DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Improving tolerance of yeast to lignocellulosic-derived feedstocks and products

Mar. 5, 2019
Biochemical Conversion

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Goal Statement

Goal: Engineer **tolerance** to **lignocellulosic hydrolysates** in yeast ***S. cerevisiae***, the industry-dominant biocatalyst

Outcome: Genetically-enhanced strains and fermentation parameters capable of:

- **Ethanol** (EtOH) titers **>100 g/L** from **wide range** of **high toxicity**, **pretreated biomass**
- **C6** (glucose) and **C5** (xylose) **sugar** utilization
- Producing plastic precursor **monoethylene glycol** (MEG) and other non-EtOH products from lignocellulose

Relevance:

- Combined **feedstock** + **product toxicity** inevitable issue for any **high volume** product (eg., alcohols) from **inexpensive lignocellulose** (eg., dilute acid pretreatment)
- Higher tolerance **enables scale** via **reduced detoxification** + greater robustness to **feedstock variety**, **production levels**, compatibility with **established fermentation infrastructure**

Quad Chart Overview

Timeline

- Project start: **Oct. 2016**
- Project end: **Mar. 2020**
- Percent complete: **60–70%**

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$0	$325k	$393k	$782k
Project Cost Share (MIT)	$50k	$1k	$191k	$199k

Barriers Addressed

- **Ct-D.** Advanced Bioprocess Development
- **Ct-E.** Improving Catalyst Lifetime

Objective

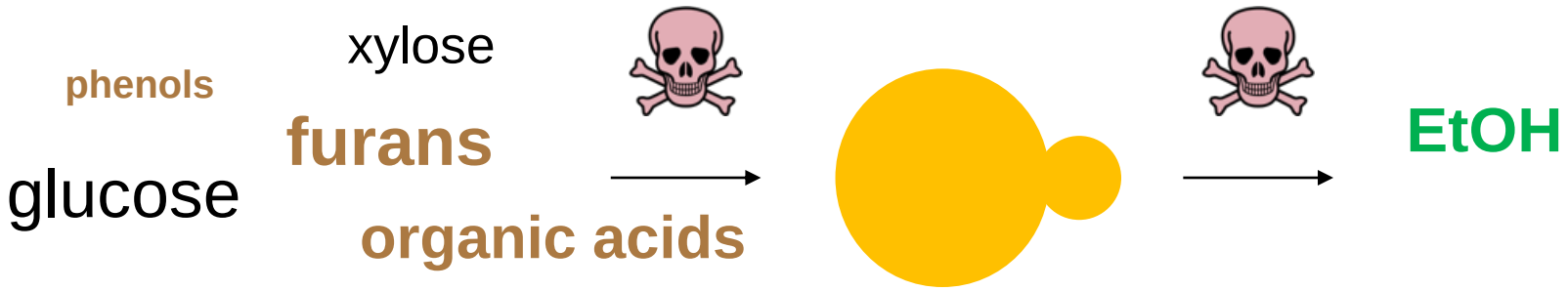
- Boost fermentation of high-toxicity hydrolysates via **enhanced biocatalytic tolerance**

End of Project Goals

- Yeast bioprocess tolerant to wide concentration range of inhibitor cocktails
- High performance cellulosic EtOH fermentation (2G) comparable to corn (1G)
- Technology demonstration of cellulosic monoethylene glycol (MEG)

1 - Project Overview

Background:

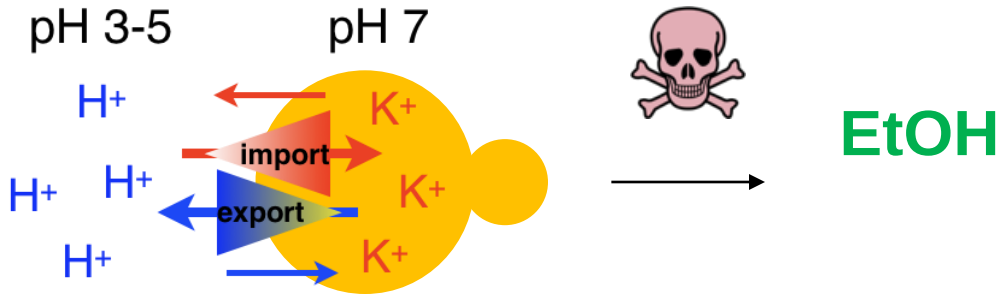


- Lignocellulosic fermentations exhibit **feedstock** + **product** **toxicity** to yeast biocatalysts
- Inhibitors *individually* sufficient to limit production; the combinatorial load exerts **synergistic effects**
- Inhibitors generally **attack cells** via **unidentified biological mechanisms**

1 - Project Overview

Previous Work (**product** toxicity):

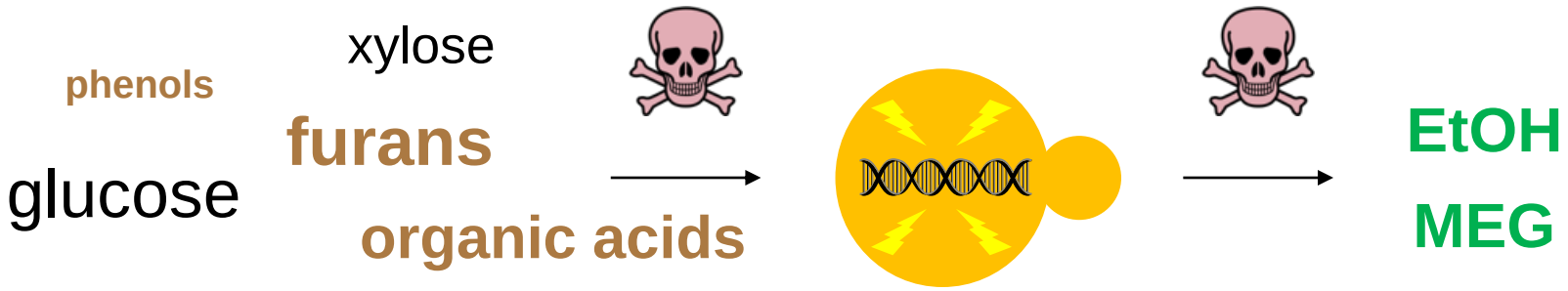
glucose



- **Upkeep of the plasma membrane potential** is a discrete, engineerable mechanism of **general alcohol tolerance** in yeast (Lam FH *et al., Science* 2014)
- Elevation of extracellular potassium K^+ + pH **strengthens membrane electrochemical gradients** → directly boost EtOH production, confers competitive advantage (Shaw AJ, Lam FH *et al.*, *Science* 2016)

1 - Project Overview

Current Work (**feedstock** toxicity):



Project Aims:

I. **Systematic deconstruction** of hydrolysate toxicity

II. Engineer **hydrolysate-tolerant strains** (cellulosic EtOH)

III. Assess **transferability** of hydrolysate **tolerance to non-EtOH product** (cellulosic monoethylene glycol / MEG)

Higher **tolerance** → higher **scale**
via **greater production** + **feedstock** range

2 – Approach (Management)

Prof. Greg Stephanopoulos, PI

Prof. Gerald Fink (Whitehead Institute), Project Collaborator

- Scientific guidance
- Financial, administrative oversight

Felix Lam, Lead Scientist

Constantinos Katsimpouras, Postdoctoral Associate

- Hydrolysate tolerance / cellulosic EtOH

Boonsom Uranukul, Graduate Researcher

- Cellulosic MEG

Weekly: Team and individual meetings (all members co-localized in same lab space for maximum interaction)

Quarterly: DOE reporting, assessment of project management plan (PMP), progress milestones

2 – Approach (Technical)

I. Systematic characterization of component hydrolysate toxicities

- Ferment wildtype yeast under increasing concentrations of individual and blends of inhibitors

➔ Equimolar dosing quantifies **relative toxicities**

➔ Combinations reveal **synergies of inhibition**

➔ Formulate **reference hydrolysate** for benchmarking in Aims II, III

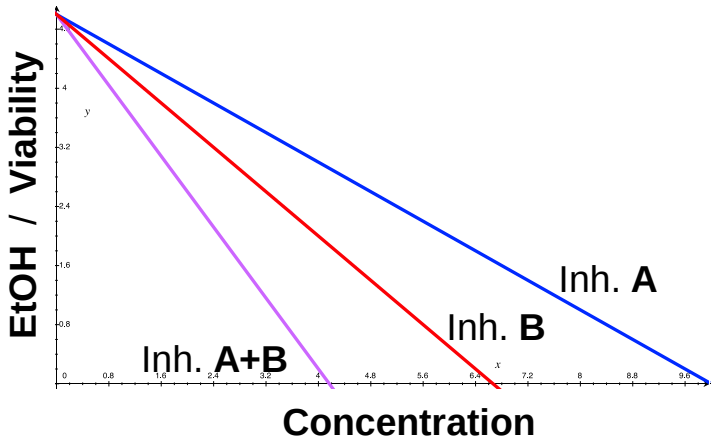
- Compare EtOH production vs. fermentation viability metrics

➔ Metabolic **inhibition** vs. **cell death**

Challenges: Few (characterization)

Critical Success Factors:

- Identify component with highest inhibition
- Insight into physiology underlying total toxicity



2 – Approach (Technical)

II. Delineation and engineering of enhanced hydrolysate tolerance

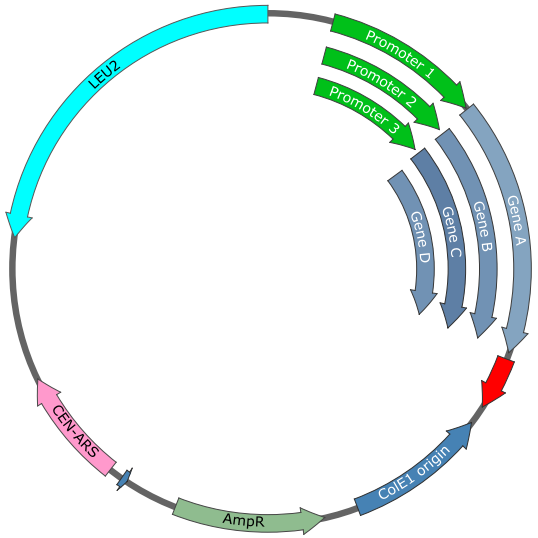
- Screen candidate **detoxification** genes (*forward* genetics)
- Screen candidate **multidrug efflux** pumps (*forward* genetics)
- **Mutagenesis** & **selection** for novel superior alleles (*inverse* genetics)
- Benchmark in reference hydrolysate **± adjustments strengthening membrane potential** (from prior work)

Challenges:

- Substrate specificity of candidate genes
- Strategy that mitigates <u>total</u> toxicity

Critical Success Factors:

- Genetic + fermentation parameters conferring cellulosic EtOH performance comparable to 1G EtOH (e.g., >100 g/L in 2-3 d)



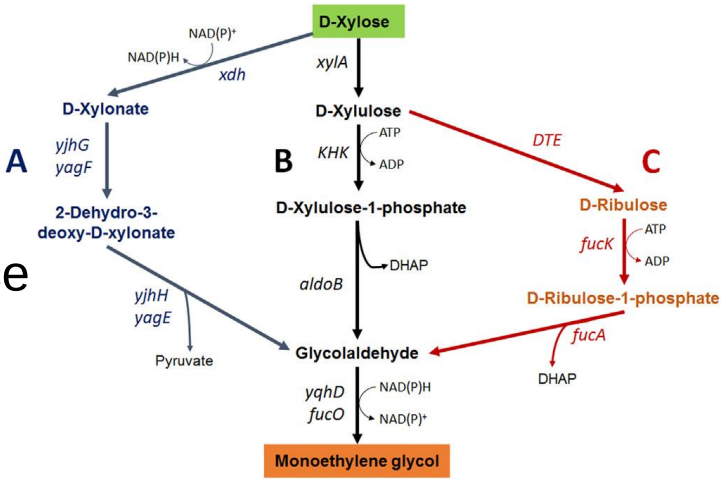
2 – Approach (Technical)

III. Engineering of cellulosic monoethylene glycol (MEG)

- **Prototype** bacterial **xylose** → **MEG** pathway in yeast
- Metabolic engineering to **delete competing fluxes**
- Metabolic engineering to **decrease EtOH byproduct**
- Add in genetic enhancements (Aim II) conferring **hydrolysate tolerance**

Challenges:

- Successful pathway expression, production of MEG (i.e., no inhibitors)
- Reduction of native EtOH flux
- Successful transfer of hydrolysate tolerance



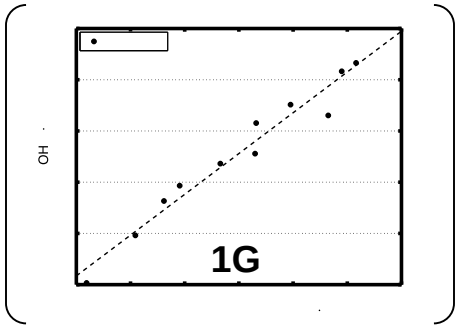
Critical Success Factors:

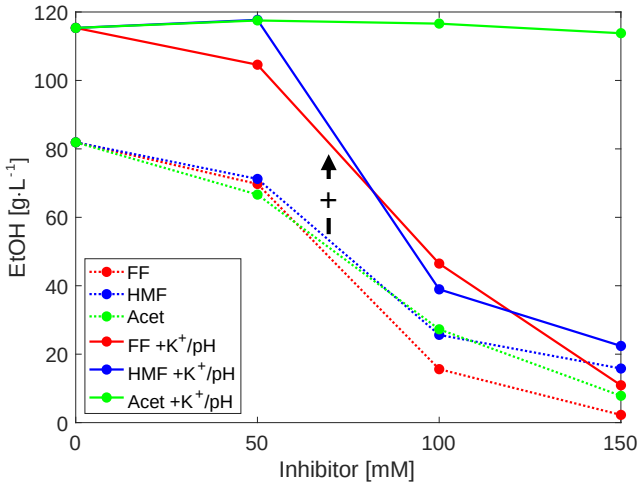
- 1–10 g/L MEG from xylose (no inhibitors)
- Proof-of-principle / any titer of cellulosic MEG

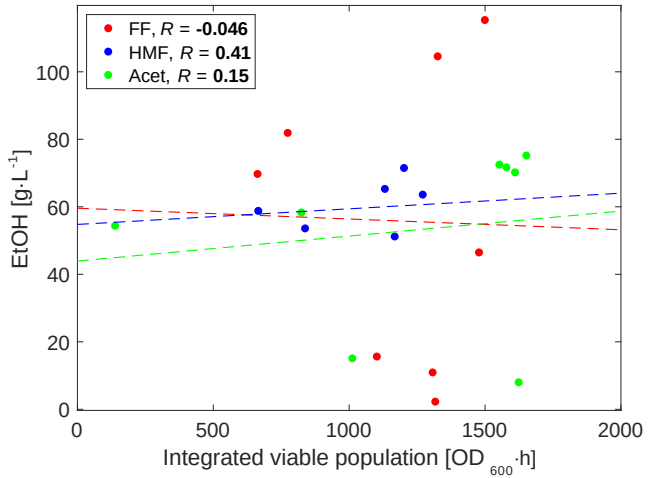
3 –Technical Accomplishments/Progress

I. Systematic characterization of component hydrolysate toxicities

- Determined toxicity order of top yeast-inhibitory components:
  1) **Furfural** (FF)
  2) **Acetic acid** (Acet)
  3) **5-Hydroxymethyl-furfural** (HMF)
- Elevated K^+ + pH confer protection
- Neutralized acetate completely non-toxic → FF, HMF primary culprits
- Unlike 1G EtOH, **NO correlation** between **EtOH** and **fermentation viability**







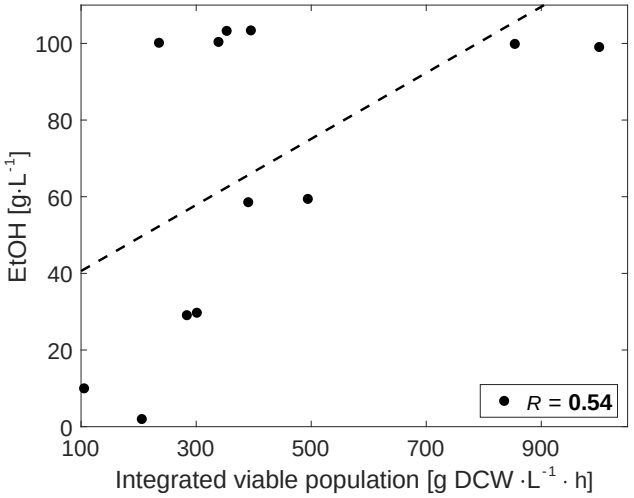
3 –Technical Accomplishments/Progress

I. Systematic characterization of component hydrolysate toxicities

- Inhibitor blends: also **no correlation** between **EtOH** vs. **fermentation viability**

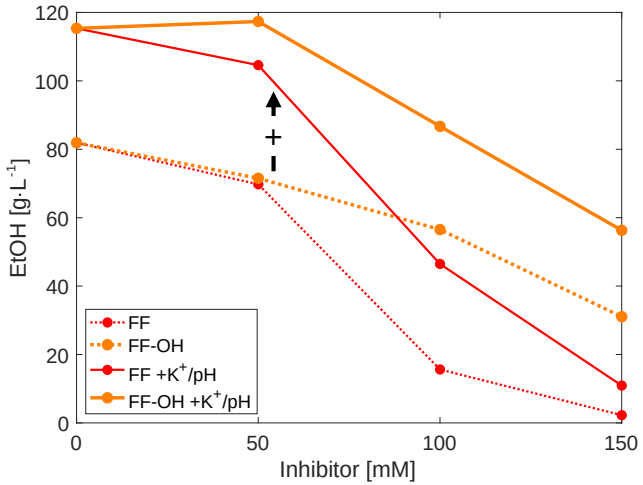
➔ Cells **metabolically inhibited**, **not dead**

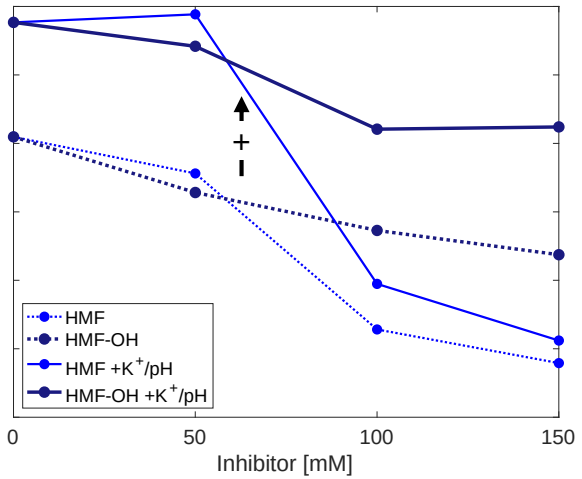
➔ If inhibition mitigated, production could potentially resume



- Comparing FF / HMF vs. FF-OH / HMF-OH, equivalent **alcohols much better tolerated!**

➔ *<u>Strategy</u>: express gene to **detoxify FF / HMF**, **elevate K$^+$ + pH**?*





3 –Technical Accomplishments/Progress

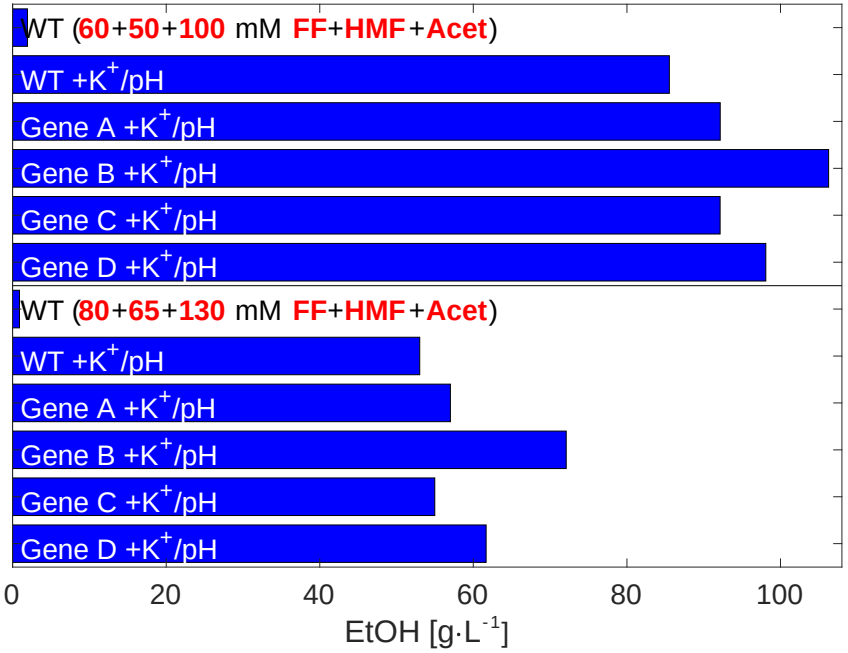
II. Delineation and engineering of enhanced hydrolysate tolerance

- Screened **native** and **heterologous** genes of:

a) Reductases – convert FF / HMF to FF-OH / HMF-OH

b) Multidrug efflux pumps – broad spectrum export of ring compounds

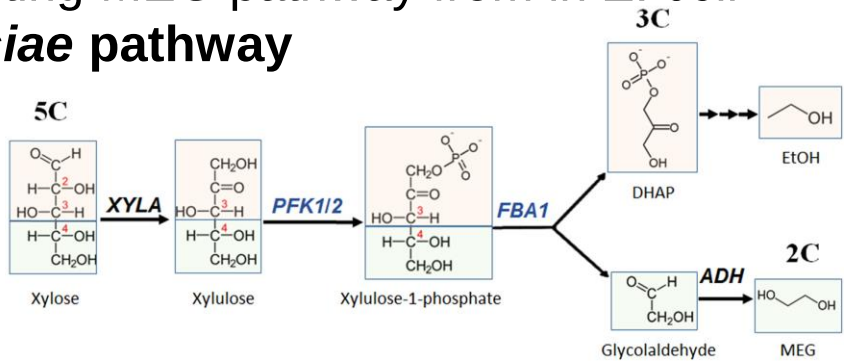
- Combined with strong promoters, several candidates increased EtOH in **full inhibitor** model **hydrolysate**
- Strain B achieved:
  - ➔ **>100 g/L EtOH** in average-strength hydrolysate
  - ➔ **24–36% gain** over WT

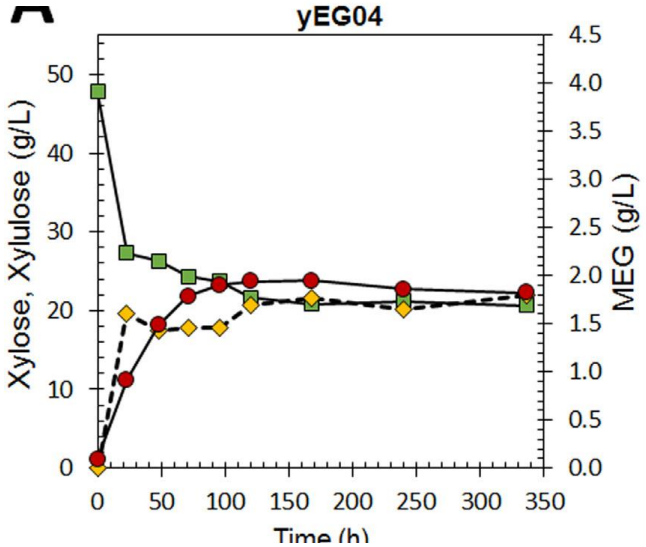


3 –Technical Accomplishments/Progress

III. Engineering of cellulosic MEG

- Started implementing MEG pathway from in *E. coli* → uncovered **native *S. cerevisiae* pathway**





- With genetic + process optimizations, achieved **4 g/L MEG** from xylose, **highest to-date** from yeast (no inhibitors)
- Published:
  Uranukul *et al., Metab Eng.* 2018

4 – Relevance

Goals

- Enhance **yeast tolerance** to **wide range** of **high toxicity biomass hydrolysates**
- Exceed current **cellulosic EtOH** tolerance of 72 g/L (MYPP, 3/2016)
- Technology demonstration of non-EtOH product: **cellulosic MEG**

Higher feedstock + product tolerance:

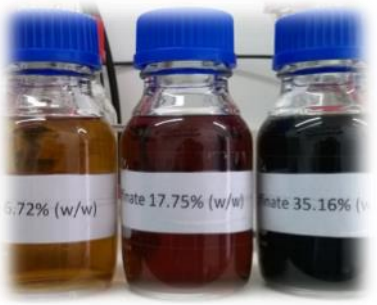
- Directly addresses BETO's 2019 challenge to "**develop robust organisms…that can achieve long efficacy times**" and for "**catalysts that are more tolerant of lower-quality feedstocks**"
- Increases **scale** via **wider diversity of biomass** and **pretreatment processes**, and **compatibility with existing infrastructure**
- **Lower CAPEX / OPEX** needed for **hydrolysate neutralization** → lower feedstock costs
- Increase production → **cost-competitiveness** of cellulosic products

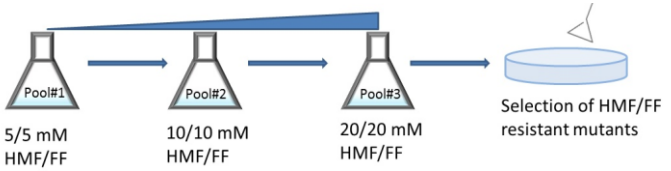
5 – Future Work

I. Systematic characterization of hydrolysate toxicity – COMPLETE

II. Engineering of enhanced hydrolysate tolerance

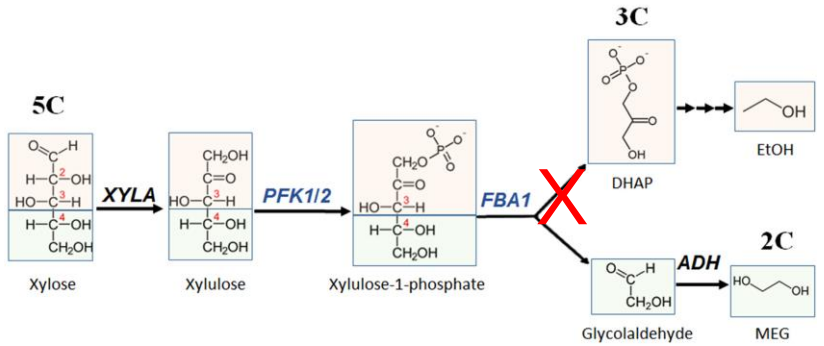
- Characterize fermentation viability in Strain B
- Further genetic + process optimizations
- Benchmark Strain B in variety of **genuine hydrolysates** / concentrates
- Screen **mutagenesis library** for novel alleles of reductases and multidrug efflux pumps





III. Engineering of cellulosic MEG

- **Reduce EtOH** byproduct / increase MEG titer, yield
- Engineer in genetic elements conferring hydrolysate tolerance
- Demonstrate cellulosic MEG (any titer)



Summary

Overview

- Enhance yeast **tolerance** to **hydrolysate toxicity** for **high production** and **feedstock diversity** of **cellulosic EtOH** and **MEG**.

Approach

- Genetic + bioprocess enhancements to boost tolerance

Technical Accomplishments / Progress / Results

- Toxicity deconstruction → cells are **not dead** but **metabolically inhibited**, identified **dominant inhibitors** to target
- Screen of reductase and multidrug efflux genes → **Strain B** conferring:
  a) >100 g/L EtOH
  b) ~30% gain over wildtype in full toxicity hydrolysate
- Successfully implemented and published **MEG synthesis** →
  a) Uncovered **native yeast pathway**
  b) 4 g/L MEG (highest reported to-date in yeast)

Summary

Relevance

- Enhanced tolerance can **increase scale** via:
  - ➔ Reduced **detoxification**
  - ➔ Robustness to **wider feedstock diversity** + **pretreatments**
  - ➔ Higher **production**
  - ➔ Potentially increased **compatibility** with **existing fermentation** infrastructure
- Understanding physiology of tolerance **enables transferability** to other synthetic pathways (eg., MEG)

Future Work

- Complete characterization, benchmarking of engineered Strain B
- Validate with variety of genuine hydrolysates
- Reduce EtOH byproduct, implement tolerance in MEG strain

Additional Slides

Publications, Patents, Presentations, Awards, and Commercialization

Publications

B. Uranukul, B. Woolston, G.R. Fink, G. Stephanopoulos. Biosynthesis of monoethylene glycol in *Saccharomyces cerevisiae* utilizing native glycolytic enzymes. *Metab Eng.* **51**, 20–31 (2019)

Responses to 2017 BETO Peer Review Comments

"Strain needs to be constructed to make MEG…starting from scratch may take longer than expected to reach 1 g/L. This should be started right away in parallel"

➔ We concurred with this recommendation and the assessment that relegating Aim III to the final year of the project was unnecessarily risky. Given that its work plan was sufficiently decoupled from Aims I and II, we indeed embarked on developing the MEG strain in BP1. As shown in Technical Accomplishments, we have now completed and published this strain (while uncovering native yeast chemistries in the process). Furthermore, we have surpassed our original estimates and achieved 4 g/L titers with a variety of optimizations.

Responses to 2017 BETO Peer Review Comments

"…team is not very connected to industrial players that may have more tolerant strains already…Is 72 g/L ethanol tolerance the industry state-of-the-art (yeast developed by Cargill, Lallemand, Purdue, etc)?…Not sure if there is actual market need for higher lignocellulosic ethanol and MEG yeast"

➔ The performance specifications of many industrial strains remain undisseminated in the literature or as verbally circulated / proprietary numbers unverified by independent testing. Thus, the actual cellulosic ethanol state-of-the-art has been difficult to ascertain. Although we have always been eager to form partnerships, our attempts to connect with industrial players have been largely met with perfunctory responses and unrealized collaborations / strain sharing. However, now that we have achieved considerable gains (Strain B), we are in a better position to reconnect as we have something to offer.

Responses to 2017 BETO Peer Review Comments

"…team is advised to use realistic lignocellulosic sugars and not mock-up [hydrolysate] *from the get-go"*

➔ We had disagreed with this for several reasons. Genuine hydrolysates are chemically undefined mixtures; without the ability to precisely control or eliminate any component, we could not determine unequivocally what any enhanced phenotype may be dependent on. Additionally, should corn stover / switchgrass / miscanthus / a mix represent the "standard" hydrolysate benchmark? Rather, we felt using an artificial but defined hydrolysate — allowing full chemical modulation of all components — during strain development has enabled a deeper understanding of toxicity (e.g., Aim I Technical Accomplishments). That said, validation on realistic lignocellulose is indeed worthwhile; thus, we have been sourcing a variety of genuine hydrolysates and will soon embark on "field-testing" of our engineered strains.