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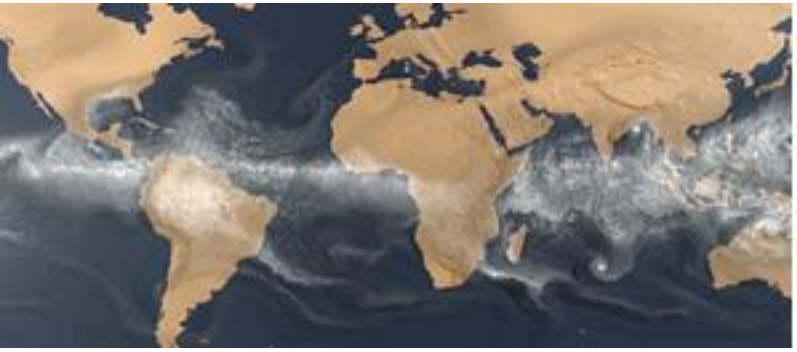

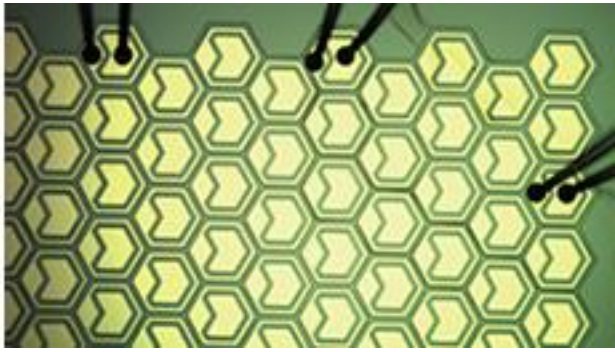

February 15, 2019
Biological Lignin Valorization – SNL
Technology Session Review Area: Lignin
Presenter: Alberto Rodriguez
PI: Ken Sale

U.S. DEPARTMENT OF ENERGY

National Nuclear Security Administration

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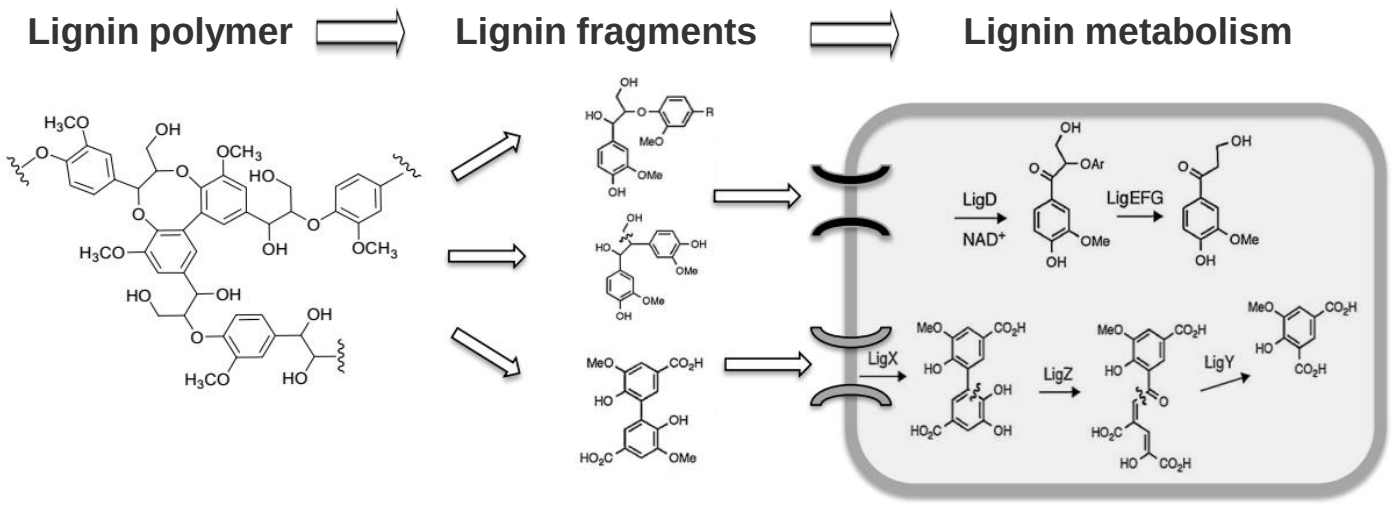
Goal Statement

Goal: Develop fundamental understanding of biological (enzymatic) depolymerization of lignin

- Identify intermediates produced during enzymatic depolymerization of lignin
- Identify which of these intermediates can be consumed by microbes and microbial consortia
- Focus on deriving intermediates amenable to biological upgrading



Outcome: Biological approach to depolymerize lignin into intermediates, a critical step to biological upgrading to valuable products

Quad chart overview

Timeline

- Start date: October 2017
- End date: September 2020

	Total Costs Pre FY17	FY17 Costs	FY18 Costs	Total Planned Funding (FY19-End Date)
DOE funded		$177k	$327k	$250k

Partners and collaborators:

Davinia Salvachúa and Gregg Beckham at National Renewable Energy Laboratory

Barriers addressed

Ct-C Process development for conversion of lignin, converting lignin into value-added products

- Development of enzyme systems for tailored depolymerization of lignin
- Depolymerizing lignin into valuable intermediates amenable to upgrading to valuable products

Objective

Fundamental understanding of biological depolymerization of lignin into defined and upgradable intermediates

End of Project Goal

Achieve >50 % enzymatic or microbial conversion of a β-O-4 and C-C bond containing synthetic lignin oligomer to compounds that can be biologically assimilated

Project overview

History: Project started in FY18, following a previously funded BETO project with NREL to develop a biological approach to depolymerize oligomeric lignin for subsequent biological conversion to value-added co-products

Context: Biorefineries do not currently utilize lignin to make value-added products

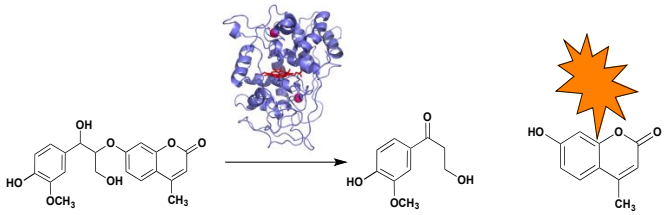
- Lignin is a complex heterogenous polymer, difficult to convert and analyze
- Biological conversion of lignin requires depolymerizing it to fragments amenable to being upgraded using engineered microbes
- Lack of knowledge on what compounds obtained from lignin streams can be consumed by microorganisms

Project Goals:

- Fundamental understanding of microbial and enzymatic lignin modification, bond cleavage and fragment utilization
- Mixtures of microbes and/or enzymes to achieve >50 % conversion of a β-O-4 and C-C bond containing dimers and oligomers

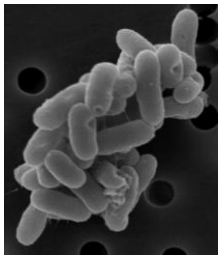
Project management

- Tasks led by subject specific experts
- Quarterly videoconferences with BETO
- Interact with NREL to help inform host engineering



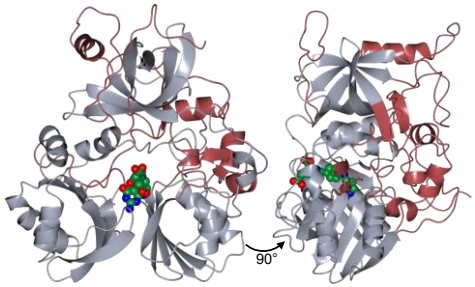
Task 1: Assay development and substrate synthesis

- Led by organic synthetic chemist Kai Deng
- Synthesis of model lignin compounds



Task 2: Microbial growth on lignin model compounds

- Led by microbiologist Alberto Rodriguez
- Identifying microbes that utilize lignin as a carbon source



Task 3: Lignin analytics and enzyme assays

- Led by biophysicist Mike Kent
- Enzymology studies

Project interactions

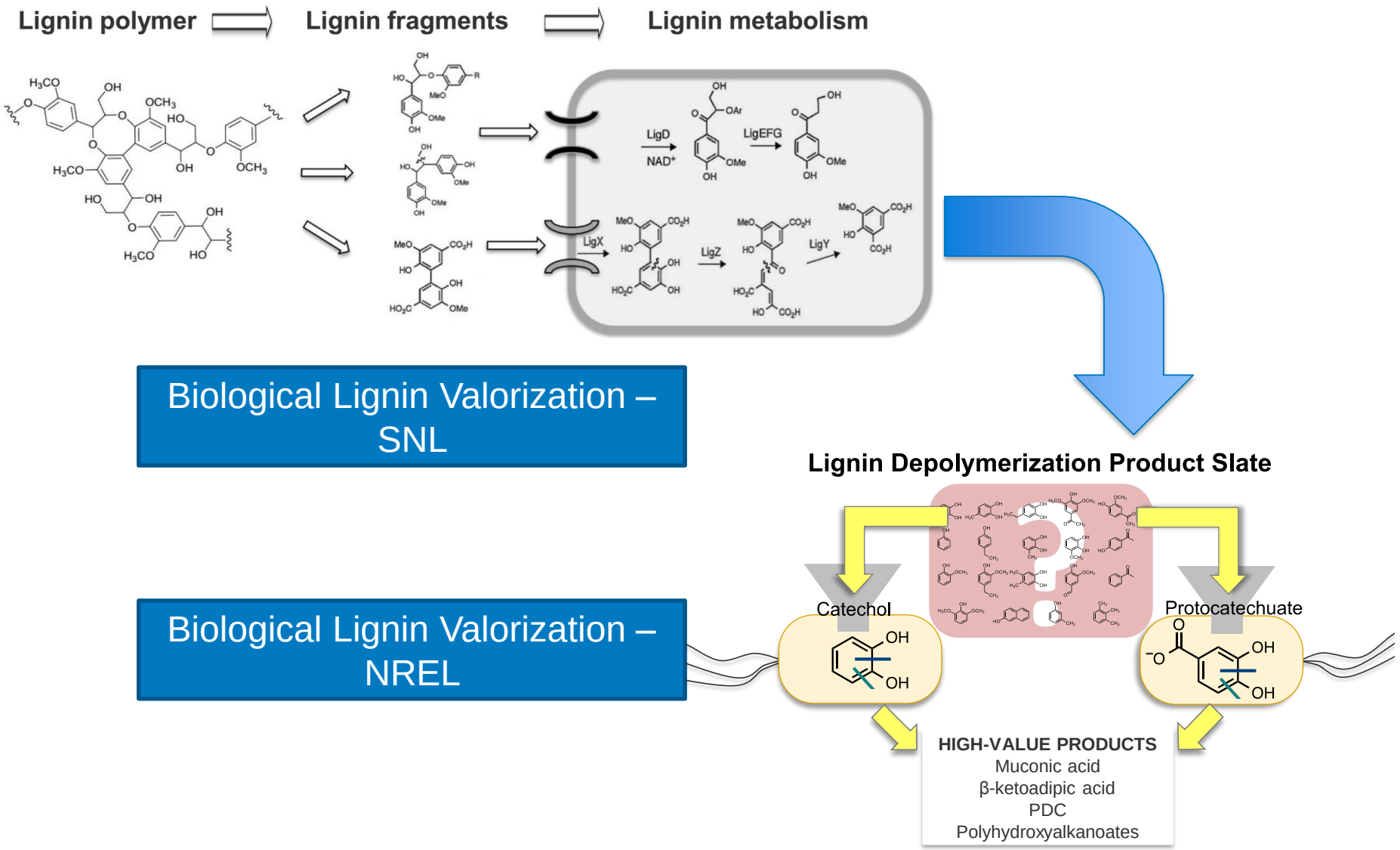
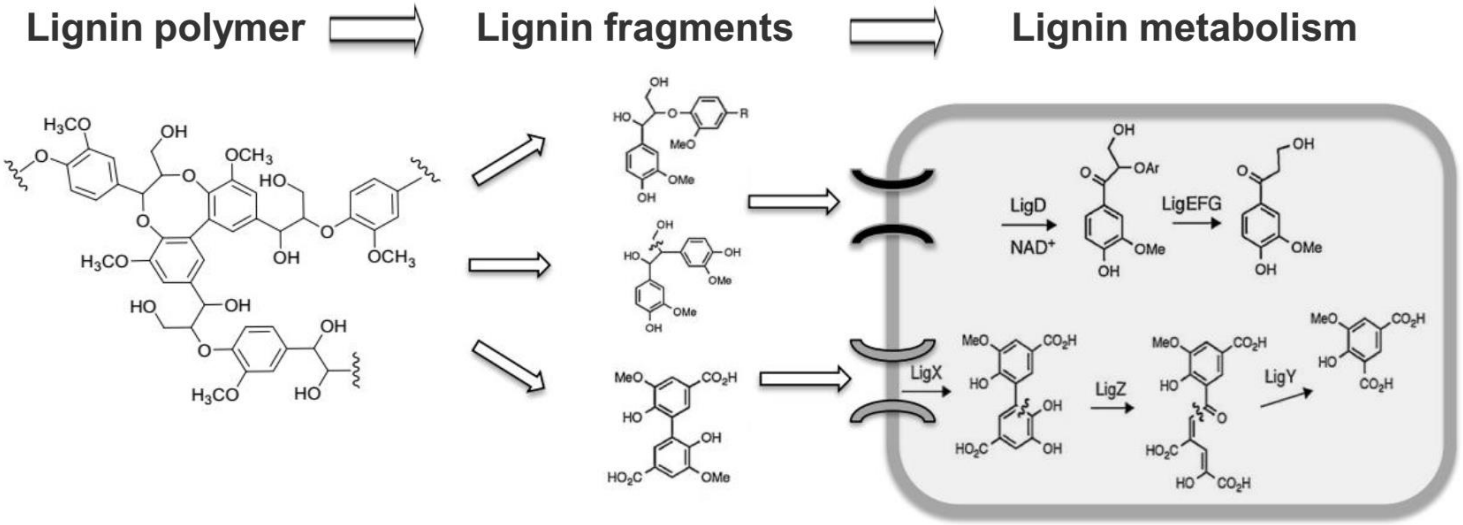


Figure by Davinia Salvachúa

Technical approach



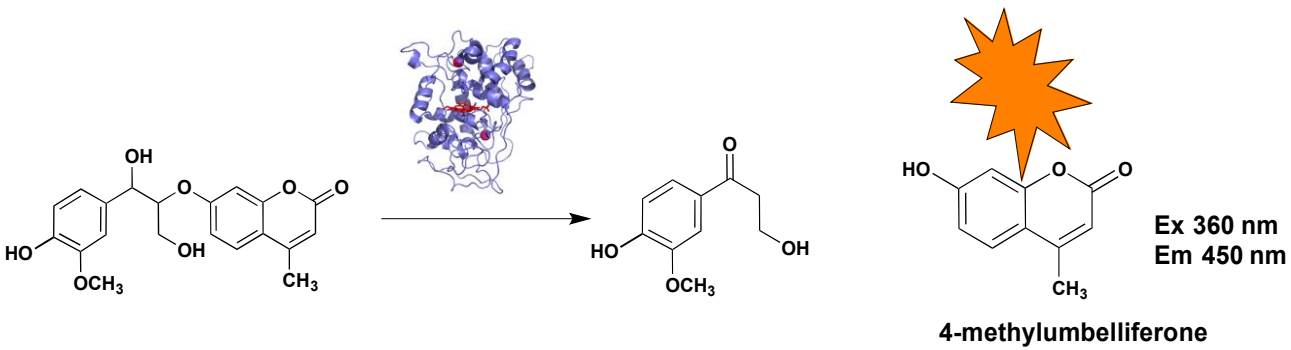
Challenges

- Assaying bond cleavage is difficult: most assays are limited to measuring oxidation events
- Most microorganisms are expected to assimilate only some monomeric or dimeric compounds
- Difficult to identify when a specific substrate is converted into compounds that can be fully metabolized

Approaches

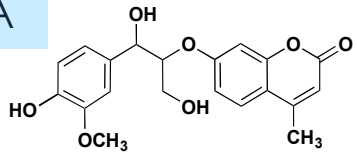
- Synthesize β-O-4 bond and C-C bond containing compounds that fluoresce upon bond cleavage
- Decouple substrate conversion from microbial growth by using a resting cell system
- Screen for combinations of enzymes and organisms that can degrade and grow on accumulated intermediates

β-aryl ether dimer substrates for fluorescence-based assays



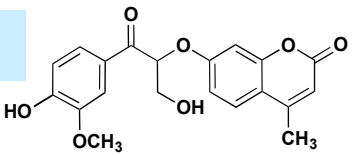
Phenolic

A



10 mg

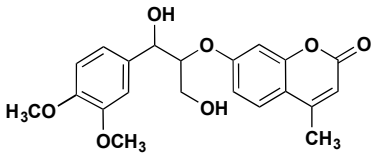
C



20 mg

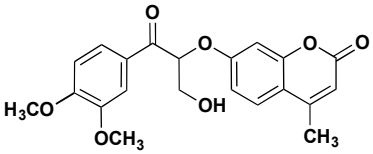
Nonphenolic

B



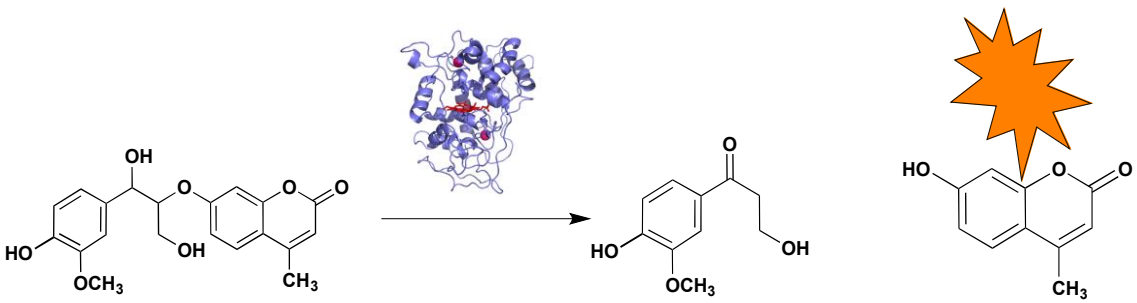
5 mg

D

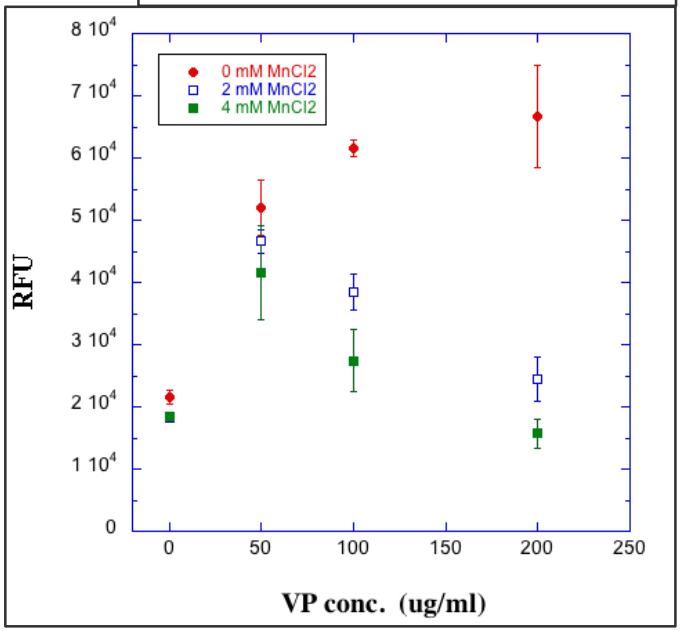


10 mg

β-aryl ether dimer substrates for fluorescence-based assays



Versatile peroxidase from *B. adusta* with compound A (10 mg/L substrate, pH 4.5, no H_2O_2)



Phenolic | ***Nonphenolic***

A — 10 mg

B — 5 mg

C — 20 mg

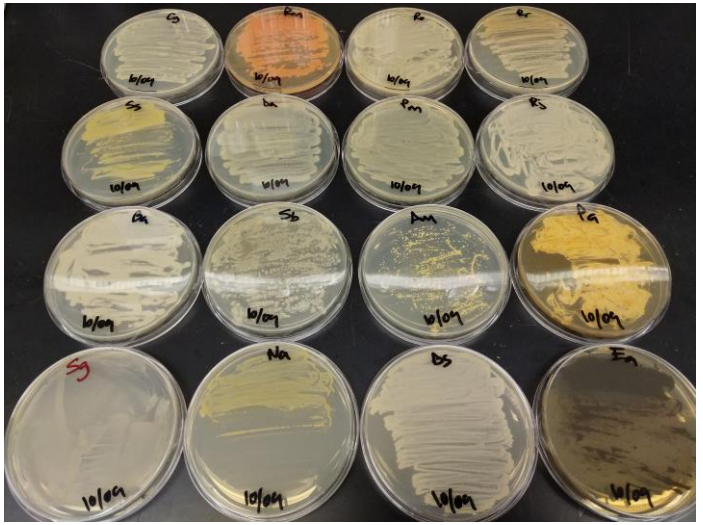
D — 10 mg

Synthesized mg quantities of phenolic and non-phenolic β-O-4 linked dimers and confirmed fluorescence readout of released MUB compound upon bond cleavage

β-aryl ether dimer substrates for fluorescence-based assays

Cg	Corynebacterium glutamicum
Rm	Rhodotorula mucilaginosa
Ro	Rhodococcus opacus
Rr	Rhodococcus rhodochrous
Ea	Exophiala alcalophila
Ss	Sphingobium sp. SYK-6
Da	Delftia acidovorans
Rj	Rhodococcus jostii

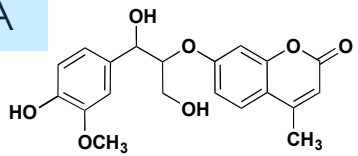
Pm	Pseudomonas putida mt-2
Bs	Bacillus subtilis
Ba	Bacillus amyloliquefaciens
Sb	Streptomyces badius
Am	Amycolatopsis sp.
Pa	Pseudonocardia autotrophica
Na	Novosphingobium aromaticivorans
Sg	Sagittula stellata



Phenolic

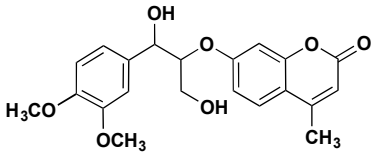
Nonphenolic

A



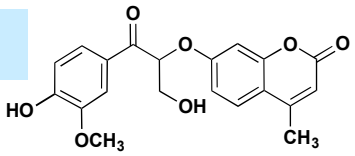
10 mg

B



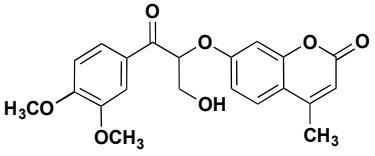
5 mg

C



20 mg

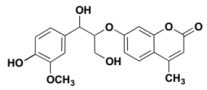
D



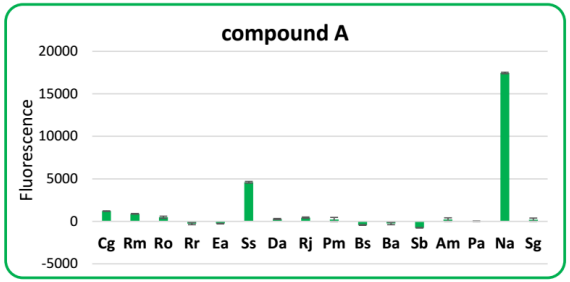
10 mg

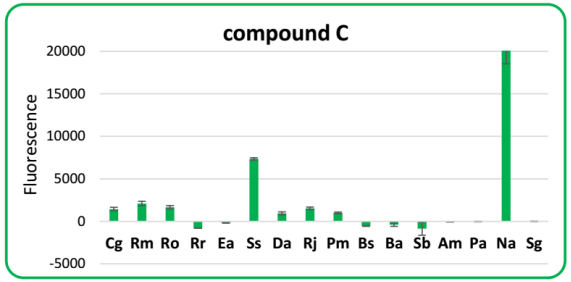
Microbial assays with fluorescent substrates

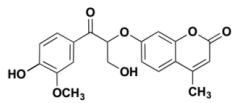
RFU values obtained after incubation of microorganisms with fluorescent dimers



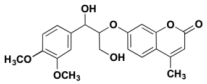
phenolic
secondary OH



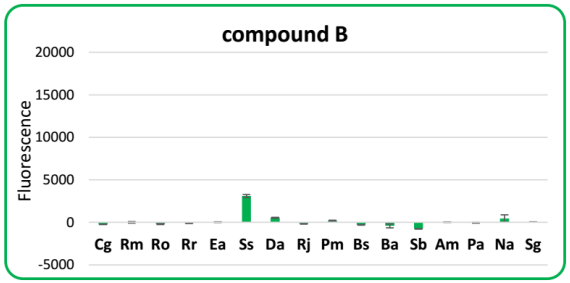


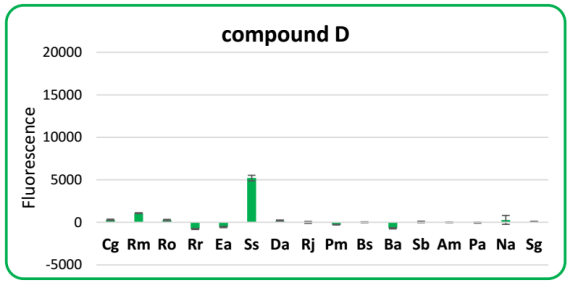


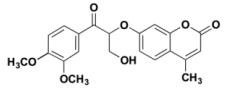
phenolic
ketone



non-phenolic
secondary OH







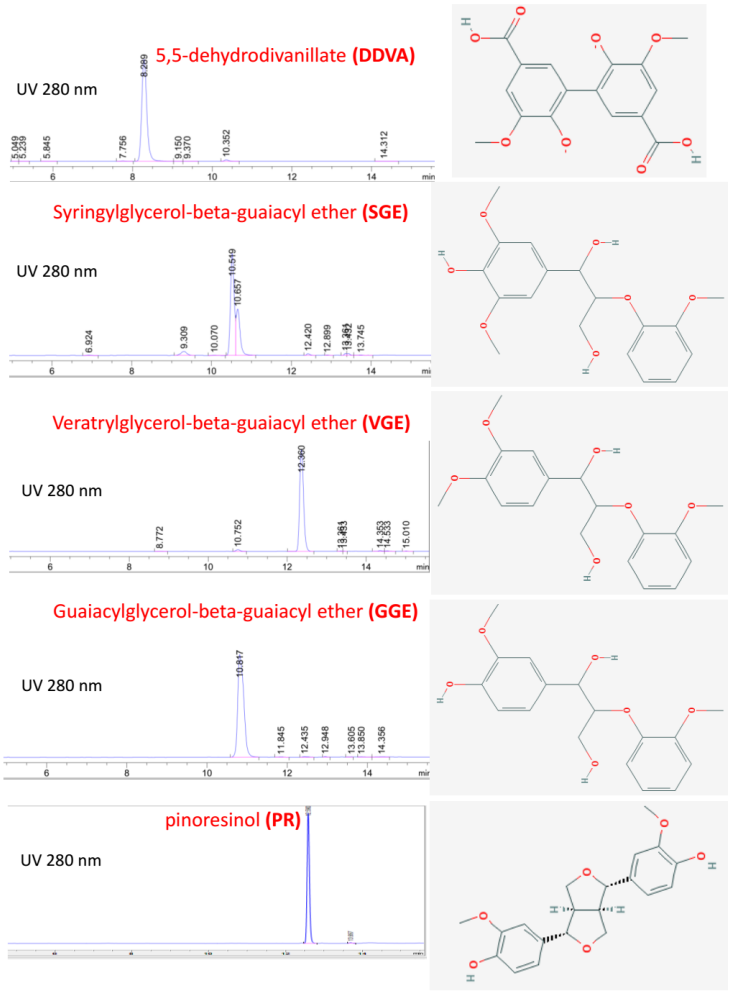
non-phenolic
ketone

Cg	Corynebacterium glutamicum
Rm	Rhodotorula mucilaginosa
Ro	Rhodococcus opacus
Rr	Rhodococcus rhodochrous
Ea	Exophiala alcalophila
Ss	Sphingobium sp. SYK-6
Da	Delftia acidovorans
Rj	Rhodococcus jostii

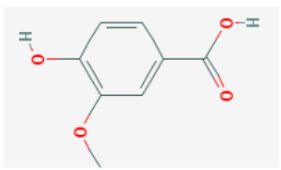
Pm	Pseudomonas putida mt-2
Bs	Bacillus subtilis
Ba	Bacillus amyloliquefaciens
Sb	Streptomyces badius
Am	Amycolatopsis sp.
Pa	Pseudonocardia autotrophica
Na	Novosphingobium aromaticivorans
Sg	Sagittula stellata

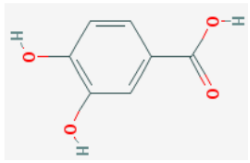
Obtained evidence of the presence of enzymes that can catalyze cleavage of some fluorescent phenolic and non-phenolic compounds

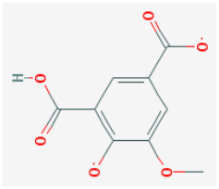
Detection of commercial lignin-related compounds

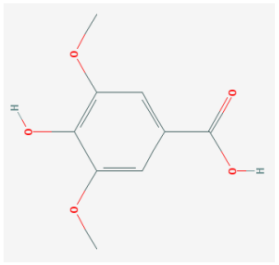


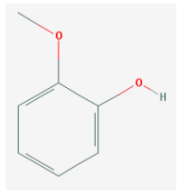
possible metabolic products

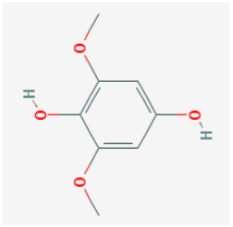

Vanillic acid

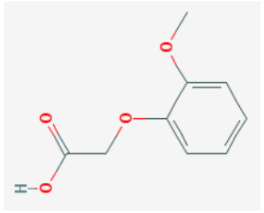

Protocatechuic acid

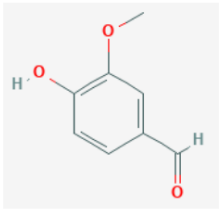

5-carboxyvanillate


Syringic acid


guaiacol


2,6-dimethoxyhydroxyquinone

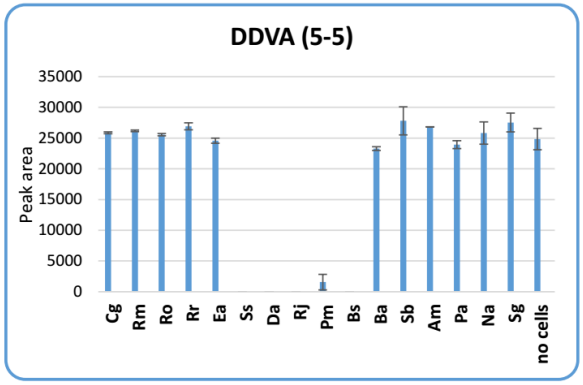

guaiacoxyacetic acid

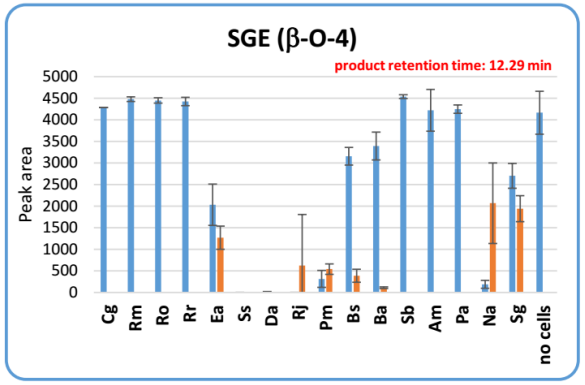

vanillin

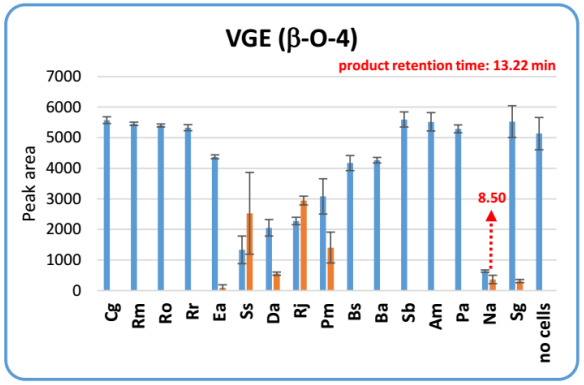
Optimized the experimental conditions to monitor the biological and enzymatic conversion of 5 model lignin-related compounds

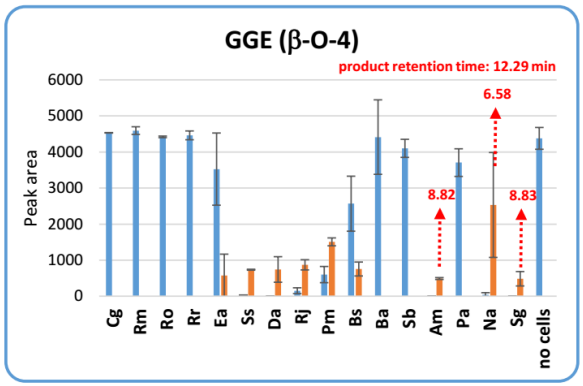
Microbial dimer degradation

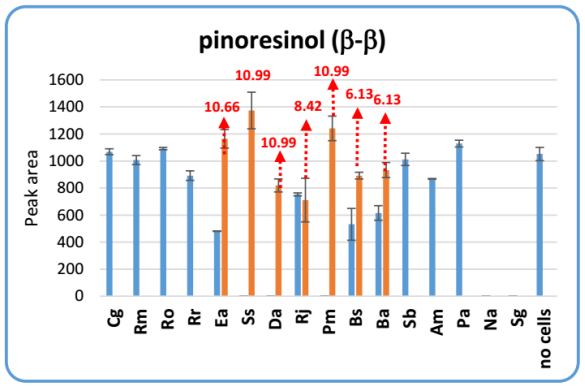
HPLC peak areas obtained after incubation of microorganisms with dimers











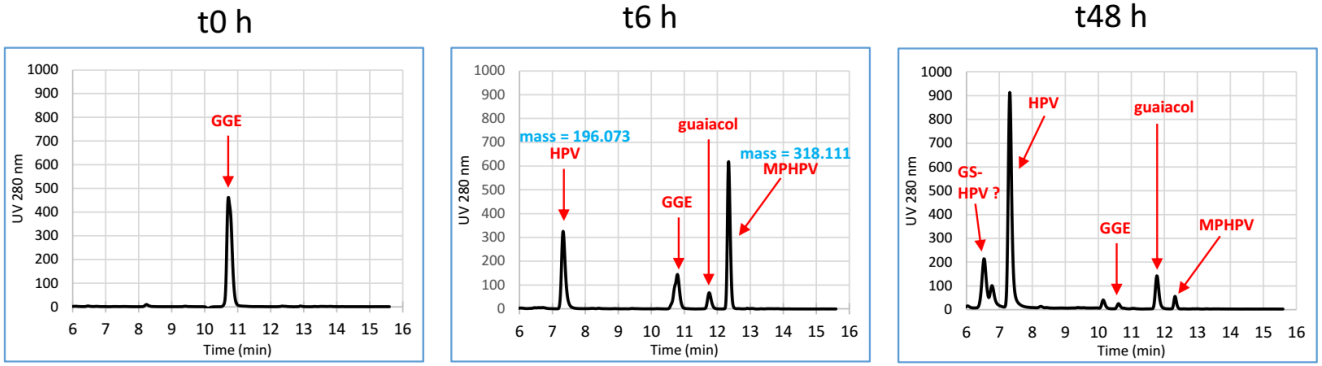
Blue bars represent substrates and orange bars represent the main product detected in each case. Numbers on top indicate compound retention time.

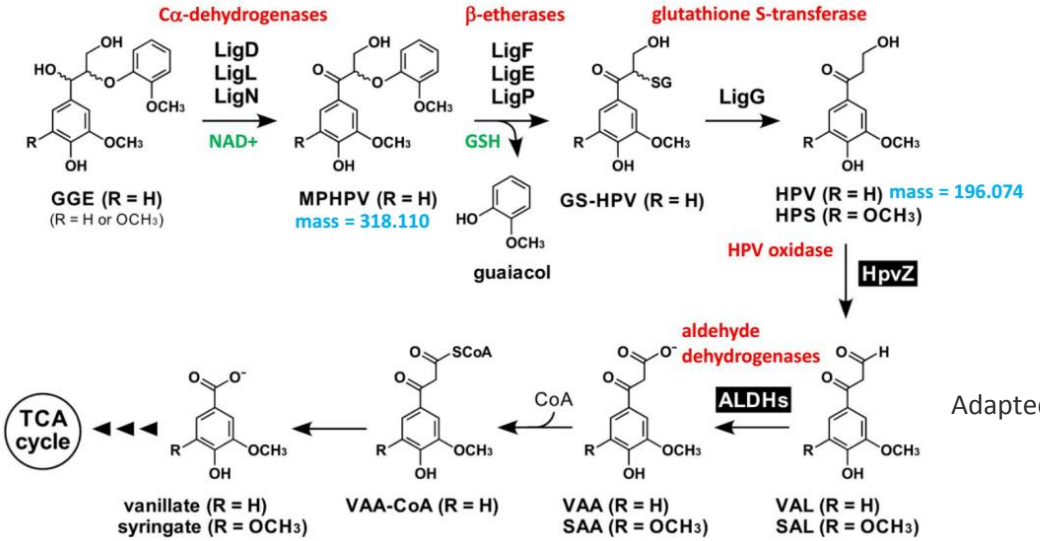
Cg	Corynebacterium glutamicum
Rm	Rhodotorula mucilaginosa
Ro	Rhodococcus opacus
Rr	Rhodococcus rhodochrous
Ea	Exophiala alcalophila
Ss	Sphingobium sp. SYK-6
Da	Delftia acidovorans
Rj	Rhodococcus jostii
Pm	Pseudomonas putida mt-2
Bs	Bacillus subtilis
Ba	Bacillus amyloliquefaciens
Sb	Streptomyces badius
Am	Amycolatopsis sp.
Pa	Pseudonocardia autotrophica
Na	Novosphingobium aromaticivorans
Sg	Sagittula stellata

Observed organism-dependent variations in dimer degradation products

Characterization of metabolic breakdown products

Novosphingobium aromaticivorans + GGE

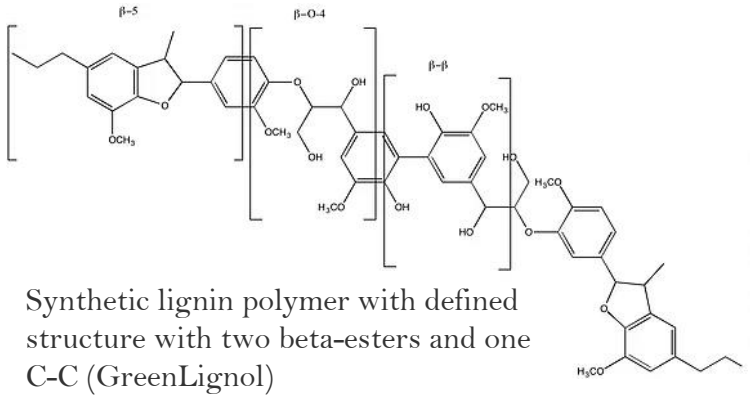




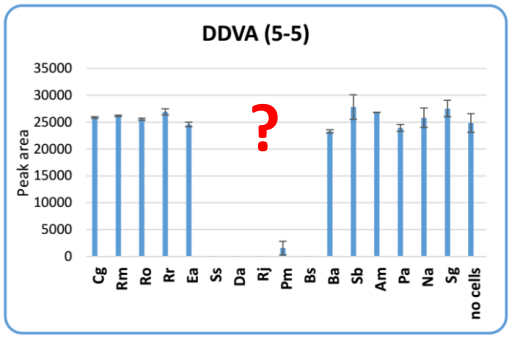
Adapted from Higuchi et al. 2018

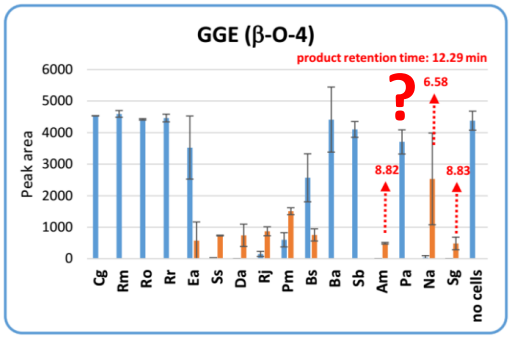
Generated kinetic information on degradation of GGE and PR to prepare solutions enriched on relevant metabolic intermediates for further screening

Future work



Synthetic lignin polymer with defined structure with two beta-esters and one C-C (GreenLignol)





Task 1: Assay development and substrate synthesis

- Synthesize a C-C bonded fluorescent model substrate

Task 2: Microbial growth on lignin model compounds

- Additional kinetic studies of degradation
- Investigate combinations of lignin modifying organisms
- Studies on a synthetic lignin oligomer

Task 3: Lignin analytics and enzyme assays

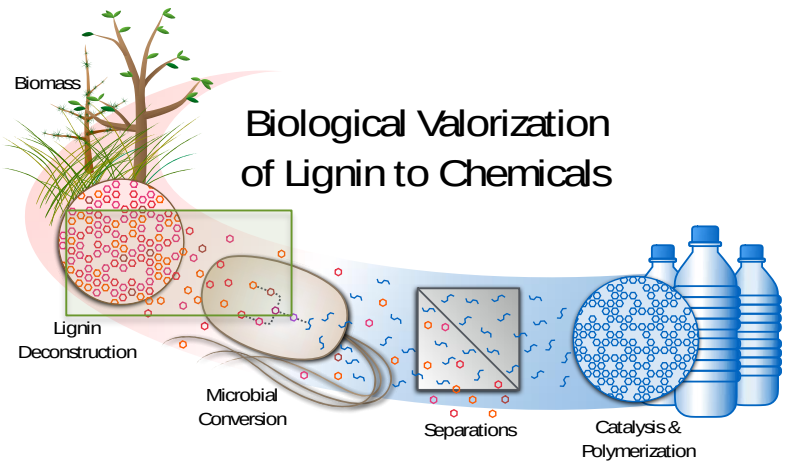
- Screen combinations of enzymes on fluorescent compounds, dimers and a lignin oligomer

Relevance

Project goal: fundamental understanding of how microbes enzymatically modify lignin and utilize lignin fragments, identification of relevant mixtures of microbes and their associated enzymes

Why is this project important, what is the relevance to BETO and bioenergy goals?

- Lignin valorization is critical to the achieving $3/gge goal.
- This project addresses two key questions for lignin valorization via biological routes:
  - What fragments can be produced from biological depolymerization of lignin?
  - Of these breakdown products, which are utilized as a carbon source by microorganisms and are thus targets for upgrading via synthetic biology?



Beckham *et al. Curr Opin Biotech* 2016

How does this project advance the SOT, contribute to biofuels commercialization?

- Advances our understanding of how biological systems break down and metabolize lignin
- Filling the gap in knowledge that limits synthetic biology efforts to valorizing lignin

Summary

Approach

- Bottom up approach utilizing model compounds to how enzymes and microbes modify lignin and utilize lignin fragments
- Investigate the synergistic effect of microbes, enzymes and substrates, to identify conditions that enable microbial growth

Technical accomplishments

- Established a screening protocol for monitoring compound degradation and analyzing metabolic intermediates
- Observed organism-dependent variations in dimer degradation products which identifies promising combinations of organisms for future studies

Relevance

- This work will provide key information for host engineering regarding substrate utilization from depolymerized lignin streams

Future work

- Investigate breakdown of oligomers by combinations of microbes and enzymes and subsequent utilization of the generated fragments
- Employ combinations of enzymes to generate defined lignin intermediates from oligomers

Acknowledgements

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BETO projects

Biological Lignin Valorization – NREL