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

March 5, 2019
Biochemical Conversion

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Quad Chart Overview

Timeline
- Project start date - 10/1/2018
- Project end date - 9/30/2021
- Project complete 7% (1/14 milestones)

<table>
<thead>
<tr>
<th></th>
<th>Total Costs Pre FY17*</th>
<th>FY 17 Costs</th>
<th>FY 18 Costs</th>
<th>Total Planned Funding (FY 19-Project End Date)</th>
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</thead>
<tbody>
<tr>
<td>DOE Funded</td>
<td>$0</td>
<td>$0</td>
<td>$0</td>
<td>$1,600,000</td>
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<td>Project Cost Share*</td>
<td>$0</td>
<td>$0</td>
<td>$0</td>
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• **Partners:** Northwestern University (34%), LanzaTech (33%), University of South Florida (25%), Georgia Institute of Technology (8%)

Barriers addressed
- Ct-D. Advanced Bioprocess Development
- Ct-H. Gas Fermentation Development
- Ct-L. Decreasing Development Time for Industrially Relevant Microorganisms
- Ct-N. Multiscale Computational Framework toward Accelerating Technology Development
- Ot-A. Availability of Quality Feedstock

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

End of Project Goal
- We will manufacture one product from engineering a reversal of the β-oxidation cycle in clostridia at a metric of >0.1g/l/h in >80L scalable pilot reactor.
- We will assess environmental, community and rural economic development impacts.
1 - Project Overview
The need for low-cost biofuels and bioproducts from sustainable resources is intensifying
Unfortunately, designing, building, and optimizing biosynthetic pathways in cells remains a complex and formidable challenge

- Development cycles to optimize biosynthetic pathways can be slow, especially for non-model organisms

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Institutions</th>
<th>Time</th>
<th>Person Years</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-Propanediol</td>
<td>DuPont, Genencor, Tate &amp; Lyle</td>
<td>15 years (1992-2007)</td>
<td>&gt;550</td>
<td>&gt;$130M</td>
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<tr>
<td>Artemisinin</td>
<td>UC Berkeley, Amyris, Sanofi</td>
<td>13 years (2000-2013)</td>
<td>&gt;130</td>
<td>&gt;$50M</td>
</tr>
<tr>
<td>Farnesene</td>
<td>Amyris, TOTAL</td>
<td>4 years (2008-2012)</td>
<td>&gt;250</td>
<td>&gt;$30M</td>
</tr>
</tbody>
</table>


- Platform organisms, accessible feedstocks, target molecules, and stable environments in which to work are limited
- Integrated computational frameworks for biodesign need improvement
Our project objective is to develop clostridia to ferment synthesis gas produced from cellulosic biomass by established gasification technologies, into a range of advanced bioproducts.

We will target products used as drop-in fuels, fuel additives, and chemical building blocks with a $14Bn US market.
2 – Approach (Management)
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
Several activities ensure efficient coordination within the team and reporting to DOE/USDA

- Northwestern and LanzaTech have **collaborated on numerous projects since 2015** and have developed successful mechanisms for technical coordination, data sharing, and integration

- Bi-weekly meetings to review the team’s progress and discuss any matters requiring action

- Project structure enables multiple, parallel paths to achieve 4 tasks and the milestones
  
  - **Task 1.** Develop and apply informatics and computer-aided design tools to choose molecules, enzymes, and pathways in clostridia (USF, LT)
  
  - **Task 2.** Establish a cell-free framework for rapid pathway prototyping and analysis (Northwestern, LT)
  
  - **Task 3.** Develop optimized production strains of clostridia (LanzaTech, NU, USF, GT)
  
  - **Task 4.** Rural economic development and sustainability analysis (GaTech, LT)

- Interface with other DOE projects to accelerate goals
  
  - Complement cBioFab project, utilize DOE user facility JGI for gene synthesis
2 – Approach (Technical)
To achieve our vision, we will:

- **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 will allow 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 (rBOX) offers unique access to thousands of molecules

- 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.

Largest collection of industrial *Clostridium* strains provides unique access to highly-evolved gene variants

- LanzaTech owns the largest collection of industrially-deployed acetone-butanol-ethanol (ABE) clostridia strains
- The collection dates back to 1944 and contains hundreds of highly evolved strains, **spanning several decades of development at several large-scale commercial plants**
- In collaboration with JGI, the entire collection have been sequenced

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

- A hybrid thermochemical (gasification) - biological (gas fermentation) pathway utilizes all biomass components (lignin and cellulose), maximizing yields and overcoming barriers such as biomass recalcitrance.
- Integrated gasification-fermentation has been demonstrated in extended continuous operations using multiple types of lignocellulosic material.
- Syngas fermentation uses the same fermentation process implemented in LanzaTech’s first commercial scale gas fermentation facility (started May 3 2018).
How are we going to achieve our goals?

**Phase 1**

**Task 1**
- Develop and apply informatics and computer-aided design tools to choose molecules, enzymes, and pathways for r-BOX in clostridia
  - Identify candidate r-BOX enzymes facilitating optimal r-BOX designs

**Task 2**
- Establish a cell-free framework for rapid pathway prototyping and analysis
- Develop optimized production strains using a clostridia biofoundry that leverages cell-free systems
  - Testing r-BOX pathway variants informed by cell-free prototyping in clostridia
  - Genome-scale model (GEM) predictions to optimize flux and inform cell-free work.
  - Iterative pathway and strain optimization
  - Process development and scale-up

**Task 3**
- Rural economic development and sustainability analysis
  - Technoeconomic analysis (TEA) and Life cycle analysis (LCA)
  - Rural economic development analysis

**Task 4**
- Cell-Free Prototyping
- Pathway Prediction
- Clostridia Development
- Clostridia Prototyping
- Genome Models
- Iterative Optimization
- Production Scale Fermentations

Legend:
- Milestones
- Interactions between tasks
- Northwestern University: (Jewett)
- LanzaTech: (Köpke, Conrado)
- Rice University: (Gonzalez)
- Georgia Tech: (Thomas)
- Go/No-go decision

Northwestern University
LanzaTech
University of South Florida
Georgia Institute of Technology
Task 1: Use pathway design to provide a focused set of r-BOX input molecules

- **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)

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

- **Milestone 1.3.** Optimize computational framework for generating novel pathways based on feedback from other Tasks and refine pathway design (Y3/Q2)
Task 2: Develop methods for accelerating systematic pathway optimization using cell-free cocktails

- **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).

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

- **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).
Task 3: Develop optimized strains for production of r-BOX products from biomass syngas

- **Milestone 3.1.** (go/no-go decision): Construct and evaluate 50 unique pathway designs for target molecules *in vivo* and *in vitro*. Our **metric is 100mg/L/d** of one target product. (Y2/Q4)

- **Milestone 3.2.** Proof of concept for additional rBOX target products on syngas. (Y3/Q2)

- **Milestone 3.3.** Construct and evaluate an additional 150 unique pathway designs for target molecules *in vivo* and *in vitro*. (Y3/Q3)

- **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 of >0.1g/l/h**. (Y3/Q3)

- **Milestone 3.5.** One selected rBOX product at a target metric of >0.1g/l/h in >80L scalable pilot reactor. (Y3/Q4)
Task 4: Assess environmental, community, and rural economic development impacts of r-BOX synthesis to facilitate design

- **Milestone 4.1.** Complete 2 workshops to inform lifecycle impact analysis using TRACI model. 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)

- **Milestone 4.1.** Completed LCA for two rBOX molecules. (Y3/Q2)

- **Milestone 4.1.** Completed assessment of infrastructure and supply chains for biomass feedstock supply of two rBOX molecules in the US southeast. (Y3/Q4)
3 – Technical Accomplishments/Progress/Results
### Task 1
- **Develop and apply informatics and computer-aided design tools to choose molecules, enzymes, and pathways for r-BOX in clostridia**
  - 1a: Determine optimal r-BOX designs in clostridia supporting maximum bioproduct synthesis
  - 1b: Identify candidate r-BOX enzymes facilitating optimal r-BOX designs

### Task 2
- **Establish a cell-free framework for rapid pathway prototyping and analysis**
  - 2: Demonstrate methods for systematic pathway optimization using cell-free cocktails.

### Task 3
- **Develop optimized production strains using a clostridia biofoundry that leverages cell-free systems**
  - 3a: Testing r-BOX pathway variants informed by cell-free prototyping in clostridia
  - 3b: Genome-scale model (GEM) predictions to optimize flux and inform cell-free work.
  - 3c: Iterative pathway and strain optimization
  - 3d: Process development and scale-up

### Task 4
- **Rural economic development and sustainability analysis**
  - 4a: Technoeconomic analysis (TEA) and Life cycle analysis (LCA)
  - 4b: Rural economic development analysis

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**Legend**
- 🌟 - Milestones
- ⬤ - Interactions between tasks
- NU - Northwestern University: (Jewett)
- LT - LanzaTech (Köpke, Conrado)
- RU - Rice University (Gonzalez)
- GT - Georgia Tech (Thomas)
- 🌟 - Go/No-go decision
Task 1: Based on initial enzyme set, mined enzyme candidates for r-BOX in *Clostridium* collection

More than 200 unique rBOX gene variants identified that have been codon adapted and are currently being synthesized through the JGI DNA synthesis program
Task 1: Genome-scale modeling (GEM) and metabolic flux balance analysis to define optimal r-BOX inputs

- *E. coli* GEM has previously been exploited to determine optimal routes for omega-functionalized product synthesis and input molecules (left) to maximize target product synthesis from glucose (right)

Reactions from *E. coli* model have been integrated to *Clostridium* GEM to enable modeling of target product synthesis in desired genetic background
Task 2: Developed cell-free platform to test homology activity and beginning to apply it to r-BOX (example for butanol pathway below)
Task 4: We plan two workshops to guide the project’s strategic direction and product emphasis

- **Workshop 1.** Atlanta, October 2019. Invitees will include Georgia Rural Economic Development Authority representatives, industry representatives, environmental NGO representatives, experts on community impacts, ethanol manufacturers.

- **Workshop 2.** Soperton area, April 2019. Invitees will include: Treutlen County Development Authority, Heart of Georgia Altamaha Regional Commission, Site Tour.
4 – Relevance
• Enable high-level, sustainable synthesis of next-generation biofuels and bioproducts by **developing clostridia to ferment synthesis gas**
  – Advance 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)
  – Expand the diversity of products that can be produced and co-produced via syngas fermentation (Ct-H)
  – Gain new knowledge of metabolism in obligate anaerobes and traits related to “industrial fitness” of clostridia (Ct-H, Ct-D)

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

• Expand the scope of biomanufacturing practice, **enabling regional and global economic growth**
  – Develop rural economic and sustainability analysis frameworks to guide product selection
  – Accelerate commercialization of new gas fermentation products from lignocellulosic biomass, with specific application to forestry residues in the Southeast (At-A)
  – Demonstrate pilot scale synthesis of one r-BOX product (Ct-H, Ct-D)
5 – Future Work
• **Key upcoming milestones (18 months):**
  – Use genome-scale metabolic models for r-BOX pathway design
  – Establish a cell-free framework for rapid pathway prototyping of r-BOX pathways to accelerate design in clostridia
  – Complete workshops to inform life cycle analysis

• **Go/No-Go Point (9/30/2020):**
  – Construct and evaluate 50 unique pathway designs for target molecules *in vivo* and *in vitro* to enable 100mg/L/d of one r-BOX product in clostridia
    • *Already created compatible vector system to facilitate evaluation*

• **Project completion steps**
  – Demonstrate synthesis of one r-BOX product in clostridia at a metric of >0.1g/l/h in >80L scalable pilot reactor.
  – Complete rural economic development & sustainability analyses
Summary
• We will develop a **new technology platform** for engineering r-BOX in clostridia for fuel and chemical synthesis from biomass syngas.

• Our approach to integrate pathway design, pathway prospecting, pathway validation, and economic analysis will **enable a new paradigm of biological design**.

• Our current project progress is **ahead of schedule**.

• The impact will be to **reduce the time required to develop new biosynthetic products** and **expand the scope of biomanufacturing practice through gas fermentation**.
Thank you!

Technology Manager: Jay Fitzgerald
Project Monitor: Clayton Rohman
Grants Management Specialist: Nicholas Oscarsson