U.S. Department of Energy (DOE)
Bioenergy Technologies Office (BETO)
2017 Project Peer Review

Improving tolerance of yeast to lignocellulosic-derived feedstocks and products

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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 of ~**100 g/L** from **unclarified, pretreated biomass**
- Utilizing **C6** (glucose) and **C5** (xylose) **sugars**
- Producing antifreeze molecule **monoethylene glycol (MEG)** and other non-EtOH products from **lignocellulose**

Relevance –

- **High tolerance** to combined **feedstock + product toxicity** (e.g., acid-hydrolyzed biomass + EtOH) **removes a primary obstacle to high production** and **cost-competitive cellulosic-based products**
- Tolerance-enhanced yeast processes (strains + specific fermentation modifications) could **leverage the established fermentation infrastructure for cellulosic economy**

Quad Chart Overview

Timeline

- Project start: **Oct. 2016**
- Project end: **Sept. 2019**
- Percent complete: <**5%**

Budget

	Total Costs FY 14 –FY 16	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$0	$633K	$428K	$439K
Project Cost Share (MIT)	$50K	$142K	$147K	$152K

Barriers

- Ct-H. Efficient Catalytic Upgrading of Sugars/ Aromatics to Fuels and Chemicals
- Ct-J. Process Integration

Partners

- Whitehead Institute (Cambridge, MA)
- National Corn-to-Ethanol Research Center (Edwardsville, IL)
- Biochemtex (Tortona, Italy)

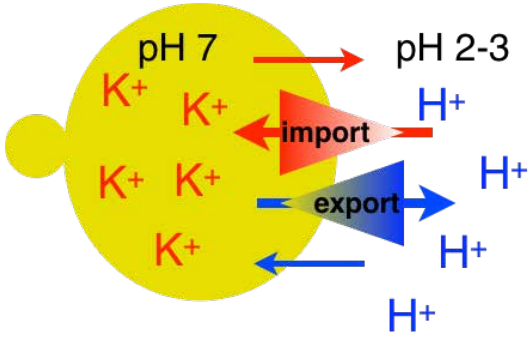
1 - Project Overview

Background

- Inhibitory compounds generally **attack microbial catalysts** via **unidentified mechanisms**
- Unlike corn/1G fermentations, lignocellulosic fermentations exhibit **combined feedstock + product toxicity** (pretreatment byproduct + EtOH toxicity)

Previous Work

- We identified **upkeep of the plasma membrane potential** as a discrete, engineerable mechanism of **general alcohol tolerance** in yeast (Lam FH *et al., Science* 2014)

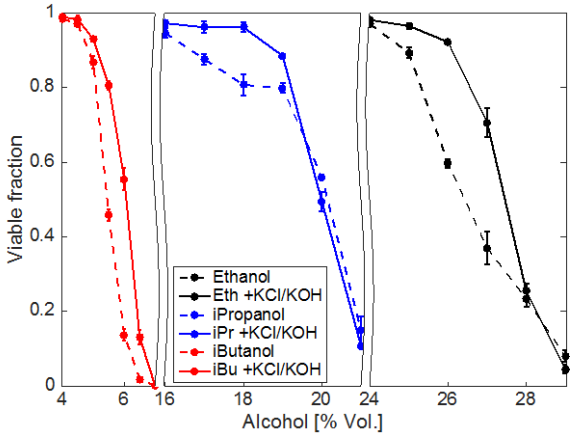


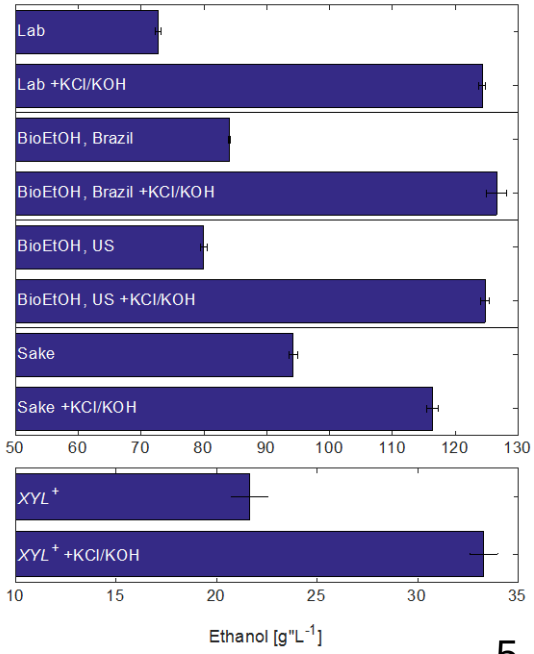
- Simultaneous elevation of extracellular potassium (K^+) + pH **strengthens the principal membrane electrochemical gradients** → directly enhances alcohol tolerance…

1 - Project Overview

Previous Work (cont.)

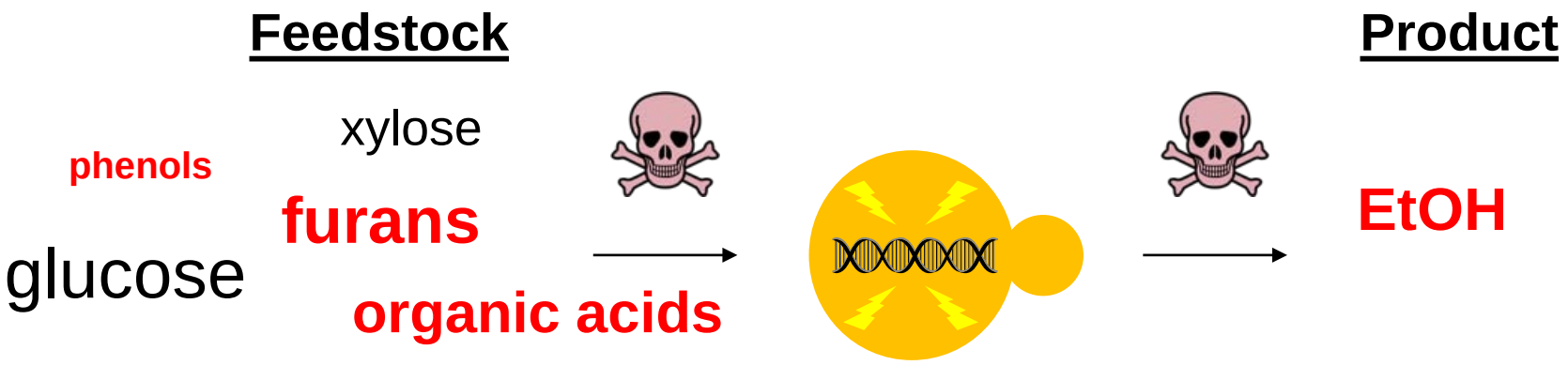
- …Boosts yeast viability against EtOH, propanol, or butanol
- …Boosts EtOH production universally in laboratory and commercial strains
- …Boosts EtOH production from either glucose (C6) or xylose (C5)
- **Strengthened membrane gradients work alongside engineered pathways**:
  - Xylose consumption
  - Xenobiotic utilization (Shaw AJ, Lam FH *et al., Science* 2016)





1 - Project Overview

Proposed Research

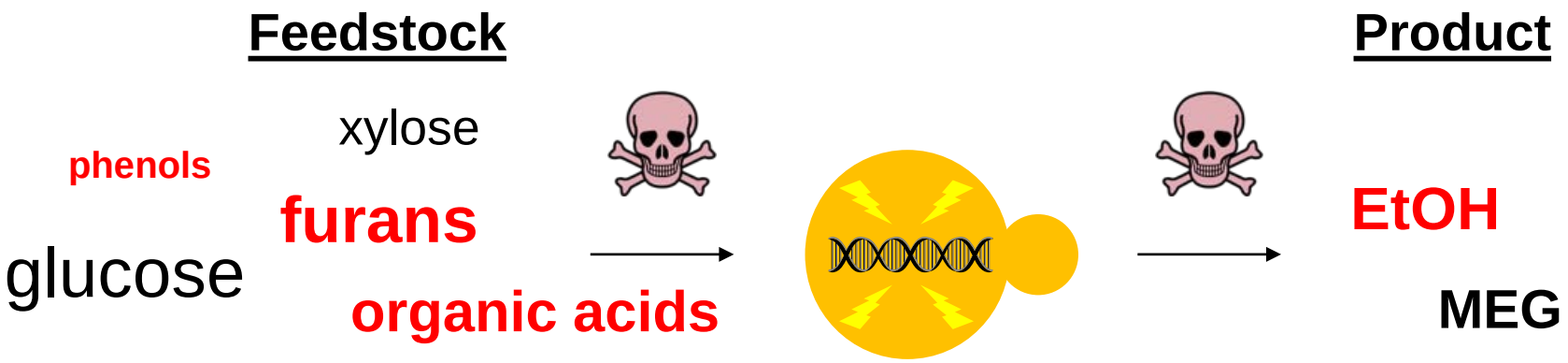


Boost lignocellulosic fermentation by **combining alcohol tolerance** advances with genetic pathways **alleviating hydrolysate toxicity**

I. Systematically **characterize** the impact of **dominant hydrolysate inhibitors** — furfural, hydroxymethylfurfural, acetic acid — on EtOH production and yeast viability

1 - Project Overview

Proposed Research (cont.)



II. Engineer **hydrolysate-tolerant strains** → target **~100 g/L** cellulosic **EtOH**, can **withstand range of toxicities**
   a. Enzymatic **detoxification** of **furfurals to furan-alcohols** → strengthen membrane potential to **increase alcohol tolerance**
   b. Screening / expression of **drug efflux pumps** to reduce intracellular inhibitor concentrations

III. Assess if hydrolysate tolerance is portable beyond EtOH: engineer yeast producing **cellulosic MEG**, an antifreeze component

2 – Approach (Management)

Prof. Greg Stephanopoulos (MIT), Principle Investigator

Prof. Gerald Fink (Whitehead Institute), Project Collaborator

- Scientific guidance
- Financial, administrative oversight

Dr. Felix Lam (MIT), Lead Scientist

Boonsom Uranukul (MIT), Graduate Researcher

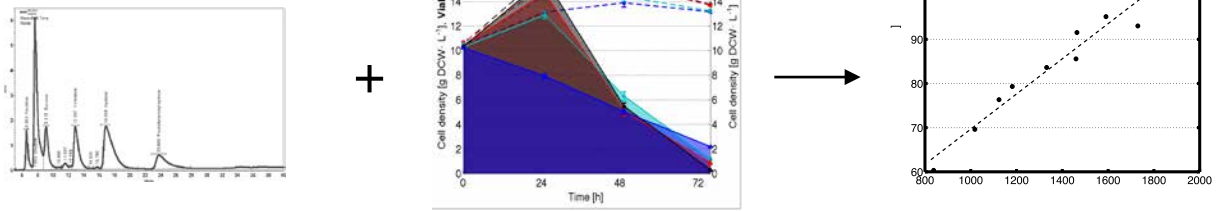
- Hydrolysate tolerance / cellulosic EtOH
- Cellulosic MEG

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)

- Formulate **reference hydrolysate**: laboratory medium containing range of individual, and varying blends, of the 3 inhibitors
- **Bench-level fermentations** monitoring EtOH / MEG production (HPLC) and yeast viability (microscopy)
- Determination of **tolerance metrics** and **correlation with EtOH / MEG** performance to assess if inhibitors impinge viability or metabolism



- **Genetic methods** (**metabolic engineering**, **CRISPR**, **gene synthesis**) to optimally express detoxification enzymes, efflux pumps, and MEG pathway
- **Pooled library selection** and **deep sequencing** to identify novel alleles of expressed genes

2 – Approach (Technical)

Top Potential Challenges

- Genes initially chosen for inhibitor detoxification or efflux may be ineffective → *mutagenesis and selection to increase efficacy*
- Activity of expressed genes may be further limited by co-factors or cytoplasmic trafficking → *metabolic engineering to tweak biochemistry*
- Enhanced alcohol tolerance dependent on medium composition → *genuine biomass hydrolysates from partners must be customized*

Critical Success Factors

- Reproducible gains in hydrolysate tolerance / cellulosic EtOH (any range of inhibitors) from engineered strains
- MEG production under any condition

4 – Relevance

Goals

- Enhance **yeast tolerance** to **unclarified lignocellulosic hydrolysates** for increased **EtOH** and **MEG** production
- Exceed current cellulosic EtOH tolerance of 72 g/L (MYPP, 3/2016)

Higher feedstock + product tolerance:

- Directly addresses BETO's MYPP Conversion R&D objectives for *"**more robust host organisms** that can **tolerate greater feedstock variability** and **accumulation of inhibitory compounds**."*
- Increases production → **cost-competitiveness** of cellulosic EtOH
- Potentially **lowers CAPEX / OPEX** needed for **hydrolysate neutralization** → lowers feedstock costs
- Technology demonstration of non-EtOH product: **cellulosic MEG**

5 – Future Work

FY17 – TOXICITY CHARACTERIZATION

- Quantify EtOH production and yeast fermentation viability as a function of the individual toxicities **furfural**, **hydroxymethylfurfural**, **acetic acid**
- Repeat study with defined stoichiometric **blends of toxicities**

Milestones

- Systematic deconstruction of toxicity to reveal **relative impact** of component inhibitors as well as **inhibitor synergism**
- Identification of **component(s) exhibiting greatest inhibition** and **requiring greatest** degree of **detoxification**
- Correlation of EtOH titers and yeast fermentation viability to **determine if toxicity impinges cell viability** or **metabolism**

5 – Future Work

FY18 – STRAIN ENGINEERING

- **Formulate reference hydrolysate** from FY17 results; characterize gains in EtOH production and yeast viability when modified with **adjustments strengthening membrane potential**
- Engineer strains expressing **alcohol dehydrogenases** that convert furan-aldehydes to -alcohols; benchmark with/without adjustments strengthening membrane potential
- **Screen "variomics" libraries** of alcohol dehydrogenase genes (e.g., *ADH6, ADH7*) to identify superior detoxification alleles
- Screen strains with deletions of annotated **multidrug efflux pumps** to identify transporters with specificity to inhibitors

Milestones

- A set of **genetic enhancements** and **fermentation conditions** that **boost hydrolysate tolerance**

5 – Future Work

FY19 – CELLULOSIC MEG

- Prototype yeast pathway synthesizing **MEG from xylose**
- Metabolic engineering to **delete competing fluxes**
- Metabolic engineering to **reduce** / **eliminate EtOH production**
- Add in genetic enhancements from FY18 that confer **hydrolysate tolerance**

Milestones

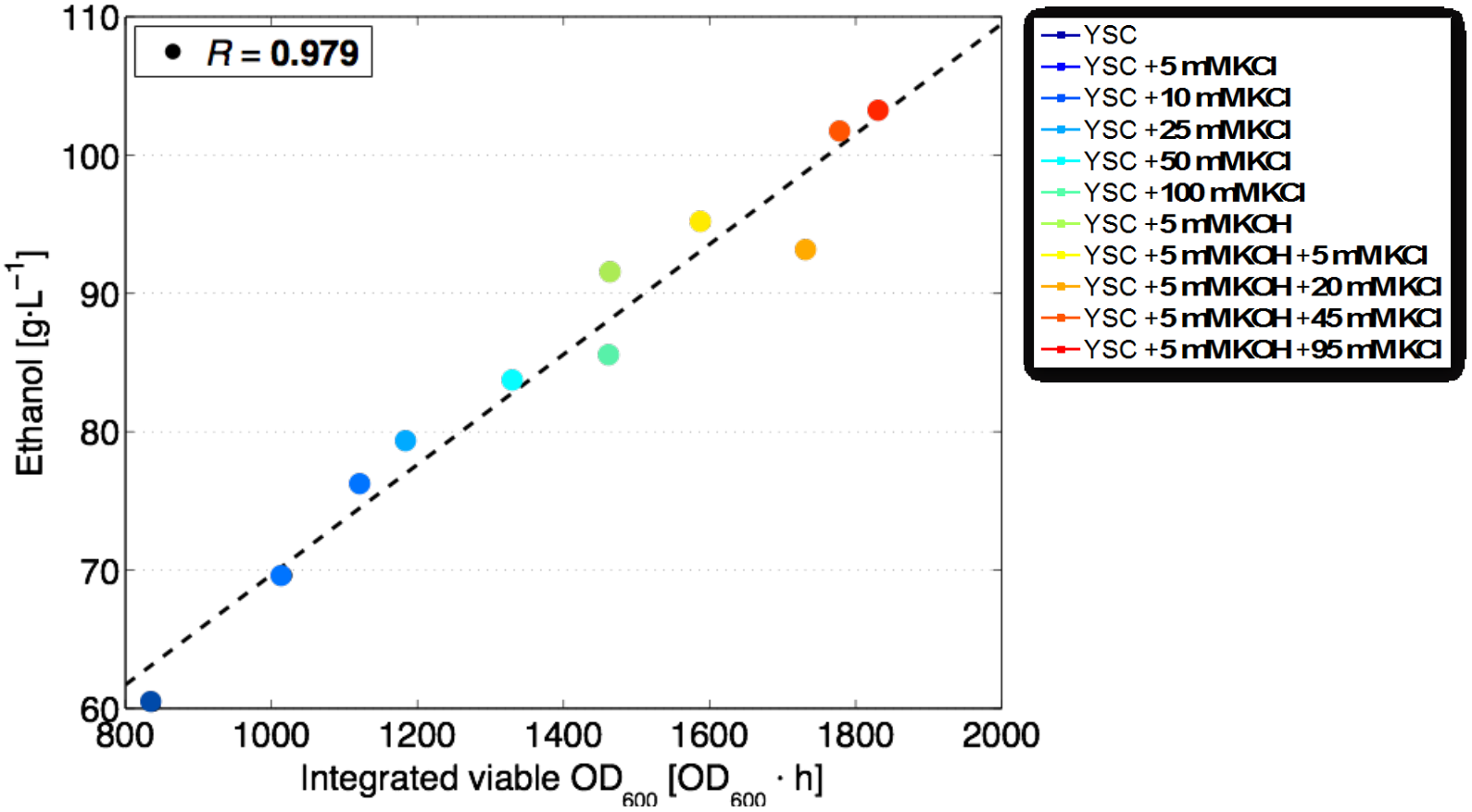
- Strains producing 1–10 g/L **MEG** from xylose **with minimal EtOH co-product**
- Strains producing **cellulosic MEG** (any titer)

Summary

1. **Overview**: engineer yeast tolerant to hydrolysate toxicity for production of lignocellulosic EtOH and MEG
2. **Approach**: bench-level fermentations, metabolic engineering, library screenings to identify genetic enhancements and fermentation specifications boosting lignocellulosic fermentation
3. Technical Accomplishments: (none yet)
4. **Relevance**: hydrolysate-tolerant yeast…
   - … Address MYPP objective for more robust biocatalysts tolerating greater feedstock variability
   - … Lower feedstock costs → boost cost-competitiveness of cellulosic products
5. **Future Work**:
   - Systematic deconstruction of hydrolysate toxicity
   - Genetic engineering for specific detoxification of inhibitors
   - Integration with previous alcohol tolerance advances for increased production from lignocellulosic hydrolysates

Additional Slides

Previous Work



Elevated K^+ and pH directly control EtOH tolerance and production

Publications, Patents, Presentations, Awards, and Commercialization

References (prior to award)

- Lam FH, Ghaderi A, Fink GR, Stephanopoulos G. Engineering alcohol tolerance in yeast. *Science*. **346**, 71–75 (2014).

- Shaw AJ, Lam FH *et al.* Metabolic engineering of microbial competitive advantage for industrial fermentation processes. *Science*. **353**, 583–586 (2016).

Patents (prior to award)

- US 14/479,118 – “Ethanol production in engineered yeast” (under examination)