Target-Host Engineering

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BETO Peer Review 2021

Technology Session Review Area: Agile BioFoundry

March 9th, 2021

Project overview

- **ABF Primary Goal:** Enable biorefineries to achieve 50% reductions in time to scale-up compared to the current average of around 10 years by establishing a distributed Agile BioFoundry that will productionize synthetic biology.

- **Target-Host Engineering Goal:** Develop hosts to efficiently, cost-effectively, and sustainably produce beachhead and exemplar pairs to aid synthetic biology commercialization

- **Heilmeier Catechism framing:**
  - **Goal:** Accelerate synbio through development of metabolic beachheads
  - **Today:** Most work done in model systems for a specific target, not for parts of metabolic space in non-model hosts
  - **Important:** This will directly aid bioeconomy commercialization efforts
  - **Risks:** Judicious molecule and host selection, not achieving TRY goals that meet economic and sustainability goals

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:

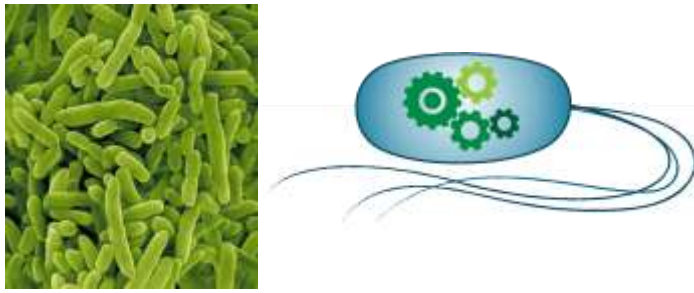
- Increased strain performance to exemplary targets via DBTL
- Use this system to improve DBTL approach
- Further develop robust, industrially relevant hosts
- Developing relevant datasets for Learn team

Relevance:

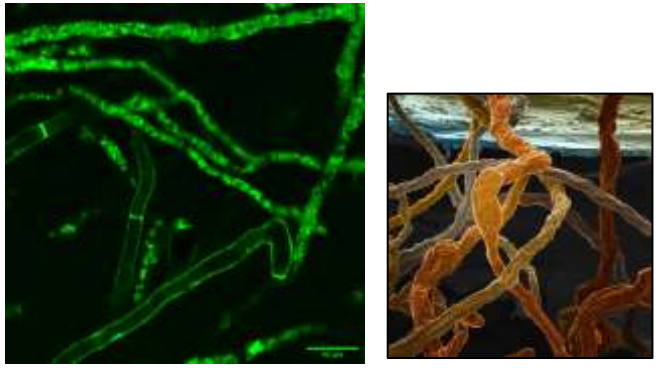
- Benchmark DBTL cycle performance and improvement across scales with real-world substrates and process configurations
- Information from DBTL and Integration efforts will be critical to predictive scale-up and scale-down

Management

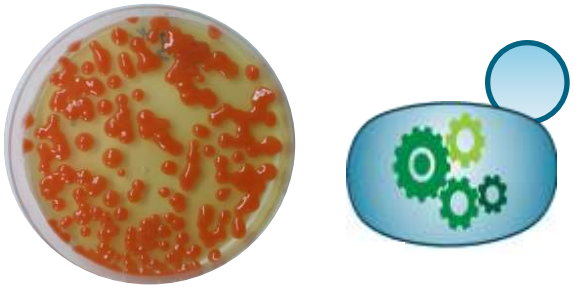
- **Team management:**
  - Leads: Bacteria (NREL), filamentous fungi (PNNL), and yeast (SNL)
  - Contributions from all labs to all teams
  - Members from IA, PISU, HOD, and DBTL to ensure efficient collaboration
- **Team meetings:**
  - Weekly: bacteria, filamentous fungi, and yeast
  - Rotating: ABF Task Lead call
- **Project risks and mitigation:**
  - BH selection – collaborate with other BETO projects and industry
  - TRY goals – three-pronged strategy ensures that at least one track will meet TRY goals



Pseudomonas putida KT2440



Aspergillus niger

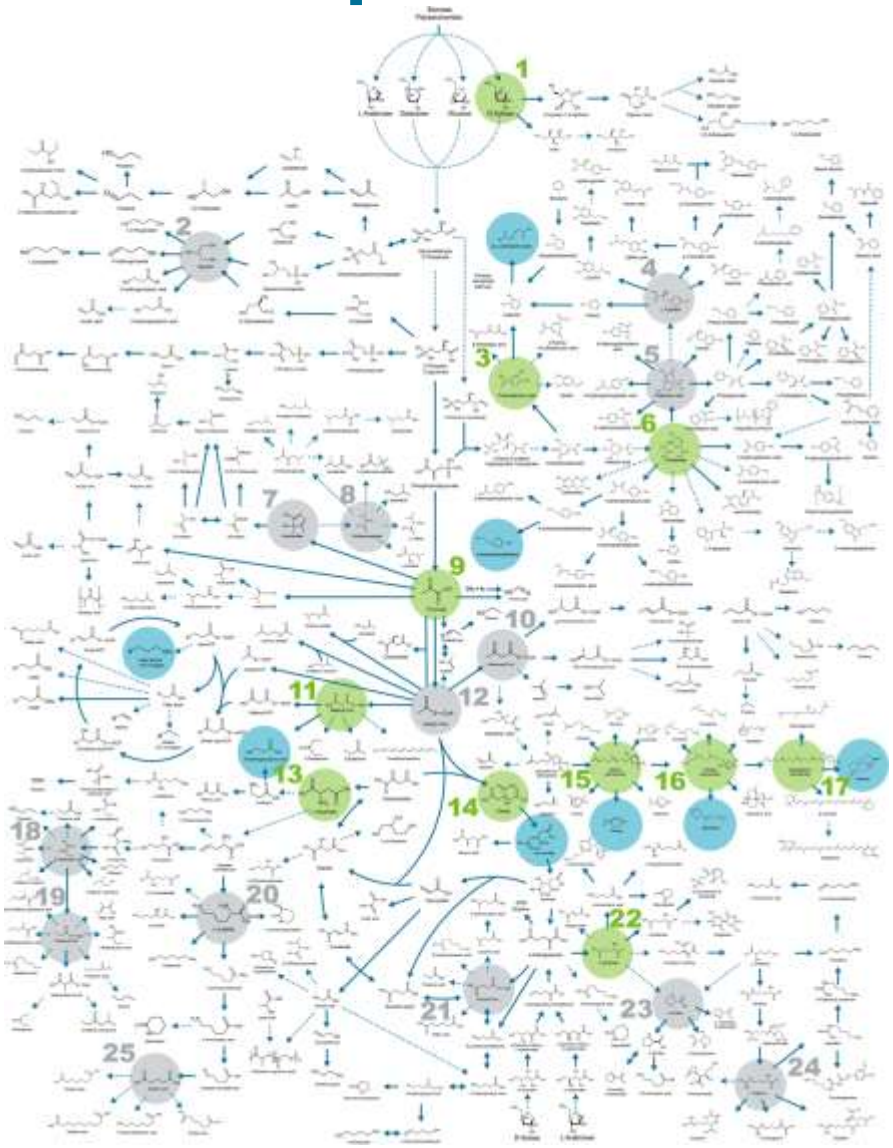


Rhodosporidium toruloides

Approach: Beachhead-exemplars

Milestones

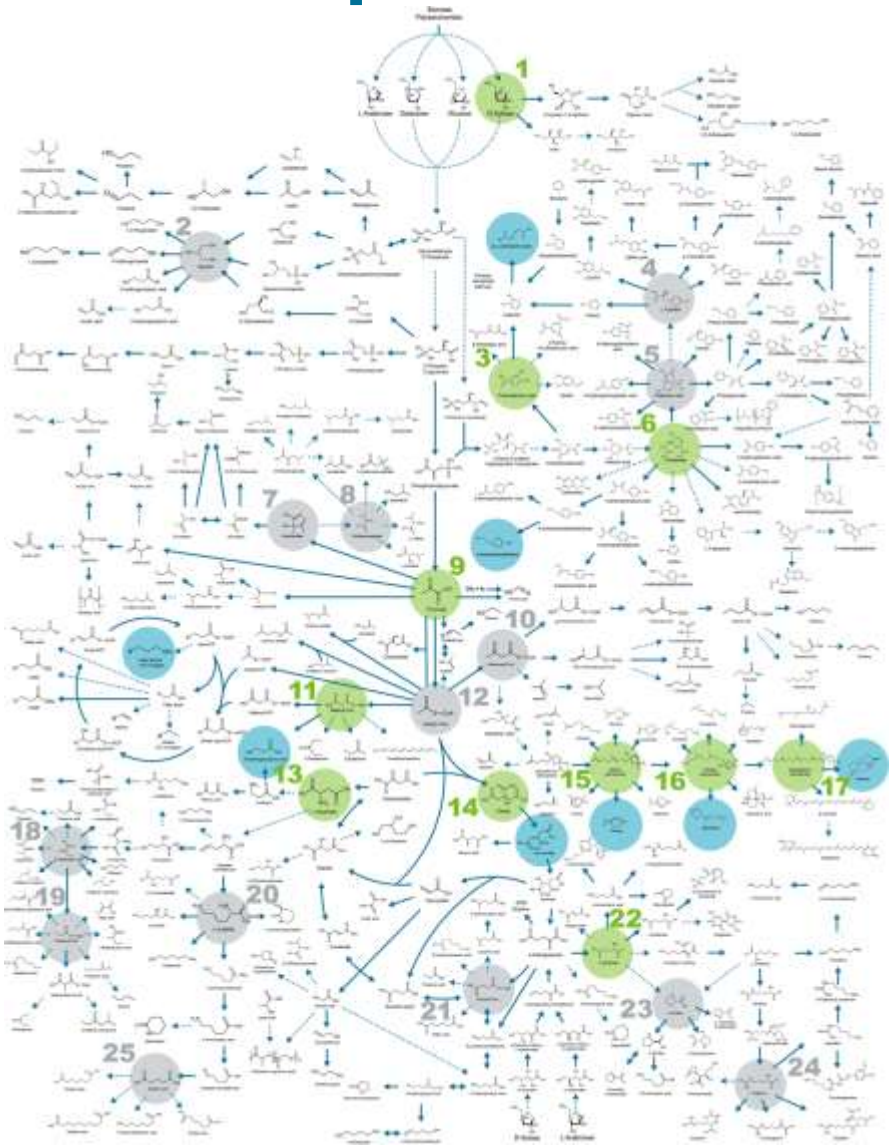
- FY20: Demonstrate an exemplar at a TRY of 20 g/L, 0.3 g/L/hr, and 50% of theoretical yield, either from DMR-EH hydrolysate or a mock hydrolysate containing hexose and pentose sugars
- FY21: 40 g/L, 0.5 g/L/hr, 60% of theoretical yield
- FY22: 80 g/L, 1 g/L/hr, 70% of theoretical yield
- TRY targets established and refined by TEA
- We use G/NG milestones based on achieving ≥1 g/L product titers for new BH-exemplars



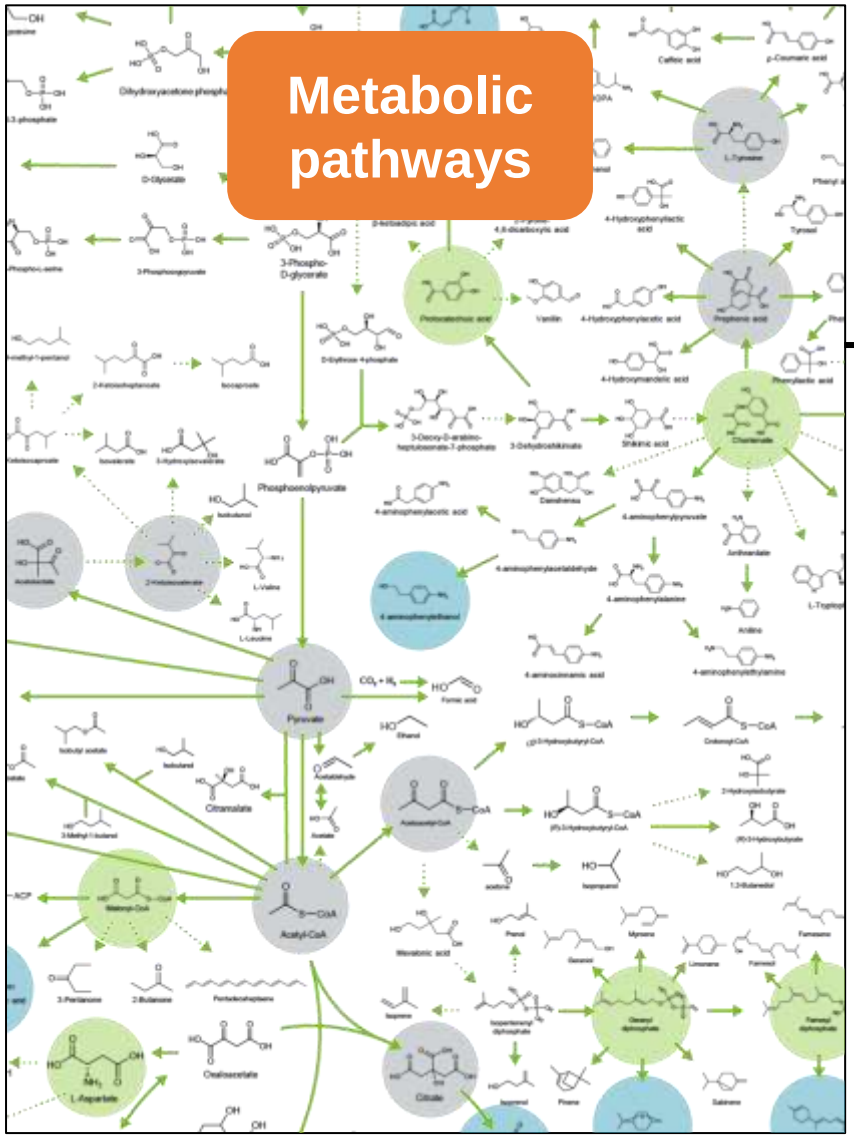
Approach: Beachhead-exemplars

Molecule selection

- Focus on areas of metabolism of interest to the bioeconomy
- Work with other BETO projects (Co-Optima, SepCon, Performance-Advantaged Bioproducts) to select targets of interest to BETO
- Act on IAB input as to metabolic space of interest to industry
- Directed Funding Opportunities also inform these decisions



Approach: Beachhead-exemplars



Beachhead molecule

Process 1

Process 2

Process n

Bioproduct 1

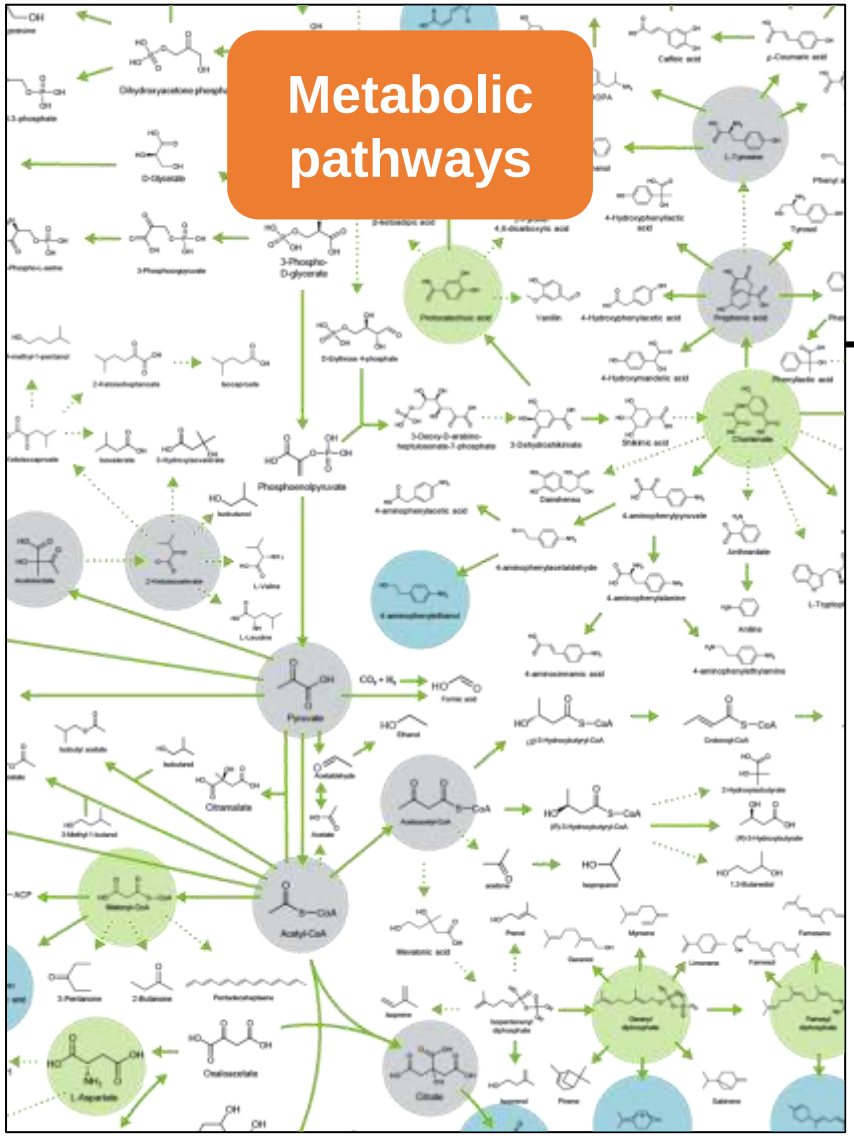
Bioproduct 2

Bioproduct n

TEA/LCA for all possible bioproducts is not feasible

Instead: select a single exemplar molecule to represent each pathway

Approach: Beachhead-exemplars



Beachhead molecule

Exemplar molecule

Similar processing parameters

- T/R/Y
- Downstream
- Aeration
- …

Objective: build a library of TEA/LCA metrics in several metabolic spaces

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

Approach: Beachhead-exemplars

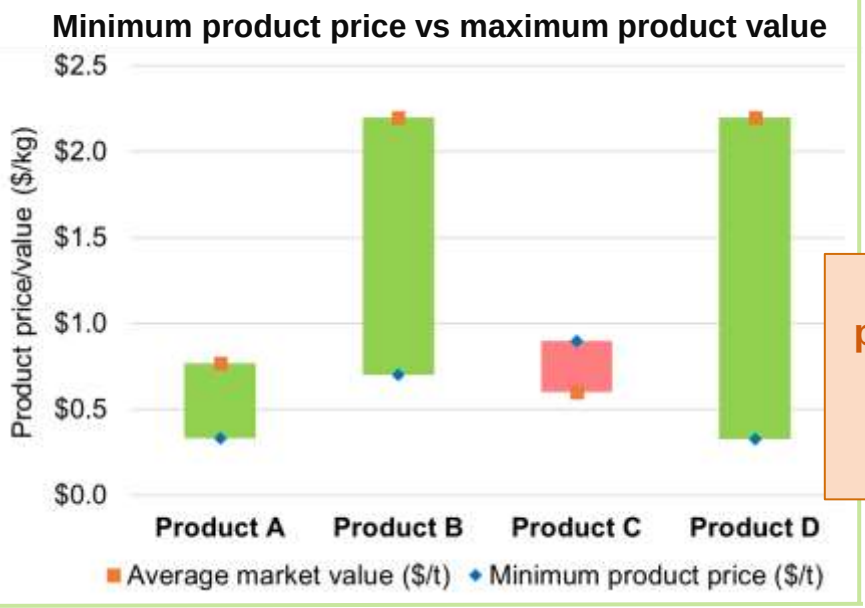
Initial list of products from a given beachhead

Product A
Product B
Product C
Product D

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

Scan for cost opportunities

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



Evaluate candidate molecules

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

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

Gather further data for comparison

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

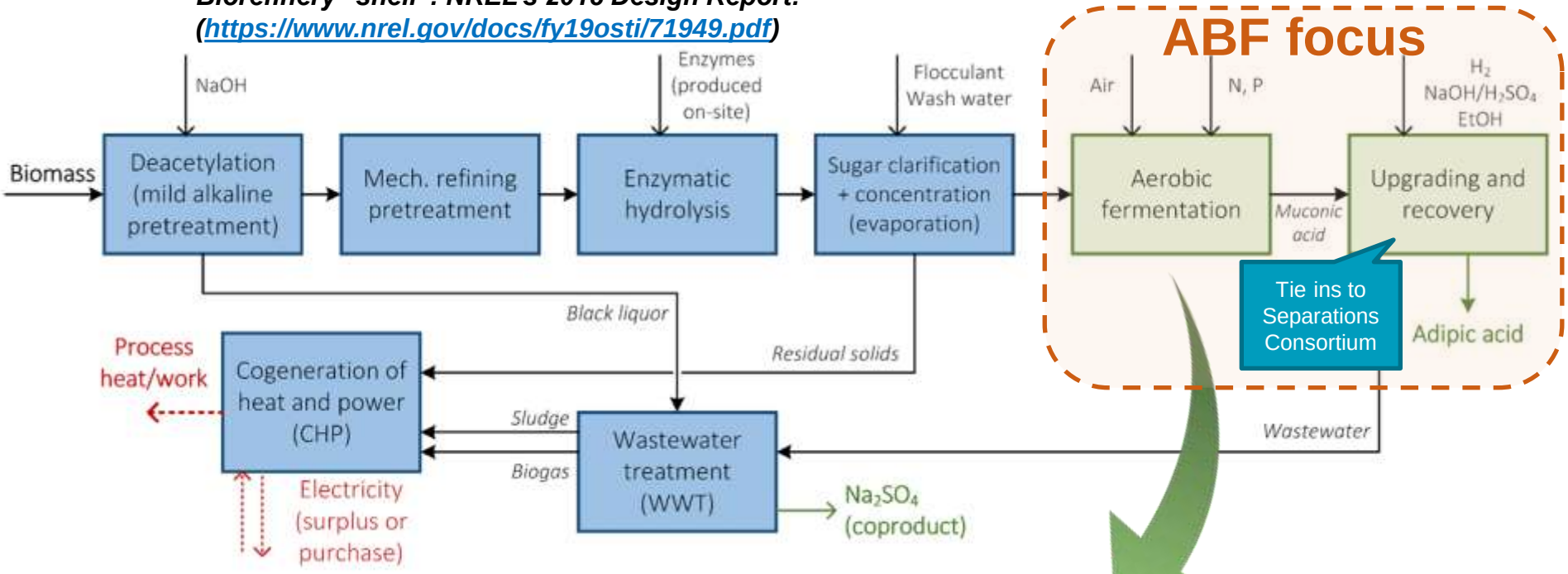
- Titer/rate/yield
- Aeration requirements
- Fermentation mode
- Separation strategy

Compare min. product price (sugar costs + theor. yields) vs market value
- **Search for promising economic margin**

Process modeling and full TEA/LCA for the exemplar

Approach: Process Modeling

Biorefinery "shell": NREL's 2018 Design Report:
(https://www.nrel.gov/docs/fy19osti/71949.pdf)



Biomass growth $\text{Glucose} + 0.28\ NH_3 + 1.18\ O_2 \rightarrow$ **4.8 M.O.** $+ 1.2\ CO_2 + 2\ H_2O$

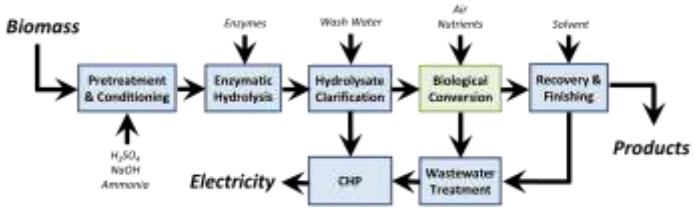
Muconic acid production $\text{Glucose} + 1.94\ O_2 \rightarrow$ **0.74 M. Acid** $+ 1.57\ CO_2 + 3.8\ H_2O$

Stoichiometries set by metabolic flux analysis from researchers

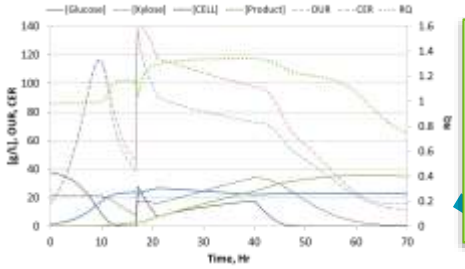
Evaluate sensitivity drivers to key fermentation parameters (rate, yield) over a range of achievable values towards impacts on MSP and GHG emissions

Approach: TEA and LCA

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

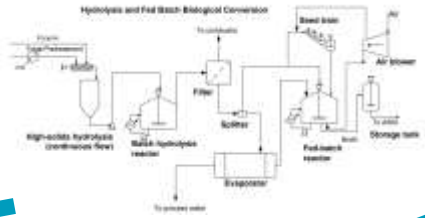


2) Individual unit operations are **designed and modeled using experimental data**. Process model outputs are used to size and cost equipment.



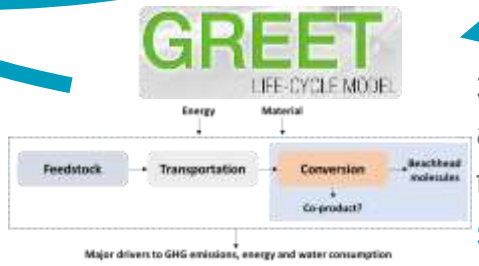
TEA:
- ✔ Yields (kg/tonne)
- ✔ Minimum selling price ($/kg)

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

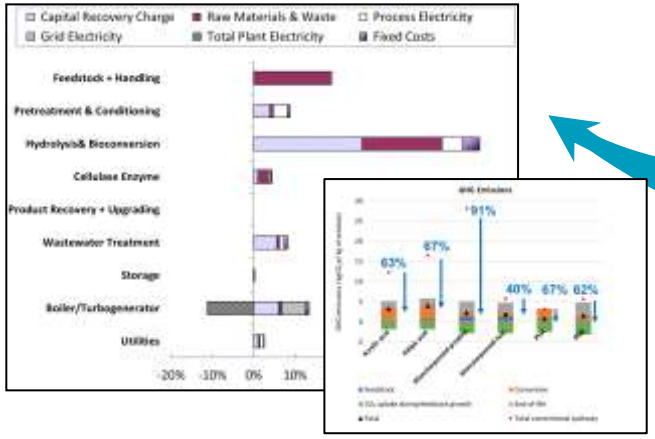


LCA:
- ✔ GHG emissions (kg CO_2e/kg)
- ✔ Fossil fuel consumption (MJ/kg)
- ✔ Water use (L/kg)

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



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



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Agile BioFoundry

Approach: Process integration and scale-up

Aim 1: Hydrolysate production

Approach:

- Deacetylation, mechanical refining, enzymatic hydrolysis at the pilot scale.
- Industrially relevant, contains C5/C6 sugars from real biomass, and is a new process relevant to the entire BETO portfolio
- Harnessing advantages in filtration at pilot scale to make clarified, concentrated sugars

Aim 2: Achieve TRY goals for BH-exemplar pairs

Approach:

- Conduct Test experiments from µL to kL scale
- Bioprocess development for DBTL/BH-exemplar activities

Impact

Scientific

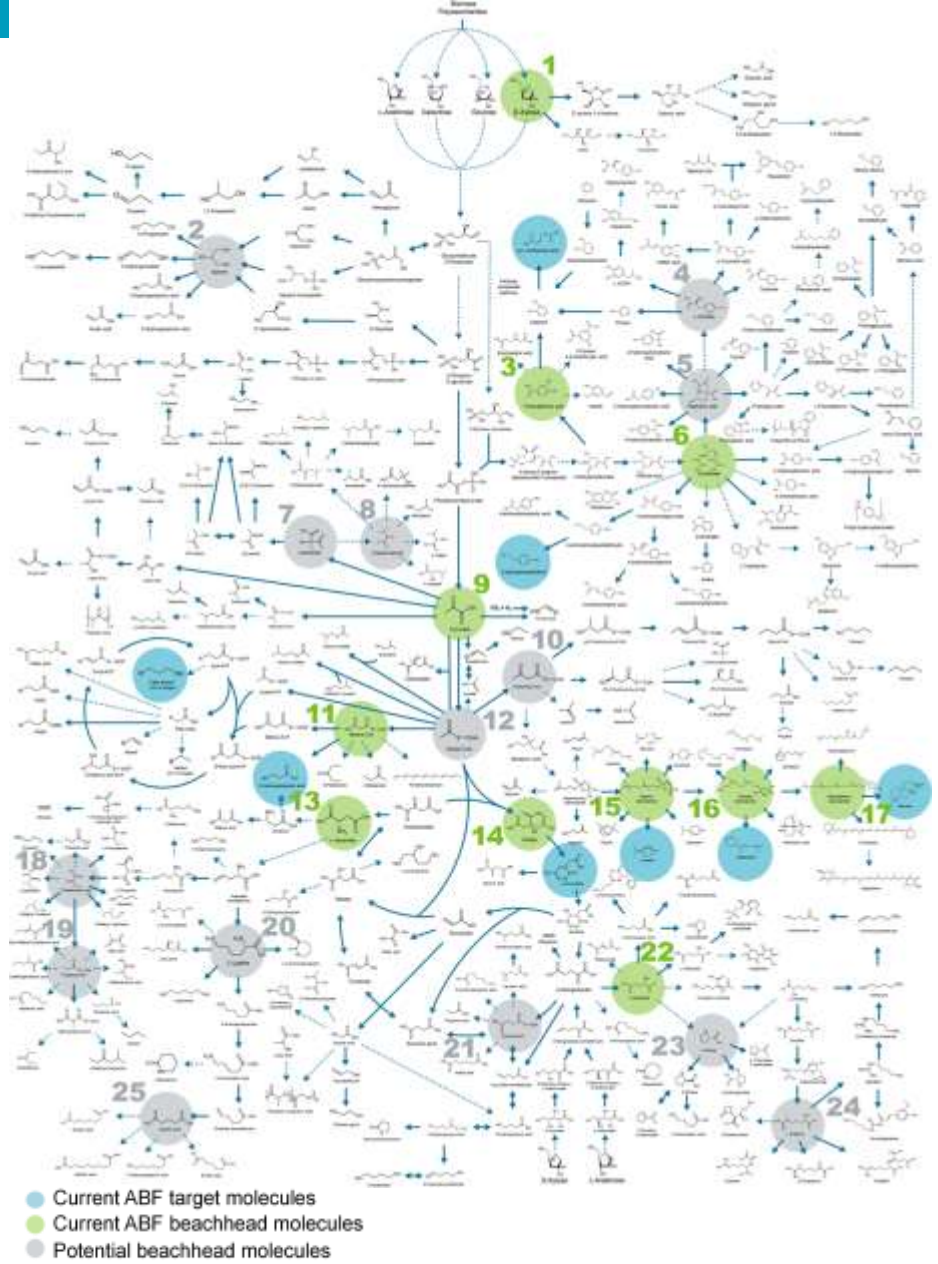
- Peer-reviewed manuscripts and patent applications reported in bacteria, filamentous fungi, and yeast

Industry

- Helping elevate *P. putida, A. pseudoterreus*, *R. toruloides* as chassis for industrial biotech. from carbohydrates
- Working with industry on these hosts via Directed Funding Opportunities and DOE FOAs

Overall

Our T-H Engineering efforts focus on developing BH-exemplar pairs for the community and demonstrating the ABF tools

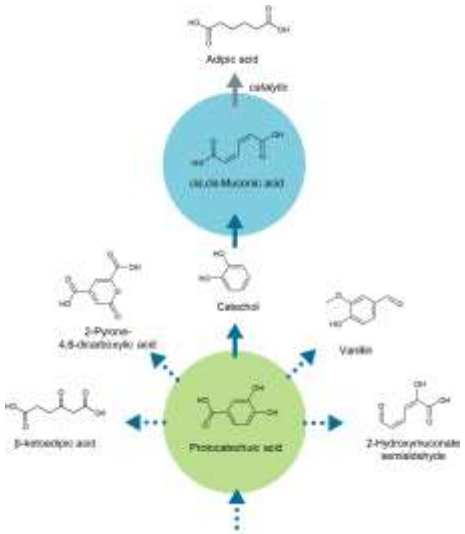


Adapted by permission from Springer Nature Customer Service Centre GmbH: Nature, Nature Calalysis, A comprehensive metabolic map for production of bio-based chemicals, Lee, S.Y., et al., © 2019

Target-Host Engineering outline

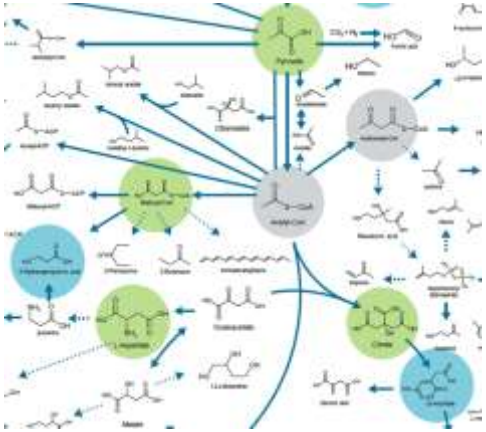
Bacteria:

- *Pseudomonas putida* KT2440
- Beachhead: protocatechuate
- Exemplar: Muconic acid
- Other hosts and targets being pursued, but will not be shown



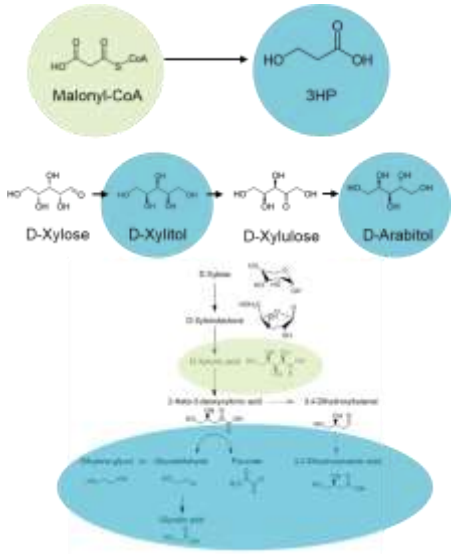
Filamentous fungi:

- *Aspergillus pseudoterreus*
- Beachhead: citric acid
- Exemplar: aconitic acid
- *Aspergillus niger*
- Beachhead: L-aspartic acid
- Exemplar: 3-hydroxypropionic acid
- Other hosts and targets being pursued, but will not be shown



Yeast:

- *Rhodosporidium toruloides*
- Beachhead: malonyl-CoA, xylose, xylonate
- Exemplar: 3HP, arabitol
- Other hosts and targets being pursued, but will not be shown



Each T-H Engineering talk will highlight impact for overall ABF goals as well

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Target and Host Engineering: Bacteria

Project overview

Context and History: Task initiated at the inception of the Agile BioFoundry

- *P. putida* KT2440-C6 diacids were first target-host pair from ABF pilot project
- Achieved Go decision in ABF pilot with muconate, ß-ketoadipate

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)
- Provide products to Performance-Advantaged Bioproducts projects

Heilmeier Catechism:

- Today: Few protocatechuate-derived products at scale
- Important: Offers PABP and direct replacements for large-scale chemicals
- Risks: Sufficient flux to muconate not able to be achieved for TRY goals

Project overview: Why *P. putida*?

Pseudomonas putida

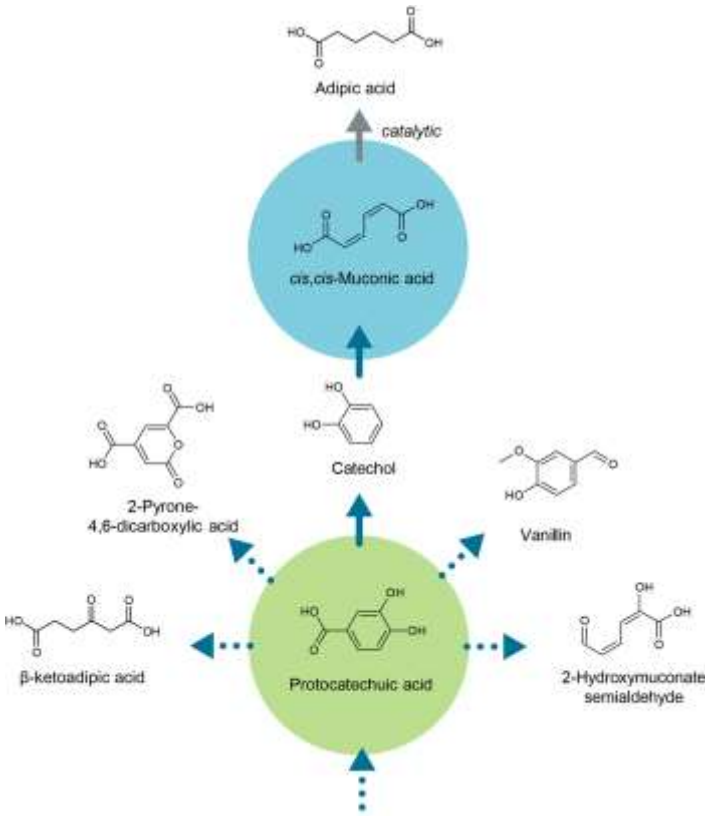
- Soil bacterium
- Gram-negative aerobe
- Fast growing
- Stress tolerant
- Metabolically versatile
- Genetically tractable

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 growth 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 bioproduct[1-3]

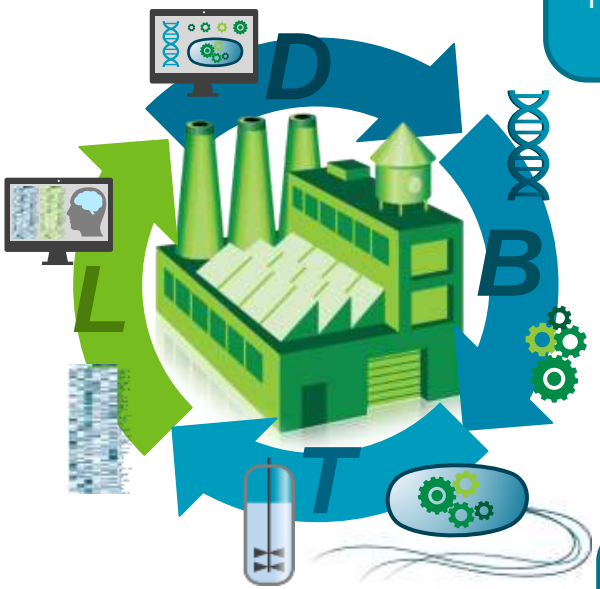
- **Beachhead molecule:** Protocatechuate
- **Host:** *Pseudomonas putida* KT2440



1. NA Rorrer *et al. ACS SusChemEng.* 2016
2. NA Rorrer *et al. Green Chem*. 2017
3. NA Rorrer *et al. Joule* 2019

Management

- **Virtual meetings:** weekly calls
- **F2F meetings**: ABF annual all-hands, during year as needed
- **Updates**: frequent team updates on task lead call, monthly DBTL tracking
- **Team Leads:** experts in fields of work in *P. putida*
- **Milestones:** product performance metrics
- **Project interfacing:** *ad hoc* meetings with IA, I&S, other BETO consortia
- **Risks**: Rate improvements potentially not attainable – leveraging full ABF tool suite to achieve the necessary flux to muconate



Gregg Beckham, Chris Johnson

Lead, DBTL tools, applications

Adam Guss

DBT applications, hydrolysate utilization

Nathan Hillson, Hector Garcia Martin

Build, Learn tools

Taraka Dale

In vivo biosensors, DBT tools, applications

Phil Laible

In vitro biosensors, Learn tools, applications

John Gladden

DBT applications

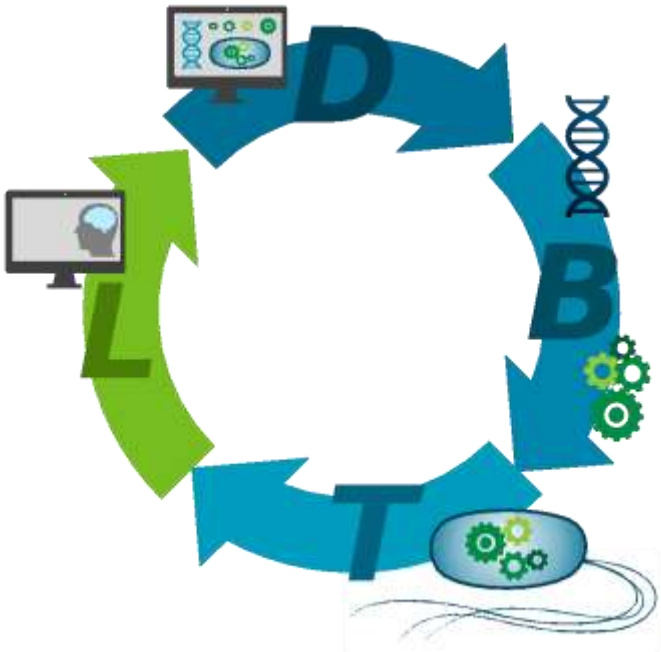
Jon Magnuson, Kristin Burnum Johnson

omics for Test

Approach

Challenges

- Deploying Learn tools in development in parallel with Learn-friendly Test experiments
- Flux achievements to obtain rate goals
- Salt and product toxicity at high muconate concentrations



Technical approach

- Employ ALE, biosensor-based screening, and metabolic modeling to overcome bottlenecks
- Use DBTL to identify rate and regulatory bottlenecks, off-target carbon sinks
- Employ advanced tools (e.g., biosensors) to increase DBTL strain generation efficiency
- On-board new Learn algorithms and co-design Learn-friendly Test experiments
- Conduct parallel bioprocess development in bioreactor cultivations to measure TRY and feedback information to DBTL team

Impact

Scientific

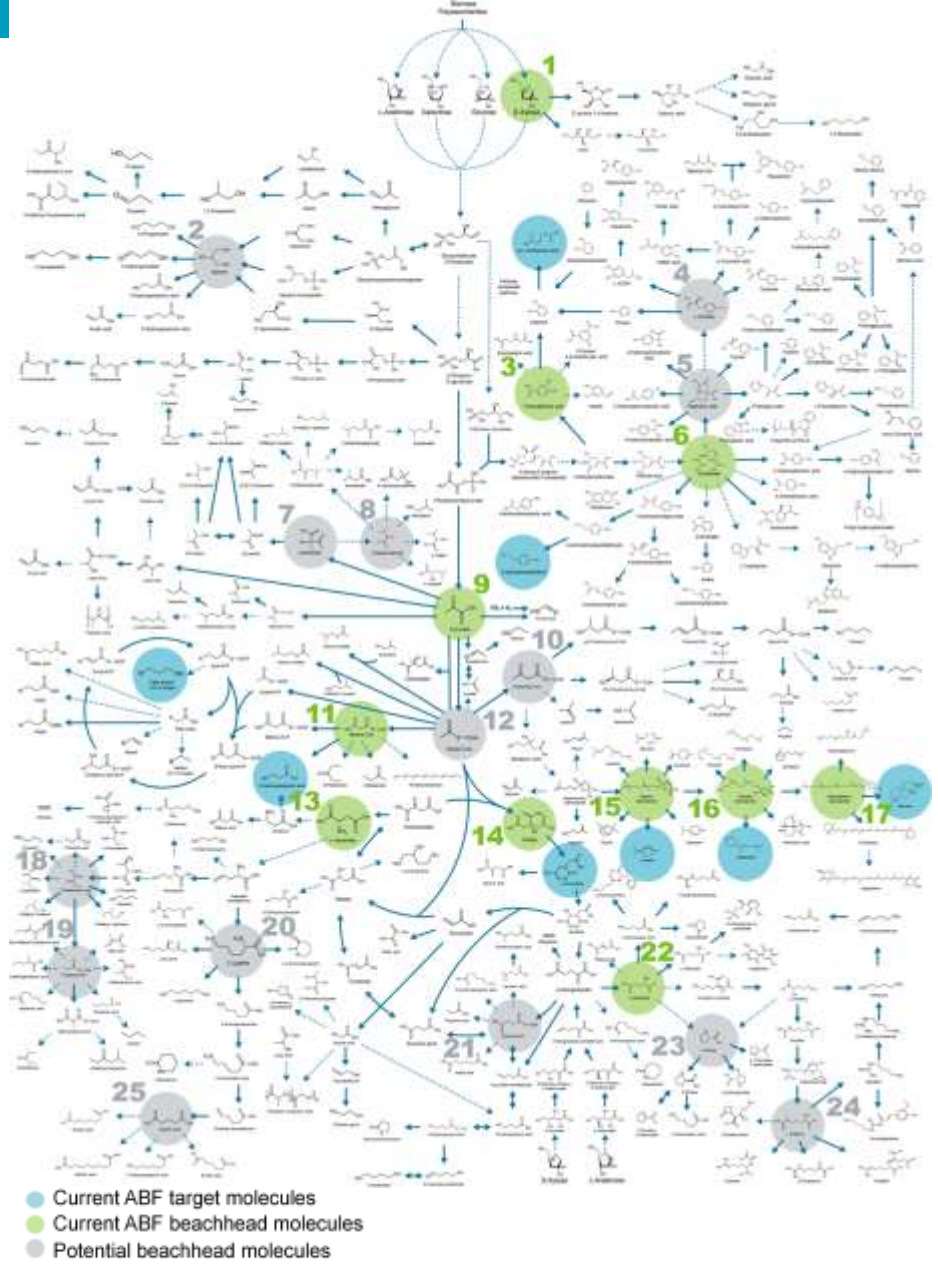
- Developed several hundred *P. putida* strains, reported in peer-reviewed publications and patent applications towards aromatic compounds (PCA)
- Helping test and improve ABF DBTL tools and overall ABF infrastructure – especially Build, Test, and Learn tools

Industry

- Helping elevate both 1) *P. putida* as a viable chassis for industrial biotechnology from carbohydrate feedstocks and 2) production of compounds near PCA

Overall

The ABF's T-H Engineering efforts focus on simultaneously developing BH-exemplar pairs for the community and demonstrating the ABF tool development efforts



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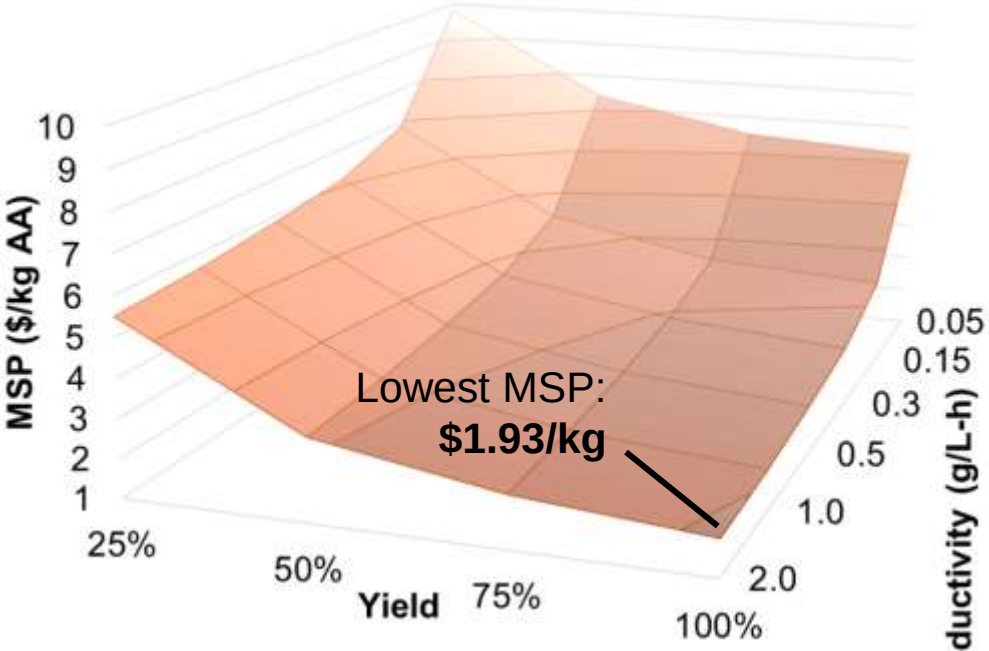
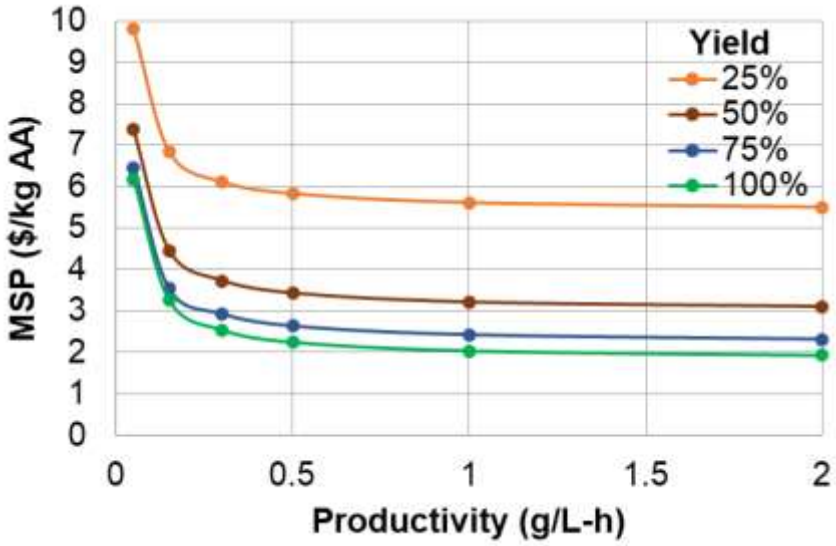
Progress and Outcomes

Muconic Acid TEA

- 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
- Strategies to further reduce MSP:
  - Lowering feedstock costs
  - Increasing biorefinery scale
  - Using lower-cost separation strategy
  - Adding value to lignin





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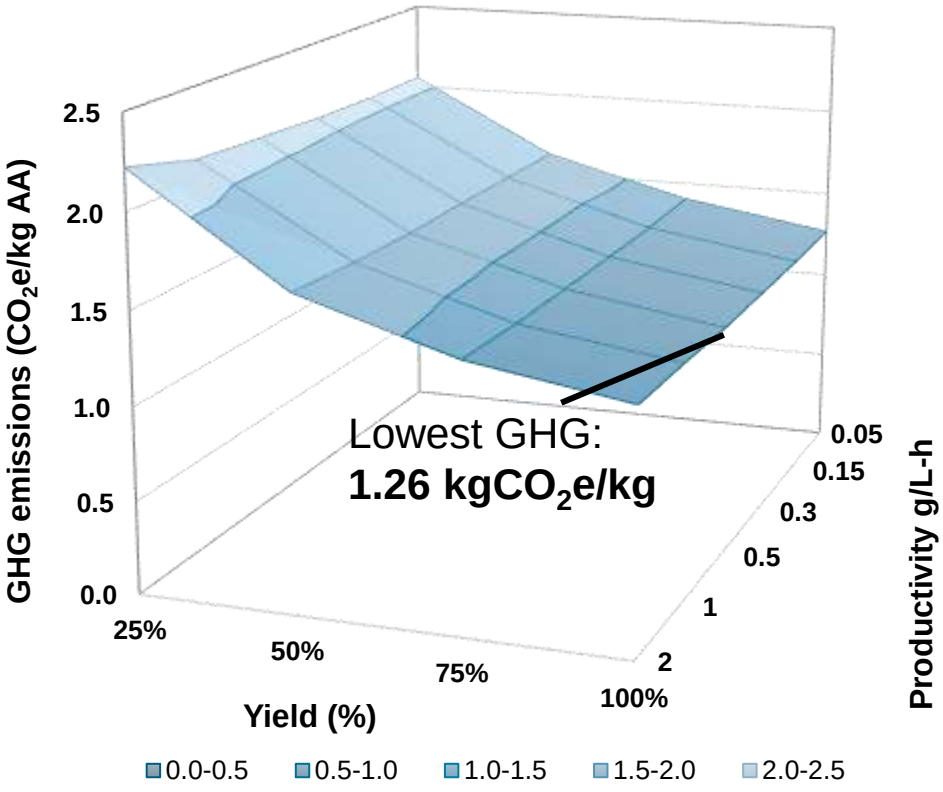
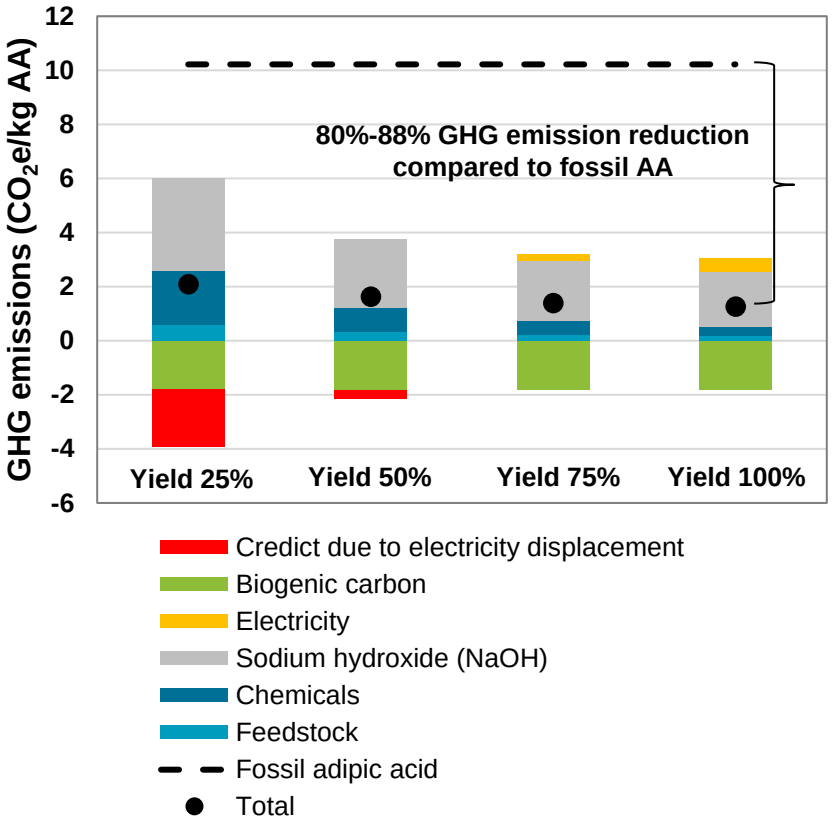
Muconic Acid LCA

GHG emissions of adipic acid



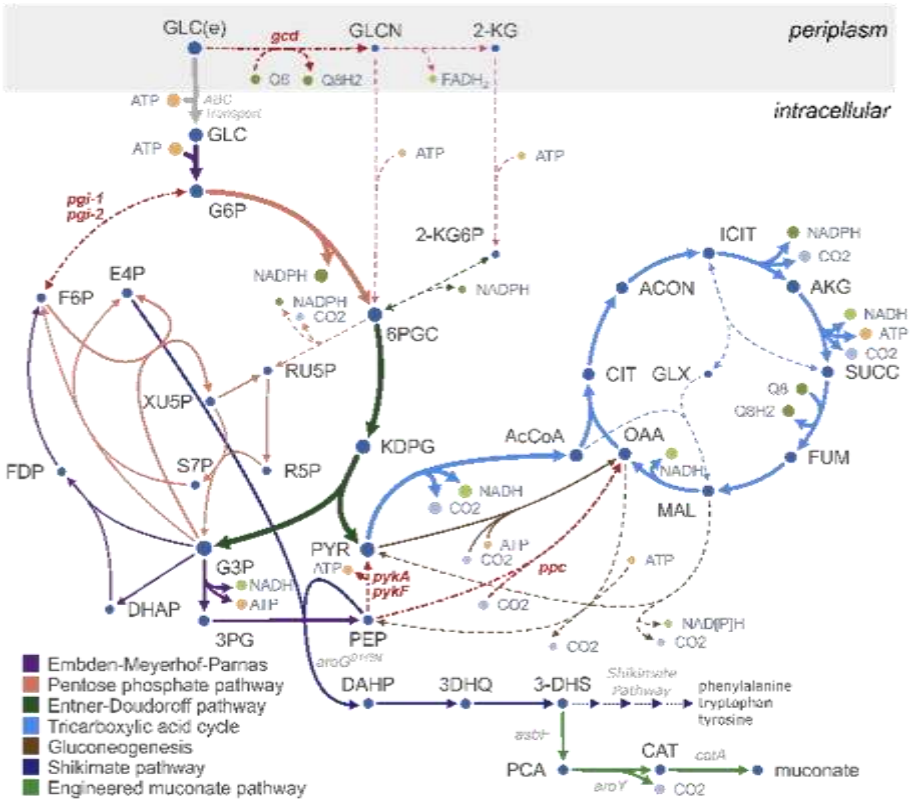
Productivity plays a considerably smaller role on LCA than it does on TEA

GHG emissions for Productivity: 0.5 g/L/h

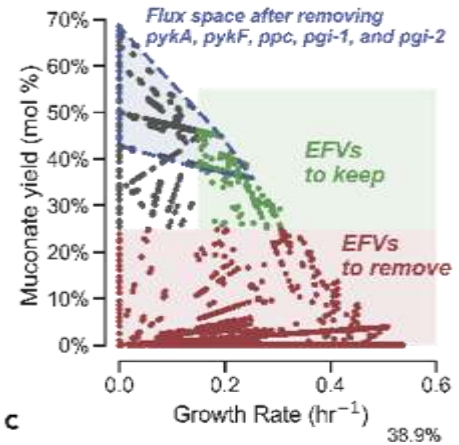


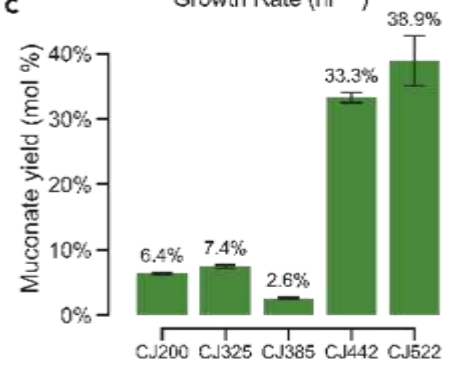
The lowest GHG emission value is obtained with the highest yield at different productivities (0.5; 0.3; 0.15)

Baseline strain for muconic acid production



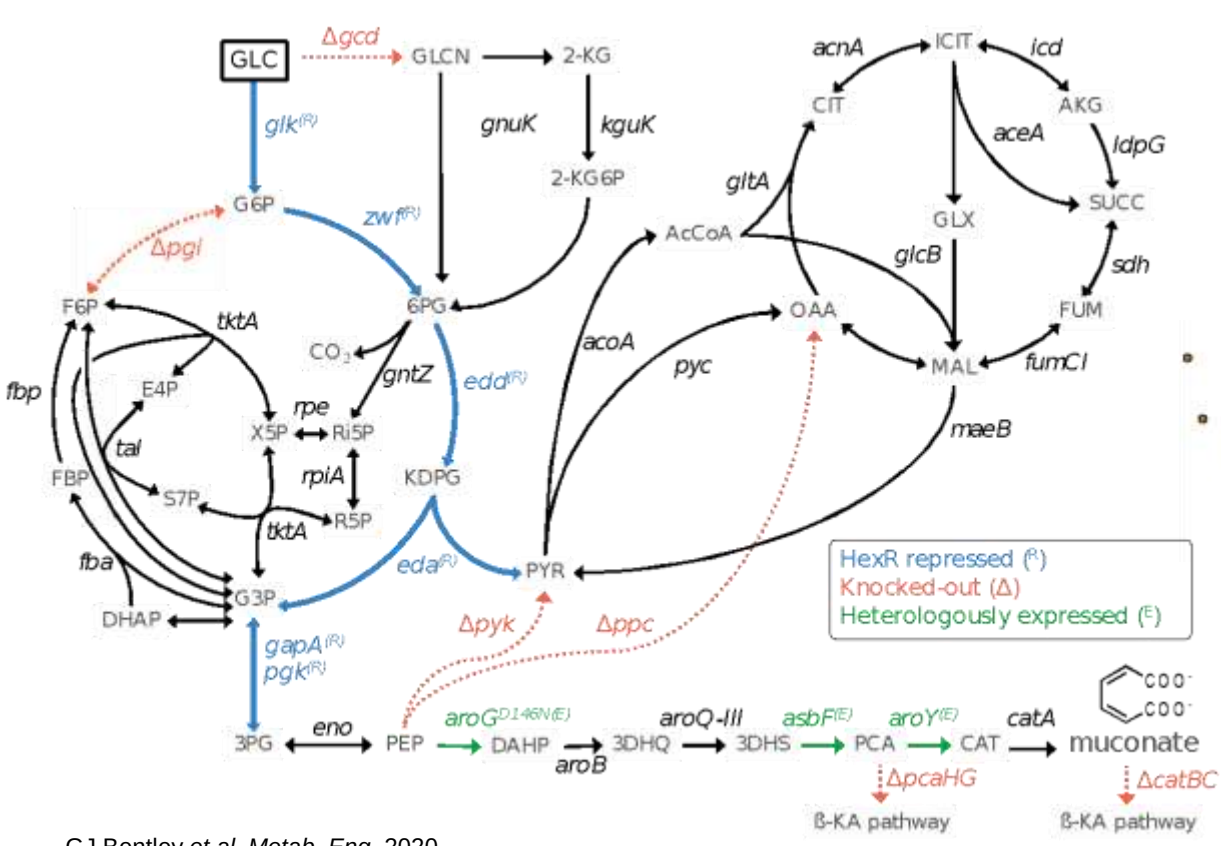
CW Johnson *et al., Joule* 2019

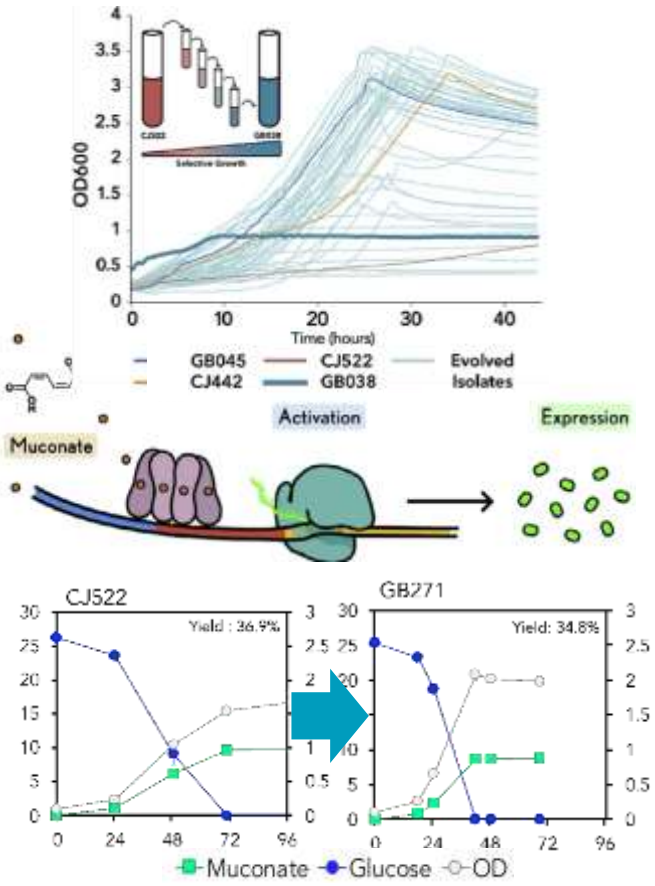




- Leverage pathway originally reported by Draths and Frost (*JACS*, 1999)
- Achieved a 39% molar yield of muconate from glucose
- **Outcome:** High-yield platform strain, but low rates from glucose

Regulatory bottlenecks to rate improvements

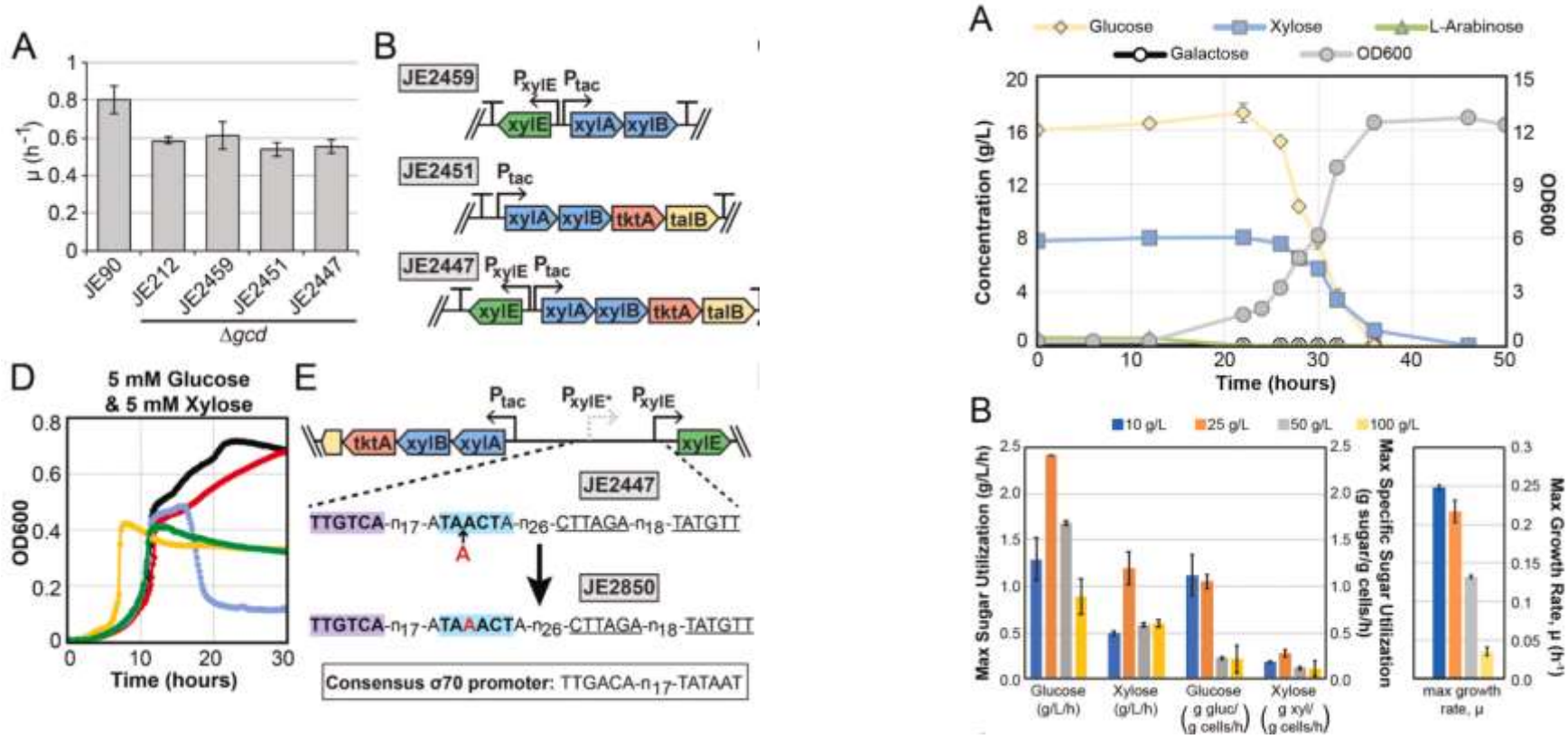




GJ Bentley *et al. Metab. Eng.* 2020

- 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

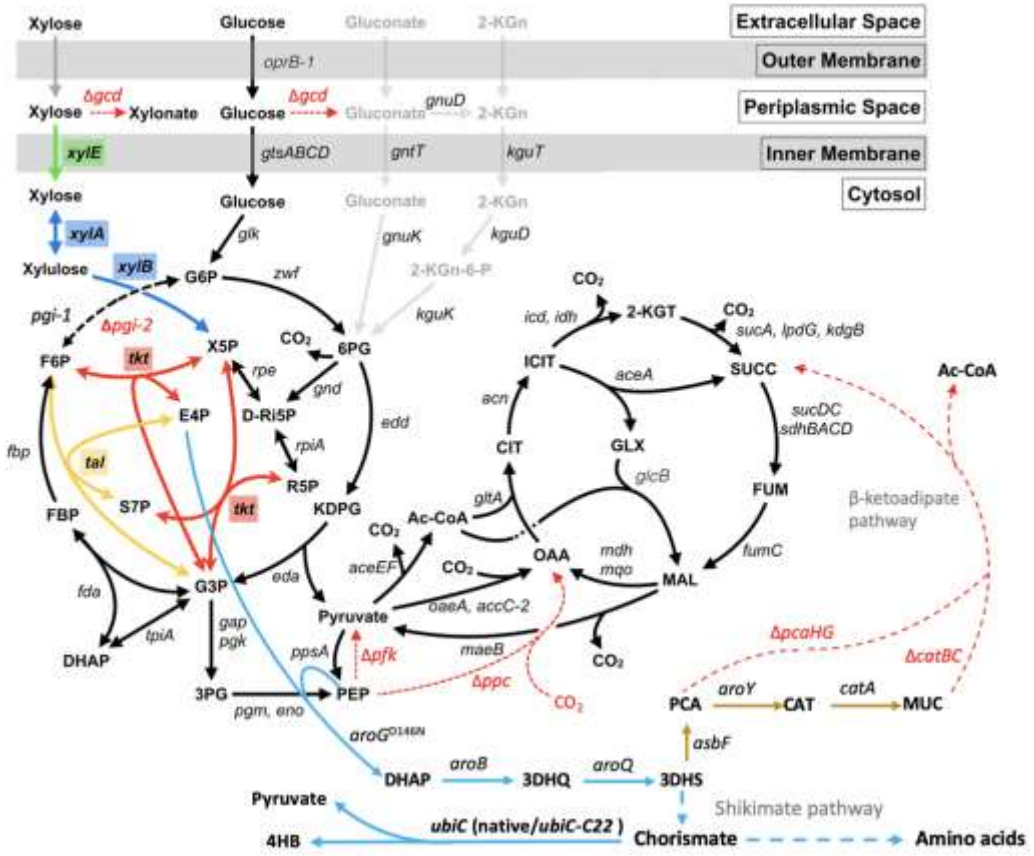
Baseline strain for hydrolysate co-utilization in *P. putida*

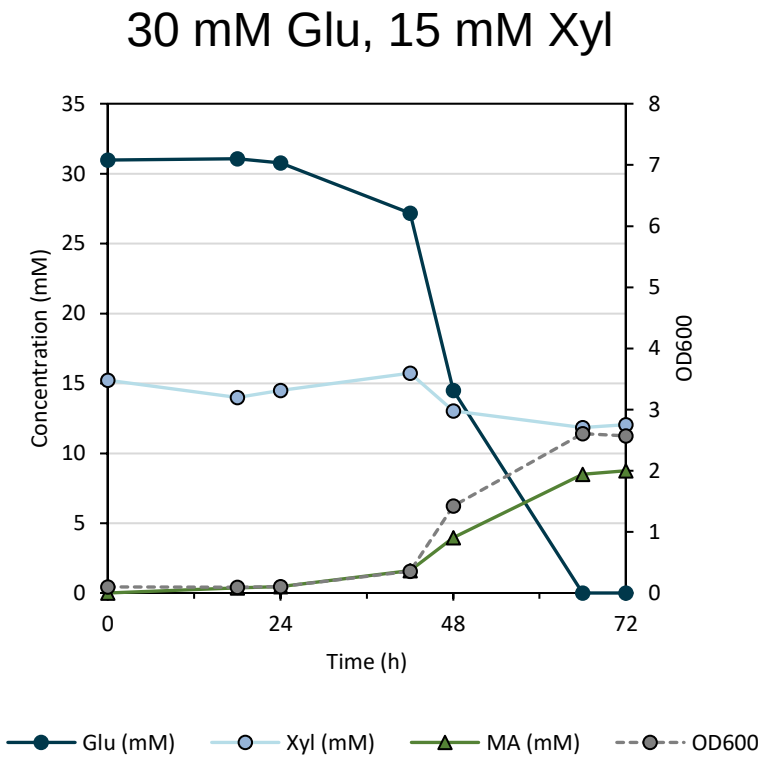


JR Elmore *et al.*, *MBE* 2020

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

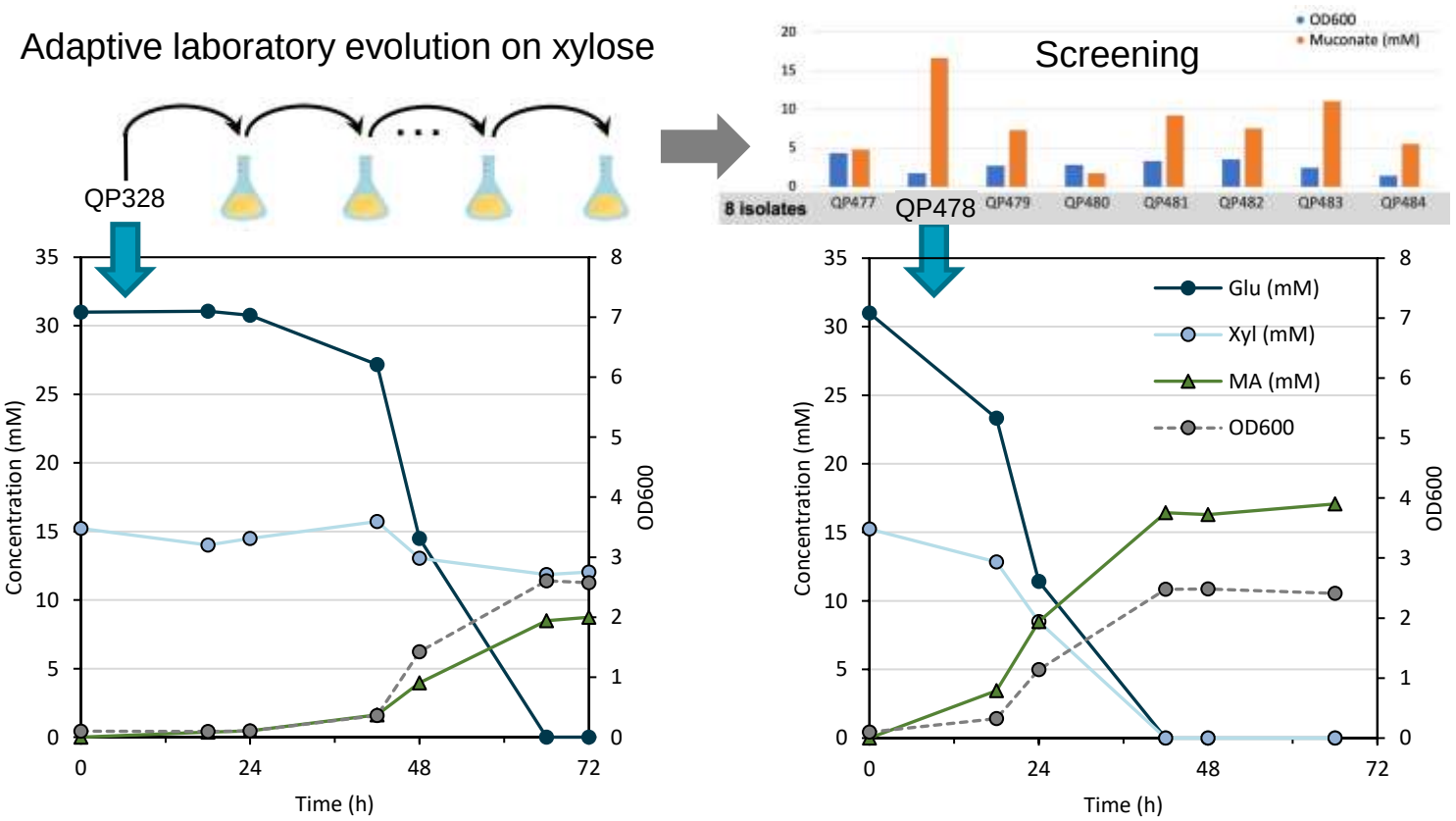
Baseline strain for muconate from hydrolysate





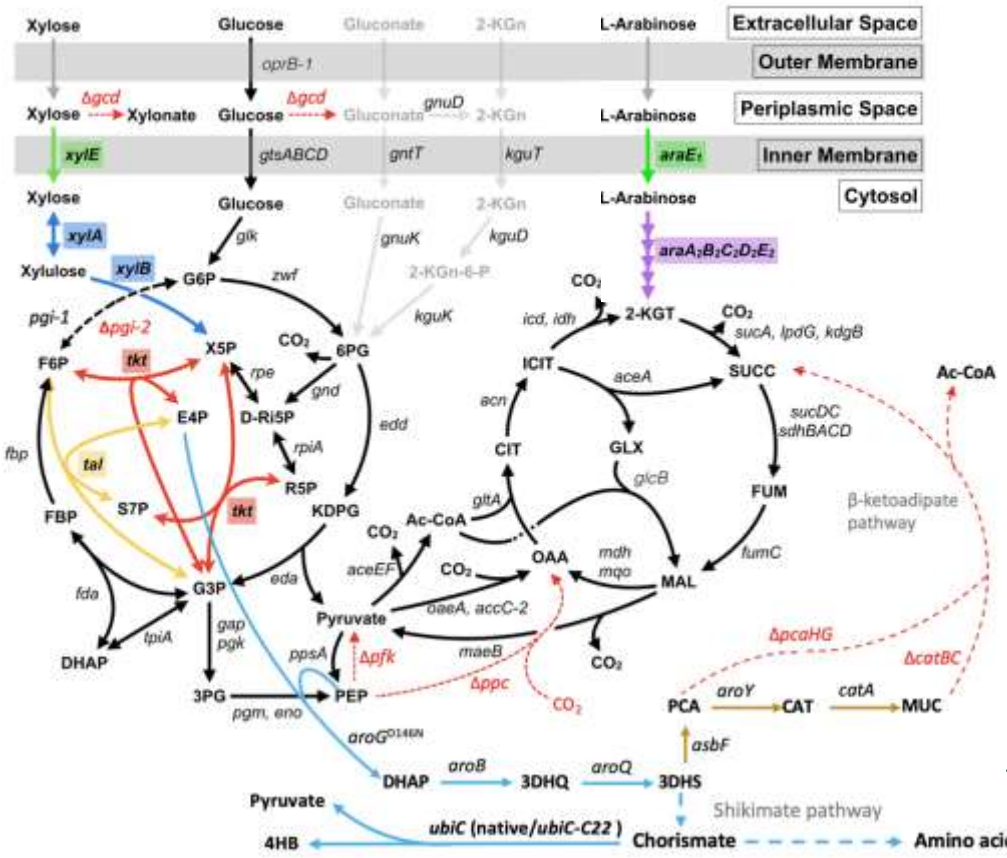
- The non-oxidative xylose pathway was integrated
- **Outcome:** Slow conversion of glucose and xylose to muconate

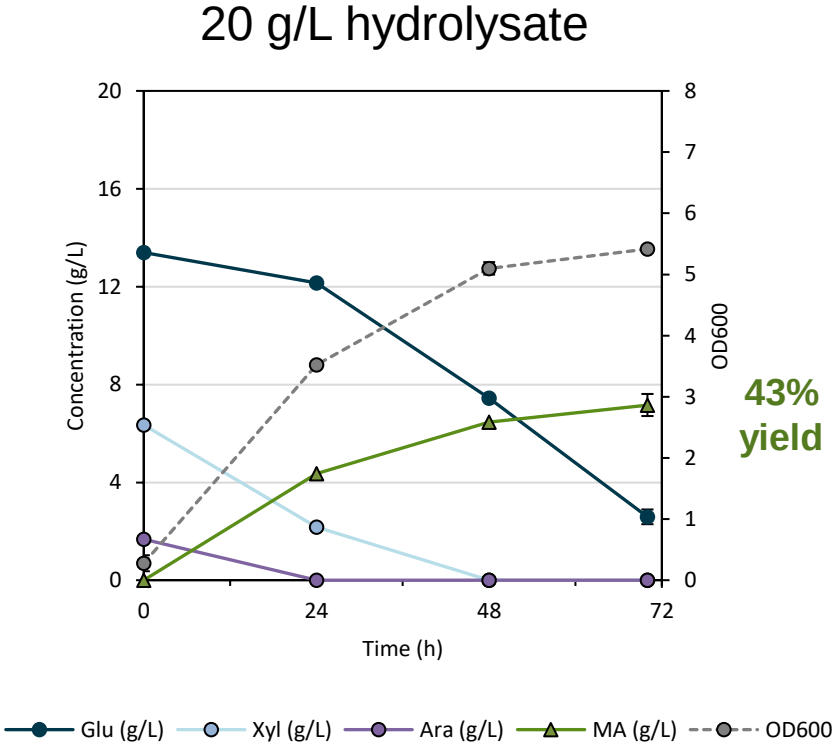
Improving muconate rate from hydrolysate



- 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

Improving muconate rate from hydrolysate





- The arabinose pathway was integrated into QP478
- Conversion of hydrolysate to muconate was demonstrated in a shake flask
- **Outcome:** Simultaneous conversion of hydrolysate sugars to muconate

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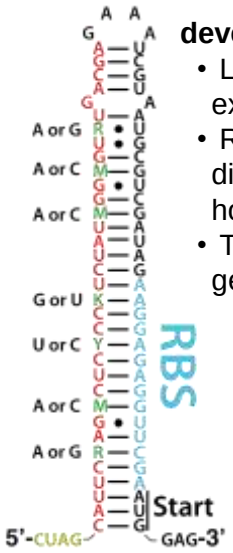
Controlling gene expression to manipulate the trade-offs between muconate productivity, titer and yield in *Pseudomonas putida*

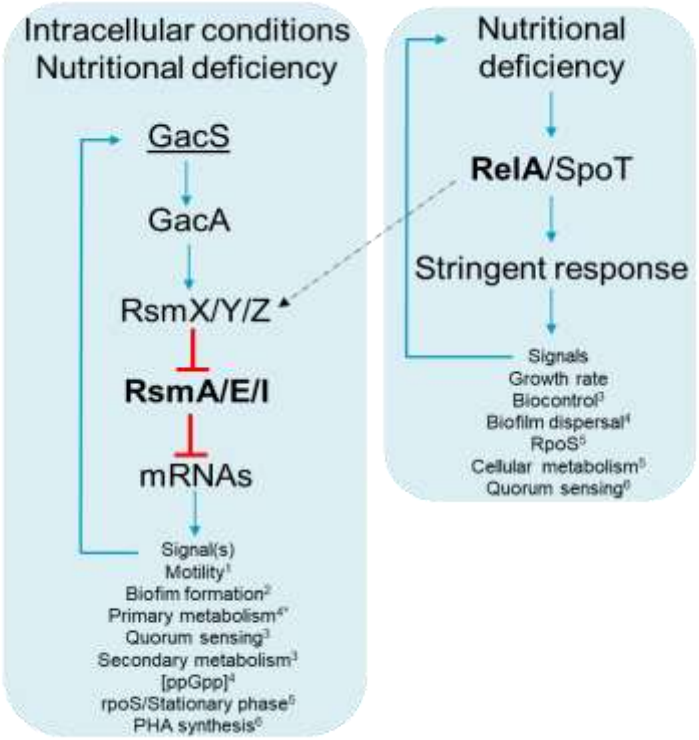


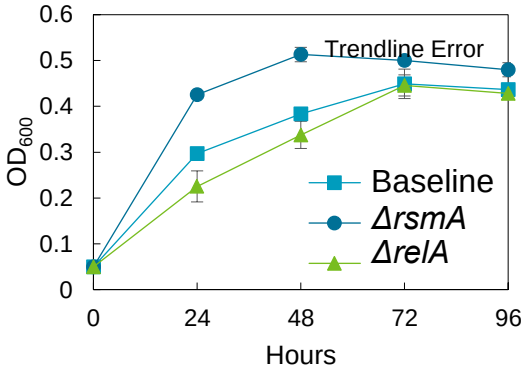
Riboregulator development and testing

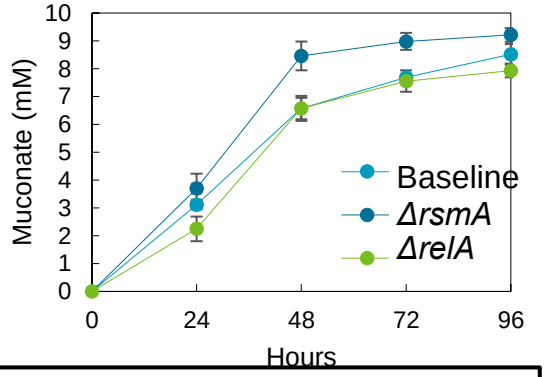
- Large dynamic range of expression control
- Reproducible near-digital regulation across hosts
- Titrate otherwise toxic genes.

Precise riboregulation for directing metabolic pathway flux
Fully regulated target molecule production

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 and RelA

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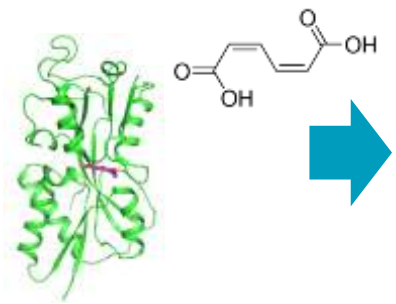
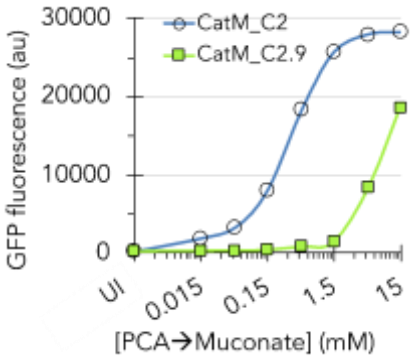
In vivo biosensor development in KT2440

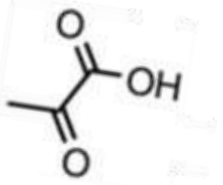
CatM for muconate

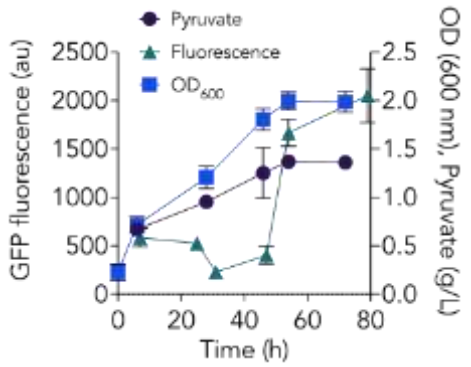


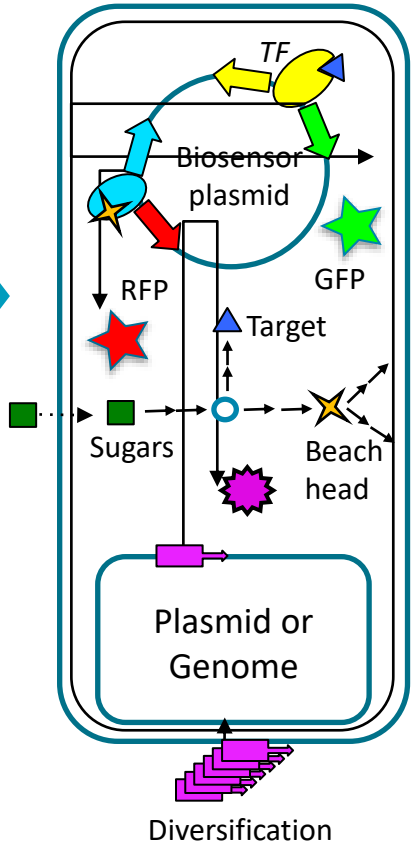
Sensors with variable sensitivity



Beachhead (pyruvate) sensor







Two transcription-factor based biosensors *simultaneously monitor* metabolism & muconate titers

Cell sorting to *isolate next-generation* production strains

- Custom sensors generated *via* promoter and protein engineering, previously used to increase muconate productivity in *P. putida*
- *Dual sensing and cell sorting* for target and beachhead → high productivity
- **Outcome:** Improved strains, high throughput **Test** and data collection for **Learn**

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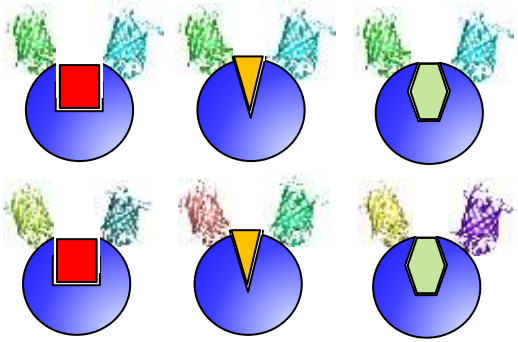
In vitro biosensors for strain engineering

Sensor selection

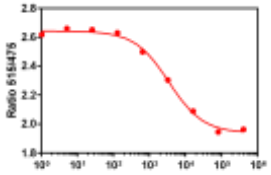
- Binding protein for precursor and/or product molecules



- FRET pairs for different applications

Diverse experimental approaches

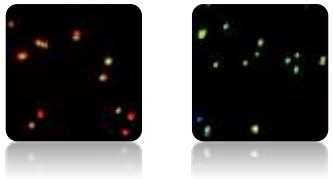
- *In vitro* measurements



Titration of biosensor with cis,cis-muconate

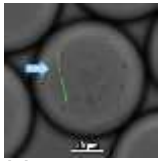
- *In vivo* measurements

- muconate + muconate



Images of P. putida *expressing biosensors*

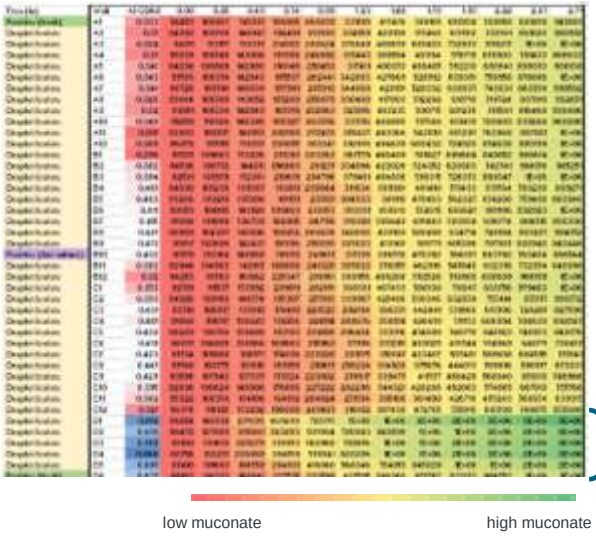
- Development of whole-cell or enzyme-linked biosensors



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 utilizaing *in vitro* biosensors
- **Ongoing:** Optimizing sensing for other targets and dALE protocols for other hosts

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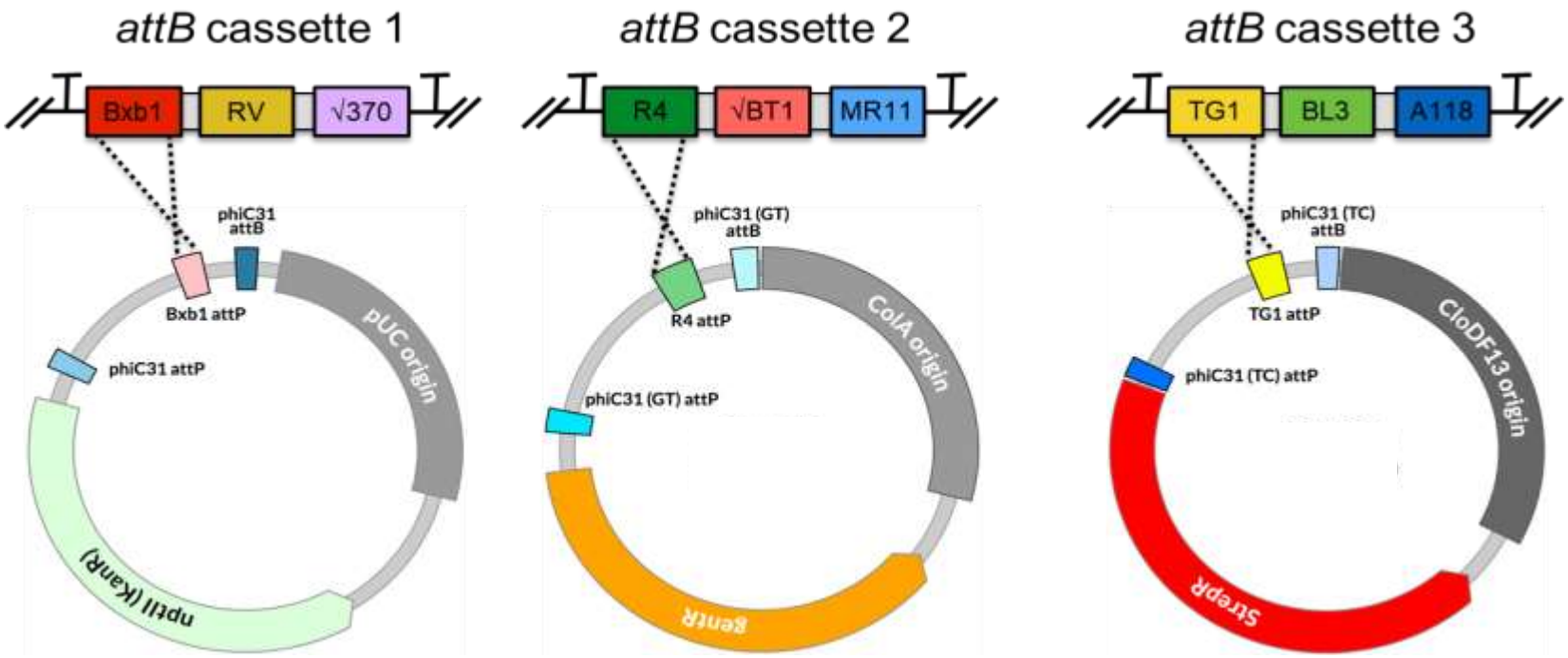
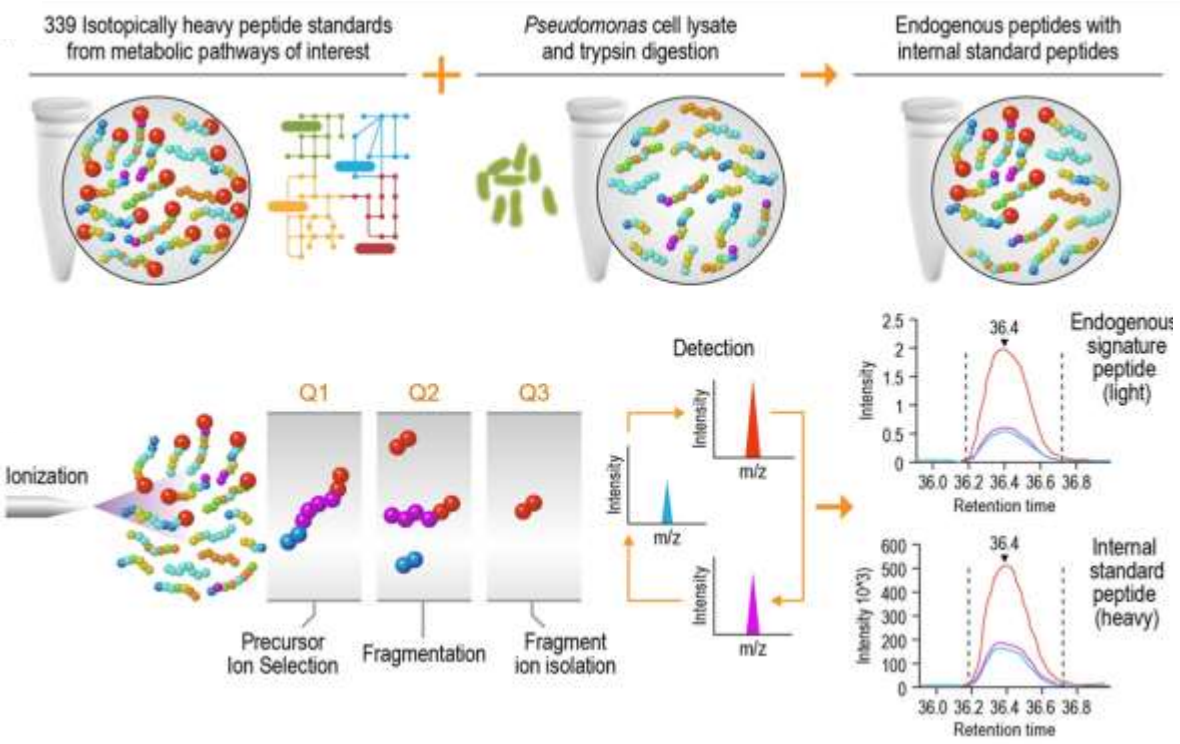
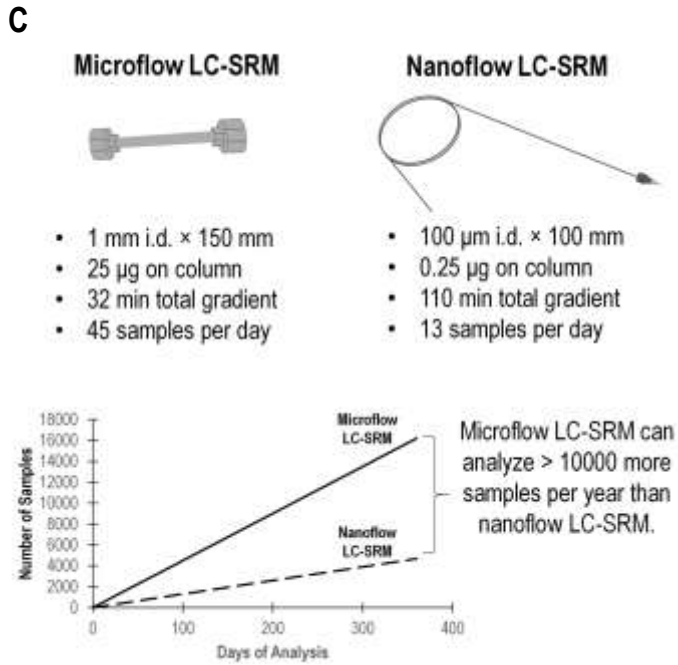
Site-specific DNA integration tool in KT2440



- Can simultaneously insert 3 plasmids into chromosome at ~10^6 cfu / µg 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

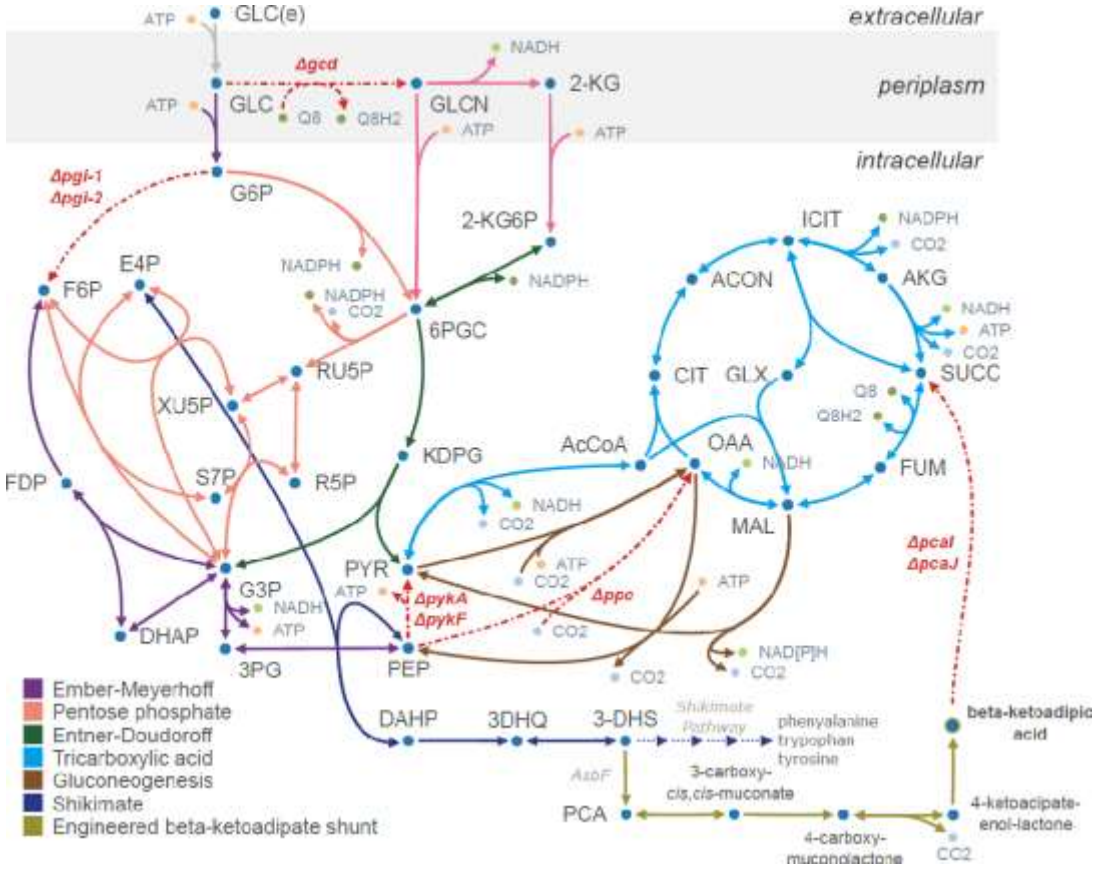


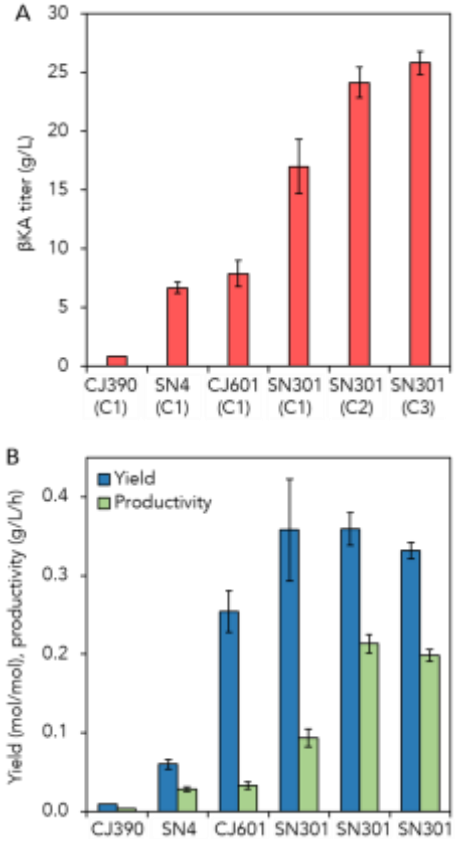


- Targeted proteomics with internal standards accurately quantified 132 enzymes.
- Using microflow LC to replace nanoflow LC greatly reduced the analysis time without sacrificing sensitivity.
- **Outcome:** Increase the throughput of protein quantification by 4 times.

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.

Second exemplar for PCA: β-ketoadipate





- Followed similar baseline approach to *cis,cis*-muconic acid production for βKA
- Collaboration with Performance-Advantaged Bioproducts projects
- **Outcome:** Base *P. putida* strain for βKA; 2° transfer target in *C. glutamicum*

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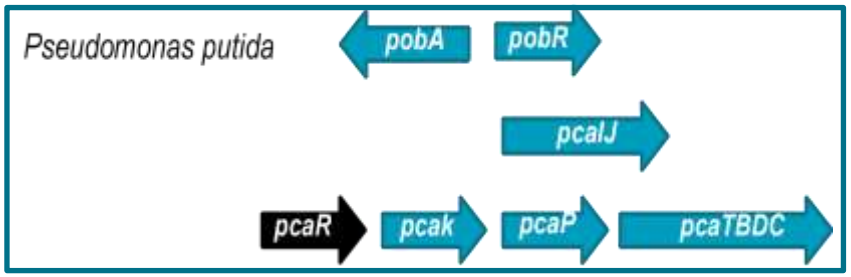
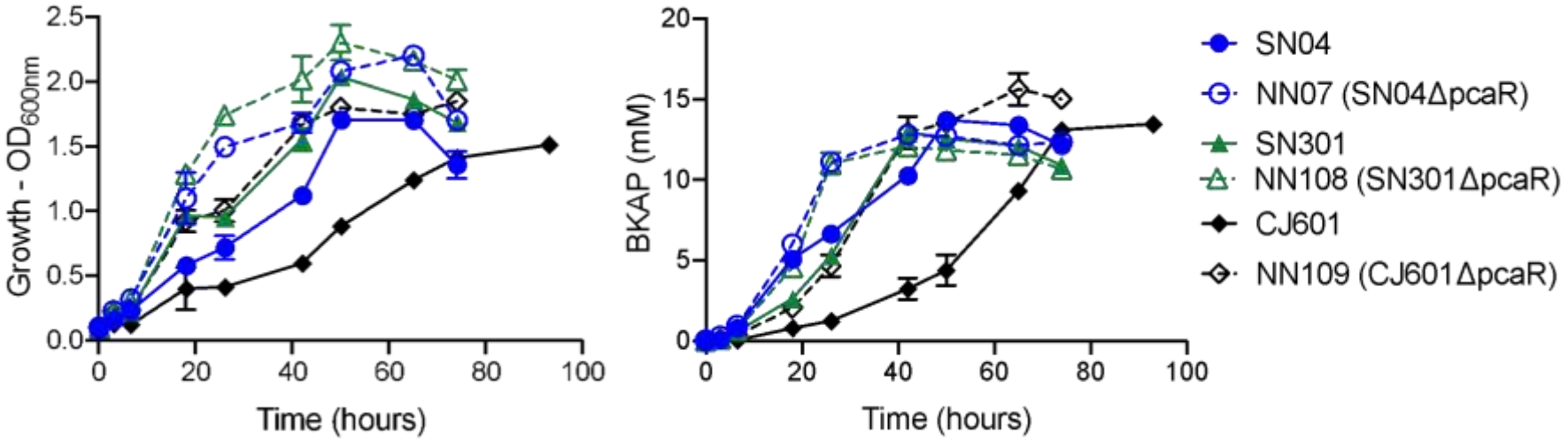
Transcriptional regulator engineering for improvement of β-ketoadipate production in *Pseudomonas putida*



pcaR deletion ("NN" strains) in 3 strain backgrounds:

- BKAP-producing baseline strain (SN04)
- SN301 = SN04 + *ΔHexR*
- CJ601 = SN04 + *Δgcd*

beneficial deletions in muconate strains



- Deletion of PcaR transcriptional regulator improves growth & productivity in 3 BKAP- producing *P. putida* strains
- RT-qPCR and RNA-Seq analysis show significant difference with pcaR deletion

Outcome: Increased BKAP productivity in flasks; sending to bioreactors

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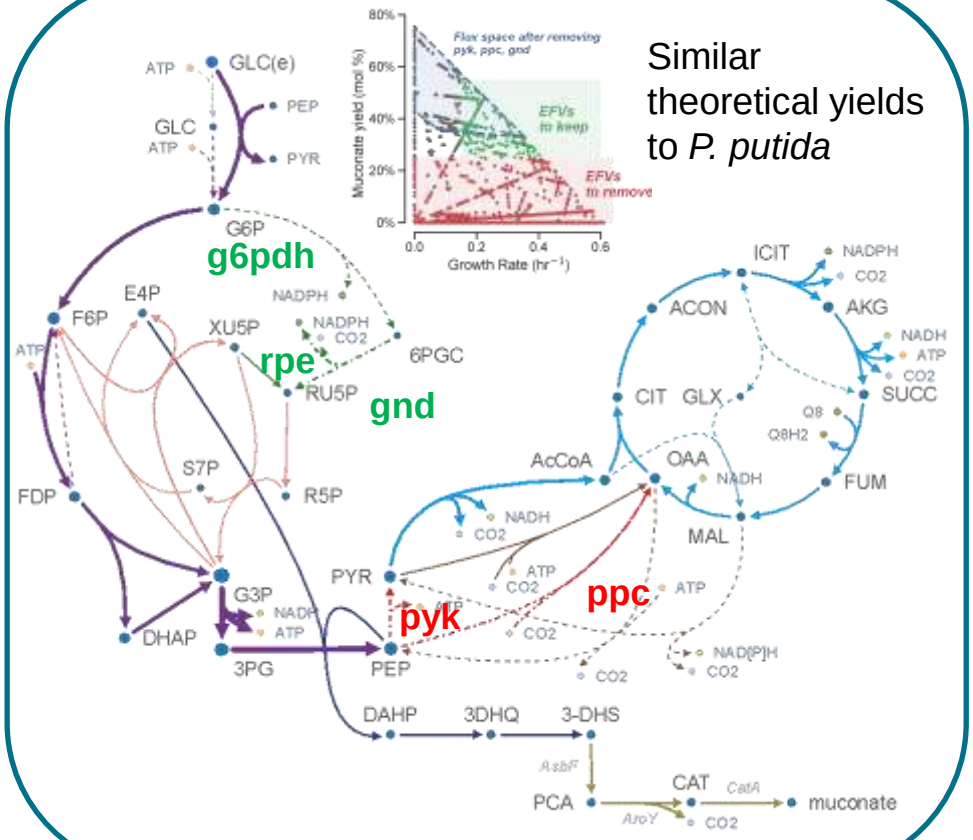
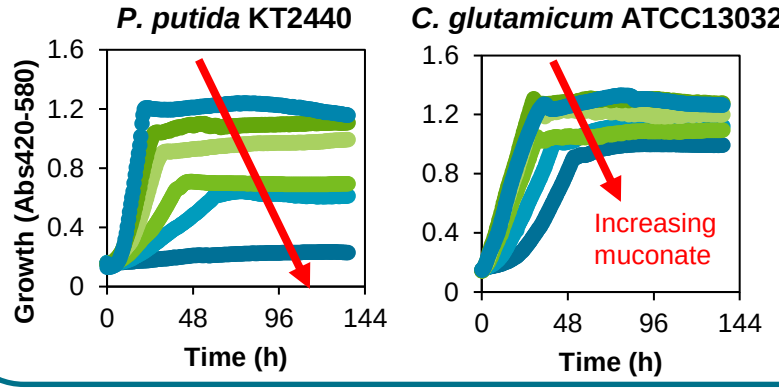
Transfer target: Muconate in *C. glutamicum*

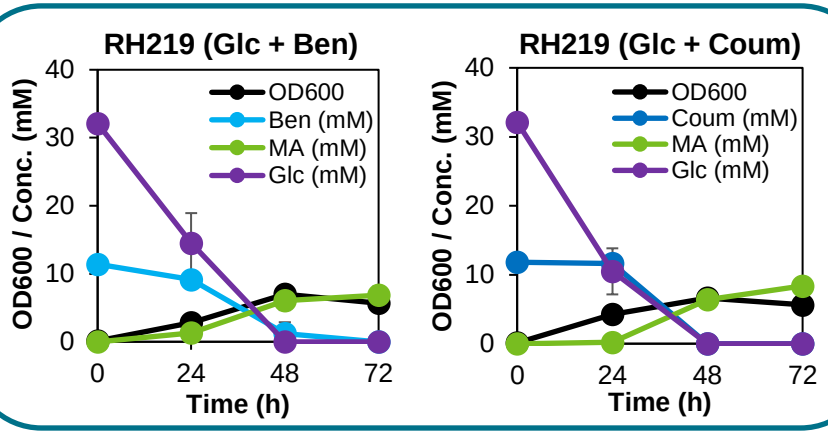
Metabolic Modeling



Tolerance to 80+ g/L muconate



Strain engineering for muconate production



- *C. glutamicum ATCC13032* has promising attributes for muconate production
- Initial engineering efforts enable muconate production from aromatics, with glucose next

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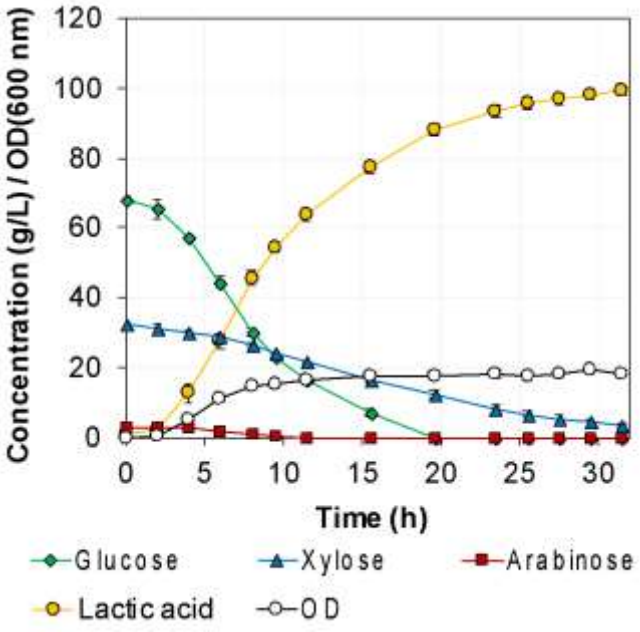
Emerging BH-exemplar pairs in bacteria

Bacillus coagulans

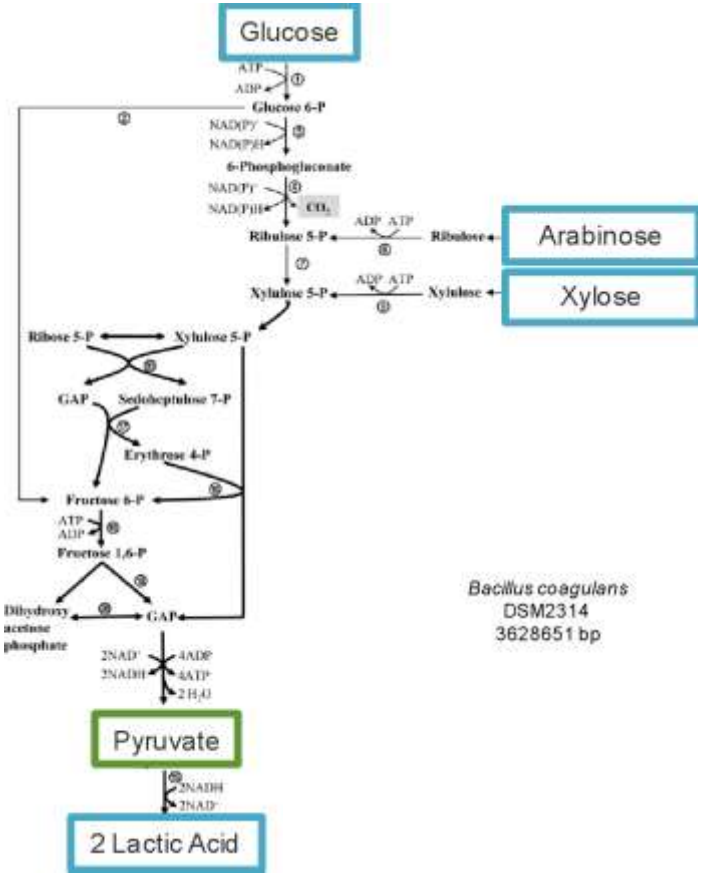


Performance in DMR-EH hydrolysate

$Q_{glucose,max}$ = 6.4 g/L h
$Q_{xylose,max}$ = 1.2 g/L h

[lactate] = 99.4 g/L
$Q_{lactate}$ = 3.1 g/L h
$Y_{lactate}$ = 0.91 g/g

μ_{max} = 1.1 h^{-1}
$Y_{biomass}$ = 0.04 g/g



- High glucose and xylose consumption rate, and lactic acid production rate
- Homolactic fermentation from C5 and C6 sugars
- **Outcome:** Pyruvate as beachhead in *Bacillus coagulans*

Summary and Next Steps

PCA/Muconate in *P. putida*

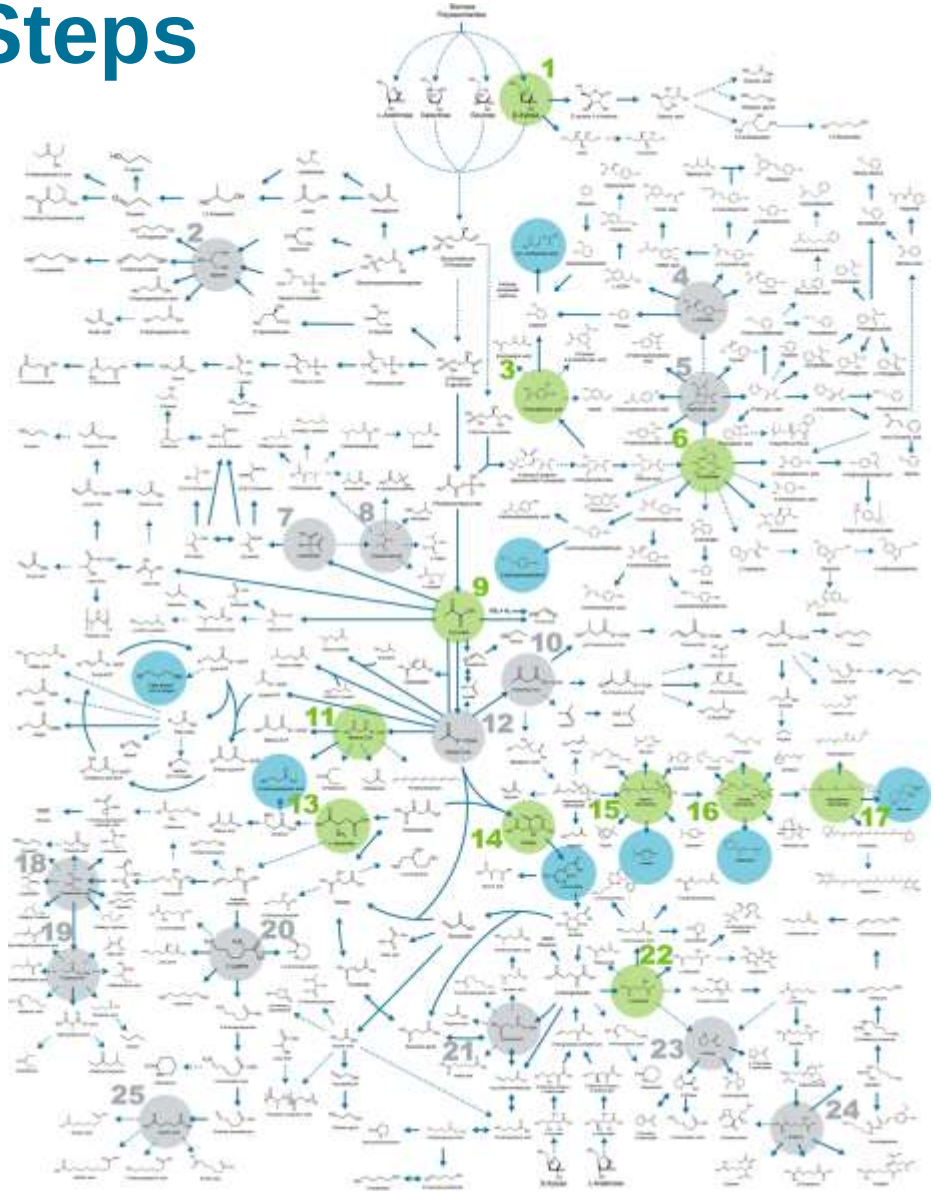
- Ramping up new DBTL cycles now towards higher titers and rates to meet FY21-FY22 TRY goals
- Gearing up for several additional Learn-friendly DBTL campaigns for *P. putida*
- Ramping up DBTL efforts to understand why strain performance is limited at [muconate] > 20 g/L

PCA/Muconate in *C. glutamicum*

- Efforts now focused on connecting sugar metabolism to muconate production

Pyruvate BH in *B. coagulans*

- Working with other BETO projects and garnering input from industry advisors as to a specific BH-exemplar pairing



Acknowledgements

LBNL: Nathan Hillson, Hector Garcia Martin, Todd Pray, Alastair Robinson, Blake Simmons, Deepti Tanjore

NREL: Gregg Beckham, Gayle Bentley, Mary Biddy, Brenna Black, Rita Clare, Ryan Davis, Annette De Capite, Graham Dominick, Rick Elander, Molly Gaddis, Nick Grundl, Stefan Haugen, Ray Henson, Chris Johnson, Kelsey Kinley, Bruno Klein, Megan Krysiak, Colin Kneucker, Chen Ling, Kevin McNaught, Bill Michener, Michela Monninger, Michelle Reed, Davinia Salvachua, Violeta Sanchez i Nogue, Amie Sluiter, Todd Schollenberger, Kiki Szostkiewicz, Frankie Tapia, Marykate O'Brien, Isabel Pardo, Darren Peterson, Peter St. John, Todd Vinzant, Sean Woodworth

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

LANL: Taraka Dale, Ramesh Jha, Scott Hennelly, Niju Narayanan, Neresh Pandey, Carol Kay Carr,

ORNL: Adam Guss, Josh Michener, Gara Wolff

PNNL: Jon Magnuson, Jeremy Zucker, Joshua Elmore, Kristin Burnum-Johnson, Young-Mo Kim , Mark Butcher

SNL: John Gladden, Jamie Meadows

Target and Host Engineering: Filamentous fungi

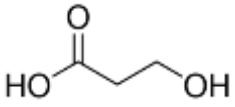
Project overview

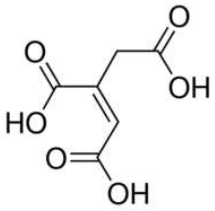
Context and History: Task initiated in 1st year of *full* Agile BioFoundry project (FY17)

- Historical expertise in genetic and bioprocess engineering of **industrial fungi**
- First target was 3-hydroxypropionic acid (3HP) via prokaryotic pathway

Project goals:

- Employ DBTL to develop *Aspergillus* strains to convert sugars (hydrolysate) into organic acids at high TRY: 3HP and aconitic acids
- Develop broadly useful **Build** tools for *Aspergillus* and other fungi

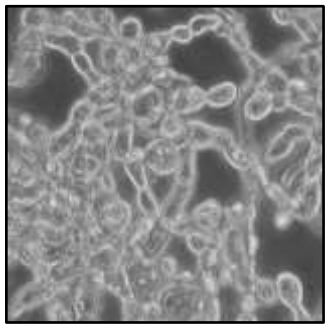




Heilmeier Catechism:

- Today: fungal processes are widely used for bioproducts but strains are derived from traditional mutagenesis and selection
- Important: organic acids are direct use products and precursors to many others
- Risks: Achieving Titer Rate Yield (TRY) goals, especially Rates

Project overview:
Why *Aspergillus*?



- *Aspergillus* spp. are **industrially relevant**: used for producing small molecules and enzymes in large bioreactors

e.g., citric acid, itaconic acid in ≥100,000L airlift reactors, ~3M ton market (citric)

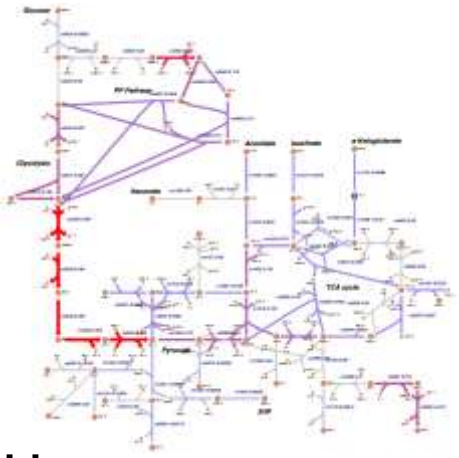
Genetically tractable, genomes sequenced, genome scale **metabolic models**

High flux from sugars toward beachhead molecules in glycolysis and the TCA cycle to **organic acids**, e.g., *A. pseudoterreus* makes 50 g/L itaconic acid

Grows and produces organic acids at pH 1-3, **free acids**, not salts

Separations: high titer, free acid, crystallization possible

No lime or sulfuric acid and hence no waste gypsum

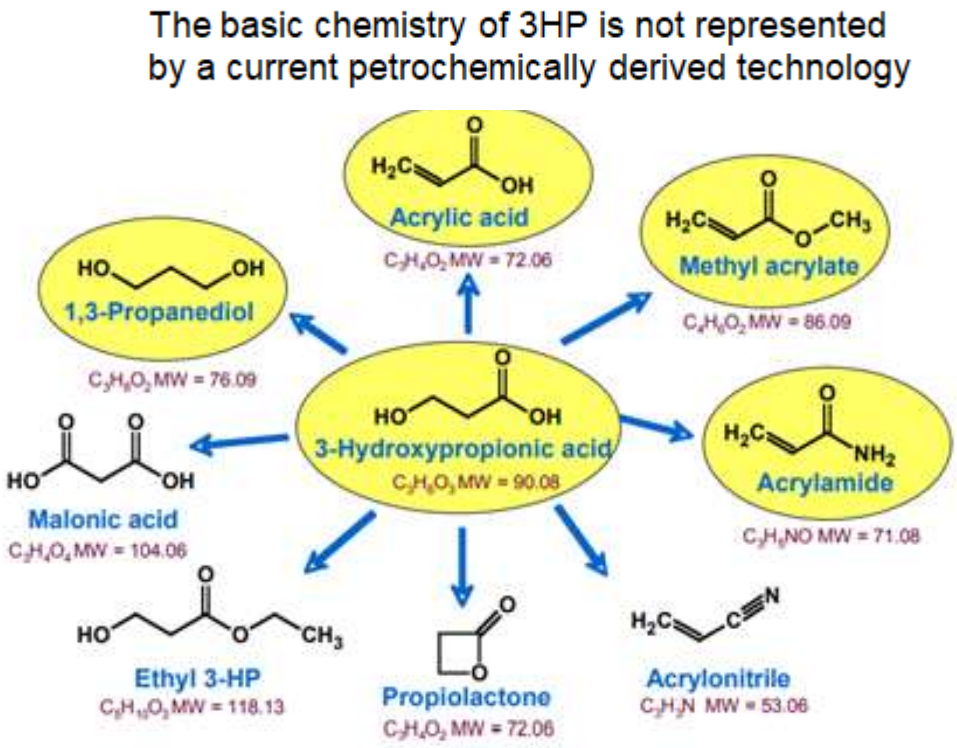


Purposes:

Develop **advanced DBTL tools** broadly applicable to *Aspergillus* spp. and other fungi

Show the value of the platform for producing beachhead molecules (pyruvate, oxaloacetate, etc.) leading to **organic acids**

Project overview: 3-hydroxypropionic acid (3HP)

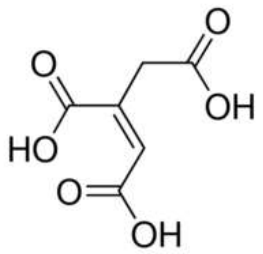


- **Beachhead: L-aspartic acid**
- **1,3-propanediol**:
  - Acrylic acid
- **Polyacrylates**:
  - Acrylonitrile
- **Carbon fiber**

- Figure from: Werpy & Petersen (2004) *Top Value Added Chemicals from Biomass*

Project overview: Aconitic acid

- **Beachhead: citric acid**
- **Bio-based plasticizers**:
  - Thermal and mechanical properties comparable to those of phthalate ester plasticizers
  - Potential safer substitute
- **Monomer in elastomers**:
  - Biomaterials for drug delivery and tissue engineering that are biocompatible
- **Direct Uses**
  - Acidulant
  - Chelator
  - Nutty flavoring

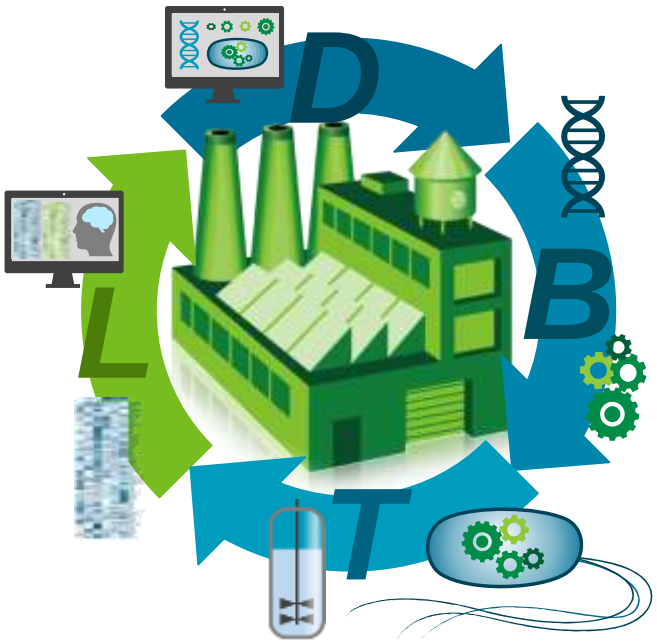


- Aconitic acid cited in: Werpy & Petersen (2004) *Top Value Added Chemicals from Biomass*

Management

- **F2F team meetings:** weekly Aspergillus Design Build and Test Learn teams
- **Virtual meetings**: Annual All-Hands Meeting, Semi-annual leadership meeting
- **Updates**: quarterly team updates on task lead call, monthly DBTL tracking
- **Team Leads:** Fungal molecular biologists for main targets, fungal bioprocess engineering
- **Milestones:** largely centered on TRY, also enabling tech (DBTL efficiency)
- **Project interfacing:** *ad hoc* meetings with Learn, PISU (round robins), Transfer (*Rhodosporidium* team)
- **Risks**: TRY targets challenging, especially Rate and Yield

Approach



Challenges

- Finding the next gene targets leading to a step change in T,R or Y; identifying non-intuitive targets
- Transporter identification and overall metabolic flux for TRY goals
- Product/intermediate degradation or diversion
- Controlling optimal fungal morphology in the bioprocess
- Accelerating the Build arc of the cycle

Technical approach

- Two workhorse *Aspergillus* species with good tools and different process advantages
- Expand and improve Design-Build tools for fungi to build more strains and stack traits
- Multiomics analysis in context of genome scale models to identify transport limitations, product or precursor degradation, bottlenecks to beachheads and exemplar targets
- Statistically robust comparison of multiple strains for deep Test-Learn analyses
- Integrated genetic *and* bioprocess engineering to optimize conditions for strains
- Transfer host team interactions accelerate idea generation and insights

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Impact

Scientific

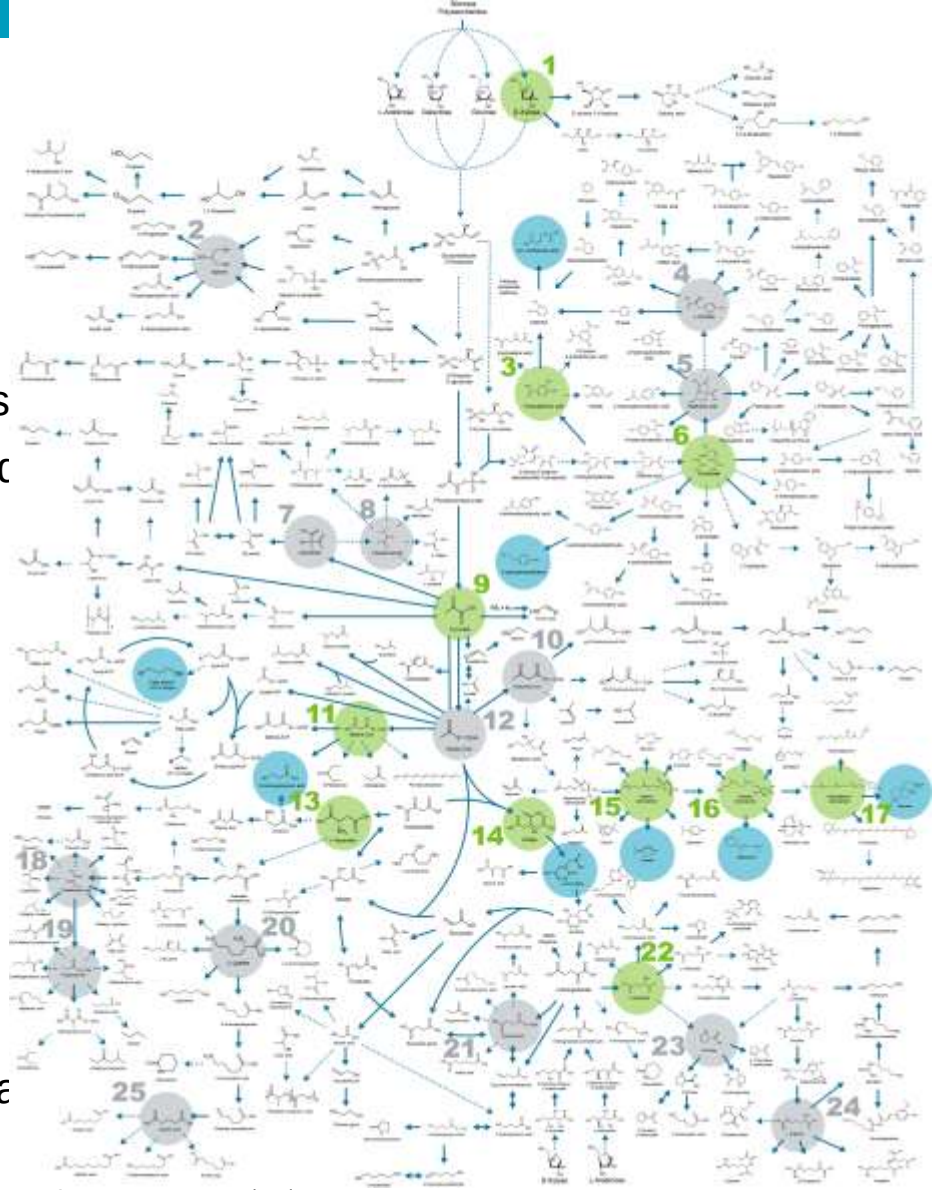
- Developed *A. niger* and *A. pseudoterreus* strains for organic acids, reported in peer-reviewed publications and patent applications
- Helping test and improve ABF DBTL tools and overall ABF infrastructure

Industry

- Developing DBTL tools for *Aspergillus* spp. used in industry
- Demonstrating the utility of the tool set by moving up the Titer Rate Yield goals
- CRADA partners through FOA/DFO

Overall

The ABF's T-H Engineering efforts focus on simultaneously developing Beachhead-exempla pairs for the community and demonstrating the ABF tool development efforts

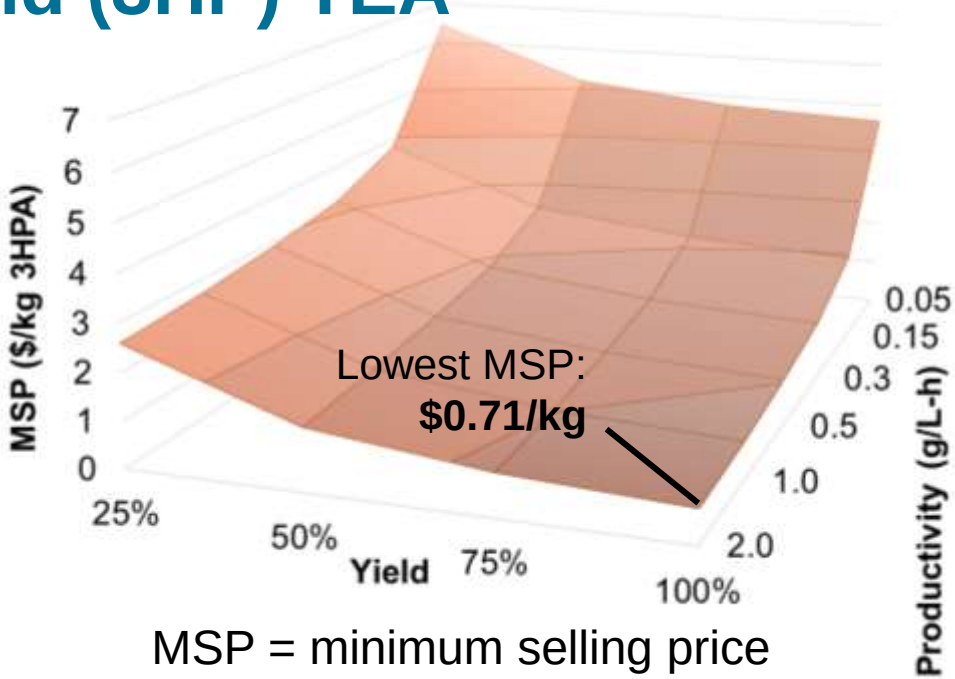


Adapted by permission from Springer Nature Customer Service Centre GmbH: Nature, Nature Calalysis, A comprehensive metabolic map for production of bio-based chemicals, Lee, S.Y., et al., © 2019

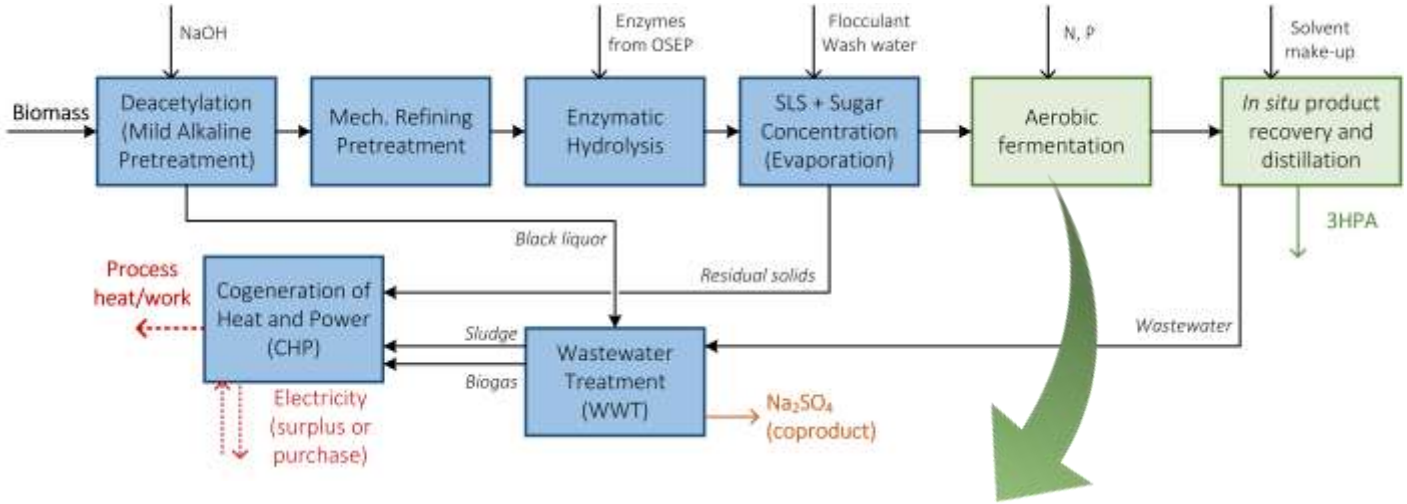
Progress and Outcomes

3-Hydroxypropionic acid (3HP) TEA

- MSP driven strongly by productivity below **0.15 g/L-h** and plateaus at productivities higher than **0.3 g/L-h**
- Higher influence of yield when passing from **25% to 50%** of theoretical yield
  - Productivity should take priority for R&D improvements if both parameters are initially shown to be at low levels
- Overall behavior of curve is similar to muconic acid but shifted lower by $1-3/kg due to higher theoretical yields, lower downstream recovery/processing costs



MSP = minimum selling price



Biomass growth Glucose + 0.8 NH_3 + 0.81 O_2 + 0.06 DAP → **4.6 M.O.** + 1.4 CO_2 + 2.6 H_2O

3HPA production Glucose → **2 3HP**

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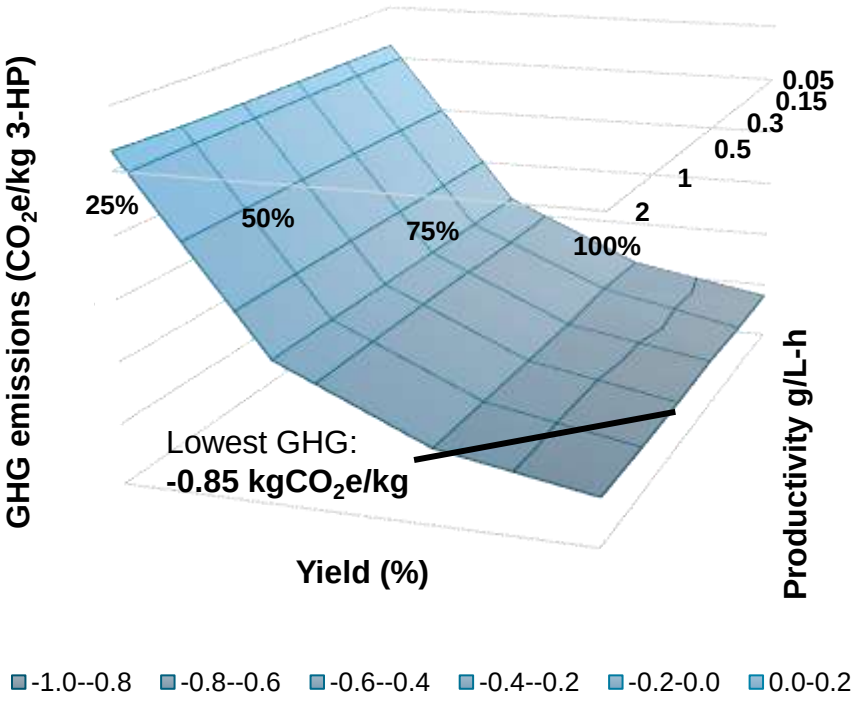
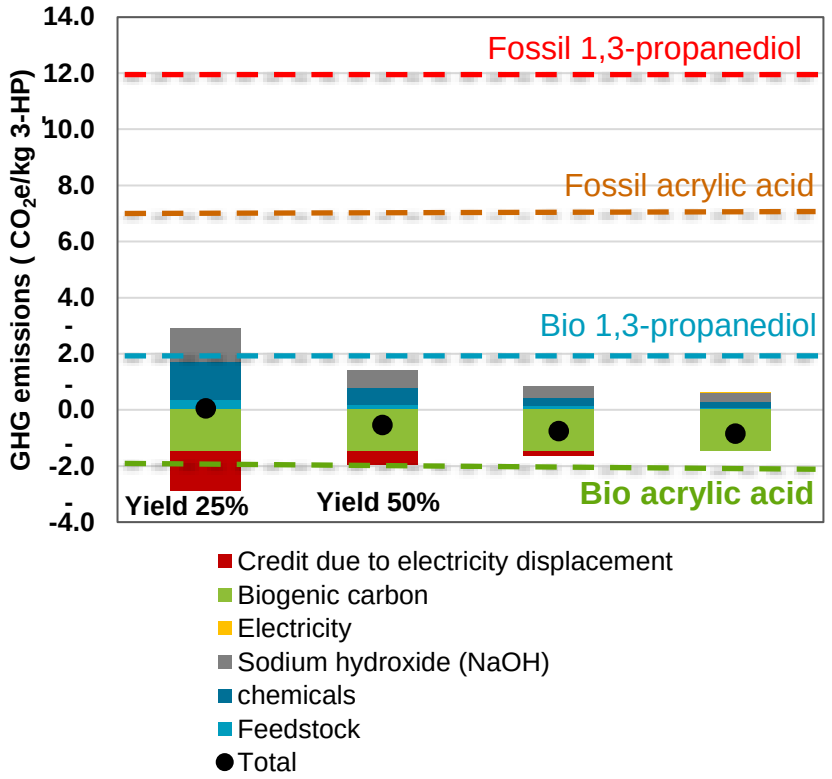
3HP LCA

GHG emissions of 3HP



GHG emissions for Productivity: 0.5 g/L-h

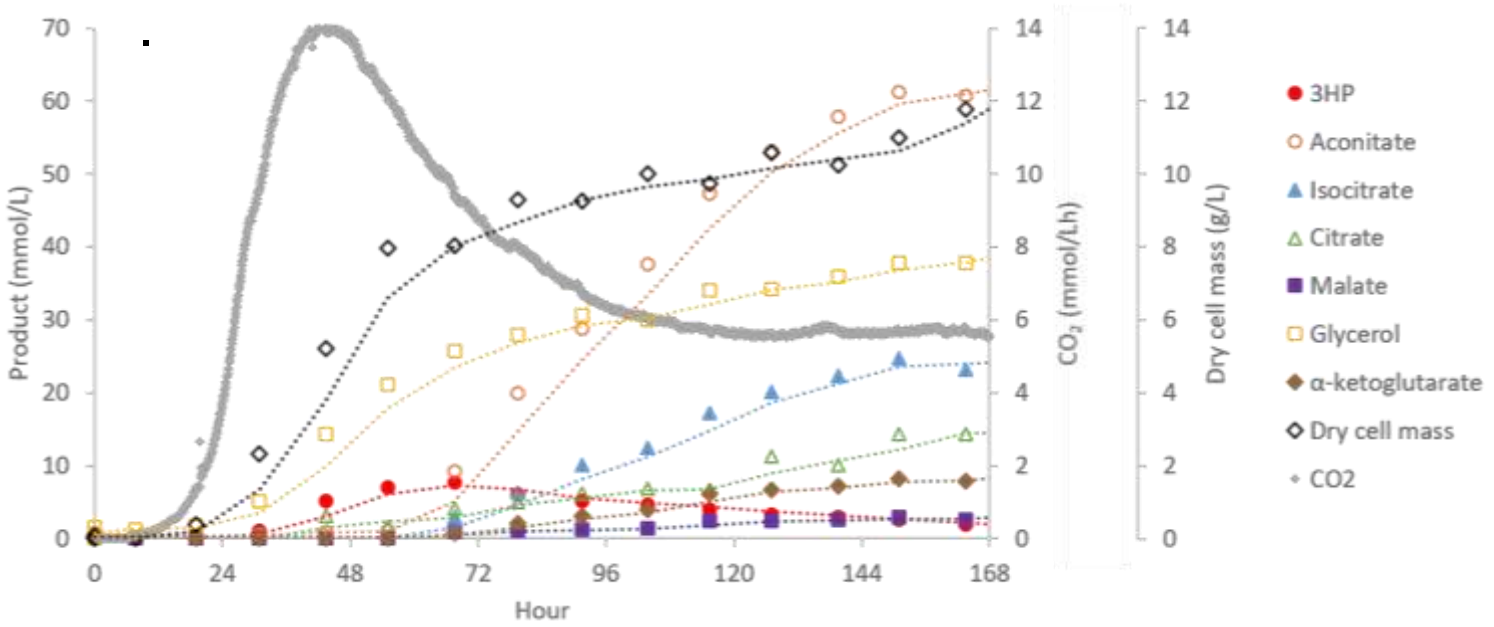


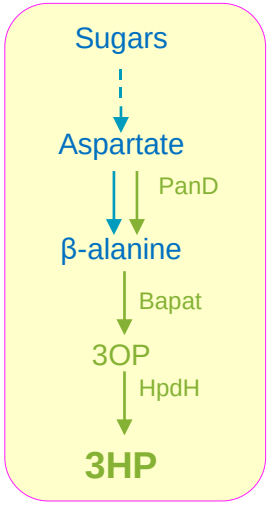
- GHG emissions varied greatly at lower yields
- GHG emissions decrease as yield improves
- This pathway can reach near-zero to negative GHG emissions

If 3HP is used as precursor of bio-1,3-propanediol or bio acrylic acid, it has the potential to reduce GHGs from 79% to 88% (depending on the yield) and 125% to 141% compared to fossil-1,3-propanediol and fossil acrylic acid, respectively

Initial 3HP Strain Development

Engineering 3HP production in *A. pseudoterreus*

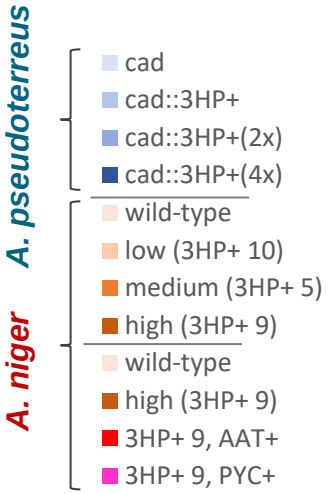




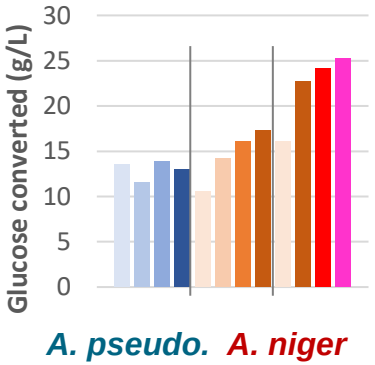
- **Goal**: Establish 3HP by inserting **three gene heterologous pathway** at *cad* locus to simultaneously knock out native itaconic acid production
- Phosphate depletion triggers itaconic acid production in wild type *A. pseudoterreus*, so the intent was to capitalize on that property for 3HP production
- **Issues in A. pseudoterreus**: many organic acids produced; **low titer of 3HP (1.3 g/L** reported at last Peer Review)
- **Outcome**: mitigation of Issues by **transferring host to *A. niger***

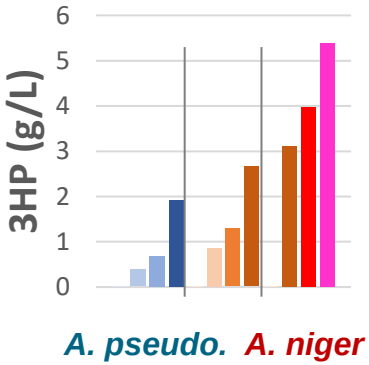
Host transfer to *A. niger*

3HP titer improved and low background of other organics

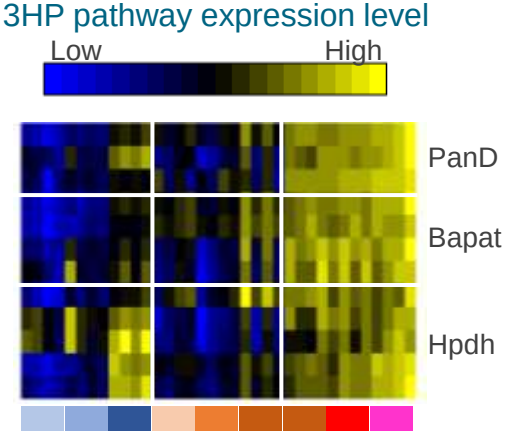


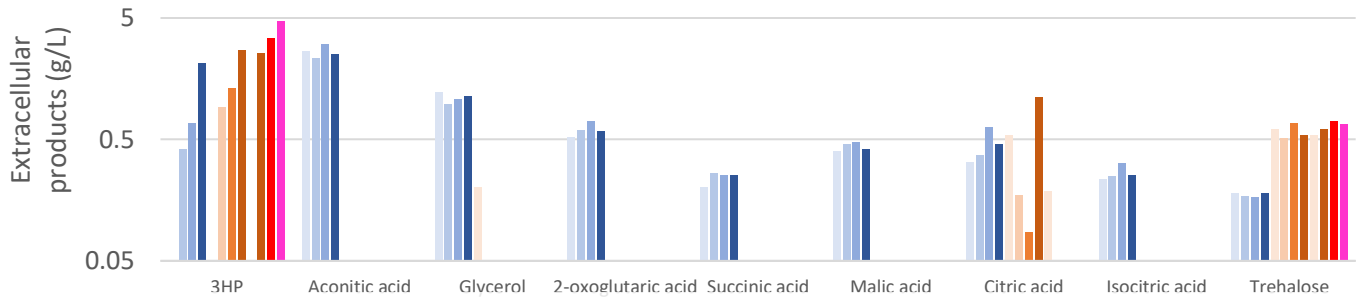
Higher glucose conversion rate and higher 3HP titers in *Aspergillus niger*





Targeted proteomics





Outcomes

- Demonstrated DBTL efficiency gains in transfer host; 4 weeks, ~**12x faster**
- *A. niger* 3HP product is cleaner and higher titer
- TEST/LEARN: increased heterologous pathway expression correlates to **improved titers of 3HP**

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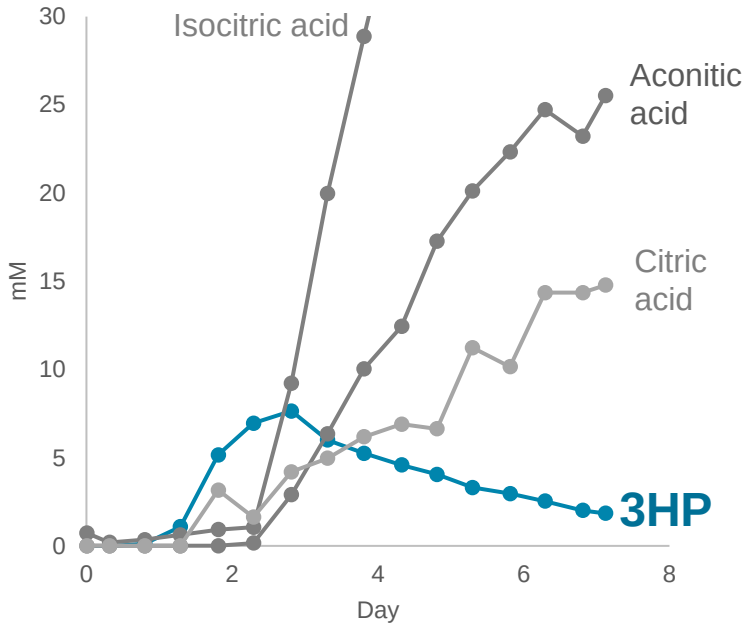
Issue identified: 3HP degradation

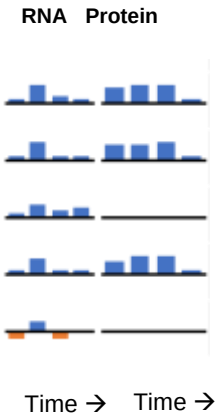
3HP is synthesized AND catabolized during a bioreactor run



3HP degradation enzyme identification

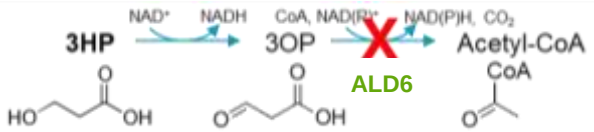
- *A. pseudoterreus* homologues to 3HP degradation pathway genes were identified by blastP
- **Test/Learn**: transcriptomics and proteomics identified multiple 3HP or intermediate catabolism gene candidates

Enzyme candidate	Gene	Evalue
3OP dehydrogenase (bacterial)	gene2	6.19E-62
3OP dehydrogenase (bacterial)	gene4	1.54E-54
3OP dehydrogenase (bacterial)	gene5	7.22E-39
3OP dehydrogenase (fungal)	gene1	2.35E-114
3OP dehydrogenase (fungal)	gene6	1.91E-82



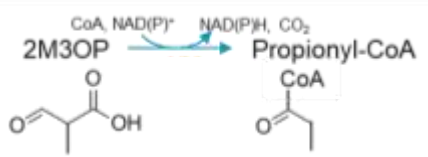
Outcome

- Candidate 3HP **catabolic enzymes identified** by TEST-LEARN

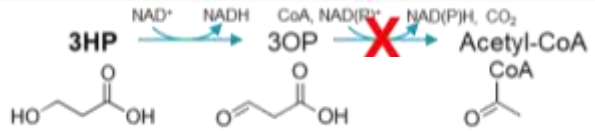


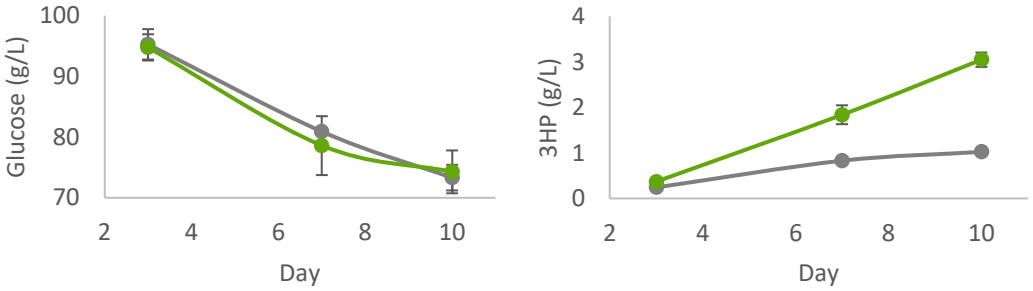
3HP degradation issue addressed!

Predicted ***native*** reaction
(methylmalonate-semialdehyde dehydrogenase)



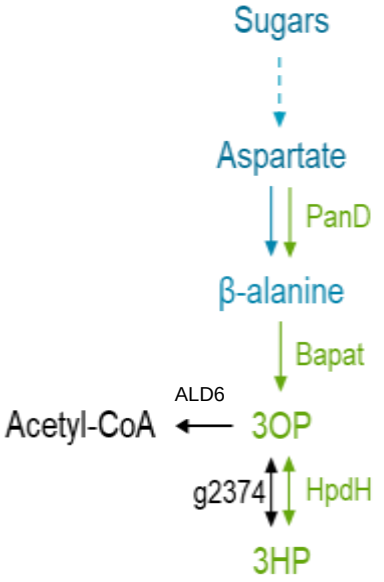
Predicted reaction with 3-oxopropanoate (3OP) substrate





3HP+ **3HP+:ΔALD6**

- Native pathways
- Engineered 3HP pathway
- **LEARN Identified pathways**



Outcomes

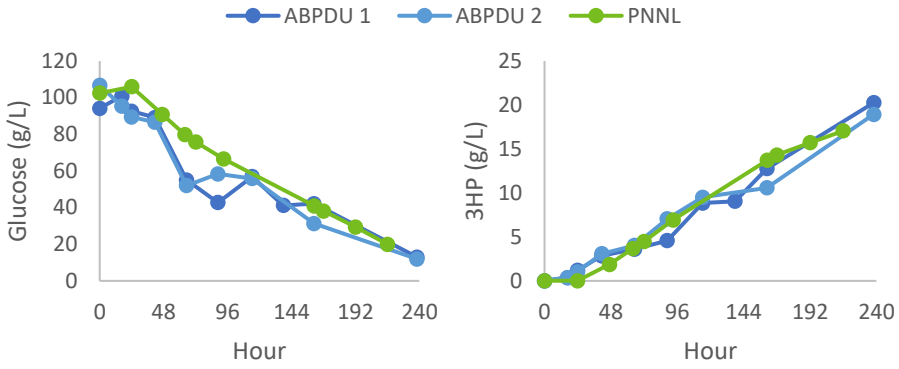
- Candidate 3OP catabolic enzyme deleted and **3HP titer improvement verified**
- **Δald6 impact on 3HP**: captured in a patent application
- Useful information for *Rhodosporidium toruloides* transfer host team

Scale-up reproducibility across facilities (round-robin)

PNNL Fungal Bioprocess Lab (0.5 L)

ABPDU (2 L)

Nearly identical substrate conversion and product synthesis rate across facilities and bioreactor types



Outcomes

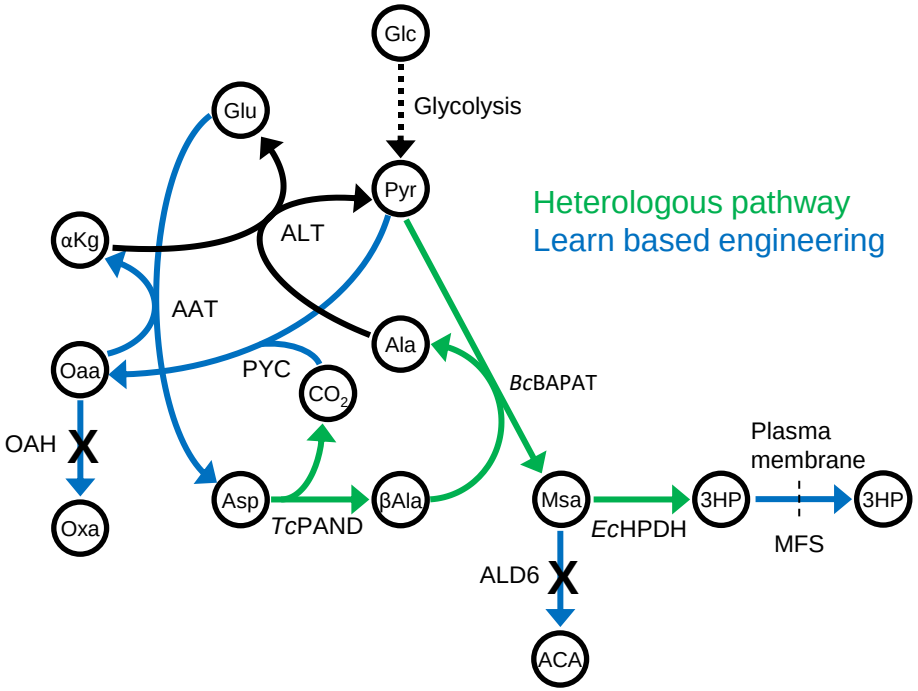
- **Round Robin testing Milestone**
- Purpose: verify that bioprocesses are **reproducible** and **transferable** across locations
- **PISU collaboration** between three facilities

Engineering a higher TRY path to 3HP production in *Aspergillus species*

Bioreactor analyses of critical DBTL cycles

Host species	DBTL purpose	Titer (g/L)	Rate (g/Lh)	Yield (g/g)
A. pseudoterreus	Pathway establishment	0.78	0.003	0.013
A. pseudoterreus	Learn: 3HP metabolism	1.18	0.004	0.016
A. niger	Host transfer	11.16	0.048	0.132
A. niger	Yield improvement	20.78	0.087	0.241

Ideal 3HP pathway



Outcomes

Direct Value of TEA/LCA

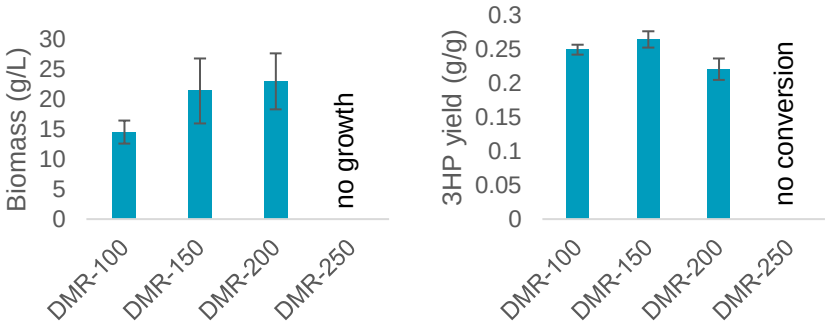
- Identified target range for Rate: 0.15 to 0.30 g/L·h. Yield: flat over 50% for TEA

General target strategies from Learn

- **Increase flux** by increasing 3HP precursors; anabolism up (pyc, aat), catabolism down (oah)
- **Increase export** of 3HP (mfs). Also a target for Rhodosporidium transfer host team
- **Decrease degradation** of heterologous pathway intermediates (ald6)

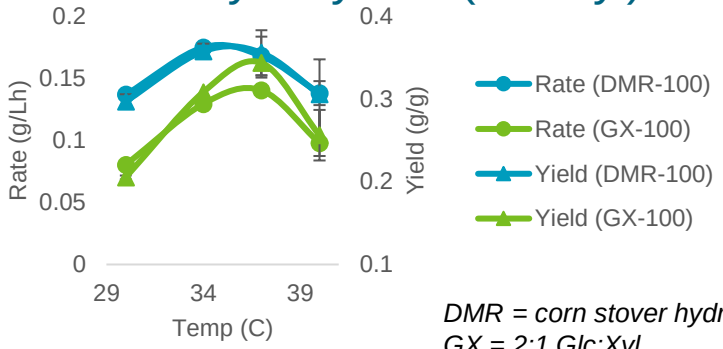
Conversion of DMR hydrolysate to 3HP

DMR maximum concentration testing (batch mode)



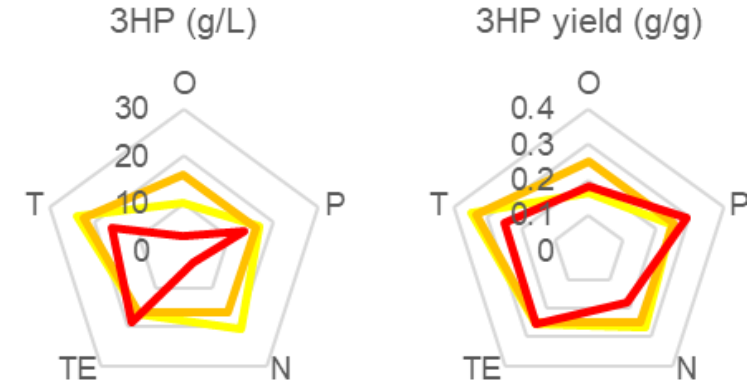
100, 150, 200, 250 = g/L total sugars

Rate on DMR is higher than mock hydrolysate (Glc:Xyl)



DMR = corn stover hydrolysate
GX = 2:1 Glc:Xyl
100 = g/L total sugars

Parameter optimization in DMR-100

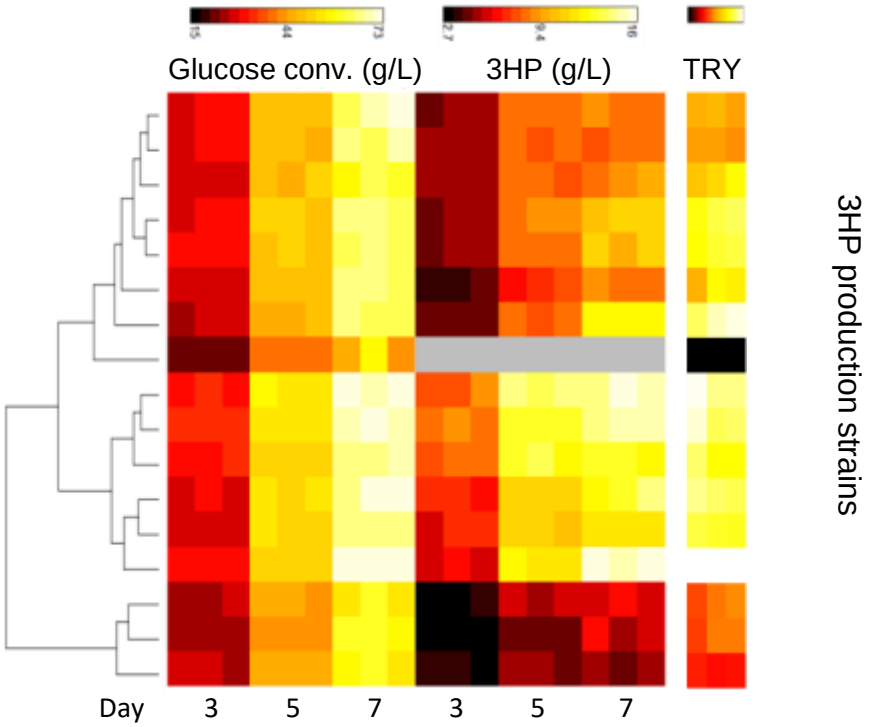


T = temperature
TE = trace elements solution
P = phosphate
N= ammonium
O = dissolved oxygen

Outcomes

- *A. niger* grows well in DMR up to 200 g/L sugars
- Temperature and nitrogen dramatically impact productivity in DMR
- Max 3HP TRY in DMR-100
  - **Titer - 29.4 g/L 3HP**
  - **Rate - 0.17 g/Lh**
  - **Yield – 0.36 g/g sugar**

TEST-LEARN In Progress: *Aspergillus niger* 3HP, diverse strains



Objectives

- **Overarching ABF Goals**
  - Increase efficiency of DBTL
  - Tool development, especially datasets to aid LEARN tool development
- **TH Team Goals**
  - Identify new gene targets for 3HP TRY improvements
  - Meet FY21/22 TRY Milestones

TEST & LEARN to ID new gene candidates

- 17 genotypes that have different phenotypes with respect to TRY
- TEST: **Multi-omics** (*in progress)*
- LEARN: Analysis in **genome scale model**
- LEARN: Non-intuitive gene candidates from **Deep Learning** approaches

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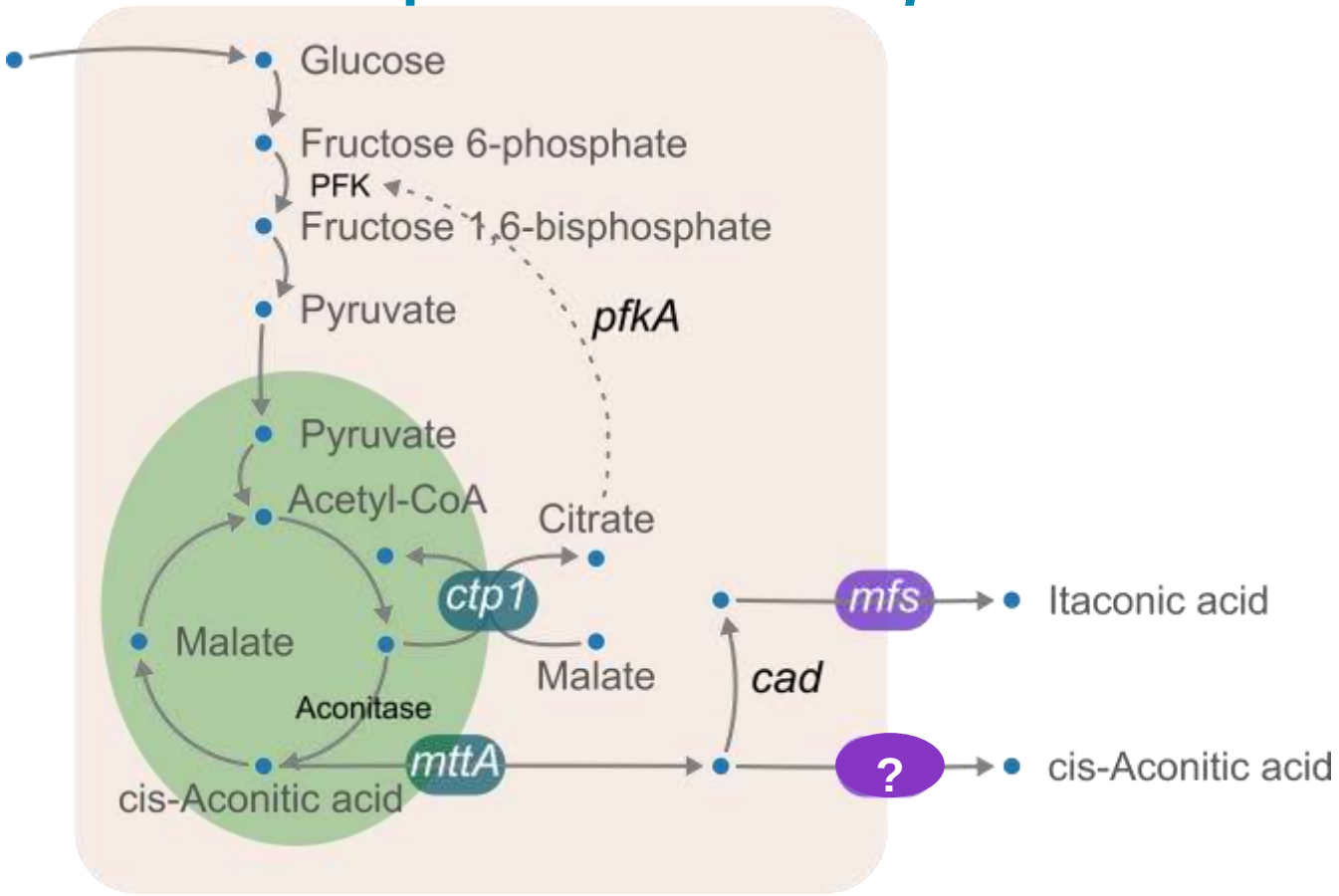
Progress and Outcomes (continued)

Aconitic Acid

Aconitic Acid Baseline:

Engineering aconitic acid production in *A. pseudoterreus*



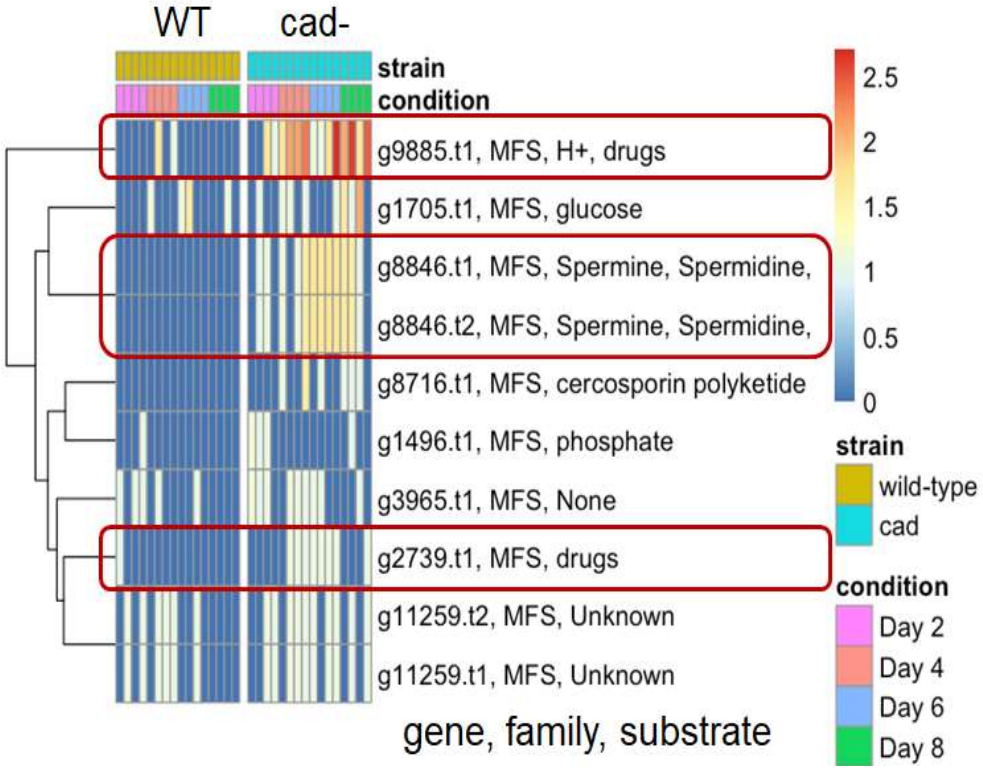
DBTL1: *cad* deletion generates aconitic acid production strain

Max titer of aconitic acid: 10 g/L (max at 2019 Peer Review)

Max titer of itaconic acid in wild type strain: 50 g/L

Hypothesis: transport of aconitic acid is limiting

Test-Learn: discovery proteomics and bioinformatics

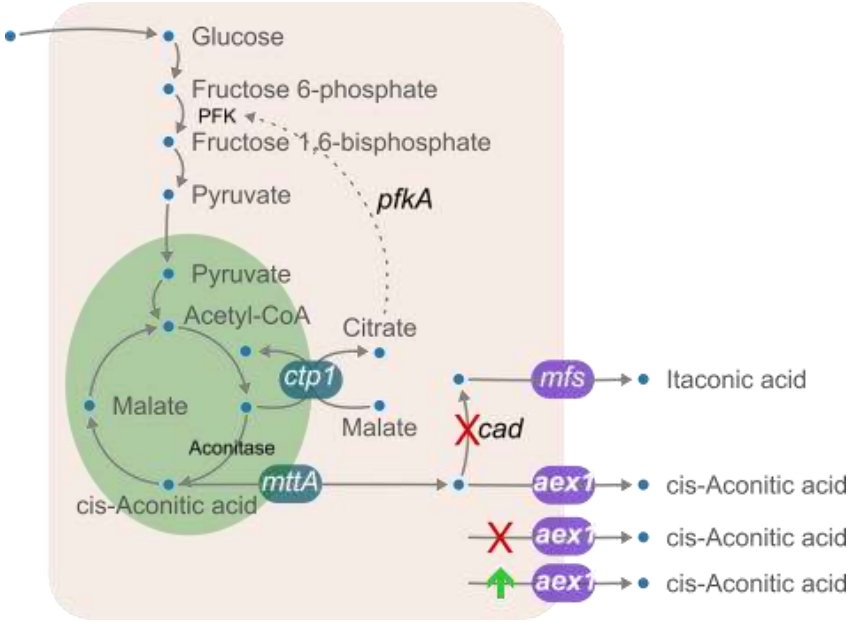


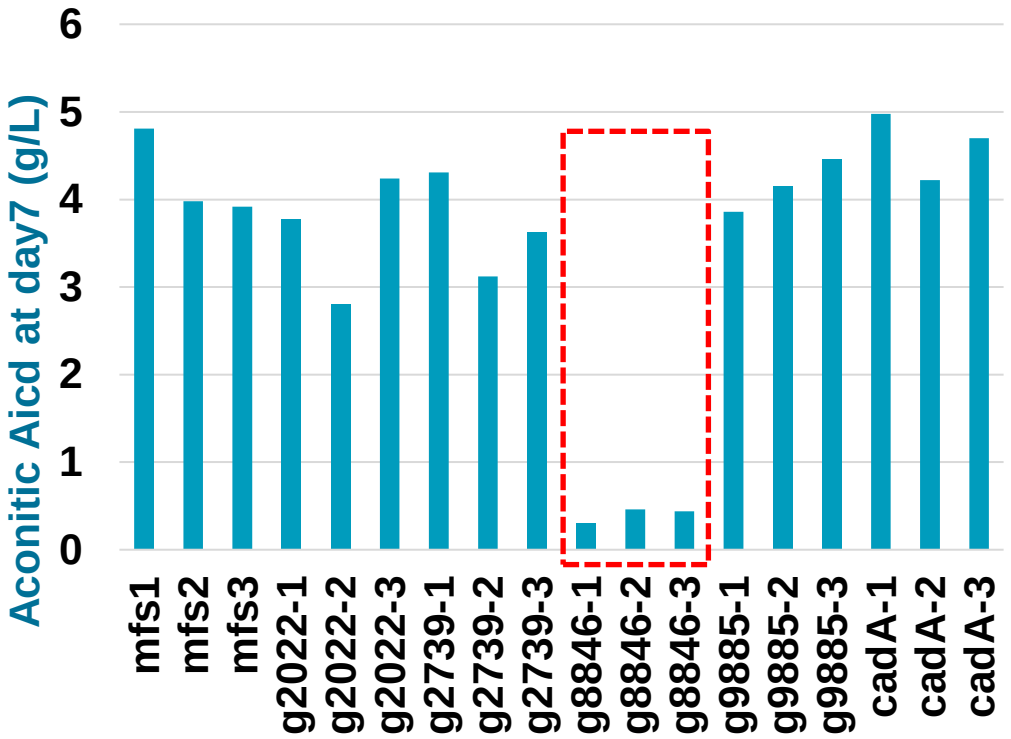
- Top 10 transporters annotated as MFS, sorted by WT vs cad- expression difference
- cis-Aconitic acid transporter candidates
  - g9885
  - g8846
  - g2739

Outcomes: DBTL Efficiency

- TEST-LEARN analysis enabled identification of a prioritized list of aconitic acid transporter candidates from poorly annotated MFSs
- These would NOT have been flagged by traditional biochemical intuition

Deletion analysis identifies transporter for aconitic acid



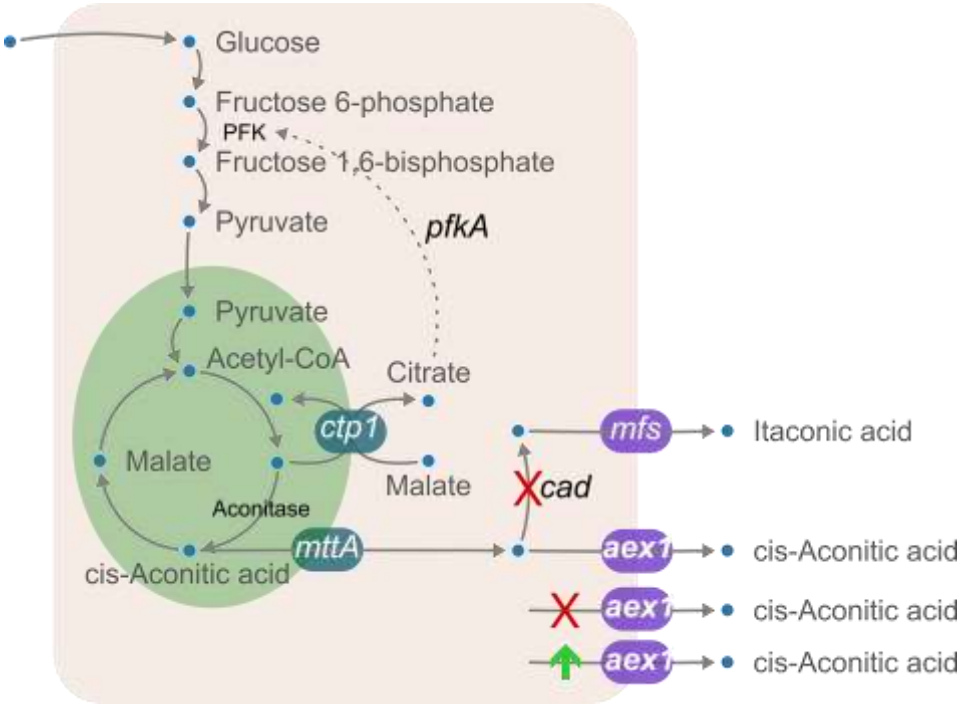


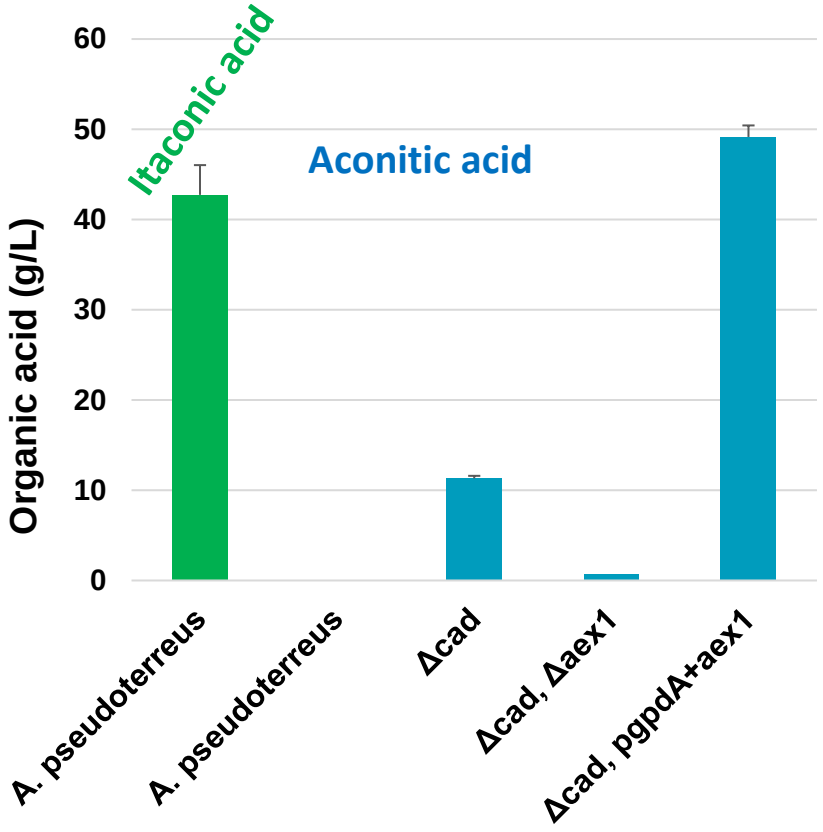
Three transformants of five candidate transporter deletions

DBTL Cycle: transporter testing

- Test/Learn – Proteomics
- Identification of *cis*-aconitic acid transporter candidates

Overexpression of transporter candidate: Aconitic acid production increased 5X





Outcomes

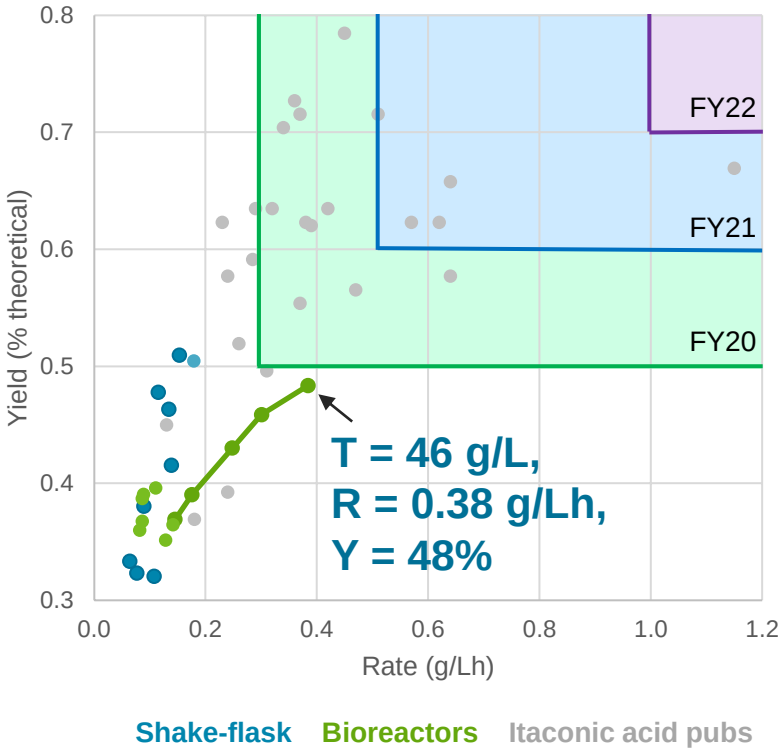
- Hypothesis regarding transport limitations verified through DBTL cycle focused on **efficiently** identifying and verifying candidate aconitate transporter genes
- **5x increase in titer**, met FY20 **Annual Milestone** and Titer target of FY21

A. pseudoterreus scale-up: Optimizing bioprocess parameters

Target-Host Annual TRY Milestones

- ***FY20:*** **Demonstrate a TRY of 20 g/L, 0.3 g/L/hr, and 50% of theoretical yield**
- *FY21:* Demonstrate a TRY of 40 g/L, 0.5 g/L/hr, and 60% of theoretical yield
- *FY22:* Demonstrate a TRY of 80 g/L, 1.0 g/L/hr, and 70% of theoretical yield

Parameters for *A. pseudoterreus* at bioreactor scale from the Literature



Outcomes

- **Met FY20 Milestone**
- **DBTL efficiency** demonstrated in rapid identification and validation of aconitic acid transporter ***aex1***
- **Patent applications**

Philosophy: Parallel Genetic and Bioprocess Engineering Strategy

TEST-LEARN In Progress: *Aspergillus pseudoterreus* aconitic acid

Test-Learn Experiment

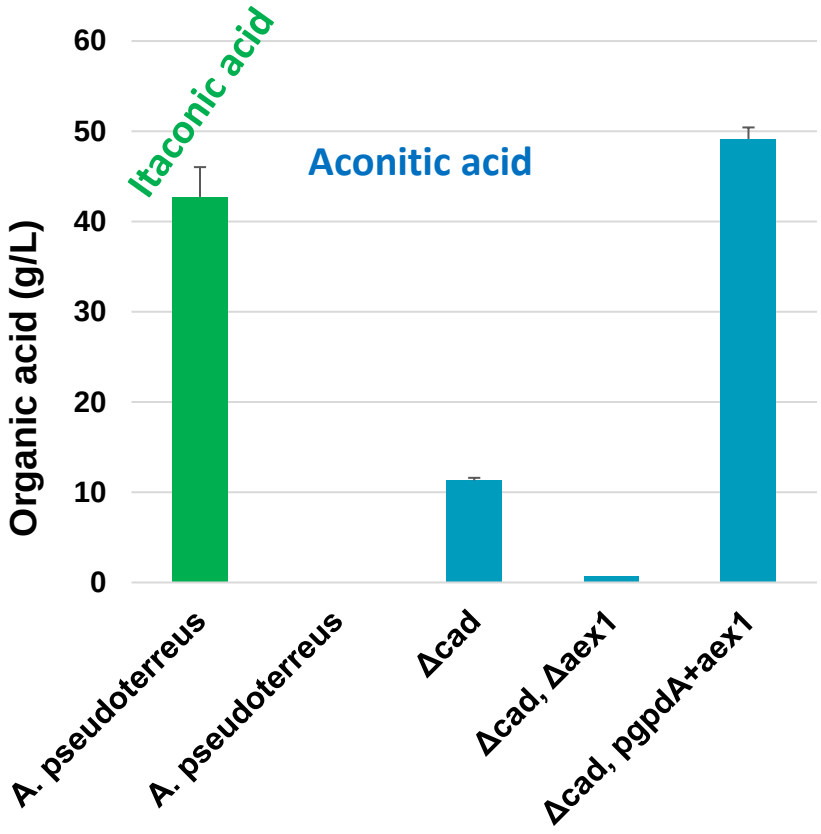
- Three strains: wt, *cad*, *cad+ aex1+*
- Two media: high P, low P. Affect cell mass and production rates

Approach

- Traditional genome modeling & bioinformatics analysis
- Deep Learning for non-intuitive gene candidates

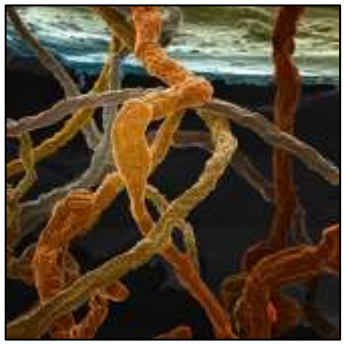
Objective

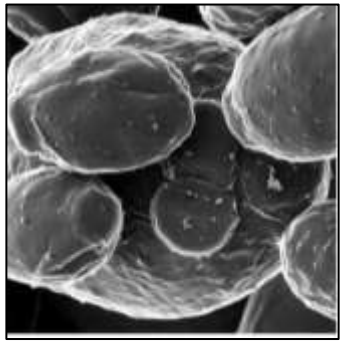
- Identify new gene candidates to increase TRY

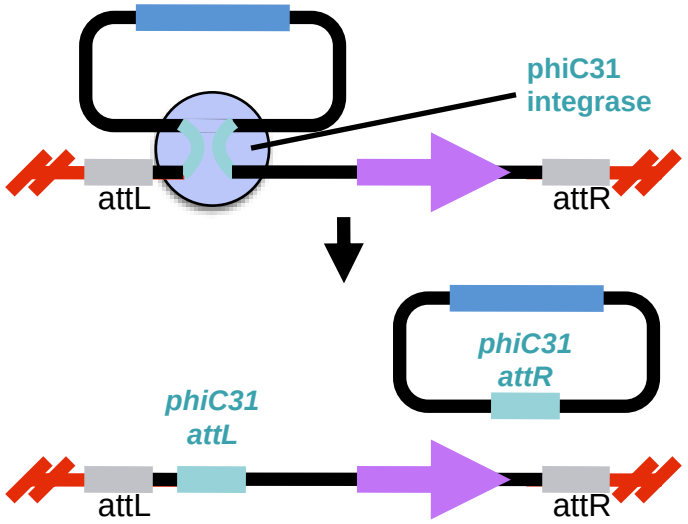


Broad Design-Build Tools for Fungi

- Improving gene manipulation & stacking tools (in progress)
  - Adaption of bacterial **serine integrase** systems to fungi to increase build throughput and decrease variability in expression level (ORNL/PNNL, in progress).
  - **Cre/Lox recombinase** and inducible promotor systems for *Aspergilli* to recycle markers (in progress).
- Improving expression level
  - High expression *Aspergillus* **promoters**
  - **Universal codon and promoter** identification for fungi. **Design** of constructs and genetic tools that function in diverse species







Progress Summary: Milestones Achieved
(Collaborations of TH, Infrastructure, HOD, IA, PISU Teams)

Purpose:Improving TRY of industrially relevant organic acid Beachhead/Exemplar targets to advance DBTL capabilities for *Aspergillus*

Milestones

Target-Host

√ •*FY20Q4_DBTL* Demonstrate a TRY of 20 g/L, 0.3 g/L/hr, and 50% of theoretical yield from DMR-EH or mock hydrolysate

√ •*FY21Q4_DBTL* Demonstrate a TRY of 40 g/L, 0.5 g/L/hr, and 60% of theoretical yield from DMR-EH or mock hydrolysate

•*FY22Q4_DBTL* Demonstrate a TRY of 80 g/L, 1.0 g/L/hr, and 70% of theoretical yield from DMR-EH or mock hydrolysate

DBTL metrics

√ •*FY20Q4_DBTL* Demonstrate a 2X improvement over 2019 performance baseline in both DBTL cycle efficiency and titer (objective: 1-2 g/L) for three compounds of direct relevance to producing Performance-Advantaged BioProducts at sufficient quantities for polymer testing (10 g) by BETO's Performance-Advantaged BioProducts mini-consortium

Host Onboarding and Development

√ •*FY20Q2_HOD* Compile a list of the top 15 most important hosts for the ABF to develop

PISU

√ •*FY20Q4_PISU R*ound robin testing of target/host pairs

Summary and Next Steps

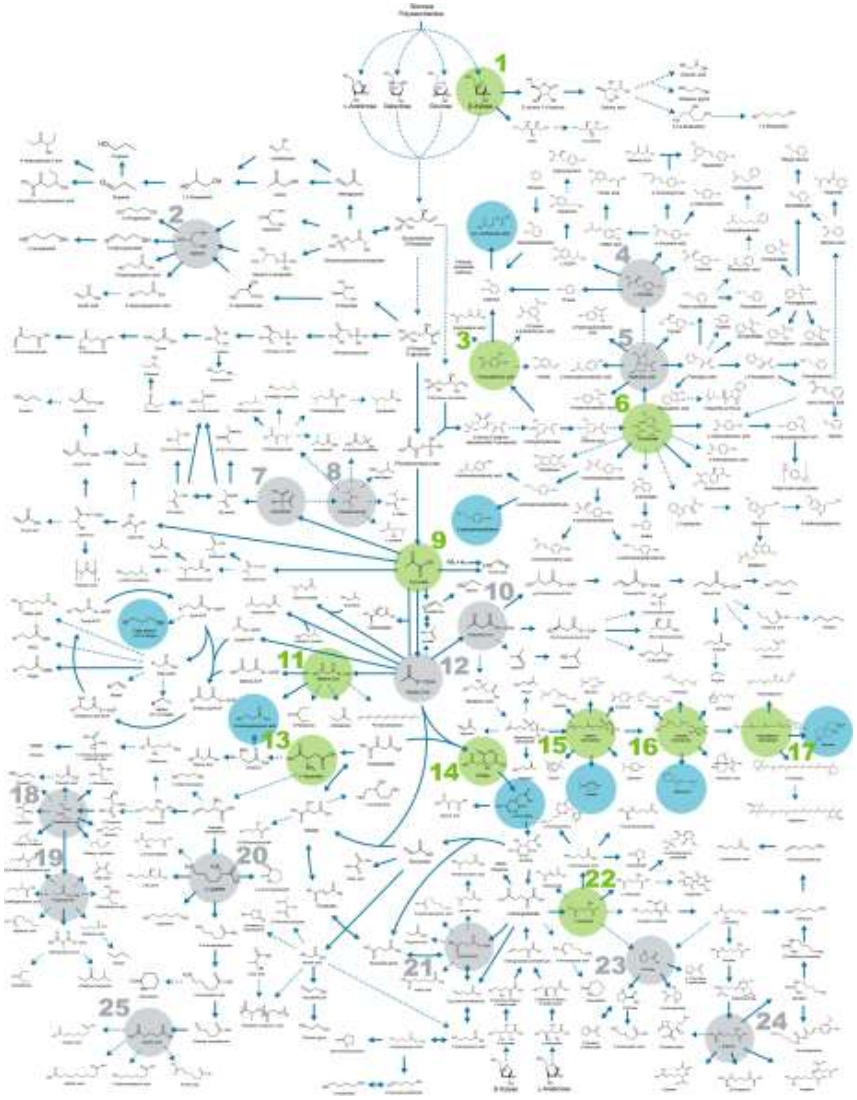
3HP in *A. niger*

- Large DBTL experiment with 17 strains to identify gene targets for achieving FY21/22 TRY goals…**TEST/LEARN**
- **TRY** on **DMR** is very encouraging:
  T = 29 g/L, R = 0.17 g/L·h, Y = 36%
- **TEA**: R = 0.15-0.30, Y = 25-50%, biggest gains

Aconitic Acid in *A. pseudoterreus*

- Large DBTL experiment to identify gene targets for achieving FY21/22 TRY goals…**TEST/LEARN**
- Rational targets: internal transporters and metabolic flux targets in the *aex1* over-expression strain
- **TRY in Bioreactor:**
  T = 46 g/L, R = 0.38 g/L·h, Y = 48%

Fungal DB Tool Development



Acknowledgements
ABF Aspergillus and Test Teams/Collaborators

PNNL
Scott Baker
Aivett Bilbao
Kristin Burnum-Johnson
Jim Collett
Ziyu Dai
Shuang Deng
Yuqian Gao
Beth Hofstad
Joonhoon Kim
Young-Mo Kim
Anna Lipton
Jon Magnuson
Nathalie Munoz Munoz
Kyle Pomraning
Ana Robles
Jeremy Zucker

NREL
Gregg Beckham
Ryan Davis
Chris Johnson
Bruno Klein
Davinia Salvachua
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LANL
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LBNL
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James Garner
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Ethan Oksen
Chris Petzold
Hector Plahar
Jan-Philip Prahl
Alastair Robinson
Blake A. Simmons
Deepti Tanjore

Target and Host Engineering: Yeast

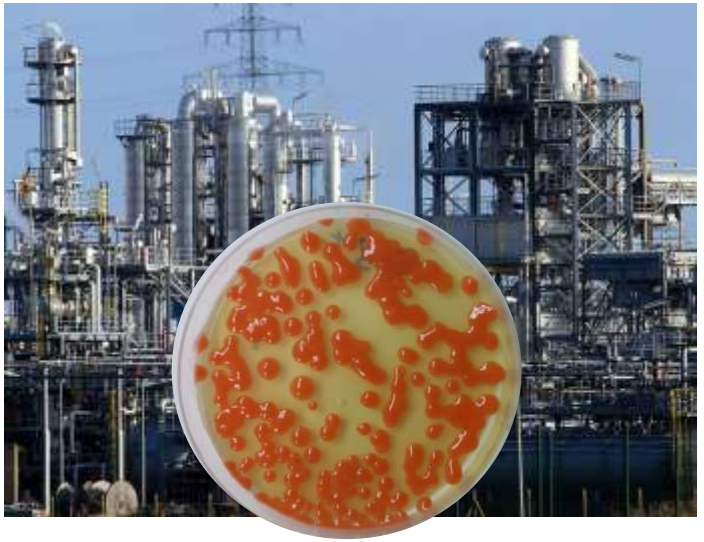
Project Overview

History: Task initiated at the beginning of the Agile BioFoundry

- *Rhodosporidium 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

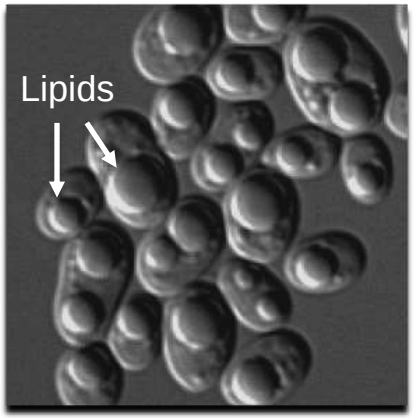
- Naturally consumes lignocellulose: pentose, hexose, aromatics
- High natural flux in terpene and lipid pathways

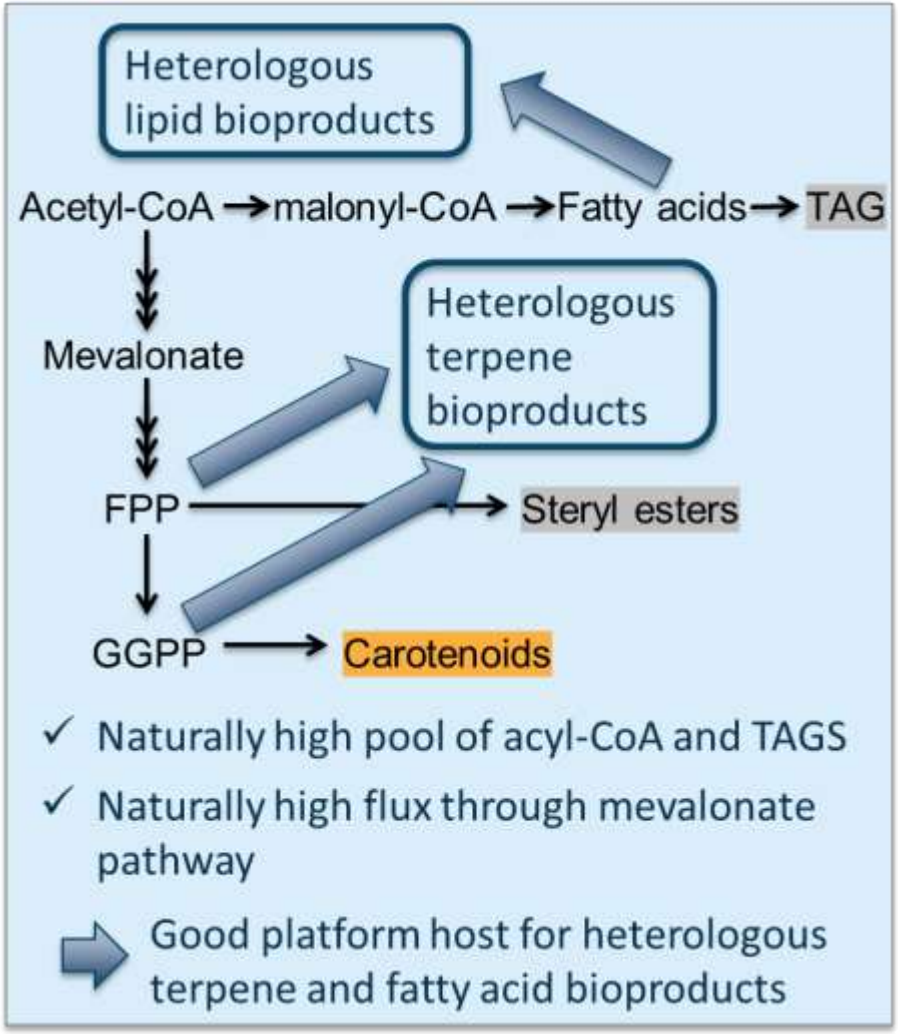


Project goals:

- 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*?





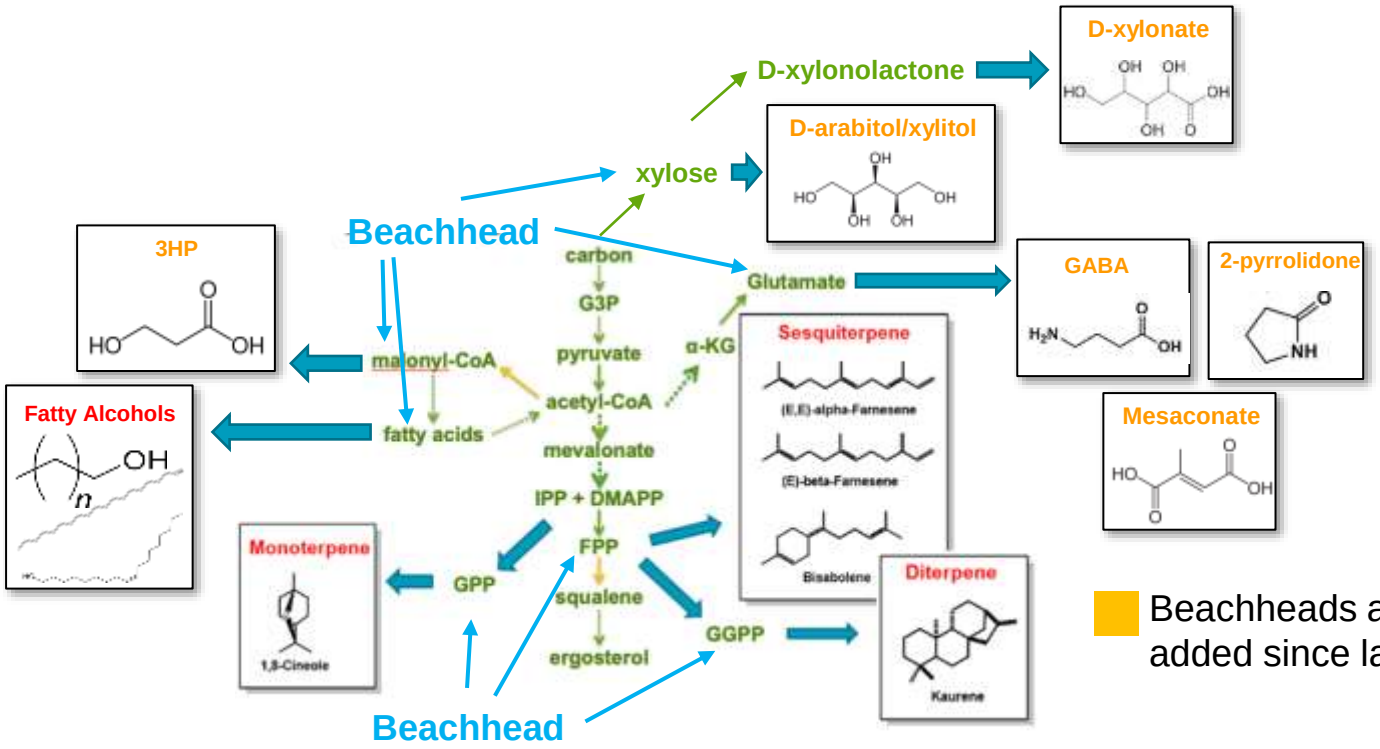
Rhodosporidium toruloides

- 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



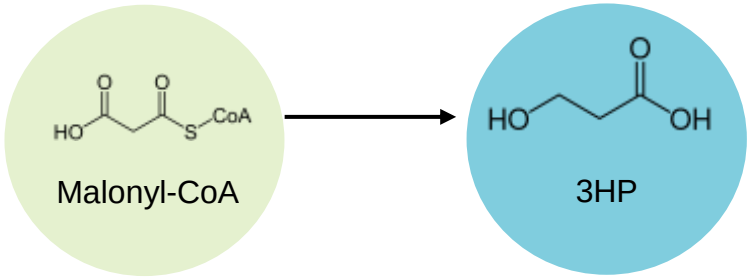
Beachheads and Targets added since last peer review

*We will provide updates on 3HP and Xylose products as an example of T/H engineering

Project overview: Why these products?

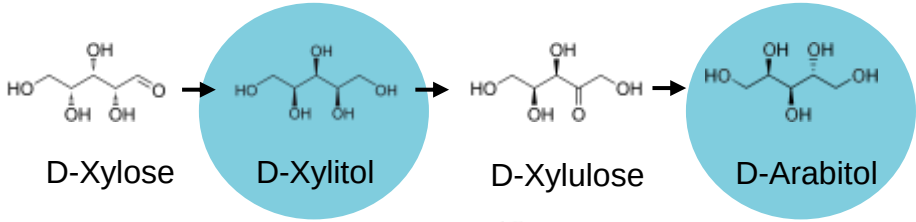
3-Hydroxypropionic acid

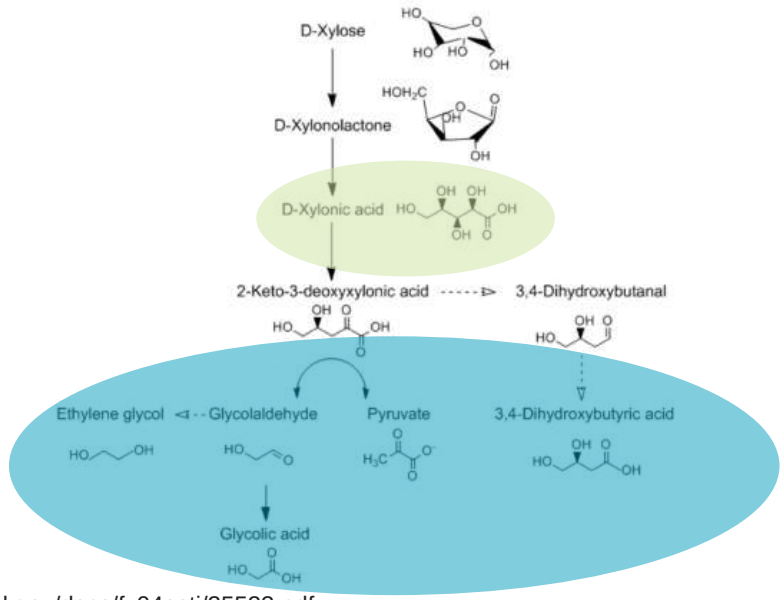
- Transfer target from *Aspergillus pseudoterreus*
- Third of 12 critical building-block chemicals for the DOE[1]
- Easily converted into acrylic acid, a market of ~$19 billion[2]



Pentose Bioproducts

- Arabitol/xylitol
  - Identified as a top value added product from biomass[3]
  - Applications in polymers, resins, plasticizers, solvents
  - market projected to reach $1.4 billion by 2025
- Xylonic acid-
  - Identified as among the top value added chemicals from biomass[3]
  - beachhead for several bioproducts, e.g. ethylene glycol, glycolic acid, 1,2,4-butanetriol, 3,4-dihydroxybutyric acid, and 3-deoxypentanoic acid
  - Applications in polymers, plasticizers, dyeing and concrete dispersal agents, adhesives, etc.





- **Beachhead molecule:** malonyl-CoA, xylonic acid
- **Host:** *Rhodosporidium toruloides* IFO0880

1. Werpy 2004
2. Kildegaard 2018
3. https://www.nrel.gov/docs/fy04osti/35523.pdf

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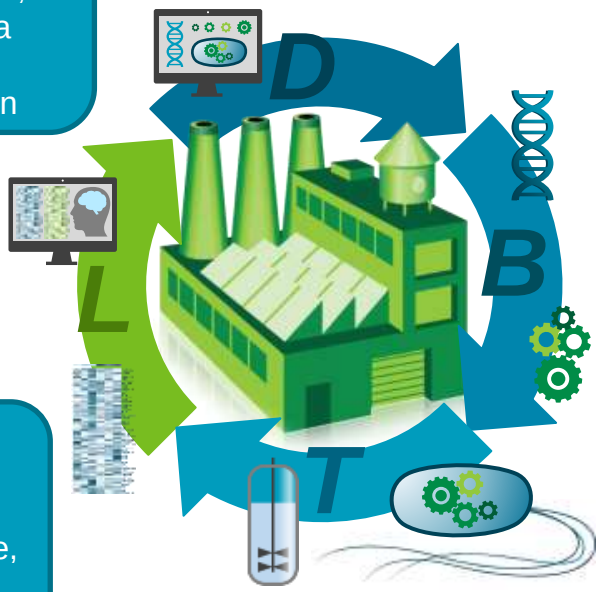
Management

- **Virtual meetings:** biweekly calls with *R. toruloides* team, monthly with Test/Learn teams
- **F2F meetings**: ABF annual all-hands and leadership
- **Updates**: regular team updates on task lead call, monthly DBTL tracking
- **Milestones:** DBTL cycle times, product performance metrics
- **Project interfacing:** *ad hoc* meetings with Integrated Analysis, Integration and Scaling, other BETO consortia



Lead, DBTL tools, applications

John Gladden

Design, Build, EDD tools

Nathan Hillson, Hector Garcia Martin
John Gladden

Scale and Integration

Deepti Tanjore, Gregg Beckham

Learn tools

Hector Garcia Martin, Phil Laible, Joonhoon Kim

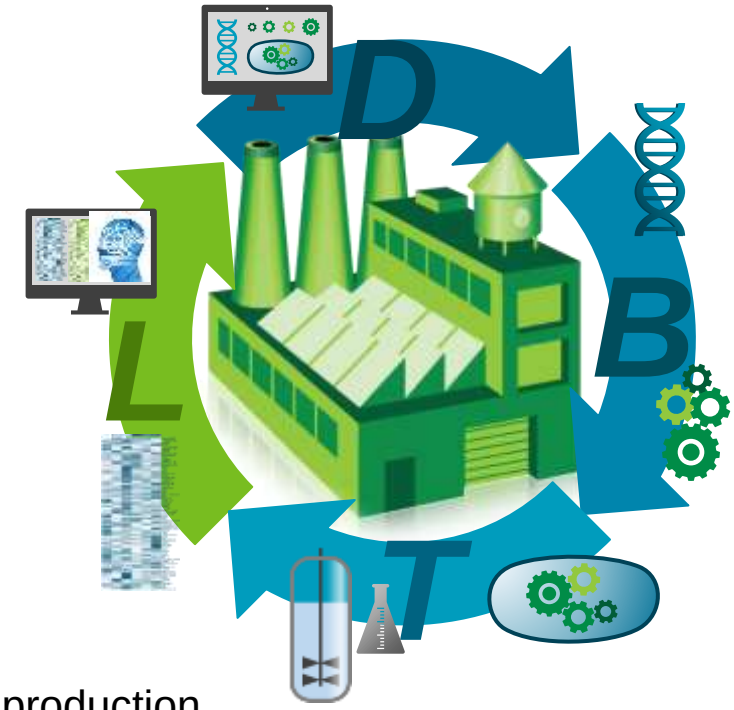
Test multi omics

Jon Magnuson, Kristin Burnum Johnson, Joonhoon Kim

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

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

Impact

Impact on state of technology and/or industry if project is successful

- Improved strain performance enabled by DBTL to reduce time-to-scale up
- Demonstrated ability to make non-intuitive predictions from Learn for strain engineering
- Contribute to overall BETO and bioeconomy goals of using non-standard strains to produce drop-in replacements and performance-advantaged bioproducts
- Learn can inform scaling activities and vice versa
- Synthetic biology towards valuable co-products will be critical for the viability of the US and global bioeconomy
- Learn activities directly advance “State of Technology” over solely rational strain engineering approaches
- *R. toruloides* is a promising chassis for bioproduction of a wide range of products

Dissemination of Results

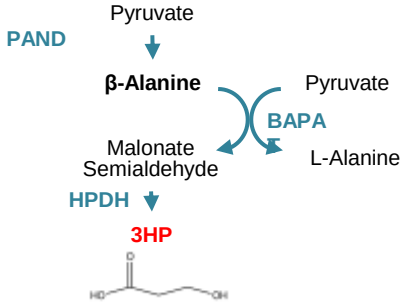
- Patent applications on engineered strains, enzymes, and new pathways
- Peer-reviewed publications in the pipeline describing work in collaboration with other BETO projects and across the ABF tasks
- Industry collaboration through DFOs and BEEPs projects: DFO and BEEPS with Lygos to engineer their yeast to produce organic acids; C16 Biosciences DFO that uses *R. toruloides* to produce an alternative palm oil leveraging tools and strains developed within the ABF to produce fatty alcohols

Progress and Outcomes

Transfer Target: 3HP from *A. pseudoterreus*

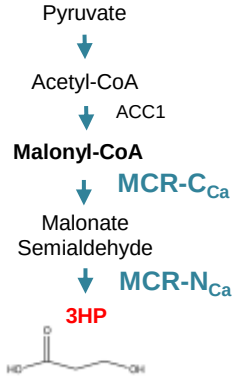
- Transfer targets help exemplify the value proposition of the ABF
- Pathways used in *A. pseudoterreus* for making 3HP

Design 1
β-Alanine Pathway



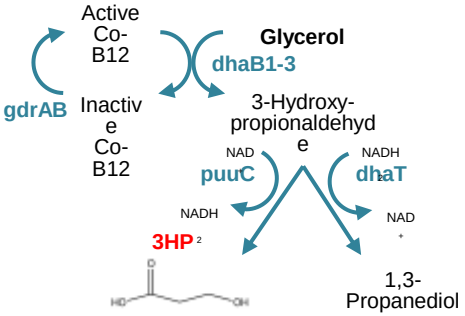
Top pathway in *A. pseudoterreus*

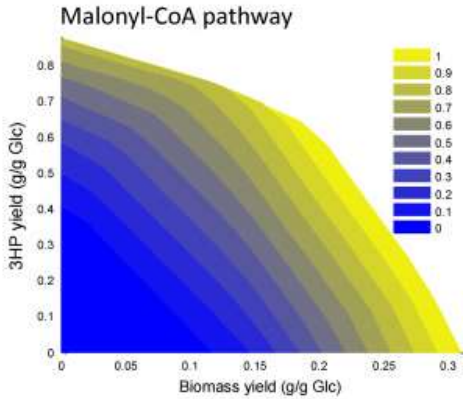
Design 2
Malonyl-CoA Pathway

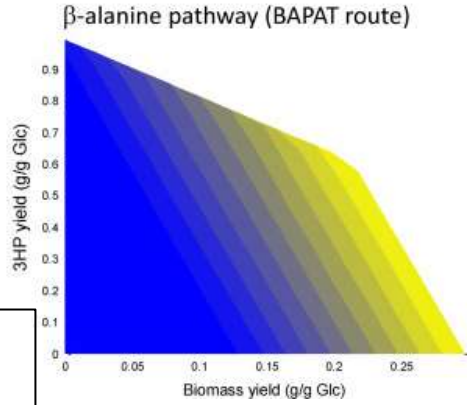


Top pathway in *R. toruloides*

Design 3
Glycerol Pathway





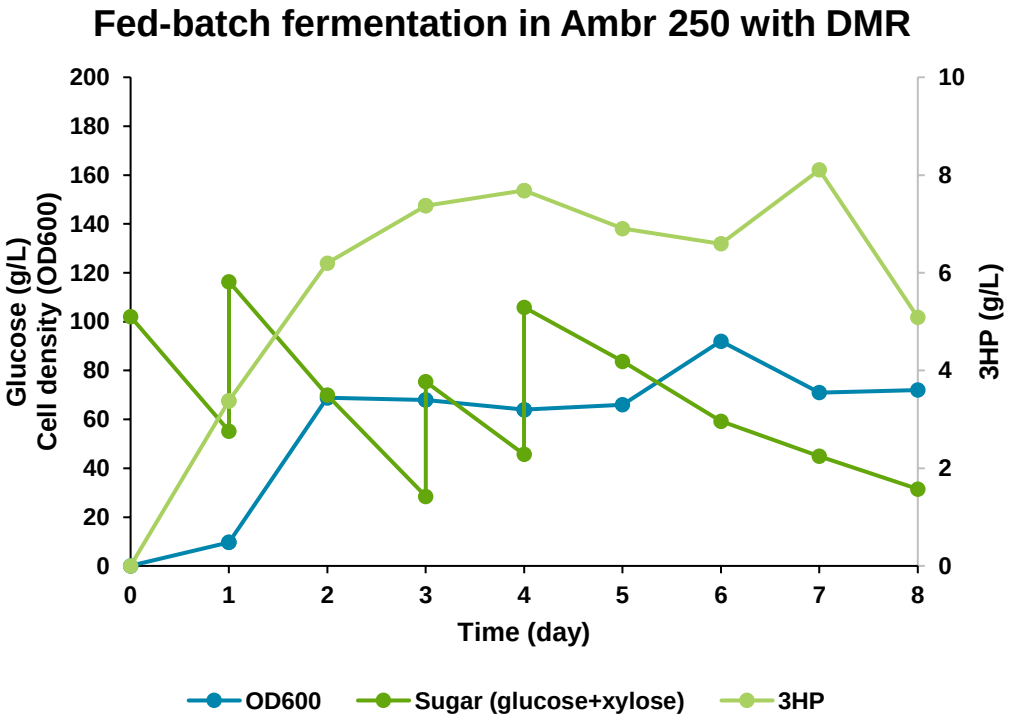


Yields from B-alanine and malonyl-CoA pathways are similar under typical oxygenation and cell biomass

FY21Q2 Go/No-Go: Transfer of 5 metabolic pathways and/or tools between hosts, with 2X improvements in second host, defining metrics for each case. Titers in both first and new host are > 1 g/L. For unsuccessful efforts, perform post-mortem, address specific reasons for why not achieved.

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



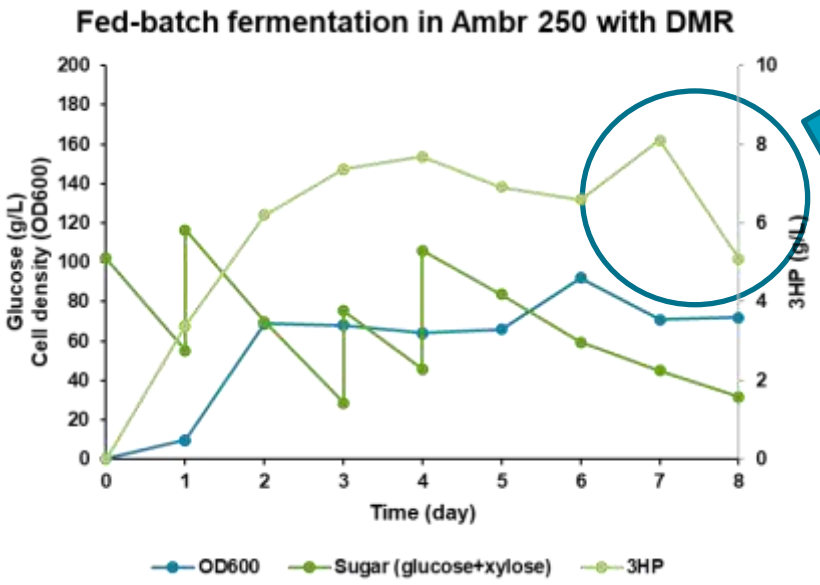
- Utilized medium: DMR with high nitrogen
- Feeding: 50 g/L glucose
- TRY
  - Maximum titer: 8.1 g/L
  - Maximum yield: 0.072 g/g sugar
  - Maximum rate: 0.14 g/L/h

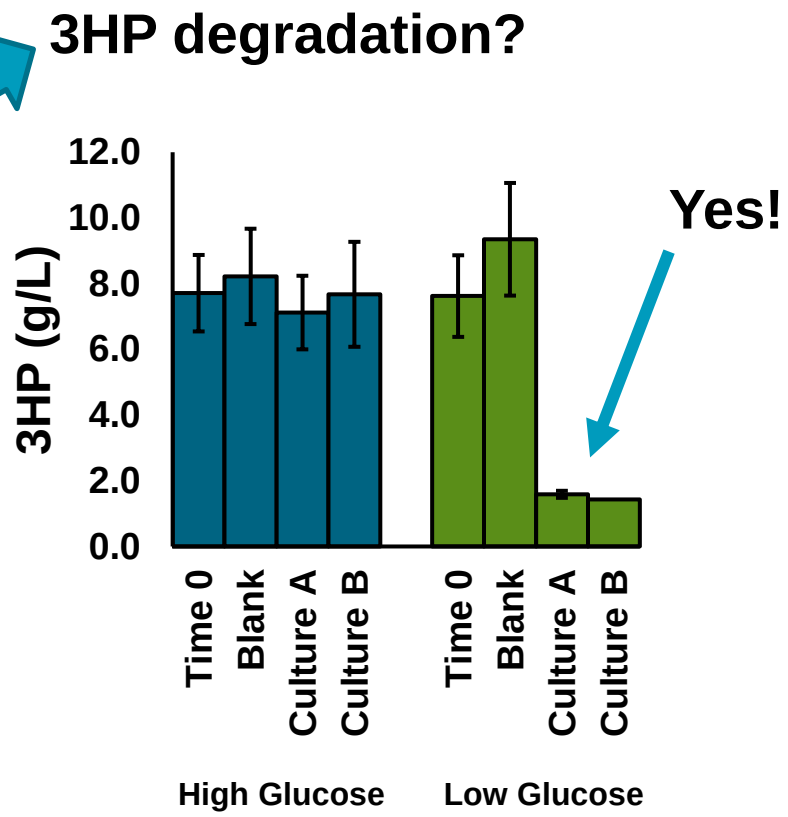
Organism	Titer	Reference
Sach. Cer.	13.7g/L	Borodina, 2014
Sach. Cer.	7.4g/L	Kildegaard, 2015
Methyloibacterium exoterquens AM1	0.07g/L	Yang, 2017
Sach. Cer.	1g/L	David, 2016
E. coli	40.6g/L	Liu, 2016

- Samples collected for multi-omic analysis with Test Team

R. toruloides consumes 3HP

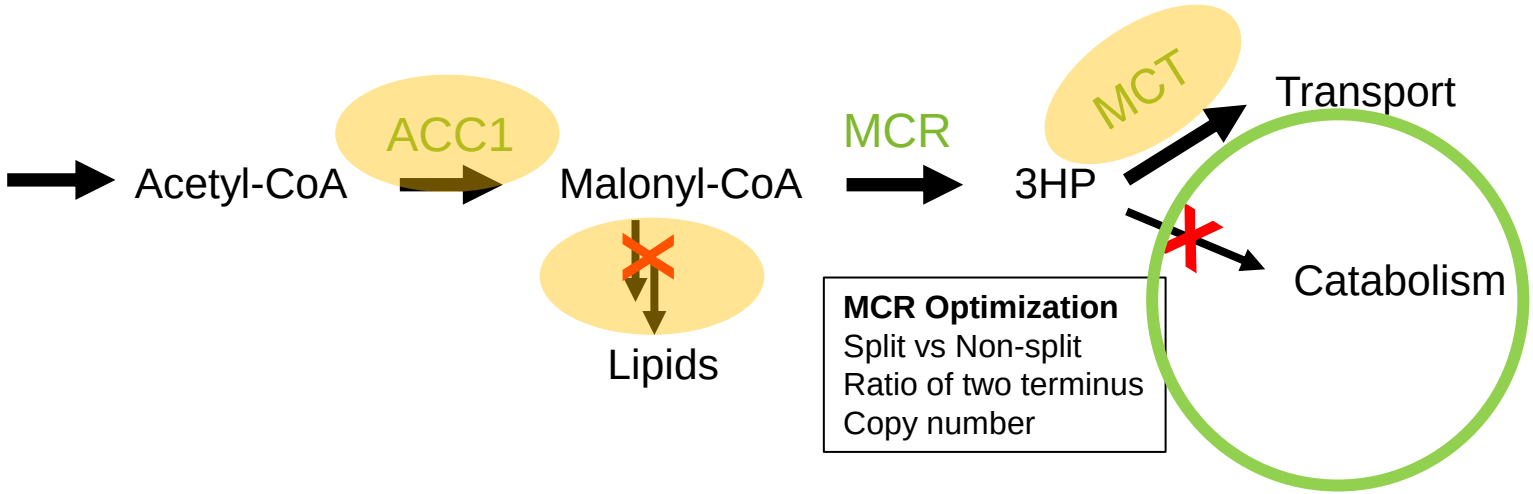
- Bioreactor Ambr 250 data suggests that 3HP may be consumed
- Tests verified 3HP is consumed by *R. toruloides*





Engineering strategies for 3HP production

Pathway Engineering

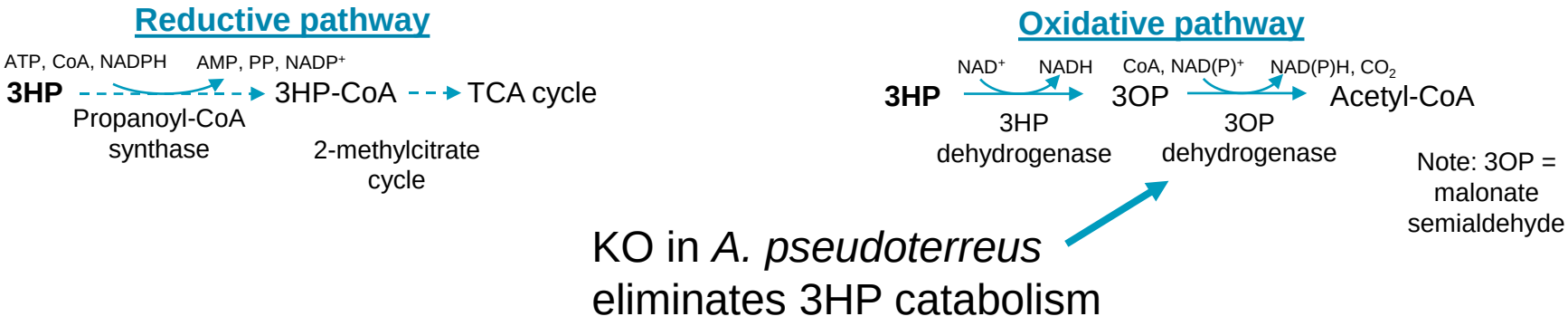


Process Development

- Medium Optimization
- Bioreactor Optimization

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

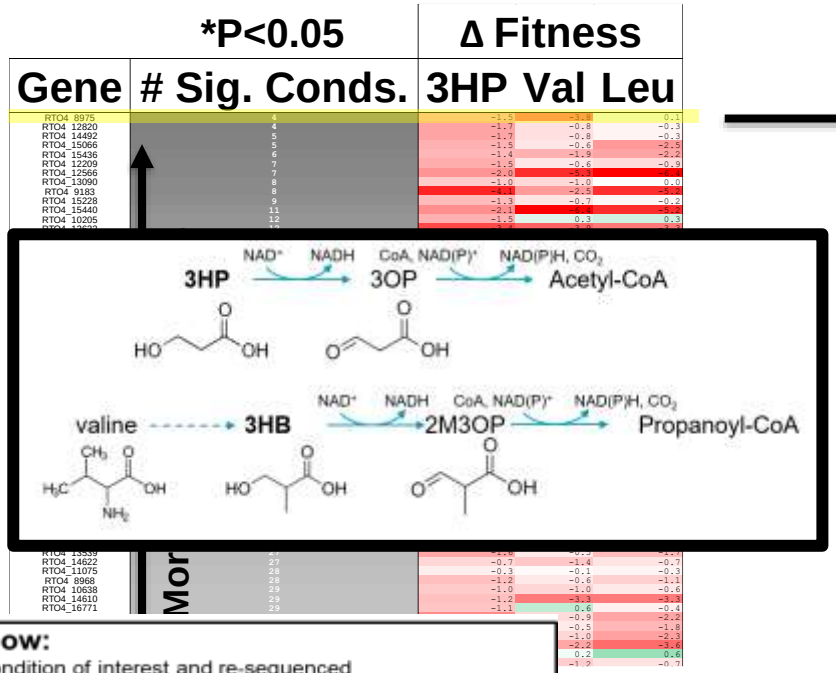


Some possible homologous to the *A. pseudo* genes were found in *R. toruloides*

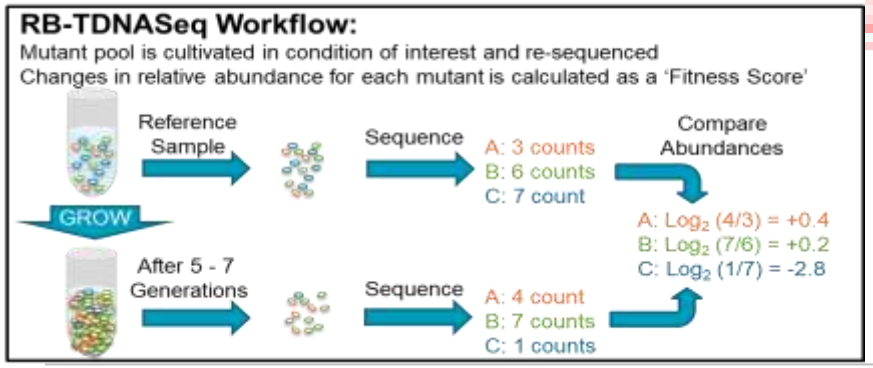
None	**RT_2MC1** **RT_2MC2**	**RT_???**	**RT_ALD1** **RT_ALD2** **….** **RT_ALD6**

Functional Genomics to elucidate 3HP catabolism

- **Discovered 65 KOs with significant fitness impacts**

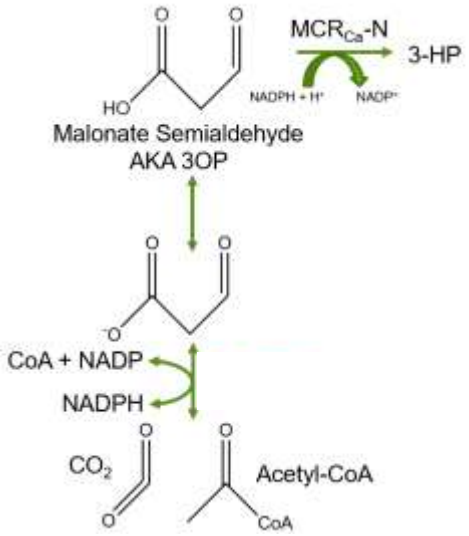


- **Fitness defects suggests the Valine degradation pathway maybe be involved in 3HP degradation**



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

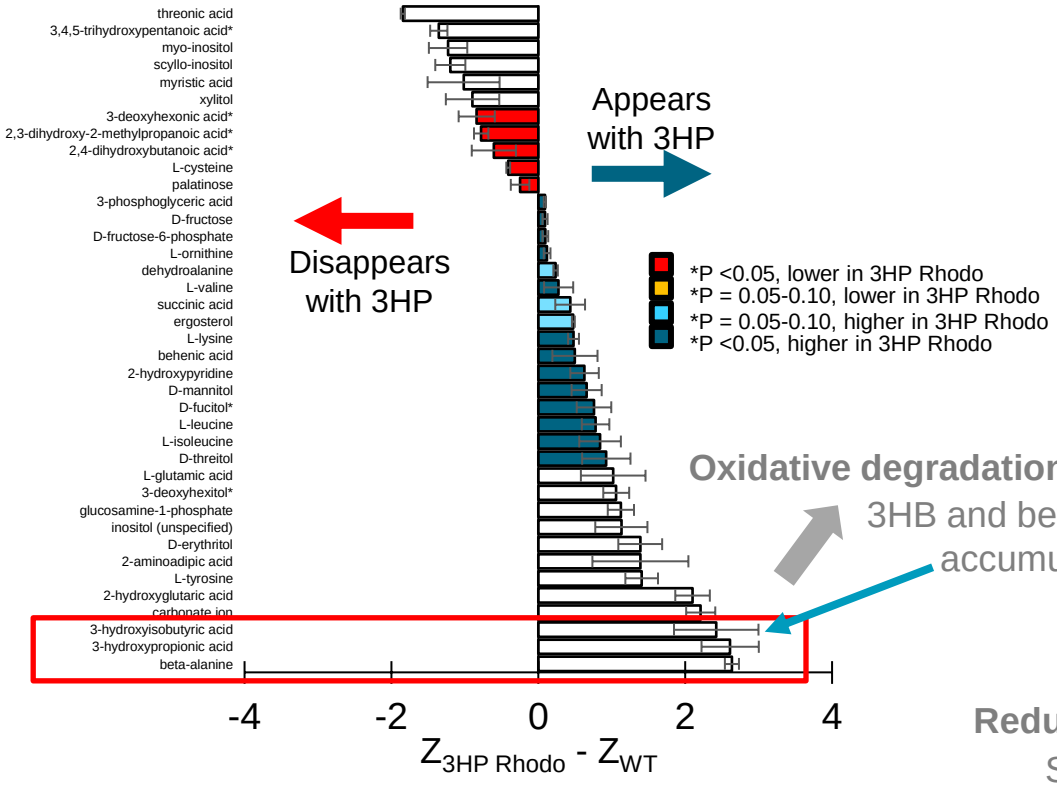
ALD6, Malonate-Semialdehyde Dehydrogenase (Acetylating)



Metabolomics inform on 3HP catabolism

- **Metabolomics results from 3HP-producing *R. toruloides* strains**
- **Some metabolites suggest that both oxidative and reductive 3HP catabolic pathways may exist**

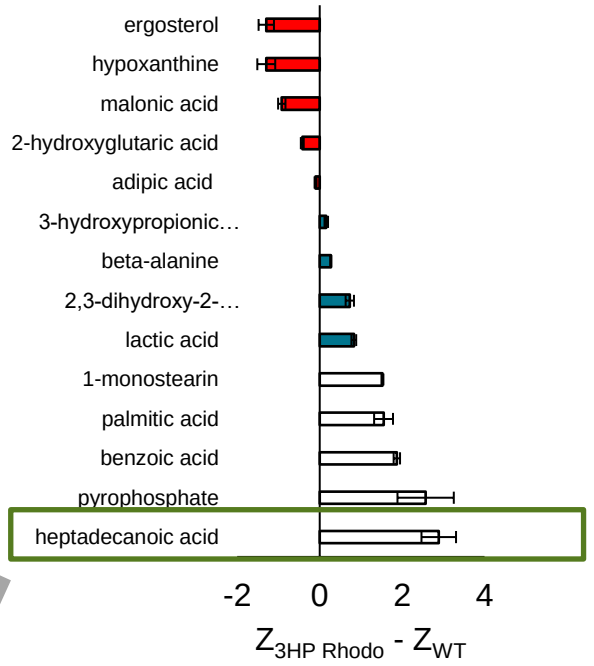
Differentially Expressed Metabolites: Day 4



Oxidative degradation pathway

3HB and beta-alanine accumulates

Differentially Expressed Metabolites: Day 1

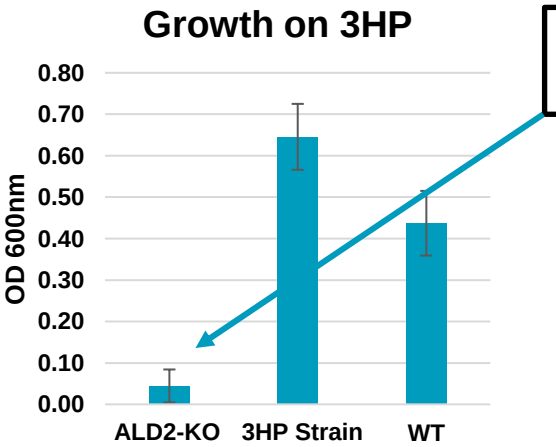


Reductive degradation pathway

Synthesized from propionyl-CoA

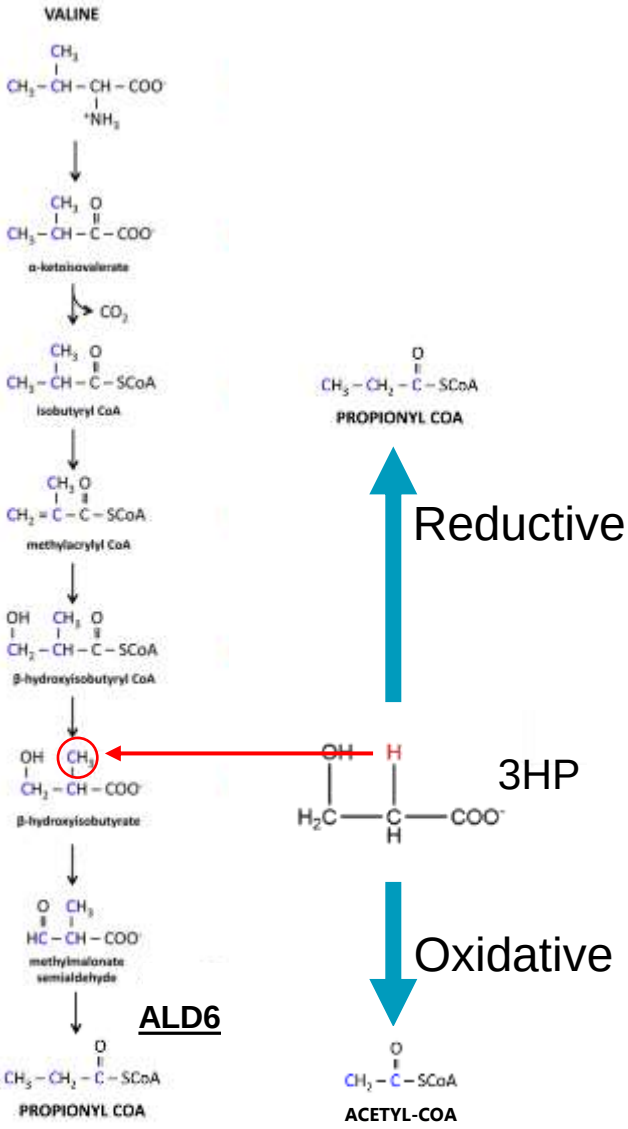
Valine catabolic pathway and 3HP

- Enzymes in Valine degradation pathway may be responsible for both oxidative and reductive 3HP degradation
- Valine pathway metabolites differ by a single methyl group (red circle)
- β-alanine accumulates, suggesting malonate semialdehyde is a key metabolite for both 3HP synthesis and degradation
- Overall, 70 KO candidates identified, with a top 10 list guided by BarSeq and Metabolomics
- KO of top candidate ALD6 eliminates 3HP consumption, suggesting oxidative 3HP degradation is the primary route
- Transfer of knowledge from *Aspergillus* pointed at ALD6



ALD6 KO eliminates 3HP degradation!

- 3HB accumulates in 3HP strains
- Is 3HP being funneled into the valine degradation pathway?



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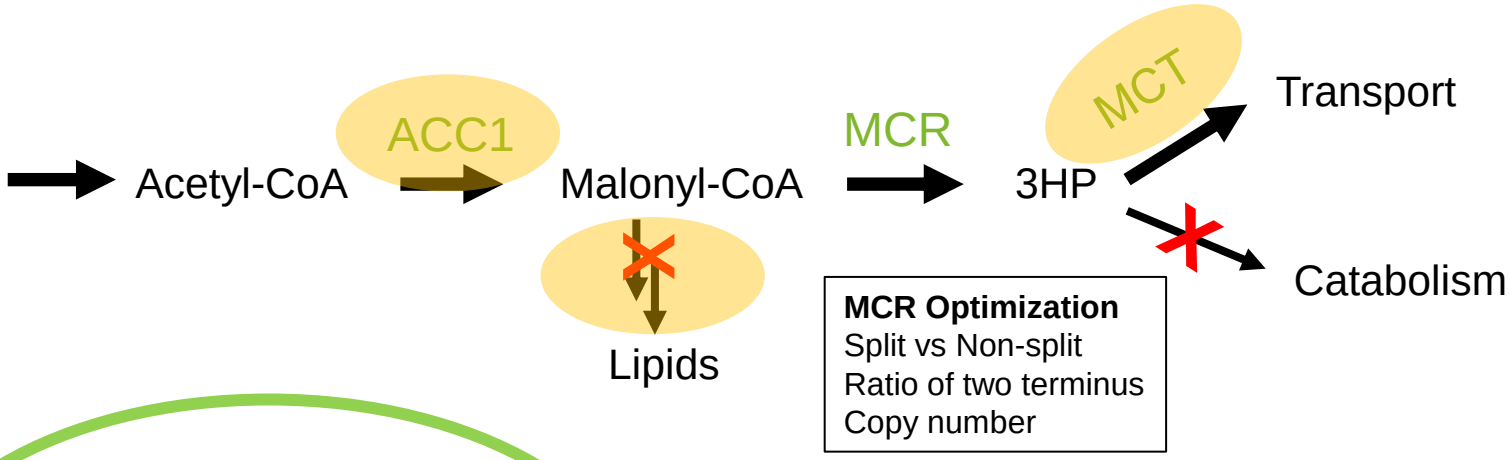
Engineering strategies for 3HP production

Pathway Engineering

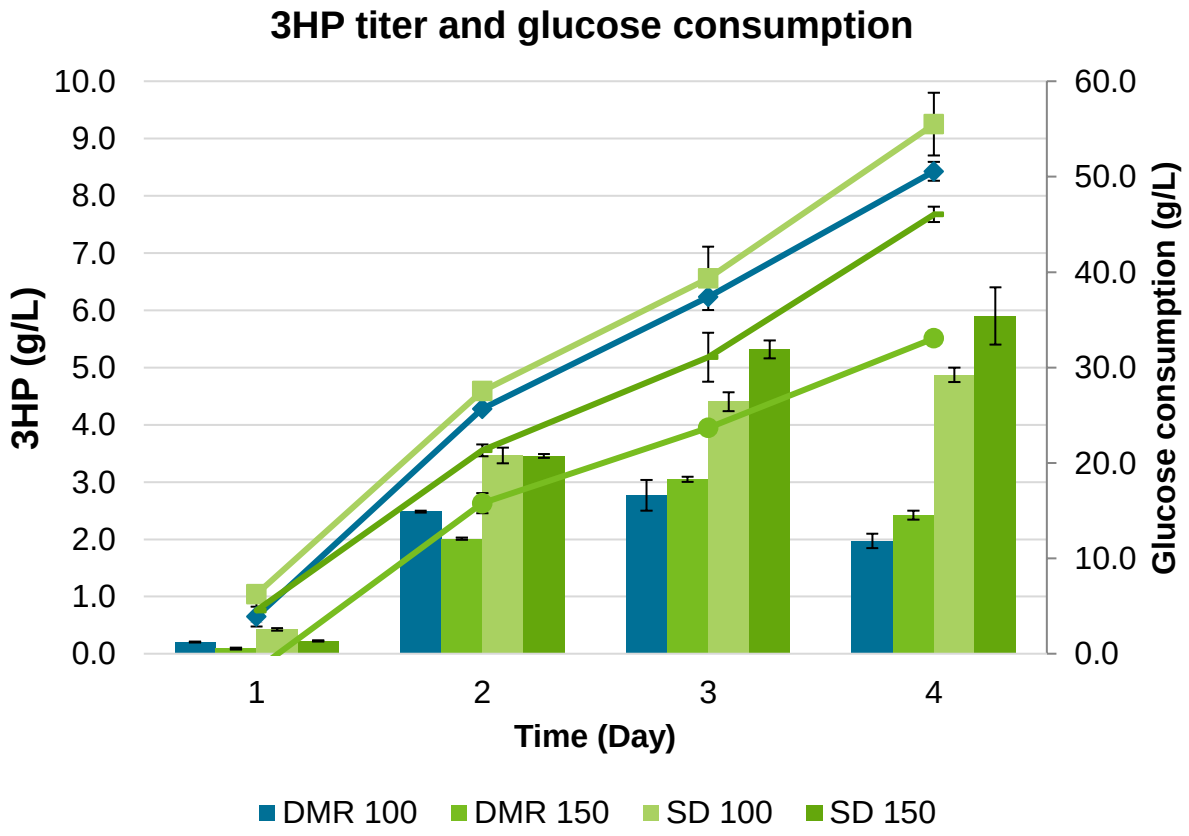


Process Development

- Medium Optimization
- Bioreactor Optimization

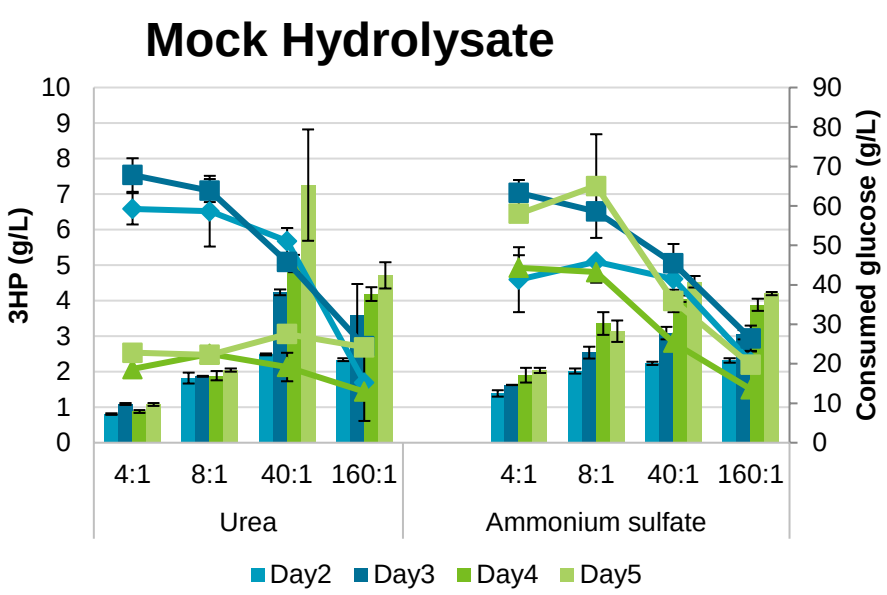
3HP media optimization – sugar concentration

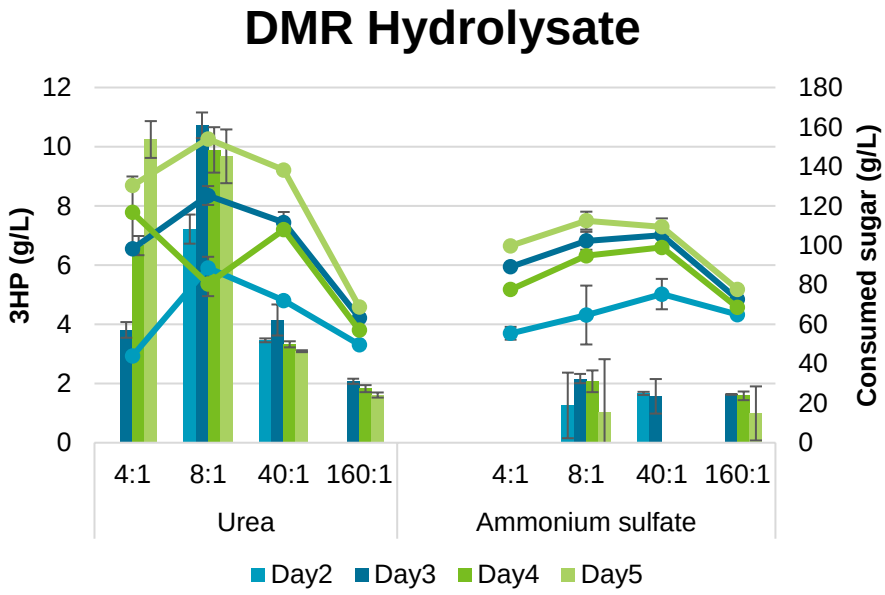
- ***R. toruloides*** **prefers higher gravity hydrolysates**
- **150 g/L sugar optimal for 3HP production, 3 g/L in DMR, 6 g/L in mock**



3HP media optimization – C/N ratio

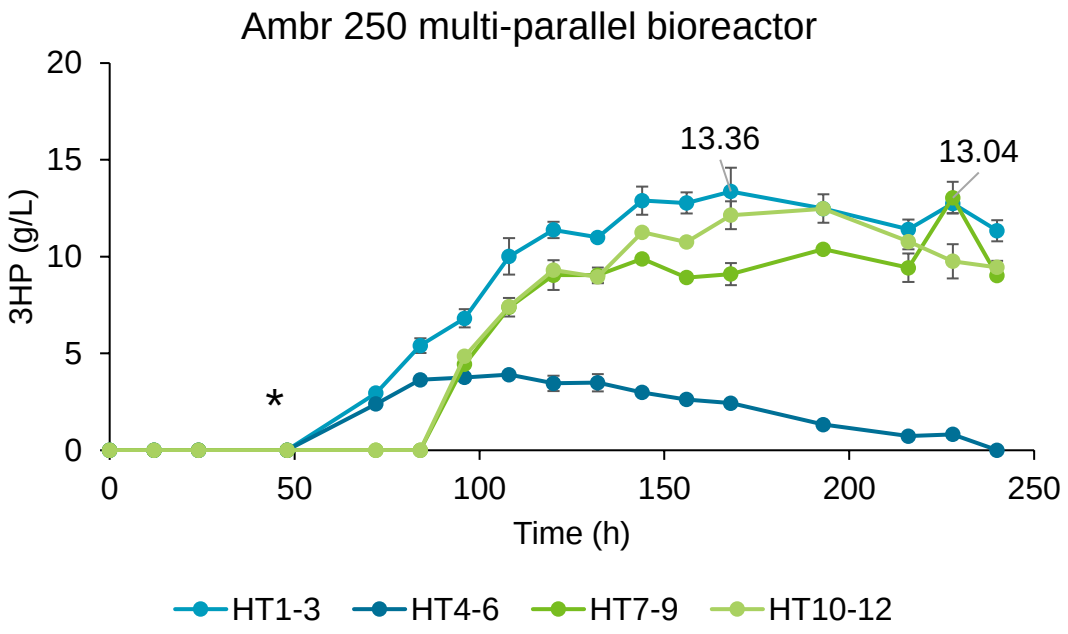
- C/N ratio optimization differs in mock (40:1) vs DMR (4:1 or 8:1)
- Max titer in DMR reach 10 g/L, a 5-fold increase
- Lesson- caution should be taken when optimizing with mock hydrolysates





Optimizing 3HP cultivation conditions

- Bioreactor optimization increased 3HP titers to 13 g/L
- Increased oxygenation has no benefit
- Addition of metal salts to restrict growth was beneficial in shake flaks but was too prominent in bioreactors and needs to be adjusted to maximize 3HP titers
- Rapid culture acidification early in cultivation is detrimental and needs to be tightly controlled



*Lag phase induced by addition of growth-restricting metal salts

Culture medium: DMR + Urea 4:1 C/N ratio

Culture conditions

HT1-3
- pH: uncontrolled
- No glucose feed
- DO: uncontrolled

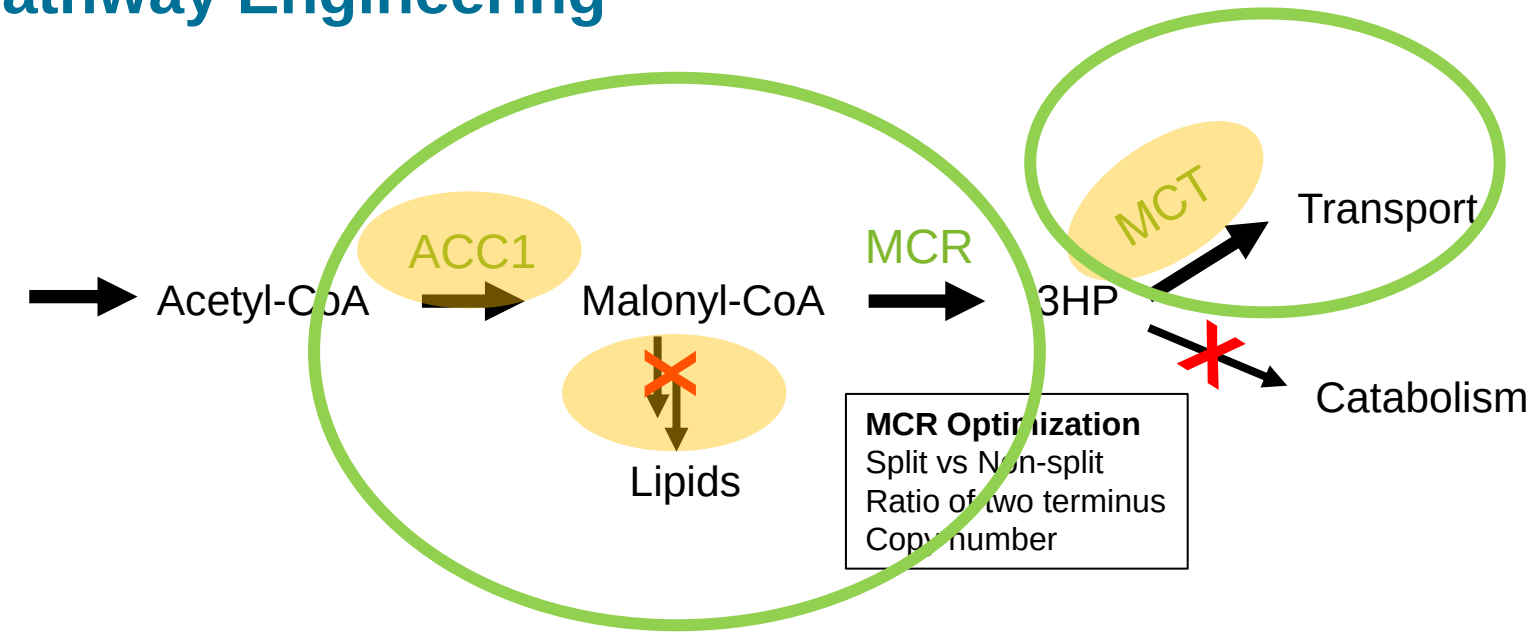
HT4-6
- pH: uncontrolled up to 48h, then slowly forced to pH4 over 24h with acid, then hold at pH4 with acid and base control
- No glucose feed
- DO: uncontrolled

HT7-9
- pH: uncontrolled
- No glucose feed
- DO: 30%

HT10-12
- pH: uncontrolled
- Continuous glucose feed when glucose conc. < 50 g/L
- DO: 30%

Engineering strategies for 3HP production

Pathway Engineering



Process Development

- Medium Optimization
- Bioreactor Optimization

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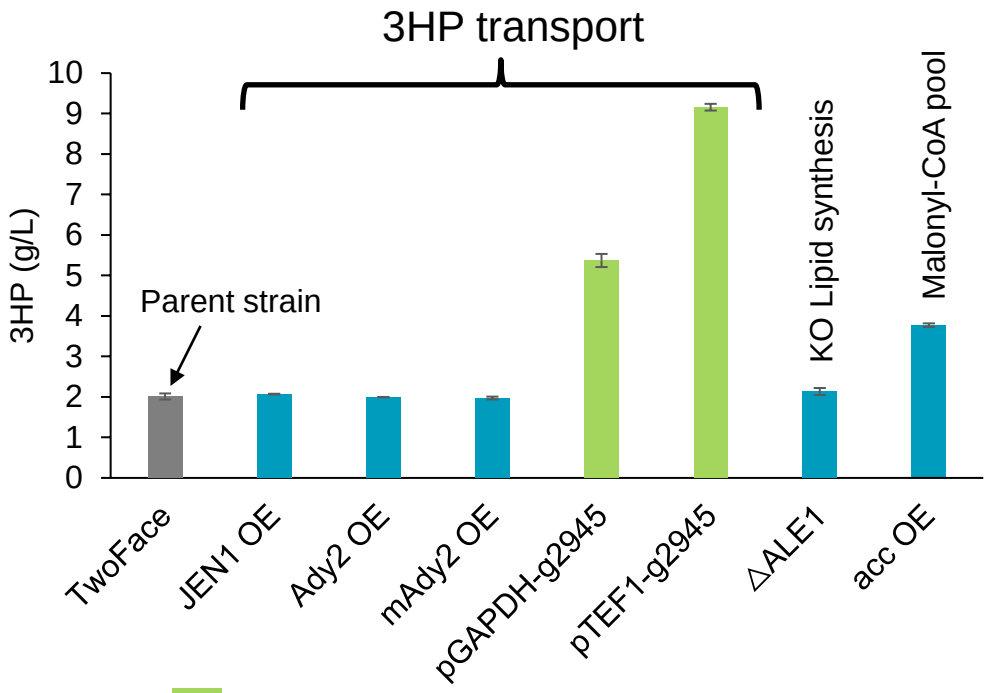
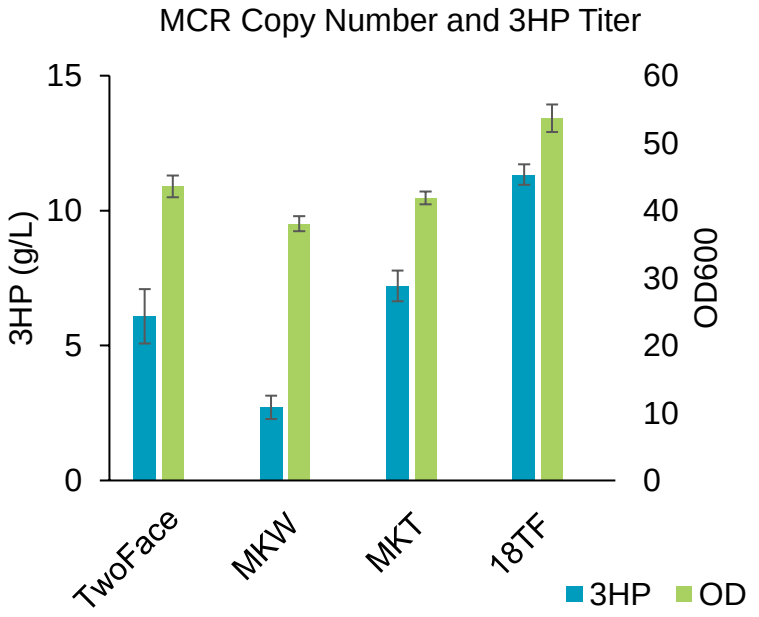
Host Engineering to Improve 3HP Titer

- 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



Knowledge transfer from *A. pseudoterreus*



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

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3HP Summary and Next Steps

- Several target transfer successes: MCR split, 3HP degradation, and transporter
- Up to 13 g/L of 3HP was produced from malonyl-CoA pathway after media optimization
- 3HP titers increased by increasing MCR copy number and malonyl-CoA pool, and expressing a monocarboxylic acid transporter
- 3HP degradation is a problem, but knowledge transfer, multi-omic analysis, and functional genomics has identified degradation pathways for KO, such as ALD6
- Next steps:
  - KO 3HP degradation in 3HP production strains
  - Combine engineering strategies (increase MCR, transport, and malonyl-CoA pool)
  - Continue media optimization on DMR

Tying into ABF Goals and Milestones

FY21 Go/No-Go: Transfer of 5 metabolic pathways and/or tools between hosts, with 2X improvements in second host, defining metrics for each case. Titers in both first and new host are > 1 g/L. For unsuccessful efforts, perform post-mortem, address specific reasons for why not achieved.

- Transfer of 3HP transfer and organic acid transporter

FY20

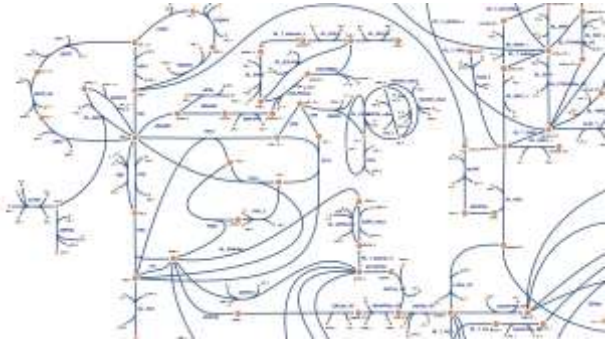
- Compile a list of the top 15 most important hosts for the ABF to develop and report on the justification for each.
- Identify at least 2 new strategic beachhead. Assess any existing ABF beachhead that does not enable 1g/L production of a downstream target and determine whether to continue or sunset.
- Compile data on and spatial structure rendering of existing beachheads for developing an ABF metabolic coverage map.
- Assess mini-DBTL cycle workflows, identify areas for efficiency improvement, and initiate workflows for at least 3 of the 8 FY17/18 target molecules, and quantify reductions.
- Identify DBTL tasks suitable for automation, undertake prioritization of identified tasks, and initiate development of automation workflows for 2 identified priorities.
- Demonstrate at least one representative target of a beachhead at a TRY of 20 g/L, 0.3 g/L/hr, and 50% of theoretical yield
- Demonstrate 2X improvement over 2019 performance baseline in titer (objective: 1-2 g/L), and rate or yield for three compounds of relevance to PAPBs at sufficient quantities for testing (10 g).

FY21

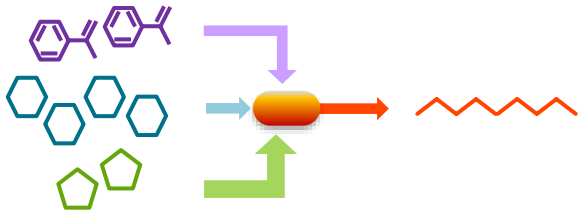
- Continue host improvement to elevate 2-3 onboarded hosts by at least one Tier.
- Develop biosensors for at least two beachhead molecules, metabolic/pathway intermediates, or indicators of cellular energy/redox status to sort and screen for improved hosts/biocatalysts for use in ABF host organisms.
- Finalize development of 1-2 DBTL automation workflows that improve efficiency by =>2X.
- Collect data for pan-scale Test and Learn efforts for 2+ target/host pairs to understand oxygen availability across 3+ bioreactor scales. Work with the Learn team to connect ‘omics results.
- Demonstrate at least one representative target of a beachhead at a TRY of 40 g/L, 0.5 g/L/hr, and 60% of theoretical yield in DMR-EH hydrolysate or a mock hydrolysate.

Elucidating Xylose Metabolism in *R. toruloides*

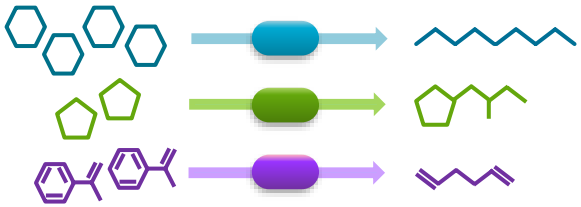
- Improving metabolic models



- Increasing xylose flux to central metabolism (one-hydrolysate / one-host / one-product)

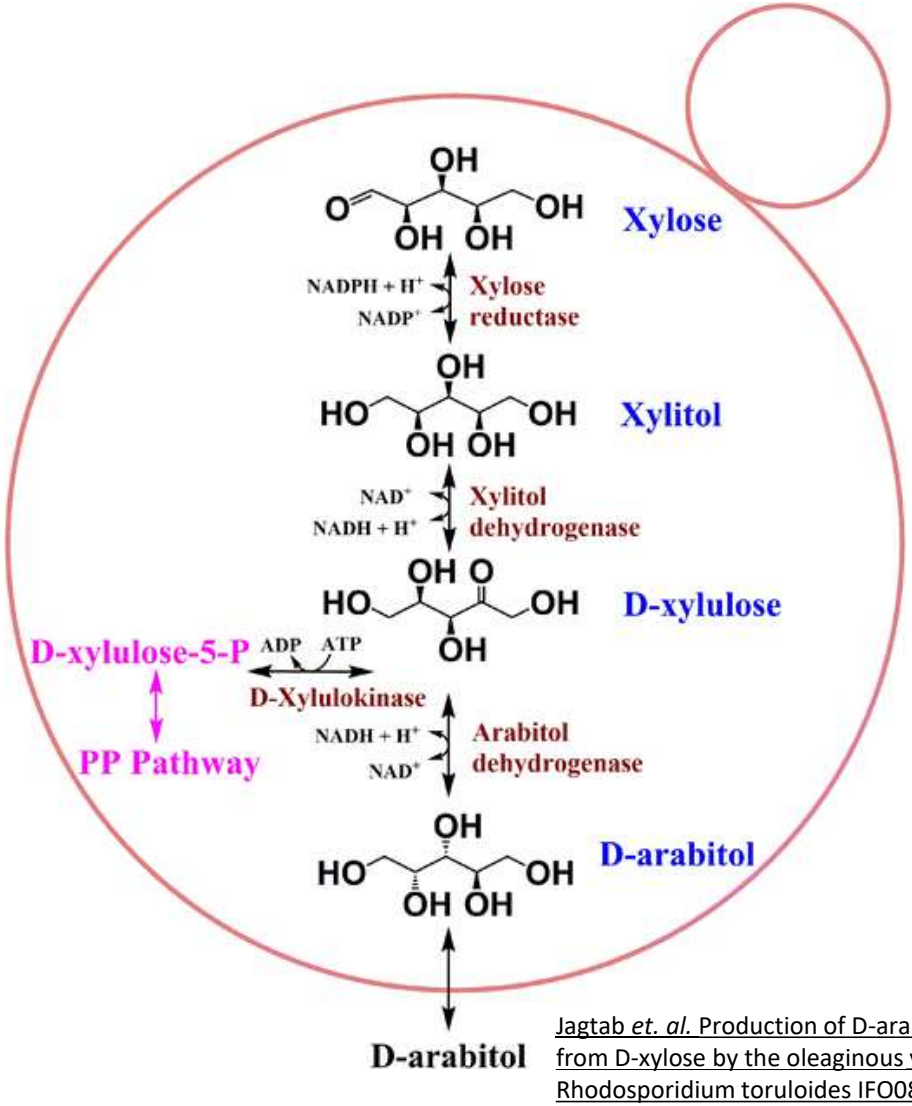


- Xylose specific products and beachheads (multi-fraction / multi-host / multi-product)



Assumed Xylose catabolic pathway

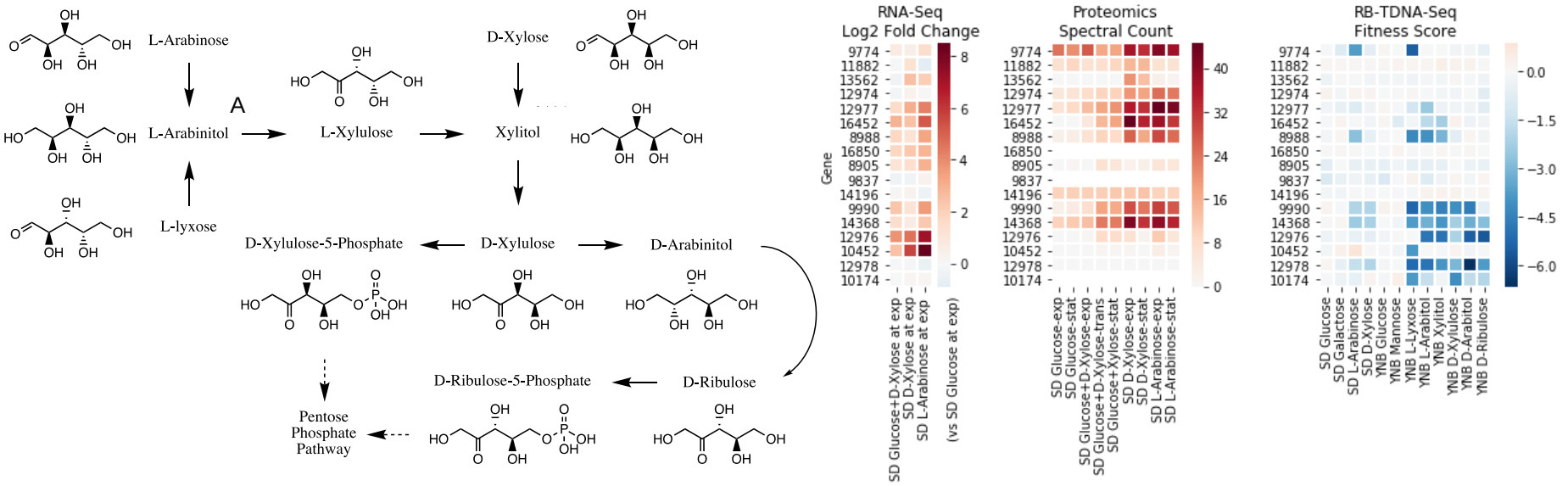
- Xylose catabolized by funneling into PP-pathway through xylose-5-P
- Arabinitol observed as a metabolic overflow product



Jagtab *et. al.* Production of D-arabitol from D-xylose by the oleaginous yeast Rhodosporidium toruloides IFO0880

Elucidation of pentose utilization pathway

- Used multi-omics and functional genomics to aid metabolic network reconstruction by the Test team
- The data suggests that *R. toruloides* has a unique xylose catabolism

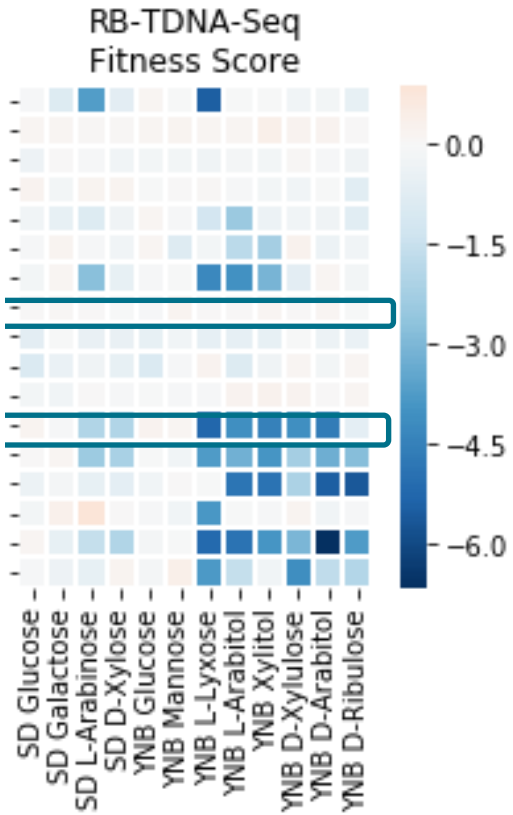


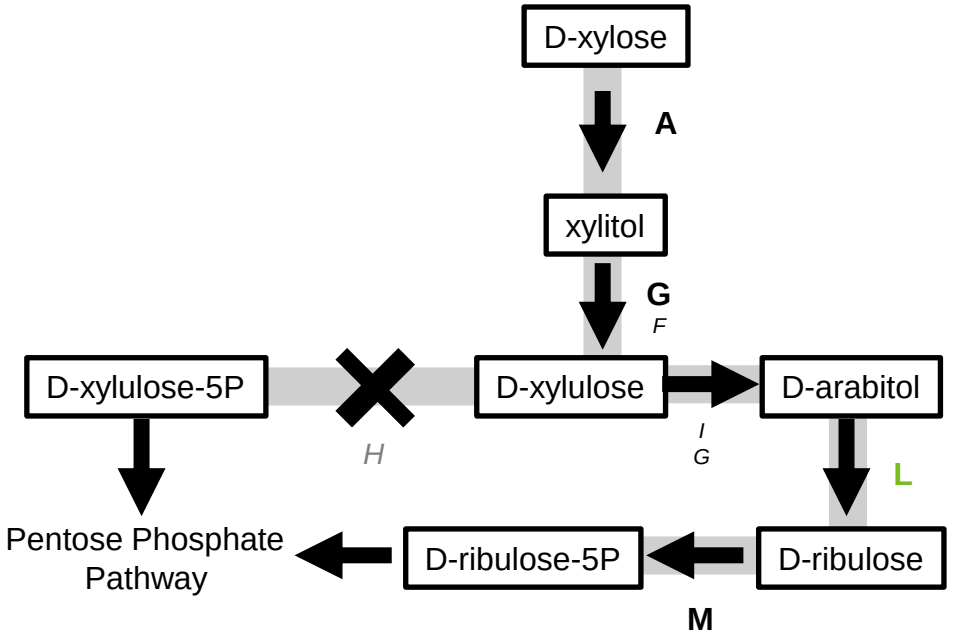
Pentose utilization pathway in *R. toruloides*. (A) Proposed pentose sugars and alcohols degradation to D-ribulose-5-phosphate via D-arabinitol dehydrogenase. (B) Gene expression, protein expression, and fitness scores for pentose utilization pathway genes (exp – exponential phase, trans – transition phase, stat – stationary phase).

Kim et al. 2021
https://www.frontiersin.org/articles/10.3389/fbioe.2020.612832/full

R. trouloides has a unique xylose catabolism

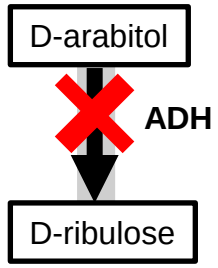
- Xylose catabolism occurs through arabitol not xylose-5P
- KO of xylulose kinase has no fitness defect on xylose
- But KO of L does (D-arabinitol 2-dehydrogenases = D-arabinitol to D-ribulose)

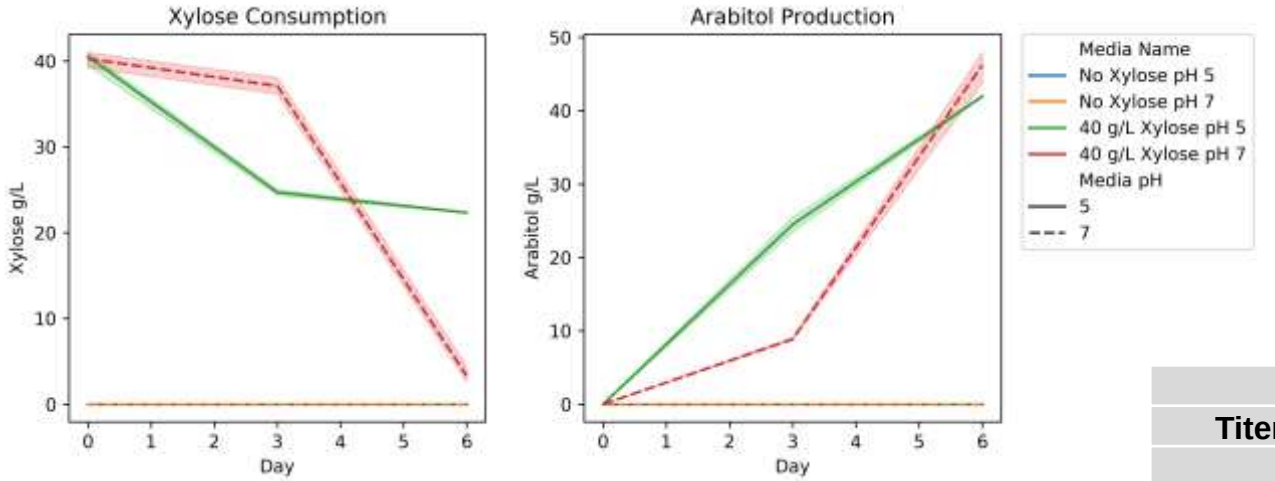




Confirming arabitol route for xylose catabolism

- Xylose catabolism prevented by an ADH knockout
- High amounts of arabitol accumulate
- Suggests *R. toruloides* is an excellent host for production both arabitol and xylitol
- Pentose sugars estabilised as a new beachhead

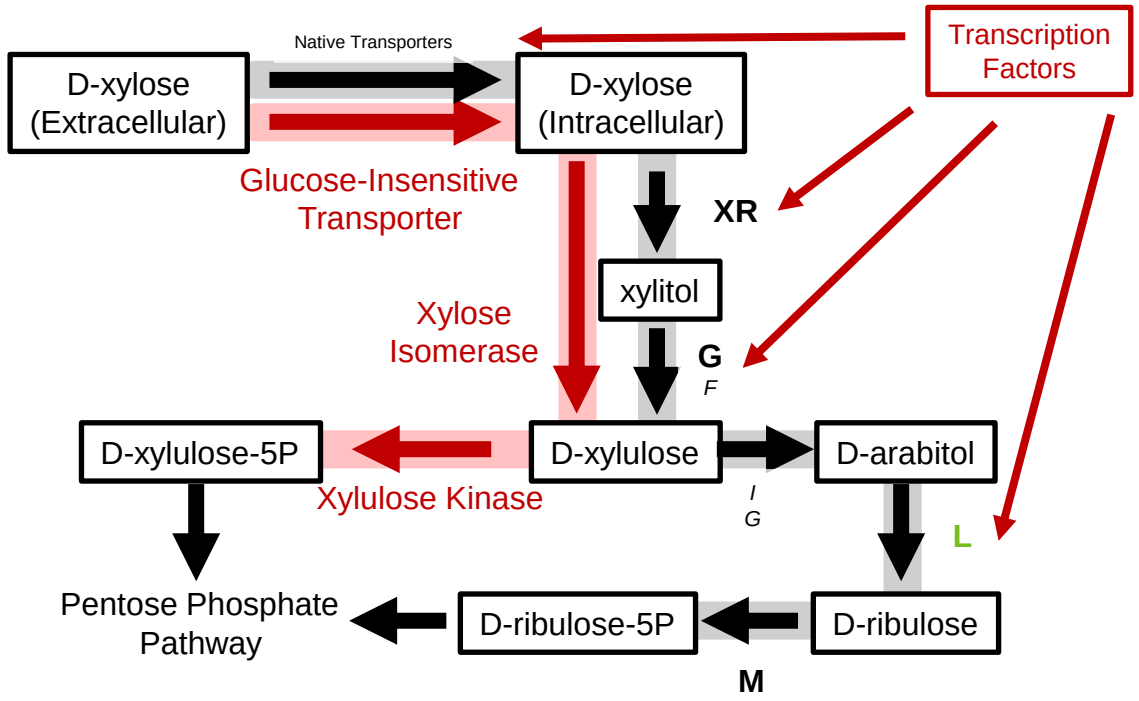




	pH 7	pH 5
Titer (g/L)	46	42
Yield	53%	62%

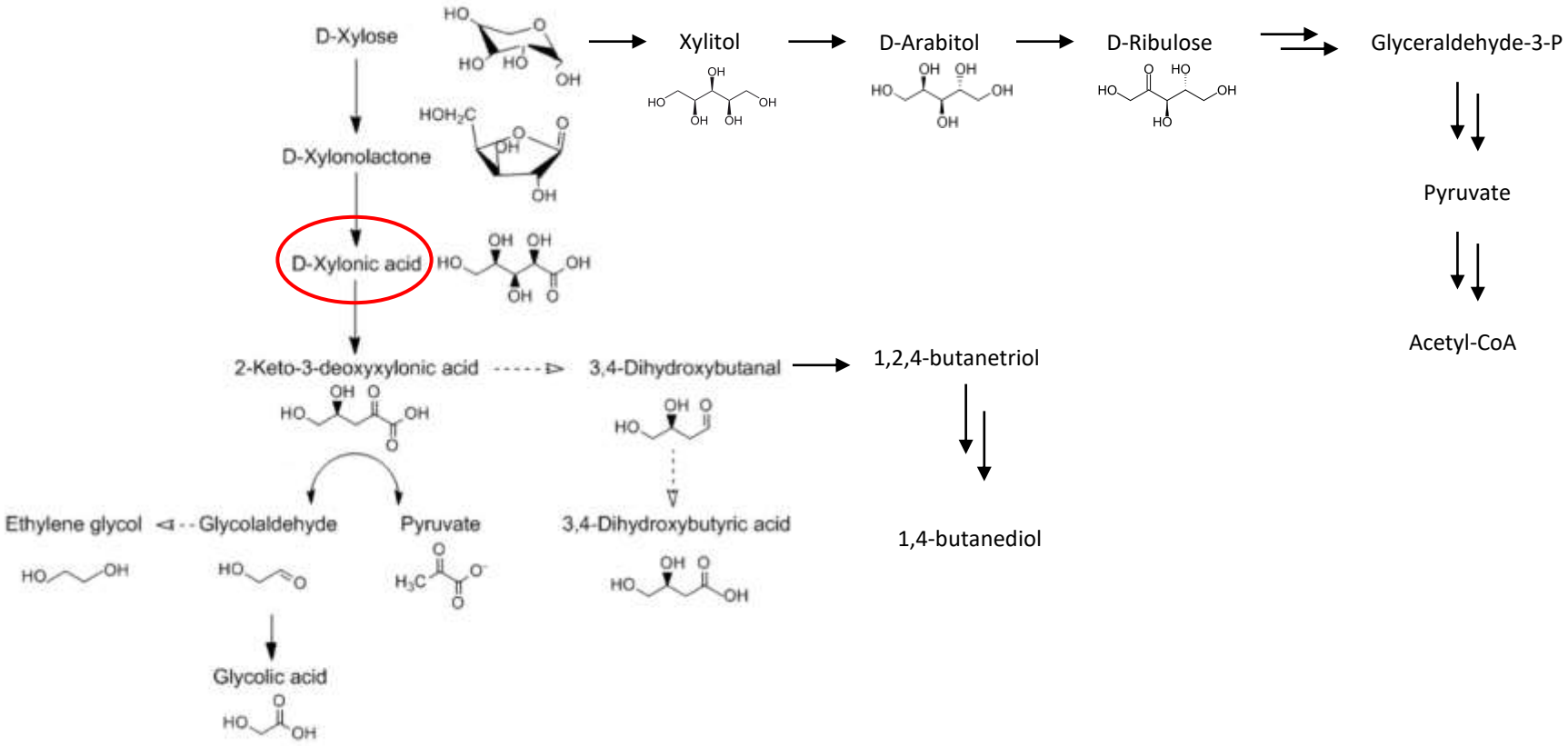
Plans to optimize xylose utilization

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



Xylose Derived Beachheads and Targets

- Xylonic acid is a good initial beachhead to explore with pentose sugars
- Xylonate can be converted into several unique bioproducts

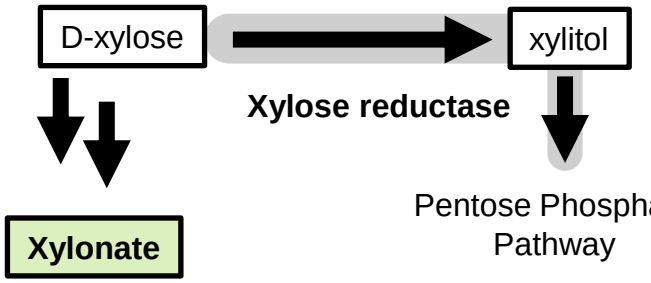


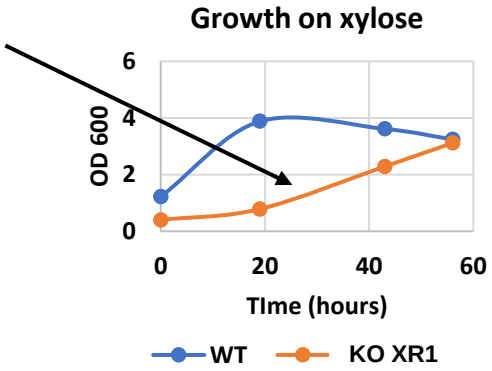
Salusjarvi et al. 2017.

Eliminating native xylose metabolism

- 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)
There are 8 more possible XRs!

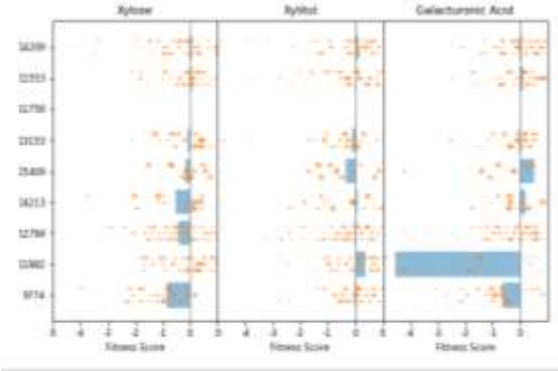




KO of top XR does not completely eliminate xylose consumption

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

BarSeq data on pentose sugars



Transcriptomics on xylose

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Xylonic acid engineering strategy

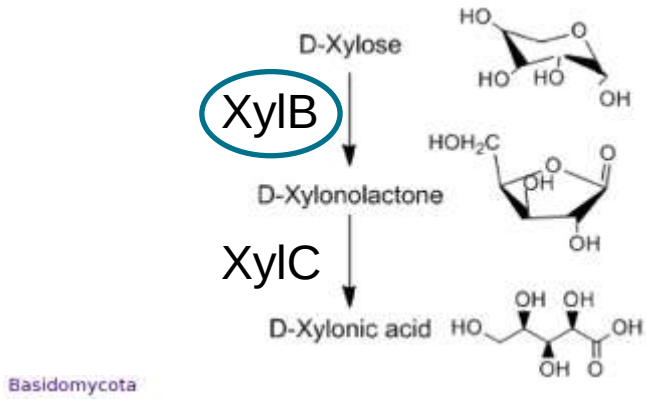
28 diverse xylose dehydrogenase(XylB) candidates chosen

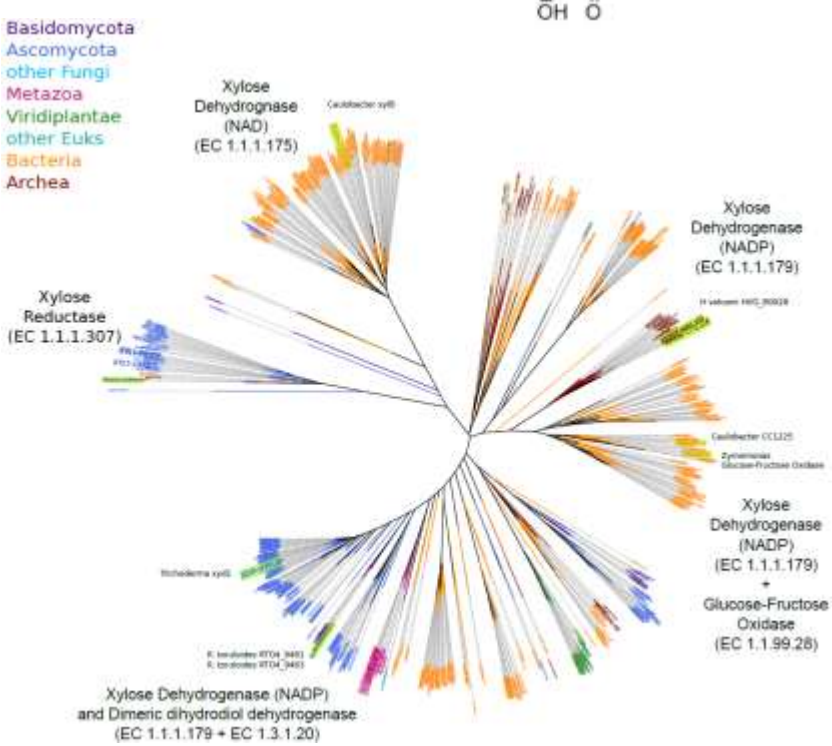
- Encompass sequence similarity to known XDHs
- Include functionally annotated XDHs

Engineering strategies

- Integrate single copy @ CAR2 locus
- Randomly integrate each of 28→test titers individually
- Randomly integrate pool of 28→highthroughput screening

FY21 Milestone: Finalize development of 1-2 DBTL automation workflows that improve efficiency by =>2X.





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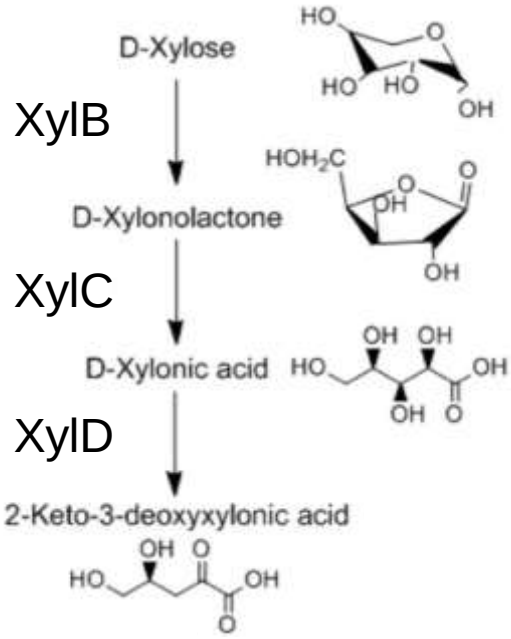
Preliminary Results and Next Steps for Xylonate Beachhead

Preliminary Results

- Integrated *C. vibriodes* XylBC into XR KO
- Top strain produced ~15 g/L xylonate
- ~75% of the xylose converted to xylonate

Next Steps

- KO xylose reductases to completely block xylose catabolism
- Screen 27 XylB genes to identify one with top xylonate TRY
- Overexpress xylose-specific transporters and transcription factors to increase xylose import and optimize media
- Initiate downstream target production by expressing XylD



Multiple Bioproducts

Tying into ABF Goals and Milestones

FY20 Beachheads: Identify at least 2 new strategic beachhead. Assess any existing ABF beachhead that does not enable 1g/L production of a downstream target and determine whether to continue or sunset.

- Xylonic acid and xylose were chosen as FY20 beachheads

FY20

- Compile a list of the top 15 most important hosts for the ABF to develop and report on the justification for each.
- Compile data on and spatial structure rendering of existing beachheads for developing an ABF metabolic coverage map.
- Assess mini-DBTL cycle workflows, identify areas for efficiency improvement, and initiate workflows for at least 3 of the 8 FY17/18 target molecules, and quantify reductions.
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FY21

- Continue host improvement to elevate 2-3 onboarded hosts by at least one Tier.
- Develop biosensors for at least two beachhead molecules, metabolic/pathway intermediates, or indicators of cellular energy/redox status to sort and screen for improved hosts/biocatalysts for use in ABF host organisms.
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Quad Chart Overview

Timeline

- Start: October 1, 2019
- End: September 30, 2022

	FY20	FY21	FY22	Total Active
DOE Funding	$7.2M	$6.4M	$6.2M	$19.8 M

Project Partners

- LBNL (17%), SNL (22%), PNNL (20%), NREL (25%), ANL (4%), LANL (6%), ORNL (5%)

Barriers addressed

- *Ct-L*. Decreasing Development Time for Industrially Relevant Microorganisms
- *Ct-D*. Advanced Bioprocess Development

Project Goal

- Develop hosts to efficiently, cost-effectively, and sustainably produce beachhead and exemplar pairs to aid synthetic biology commercialization

End of Project Milestone

- At least one representative target of a beachhead at a TRY of 80 g/L, 1 g/L/hr, and 70% of theoretical yield
- 10-15 beachheads of strategic interest to BETO in at least 3 onboarded hosts

Funding Mechanism

AOP

Acknowledgements

SNL
Derek Clay
Sam Coradetti
Gina Geiselman
John Gladden
HeeJin Hwang
James Kirby
Di Liu
Peter Otoupal
Anne Ruffing
Tessily Hogancamp
Mary Tran-Gymafi
Xun Zhuang
Anthe George
Tylyn Turner

NREL
Gregg Beckham
Davinia Salvachua
Violeta Sanchez i Nogue
Chris Johnson

PNNL
Jon Magnuson
Kyle Pomraning
Kristin Burnum-Johnson
Ziyu Dai
Shuang Deng
Yuqian Gao
Beth Hofstad
Joonhoon Kim
Young-Mo Kim
Nathalie Munoz Munoz
Jeremy Zucker

ANL
Phil Laible
Peter Larsen

ORNL
Adam Guss

LBNL
Edward Baidoo
Jennifer Chiniquy
Henrique De Paoli
Nathan Hillson
Chris Petzold
Hector Plahar
Alastair Robinson
Blake A. Simmons
James Gardner

LBNL-ABPDU
Ethan Oksen
Jan-Philip Prahl
Deepti Tanjore

LANL
Taraka Dale
Chris Yeager
Ramesh

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Additional Slides

Responses to Previous Reviewers' Comments, Pseudomonas (Bacteria)

- **C:** As a testbed for the Agile BioFoundry effort, this team reports an impressive number of P. putida developments and innovations. Through a combination of different kinds of DBTL and min-DBTL cycles, they have enhanced production of two target compounds, improved sugar utilization in the host, and made inroads into taking greater advantage of omics data, machine learning, and biosensors to further enhance development and to expand into new target compounds. It will be important to evaluate the impact of these latter efforts as they are completed during the next phase of the performance period.
- **R:** We thank the reviewer for the positive feedback. We completely agree that we need to evaluate the impact of the tool development as part of the DBTL cycle and overall Foundry concept and we are actively doing that now. We note, for example, that the biosensor work has already led directly to improved muconate strains, so investment in those tools are generating improvements and accelerated DBTL progress.

- **C:** With less than a year remaining in this project, future work seems ambitious. prioritizing task and research to support key outcome is needed.
- **R:** We agree that the future work is indeed ambitious, but we have an aggressive timeline and a large team working in close coordination to be able to be successful in our objectives.

Responses to Previous Reviewers' Comments, Pseudomonas (Bacteria)

- **C:** Pseudomonas putida is being developed as a host, with the model products being C6 diacids and branched-chain polyhydroxyalkanoates. Actual hydrolysate is being used to evalate performance. The overall objectives are to develop a robust bacterial host while demonstrating improvements to the DBTL cycle time and generating datasets to be used in modeling and learning. The team has demonstrated that the DBTL cycle can be applied iteratively to overcome bottlenecks and make improvements. Omics and computational work led to specific targets. However, improvement so far seems to be a result of improved sugar uptake alone, rather than any pathway enhancements. It may be that enzyme engineering is needed to improve pathway flux. In addition, the DBTL cycle is also quite slow. There are inherent limitations in the speed of conducting genetic manipulations and other tasks, but more workflow management could streamline progress.
- **R:** We thank the reviewer for the positive feedback, and we agree that the initial DBTL cycle timelines were very long. This was the case mostly because we were setting up the tools, workflows, etc. We anticipate that the timelines will be reduced significantly in future DBTL cycles..

Responses to Previous Reviewers' Comments. Aspergillus (Filamentous Fungi)

- **C:** The goal of this subproject is to produce 3HP and aconitic acid in Aspergillus pseudoterreus. The inclusion of a filamentous fungus provides a useful test for the Agile Biofoundry. The team is making solid progress. It is difficult to evaluate the learn component of this project at this stage, though it is clearly an important step. More details should be provided given the focus on learn. While clear milestones are presented, the project would benefit from competitive benchmarks, particularly with regards to 3HP.
- **R:** Thank-you, we believe filamentous fungi are very important, as evidenced by their widespread use within industry. "Traditional" Learn using metabolic models as a framework for analyzing omics data and to support flux balance analysis for identifying bottlenecks and targets has been a major emphasis, and has resulted in both intuitive and non-intuitive targets. Advanced Learn techniques, such as ANN (Artificial Neural Networks), have been employed in the last year to identify non-intuitive targets, and this effort will expand in the future with increasing numbers of large Test data sets. A number of targets already suggested by Learn remain to be Designed, Built and Tested. We will be able to address the highest priority targets in FY19. Milestones pertaining to all of the targets have been satisfied, but we realize we are still well short of handing off the 3HP strain for commercialization.

- **C:** Overall, significant portion of work was done (more than 80%). however future work still pictured significant research which does not seem to be achievable within less than a year left in the project. IP was mentioned in the first slide, if there is any IP associated with this project, it would be needed for PI to touch base on potential IP.
- **R:** The demarcation between work to be accomplished in the remainder of FY19 and moving beyond into FY20 could have been clearer (see previous response). A provisional patent was filed and a full patent will be filed before the end of April. Since this was a public presentation, few details were discussed.

Responses to Previous Reviewers' Comments. Aspergillus (Filamentous Fungi)

- **C:** As a testbed for the Agile BioFoundry, this team reports in-process results in applying DBTL to aid in the development of the expression of organic acids in an industrially relevant fungus, A. pseudoterius. Initial mini-DBTL cycles have established baseline strain performance of 1.3 g/L and 10 g/L for respective targets, and they have started to analyze omics data in order to guide genetic modifications for subsequent design cycles. The group has also started to develop new genetic constructs in order to streamline strain construction.
- **R:** Thank-you, no response necessary.

- **C:** Aspergillus pseudoterreus is a fungal host related to strains historically used for enzyme expression and production of citric acid. Due to its acid tolerance, it is a suitable host for producing organic acids in the free acid form. Here it is being considered for aconitic acid and 3-hydroxypropionate (3HP). The pathways to these molecules are well established, so the team is focused on solving the key challenges of improving timelines for genetic manipulation and reducing byproduct formation. For the latter, they have leveraged a variety of omics techniques in conjunction with metabolic modeling and machine learning. This has led to identification of targets for knockout or overexpression, many of them not intuitive. However, the value of these techniques cannot really be evaluated until these manipulations are made and results provided. The team has also greatly reduced the amount of time needed for testing strains, and has taken steps to improve translation and reproducibility from shake flask to fermentation.
- **R:** Thank-you. We agree with this assessment and we have a significant number of high priority targets in regard to improving TRY for both target molecules. The highest priority targets will be constructed and tested before the end of FY19 (see response above). We are excited to have the opportunity to report on those results in the literature and at Peer Review two years hence.

- **C:** Aspergillus
- **R:** niger.

Responses to Previous Reviewers' Comments. Aspergillus (Filamentous Fungi)

- **C:** The selection of a filamentous fungus, especially Aspergillus, is an excellent inclusion in the host-target development programs due to industrial relevance and as an opportunity to optimize a DBTL cycle. This organism has inherent handling and growth challenges that impose time and throughput limitations, which perhaps can be overcome with improved Learn (including deep learn) capabilities that seem to be the focus. In this respect it may be a good example of how DBTL can be flexible to garner improvements by emphasizing certain elements of the cycle because of limitations in others. Progress has been good and the outcome of potentially exciting unintuitive learnings is pending. It might be of benefit to develop relationships with external entities who are developing (other but related) strains and tools for similar hosts to move towards HTP use.
- **R:** We appreciate the guidance with regard to exploring external collaborations for development of HTP tools for these challenging but highly useful hosts. We also have work in a project outside of BETO exploring high throughput methods for transformation that will be leveraged within the ABF.

Responses to Previous Reviewers' Comments Rhodosporidium (Yeast)

- **C:** As a testbed for the Agile BioFoundry, this team reports significant process in using DBTL to aid in the development of the expression of terpenes and fatty alcohols in R. toruloides. Through large scale testing (100-200 strains constructed), they have shown improvements in production of both of these target compounds. Particularly noteworthy are the impacts of computational methods on their progress: Simple regression models validate predictions regarding the impact of expression level on yield, and by developing a new, quantitative metabolic models and kinetic models for this organism and combining them with omics data, they have shown that they can identify non-obvious genetic targets for optimization, some of which have been validated experimentally.
- **R:** Thanks for your comments. The ABF has certainly accelerated development of this organism..

- **C:** This goal of the subproject is to produce terpenes and fatty alcohols in Rhodosporidium toruloides. This is a great target organism as little is known about its metabolism. The team is making good progress. The dynamic proteomic datasets will be very useful for the learn step. The initial learn prediction regarding transcription factor overexpression seems promising.
- **R:** Thanks for your comments. We agree that this is a great target organism for the ABF..

Responses to Previous Reviewers' Comments Rhodosporidium (Yeast)

- **C:** Great progress so far. Same as other subsections in ABF projects, it would be great if PI can expand on time reduction (10X) and their proposed approach claiming to achieve that reduction. Overall, sub-projects missing connecting their project to the overall goal/target in a clear way.
- **R:** Our ultimate goal within the ABF is to reduce the time to bioprocess scale-up by 50% through improvements in DBTL cycle efficiency. Reduction of DBTL cycle time is one of several metrics that will contribute to increased DBTL cycle efficiency. One of our goals for the initial DBTL cycles implemented for each target/host pair was to identify areas where efficiency gains can be made. This exercise has allowed us to identify many areas for improvement. Specifically regarding time, improvements can be made in Design through the assembly and validation of a library of engineering parts, which can then be more rapidly refactored into new Designs with a greater likelihood of being functional. Within Build, improvements can be made with streamlined transformation and screening protocols as well as development of high-throughput plate-based protocols to enable examination of a greater number of samples in a shorter timeframe. For Test, now that analysis of baseline samples has been conducted and protocols validated for this host, sample analysis can be expediated. In addition, reliance on mini-DBTL Test analysis (HPLC, GC-MS, etc.) can expediated by high-throughput Testing of strains constructed in the aforementioned plate-based Build efforts. For Learn, we now have a metabolic model to put our multi-omic data into context, which can be analyzed both manually and computationally. We are in the process of assessing several machine learning platforms for data analysis, which should also expediate Learn and lead to better predictions for improvements to be made in subsequent DBTL cycles, reducing the time it takes to optimize TRY.

- Regarding connection to the broader ABF milestones, while not specifically called out in our presentations, all the ABF hosts have multiple specific milestones within the ABF. To assess the overall progress of the ABF, these milestones were specified to be met through the combination of all three ABF host organisms and their targets. So, rather than host-specific milestones, we had aggregated host milestones. The milestones defined both the of number of target molecules selected to be engineered into these host organism, and specific TRY targets.

Responses to Previous Reviewers' Comments Rhodosporidium (Yeast)

- **C:** Rhodosporidfium toruloides is a fungal host being developed for terpenes and long-chain fatty alcohols. The organism readily consumes not only C5 and C6 sugars, but also aromatics; thus it can be used in lignin conversion. This project showcases several capabilites of the ABF: iterative rounds of strain construction and screening, metabolic modeling and machine learning, multi-omics analysis, and fermentation development. One particular novel application of machine learning was the 'kinetic learn' method, using protein time series data in conjuction with a metabolic model to predict metabolite levels. The first DBTL cycle was completed for both products, and used to inform designs for the next cycle. Mini cycles were also used to screen strains and conditions prior to a full experiment where omics data was collected. It is still uncertain how useful the machine learning approach was compared to rational intuition and the metabolic model alone. This will be more clear once the recommended modifications are tested and evaluated.
- **R:** While we are very excited about some of the machine learning Learn methods, we are still in the early phases of validating and optimizing these approaches in R. toruloides. The ABF offers a great test-bed for these approaches and as time goes on, will provide the large data-sets some of these methods require. We are in the process of dedicating a significant portion of our Test and Learn resources to generate that data, in consultation with the Learn team experts. We are also assessing less data intensive Learn approaches in parallel to ensure we have multiple routes to success. Where possible, we will leverage the same datasets for different Learn approaches but will also generated tailored data as needed. We are in the process of vetting various different Learn approaches, and will focus resources toward those that are determined the most promising after this initial vetting phase. We are excited to experimentally validate these approaches in upcoming DBTL cycles.
- **C:** R. toruloides is a good host choice for ABF objectives (e.g. for DBTL demonstration) and compliments other host choices across the ABF. Target choices are also sensible and reflect pathways that lead to target-rich classes of compounds (terpenes and FAs) with wide commercialization potential.
- **R:** Thanks for your comments. We agree that this is a good host to help us meet our ABF objectives.

Responses to Previous Reviewers' Comments Integration and Analysis

- **C:** Weakness: It remains to be seen whether the quality of the inputs will be sufficient to enable quality outputs.
- **R:** We appreciate the reviewer for this comment. The development of TEA and LCA has been consistent with previous analysis under the BETO portfolio. In addition, the LCA results of some of the target studied in the ABF project are in good agreement with what we have found in the literature.
- **C:** Meaningful TCAs and LCAs are the currency of fact-based decisions for downselection and prioritization within and across projects. The value that this program is bringing is critical to the success of the ABF, provided that inputs are sound, and that the results and recommendations are acted upon by those making program decisions. This program is not only important, but should probably be expanded.
- **R:** We thank the reviewers for their helpful and supportive feedback. We will work to incorporate these suggestions in future analysis efforts.

Responses to Previous Reviewers' Comments Process Integration & Scale-Up

- **Comments:**
  - This subproject focuses on process scale up and providing standardized DMR hydrolysates for members and external partners. Overall, the team is making good progress. Major suggestion would be to develop a more systematic procedure for facilitating scale up. In particular, the team should focus on identifying the key factors involved in scale up and optimization, in order to accelerate this process and improve the broader impact of this work.
  - This group focuses on providing hydrolysate feedstock, testing ABF strains at various scales, and providing fermentation data to the Learn team. Scaling up a process is an important step in de-risking, and collecting fermentation data at bench scale is important for guiding project decisions. The team is making a strong effort to generate high quality data and fully characterize the system; for example omics analysis at multiple scales, closing the carbon balance, and cross-site validation. They are working on better understanding the impact of different scales on cell physiology, and are implementing new small scale culturing techniques. Improvements could be made by defining clear goals, and integrating this team better into the overall DBTL workflow.
  - Process and integration is an underserved area and often an afterthought in new projects and products. In particular, a lack of scale-up and scale-down consistency is often hard to come by. This project is developing a core competency by standardizing substrates (especially relevant to biomass-derived complex substrates) and pan-scale test methods. So-called "round-robin" testing across sites is helping to develop what is more broadly known to industry as technology transfer expertise, which is how success (or failure) is often determined (e.g. for milestone testing, Go-No Go decisions). In a multi-site environment, this is an absolute must-have competency for measurable and fact-based success. As more projects come on line, more host-target pairs and substrates will need to be put through this process, so it is likely this area will emerge as a priority for additional funding and effort.

Responses to Previous Reviewers' Comments Process Integration & Scale-Up

- **Response:** We thank the reviewers for their time and constructive feedback. One reviewer suggested that we develop a systematic procedure to facilitating scale up. We agree with this suggestion. At this time, while we are working with non-canonical hosts, we are still developing unique cultivation protocols for each target-host pair. During the process of achieving our end-of-project goal: at least 3 molecules at 10 g/L, 100 mg/L/hr, at 40% of theoretical yield from DMR-EH at 10 L, we will learn many nuances to scale-up, some of which may be broadly applied to both canonical and non-canonical hosts. We are hopeful that the -omics approach to identifying variances in microbial culture behavior and thereby performance with scale will form the basis of a systematic template. Another reviewer identified integration with DBTL cycle as essential to this task. We agree with this comment and are actively working on this with the pan-scale Test and Learn activities. We consider the scale-up task to be a part of the Test arc of the DBTL cycle. Our -omics and fermentation data from our most recent scale-up campaigns (muconic acid at 600 L and fatty alcohols at 300 L) will enable us in engaging with the Learn team. We expect to be demonstrably integrated in the full DBTL cycle by the end of FY21, by when some of the design principles would be based on our learnings from the scale-up studies. Another reviewer suggested that we further our round-robin studies by including more target-host pairs. In FY20, we will be including 3-hydroxypropionic acid-Aspergillus niger into this effort. The FY20 milestone of ABF is being constructed such that reproducibility at separate locations is a prime subject: "Reproducibility of 3 distributed Test unit operations including bioreactor scale-up quantified through comparison of results post data quality assurance for on-site vs. off-site sample analysis." This task will stay focussed on integrating with ABF's DBTL cycle and achieve BETO goals.

Publications, Patents, Presentations, Awards, and Commercialization

- **50 publications, 126 presentations to date**
  - 16 publications and 20 presentations since FY20
    - The following slides provide explicit lists thereof

- **2020 R&D 100 Award**
  - Awarded to Smart Microbial Cell Technology for rapid optimization of biocatalysts
  - Special Recognition (Silver Medal) for Market Disruptor in the Services category

- **36 patents, records of invention, software disclosures, & licenses**
  - The following slides list these intellectual property assets

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Publication)** Peabody GL, Elmore JR, Martinez-Baird J, and Guss AM. “Engineered Pseudomonas putida KT2440 co-utilizes galactose and glucose.” Biotechnol Biofuels 12, 295 (2019).
- **(Publication)** Christopher B. Eiben, Tristan de Rond, Clayton Bloszies, Jennifer Gin, Jennifer Chiniquy, Edward E. K. Baidoo, Christopher J. Petzold, Nathan J. Hillson, Oliver Fiehn, Jay D. Keasling. "Mevalonate Pathway Promiscuity Enables Noncanonical Terpene Production", ACS Synth. Biol. (2019).
- **(Publication)** Yan Chen, Deepwanita Banerjee, Aindrila Mukhopadhyay, Christopher J. Petzold. “Systems and synthetic biology tools for advanced bioproduction hosts”, Curr. Op. Biotechnol. (2020).
- **(Publication)** Jacquelyn M. Blake-Hedges,Jose Henrique Pereira, Pablo Cruz-Morales, Mitchell G. Thompson, Jesus F. Barajas, Jeffrey Chen, Rohith N. Krishna, Leanne Jade G. Chan, Danika Nimlos, Catalina Alonso-Martinez, Edward E. K. Baidoo, Yan Chen, Jennifer W. Gin, Leonard Katz, Christopher J. Petzold, Paul D. Adams, Jay D. Keasling. “Structural Mechanism of Regioselectivity in an Unusual Bacterial Acyl-CoA Dehydrogenase”, J. Am. Chem. Soc. (2019).

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Publication)** Thompson, Mitchell G., Allison N. Pearson, Jesus F. Barajas, Pablo Cruz-Morales, Nima Sedaghatian, Zak Costello, Megan E. Garber et al. "Identification, characterization, and application of a highly sensitive lactam biosensor from Pseudomonas putida." ACS Synthetic Biology (2019).
- **(Publication)** Geiselman GM, Zhuang X, Kirby J, Tran-Gyamfi MB, Prahl JP, Sundstrom ER, Gao Y, Munoz Munoz N, Nicora CD, Clay DM, Papa G, Burnum-Johnson KE, Magnuson JK, Tanjore D, Skerker JM, Gladden JM. “Production of ent-kaurene from lignocellulosic hydrolysate in Rhodosporidium toruloides.” Microb Cell Fact. 19(1):24. (2020).
- **(Publication)** Gayle J. Bentley, Niju Narayanan, Ramesh K. Jha, Davinia Salvachúa, Joshua R. Elmore, George L. Peabody, Brenna A. Black, Kelsey Ramirez, Annette De Capite, William E. Michener, Allison Z. Werner, Dawn M. Klingeman, Heidi S. Schindel, Robert Nelson Lindsey Foust, Adam M. Guss, Taraka Dale, Christopher W. Johnson*, Gregg T. Beckham*, “Engineering glucose metabolism for enhanced muconic acid production in Pseudomonas putida KT2440,” Metabolic Eng. (2020).

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Publication)** Chen, Y; Guenther, J.; Gin, Jennifer; Chan, Leanne J.; Costello, Z.; Ogorzalek, T.; Tran, Huu; Blake-Hedges, J.; Keasling, J. D; Adams, P.; Garcia Martin, H.; Hillson, N.; Petzold, C. "An automated 'cells-to-peptides' sample preparation workflow for high-throughput, quantitative proteomic assays of microbes" Journal of Proteome Research (2019)
- **(Publication)** Isabel Pardo, Ramesh K. Jha, Ryan E. Bermel, Felicia Bratti, Molly Gaddis, Emily McIntyre, William Michener, Ellen L. Neidle, Taraka Dale, Gregg T. Beckham, Christopher W. Johnson. "Gene amplification, laboratory evolution, and biosensor screening reveal MucK as a terephthalic acid transporter in Acinetobacter baylyi ADP1." Metabolic Engineering, (2020), Vol 62, 260-274
- **(Publication)** Radivojević, T., Costello, Z., Workman, K., & Martin, H. G. "A machine learning Automated Recommendation Tool for synthetic biology." Nature Communications, 11(1), 1-14.(2020).
- **(Publication)** Zhang, J., S. D. Petersen, T. Radivojevic, A. Ramirez, Andrés Pérez-Manríquez, E.Abeliuk, B. J. Sánchez et al. "Combining mechanistic and machine learning models for predictive engineering and optimization of tryptophan metabolism." Nature Communications 11, no. 1 (2020): 1-13.

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Publication)** Ernst Oberortner, Robert Evans, Xianwei Meng, Sangeeta Nath, Hector Plahar, Lisa Simirenko, Angela Tarver, Samuel Deutsch, Nathan J. Hillson, and Jan-Fang Cheng. "An Integrated Computer-Aided Design and Manufacturing Workflow for Synthetic Biology". In: Chandran S., George K. (eds) DNA Cloning and Assembly. Methods in Molecular Biology, vol 2205. (2020).
- **(Publication)** Gledon Doçi, Lukas Fuchs, Yash Kharbanda, Paul Schickling, Valentin Zulkower, Nathan Hillson, Ernst Oberortner, Neil Swainston, Johannes Kabisch. "DNA Scanner: a web application for comparing DNA synthesis feasibility, price, and turnaround time across vendors". OUP Synthetic Biology, ysaa011 (2020).
- **(Publication)** Somtirtha Roy, Tijana Radivojevic, Mark Forrer, Jose Manuel Marti, Vamshi Jonnalagadda, Tyler Backman, William Morrell, Hector Plahar, Joonhoon Kim, Nathan Hillson, and Hector Garcia Martin. "Multiomics Data Collection, Visualization, and Utilization for Guiding Metabolic Engineering". Frontiers in Bioengineering and Biotechnology 9, 45 (2021).

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Publication)** Chris Lawson, Jose Manuel Martí, Tijana Radivojevic, Sai Vamshi R. Jonnalagadda, Reinhard Gentz, Nathan J. Hillson, Sean Peisert, Joonhoon Kim, Blake A. Simmons, Christopher J. Petzold, Steven W. Singer, Aindrila Mukhopadhyay, Deepti Tanjore, Josh Dunn, and Hector Garcia Martin. "Machine learning for metabolic engineering: A review" Metabolic Engineering (2020)
- **(Publication)** Riley LA and Guss AM*. “Approaches to genetic tool development for rapid domestication of non-model microorganisms”. Biotechnol 14:30 (2021)

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Presentation)** Nathan J. Hillson “U.S. DOE Agile BioFoundry: Organization and Capabilities”, Invited Talk, ABF Industry Day 2019, Emeryville, CA October 4, 2019
- **(Presentation)** Garcia Martin, H. “Machine Learning, Synthetic Biology and Automation: Engineering Life for the Benefit of Society”. NERSC data seminar, Berkeley CA, November 1st, 2019
- **(Presentation)** Benavides PT, Davis R, Klein, B. “Economic and environmental assessment of biological conversions of Agile BioFoundry (ABF) bio-derived chemicals”. 2nd Bioenergy Sustainability Conference 2020, Virtual meeting, October 15th, 2020
- **(Poster)** Tijana Radivojevic, Zak Costello, Kenneth Workman, Soren Petersen, Jie Zhang, Andres Ramirez, Andres Perez, Eduardo Abeliuk, Benjamin Sanchez, Yu Chen, Mike Fero, Jens Nielsen, Jay Keasling, Michael K. Jensen, Hector Garcia Martin, “ART: A machine learning Automated Recommendation Tool for synthetic biology”, BRC Workshop on AI and ML for Biosystems Design, Washington, DC, February 27, 2020

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Presentation)** Garcia Martin, H. “ART: a machine learning Automated Recommendation Tool for guiding synthetic biology”. AI4Synbio Symposium, Arlington VA, November 8th, 2019.
- **(Presentation)** Garcia Martin, H. “Opportunities in the intersection of:Artificial Intelligence & Synthetic Biology & Automation”. Army Science Planning and Strategy Meeting, Burlington MA, November 13th, 2019.
- **(Presentation)** “ART: A machine learning Automatic Recommendation Tool for guiding synthetic biology”, Invited Talk, Computational Bio-Science Meeting, Berkeley, CA, April 23, 2020
- **(Presentation)** Garcia Martin, H. “Opportunities in the intersection of machine learning, synthetic biology, and automation”. ABLC 2020, Virtual meeting, July 10th, 2020.
- **(Presentation)** Garcia Martin, H. “Leveraging machine learning and automation to make synthetic biology predictable”. SPIE Optics + Photonics 2020, Virtual meeting, August 24th, 2020.
- **(Panel)** Garcia Martin, H. “Sustainable Living Systems”. LA Life Summit, Virtual meeting, October 15th, 2020.

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Presentation)** T. Radivojevic, “Automatic Recommendation Tool”, Invited Talk, Agile BioFoundry Learn Summit 2020, Argonne/Lemont, IL, March 4, 2020
- **(Presentation)** T. Radivojevic, “Using ART to improve tryptophan production”, Invited Talk, Agile BioFoundry Learn Summit 2020, Argonne/Lemont, IL, March 4, 2020
- **(Presentation)** T. Radivojevic, “Guiding synthetic biology via machine learning”, Invited Talk, Biofuels & Bioproducts Division Meeting, JBEI, Emeryville, CA, March 11, 2020
- **(Presentation)** T. Radivojevic, “ART: A machine learning Automatic Recommendation Tool for guiding synthetic biology”, Invited Talk, Computational Bio-Science Meeting, Berkeley, CA, April 23, 2020
- **(Presentation)** Nathan J. Hillson, "FY20 ABF CRADA Call: Process, Applications, and Selections", Conversion R&D Standing Lab Update Call, via WebEx, July 27, 2020

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **(Presentation)** Nathan J. Hillson, "Perspectives from the U.S. DOE Agile BioFoundry", OECD BNCT Virtual Workshop, Session 1: Biofoundries and COVID-19, via Zoom, July 29, 2020
- **(Presentation)** Garcia Martin, H. "Opportunities in the intersection of machine learning, synthetic biology, and automation". ABLC 2020, Virtual meeting, July 10th, 2020.
- **(Presentation)** Garcia Martin, H. "Leveraging machine learning and automation to make synthetic biology predictable". SPIE Optics + Photonics 2020, Virtual meeting, August 24th, 2020.
- **(Presentation)** Nathan J. Hillson, "FY20 ABF CRADA Call: Process, Applications, and Selections", Conversion R&D Standing Lab Update Call, via WebEx, July 27, 2020
- **(Presentation)** Nathan J. Hillson, "Perspectives from the U.S. DOE Agile BioFoundry", OECD BNCT Virtual Workshop, Session 1: Biofoundries and COVID-19, via Zoom, July 29, 2020

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **License partners**
  - University of Georgia
  - Kiverdi, Inc.
  - LanzaTech, Inc.
  - Visolis, Inc.
  - Danimer Scientific
- **Patent Applications**
  - Terephthalate biosensor and applications thereof
  - Mutant transporters for bacterial uptake of terephthalic acid
  - Alleviating the bottleneck in enzyme evolution and pathway optimization using novel biosensors (Disclosure Title) Modified Biosensors and Biocatalysts and Methods of Use (Application Title)
  - Mutant transporters for bacterial uptake of terephthalic acid
  - ART: A machine learning Automated Recommendation Tool for guiding synthetic biology

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **Patent Applications (cont.)**
  - A Generative Model for Protein Sequences for the Purpose of Protein Design or Phenotypic Inference
  - Predicting Metabolic Pathway Dynamics from Time Series Multiomics Data Using Machine Learning Techniques
  - Use of Statistical Learn Approaches to Predict Next Generation Sequencing Subsequence Depth of Coverage
  - Mutant transporters for bacterial update of terepthalic acid
  - Method and strain for sugar conversion
  - Engineered Microorganisms for the Production of Intermediates and Final Products (1st)
  - Engineered Microorganisms for the Production of Intermediates and Final Products (2nd)
  - Production of organic acids from *Aspergillus pseduoterreus* cadA deletion strain (1st)
  - Production of organic acids from *Aspergillus pseduoterreus* cadA deletion strain (2nd)

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **Patent Applications (cont.)**
  - Genetically engineering an industrial filamentous fungus *Aspergillus niger* for 3-hydroxypropionic acid production
  - A specific exporter responsible for aconitic acid high production in *Aspergillus pseduoterreus*
- **Records of Invention**
  - Bioproduction of limonene from syngas
  - Mutant transporters for bacterial update of terepthalic acid
  - Method to produce branched chain polyhydroxyalkanoates and branched chain 3-hydroxyacids
  - A genetic circuit to reduce cell-to-cell production heterogeneity
  - High yield conversion of D-xylose to D-arabitol in *R. toruloides*
  - Manipulation of tRNA thiolation gene ncs2 for enhanced production of fatty-acyl-CoA derived chemicals in *R. toruloides*

Publications, Patents, Presentations, Awards, and Commercialization (cont.)

- **Software Disclosures**
  - Automated Recommendation Tool (ART) v2.0
  - Kinetic Learning v0.1
  - Automated Recommendation Tool (ART): v1.0
  - PIACE: Parallel Integration and Chromosomal Expansion of Metabolic Pathways
  - OMG, Omics Mock Generator Library: v0.1.1
  - Fermentation Data Processing
  - Fermentation Data Manipulation and Analysis Once imported