DOE Bioenergy Technologies Office (BETO)
2019 Project Peer Review

Second-Generation Mixotrophy for Highest Yield and Least Expensive Biochemical Production

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Biochemical Conversion

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

Goal Statement

- The goal for this project is to develop and demonstrate a fermentation process to produce acetone from a cellulosic feedstock at a mass yield at least 130% the previous theoretical maximum.
- This is a **platform technology** that can be adapted to almost any biochemical or biofuel of interest.
- Improving carbon yields from expensive cellulosic sugars can help lower the operating costs of cellulosic-based processes and lead to greater adoption of the technology.

Quad Chart Overview

Timeline

- September 1, 2016
- December 31, 2018
- 100%

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$230k	$544k	$567k	$160k
Project Cost Share*	$58k	$138k	$186k	$48k

• Partners: No partners received funding but worked with different cellulosic producers

Barriers addressed

- Ct-D – Advanced Bioprocess Development
- Ct-L – Decreasing Development Time for Industrially Relevant Microorganisms

Objective

Demonstrate a process to produce acetone from cellulosic hydrolysates at improved mass yields

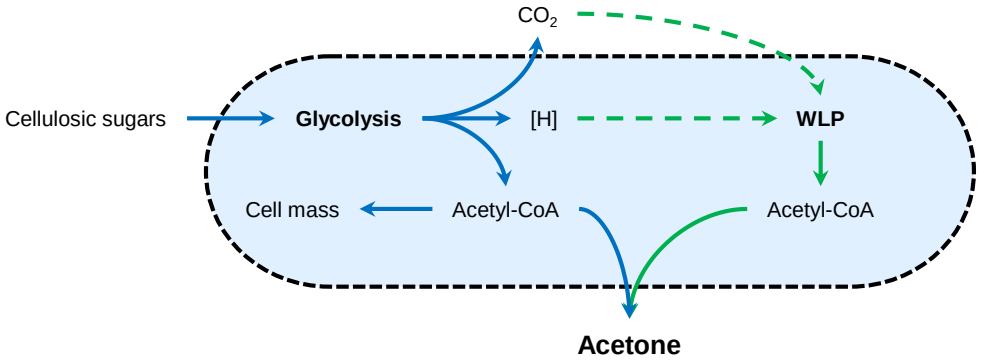
End of Project Goal

A 3L demonstration fermentation with an acetone yield of 43wt%, 40 g/L titer, and a productivity of 3 g/L/hr

1 – Project Overview

The underlying technology is WDL's **MixoFerm™ platform**.

MixoFerm™ combines aspects of both conventional fermentation and gas fermentation into a single microbe. The microbes are able to fix CO_2 produced during catabolism of sugar to produce more product and increase *Carbon Yields*.



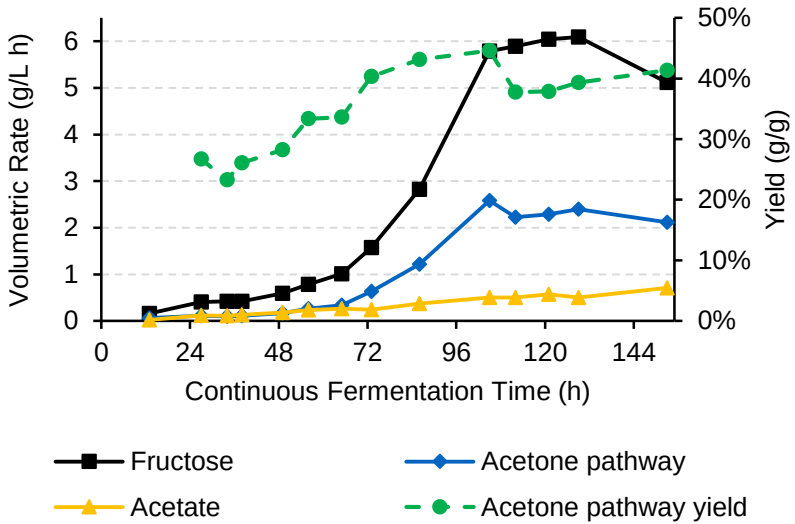
By improving yields from cellulosic sugars, we can improve the overall economics of a cellulosic biorefinery.

Demonstrate improved performance on cellulosic sugars

1 – Project Overview

Demonstrate proof-of-principle:

- Constructed a plasmid-based strain to produce acetone and a continuous, cell-retention process
- Preferred C6 sugar is fructose
- Achieved >90% MixoFerm maximum yield



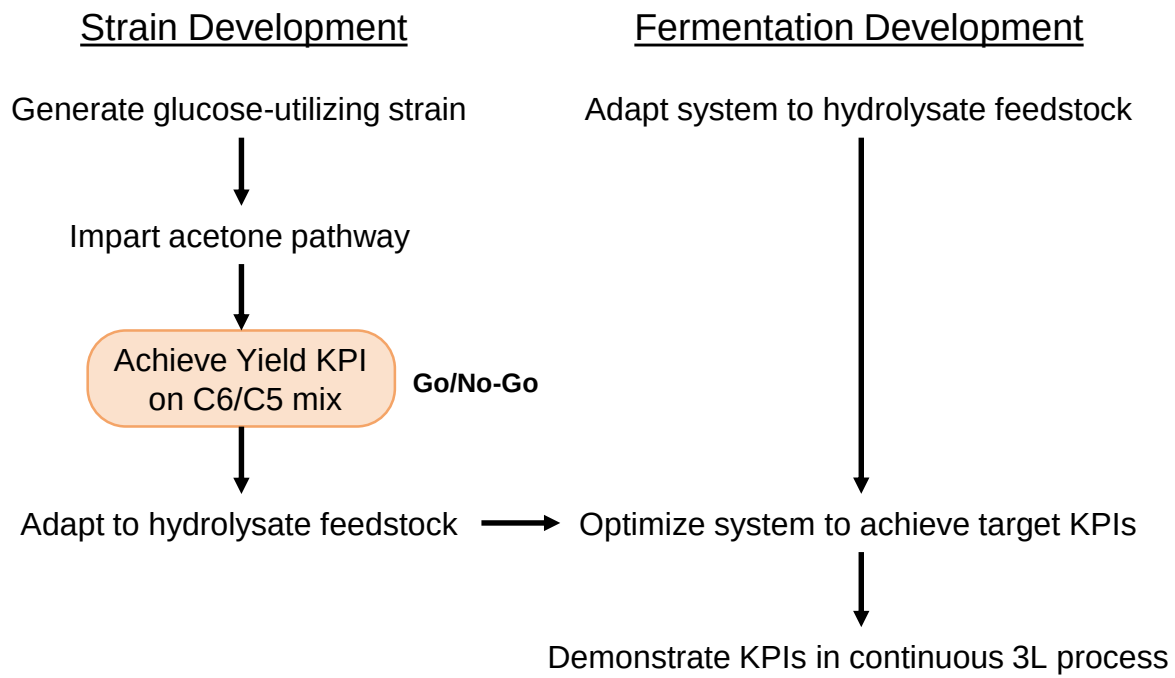
Issues addressed in project:

- Construct a fully integrated strain (no plasmids) that could consume all C6 and C5 sugars
- Adapt cell-retention system to use cellulosic hydrolysates
- Achieve stable, long-term production of acetone

2 – Approach (Management)

- WDL performed all tasks in this project
- Two WDL locations:
  - Microbiology lab – strain selection and improvement
  - Fermentation facility – fermentation optimization and SCP production and processing
- Weekly meetings were held between the PI and project leads
- Monthly project update meetings of all team members were held to assess progress and discuss critical issues
- Updated DOE program manager quarterly on progress and any delays

2 – Approach (Technical)



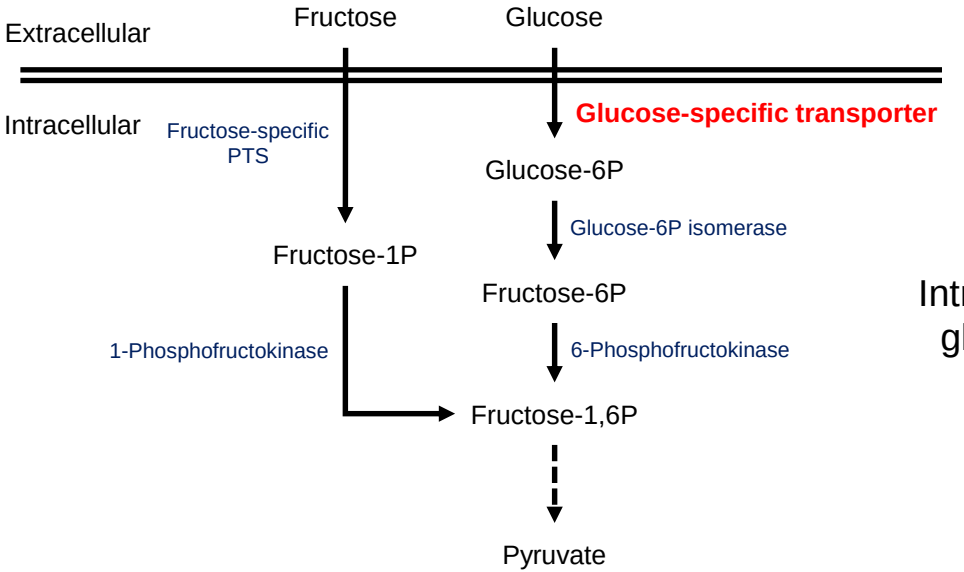
Major Challenges

1. Adapting fermentation system to use hydrolysate feedstocks which contain higher solids content. Solids could clog cell-retention system → Modify Cleaning In Place (CIP) procedures or perform more frequently

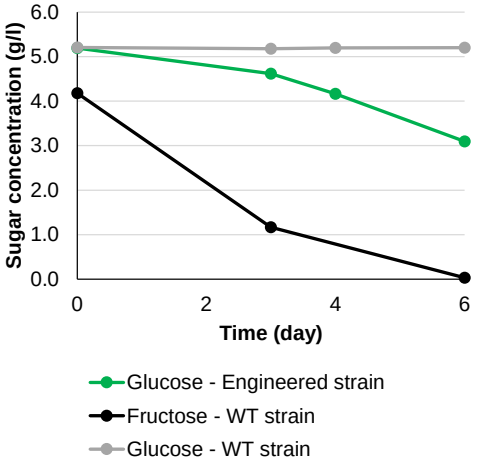
2. Adapting strain to grow on hydrolysates. Hydrolysates can contain inhibitory molecules → Adaptively evolve strain to overcome inhibition

3 – Technical Accomplishments

Generate glucose-utilizing, acetone-producing *C. ljungdahlii* strain



Introduced recombinant glucose-specific PTS genes

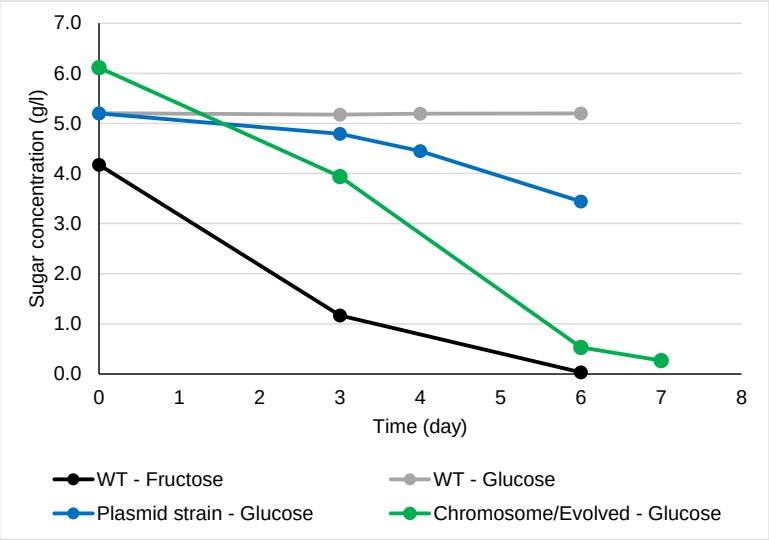


Permanently integrated the best PTS gene into the chromosome

3 – Technical Accomplishments

Generate glucose-utilizing, acetone-producing *C. ljungdahlii* strain

Evolved integrated strain in chemostat to select for faster growing/faster consuming strain



Estimated sugar consumption rates:

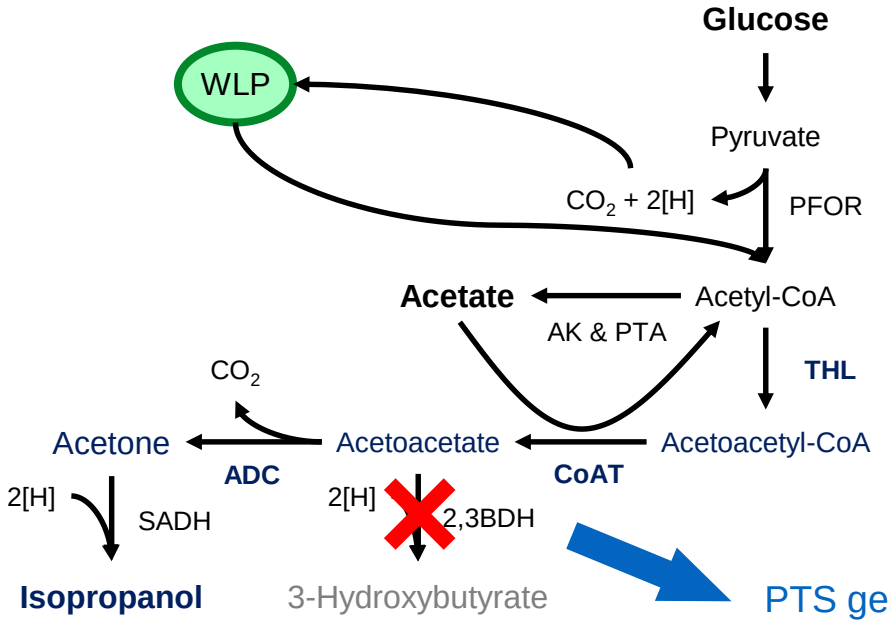
- WT on fructose – 0.042 g/L/hr
- Plasmid strain on glucose – 0.012 g/L/hr
- Evolved strain on glucose – 0.047 g/L/hr

Next step: introduce the acetone pathway

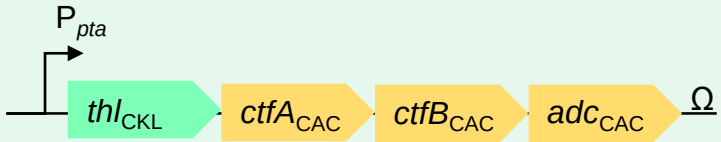
3 – Technical Accomplishments

Generate glucose-utilizing, acetone-producing *C. ljungdahlii* strain

Transformed glucose strain (*Clj* ΔBDH::CSB-PTS) with acetone pathway plasmid



Expressed on a plasmid:



PTS gene integrated into the 2,3BDH locus

3 – Technical Accomplishments

Generate glucose-utilizing, acetone-producing *C. ljungdahlii* strain

		Product profile				
Strain	Sugar consumed	Isopropanol	Acetone	3-Hydroxybutyric acid	Acetic acid	Solvent yield (g/g)
Clj (pTCtA-Ckl)	9.38 g/L (fructose)	2.72 g/L	0.58 g/L	0.06 g/L	1.63 g/L	33.4%
Clj ΔBDH::CSB-PTS (pTCtA-Ckl)	8.94 g/L (glucose)	2.22 g/L	0.43 g/L	0.00 g/L	2.66 g/L	29.7%

Transformation efficiency of *Clj* ΔBDH::CSB-PTS was significantly reduced to ~10 CFU/µg DNA.

Sequencing identified several SNPs in membrane synthesis genes, but no obvious correlation with reduced transformation efficiency.

Next step: delete *sadh* gene for acetone production

3 – Technical Accomplishments

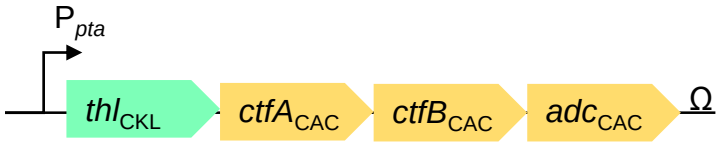
Generate glucose-utilizing, acetone-producing *C. ljungdahlii* strain

Despite many attempts and modifications to our transformation protocol, we were unable to transform *Clj* ΔBDH::CSB-PTS with a deletion plasmid for *sadh*.

Alternative strategy – integrate acetone pathway in WT *Clj* and then evolve for glucose utilization

Targeted two different loci for integration:

- *sadh* – needed to be deleted for acetone production; previously successful in deleting gene
- *adhE2* – part of ethanol pathway; previously successful in integrating large DNA fragments into locus



3 – Technical Accomplishments

Generate glucose-utilizing, acetone-producing *C. ljungdahlii* strain

Successfully integrated operon into *adhE2* locus to generate *Clj* ΔADHE2::A04

	Product profile						
Strain	Fructose consumed	Isopropanol	Acetone	3-Hydroxybutyric acid	Acetic acid	Solvent yield (g/g)	Operon copy #
Clj (pTCtA-Ckl)	5.24 g/L	1.35 g/L	0.13 g/L	0.14 g/L	1.83 g/L	30.1%	~8
Clj ΔADHE2::A04	4.16 g/L	0.20 g/L	0.07 g/L	0.11 g/L	3.74 g/L	9.1%	1

When compared to the plasmid strain *Clj* (pTCtA-Ckl), *Clj* ΔADHE2::A04 had much lower acetone pathway products.

While a drop in production was expected a drop in copy number, we did not anticipate the drop to be so significant.

Still working to understand the behavior of integrated strain

3 – Technical Accomplishments

Adapting *C. ljungdahlii* to grow on cellulosic hydrolysates

WDL acquired hydrolysates from four commercial producers for testing:

- Renmatix – two hydrolysates (a C6 sugar hydrolysate and a C5 sugar hydrolysate)
- Sweetwater – one hydrolysate (a blend of C6 and C5 sugars)
- BetaRenewables – one hydrolysate (a blend of C6 and C5 sugars)
- American Process Inc. (API) – two hydrolysates (a C6 sugar hydrolysate and a C5 sugar hydrolysate)

Tested sugars in standard media at ~15 g/L total sugars with *Clj* ΔBDH::CSB-PTS.

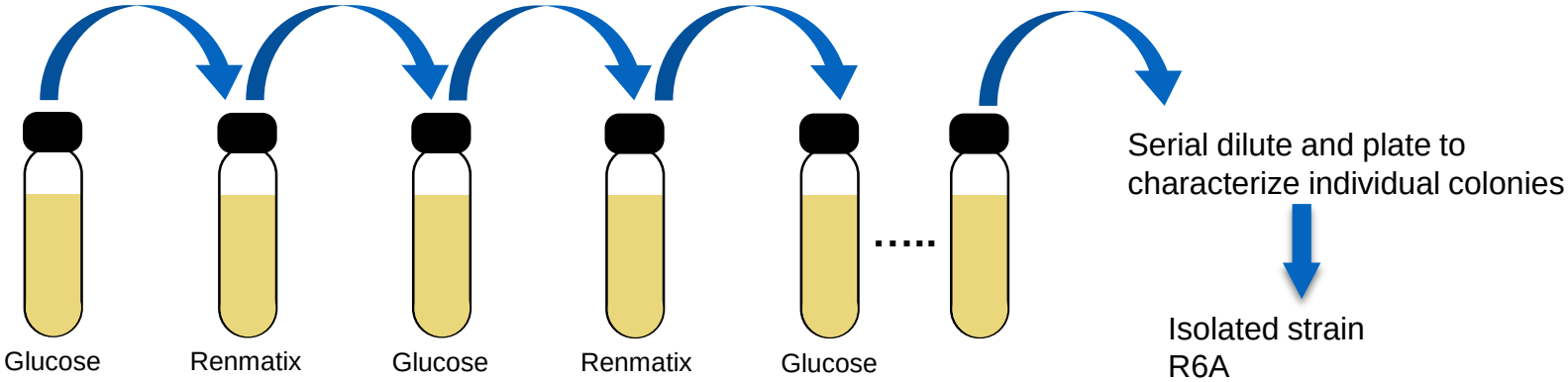
	Concentration (g/L)		
Sugar feedstock	C6 consumed	C5 consumed	Total sugar consumed
Glucose	8.39	0.00	8.39
Renmatix-C6	2.48	0.17	2.66
Renmatix-C5	0.60	4.81	5.42
Sweetwater	3.40	3.23	6.62
BetaRenewables	2.85	3.02	5.87
API-C6	5.30	1.14	6.44
API-C5	1.47	4.90	6.37

Cultures grown on **Renmatix-C6 hydrolysate** grew the least of the hydrolysates tested.

3 – Technical Accomplishments

Adapting *C. ljungdahlii* to grow on cellulosic hydrolysates

Chose to adaptively evolve the strain on Renmatix-C6 hydrolysate



3 – Technical Accomplishments

Adapting *C. ljungdahlii* to grow on cellulosic hydrolysates

Tested isolated strain under same conditions as before (~15 g/L total sugar)

	Concentration (g/L)
Sugar feedstock	Total sugar consumed
Glucose	8.39
Renmatix-C6	2.66
Renmatix-C5	5.42
Sweetwater	6.62
BetaRenewables	5.87
API-C6	6.44
API-C5	6.37

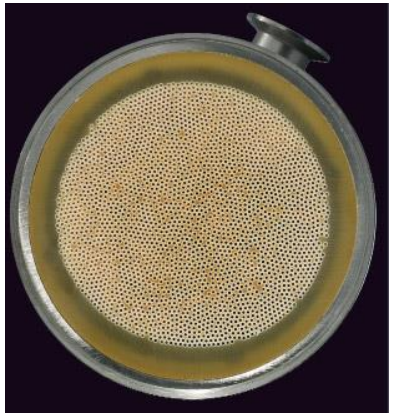
	Concentration (g/L)		
Sugar feedstock	C6 consumed	C5 consumed	Total sugar consumed
Glucose	9.76	0.00	9.76
Renmatix-C6	4.93	1.26	6.18
Renmatix-C5	0.94	6.99	7.93
Sweetwater	6.70	3.34	10.05
BetaRenewables	4.22	4.66	8.88
API-C6	6.29	1.31	7.60
API-C5	1.26	7.17	8.43

Evolved strain showed improved performance on all hydrolysates

3 – Technical Accomplishments

Optimization of cell-recycle fermentation

Cell-recycle was using a **hollow fiber membrane**.



Membrane would quickly become clogged with solids in hydrolysate.

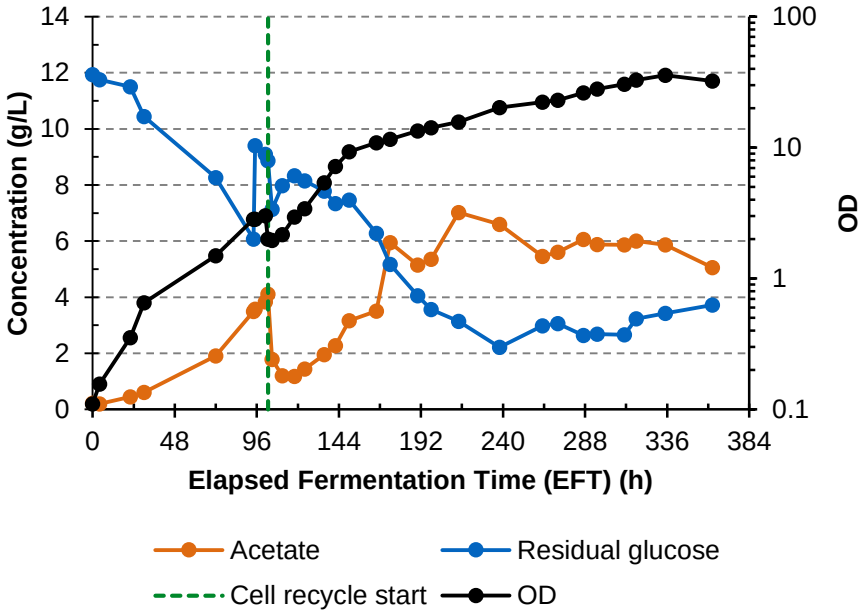
System has larger inlets and operates under higher pressure to reduce fouling.

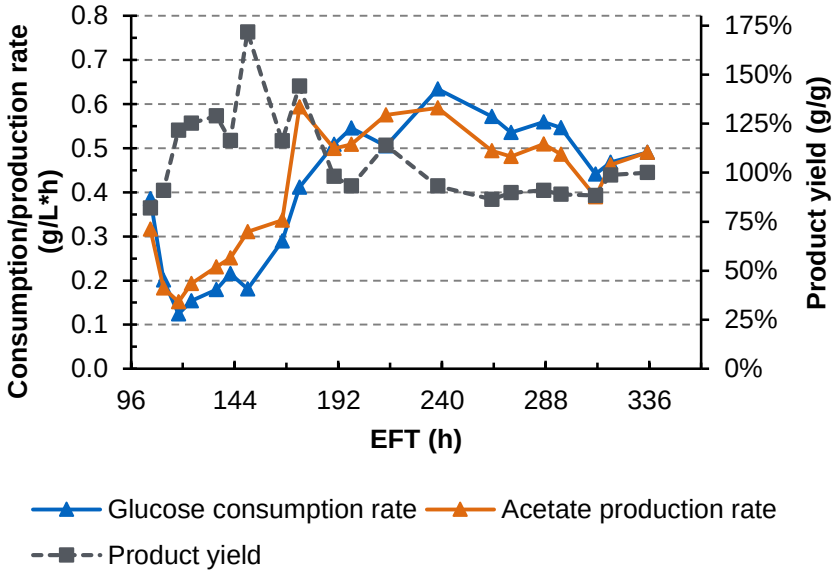
Decided to use the Graver system

3 – Technical Accomplishments

Optimization of cell-recycle fermentation

Operated system with R6A strain and Renmatix-C6 hydrolysate



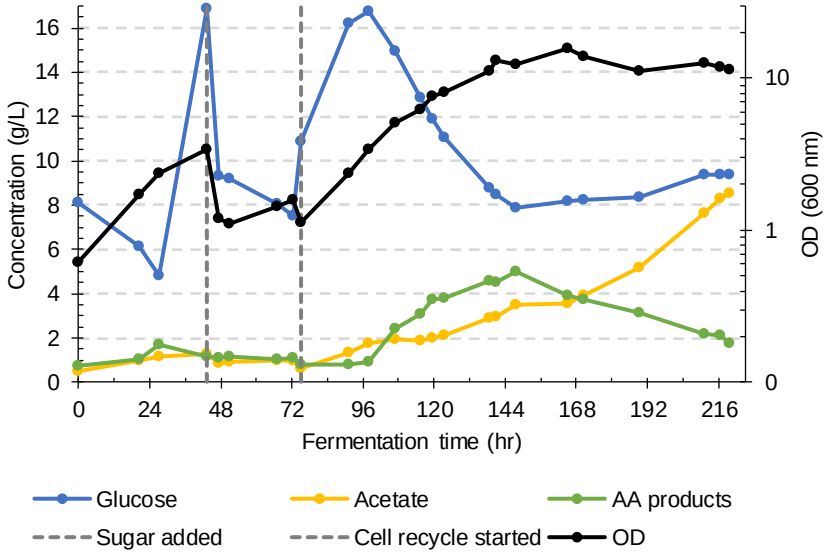


Achieved >250 hrs of continuous operation

3 – Technical Accomplishments

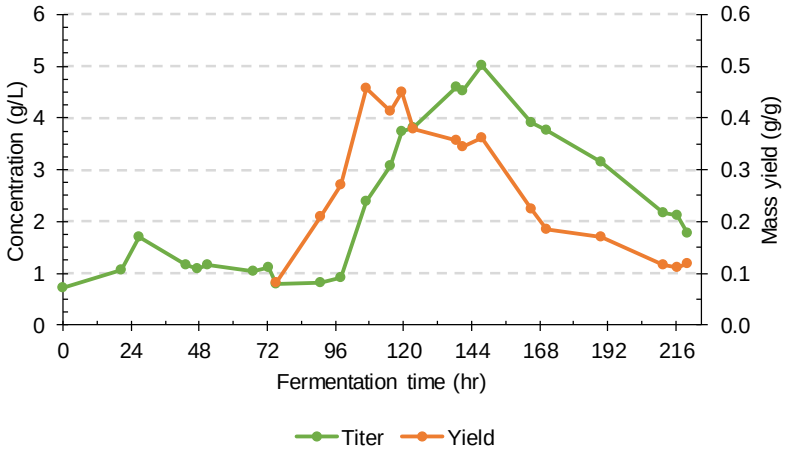
Optimization of cell-recycle fermentation

Tested bioprocessing parameters with *Clj* ΔBDH::CSB-PTS (pTCtA-Ckl) and glucose feedstock



- Cell recycle initiated and OD grew to >10
- Peak product titer of 5 g/L with a yield of about 0.45 (g/g)

After 144 hours, lost productivity (drop in AA product titer and rise in acetic acid).

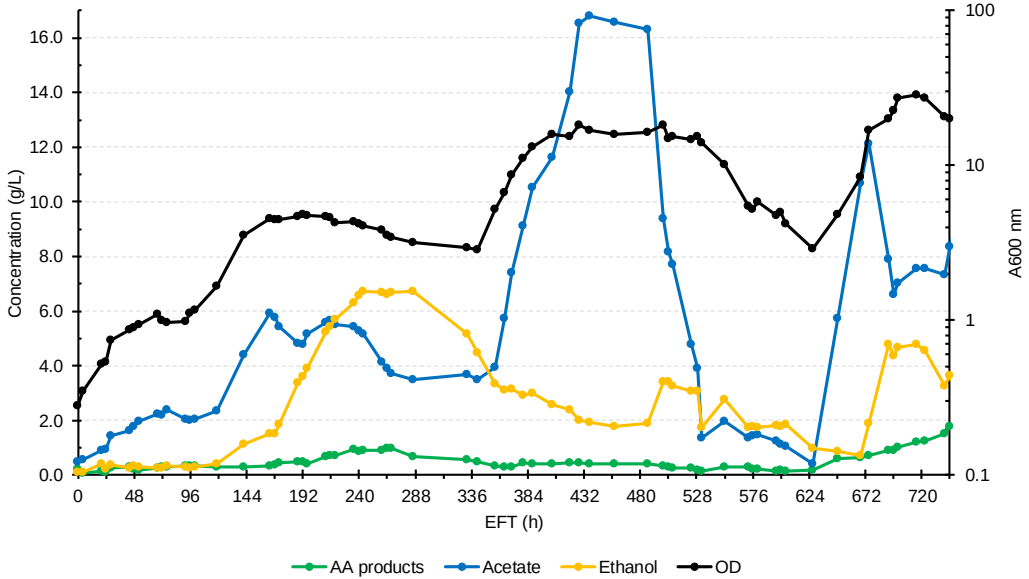


Major issue across multiple runs was loss of AA productivity

3 – Technical Accomplishments

Optimization of cell-recycle fermentation

Tested bioprocessing parameters with *Clj* ΔADHE2::A04 and fructose feedstock



Retained AA pathway production but unable to increase titer by bioprocessing parameters

Still working to maintain high productivity and yield

4 – Relevance

Lower operating costs of cellulosic-based processes by **improving carbon yields** of biochemicals and biofuels

- Addresses a critical issue identified by BETO:
  "Develop strategies for **conserving carbon** and hydrogen in conversion and upgrading processes"
- Improving carbon yields can directly lower operating costs and thus costs of final fuels by producing more unit product per unit feedstock.
- This is a ***platform technology*** to produce a wide array of biofuels and biochemicals using a common chassis microorganism and process. Success will demonstrate a proof-of-concept process to be replicated and adapted to other specific fuel molecules.
- Goal is to demonstrate industrially-relevant production metrics (3 g/L/hr productivity and 40 g/L titer) at the 3 L fermentation-scale.

Increase efficiency of fermentation processes

5 – Future Work

- The project work is completed
- WDL is continuing to develop its biochemical MixoFerm platform with industrial partners

We have an on-going Joint Development Agreement (JDA) with **Air Liquide** with the MixoFerm platform.

As one of the largest industrial gas suppliers in the work, Air Liquide is particularly interested in advancing the use of gas addition to fermentations to improve product yields.

Summary

- Goal was to develop a cellulosic acetone process using WDL's MixoFerm technology for enhanced mass yields
- Successfully generated a glucose-consuming *C. ljungdahlii* strain but had difficulties engineering a fully integrated strain
- Adapted both *C. ljungdahlii* and our cell-recycle platform to use cellulosic hydrolysates
- Remaining issues:
  - Achieving high acetone production in a fully integrated strain
  - Maintaining high productivities and yield of acetone during fermentation

Additional Slides

Responses to Previous Reviewers' Comments

Previous Reviewers' Comments

- "The business decision of focusing on acetone needs to be revisited, as this commodity chemical will be hard to replace with a biologically derived one."

  Response: Acetone is a difficult market to compete in. As discussed, there is a high-quality premium market we can enter since our product does not come into contact with phenol during the production process. However, in the commercialization efforts with Air Liquide and the other European partner, we are focusing on products other than acetone. The proof-of-concept data using acetone did convince them that the platform works, and they have specified other desirable products target.

- "The approach to future work looks oversimplified and overly optimistic."

  Response: We were aware of the difficulties of the project, and considered multiple approaches to achieve success. However, in hindsight, there were multiple challenges to different aspects of the project which has hindered success. Despite these, we have made great progress on both the strain and fermentation and continue to develop both under commercial partnerships.

Publications, Patents, Presentations, Awards, and Commercialization

Patents:
“Mixotrophic fermentation method for making acetone, isopropanol, butyric acid and other bioproducts, and mixtures thereof.” US 9,938,542 B2.

Publications:
Maru, B.T., Munasinghe, P.C., Gilary, H., Jones, S.W., & Tracy, B.P. 2018. “Fixation of CO_2 and CO on a diverse range of carbohydrates using anaerobic, non-photosynthetic mixotrophy.” *FEMS Microbiol Lett* 365(8).

Presentations:
“Improving biochemical yields with MixoFerm.” Presented at Microbial Engineering conference on March 5, 2018.
“Production of biochemicals and biofuels with no CO_2 production and improved product yields.” Presented at Biochemical and Molecular Engineering XX conference on July 19, 2017.

Publications, Patents, Presentations, Awards, and Commercialization

Commercialization:
Joint Development Agreement with Air Liquide to pursue biochemical production with MixoFerm platform (in Phase I with a Go/No-Go decision on Phase II April 2019)

Joint Development Agreement with major European petrochemical company (prefers not to be named) to produce a specific biochemical with MixoFerm platform