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 end: Sept. 2019
- Percent complete: <5%

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

Budget

<table>
<thead>
<tr>
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<th>Total Costs FY 14–FY 16</th>
<th>FY 17 Costs</th>
<th>FY 18 Costs</th>
<th>Total Planned Funding (FY 19-Project End Date)</th>
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</thead>
<tbody>
<tr>
<td>DOE Funded</td>
<td>$0</td>
<td>$633K</td>
<td>$428K</td>
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<td>Project Cost Share (MIT)</td>
<td>$50K</td>
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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…
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)
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
Elevated K⁺ and pH directly control EtOH tolerance and production
Publications, Patents, Presentations, Awards, and Commercialization

References (prior to award)


Patents (prior to award)

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