OAK RIDGE National Laboratory

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DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

[2.3.2.104] Metabolic engineering for lignin conversion

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Biochemical Conversion Review

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 flexibility of biorefineries to meet market demand

Quad Chart Overview

Timeline

- Project start: 10/01/2018
- Project end: 9/30/2021
- Percent complete: 14%

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$877k	$300k	$339k	$1050k
Project Cost Share				

•Separately-funded collaborators:
BETO Projects: Lignin Utilization, Biological Lignin Valorization, Biochem Platform Analysis

Barriers addressed

Ct-C. Process Development for Conversion of Lignin

Ct-D. Advanced Bioprocess Development

Objective

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

End of Project Goal

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.

1 - Project Overview

Context

- Lignin accounts for ~25% of plant biomass by weight and 40% of the carbon
  - Underutilized during biofuel production
- Primary current use is for process heat and electricity
- A small number of biorefineries will saturate the market with for most single co-products

Project Objectives

- Develop a biological platform for production of a **portfolio of chemicals** from lignin-rich streams
- Engineer *Pseudomonas putida* to convert aromatic compounds to value-added products
  - *P. putida* can catabolize many aromatic compounds derived from lignin
  - PHAs (proof of concept molecule for products made through fatty acid chain elongation pathway)
  - Medium chain length (mcl)-alcohols (plasticizers, lubricants, surfactants and solvents; future possible fuel)
  - Itaconic acid (“green” alternative to acrylates) and new molecules

2 – Approach (Management)

Management approach

- Regular interactions with NREL collaborators (e.g., Beckham, Biddy)
  - Close experimental collaboration (e.g., NREL tests our strains for conversion of real lignin streams in bioreactors)
  - Regular phone calls and site visits to discuss progress
  - Routine data sharing, strain exchanges
- Milestones
  - Go/No-go FY17 Q4 – Demonstrated production of 1 g/L mcl-PHAs titer from aromatic substrate
  - FY18 Q4 (end of project cycle) – Production of at least one product (itaconic acid) in *P. putida* from p-coumarate at a yield of at least 25% of the theoretical maximum and titer of at least 2 g/L. Production from lignin-derived substrate of at least 1 g/L.

Project structure

- Adam Guss, PI
- Postdoc Thom Mand (previously Joshua Elmore) leads itaconic acid production
- Student Jay Huenemann leads PHA and alcohol production

2 – Approach (Technical)

Main approach

- Microbial genetics and synthetic biology: Combination of gene deletion and heterologous gene overexpression to optimize metabolic pathways

Potential challenges to be overcome for achieving successful project results

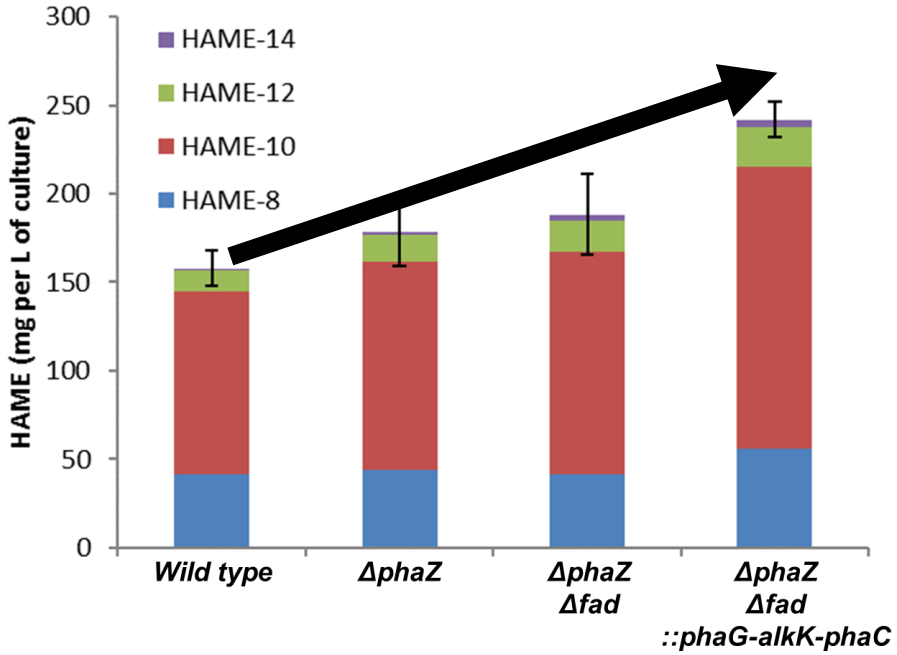
- Genetic modifications can be toxic or lethal
- Flux through heterologous pathways may not be sufficient

Critical success factors (technical, market, business) that will define technical and commercial viability

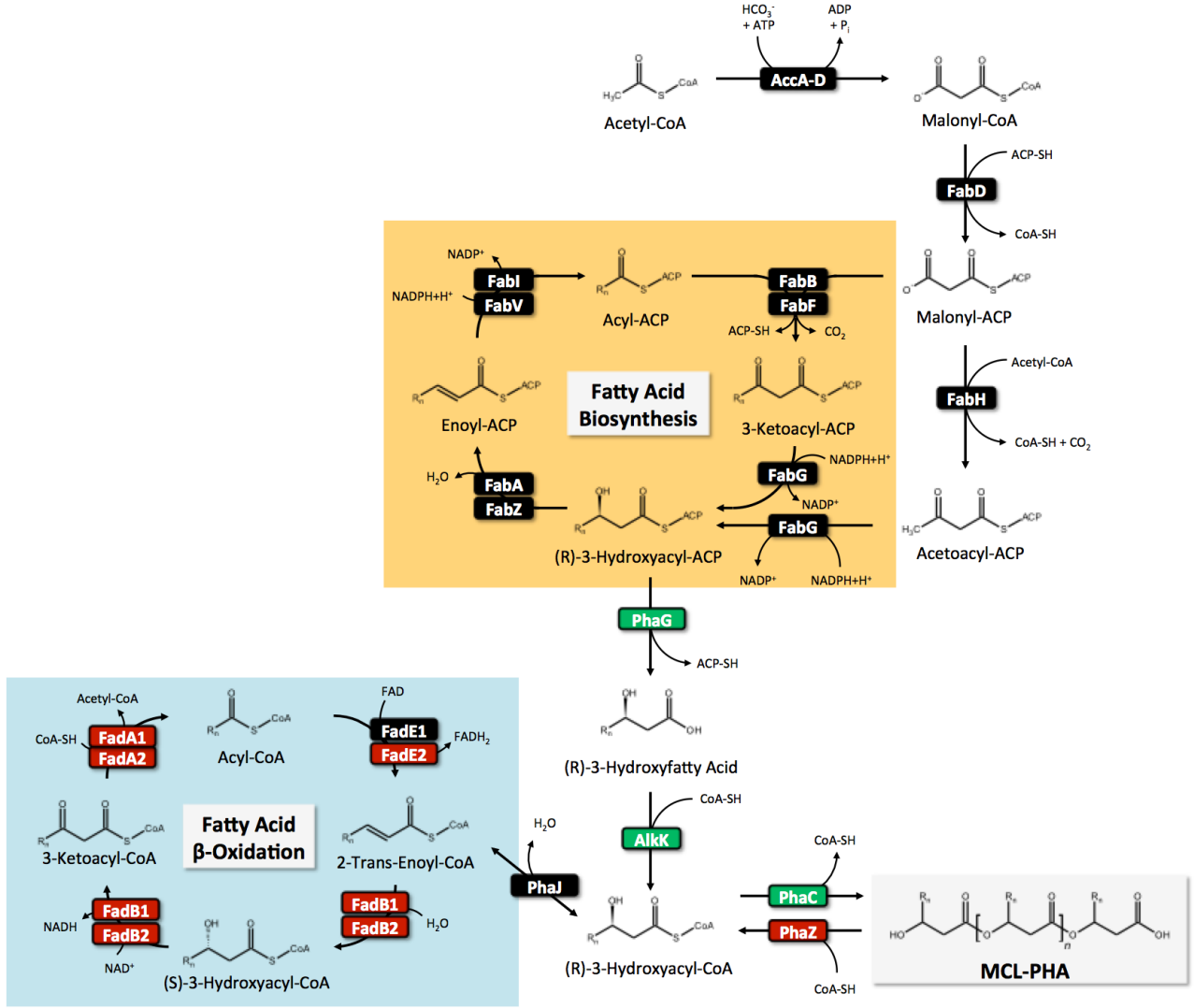
- Developing pathways with high flux to critical metabolic nodes for industry to take the final step(s) to desired product
- Availability of aromatic streams capable of supporting bioconversion

3 – Technical Accomplishments/ Progress/ Results

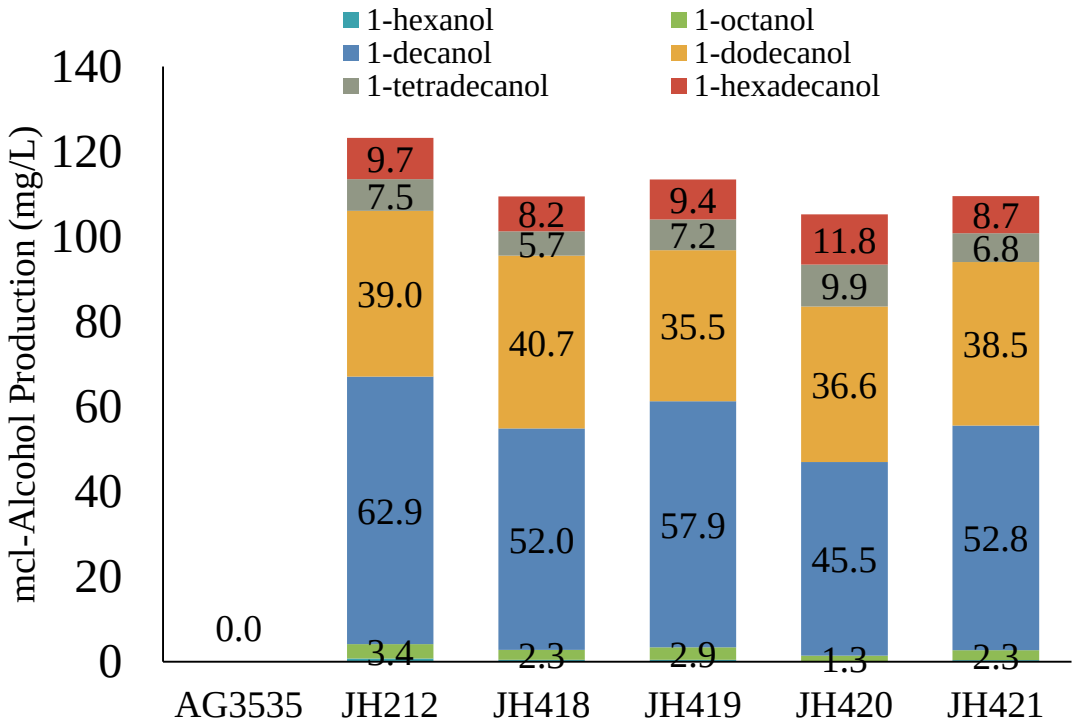
Enhance PHA production from aromatic substrates

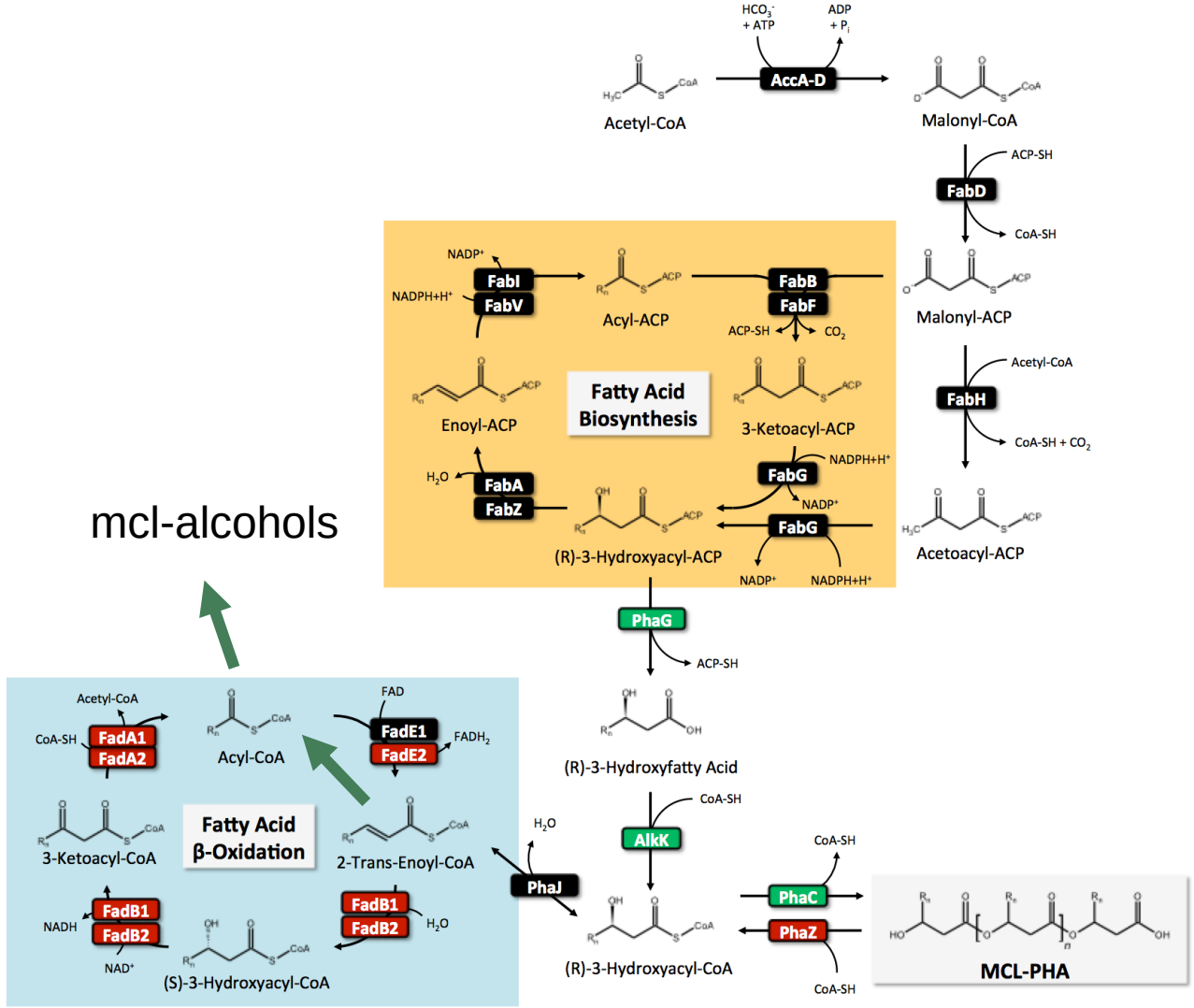


- Early work targeted mcl-PHA production as proof of concept with native product
- Deleted competing pathways
- Overexpressed production pathway
- 40% increase in PHA yield and titer



mcl-alcohol production from aromatic substrates

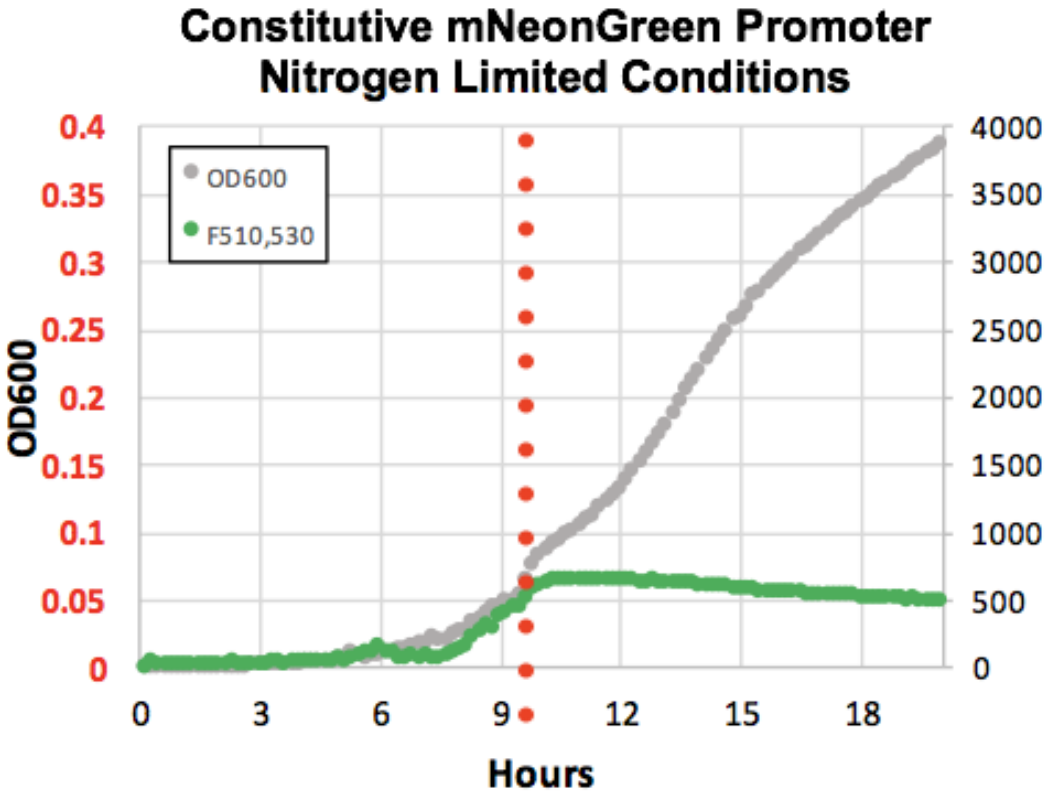


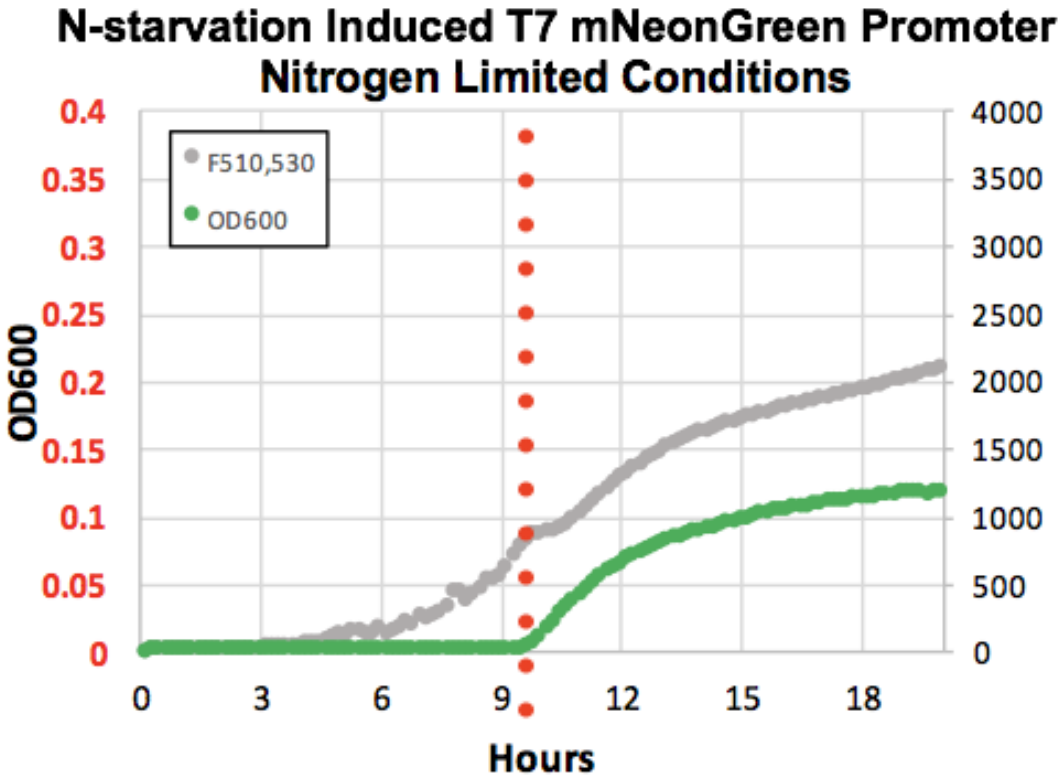


- Many of the same modifications as PHAs
- Tested multiple modifications and conditions
- Achieved titers > 100 mg/L

Developed promoters that function in production phase

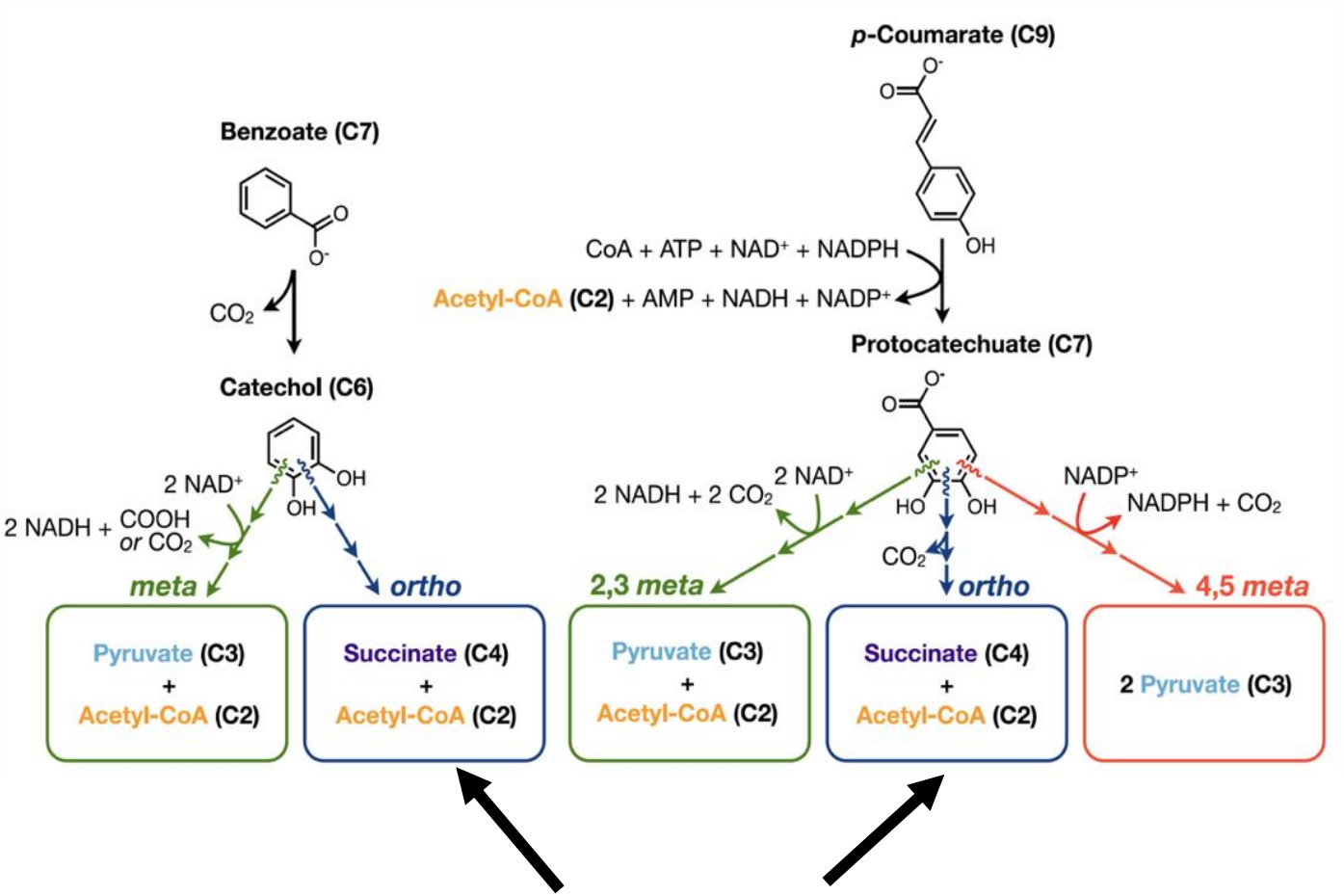
- We were concerned that yields could be limited by lack of stationary (N-starvation) phase gene expression



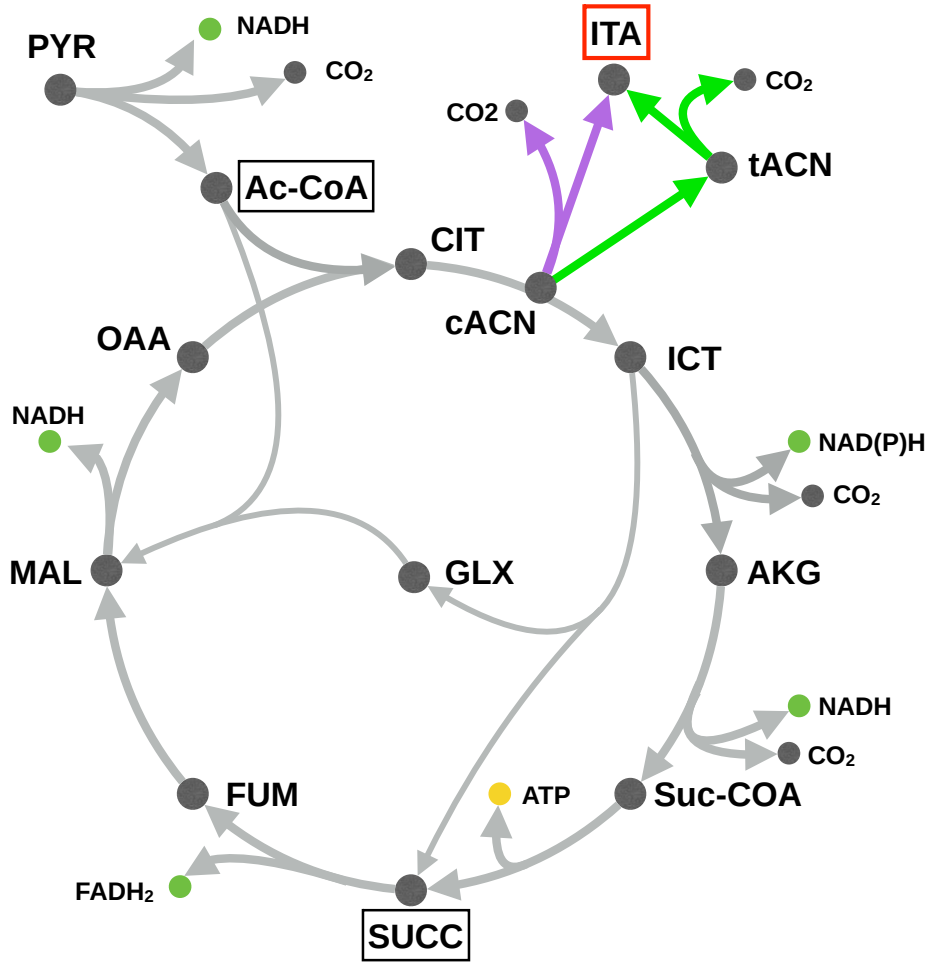


- We created promoters that are specifically induced during N-starvation
- Also created hybrid promoters that express in both log and stationary phase

Accessing different metabolic nodes: itaconic acid as an additional target molecule

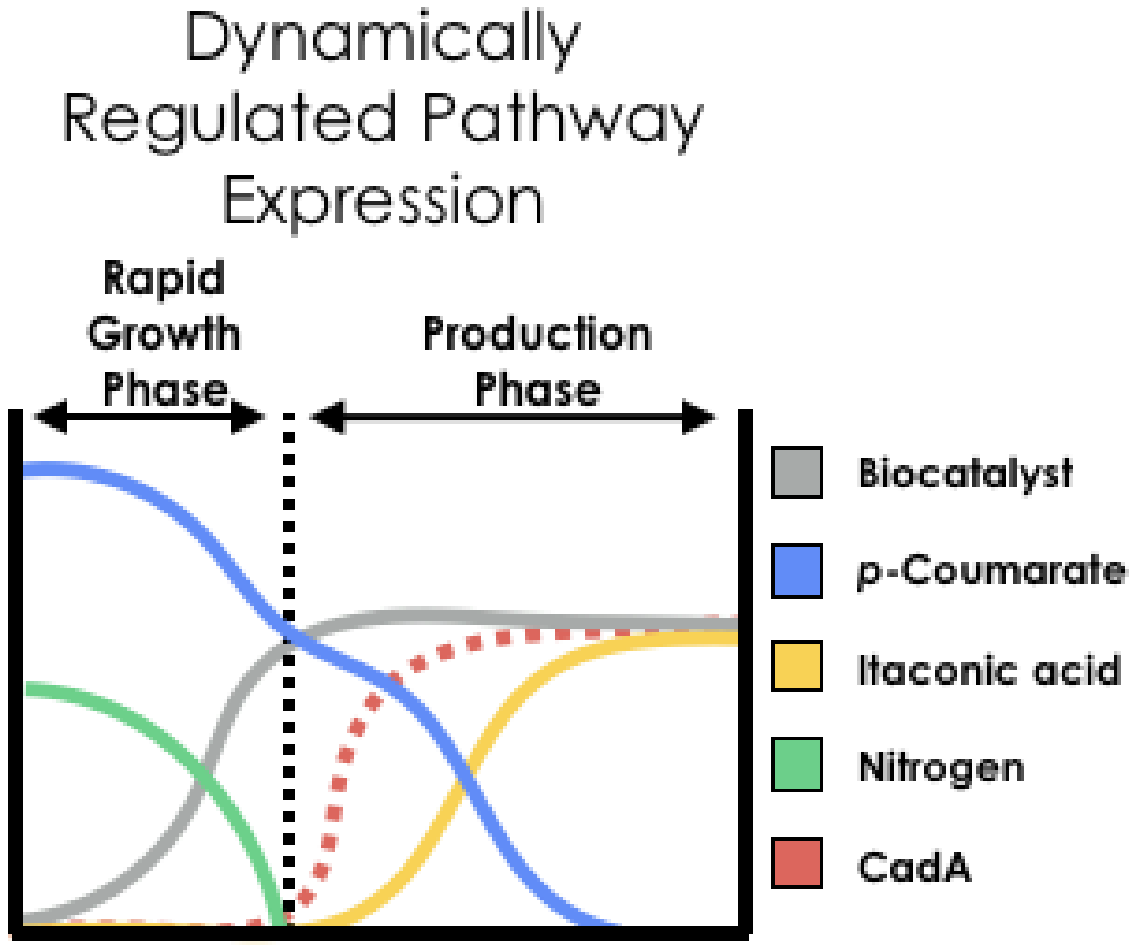


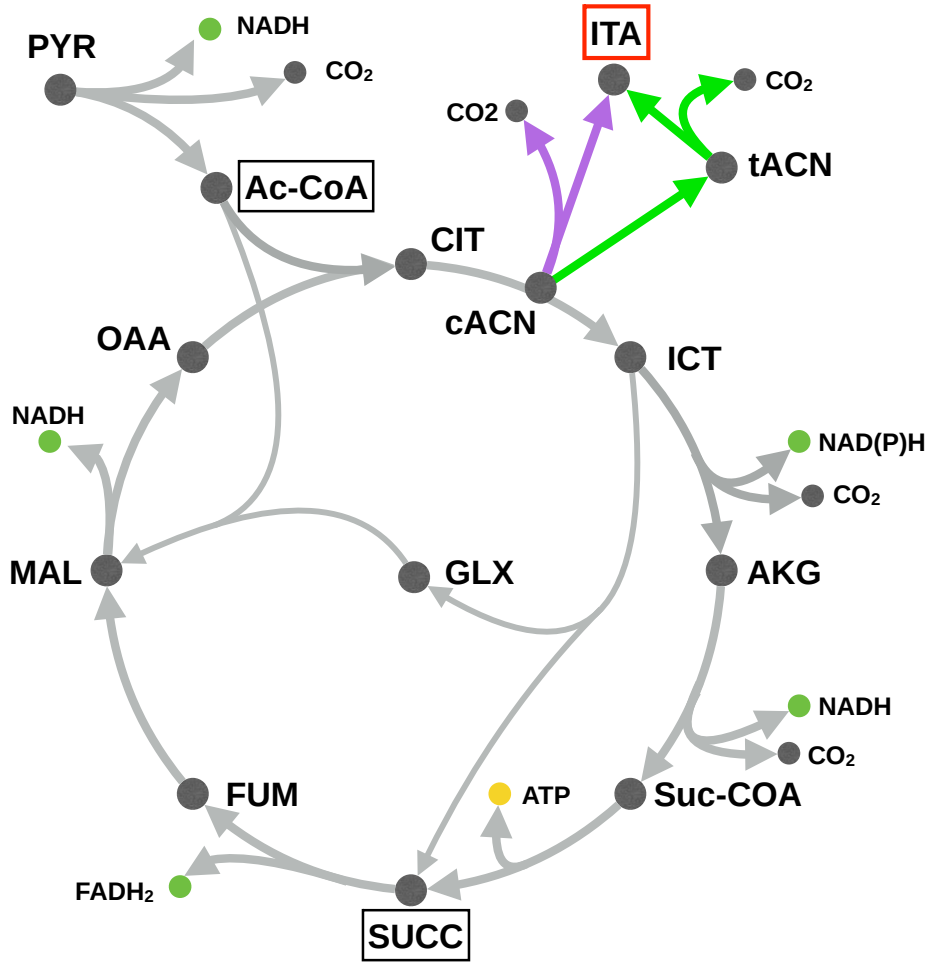
Native to *P. putida* KT2440
Feeds directly into TCA cycle



Purple = cis-pathway
Green = trans-pathway

Accessing different metabolic nodes: itaconic acid as an additional target molecule

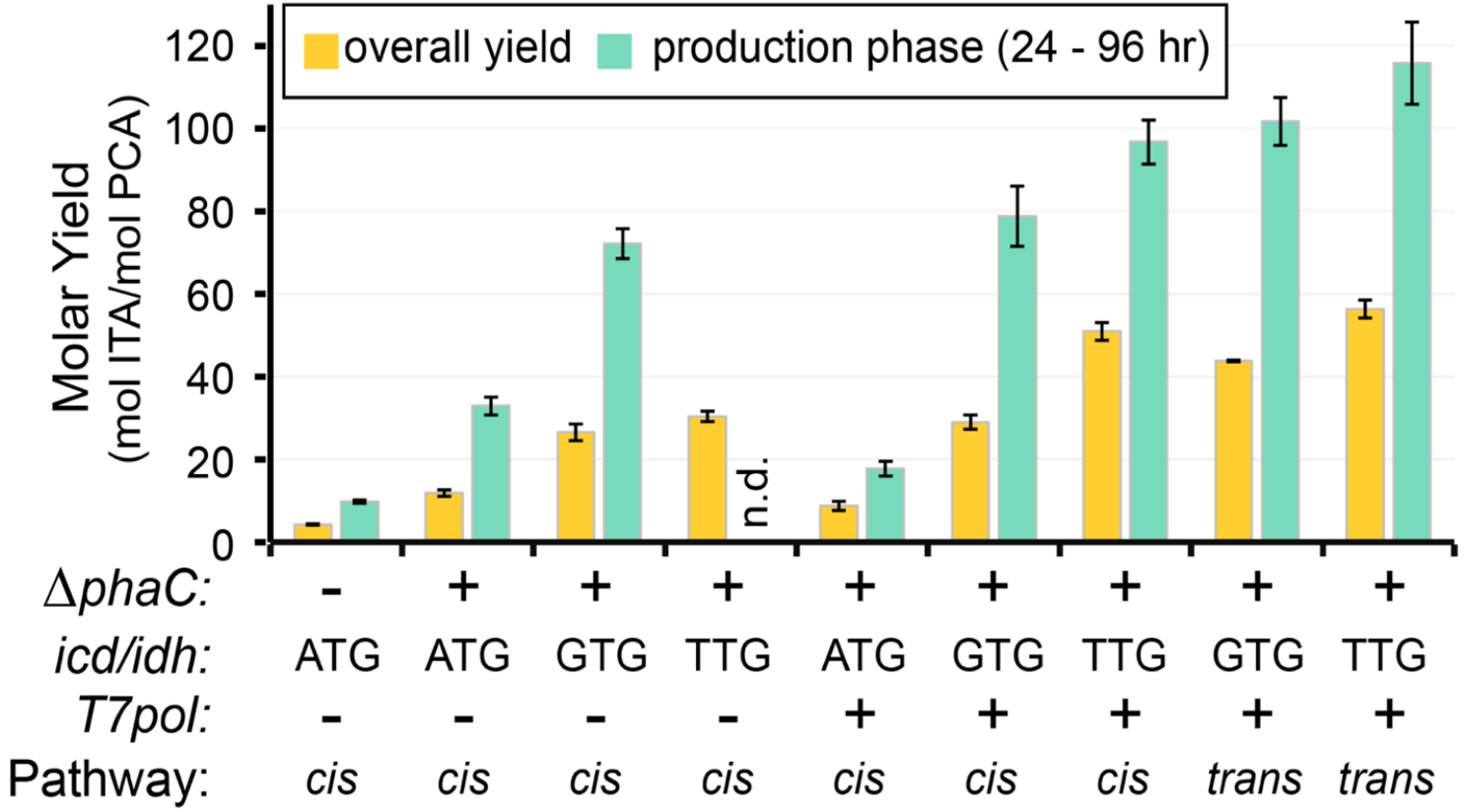




Purple = cis-pathway
Green = trans-pathway

Itaconic acid yields from aromatics approaching 60% mol/mol

- We changed the *icd* start codons to less favorable ones to decrease translation
- 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
- Production phase yields approaching 1.2 mol/mol (Theoretical max = 1.33 mol/mol)

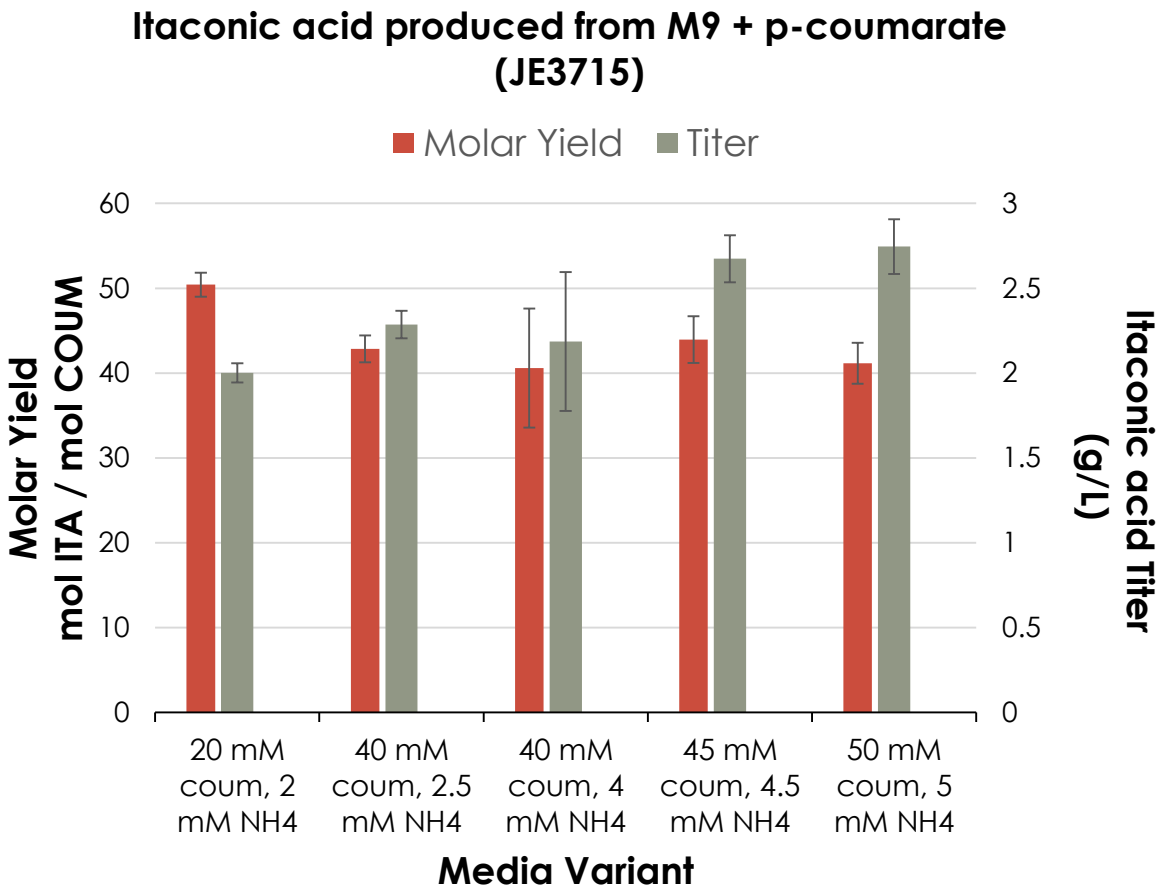


Δ*phaC* – deletion of PHA synthesis genes
icd/idh – start codon used for isocitrate dehydrogenases
T7pol – presence or absence of N-starvation inducible pathway
Pathway – cis- or trans- pathway to itaconic acid

Exceeded 2 g/L itaconic acid titer from model substrate

- Tested increased substrate and nitrogen loading to increase titer
- With both 45 and 50 mM coumarate, exceeded milestone target (up to 2.75 g/L itaconic acid)

Milestone: Demonstrate production of at least one product in *P. putida* from p-coumarate at a **yield of at least 25% of the theoretical maximum and titer of at least 2 g/L.** Production from APL or another lignin-derived substrate of at least 1 g/L.

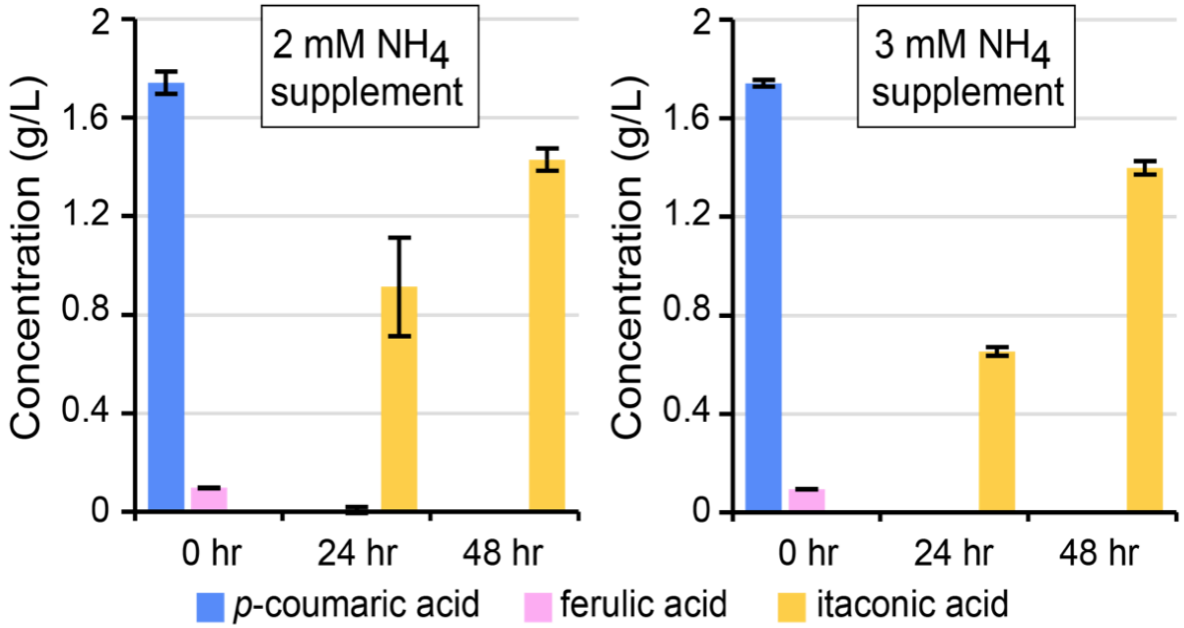


Exceeded 1 g/L itaconic acid titer from BCD lignin

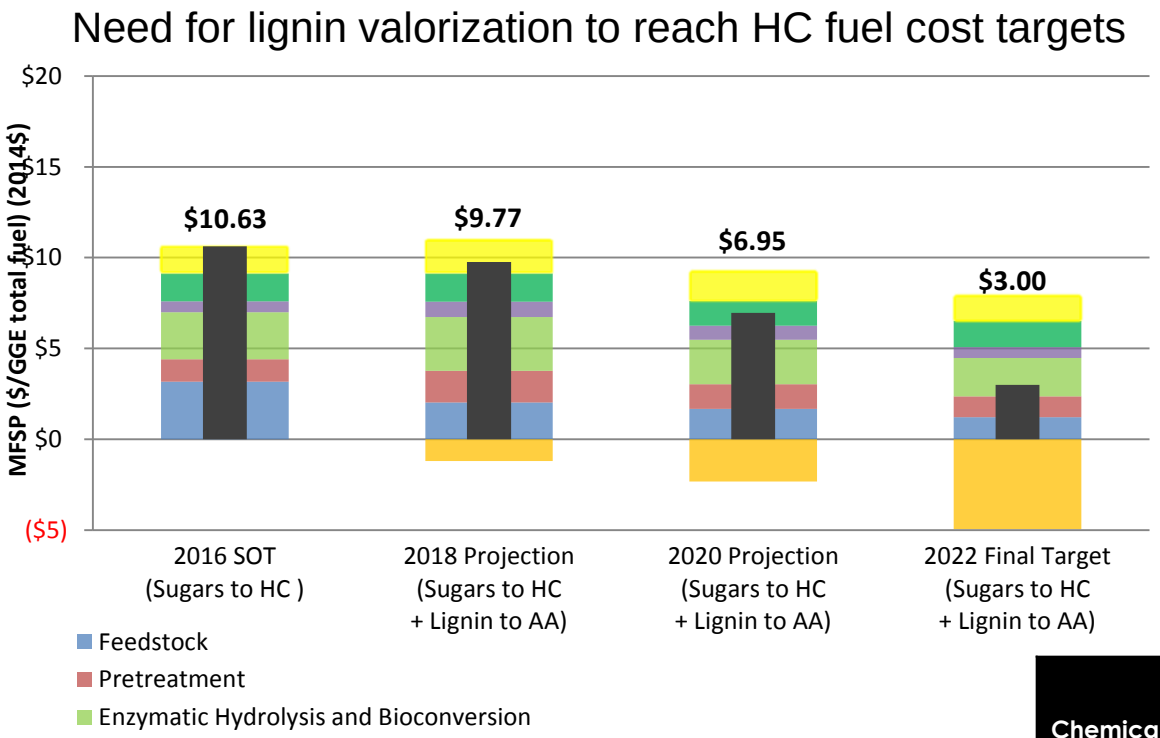
- Tested real-world lignin-derived substrate
  - Corn stover pretreated by deacetylation and mechanical-refining and enzymatic hydrolysis
  - Lignin-rich solid fraction subjected to Base-Catalyzed Deconstruction (BCD)

- With both 2 and 3mM added NH_4, exceeded milestone target (<u>up to 1.4 g/L itaconic acid</u>)

Milestone: Demonstrate production of at least one product in *P. putida* from p-coumarate at a yield of at least 25% of the theoretical maximum and titer of at least 2 g/L. **Production from APL or another lignin-derived substrate of at least 1 g/L**.



Techno-Economic Analysis of mcl-alcohol production



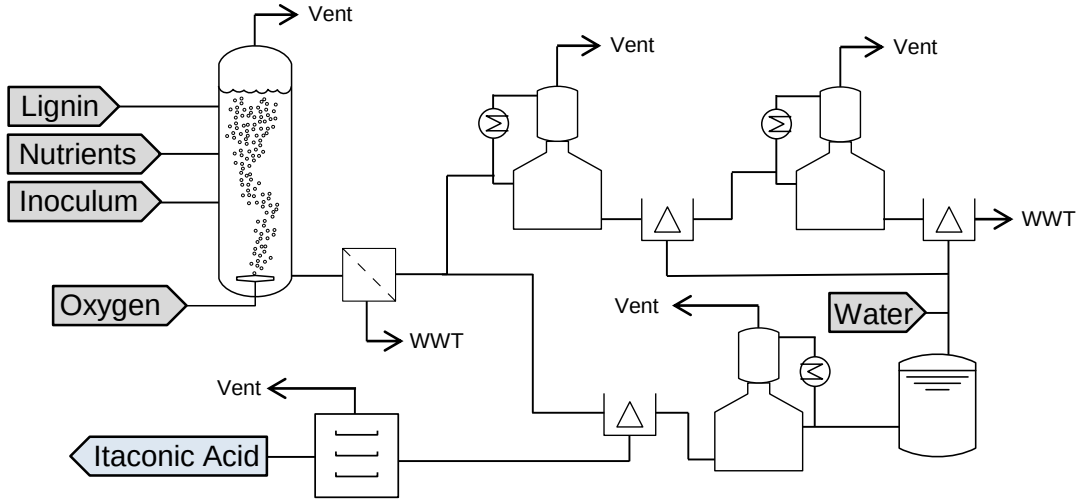
Biddy et al. 2016

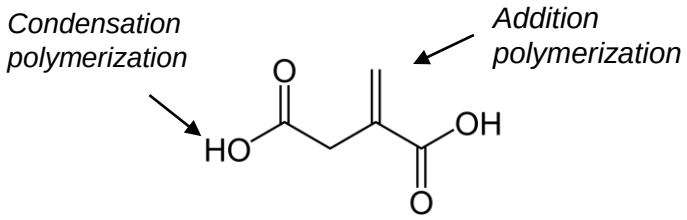
- Comparison to the adipic acid TEA
- mcl-alcohol production has very similar economics
  - Similar market size (~3MM metric tons/year)
  - Lower capital and operating costs for mcl-alcohols
  - Lower mass yield for mcl-alcohols

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]

Preliminary TEA: itaconic acid

Aerobic fermentation, primary crystallization, and recrystallization

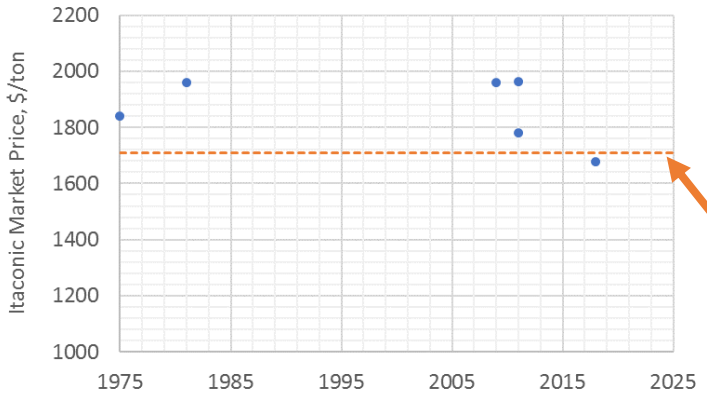




Coproduct	MFSP*	Market Price	Productivity
	$/GGE	$/ton	g/L/hr
Itaconic	$2.08	1704	1
Adipic	$2.49	1710	1

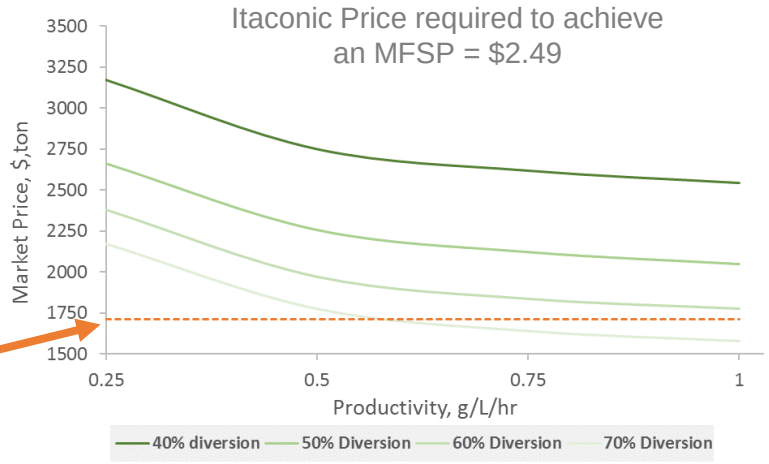
*Upstream and process steps not depicted here are consistent with the BC 2018 Design Report acids pathway. More details can be found at: (https://www.nrel.gov/docs/fy19osti/71949.pdf)

- Appealing bio-based unsaturated monomer (trifunctional)
- Estimated market prices are similar or higher than adipic acid
- Target MFSP achievable even at the lower bound of historical market price estimates
- **Maintaining high diversion of substrate to product instead of growth is a key consideration**



De Carvalho, Julio C., ANTONIO I. MAGALHÃES JR, and Carlos R. Soccol. "Biobased itaconic acid market and research trends-is it really a promising chemical?." Chimica Oggi-Chemistry Today 36 (2018): 4.

Adipic Acid



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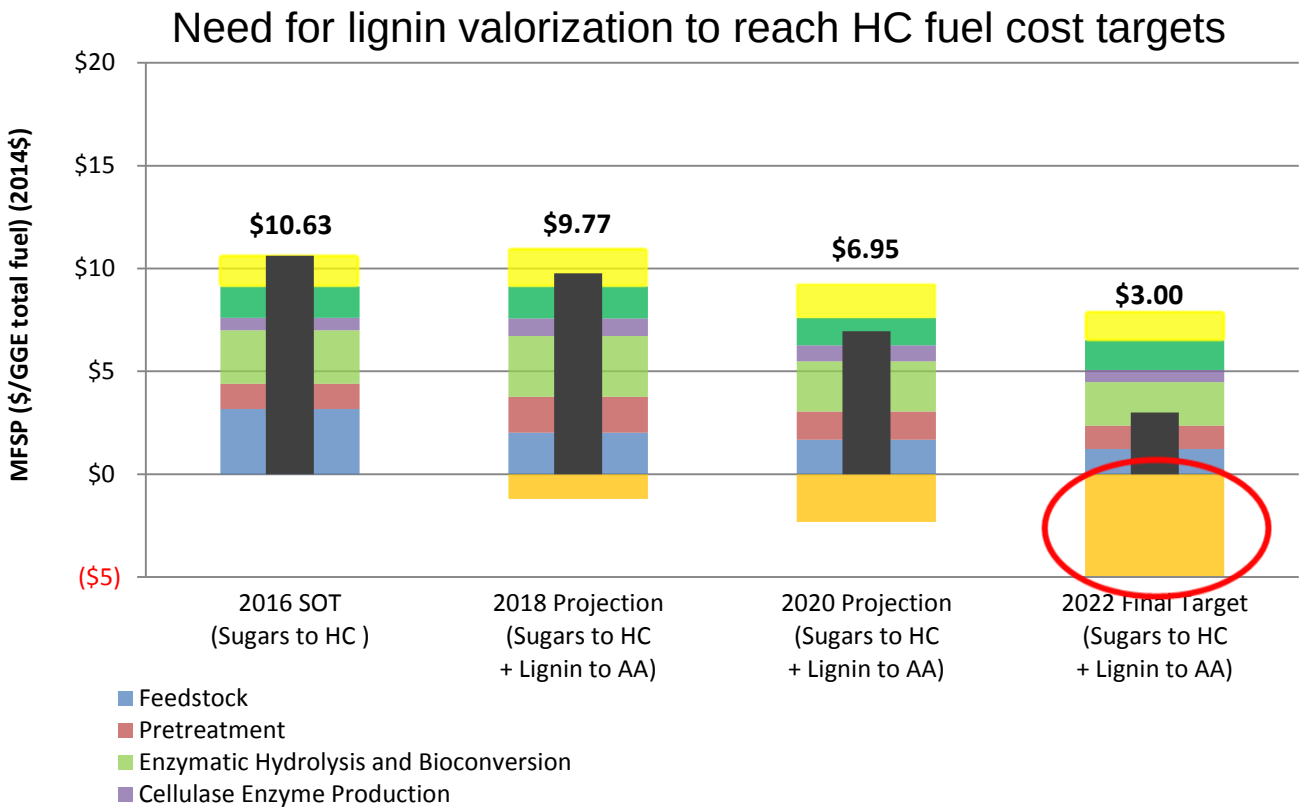
Nick Grundl, NREL

4 – Relevance

- **Goal:** Engineer microorganisms to increase the number of chemicals that can be made from lignin
- **Importance:** Lignin valorization to diverse products makes a critical contribution to economic production HC fuels
- **Contribution to BETO goals:** Lignin valorization directly contributes to cost reduction of HC fuels, contributing to lower MFSP for HC biofuels
- **Relevance to the bioenergy industry:** Industry needs multiple technologies for converting lignin into diverse value-added chemicals at high flux to provide flexibility and prevent market saturation
- **Advancing the state of technology and impact:** This project will expand the portfolio of products that can be made from lignin



5 – Future Work

Develop organisms that have high flux of aromatic substrates to value-added products at diverse metabolic nodes

- Test more genetic modifications to increase flux to fatty acid-derived products
- Understand stationary phase to enhance production
- Proof of concept production of other molecules
- Work with NREL collaborators to test bioreactor-scale production and lignin-derived substrates
- Upcoming key milestones:
  - FY19Q3: In collaboration with NREL project Biochemical Platform Analysis (WBS 2.1.0.100), complete preliminary Techno-Economic Analysis of itaconic acid production from lignin.
  - FY19Q4: Demonstrate production of at least 0.5 g/L of a new target molecule from an aromatic substrate.
- Go/No-Go Point: Increase titer by at least 5-fold over the current state of the art using model substrates and a real depolymerized lignin such as DMR-EH BCD lignin (March 2020)

Summary

Overview

Engineering microbes to produce diverse chemicals from lignin to produce valuable co-products to help reach HC fuel cost targets

Approach

Synthetic biology and metabolic engineering of *Pseudomonas putida* to optimize pathways for converting lignin-derived substrates into chemicals

Technical Accomplishments/Progress/Results

Demonstrated g/L production of itaconic acid from lignin-derived aromatics and >100 mg/L mcl-alcohols from model and lignin-derived substrates

Relevance

Industry needs approaches to convert lignin into multiple value-added products, which will enable more economical production of sugar-based fuels

Future work

Pathway optimization to increase titer/rate/yield, new products from aromatic substrates

Acknowledgements

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NREL collaborators

Gregg Beckham (and team)
Davinia Salvachúa (and team)
Chris Johnson
Mary Biddy
Ryan Davis
Nick Grundl

Additional Slides

Responses to Previous Reviewers' Comments

- Comment: The medium chain length alcohols look like a good target for an output molecule. Getting an early read on the toxicity of these products to the P. putida system would be a good idea, to make sure there isn't another large technical challenge out there that will limit progress towards economic levels of production, in addition to the metabolic engineering required for use of lignin derivatives.
- Response: We have tested toxicity of medium chain alcohols, and *P. putida* is tolerant to at least 1% w/v (~8 g/L) octanol and decanol, which is well beyond the solubility limit for these compounds, so we do not anticipate toxicity to be a substantial barrier. Extending this to newer targets, we have also tested toxicity of itaconic acid, and *P. putida* can grow in 0.5M sodium itaconate, and this inhibition is likely due to the accumulation of sodium rather than itaconate.

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 DRb, Beckham GT*, Guss AM*. Metabolic engineering of Pseudomonas putida for increased polyhydroxyalkanoate production from lignin. Submitted.

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.

Patents:

- Provisional patent application (US 62/664,570): "Production of Itaconic Acid and Related Molecules from Aromatic Compounds"