NREL
Transforming ENERGY

DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

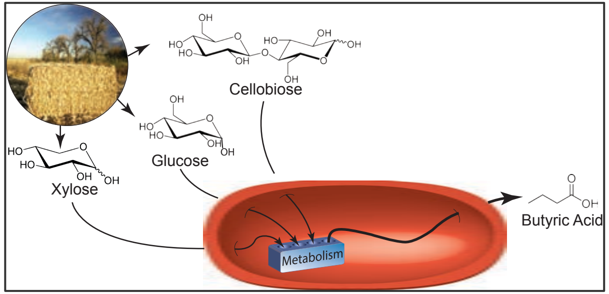
Biological Upgrading of Sugars

April 6
Biochemical Conversion
Jeff Linger
National Renewable Energy Laboratory

Photo from iStock-627281636

Approach: Project Overview- Biological Upgrading of Sugars (BUS)

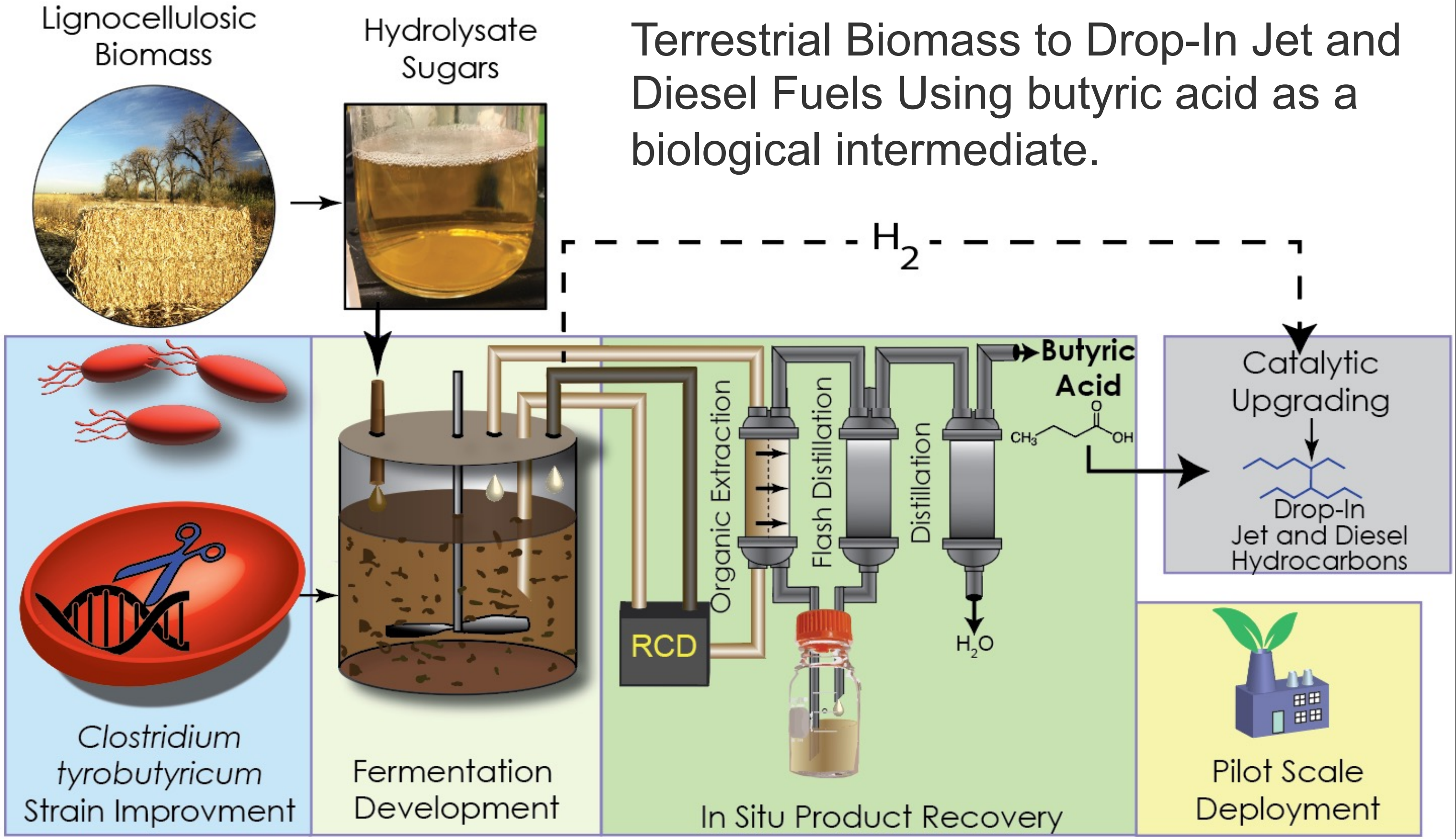
Overall Mission: Develop an integrated pilot scale process for the production of biological intermediates from terrestrial biomass that is catalytically upgradeable to sustainable aviation fuel and other valuable molecules.



Current Focus: Leveraging strain development, fermentation engineering, separations and design/build of pilot scale equipment. We are developing an **integrated and scalable process for the production of butyric acid,** and biologically derived intermediates towards the production of **Sustainable Aviation Fuel**.

Approach: Integrated Research Activities to Enable a Biomass to SAF Pathway

Terrestrial Biomass to Drop-In Jet and Diesel Fuels Using butyric acid as a biological intermediate.



Approach: Project Management-Senior Level Scientists

Jeff Linger (PI)
Overall Coordination

Chenlin Li
(TM-BETO)
Strategic Guidance

Interaction Highlights

- Formal biweekly full-team meetings
- Regular interproject collaboration
- Frequent engagement with external partners for commercialization

Davinia Salvachúa

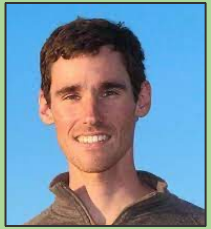
Fermentation & Proteomics

Violeta Sànchez

Fermentation

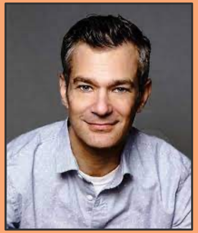
Eric Karp

Jake Kruger



Patrick Saboe

Pilot Process Development



Michael Guarnieri

Strain Development

Approach: Diversity, Equity and Inclusion

While a formalized DEI strategy was not a required component at the onset of BUS, we are collectively committed to promoting diversity, equity, and inclusion in all aspects of our scientific project management. We strive to create an environment where all team members feel valued, respected, and empowered to contribute their unique perspectives and experiences. Through intentional efforts to increase diversity and promote equity, we aim to advance scientific discovery and innovation while addressing the complex challenges facing our world today.

Greater than half of the members of this project are women and/or minorities

Approach: Risks and Mitigation

Risk	Description	Response
Organic Extractant Toxicity and performance of Liquid-liquid extraction	The organic extractant that we had been using (Cyanex) has shown toxicity to C. tyrobutyricum and has a negative impact on its productivity. Less toxic extractants have shown reduced extractability of butyric acid.	We have evaluated additional proprietary organic extractants and mixes that have shown reduced biological toxicity. We are finalizing selection of this extractant currently.
Installation and commissioning of pilot reactor	Final installation and Readiness Verifications are ongoing. These activities require collaboration with pilot plant operations, Safety Professionals and outside contractors.	We are evaluating individual components and making modest adjustments to improve performance. We anticipate being fully commissioned within 1-2 months.
Performance of the pilot reactor	This reactor is a first of its kind system with dozens of control points and fully integrated control systems. While we have no concerns that this system will fail to operate, there are risks with operating, trouble shooting and modifying the system to be fully optimized.	We are currently evaluating individual components of the and are making modifications to. Each Skid is independent, and can be individually modified or upgraded as needed to improve the overall performance

Approach: Increasing Impact Through Collaborations & Commercialization

-Genetic Tools
-Strain Construction

Agile BioFoundry

Catalytic upgrading of carboxylic acids

ChemCatBio
Chemical Catalysis for Bioenergy

Advancing carboxylic acid separations

Bioprocessing Separations Consortium

Proteomics

Oak Ridge National Laboratory

BUS Project

NREL
National Renewable Energy Laboratory

- BPMS-Metabolic Modeling
- Several BER/LDRD-funded Projects
- NREL/Quasar FOA Project
- Biochemical Platform Analysis

fcic
Feedstock-Conversion Interface Consortium

Understanding effects of feedstock variability

Company X

Commercializing Bio-butyric acid derivatives for human health

CRADA

Crysalis
Advancing Zero Carbon Futures

Commercializing Bio-butyric acid derivatives for SAF & chemicals

CRADA (TBD)

Approach: Integrating Research to Improve Overall Process

Proteomics

NREL
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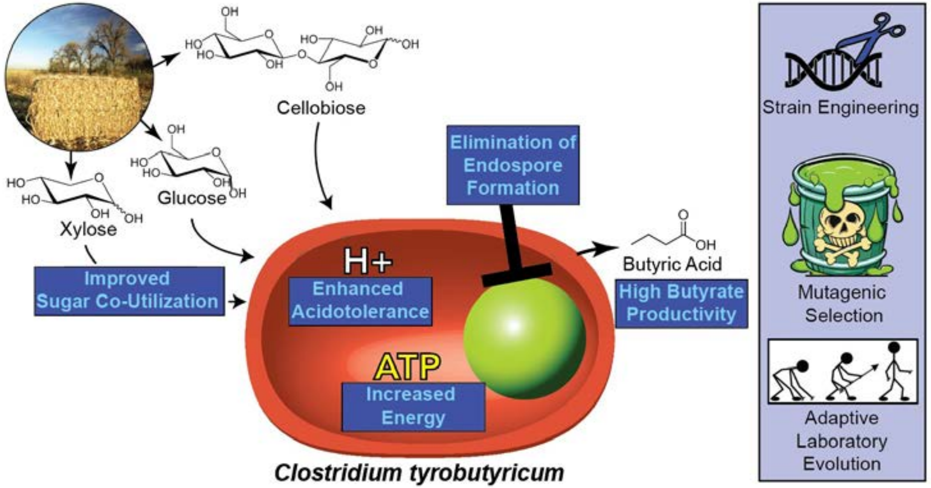
OAK RIDGE National Laboratory

Fermentation

Strain Development



Pilot Process Development

Approach: Fermentation Engineering Leads to Increased Process Efficiency

Proteomics

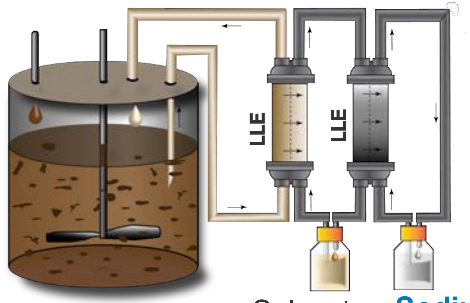
Strain Development

Fermentation

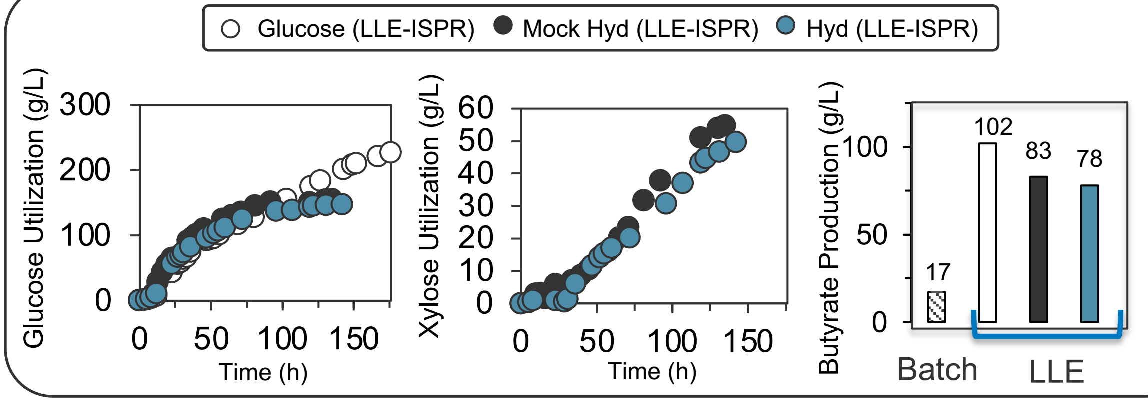
Pilot Process Development

Progress and Outcomes (FY21): In Situ Product Recovery By LLE Dramatically Improves Overall Butyrate Production

Liquid-Liquid Extraction (LLE)-ISPR



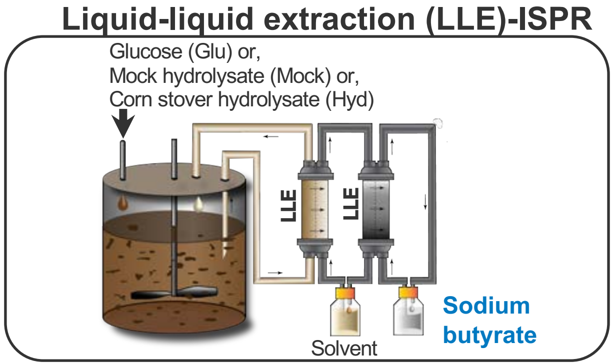
Substrate

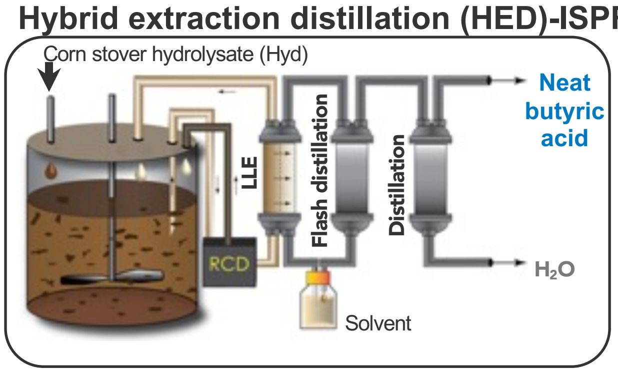


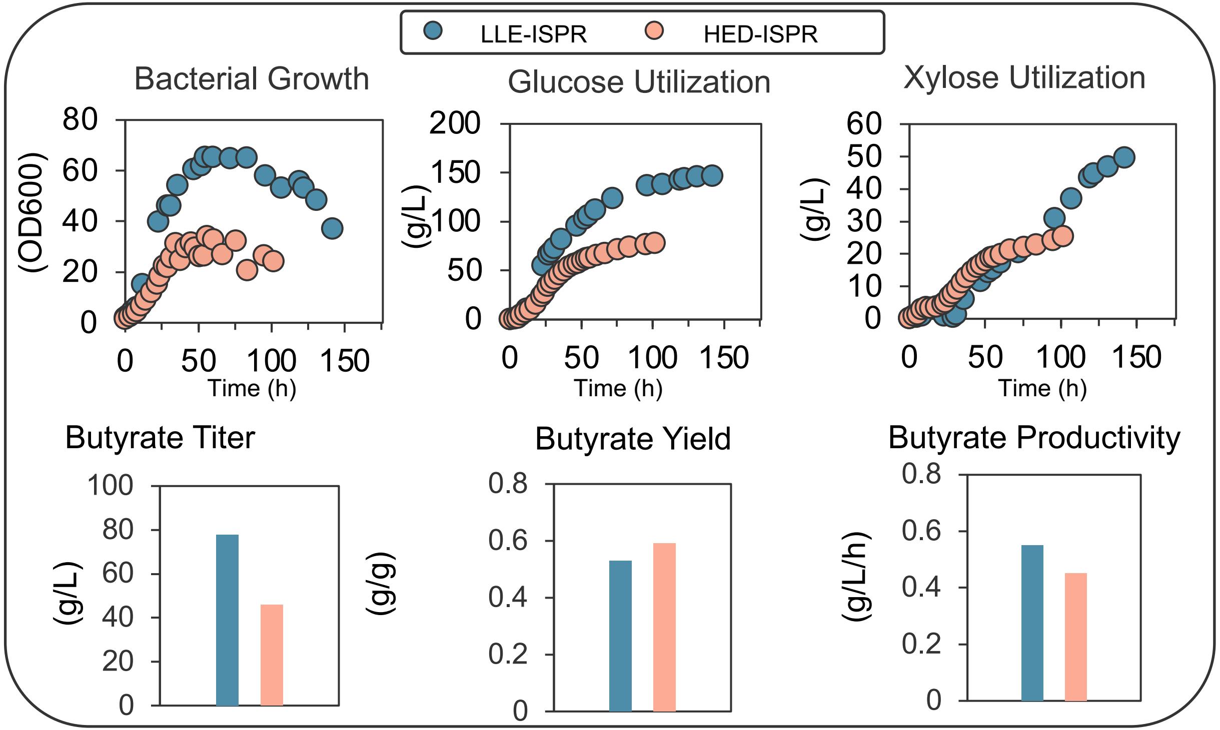
Key Finding: Butyrate is produced >100 g/L from Glucose in an LLE system. Butyrate is produced at ~80 g/L from hydrolysate or mock hydrolysate.
Challenge: Presence of xylose causes premature cessation of fermentation

Salvachúa, D., Saboe, P, et al. 2021

Progress and Outcomes (FY21): In Situ Product Recovery By HED is Challenged by Solvent Toxicity

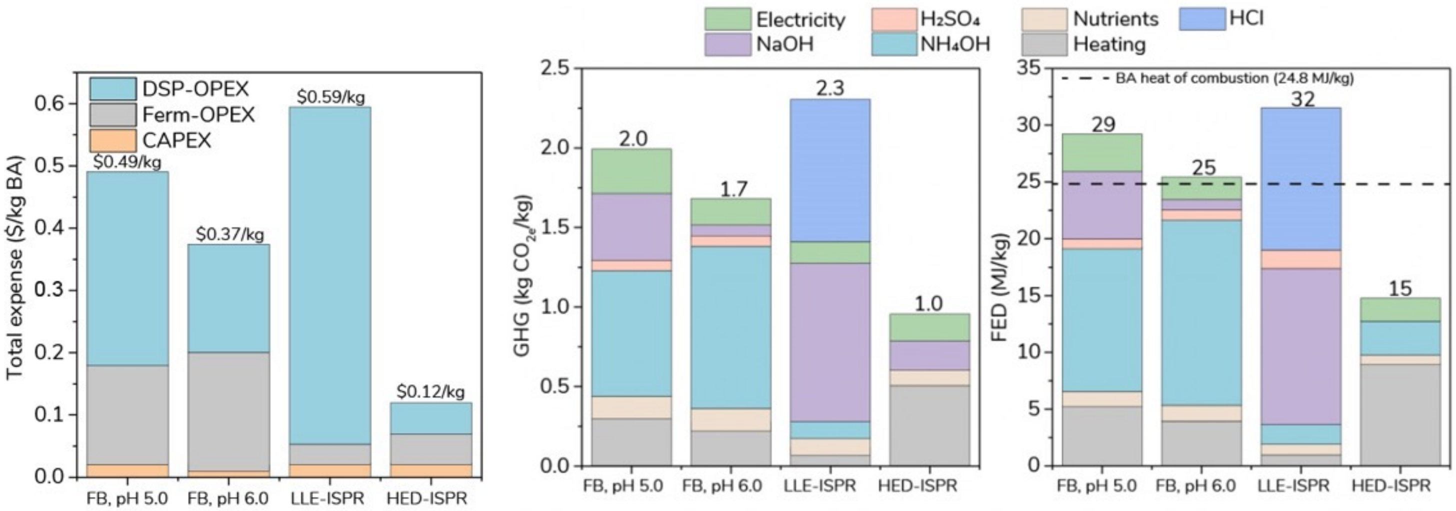






Key Finding: Hybrid Extraction Distillation (HED) works, but solvent is toxic
Challenge: There is a need to identify less-toxic solvents

HED-ISPR Dramatically Improves Economics and Reduces Environmental Impact



- The higher cost of LLE-ISPR results from the chemical consumption of sodium hydroxide in back extraction
- $0.12/kg represents 14% of the total expense.
- **Total MSP is $0.99/kg. Current biobased butyric acid market price is ~$1.80/kg.**
- Clear targets to reduce projected expenses further:

Progress & Outcomes: Proteomics to Understand Effects of pH and Xylose

Proteomics

NREL
NATIONAL RENEWABLE ENERGY LABORATORY

OAK RIDGE
National Laboratory

Fermentation

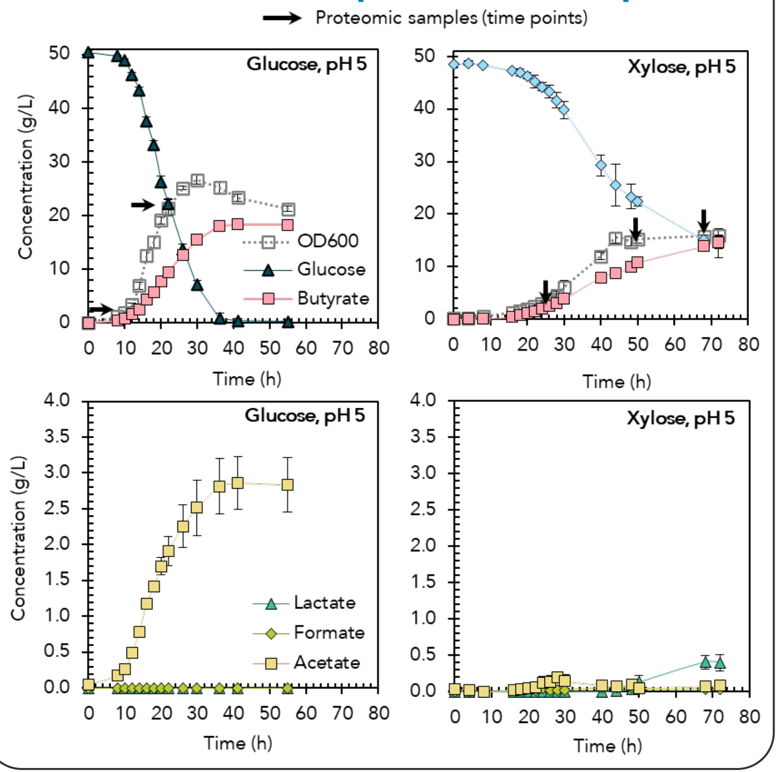
Strain Development

Pilot Process Development

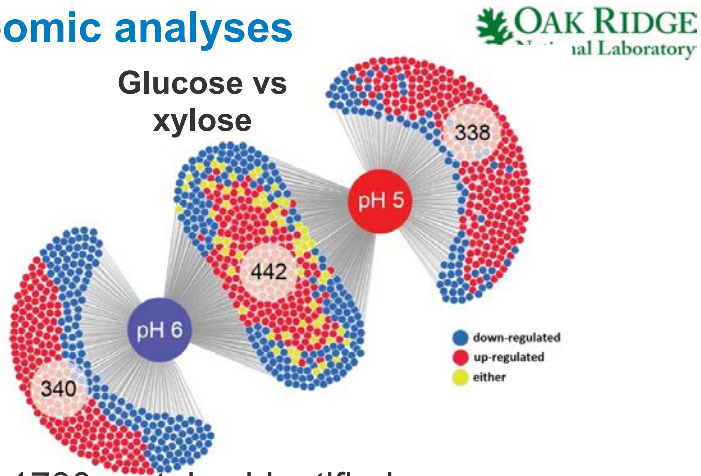
Progress & Outcomes: Proteomics Reveals Key Differences Between Carbon Sources and pHs

Proteomics in xylose-containing media and low pHs has not been conducted in *C. tyrobutyricum* to date (in collaboration with Robert Hettich' lab at Oak Ridge National Laboratory).

Generation of proteomic samples



Overall proteomic analyses



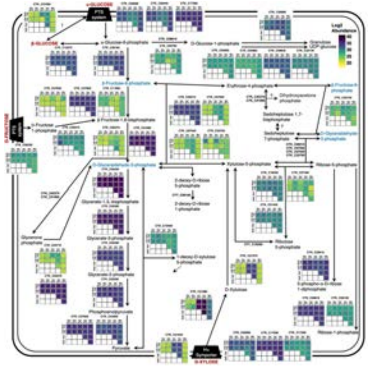
- Between 1400-1700 proteins identified
- 56 and 61% of the proteins showed significant abundance differences between glucose and xylose medium in exponential and stationary phase, respectively.

Progress & Outcomes: Proteomics Reveals Key Differences Between Carbon Sources and pHs

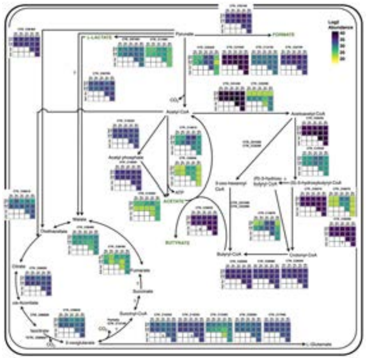
Targeted proteomic analyses in central carbon metabolism

- Enzyme abundances are variably dependent on Carbon Source, pH.
- Identification of potential **key xylose transporters**.
- Xylose containing media leads to a reduced flux to acetate, this is *not* likely due to central metabolism but rather **a novel suite of genes not previously characterized** in this regard.
- **Metabolic modeling is currently being conducted for *C. tyrobutyricum*** in collaboration with the Biochemical Process Modeling and Simulation project.

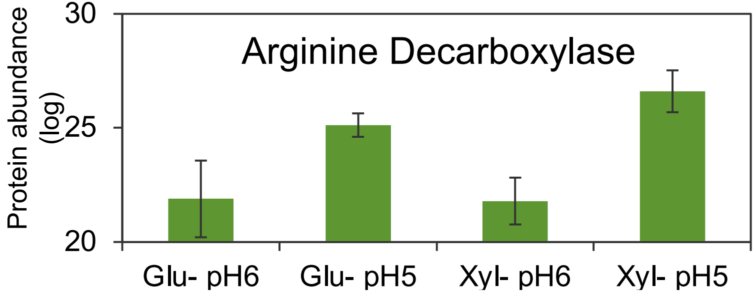
Sugars to Pyruvate



Pyruvate to Butyric Acid



Amino Acid Decarboxylases to Raise Intracellular pH



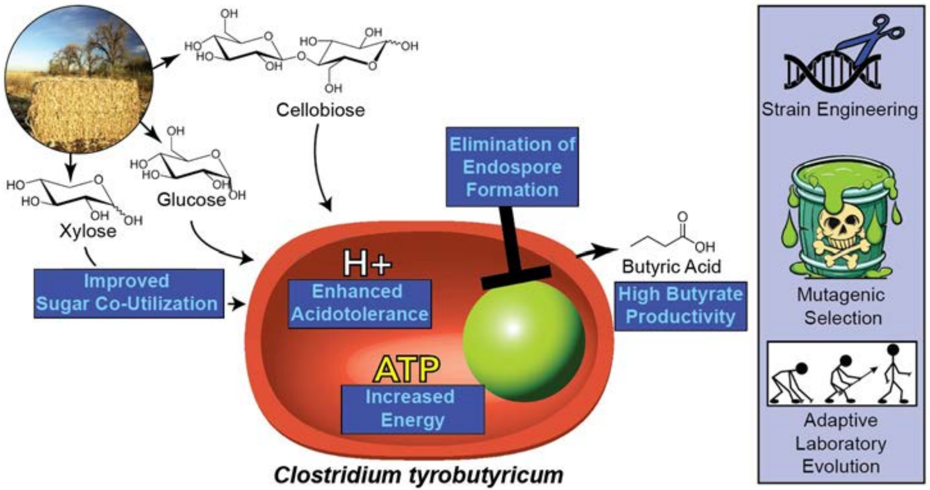
Manuscript in preparation

Progress & Outcomes: Strain Development To Improve Overall Performance

Proteomics

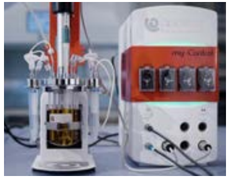
Fermentation

Strain Development



Pilot Process Development

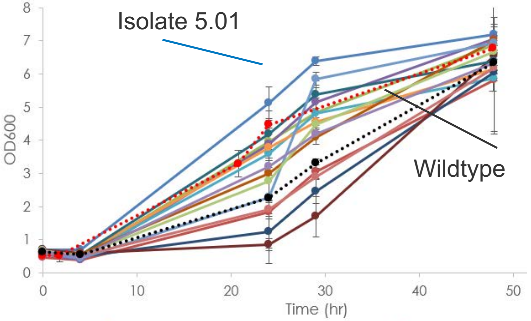
Progress & Outcomes: Evolution Generates Strains with Improved Characteristics at low pH in Xylose-Containing Medium



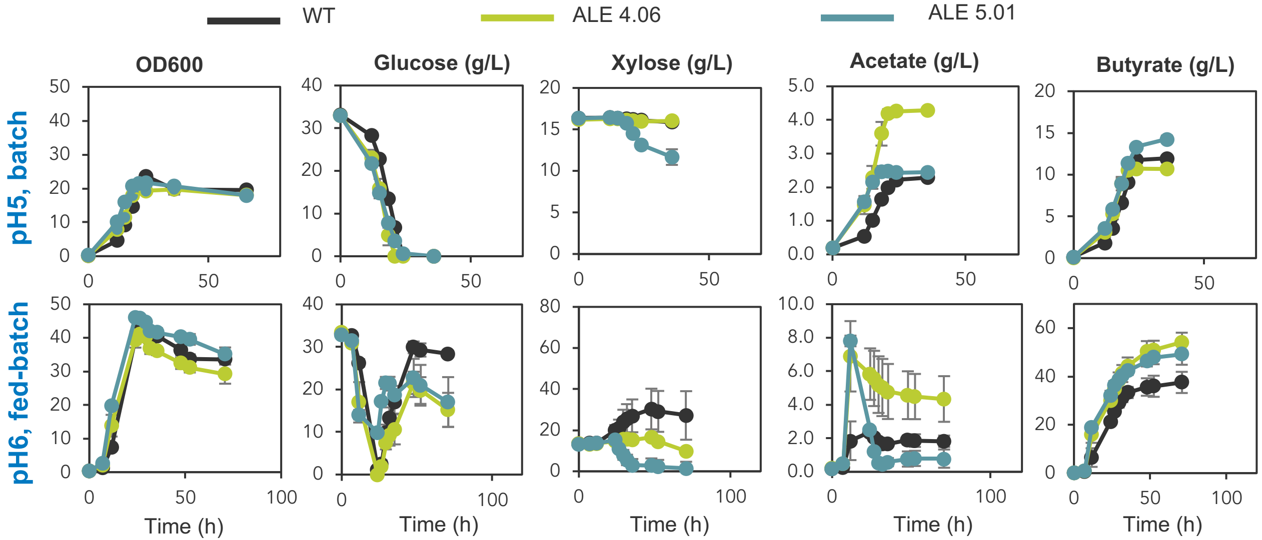
ALE

- pH 5.0, mixed glucose and xylose
- 2,500 h cultivation 396 generations

Preliminary Screens Identified Top Candidates (tested at pH 5.0)

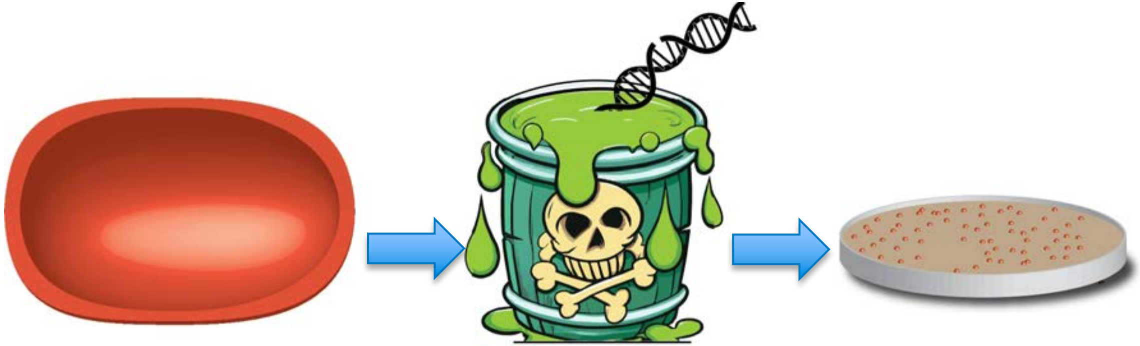


Evolved strains have higher initial growth and sugar utilization rates and overall productivity than the wild-type strain

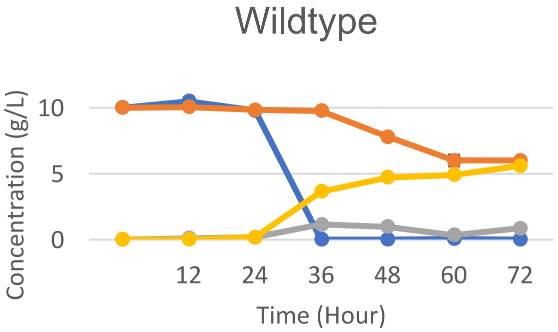


Key Finding: Genomic sequencing reveals mutations in key metabolic enzymes, sporulation system, flagellar machinery, and sugar utilization.

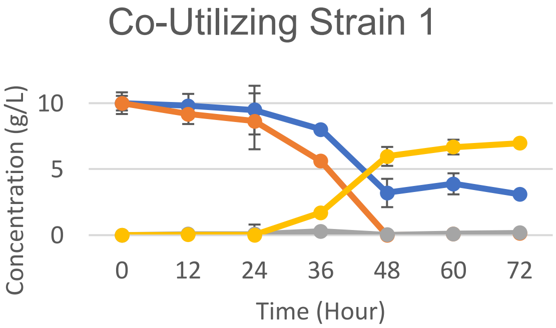
Progress & Outcomes: Mutagenesis Improves Sugar Co-Utilization

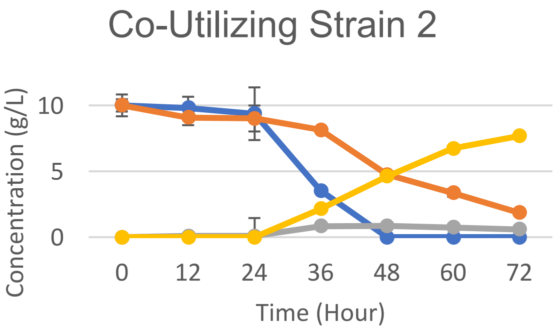


C. tyrobutyricum → NTG Mutagen → Xylose plus 2-deoxyglucose



Glucose Xylose Acetate Butyrate

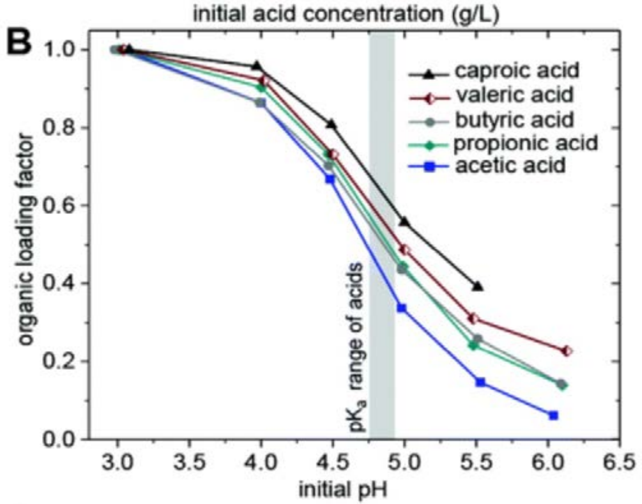




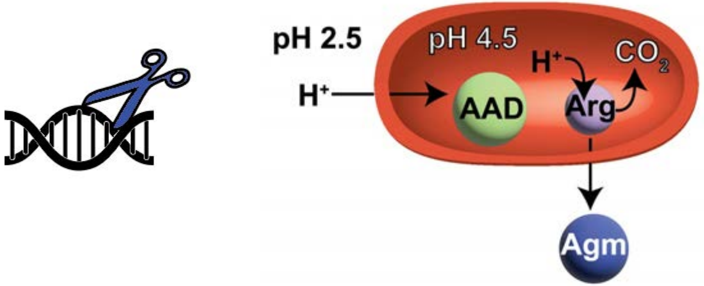
Key Finding: Genome Resequencing of mutagenized strains revealed novel targets in sugar metabolism

Progress & Outcomes: Increasing Acidotolerance to Improve Process Efficiency

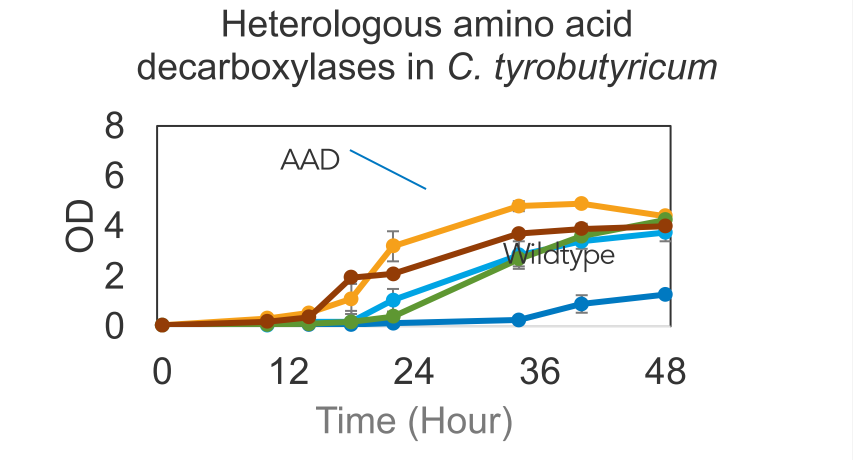
Acid Extraction Increases as pH Decreases



Heterologous Amino Acid Decarboxylases Raise Intracellular pH

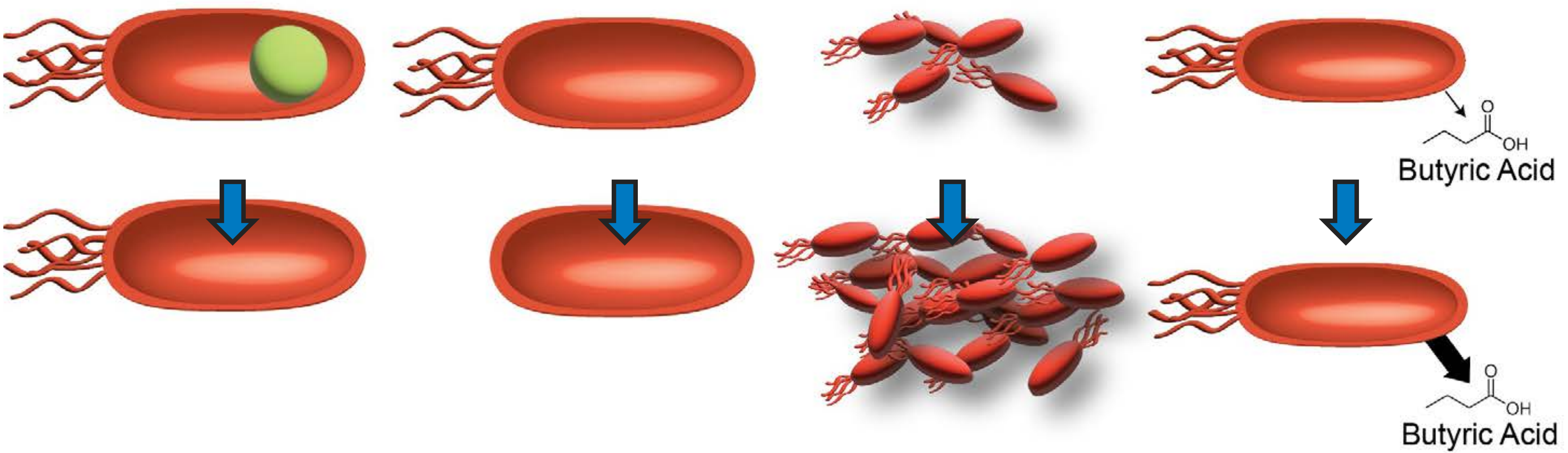


Strain Improvement by Engineering (pH 4.5)



Key Finding: Engineered strains have improved acidotolerance which can Improve overall process performance

Progress & Outcomes: Additional Engineered Strains Constructed Currently Being Evaluated Fermentatively



Sporulation Eliminated

Flagella Eliminated

Quorum Sensing Eliminated

Increased Butyrate Flux

(In Collaboration with Adam Guss)

Oak Ridge National Laboratory

Progress & Outcomes: Integrated Research to Improve Overall Process

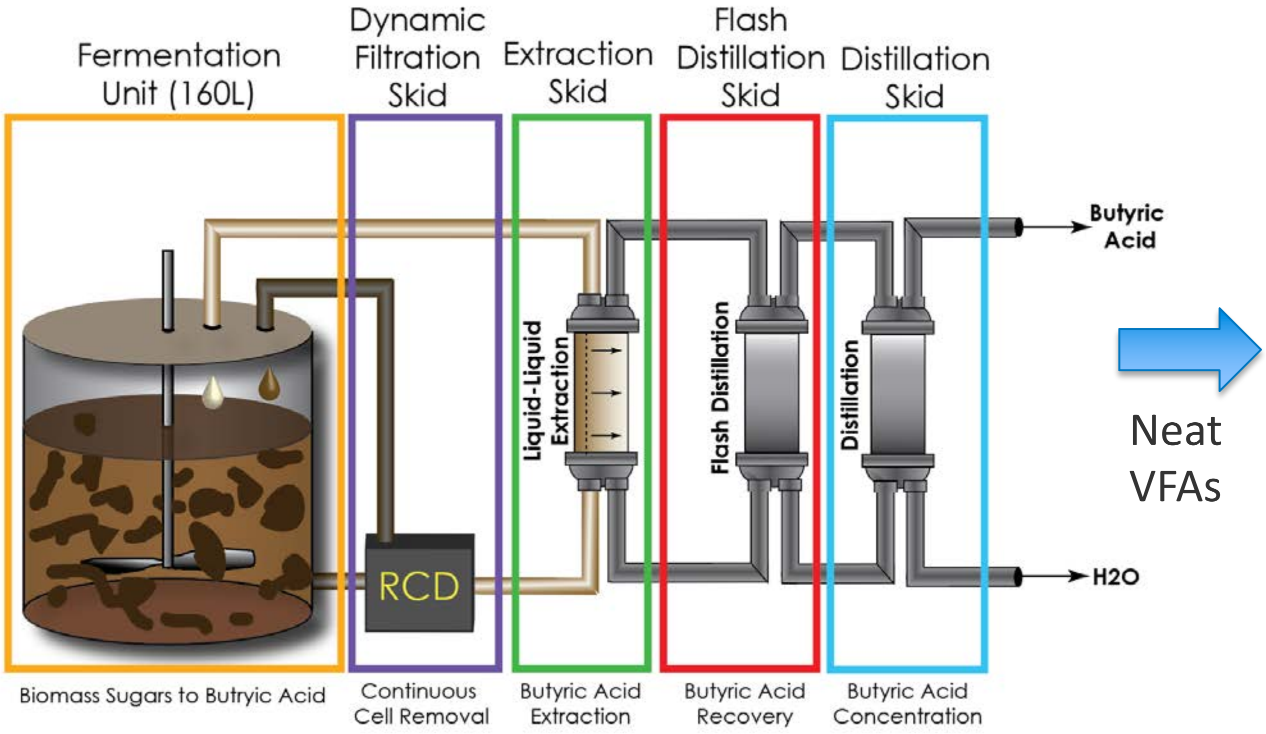
Proteomics

Fermentation

Strain Development

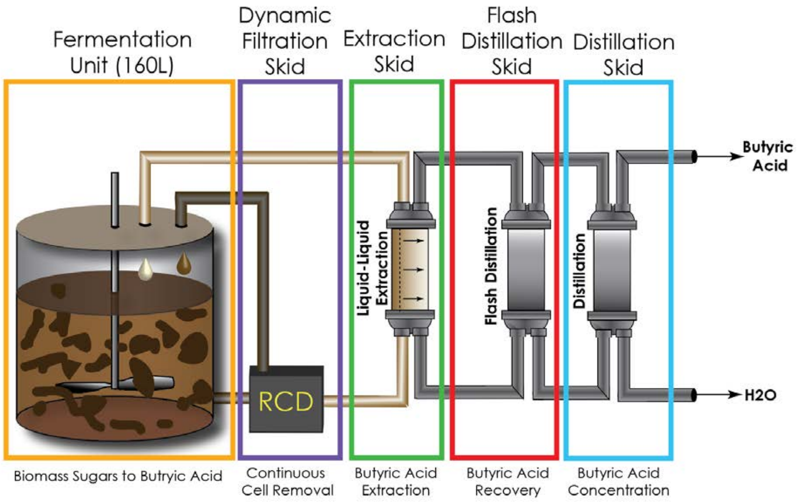
Pilot Process Development

Progress & Outcomes: Design and Construction of Pilot Plant Skids Enables Scale-Up



- ~5-10 kg of neat VFA's per run
- Automated control system
- Future catalysis skids are tentatively planned

Progress & Outcomes: Construction of the Pilot Reactor is Complete



160L Bioreactor

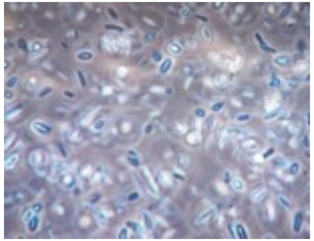
Filtration Skid

Extraction Skid

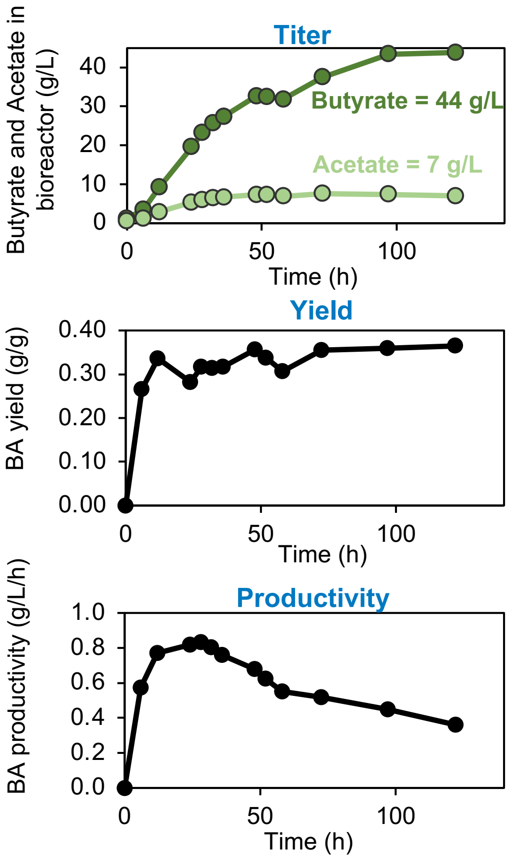
Flash Distillation

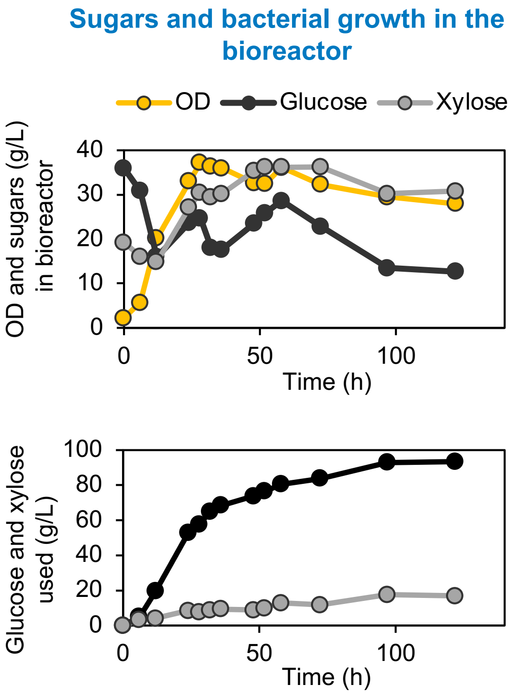
Distillation

C. tyrobutyricum Performs Similarly at 100L Scale and Bench Scale

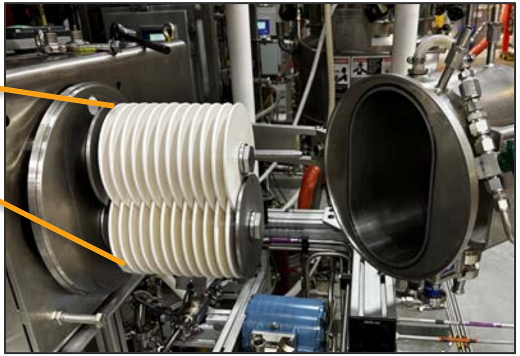


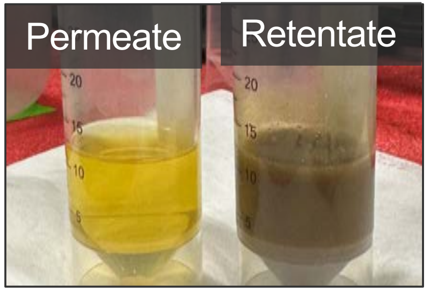
100 L Fed-Batch Fermentation (Non-Pertractive)





Progress & Outcomes: Cells and Solids Retained via Rotating Ceramic Disk (RCD) Filtration Unit





Filtered Hydolysate (DMR-EH)

Filtered Fermentation Broth (40L, <2h)

Cross-filtration RCD unit

- 24 membrane disks
- Total membrane area of 0.81 m^2
- Maximum disk rotating speed 750 rpm
- Membrane disk pore size 10 kDa - 0.5 μm
- Clean membrane permeability 118.6 $L/m^2 \cdot h \cdot bar$
- Maximum operation process 400 L/h feed solution

Progress & Outcomes: Pilot-Scale Membrane Contactor and Flash Distillation System


Liquid-Liquid Extraction (LLE)

Flash Distillation

Initial validation is underway for both the LLE and flash distillation systems

Impact:

- We have developed a fully integrated pilot-scale process for the production, separation and purification of volatile fatty acids or other carboxylic acids (*Salvachua, Saboe et al, 2021*) Cell Reports Physical Science

- While validated here with butyric acid from hydrolysate, this system will work with longer chain or mixed VFAs and from high-solids wet wastes (Collaborative effort).

- Proteomics and strain development efforts have led to novel strains with exceptional performance and novel insights into *C. tyrobutyricum* metabolism. Several manuscripts in preparation.

- Actively licensing intellectual property and are working with two entities commercializing technology developed on BUS.

3. Impact Since FY21

Commercialization:

- We have developed two collaborations with industrial entities for the Technology Commercialization.

Company X

Commercializing Bio-butyric acid derivatives for human health

- Intellectual property has been licensed to both entities.
- One Cooperative Research & Development Agreement (CRADA) has been executed and another is currently being negotiated.

Summary

- We have advanced technology of carboxylic acid production through fermentation engineering, strain development and establishing a first-in-class pilot scale reactor for the production of carboxylic acids coupled with in situ product recovery.
- Strains and technology developed on BUS is being commercialized by two independent companies: The first for chemical and eventually SAF production (initial pilot facility has 8M gallon capacity), and the second for human health and nutrition.
- The goals of this project have largely been met or will be met by the end of the project ending at the end of the fiscal year and strongly support BETOs mission of commercializing technologies that can push SAF to market.

Acknowledgements

Core Team

Davinia Salvachúa
Violeta Sànchez i nogué
Michael Guarnieri
Eric Karp
Patrick Saboe
Jacob Kruger
Christine Singer
Colin Kneucker
Lauren Riley
Yian Chen
Yat-Chen Chou
Wale Aromolaran
Anahita Bharadwaj
Michelle Reed

Key Collaborators

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Ryan Ferguson
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Stefan Haugen
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Ed Jennings
Justin Kastner
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Jacob Miller
Pam Motyl
Shelly Raemer
Kelsey Ramirez
Dan Schell
Ryan Spiller
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Frankie Tapia
Jason Thibodeaux
Sean Woodworth

ORNL

Adam Guss
Melissa Tumen-Velasquez
Robert Hettich
Paul Abraham
Richard Giannone
Dana Lynn Carper

Thank you !!

Quad Chart Overview

Timeline

- Active Project Duration: 10/1/2020 – 9/30/2023
- Total Project Duration: 10/1/2014 – 9/30/2023

	FY22 funding	Total Award
DOE Funding	$1,300,000	$950,000 – FY23 $3,200,000 – Active Project (FY21-23)

Project Partners

BETO Projects: Agile BioFoundry, Separations Consortium, and Biochemical Platform Analysis

Project Goal

Develop microbial lignocellulosic conversion processes to enable the cost-effective production of sustainable aviation fuels at bench and pilot scales

End of Project Milestone

Demonstrate the production of >5 kg of butyric acid at >98% purity from DMR-EH utilizing glucose and xylose enabling a modeled cost below $3/GGE.

Funding Mechanism

Bioenergy Technologies Office FY21 AOP Lab Call (DE-LC-000L079) – 2020

TRL at Project Start: 2
TRL at Project End: 7

Additional Slides

RCD effectively rejects insoluble contents and let through only liquid fraction

Demonstration production run

Produced 38 L filtered fermentation broth in under 2 hr

Enzymatic Hydrolysate (EH) filtration

Permeate Retentate

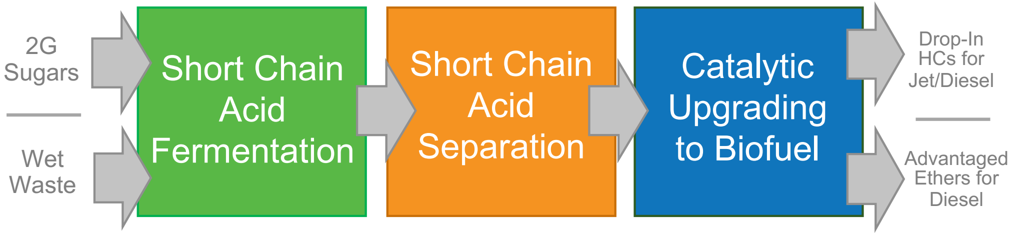
<u>EH feed:</u>
Total solid content: 6.66 wt%
Insoluble solid content: 1.77 wt%

<u>EH permeate:</u>
Insoluble solid content: 0.01 wt%

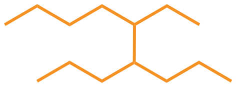
<u>EH retentate:</u>
Insoluble solid content: up to 11.3 wt%

<u>RCD performance:</u>
Insoluble solid rejection: 99.5-100%
Total water recovery: 75%

Approach: - Catalytic Upgrading of Carboxylic Acids (ChemCatBio-CUBI)



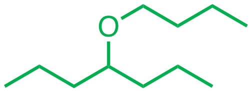
Drop-In C14 Isoparaffin for Diesel & Jet



5-Ethyl-4-Propylnonane

Property	Fossil Diesel	C14 HC
Energy	45 MJ/kg	44 MJ/kg
Freeze pt	- 10 °C	< - 80 °C
Flash pt	55 °C	74 °C
Cetane	47	48
Soot YSI	215	98

Advantaged C11 Ether Diesel Blendstock

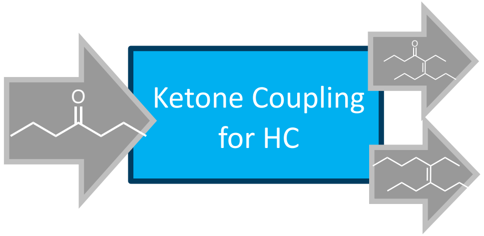


4-Butoxyheptane

Property	Fossil Diesel	C11 Ether
Energy	45 MJ/kg	39 MJ/kg
Freeze pt	- 10 °C	< - 80 °C
Flash pt	55 °C	74 °C
Cetane	47	80
Soot YSI	215	58

Neat Acid Ketonization

Ketone Elongation - HC

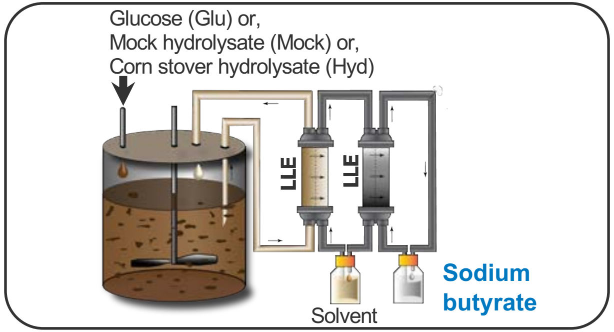


Ketone Elongation - Ether

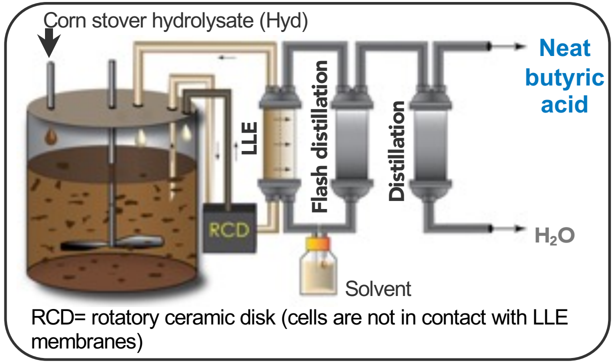
(Jacob Miller, ChemCatBio)

Progress and Outcomes: (Previous)

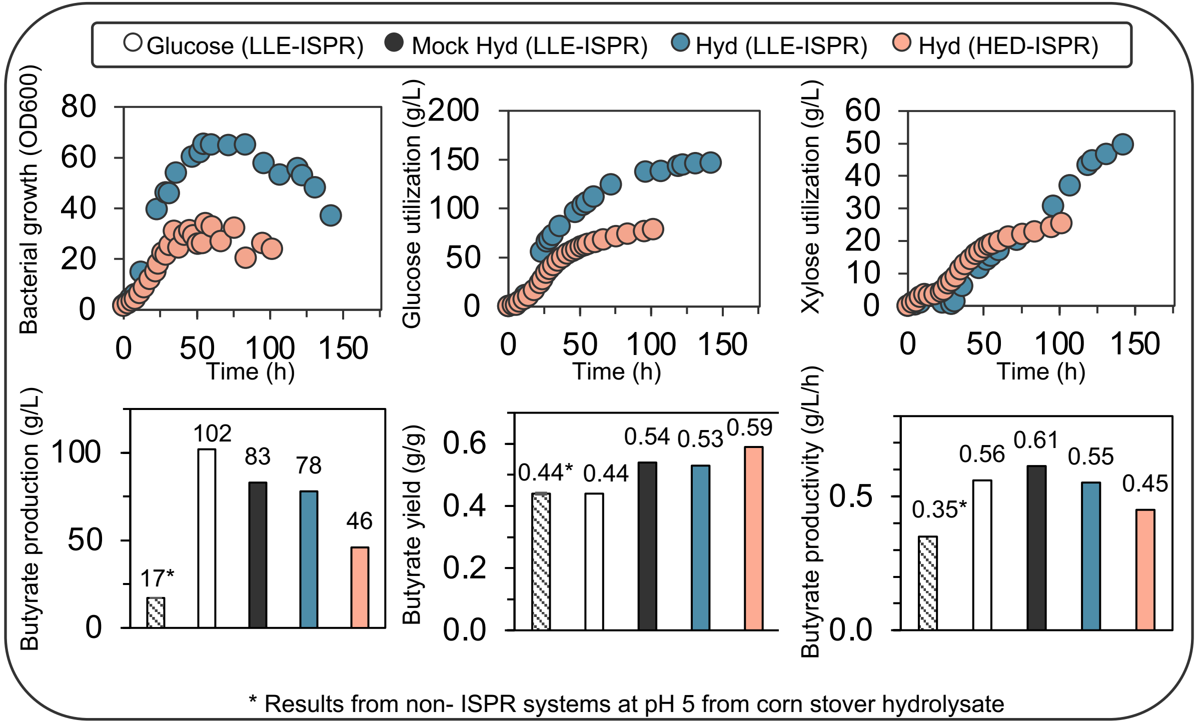
Liquid-liquid extraction (LLE)-ISPR



Hybrid extraction distillation (HED)-ISPR



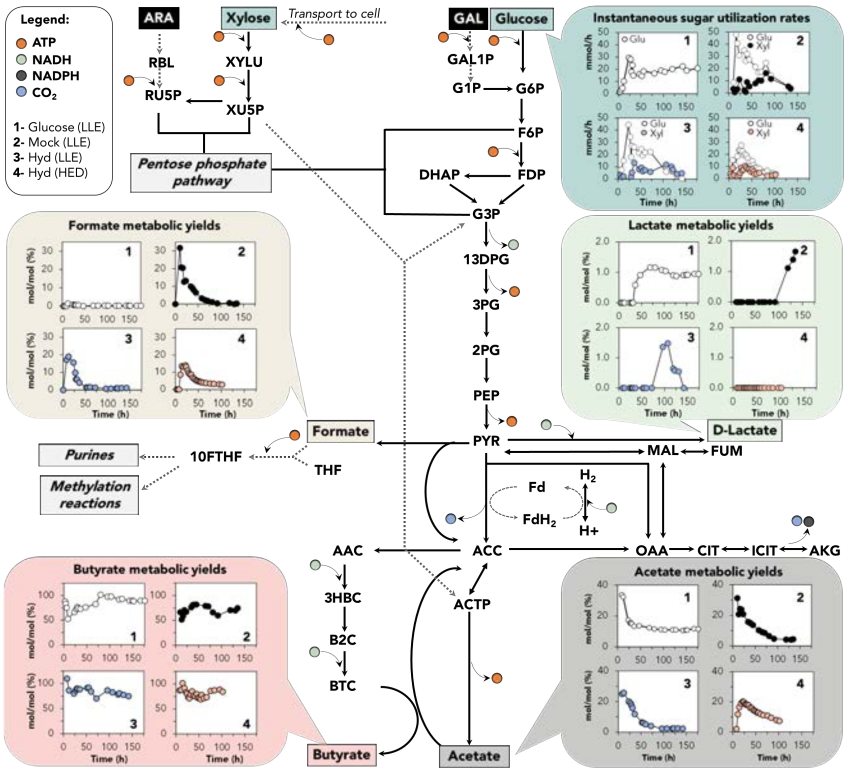
Bacterial performance on different substrates in LLE- and HED- ISPR systems



Salvachúa, D., Saboe, P, et al. 2021

Progress and Outcomes: Metabolic challenges that *C. tyrobutyricum* presents

Product and byproduct metabolic yields in ISPR systems at pH5 with various carbon sources



Salvachúa, Saboe et al (2021). Process intensification for the biological production of the fuel precursor butyric acid from biomass. *Cell Reports Physical Science*, 10, 100587.

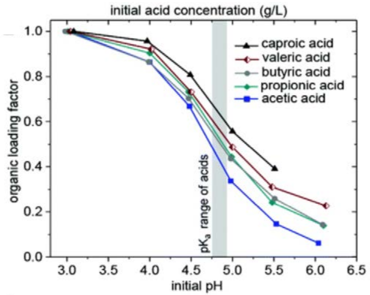
Xylose-containing media reduces the fermentation length

- Sugar utilization ceases earlier in ISPR systems (at pH5) in the presence of xylose compared to cultivations containing glucose as the sole carbon source.
- Flux to acetate decreases in the presence of xylose, which is necessary for the elongation to butyric acid and generation of ATP.

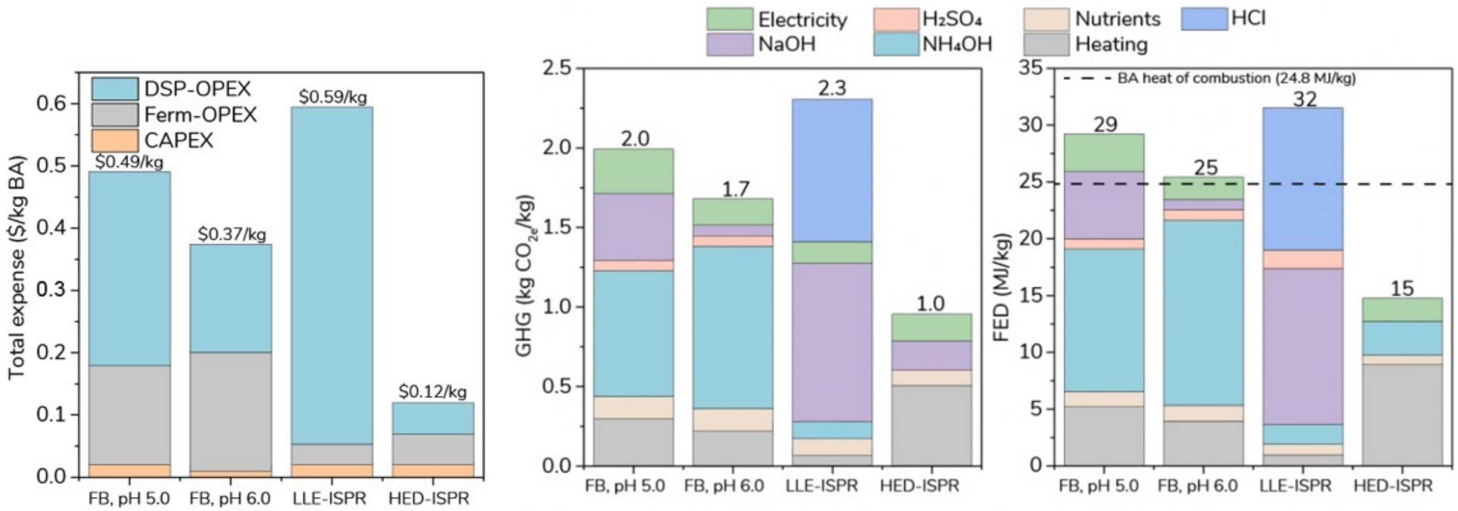
Butyrate productivity decreases at pH 5 compared to pH 6

- Butyrate productivity significantly decreases at pH 5 compared to pH 6 (the latter is optimal for *C. tyrobutyricum* growth) (Salvachúa, Saboe et al (2021). *Cell Reports Physical Science*). However, extraction in ISPR systems increases as pH decreases.

Extraction of carboxylic acids at different pHs



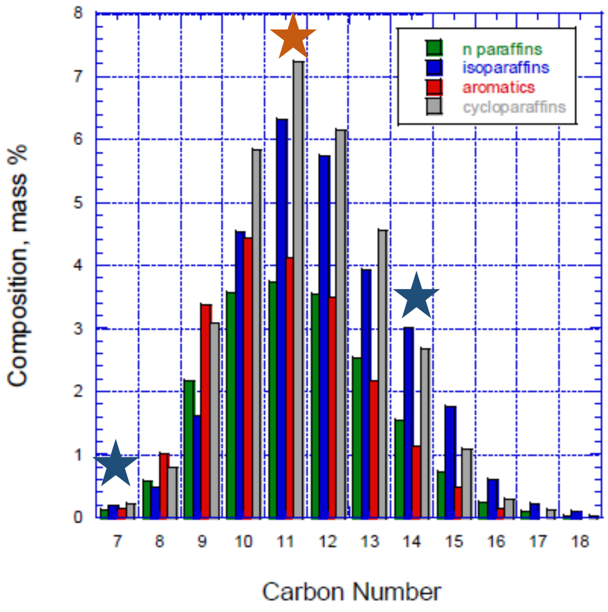
HED-ISPR Dramatically Improves Economics and Reduces Environmental Impact



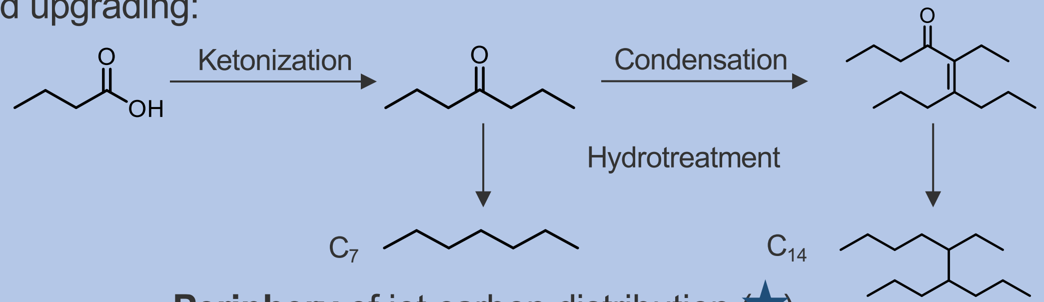
- The higher cost of LLE-ISPR results from the chemical consumption of sodium hydroxide in back extraction
- $0.12/kg represents 14% of the total expense.
- **Total MSP is $0.99/kg. Current biobased butyric acid market price is ~$1.80/kg.**
- Clear targets to reduce projected expenses further:

Future Directions: C_6 Acids are more readily upgradeable to SAF

Aviation fuel has **extremely specific** fuel property/composition requirements, most prominently **carbon distribution** centered around **C_{11}** alkanes.

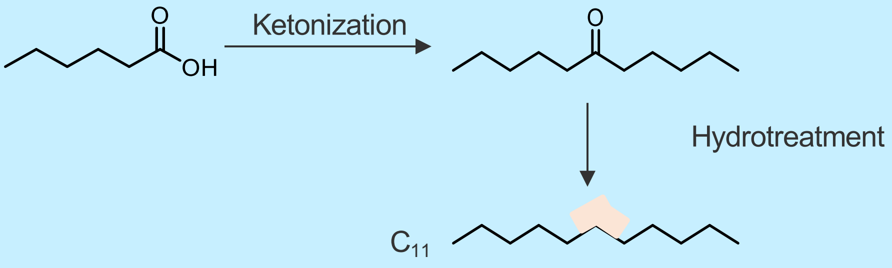


C_4 acid upgrading:



Periphery of jet carbon distribution (★)

C_6 acid upgrading:



Center of jet carbon distribution (★)

Holladay et al. "Sustainable Aviation Fuel: Review of Technical Pathways" DOE (2020)

Responses to Previous Reviewers' Comments

The reviewer is also somewhat skeptical about developing co-utilizing C5/C6 strains.

We apologize for the lack of clarity in our description of *C. tyrobutyricum* metabolism. *C. tyrobutyricum* is a native utilizer of xylose, and through our development of a high-xylose-containing seed culture, as well as a fed-batch operation, we can achieve efficient co-utilization of glucose and xylose. Enhanced glucose/xylose co-utilization in *C. tyrobutyricum* has also been demonstrated via genetic engineering approaches, and we have replicated similar approaches in our lab. The initial co-utilization of sugars in the batch phase is poor. To overcome this problem, we initiate the batch phase with diluted hydrolysates. However, this dilution would add an additional cost to the process. Thus, if we improve the co- utilization, fermentations could be initiated at higher initial sugar concentrations.

Responses to Previous Reviewers' Comments

Lastly, one of the potential benefits of in situ product recovery in bioreactors producing organic acids is that pH control will require the use of lower amounts of base to keep the pH in the desired range. This was not highlighted in the techno-economic analysis. Is it not a significant factor on cost, or was it just not included in the analysis?

We apologize that this was not sufficiently explained. You are correct, this represents a drastic cost savings provided by the in situ product recovery system, and we fully take this into account. In fact, during the fermentations, the pertraction of butyric acid is able to auto-control the fermenter pH so that no expensive base addition is required.

Responses to Previous Reviewers' Comments

Q7: The butyric acid market is reasonably large (slide 10), along with its utility as a precursor to specialty chemicals and materials. These would seem to be better biorefinery targets, to be added to the portfolio of developing lignin coproducts. Thus, it is not clear why butyric is ultimately being down- valued to fuel components or precursors, and how the economics hang together.

This is a fantastic and central point to our project philosophy and warrants further discussion. The primary objective of this project is to identify economically and environmentally feasible routes towards making biofuels. However, it should be noted that while we are modeling the production of a biofuel from butyric acid, we fully support using it as an intermediate for alternative bio-derived chemicals and materials. The entire research portfolio of this project is focused on the production of butyric acid as an intermediate, and we are end-product-agnostic. In fact, we currently have industrial collaborations for numerous offtake directions for butyric acid including specialty chemicals, materials, fuels and as an intermediate towards human health and nutrition.

Publications, Patents, Presentations, Awards, and Commercialization

Publications & Presentations:

- Salvachua, D., Saboe, P. O., Nelson, R. S., Singer, C., McNamara, I., del Cerro, C., ... & Linger, J. G. (2021). Process intensification for the biological production of the fuel precursor butyric acid from biomass. *Cell Reports Physical Science*, *2*(10), 100587.
- Process Integration for the Production of Sustainable Aviation Fuel Precursors, Frontiers in Biorefining. October, 2022
- Five Manuscripts currently in preparation *(Novel genetic tools in C. tyrobutyricum, mechanisms of acid tolerance in C. tyrobutyricum, proteomic analyses as a function of pH and carbon source, mechanisms of acid tolerance in non-model yeast.)*

ROIs: Four Records of Inventions surrounding strain development efforts

Patents: Three provisional patent applications submitted or soon to be submitted

Commercialization: We have licensed several patents to two separate companies: Crysalis and one which wishes to remain anonymous. We are actively working on one funds-in CRADA ($200k) and have established the framework for the second CRADA ($200k) and are developing the work scope. Crysalis is currently retrofitting an 8M gallon ethanol facility for the production of butyric acid among other chemicals.