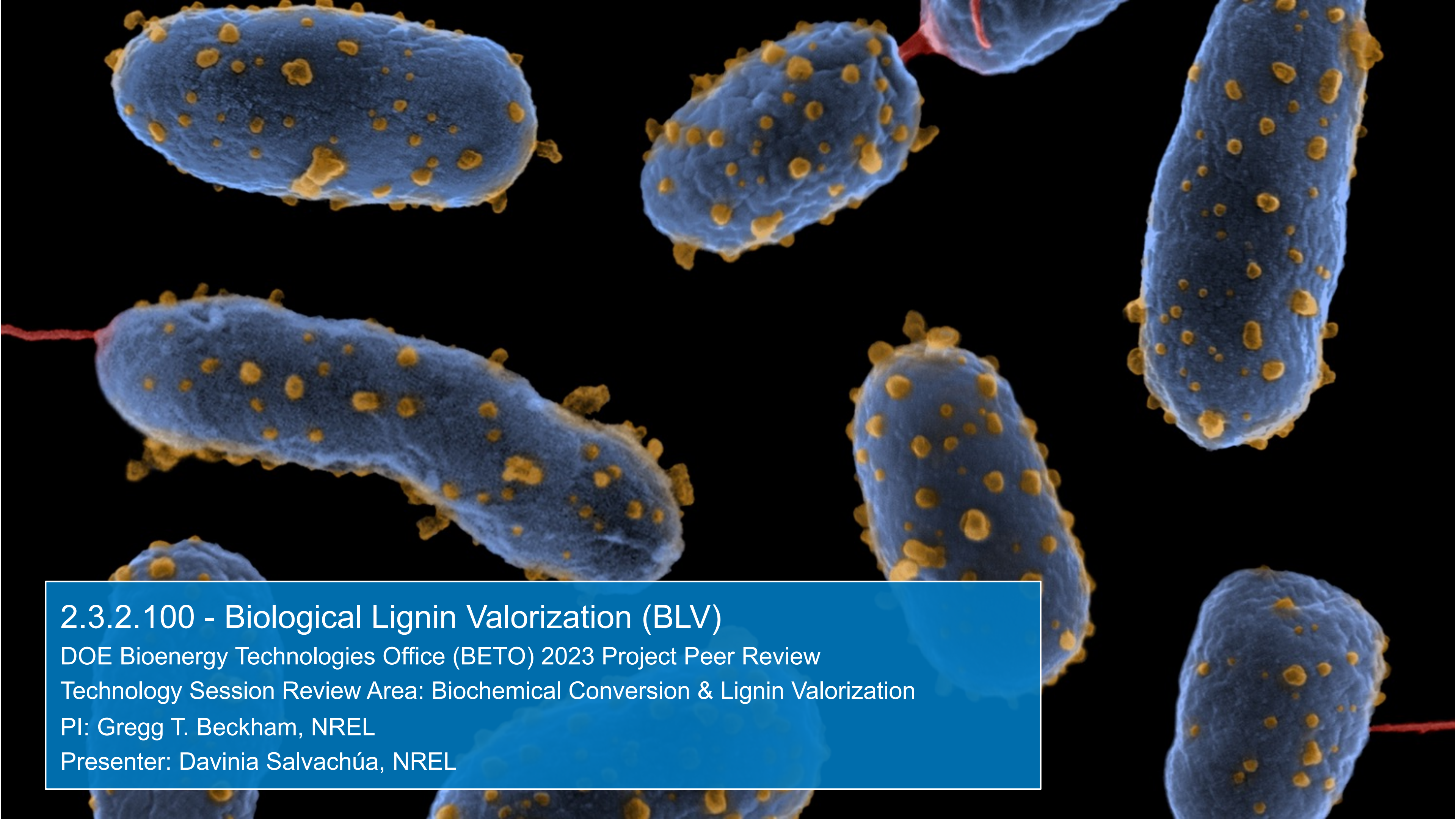


2.3.2.100 - Biological Lignin Valorization (BLV)

DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

Technology Session Review Area: Biochemical Conversion & Lignin Valorization

PI: Gregg T. Beckham, NREL

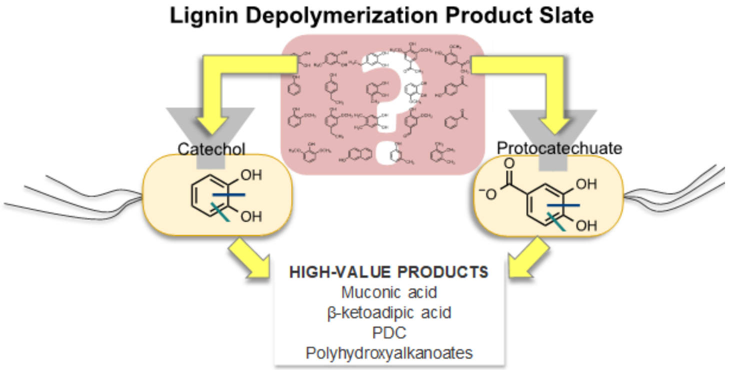
Presenter: Davinia Salvachúa, NREL

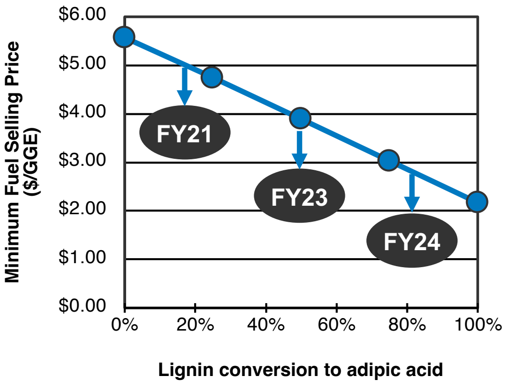
Project overview

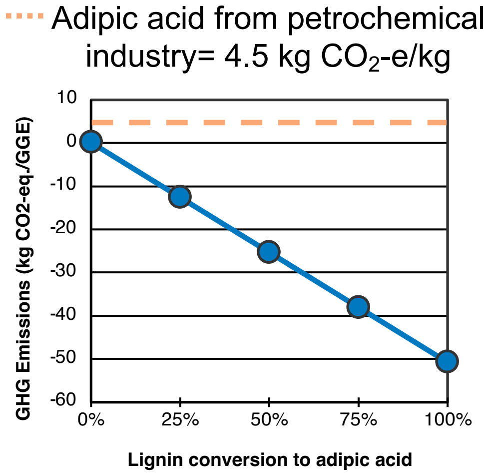
Linger *et al. PNAS* 2014
Davis *et al. NREL Design Report* 2018

Goal: Develop strains and bioprocesses to funnel heterogeneous lignin-derived aromatics to single, value-added products (BETO 2030 goal)

- **History:** reported concept of biological funneling in FY14, BLV project started in FY16
- **Focus:** products with market sizes and selling prices to aid biofuels production (e.g., adipic acid) that can contribute $2-3/gge and be cost-competitive with petrochemical baselines
- **BETO project collaborations:** Lignin Utilization and Separation Consortium for lignin substrates, Performance Advantaged Bioproducts for products







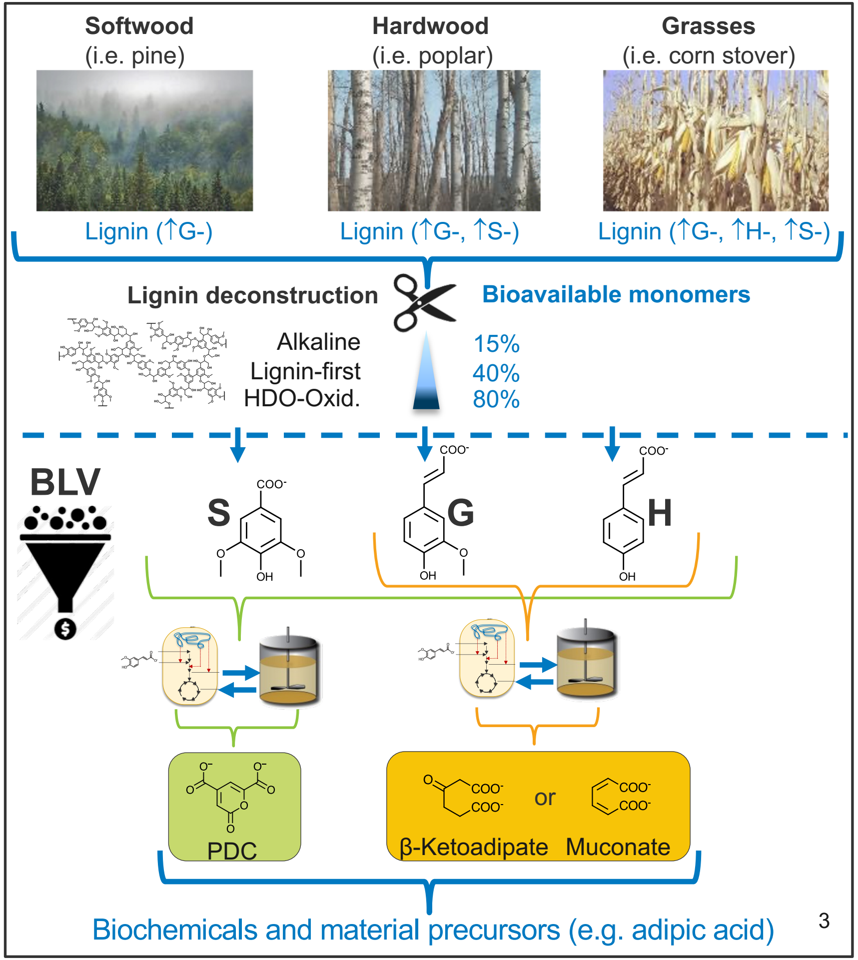
Approach: Focus areas for BLV

Technical approach:

- Use **model compounds** for strain evaluation
- Use **lignin streams** from Lignin Utilization and Separations Consortium projects and collaborators for process demonstration
- *Pseudomonas putida* KT2440
- Strains for S, G, H-lignin conversion
- Iterate between strain and bioprocess development
- Target atom-efficient products (e.g., adipic acid replacements)
- Techno-economic analysis (TEA) and life cycle assessment (LCA) to identify process drivers

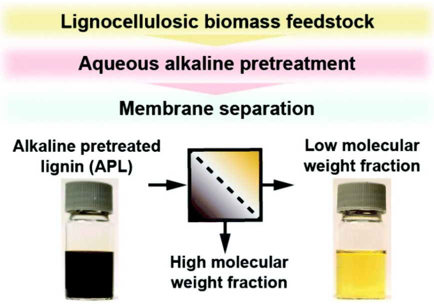
Major challenges and areas of focus:

- Bio-available lignin to achieve bioprocess performance seen with model compounds **(major progress from FY21)**
- Industrially relevant titer, rate, yield on model compounds
- Strains and bioprocesses for high titers, rates, and yields from lignin streams



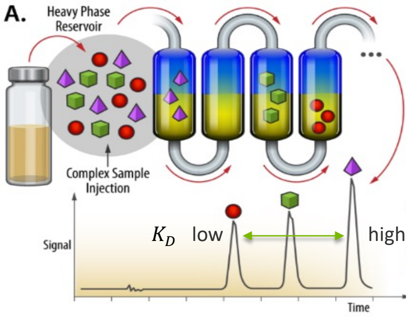
Approach: Integration with upstream lignin catalysis and separations

Membrane separations to isolate low molecular weight lignin (Separation Consortium)



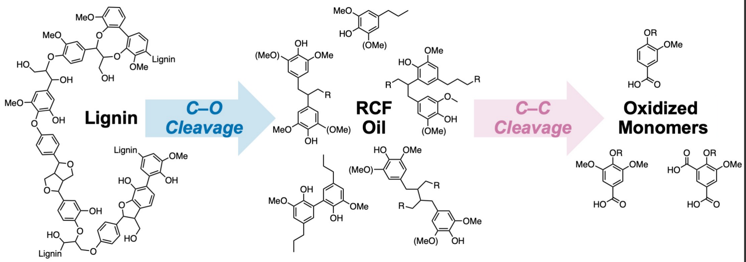
Saboe *et al.*, *Green Chem.* 2022

Counter-current chromatography for lignin monomer isolation (Separation Consortium, Lignin Utilization, University Wisconsin Madison)

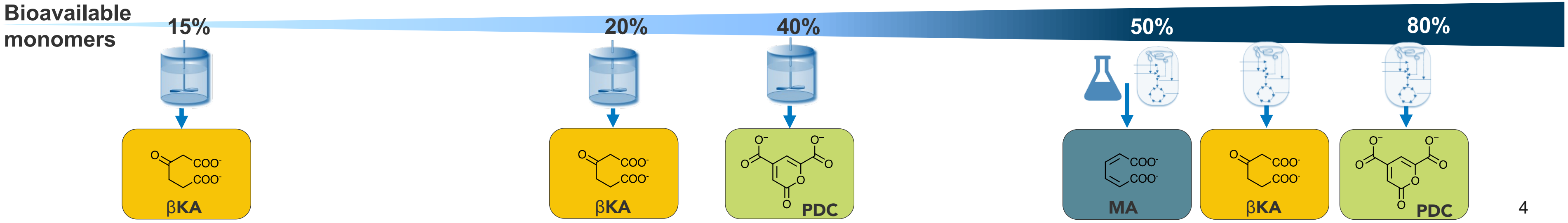


Alherech *et al.*, *ACS Central Science* 2021; Choi *et al.*, *in review*

Oxidation to cleave lignin C–C bonds to bio-available compounds (Lignin Utilization)



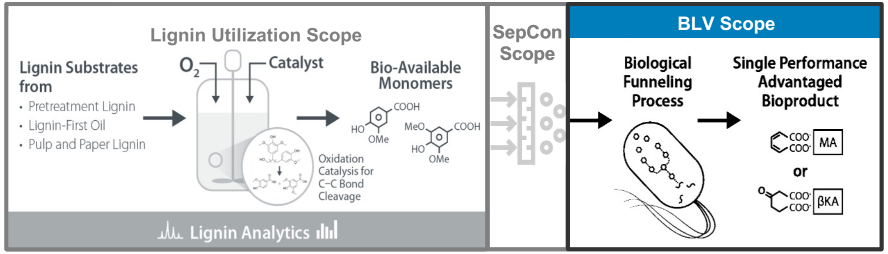
Gu *et al.*, *in review*
Palumbo *et al.*, *in review*

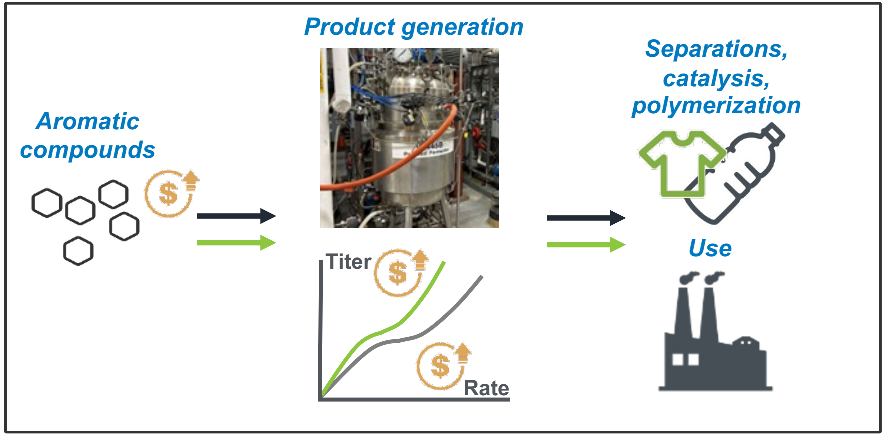


Approach: Risks, management, and milestones

Risks and mitigation strategies:

- **Risk**: accessing lignin streams with high yields of bio-available aromatic compounds
- **Mitigation**: Lignin Utilization and Separation Consortium to deliver lignin with high yields of bio-available monomers
- **Risk**: titer, rate, and/or substrate/product toxicity limit the strain performance
- **Mitigation**: TEA and LCA to understand impactful parameters, synthetic biology tools to address strain performance





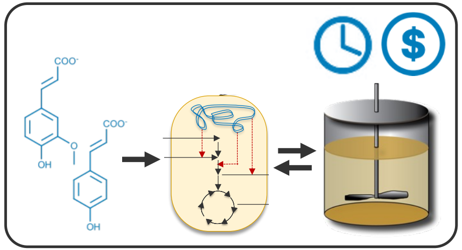
Management, communication, & DEI:

- Monthly project meetings
- *Ad hoc* meetings with other BETO projects
- Dedicated Project Managers – lab space, equipment, reporting, finances
- Focused on creating physically and psychologically safe research environments

Major project milestones:

- **FY22 G/NG**: 10 g/L product from lignin (FY20: 4 g/L)
- **FY23**: 40 g/L product from lignin

Progress and outcomes: Outline



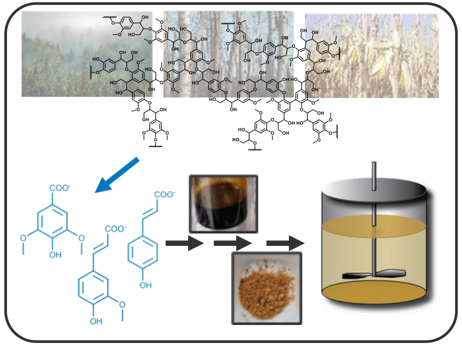
TEA highlights economic drivers

- Strain and bioprocess development opportunities

Strain and bioprocess development using model lignin-related aromatic compounds (LRCs)

- Muconate production
- β-Ketoadipate production

Overcoming metabolic bottlenecks to concurrently increase titers, rates, and yields

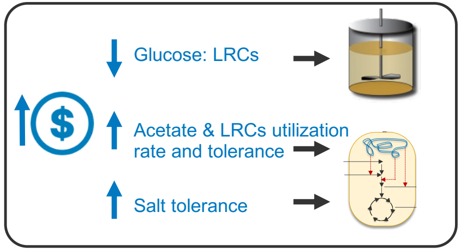


BLV integrates with upstream lignin catalysis and separations to produce bio-available lignin streams

Lignin from:

- Corn stover (alkaline pretreatment)
- Pine (lignin-first oxidation)
- Poplar (lignin-first oxidation)
- Poplar (reductive catalytic fractionation (RCF) + autoxidation)
- Feedstock-agnostic (hydrodeoxygenation + autoxidation)

Conversion to product with optimal strains

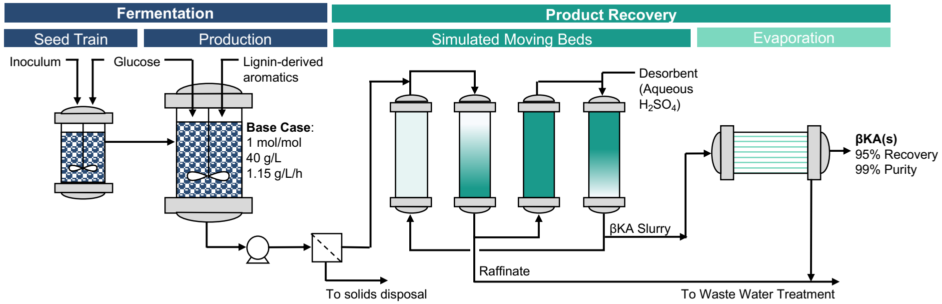


Improvement of bioprocess and strain features based on economic drivers

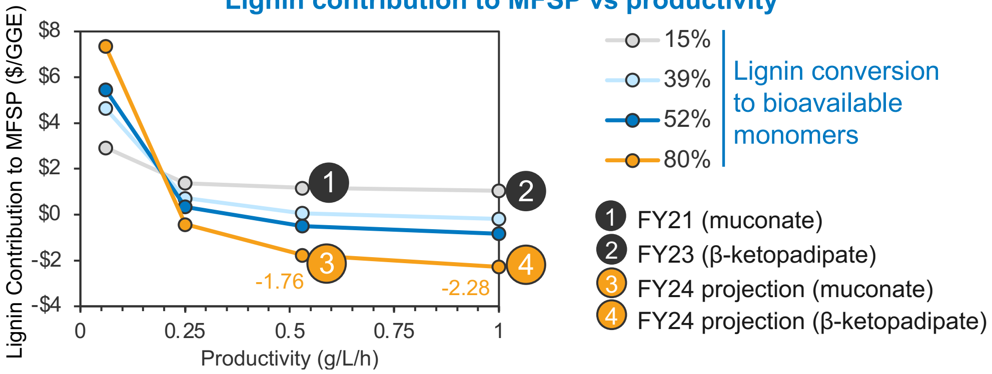
- Reduce cost of supplemental carbon source
- Improve tolerance to supplemental carbon sources
- Improve tolerance to aromatic compounds to increase productivity
- Improve tolerance to salt to increase product titers

TEA highlights important economic drivers

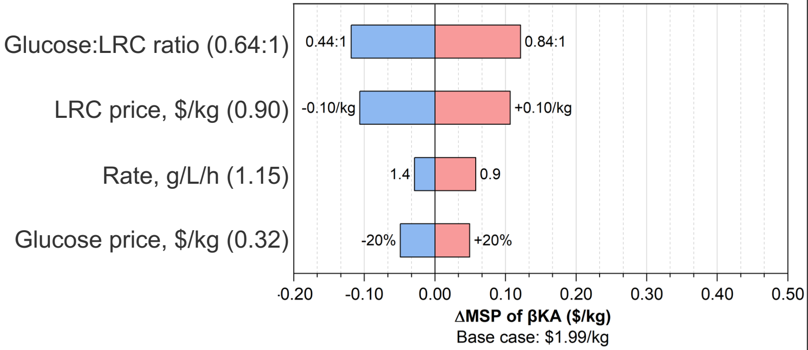
Modeled process configuration



Lignin contribution to MFSP vs productivity



Select key cost drivers for β-ketoadipate production



LRC = lignin-related compounds

Werner, Cordell *et al.*, *in review*

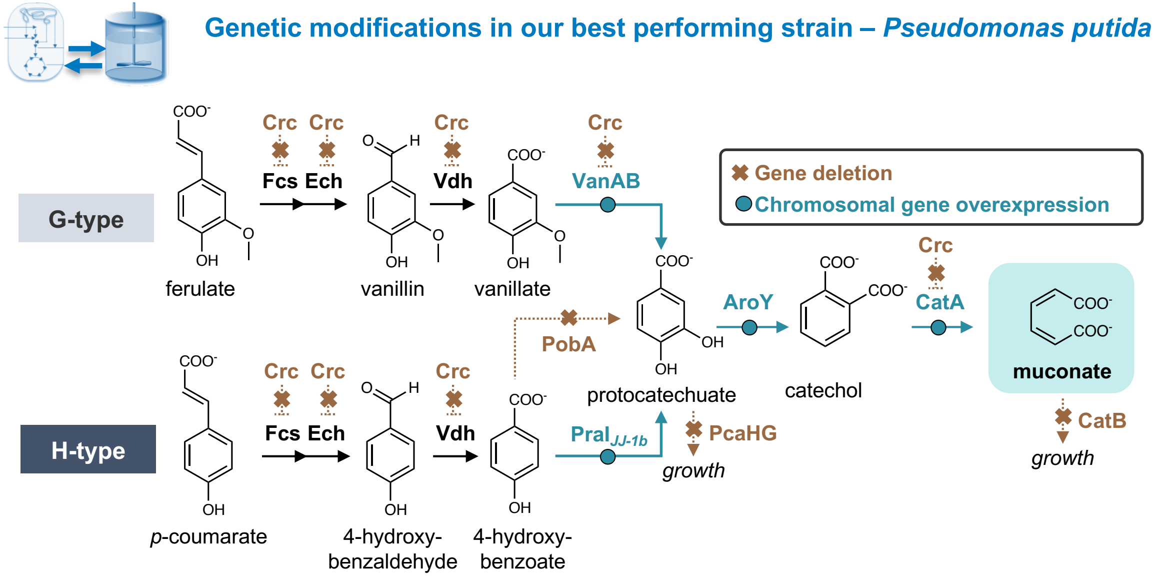
Our FY24 projection aims to reduce the MFSP by ≥$2/gge and produce βKA (currently $1.99/kg) at a competitive cost compared to adipic acid ($1.71/kg) from petrochemical industry

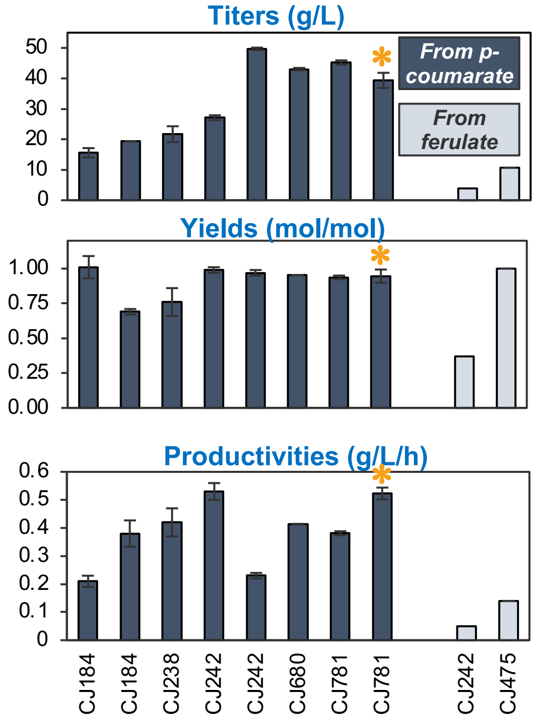
- Lignin conversion to bioavailable aromatic compounds is ~80% (Lignin Utilization)
- Key cost drivers are glucose:aromatic compounds (LRC) ratio and productivity

Muconic acid production status on model compounds

Kuatsjah, Werner *et al.*, *Metabolic Engineering* 2022

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





Muconate: 40 g/L, 0.5 g/L/h, and ~100% molar yield

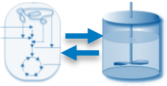
- Performance-advantaged bioproduct or direct replacement for adipic acid, adiponitrile, caprolactam, and terephthalic acid
- Titer, rate, and yield enables $1.8/gge decrease in the minimum fuel selling price (MFSP) at 80% lignin conversion to bio-available monomers

2015 → 2022 Key genotypes

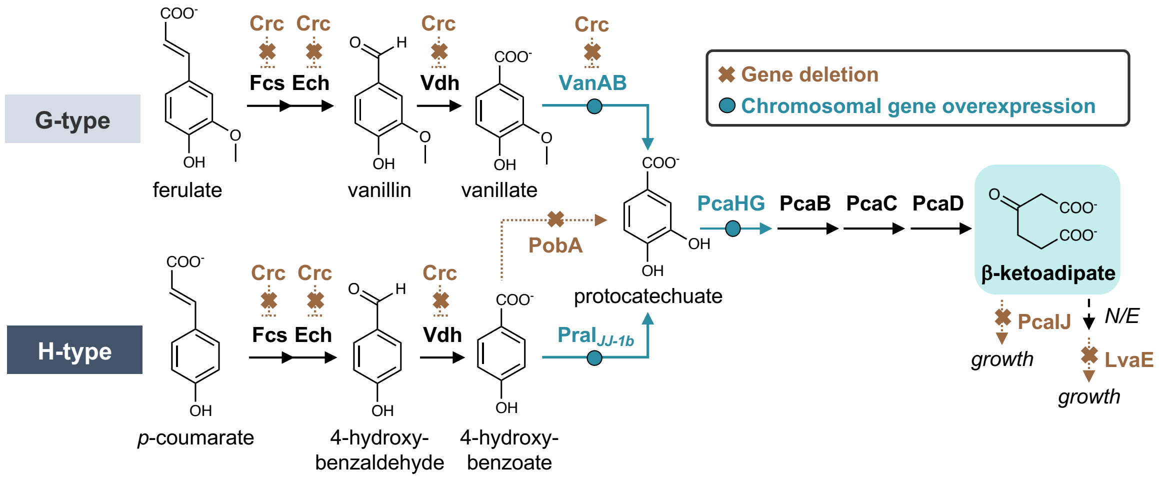
All strains: *ΔpcaHG, ΔcatB*
CJ184: *aroY, ecdBD*
CJ238: *aroY, Δcrc*
CJ242: *aroY, ecdBD, Δcrc*
CJ475: *aroY, ecdBD, Δcrc, ↑vanAB*
CJ680: *aroY, ecdBD, Δcrc, ↑vanAB, praI*
CJ781: *aroY, ecdBD, Δcrc, ↑vanAB, praI, ΔpobA*

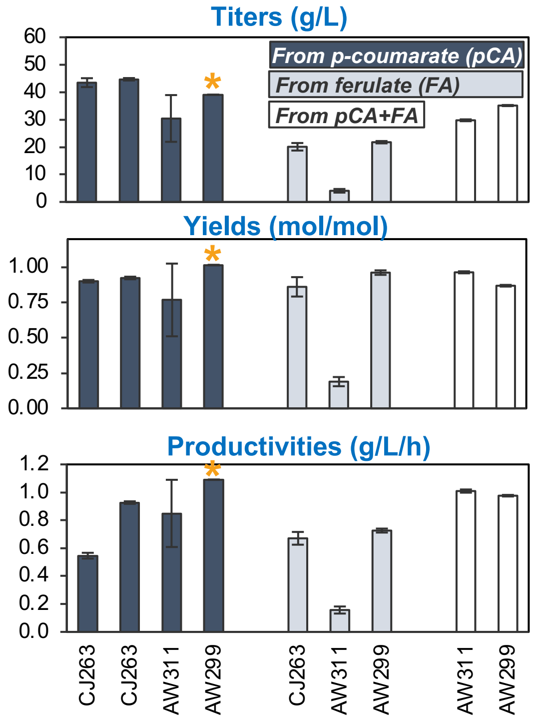
β-Ketoadipic acid production status on model compounds

Rorrer *et al. Cell Rep. Phys. Sci.* 2022
Werner, Cordell *et al., in review*



Genetic modifications in our best performing strain – *Pseudomonas putida* AW299





2022 ← 2020 Key genotypes

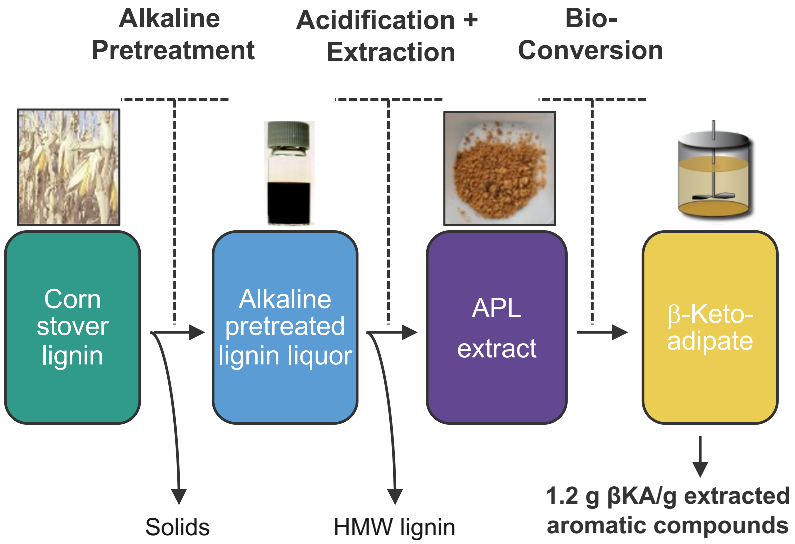
CJ263: *ΔpcaIJ*
AW299: *ΔpcaIJ, ΔpobAR, ΔlvaE, Δcrc, fpvA:Ptac:pralJJ-1b:vanAB*
AW311: ΔpcaIJ, ΔpobAR, Δlva,E, Δcrc, fpvA:Ptac:pralJJ-1b:vanAB, Ptac:pcaHG

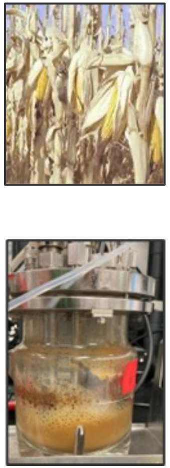
β-Ketoadipic acid: 40 g/L, 1 g/L/h, and ~100% molar yield

- **Performance-advantaged monomer in nylons** (~sebacic acid) and polyesters
- Higher strain performance achieved in shorter time with learnings from muconate
- Titer, rate, and yield enable \$2.2/gge decrease in the minimum fuel selling price (MFSP) at 80% lignin conversion to bio-available monomers
- Current **strain bottlenecks** are substrate import/utilization, which limits productivity, and product and salt tolerance, which limits titers

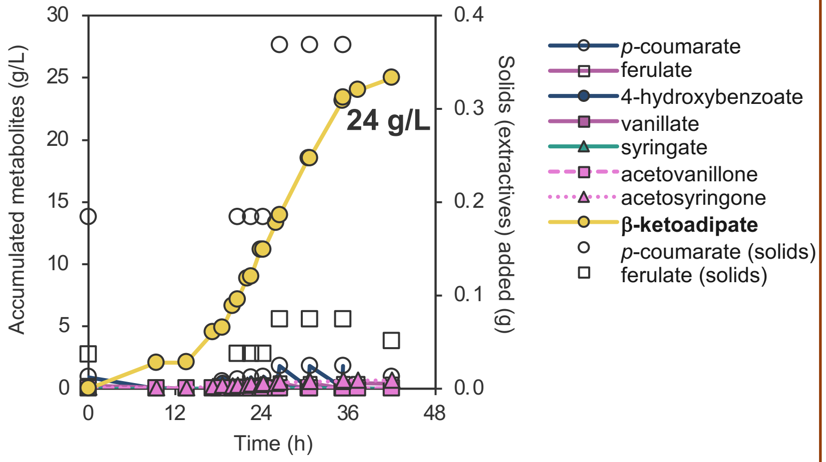
β-Ketoadipic acid production from corn stover lignin

Werner, Cordell *et al., in review*





β-Ketoadipate production in bioreactors
(fed- batch feeding with lignin solids)



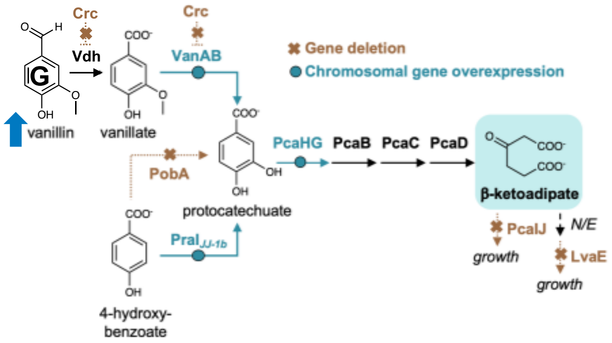
Highest titer (24 g/L) and productivity (0.66 g/L/h) achieved from a lignin-derived stream to our knowledge

- The production strain does not show any measurable metabolic bottleneck
- Main limitation is the yield of lignin-related compounds from alkaline treatment **(bioavailable compounds = ~15%)**
- Ongoing work with Lignin Utilization and collaborators to achieve higher bio-available aromatic content in feed streams

β-Ketoadipic acid production from pine (lignin-first oxidation)

Work with Shannon Stahl, UW Madison

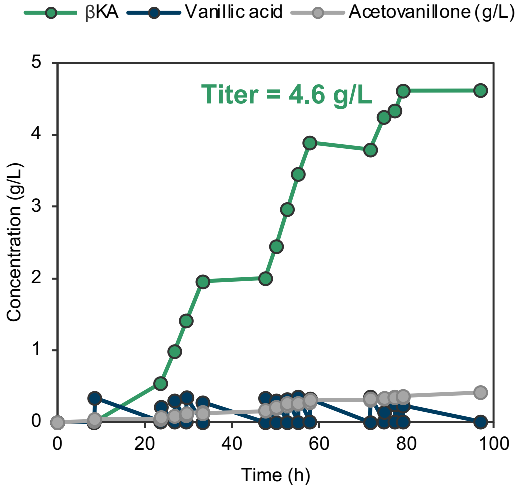
Metabolic pathway *P. putida* AW311



Composition of lignin oil fed to the bioreactor

Aromatic monomer	Amount (g)
Vanillic acid	0.55
Vanillin	1.33
Acetovanillone	0.24

β-Ketoadipate (βKA) production in bioreactors (fed-batch feeding with lignin oil)



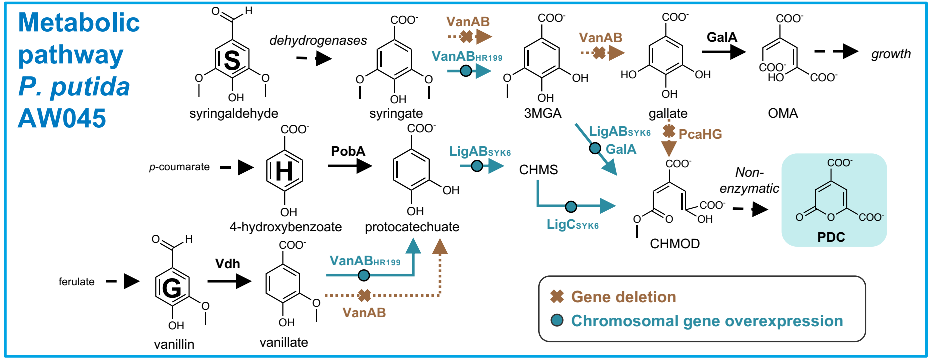
Acetovanillone was the only aromatic compound that accumulated overtime

Conversion of lignin oil from pine resulted in 4.6 g/L of β-ketoadipate

- Aromatic monomers from oxidative lignin-first process, refined with centrifugal partitioning chromatography to remove dimers
- Increased extent of lignin depolymerization **(bioavailable compounds = ~20%)**
- Work ongoing to **scale-up production** and separations of lignin monomers to reach higher β-ketoadipate titers and rates

2-Pyrone-4,6-dicarboxylic acid from poplar (lignin-first oxidation)

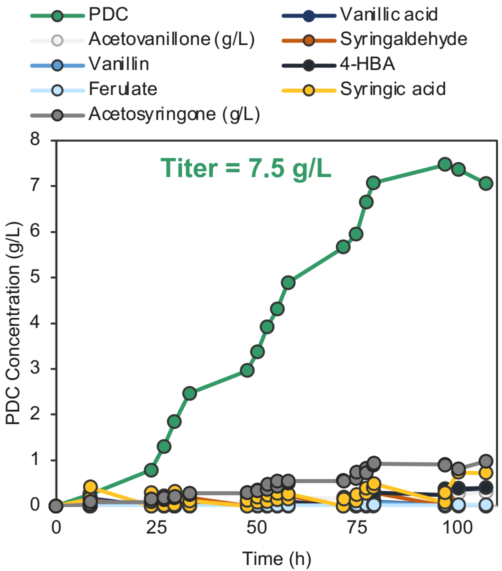
Work with Shannon Stahl, UW Madison

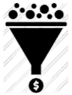


Composition of lignin oil fed to the bioreactor

Aromatic monomer	Amount (g)
4- Hydroxybenzoic acid	0.292
Vanillin	0.525
Vanillic acid	0.145
Syringaldehyde	1.294
Syringic acid	0.254
Acetosyringone	0.235
Acetovanillone	0.084

2-Pyrone-4,6-dicarboxylic acid (PDC) production in bioreactors (fed-batch feeding with lignin oil)



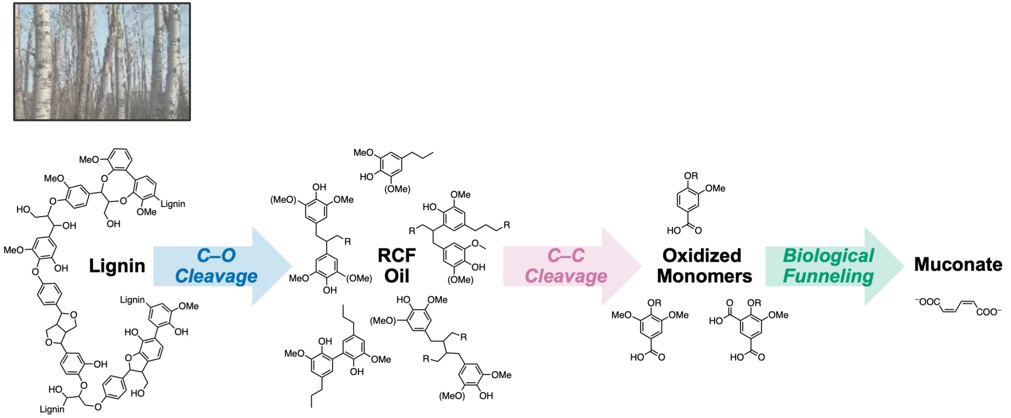


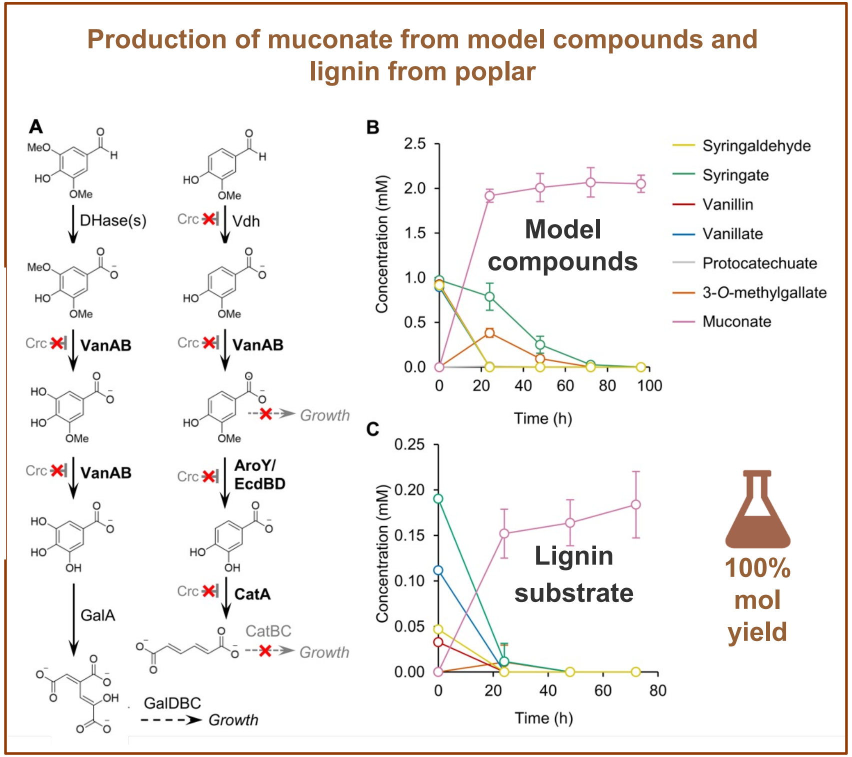
Six different aromatic compounds were simultaneously utilized

Conversion of lignin oil from poplar resulted in 7.5 g/L of PDC

- Aromatic monomers from oxidative lignin depolymerization and refined by centrifugal partitioning chromatography
- Increased extent of lignin depolymerization **(bioavailable compounds = ~40%)**
- Work ongoing to scale-up production and separations of lignin monomers to reach higher product titers and rates

Proof-of-concept muconic acid production from poplar lignin autoxidation





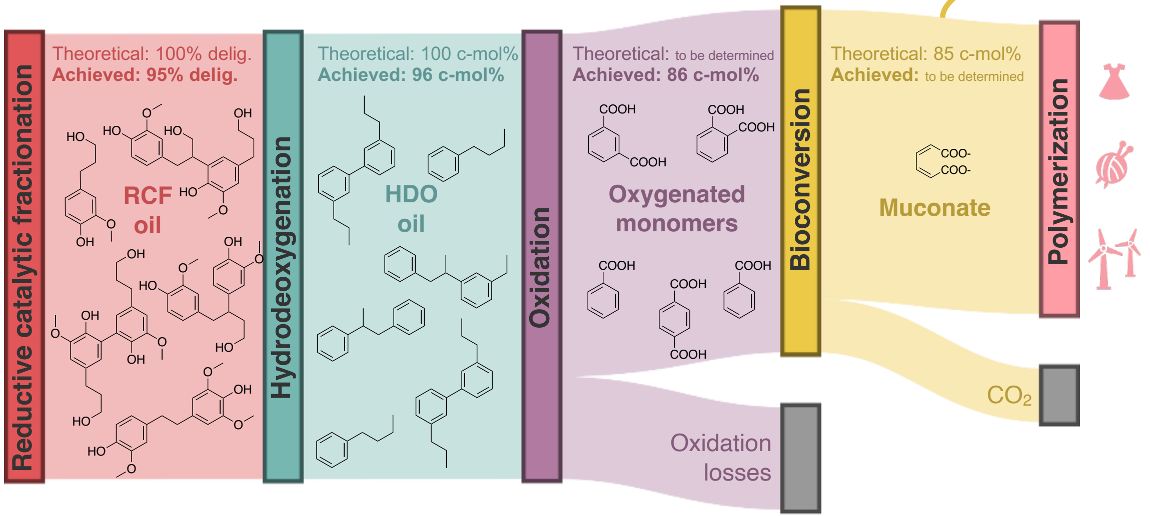
Strain performance in shake flasks with autoxidation substrates mirror model compound results

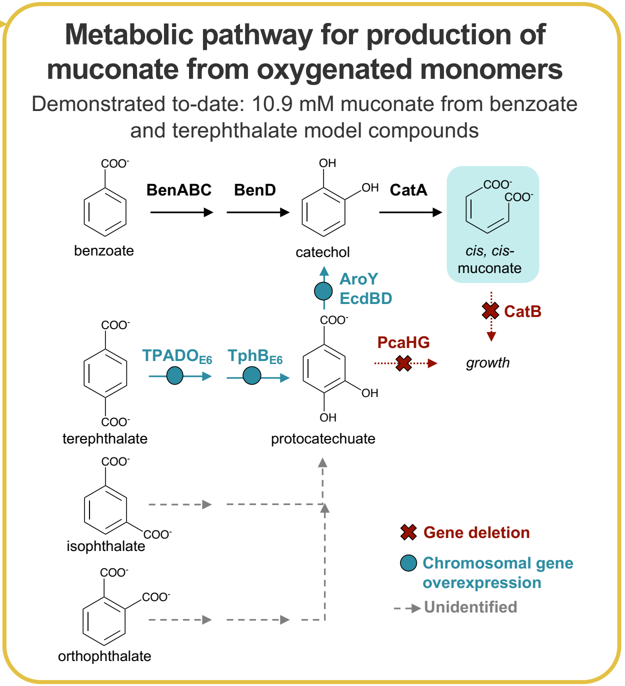
- The Lignin Utilization project is scaling up this chemistry to produce larger quantities of material for bioreactor cultivations
- Significantly increased extent of lignin depolymerization **(bioavailable compounds = ~50%)**

Gu, Palumbo, Bleem *et al., in review*

Proof-of-concept muconic acid production from lignin HDO/oxidation

Sankey diagram showing theoretical and achieved carbon yields from lignin to muconate





Autoxidation of HDO lignins offers a feasible route to ≥80% yield of a single product from lignin

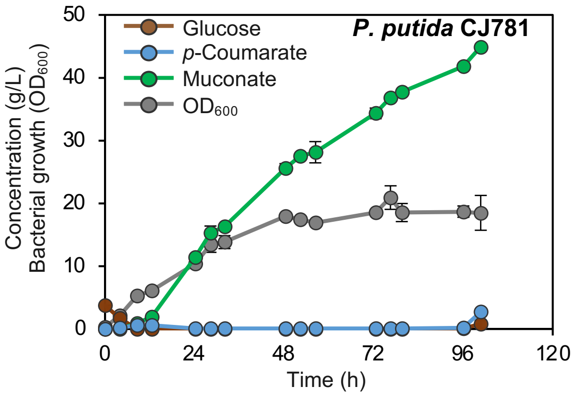
- Work ongoing with Lignin Utilization to scale this approach to bioreactors to reach industrially relevant performance for muconic acid (FY23 Q4 milestone – 40 g/L)
- Incorporating new metabolic pathways relative to conventional lignin aromatics to enable conversion to muconate

Palumbo, Werner *et al.*, *ongoing*

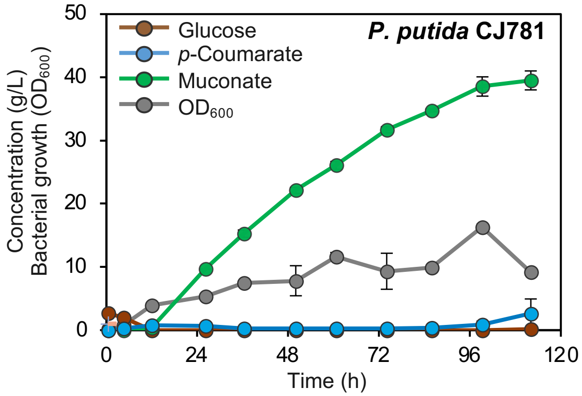
Reducing supplemental carbon source inputs

Effect of glucose-to-aromatic compound ratio on muconate production

Glucose (1 mmol): *p*-CA (2 mmol)

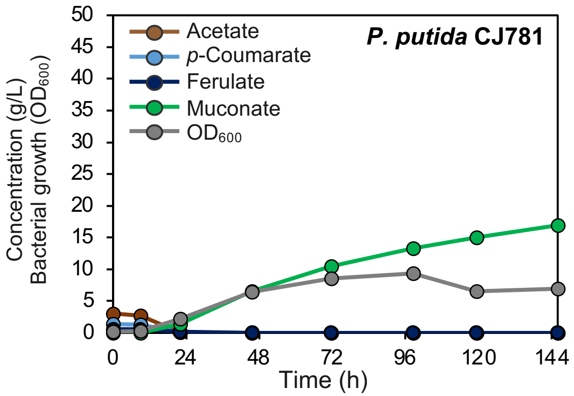


Glucose (1 mmol): *p*-CA (8 mmol)



Muconate production using acetate as the sole carbon source

Acetate (17 mmol): *p*-CA (3 mmol) + FA (1 mmol)



Acetate source

- Black liquor
- Chemical catalysis

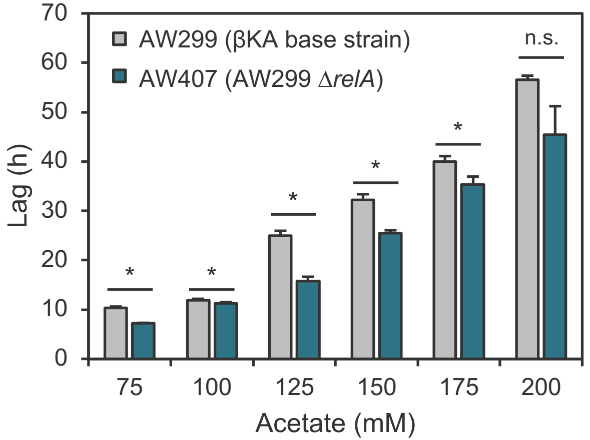
We can demonstrate further process improvements by reducing glucose content in the media

- Based on TEA (**key cost drivers**), at a ratio <0.44:1, reducing the MSP of βKA by \$0.12/kg
- Proof of concept for the utilization of acetate as the sole carbon source – experimental setup can be further enhanced to increase productivity and titers

Improved tolerance to aromatics, acetate, and salt

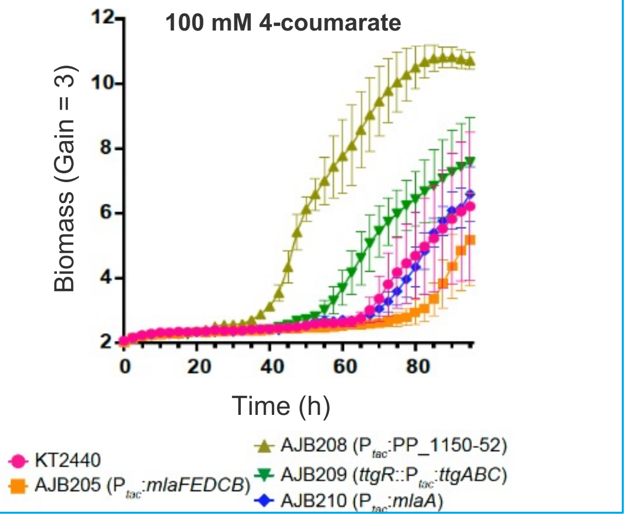
Borchert *et al.*, *In revision*
Wilkes *et al.*, *In preparation*

RelA knockout improves acetic acid tolerance in βKA production strains

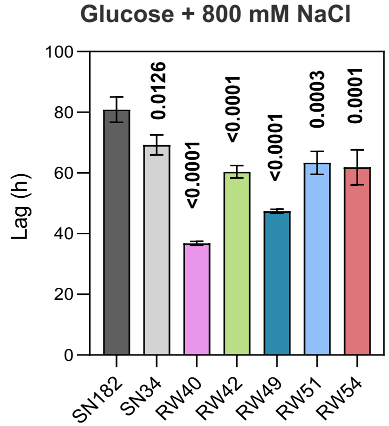


AW299: *P. putida* KT2440 Δ*pcaIJ* *fpvA*:P*tac*:*praI*:*vanAB* Δ*pobAR* Δ*lvaE* Δ*crc*
AW407: AW299 Δ*relA*

Overexpression of *PP_1150-52* improves tolerance to *p*-coumarate



Overexpression of five genes improves tolerance to NaCl



RW40: P_{tac}:PP_4799
RW42: P_{tac}: PP_2714
RW49: P_{tac}:*nhaA-I*
RW51: P_{tac}: PP_2444
RW54: P_{tac}: *nhaP*

Identified genes to improve *P. putida* performance via Randomly Barcoded Transposon insertion Sequencing (RB-TnSeq)

- Boosting acetate tolerance will address substrate toxicity (e.g., black liquor) and increase productivity
- Improving strain aromatic tolerance will address substrate bottlenecks
- Increasing strain salt tolerance will address the titer ceiling (salt is used to neutralized diacids)

Impact

Overall:

BLV is at the cutting-edge of a promising direction to valorize lignin to single products that can contribute to biorefinery economics

Scientific:

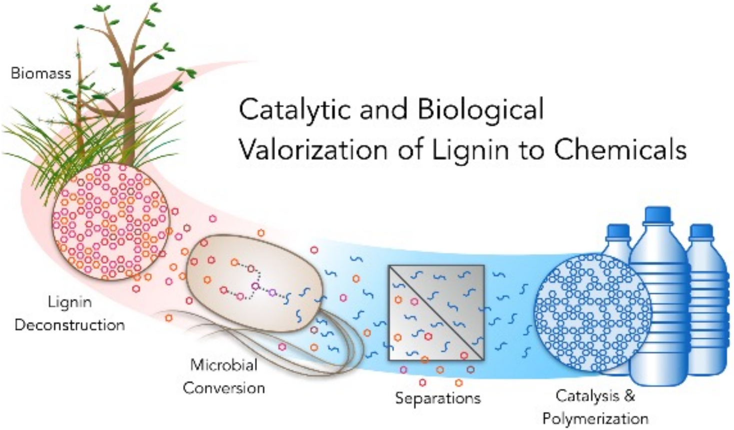
- BETO has enabled lignin bioconversion as a major thrust in metabolic engineering and bioprocess development
- Strain performance on lignin at the forefront of the field
- High-impact, field-leading publications and patents

Industrial:

- Could enable **cost-competitive production at lower GHG emissions** than today's petrochemicals from lignin
- Work with industry including **strains**, **bioprocess development**, **upstream separations**, and **industrial lignin streams**
- Industrial and academic interactions inform project aims

Publications

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, Werner, ***Met. Eng***. 2021; Erickson ***Nature Catal***. 2022, Kuatsjah ***Met. Eng.*** 2022; Werner, Cordell, *in review*, Borchert, *in review*



Summary

Overview

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

Approach

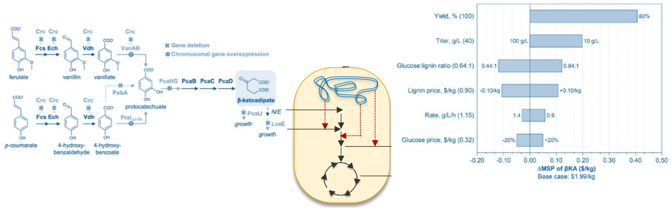
- We use TEA to guide R&D towards meaningful and impactful strains, bioprocesses, and target molecules

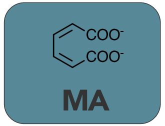
Progress and outcomes

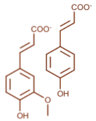
- Developed *P. putida* strains and bioprocesses moving towards industrially-relevant performance
- Ongoing work focused on continued debottlenecking, improving toxicity tolerance, and process integration with lignin deconstruction

Impact

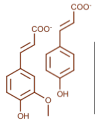
- BLV efforts consistently at the forefront of the growing microbial lignin conversion field



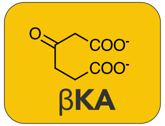




Titer = 39 g/L
Productivity= 0.5 g/L/h

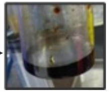


Titer = 40 g/L
Productivity= 1 g/L/h



Titer = 24 g/L
Productivity= 0.66 g/L/h

Titer = 4.6 g/L
Productivity= 0.06 g/L/h



Titer = 7.4 g/L
Productivity= 0.08 g/L/h

Quad chart overview

Timeline

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

	FY22 funding	Total Award
DOE Funding	$700,000 (10/01/2021–9/30/2022)	$700,000 – FY23 $2,100,000 – Active Project (FY21-23)

Project Partners

Nat'l labs: ORNL

BETO Projects: Lignin Utilization, Separations Consortium, Biochemical Platform Analysis, and Synthesis and Analysis of Performance-Advantaged Bioproducts

Project Goal

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

End of Project Milestone

Deliver a strain and bioprocess that is able to convert >60% of bio-available aromatic monomers from oxidized HDO lignin streams generated in the Lignin Utilization project to a single product.

Funding Mechanism

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

TRL at Project Start: 3
TRL at Project End: 4-5

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Q&A

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Additional Slides

Responses to previous reviewers' comments

The bioavailability of lignin is a critical issue. Since the only substrates that seem to undergo conversion are coumarate and ferulate, there is a concern with diminishing returns and mass loss as one goes from biomass to lignin-to-lignin monomers.

- In terms of the overall mass conversion of lignin to exemplary products like muconate, the BLV project alone cannot address the question of bioavailability of lignin. As presented in the Lignin Utilization project, oxidation chemistry is being applied to produce high yields of bioavailable molecules such as 4-hydroxybenzoate, vanillate, and syringate from lignin to far exceed the 28% of corn stover lignin that is ferulate and p-coumarate.

If DMR is not chosen as a pretreatment by a given biorefinery, do other lignin sources contain sufficient amounts of coumarate and ferulate to be useful?

- The feasibility and ultimate success of the BLV project is not tied to the DMR process. The intention in this project is to generate chassis strains and associated bioprocesses that can take streams of bioavailable lignin and convert them to value-added products in a cost-effective and energy-efficient manner. The key collaboration with the Lignin Utilization project does not solely focus on DMR, but rather is attempting to take lignin from the kraft process, the DMR process, and many others to oxidize lignin catalytically to produce monomers useful for biological funneling.

There is little or no TEA data for any of these approaches, in particular a comparative evaluation of the production (not sales) cost of conventional adipic vs. lignin-derived adipic.

- We have conducted rigorous TEA and LCA of muconic acid and conventional adipic acid production cases showing that these bio-based processes from lignin offer both a cost and environmental impacts advantage relative to petroleum-based adipic acid.

Publications, patents, and presentations

Publications

In preparation, revision, or review

Allison Z. Werner, William T. Cordell, Ciaran W. Lahive, Bruno C. Klein, Christine A. Singer, Morgan A. Ingraham, Kelsey J. Ramirez, Dong Hyun Kim, Jacob Nedergaard Pedersen, Christopher W. Johnson, Brian F. Pfleger, Gregg T. Beckham, Davinia Salvachúa, Lignin conversion to β-ketoadipic acid by *Pseudomonas putida* via metabolic engineering and bioprocess development, in review.

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Patents (issued)

21-100	Microorganisms Engineered for Production of Beta-Ketoadipic Acid

Patents (pending)

20-131	Engineered Pseudomonas putida strain for production of 2-pyrone-4-6-dicarboxylic acid guaiacyl and p-hydroxyphenyl and syringyl lignin-derived aromatic species.
20-28	Conversion of lignin-derived monomers to muconate by engineered pseudomonas
20-48	Microorganisms engineered for muconate production

Publications, patents, and presentations

Presentations (2021 - 2023)

Conversion of lignin-derived streams to performance-advantaged bioproducts by engineered *Pseudomonas putida* in bioreactors, 45th Symposium on Biomaterials, Fuels and Chemicals (poster presentation), May 2023

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Biological funneling of lignin and plastics via engineered *Pseudomonas putida*, Synthetic Biology Young Speaker Series (via webinar), Jan 2022

Publications, patents, and presentations

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