> Dr. Shivani Garg, PM Synthetic Biology
LanzaTech
DOE BETO Peer Review, April 2023

DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

Engineered reversal of the β-oxidation cycle in clostridia for the synthesis of fuels and chemicals

April 7, 2023
Biochemical Conversion & Lignin Valorization

Michael C. Jewett, Northwestern University (PI)
Michael Köpke, LanzaTech (Co-PI)
Shivani Garg, LanzaTech (Project Manager, Presenter)

This presentation does not contain any proprietary, confidential, or otherwise restricted information

Project Overview

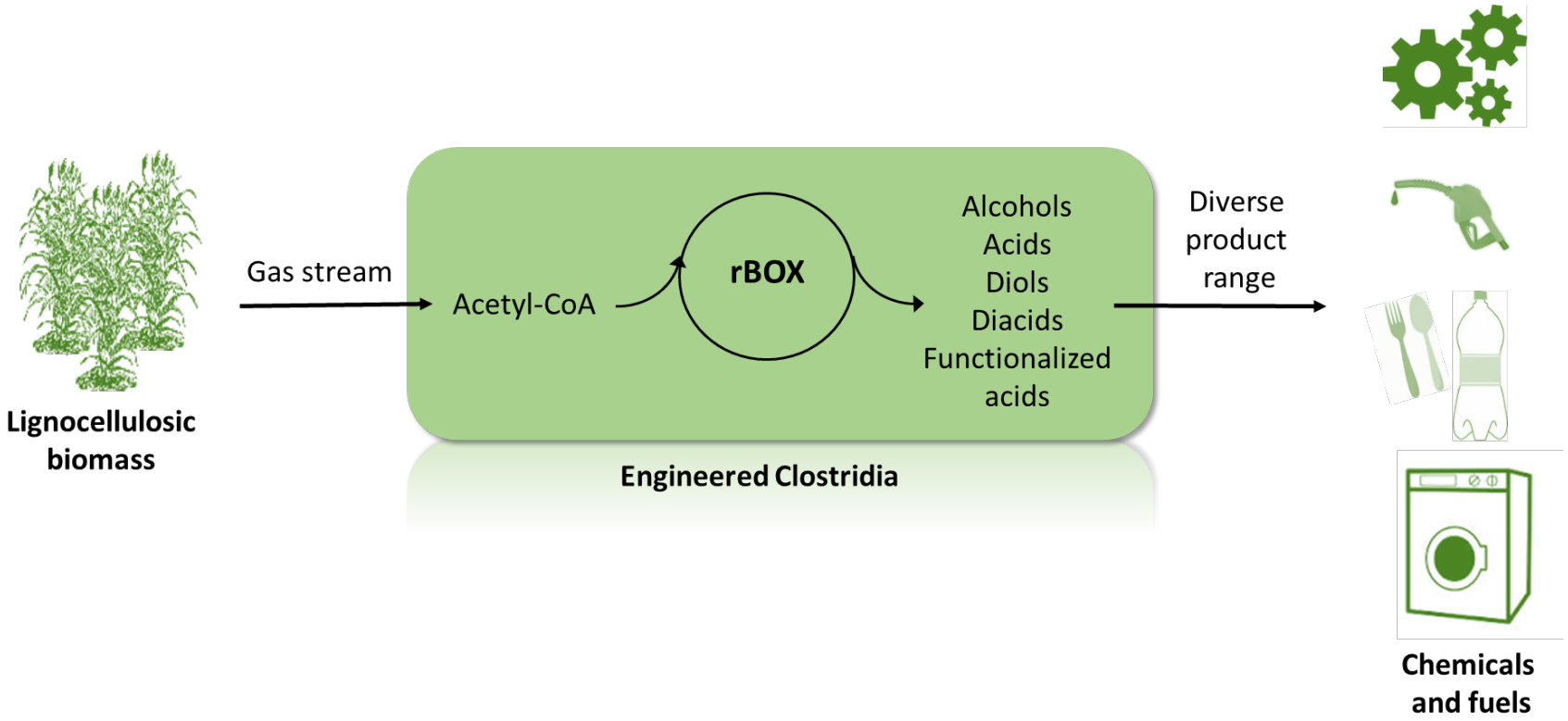
Biology is Uniquely Suited For Today's Challenges

=

Not so different...

Life on Earth originated in a reduced environment consisting mostly of carbon oxide gases

Engineered Reversal of β-oxidation in Clostridia to Synthesize Fuels and Chemicals



- **DOE funded collaboration between:**

Northwestern CENTER FOR SYNTHETIC BIOLOGY

USF

LanzaTech

Georgia Institute of Technology

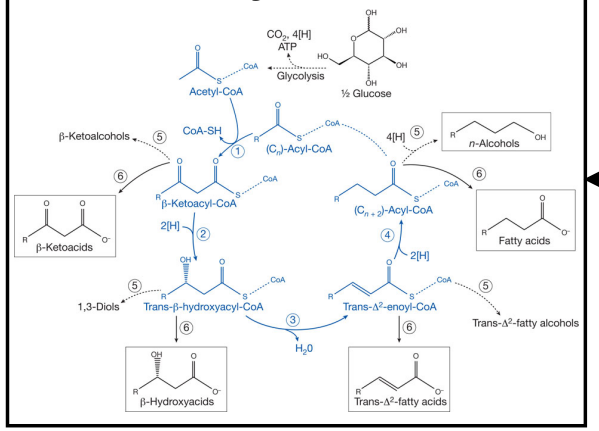
- **End goal:**
  - Produce one product at pilot scale
- **Timeline:**
  - Oct 2018 – Aug 2023

Project Objective: Develop Clostridia To Ferment Syngas From Cellulosic Biomass By Established Gasification Technologies, Into A Range Of Advanced Bioproducts

Pathway Design

University of South Florida LanzaTech

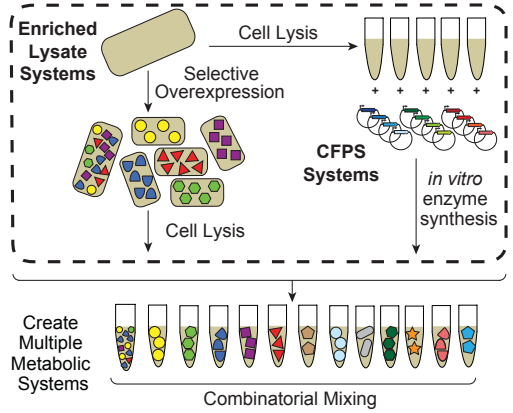
r-BOX Biosynthesis



Pathway Prospecting

Northwestern University | LanzaTech

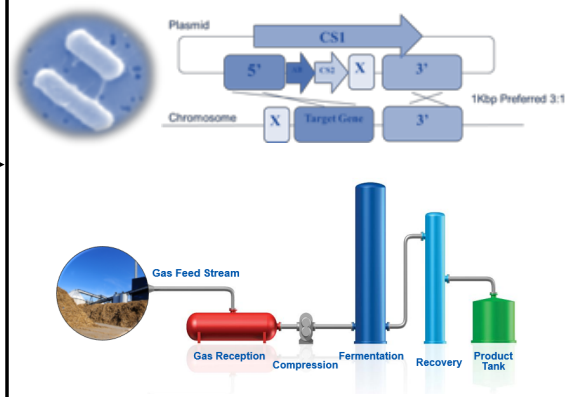
Cell-Free Framework



Pathway Validation

University of South Florida | Northwestern University | LanzaTech

clostridia Biofoundry



Rural Economic Development & Sustainability Analysis

Georgia Tech LanzaTech

We target products used as **drop-in fuels, fuel additives, and chemical building blocks with a $14Bn US market**.

An Interdisciplinary Team Across Academia and Industry is Pursuing our Objectives

Northwestern:
Michael Jewett
Bioengineering

LanzaTech:
Michael Koepke
Industrial Biotech

Univ. South Florida:
Ramon Gonzalez
Chemical Engineering

LanzaTech:
Robert Conrado
Technoeconomic Analysis

GaTech:
Valerie Thomas
Technology Assessment

LanzaTech:
Shivani Garg
Project Manager

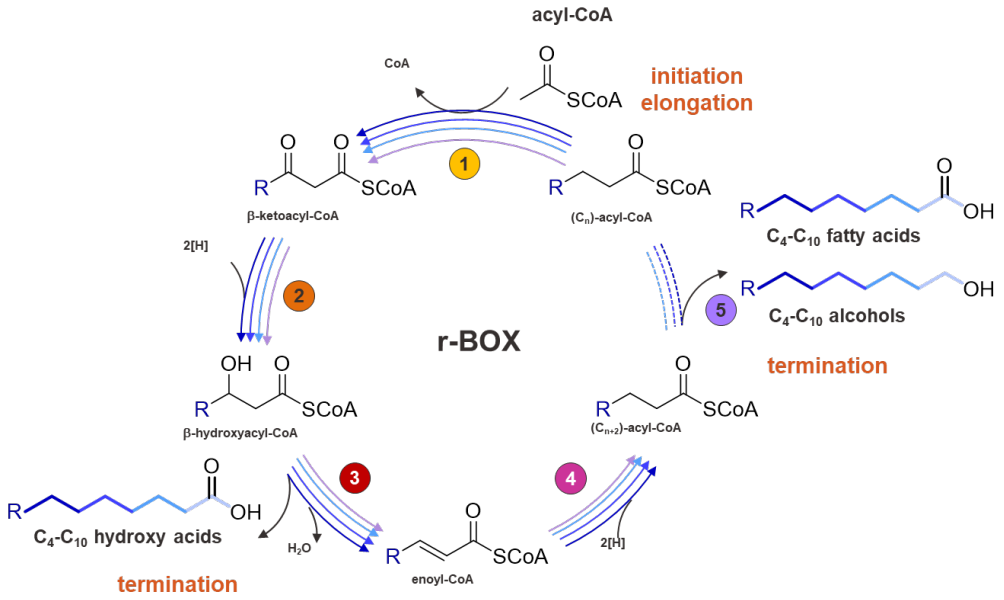
1 – Technical Approach

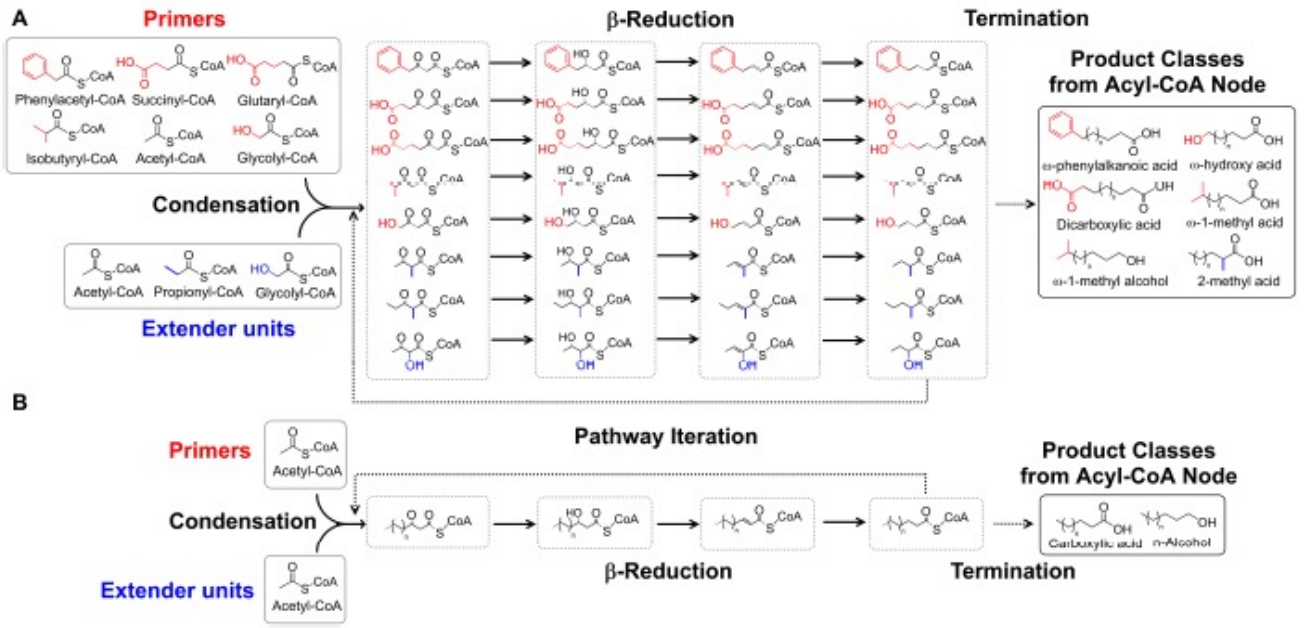
To Achieve our Vision, the Following Approach has been Taken:

- ***Aim 1.*** *Develop and apply informatics and* ***computer-aided design tools*** *to choose molecules, enzymes, and pathways for reverse β-oxidation cycle (r-BOX) in clostridia.*
- ***Aim 2.*** *Establish a* ***cell-free framework*** *for rapid pathway prototyping and analysis*
- ***Aim 3.*** *Develop optimized production strains of* ***gas-fermenting clostridia****.*
- ***Aim 4.*** ***Technoeconomic and rural economic development*** *and sustainability analysis.*

Embedded in these aims, are several key innovations that allowed us to combine *in vitro* (cell-free) and *in vivo* work to interweave and advance state-of-the-art pathway design, prospecting, validation, and production in an integrated framework

Reversal of the β-oxidation cycle (r-BOX) Offers Unique Access to Thousands of Molecules



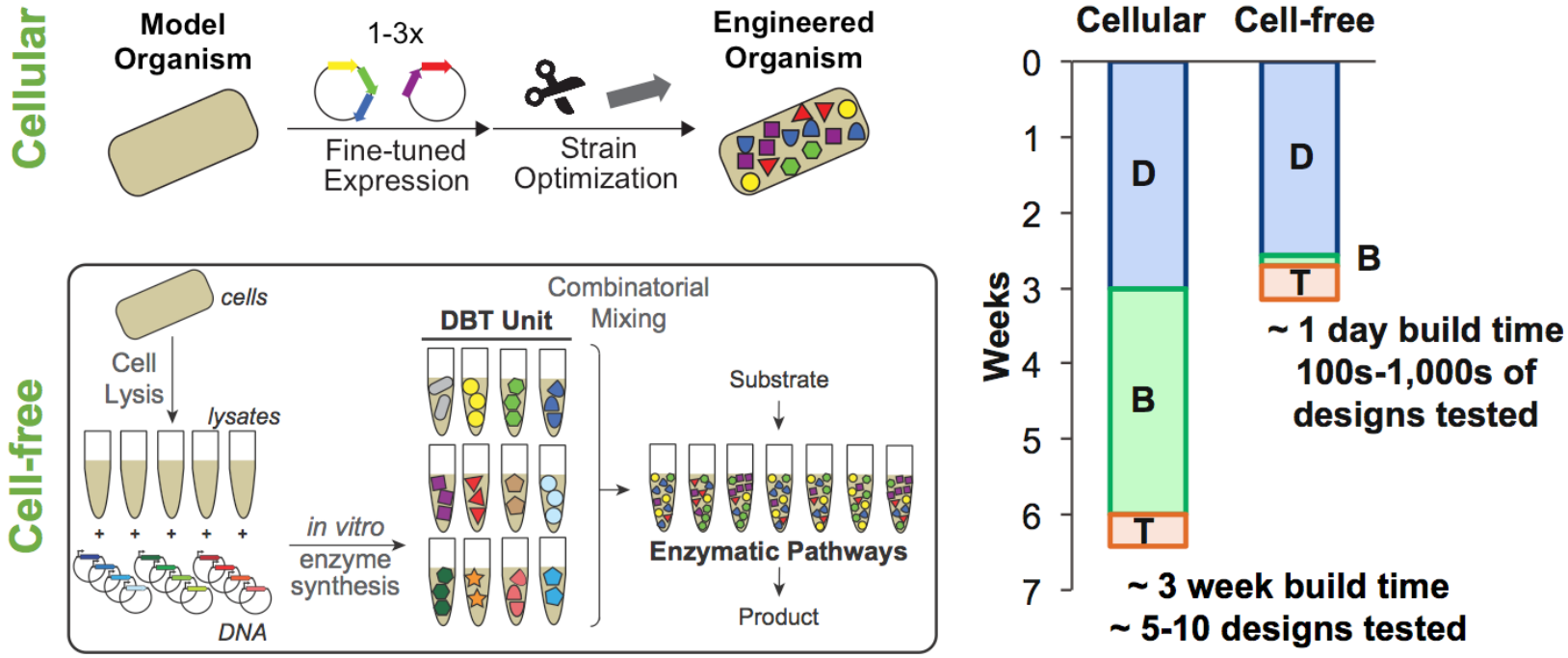


Cheong S, Clomburg JM, Gonzalez R, *Nature Biotech* (2016) DOI: 10.1038/nbt.3505

- Most bioengineering approaches to date rely on linear pathways specifically designed for a single molecule.
- The cyclic and iterative r-BOX pathway with **highly modular architecture and combinatorial nature** offer the unique advantage of providing **access to thousands of molecules** with different chemistries and chain lengths.

Cell-free Framework Allows for Rapid Pathway Prototyping

- Cell-free systems enable **acceleration of new enzyme and pathway assessments**, bypassing transformation idiosyncrasies and low-throughput workflows impeding progress on many non-model microorganisms

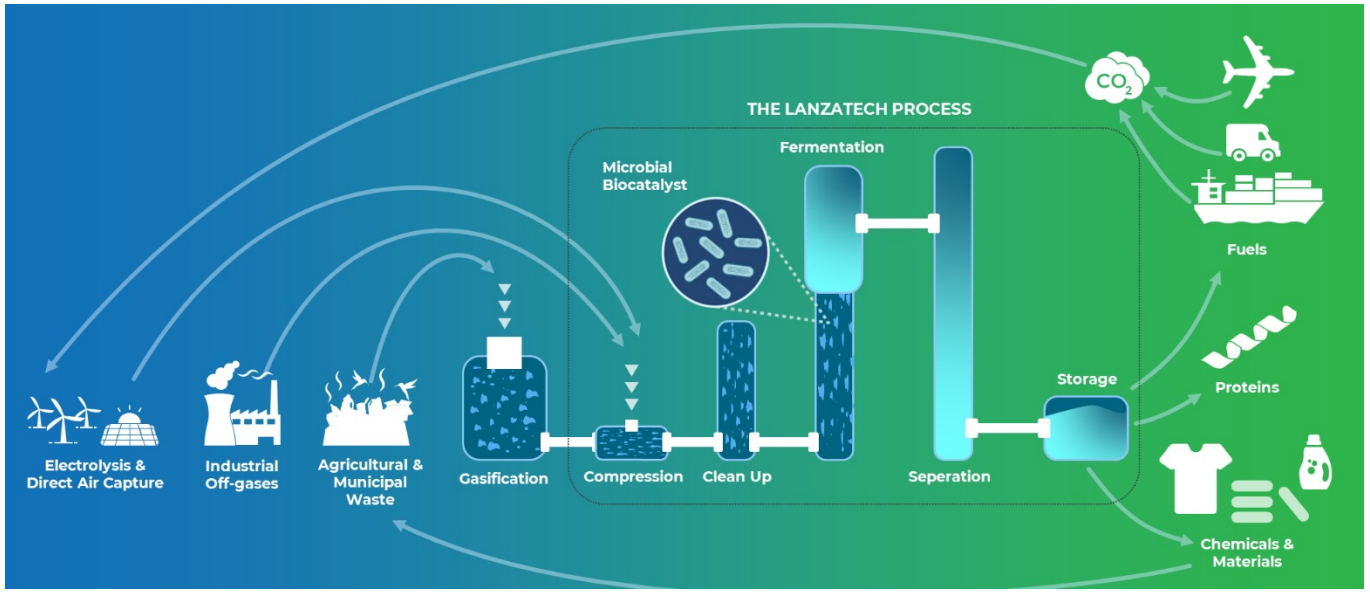


Clostridia Gas Fermentation Allows High Yield Conversion of Lignocellulosic Feedstocks

- Integrated gasification-fermentation has been demonstrated:
  - in extended continuous operations
  - with multiple types of lignocellulosic material.

- Syngas fermentation uses the same fermentation process implemented in LanzaTech's first **commercial scale gas fermentation** facility (started 5/3/2018).

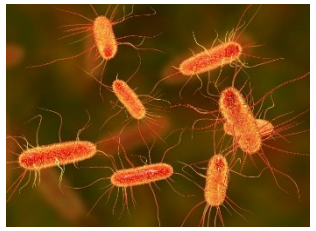
- LanzaTech has demonstrated successful scale up of gas fermentation technology at multiple plants across the globe.



Correlation Between Three Platforms Allows for Detailed Understanding of the Pathway

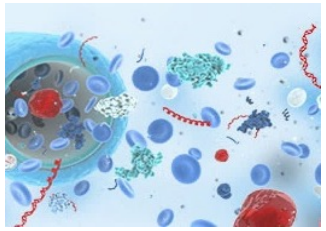
Three Platforms

E. coli
Established rBOX



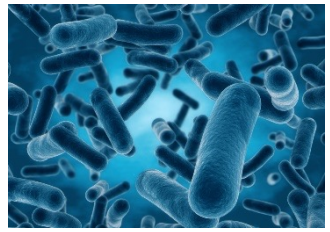
- Modelling predictions
- Generate & validate chassis optimizations

Cell-Free
Rapid Prototyping



- Rapid prototyping of rBOX pathway

Clostridium
Industrial Production

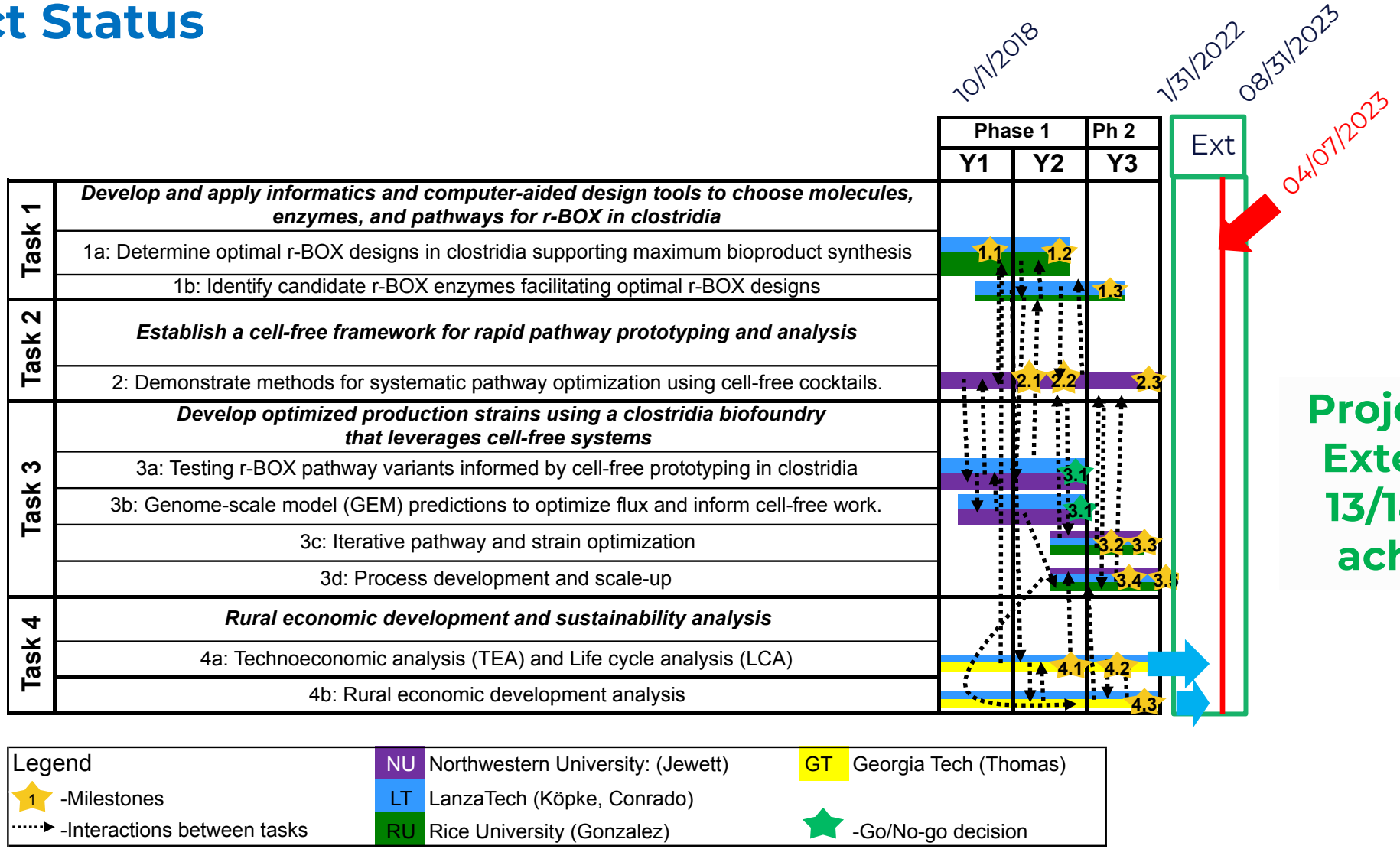


- Production system, combining optimal pathways and chassis designs

Establish correlation between platforms

2 – Progress and outcomes

Project Status



Project in No Cost Extension Phase 13/14 milestones achieved so far

Progress Summary – Task 1

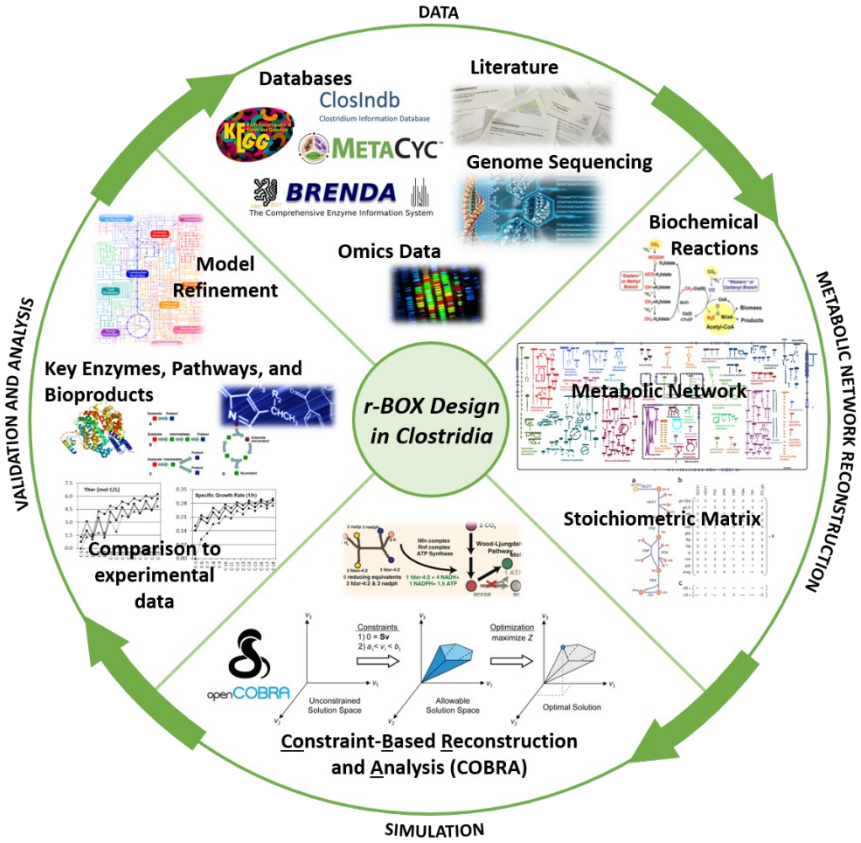
- **Milestone 1.1**. Identify and compare r-BOX enzyme variants by data-mining complete *Clostridium* collection and public databases: **>100 additional unique r-BOX and Ptb-Buk putative gene variants over the public domain identified** (Y1/Q4)

  Completed (>200 gene variants synthesized).

- **Milestone 1.2**. Quantify theoretical product yields and **generate optimal strain designs** with ***> 100,000 simulations*** carried out per design (Y2/Q2)

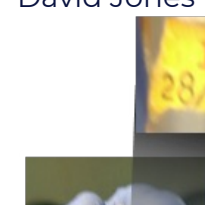
  Completed (261,000 simulations per design).

- **Milestone 1.3**. **Optimize computational framework** for generating novel pathways based on feedback from other Tasks and refine pathway design (Y3/Q2) **Completed.**



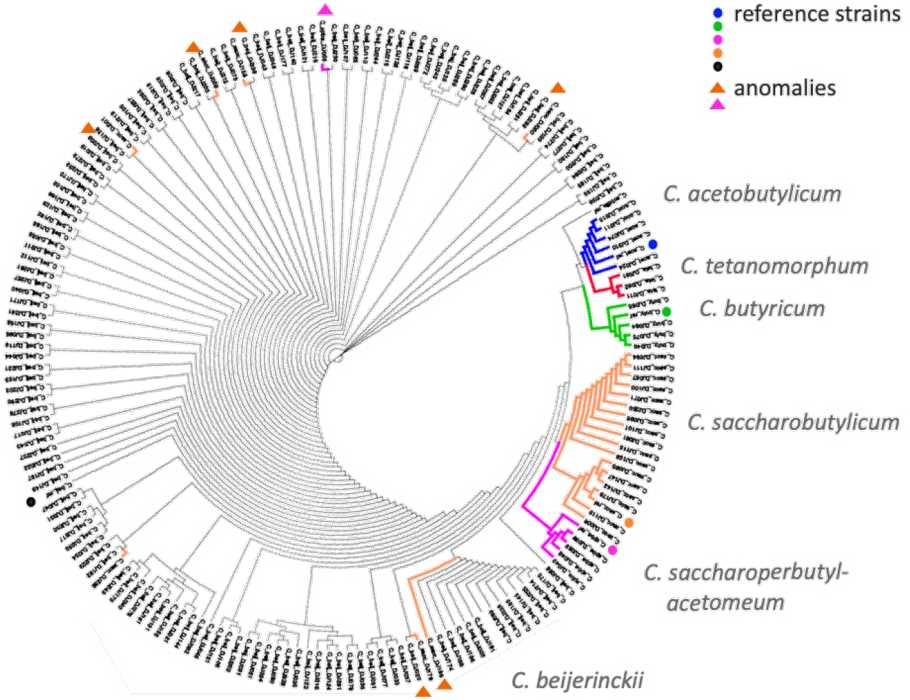
Key Achievements from Task 1

Chaim Weizmann



David Jones

Mining of an historical industrial strain collection



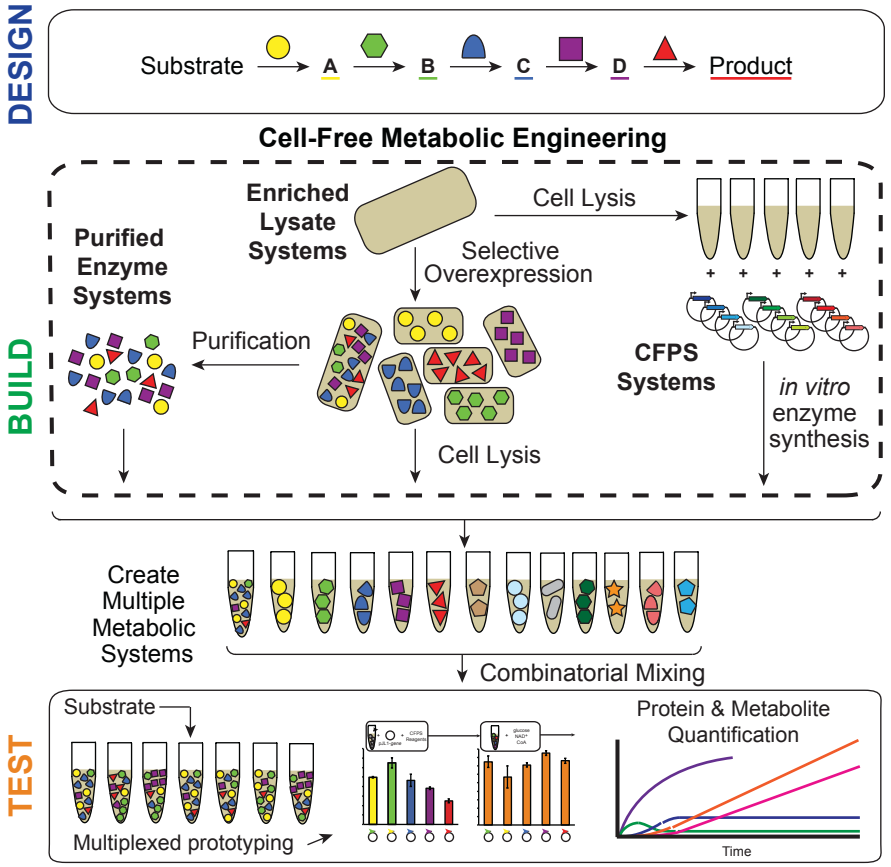
>200 genes mined from David Jones collection

Progress Summary – Task 2

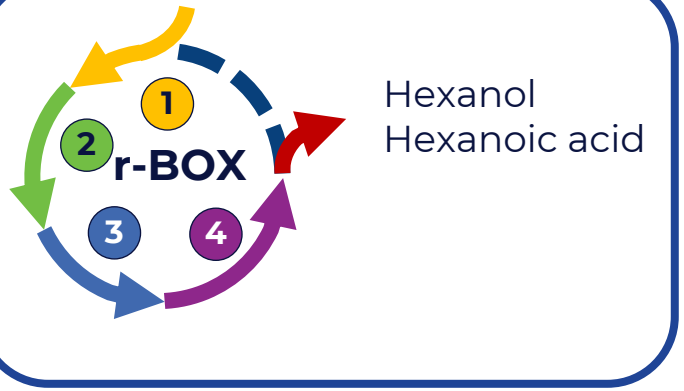
- **Milestone 2.1**. Develop, implement, and demonstrate methods for the **cell-free mix-and-match approach to optimize biosynthetic pathways** that are ***2x faster than the state of the art*** *in vivo* approach (Y2/Q2). **Completed (>10x faster)**

- **Milestone 2.2.** Demonstrate **expression of pathway enzymes** for at least one r-BOX pathway at levels of ***greater than 50 mg/mL*** using the cell-free framework (Y2/Q3). **Completed (>80 enzymes at > 50mg/mL)**

- **Milestone 2.3.** Study and **optimize pathways using our cell-free framework**, and refine and optimize pathways with ***at least 2-fold improvement*** (Y3/Q2). **Completed (>100x improvement of 1-hexanol and hexanoic acid)**



Hexanol Pathway Optimization Guided by Cell-free Systems

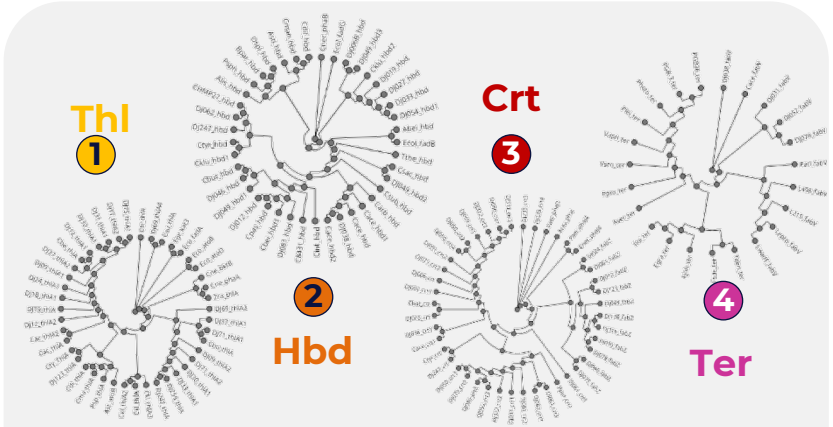


Hexanol
Hexanoic acid

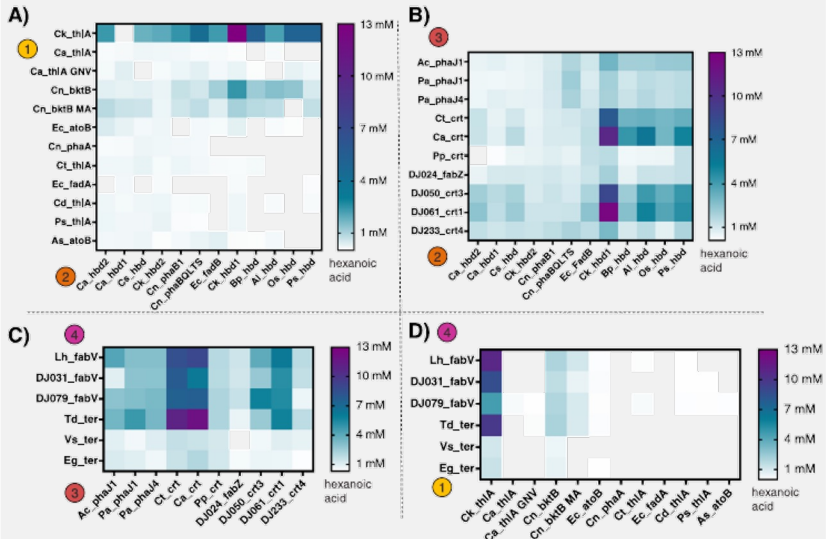
Cell-free prototyping enables implementation of optimized reverse β-oxidation pathways in heterotrophic and autotrophic bacteria

Vogeli et al, **Nature Communications**, 2022

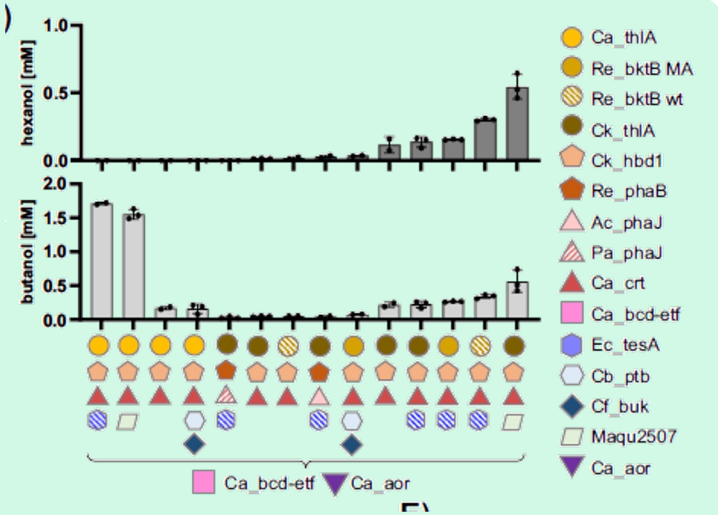
https://doi.org/10.1038/s41467-022-30571-6



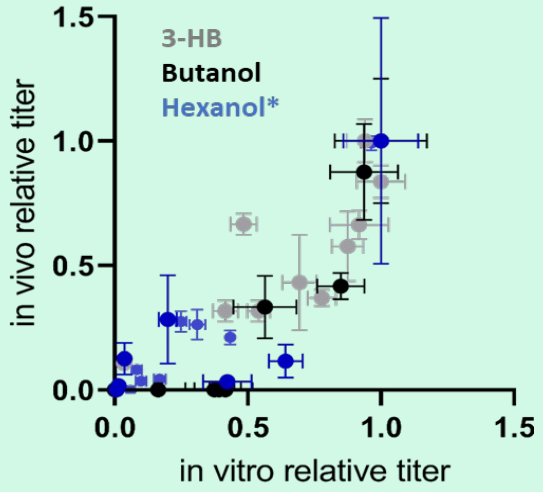
Mined >300 gene variants from DJ collection and public databases



Systematically evaluated each enzyme variant against others (>400 combinations tested)



Transferred the top pathways into *C. autoethanogenum*



Good correlation between in vivo and in vitro systems

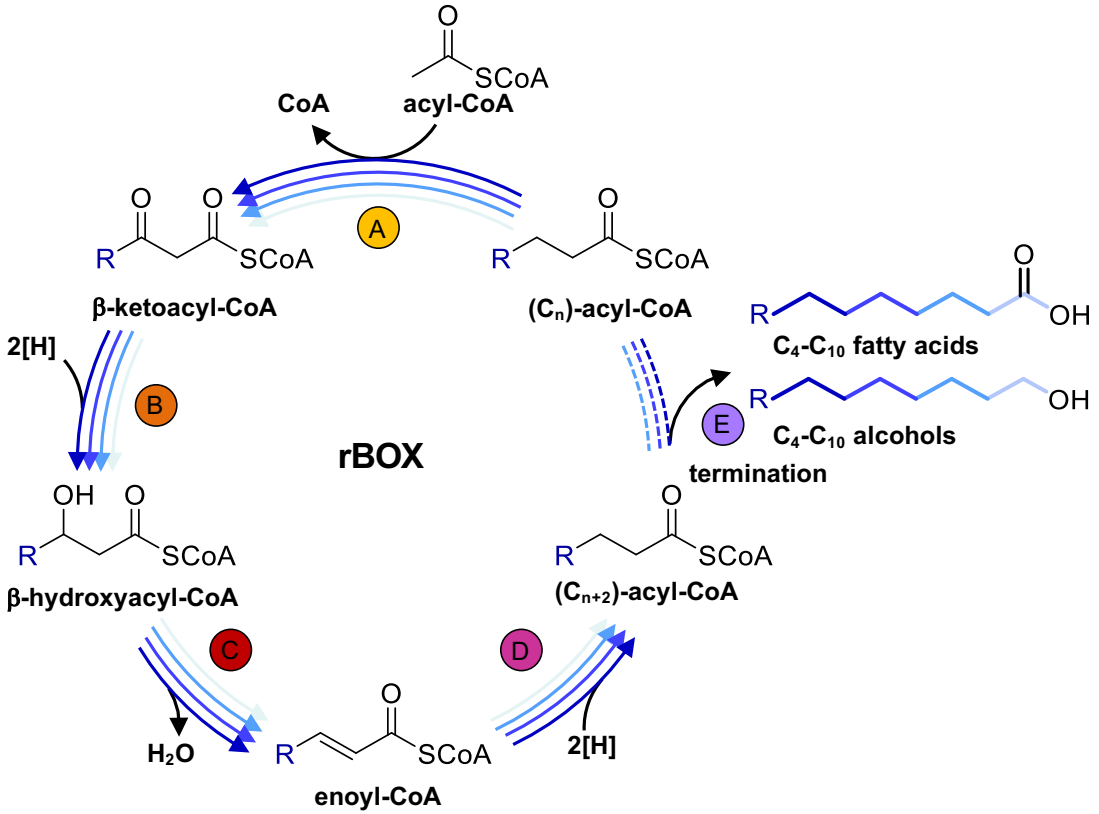
Progress Summary – Task 3

- **Milestone 3.1. (go/no-go decision):** Construct and **evaluate 50 unique pathway designs** for target molecules *in vivo* and *in vitro*. (Y2/Q4) **Completed**
- **Milestone 3.2.** Proof of concept for **additional r-BOX target products** on syngas. (Y3/Q2) **Completed**
- **Milestone 3.3.** Construct and evaluate an **additional 150 unique pathway designs** for target molecules *in vivo* and *in vitro*. (Y3/Q3) **Completed**
- **Milestone 3.4.** Comparison of best performing engineered strain for on **synthetic syngas against real biomass syngas** in 1.5L lab scale reactor and demonstration of a target ***metric***. (Y3/Q3) **Completed**
- **Milestone 3.5.** One selected r-BOX product at a target ***metric*** in **>80L scalable pilot reactor**. (Y3/Q4) **Progress ongoing**

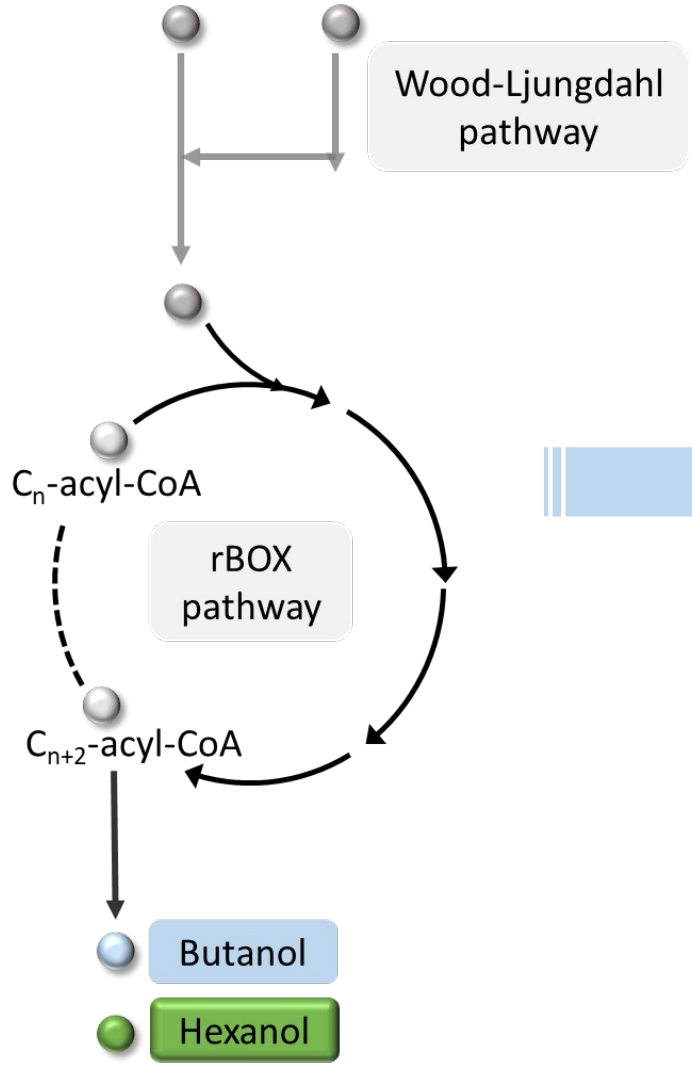
Proof of Concept of Multiple Products

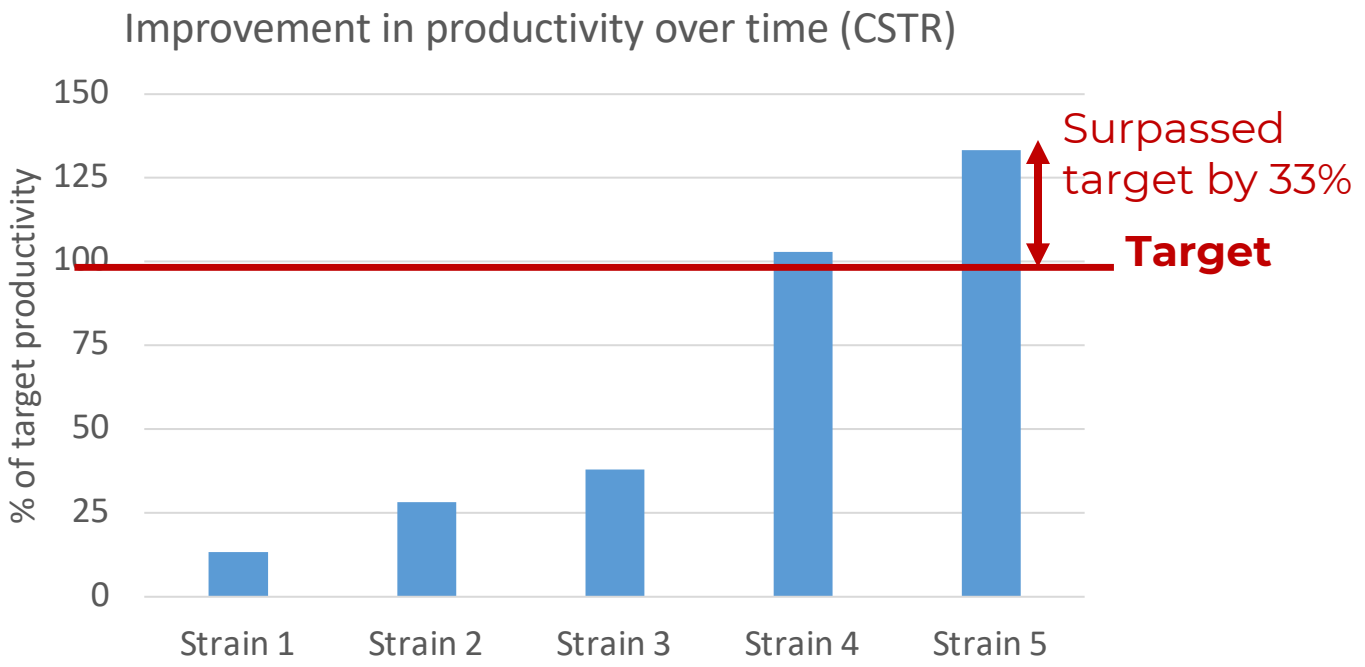
Demonstrated synthesis of multiple products across 3 platforms (cell-free, *E. coli*, *Clostridium*) through rBOX

- 3-Hydroxybutyrate
- Butanol
- Butanoate
- Hexanol
- Hexanoate
- Octanol
- Octanoate
- Decanol
- Decanoate
- Dodecanol



Surpassed Target Productivity In Lab Scale Reactors





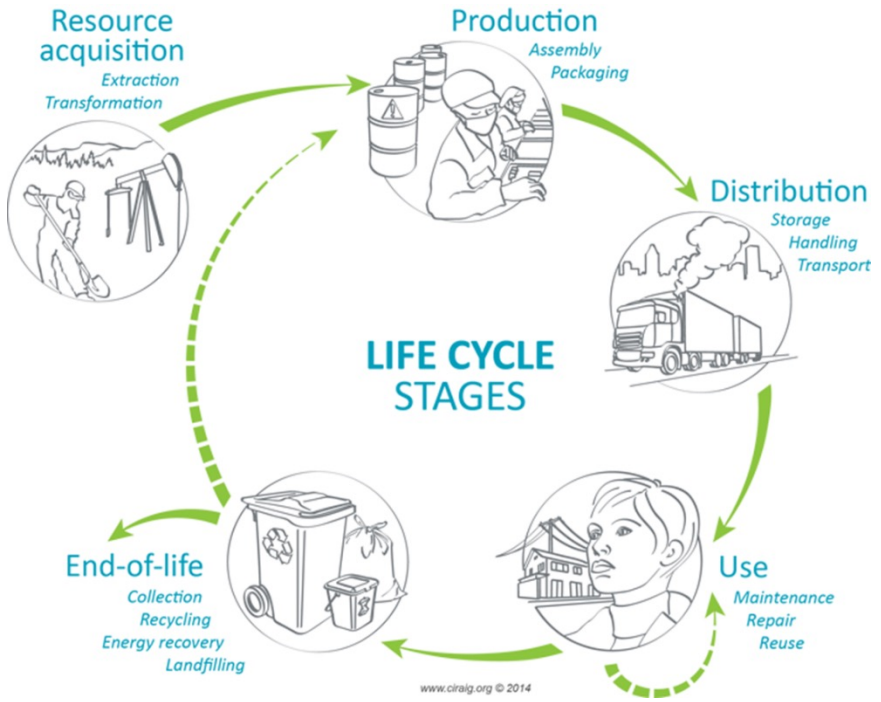
Surpassed target by 33% in lab scale reactors.
Ready for pilot scale.

Progress Summary – Task 4

- **Milestone 4.1**. Complete **2 workshops to inform environmental community and rural economic development analysis**. All stakeholders will be invited to both workshops; aims are to gather input from multiple parties on potential economic, community, and environmental impacts. (Y2/Q4) **Completed**

- **Milestone 4.2**. LCA for two r-BOX molecules. (Y3/Q2) **Completed**

- **Milestone 4.3**. Completed assessment of infrastructure and supply chains for biomass feedstock supply of two r-BOX molecules in the US southeast. (Y3/Q4) **Completed**

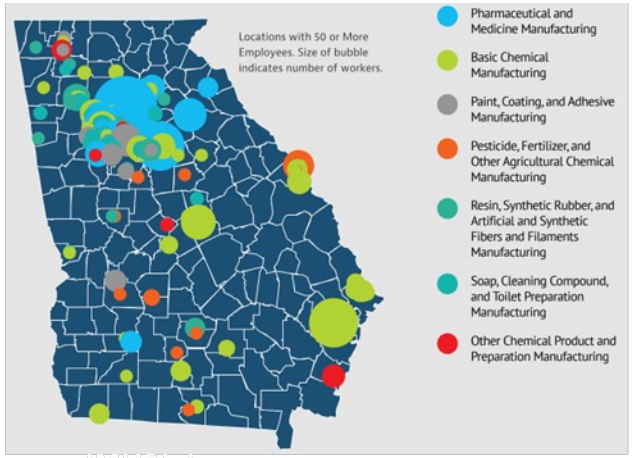


LCA and Supply Chain

	GHG (kgCO2e/kg)	Energy (MJ/kg)	Water (liters/kg)	Particulate Formation (kg PM10e/kg)	Photochemical Oxidant Formation (kg NMVOC/kg)
rBox Hexanol	0.54	53	12	3.6E-03	1.3E-02
rBox Decanol	0.69	55	12	3.6E-03	1.3E-02
Fossil Hexanol	5.56	25	2710	3.7E-03	1.3E-02
Fossil Decanol	5.75	26	2710	3.7E-03	1.3E-02

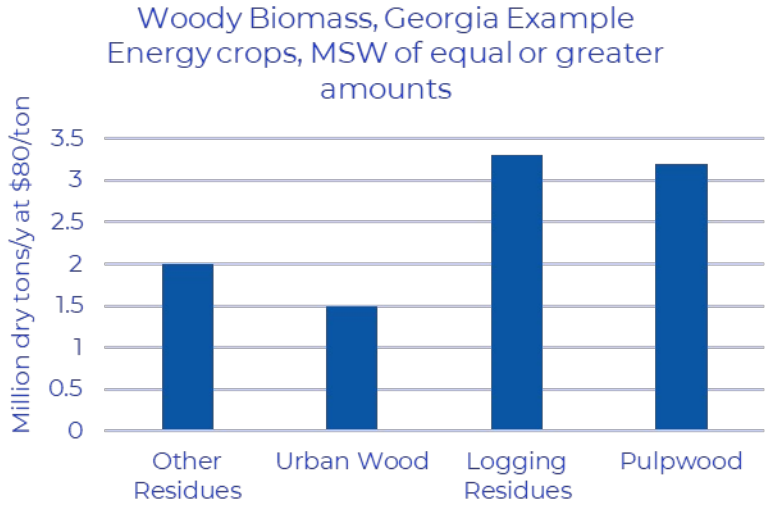
Production of rBox molecules tend to have lower GHG emissions, more energy consumed (largely biomass), less water consumption, and similar criteria air pollutant emissions such as particulates and VOCs.

Regional biomass sufficient for production at the scale of, e.g., US nylon (0.6 million tons/y). Nationally, it is possible that perhaps 1/3 of the ~ 100 M ton/y US chemical industry could be sourced from biomass.



Substantial southeast chemical manufacturing facilities (Georgia example)

Multiple opportunities for entry into chemical manufacturing supply chain; low-carbon, biobased, sustainability pull



3 – Impact

Impact of the Project

- Enabled high-level, sustainable synthesis of next-generation biofuels and bioproducts by **developing clostridia to ferment synthesis gas**
  - **Advanced syngas fermentation**, a cost-effective technology for the use of cellulosic biomass that is broadly applicable in the production of biofuels and biobased products (Ct-H)
  - **Expanded the diversity of products** that can be produced and co-produced via syngas fermentation (Ct-H)
  - **Gained new knowledge of metabolism** in obligate anaerobes and traits related to "industrial fitness" of clostridia (Ct-H, Ct-D)

- Created a cell-free framework to **decrease development time for industrially relevant microorganisms**
  - **Advanced bioprocess development** by reducing the time to new biosynthetic pathways (Ct-D, Ct-L, Ct-N)
  - **Provided a key case study** for the bioenergy industry by establishing r-BOX for production of advanced biofuels and value-added chemicals

- Expanded the scope of biomanufacturing practice, **enabling regional and global economic growth**
  - **Developed rural economic and sustainability analysis frameworks** to guide product selection
  - **Accelerated commercialization of new gas fermentation products** from lignocellulosic biomass, with specific application to forestry residues in the Southeast (At-A)
  - **Demonstrated pilot scale synthesis** of one r-BOX product (Ct-H, Ct-D)

High Impact Publications, and JDA with Leading Chemical Company

In vitro prototyping and rapid optimization of biosynthetic enzymes for cell design

Karim et al, **Nature Chemical Biology,** 2020

https://doi.org/10.1038/s41589-020-0559-0

Cell-free prototyping enables implementation of optimized reverse β-oxidation pathways in heterotrophic and autotrophic bacteria

Vogeli et al, **Nature Communications**, 2022

https://doi.org/10.1038/s41467-022-30571-6

Press release

https://www.mccormick.northwestern.edu/news/articles/2022/06/closing-gaps-to-find-new-energy-sources/

BASF
We create chemistry

https://basf.gomexlive.com/ext/rpcweb22/?lang=en

Joint Development Agreement between LanzaTech and BASF using rBOX pathway for commercialization of a target rBOX product

4 – Summary

Summary

Task 1:

- >300 new gene variants mined and synthesized
- >250,000 GEM simulations per design, confirmed growth coupling being possible
- Optimized *E. coli* chassis strain generated as basis for cell-free prototyping and blueprint for *Clostridium*

Task 2:

- Cell-Free platform for rBOX testing established
- ~1000 designs tested, >15,000 Assays run
- SAMDI-CoA method established

Task 3:

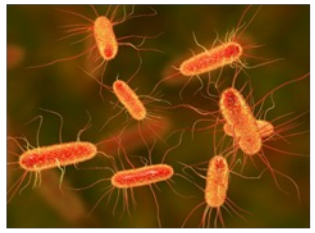
- rBOX implemented into *Clostridium*, 10 new products demonstrated
- Surpassed target metrics for lab scale production of a target molecule
- Ready for pilot scale production

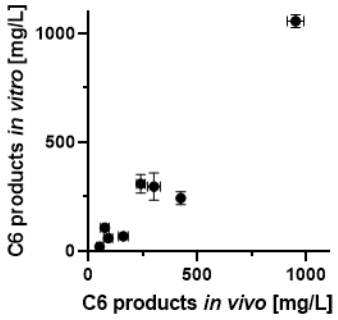
Task 4:

- Workshops completed, online platform available to the public, network with stakeholders established

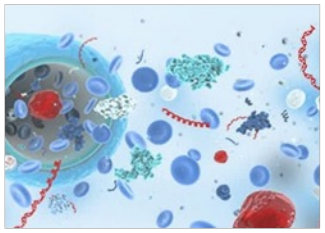
Summary

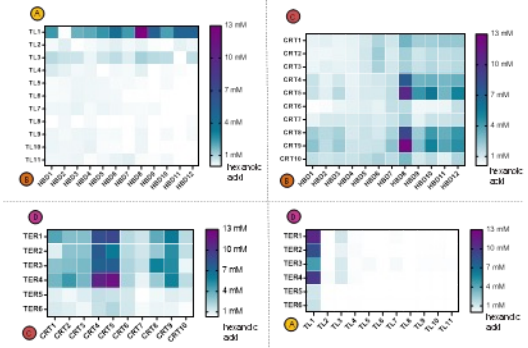
E. coli
Established rBOX



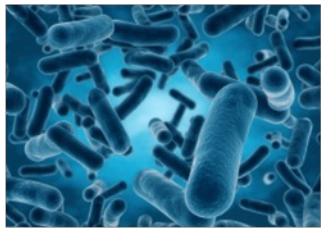


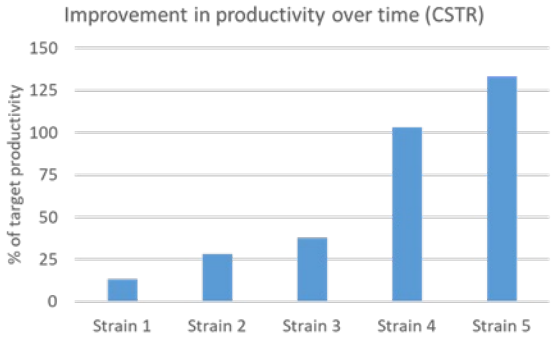
Cell-Free
Rapid Prototyping





Clostridium
Industrial Production





Advanced a new vision for accelerating biodesign of rBOX production platforms

Quad Chart Overview

Timeline

- Start - 10/1/2018
- End - 1/31/2022 (Extended to 08/31/2023)
- Status - 93% complete (13/14 milestones)

	FY22 Costed	Total Award
DOE Funding	*(10/01/2021 – 9/30/2022): $180,130*	*$1,600,000*
Project Cost Share *	Total: $400,000	

TRL at Project Start: 2
TRL at Project End: 5

Project Goal

Our project objective is to develop clostridia to ferment synthesis gas into a range of advanced bioproducts.

End of Project Milestone

- We will manufacture one product from engineering a reversal of the β-oxidation cycle in clostridia at a target metric of in a scalable pilot reactor.
- We will assess environmental, community and rural economic development impacts

Funding Mechanism

DE-FOA-0001637

B: Biofuels and Biobased Products Development

Project Partners*

- Northwestern University (34%), LanzaTech (33%), University of South Florida (25%), Georgia Institute of Technology (8%)

*Only fill out if applicable.

Thank you!

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy

BIOENERGY TECHNOLOGIES OFFICE

Project funding made possible by the Department of Energy EERE in partnership with USDA NIFA, under Fiscal Year 2017 Biomass Research and Development Initiative (DE-FOA-0001637)

Technology Manager: Ian Rowe
Project Monitor: Frank Fields
Grants Management Specialist: Nicholas Oscarsson

Additional Slides

Responses to Previous Reviewers' Comments

- **Reviewer's comment (2021):** The commercial viability is not within reach as the productivity target is low. As such this is a high-risk project but hard to assess return on the investment in R & D.
  - **Addressal:** We have demonstrated lab scale production of one target molecule at a level that exceeds the defined metric in the project by 33%. We have brought TRL of the project from 2 to 4, and are ready for pilot scale production that will improve the TRL to 5.

- **Reviewer's comment (2021):** The presentation makes no mention of TEA, production costs, etc. This omission gives the reader no sense of the commercial opportunity, which is critical in a BETO-supported program.
  - **Addressal:** Under this project, TEA was conducted but not a required deliverable. TEA was used to identify the critical performance metrics that the project is focused on.

- **Go/No-Go Review (2021):** The team successfully completed our Go/No-Go Review. This centered on Milestone 3.1: Construct and evaluate 50 unique pathway designs for target molecules in vivo (growth and metabolite profiles of *C. autoethanogenum* harboring the pathway designs on a plasmid growing on synthetic syngas) and in vitro (protein expression and metabolite data).

Publications, Patents, Presentations, Awards, and Commercialization

Publications, patents

- Silverman, A.D., Karim, A.S., and **Jewett, M.C.** Cell-free gene expression systems: An expanding repertoire of applications. *Nature Reviews Genetics*. 2019; DOI: https://doi.org/10.1038/s41576-019-0186-3
- A. S. Karim, F. (Eric) Liew, S. Garg, B. Vögeli, B. J. Rasor, A. Gonnot, M. Pavan, A. Juminaga, S. D. Simpson, **M. Köpke, M. C. Jewett**. Modular cell-free expression plasmids to accelerate biological design in cells. *Synthetic Biology*, Volume 5, Issue 1, 2020; https://doi.org/10.1093/synbio/ysaa019
- B.J. Rasor, B. Vögeli, G. M. Landwehr, J. W. Bogart, A. S. Karim, **M.C. Jewett**. Toward sustainable, cell-free biomanufacturing. Curr Opin Biotechnol. 2021 Jan 13;69:136-144, DOI: 10.1016/j.copbio.2020.12.012
- Fackler, N., Heijstra, B.J., Rasor, B.J., Brown, H., Martin, J., Ni, Z., Shebek, K.M., Rosin, R.R., Simpson, S.D., Tyo, K.E, Giannone, R.J., Hettich, R.L, Tschaplinski, T., Leang, C., Brown, S.D., **Jewett, M.C**., and Köpke, M.* 2021. Stepping on the gas to a circular economy: accelerating development of carbon-negative chemical production from gas fermentation. *Annual Reviews in Chemical and Biomolecular Engineering*. 12:439-470.
- Vögeli, B., Schulz, L., Garg, S., Tarasava, K., Clomburg, J.M., Lee, S.H., Gonnot, A., Moully, E. H., Kimmel, B.R., Tran, L., Zeleznik, H., Brown, S.D., Simpson, S.D., Mrksich, M., Karim, A.S., Gonzalez, R., **Köpke, M., Jewett, M.C**. 2022. Cell-free prototyping enables implementation of optimized reverse β-oxidation pathways in heterotrophic and autotrophic bacteria. *Nature Communications*. 13, 3058 (2022). https://doi.org/10.1038/s41467-022-30571-6

Patents

- U.S. Provisional Patent No. 63/158336
- U.S. Patent Application No. 17/653,913
- PCT/US22/71020

Presentations

- Clostridium XVI, 2022, Tolouse, France (Presentation by S. Garg)
- 2022 Synthetic Biology: Engineering, Evolution, and Design, Arlington, Virginia (Keynote by M. Kopke)
- Microbial Engineering II, 2022, Portugal (Presentation by M. Kopke)
- C1 Gordon Research Conference, 2022, Boston (Presentation by M. Kopke)
- Departmental Seminars (MIT, Wisconsin, Rice, University of Florida, University of Virginia), 2021-2022 (Presentation by Michael Jewett)
- White House Biomanufacturing Summit, November 2022, Washington D.C., (Presentation by Michael Jewett)
- 2022 SIMB Annual meeting. August 2022, San Francisco (Presentation by Michael Jewett)

Technology transfer/Commercialization efforts

- Joint Development Agreement between LanzaTech and BASF to commercialize one target rBOX product