Biological Lignin Valorization

Technology Session Review Area: Biochemical Conversion & Lignin Utilization

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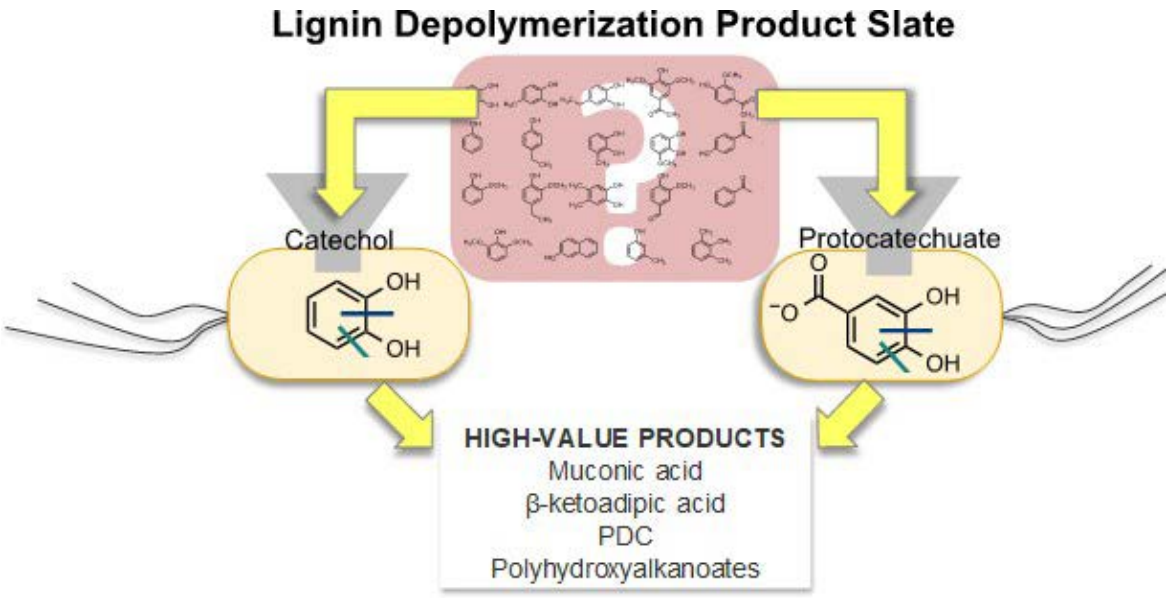
Presenter: Davinia Salvachúa, NREL

Project overview

Goal: Develop bioprocesses to funnel heterogeneous lignin-derived streams to single value-added co-products

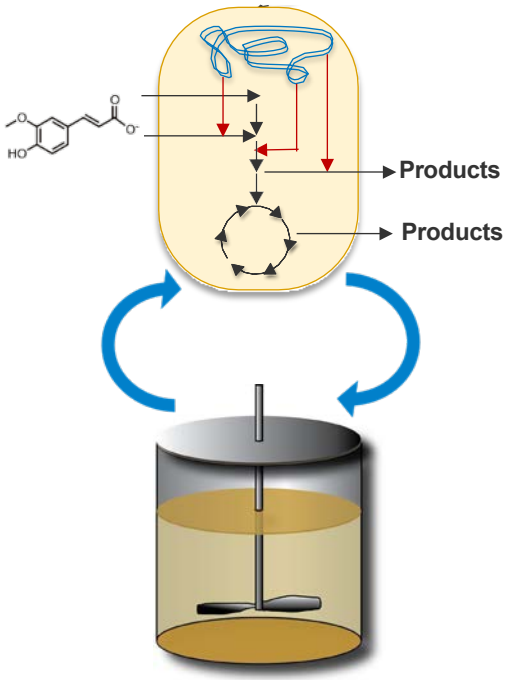
- Focus on commodity products with sufficient market sizes to aid biofuels production (e.g., adipic acid)
- Work with Lignin Util. & SepCon for lignin substrates
- Develop innovative biocatalysts and bioprocesses for lignin valorization



Heilmeier Catechism:

- **Goal**: enabling biology and bioprocess engineering to bioconvert lignin intermediates to co-products
- **Today**: lignin bioconversion is a relatively new approach (~2014)
- **Important**: contribute $2-3/gge to MFSP for biofuels production through biological lignin valorization
- **Risks**: lignin not bioavailable, bioprocess performance insufficient for real-world implementation

Management



Task 1: Strain Development

- Led by *P. putida* metabolic engineering expert (C. Johnson)
- Milestones for overcoming bottlenecks, expanding substrate specificity, and improving TRY

Task 2: Bioprocess Development

- Led by *P. putida* bioprocess expert (D. Salvachúa)
- Milestones for TRY and toxicity tolerance improvement
- Work with ORNL project (A. Guss) to evaluate strains

Project organization:

- Meetings: monthly for project, monthly with PI-task leads & PI-task leads-postdocs
- *Ad hoc* meetings with other BETO projects (*next slide*)
- Ops & Project Managers – lab space, equipment, reporting, finances

Risks:

- Substrate and product toxicity limiting TRY – evaluating multiple lignin streams, other strains
- Bio-availability of aromatics from lignin – key link to Lignin Utilization

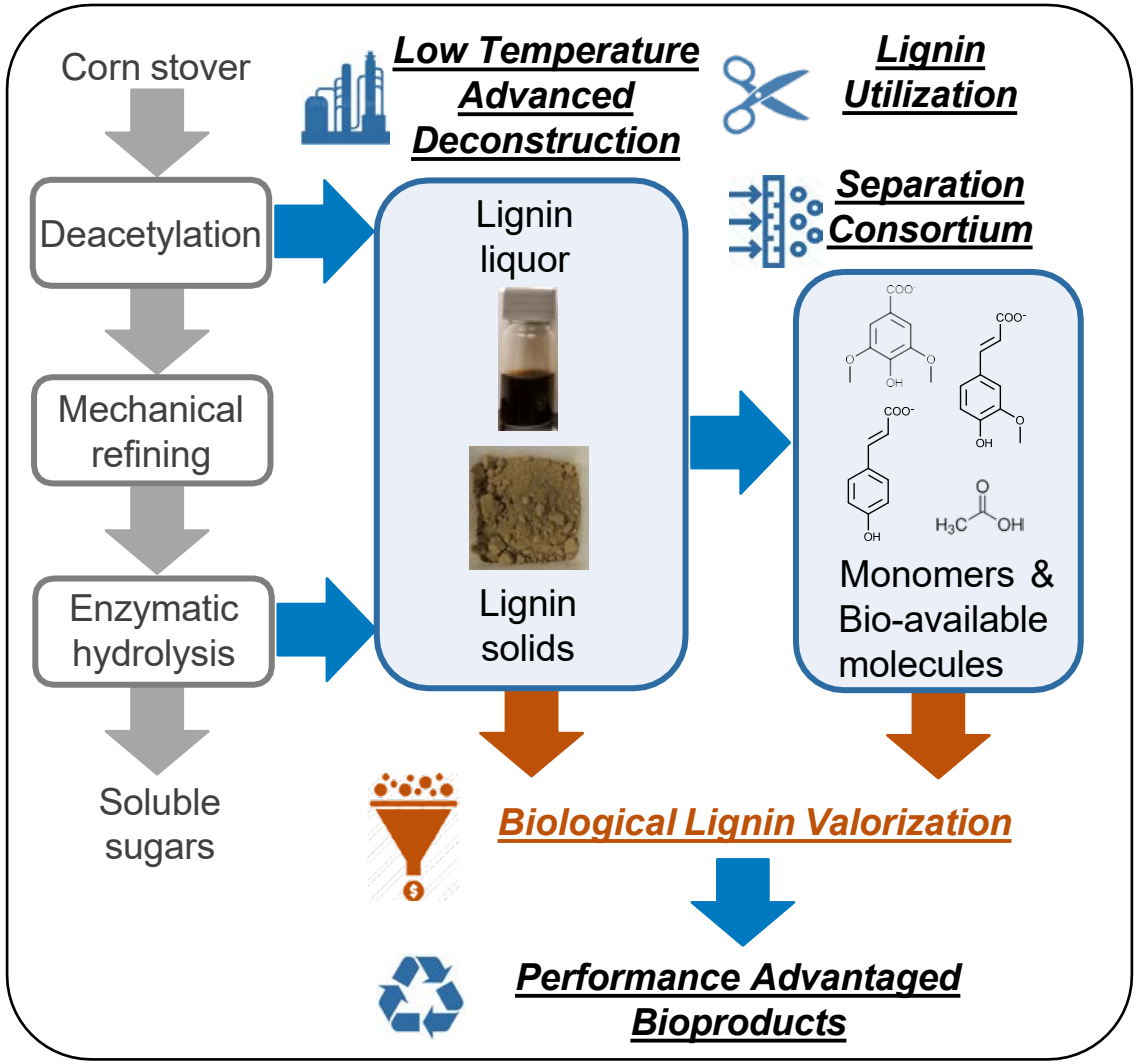
Management: Project interactions

Oxidative chemistry for lignin depolymerization is desirable to generate bio-available, water-soluble lignin streams

- BLV can convert lignin streams by expanding the biological funnel and designing bioprocess to convert toxic streams into single products

Challenge:
Lignin deconstruction

Current process configuration and projects interchange



Approach

Overall approach:

- **Model compounds** for evaluations, **lignin** from LigU, LTAD, and SepCon
- *Pseudomonas putida* KT2440 as robust chassis
- Atom-efficient targets as performance-advantaged bioproducts
- Collaborate for advanced tools (ALE/DTU, omics/ORNL)
- Work with Biochem. Analysis project to identify process drivers

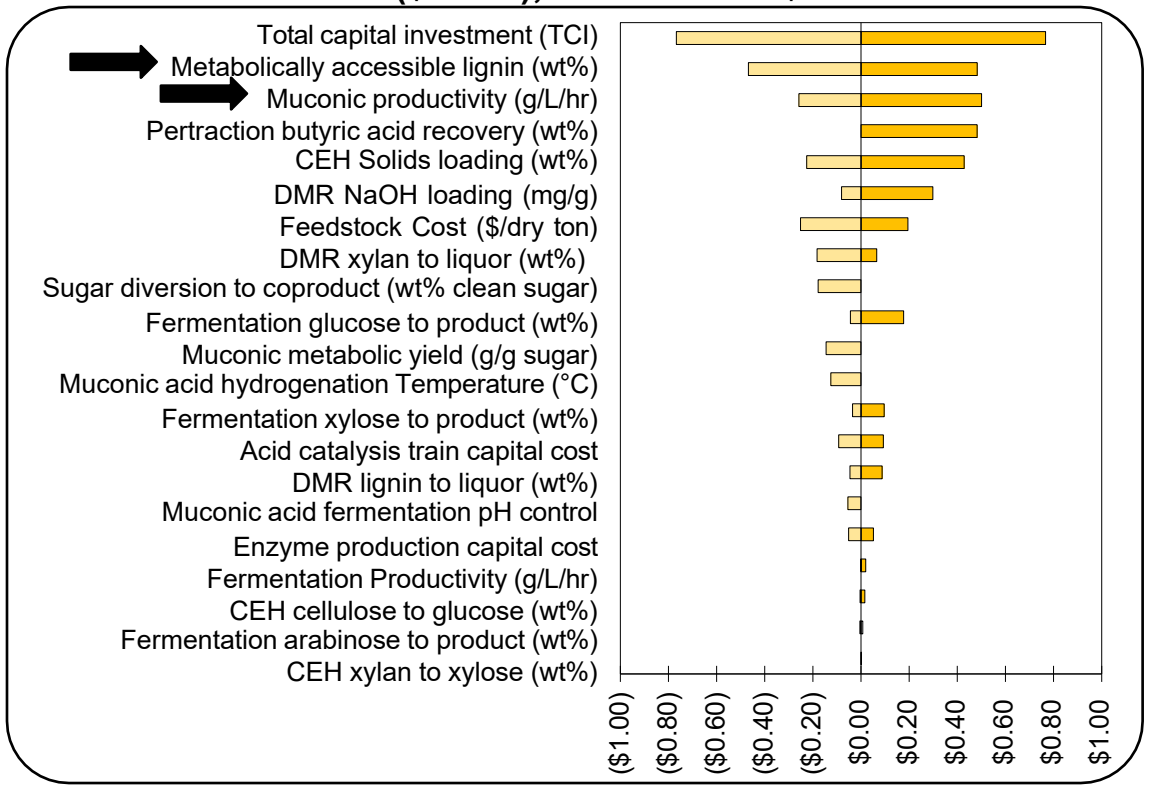
Challenges:

- Lignin streams with bio-available aromatics (LigU, SepCon)
- Translation from model to real substrates

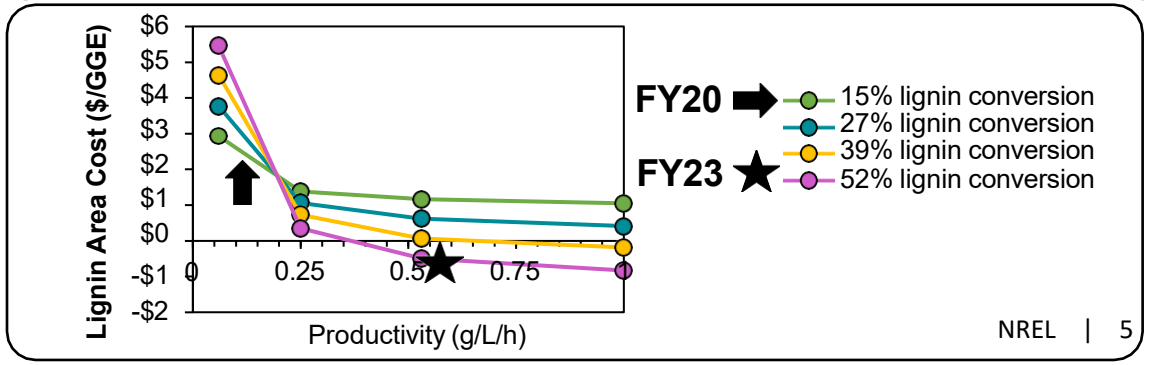
Major milestones, Go/No-Go Decisions:

- FY21: 60 g/L muconate from aromatic monomers (benchmark: ~49 g/L)
- FY22 G/NG: 10 g/L product from lignin (benchmark : 4 g/L muconate in FY20)
- FY23: 40 g/L product from lignin

MFSP ($/GGE), Base Case = $2.49



Lignin contribution to MFSP as a function of muconate productivity



Impact

Scientific:

- BETO has enabled lignin bioconversion as a new addition to metabolic engineering
- High-impact publications and patents from BLV consistently at forefront of the field
- Titer, rate, and yield achievements and fundamental discoveries at forefront of the field

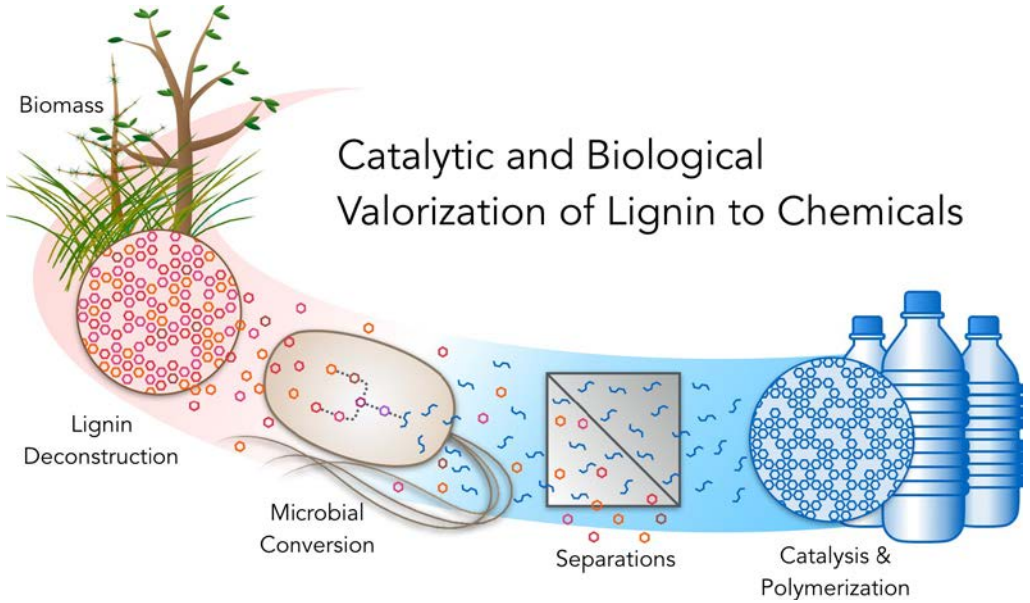
Industrial:

- Could enable $2-3/gge contribution to biorefineries through performance-advantaged bioproducts from lignin
- Work with startups to apply aromatic bioconversion for industrial applications
- Interactions inform project deliverables

Spero from nature

SUSTAINABLE FIBER TECHNOLOGIES

TECHNOLOGY HOLDING SUSTAINABLE SOLUTIONS

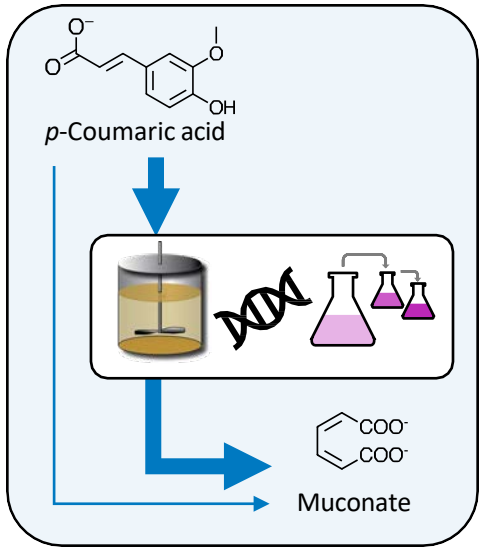


Linger ***PNAS*** 2014; Vardon ***Energy Env. Sci***. 2015; Johnson ***Met. Eng***. 2015; Salvachúa ***Green Chem***. 2015; Beckham ***Curr. Opin. Biotech***. 2016; Johnson ***Met. Eng. Comm*** 2016, 2017; Salvachúa ***Green Chem*** 2018; Johnson ***Joule*** 2019; Salvachúa ***Microb. Biotech***. 2019; Salvachúa ***PNAS*** 2020; Morya ***Trends Biotech***. 2020; Werner ***Met. Eng. Comm***. 2020; Notonier in revision at ***Met. Eng***. 2021; Erickson in review at ***Nature Catal***. 2021

Overall:

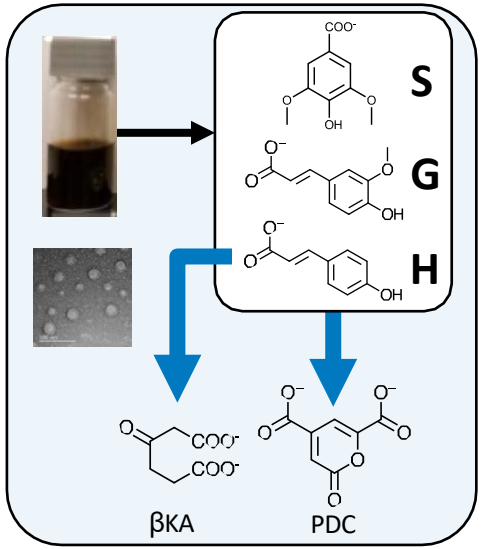
BLV at the cutting-edge of a new, promising direction in bioeconomy R&D to valorize lignin

Outline of Progress and Outcomes



Muconic acid production

- Muconic acid **analytics**
- Muconic acid production from *p*-coumarate and ferulate
- Overcoming the **hydroxylation** bottleneck
- Overcoming the **decarboxylation** bottleneck
- Overcoming **substrate toxicity**
- Overcoming **product toxicity**



Beyond muconic acid

- Engineering **β-ketoadipic acid** production
- Engineering convergent metabolism from S, G, and H lignin to **2-pyrone-4,6-dicarboxylic acid** (PDC)
- Evaluation of lignin streams and fundamental discoveries

Improved analytical method for muconic acid quantification

- Muconic acid method developed and published as a Laboratory Analytical Procedure (LAP) for accurate quantitation of *c,c*-muconic & *c,t*-muconic acids
- As substrates become more complex, analytical methods were adjusted to obtain better chromatographic resolution and rapid sample throughput

Enabled accurate total muconic acid quantitation

Methods continually being tailored for more accurate and faster data acquisition (analytical developments conducted in the LigU project)

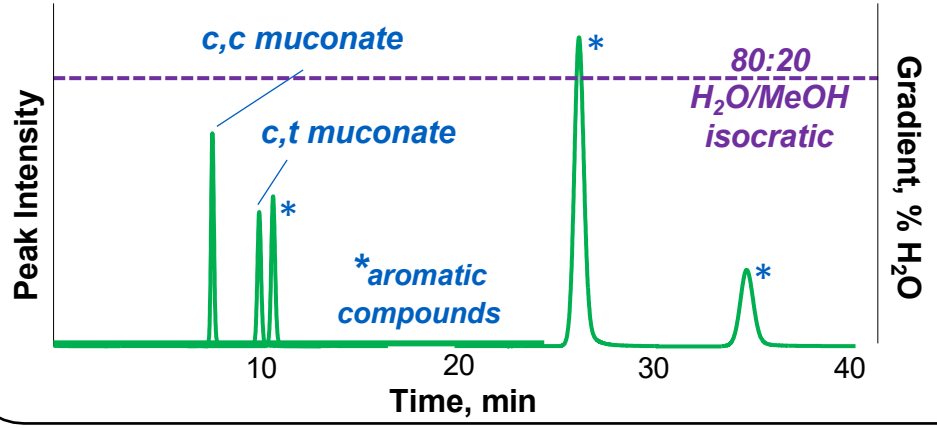
Determination of *cis,cis*- and *cis,trans*-Muconic Acid from Biological Conversion

Laboratory Analytical Procedure (LAP)

Issue Date: September 4, 2019

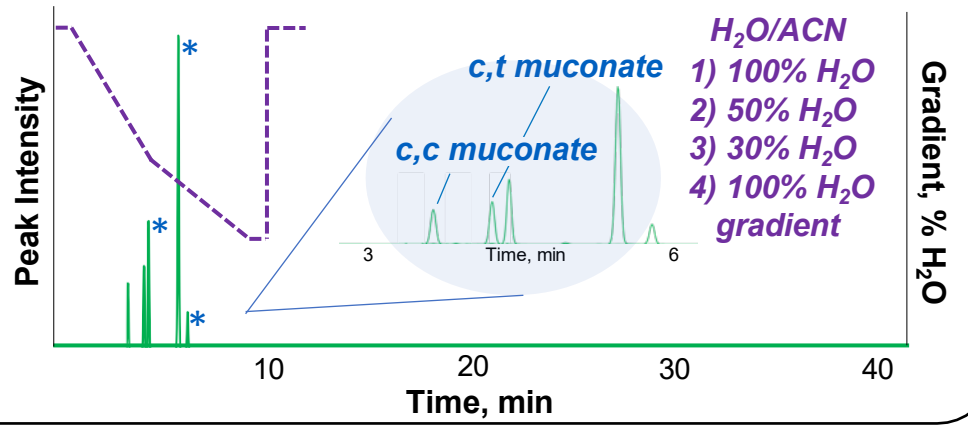
Brenna A. Black, William E. Michener, Courtney E. Payne, and Gregg T. Beckham

HPLC – simple (isocratic) but long chromatography (LAP)



Improved chromatographic resolution

UHPLC – more complex (gradient) but rapid chromatography

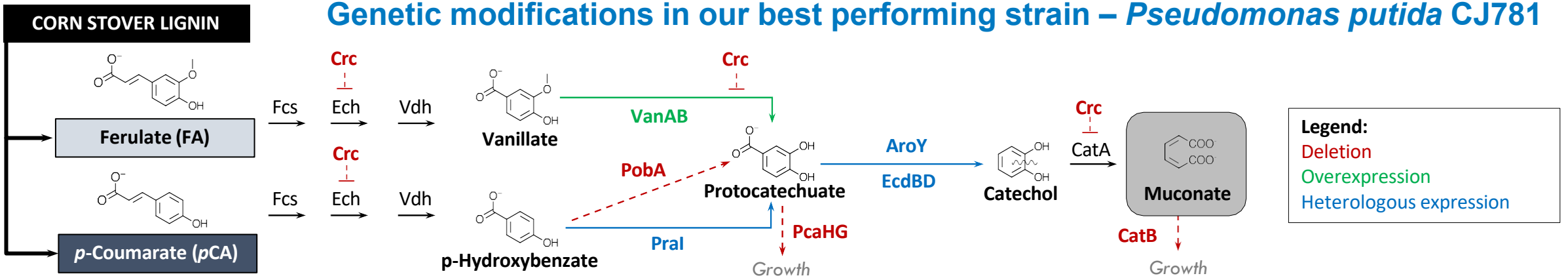


Black, B.A. *et al.*, 2019. NREL/TP-5100-74473

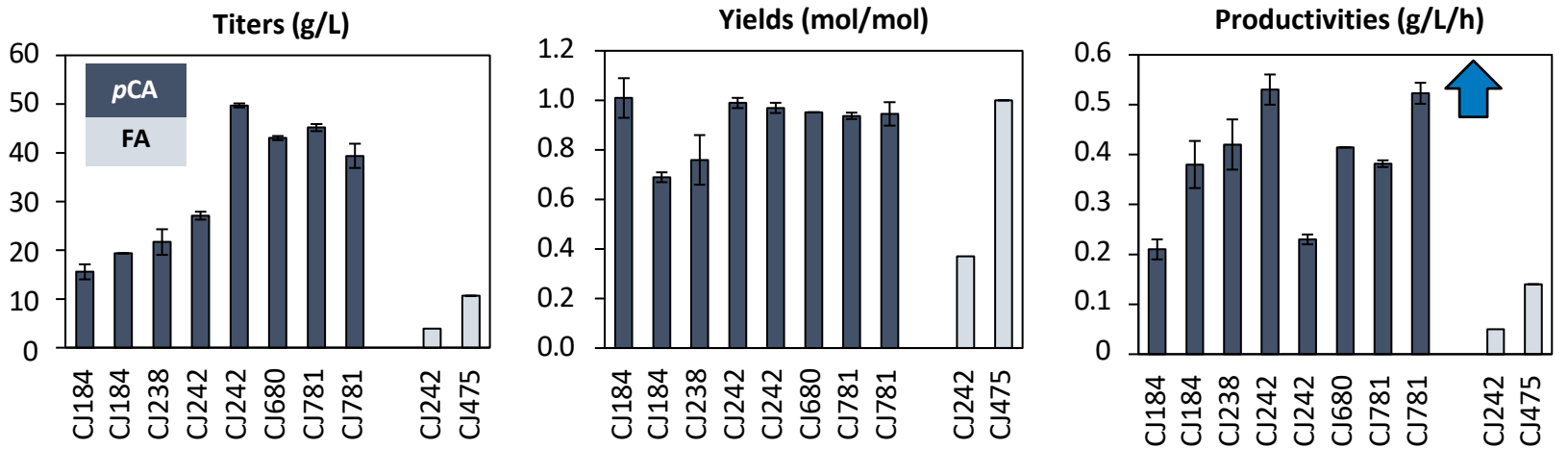
Ruhl, I. & Ramirez, K.J. *et al.*, In preparation

Muconic acid production – baseline strain performance

Genetic modifications in our best performing strain – *Pseudomonas putida* CJ781



Muconate production from *p*-coumarate and ferulate



Genotypes:
All strains: Δ*pcaHG*, Δ*catB*
CJ184: *aroY*, *ecdBD*
CJ238: *aroY*, Δ*crc*
CJ242: *aroY*, *ecdBD*, Δ*crc*
CJ475: *aroY*, *ecdBD*, Δ*crc*, ↑*vanAB*
CJ680: *aroY*, *ecdBD*, Δ*crc*, ↑*vanAB*, *praI*
CJ781: *aroY*, *ecdBD*, Δ*crc*, ↑*vanAB*, *praI*, Δ*pobA*

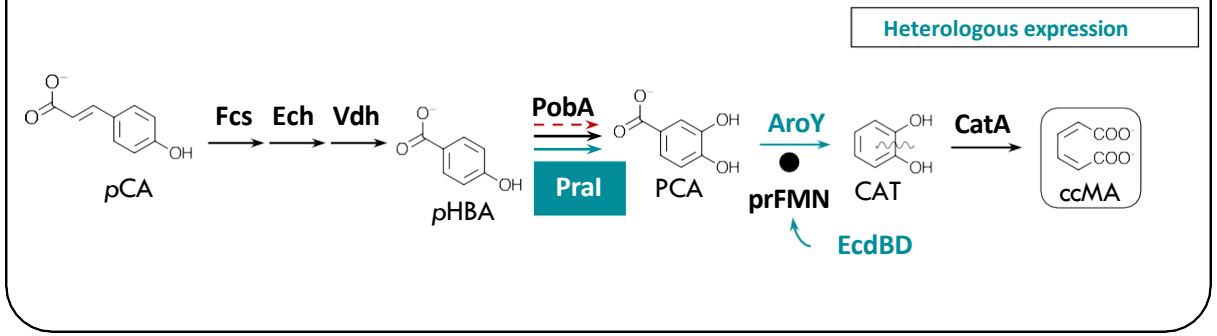
Metabolic engineering and bioprocess development enhanced productivity by 66% while maintaining high titers (40 g/L) and yields (>95%)

Salvachúa *Green Chem*. 2018
Kuatsjah *et al.*, in preparation

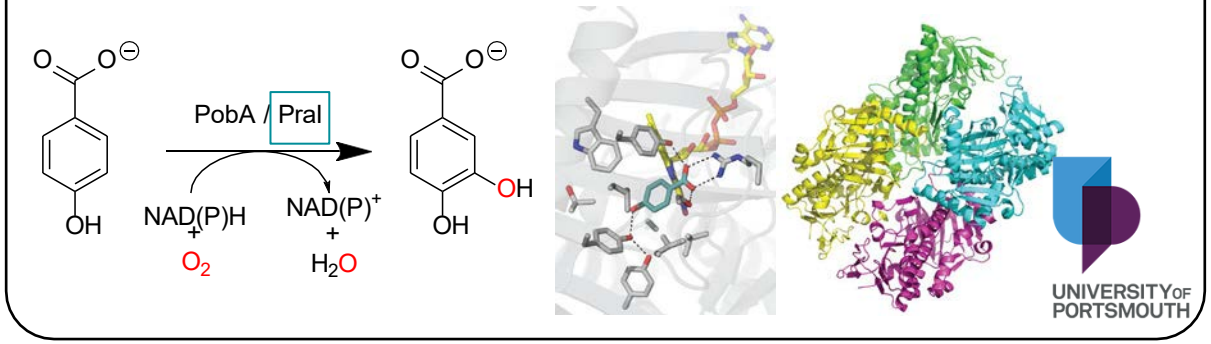
Overcoming the hydroxylation bottleneck

Kuatsjah *et al.*, in preparation

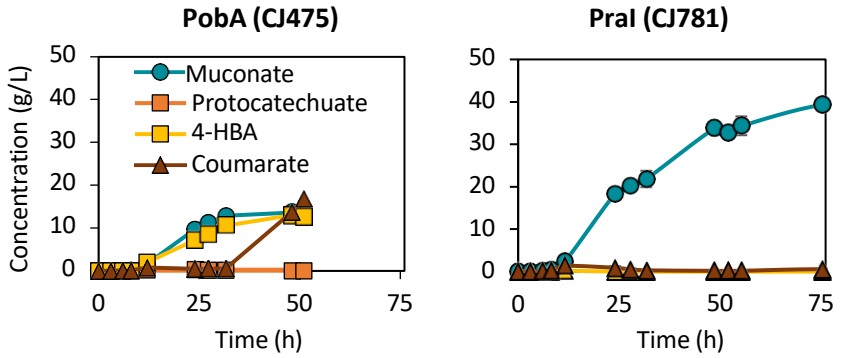
Catabolic pathways



Pral: Enzymology and Crystal Structure

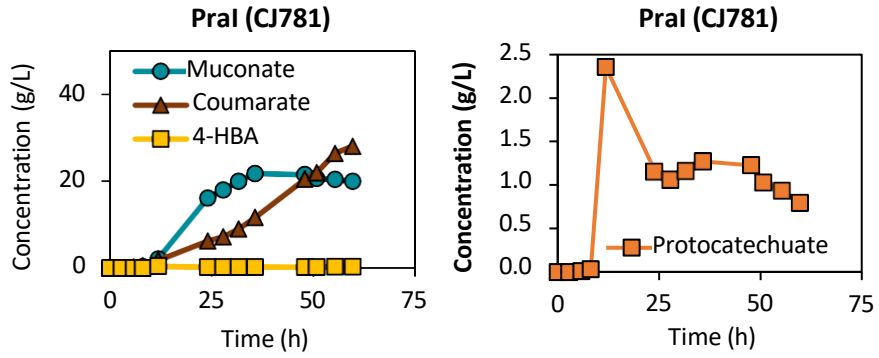


Muconate production from *p*-coumarate fed at 9 mmol/h



Increasing feeding rates to enhance productivity

Identification of new bottlenecks in CJ781 (*p*-coumarate fed at 12 mmol/h)



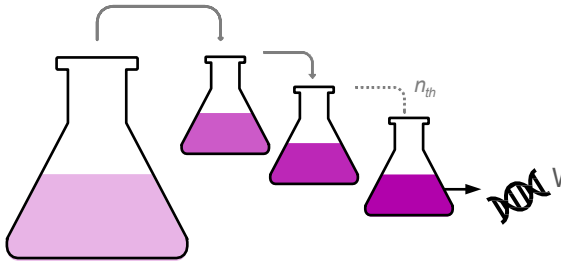
Replacing PobA with Pral eliminated the 4HBA bottleneck, improving muconate titers, rates, and yields

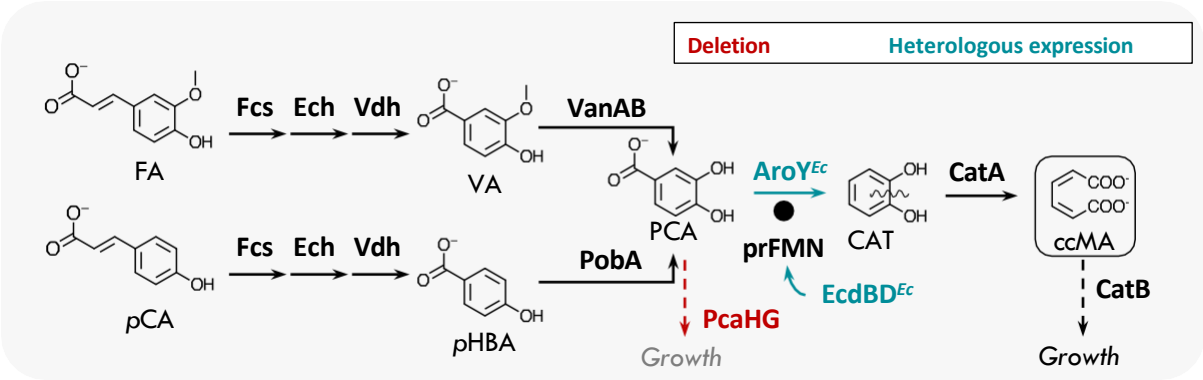
- The native *p*HBA hydroxylase, PobA, represents a bottleneck, resulting in accumulation of *p*HBA during the conversion of *p*CA
- **PobA** requires NADPH as a cofactor while the *p*HBA hydroxylase from *Paenibacillus* sp. JJ-1b, **Pral**, can use NADPH *or* NADH
- Increased pCA feeding rates revealed the PCA decarboxylase and pCA transport as the next bottlenecks

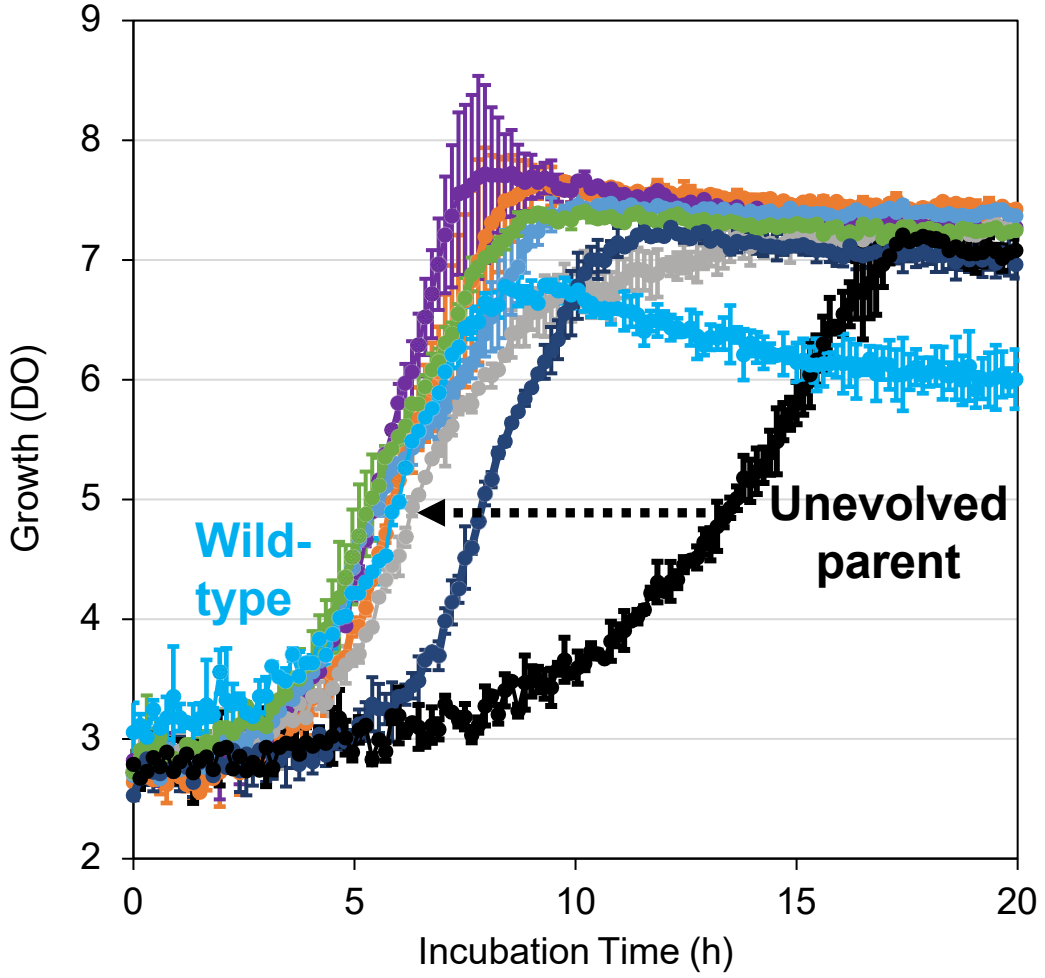
Overcoming the protocatechuate decarboxylase bottleneck

Strains evolved that are presumed to have improved PCA decarboxylase activity

- *p*-coumarate was routed through AroY for growth
- ALE performed and improved strains isolated
- Evaluation of these mutations are ongoing
- Whole genome sequencing ongoing

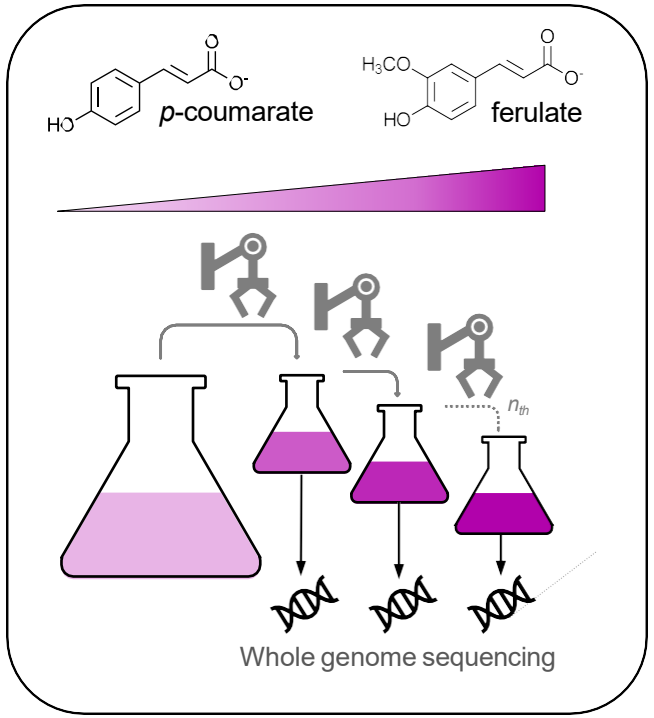




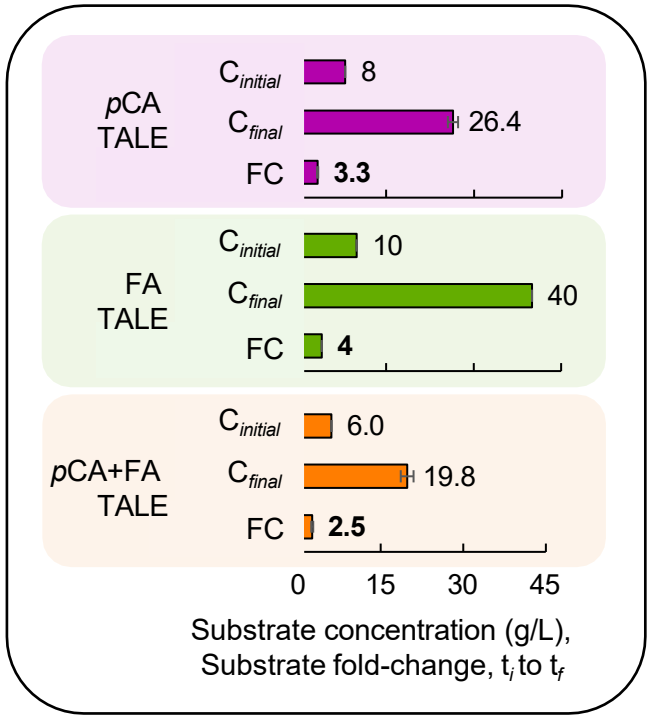


Overcoming substrate toxicity

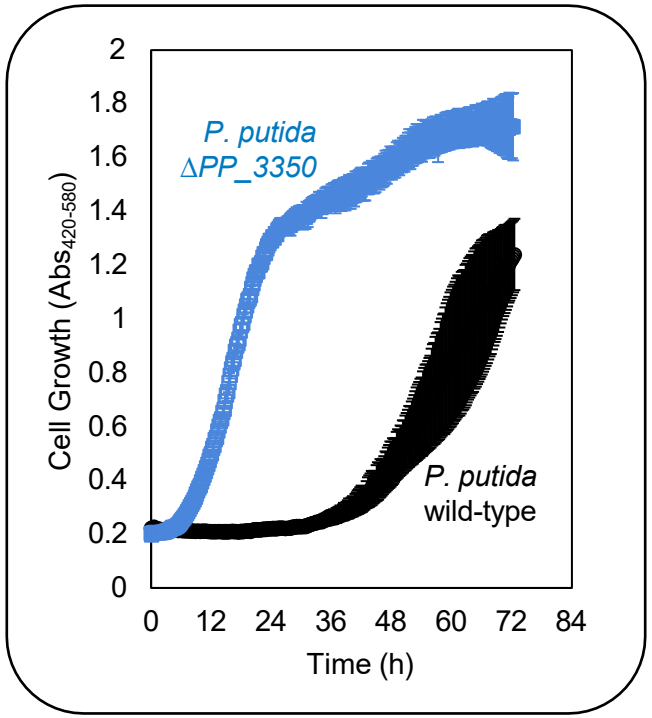
Automated tolerance adaptive laboratory evolution (TALE)



2.5- to 4-fold increase in tolerated aromatic concentrations



Reverse engineering for reduced lag phase

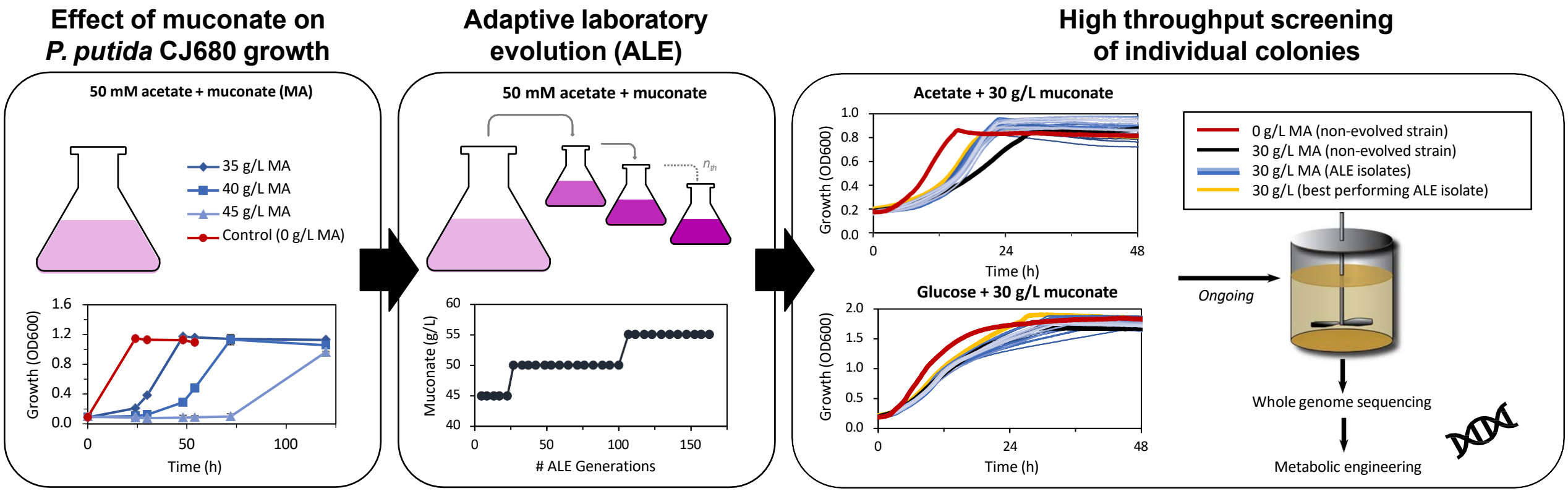


Achieved increased toxicity tolerance to *p*-coumarate and ferulate via TALE

- Identified genetic targets (e.g., *PP_3350*) to decrease lag phase in high concentrations of *p*-coumarate and ferulate
- Discovered PP_3350 PP_3349 are involved in the uptake of *p*-coumarate and ferulate
- Ongoing work focused on transporter engineering for improving productivity

Mohamed, Werner, *et al*. (2020) *Metabolic Engineering Communications*
Wada *et al*. *Metabolic Engineering* (in press)

Overcoming product (muconate) toxicity

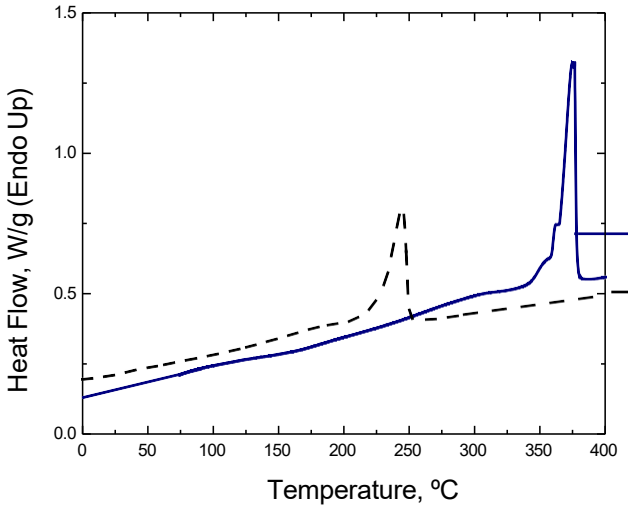


Identified isolates with reduced lags and improved growth rates at high muconate concentration

- Comparison of HTP screening with acetate or glucose revealed evolution for higher acetate utilization rates also occurred
- Best performing ALE isolates being evaluated in bioreactors now

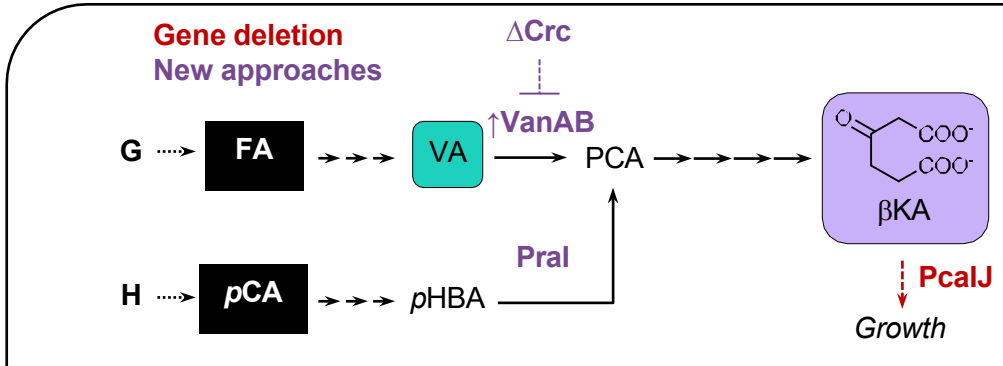
Engineering β-ketoadipic acid (βKA) production

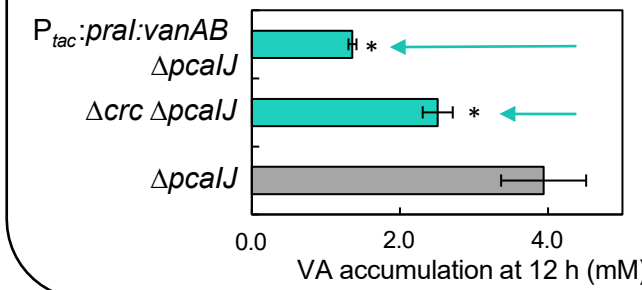
β-ketoadipic acid shows performance-advantaged properties over adipic acid in the polyamide nylon-6,6



Nylon permeability from:
Adipic acid – 10.1 g*mm/m²*day
βKA – 8.0 g*mm/m²*day

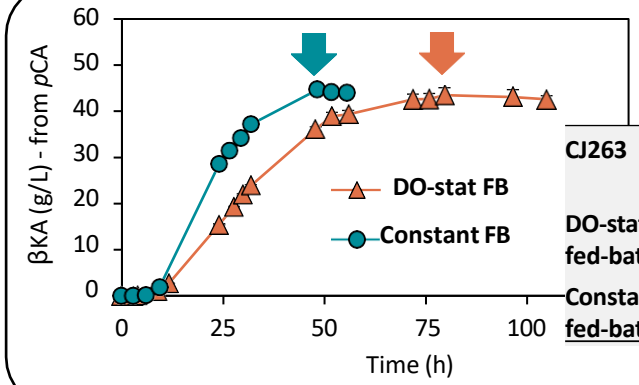
Metabolic engineering decreases VA accumulation





Currently constructing and evaluating additional targets, and stacking successful modifications into a single strain

Bioprocess development for βKA production



CJ263	T (g/L)	Y (mol/mol)	P (g/L/h)
DO-stat fed-batch	43.5	0.90	0.55
Constant fed-batch	44.7	0.92	**0.93**

βKA titers and productivities higher than those achieved for muconate with *P. putida* CJ263

- Based on the improvements in muconate-producing strains, we anticipate that modifications applied to CJ263 will enable βKA productivities over 1 g/L/h

Johnson, *et al.* Joule, 2019
Rorrer, Notonier, Black, *et al.* in review

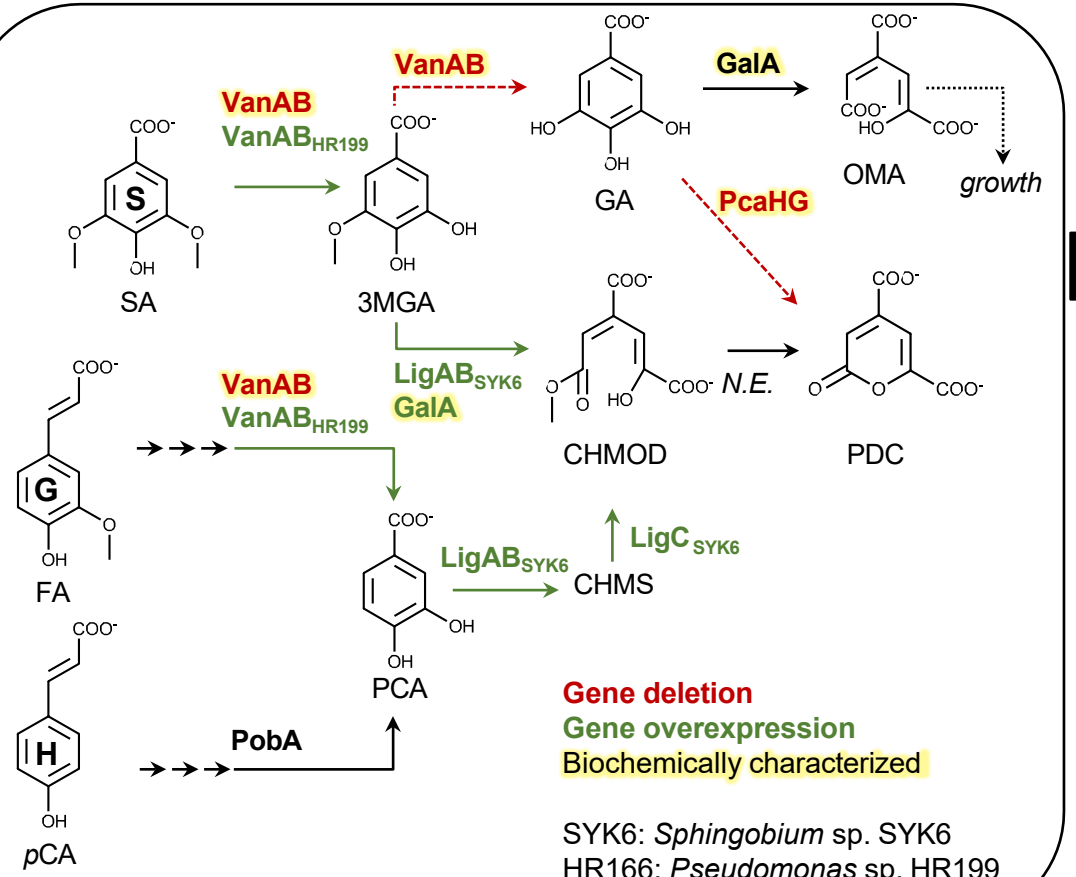
Engineering convergent metabolism from S, G, H lignin

Cell biology/ genetic engineering

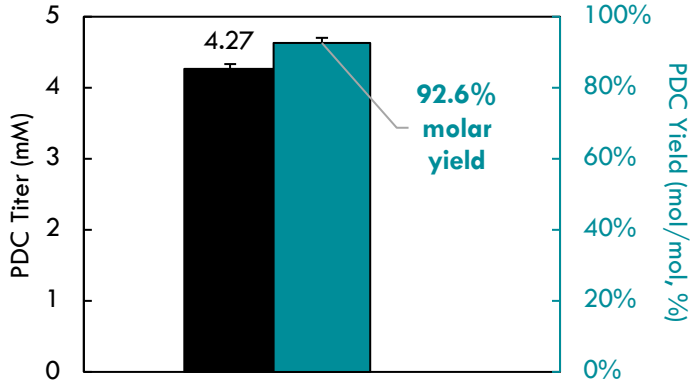
Systems biology (RNAseq, proteomics)

Biochemistry

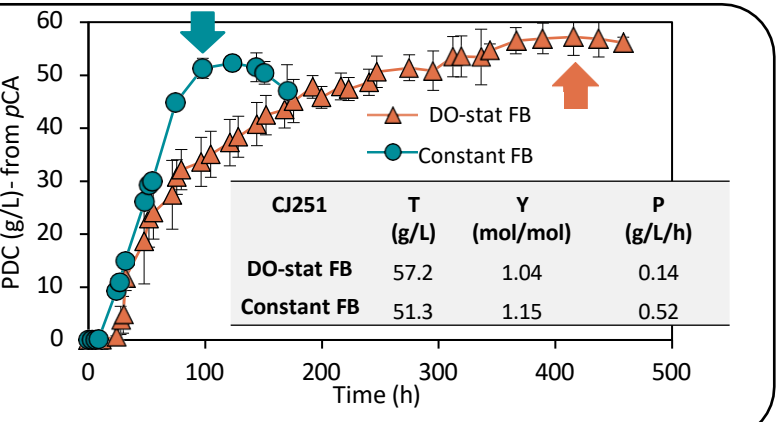
Pathway discovery & engineering



PDC production from S, G, and H substrates



Bioprocess development for PDC production



CJ251	T (g/L)	Y (mol/mol)	P (g/L/h)
DO-stat FB	57.2	1.04	0.14
Constant FB	51.3	1.15	0.52

Achieved simultaneous conversion of *p*CA, FA, and SA to PDC

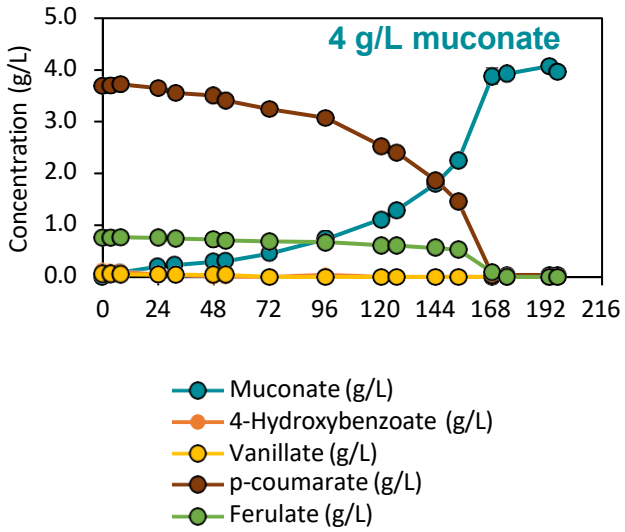
- Resulted in fundamental biochemical advances
- TRY similar to those obtained for muconate with our best performing strain

Notonier, Werner, *et al*. *Metabolic Engineering,* in revision

Evaluation of diverse lignin streams

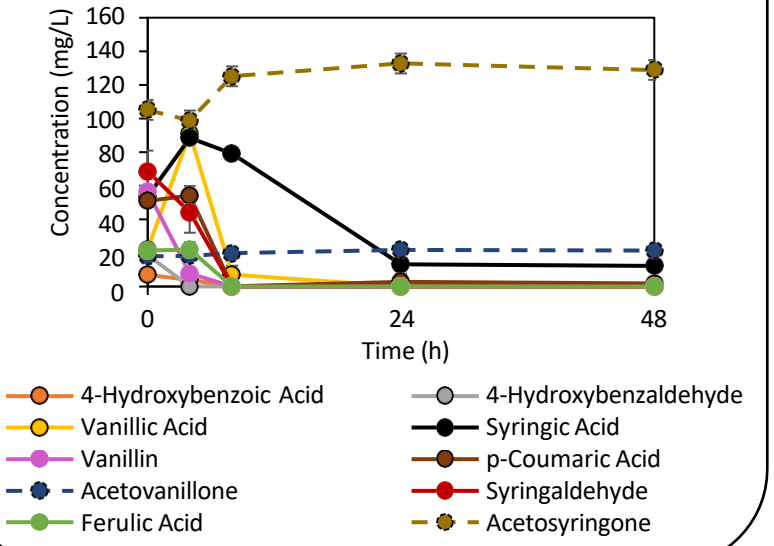
Collaboration with Low Temperature Advanced Deconstruction (LTAD)

Black liquor recycling (deacetylation of corn stover with NaOH) increases concentration of aromatic compounds. *P. putida* produces 4 g/L of muconate from this stream

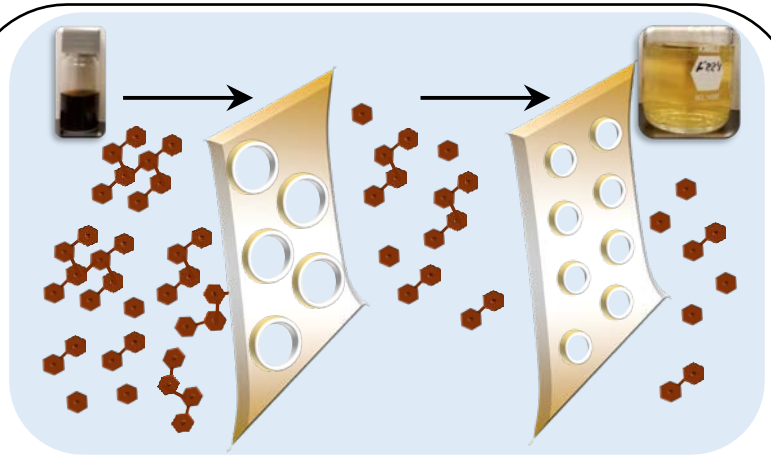


Collaboration with Lignin Utilization

$Sr(OH)_2$ depolymerization increases yields of monomeric aromatic compounds from solid lignin. *P. putida* utilizes most of the compounds as a carbon source



Collaboration with Separations Consortium



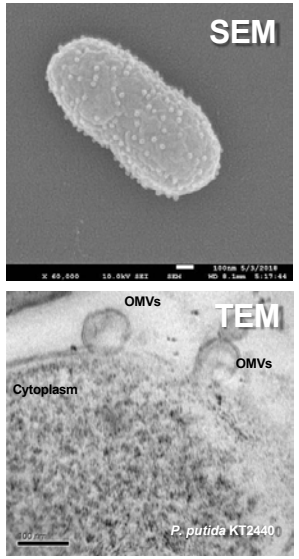
- Generation of low MW lignin and acetate stream from black liquor (generated in deacetylation of corn stover)
- Monomeric aromatic compounds and acetate highly concentrated
- Strategy key to achieve high product titers and rates

Systems bio, metabolic eng., and bioprocess development being pursued to convert lignin streams

- Elucidating additional relevant catabolic pathways
- Currently optimizing fed-batch processes to feed low MW lignin plus acetate to *P. putida*

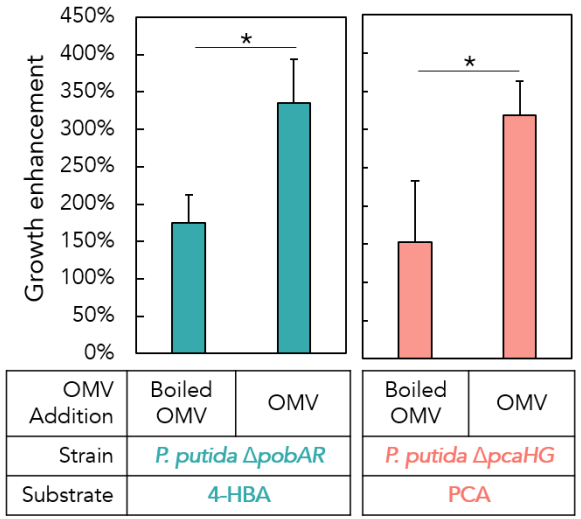
Additional work with lignin streams and *P. putida*

P. putida releases outer membrane vesicles



Are OMVs involved in the extracellular catabolism of lignin?

- *P. putida* releases OMVs in lignin
- Via proteomics, identified oxidoreductases and aromatic catabolic enzymes in OMVs
- OMVs were shown to catabolize aromatic compounds



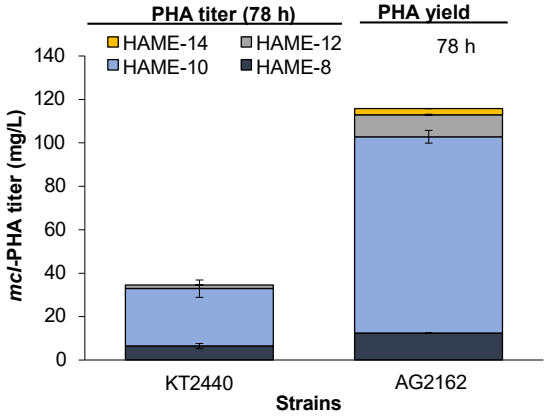
Salvachúa, Werner, Pardo ***PNAS*** 2020

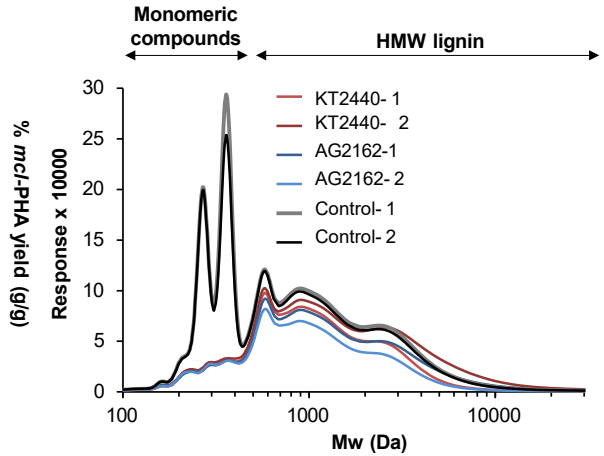
mcl-PHA production from lignin depolymerized via base catalyzed depolymerization

OAK RIDGE National Laboratory

Collaboration with Dr. Adam Guss (ORNL)

- *P. putida* engineered to enhanced PHA production from aromatic compounds.
- *P. putida* AG2162 significantly enhances PHA production from lignin streams





Salvachúa ***Microb. Biotech.*** 2020

OMVs could potentially be useful tools for synthetic biology and biotechnological applications

Ongoing bioprocess development with ORNL lignin project (A. Guss)

Summary

Overview

- BLV is developing strains and bioprocesses to funnel lignin-derived streams to co-products for the biorefinery

Management

- Strain Engineering and Bioprocess Development tasks work closely together, and with other projects upstream and downstream of BLV

Approach

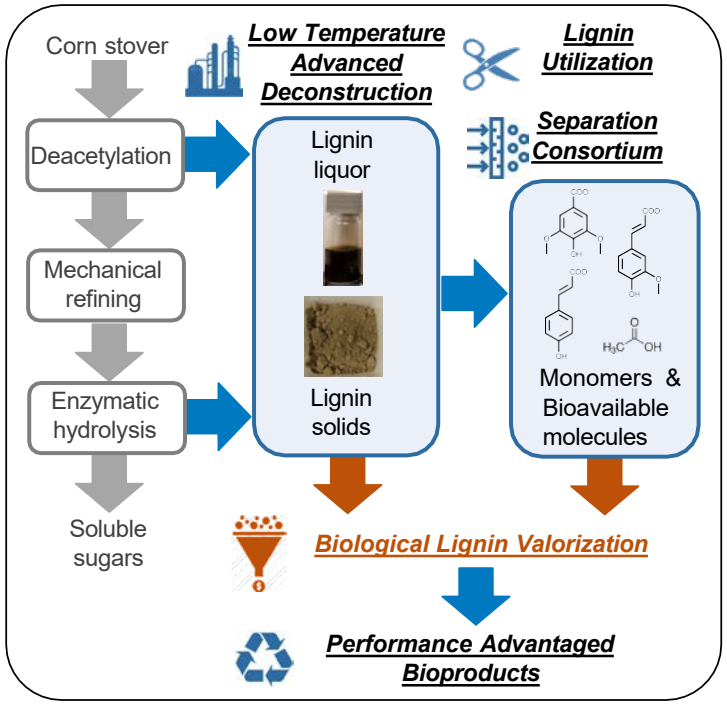
- Use analysis to guide R&D towards meaningful and impactful bioprocess target molecules

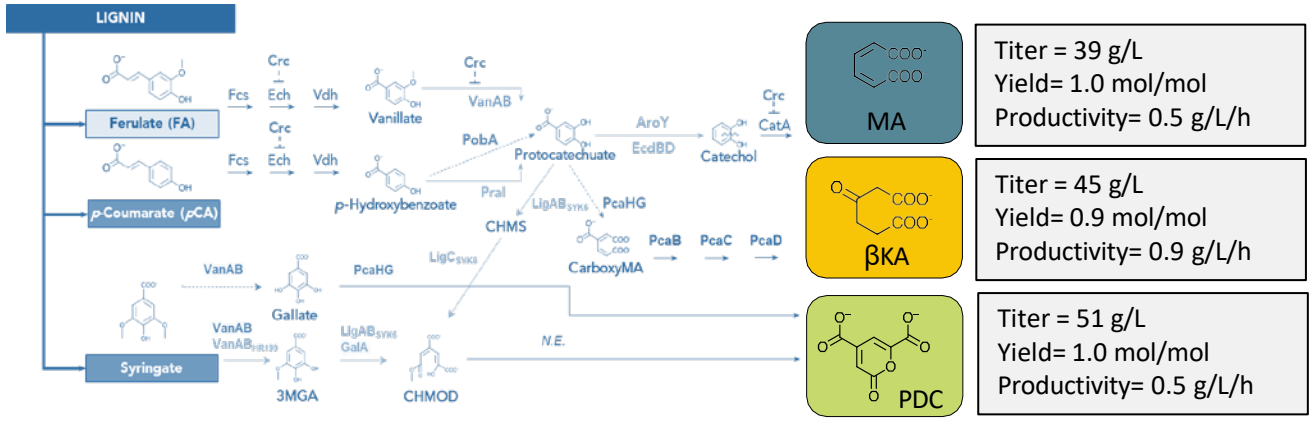
Impact

- BLV efforts consistently at the forefront of the burgeoning microbial lignin conversion field

Progress and Outcomes

- Developed *P. putida* strains and bioprocesses able to achieve near industrially-relevant TRY; ongoing work focused on continued debottlenecking and process integration with lignin deconstruction





Quad charts

Timeline

- Active Project Duration: 10/1/2020 – 9/30/2023
- Total Project Duration: 10/1/2015 – 9/30/2023

	FY20	Active Project (FY21-23)
DOE Funding	$700,000	$2,100,000

Project Partners

Nat'l labs: ORNL

BETO Projects: Lignin Utilization (2.3.4.100), Separations Consortium (2.5.5.502), Biochemical Platform Analysis (2.1.0.100), Synthesis and Analysis of Performance-Advantaged Bioproducts projects (2.3.4.501), and Low-Temperature Advanced Deconstruction (2.2.3.100)

Barriers addressed

- Ct-C Process development for conversion of lignin
- Ct-D Advanced Bioprocess Development

Project Goal

Develop biological processes to produce co-products from lignin-derived compounds

End of Project Milestone

Enable production of >40 g/L and >80% yield and 0.5 g/L/h productivity of an exemplary bioproduct from a process-derived lignin stream in collaboration with the Separations Consortium and Lignin Utilization. Demonstrate 2 products that contribute >$1/gge to HC fuel MFSP.

Funding Mechanism

Bioenergy Technologies Office FY21 AOP Lab Call (DE-LC-000L079) – 2020

Acknowledgements:
DOE Technology Managers Jay Fitzgerald and Beau Hoffman

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Caroline Amendola, Brenna Black (LigU), Ryan Davis (Analysis), Rick Elander, Christopher Johnson, Rui Katahira (LigU), Donghyun Kim, Colin Kneucker, Megan Krysiak, Jake Kruger (LigU), Eugene Kuatsjah, Kelsey Ramirez, Michelle Reed, Davinia Salvachua, Christine Singer, Kiki Szostkiewicz, Allison Werner, Sean Woodworth, Bruno Klein (Analysis)

Q&A

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NREL Transforming ENERGY

Additional Slides

Responses to previous reviewer comments

- This program describes an important strategy to build an organism to valorize lignin-derived compounds biologically. The work is in a good position to benefit from progress in the Lignin Utilization program and appears to be doing so, for example by utilizing model substrates and potentially enabling the building of mock substrate mixtures to focus on limiting compounds as it moves toward authentic substrate compositions. The Agile BioFoundry is mentioned briefly in the project description but it is not clear to what extent information is flowing and in what direction. Discussion with the Agile BioFoundry regarding P. putida development is a clear win-win, as the Agile BioFoundry is working on building a P. putida chassis. One could easily see implementation of a Design-Build-Test-Learn strategy (or at least a mini-Design- Build-Test-Learn in the early stages of discovery) for gene dosing (copy number) and stacking of tolerance factors and "on-pathway" substrate utilization/product genes as they emerge, and are likely to add value to move the program quickly. The discovery of a secreted enzyme system is interesting and may provide additional strategies to exploit this for extracellular degradation of potentially cytotoxic compounds such as soluble lignin-derived oligomers. The team is encouraged to further explore the secretome of the organism to determine its potential and limitations, and optimize key enzymes.
    - We appreciate the positive comments and feedback. Indeed, we will on-board some of the tools developed in the Agile BioFoundry (e.g., biosensors and transformation tools), and much of the work originating in this project has informed engineering in the Agile BioFoundry as well, so synergies will exist in both directions. We are certainly investigating the secretome in more detail as bandwidth permits and also leveraging fundamental discoveries made in the DOE Office of Science-funded and academic efforts.
- The team has done an outstanding job making progress across many challenges that arose during the project for such stretching goals. This team worked well together, showing solid management of program and good collaboration across the various other teams inputting information and product streams for evaluation. Again, from experience, biological conversion on such complex materials is highly challenging and the team is commended for taking on this challenge and making great progress. This program has a chance to contribute substantially to BETO goals by funneling many products into single key materials when lignin processing produces so many compounds. I would hope this project could find a way to continue beyond the September end date.
- The PIs continue a scientifically excellent project for the biochemical transformation of lignin into muconate, and ultimately adipic acid. High-yield success in this project through biological funneling will be a nice addition to biorefinery development. Demonstration of this approach on a wider range of lignins and a clear TEA will further improve the project's impact.
    - We completely agree that this approach needs to be demonstrated on a wide range of lignins. We are actively engineering a Pseudomonas putida chassis that is able to convert syringyl (S), guaiacyl (G), and hydroxyphenyl (H)-type lignin monomers, and hence will ideally be useful for lignin samples from multiple upstream catalytic treatments and biomass sources.
- This project has made excellent progress towards identifying new enzymes able to cleave the five most relevant linkages in lignin, and then transferring that capability to P. putida to increase lignin bioconversion into valued biochemicals. In addition to using lignin model compounds, the team developed P. putida strains that can utilize syringyl lignin, transform syringol, and produce muconic acid from base-catalyzed depolymerized lignin. As a next step, strain tolerance to fluctuations in feedstock composition and slight changes in pretreatment conditions will be important to quantify.
    - We agree that upstream changes will modify the organism needs and balance. At present, we are attempting to establish a baseline strain that is able to catabolize H-, G-, and S-type lignin monomers that will be produced from a wide variety of biomass samples.
- This project fits nicely in the lignin valorization portfolio and is well integrated with other relevant programs. The team appears to have made very substantial progress on titer, rate, and yield performance in model systems, which presumably is carrying over to the performance in "real" lignin streams. It appears that currently the levels of rate and titer they can achieve in these real streams is limited by the upstream processes' abilities to produce a high-enough concentration of addressable carbon.
    - The comment regarding the upstream process abilities dictating the titers, rates, and yields is absolutely correct: namely, the upstream lignin depolymerization process dictates the amount of "bioavailable" aromatic carbon for the microbe to convert. We are working in close collaboration with the Lignin Utilization project to enable higher amounts of aromatic carbon that is biologically accessible now.

Publications, patents, presentation, awards, and commercialization

Publications

In review or revision:

Joshua R. Elmore, Gara N. Dexter, Davinia Salvachúa, Jessica Martinez-Baird, E. Anne Hatmaker, Jay D. Hueneman, Dawn M. Klingeman, George L. Peabody V, Darren J. Peterson, Christine Singer, Gregg T. Beckham, Adam M. Guss*, Lignin valorization to itaconic acid at high yield by dynamic, two-stage conversion, in review at *Nature Comm*.

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Publications, patents, presentation, awards, and commercialization

Presentations (2018-2020)

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Performance-advantaged bioproducts from lignin, BioEnergy Society of Singapore (via webinar), December 14th, 2020

Efforts towards sustainable performance-advantaged bioproducts and plastics upcycling, Materials Life-Cycle Management Mini-Symposium, University of Delaware (via webinar), October 1st, 2020

Bacterial aromatic catabolism for lignin and plastics conversion, BioDiscovery Institute, University of North Texas (via webinar), August 20th, 2020

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Bacterial aromatic catabolism for lignin and plastics conversion, University of Minnesota BioTechnology Institute, March 5th, 2020

Enzymes for lignin and plastics conversion, Enzymes, Coenzymes and Metabolic Pathways, July 23rd, 2019

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Patent applications

Engineered Pseudomonas putida strain for production of 2-pyrone-4,6-dicarboxylic acid guaiacyl and p-hydroxyphenyl and syringyl lignin-derived aromatic species: ROI-20-131, pending