DOE Bioenergy Technologies Office (BETO)
2023 Project Peer Review

Development of *Bacillus* as an industrial host for the microbial production of biopolymers

April 4, 2023
Agile BioFoundry

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ZymoChem

Project Overview

- Development of *Bacillus* as an industrial host for the microbial production of biopolymers of industrial interest from lignocellulosic hydrolysates

- Project Goals
  - Enable ZymoChem's Carbon Conservation (C^2) technology in non-model organisms to maximize carbon efficiencies (yields) to biopolymers via minimizing loss of CO_2 from lignocellulosic hydrolysates
  - Apply ABF's state of the art metabolic engineering tools/strategies to engineer strains with improved Titer and Rate, metrics by >4x over base case performance
  - Partner with ABF to utilize fermentation and process development & scale up expertise to scale up production process to pilot scale

Project Overview

- Project Importance
  - Establish non-model organisms as microbial hosts for making biopolymers of industrial interest
  - Develop a scalable production process for making biopolymers using non-model organisms
  - Commercialize bio-based polymers as sustainable and cost-competitive replacements of current fossil-based polymers

- Project Risks
  - Non-model hosts are difficult to engineer due to a variety of reasons
  - Innate challenges to scaling fermentation and downstream processing

- Project Tasks
  - Task 1: Establish Carbon Conserving Technology in *Bacillus*
  - Task 2: Genetic Engineering Tool Development
  - Task 3: Strain Engineering for High Flux Biopolymer Production
  - Task 4: Fermentation and DSP Scale Up

1 – Approach

Project Structure

ZYMOCHEM

1) Project lead

2) Co-lead C5→ Biopolymer engineering

3) Co-lead strain of high flux Biopolymer production

4) Support fermentation and Biopolymer recovery scaleup

5) Support all tasks

6) Prepare DOE reports

BERKELEY LAB

1) Metabolomics lead

2) Co-lead strain of high flux Biopolymer production

3) Prepare DOE reports

OAK RIDGE National Laboratory

1) Lead genetic tools development early in project

2) Lead transcriptomics efforts

3) Support other engineering efforts as needed

4) Co-lead C5→ Biopolymer engineering

5) Prepare DOE reports

ABPDU

1) Lead scale up of fermentation and Biopolymer recovery

2) Prepare DOE reports

1 – Approach

- Task 1 – Establish Carbon Conserving Technology in *Bacillus*
  - To be accomplished in coordination with Tasks 2 and 3
  - Enables carbon lossless conversion from feedstock to product improving theoretical maximum yields by > 20%
- Task 2 – Genetic engineering tool development
  - Robust systems to enable engineering within our non-model *Bacillus* host including recombinant biosynthetic pathway expression, gene deletions, and chromosomal gene integrations
  - Critical capability that enables all tasks
- Task 3 – Strain engineering
  - Multi-omics & metabolic flux analysis to understand what's going on inside the cell and help identify strain engineering targets
- Task 4 – Fermentation and DSP scale up
  - Demonstrate scalability of the entire production process and make product samples for testing

1 – Approach

Go/No-Go Milestones

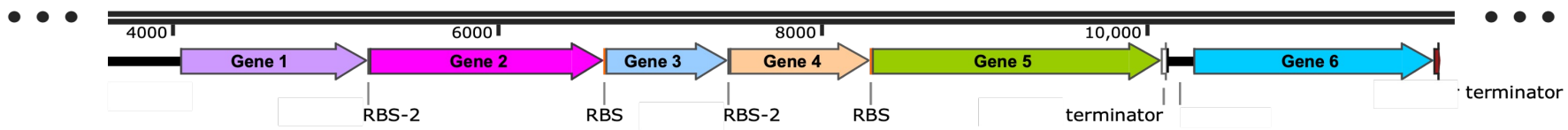
- By 30 June 2021, demonstrate >2x improvement in both titer and rate metrics over benchmarking values.

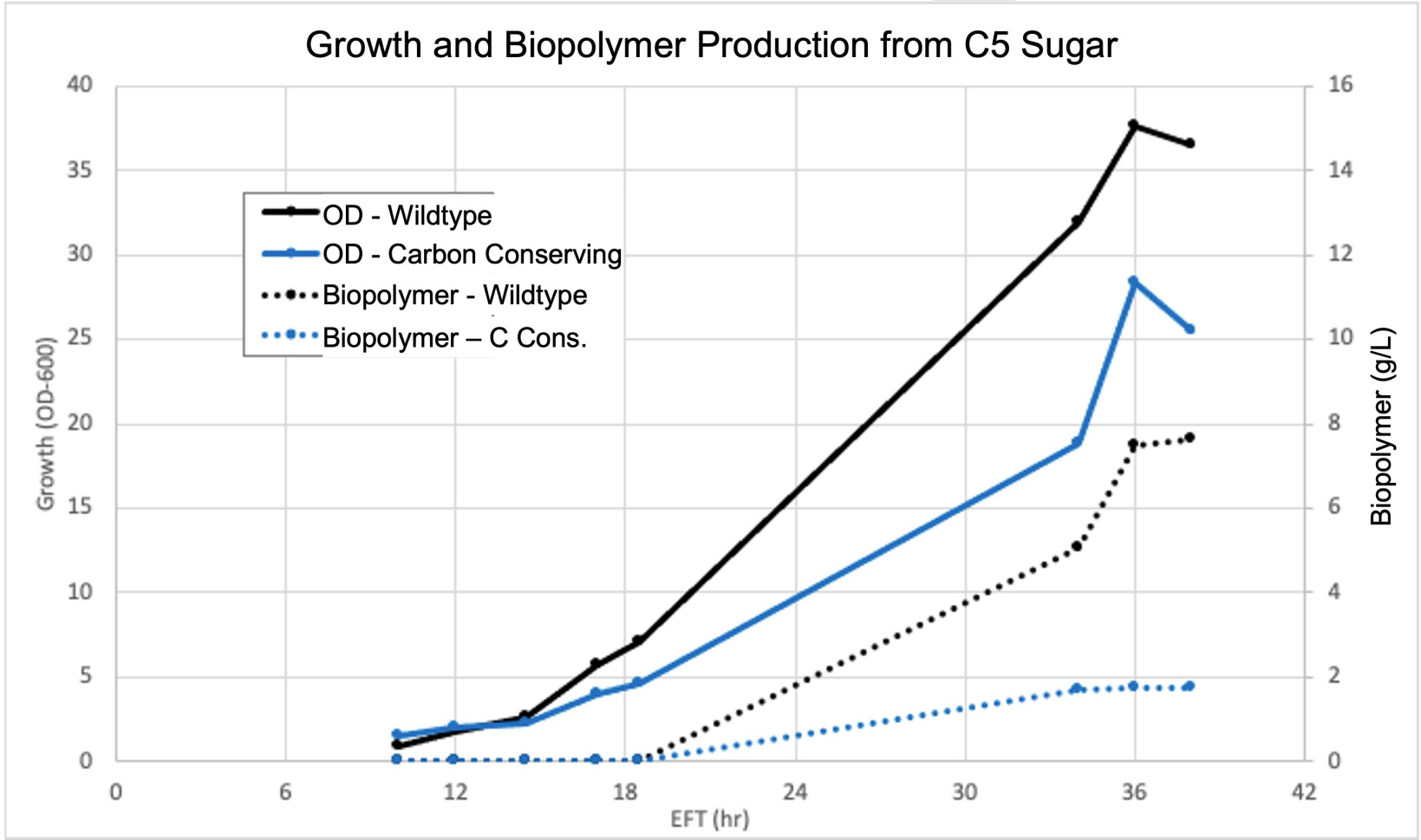
- By 30 June 2022, demonstrate >4x improvement in both titer and rate metrics over benchmarking values.

2 – Progress and Outcomes

- All Go/No-Go Milestones accomplished ahead of schedule.
- Significant progress has been achieved in all Tasks, with 100% completion reached for nearly every Task.
- Examples of progress in each task in the following slides.

Task 1: Carbon-conserving metabolism of C5 sugar in *Bacillus*

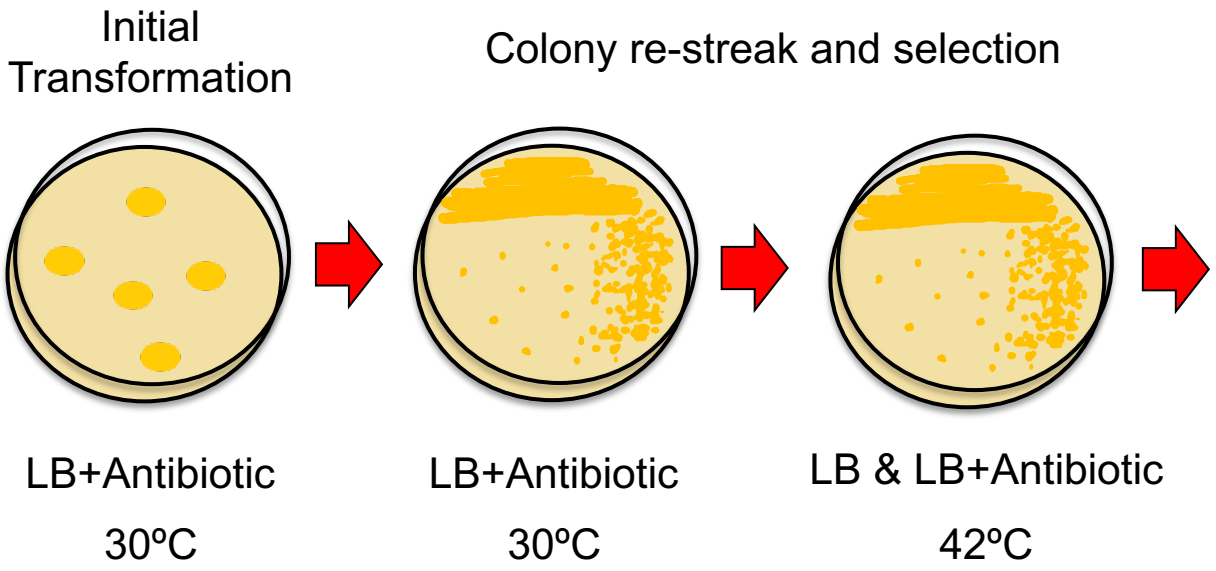


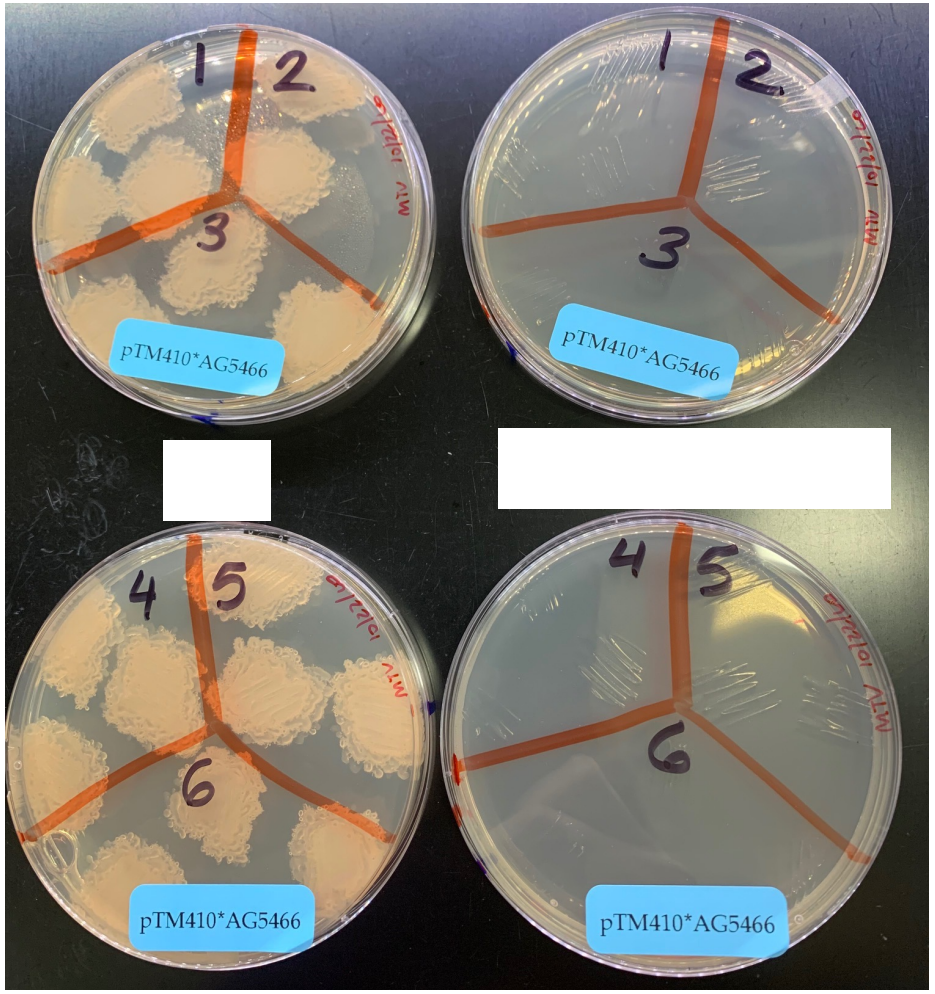


- First ever demonstration of Biopolymer production via the 5-step Carbon Conserving (C^2) pathway.
- The native C5 metabolic pathway was replaced with an optimized 6-gene C^2 operon.
- The C^2 pathway enabled growth and Biopolymer production, however, diminished growth and titer as compared to the wildtype pathway leaves room for further improvements.

Task 2: An example of genetic tool development

Temperature-sensitive plasmids as a means of engineering *Bacillus* genomes.

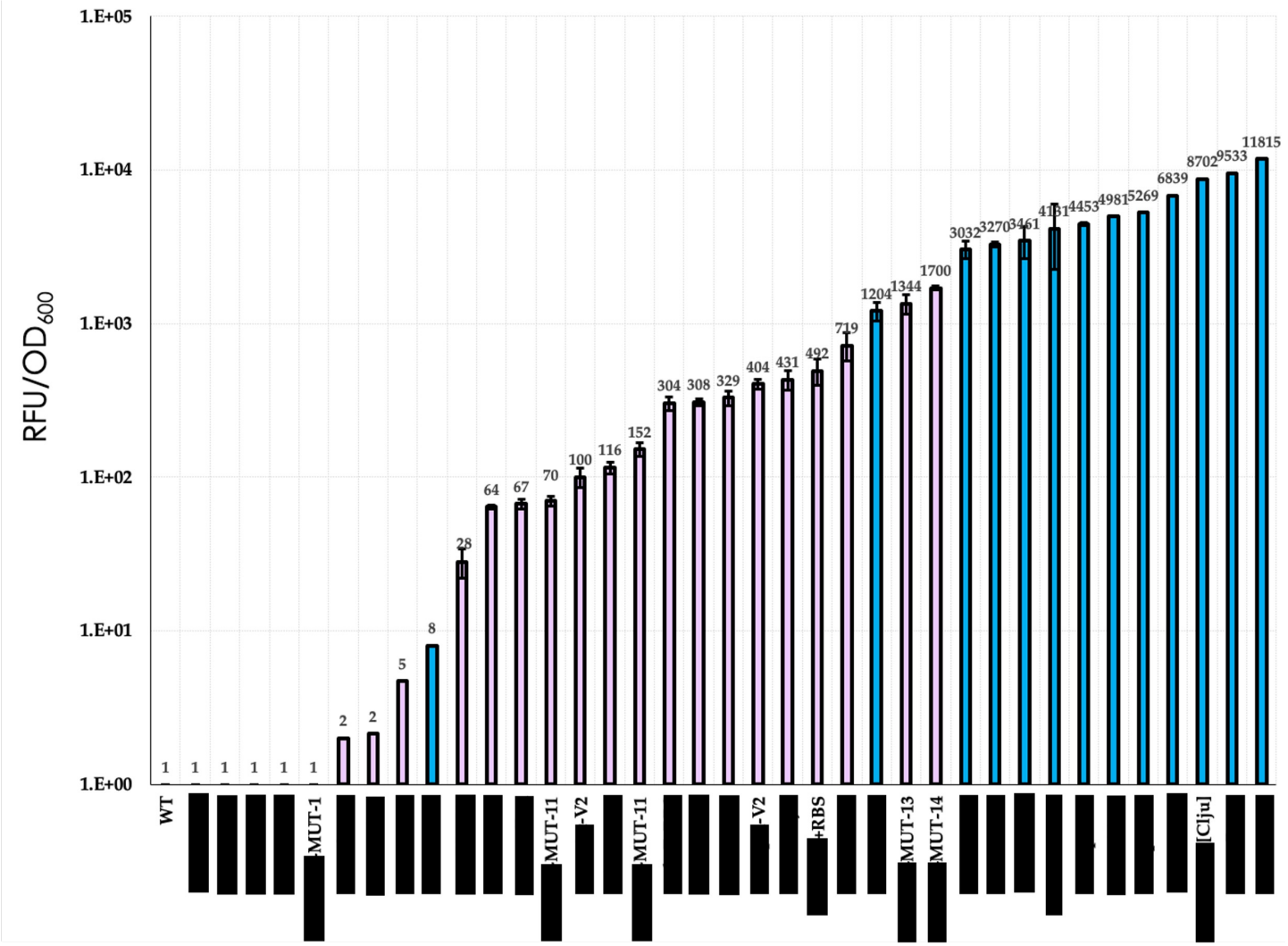


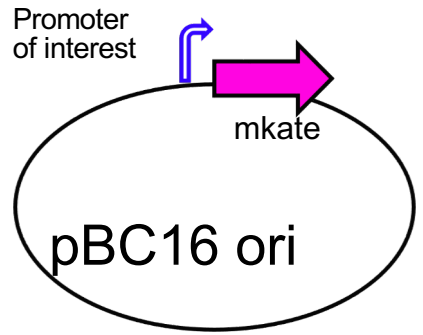


After incubation at 42ºC

This system has been used to delete genes and insert genetic elements into the chromosome.

Task 2: Promoter construction and validation in *Bacillus*

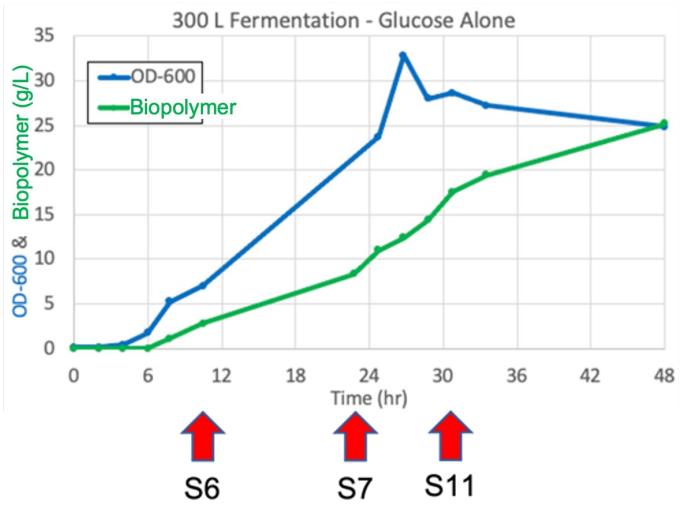




- 37+ promoter variations constructed and tested
- Expression levels span >1000-fold
- Plasmid & chromosomal expression

Task 3: Strain engineering for high flux biopolymer production

Transcriptomics used to identify targets for gene deletion or amplification.

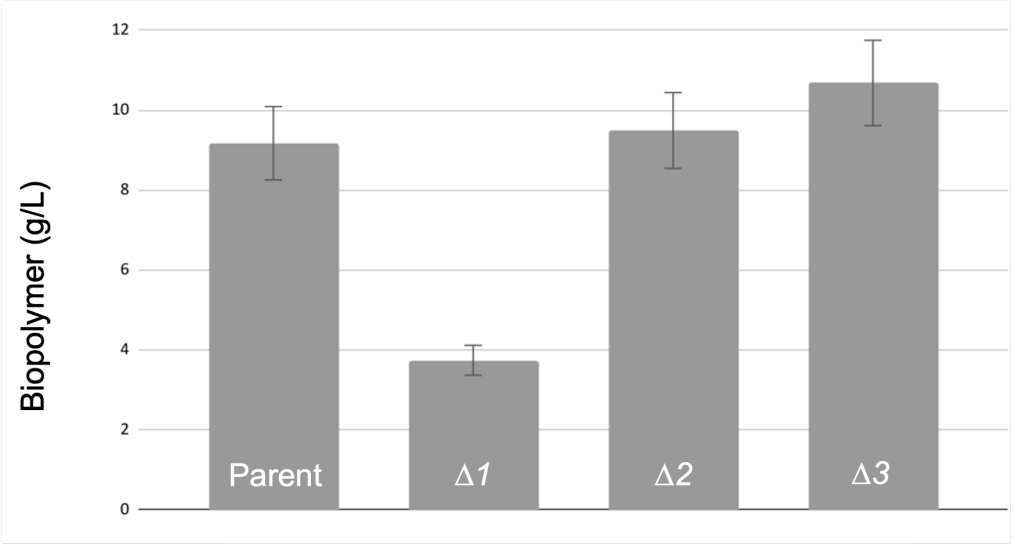


Gene	Description
bioB	Biotin Synthase
bioF	Biotin synthesis: 8-amino-7-oxonona
[redacted]	Putative heterocycle-containing bact
bioD	Biotin synthesis: ATP-dependent det
[redacted]	DNA starvation/stationary phase
[redacted]	Spore Coat CotJA
[redacted]	General stress protein, YflT
[redacted]	ABC Transporter
[redacted]	Spore Protein
[redacted]	Cytochrome P450
[redacted]	DUF2197
bioA	Biotin synthesis: Adenosylmethionine
[redacted]	ABC Transporter
mutL	DNA Repair
[redacted]	Photosynthetic Reaction Center H-ch
opuCC	Glycine betaine/carnitine/choline bin
[redacted]	Manganese catalase
[redacted]	Sporulation
[redacted]	NAD(P)-dependent oxidoreductase
[redacted]	Spore Coat
[redacted]	Membrane protein
[redacted]	6-carboxyhexanoate-CoA ligase
[redacted]	Hypothetical
[redacted]	Spore Coat
[redacted]	Hypothetical
[redacted]	Hypothetical
[redacted]	DUF378
[redacted]	Pyruvate Oxidase
[redacted]	Hypothetical
[redacted]	RNA Binding Protein S4
[redacted]	Hydroperoxide Resistance Protein
yqfC	Sporulation
[redacted]	Germination protein GerT
[redacted]	Sodium:alanine symporter

- Samples from distinct growth phases of a 300 L fermentation were submitted for transcriptomic analysis.
- Differentially regulated genes grouped by putative function and used to inform genetic and process engineering targets.
  - Biotin supplementation
  - Sporulation elimination

Task 3: Ex. of genetic modification based on ’omics data

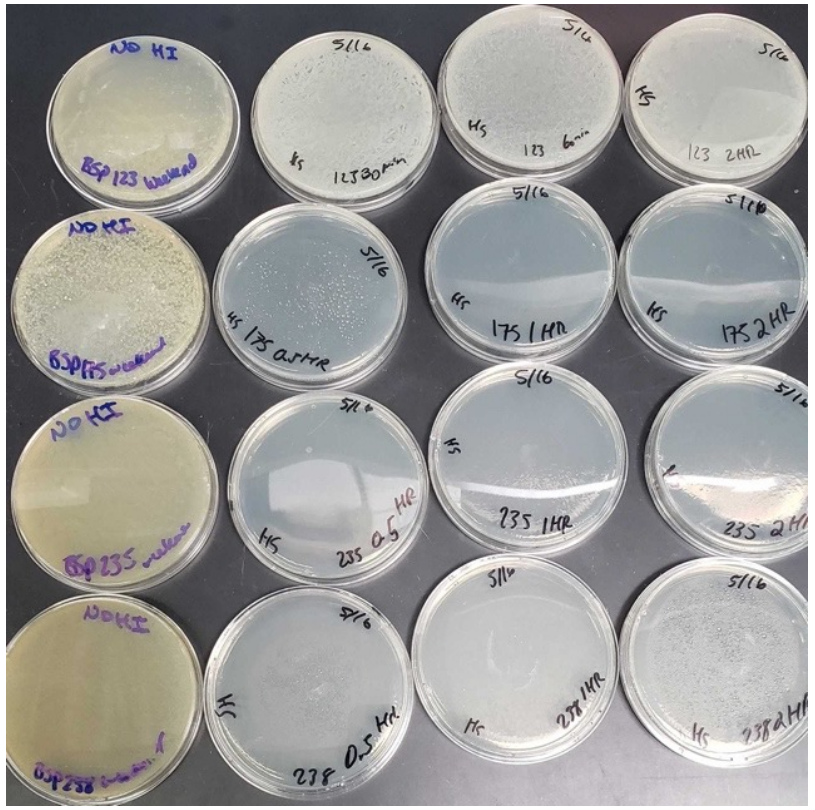
Elimination of Sporulation



- Biopolymer production of Δ*2* & Δ*3* strains is equivalent to that of the parental strain (via BioLector).
- Whereas the parental strain had robust growth after heat inactivation, none of the modified strains were able to grow, indicating sporulation had been eliminated.

Heat Inactivation at 85˚C

0 min 30 min 60 min 120 min



Parent

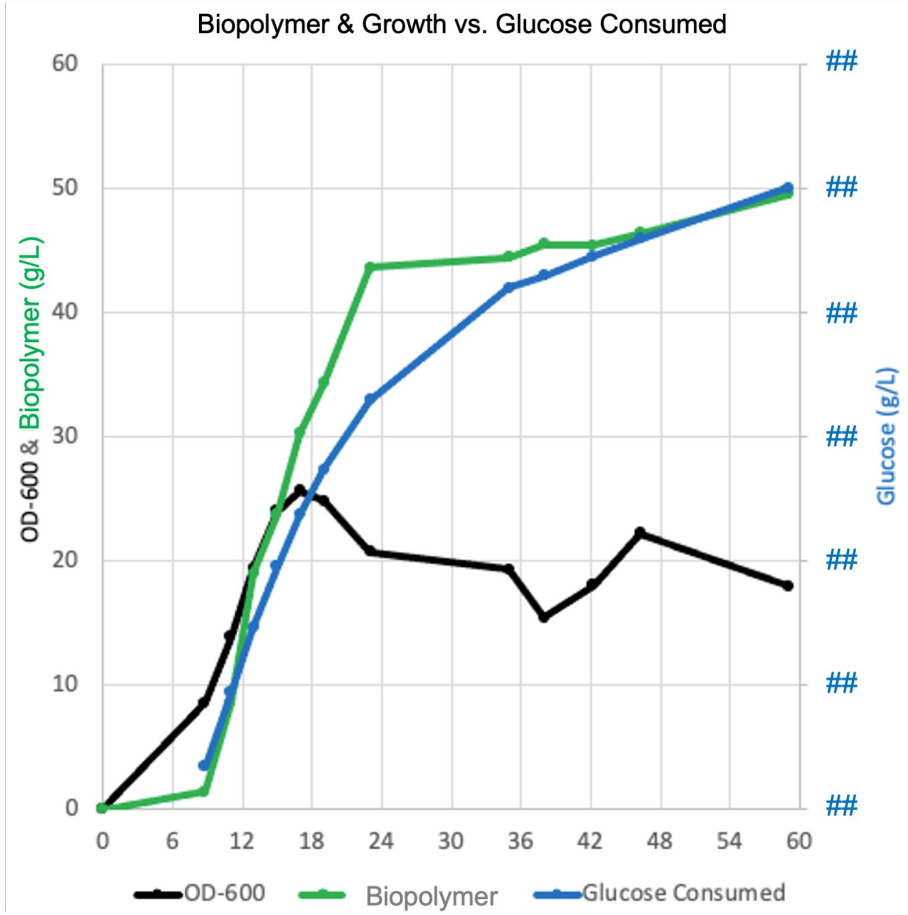
Δ*1*

Δ*2*

Δ*3*

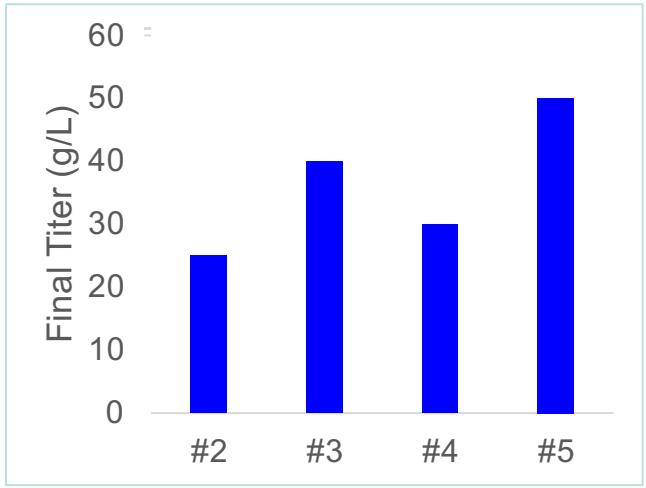
Task 4: Fermentation Scaleup to Pilot Level (300 L)

300 L Fermentation #5



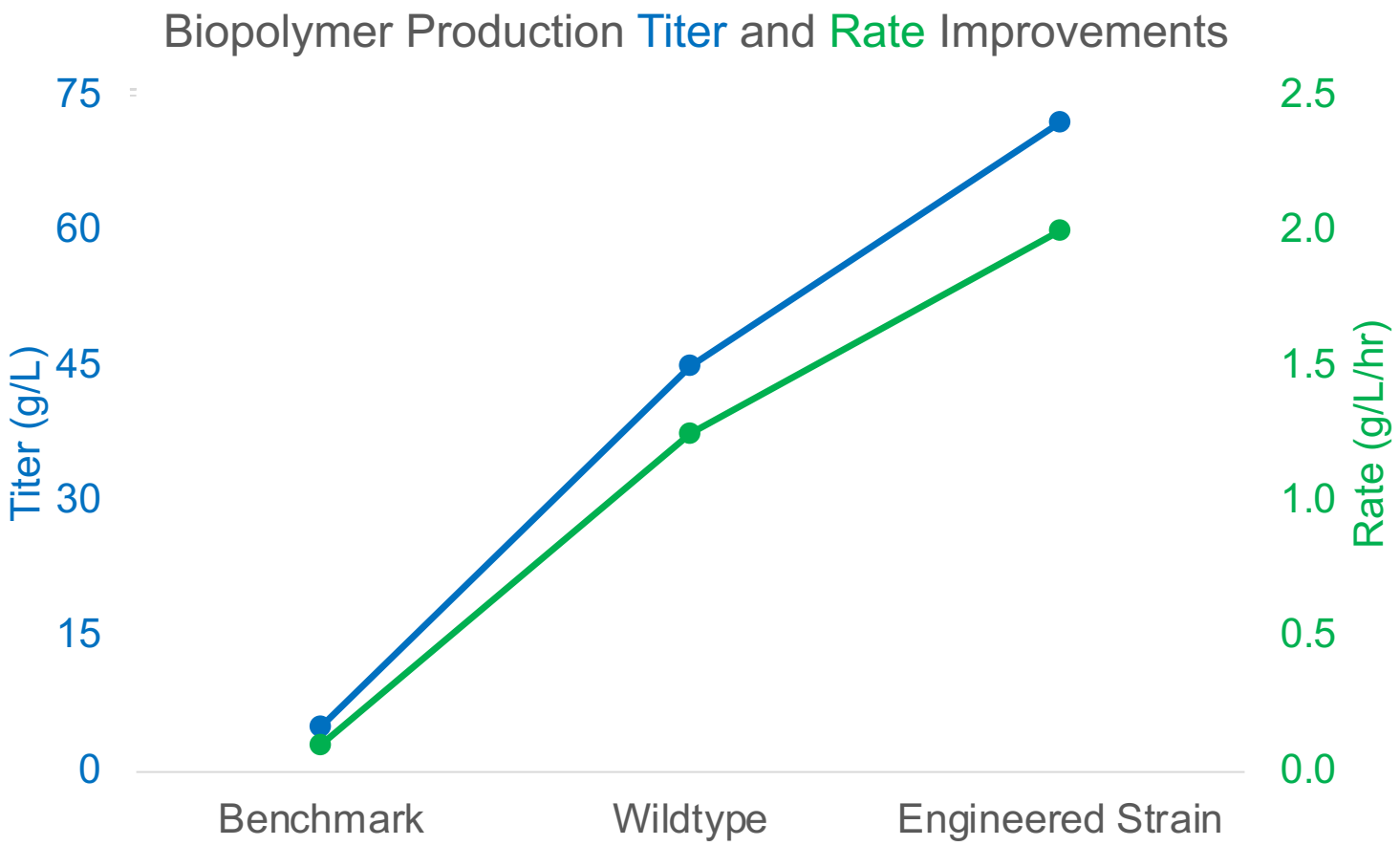
- Final Biopolymer Titer from 300 L #5 = **50 g/L**
  - 25% increase over prior fermentations at this scale
  - Approaching titers achieved at 3 L scale (60-70 g/L)

- Improvements due to process development, ensuring sufficient glucose throughout fermentation.
  - Feed initiation and rate of addition
  - Continuous glucose monitoring and adjustment



Final Go/No-Go Milestone

Demonstrate >4x improvement in both titer (>20 g/L) and rate (>0.44 g/L/hr) metrics over benchmarking values.



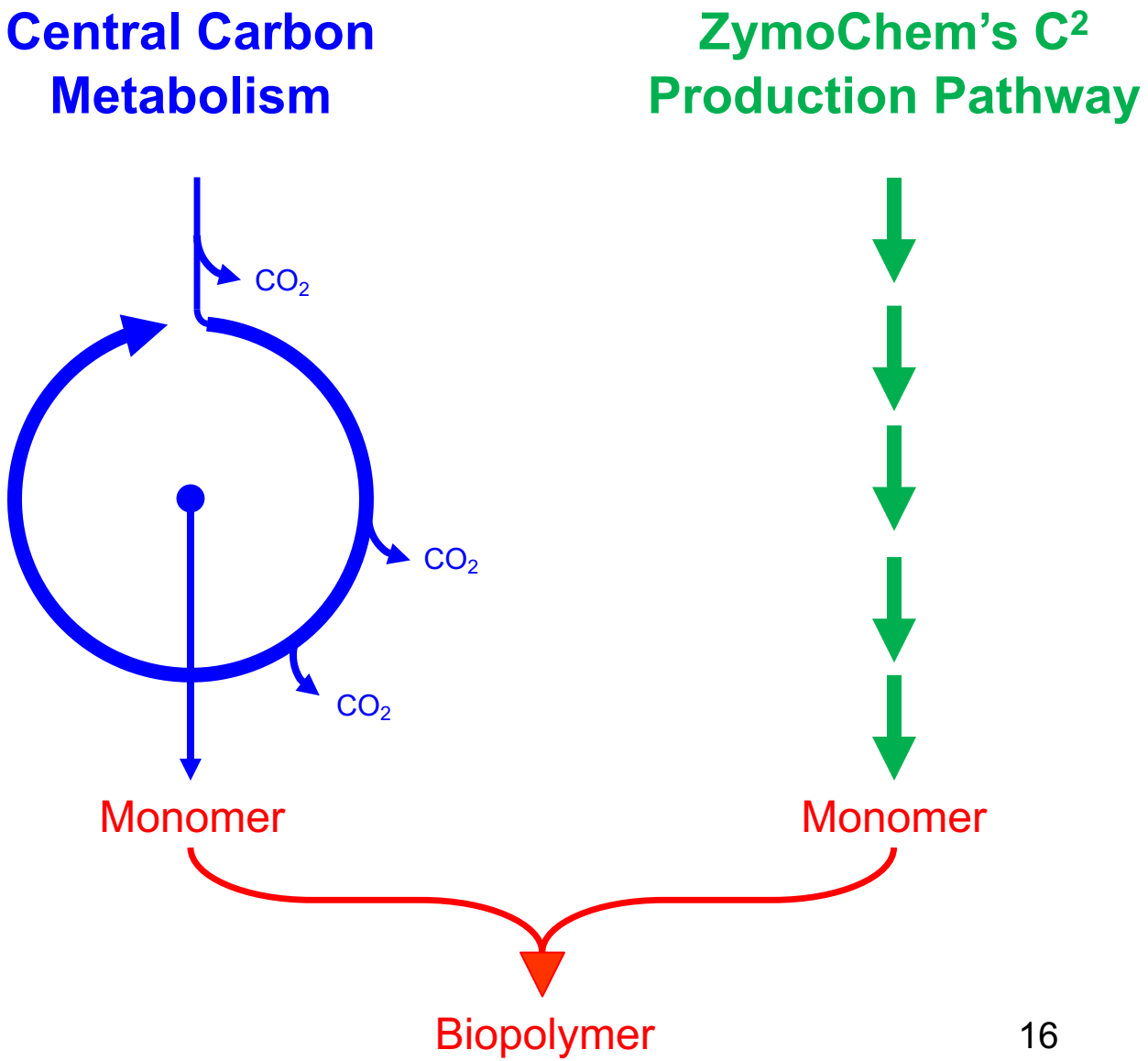
- Through process optimization, **45 g/L** Biopolymer produced in WT *Bacillus.*
- Combined with strain engineering, **72 g/L** Biopolymer produced.
  - **3.6X final project milestone**.
  - Overall rate: 2 g/L/hr

3 – Impact

- Results from this project have generated follow-on funding for ZymoChem to further develop and scale this technology.
- Genetic tools, resources, and expertise created as part of this project for engineering this *Bacillus* species will enable researchers of future engineering endeavors with other closely-related species of this genus, as they are great production hosts for proteins, biopolymers, and small molecules.
- Successful development of a Carbon-Conserving biotechnology that is designed to improve yields by minimizing or eliminating carbon loss will help change the paradigm and motivate future researchers to pursue novel ideas on pathway and microbial designs tailored for carbon conservation to enable more economical production of products.
- Successful demonstration of a functional C5-utilizing Carbon-Conservation pathway enables implementation of lignocellulosic feedstocks and potential co-feeding of C5 & C6 sugars, as the novel pathway is likely not subjected to catabolite repression.

Summary

- *ZymoChem's Carbon Conserving (C^2) Pathway minimizes carbon loss, thereby lowering overall production costs.*
- *Demonstrated success in all 4 project tasks.*
- *Accomplished Go/No-Go milestones months ahead of schedule.*
- *Successfully demonstrated key enabling technologies in a non-model organism, thereby supporting application of Design/Build/Test/Learn tools to further develop improved strains and processes.*
- *Process successfully scaled to Pilot level, and further increases to Production scale are being implemented.*



Quad Chart Overview

Timeline

- *January 1, 2019*
- *March 31, 2023*

	FY22 Costed	Total Award
DOE Funding	*$123,267.20*	*$1,321,381*
Project Cost Share *	$88,613.80	$331,615

Project Goal

The overall goal of this project is to develop a *Bacillus*-based bioprocess for production of biopolymer from lignocellulosic-derived C5 and/or C6 sugars at 4x the titer and rate over benchmarking values

End of Project Milestone

Demonstrate >4X improvement in both titer and rate metrics over benchmarking values.

Funding Mechanism

DE-FOA-0001916, BioEnergy Engineering for Products Synthesis (BEEPS), 2019.

TRL at Project Start: 3
TRL at Project End: 4

Project Partners*

- Lawrence Berkeley National Laboratory
- Oak Ridge National Laboratory

*Only fill out if applicable.

Additional Slides

(Not a template slide – for information purposes only)

- *The following slides are to be included in your submission for evaluation purposes, but will **not** be part of your oral presentation –*
- *You may refer to them during the Q&A period if they are helpful to you in explaining certain points.*

Responses to Previous Reviewers' Comments

- If your project has been peer reviewed previously, address 1-3 significant questions/criticisms from the previous reviewers' comments which you have since addressed
- Also provide highlights from any Go/No-Go Reviews

Note: This slide is for the use of the Peer Reviewers only – it is not to be presented as part of your oral presentation. These Additional Slides will be included in the copy of your presentation that will be made available to the Reviewers.

Publications, Patents, Presentations, Awards, and Commercialization

- List any publications, patents, awards, and presentations that have resulted from work on this project
- Use at least 12 point font
- Describe the status of any technology transfer or commercialization efforts

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