



U.S. DEPARTMENT OF
ENERGY

Energy Efficiency &
Renewable Energy

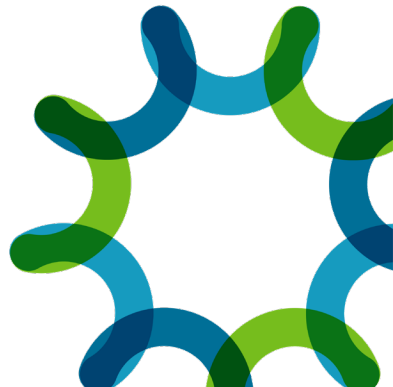
BIOENERGY TECHNOLOGIES OFFICE

Welcome

Nathan J. Hillson
njhillson@lbl.gov

Lead PI, DOE Agile BioFoundry

ABF Webinar
April 29, 2022



Webinar agenda

- **Agile BioFoundry overview:** Nathan Hillson
- **Beachheads:** Nathan Hillson (for Christopher Johnson)
- **Bacterial demonstration projects:** Gregg Beckham
- **Yeast demonstration projects:** Di Liu (for John Gladden)
- **Fungal demonstration projects:** Jon Magnuson
- **TEA/LCA:** Bruno Klein / Hui Xu (for Thathiana Benavides)
- **Future directions:** Nathan Hillson



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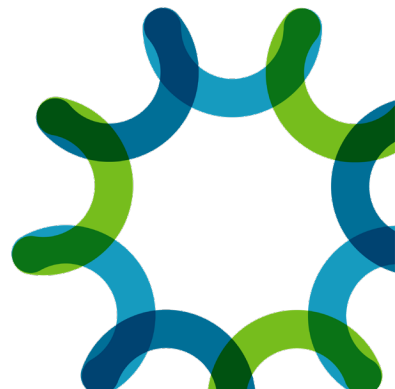
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ABF Overview

Nathan J. Hillson
njhillson@lbl.gov

Lead PI, DOE Agile BioFoundry

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ABF's goal

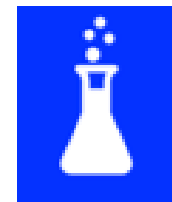
- **Goal:** Enable biorefineries to achieve 50% reductions in time to bioprocess scale-up as compared to the current average of around 10 years by establishing a distributed Agile BioFoundry to productionize synthetic biology
- **Outcomes:** Development and deployment of technologies enabling commercially relevant biomanufacturing of a wide range of bioproducts by both new and established industrial hosts
- **Relevance:** \$21M/year public infrastructure investment that increases U.S. industrial competitiveness and enables opportunities for private sector growth and jobs
- **Risks:** Past learnings do not transfer well across target molecules and microbial hosts. Experiment data sets are of insufficient quality/quantity/consistency to learn from



ABF supports DOE EERE's decarbonization strategies and emphasis on diversity in STEM

- **Decarbonizing energy-intensive industries:**

- ABF metabolic beachheads supporting and optimized routes to direct replacement chemicals, Performance-Advantaged BioProducts (through PABP mini-consortium collaborations), and CO2 utilization for chemicals (through ABF Direct-Funding Opportunity supported industry collaborations)
- FY22 goal: achieve at least one target molecule within 20% of the fossil feedstock incumbent minimum sales
- The ABF is identifying which chemical markets should be prioritized by adapting metabolic models in an TEA / LCA framework



- **Decarbonizing transportation:**

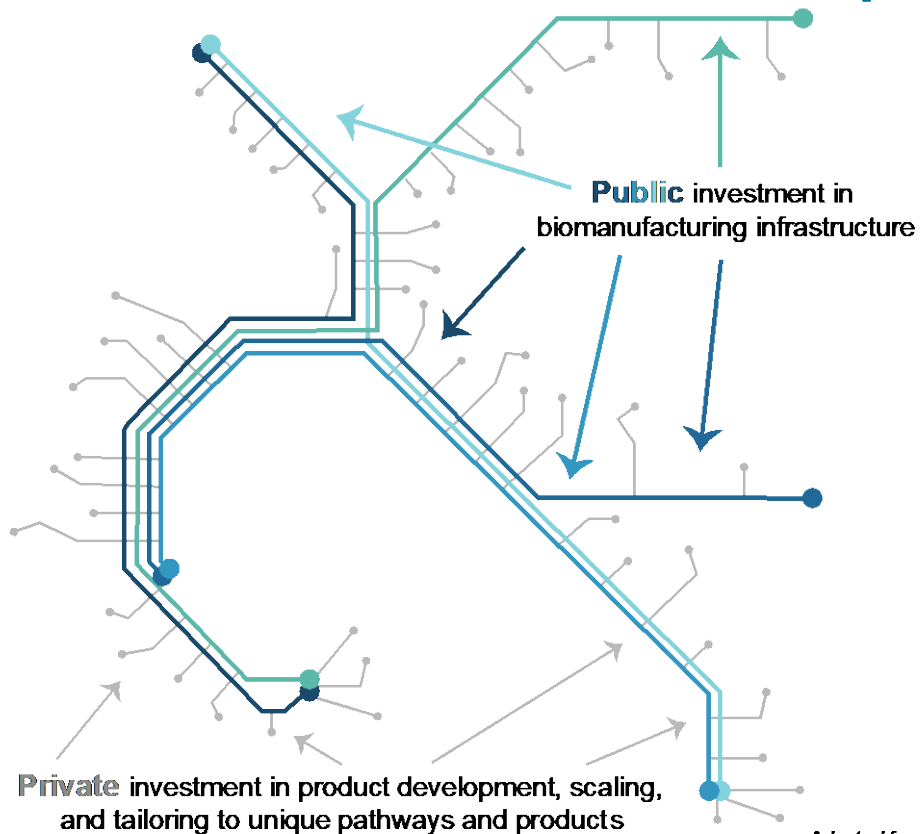
- The ABF will be leveraging its Design-Build-Test-Learn infrastructure (and data) to the challenges and opportunities of Sustainable Aviation Fuels



- **Diversity in STEM:**

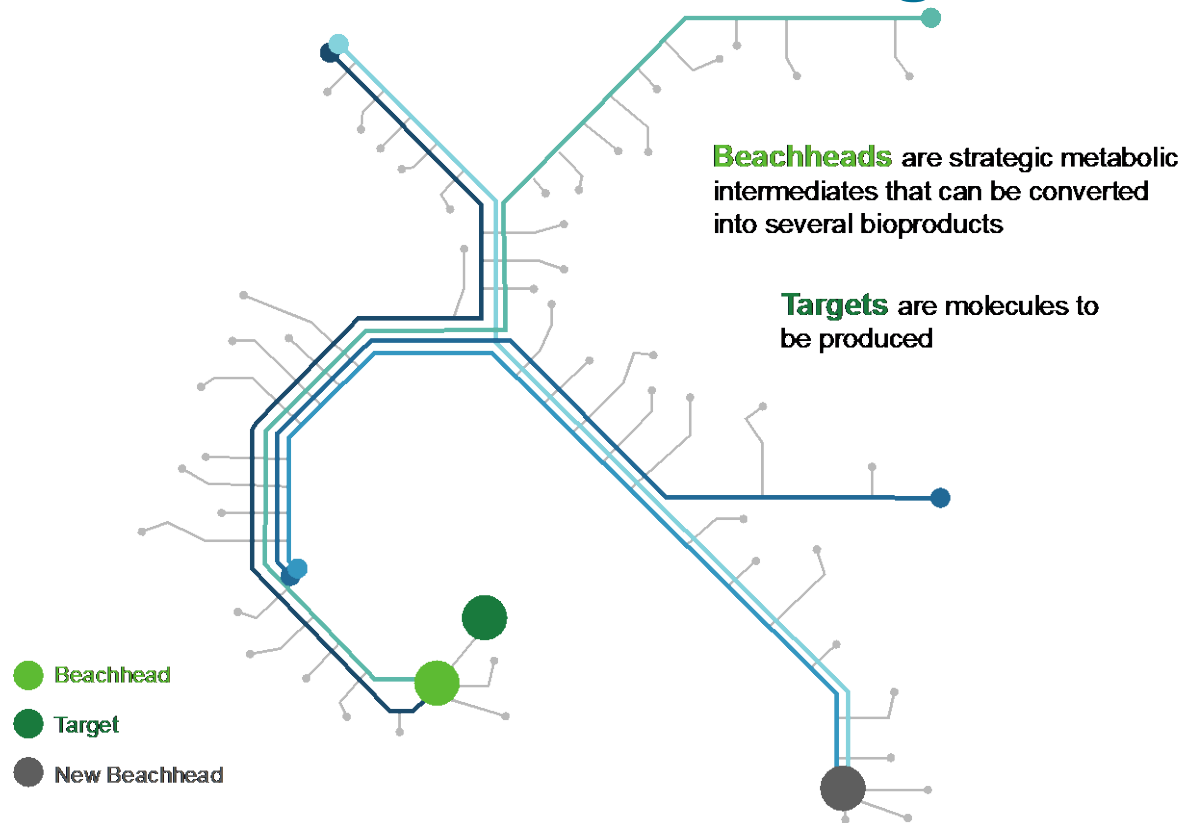
- The ABF is allocating \$1M in Directed-Funding Opportunity resources to collaborate with the Minority Supporting Research and Development consortium

Public infrastructure investment enables private industry

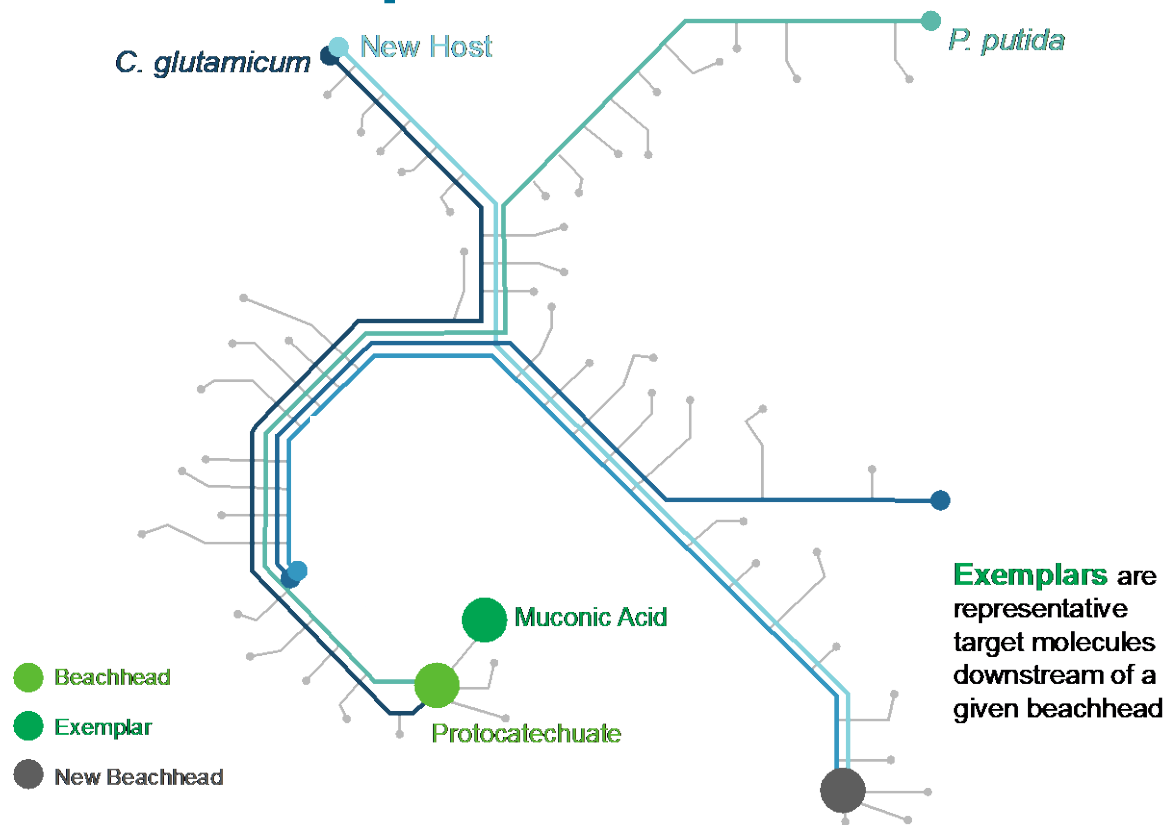


Adapted from Lyft and BART

Metabolic beachheads and targets



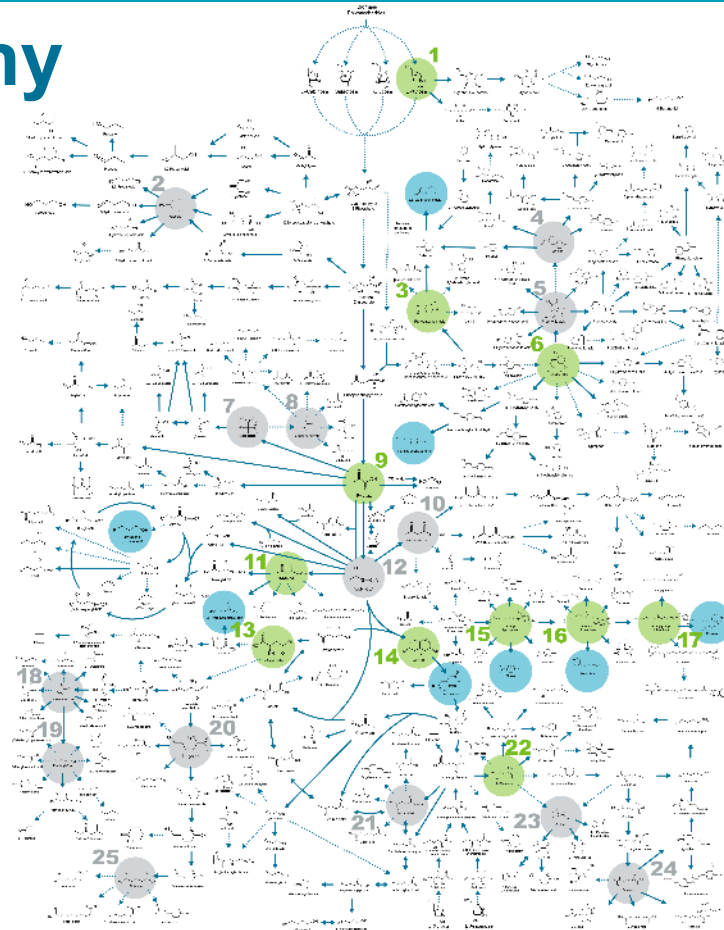
Hosts and exemplar molecules



ABF metabolic cartography

- | | |
|------------------------|-------------------------------|
| 01 Xylose | 15 Geranyl diphosphate |
| 02 Glycerol | 16 Farnesyl diphosphate |
| 03 Protocatechuic acid | 17 Geranylgeranyl diphosphate |
| 04 L-Tyrosine | 18 2-ketobutyric acid |
| 05 Prephenic acid | 19 Propionyl-CoA |
| 06 Chorismate | 20 L-Lysine |
| 07 Acetolactate | 21 Succinyl-CoA |
| 08 2-Ketoisovalerate | 22 L-Glutamate |
| 09 Pyruvate | 23 L-Proline |
| 10 Acetoacetyl-CoA | 24 L-Arginine |
| 11 Malonyl-CoA | 25 Glutaric acid |
| 12 Acetyl-CoA | |
| 13 L-Aspartate | |
| 14 Citrate | |

- Current ABF target molecules
- Current ABF beachhead molecules
- Potential beachhead molecules



Adapted by permission from Springer Nature Customer Service Centre GmbH: Nature, Nature Catalysis, A comprehensive metabolic map for production of bio-based chemicals, Lee, S.Y., et al., © 2019

ABF hosts and tier system

Six Hosts Onboarded to Tier 1:

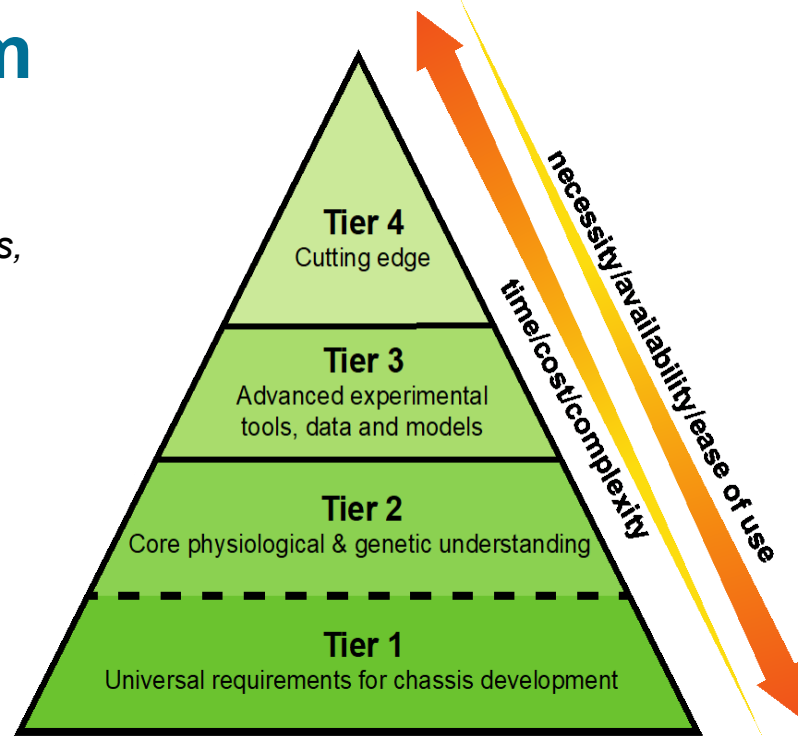
Bacteria - *Cupriavidus nector*, *Rhodobacter sphaeroides*,
Clostridium ljungdahlii, *Zymomonas mobilis*

Fungi - *Lipomyces starkeyi*, *Aspergillus pseudoterreus*

Four Hosts Elevated to Tier 2:

Bacteria - *Pseudomonas putida*,
Corynebacterium glutamicum

Fungi - *Rhodospiridium toruloides*, *Aspergillus niger*

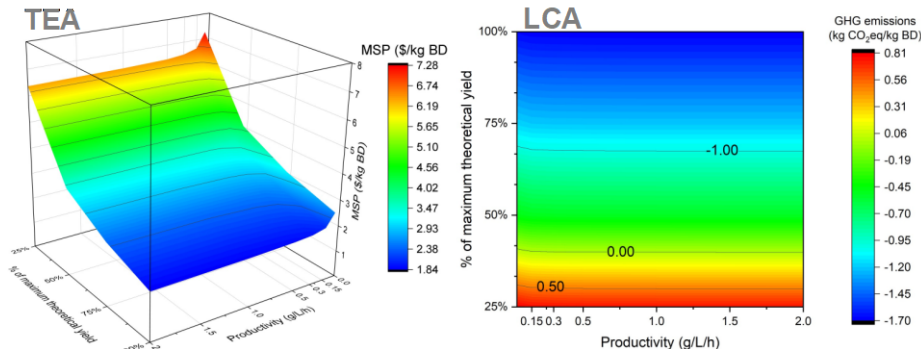


Tier 1 represents the fundamental tools & information needed for any rational DBTL cycle; these basics must be achieved to be “onboarded”.

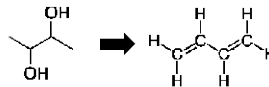
Tier 2 criteria consist of the tools and knowledge needed for rapid and robust DBTL cycles.

Exemplar TechnoEconomic Analyses and Life Cycle Assessments

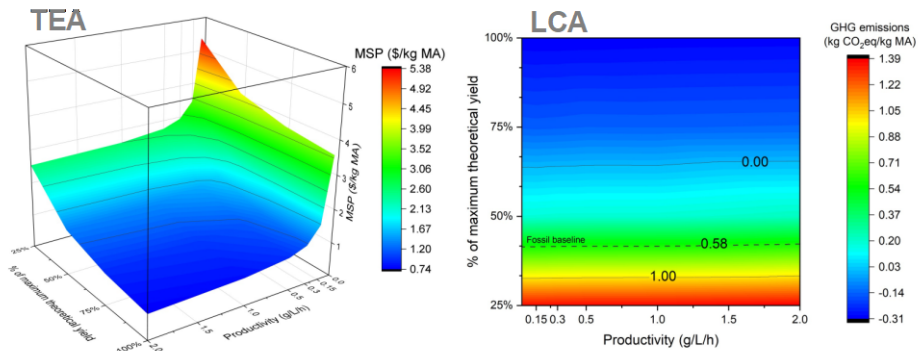
2,3-butanediol (BDO) converted to butadiene (BD)



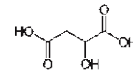
- Lowest possible MSP: **\$1.85/kg**
- Current market price: **\$1.06/kg**
- Reduction in GHG emissions for any case



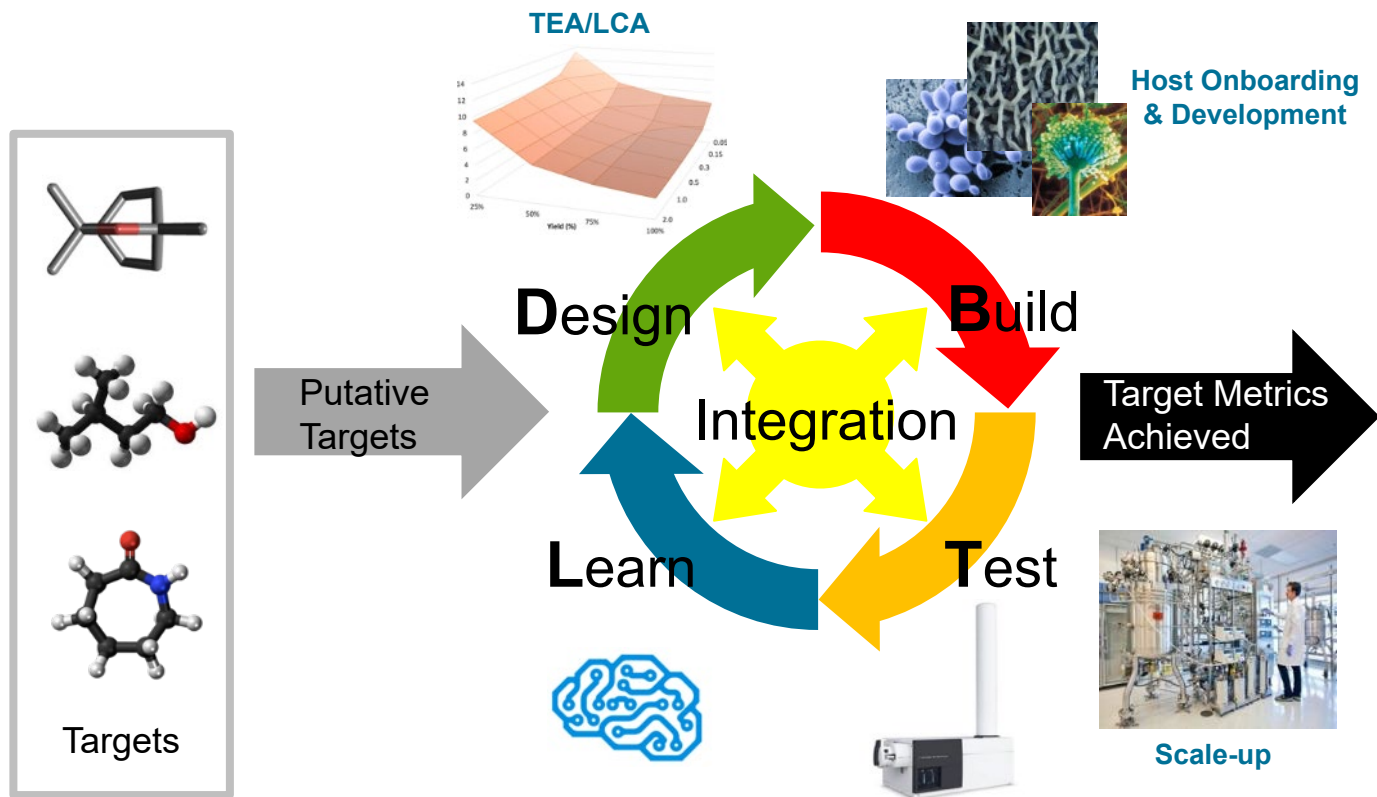
Malic acid



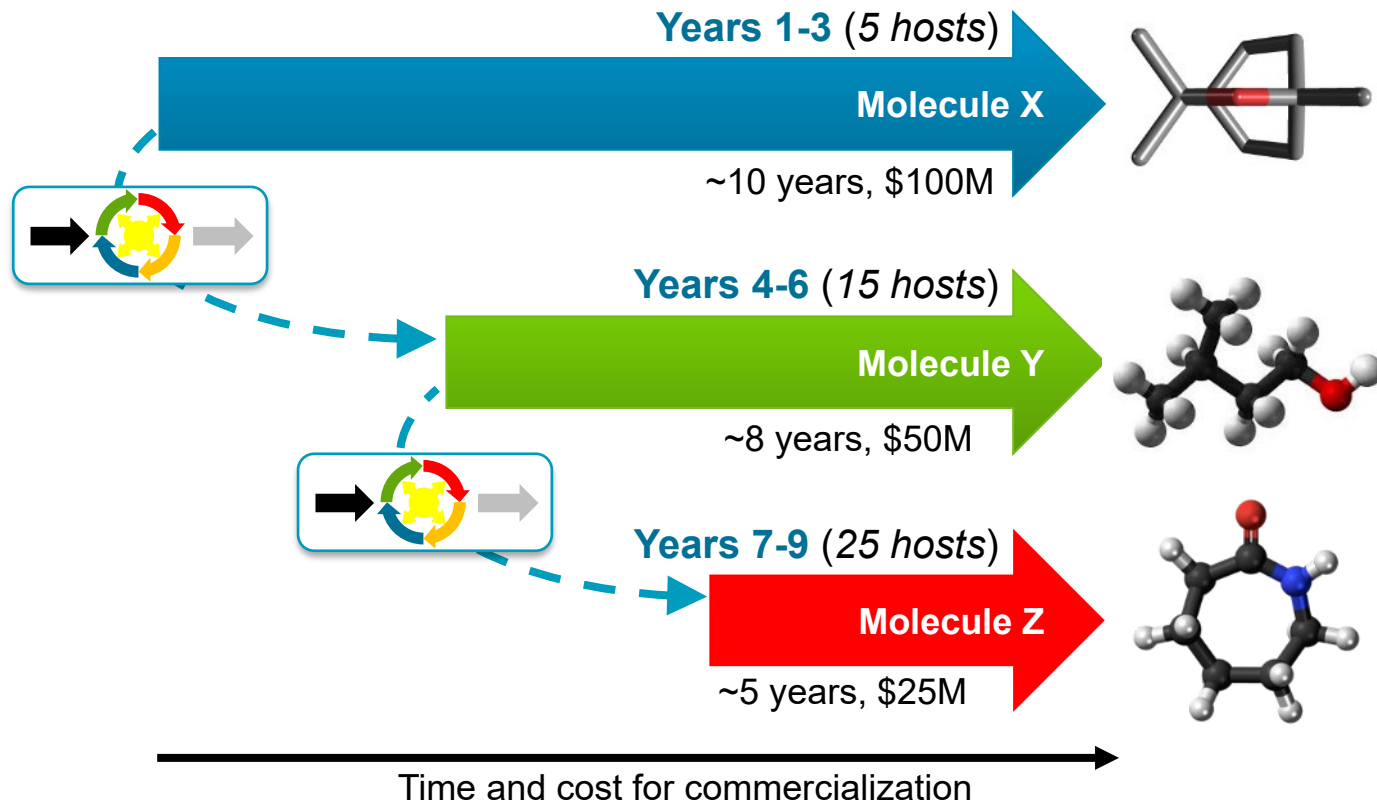
- Lowest possible MSP: **\$0.75/kg**
- Current market price: **\$2.00/kg**
- Reduction in GHG emissions for yields higher than 42% of the theoretical maximum



The Agile BioFoundry approach



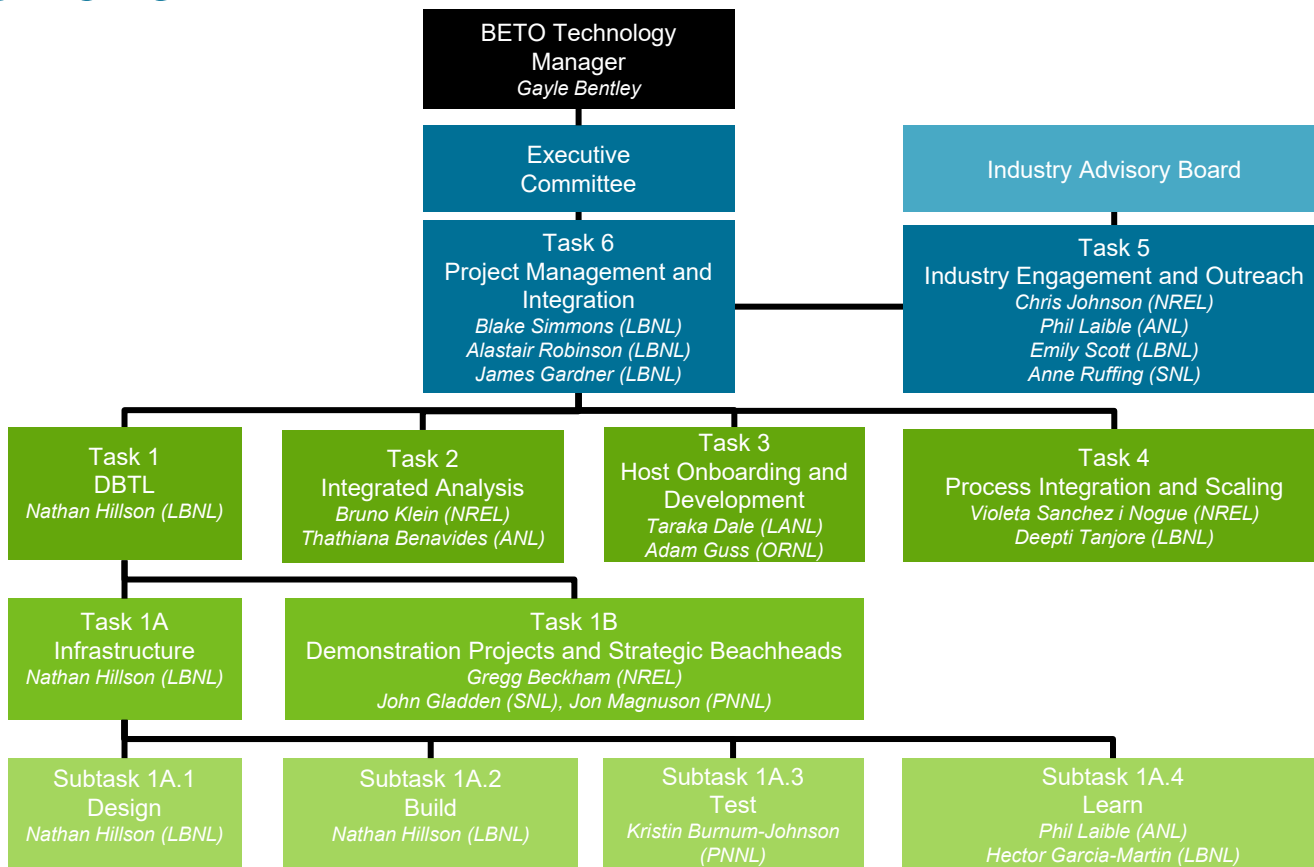
Agile BioFoundry will reduce time-to-scale up



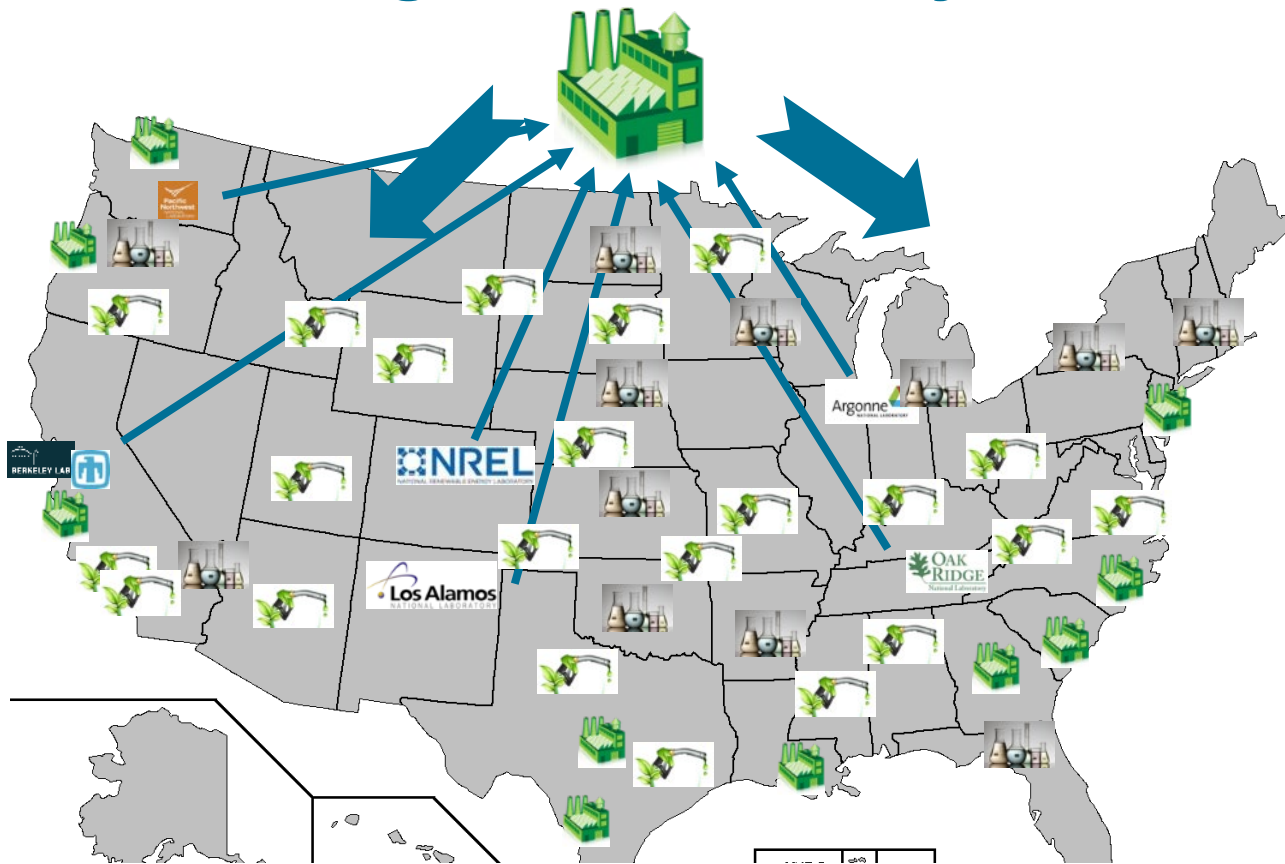
Six Tasks

- **Task 1: Design-Build-Test-Learn** (*Nathan Hillson* - lead)
 - **Infrastructure:** Integrate design-build-test-learn cycle with process automation
 - **Demonstration Projects and Strategic Beachheads:** Demonstrate uses of DBTL infrastructure and establish and improve routes in microbial hosts to beachhead molecules of high strategic interest
- **Task 2: Integrated Analysis** (*Bruno Klein / Thathiana Benavides* – co-leads)
 - Analyze proposed target and beachhead molecules with TEA and LCA methodologies
- **Task 3: Host Onboarding & Development** (*Taraka Dale / Adam Guss* – co-leads)
 - Onboard additional microbial host organisms and further develop them to higher capability tiers through tool development and data collection
- **Task 4: Process Integration & Scale-up** (*Violeta Sanchez i Nogue / Deepti Tanjore* – co-leads)
 - Provide DMR-EH hydrolysates, and test and scale fermentation to improve titer, rate, and yield
- **Task 5: Industry Engagement & Outreach**
(*Chris Johnson / Phil Laible / Emily Scott / Anne Ruffing* – co-leads)
 - Identify barriers to industry adoption of ABF technologies, expand number and diversity of industry partnerships, and establish a set of metrics for determining impact of ABF technologies on industry
- **Task 6: Management** (*Blake Simmons* - lead)
 - Manage project management, develop internal and external communications, provide deliverables to BETO, and make capital equipment purchases

Org Chart



A distributed Agile BioFoundry



ABF 2022 budget / resource planning

- **Budget:** \$21M
 - \$15M internal projects
 - \$6M collaborations (e.g. Funding Opportunities)
 - \$3-4M Open
 - \$1M+ NSF
 - \$1M+ MSRDC
- **AI/ML related resource allocation:** \$3M
 - \$1.5M internal projects
 - \$1.5M collaborations

Funding Opportunities

[Agile BioFoundry 2022 Funding Opportunity](#)

The Department of Energy's Office of Energy Efficiency & Renewable Energy's Bioenergy Technologies Office (BETO)-funded Agile BioFoundry (ABF) consortium is overseeing a funding opportunity for industry and academic partners to utilize [ABF capabilities](#). This funding opportunity provides resources for partners to collaborate with ABF's investigators for developing novel microbial hosts, augmenting titer, rate and yield of bioproducts, and creating new capabilities and approaches to improve the Design-Build-Test-Learn biomanufacturing cycle. [Full details are available here.](#)

[Accelerating Innovations in Biomanufacturing Approaches through Collaboration Between NSF and the DOE BETO funded Agile BioFoundry \(NSF-DOE/ABF Collaboration\)](#)

To help advance the U.S. bioeconomy, the National Science Foundation and the Department of Energy's Bioenergy Technologies Office [invite proposals](#) from researchers at institutions of higher education and non-profit organizations (eligible PIs). The proposals must leverage the unique [Design-Build-Test-Learn capabilities](#) available at the Agile BioFoundry to translate the latest advances in synthetic biology and engineering biology basic research into testable prototype processes and products that are potentially scalable and manufacturable and can be appropriately validated. [Full details are available here.](#)

Capabilities

Uniting world-class national laboratory facilities through a
Design-Build-Test-Learn platform



Design

Develop bioprocesses for your desired target molecules, as well as the necessary tools to build out pathways in a host organism.



Build

Transform Design concepts and specifications into physical engineered microbial host organisms, ready to be Tested.



Test

Understand how an engineered pathway behaves in your host organism and measure performance.



Learn

Use various methods to translate your experimental data into predictions for the design of future pathways and processes.



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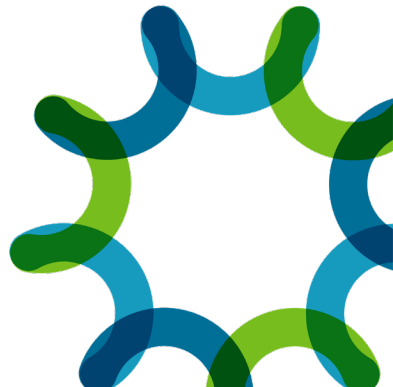
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Pathway Development and Evaluation

Nathan J. Hillson (for Christopher Johnson)
njhillson@lbl.gov (christopher.johnson@nrel.gov)

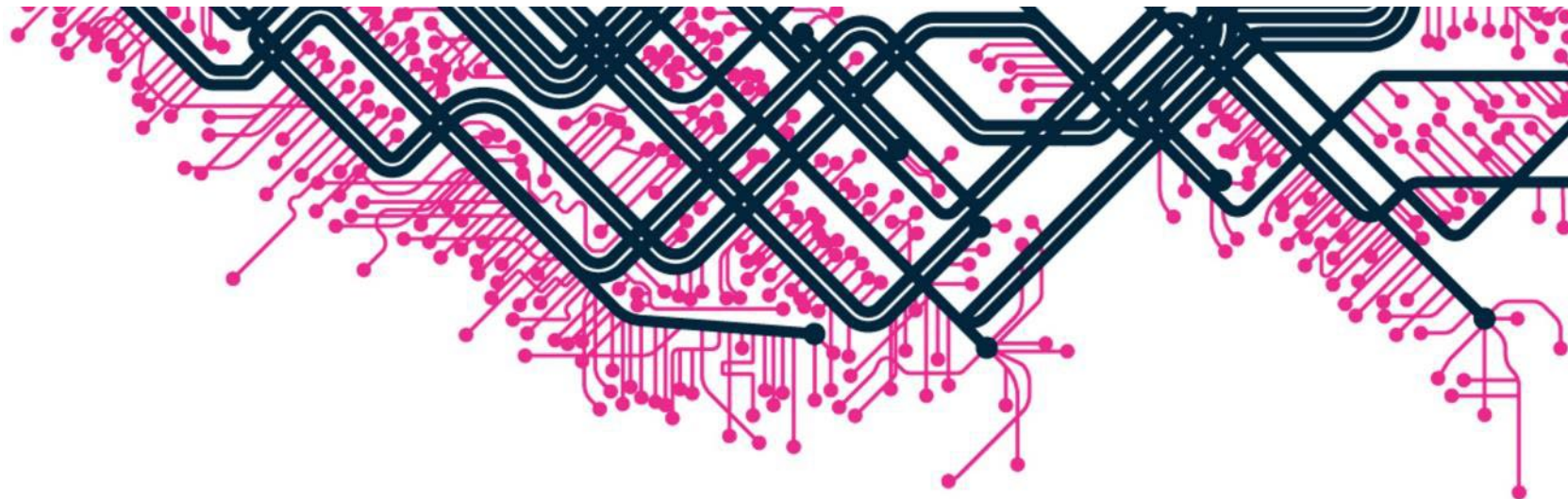
Lead PI, DOE Agile BioFoundry

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Infrastructure Investment Enables Private Industry

Infrastructure investment

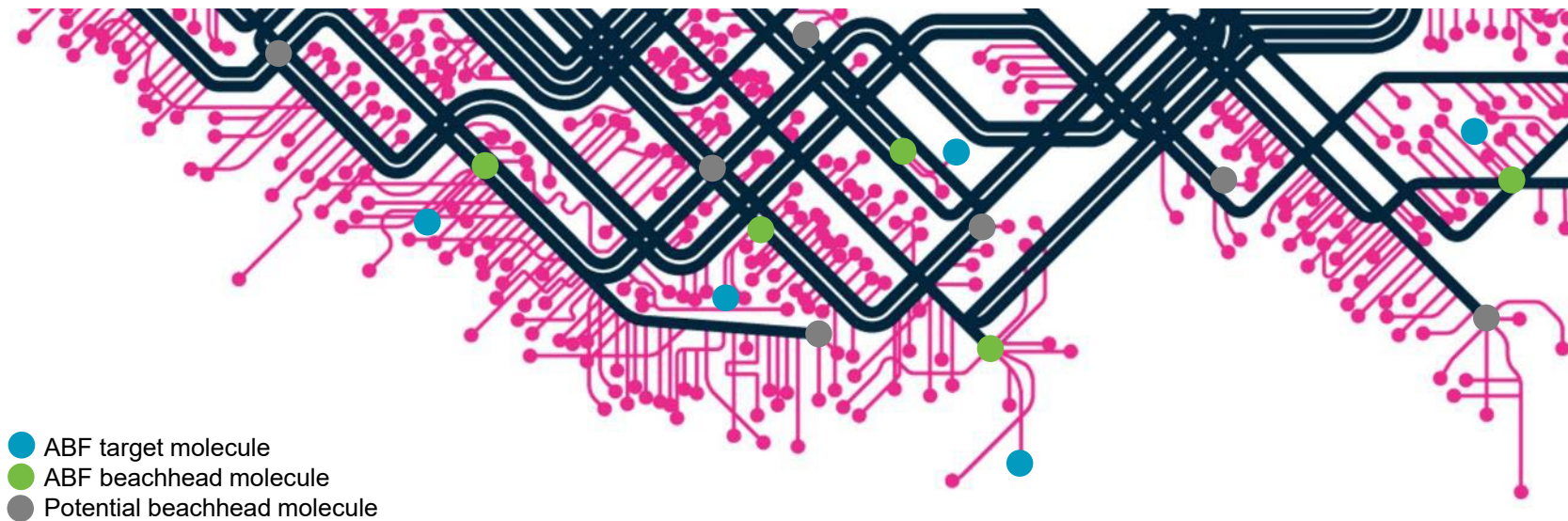


Private investment

Adapted from Lyft

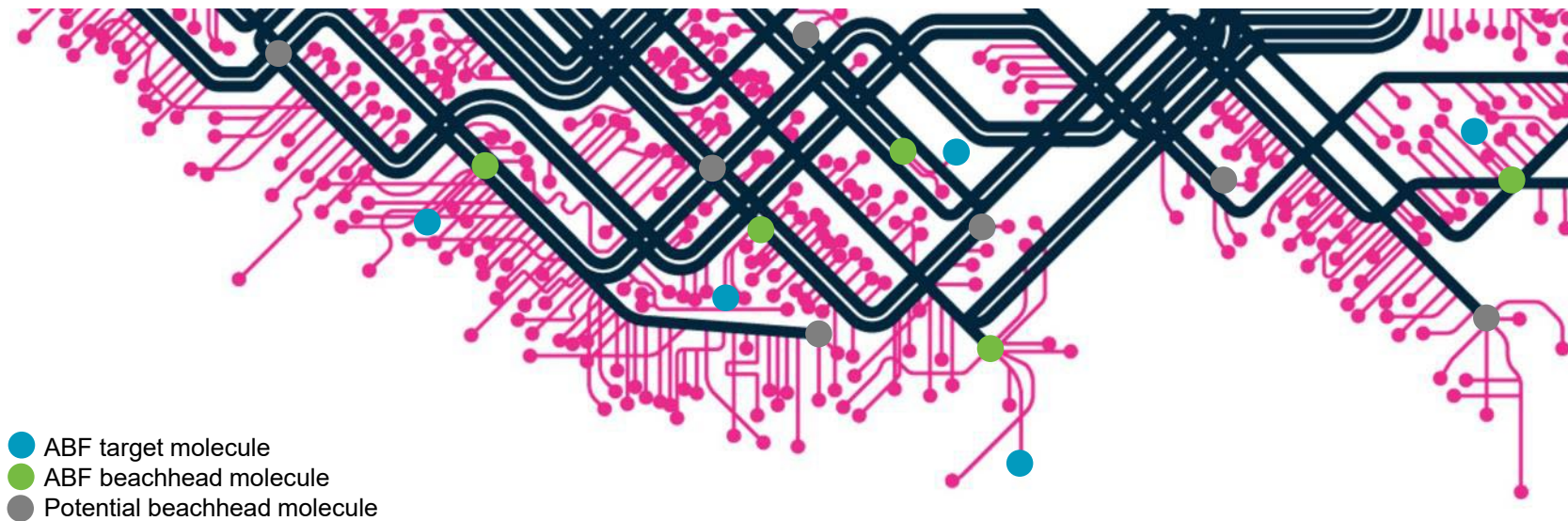
Beachheads

- **Beachheads are metabolic intermediates that can be converted into several bioproducts**
- The development of a strain for production of target associated with certain beachhead will enable rapid development of related bioproduct



Beachheads

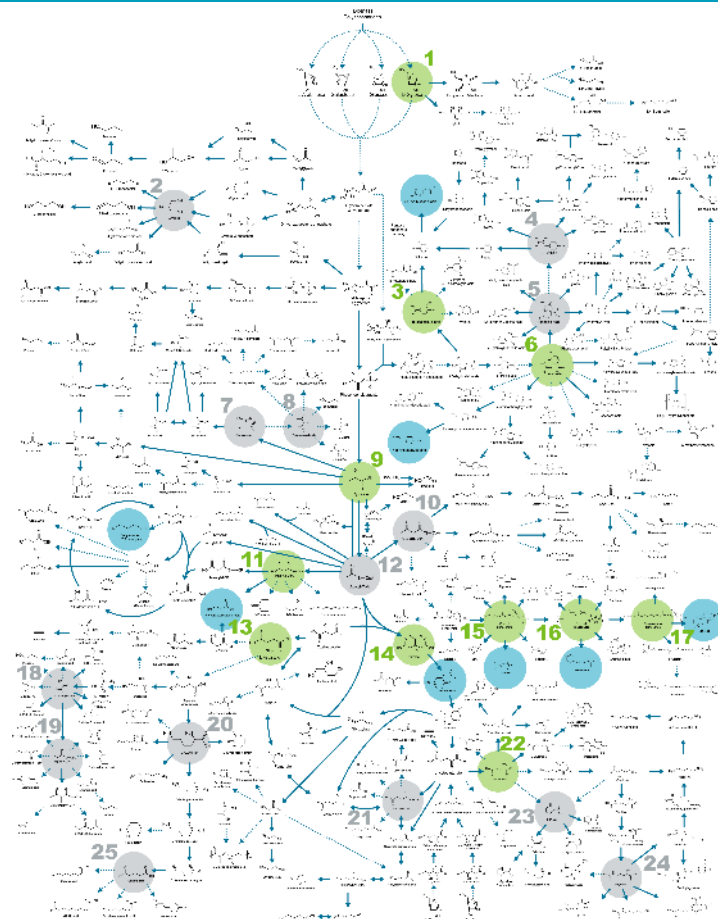
- Metabolic engineering strategies developed for a given target can be applied to targets associated with the same beachhead
- Similar theoretical yields and processing parameters enable TEA and LCA of a single exemplar target product to extend to related products



ABF Metabolic Coverage

- | | |
|------------------------|-------------------------------|
| 01 Xylose | 15 Geranyl diphosphate |
| 02 Glycerol | 16 Farnesyl diphosphate |
| 03 Protocatechuic acid | 17 Geranylgeranyl diphosphate |
| 04 L-Tyrosine | 18 2-ketobutyric acid |
| 05 Prephenic acid | 19 Propionyl-CoA |
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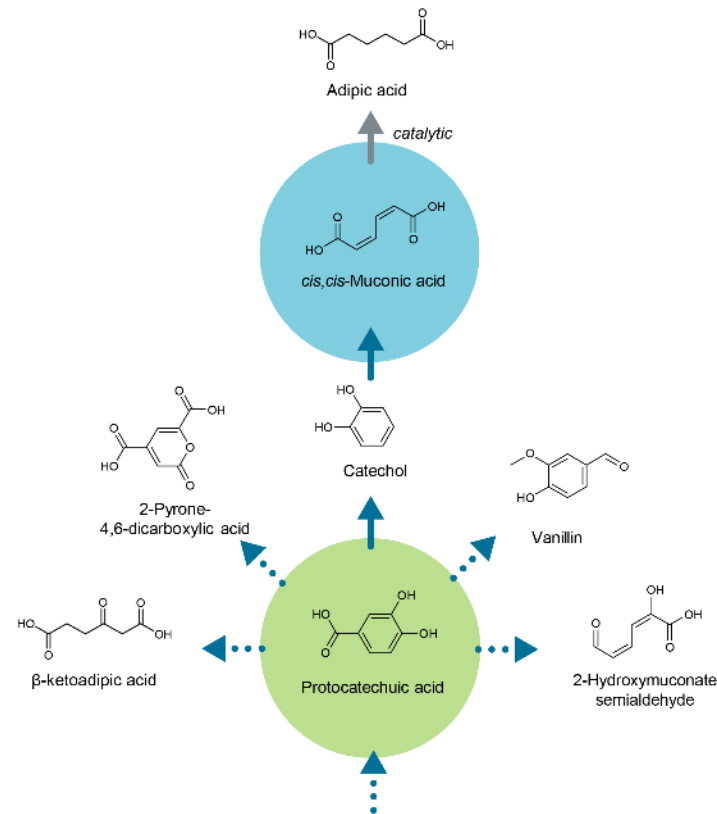
- Current ABF target molecules
- Current ABF beachhead molecules
- Potential beachhead molecules



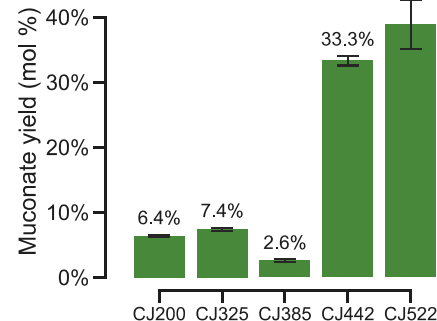
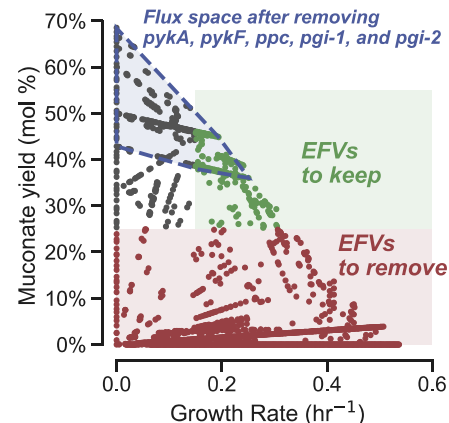
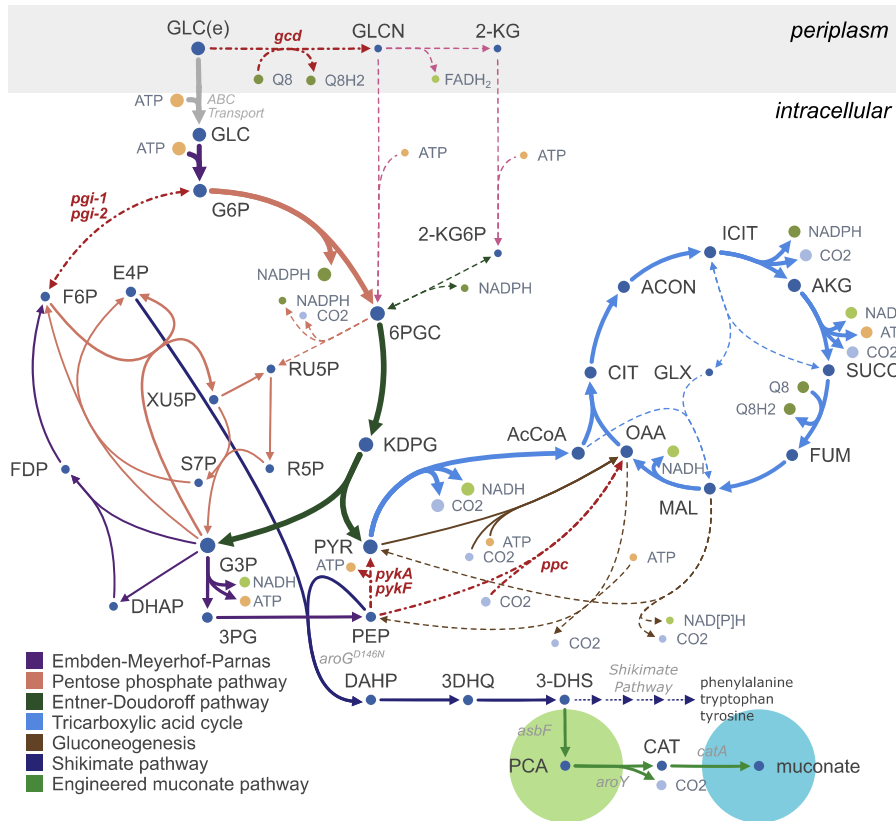
Adapted by permission from Springer Nature Customer Service Centre GmbH: Nature, Nature Calalysis, A comprehensive metabolic map for production of bio-based chemicals, Lee, S.Y., et al., © 2019

Adipic acid

- Widely used aliphatic diacid
- High-value chemical with a market volume of ~2.6 million tons per year
- Demand expected to growth 3-5% globally
- Industrial applications include production of Nylon 66, polyurethanes, plasticizers, and polyethylene terephthalate (PET)
- US is the leading producer (net exporter) and consumer of the compound
- **Beachhead molecule:** Protocatechuate
- **Host:** *Pseudomonas putida*

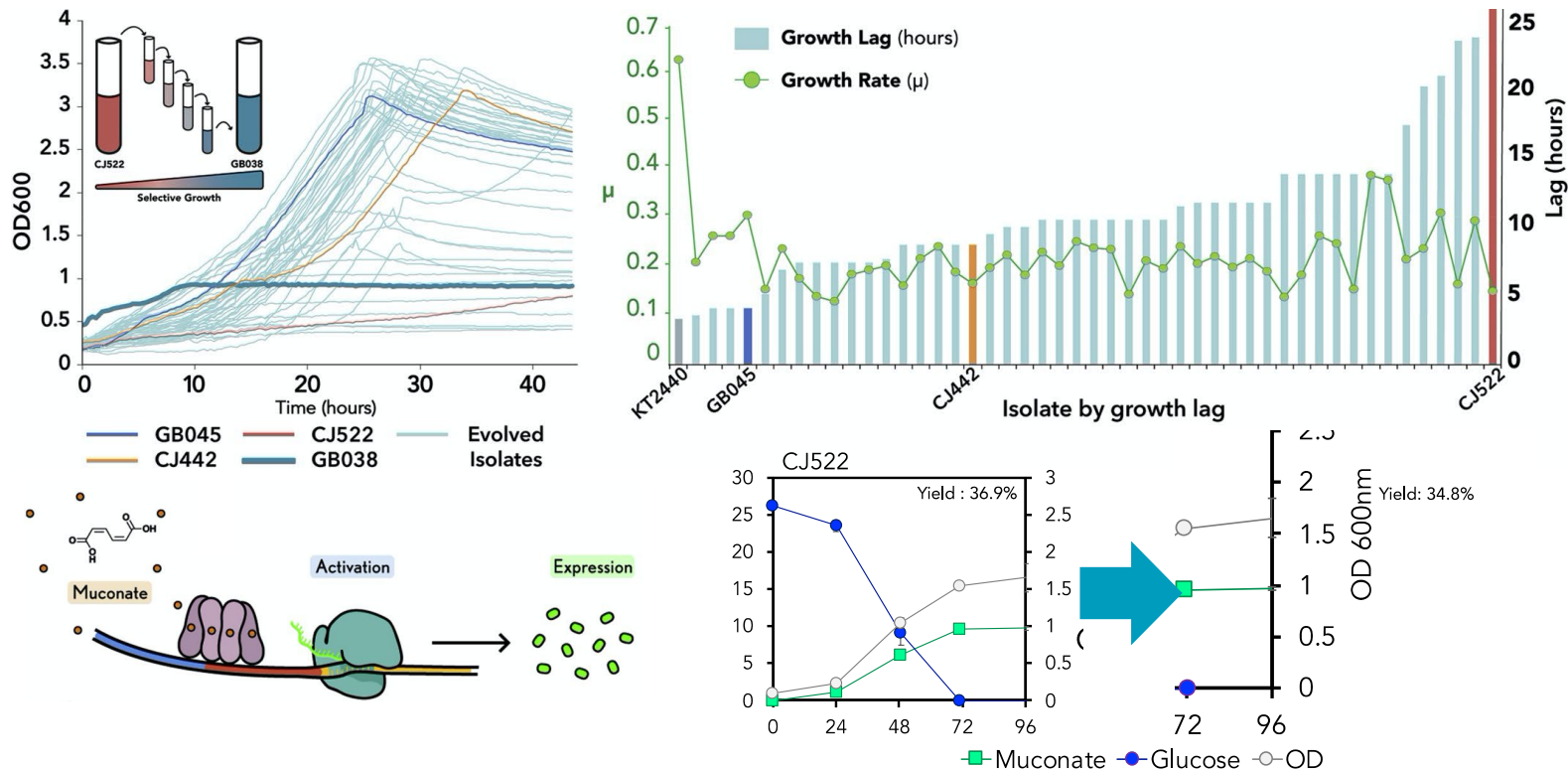


Pathway Development: Protocatechuete / Adipic acid



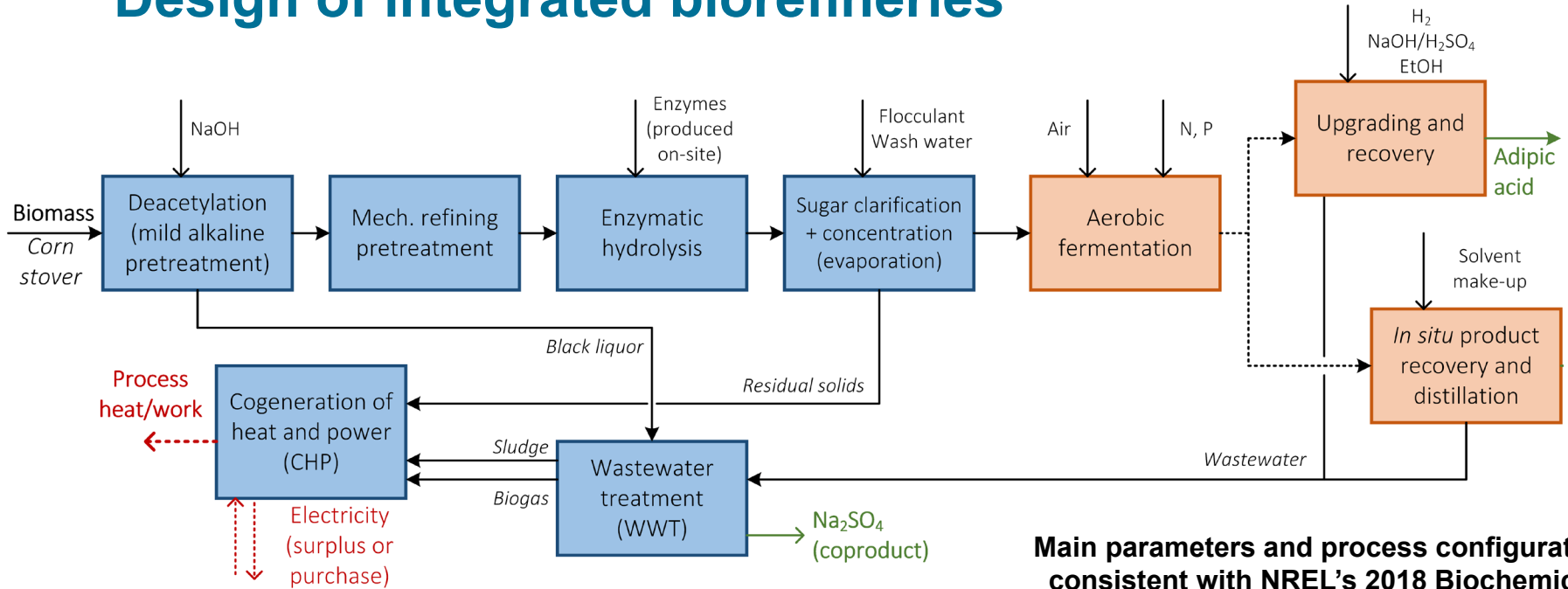
Johnson, C.W., et al., 2019. Innovative Chemicals and Materials from Bacterial Aromatic Catabolic Pathways. *Joule* 3, 1523–1537.

Pathway Development: Protocatechuate / Adipic acid



Bentley, G.J. et al., 2020. Engineering glucose metabolism for enhanced muconic acid production in *Pseudomonas putida* KT2440. Metab. Eng. 59, 64–75.

Design of integrated biorefineries



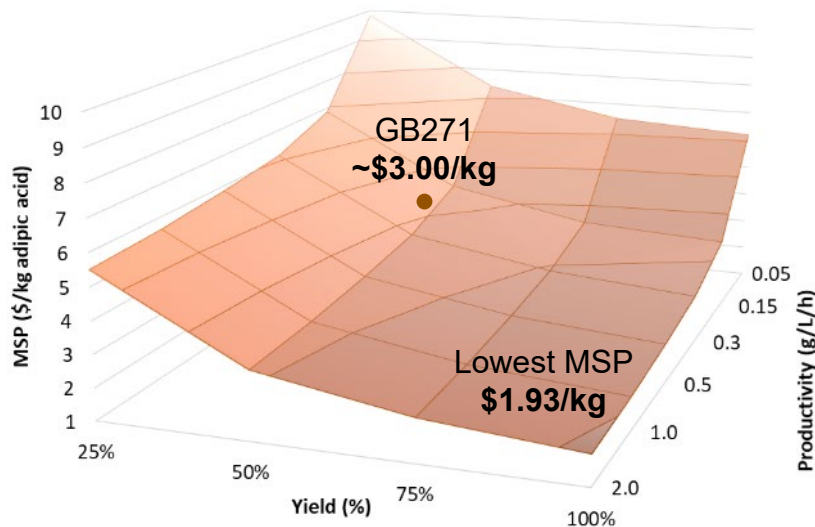
**Main parameters and process configuration
consistent with NREL's 2018 Biochemical
Design Report**

<https://www.nrel.gov/docs/fy19osti/71949.pdf>

Evaluate sensitivity drivers of minimum selling price (MSP) and greenhouse gas (GHG) emissions over a range of achievable fermentation parameters (rate, yield)

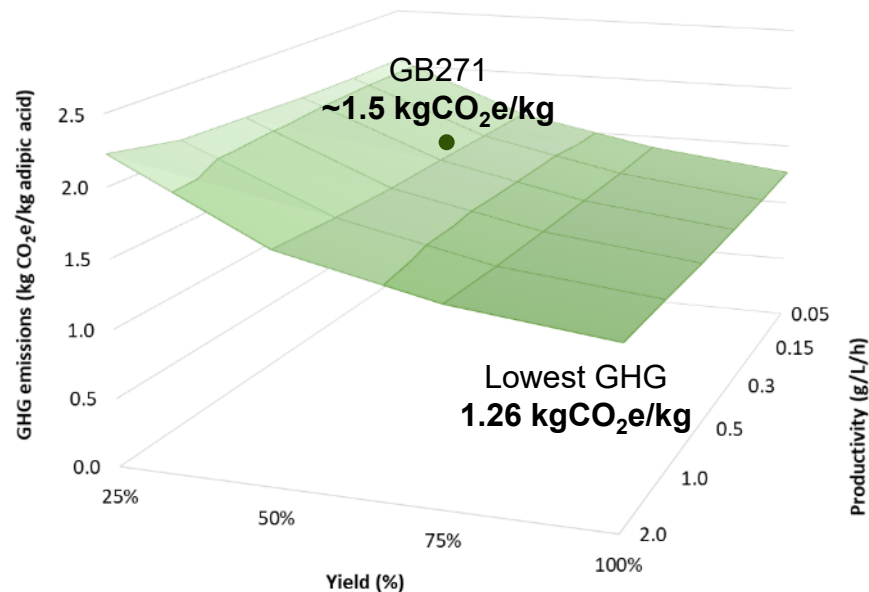
Technoeconomic Analysis (TEA) and Life Cycle Assessment (LCA)

Minimum selling price (MSP)
(\$/kg adipic acid)



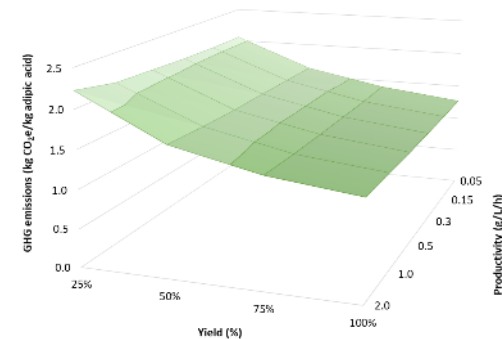
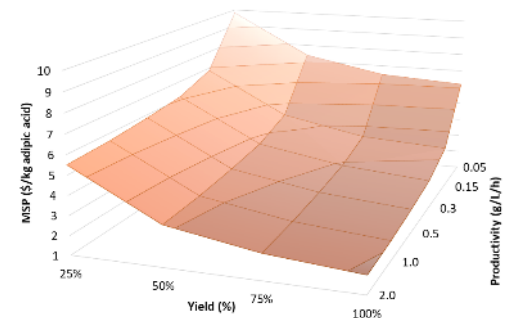
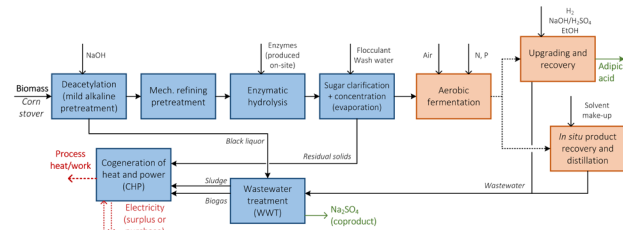
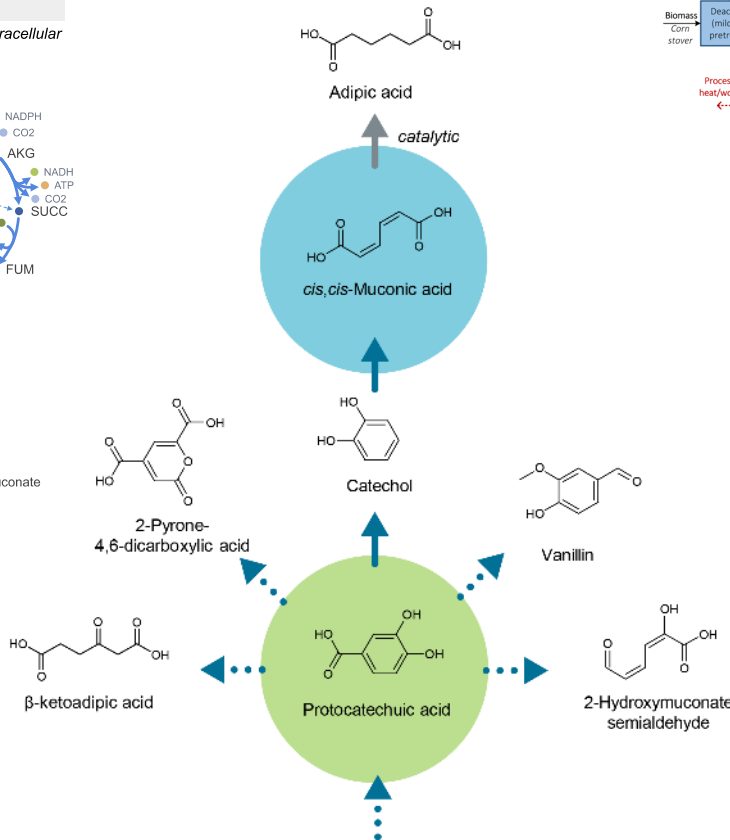
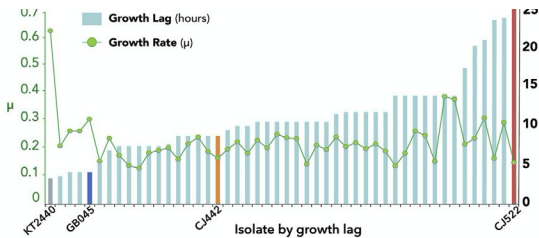
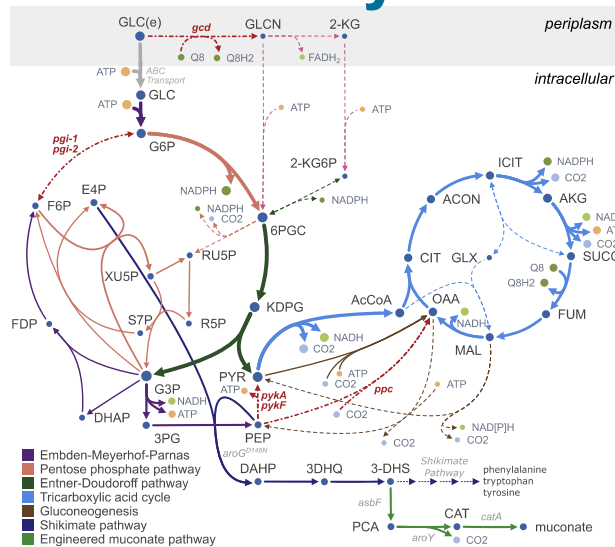
Reference market price: \$1.89/kg AA

Greenhouse gas emissions
(kg CO₂e/kg adipic acid)



Fossil-based: 10.22 KgCO₂e/Kg AA

Summary





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Select ABF efforts in bacterial systems

Presenter: Gregg Beckham, with contributions from **many** ABF colleagues from across the DOE national laboratory complex

ABF Webinar
April 29th, 2022



Beachhead (BH)-exemplar pair overview

Goal:

- Validate the Foundry concept by testing the ABF DBTL infrastructure using beachhead-exemplar pairs
- Demonstrate improved efficiency of DBTL cycle and Foundry via target-host pair work in bacteria, filamentous fungi, yeast



Outcome:

- Increased strain performance to exemplary targets via DBTL
- Use this system to improve DBTL approach
- Further develop robust, industrially relevant hosts
- Developing relevant datasets for Learn team



Relevance:

- Benchmark DBTL cycle performance and improvement across scales with real-world substrates and process configurations
- Information from DBTL and Integration efforts will be critical to predictive scale-up and scale-down



Management

- **Team management:**

- Leads: Bacteria (NREL), filamentous fungi (PNNL), and yeast (SNL)
- Contributions from all labs to all teams
- Members from Integrated Analysis, Process Integration and Scale-Up, Host Onboarding, and DBTL-Infrastructure to ensure effective collaboration

- **Team meetings:**

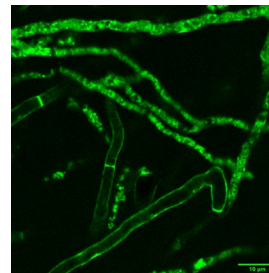
- Weekly: bacteria, filamentous fungi, and yeast
- Rotating: ABF Task Lead call

- **Project risks and mitigation:**

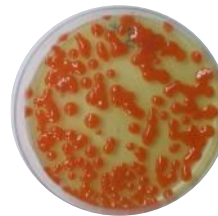
- BH selection – collaborate with other BETO projects and industry partners



Pseudomonas putida KT2440



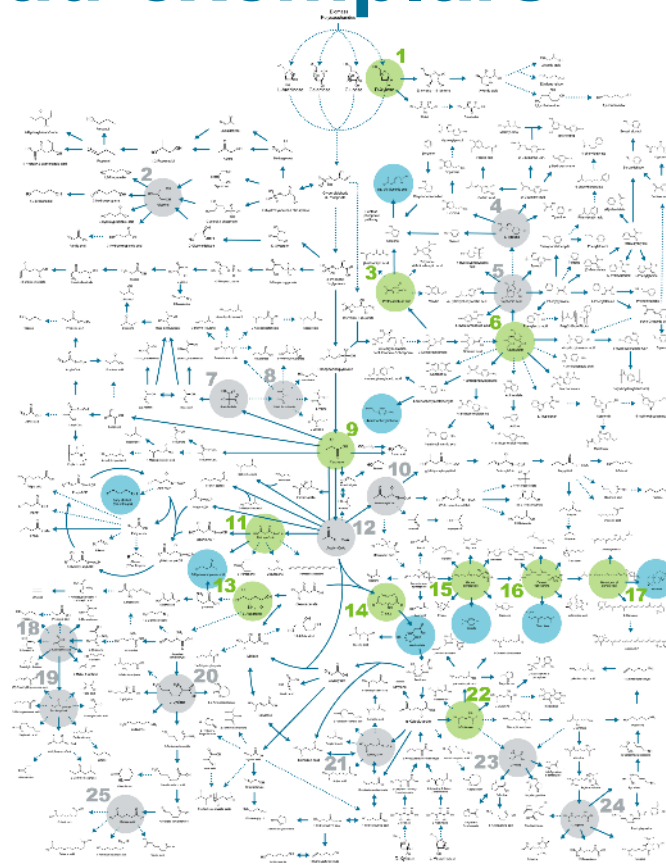
Aspergillus niger



Rhodosporidium toruloides

Approach: Beachhead-exemplars

- FY20: Demonstrate an exemplar at a TRY of 20 g/L, 0.3 g/L/hr, and 50% of theoretical yield, either from hydrolysate or a mock hydrolysate containing hexose and pentose sugars
- FY21: 40 g/L, 0.5 g/L/hr, 60% of theoretical yield
- FY22: within 20% of fossil-based incumbent molecule minimum selling price
- TRY targets established and refined by techno-economic analysis and life cycle assessment
- We use G/NG milestones based on achieving ≥ 1 g/L product titers for new BH-exemplars



Project overview

History: Task initiated at the inception of the Agile BioFoundry

- *Pseudomonas putida* KT2440-C6 diacids were first target-host pair from ABF pilot project

Project goals:

- Engineer KT2440 to convert hydrolysate into protocatechuate-derived products, among several others
- Main initial target is muconate productivity (shown to be a key cost driver)
- Expanded to fatty acid-related products in FY21
- Provide products to Performance-Advantaged Bioproducts projects
- Expanded to *C. glutamicum*, *B. coagulans*, and several other bacteria in recent years



Project overview: Why *P. putida*?

Pseudomonas putida

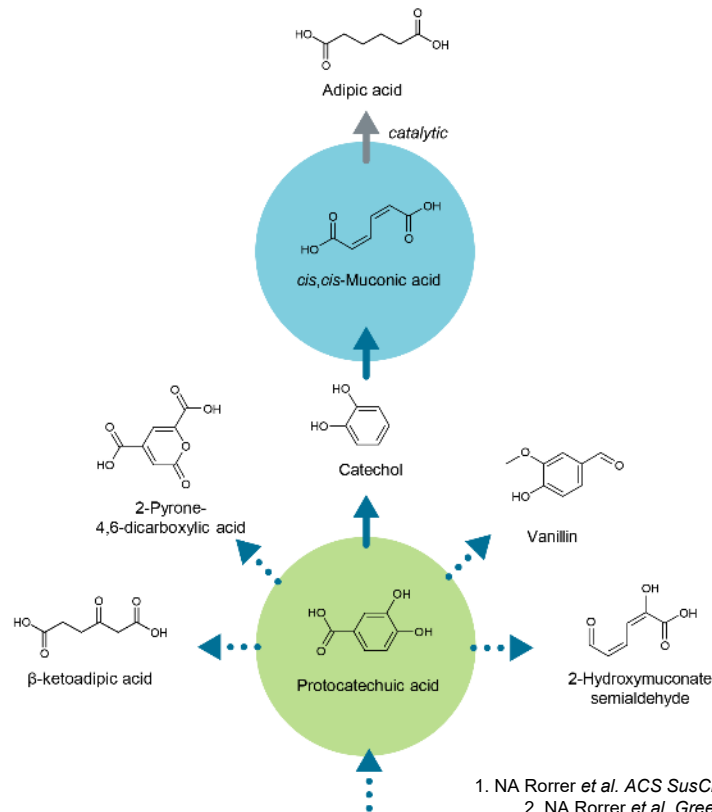
- Soil bacterium
- Gram-negative aerobe
- Fast growing
- Stress tolerant
- Metabolically versatile
- Genetically tractable



Project overview: Why these products?

Muconic acid

- Easily converted to adipic acid
- Adipic acid is a high-value chemical with a market of ~2.6 million tons per year
- AA: Demand expected to grow 3-5% globally
- AA: Industrial applications include production of Nylon 66, polyurethanes, and plasticizers
- AA: US is the leading producer (net exporter) and consumer of the compound
- Muconate itself can be used as a performance-advantaged bioproduct¹⁻³
- **Beachhead molecule:** Protocatechuic acid
- **Host:** *Pseudomonas putida* KT2440



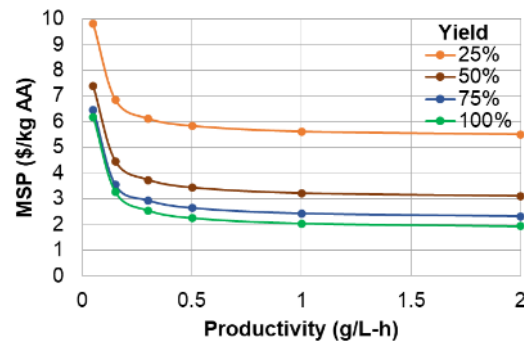
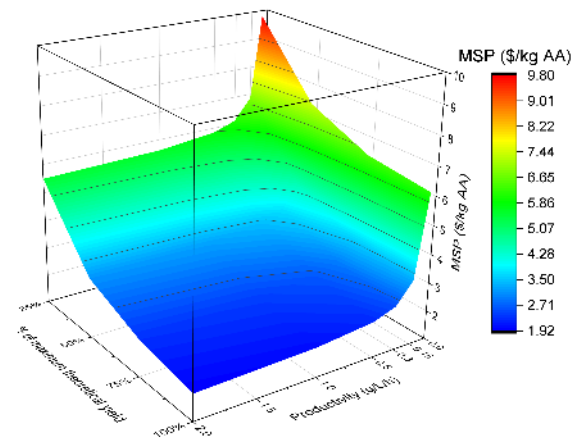
1. NA Rorrer *et al.* ACS SusChemEng. 2016

2. NA Rorrer *et al.* Green Chem. 2017

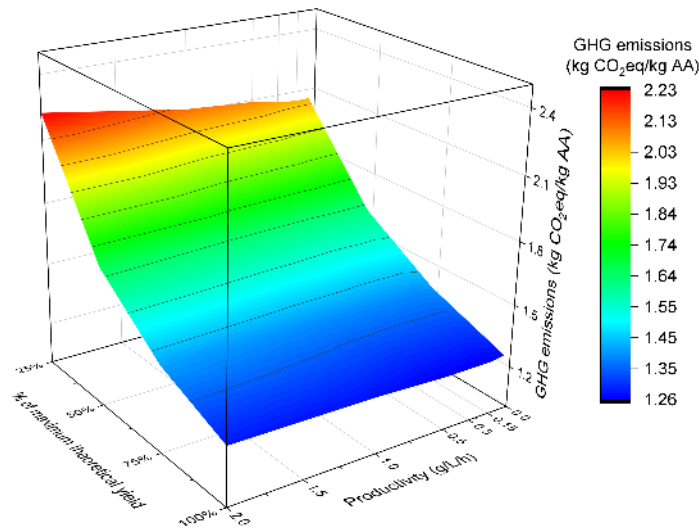
3. NA Rorrer *et al.* Joule 2019

Muconic acid techno-economic analysis

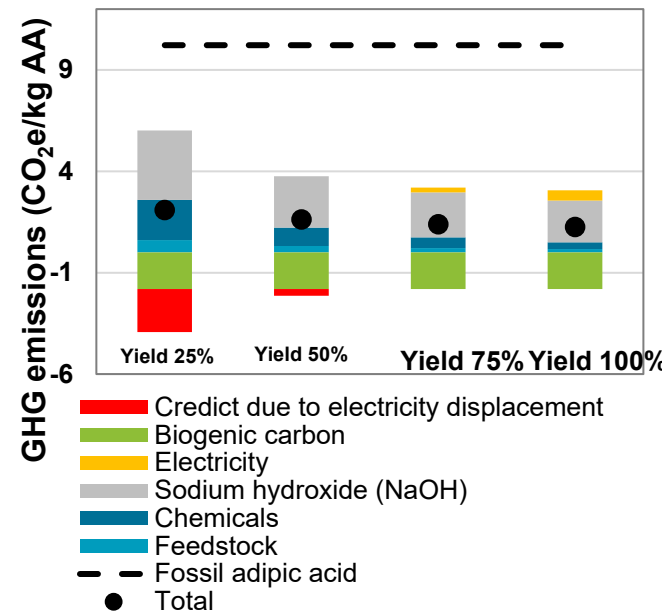
- MSP driven strongly by productivity below 0.3 g/L-h, starts to plateau at productivities higher than 0.3 – 0.5 g/L-h
- Considerable influence of MA yield when passing from 25% to 50% of theoretical yield



Muconic acid life cycle assessment

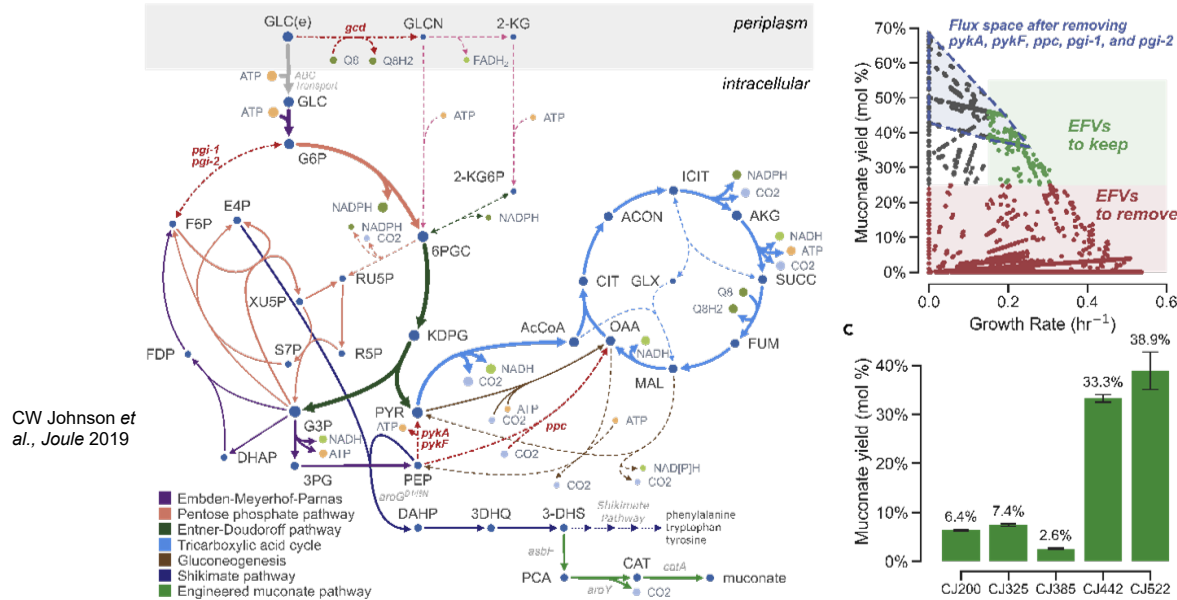


Productivity plays a considerably smaller role on LCA than it does on TEA



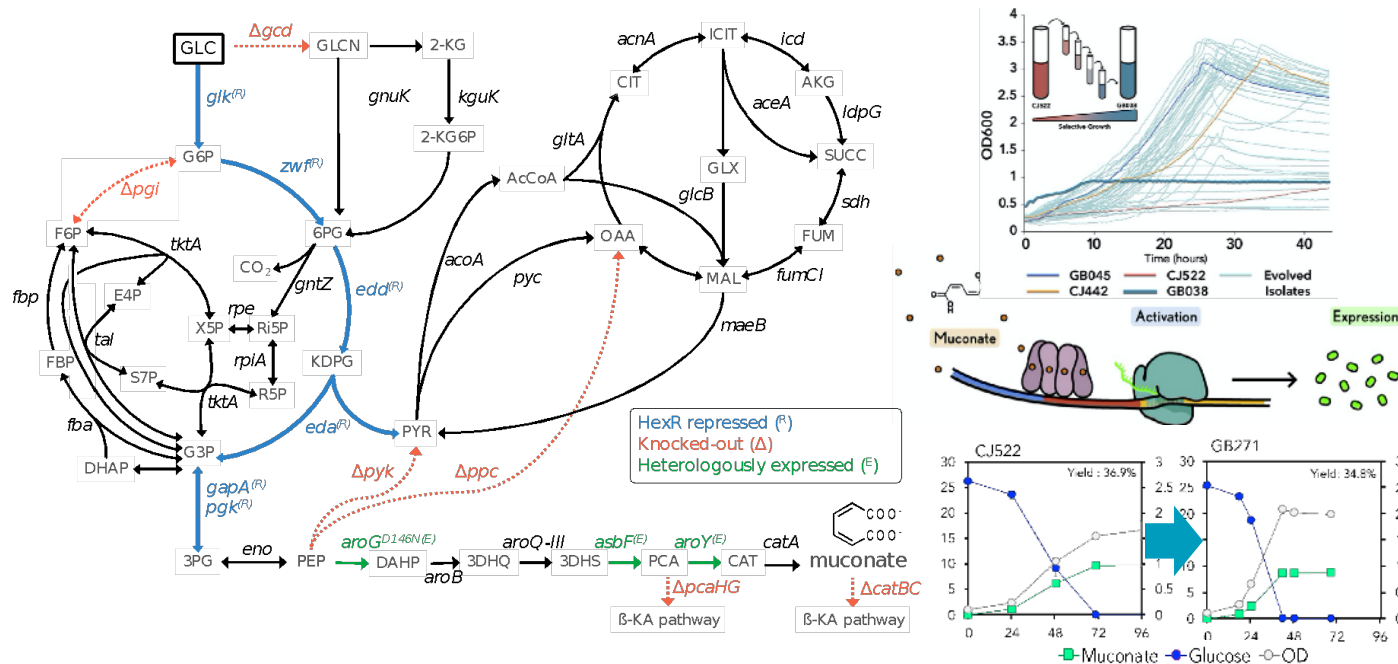
The lowest GHG emissions are obtained with the highest yield at different productivities (0.5; 0.3; 0.15)

Baseline strain for muconic acid production



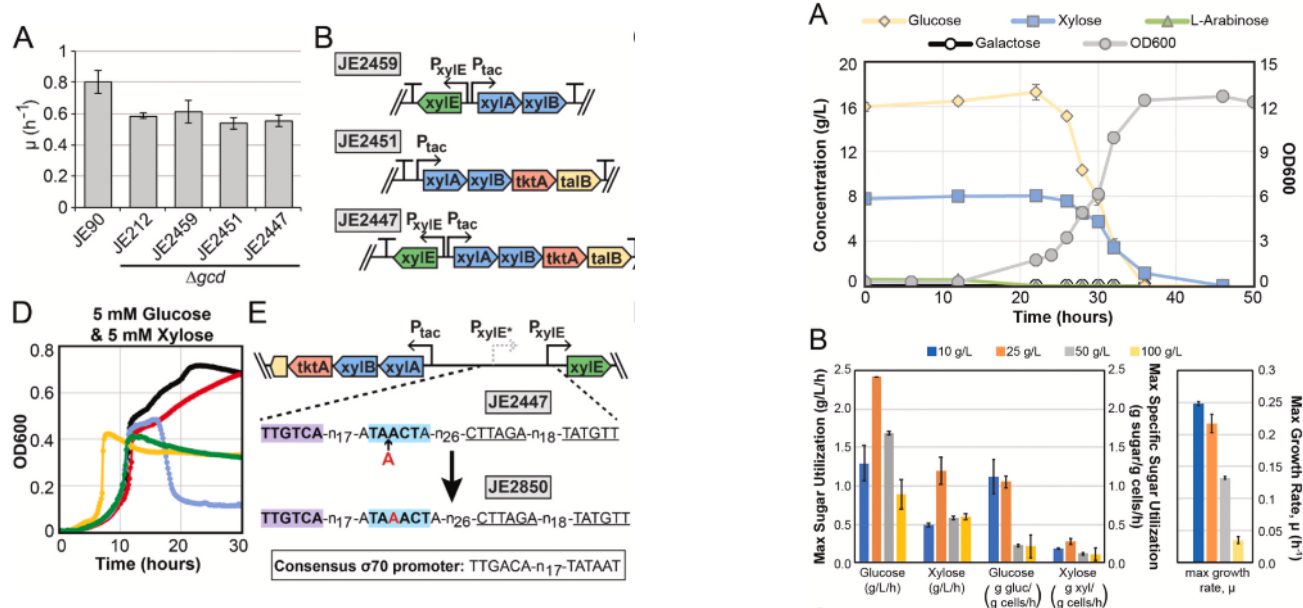
- Leverage pathway originally reported by Draths and Frost (JACS, 1999)
- Achieved a 39% molar yield of muconate from glucose
- **Outcome:** High-yield platform strain, but low rates from glucose

Regulatory bottlenecks to rate improvements



- Developed and leveraged a specific muconate biosensor
- Identified and engineered key regulators of conversion of glucose to muconate
- **Outcome:** Doubled the productivity while maintaining high yield

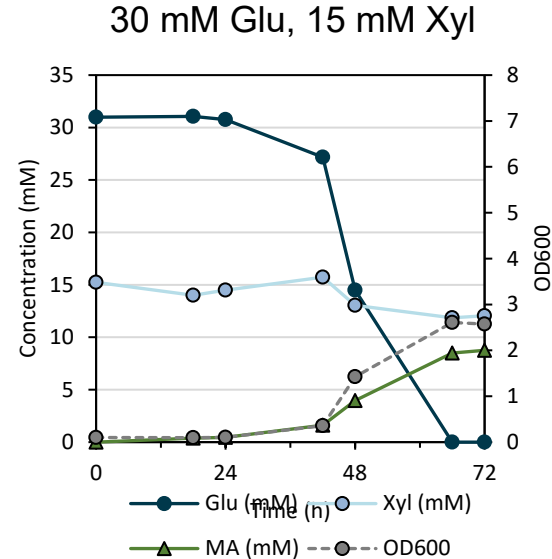
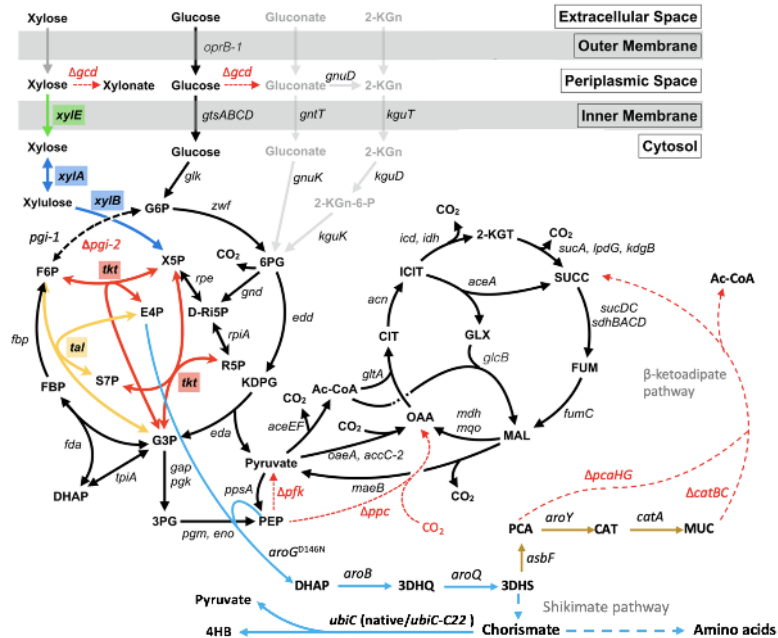
Baseline strain for sugar utilization in *P. putida*



JR Elmore et al., *Metabolic Engineering* 2020

- Xylose and arabinose utilization via rational engineering and laboratory evolution
- Max sugar utilization rate of $3.3 \text{ g L}^{-1} \text{ h}^{-1}$
- **Outcome:** *P. putida* strain capable of co-utilization of hydrolysate sugars

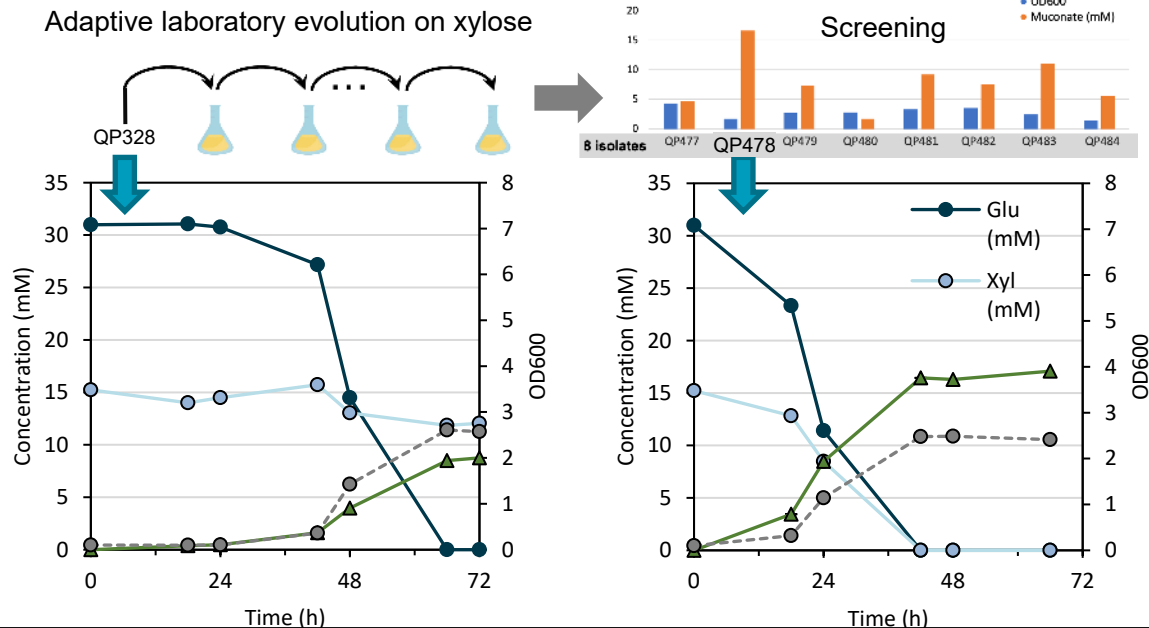
Baseline strain for muconate from hydrolysate



Chen Ling et al. in revision

- The non-oxidative xylose pathway was integrated
- **Outcome:** Slow conversion of glucose and xylose to muconate

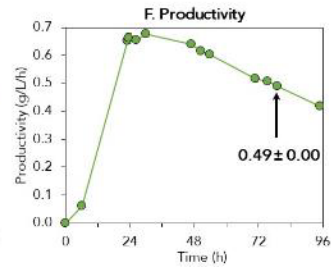
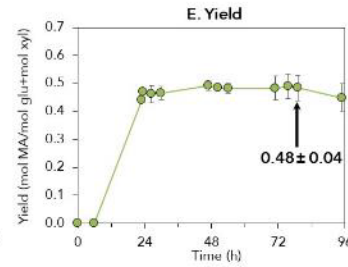
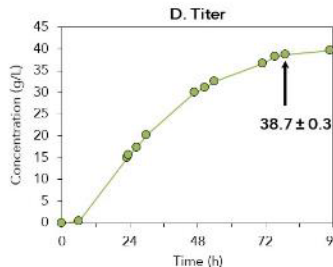
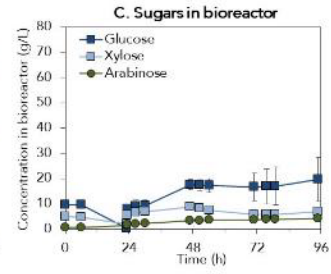
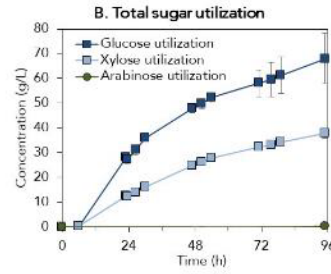
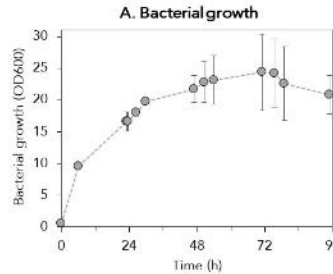
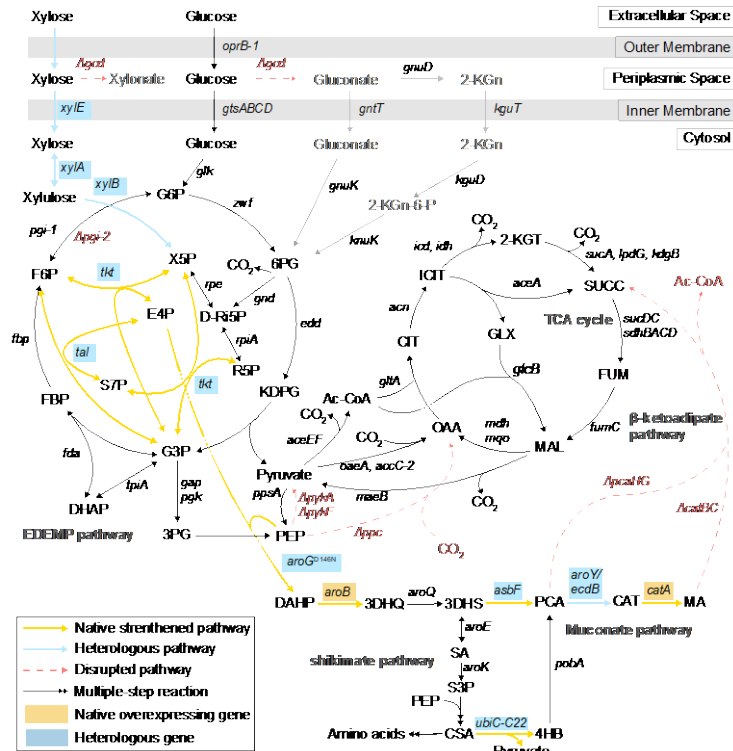
Improving muconate rate from hydrolysate



Chen Ling *et al.* in revision

- QP328 was subjected to adaptive laboratory evolution on xylose
- Isolates with improved xylose consumption were screened
- **Outcome:** Rapid, simultaneous conversion of glucose and xylose to muconate

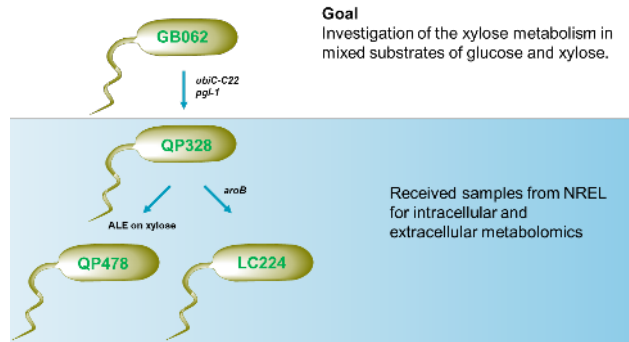
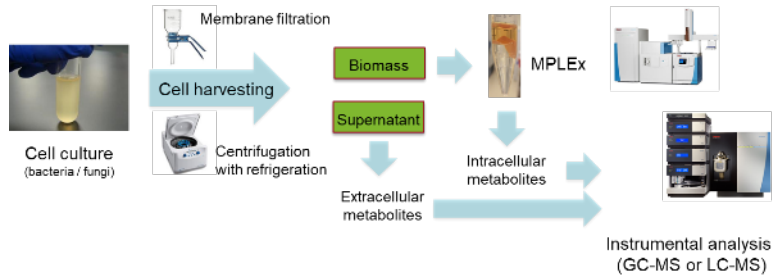
Reverse engineering to create new strain, LC224



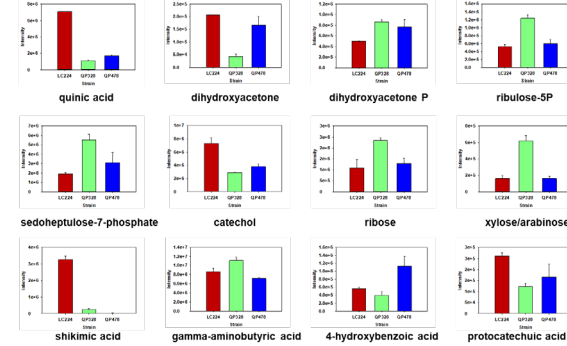
Chen Ling et al. in revision

Discovery metabolomics on ALE strains

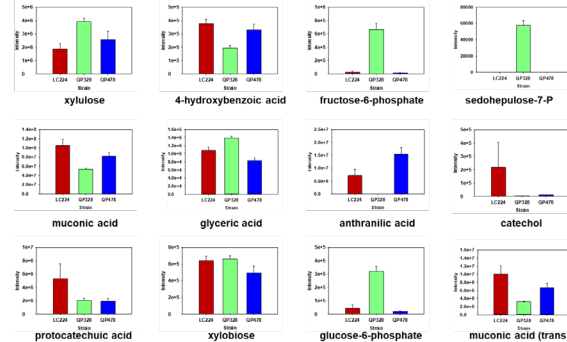
Intracellular vs extracellular metabolomics



Intracellular metabolites (selected)



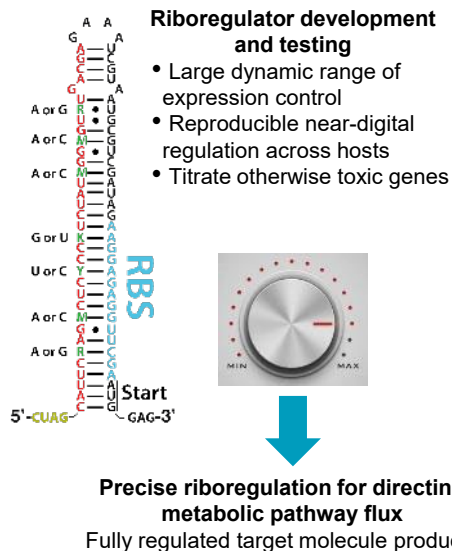
Extracellular metabolites (selected)



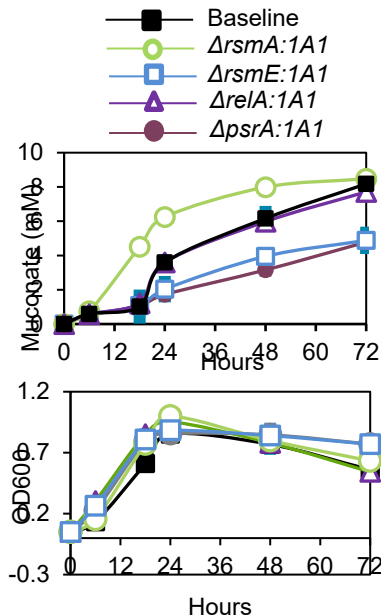
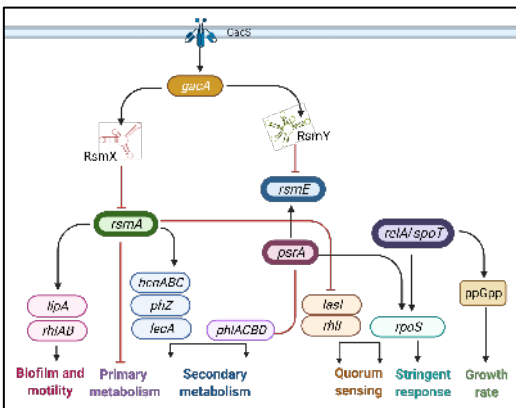
Chen Ling *et al.* in revision

Outcome: 86 intracellular and 24 extracellular metabolites were detected and quantified from the study

Tuning gene expressions for muconate TRY in *P. putida*

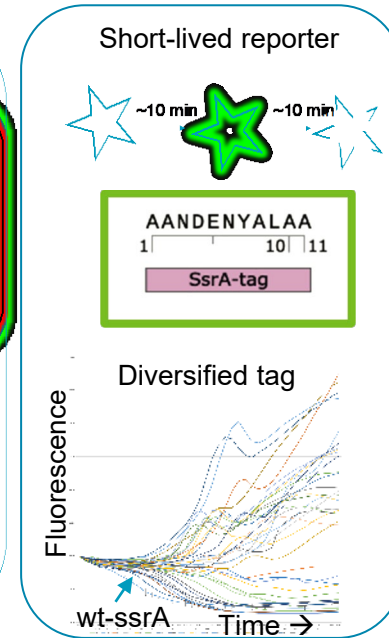
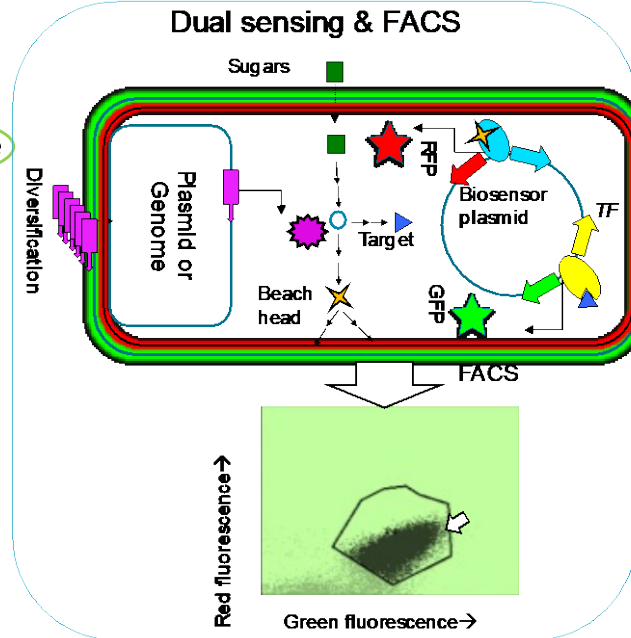
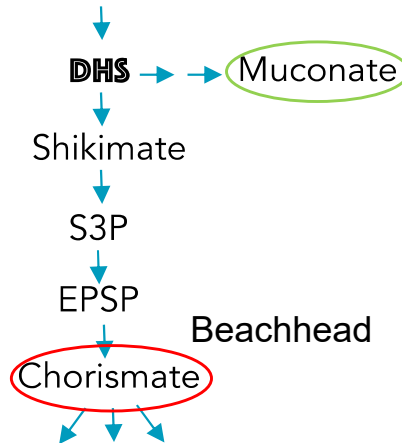


Example: Targeting global responses



- RNA-based elements are used to tune gene expression at translation level, from knockdown to high expression phenotypes
 - Currently using to test effects of tuning expression of global metabolic regulators
- Outcome:** Increased muconate upon knockdown of RsmA

Beachhead sensor and dual sensing

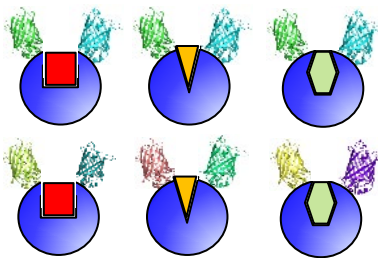


- Custom sensors generated *via* promoter and protein engineering
 - Introduce short-lived reporters for beachhead for transient sensing
 - **Goal:** Dual sensing and cell sorting for target and beachhead → high productivity
- Expected Outcome:** Improved strains, high throughput **Test** and data collection for **Learn**

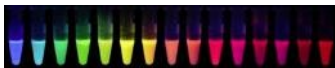
In vitro biosensors for strain engineering

Sensor selection

- Binding protein for precursor and/or product molecules

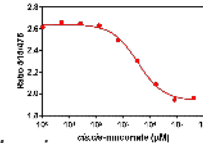


- FRET pairs for different applications



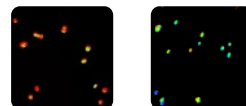
Diverse experimental approaches

- *In vitro* measurements



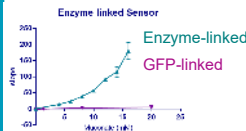
Titration of biosensor with *cis,cis*-muconate

- *In vivo* measurements
 - muconate + muconate



*Images of
P. putida
expressing
biosensors*

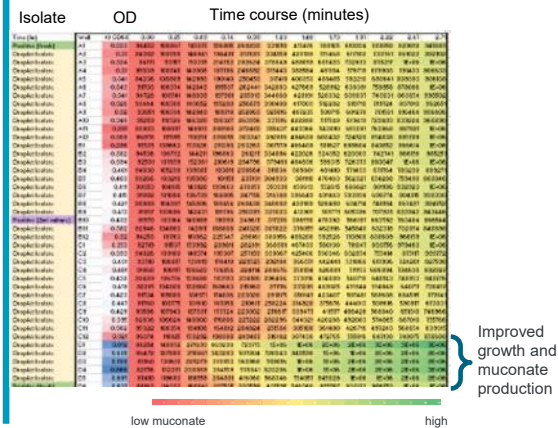
- Development of whole-cell or enzyme-linked biosensors



Whole-cell biosensor detecting muconate in droplet

Example application

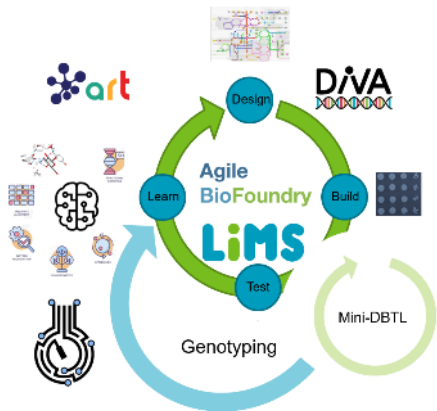
- Scalable HTP screening of isolates from Droplet-based Adaptive Laboratory Evolution (dALE) experiment



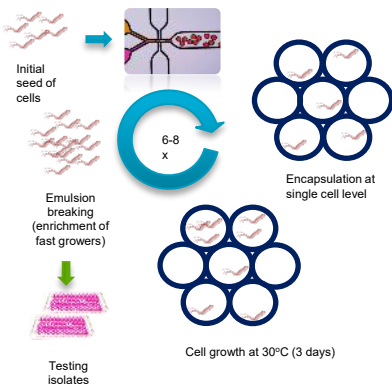
- *In vitro* biosensors can be used for strain engineering and pathway optimization
- **Outcome:** Improved strains identified from dALE utilizing *in vitro* biosensors
- **Ongoing:** Optimizing sensing for other targets and dALE protocols for other hosts

Microfluidic Droplet-based Adaptive Laboratory Evolution (dALE)

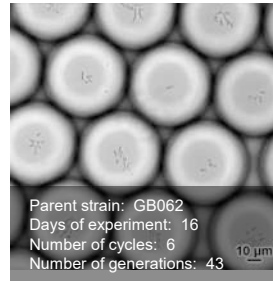
Enables rapid mini-DBTLs










General dALE workflow



~ 2.8 million 70 pL droplets

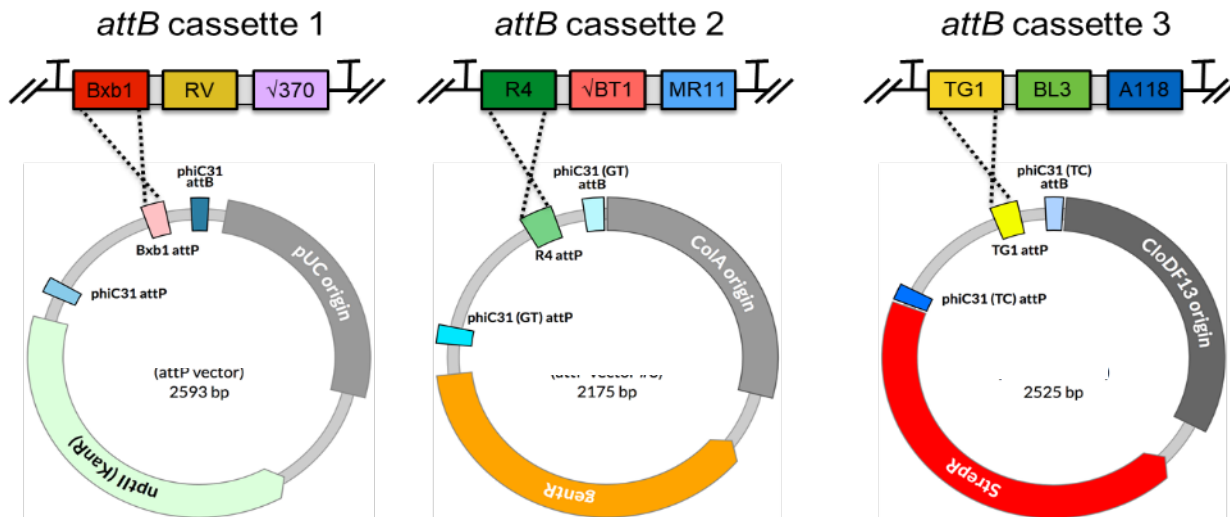


Miniaturization

Strain	dALE 3 weeks	Initial Selection Biosensor and/or HTP MS for CCM	Scaled characterization					Geno- typing
			Multi-well plates			Shake flasks	Bioreactor	
			Production HTP LC-MS (96-well)	Growth (48-well)	Initial mini-DBTL duration			
								
GB062	M9-glucose (30 mM)	92	15	15	14 weeks	5	0	finished
GB062	M9-DMR (30 mM equiv)	92	9	9	9 weeks	5	1	finished
LC040	M9-DMR (30 mM equiv)	180	15	18 ongoing	11 weeks	5	2	ongoing
LC071	M9-DMR (30 mM equiv)	180	3					
NREL baseline mutant	M9-Mock planned							

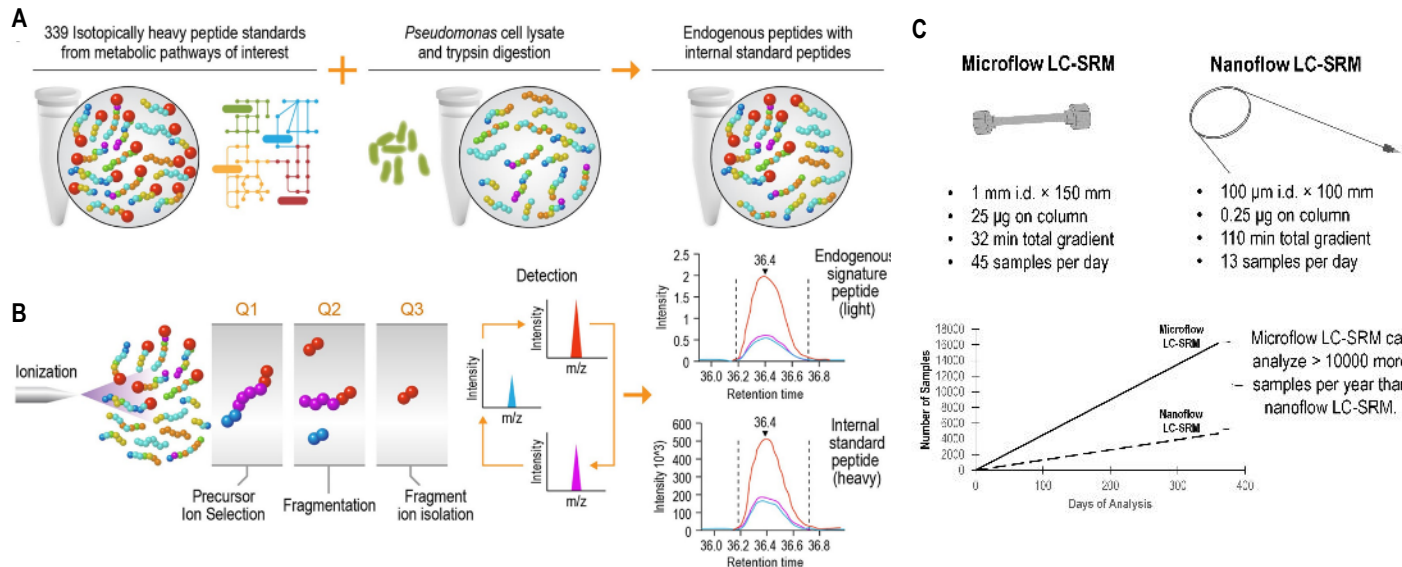
Applications in *P. putida*

Site-specific DNA integration tool in KT2440



- Can simultaneously insert 3 plasmids into chromosome at $\sim 10^6$ cfu / μ g DNA
- Backbone excision allows marker removal and repeated use
- **Outcome:** Highly efficient tool enables rapid Build of large libraries for screening of pathway variants

Test methods improvement in KT2440



- Targeted proteomics with internal standards accurately quantified 132 enzymes.
- Using microflow LC to replace nanoflow LC greatly reduced the analysis time without sacrificing sensitivity.
- **Outcome:** Increase the throughput of protein quantification by 4 times

Gao, Yuqian, et al. "High-Throughput Large-Scale Targeted Proteomics Assays for Quantifying Pathway Proteins in *Pseudomonas putida* KT2440" Front. Bioeng Biotechnol. 2020 Dec 2;8:603488. doi: 10.3389/fbioe.2020.603488.

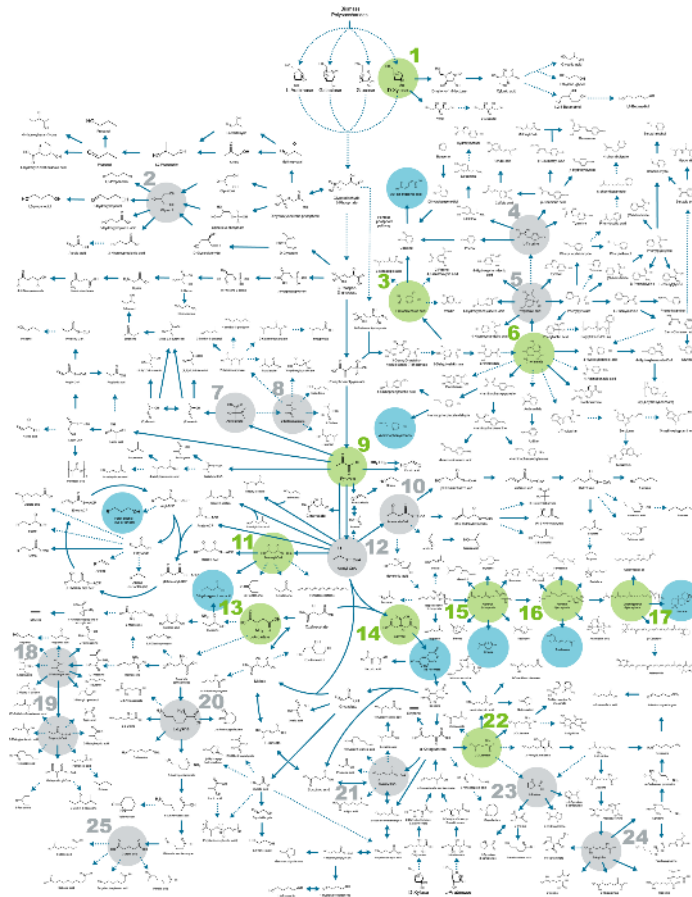
Summary and next steps

PCA/Muconate *et al.* in *P. putida*

- Ramping up new DBTL cycles now towards higher titers and rates to meet FYFY22 TRY goals
- Ramping up DBTL efforts to understand why strain performance is limited at [muconate] ~ 40 g/L

BHs related to performance-advantaged bioproducts and sustainable aviation fuel

- Working with other BETO projects for a specific BH-exemplar pairing related to SAF-relevant intermediates in anaerobic thermophiles



Acknowledgements

DOE: Gayle Bentley, Jay Fitzgerald

LBNL: Nathan Hillson, James Gardner, Hector Garcia Martin, Alastair Robinson, Blake Simmons, Deepti Tanjore

NREL: Ryan Davis, Stefan Haugen, Ray Henson, Kelley Hestmark, Christopher Johnson, Kelsey Kinley, Bruno Klein, Colin Kneucker, Chen Ling, Kevin McNaught, Bill Michener, Michela Monninger, Michelle Reed, Davinia Salvachua, Violeta Sanchez i Nogue, Peter St. John, Sean Woodworth

ANL: Phil Laible, Peter Larsen, Gyorgy Babnigg, Rosemarie Wilton, Deborah Hanson, Martyna Michalska, Thathiana Benevides

LANL: Taraka Dale, Ramesh Jha, Scott Hennelly, Chris Yeager, Niju Narayanan, Neresh Pandey, Herbert Huttanus, Ellin Kristina-Triola, Steffi Davison, Katherine Wozniak, Jeanette Velasquez, Claire Sanders, Carol Kay Carr, Tari Kern

ORNL: Adam Guss, Carrie Eckert, Josh Michener, Jay Huenemann, Austin Carroll

PNNL: Jon Magnuson, Jeremy Zucker, Joshua Elmore, Kristin Burnum-Johnson, Young-Mo Kim, Nathalie Munoz, Yuqian Gao, Brenton Poirier

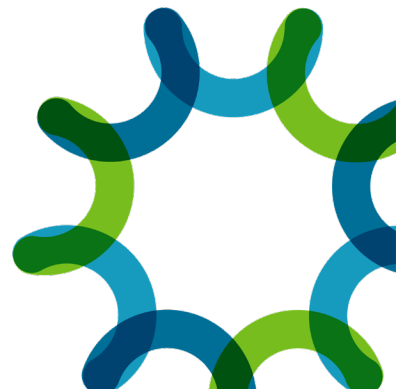
SNL: John Gladden, Jamie Meadows



Yeast Demos: *R. toruloides*

Presenter: Di Liu

Date: 04/29/2022



Project Overview

History: Task initiated at the beginning of the Agile BioFoundry

- *Rhodospiridium toruloides* is a new host introduced in FY17
- Heterologous terpene production had just been demonstrated prior

Context: *R. toruloides* offers a robust host for producing terpene, lipid, and other bioproducts

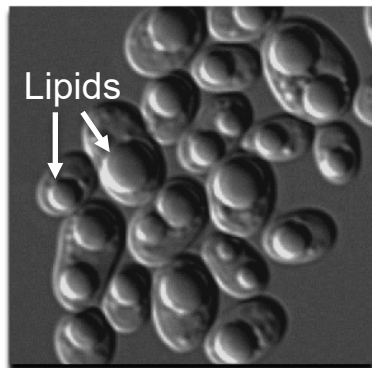
- Naturally consumes lignocellulose: pentose, hexose, aromatics
- High natural flux in terpene and lipid pathways

Project goals:

- Employ the ABF DBTL to produce multiple bioproducts.
- Expand knowledgebase, engineering tools/strategies, and beachheads
- Use Target/Host pairs to identify areas to improve DBTL cycle efficiency
- Exemplify ABF value by transferring knowledge between hosts

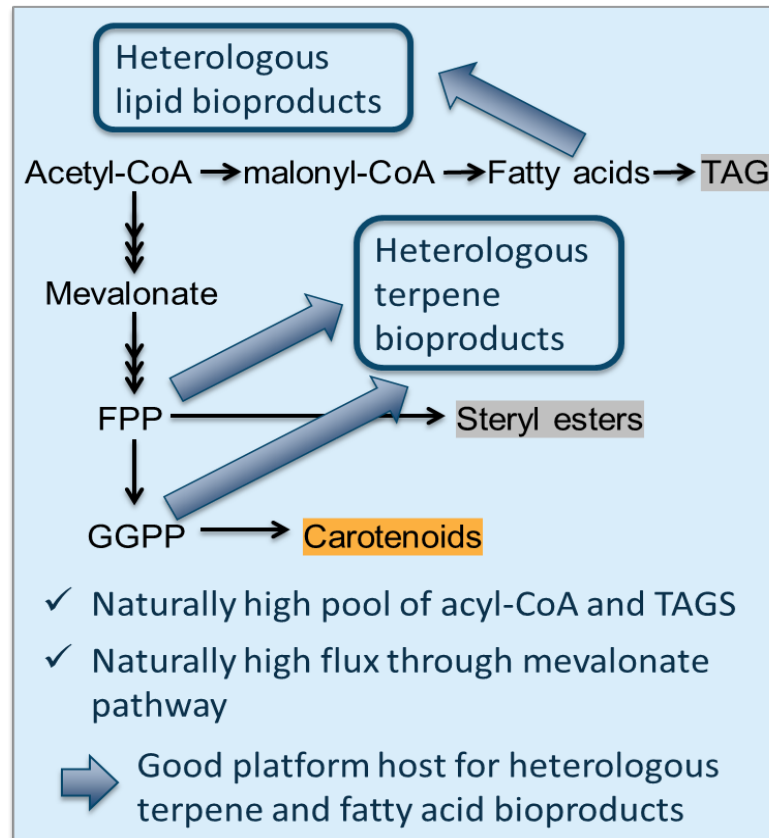


Project Overview: Why *R. toruloides*?



Rhodospiridium toruloides

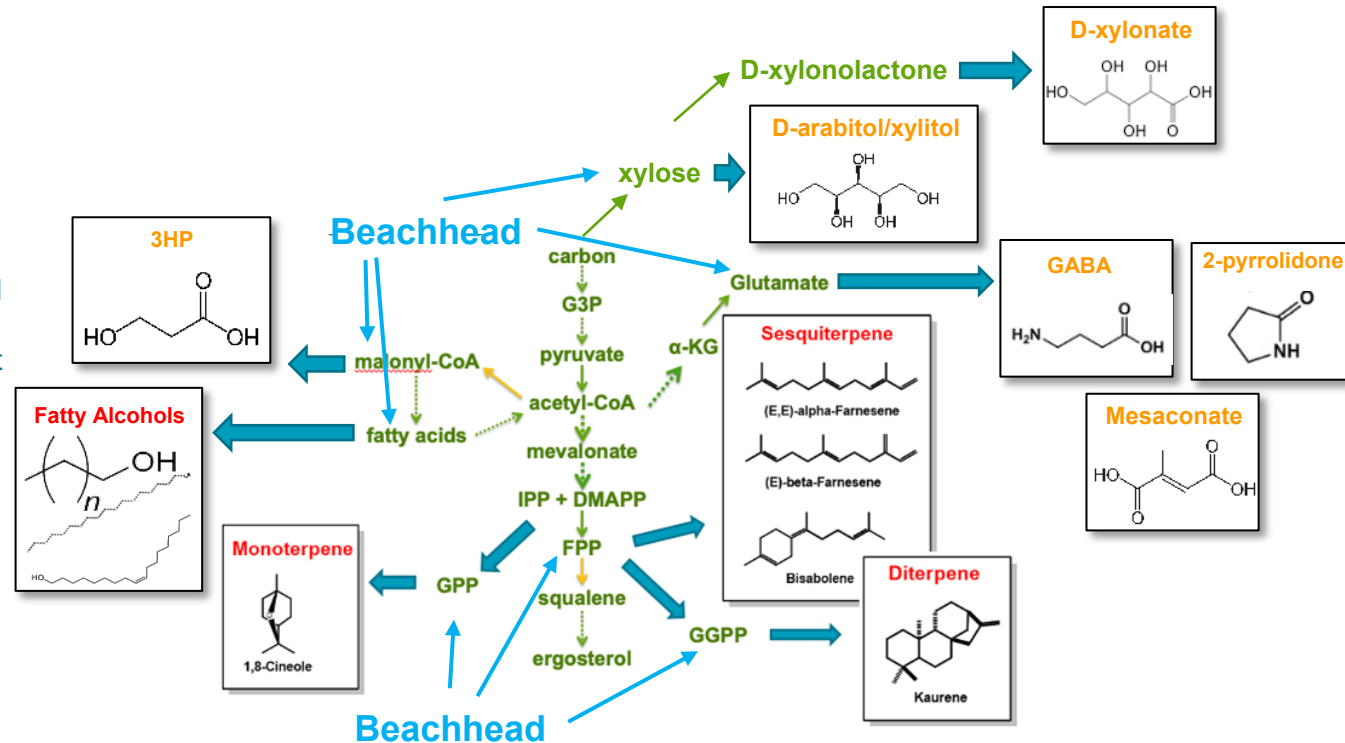
- Utilizes lignocellulose
- Fast growing
- Oleaginous, carotenogenic
- Metabolically versatile
- Genetically tractable



Project Overview: Current Targets and Beachheads

Complete List of Beachheads and Targets in *R. toruloides*

- **Pyrophosphates: Terpenes-** biofuels and bioproducts (adhesives, insect repellents, polymers, fragrances, food additives)
- **Malonyl-CoA: Fatty Alcohols-** Detergents, lubricants, plastics and cosmetics. \$5.2 billion in 2011 globally. Grow at 4% CAGR in next decade. **3HP transfer target-** acrylate polymers, biodegradable polymers
- **Xylose: sugar alcohols and xylonic acid-** top value-added chemicals from biomass to make polymers, plasticizers, concrete dispersal agents, adhesives, etc.
- **Glutamate: mesaconate, GABA, and pyrrolidone-** polymers, solvents, hydrogels, dyes, and d flame-retardant materials



Approach

Critical success factors

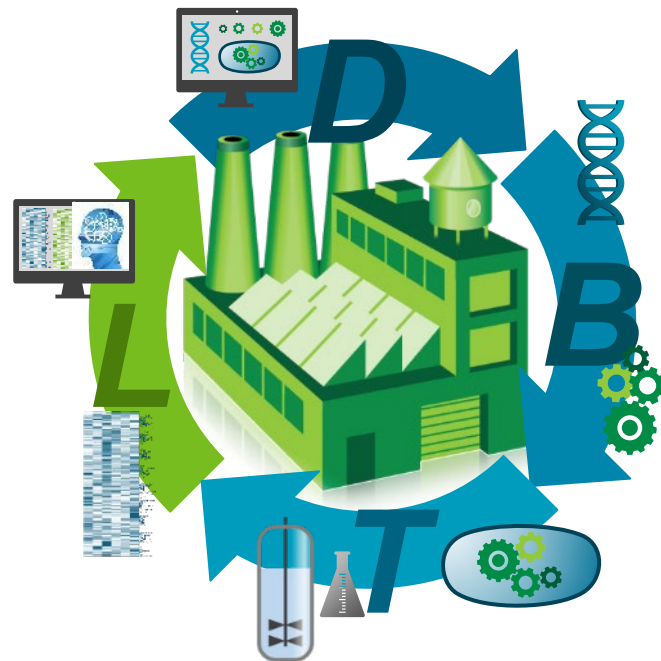
- Demonstrate DBTL works through improvements multiple targets
- Meaningful DBTL cycles with output from Learn leading to strain improvements
- Identification and mitigation of key DTBL bottlenecks

Challenges

- Developing a versatile host for producing both a wide variety of bioproducts
- Limited knowledgebase, needs improvement to enable more efficient DBTL
- Limited set of engineering tools and strategies can limit Design/Build space, e.g. no plasmids

Technical approach

- Expand knowledgebase and tools by acquiring systems level multi-omic and functional genomic data, developing a metabolic model, testing new parts and engineering strategies
- Engineer target biosynthetic genes into *R. toruloides*
- Use DBTL understand metabolism and optimize target production
- Optimize cultivation conditions and examine scalability in DMR-EH hydrolysate

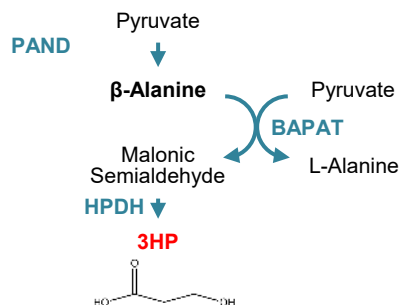


Progress and Outcomes

Transfer Target: 3HP from *A. pseudoterreus*

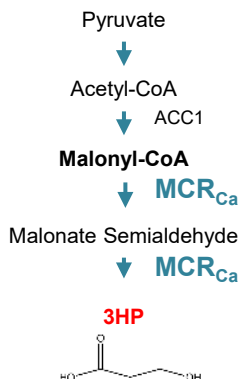
- Transfer targets help exemplify the value proposition of the ABF
- Pathways used in *A. pseudoterreus* for making 3HP

Design 1 β-Alanine Pathway



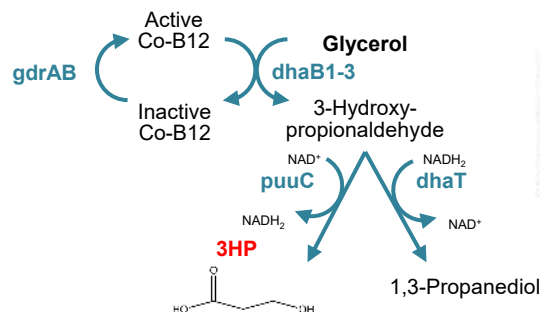
Beta-alanine designs
from *A. pseudoterreus*

Design 2 Malonyl-CoA Pathway

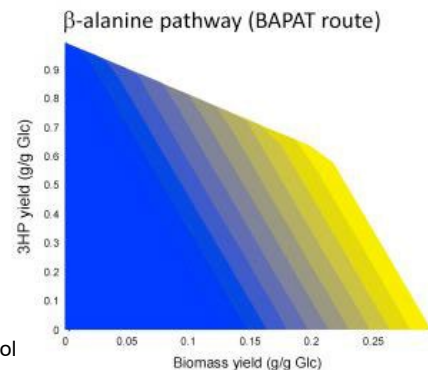
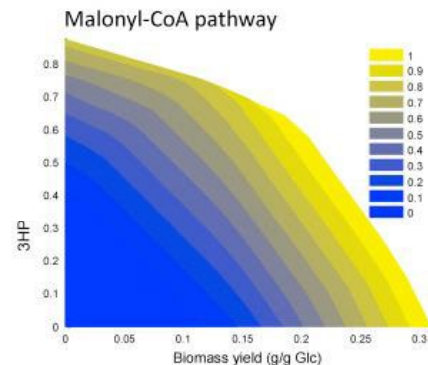


Rhodo naturally has high
flux toward malonyl-CoA

Design 3 Glycerol Pathway



Widely engineered
pathway in bacteria

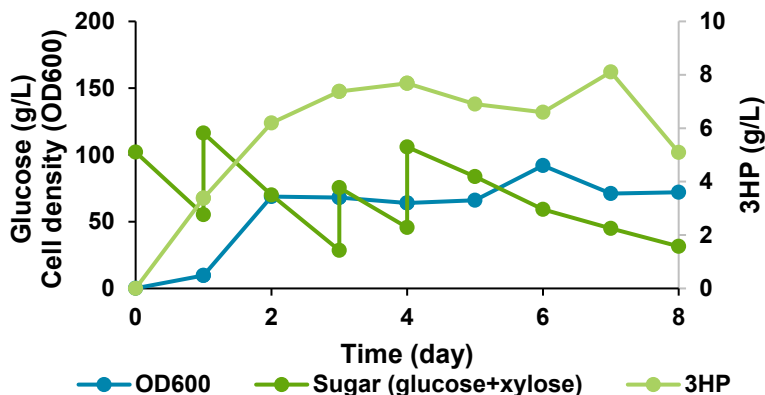


Yields from β-alanine and
malonyl-CoA pathways are
similar under typical
oxygenation and cell biomass

Demonstrate 3HP production with a split MCR

- Lessons from *A. pseudoterreus* suggested to split MCR gene into two functional enzymes
- Initial titer of 3HP split MCR was 2 g/L in test tube
- Bioreactor Ambr 250 runs of 3HP split MCR performed at ABPDU resulted in 8 g/L 3HP

Fed-batch fermentation in Ambr 250 with DMR



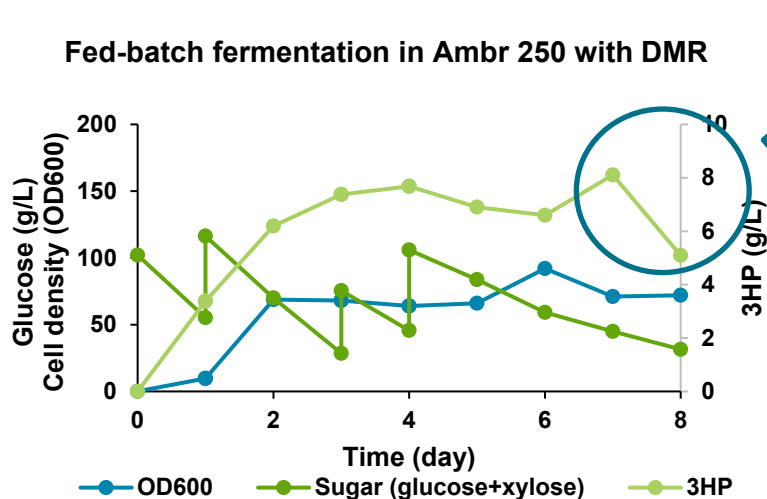
- Utilized medium: DMR with high nitrogen
 - Feeding: 50 g/L glucose
 - TRY
- Maximum titer: 8.1 g/L
Maximum yield: 0.072 g/g sugar
Maximum rate: 0.14 g/L/h

Organism	Titer	Reference
Sach. Cer.	13.7g/L	Borodina, 2014
Sach. Cer.	7.4g/L	Kildegaard, 2015
Methylobacterium exotetrans AM1	0.07g/L	Yang, 2017
Sach. Cer.	1g/L	David, 2016
E. coli	40.6g/L	Liu, 2016

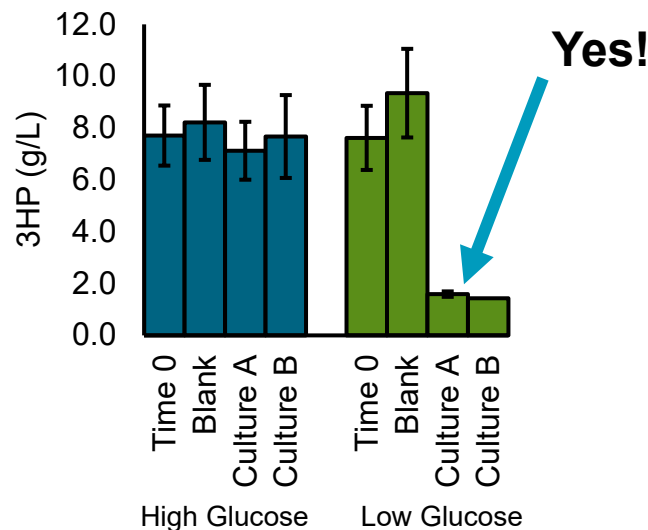
- Samples collected for multi-omic analysis with Test Team

R. toruloides consumes 3HP

- Lessons from *A. pseudoterreus* suggested to split MCR gene into two functional enzymes
- Initial titer of 3HP split MCR was 2 g/L in test tube
- Bioreactor Ambr 250 runs of 3HP split MCR performed at ABPDU resulted in 8 g/L 3HP



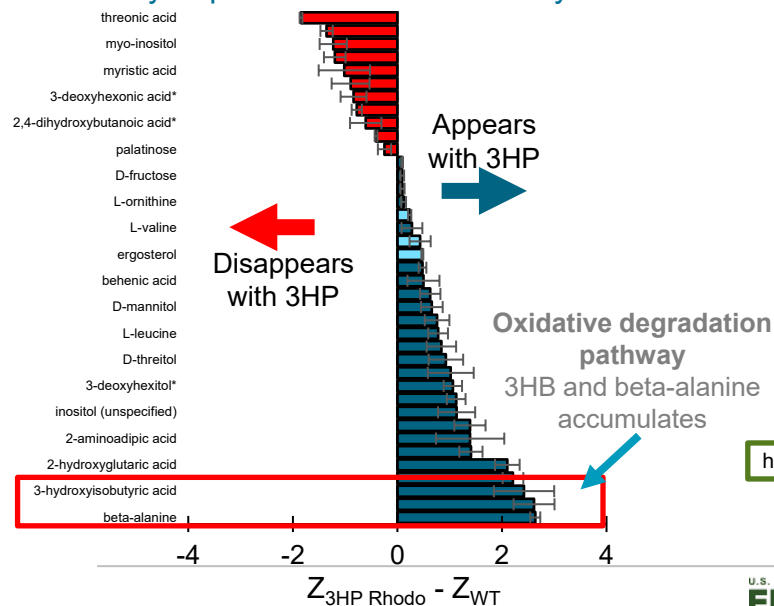
3HP degradation?



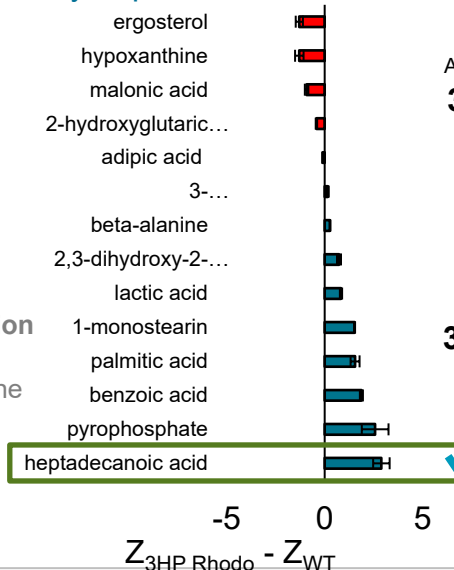
3HP catabolism – transfer lessons

- 3HP is also consumed by *A. pseudoterreus*
- *A. pseudoterreus* team identified genes potentially involved in 3HP catabolism
- Two potential pathways were identified, an oxidative and a reductive
- Homologues in *R. toruloides* were identified for to see if similar pathways exist

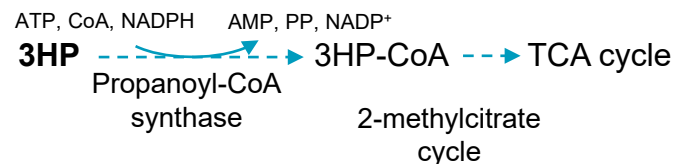
Differentially Expressed Metabolites: Day 4



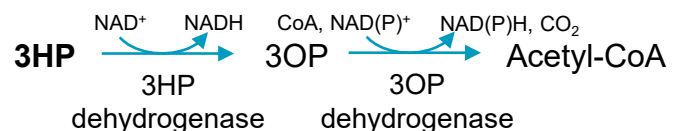
Differentially Expressed Metabolites: Day 1



Reductive pathway



Oxidative pathway



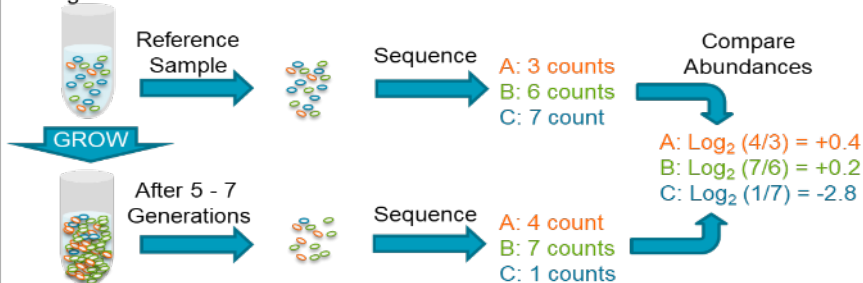
Note: 3OP = malonate semialdehyde

Reductive degradation pathway
Synthesized from propionyl-CoA

Functional Genomics to elucidate 3HP catabolism

RB-TDNASeq Workflow:

Mutant pool is cultivated in condition of interest and re-sequenced
Changes in relative abundance for each mutant is calculated as a 'Fitness Score'



Δ Fitness			
s. 3HP Val Leu			
	0.0	-0.4	-0.8
	-0.1	-0.5	-0.9
	-0.2	-0.6	-1.0
	-0.3	-0.7	-1.1
	-0.4	-0.8	-1.2
	-0.5	-0.9	-1.3
	-0.6	-1.0	-1.4
	-0.7	-1.1	-1.5
	-0.8	-1.2	-1.6
	-0.9	-1.3	-1.7
	-1.0	-1.4	-1.8
	-1.1	-1.5	-1.9
	-1.2	-1.6	-2.0
	-1.3	-1.7	-2.1
	-1.4	-1.8	-2.2
	-1.5	-1.9	-2.3
	-1.6	-2.0	-2.4
	-1.7	-2.1	-2.5
	-1.8	-2.2	-2.6
	-1.9	-2.3	-2.7
	-2.0	-2.4	-2.8
	-2.1	-2.5	-2.9
	-2.2	-2.6	-3.0
	-2.3	-2.7	-3.1
	-2.4	-2.8	-3.2
	-2.5	-2.9	-3.3
	-2.6	-3.0	-3.4
	-2.7	-3.1	-3.5
	-2.8	-3.2	-3.6
	-2.9	-3.3	-3.7
	-3.0	-3.4	-3.8
	-3.1	-3.5	-3.9
	-3.2	-3.6	-4.0
	-3.3	-3.7	-4.1
	-3.4	-3.8	-4.2
	-3.5	-3.9	-4.3
	-3.6	-4.0	-4.4
	-3.7	-4.1	-4.5
	-3.8	-4.2	-4.6
	-3.9	-4.3	-4.7
	-4.0	-4.4	-4.8
	-4.1	-4.5	-4.9
	-4.2	-4.6	-5.0
	-4.3	-4.7	-5.1
	-4.4	-4.8	-5.2
	-4.5	-4.9	-5.3
	-4.6	-5.0	-5.4
	-4.7	-5.1	-5.5
	-4.8	-5.2	-5.6
	-4.9	-5.3	-5.7
	-5.0	-5.4	-5.8
	-5.1	-5.5	-5.9
	-5.2	-5.6	-6.0
	-5.3	-5.7	-6.1
	-5.4	-5.8	-6.2
	-5.5	-5.9	-6.3
	-5.6	-6.0	-6.4
	-5.7	-6.1	-6.5
	-5.8	-6.2	-6.6
	-5.9	-6.3	-6.7
	-6.0	-6.4	-6.8
	-6.1	-6.5	-6.9
	-6.2	-6.6	-7.0
	-6.3	-6.7	-7.1
	-6.4	-6.8	-7.2
	-6.5	-6.9	-7.3
	-6.6	-7.0	-7.4
	-6.7	-7.1	-7.5
	-6.8	-7.2	-7.6
	-6.9	-7.3	-7.7
	-7.0	-7.4	-7.8
	-7.1	-7.5	-7.9
	-7.2	-7.6	-8.0
	-7.3	-7.7	-8.1
	-7.4	-7.8	-8.2
	-7.5	-7.9	-8.3
	-7.6	-8.0	-8.4
	-7.7	-8.1	-8.5
	-7.8	-8.2	-8.6
	-7.9	-8.3	-8.7
	-8.0	-8.4	-8.8
	-8.1	-8.5	-8.9
	-8.2	-8.6	-9.0
	-8.3	-8.7	-9.1
	-8.4	-8.8	-9.2
	-8.5	-8.9	-9.3
	-8.6	-9.0	-9.4
	-8.7	-9.1	-9.5
	-8.8	-9.2	-9.6
	-8.9	-9.3	-9.7
	-9.0	-9.4	-9.8
	-9.1	-9.5	-9.9
	-9.2	-9.6	-10.0
	-9.3	-9.7	-10.1
	-9.4	-9.8	-10.2
	-9.5	-9.9	-10.3
	-9.6	-10.0	-10.4
	-9.7	-10.1	-10.5
	-9.8	-10.2	-10.6
	-9.9	-10.3	-10.7
	-10.0	-10.4	-10.8
	-10.1	-10.5	-10.9
	-10.2	-10.6	-11.0

- RB-TDNaseq library grown in 3HP media to identify potential genes responsible for 3HP catabolism

Functional Genomics to elucidate 3HP catabolism

- Discovered 65 KOs with significant fitness impacts

- ALD6 is the same enzyme as the *A. pseudo* team's proposed oxidative pathway

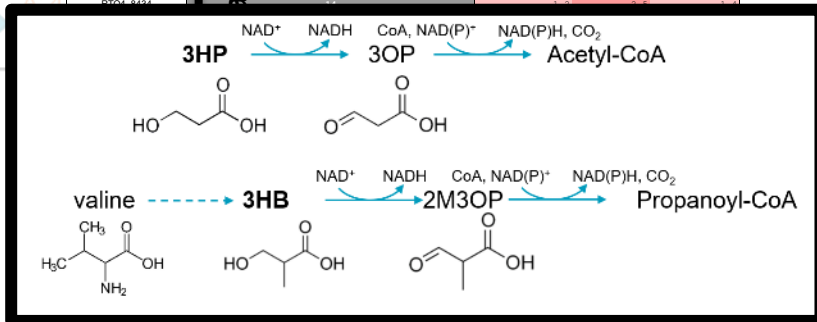
RB-TDNaseq Workflow:

Mutant pool is cultivated in condition of interest and Changes in relative abundance for each mutant is calculated



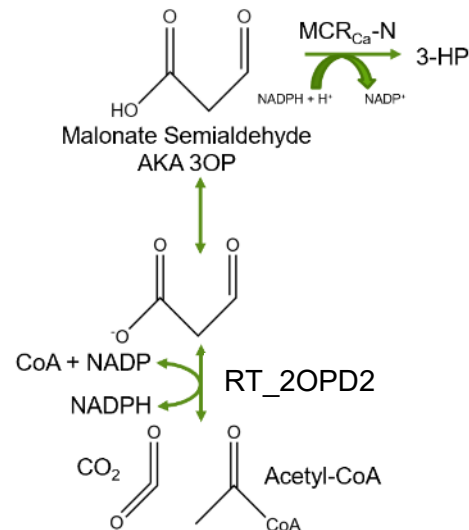
- RB-TDNaseq library grown in 3HP media to identify potential genes responsible for 3HP catabolism

Gene	# Sig.	Conds.	Δ Fitness		
			3HP	Val	Leu
RT04_8975	4	4	-1.5	-0.8	-0.1
RT04_12820	4	4	-1.7	-0.8	-0.3
RT04_14492	4	4	-1.7	-0.8	-0.3
RT04_15066	5	5	-1.5	-0.6	-2.5
RT04_15436	6	6	-1.4	-0.9	-2.2
RT04_12209	7	7	-1.5	-0.6	-2.2
RT04_12566	8	8	-2.0	-1.0	-0.9
RT04_13060	8	8	-1.0	-1.0	-0.0
RT04_9193	8	8	-2.2	-2.5	-0.4
RT04_15228	9	9	-1.3	-0.7	-0.3
RT04_15440	11	11	-2.1	-0.7	-0.3
RT04_10205	12	12	-1.5	-0.3	-0.3
RT04_12552	13	13	-1.4	-0.9	-2.2
RT04_12034	13	13	-2.8	-1.3	-1.6
RT04_14699	13	13	-0.9	-1.5	-0.6
RT04_15218	13	13	-1.6	-3.6	-0.5
RT04_11980	14	14	-1.2	-0.9	-0.9
RT04_9424	14	14	-1.2	-0.9	-0.9



RT04_16010	28	28	-1.1	-0.6	-0.4
RT04_16771	29	29	-2.1	-0.9	-2.2
RT04_0506	31	31	-1.2	-0.5	-1.8
RT04_12594	31	31	-2.0	-1.0	-2.3
RT04_16027	32	32	-2.0	-2.2	-0.6
RT04_16858	32	32	-0.6	-0.2	-0.6
RT04_16107	33	33	-0.7	-1.2	-0.7

ALD6, Malonate-Semialdehyde Dehydrogenase (Acetylating)



Functional Genomics to elucidate 3HP catabolism

- Discovered 65 KOs with significant fitness impacts

- ALD6 is the same enzyme as the *A. pseudo* team's proposed oxidative pathway

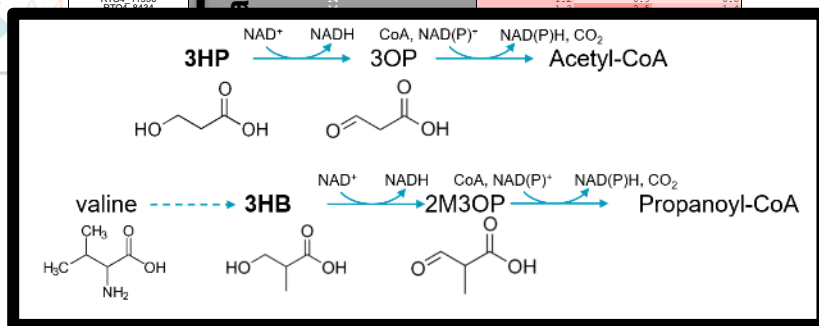
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- RB-TDNaseq library grown in 3HP media to identify potential genes responsible for 3HP catabolism

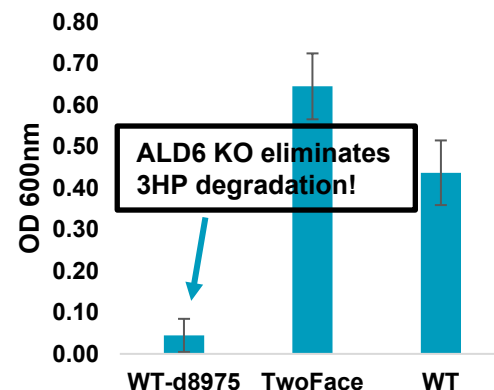
Gene	# Sig. Conds.	Δ Fitness		
		3HP	Val	Leu
RT04_8975	4	-1.5	-0.8	-0.1
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RT04_15066	5	-1.5	-0.6	-2.5
RT04_15436	6	-1.4	-0.9	-2.2
RT04_12209	7	-1.5	-0.6	-2.2
RT04_12566	7	-2.0	-1.0	-0.0
RT04_13060	8	-1.0	-1.6	-0.0
RT04_9193	8	-1.2	-2.5	-0.4
RT04_15228	9	-1.3	-0.7	-0.3
RT04_15440	11	-2.1	-0.7	-0.3
RT04_10205	12	-1.5	0.3	-0.3
RT04_12652	13	-1.4	-1.4	-1.8
RT04_12034	13	-2.8	-1.3	-1.6
RT04_14699	13	-0.9	-1.5	-0.6
RT04_15218	13	-1.6	-3.6	-0.5
RT04_11980	14	-1.2	-0.9	-0.5
RT04_16107	14	-1.2	-0.9	-0.5



RT04_16110	28	-1.1	-0.6	-0.4
RT04_16771	29	-2.1	-0.9	-2.2
RT04_0506	31	-1.2	-0.5	-1.8
RT04_10025	31	-2.0	-1.0	-2.3
RT04_12594	31	-2.0	-1.0	-2.3
RT04_16027	32	-2.0	-2.2	-0.6
RT04_16858	32	-0.6	0.2	-0.6
RT04_16107	33	-0.7	-1.2	-0.7

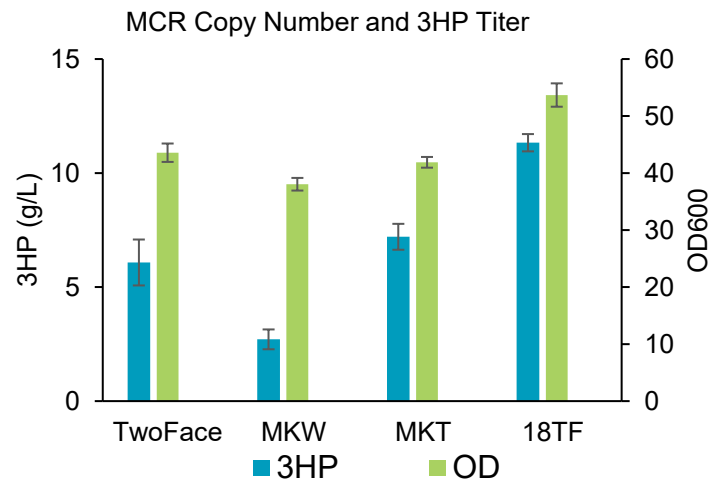
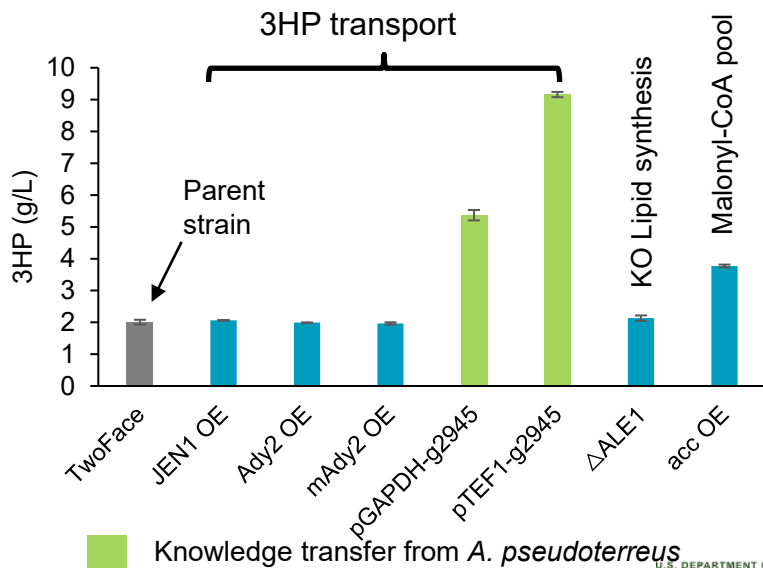
ALD6, Malonate-Semialdehyde Dehydrogenase (Acetylating)

Growth on 3HP



Host Engineering to Improve 3HP Titer

- Increasing malonyl-CoA pool by ACC1 overexpression doubles 3HP titer
- Interrupting TAG biosynthesis (KO of ALE1) has no impact on 3HP
- Increasing MCR copy number to 3 doubles 3HP titer
- Overexpressing a 3HP transporter increases 3HP titers 4-5 fold
- The g2945 transported from *A. pseudoterreus* works best

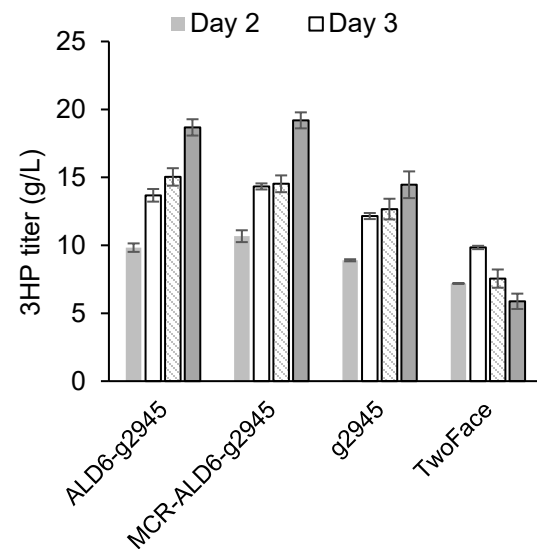
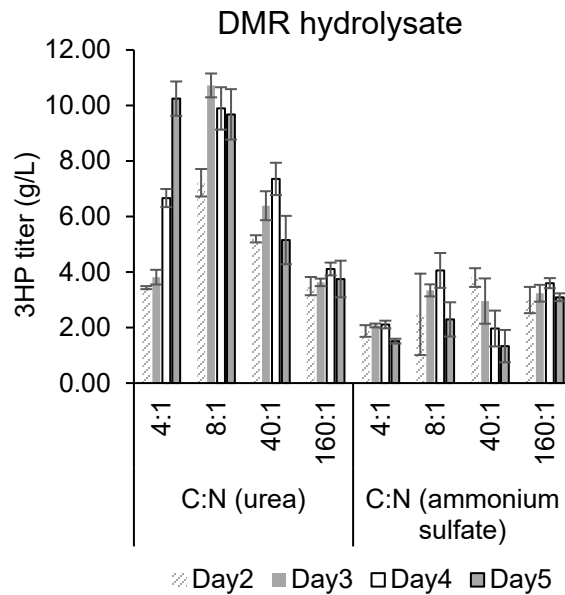
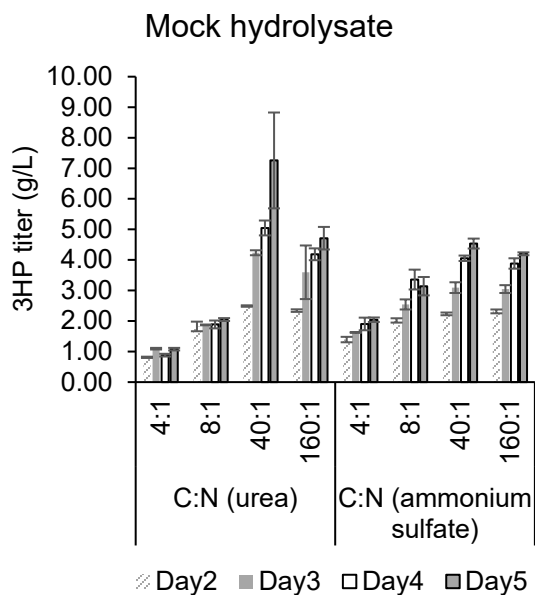


MCR Copies

TwoFace- 1 copy; MKW- 1 copy; MKT- 2 copies; 18TF- 3 copies

3HP media optimization – C/N ratio

- C/N ratio optimization differs in mock (40:1) vs DMR (4:1 or 8:1)
- Max titer in DMR reach 10 g/L, a 5-fold increase
- Lesson- caution should be taken when optimizing with mock hydrolysates

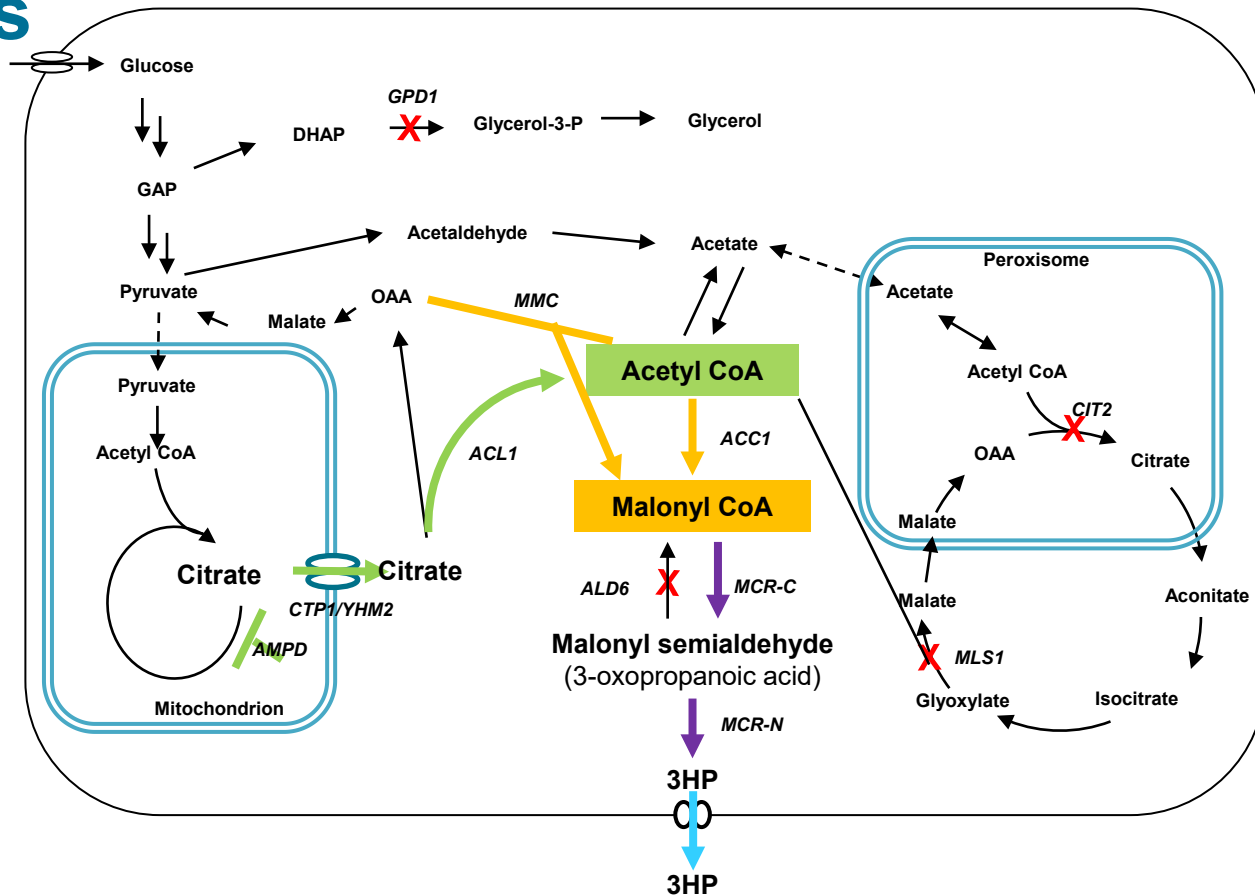


Titer: 2 g/L -> 19.2 g/L, 9.6-fold increase

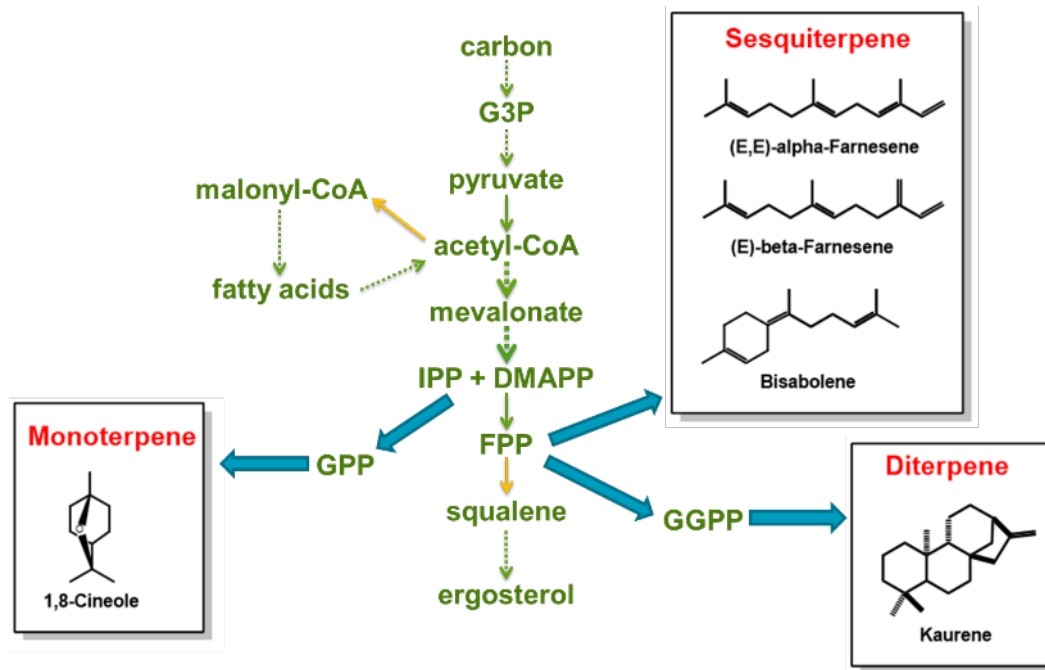
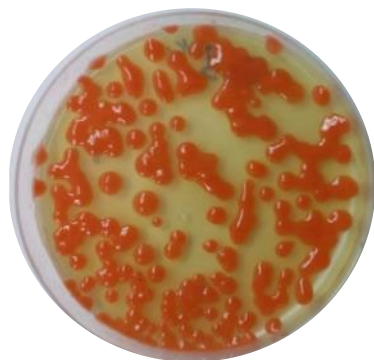
Next Steps

Strain engineering to optimize global metabolism

- Enhance pools of key precursors
- Turn down competing pathways








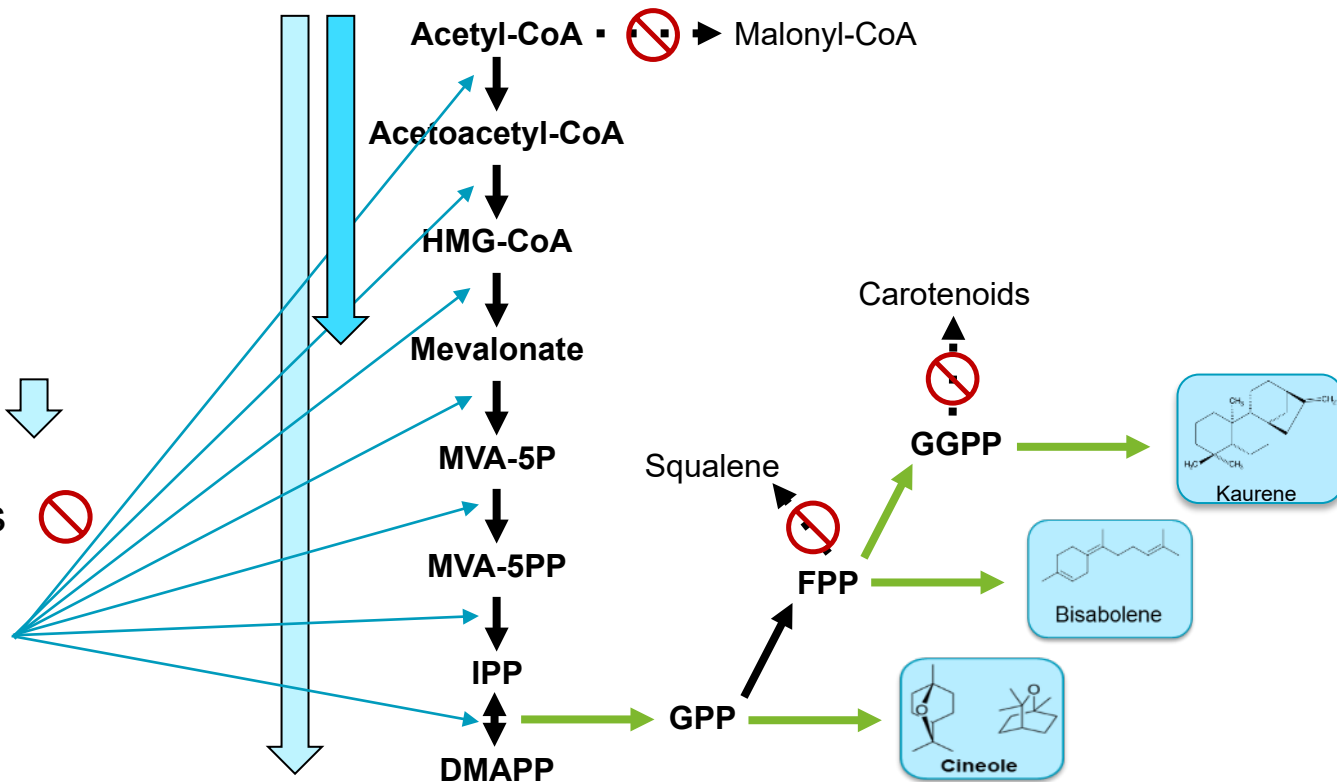
Terpene targets in *R. toruloides*



Strategy for MEV Pathway Optimization

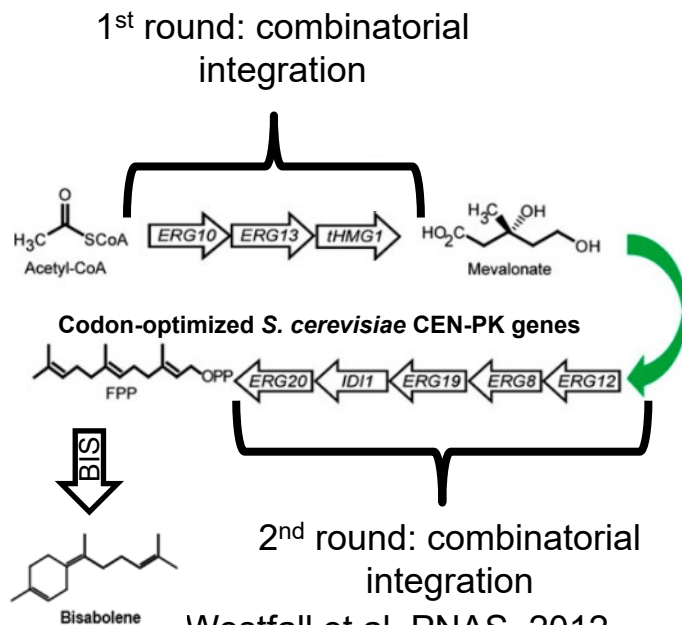
Overall Strategy

1. Flux Out 
2. Flux In 
3. Flux Through 
4. Carbon Shunts 
5. Optimize Flux 



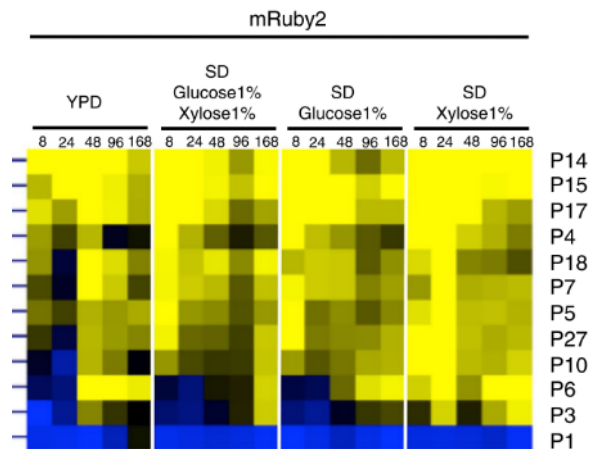
Complete overexpression MEV pathway

- Will split the pathway into two parts for flux in (to mevalonate) and flux through (to FPP)
- Designs are informed by “Optimize Flux” activity, currently in construct build.



Westfall et al. PNAS. 2012

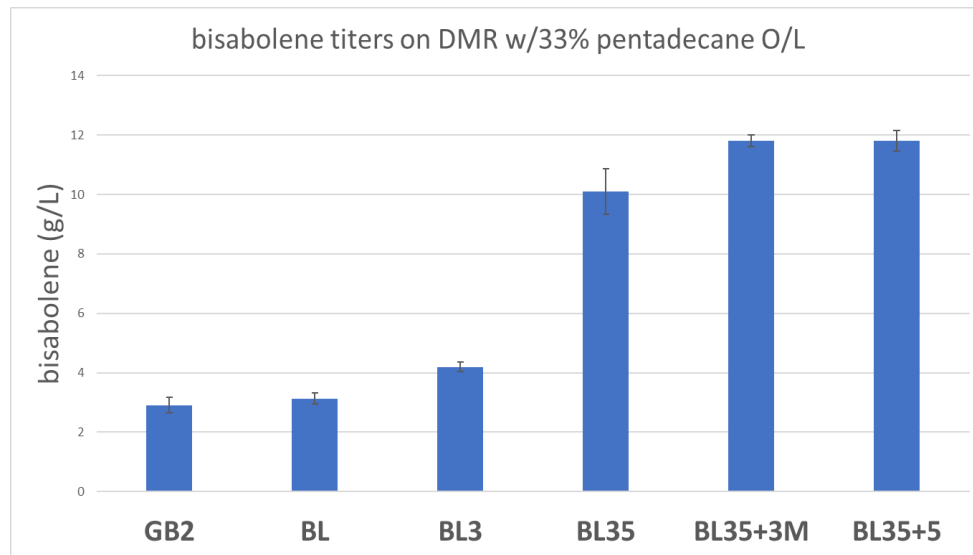
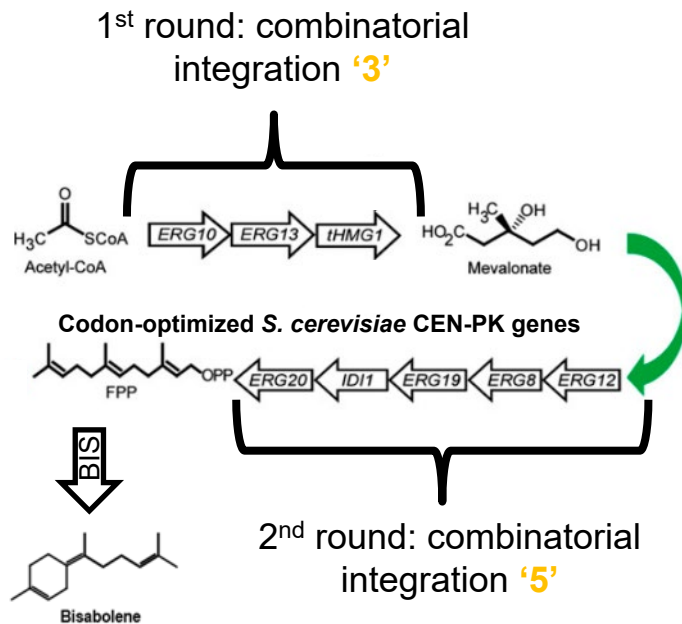
Using newly characterized constitutive *R. toruloides* promoters



Nora et al. Microb Cell Fact. 2019.

Complete overexpression MEV pathway

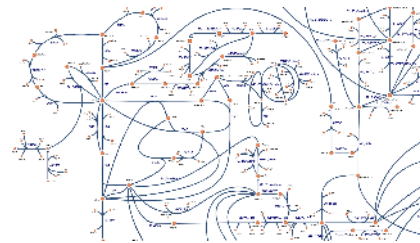
- Will split the pathway into two parts for flux in (to mevalonate) and flux through (to FPP)
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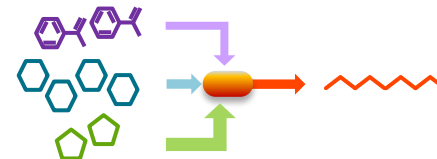
Bench scale: 0.26 g/L -> 11.8 g/L, 45-fold increase

Elucidating xylose metabolism in *R. toruloides*

➤ Improving metabolic models

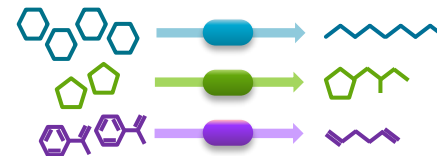


➤ Increasing xylose flux to central metabolism



➤ Xylose specific products and beachheads

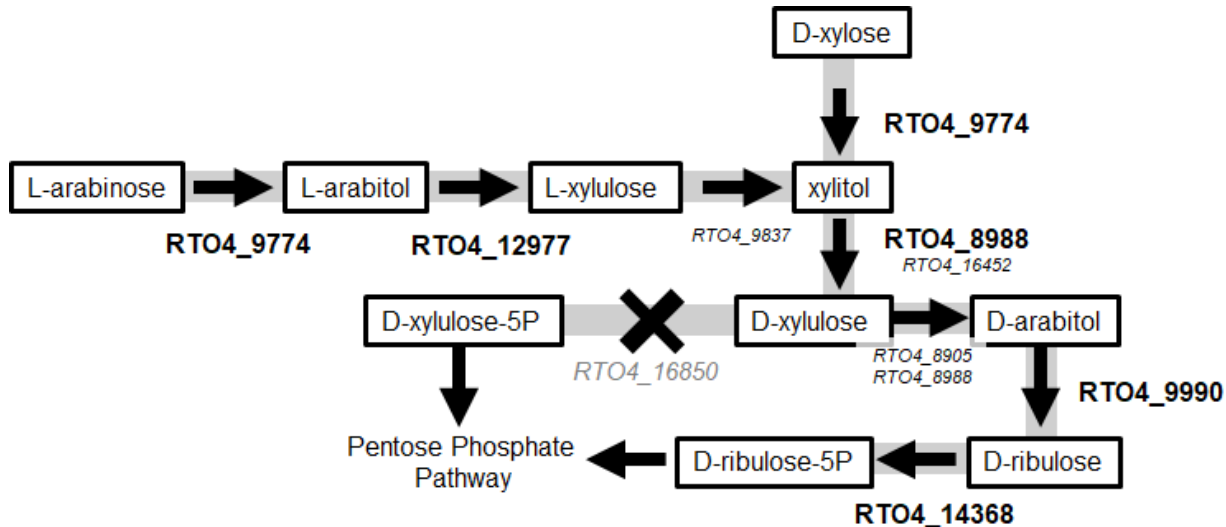
(arabitol, xylitol, xylonic acid)



FY22Q3_DBTLDPB_R1 - Provide three additional compounds of interest to BETO's Performance-Advantaged BioProducts projects at sufficient quantities for property testing

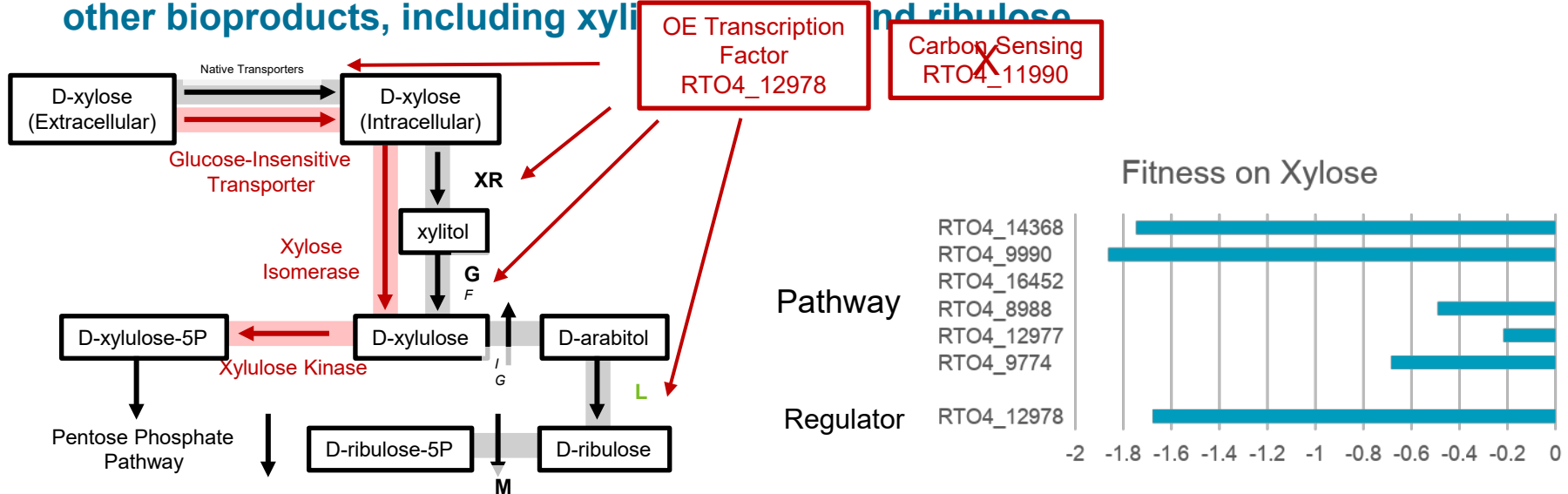
Xylose catabolic pathway

- Pathway uses a unique route through arabitol
- May be useful for xylose beachhead and product development
- Targets: xylitol, arabitol, xylonate



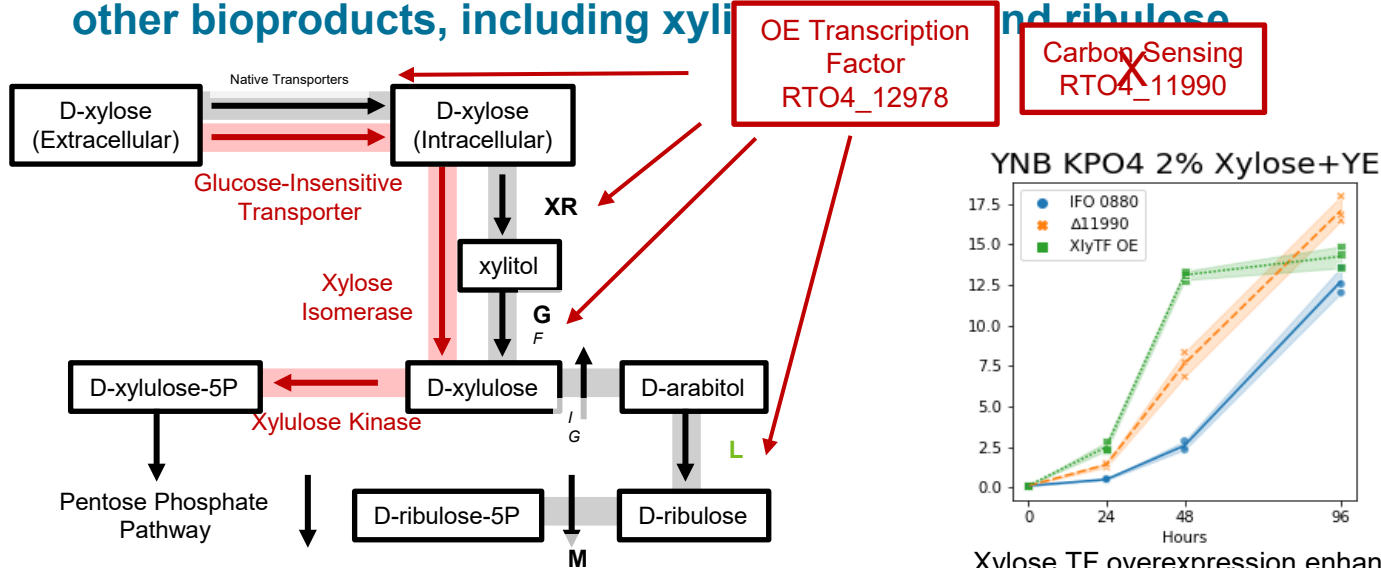
Plans to optimize xylose utilization

- Xylose uptake will be improved by overexpressing transporters, transcription factors, and heterologous catabolic genes & deletion of carbon sensing
- Other pathway genes will be KOed to validate pathway and enable production of other bioproducts, including xylitol and ribulose

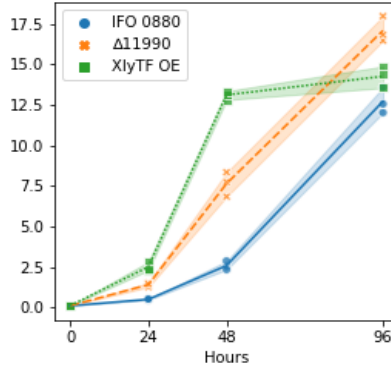


Plans to optimize xylose utilization

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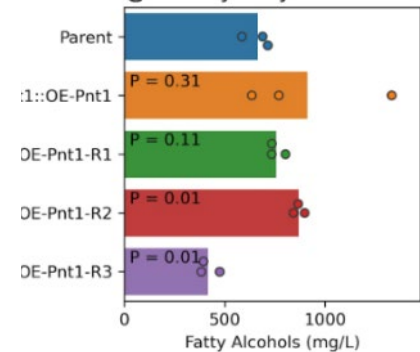


YNB KPO4 2% Xylose+YE



Xylose TF overexpression enhances cell growth and fatty alcohol titer

C Hydrolysate



Identification of RT04_12978 TF regulon

- Global proteomics confirms most TF targets belong to xylose pathway
- Two putative new transporters identified
- Additional few unknowns to be characterized

Protein ID Predicted Function

RT04_9774[†] Xylose Reductase

RT04_12977[†] Putative L-arabinose reductase

RT04_16452[†] Similar to xylitol dehydrogenases

RT04_8988[†] Similar to sorbose dehydrogenase

RT04_9990[†] D-arabitol dehydrogenase

RT04_14368[†] D-ribulose kinase

RT04_8504[†] Similar to aldose-1 epimerase

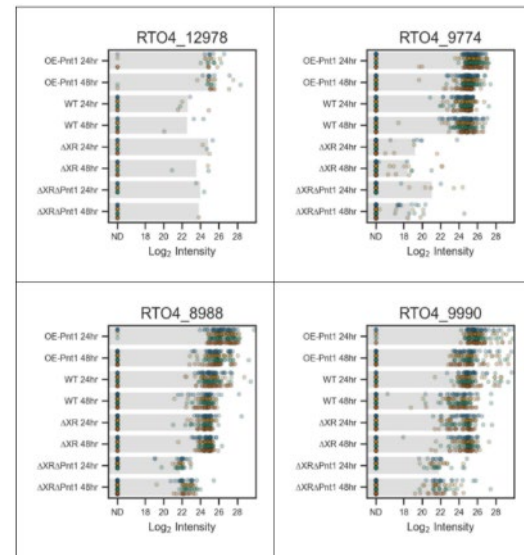
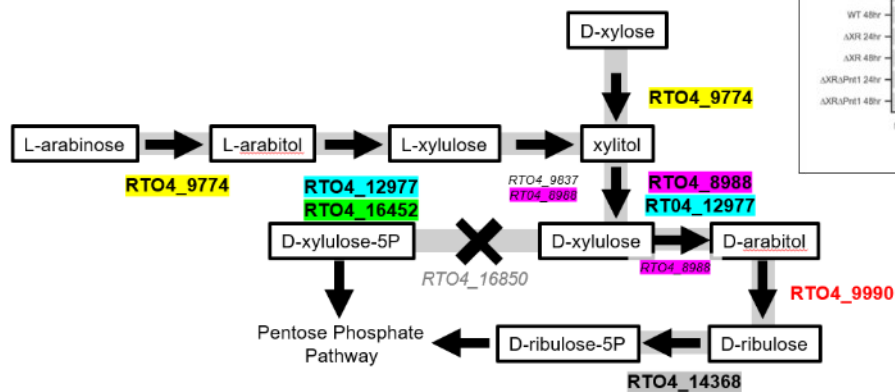
RT04_13524 Similar to glucose dehydrogenase

*RT04_11216[†] Similar to glucose dehydrogenase

RT04_10421 Aldehyde dehydrogenase

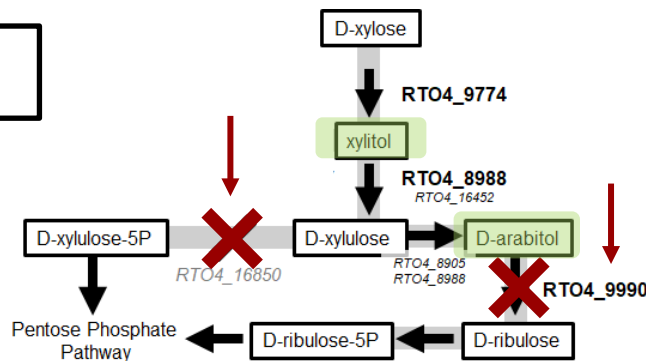
RT04_10463 Uncharacterized protein

*RT04_11368 Amidase

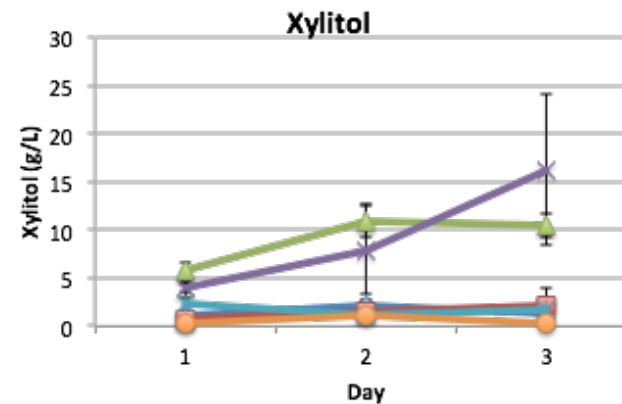
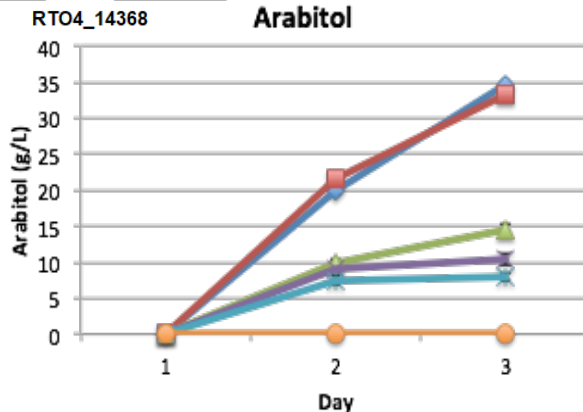
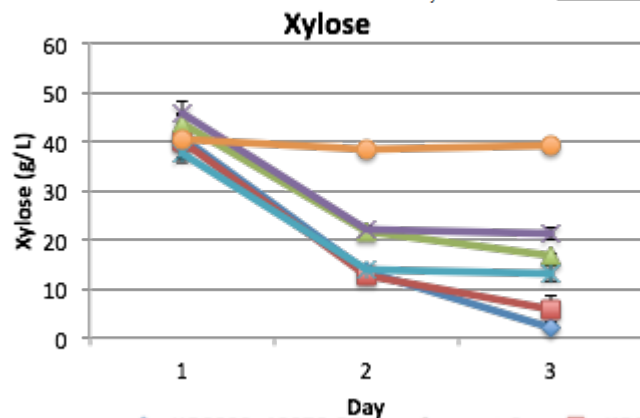


Xylitol & arabitol production

OE Transcription
Factor
RTO4_12978



Overexpression of xylose transcription factor significantly improves arabitol productivity



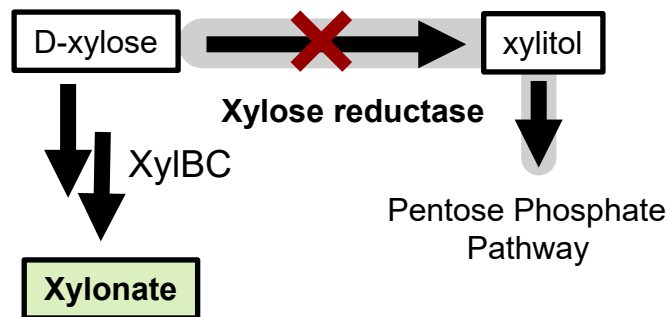
FY22Q3_DBTLDPB_R1 - Provide three additional compounds of interest to BETO's Performance-Advantaged BioProducts projects at sufficient quantities for property testing

Xylonate production

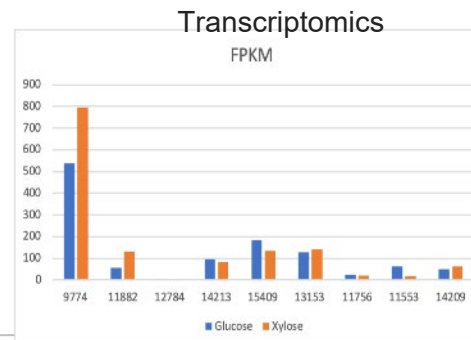
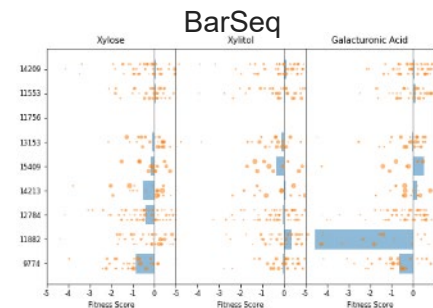
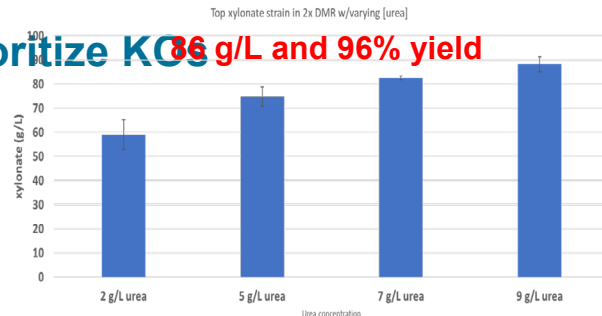
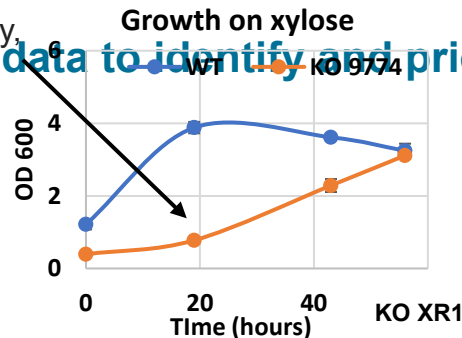
- First step for xylonate engineering is to KO xylose reductase
- Problem: there are a lot of potential XRs!!

- Use BarSeq and expression data to identify and prioritize KOs

KO of Top XR (enzymology, fitness, expression)

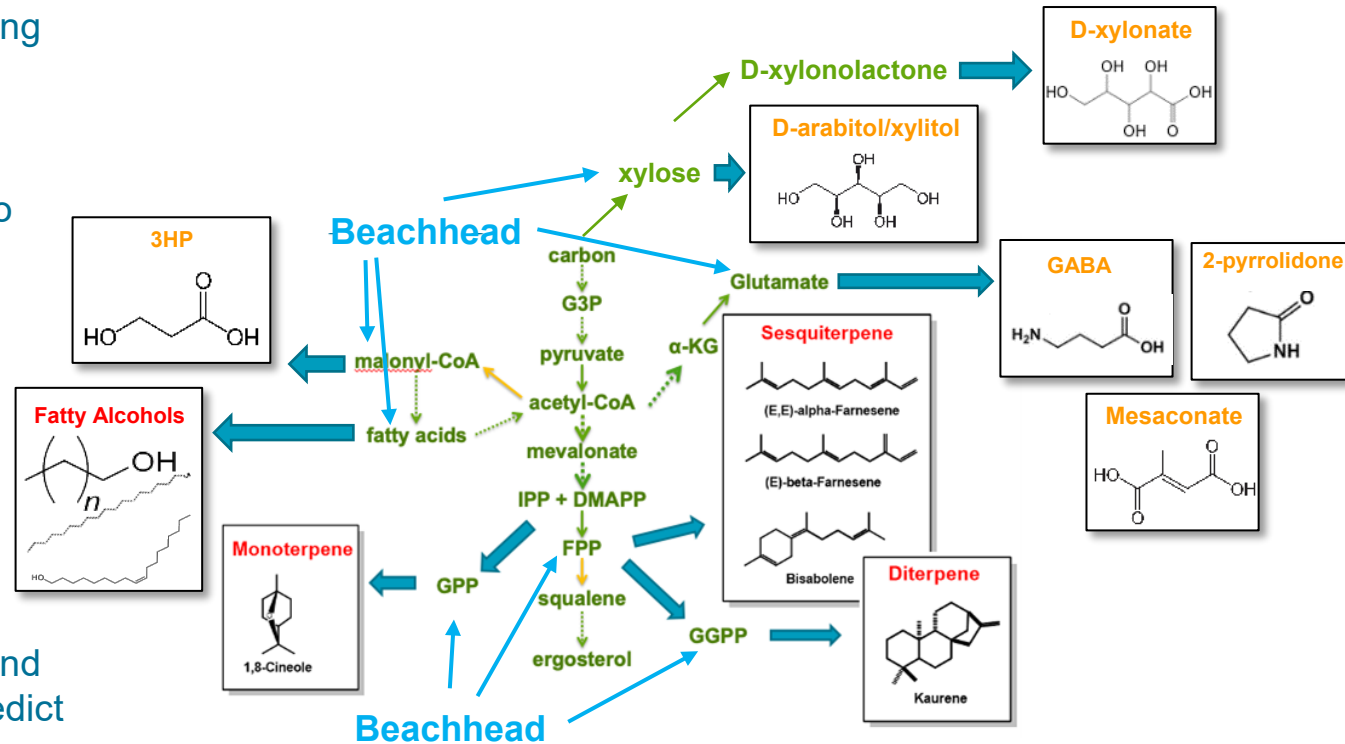


BarSeq and transcriptomics will be used to prioritize KOs of the 8 additional possible XRs



Yeast Demo Summary

- Expanded genetic engineering tools and knowledge in *R. toruloides*
- Implemented DBTL cycles to engineered the organism to produce a wide range of bioproducts at high TRYs
- Optimized fermentation conditions by process development
- Leveraged multiomics and functional genomics to expand the knowledge base and predict engineering targets



Acknowledgements

SNL

Derek Clay
Sam Coradetti
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Di Liu
Peter Otoupal
Anne Ruffing
Tessily Hogancamp
Mary Tran-Gymafi
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Shuang Deng
Yuqian Gao
Beth Hofstad
Joonhoon Kim
Young-Mo Kim
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Jeremy Zucker

ANL

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Peter Larsen

ORNL

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LBNL-ABPDU

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Deepti Tanjore

LANL

Taraka Dale
Chris Yeager
Ramesh



U.S. DEPARTMENT OF
ENERGY

Energy Efficiency &
Renewable Energy

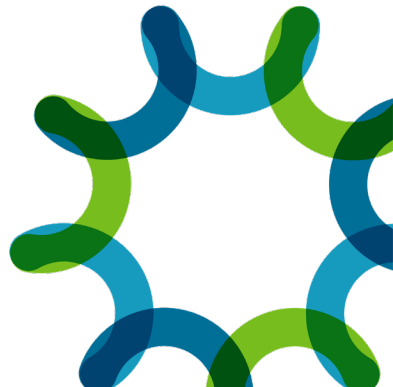
BIOENERGY TECHNOLOGIES OFFICE

Filamentous Fungi at the ABF

Presenter: Jon Magnuson

ABF Webinar

Date: April 29, 2022



Introduction to our Favorite Filamentous Fungi

Aspergillus pseudoterreus & *A. niger*



- ***Aspergillus* spp. are industrially relevant:** used for producing small molecules and enzymes in large bioreactors
Examples: citric acid, itaconic acid in $\geq 100,000\text{L}$ airlift reactors, ~3M ton market (citric)
- **Genetic tools developed, genomes sequenced, genome scale metabolic models built**
- **High flux** from sugars toward *beachhead* molecules in glycolysis and the TCA cycle, **organic acids**.
- Grows and produces organic acids at pH 1-3, **free acids, not salts**
 - **Separations:** high titer, free acid, fewer entrained impurities
 - No lime or sulfuric acid and hence no waste gypsum

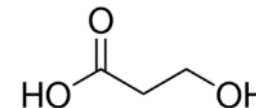


Hosts: Acid Tolerant *Aspergillus* spp.

Targets: Organic Acids

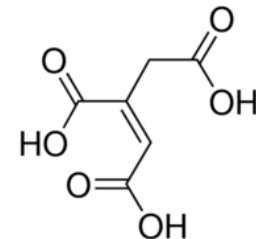
– Target 1: 3-Hydroxypropionic Acid (3HP)

- Uses: intermediate to monomers acrylic acid and acrylonitrile, which lead to polyacrylate and carbon fiber
- **Heterologous pathway** (prokaryotic)
- **Issue: degradation of 3HP observed in culture**



– Target 2: Aconitic Acid

- A 6-carbon tricarboxylic acid, like citric acid
- Uses: acidulant, chelator (cement), comonomer
- **Issue: apparent transport limitations**



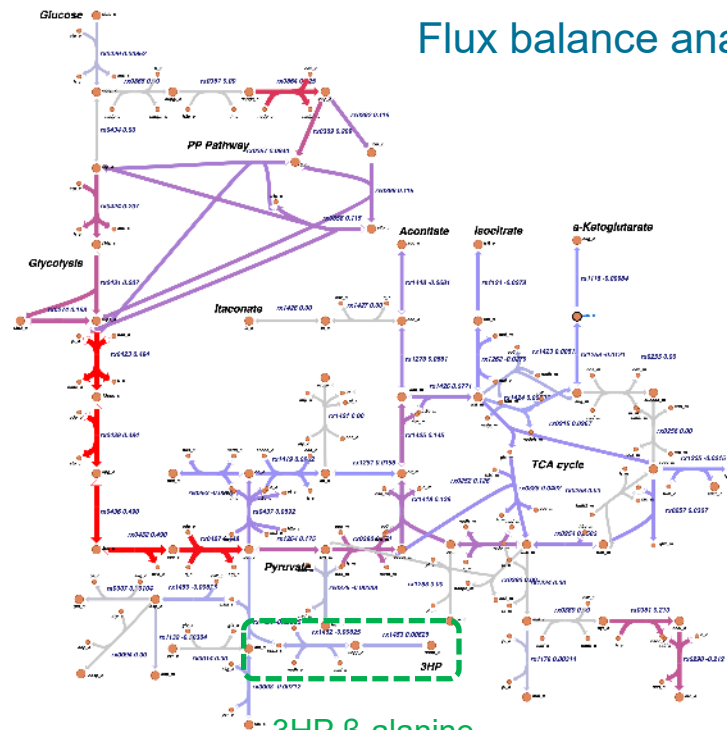
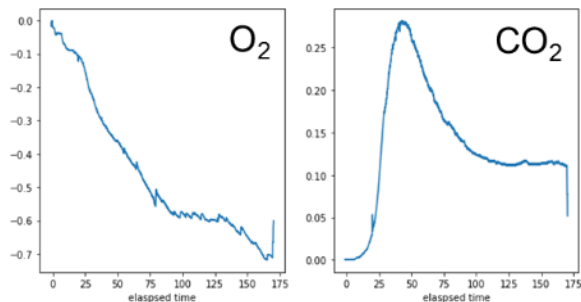
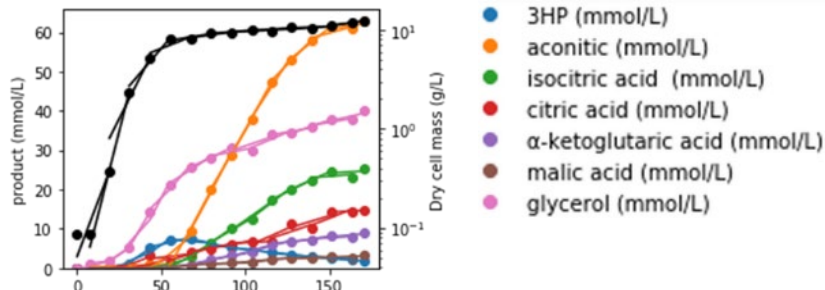
- **Purpose:** use industrially relevant organic acid demonstration targets emanating from useful beachhead molecules to advance DBTL capabilities for *Aspergillus* spp. (and other fungi) applicable to the entire bioprocess and TRY development range

Established the β -alanine 3HP pathway in *A. pseudoterreus*

Scaled up in 20L bioreactor

Issue 1: background of organic acids in *A. pseudoterreus*

Solution: transfer to *A. niger*



Flux balance analysis

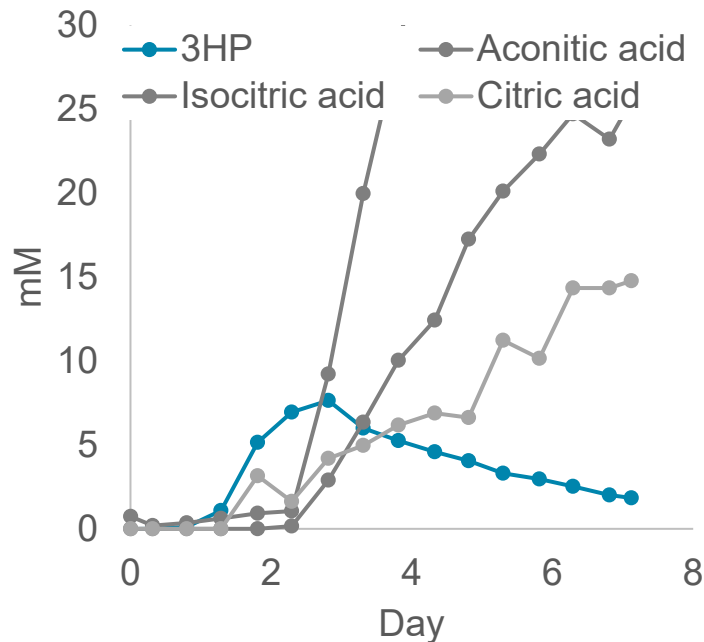
3HP β -alanine
pathway



Targeted (quantitative) metabolomics

Issue 2: the rise and fall of 3HP

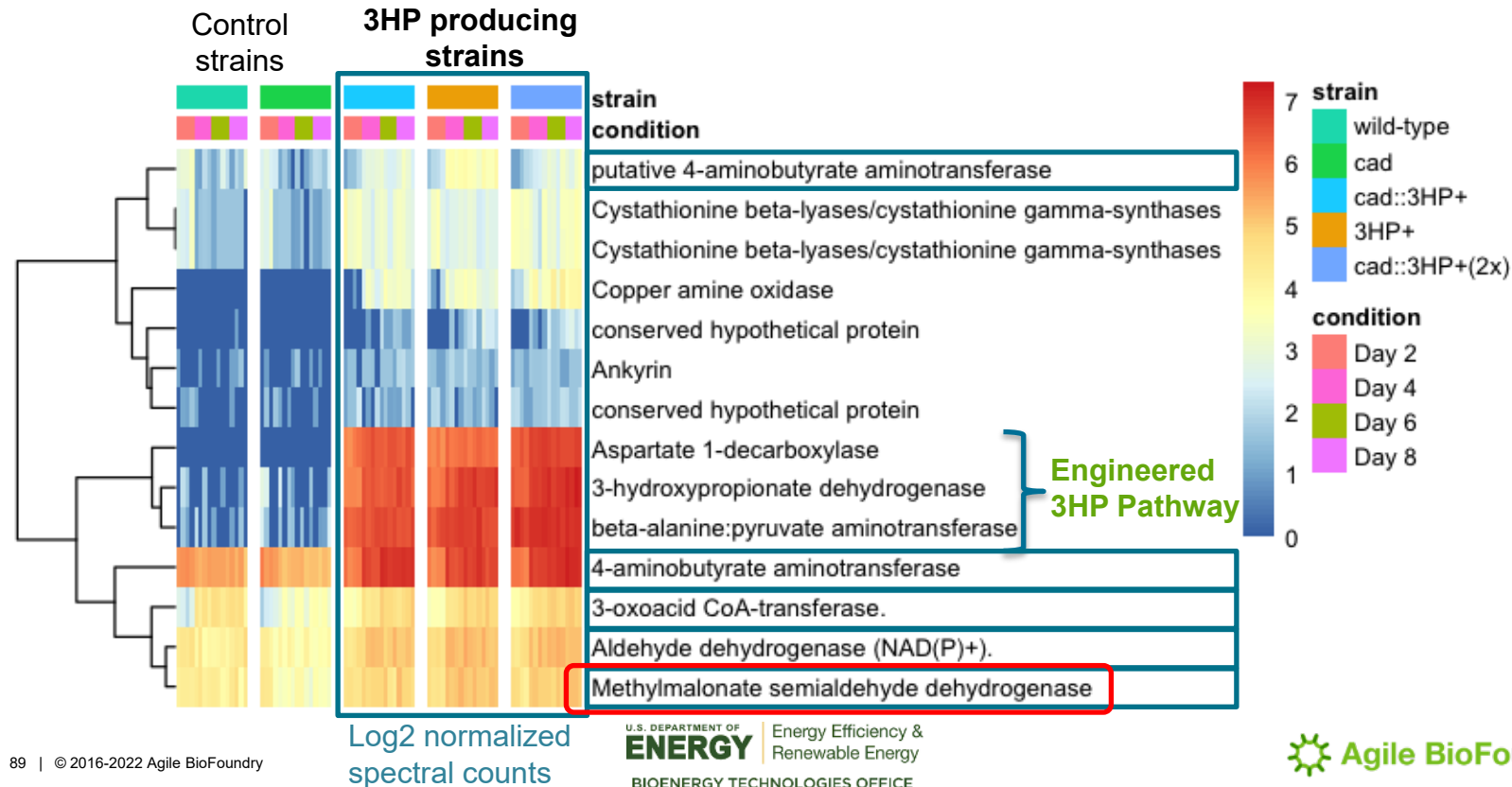
3HP is synthesized **AND** degraded during a bioreactor run of engineered *A. pseudoterreus*



Test/Learn: Candidate genes for 3HP degradation

Test: Discovery (global) proteomics

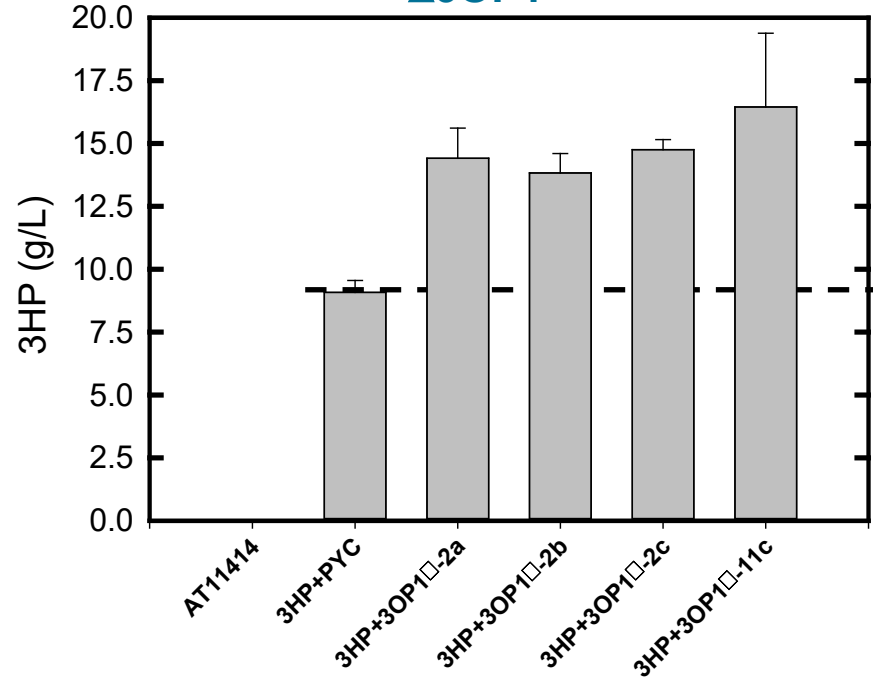
Learn: Genes upregulated in 3HP producing *A. pseudoterreus* strains



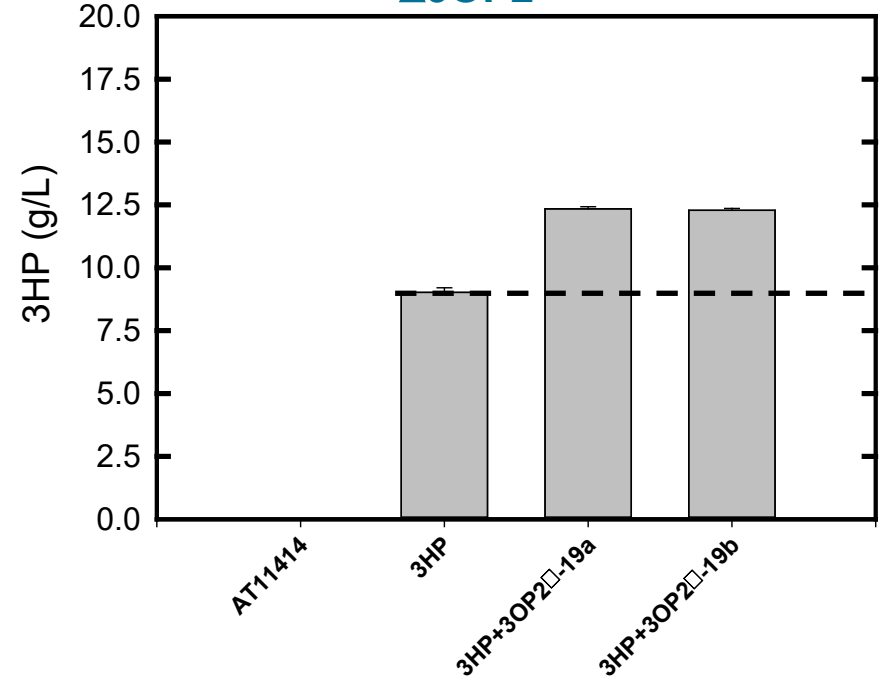
Design/Build/test:

3HP titer improved by 3-oxopropanoate DH (3op) gene deletions
in *A. niger* 3HP production strain

$\Delta 3OP1$



$\Delta 3OP2$



A. *niger* Strains from Design-Build for Major Test-Learn Campaign

- 15 strains with different genotypes and phenotypes (3HP TRY)
 - Increasing flow of carbon to 3HP, including carbon fixation
 - Decreasing degradation/diversion
 - Increasing export
- Identify new gene targets
 - FBA approaches
 - Advanced Learn to identify non-intuitive targets
- **Goal:** Identify gene targets to increase TRY (titer, rate, yield)

ICE ID	Strain (built/tested)	3HP Titer (g/l)
ABF_008340	Parent strain (ATCC11414)	0
ABF_008343	3HP-9	6
ABF_008344	3HP+AAT	>8
ABF_008345	3HP+PYC	10
ABF_008346	3HP+PYC+3HP	15
ABF_008347	3HP+PYC+ <i>oahA</i> Δ	12.5
ABF_008348	3HP+PYC+ <i>3op1</i> Δ	>15
ABF_008351	3HP+PYC+ <i>uga2</i> Δ	6.0
ABF_008354	3HP+PYC+AAT	11
ABF_008355	3HP+PYC+3HP+AAT	16.5
ABF_008356	3HP+PYC+ <i>3op1</i> Δ+AAT	15.8
ABF_008897	3HP+PYC+3HP+MFS	17.5
ABF_008898	3HP+PYC+MFS	12.1
ABF_008899	3HP+PYC+3HP+ <i>oahA</i> Δ	17.1
ABF_008900	3HP+PYC+3HP+ <i>uga2</i>	16.1

Learn: Bayesian Metabolic Control Analysis

Inputs: Multiomics measurements for different perturbations (genetically altered strains, culture conditions) relative to a reference strain

Outputs: which enzyme perturbations are most likely to improve TRY

Assumptions:

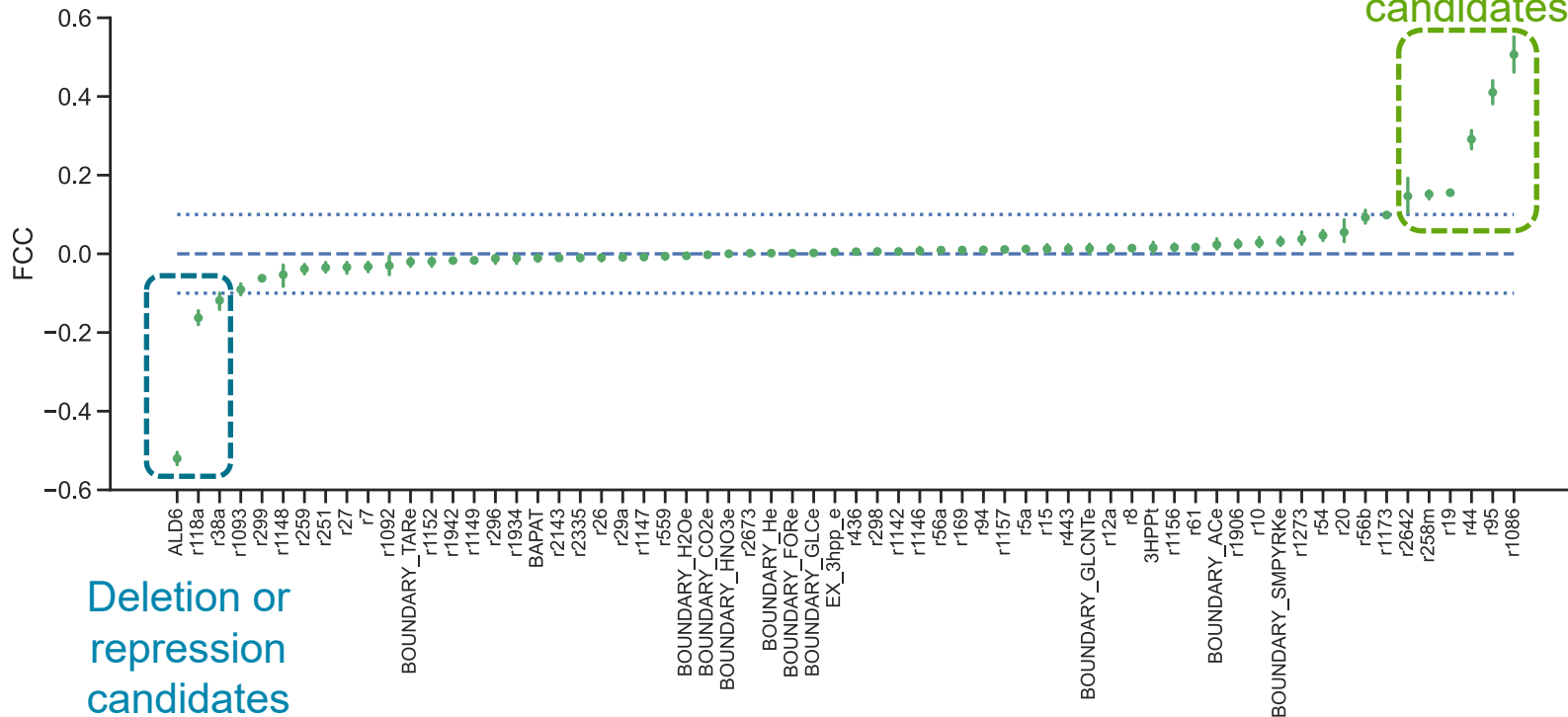
- Use steady-state assumption to constrain reaction fluxes
- Use lin-log kinetics to predict reaction fluxes from metabolite and protein (enzyme) measurements
- Use knowledge of reaction stoichiometry to generate prior beliefs about the effect of change of each metabolite to a change in each reaction flux

Limitations: Input perturbations restricted to different media and overexpression/repression/deletion of genes

ABF - *Aspergillus* 3HP, multiomics of 14 3HP strains + control

Bayesian Metabolic Control Analysis

Over-expression
candidates



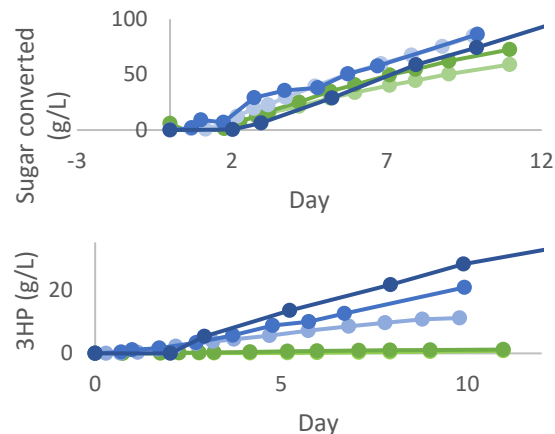
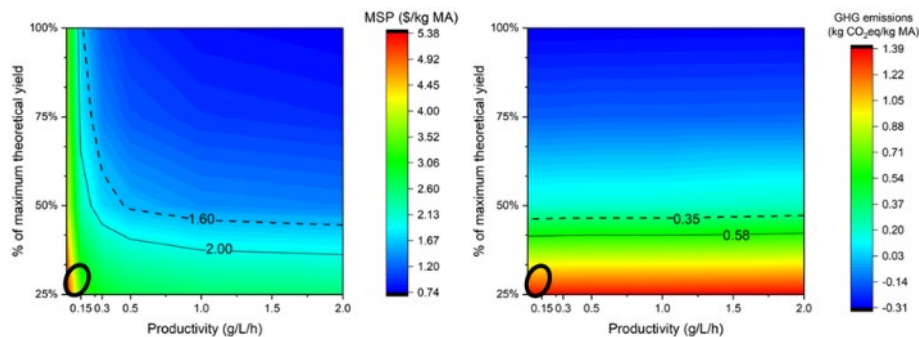
TEA/LCA: TechnoEconomic Analysis, Life Cycle Analysis & Scale-Up

FY21 – Best observed TRY in 0.5L bioreactors

3-HP strain development at bioreactor scale

Aspergillus niger – 3-hydroxypropionic acid

- Titer - 39.8 g/L
(batch end-point)
- Rate - 0.137 g/Lh
(batch growth phase)
- Yield - 0.336 g/g sugar
(batch growth phase)



Species	Genotype	Titer (g/L)	Rate (g/Lh)	Yield (g/g)
<i>A. pseudoterreus</i>	3HP+	0.8	0.003	0.01
<i>A. pseudoterreus</i>	3HP+, Δ ald6	1.2	0.004	0.02
<i>A. niger</i>	3HP+, PYC+	11.2	0.048	0.13
<i>A. niger</i>	3HP+, PYC+, Δ ald6	20.8	0.087	0.24
<i>A. niger</i>	3HP++, PYC+, Δ ald6	39.8	0.080	0.30

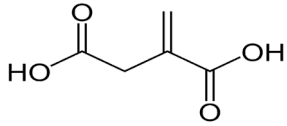
3-HP Summary

- **Mutiple DBTL cycles:** establish 3HP pathway, identify issues, transfer to another host, push on filling the precursor pool, increase 3HP pathway copies, delete genes that divert intermediates, ID and over-express 3HP transporter
- **Test:** Discovery/global proteomics, transcriptomics and metabolomics in a genome modeling context
 - **Learn** Genome modeling context for analysis of omics results helps identify gene candidates
 - Deeper **Learn**, MCA, helps identify intuitive and non-intuitive gene candidates

A. pseudoterreus Aconitic Acid Production Transporter Identification

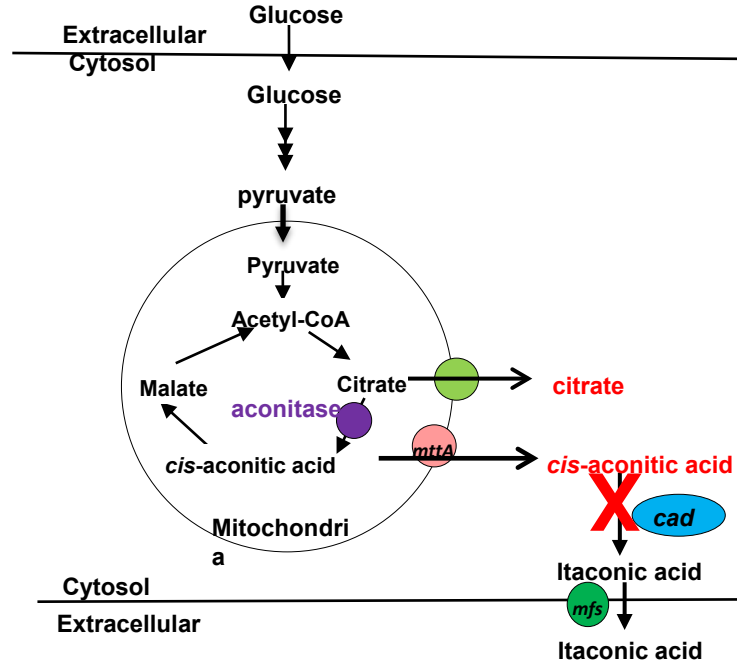
Organic Acid production in *A. pseudoterreus*

Wild type
(ATCC32359)



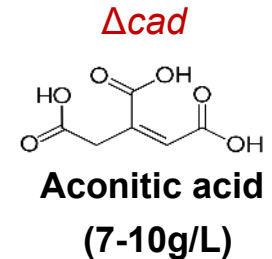
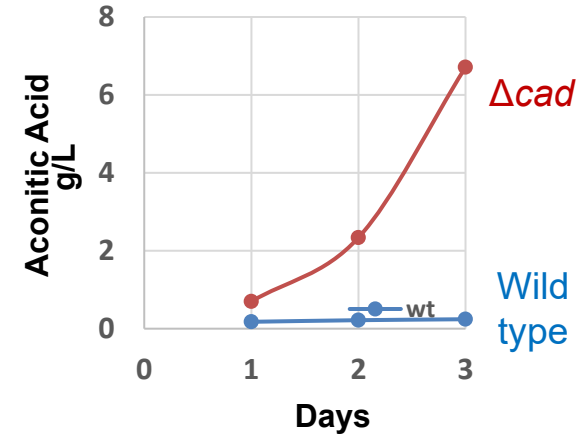
Native product:
Itaconic acid
(50g/L)

Key enzyme: ***cad*** =
cis-aconitate
decarboxylase



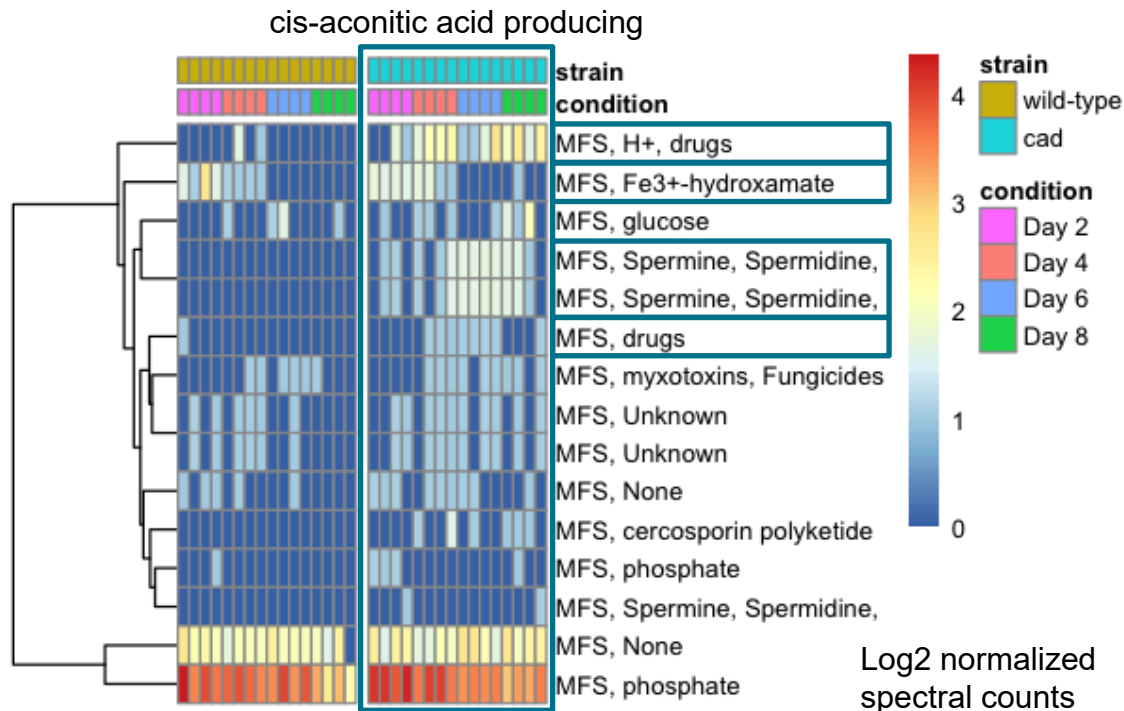
Question: Why is the itaconic acid titer in WT much greater than aconitic acid titer in Δcad ?

Hypothesis: Transport of aconitic acid across the plasma membrane is limiting.

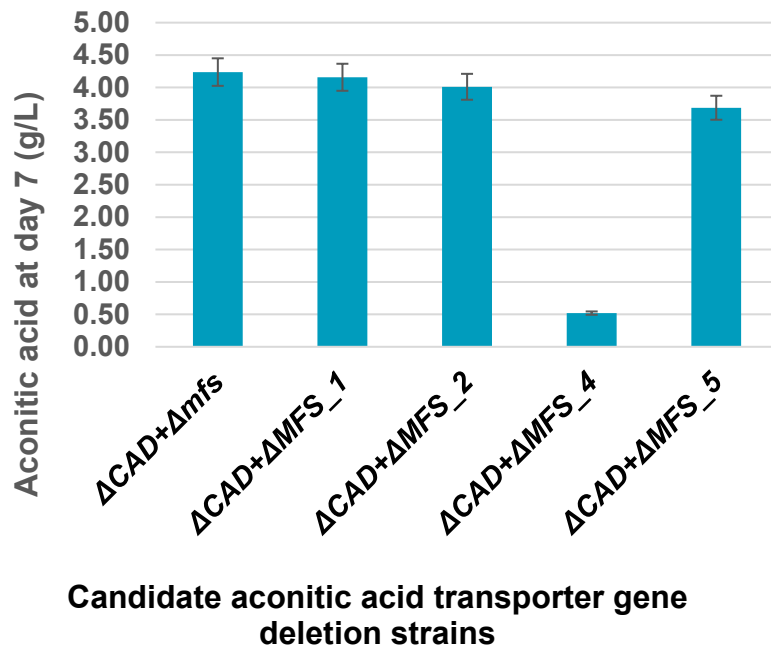


Potential *cis*-aconitic acid transporters

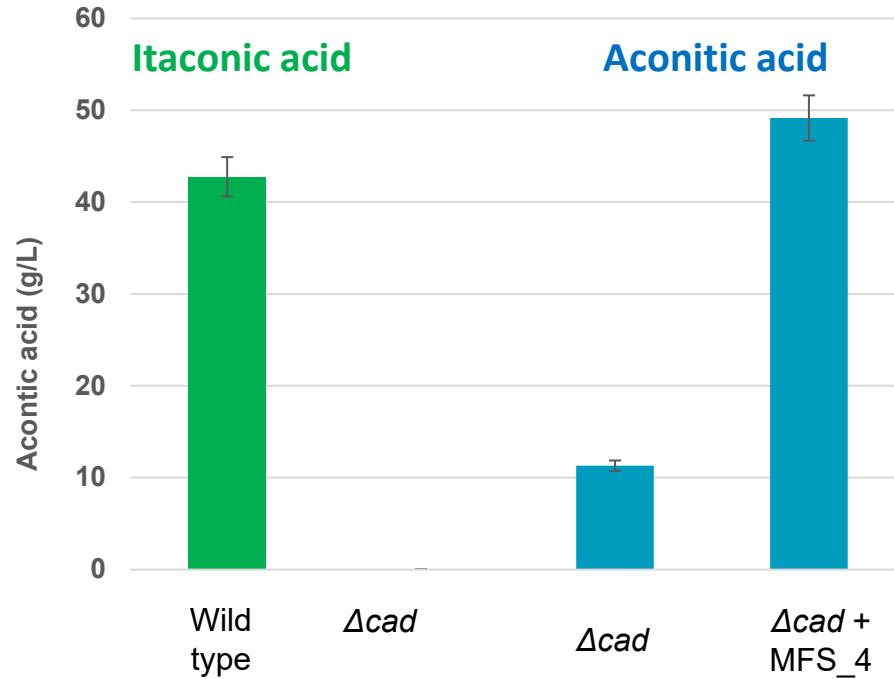
- Comparative **Discovery Proteomics** Analysis: ID'd **MFS transporters** upregulated in *cad* mutant vs. wild type (*cis*-aconitic acid producing and non-producing strains)
- Top 15 MFS transporters sorted by the difference of log2 normalized spectral counts between the wild-type and *cis*-aconitic acid producing (Δcad) strains are shown
- Most significantly changing MFS transporter genes were selected for deletion analysis



Deletion analysis objective: Identify potential aconitic acid transporter



Transporter gene *MFS_4* overexpression Effect on aconitic acid titer



4x increase in titer with
aconitic acid transporter
MFS_4 over-expressed

A. pseudoterreus Aconitic Acid Production

Transporter Identification

- Discovery/global proteomics with analysis in a genomic context
 - Helps identify pools of gene candidates
 - Helps prioritize those candidates for more efficient analysis by labor intensive gene deletion & over-expression
- **General Theme: Transporters** are often crucial to TRY improvements and are poorly annotated

Acknowledgements: ABF Aspergillus and Test Teams & Collaborators

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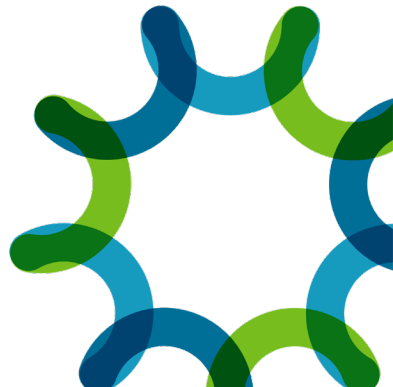
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Integrated Analysis: Techno-Economic and Life Cycle Assessment

ANL: P. Thathiana Benavides, Taemin Kim, Hui Xu
NREL: Bruno Klein, Ryan Davis



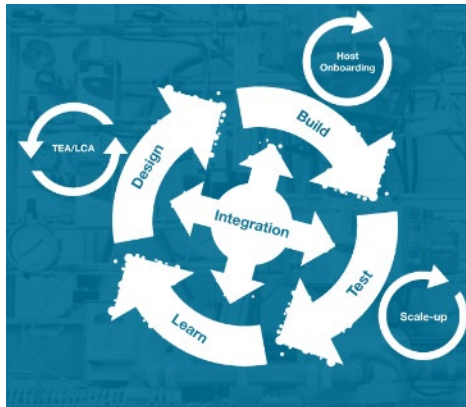
Introduction

The **Agile BioFoundry (ABF)** consortium goal: enable biorefineries to achieve **50% reductions** in time to **bioprocess scale-up** as compared to the current average of around 10 years by establishing a distributed Agile BioFoundry to productionize synthetic biology. <https://agilebiofoundry.org/>

Integrated Analysis team goal

- Help to quantify the ultimate **economic and environmental** sustainability potential for a given beachhead molecule/ product pathway of interest,
- Compare different products or synthesis routes to understand relative merits or drawbacks,
- Highlight key TEA/LCA drivers for prioritizing R&D focus areas

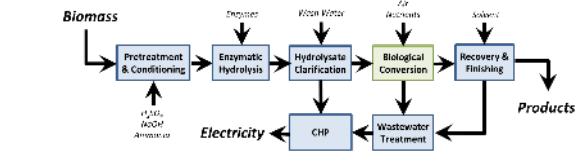
*Integration with the
DBTL cycle*



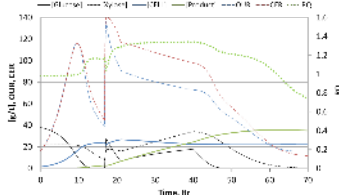
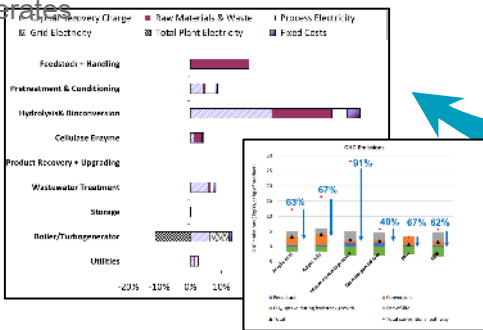
Techno-economic and life cycle assessment approach

1) Conceptual process is **formulated or refined based on current research** and expected chemical transformations. Process flow diagram is synthesized.

2) Individual unit operations are **designed and modeled using experimental data**. Process model outputs are used to size and cost equipment.



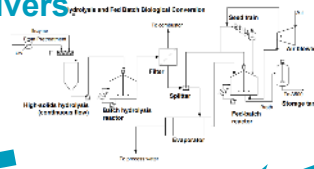
4) Results and **new understanding is fed back** into step 1) and the process iterates.



3a) Capital and operating costs are input into an economic model to **identify the major cost drivers**.



GREET
LIFE-CYCLE MODEL

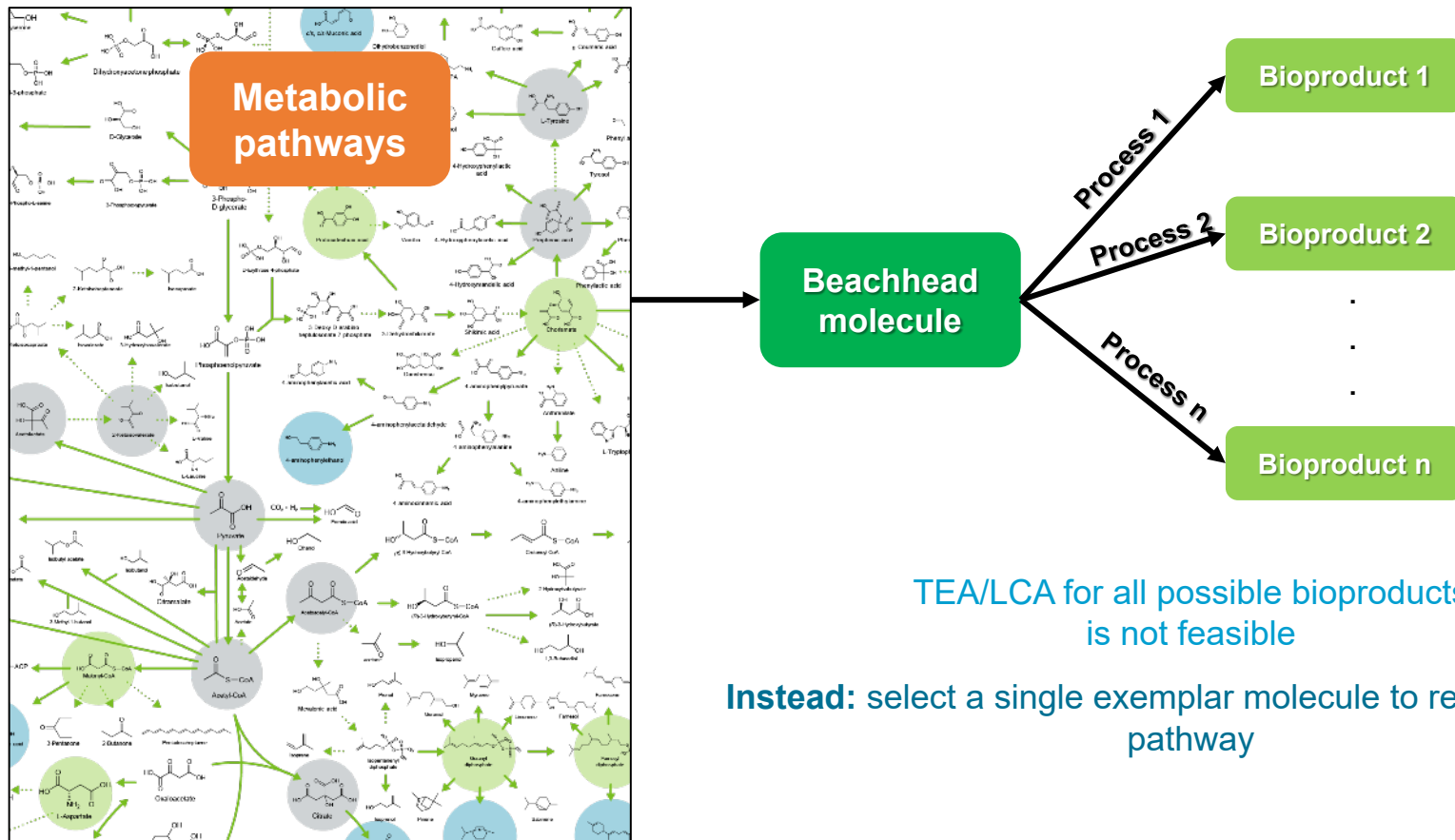


3b) Material and Energy flows are input into a life cycle model to **identify the major sustainability drivers**.

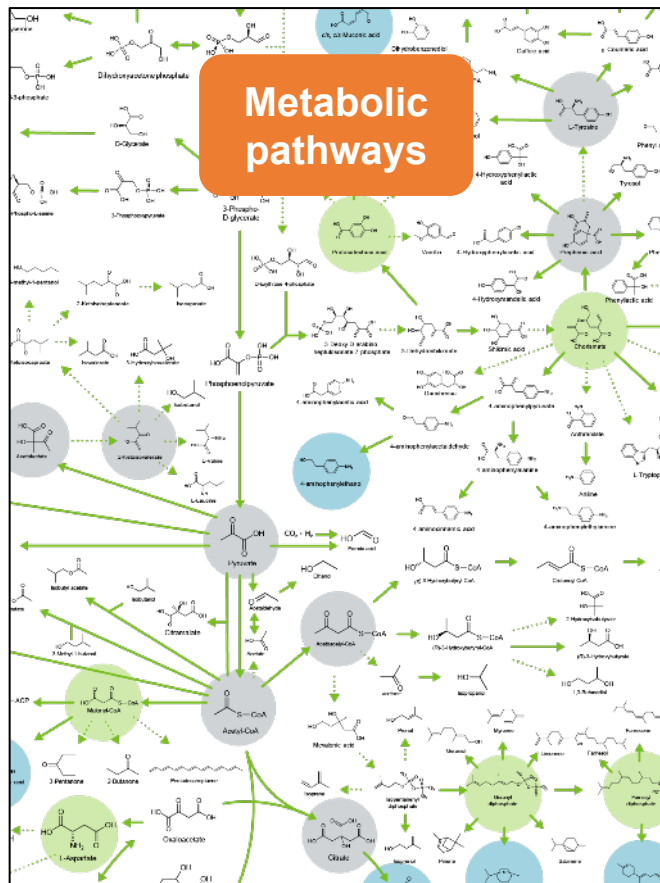
TEA
Minimum selling price
\$/kg

LCA
GHG emissions
kg CO₂e/kg

Assessment of beachhead intermediates



Assessment of beachhead intermediates



Beachhead molecule

Exemplar molecule

Similar processing parameters

- T/R/Y
- Downstream
- Aeration
- ...

Fatty acids Isoprenoids Organic acids
Shikimate-derived compounds PHAs
Polyketides Flavonoids others

Strategy for exemplar selection

Initial list of products from a given beachhead

Product A
Product B
Product C
Product D

Methodology is used to select exemplar molecules to represent beachhead in cases without a clear exemplar already identified

Scan for commercial viability

Product A
Product B
~~Product C~~
Product D

Evaluate candidate molecules

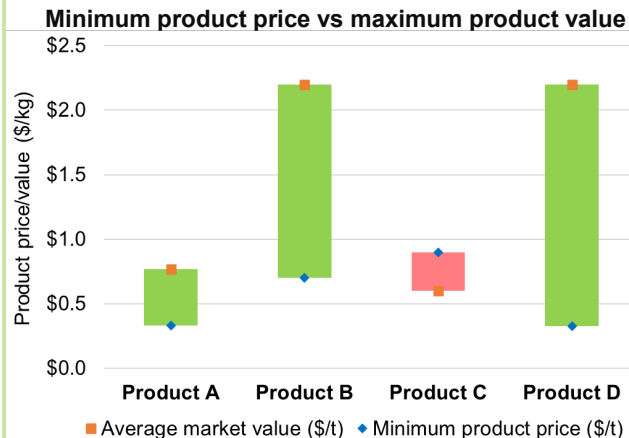
~~Product A~~
Product B
~~Product C~~
Product D

Gather further data for comparison

~~Product A~~
Product B
~~Product C~~
~~Product D~~

- Titer/rate/yield
- Aeration requirements
- Fermentation mode
- Separation strategy

Process modeling and full TEA/LCA for the exemplar



- Known TEA/LCA drivers
- Commercial/DFO interest
- ABF research relevance

Compare minimum product price (sugar costs + theoretical yields) vs market value

Identify promising economic margins

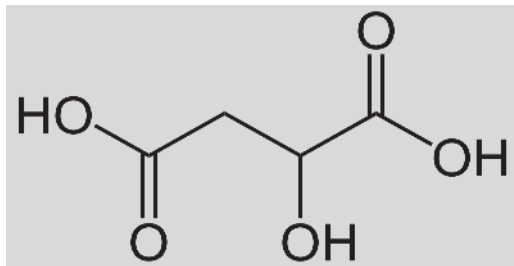
Case study: Malic acid

Background

- Currently produced from fossil sources at commercial scale
 - *Market price: ca. \$2.00/kg*
- Potential use as a precursor to maleic anhydride (global market of 1.5 MMT/year)

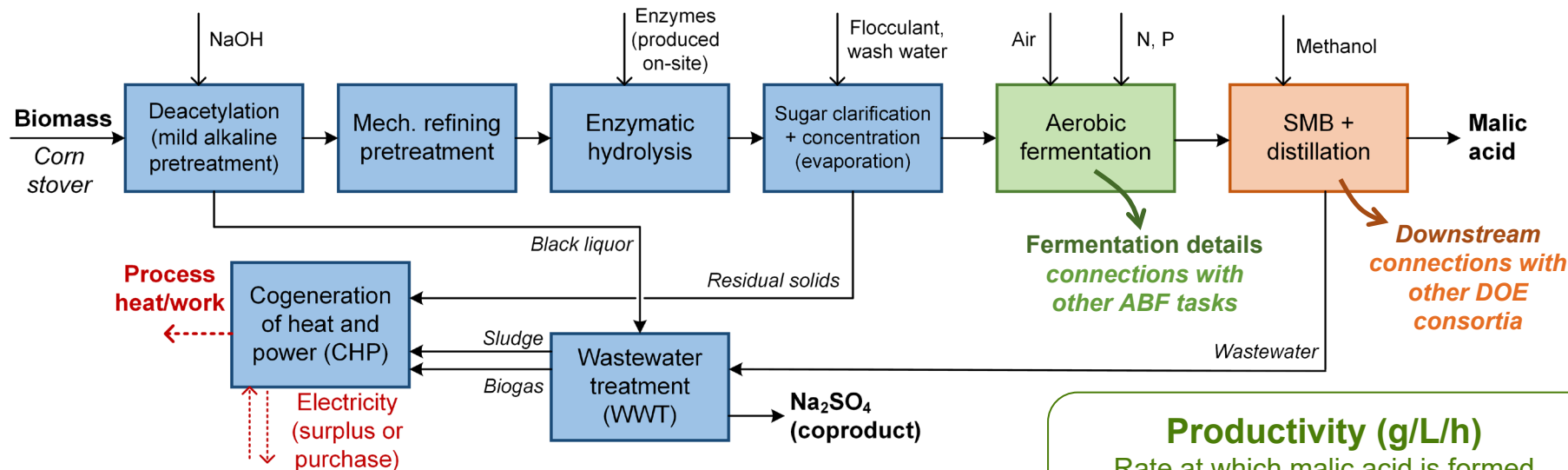
Bioprocess design

- **Beachhead molecule:** pyruvate
- **Microorganism:** *Lipomyces starkeyi*
- Downstream strategy: Simulated Moving Bed (SMB) adsorption followed by desorption with methanol



Malic acid

Design of integrated biorefineries



Main parameters and process configuration consistent with NREL's 2018 Biochemical Design Report: <https://www.nrel.gov/docs/fy19osti/71949.pdf>

Goal: Evaluate sensitivity drivers to key fermentation parameters (productivity, yield) over a range of achievable values towards impacts on MSP and GHG emissions

Productivity (g/L/h)

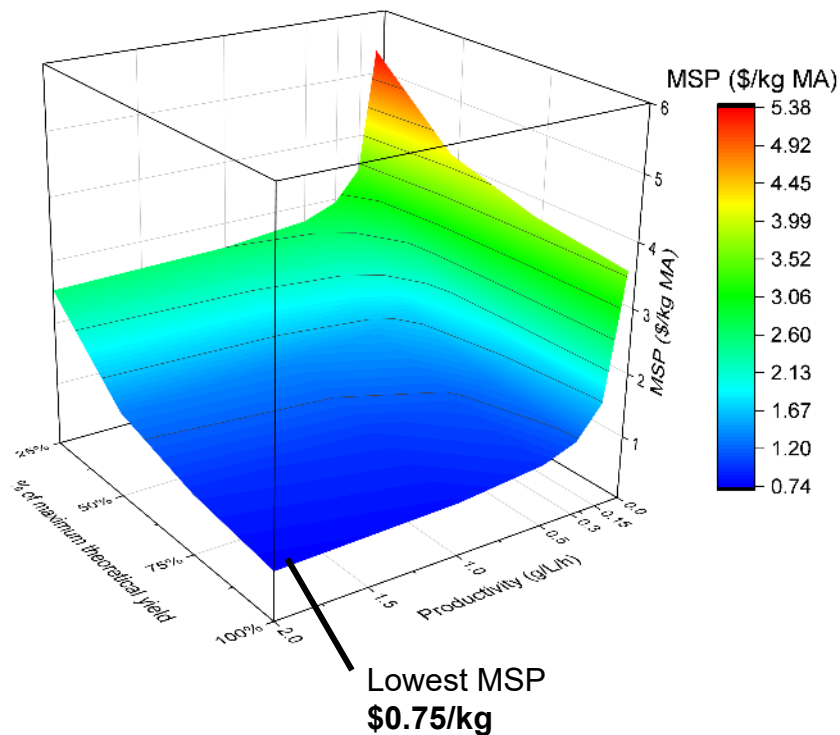
Rate at which malic acid is formed
Affects the # of fermentation vessels
Key parameter for TEA

Yield to product (%)

Calculated as a % of the maximum yield
Determines the output of malic acid
Key parameter for both TEA and LCA

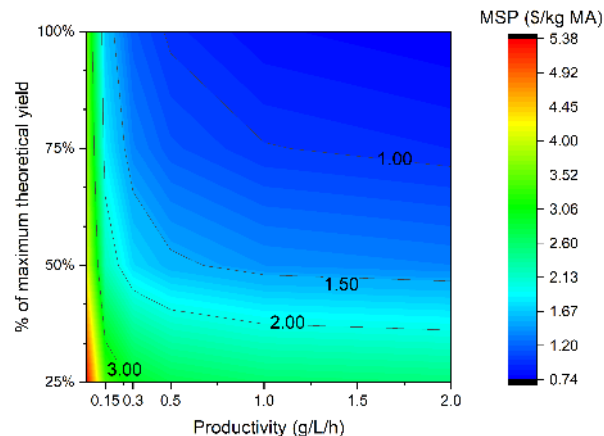
TEA of malic acid

MSP (\$/kg malic acid)



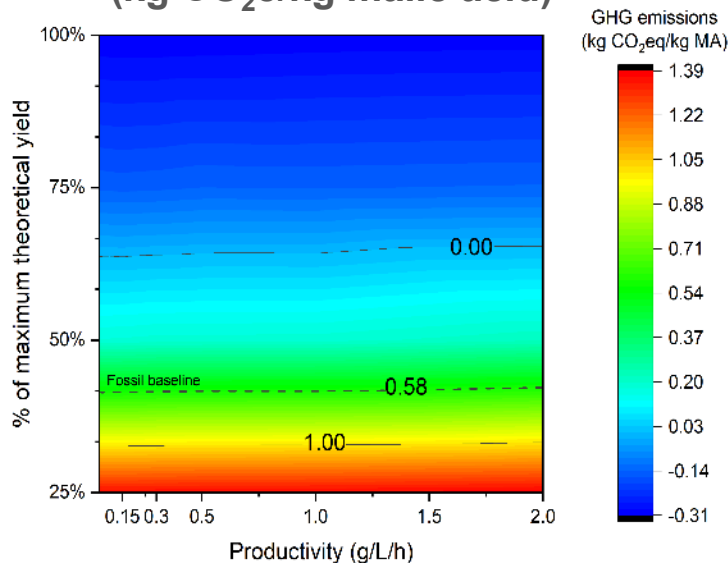
Reference market price: \$2.00/kg

- **Key driver:** productivity
- Several conditions can yield MSPs below the current market price for fossil malic acid
 - **Productivity > 0.3 g/L.h**
 - **Yield > 50% of max yield**

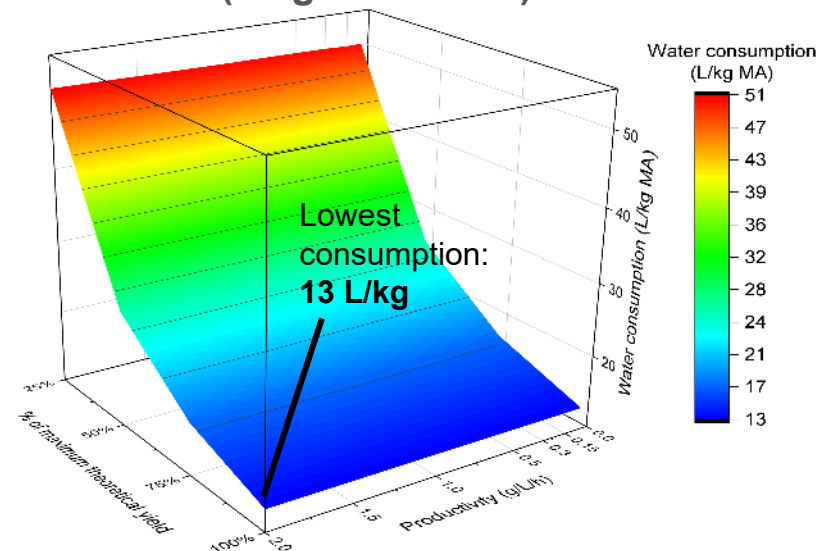


LCA of malic acid

GHG emissions
(kg CO₂e/kg malic acid)



Water consumption
(L/kg malic acid)



- Yield is the dominant parameter determining environmental impact metrics
- GHG emissions and water consumption decrease as yield improves, insignificant impact of productivity
- At about 42% theoretical yield, bio-based malic acid emits less GHG than its fossil-based counterpart
- Although water consumption decreases for higher yields, it is always higher than that of fossil-fuel derived malic acid



Thank you!

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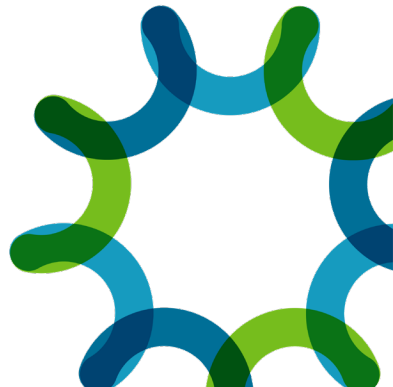
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Future Directions

Nathan J. Hillson
njhillson@lbl.gov

Lead PI, DOE Agile BioFoundry

ABF Webinar
April 29, 2022



Future directions (representative / pending)

• FY23

- Capabilities development (software, automation, AI/ML, analytical methods)
- Efforts supporting predictive scale-up/scale-down
- Identification of new non-model fungal/yeast strains for potential onboarding
- Collaborations with BETO consortia/projects (e.g. FCIC, BOTTLE, PABP, SepCon)
- TEA/LCA prioritization / assessments of activities supporting DOE decarbonization goals
- Emphasis on DEI (including MSRDC funding opportunity)

• FY24

- Engineering biology cycle efficiency assessment
- TEA/LCA identification of additional high-impact process targets
- Development of additional beachheads prioritized by TEA/LCA
- Development of dynamic beachhead map web application

• FY25

- 50% acceleration of bioprocess development timelines
- Industry adoption of ABF onboarded hosts
- Completed transition to 50:50 internal:collaboration project activities

This project is a part of the



A consortium of seven national laboratories
dedicated to accelerating biomanufacturing

