Biological Upgrading of Sugars

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2019 DOE-BETO Peer Review Meeting

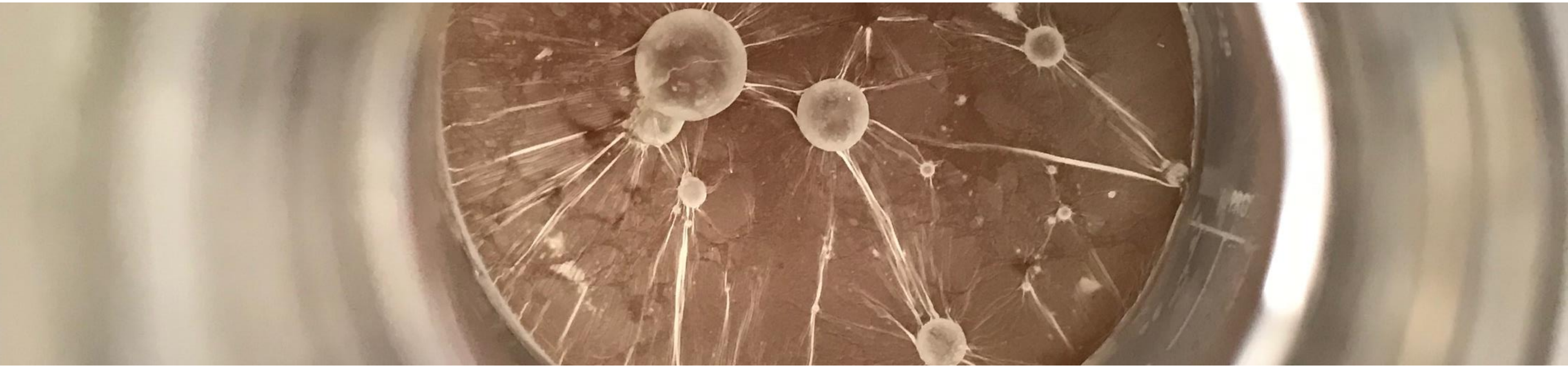
03.04.2019

Goal Statement

Goal: To develop robust microbial strains to convert lignocellulosic sugars to fuel precursors at titer, rate, and yield targets set by techno-economic analysis and which are fully integrated into a complete process to produce biofuels at BETO's MYPP cost targets.

End of Merit Review Cycle Outcome: In collaboration with partner projects, we will have developed fully integrated processes **demonstrating cost-effective tailored hydrocarbon jet and diesel blendstock production**. The BUS end of project target is to achieve 150 g/L butyric acid anaerobically from hydrolysate to serve as the biological intermediate towards finished fuel production.

Relevance to Bioenergy industry: This project seeks to develop and optimize hybrid biological routes to be coupled with downstream catalytic approaches towards cost efficient jet and diesel fuel molecules. Additionally, methods to upgrade sugars to organics acids can be leveraged well beyond fuel precursors.



Quad Chart Overview

Timeline
- Project Start: 10/01/2017
- Project End: 09/20/2020
- Percent Completion (by March 2019): ~50%

	Total Costs Pre FY17 **	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	N/A	$1.75M	$1.35M	$4.05M
Project Cost Share*	N/A	N/A	N/A	N/A

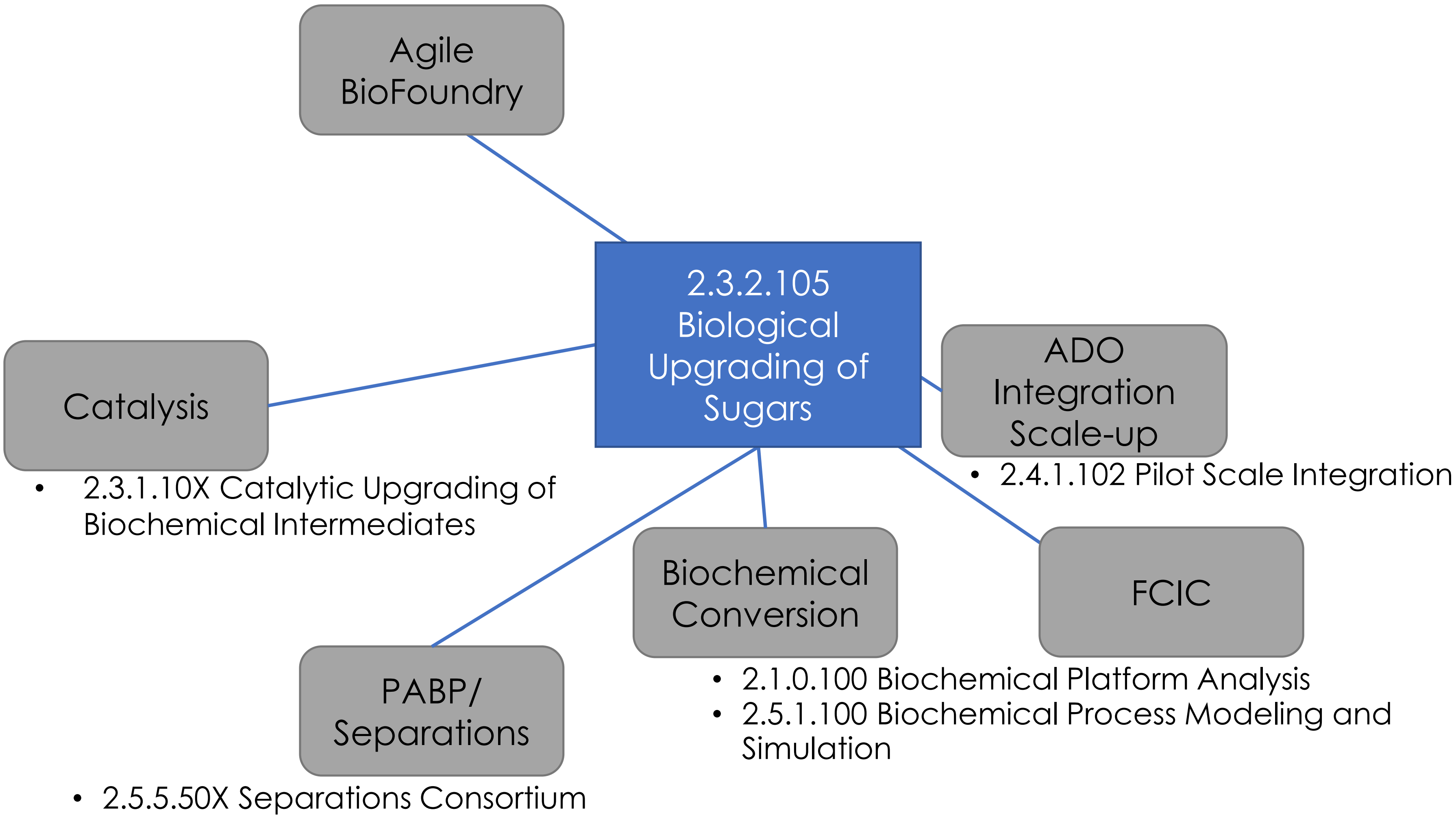
Partners: Internal partners include the Separations Consortium, Catalytic Upgrading of Biological Intermediates, Biochem platform analysis , Biochemical Process Modeling and Simulation projects, Feedstock Conversion Interface Consortium. **External collaborators** include Adam Guss- Agile Biofoundry, Lygos, Paola Branduardi-University of Milan, Nicholas Sandoval-Tulane University, Ken Reardon-Colorado State University, Neol BioSolutions, Volkmar Passoth, Swedish University of Agricultural Sciences

Barriers addressed
- Ct-D: Advanced Bioprocess Development:
  - Increasing titer, rates, and yields of bioproducts.
- Ct-L: Decreasing Development Time for Industrially Relevant Microorganisms
  - Microbiology techniques to enable better bioprocesses with predictable performance and scaling.
- Ct-O: Selective Separations of Organic Species
  - Separation of organic species in biomass processes for upgrading.
- Ot-B: Cost of Production
  - Research in process integration, systems efficiencies, and advanced, robust separations and molecular efficiency.

Objective
- To develop robust microbial strains to convert lignocellulosic sugars to fuel precursors at titer, rate, and yield targets set by techno-economic analysis and which are fully integrated into a complete process to produce biofuels at the BETO 2022 goal of $3/gasoline-gallon equivalent (gge).

End of Project Goal
- Demonstrate production butyric acid at 150 g/L from DMR-EH hydrolysate in a bioreactor utilizing both glucose and xylose fractions.

Project Interactions



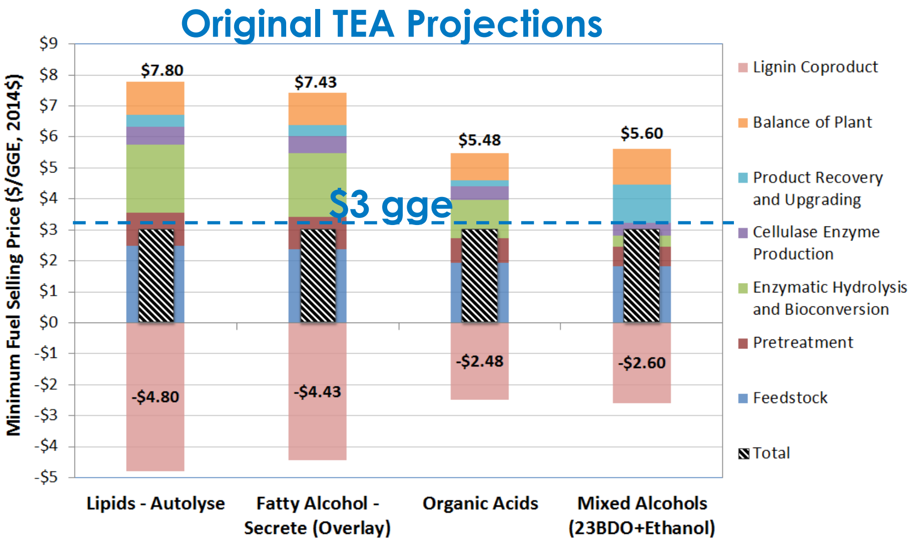
Project Overview

History:

- BUS project began in 2015 i**nitially focusing on the production of fatty acid derived fuels and cost-target enabling coproducts including succinic acid.**
- In depth TEA analysis suggested a difficult challenge to meet DOE cost targets using aerobic production of fatty acids.
- **In FY17 the BUS project began pivoting to focus on carboxylic acids** as a biologically derived intermediate that can be catalytically upgraded to hydrocarbon fuels.

Context: "Beyond ethanol" to produce a portfolio of biofuels

- Produce direct replacements or blendstocks for diesel and jet fuel
- Shifted to intermediates that can be upgraded to diesel and jet fuel.



Current Project Objectives:

- Develop industrially relevant strains and fermentation processes to generate short-chain carboxylic acids to meet MYPP cost targets.
- Focus efforts toward titer, rate, and yield targets set by TEA/LCA modeling.
- Deliver biologically derived carboxylic acids to the CUBI project for catalytic upgrading to finished fuels.

Management Approach

Experienced task leads in fermentation and strain development

Biological Upgrading of Sugars

Quarterly milestones prioritized to enable 2022 process deployment

- Acid Tolerant Yeast Development — Violeta Sànchez i Nogué
- Anaerobic Bacterial Strain Development — Michael Guarnieri
- Anaerobic Bacterial Fermentation Development — Davinia Salvachúa

Approach:

- Collaborative fuel target selection using TEA/LCA and downstream processing (separations and catalysis).
- Monthly meetings with cross-disciplinary teams to ensure efficiency in the entire process.
- Initial approach casts a wide net to evaluate numerous biocatalysts and fermentation parameters.
- Continuously narrow the research focus in order to concentrate efforts on most promising strategies.
- Identify research bottlenecks and establish internal and external collaborations to accelerate progress and minimize duplicated efforts.

Technical Approach: Demonstrated Integrated Diesel Production (Research path redirected in 2017)

Shake flask screening → Bioreactor evaluation → Conversion of lipids into diesel blendstock

32 strains:
7 genus, 12 species

3 strains

1 strain

**DDA-EH*
corn stover
at 100 g/L**

pH 5.5
30°C
225 rpm

**DDA-EH
corn stover
at 100 g/L**

pH 5.2
30°C
1 vvm air
dO_2 25%

Pretreatment

Hydrotreating
Hydrodeoxygenation
Hydroisomerization

Engineered
Secreted
Fatty Alcohols

Green Chemistry

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Cite this: Green Chem., 2018, 20, 4349

Integrated diesel production from lignocellulosic sugars *via* oleaginous yeast†

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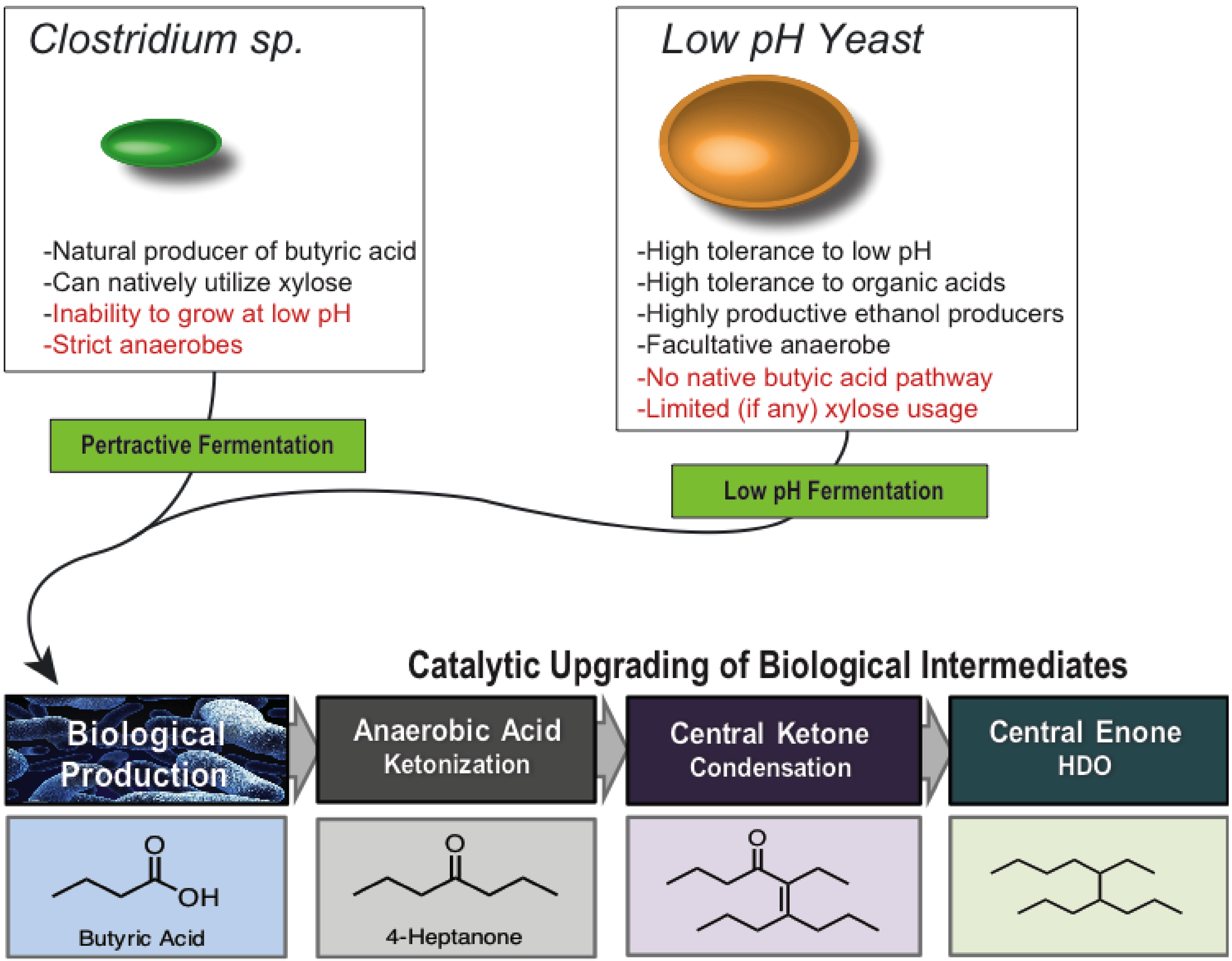
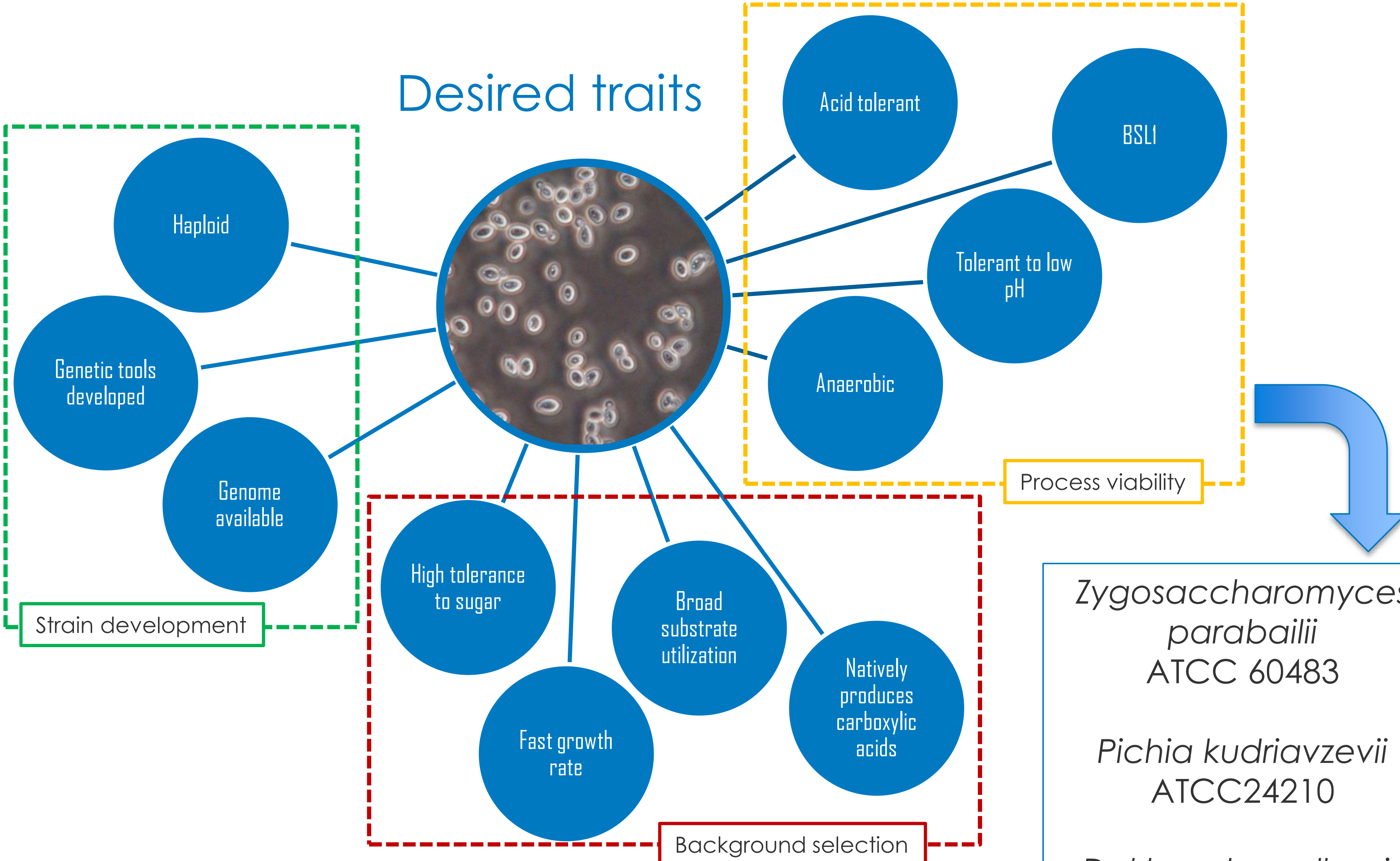
In partnership with:
NEOLBIO

*-Deacetylated dilute acid pretreated and enzymatically hydrolyzed

Technical Approach: Carboxylic Acid Upgrading Overview



Identifying Candidate Yeast Strains



Zygosaccharomyces parabailii
ATCC 60483

Pichia kudriavzevii
ATCC24210

Dekkera bruxellensis
CBS 11270

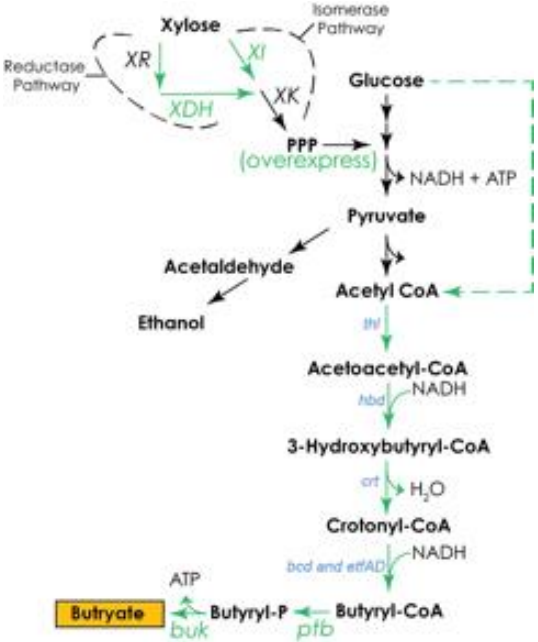
Technical Accomplishments: Engineering Acid Tolerant Yeasts

Parallel strain engineering

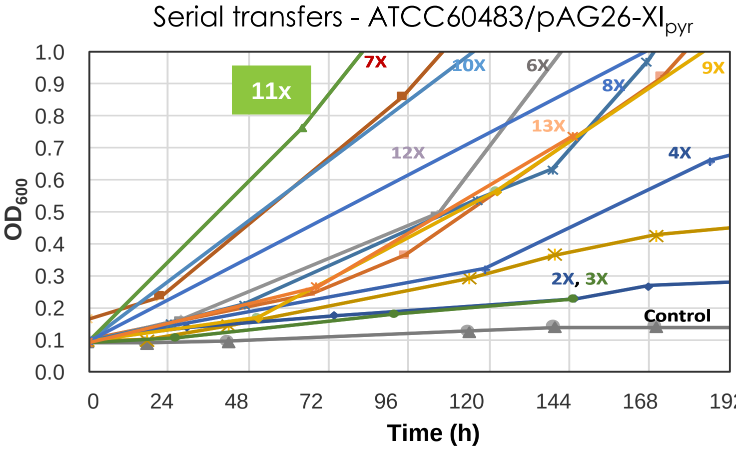
P. kudriavzevii *Z. parabailii*

- Established fundamental genetic methods for both acid-tolerant yeasts
- Engineered and adapted xylose usage for *Z. parabailii*.
- Initiated CRISPR efforts to enable efficient and multiplexed engineering.

Engineering targets



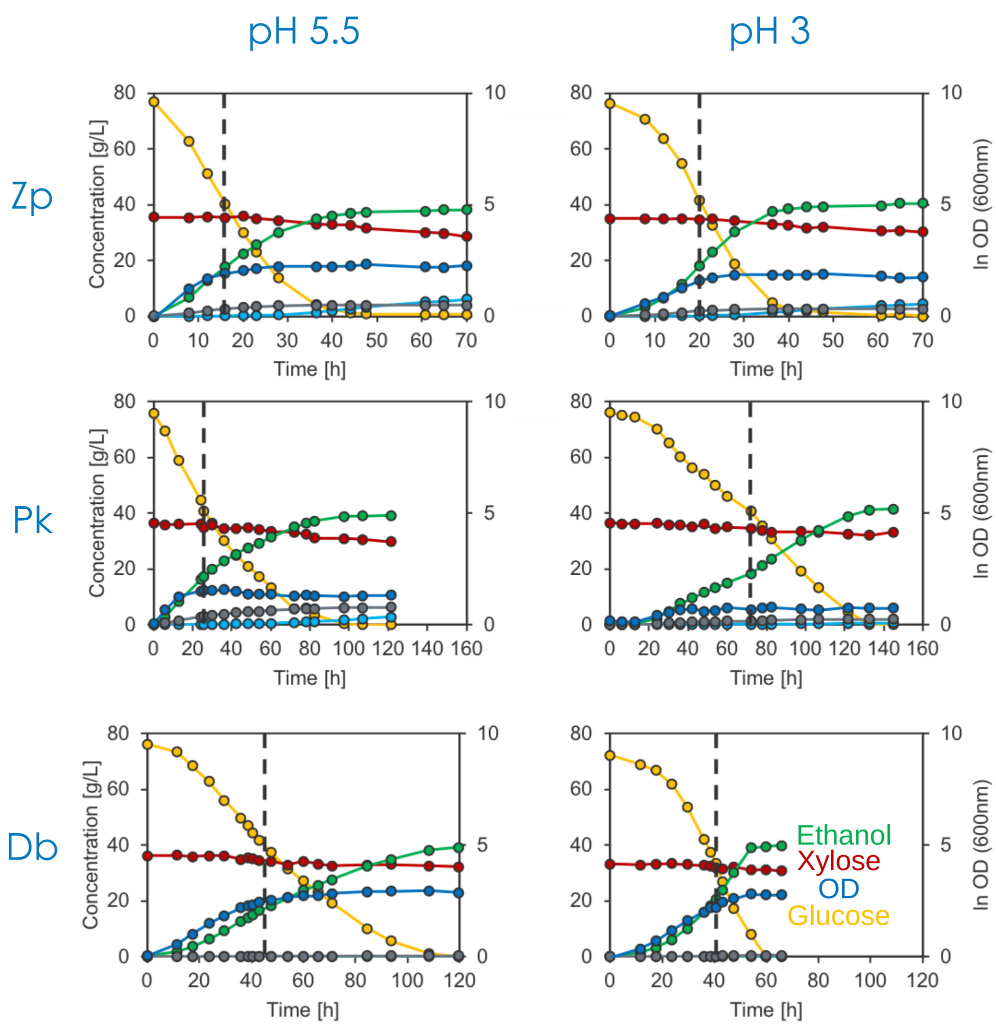
Engineered and evolved xylose usage



Collaborators: LYGOS, CDPHE Colorado Department of Public Health & Environment, Università degli Studi di Milano Bicocca, SLU

We have successfully identified top candidate acid-tolerant yeast strains implemented fundamental genome engineering strategies and generated xylose assimilating yeast strains

Technical Accomplishments: Understanding Acid Tolerance in Yeast Species

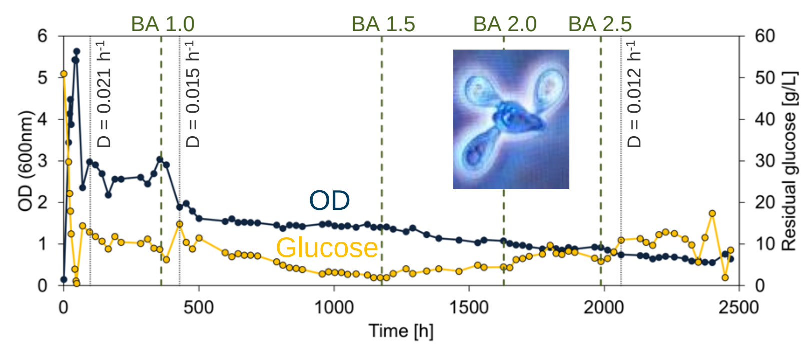


Transcriptomic and Proteomic Analyses

Goal: Understand both inter- and intra- strain physiological differences at low pH (3.0) and standard pH (5.0) conditions.

- Samples taken at period of maximum growth rate at each condition.
- Proteomic and RNAseq data currently being analyzed.

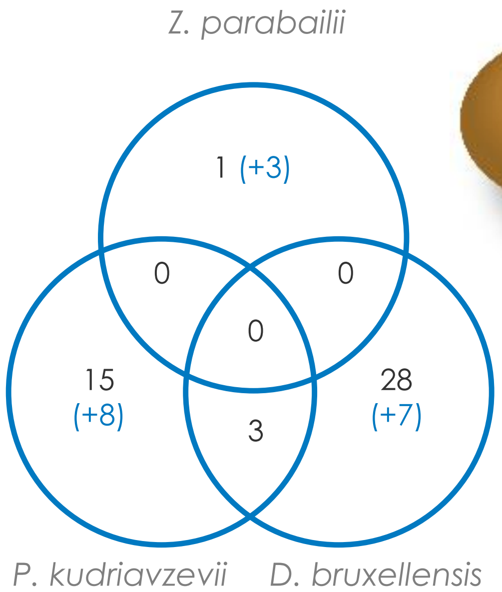
Chemostat based adaptation at pH 4.0 with increasing butyric acid concentration

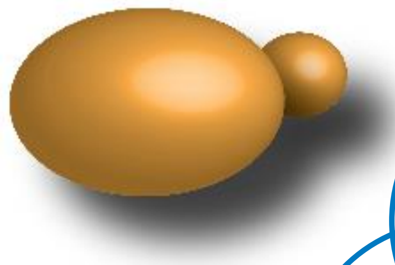


<u>158 generations</u>

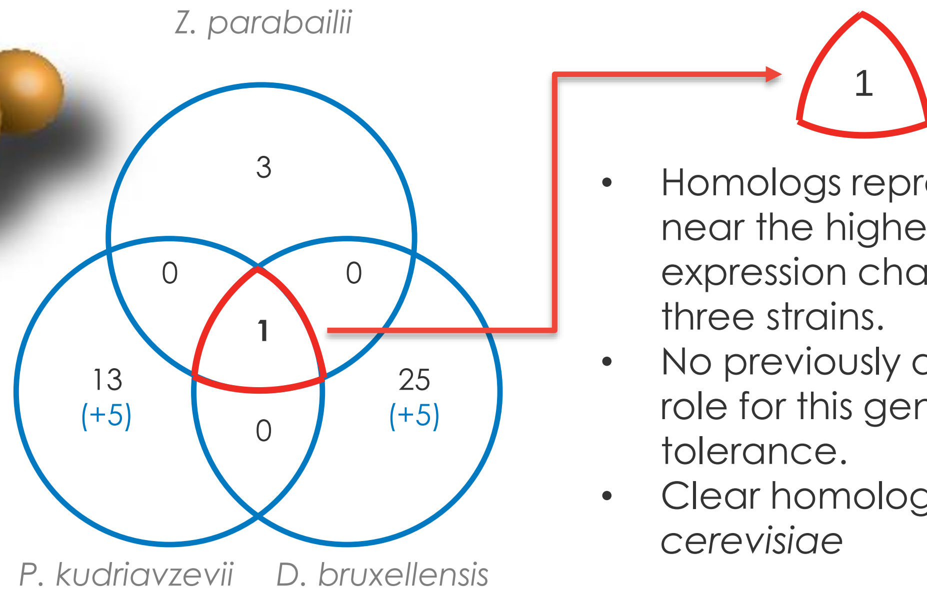
Technical Accomplishments: Understanding Acid Tolerance in Yeast Species

pH 5.5





pH 3.0



- Homologs represent at or near the highest fold expression change in all three strains.
- No previously defined role for this gene in pH tolerance.
- Clear homolog in *S. cerevisiae*

- Three distinct yeast lineages appear to have **three distinct mechanisms to adapting to low pH conditions.** Modulating these responses may increase pH and acid resistance.
- Several novel gene targets being screened in *S. cerevisiae* as knockout and overexpression targets to identify genetic modifications to enable elevated resistance to low pH environments.
- Transcriptomics data will be integrated and follow on -omic studies on organic acid sensitivities have been initiated.

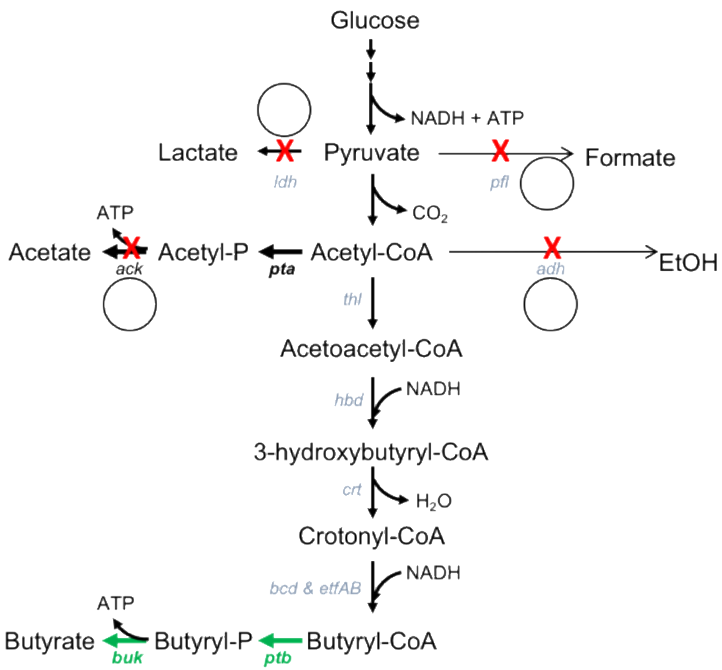
Technical Accomplishments: Anaerobic bacteria for the production of butyric acid

C. butyricum & C. tyrobutyricum

- Strict anaerobes
- Native butyrate and acetate producers
- Glucose and xylose utilization

- High tolerance to butyric acid
- Relatively rapid growth
- Limited genetic tools developed
- Inability to tolerate low pH

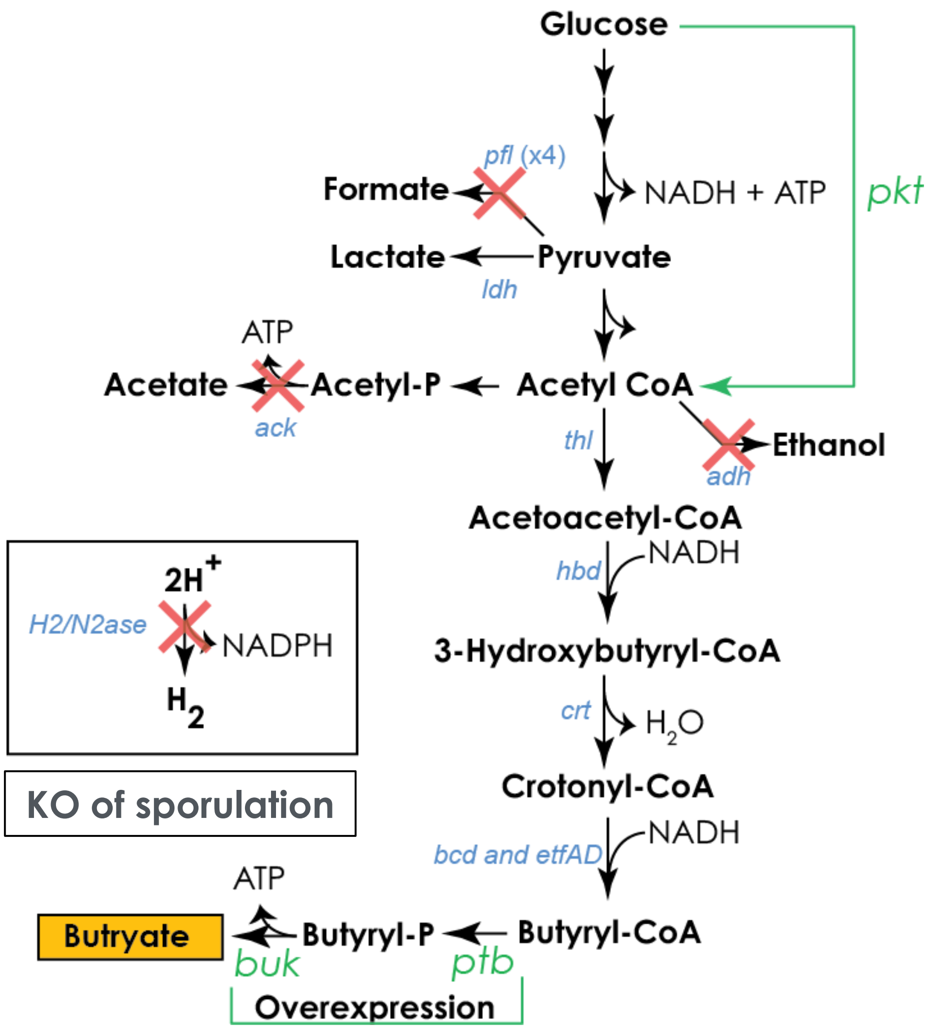
Task 1: Strain Engineering

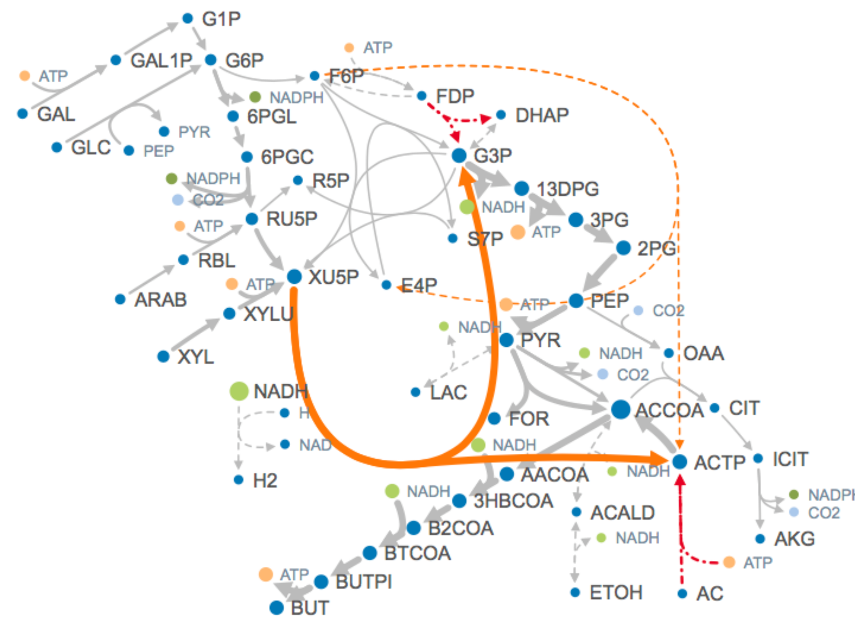


Task 2: Fermentation Engineering

Technical Accomplishments: Model-Guided Strain Engineering

Initial targets to increase flux to butyrate

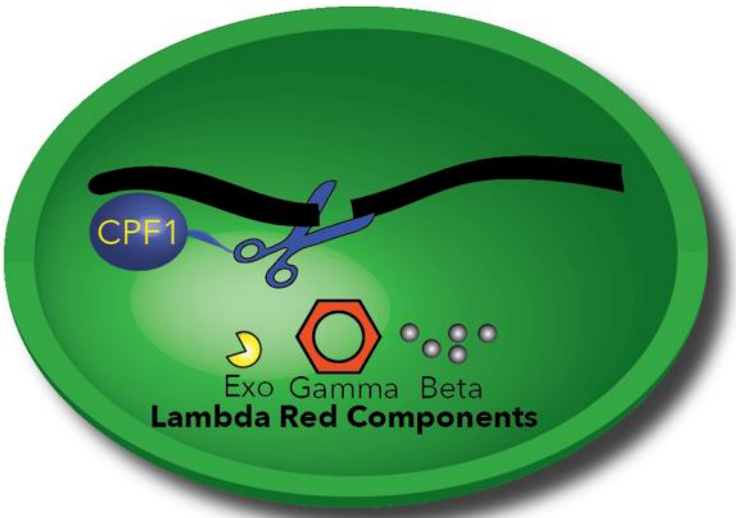




(Collaboration with BPMS Task)

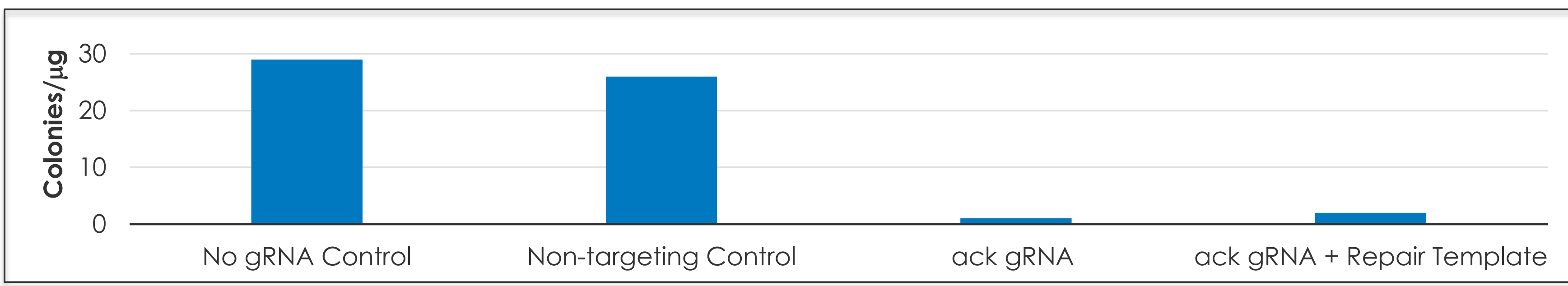
- Individual gene targets have been knocked out or overexpressed and tested.
- Combinatorial knockouts are underway using a Cre/Lox system. Currently developing tools in-house and in collaboration to improve engineering in both strains.

Technical Accomplishments:
Genome engineering through CRISPR/Cpf1 *ack* targeting



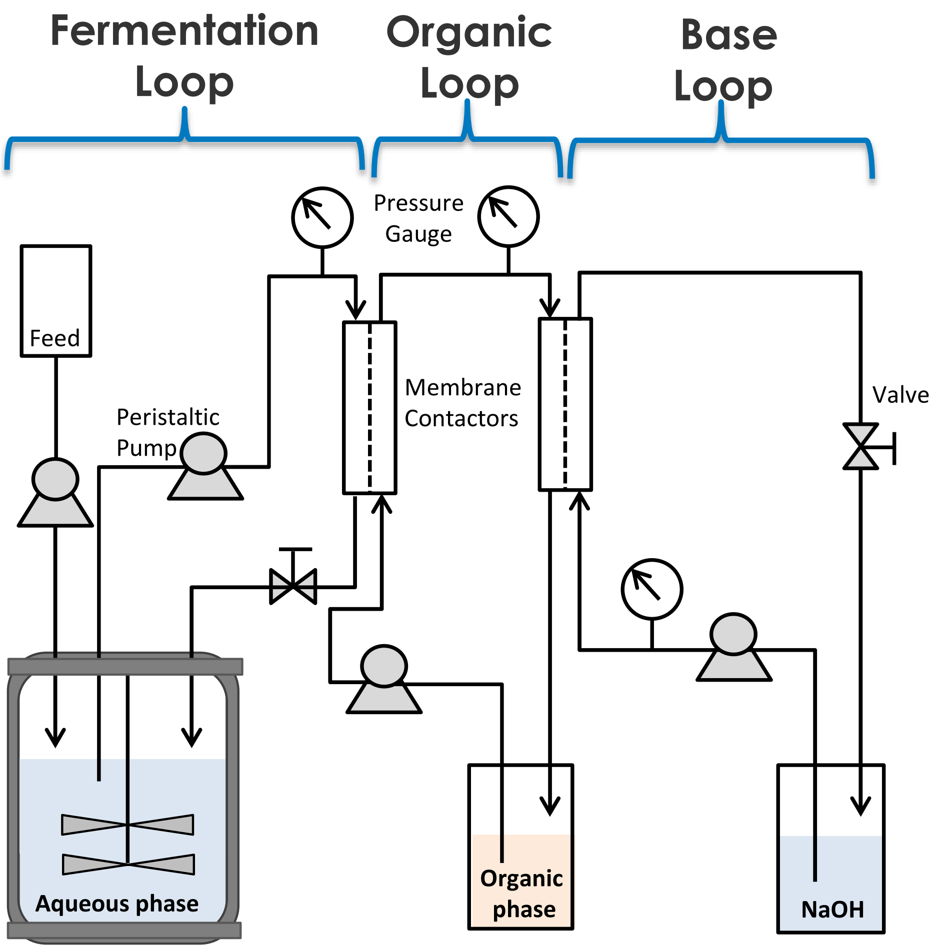
Clostridium butyricum

- Initial CRISPR-Cpf1 trials have indicated efficient genome cutting.
- Native, DNA repair efficiency is likely prohibitive.
- Currently testing Lambda Red components to facilitate DNA repair.
- Cre/LoxP Recombination is functioning.



We have had early success in establishment of genome engineering tools in both *Clostridium* species.

Technical Accomplishments: Pertractive Fermentation for Butyric Acid

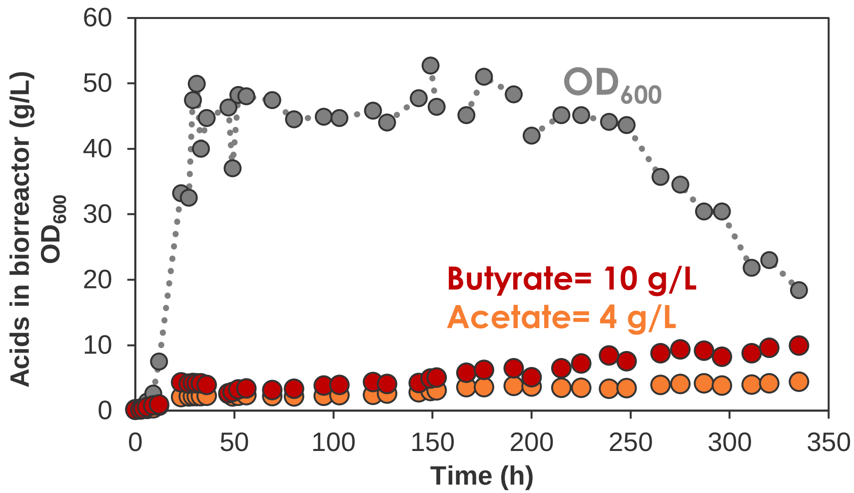


- Pertractive fermentation enables the continuous stripping of butyric acid using an organic phase.
- Base loop serves to back extract and collect acids. **Future plan involves replacing base extraction loop with continuous distillation to dramatically reduce process costs**.
- Continuous acid stripping reduces product toxicity and enables pH maintenance of fermentation media without base addition.

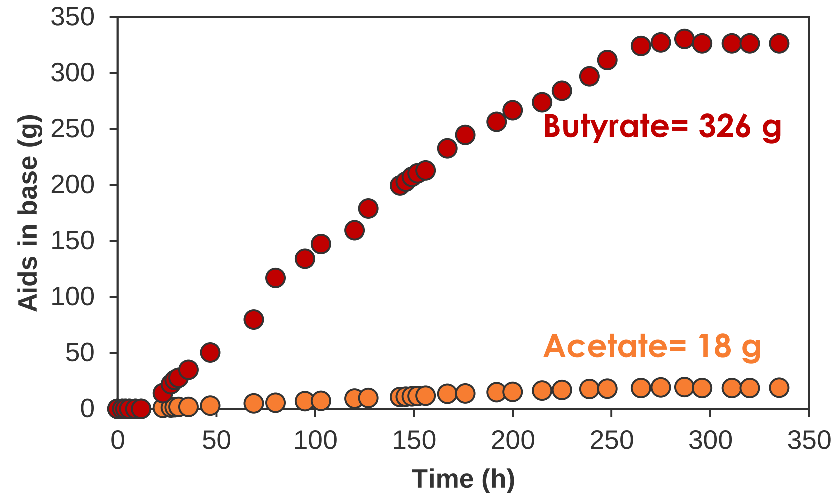
In collaboration with the 'Separations Consortia' led by Eric Karp

Technical Accomplishments:
Pertraction of Butyric Acid from Glucose

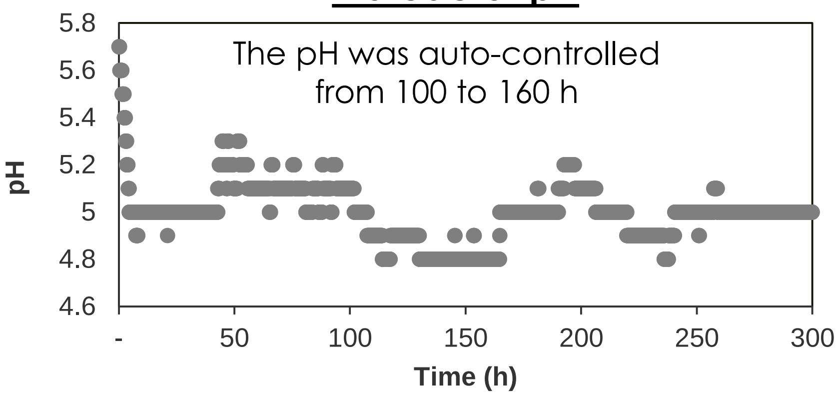
Bacterial growth and acids in bioreactor



Total acids collected from the base loop



Bioreactor pH



- Fermentation length (h)= 335
- **Glucose consumed (g)= 873**
- Acetate production (g)= 29.5
- **Butyrate:**
  - Total production (g)= 357
  - Yield (g/g glucose)= 0.41
  - Overall productivity= 0.44 g/L/h
  - Maximum productivity= 0.68 g/L/h

Over 300 g butyric acid produced during a 336 h pertractive fermentation with limited acetic acid production from pure glucose

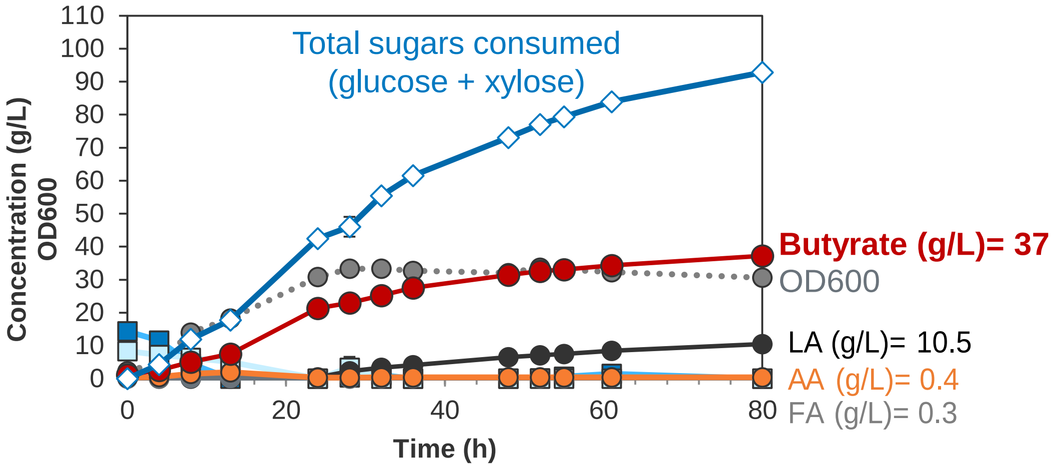
In collaboration with the 'Separations Consortia' led by Eric Karp

Technical Accomplishments:
Fed-batch Production of Butyric Acid from DDR hydrolysate

Fed-batch fermentations in DDR hydrolysate

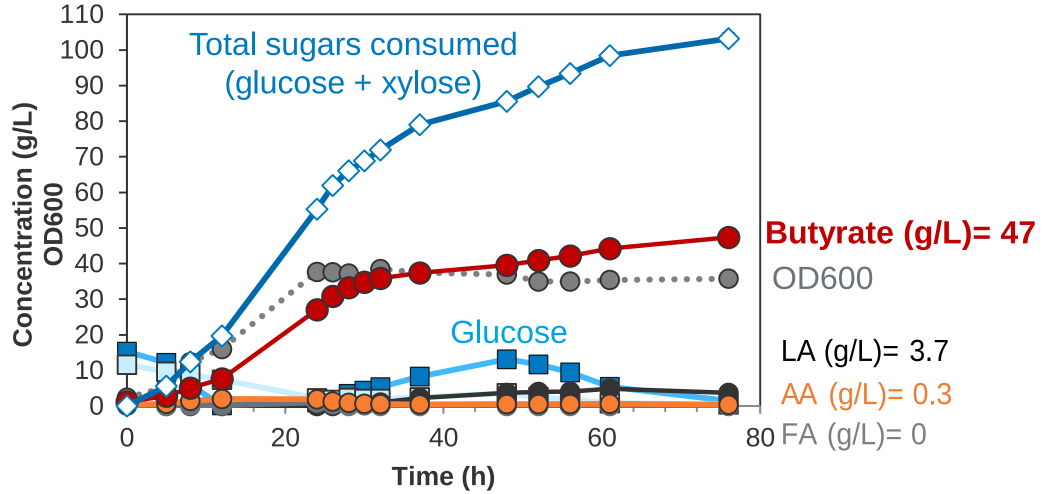
Batch phase:
25 g/L total sugars (glucose, xylose)
Fed-batch phase:
sugars maintained at <1 g/L/h
(feed rate= 2-3 g/L/h)

Butyric Acid Yield: 39%



Batch phase:
25 g/L total sugars (glucose, xylose)
Fed-batch phase:
sugars maintained at >1 g/L/h
(feed rate= 3-5 g/L/h)

Butyric Acid Yield: 45%



Optimized sugar feed rate enables:
1. **Xylose co-utilization**
2. **Minimization of byproducts (lactic, formic, acetic acids)**

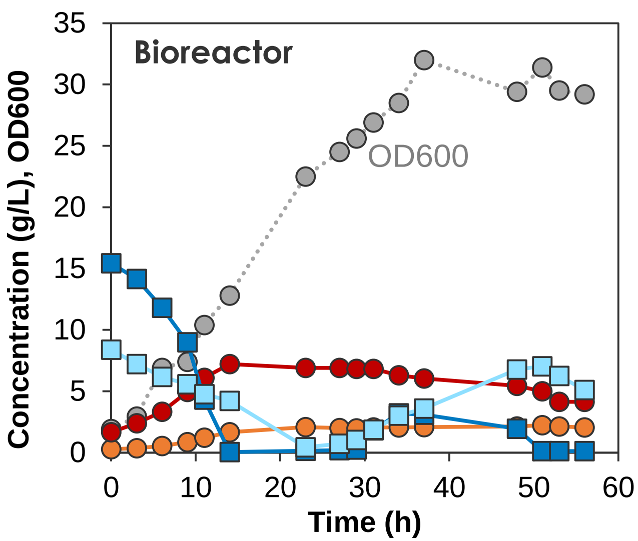
Technical Accomplishments:
Pertraction of Butyric Acid from Mock Hydrolysate

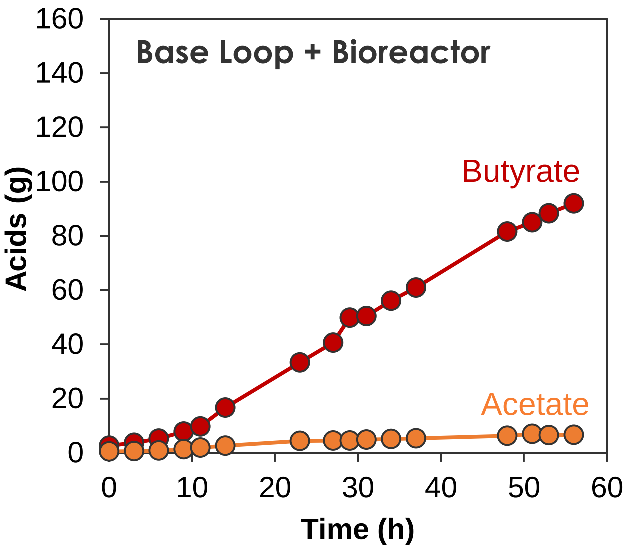
Initial Pertractive Trial

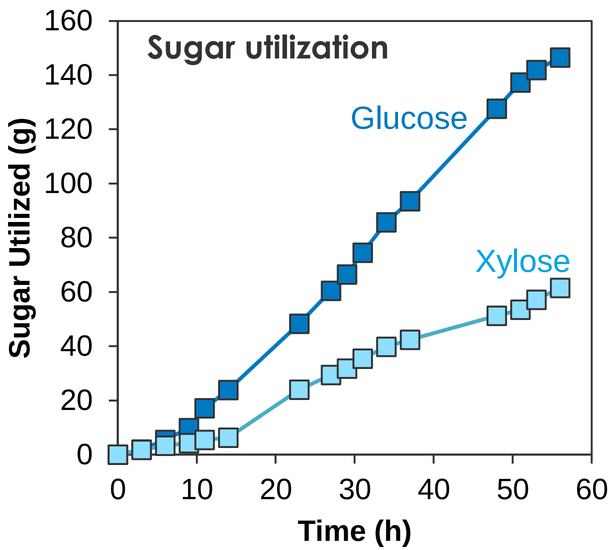
Sugar Co-utilization

- Glucose usage 1.6 g/L/h
- Xylose usage 0.8 g/L/h

- **Butyric Acid Productivity: ~1 g/L/h**
- **Butyric Acid Yield: 43%**
- Acetic Acid production minimal







- **Initial pertractive fermentation using <u>mock hydrolysate</u> successfully demonstrated co-utilization of glucose and xylose, efficient extraction, and auto pH balancing.**
- **We are currently optimizing our system on DDR hydrolysate.**

Relevance

- Our primary goal is to develop robust microbial strains and fermentation approaches to convert lignocellulosic sugars to fuel precursors at titer, rate, and yield targets set by techno-economic analysis and which are fully integrated into a complete process to produce cost competitive jet and diesel blendstocks.

- The final branched alkane molecule targeted directly target BETO's current and future MYPP targets for renewable diesel and jet fuels.

- Success of this project will advance the state of technology on hybrid biological/catalytic approaches towards biofuel production and is supported by TEA and LCA analysis.

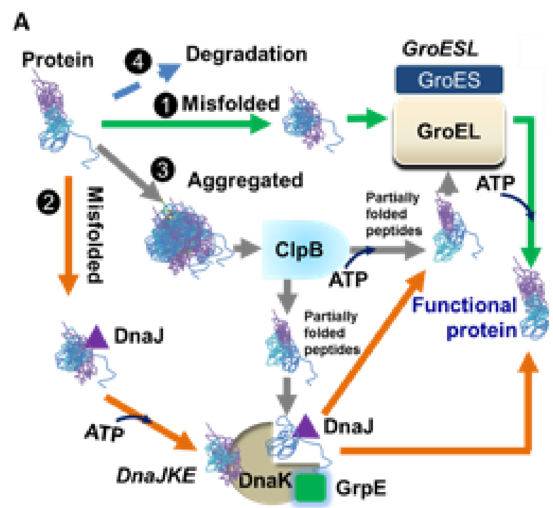
- The BUS project principally focuses on biocatalyst and fermentation development within the context of an end-to-end process. This project is fully committed to integrating with TEA and LCA analysis, upstream processes, downstream separations, catalytic upgrading and fuel testing in order to demonstrate the viability of our entire process from feedstock to finished fuels.

Future Work-Acid Tolerant Yeast

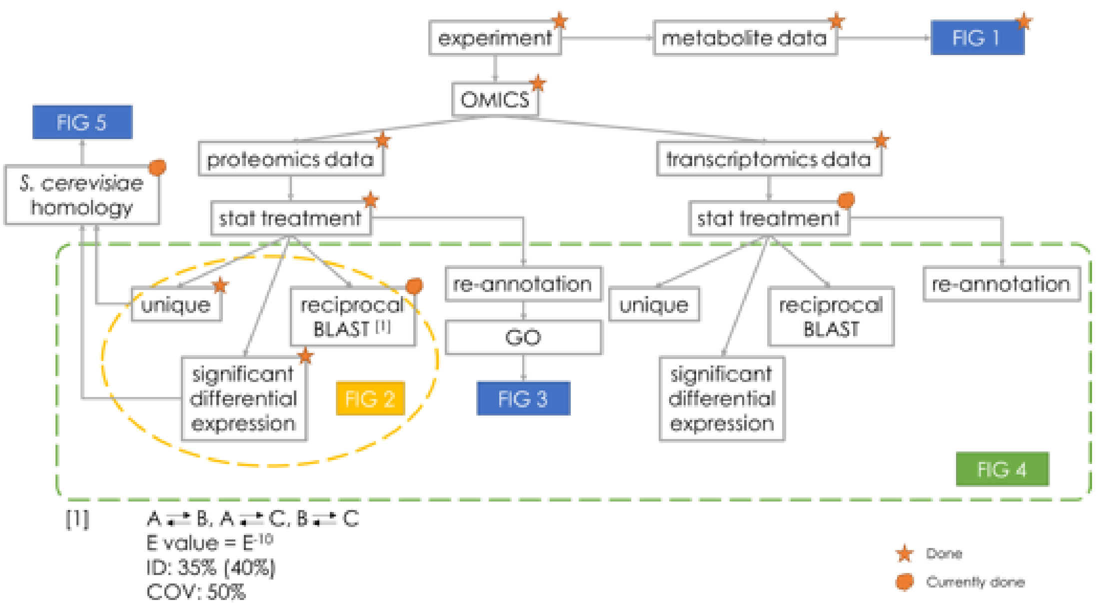
Acid-tolerant yeast development:

- Go/No-go decision (March 2019) Engineer, evolve or identify novel yeast strains capable of tolerating at least 4 g/L butyric acid at ≤ pH 4.0. (March, 2019) via adaptation or engineering.
- Finalize multi-omic analysis to understand physiological responses to low pH and high organic acid concentrations.

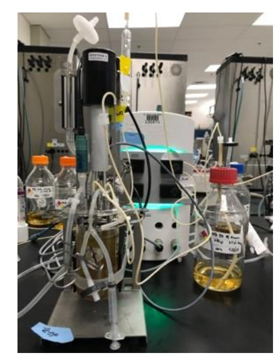
Engineering Yeast For Organic Acid Toxicity Mitigation



Omics-based understanding of acid resistance

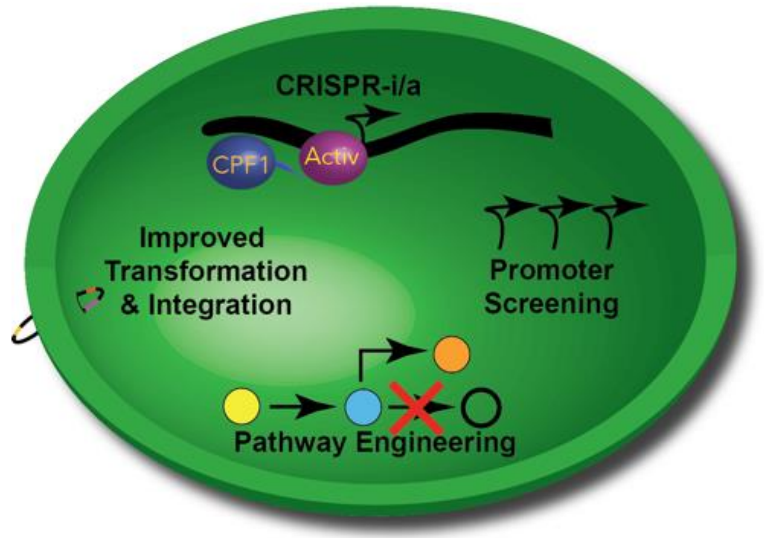


Lab –Adapted Evolution



Future Work-Bacterial strain development

FY19 Q4 Milestone: Perform quantitative and qualitative comparison of top native and/or engineered *Clostridium* strains (*butyricum* and *tyrobutyricum*) using hydrolysate as a feedstock to enable a down-selection to a single organism.



Research Priorities

- Further developing CRISPR Tools (CRISPR, CRISPRi/a)- *With Nicholas Sandoval*
- Counter-selectable markers-*With Adam Guss*
- Bypassing R/M systems (methylomics)-*With Adam Guss*
- Developing gene expression controls
- Alleviating catabolic repression
- Implement arabinose utilization
- Multiplex pathway engineering
- Improving flux towards butyric acid
- Improving low pH tolerance
- Harnessing flux to alternative acids and/or esters

Future Work-Bacterial Fermentation Development

Joint Milestone between BUS and SepCon in collaboration with CUBI 6/30/2019. Produce at least 150 g of butyric acid by a *Clostridium* strain, at productivities > 0.5 g/L/h and yields > 0.35 g/g sugars at greater than 80% purity from DMR-EH hydrolysate for further characterization and upgrading by the Catalytic Upgrading of Biological Intermediates (CUBI) Project.

Research Priorities: Continue improving processes in order to meet current and outyear MYPP targets.

- Tuning pertractive fermentation setup and parameters to improve performance with hydrolysate.
- Demonstrate successful prolonged pertractive fermentation in DDR hydrolysate.
- Assess performance of engineered strains delivered by Bacterial Strain Development Task.
- Further optimizing xylose usage through modification of seed protocol and feed parameters
- Deliver high-purity bio-derived butyric acid to the Catalytic Upgrading of Biological Intermediates project.

Summary

1. **Overview-** The BUS project seeks to deliver strains and processes that enable the production of cost efficient jet and diesel blendstocks.

2. **Approach-** We identify microbial strains with desirable characteristics, engineer these strains for improved performance, and optimize process configurations through productive collaborations to maximize our impact on the development of cost-effective biofuels.

3. **Technical Accomplishments/Progress/Results-**We have identified and engineered diverse microbial strains and optimized processes to demonstrate a high production of biological intermediates (Including fatty acid based molecules and carboxylic acids) in an integrated process.

4. **Relevance-** This project directly attacks the challenge of identifying Technoeconomically-viable routes towards the production of branched alkanes that are useful for jet and diesel blendstocks.

5. **Future work-** This project will continue to improve our baseline progress through strain engineering, fermentation development and through identifying attractive alternative routes to biological intermediates. All future research is directly tied to meeting BETO's 2022 and 2030 MYPP cost targets using biomass hydrolysate.

NATIONAL RENEWABLE ENERGY LABORATORY

ACKNOWLEDGEMENTS

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- Eric Karp
- Michela Monninger
- Patrick Saboe
- Dan Schell
- Derek Vardon

External collaborators

- Paola Branduardi, University of Milano
- Adam Guss, Oak Ridge National Laboratory
- Ken Reardon, Colorado State University
- Nicholas Sandoval, Tulane University
- Eric Steen & Jeffrey Dietrich, Lygos
- Jose Luis Adrio Fondevilla, Neol BioSolutions
- Volkmar Passoth, Swedish University of Agricultural Sciences

Thank you!

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BIOENERGY TECHNOLOGIES OFFICE

Additional Slides

Responses to Previous Reviewers' Comments

"The PI, together with the TEA team, needs to evaluate the production cost of C16–18 fatty alcohols as fuel. I doubt if hydrolysate feed of C6/5 stream will cut it compared to alternatives in the market (i.e., plant oil and petro-derived long-chain alcohols)." While we successfully generated strains capable of secreting fatty alcohols, we ultimately learned that poor carbon efficiency of FA pathways and significant TEA hurdles made this approach a daunting challenge. Accordingly, we abandoned this route and heightened our focus on carboxylic acid production routes.

"Another recommendation will be to try to find know-how (which exists) in industry in the United States as way to accelerate this R&D effort." We have broadened our outreach in this field in trying to leverage experts in areas that represent bottlenecks towards our success. We formed a cooperative research agreement (CRADA) with Lygos, established a working collaboration with Nicholas Sandoval at the University of Tulane, Adam Guss at Oakridge, Ken Reardon at Colorado State University, Paola Branduardi at the University of Milan.

Publications, Patents, Presentations, Awards, and Commercialization

Publications:

- R Nelson, D Peterson, EM Karp, GT Beckham*, D Salvachúa* **(2017)** Mixed carboxylic acid production by *Megasphaera elsdenii* from glucose and lignocellulosic hydrolysate, Fermentation. 3, 10.
- MT Guarnieri‡, YC Chou‡, D Salvachua‡, A Mohagheghi, P St. John, YJ Bomble, GT Beckham* **(2017)** Metabolic engineering of *Actinobacillus succinogenes* for enhanced succinic acid production. Applied Environmental Microbiology. 83, 00996-17.
- EM Karp, R Cywar, L Manker, P Saboe, C Nimlos, D Salvachúa, X Wang, BA Black, M Reed, W Michener, N Rorrer, GT Beckham **(2018)** Post-fermentation recovery of bio-based carboxylic acids. ACS Sustainable Chemistry & Engineering. 6, 15273–15283.
- V Sànchez i Nogué, BA Black, JS Kruger, CA Singer, KJ Ramirez, ML Reed, NS Cleveland, ER Singer, X Yi, RY Yeap, JG Linger and GT Beckham (**2018**) Integrated renewable diesel production from lignocellulosic biomass via oleaginous yeast. Green Chemistry. 20, 4349-4565

Patent applications:

- M Guarnieri, YC Chou, D Salvachúa, G Beckham. Metabolic engineering for enhanced succinic acid biosynthesis. U.S. non-provisional patent application. Filed on April 25, **2017** at the United States Patent & Trademark Office (USPTO), Application No. 15/496,944. 30, 2018 at the United States Patent & Trademark Office (USPTO). Application No. 62/664,445.

Publications, Patents, Presentations, Awards, and Commercialization

Presentations

- 34th International Specialized Symposium on Yeasts (1- 4 October 2018 · Bariloche, Argentina) V Sànchez i Nogué, CA Singer, AT Gerritsen, C del Cerro, ML Reed, GT Beckham, JG Linger. Comparative Proteomics of non-model yeasts to low pH in hydrolysate cultivations. Oral presentation.
- 38th Symposium on Biotechnology for Fuels and Chemicals (25 – 28 April 2016 · Baltimore, MD – USA) V Sànchez i Nogué, T Trinh, M Sanchez, K Ramirez, ML Reed, JG Linger, N Dowe and GT Beckham. Improving lipid and biomass production by Rhodosporidium toruloides. Poster presentation.
- 39th Symposium on Biotechnology for Fuels and Chemicals (1 – 4 May 2017 · San Francisco, CA – USA) V Sànchez i Nogué, BA Black, JS Kruger, CA Singer, KJ Ramirez, MI Reed, NS Cleveland, JG Linger and GT Beckham. Lipid production from biomass via oleaginous yeasts. Oral presentation.
- Rocky Mountain Yeast Meeting (January 8, 2018 · Coors Brewery – Golden, CO). V Sànchez i Nogué. Integrated renewable diesel production from lignocellulosic biomass via oleaginous yeasts. Oral presentation.
- Rocky Mountain Yeast Meeting (January 11, 2019 · Colorado State University – Fort Collins, CO). C del Cerro. Comparative proteomic studies of non-model yeasts to understand low pH tolerance mechanisms in hydrolysate cultivations. Oral presentation.
- R Nelson, DJ Peterson, EM Karp, G Beckham, D Salvachúa. Enhanced volatile fatty acid production by *Megasphaera elsdenii* via fed-batch, pertractive fermentation. Recent Advances in Fermentation Technology (RAFT). October 29-November 1, **2017**. Bonita Springs (FL).