OAK RIDGE National Laboratory

DOE Bioenergy Technologies Office (BETO) 2021 Project Peer Review

[2.3.2.104] Metabolic engineering for lignin conversion

2:55 pm (ET) on March 9, 2021

Biochemical Conversion and Lignin Utilization

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ORNL is managed by UT-Battelle, LLC for the US Department of Energy

U.S. DEPARTMENT OF ENERGY

Goal Statement

Goal: To develop microbial biocatalysts for the conversion of lignin-derived aromatics to value-added chemicals, increasing the portfolio of products that can be produced from lignin-rich streams

Project outcome: Microbial strains capable of producing itaconic acid, medium chain length alcohols, and other molecules from lignin-derived aromatic compounds

Relevance: Chemical markets are small compared to fuel markets, so the ability to produce a range of products from lignin will increase the diversity of lignin valorization options for biorefineries

1 - Project Overview

Context

- Lignin accounts for ~25% of plant biomass by weight and 40% of the carbon
- Lignin is under-utilized – primary current use is for process heat and electricity
- Lignin valorization will be critical for the economics of biomass fermentation to fuels and is important for carbon efficiency
- A small number of biorefineries will saturate the market for most single co-products

Heilmeier Catechism

- *What are you trying to do?* Create pathways and strains to convert lignin-derived aromatics into industrial chemicals
- *How is it done today and what are the limits?* Lignin is burned. Biological routes currently target carbon storage molecules (e.g., lipids) or aromatic derivatives (e.g., muconate). Most biomolecules are made from sugars.
- *Why is it important?* Lignin co-products will be critical to the economics of future biorefineries
- *What are the risks?* Achieving sufficient TRY, accessing sufficient amounts of aromatic carbon in lignin

1 – Management

Project structure

- Adam Guss, PI
- Postdoc Austin Carroll leads itaconic acid production, metabolic engineering expertise
- Student Jay Huenemann leads PHA and alcohol production, metabolic engineering expertise

Management approach

- Weekly ORNL team meetings to monitor progress
- Coordination with NREL collaborators (e.g., Beckham, Salvachúa, Davis)
  - Close experimental collaboration with NREL Lignin projects (e.g., Lignin Utilization, Lignin-First Biorefinery Development, Biological Lignin Valorization), which have led to shared publications and intellectual property
  - Example: NREL tests our strains for conversion of real lignin streams in bioreactors
  - Regular site visits (pre-COVID) and conference calls to discuss progress, routine data sharing, strain exchanges

1 – Management

Risk identification

- Too low titer, rate and yield (TRY)
- Inefficient pathways
- Incomplete understanding of metabolism

Mitigation

- Pursue multiple target molecules
- Use HTP genetic tools to explore gene expression levels and enzyme orthologs with potentially different kinetic properties and specificities
- Use systems biology (omics) to understand native pathways and the effects of engineering strategies
- Leverage lignin and bioreactor expertise at NREL via close collaboration

2 – Approach

Main approach

- Microbial genetics and synthetic biology: Combination of gene deletion and heterologous gene overexpression to optimize metabolic pathways
- Systems biology (omics) as needed to identify bottlenecks

Potential challenges to be overcome for achieving successful project results

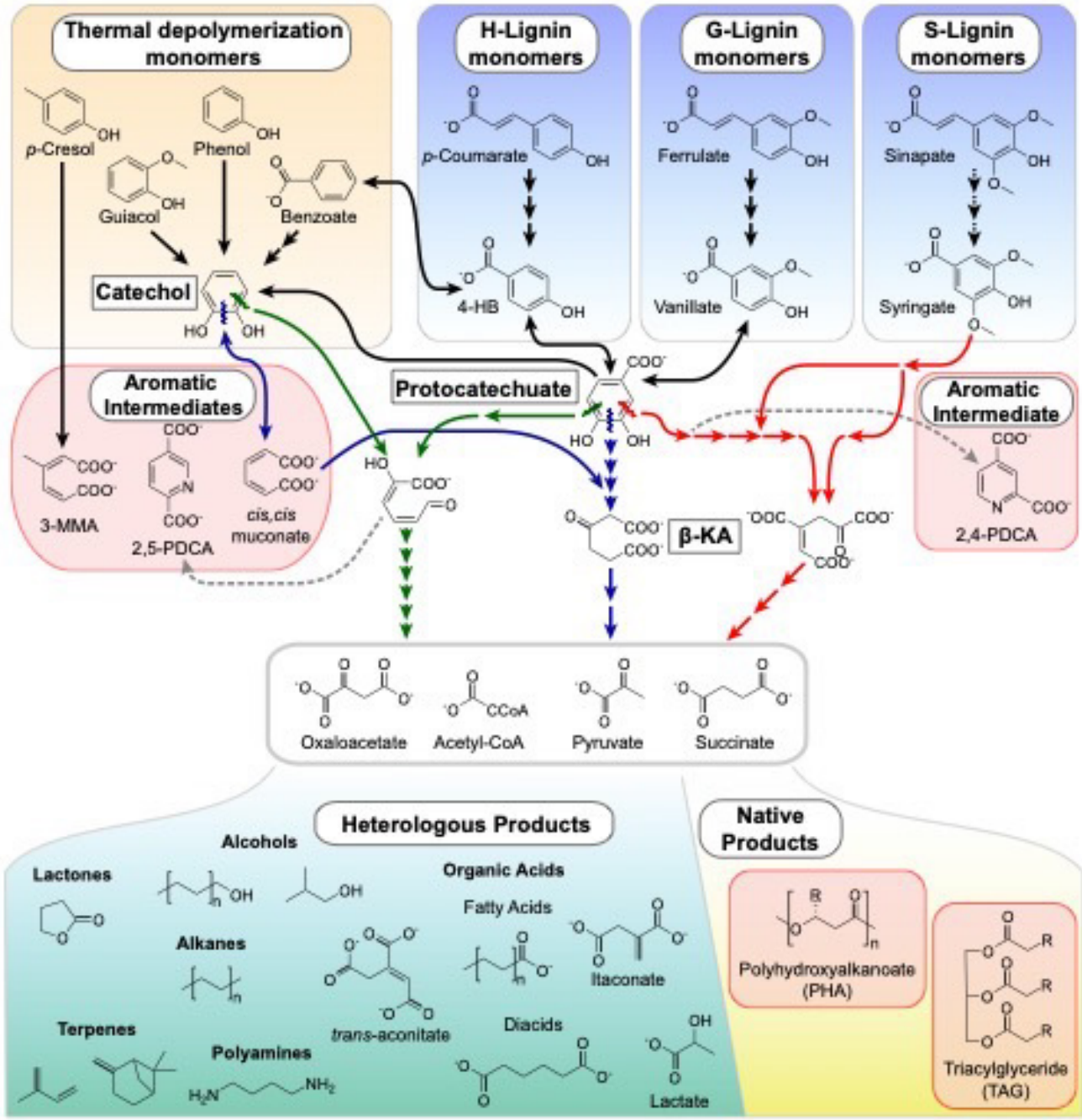
- Genetic modifications can be toxic or lethal
- Flux through native or heterologous pathways may not be sufficient

Major milestones

- Go/No-go FY20 Q2 – Produce at least 5 g/L of a target molecule such as itaconic acid from a model aromatic substrate and 2 g/L from a real depolymerized lignin such as DMR-EH BCD lignin **[Achieved, March 2020]**
- FY21 Q4 (end of project cycle) – Produce a target molecule such as itaconic acid at titer of at least 20 g/L from a model aromatic substrate with a yield of at least 30% of the theoretical maximum, and 5 g/L product from a real depolymerized lignin stream such as DMR-EH BCD lignin.

2 – Approach

- Most lignin valorization to date has targeted aromatic intermediates and native carbon storage compounds
- Our goal is to make new products after the carbon has entered central metabolism
- This will greatly expand the number and types of molecules that can be made from lignin
- Our initial targets have focused on products derived from fatty acid biosynthesis (e.g., PHAs and alcohols) and the TCA cycle (e.g., itaconic acid)



2 – Approach: Preliminary Techno-Economic Analysis of mcl-alcohol production

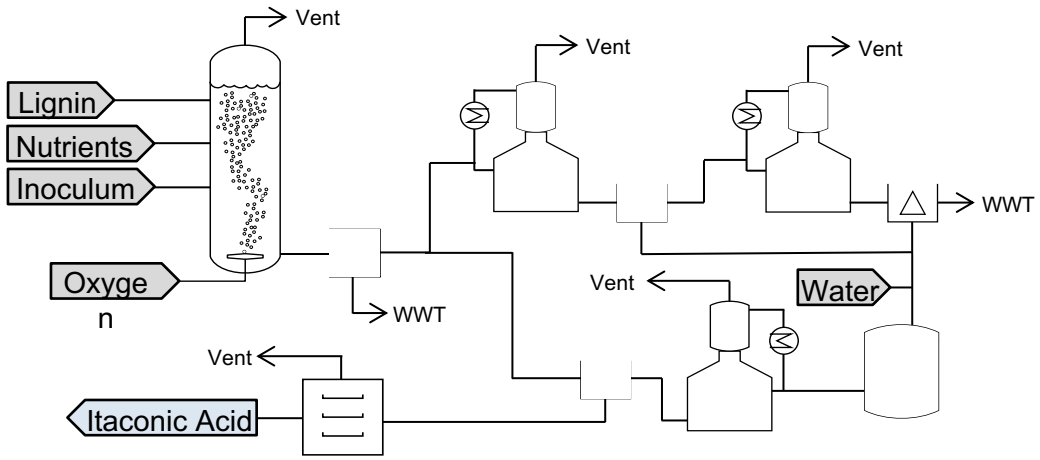
Chemical coproduct	Chemical price ($/lb, 2014$)	Lignin train capital cost (MM$, 2014$)	Lignin train upgrading raw materials cost (MM$/yr, 2014$)	Lignin train carbon efficiency required to meet $3/GGE target	Coproduct Revenue (MM$/yr, 2014$)	Substrate Conversion to products (Lignin/ Sugars)
Octanol	1.20	48	15	20.5 %	109	94.5%/89.4%
Decanol	1.20	48	15	21.1 %	109	94.5%/83%
Dodecanol	1.20	49	15	21.6 %	109	94.5%/72.3%
30% Decanol, 70% Dodecanol	1.20	49	15	21.3 %	109	94.5%/80.3%
33% Octanol, 33% Decanol, 33% Dodecanol	1.20	49	15	21.1 %	109	94.5%/82%
Adipic Acid [3]	0.86	113	27	26.9%	153	94.5%/0%[1]

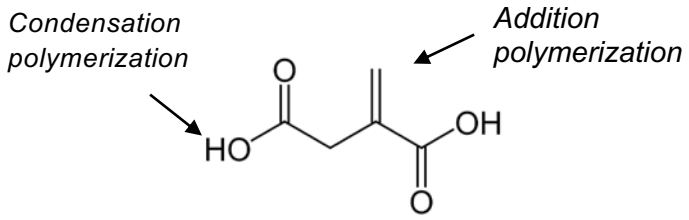
- Used preliminary TEA to ask if the mcl-alcohols are a reasonable target
- Comparison to the adipic acid TEA
- mcl-alcohol production has very similar economics
  - Similar market size (~3MM metric tons/year)
  - Lower capital and operating costs for mcl-alcohols
  - Lower mass yield for mcl-alcohols
- **Outcome:** mcl-alcohols are a reasonable target - alcohol production has very similar economics to adipic acid production

Biddy et al. 2016

2 – Approach - Preliminary TEA: itaconic acid

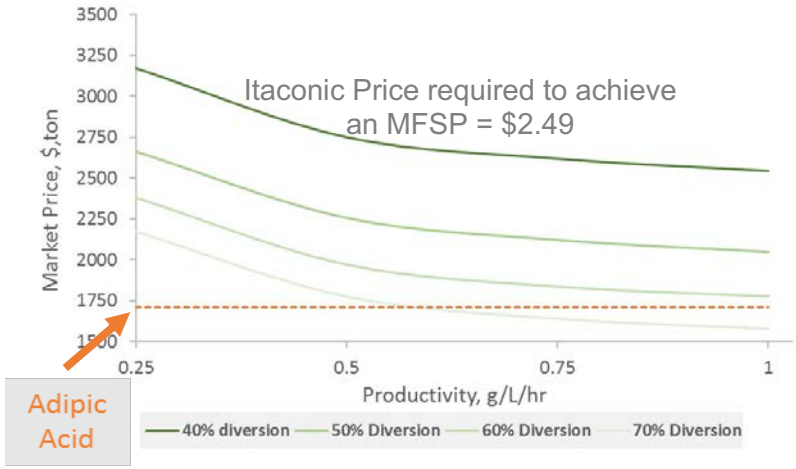
Aerobic fermentation, primary crystallization, and recrystallization





*Upstream and process steps not depicted here are consistent with the BC 2018 Design Report acids pathway. More details can be found at: (https://www.nrel.gov/docs/fy19osti/71949.pdf)

- Versatile performance advantaged bioproduct
- Currently, primarily sourced from glucose (and starch)
- Appealing bio-based unsaturated monomer (trifunctional)
- “Green” alternative to acrylates
- Historical and predicted market prices are similar to or higher than adipic acid
- Target MFSP achievable even at the lower bound of historical market price estimates
- **Outcome:** Maintaining high conversion of substrate to product instead of growth is a key consideration for economic production of itaconic acid



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Nick Grundl, NREL

3 – Impact

Impact on state of technology

- Commercial lignin valorization will require advances on multiple fronts, including better biocatalysts for production of diverse industrially useful molecules
- This work demonstrates that lignin can be converted to new types of molecules derived from central metabolism at high yield and titer

Dissemination of results

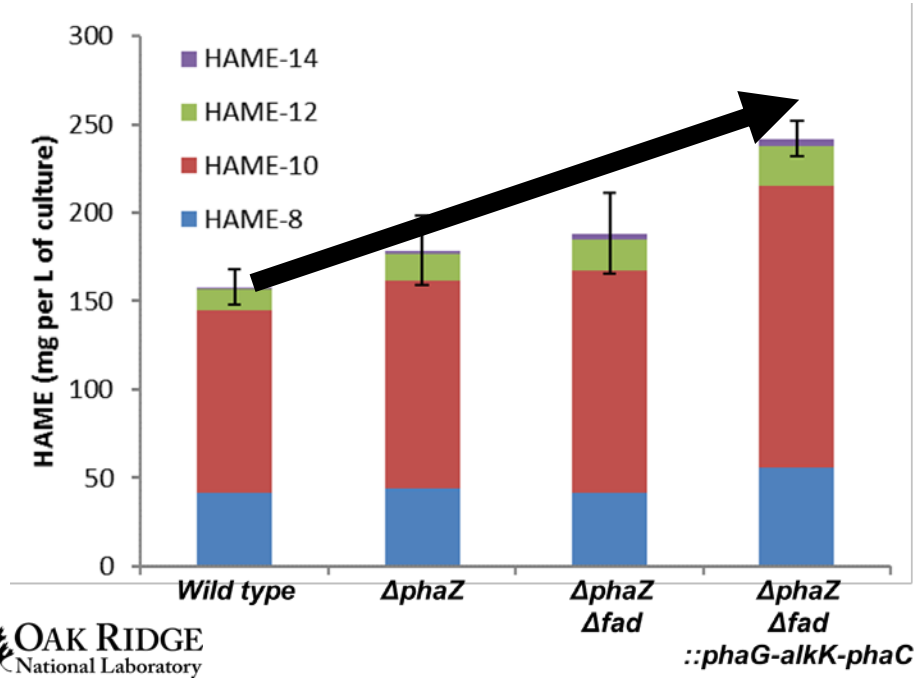
- Publications, including the itaconic acid work that is in final revision at *Nature Communications*
- Presentations at scientific meetings
- Patents
  - Granted: *Elmore JR, Huenemann J, Salvacha D, Beckham GT, and Guss AM. "Production of itaconic acid and related molecules from aromatic compounds" Application Number: 16/397256. Patent Number: 10,738,333. Date Issued: August 11, 2020*
  - *Non-provisional patent application submitted on mcl-alcohol work*

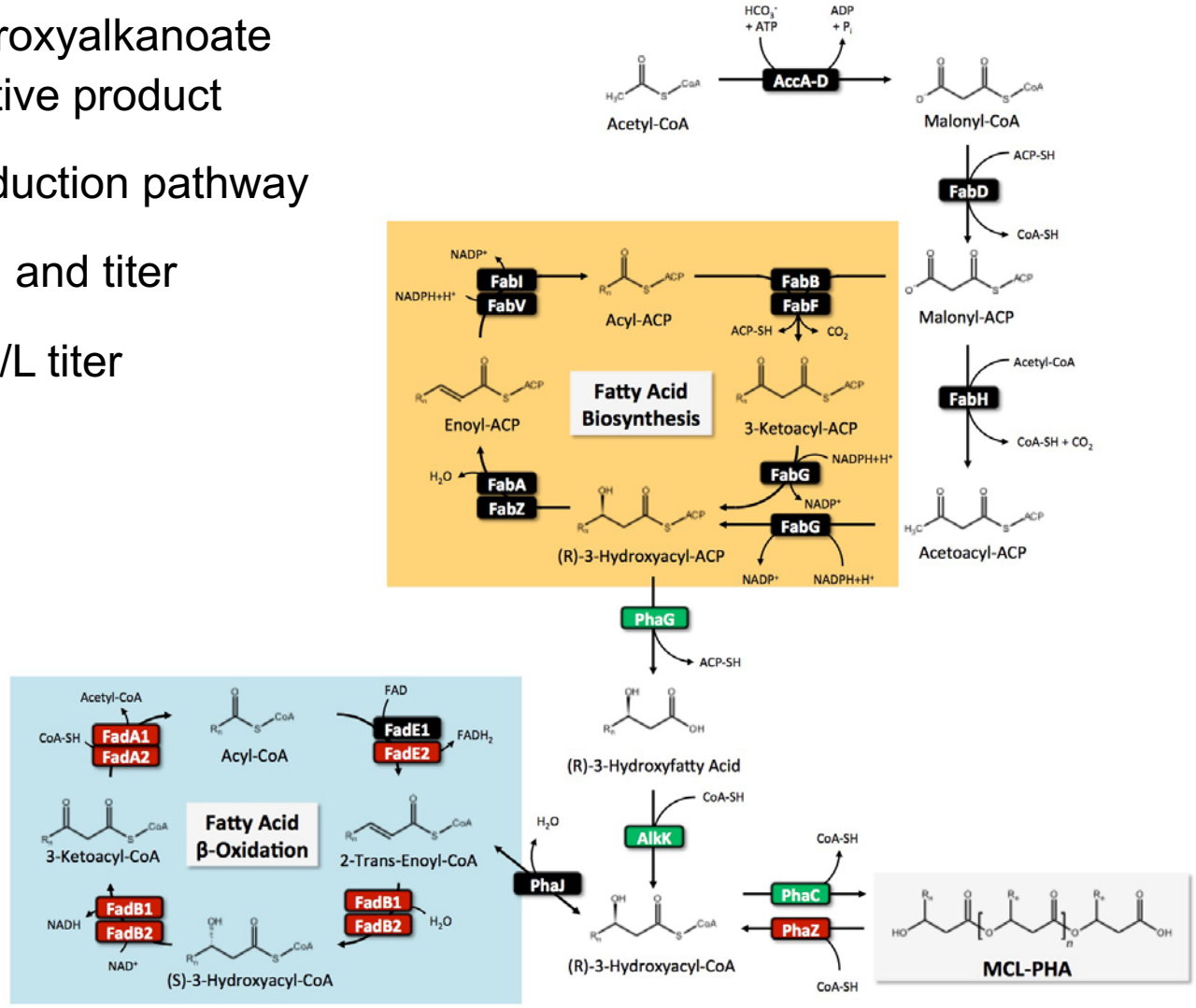
4 – Progress and Outcomes: Outline

- Early work: medium chain length polyhydroxyalkanoate (mcl-PHA) production as proof of concept with native product
- Medium chain length alcohols
- TCA cycle-derived itaconic acid
- Additional new products

4 – Progress and Outcomes: Enhanced PHA production from aromatic substrates

- Early work targeted medium chain length polyhydroxyalkanoate (mcl-PHA) production as proof of concept with native product
- Deleted competing pathways, overexpressed production pathway
- **Outcome:** Engineered 40% increase in PHA yield and titer
- **Outcome:** Separately, we have also achieved 1 g/L titer

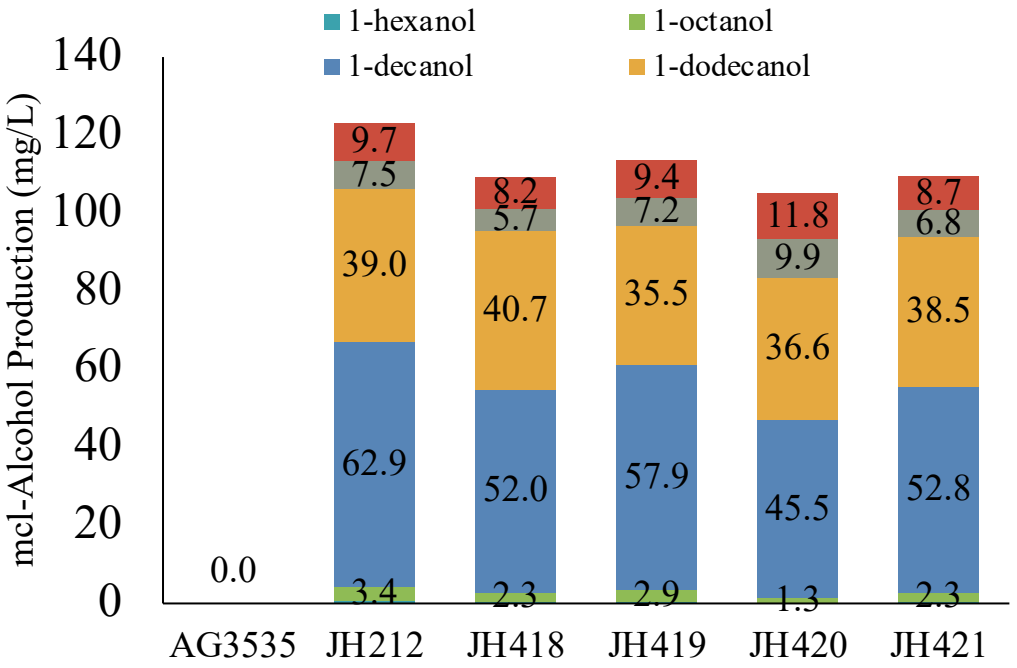


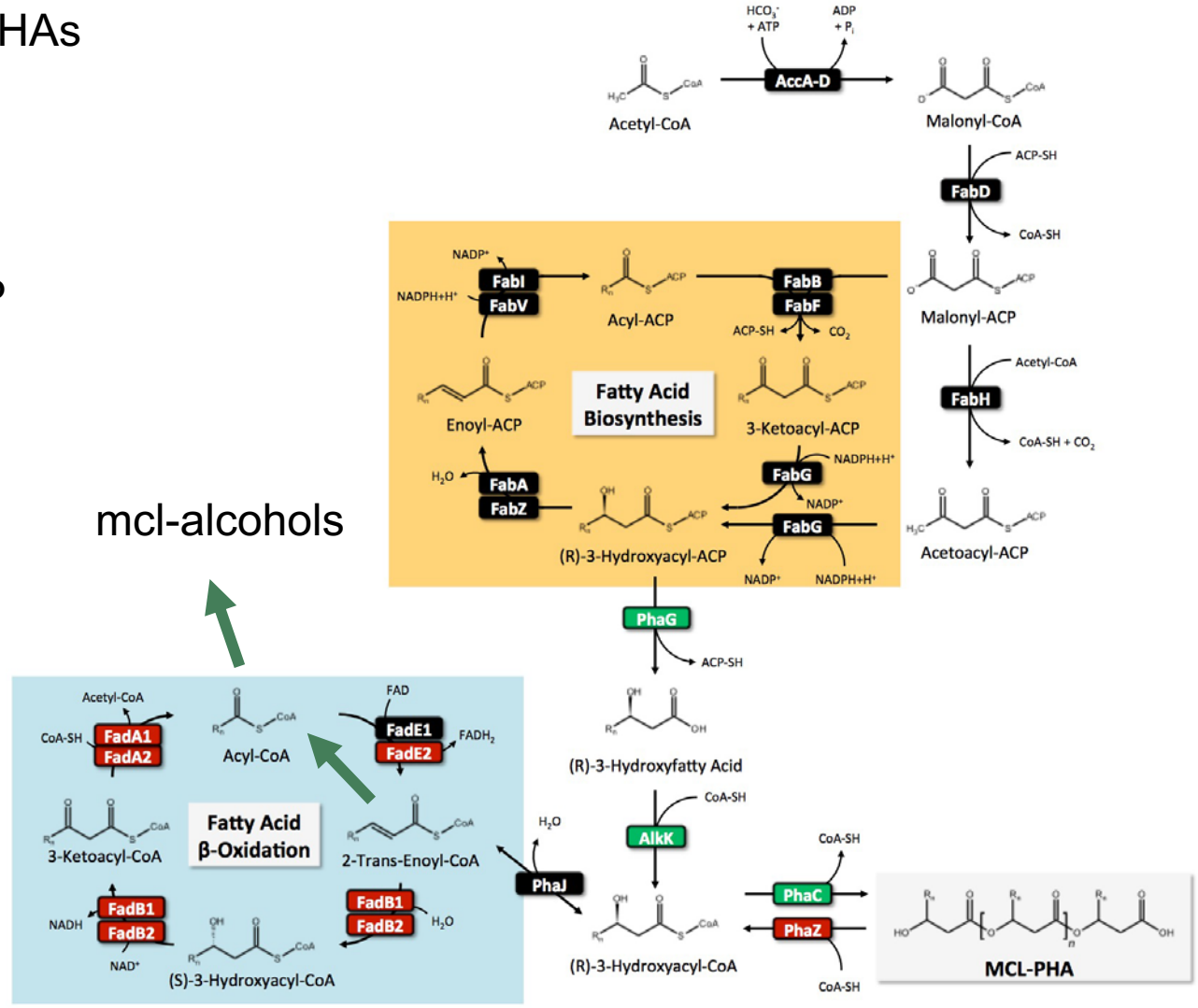


Salvachúa et al DOI: 10.1111/1751-7915.13481

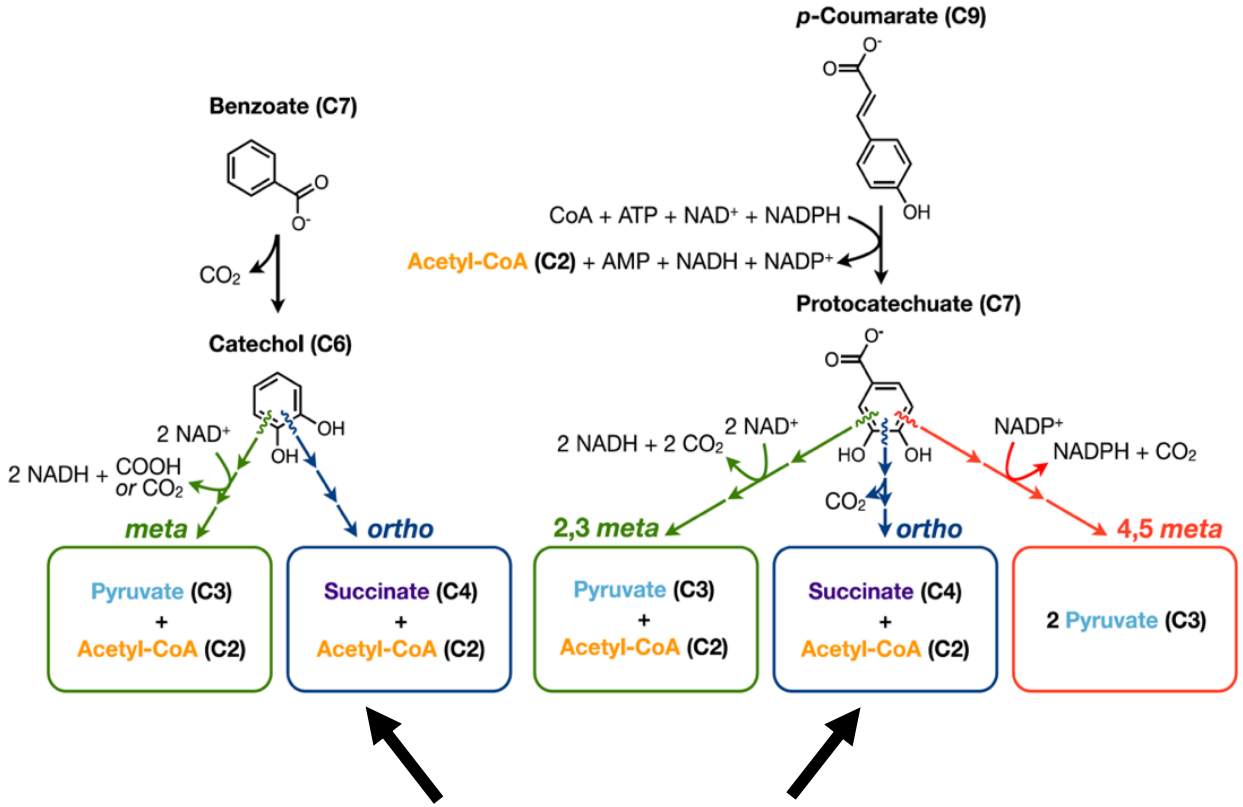
4 – Progress and Outcomes: mcl-alcohol production from aromatic substrates

- Leveraging many of the same modifications as PHAs
- Tested multiple modifications and conditions
- **Outcome:** Achieved titers > 100 mg/L
- **Ongoing:** Exploring alternate pathways and HTP genetics to push yields and titers higher

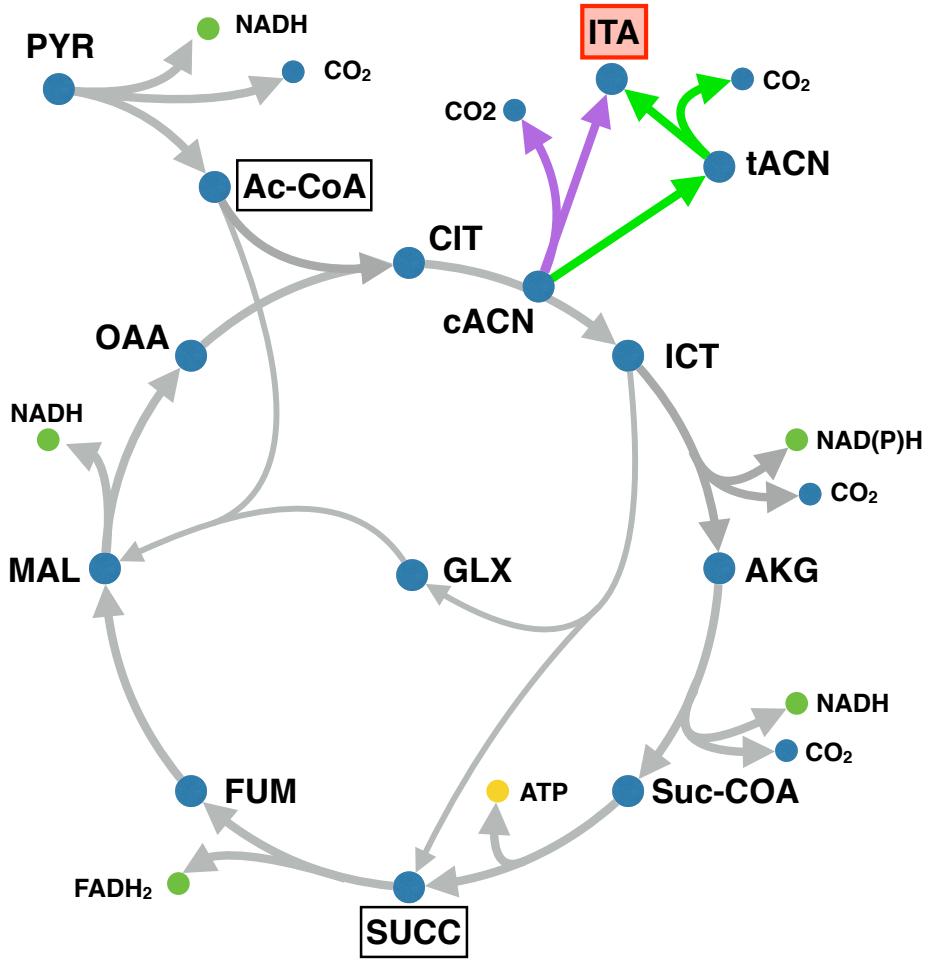




4 – Progress and Outcomes: Accessing different metabolic nodes: itaconic acid as a target molecule from TCA cycle



- The ortho-cleavage pathways native to *P. putida* KT2440 feed directly into TCA cycle
- Itaconic acid is one or two metabolic steps away from the TCA cycle



Purple = cis-pathway
Green = trans-pathway

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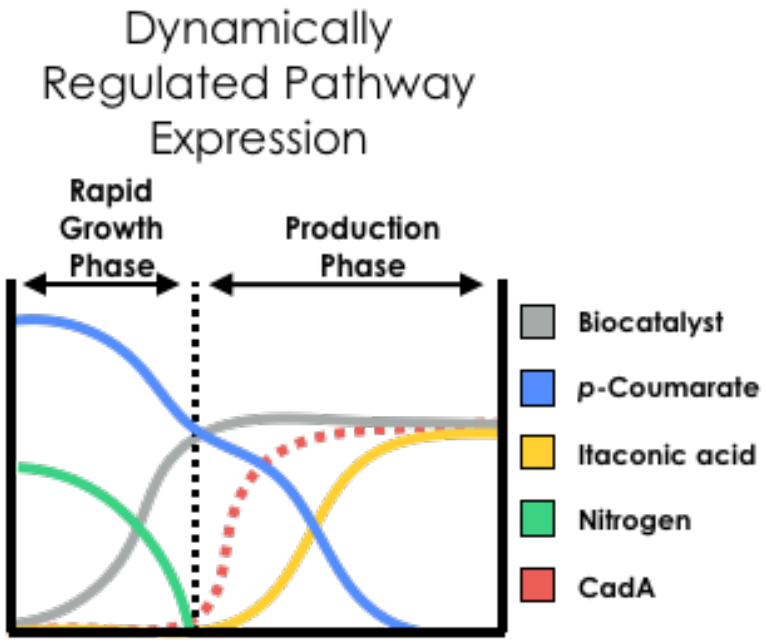
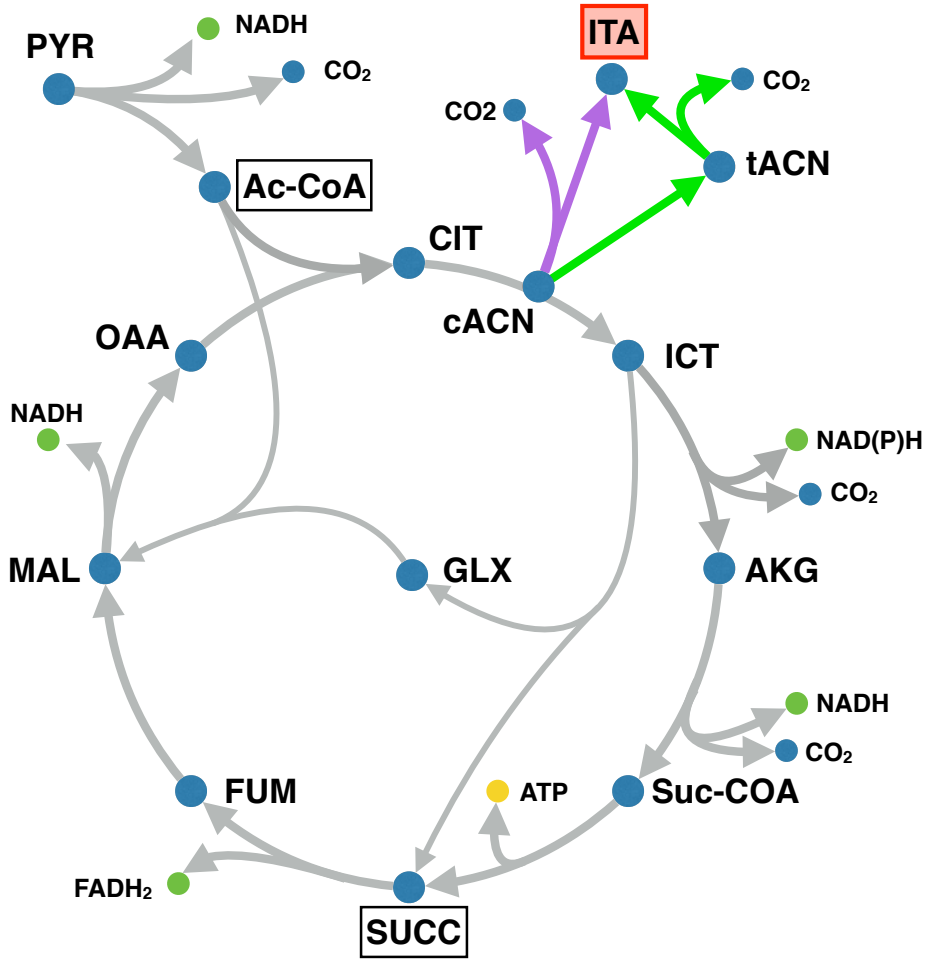
4 – Progress and Outcomes: Accessing different metabolic nodes: itaconic acid as a target molecule from TCA cycle



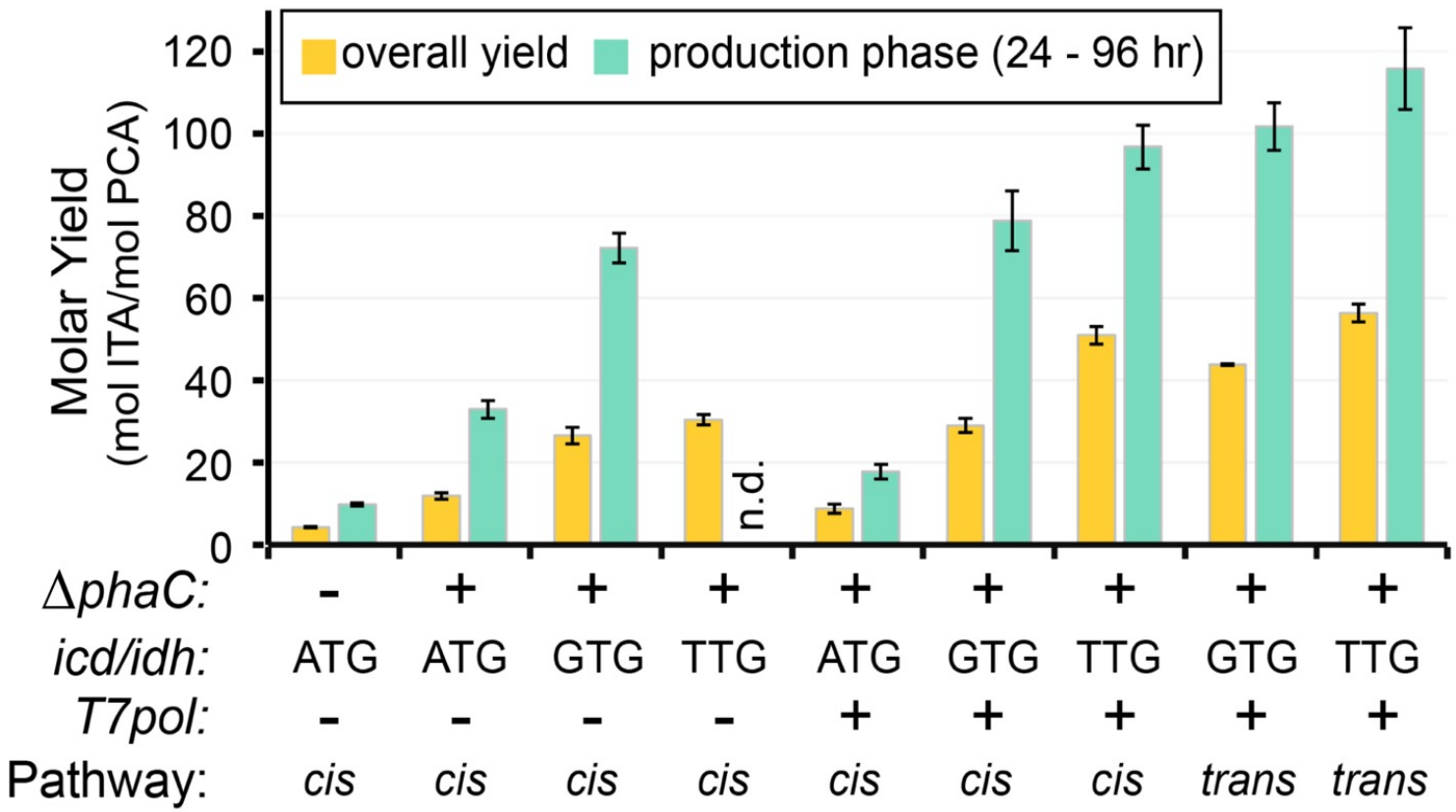
- Dynamic regulation of itaconic acid production allows uncoupling of growth and production
- Addresses a major outcome of Techno-Economic Analysis
- Understanding the physiology of this production phase will enable extended production without carbon going to the production of new cells



Purple = cis-pathway
Green = trans-pathway

4 – Progress and Outcomes: Previous results: Itaconic acid yields from aromatics approaching 60% mol/mol

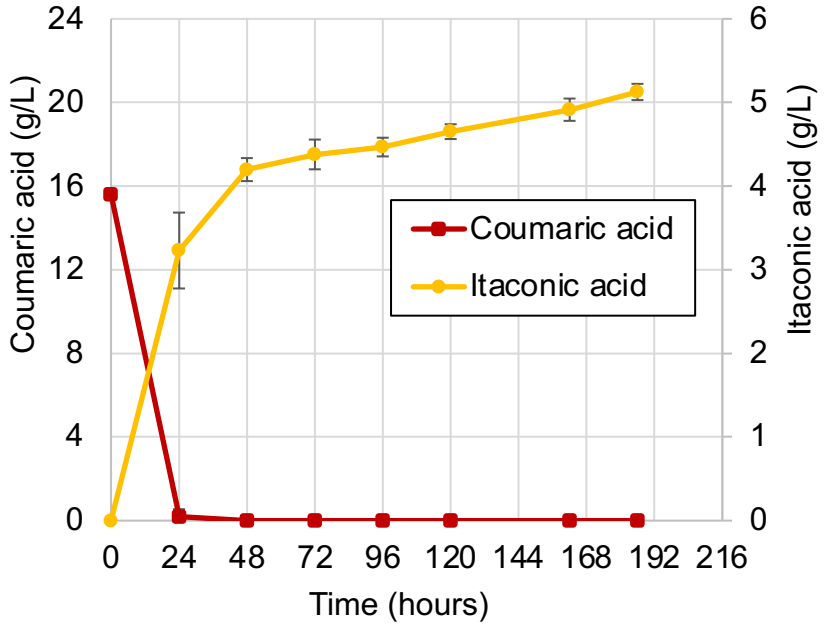
- We changed the *icd* start codons to less favorable ones to decrease translation rate
- Strains with lower efficiency start codons produced more itaconic acid
- Because production is coupled to nitrogen starvation, we can look at post-growth product formation
- **Outcome:** Overall yield nearly 0.6 mol itaconic acid/ mol p-coumarate
- **Outcome:** Production phase yields approaching 1.2 mol/mol (theoretical max = 1.33 mol/mol)



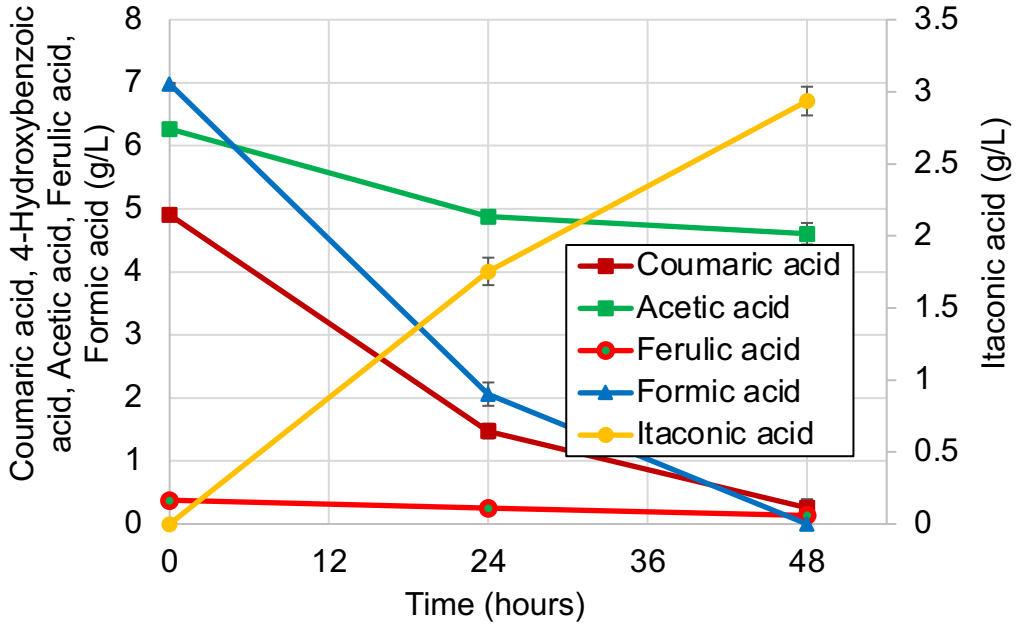
Δ*phaC* – deletion of PHA synthesis genes
icd/idh – start codon used for isocitrate dehydrogenases
T7pol – presence or absence of N-starvation inducible pathway
Pathway – cis- or trans- pathway to itaconic acid

4 – Progress and Outcomes: Increasing titer of itaconic acid from aromatics

FY20 Q2 Go/No Go: Produce at least 5 g/L of a target molecule such as itaconic acid from a model aromatic substrate and 2 g/L from a real depolymerized lignin such as DMR-EH BCD lignin **[Achieved]**

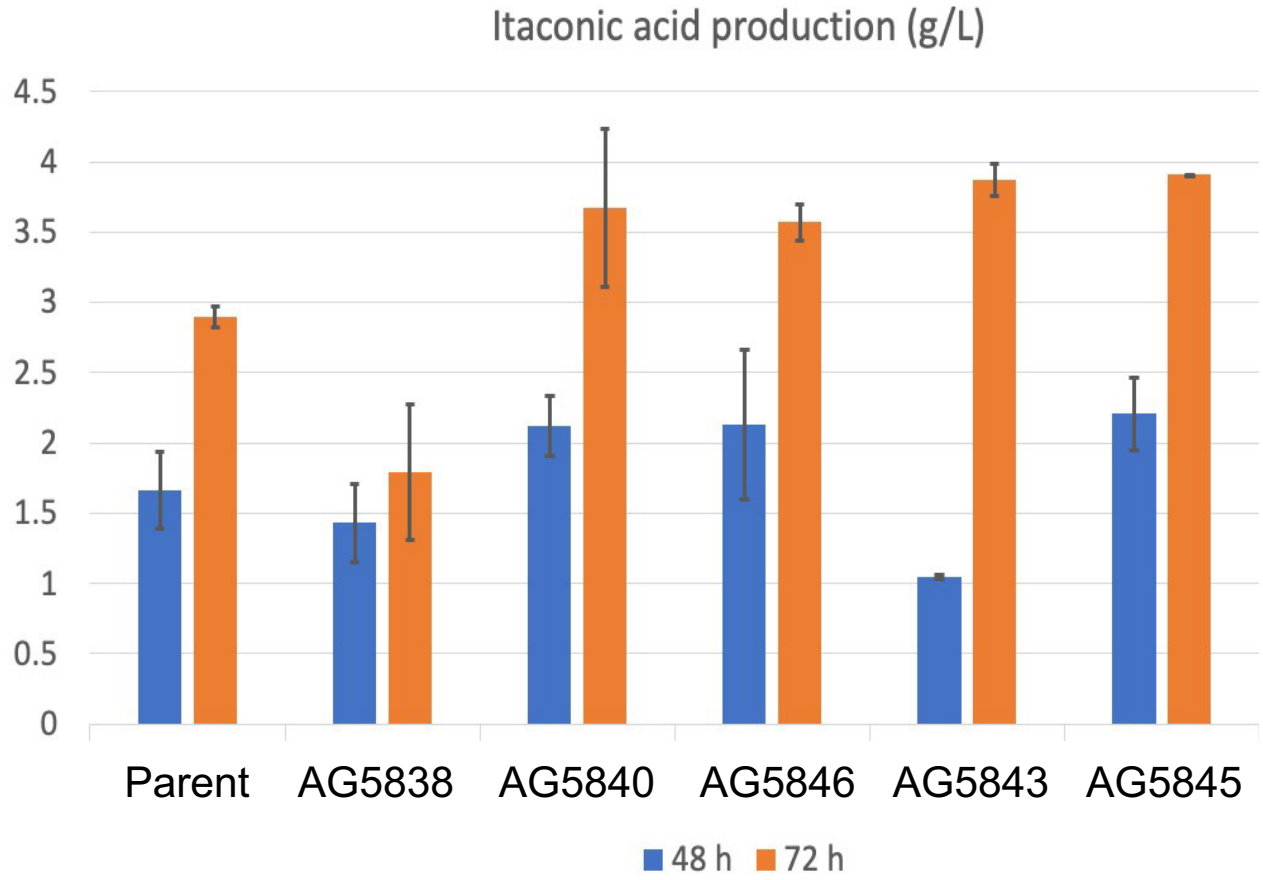


Outcome: Production of 5 g/L itaconic acid from a model aromatic substrate



Outcome: Production of >2 g/L itaconic acid from real depolymerized lignin (Alkaline Pretreated Liquor, NREL)

4 – Progress and Outcomes: Increasing titer of itaconic acid from aromatics



Progress toward final milestone

- Further genetic modifications have increase yield and titer at higher substrate loadings up to 35%
- Additional genetic modifications are ongoing
- We are using proteomics to understand the physiological changes that occur during production phase
- We will also need to implement fed batch cultivation to provide enough substrate to reach our target

Upcoming Milestone
FY21 Q4 (end of cycle): Produce 20 g/L from model aromatics and 5 g/L from lignin stream

4 – Progress and Outcomes: Exploring other products to expand the portfolio

FY19 Q4 SMART Milestone: Demonstrate production of at least 0.5 g/L of a new target molecule from an aromatic substrate. **[Achieved]**

Approach for new products

- Expressed heterologous pathway in *P. putida*
- Modified flux through competing pathways
- **Outcome:** >1 g/L new product from aromatics
- **Ongoing:** Additional products are also being evaluated

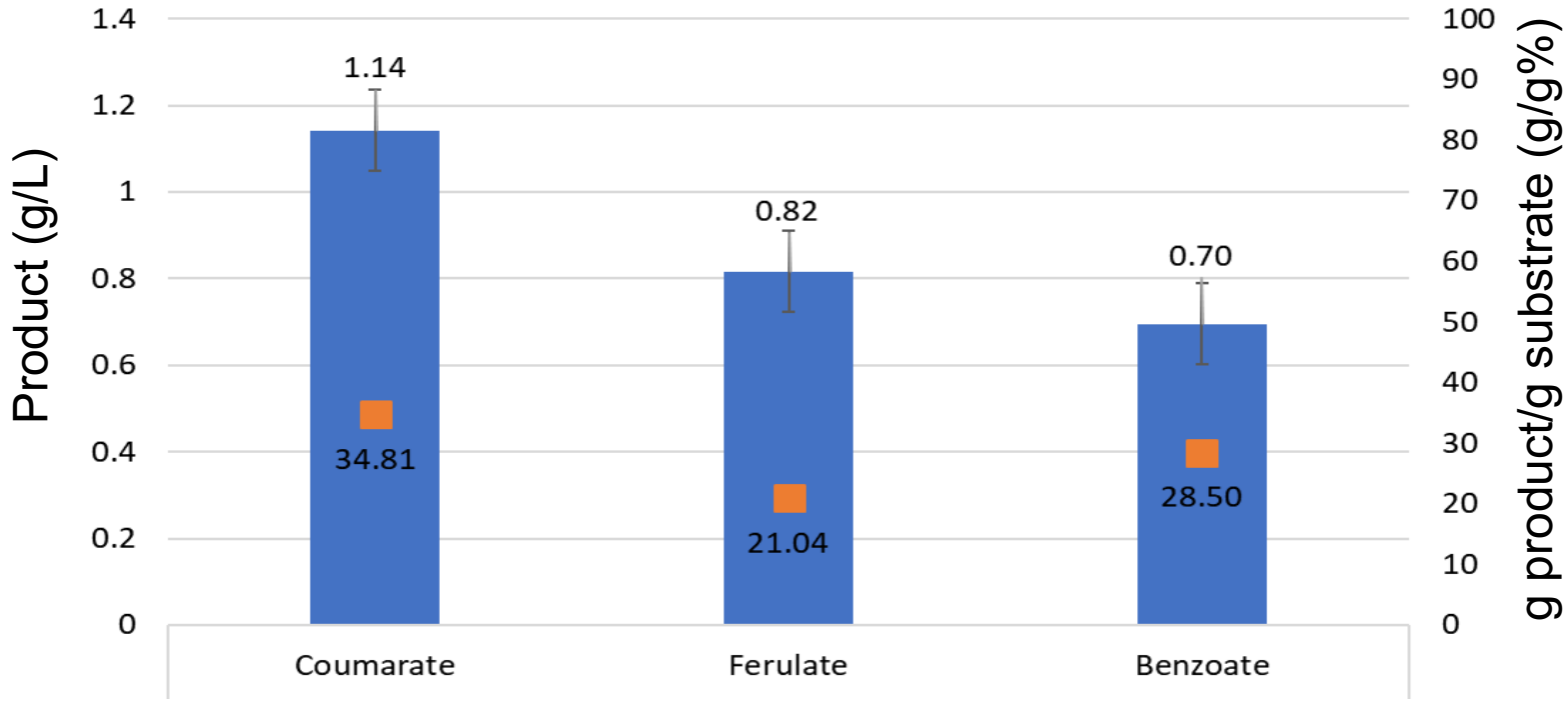


Figure 1: Production of Product from aromatic compounds. Titers (g/L) and yield (g/g %) produced by *P. putida* strain AG4116 from p-coumarate, ferulate, and benzoate.

Summary

- The ability to valorize lignin to a suite of products will be critical to the success of future biorefineries
- We are diversifying the portfolio of products that can be made from lignin
- mcl-alcohol production is still early-stage and will require substantially higher flux through fatty acid biosynthesis to achieve success
- Itaconic acid titers exceed 5 g/L from aromatic substrates; near-term goal of 20 g/L
- New efforts toward additional products is promising, with early titers exceeding 1 g/L
- Understanding nitrogen starvation-based production phase will be critical to extended production and corresponding high yields, titers, and productivities
- The main focus going forward will be pushing to higher TRY for itaconic acid and other molecules

Quad Chart Overview

Timeline

- Project start: 10/01/2018
- Project end: 9/30/2021

	FY21	Active Project
DOE Funding	$350,000	$1,050,000

Separately-funded collaborators:

BETO Projects: Lignin-First Biorefinery Development, Lignin Utilization, Biological Lignin Valorization, Biochem Platform Analysis

Barriers addressed

Ct-C. Process Development for Conversion of Lignin

Ct-D. Advanced Bioprocess Development

Project Goal

Develop microbial strains capable of producing value added products from lignin-derived aromatic substrates

End of Project Milestone

Produce a target molecule such as itaconic acid at a titer of at least 20 g/L from a model aromatic substrate with a yield of at least 30% of the theoretical maximum, and 5 g/L product from a real depolymerized lignin stream such as DMR-EH BCD lignin.

Funding Mechanism

FY19 AOP

Additional Slides

Responses to Previous Reviewers' Comments

- Weakness: It is not clear whether the work to date has clarified product choices or ranking, or what other inputs might be considered to product ranking. Rate should perhaps be considered in metrics at some point to take into account CapEx impact in a biorefinery setting.
- Answer: For prioritization of target molecules, our goal is to demonstrate the ability to biologically produce diverse molecules from lignin, and in particular molecules from diverse nodes of metabolism. This is a major driver of our product ranking, and why we chose itaconic acid (it is derived from the TCA cycle). As we move down the road of pathway and strain optimization, we agree that rate will increasingly become a critical parameter for us to consider. For this reason, we are trying to understand cellular physiology during the non-growth-associated production phase, which could allow for high cell densities and therefore high rates of conversion.

- The Go/No-go milestone was discussed on slide 17. We were successful in meeting this milestone.

Publications, Patents, Presentations, Awards, and Commercialization

Publications (*=corresponding author)

- Elmore J, Furches A, Wolff G, Gorday K, and Guss AM*. Development of a high efficiency integration system and promoter library for rapid modification of Pseudomonas putida KT2440. Metabolic Engineering Communications 5 (2017) 1–8. DOI: j.meteno.2017.04.001
- Salvachúa D, Rydzak T, Auwae R, De Capite A, Black BA, Bouvier JT, Cleveland NS, Elmore JR, Huenemann JD, Katahira R, Michener WE, Peterson DJ, Smith H, Vardon DR, Beckham GT, Guss AM*. (2019) Metabolic engineering of Pseudomonas putida for increased polyhydroxyalkanoate production from lignin. Microbial Biotechnology. DOI: 10.1111/1751-7915.13481
- Elmore JR, Dexter GN, Hatmaker EA, Klingeman DM, Salvachúa D, Singer C, Peterson DJ, Hueneman JD, Peabody GL, Martinez-Baird J, Beckham GT, Guss AM*. Lignin valorization to itaconic acid at high yield by dynamic, two-stage conversion. In final revision at Nature Communications.

Presentations (*=presenting author)

- Guss AM*. "Diversifying the portfolio of products that can be made from lignin using engineering *Pseudomonas putida*" Lignin Gordon Research Conference, Easton, MA, August 9, 2018. Oral presentation.
- Elmore JR*, Dexter G, Hatmaker EA, Klingeman DM, Al-Rashid S, de Capite A, Guss AM. Dynamic pathway regulation for two-stage conversion of aromatics to itaconic acid for lignin valorization. Lignin Gordon Research Conference, Easton, MA, August 7, 2018. Poster presentation.
- Elmore JR* and Adam Guss. **"**Dynamic pathway regulation for two-stage conversion of aromatics to itaconic acid for lignin valorization" Society for Industrial Microbiology and Biotechnology Annual Meeting, Chicago, IL, August 16, 2018. Oral Presentation.
- Huenemann J*, Cowan D, De Capite A, Elmore JR, Hatmaker EA and Guss AM. Development of nitrogen starvation response and bi-phasic hybrid promoters in *Pseudomonas putida.* Society for Industrial Microbiology and Biotechnology Annual Meeting, Chicago, IL, August 13, 2018. Poster Presentation.
- Guss AM*. Synthetic biology and metabolic engineering of non-model microbes for the production of renewable fuels and chemicals. Department of Chemical and Biomolecular Engineering. University of Nebraska-Lincoln. Lincoln, NE. January 25, 2019.

Publications, Patents, Presentations, Awards, and Commercialization

Patents:

- Issued patent: Elmore JR, Huenemann J, Salvacha D, Beckham GT, and Guss AM. "Production of itaconic acid and related molecules from aromatic compounds" Application Number: 16/397256. Patent Number: 10,738,333. Date Issued: August 11, 2020
- Non-provisional patent application: Huenemann J and Guss AM. "Engineered microbes for conversion of organic compounds to medium chain length alcohols and methods of use" ID 20194425. Non-provisional filed July 20, 2020 and assigned US Application Serial No. 16/934,570.