Endurogenetics ABF DFO

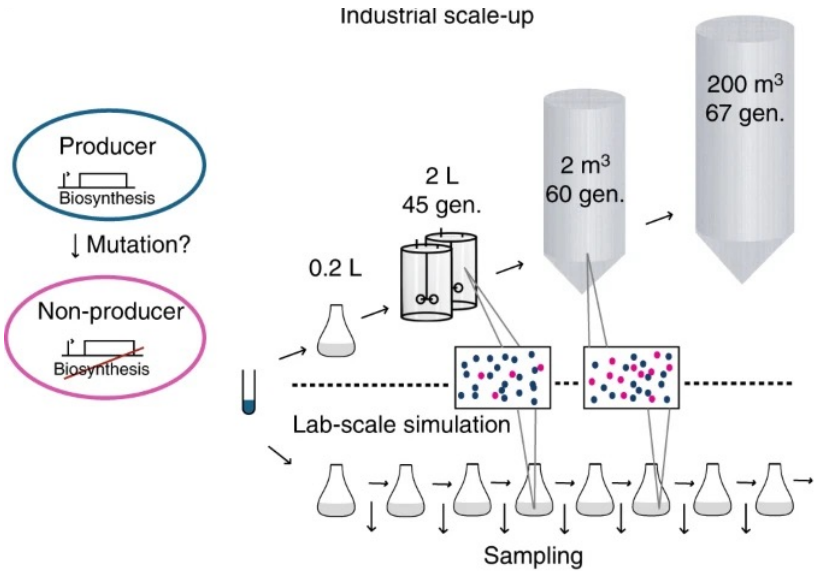
Lawrence Berkeley National Laboratory
National Renewable Energy Laboratory
Los Alamos National Laboratory

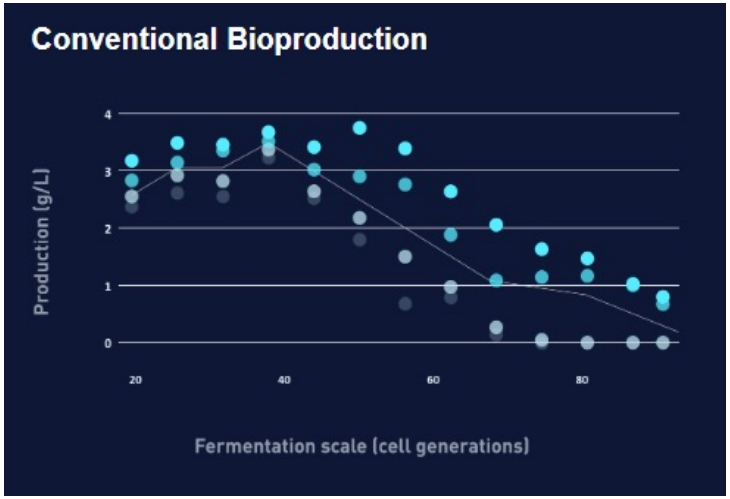
FY23 – FY24

Project Overview

Biological Heterogeneity -

Large scale fermentations tend to select for Non-producers





Rugbjerg et al, 2018. Diverse genetic error modes constrain biological production, Nature Communications

Project Overview

Enduro Genetics' Technology Aims at Reducing Biological Heterogeneity at Scale

Location: Copenhagen, Denmark

Mission: Service and tech provider focusing on Improving large-scale fermentation

History: Peter met JP Prahl at RAFT conference in November 2019 and discussed ABF DFO opportunities. Enduro was initially funded by Novo Nordisk Foundation Center for Biosustainability, TU Denmark

DFO proposal objective: To demonstrate Enduro's technology with ABF partners in a 3rd party location in novel hosts: Enduro's *B. subtilis* and ABF's *P. putida* (previously *C. glutamicum*). DFO was funded in August 2020

ENDURO

Peter Rugbjerg (CEO)

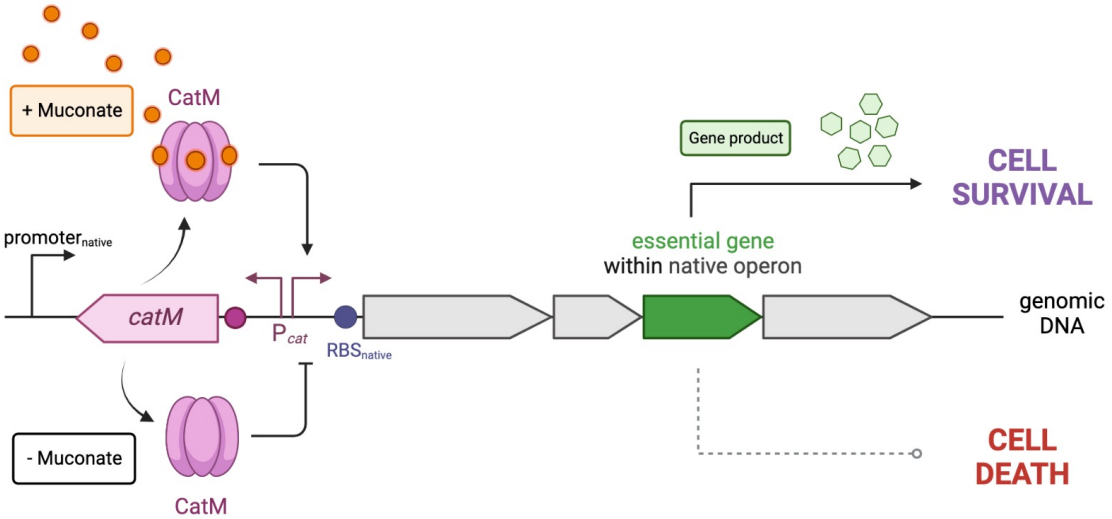
ABPDU

JP Prahl (Pr. Process Engineer)

RAFT®
Recent Advances in Fermentation Technology

1 – Approach

- **Solution: By coupling growth and production, cells are forced to continue making bioproducts to survive**
- 7 genes essential to survival ("**essential genes**") were selected based on 1) literature and 2) prior experience in *E. coli* to be expressed under the control of the muconate biosensor.
- The genomic context is preserved such that the sensor, CatM transcription factor, is placed between the native promoter and RBS of the essential gene.



1 – Approach

- The original proposal suggested engineering in *C. glutamicum* and *P. putida*.
- A key **Go / No-Go Milestone** decision was made to shift focus to exclusively *P. putida*
- *P. putida* has better genetic tractability and significantly higher TRY values for muconate production compared to *C. glutamicum*.
- Enduro, as a part of their cost share, developed a B. subtilis that was tested in fermentation studies at the ABPDU

Organism	Titer (g/L)	Rate (g/L/h)	Molar yield (%)	Carbon source
C. glutamicum	~1	No data	3-4	Glucose
P. putida	33.7	0.18	46	Glucose & xylose

1 – Approach

- **High Throughput R&D**
  - Tech transfer
  - Process optimization
  - Strain screening
  - Non-optimized strain vs. Optimized
- **Scale-up to 300L bioreactor**
  - Non-optimized strain vs. Optimized

Enduro Team at ABPDU

On-Site Visit for Planning & Kick-off for Enduro's B. subtilis strain

Left: Peter Rugbjerg and JP Prahl discussing the first Ambr®250 experiment in Spring 2022. **Right:** Kristoffer Falkenberg and ABPDU team after 300L pilot scale demonstration in Fall 2022.

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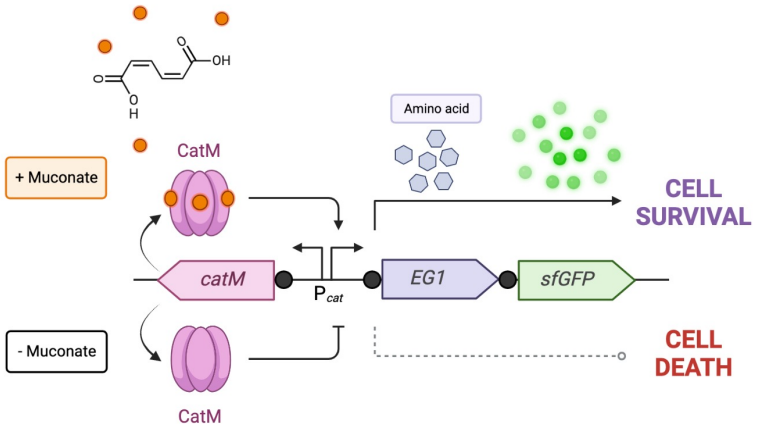
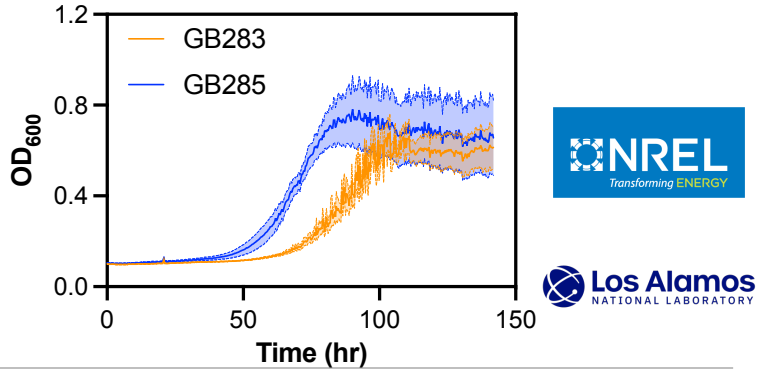
2 – Progress and Outcomes

- Preliminary results using a gene essential to amino acid biosynthesis (*EG1*) demonstrates a clear growth advantage when *EG1* expression is controlled by P_{cat} in a muconate production strain (**CJ522**).

Strain	Genotype
GB283	CJ522 + pBTL2-CatM:P_{cat}:*sfGFP*
GB285	CJ522 Δ*EG1* + pBTL2-CatM:P_{cat}:*EG1*:*sfGFP*





2 – Progress and Outcomes

- Homologs of the proposed essential genes have been evaluated using transposon (Tn) knockout studies in related bacterium, *Pseudomonas aeruginosa.*
- Of the 7 essential genes chosen, 3 are ready to be integrated into the *P. putida* genome for testing.
- The biosensor is intended for strains CJ522 and KH083. CJ522 utilizes glucose and is a poor muconate producer as compared to KH083. KH083 utilizes glucose and xylose and is an excellent muconate producer with higher TRY values than CJ522.

Essential gene (EG)	Target	Status
EG 1	Amino acid biosynthesis	Being built
EG 2	Transcription	Ready for integration
EG 3	Transcription	Ready for integration
EG 4	Lipid metabolism	Ready for integration
EG 5	Nucleotide synthesis	Being built
EG 6	Folate synthesis	Being built
EG 7	-	Being built
EG 7	Cell wall biosynthesis	Being built

2 – Progress and Outcomes

Date	Platform	Objective	Status
	Track #1 - Task# 1 - 3		
	T: Characterization of three Bacillus strains in 24 X 250 mL bioreactors (ABPDU)		
	O: Deep-seq analysis of population heterogeneity. Identification of lead strain for scale-up		
May 2022	1st Ambr®250 Campaign	R&D	Completed
June 2022	2nd Ambr®250 Campaign	R&D	Completed
July 2022	3rd Ambr®250 Campaign	R&D	Completed
	Track# - Task# 1 - 4		
	T: Scale-up lead strain to 300L campaigns; compare with non-optimized strain (ABPDU)		
	O: (i) Deep sequencing analysis of population heterogeneity. Impact of product addiction demonstrated in commercial Bacillus and (ii) Enduro site visit.		
October 2022	1st ABEC 300L Campaign	Demonstration	Completed
November 2022	2nd ABEC 300L Campaign	Demonstration	Completed
January 2023	3rd ABEC 300L Campaign	Demonstration	Completed
Summer 2023	4th ABEC 300L Campaign	Demonstration	Pending

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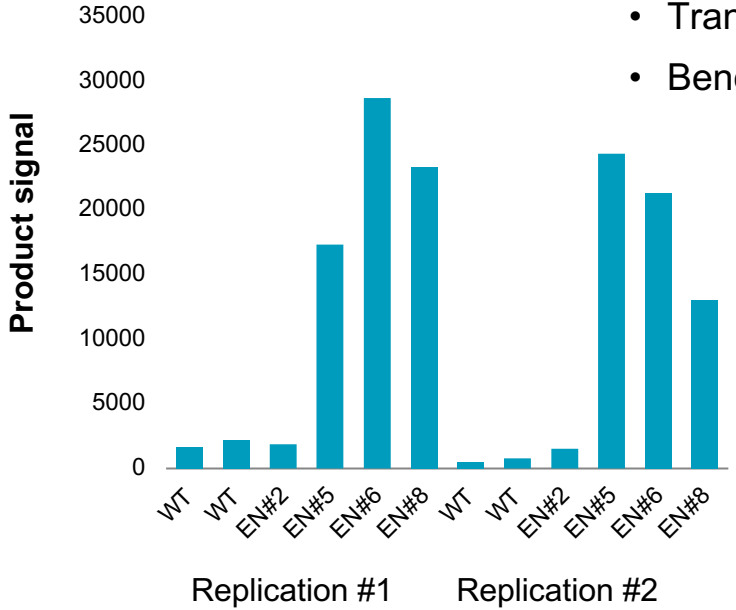
3 – Impact

R&D: Engineered Strains outperformed Wildtype in 250 mL bioreactors



Demo: Work in Progress

- Transfer of Enduro Strains from 0.25 L to 300L is completed
- Benchmarking Wildtype at 300L is Pending

Expected Impact and Dissemination

- First pilot scale dataset
- Complementary to Enduro R&D
- Peer-Review Publication

EN = Enduro Strains

WT = Wildtype

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Summary

- Out of the seven essential genes, three are ready to be integrated into the *P. putida* genome for testing.
- Other genes are being built
- Enduro's *B. subtilis* strain outperformed wild-type in 250 mL Ambr studies
- Preliminary results indicate significant performance of Enduro's *B. subtilis* strain over wild-type at 300L scale as well.
- Fermentation studies of *P. putida* will commence in fall 2023.

Quad Chart Overview

Timeline
- *Project Start Date: 10/01/2021*
- *Project End Date: 09/30/2023*

	FY22 Costed	Total Award
DOE Funding	*(10/01/2021 – 9/30/2022)* *250K*	*(negotiated total federal share)* *500K*
Project Cost Share *Only fill out if applicable.	100K	172K

Project Goal

Porting and scaling product addiction, followed by culture heterogeneity analysis in commercial Bacillus strain. Adapting muconate biosensor to P. putida (C. glutamicum) for porting and scaling product addiction and culture heterogeneity analysis.

End of Project Milestone

Deep sequencing analysis of population heterogeneity. Impact of product addiction demonstrated in P. putida (previously C. glutamicum)

Funding Mechanism

Agile Biofoundry DFO FY20

Project Partners*
- Lawrence Berkeley National Laboratory
- National Renewable Energy Laboratory
- Los Alamos National Laboratory