U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy

2017 DOE BioEnergy Technologies Office (BETO) Project Peer Review

Date: March 7th, 2017

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

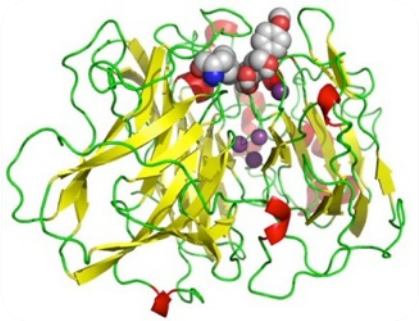
Goal: develop a biological approach to depolymerize lignin for upgrading of aromatic compounds to co-products

Outcome: biological solutions to break down lignin polymers

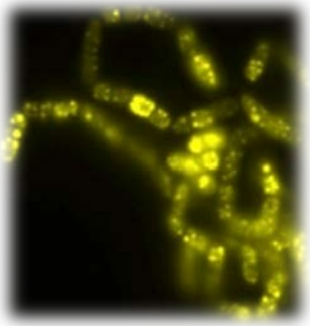
Relevance to BC Platform, BETO, industry: lignin valorization is key for meeting HC fuel cost and sustainability targets in integrated biorefineries

Residual Biorefinery Lignin

Ligninolytic Enzymes



Aromatic-catabolizing bacteria



NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Sandia National Laboratories

Quad Chart

Timeline

- Start date: October 2014
- End date: September 2017
- Percent complete: ~83%

Barriers

- Ct-E Efficient Low Temperature Deconstruction
  - Evaluating enzymatic routes to lignin depolym.
- Ct-H Efficient Catalytic Upgrading of Aromatics
  - Incorporating enzymes into aromatic-catabolic microbes
- Ct-J Process Integration
  - Working with process-relevant substrates

Budget

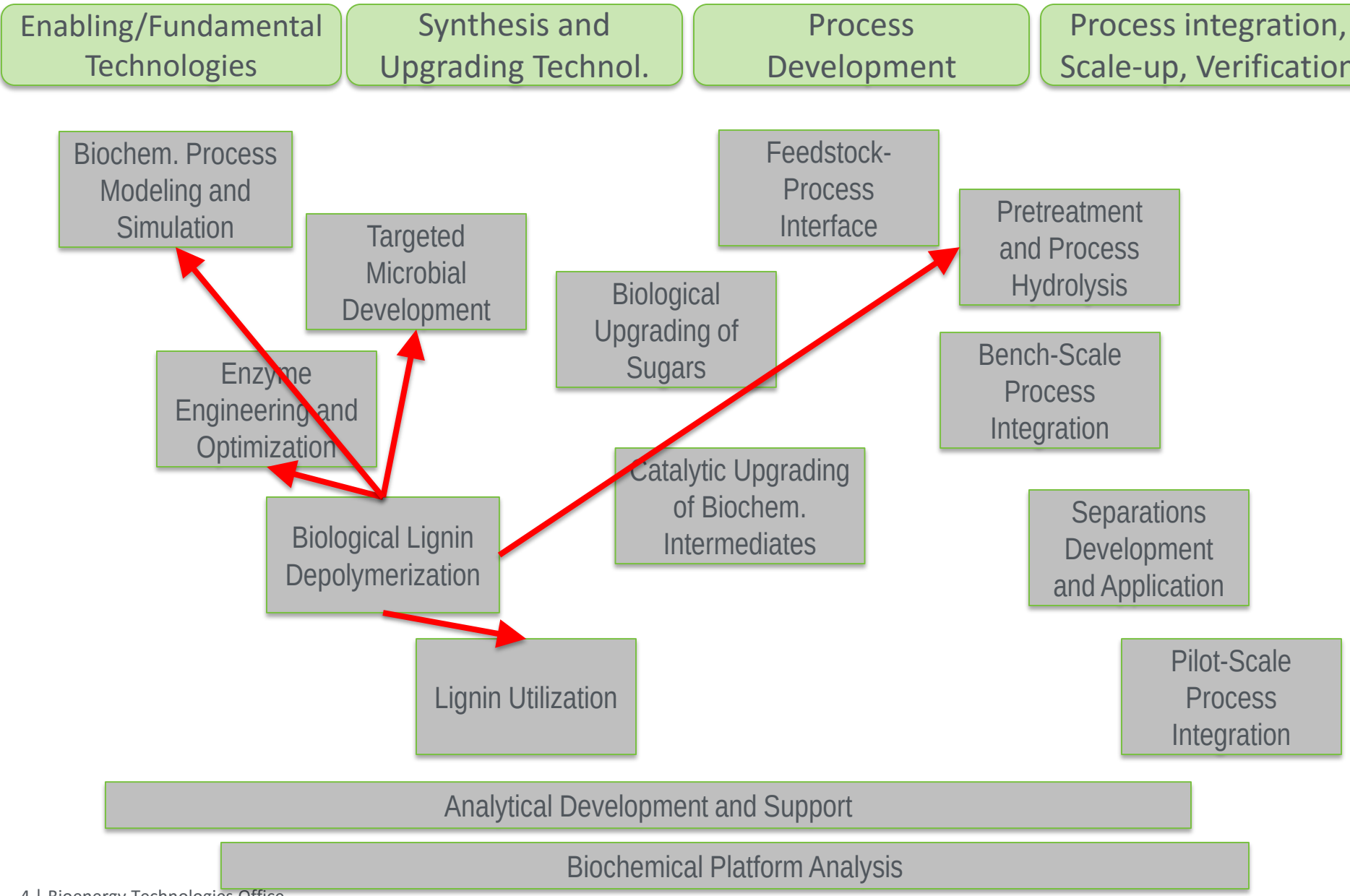
	FY14 Costs	FY15 Costs	FY16 Costs	Total Planned Funding (FY17-Project End Date)
DOE funded	$229k	$443k	$581k	$550k

Funds are split 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

Biochemical Conversion Projects - NREL



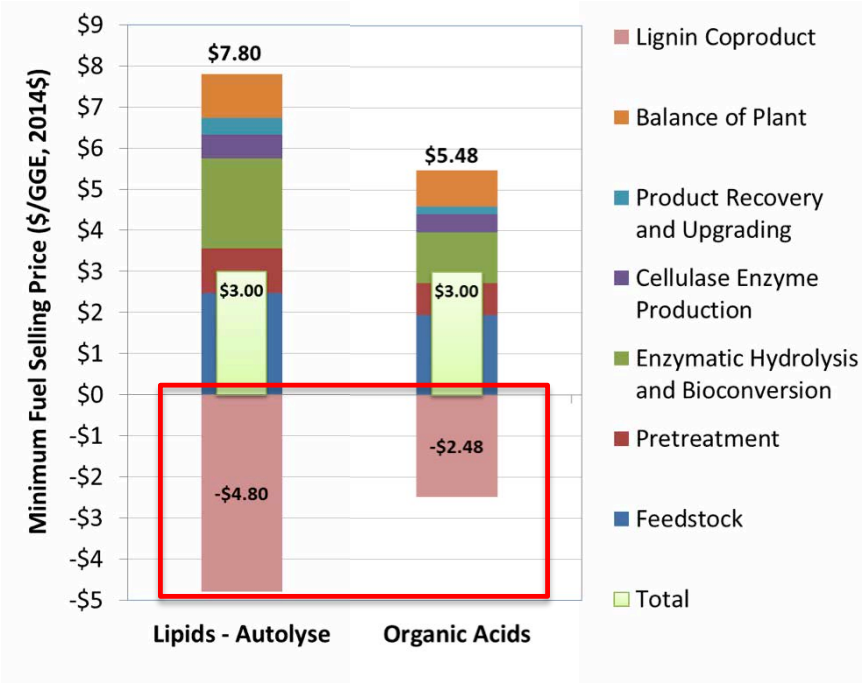
Project Overview

Project History:

- Began as a BETO seed project in FY14
- Achieved major milestone end of FY14
- Complementary to other BETO-funded lignin projects

Project Context:

- Lignin valorization is key for $3/gge targets
- Potential for $2.50-5/gge credit



High-Level Objective: Understand and employ biology for lignin depolymerization

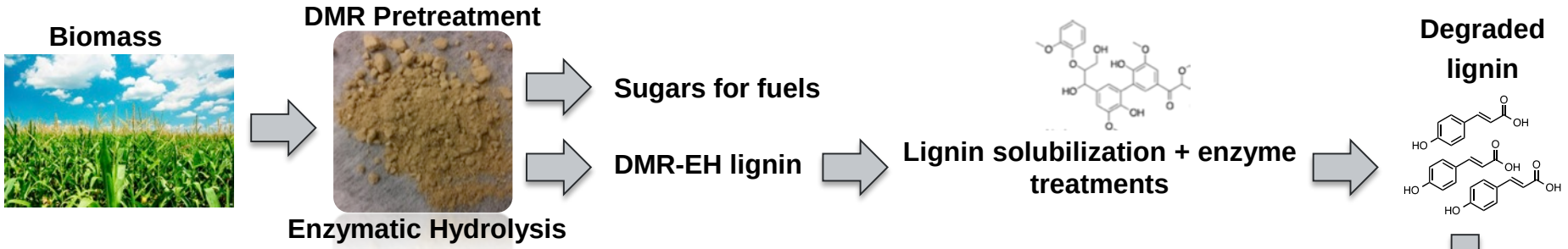
Aim 1: Lignin Solubilization

- Focus on residuals from Deacetylation-Mechanical Refining Prt.
- Examine secretomes for solid and soluble lignin depolymerization ("top down")

Aim 2: Biological Depolymerization

- Understand catabolism of lignin linkages
- Identify necessary enzymes for breaking specific linkages ("bottom up")
- Engineer improved depolymerization activity into microbes

Technical Approach



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

Approach:

- Characterize enzymatic degradation of DMR-EH lignin
- Identify parameters for optimal biological lignin degradation
- Engineer organisms depolymerize lignin polymers and oligomers

Primary challenges:

- Effective **analytical tools** to quantify lignin depolymerization
- DMR-EH lignin is not water soluble, possibly limiting enzyme accessibility
- Minimizing **repolymerization** of high MW lignin during depolymerization

Success factors:

- Achieving high yields of low MW lignin species
- Generating aromatics that can be metabolized by microbes

Management Approach

Critical Success Factors:

- Employ tools of synthetic biology and lignin analytical chemistry
- Leverage complementary experience from two biomass conversion centers
- **Strategic collaboration** 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 6-9 months
- Contributions from both partners on lignin analytical chemistry leveraging respective expertise
- Access EMSL (PNNL) capabilities via competitive proposals

Technical Results – Outline

1- White-rot FUNGI

Optimal for depolymerizing lignin
Difficult to genetically manipulate

2- BACTERIA

Poor lignin degraders
- Genetically tractable
- Production of value added-compounds from aromatics

3- ENZYMES from white-rot fungi

Novel approach to depolymerize lignin
- Peroxidases
- Laccases
- Oxidases
- Reductases

4- ENZYMES + BACTERIA

Fungal enzymes + bacteria
- Recombinant bacteria secreting ligninolytic enzymes

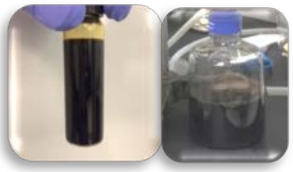
Project Storyline

Black liquor conversion by bacteria

Fungal enzymes (+ bacteria) for DMR-EH lignin depolymerization

Secretion of recombinant ligninolytic enzymes by bacteria

BCD-DMR-EH lignin conversion by bacteria



-Omics: searching for new enzymes

FY14 → **FY17**

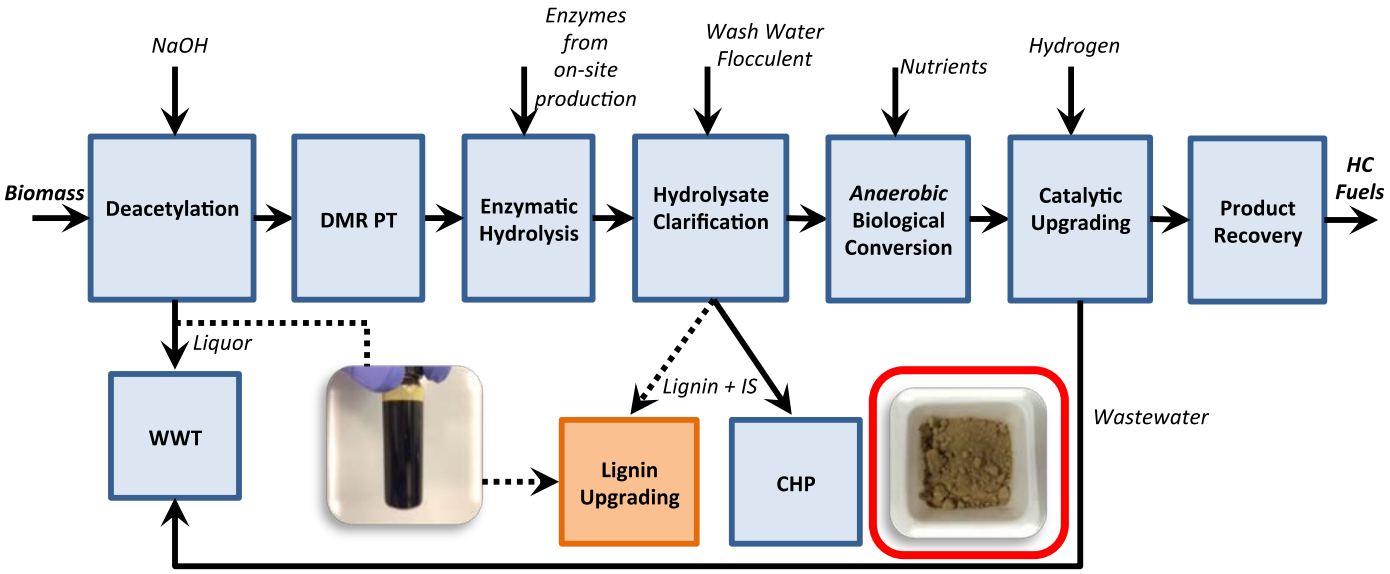
Conversion=30%

Lignin Mw=↓75%

Secretion = ✓
Lignin conversion = ✗

Solubilization = 80%
Conversion = 15%

Working with a relevant biorefinery substrate

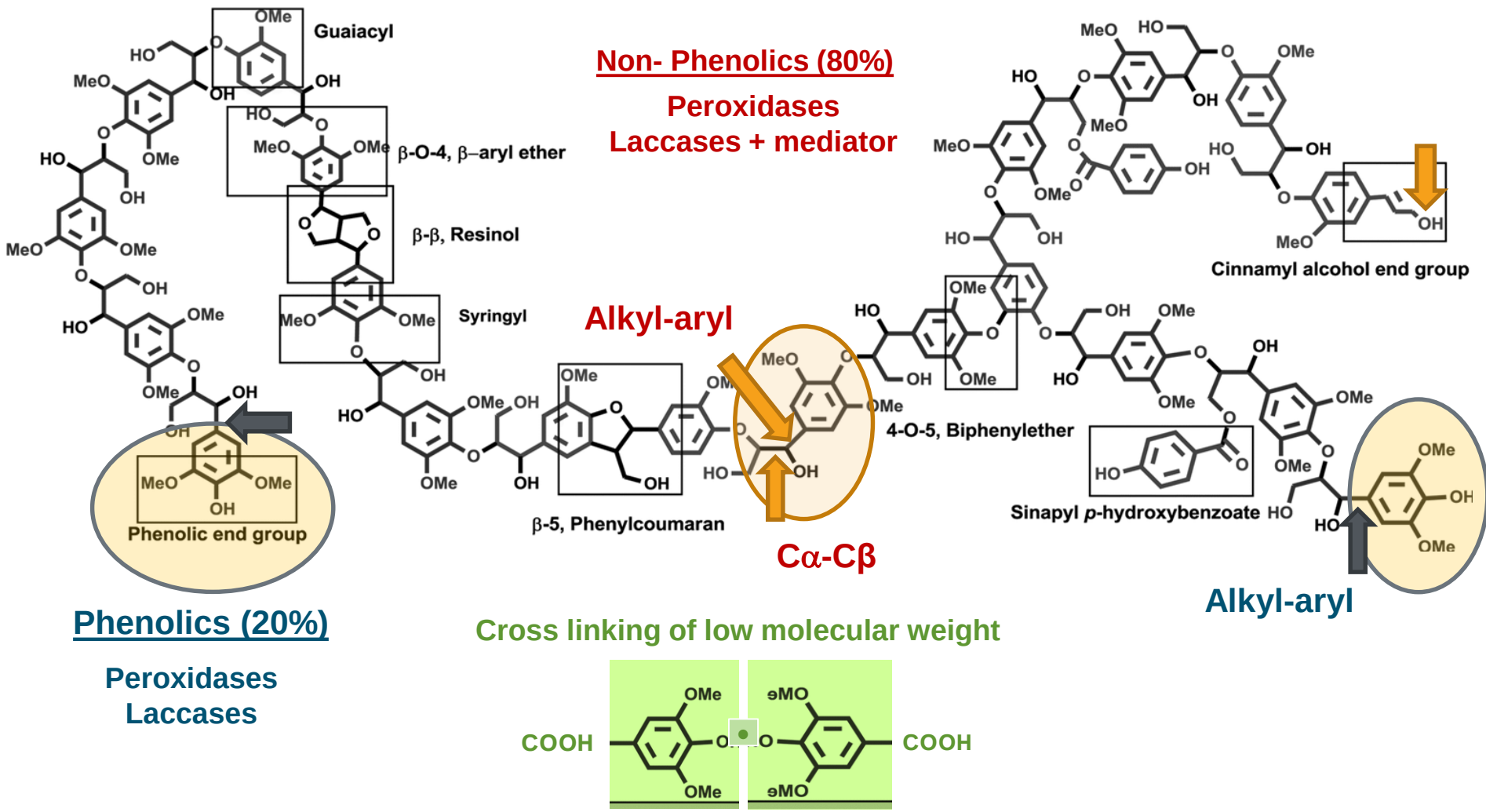


	Corn stover (%)	Black liquor (g/L)	DMR-EH lignin (%)
Lignin	**14.8**	**11.2**	**60.0**
Ash	2.5	13.1	2.2
Glucan	36.5	**0.3**	**9.24**
Xylan	30.2	1.6	**9.4**
Galactan	1.8	0.6	1.0
Arabinan	3.5	1.1	1.6
Acetate	2.7	**2.6**	0.7
Proteins	NA	NA	6.1
Total mass balance	**92.0**	30.6 (**95.5%**)	**90.2**

DMR-EH lignin exhibits native-lignin like properties

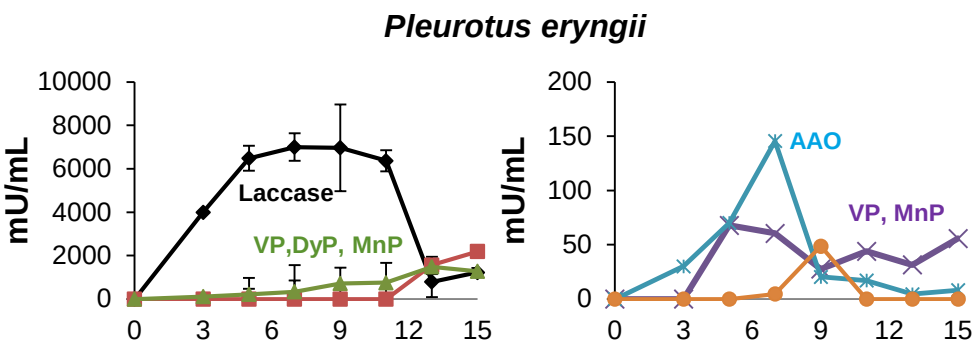
- DMR pretreatment generates a highly digestible substrate, enabling effective sugar recovery.
- Lignin content is up to 60 % and its **average** molecular weight is **>6,000 Da**

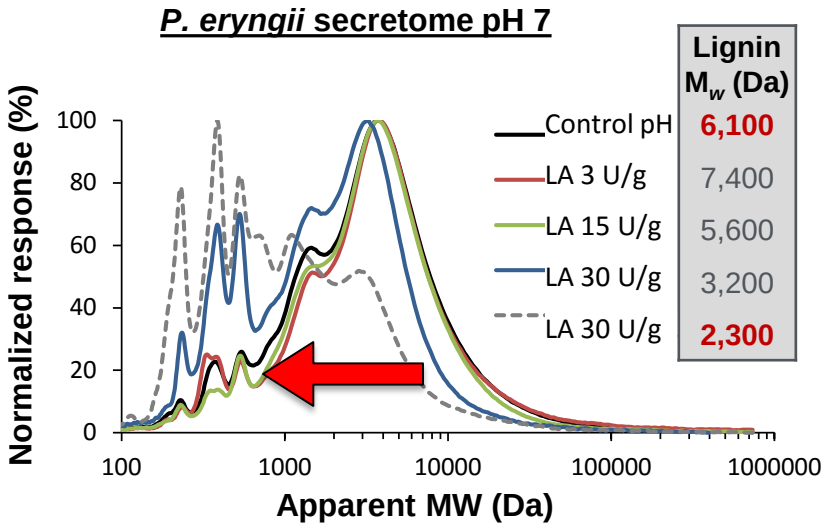
Lignin depolymerization by ligninolytic enzymes



Laccases and peroxidases generate radicals that can further initiate alkyl-aryl cleavage, Cα-Cβ, cross linking, demethoxylation, and ring cleavage of aromatic compounds.

Enzyme screening in fungal cocktails



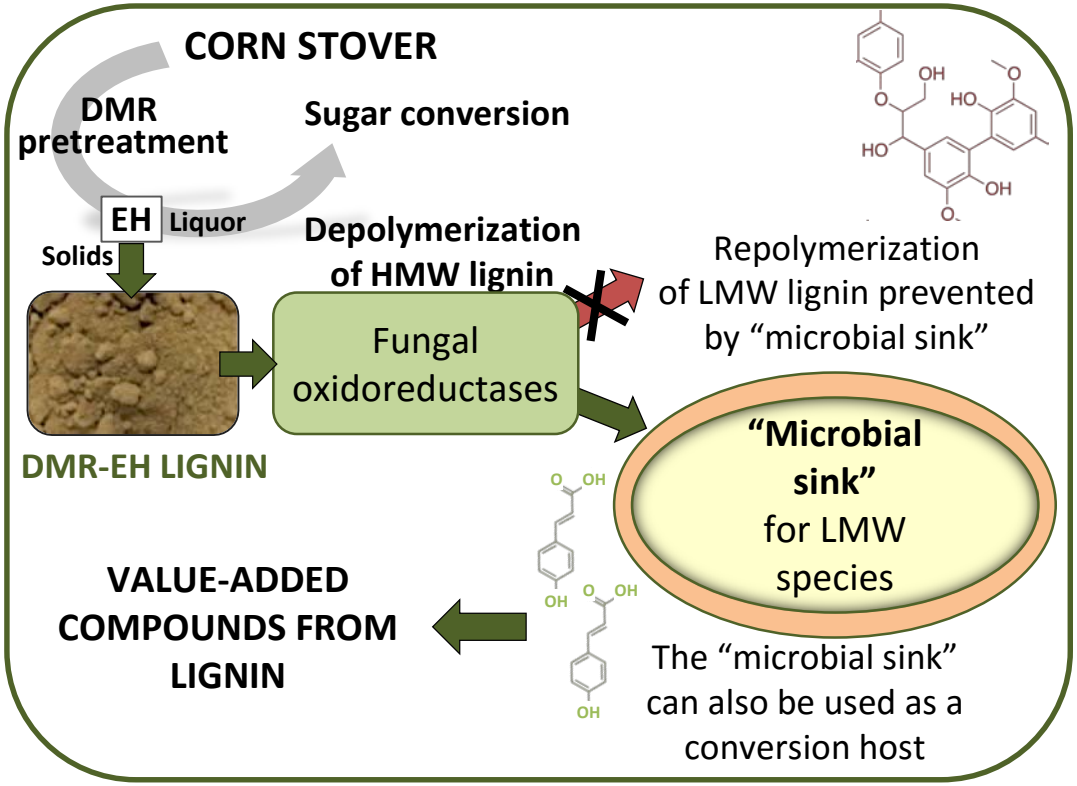


Screened 12 white-rot fungi secretomes with solid DMR-EH lignin as a sole carbon and energy source

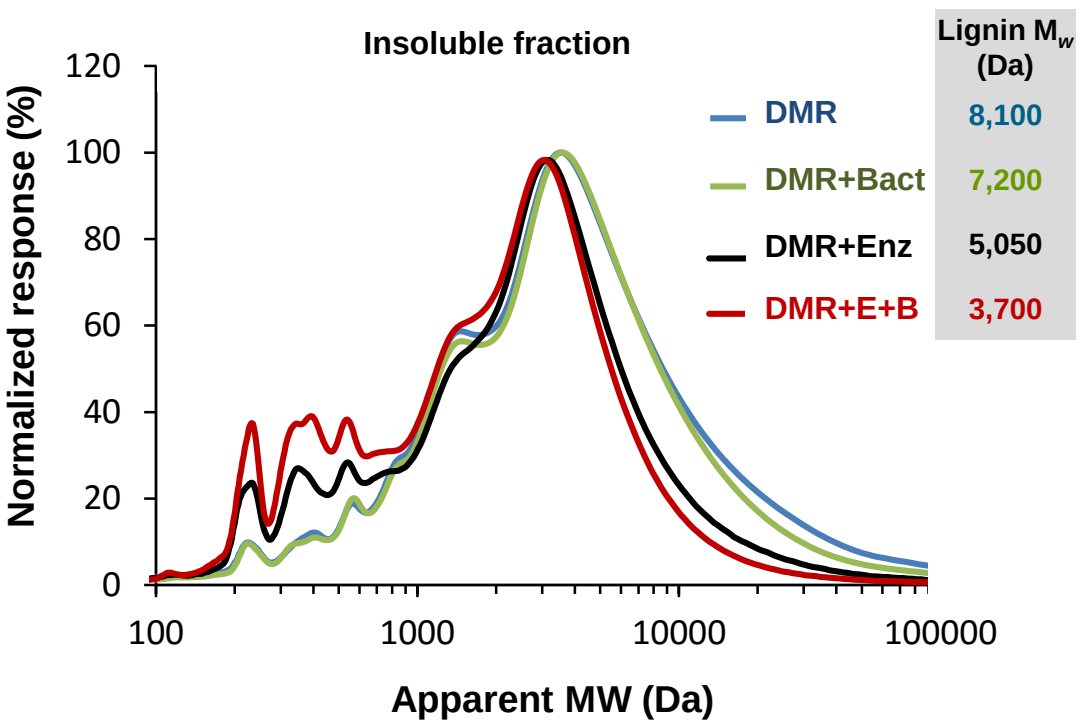
- *Pleurotus eryngii* produced the highest laccase levels on solid DMR-EH lignin
- Isolated and evaluated secretome (sans fungus) on DMR-EH lignin at pH 7 (compatible with bacteria)
- Achieved substantial MW reduction (6,100 Da to 2,300 Da) but observe **repolymerization**
- **Outcome:** Identified a lignin depolymerizing fungal secretome, but need to prevent repolymerization

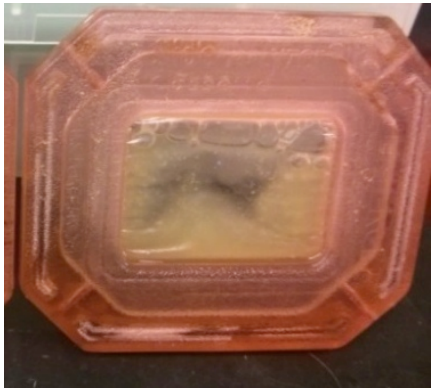
Avoiding repolymerization via a microbial sink

- **Polymerization** of low MW lignin is "unavoidable" when laccases and peroxidases are utilized as they generate aromatic radicals that can repolymerize LMW lignin
- **Hypothesis**: bacteria can inhibit repolymerization by scavenging LMW lignin compounds as they are generated by ligninolytic enzymes



Evaluating the microbial sink hypothesis





Fungal enzymes with a microbe enhance depolymerization of insoluble lignin

- Lignin depolymerization is significant, but overall solubilization is not drastically improved
- Lignin "solubilization" by **chemical means** is needed to enhance lignin access for further biological upgrading

Solubilizing lignin streams with NaOH

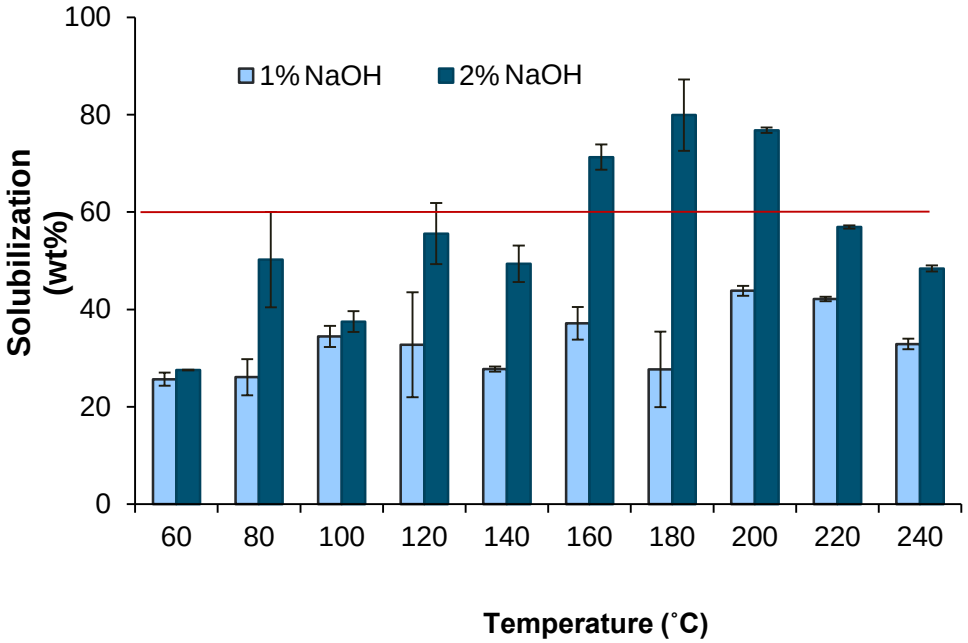
DMR-EH lignin

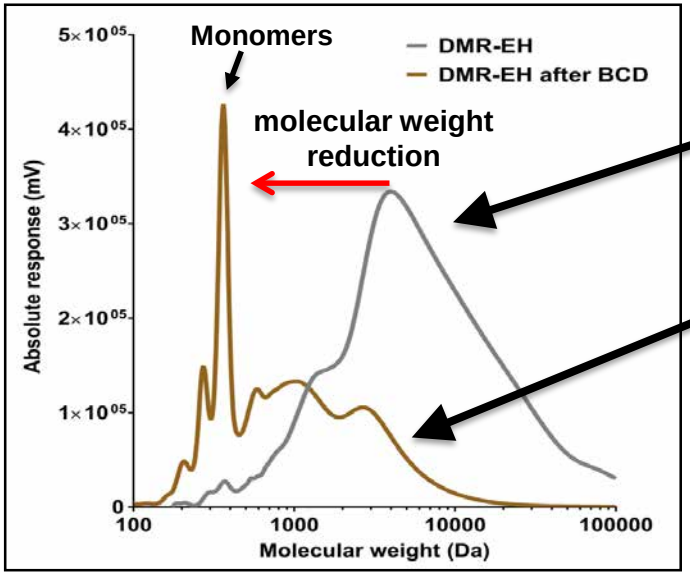
10% lignin loading, 1, 2%NaOH, 120°C, 45 min

Few solids are left after the reaction (150 mL/reactor)

Neutralization with H_2SO_4 to pH 7 and centrifugation to remove solids

Leveraging work from the Lignin Utilization project for lignin solubilization

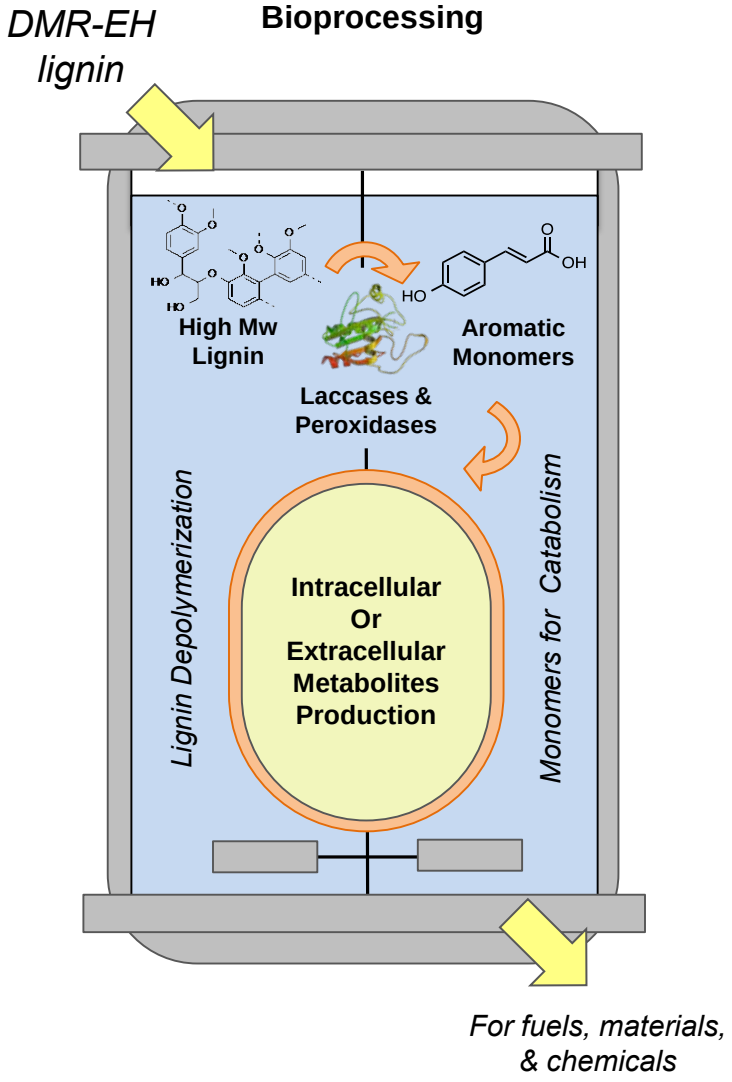




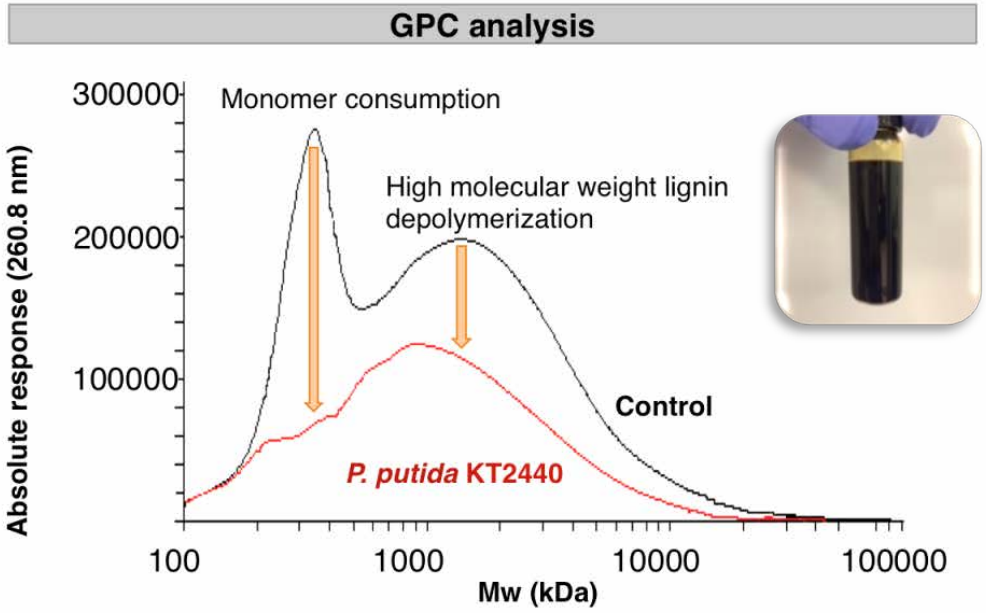
P. putida can partially depolymerize soluble lignin

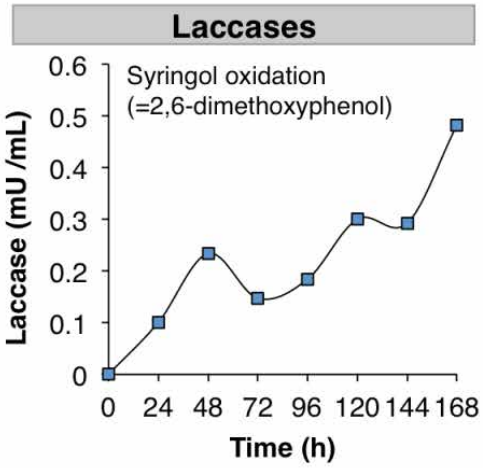
L-CBP

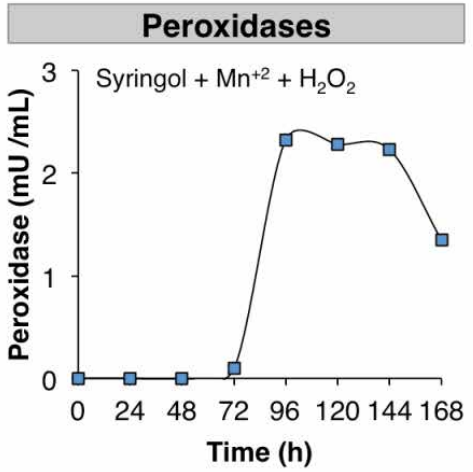
Lignin Consolidated Bioprocessing



Alkaline treated liquor from corn stover (30% conversion)

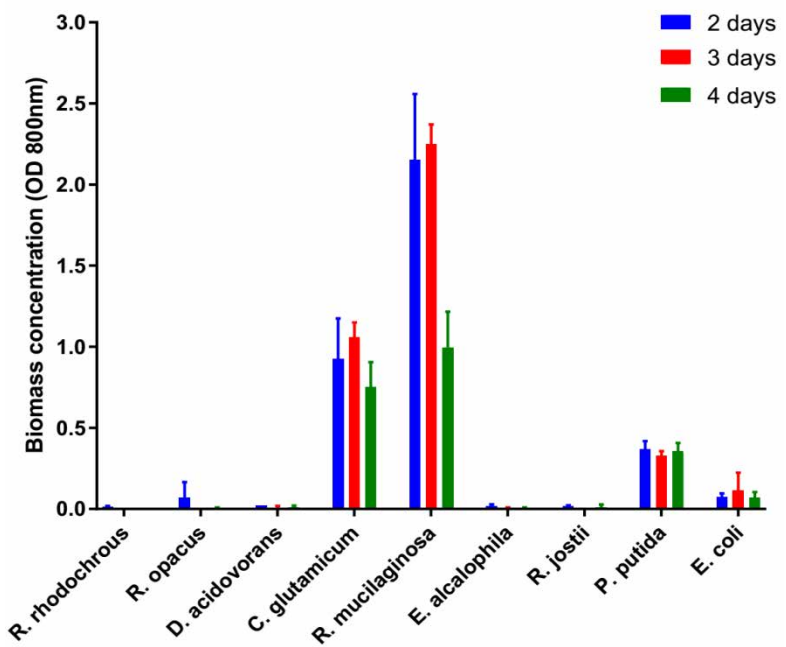




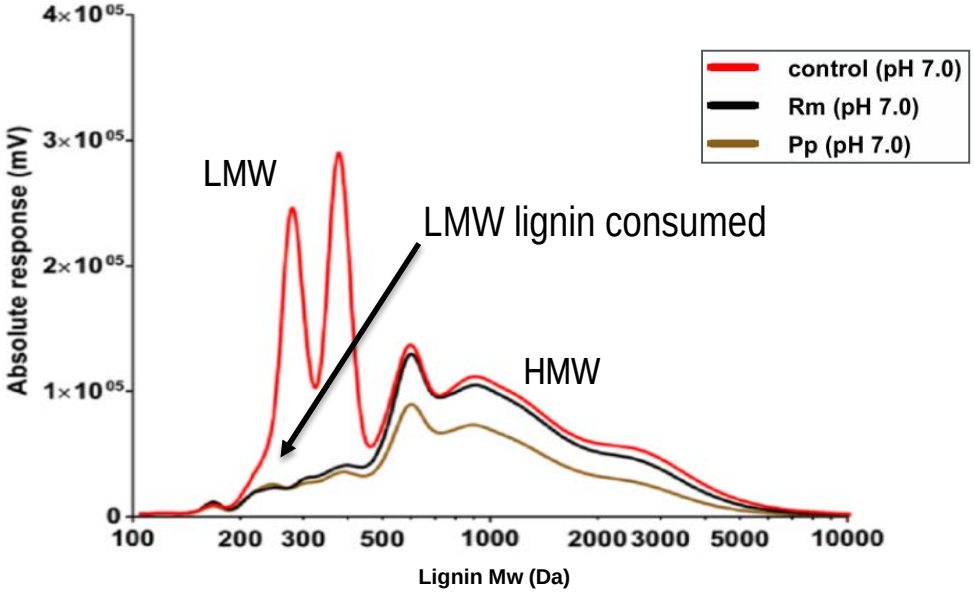


Multiple microbes can consume solubilized DMR-EH lignin

Three microbes grow on BCD lignin



And consume the LMW lignin…



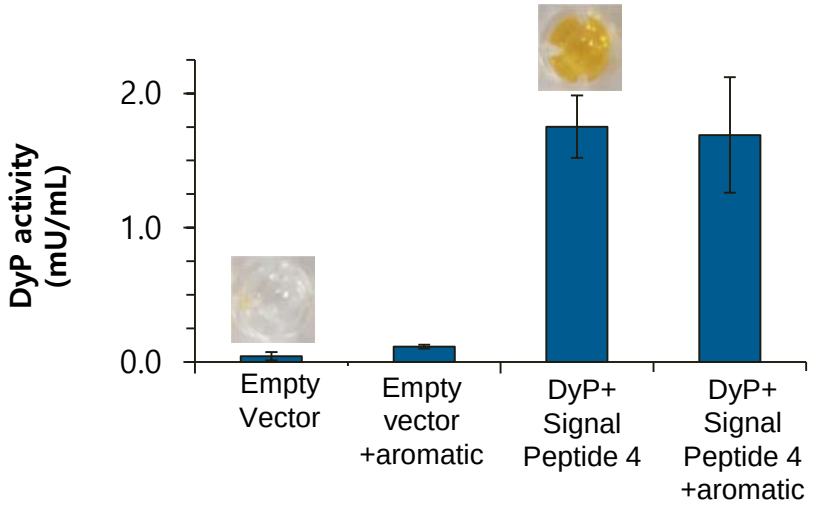
- Three organisms grow on concentrated BCD lignin: *P. putida, Corynebacterium glutamicum,* and *Rhodotorula mucilaginosa*
- The consumption of the LMW lignin can decrease the total lignin content by up to 15%

- The three microbial sinks can remove most of the LMW BCD lignin products
- Leave HMW lignin for further biological depolymerization

Can we engineer bacteria to **depolymerize** more lignin?

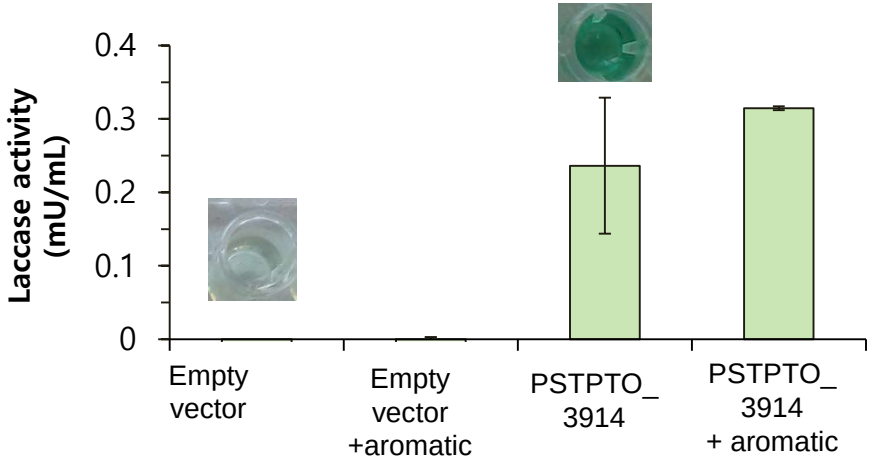
Recombinant expression of bacterial Dye-Decolorizing Peroxidase (DyP) from *Amycolatopsis* sp. in *P. putida* (pH 4)

4 potential signal peptides were tested for DyP secretion



Recombinant expression of bacterial laccases in *P. putida* (pH 7 and 8)

5 potential bacterial laccases were tested with the selected signal peptide



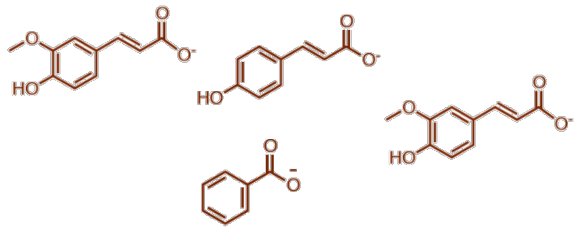
Goal: increase production of ligninolytic enzymes compared to the native species

- As demonstrated, ligninolytic enzymes plus bacteria enhance lignin depolymerization. Another approach: **are bacteria secreting ligninolytic enzymes able to grow on solid lignin**?
- *P. putida* secreted both enzymes but still at low levels
- Assays on BCD solubilized lignins do not lead to higher extents of depolymerization

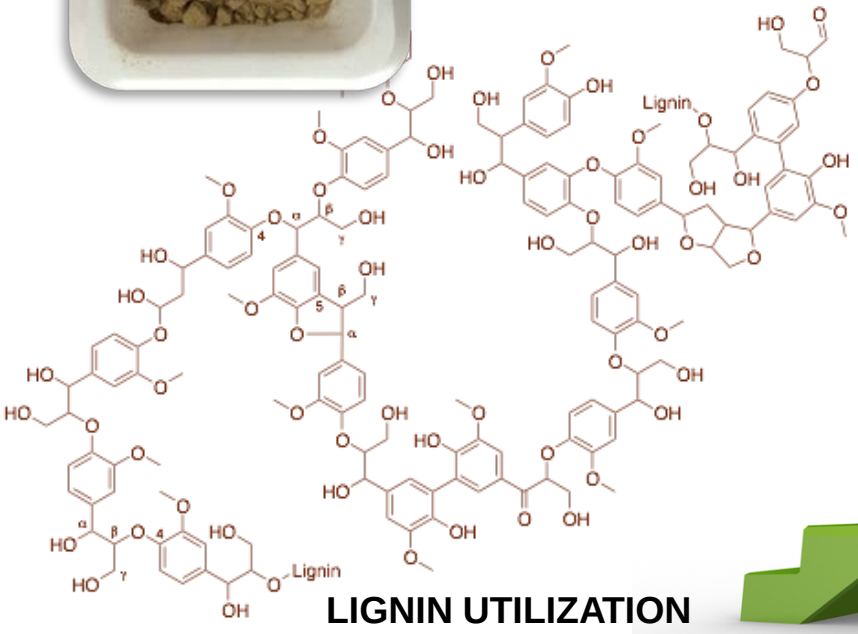
Catalysis to solubilize lignin, then biology to break down oligomers

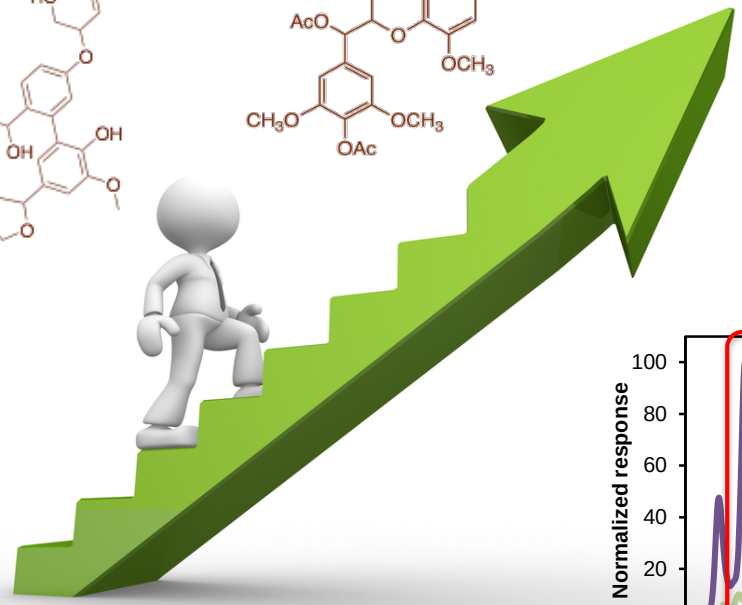
- We need high yields of monomeric compounds for lignin conversion
- We need improved enzymes and strains to depolymerize oligomeric lignin
- To date, lignin depolymerized by **chemical catalysis still contains lignin oligomers** that might be broken down by enzymes released by bacteria

BIOLOGICAL LIGNIN DEPOLYMERIZATION
Project: improve lignin conversion of oligomeric lignin

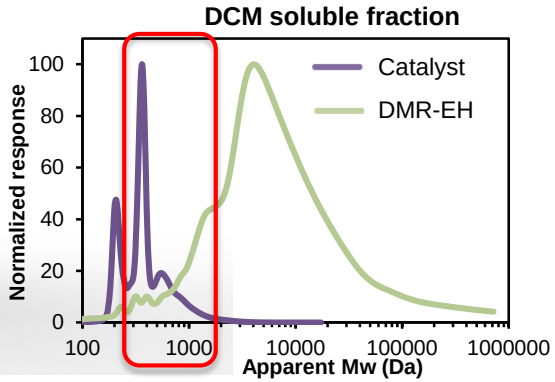


TMD and LigUtil projects: bacterial upgrading of monomeric aromatics





LIGNIN UTILIZATION
Project: depolymerize high molecular weight lignin



Relevance

Lignin valorization essential to achieve 2022 HC fuel cost targets and to realize economic viability of biorefineries

Key MYPP areas for process improvement via lignin utilization:

Efficient Low Temp Deconstruction

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

Efficient Upgrading of Aromatics

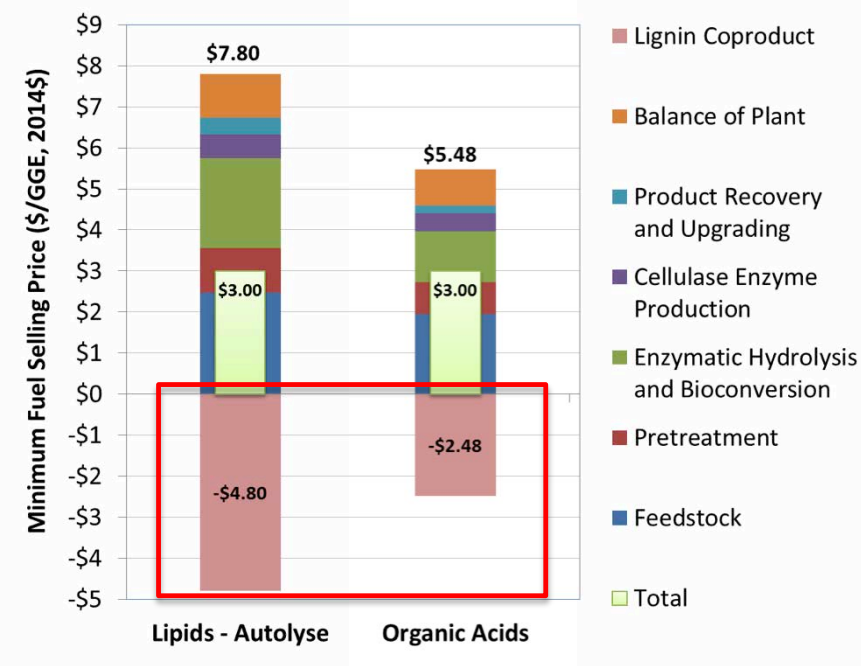
- Examining selective enzymatic bond cleavage reactions on lignin oligomers

Process Integration

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

Key Stakeholders and Impacts:

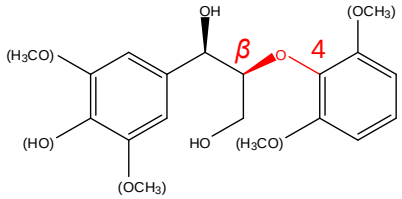
- Lignocellulosic biorefineries: TEA has shown that biorefineries must valorize lignin to be competitive
- Efficient lignin depolymerization is a major barrier to lignin utilization
- Catalytic depolymerization commonly yields lignin oligomers

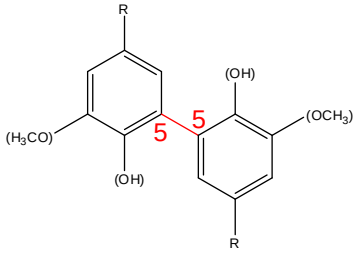


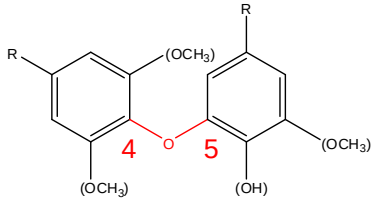
Future Work

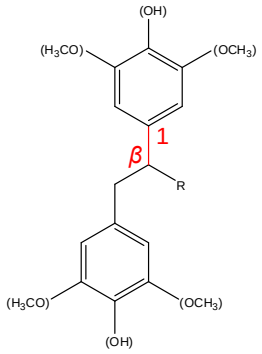
Identify and engineer ligninolytic enzymes into relevant strains

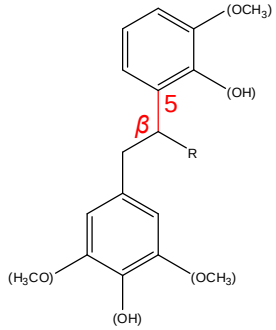
- Use omic analysis (top down) and enzyme assays (bottom up) to identify ligninolytic enzymes to cleave oligomers present in lignin streams
- Engineer these enzymes into production hosts (*P. putida*) with TMD



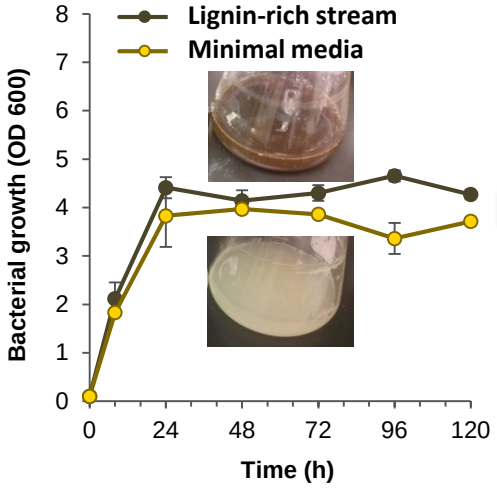








Omics workflow:



Analysis of extracellular proteins.

Which enzymes are up-regulated in the presence of lignin?

Search and selection of potential (new) enzymes involved in lignin breakdown

Enzyme assays with model compounds

EMSL

Summary

1) Approach:
   - Develop a biological approach to depolymerize solid lignin for upgrading of low MW aromatic compounds to co-products
2) Technical accomplishments
   - Screened multiple lignin secretomes for solid lignin depolymerization of a biorefinery-relevant substrate
   - Found microbial aromatic “sinks” may be critical for extensive lignin depolymerization
   - Determined that some bacteria can depolymerize solubilized lignin with native enzymes already
3) Relevance
   - TEA shows lignin valorization is critical for lignocellulosic biorefineries
   - Low molecular weight aromatics and subsequent bio-derived products can enable economics of fuel production from sugars
4) Critical success factors and challenges
   - Achieving high yields of low MW, upgradeable species
   - Minimizing lignin monomer repolymerization
   - Understanding mechanisms for oligomer bond cleavage by microbes
5) Future work:
   - Use –omics and enzyme assays to identify enzymes that cleave oligomers in lignin
   - Over-express enzymes in microbial hosts for rapid lignin depolymerization to enable more facile and rapid lignin conversion by microbes (in collaboration with Lignin Utilization and Targeted Microb. Dev.)
6) Technology transfer:
   - Generate IP around lignin depolymerization methodologies
   - Generate novel microbial lignin conversion strategies

Acknowledgements

- **BETO**: Jay Fitzgerald and Brandon Hoffman
- Edward Baidoo
- Brenna Black
- Florent Bouxin
- Adam Bratis
- Xiaowen Chen
- Nicholas Cleveland
- Tanmoy Dutta
- Rick Elander
- Mary Ann Franden
- Chris Johnson
- David Johnson
- Rui Katahira
- Eric Karp
- Payal Khanna
- Chelsea Martinez
- Bill Michener
- Claire Nimlos
- Michael Resch
- Alberto Rodriguez
- Davinia Salvachua
- Blake Simmons
- Priyanka Singh
- Melvin Tucker
- Derek Vardon

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy BIOMASS PROGRAM

External Collaborators

- Adam Guss, Oak Ridge National Laboratory
- R. Robinson, E. Zink, S. Purvine EMSL Pacific Northwest National Laboratory
- John McGeehan, University of Portsmouth
- Jerry Ståhlberg, Mats Sandgren, Swedish University of Agricultural Sciences
- Angel Martínez, María J Martínez, Alicia Prieto, Center of Biological Research (CIB-CSIC), Madrid, Spain.

Additional slides

- Publications
- Acronyms
- Previous reviewer comments

Publications

Publications in print:

1. D Salvachúa, R Katahira, NS Cleveland, P Khanna, MG Resch, BA Black, SO Purvine, EM Zink, A Prieto, MJ Martínez, AT Martínez, BA Simmons, JM Gladden, GT Beckham, "Lignin depolymerization by fungal secretomes and a microbial sink", *Green Chemistry* **(2016)**, 18 (22), 6046-6062

2. D Salvachúa, EM Karp, CT Nimlos, DR Vardon, GT Beckham "Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria" *Green Chemistry* **(2015),** 17 (11), 4951-4967

3. A Ragauskas, GT Beckham, MJ Biddy, R Chandra, F Chen, MF Davis, BH Davison, RA Dixon, P Gilna, M Keller, P Langan, AK Naskar, JN Saddler, TJ Tschaplinski, GA Tuskan, CE Wyman, "Lignin Valorization: Improving Lignin Processing in the Biorefinery", *Science* **(2014),** 344, 1246843

Publications in preparation:

1. A Rodriguez, D Salvachua, R Katahira, NS Cleveland, BA Simmons, GT Beckham, J Gladden, "Low-temperature base-catalyzed depolymerization of solid biorefinery enriched-lignin streams enables effective microbial conversion". Soon to be submitted **(2017)**.

Acronyms

- DMR-EH Lignin: Deacetylated, Mechanically-Refined, Enzymatically Hydrolyzed Lignin
- DyP: Dye-decolorizing Peroxidase
- LiP: Lignin Peroxidase
- MnP: Manganese Peroxidase
- MW: Molecular Weight
- VP: Versatile Peroxidase
- BCD: Base-catalyzed depolymerization
- APL: Alkaline pretreated liquor

Previous Review Comments

Project Overview

- The project performers have communicated the projects history, the context in which the project fits into the portfolio, and its high level objectives.
- Biological depolymerization of lignin.
- Joint with NREL and SNL focus on solid lignin.
- Focus on deacetylated and refined.
- Seed project. Complementary to other BETO-funded work on lignin depolymerization and lignin upgrading.
- Fungal and bacterial lignolytic enzymes.
- The background of the project was very well described with sufficient detail to understand how it fits into BETOs goals.
- Good overview. More on the project's history would have been appreciated, but clear goal discussion and project objectives and context.
- Bio approach to breaking down lignin. Good overview of project and need.
- Solid lignin depolymerization using biological system.
- Good overview of concept, context and objectives.
- Depolymerizing solid lignin out of the back of the process is important to the ultimate economics.
- May not be practical to add an alkaline pre-treatment up front of the process.
- Acid pre-treatment is a reality in the industry so needs to be included in research.
- Collaboration with other labs is encouraged and great to see.
- Complements the other chemical lignin depolymerization work presented.

Previous Review Comments

Project Approach

- The project performers have implemented technically sound research, development, and deployment approaches, and have demonstrated the results needed to meet their targets.
- The project performers have identified a project management plan that includes well-defined milestones and adequate methods for addressing potential risks.
- The project performers have clearly describe critical success factors which will define technical and commercial viability, and that they have explained and understand the challenges they must overcome to achieve success.
- DMR-EH lignin. Laccases and peroxidases minimize repolymerization made a strategic hire in lignin degradation id-ed and overcoming key risks.
- Enzymatic deconstruction of lignin is very challenging due to the low catalytic rates of the enzymes as well as the tendency of lignin fragments to repolymerize. The problem is further exacerbated by the fact that the most common commercially available lignin is very recalcitrant. Lignin potentially represents a very valuable raw material if processes can be designed to utilize it (besides low value boiler fuel).
- Great approach. Use of pretreated feedstock lignin and post-hydrolysis solids is excellent and should be informative; it's great to see a project focusing on very realistic feedstocks. Discussion of kraft, acid, and caustic lignin showed depth of knowledge that clearly is guiding the project. Biological/catalytic upgrading approach of producing a single chemical precursor for upgrading is novel and has good potential. Excellent integration with TEA.
- Analytical tools identified as a need. Issues with repolymerization identified. Good overall.
- Clear structure of approach, challenges and CSFs.
- Enzymatic depolymerization engineer organisms to make bioproducts. Challenges outlined approach on track – fungal.
- Collaborations with sandia is excellent.

Previous Review Comments

Technical Progress and Accomplishments

- The project performers have made progress in reaching their objectives based on their project management plan.
- The project performers have described their most important accomplishments in achieving milestones, reaching technical targets, and overcoming technical barriers.
- The project performers have clearly described the progress since the period of the last review.
- Extensive characterization of DMR-EH investigated how nature depolymerized lignin; laccases (O2 dependent); peroxidases (H2O2 dependent) fungal and bacterial characterization of lignolytic enzymes optimal time since will start to repolymerize microbes as a "sink" to keep them from repolymerizes coordinating reaction times; free radical mechanisms pulp bleaching applications.
- Excellent progress is being made addressing the technical challenges.
- Technical progress is excellent, lots of detail provided and discussion of improvement since last review. Very happy to see transition to high-solids pretreatment, this is highly relevant to industrial application. Even more happy to see clearly that the TEA is driving research (e.g. omission of organosolv, hydrotalcite pathways). As a side note, slow down! There was a LOT of data on a LOT of slides, and it could be hard to keep up. Be assured the project stands on its own, no need for the "fire hose" approach.
- Surpassed MW reduction before 1 year go/no go, great. Overall results real good.
- Characterize substrate.
- Commercial enzymes and fungal secretomes.
- Ggood progress on identifying and demonstrating performance of lignin degrading enzymes.
- Well designed experiments for selecting optimal conditions and understanding kinetics/fundamentals.
- Depolymerization research included key process variables and sensitivities.
- 50% target of degregation met. Quantified targets for go no decisions encouraged.
- Microbial sink to stop re-polymerization path is intriguing.
- Leveraging the work from PNNL excellent.

Previous Review Comments

Project relevance

- The project performers have describes how the project contributes to meeting Program/Technology Area goals and objectives and the Bioenergy Technologies Office, as cited in the MYPP.
- The project performers have considered applications of their expected outputs.
- The project performers have presented the relevancy of this project and how successful completion of the project will advance the state of technology and impact the viability of commercial bioenergy applications.
- Lignin valorization will be essential to achieve 2022 HC fuel costs however can't lose sight of the LCA
- The project has been highlighted in the past as filling a key gap in the biochemical conversion platform, and it is even more relevant today as more and more economic analyses are showing the need to include lignin utilization in the final process. Impacts the "whole barrel" initiative.
- Very much needed to enable other uses for lignin besides burning. Would only question whether or not they shouldn't be looking at lignin from one of the commercial plants. Especially seeing as Gregg mentioned that the dilute acid lignin is more difficult to breakdown. Maybe work with both.
- Good strategic fit for lignin utilization.
- Highly relevant . Important research.

Previous Review Comments

Future work

- The project performers have outlined adequate plans for future work, including key milestones and go/no go decision points through September 30, 2016.
- The project performers have communicated key planned milestones and addressed how they plan to deal with upcoming decision points and any remaining issues.
- Evaluate "microbial" sinks
- CBP-like approach.
- Proteomics to fingerprint enzymes produced mine genomes of microbial sinks.
- Genetics parts list how to tune reaction rates of depoly- and re-polymerization vs microbial sinks?
- Future work looks good; working on additional feedstocks will inform additional research, both inside and outside the project. Emphasizing upgrading and integration is of course critical to the project, but seeing the incredible integration with other collaborators and national labs is very encouraging.
- Good plan, should consider some "commercial" lignin too.
- Clear goals and tasks for future work. Quantitative milestones?
- Would like to see a process engineer brought into the future research to start understanding the process and cost to recover the lignin in a plant. This may be a year out but it is important to understand how to turn this excellent research into reality. The industrial partners are possibly working on that.

Previous Review Comments

Overall impressions

- This is an extremely relevant project since it is commonly believed that a high value co-product will be needed in order to achieve fuel economics for hydrocarbons from lignocellulosic biomass. Lignin is a valuable raw material that to date is being used as boiler fuel, however it can be converted into higher value products under the right processing conditions.
- This is an outstanding project. The work being done is not only commercially relevant, but utilizes lignin from likely commercial processes, rather than from unrealistic sources. There is a large number of publications being produced to help drive additional research, the process impacts the "whole barrel" initiative, there is strong integration with other BETO projects, heavy focus on technoeconomic analysis to drive the work; it has the potential to contribute more to the portfolio than perhaps most projects out there.
- Great potential, project is needed by the industry. Result could have immediate use with very large initial volume, but to get that will need to include some "commercial" lignin in the development. Ok to be working on the new process configuration for lignin fractionation that NREL has envisioned, but should leave out the industrial lignin that is being produced.
- Peroxidases and laccases have been well studied for lignin degradation. This project uses a novel approach to using base catalyzed decomposition or alkaline extracted lignin liquor to generate higher quality, more useful lignin feedstocks for conversion/upgrading. Plan and experimental approach are very well developed and designed.
- A lot of challenges ahead but really great research.

Exploring methods to solubilize lignin: BCD

- DMR-EH lignin is insoluble. Solubilization of lignin may increase its accessibility to microbes and enzymes, possibly enhancing depolymerization
- To explore this possibility, mild base treatment (in collaboration with **Lignin Utilization project**) was used to solubilize a significant portion of the DMR-EH lignin and partially depolymerize it