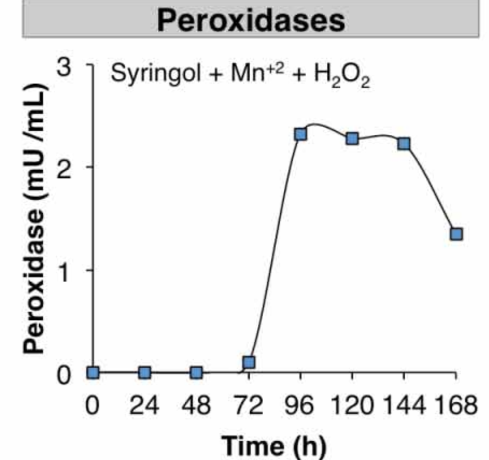
Salvachua *et al., in review*



ALKALINE PRETREATED LIQUOR FROM CORN STOVER







Proteomics to fingerprint ligninolytic enzymes

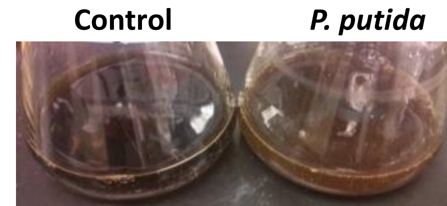
EMSL

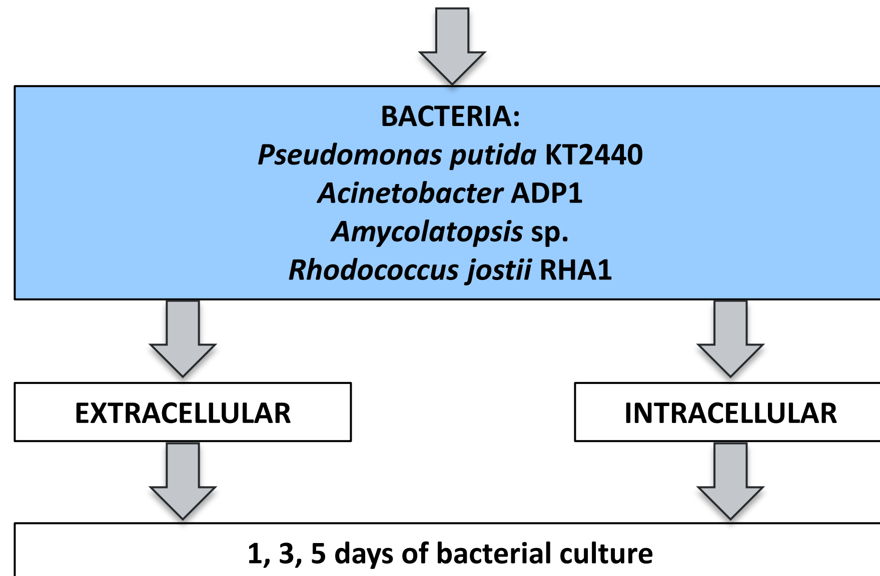
- **Bacteria also depolymerize lignin with laccases and peroxidases**, but produce much smaller amounts of enzymes than fungi
- **Proteomics studies** will provide detailed information about fungal and bacterial enzymes implicated in lignin depolymerization and catabolism
- Awarded **competitive allocation for proteomics at EMSL**, analysis ongoing

FUNGI
Pleurotus eryngii

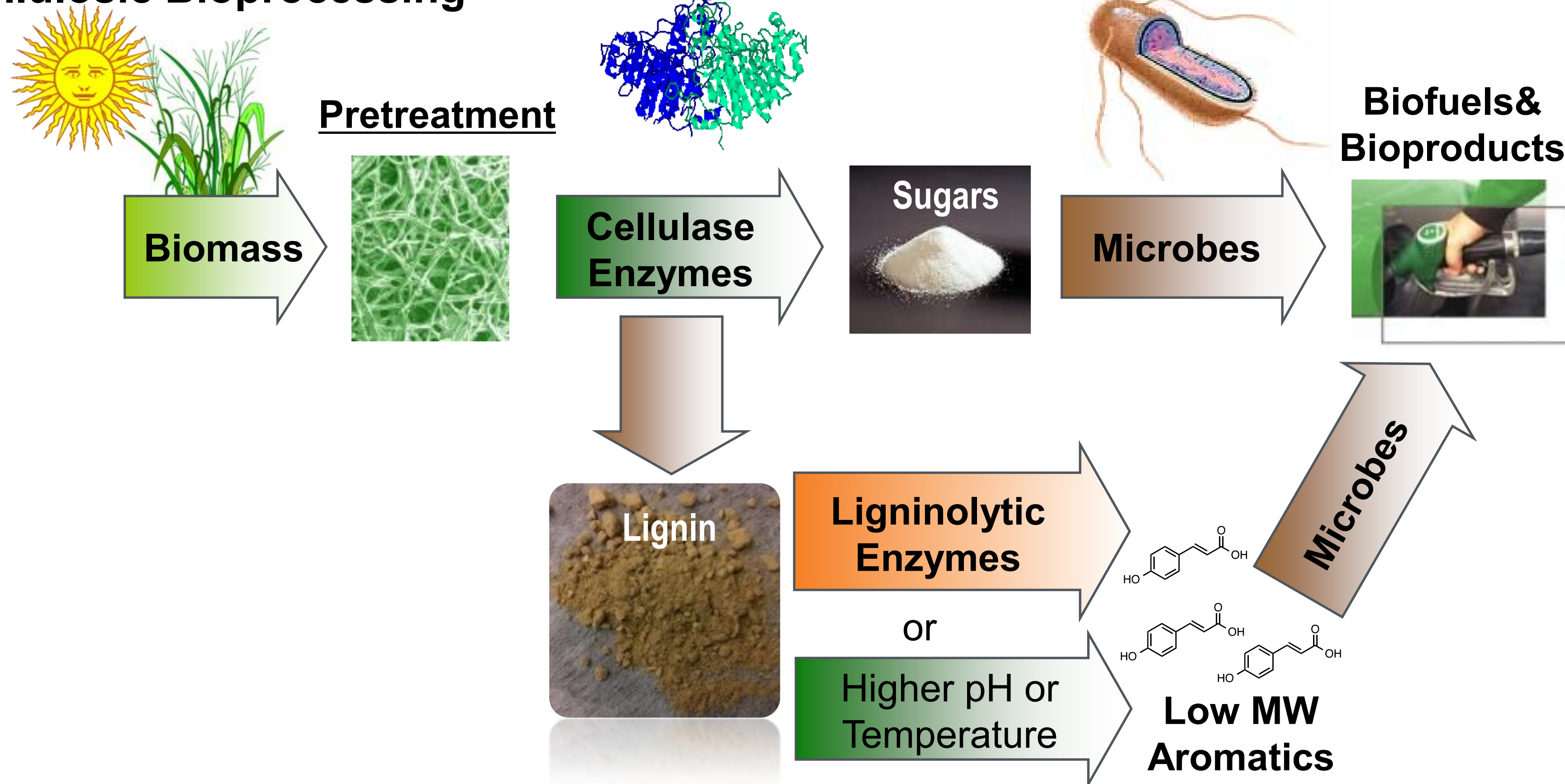
Analysis of the secretome at 9 and 15 days

Control **_P. putida_**





Evaluating optimal depolymerization and sink



- Develop an effective lignin depolymerization approach that enables near complete conversion to co-products
- Determine the optimal configuration of microbes and enzymes: multiple enzymes, multiple microbes, L-CBP

Relevance

Lignin valorization will be essential to achieve 2022 HC fuel cost targets

Highlighted in 2011 Review/CTAB as a key gap in BC Platform
Key MYPP areas for process improvement via lignin utilization:

Hydrolytic enzyme production

- Evaluating ligninolytic enzymes
- Demonstrated effectiveness of fungal secretomes at neutral pH on DMR-EH substrate

Catalyst efficiency

- Combined depolymerization and sink concept for lignin
- Selective transformation of lignin to advanced fuels and chemicals

Biochemical Conversion Process Integration

- Examining ability of biological catalysts to function on a process-relevant substrate

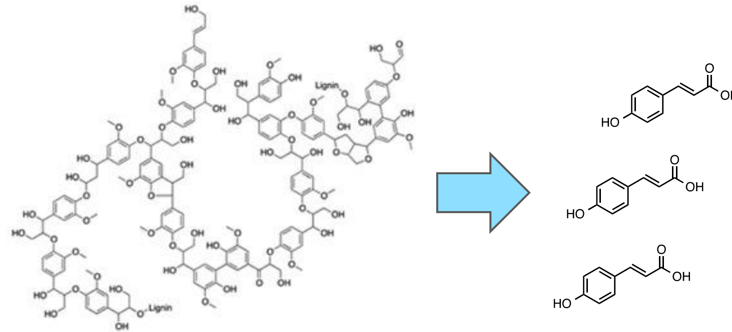
Key Stakeholders and Impacts:

- Lignocellulosic biorefineries: TEA has shown that refineries must valorize lignin to be competitive with petroleum
- Lignin-derived aromatics could be useful for the fuels, chemical, and material science industries
- Efficient lignin depolymerization is a major barrier to lignin utilization
- A successful outcome of this project will lead to technologies that can efficiently depolymerize lignin and simultaneously convert it into value-added bioproducts via microbial cell factories

Future Work

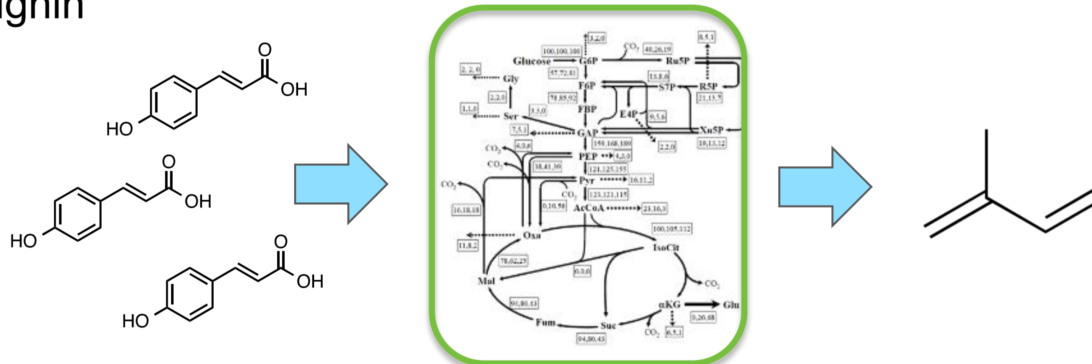
Lignin Depolymerization

- Use omic analysis to understand microbial lignin depolymerization
- Engineer microbial sinks to depolymerize lignin by expression of heterologous enzymes
- Examine ability of microbial “sinks” to depolymerize DMR-EH alone or with fungal enzymes



Lignin Transport and Catabolism

- Mine genomes of microbial “sinks” to identify catabolic pathways to leverage for products
- Conduct metabolomics analysis with ^{13}C labeled aromatics to identify intermediates
- Identify genetic “parts” that can be used from metabolic engineering of microbes to produce bioproducts from lignin



Summary

1) Approach:
   - Develop a biological approach to depolymerize solid lignin for upgrading of low MW aromatic compounds to co-products
2) Technical accomplishments
   - Examined purified enzymes and fungal-derived secretomes on several lignin substrates for ability to depolymerize lignin
   - Determined that reaction conditions and enzyme composition are key factors in promoting lignin depolymerization
   - Found microbial aromatic “sinks” may be critical for extensive lignin depolymerization
3) Relevance
   - TEA shows lignin valorization is critical for lignocellulosic biorefineries
   - Low molecular weigh aromatics have value in several industries
4) Critical success factors and challenges
   - Achieving high yields of low MW, upgradeable species
   - Minimizing lignin monomer repolymerization
   - Overcoming aromatic transport and catabolic rate limitations
5) Future work:
   - Increase efficiency of biological lignin depolymerization
   - Implement a microbial aromatic “sink” to prevent lignin repolymerization and produce valuable bioproducts
6) Technology transfer:
   - Generate IP around lignin depolymerization methodologies
   - Generate novel microbial lignin conversion strategies

Acknowledgements

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External Collaborators

- Adam Guss, Oak Ridge National Laboratory
- R. Robinson, E. Zink, EMSL Pacific Northwest National Laboratory
- John McGeehan, University of Portsmouth
- Jerry Ståhlberg, Mats Sandgren, Swedish University of Agricultural Sciences

Additional slides

- Publications
- Acronyms
- Additional Technical Accomplishment Slides

Publications

Publications in review:

1. D. Salvachua *et al.*, “Lignin Consolidated Bio-Processing: Simultaneous lignin depolymerization and co-product generation by bacteria”, in review.

Publications in print:

2. A. Ragauskas, G.T. Beckham, M.J. Biddy, R. Chandra, F. Chen, M.F. Davis, B.H. Davison, R.A. Dixon, P. Gilna, M. Keller, P. Langan, A.K. Naskar, J.N. Saddler, T.J. Tschaplinski, G.A. Tuskan, C.E. Wyman, “Lignin Valorization: Improving Lignin Processing in the Biorefinery”, *Science* (2014), 344, 1246843.

Acronyms

- DDR-EH Lignin: Deacetylated, Disk-Refined, Enzymatically Hydrolyzed Lignin
- DyP: Dye-decolorizing Peroxidase
- LiP: Lignin Peroxidase
- MnP: Manganese Peroxidase
- MW: Molecular Weight
- VP: Versatile Peroxidase

Characterization of ligninolytic cocktails

To measure enzyme activities, we utilized UV- and colorimetric assays to follow substrate oxidation

Laccases:
ABTS at pH 5
Reactive blue 19 at pH 3

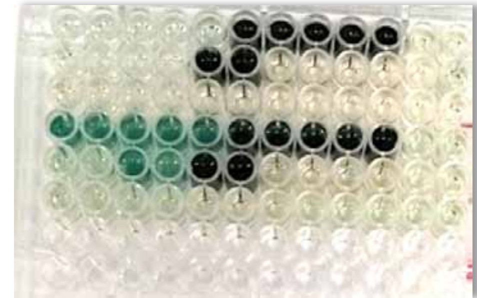
Manganese Peroxidase (MnP):
ABTS + H_2O_2 at pH5 (also for DyP and VP)
ABTS + H_2O_2 + Mn^{2+} at pH 5
$MnSO_4$ + H_2O_2 at pH 5

Lignin Peroxidase (LiP):
Veratryl alcohol + H_2O_2 at pH 3

Dye-decolorizing peroxidase (DyP):
Reactive Blue 19 + H_2O_2 at pH 3

Versatile peroxidase (VP):
Reactive black 5 + H_2O_2 at pH 5

Aryl-alcohol oxidase (AAO):
Veratryl alcohol at pH 5



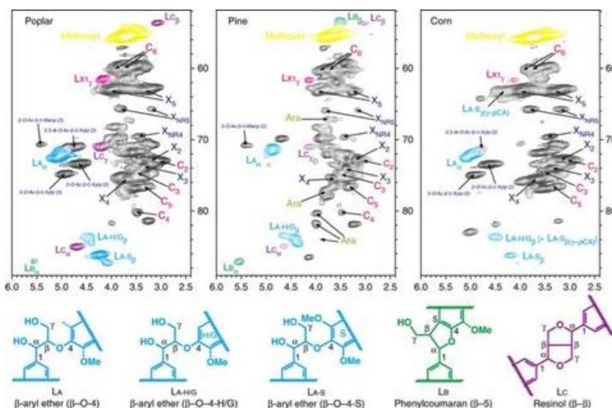
ABTS assay

Reactive Blue 19 assay

Detailed characterization of lignin depolymerization

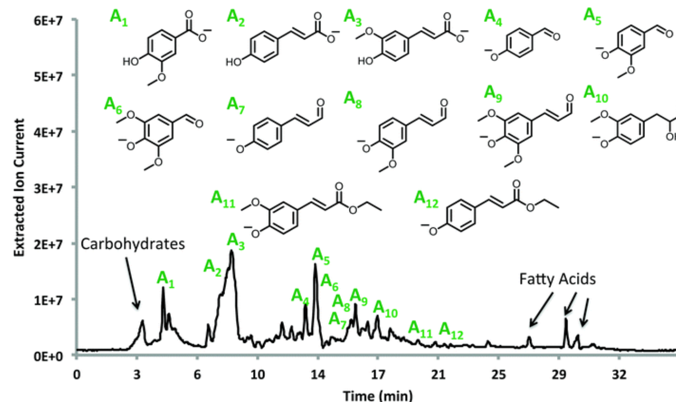
- In order to further develop depolymerization strategies and microbial aromatic "sinks" we must first have a detailed understanding of the DMR-EH lignin macromolecule and degradation products
- Will employ a variety of analytical techniques to characterize the lignin, including mass spectrometry (MS), size exclusion chromatography (SEC), and nuclear magnetic resonance (NMR)

NMR- linkages



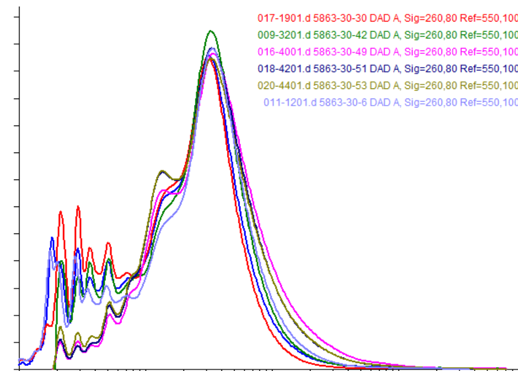
Nature Protocols **7**, 1579–1589 (2012)

MS- identification



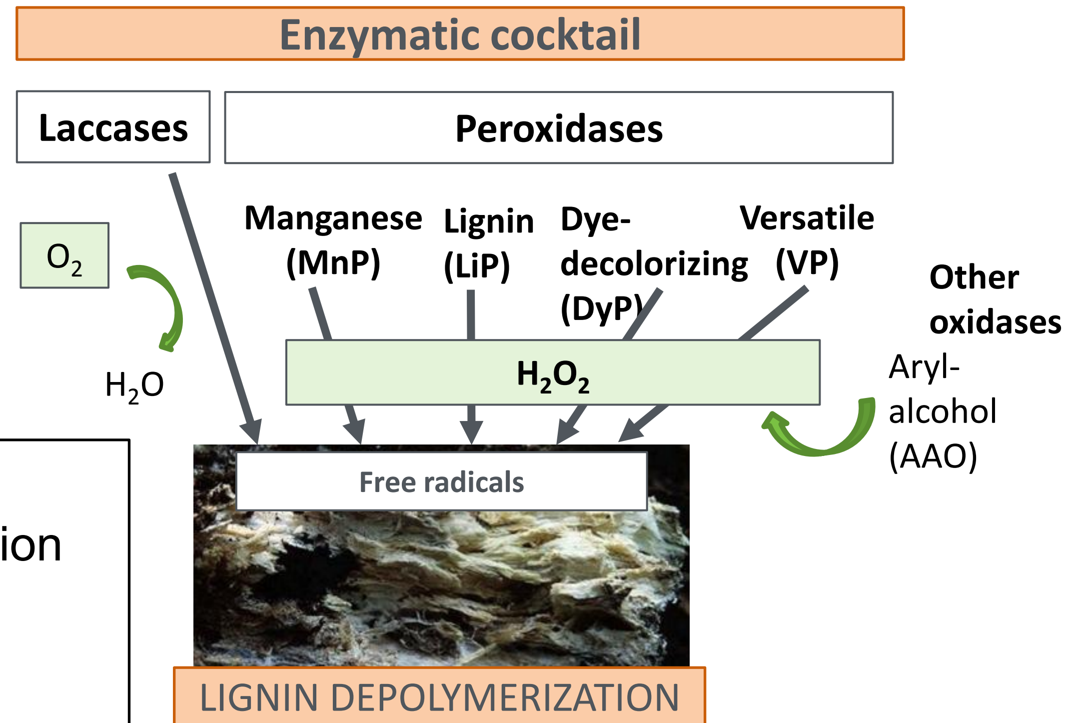
Green Chem., 2014, **16**, 2713-2727

SEC- size



Biological Lignin Depolymerization: Fungi

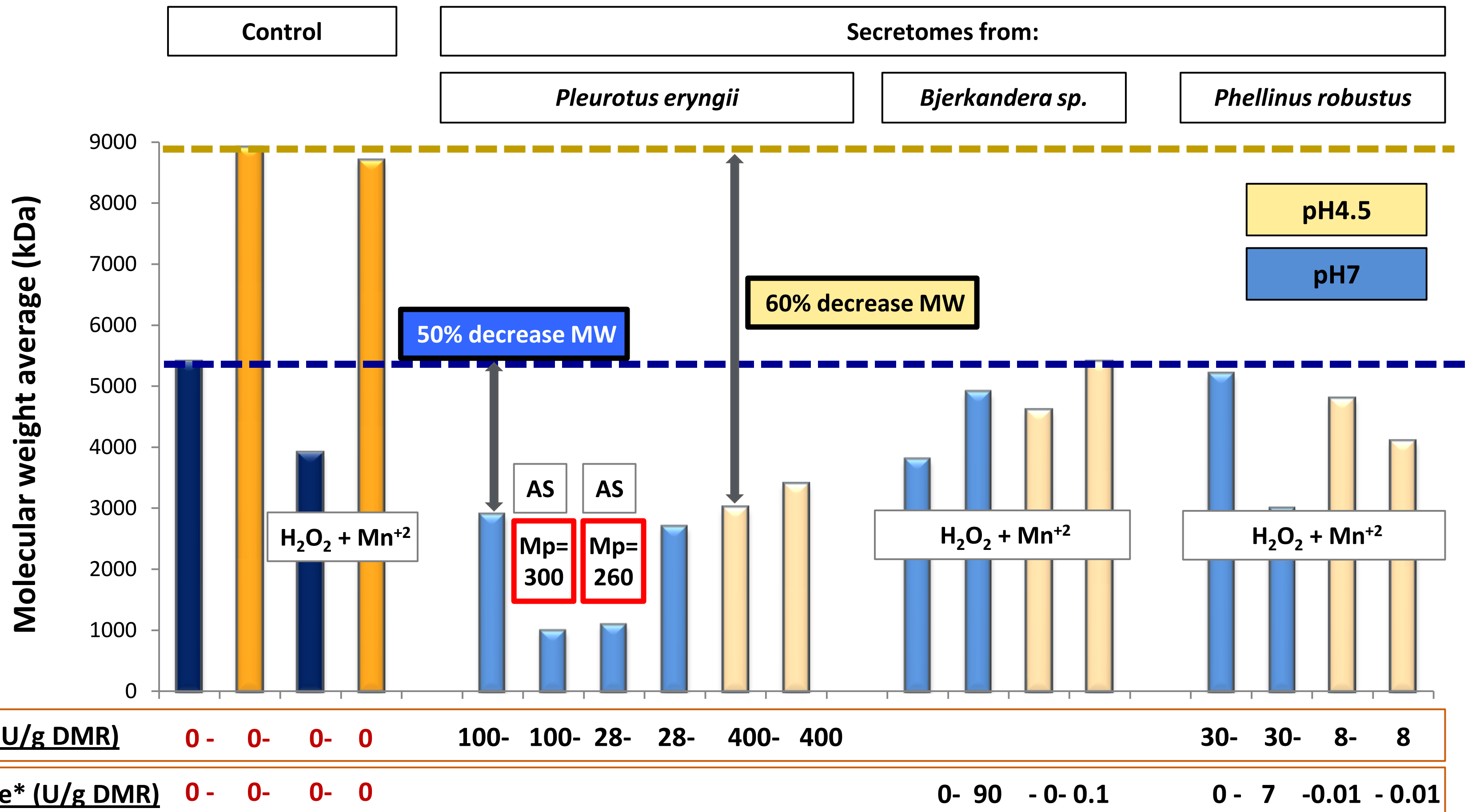
- Laccases and peroxidases are the major enzymes involved in microbial lignin depolymerization
- Require O_2 and H_2O_2 respectively for activity



Approach:
- Characterized microbial degradation of DDR-EH lignin
- Identify parameters for optimal biological lignin degradation

FY14 Research: Start with Fungi
- Use **purified fungal enzymes** to optimize conditions for lignin depolymerization
- Examine the **lignolytic enzyme cocktails produced by basidiomycete fungi** to optimize conditions and identify the enzymes present in natural secretomes

DMR-EH lignin depolymerization

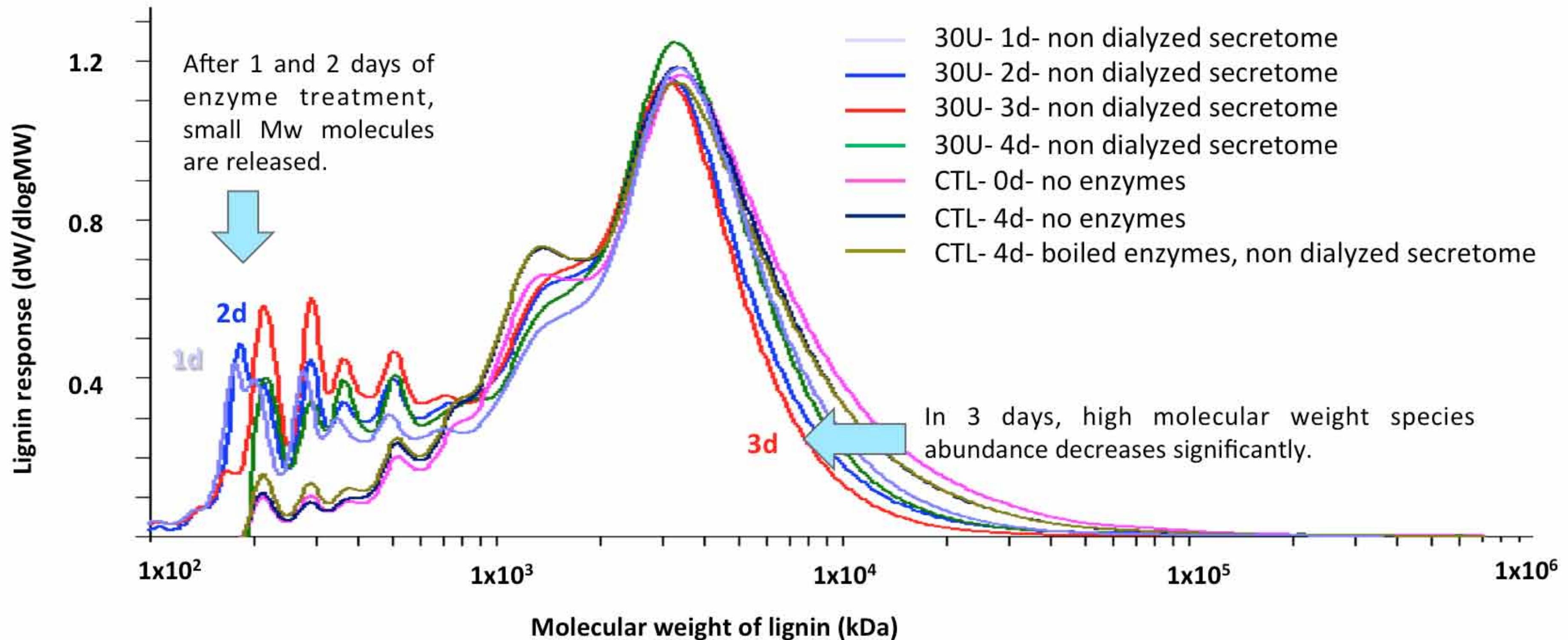


***P. eryngii* secretome is an effective lignin degrading enzyme cocktail**

- Obtained major difference between pH 4.5 and 7 in both initial MW and MW change
- *P. eryngii* samples seem able to produce a substantial amount of low MW species
- Selected *P. eryngii* secretome at pH 7 for more in-depth experimental studies

Treatment of DMR-EH lignin with the *P. eryngii* secretome

GPC chromatogram

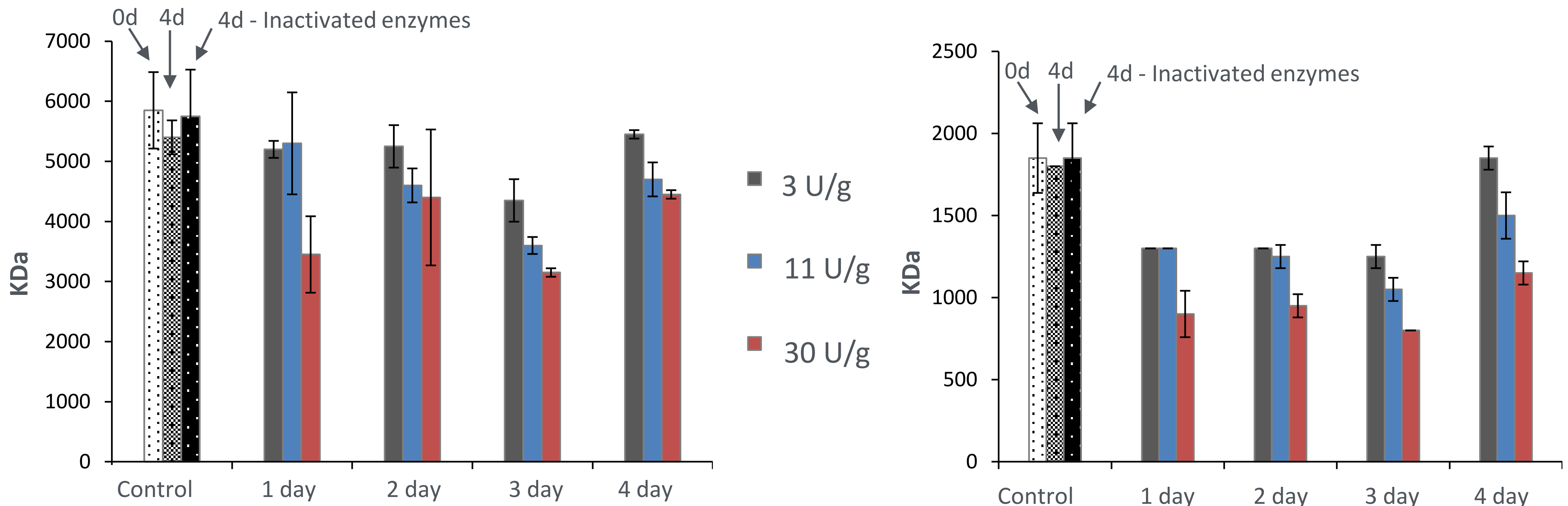


Fungal ligninolytic enzymes are clearly depolymerizing lignin, however, as we can see at 4 days of incubation no more depolymerization is observed although the enzymes are still active. Is it due to repolymerization?

Treatment of DMR-EH lignin with the *P. eryngii* secretome

P. eryngii **secretome** → **Different enzyme dosages at pH 7** (3, 11, 30 U laccase/g substrate) → **DDR-EH lignin (2%)** *Autoclaved* → **Time-course analysis**

GPC results: Mw and Mn are the values at which there are equal **masses** or **number** of molecules on each side, respectively.



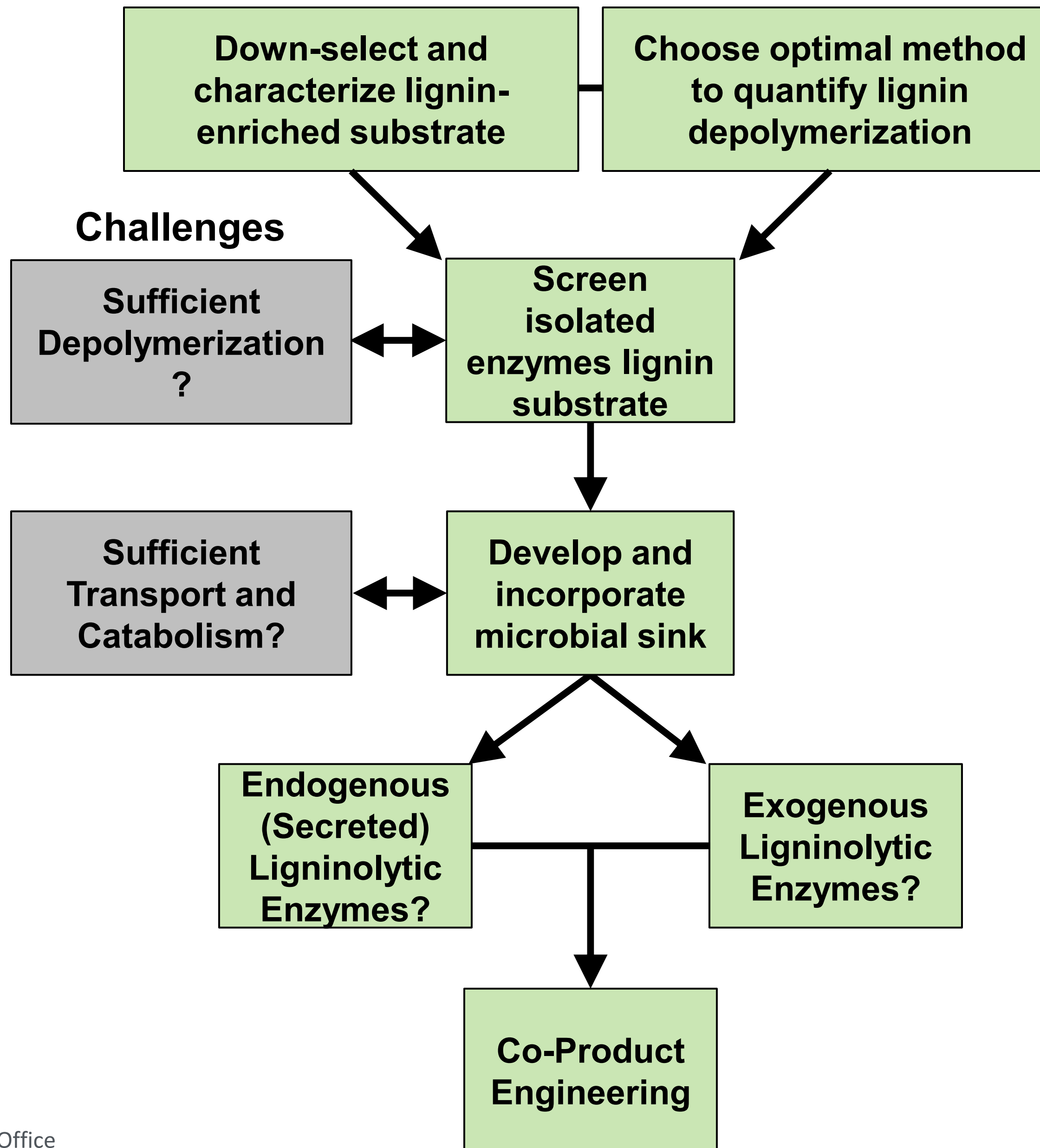
P. eryngii secretome shows rapid DDR-EH lignin depolymerization

- 50% of lignin species are smaller than the control (M_n) in a single day of treatment
- Control experiments with boiled enzymes and without enzymes show no depolymerization
- Analyzed detailed molecular weight distributions (next slide)

Current Key Milestones and Findings

- Examined purified enzymes and fungal-derived secretomes on several lignin substrates for ability to depolymerize lignin
- Determined that reaction conditions and enzyme composition are key factors in promoting lignin depolymerization
- Found evidence that lignin repolymerization is an issue that must be addressed to achieve maximum lignin depolymerization
- Repolymerization may be prevented using a microbial “sink” to remove low molecular weight lignin products form the reaction
- Superseded our FY14 Go/No-Go of attaining 20% lignin depolymerization
- For FY15, we have assembled a list of microbial “sinks” and initiated a detailed characterization of DMR-EH lignin

Workflow

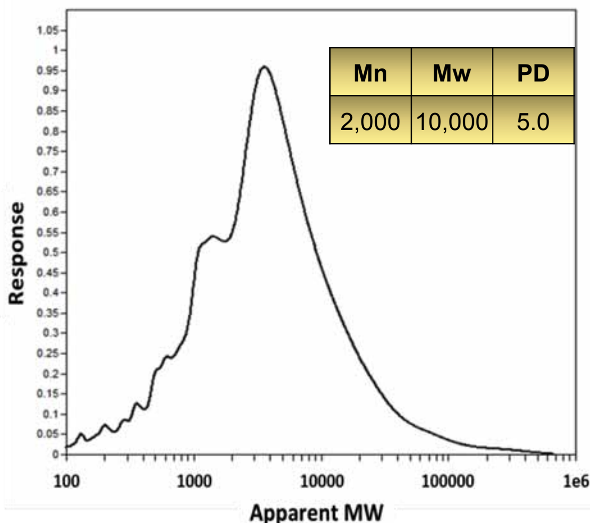


FY15 Milestone: Characterization of solid DMR-EH

Compositional analysis

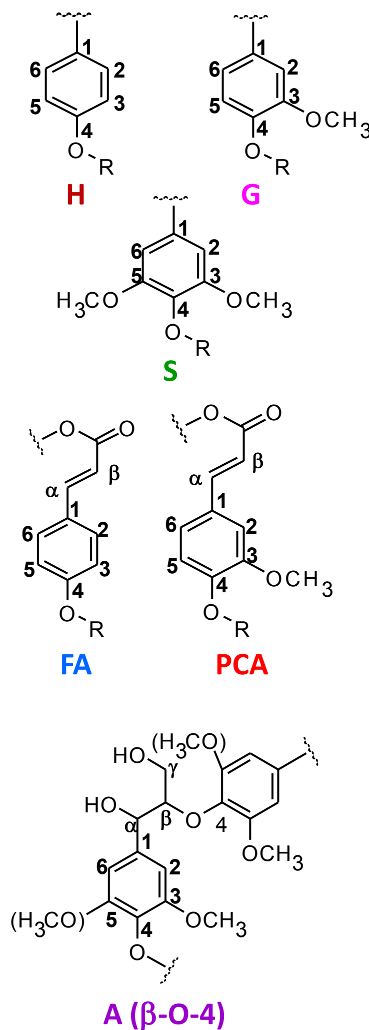
Sample ID	Content (%)
Ash	2.18
Lignin	66.0
Glucan	9.24
Xylan	9.36
Galactan	1.04
Arabinan	1.62
Fructan	0.00
Acetate	0.72
Total sugar	21.26
Total	90.2

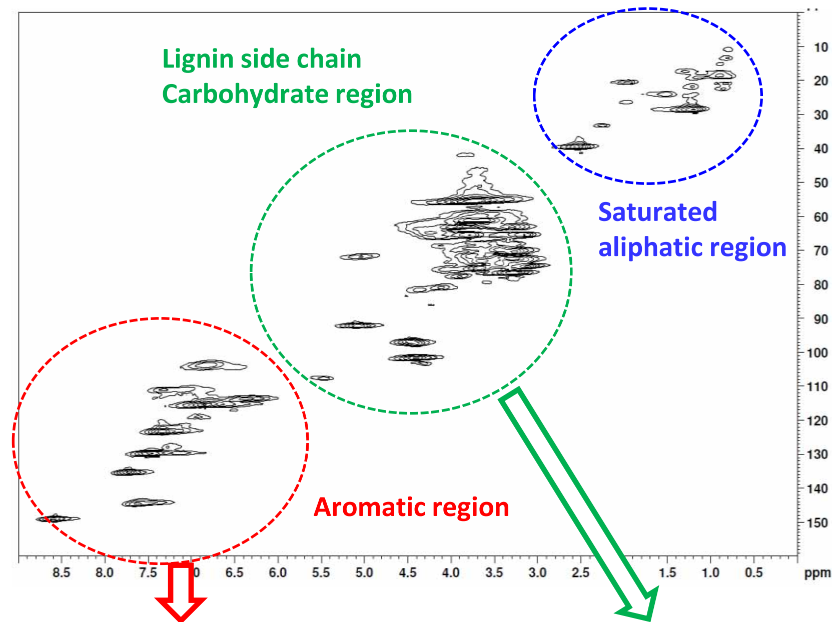
Molecular weight distribution

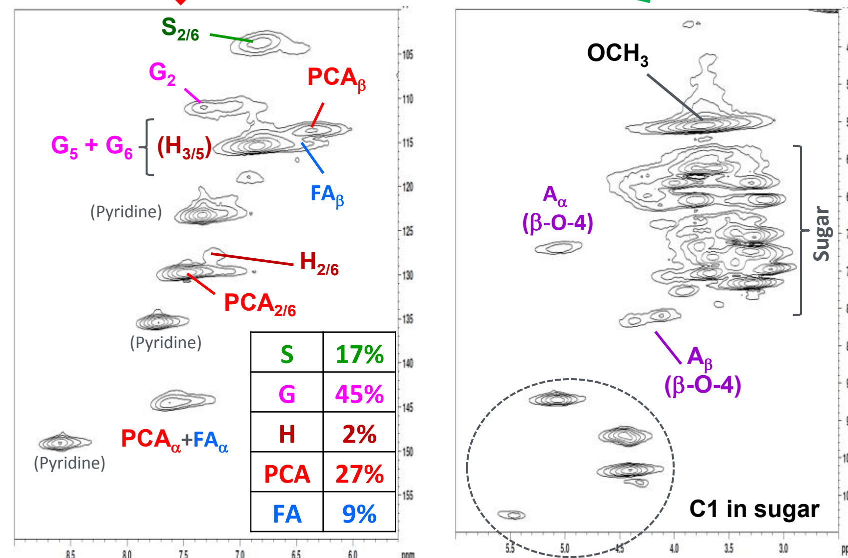


Mn	Mw	PD
2,000	10,000	5.0

2D-NMR (HSQC)







S	17%
G	45%
H	2%
PCA	27%
FA	9%