DOE Bioenergy Technologies Office (BETO) 2021 Project Peer Review

Improving tolerance of yeast to lignocellulosic-derived feedstocks and products

March 10, 2021
Biochemical Conversion and Lignin Utilization

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Quad Chart Overview (Competitive Project)

Timeline

- Project start: **10 / 2016**
- Project end: **12 / 2020**

	FY20 Costed	Total Award
DOE Funding	$81k	$1.5M
Project Cost Share	$17k	$441k

Project Partners

(None)

Project Goal

Engineer **enhanced microbial tolerance** to high-toxicity lignocellulosic hydrolysates for production of **cellulosic fuels** (ethanol) and **non-fuel chemicals.**

End of Project Milestone

At minimum, we anticipate **engineered yeast strains** and **bioprocess specifications** that enable **ethanol** production in the range of **100 g/**L from model or genuine **lignocellulosic hydrolysates**. Should the efforts of Budget Period 3 be successful, we also anticipate proof-of-concept yeast strains capable of synthesizing **MEG from lignocellulosic** material (titers likely <1 g/L).

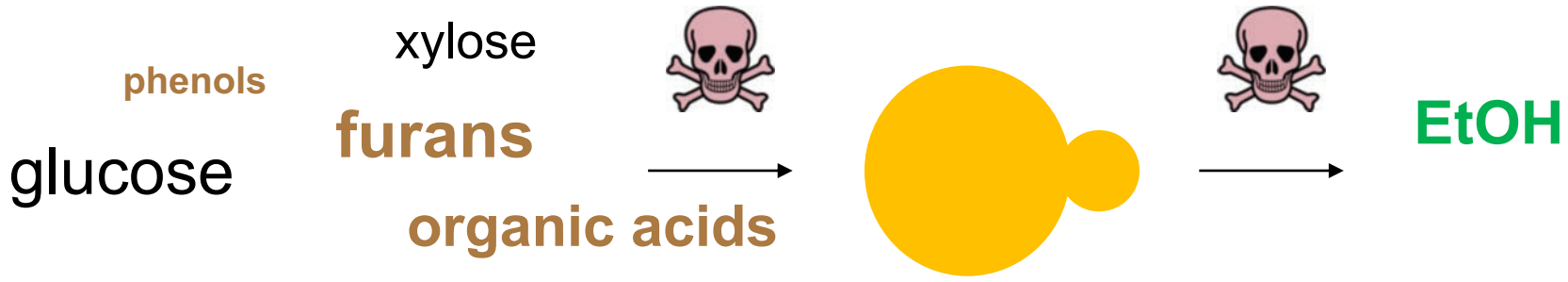
Funding Mechanism

FOA: USDA-NIFA-9008-004957 (FY2015)

Topic: Biofuels and Biobased Products Development

Project Overview

Background:



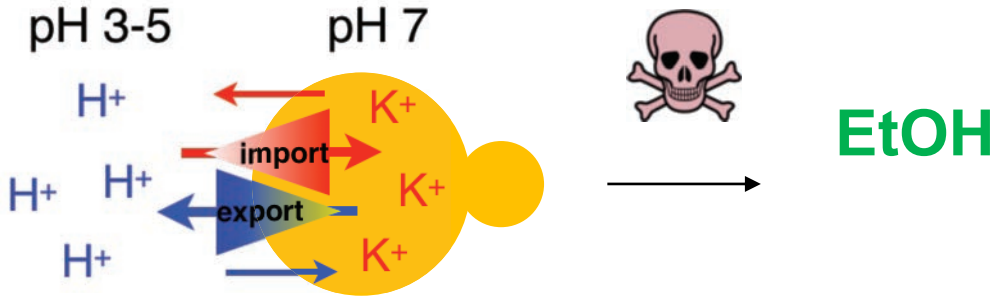
- Lignocellulosic fermentations exhibit **feedstock** + **product** **toxicity** to **yeast** biocatalysts
- Inhibitors *individually* limit production; the combination exerts **synergistic** toxicity
- Inhibitors typically **attack cells** via **unidentified biological mechanisms**

→ Present solution: detoxify feedstock; refine processing / pretreatment → *complexity limits scalability, cost-effectiveness, adoption*

Project Overview

Previous Work (**product** toxicity):

glucose

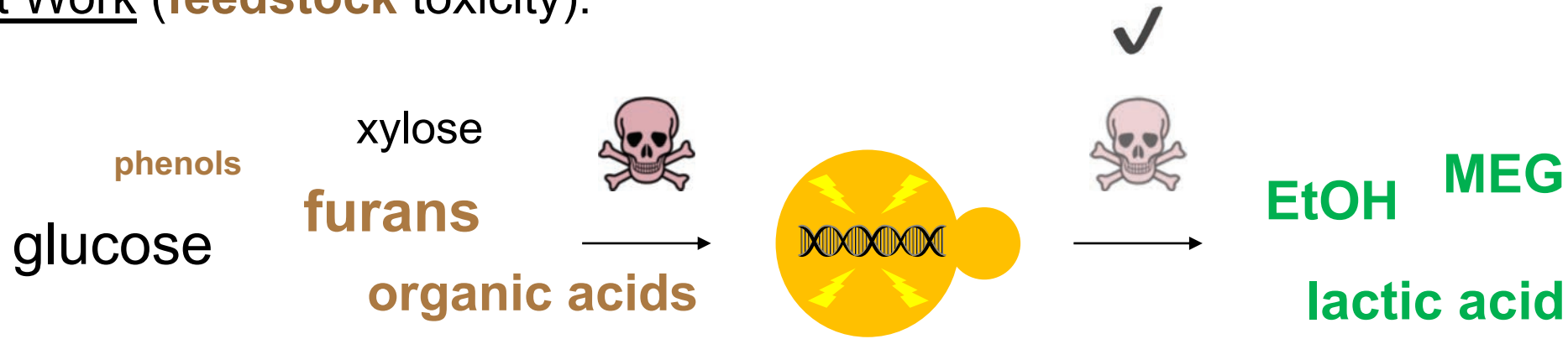


- **Maintenance** of plasma **membrane potential** is discrete, engineerable mechanism of yeast **general alcohol tolerance** (Lam FH *et al.*, *Science* 2014)

- High extracellular potassium **K^+** + **pH** **strengthen membrane** electrochemical **gradients** → directly boost **EtOH** production, confer competitive advantage (Shaw AJ, Lam FH *et al.*, *Science* 2016)

Project Overview

Present Work (**feedstock** toxicity):



Project Aims:

I. **Systematically characterize** yeast hydrolysate toxicity (furans, organic acids)

II. Engineer **total cellulosic hydrolysate tolerance** (cellulosic EtOH)

III. **Transfer** tolerance to **non-native** end-product → cellulosic MEG (monoethylene glycol), cellulosic lactic acid

Higher **tolerance** → lower detoxification + complexity, higher **scalability**, **greater production** + **feedstock** range

1 – 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

Boonsom Uranukul, Graduate Researcher

- Hydrolysate tolerance, cellulosic EtOH / MEG / lactic acid

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

I. Systematically characterize yeast hydrolysate toxicity

Map EtOH production vs. increasing concentrations of individual, blends of inhibitors:

→ Equimolar dosing quantifies **relative toxicities**

→ Combinations reveal **synergies** of inhibition

→ **Formulate benchmark hydrolysate** for strain engineering in Aims II, III

Compare EtOH production, fermentation viability, yield metrics:

→ Metabolic **inhibition** vs. **cell death**

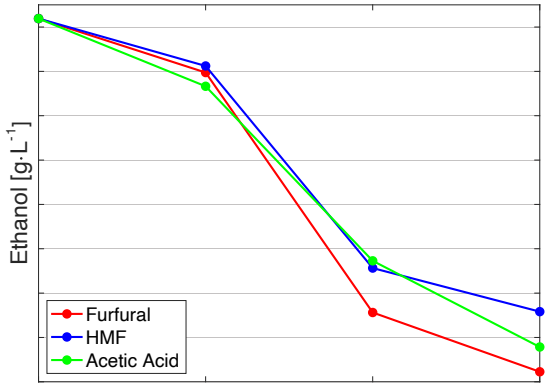
Challenges:

→ Few; characterization only

Go/No-Go Decision Points:

→ Identify components, combinations exerting greatest inhibition

→ Gain insight into physiology underlying component, total toxicity



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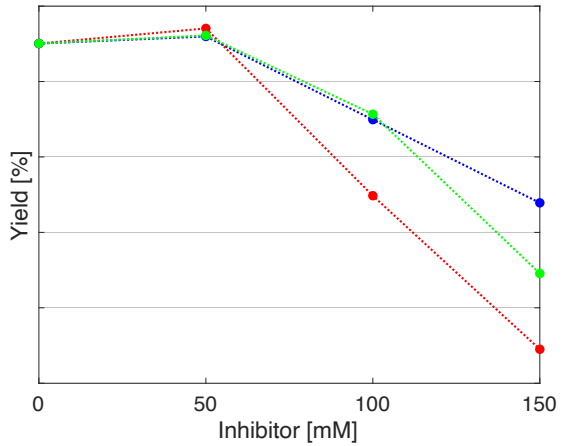
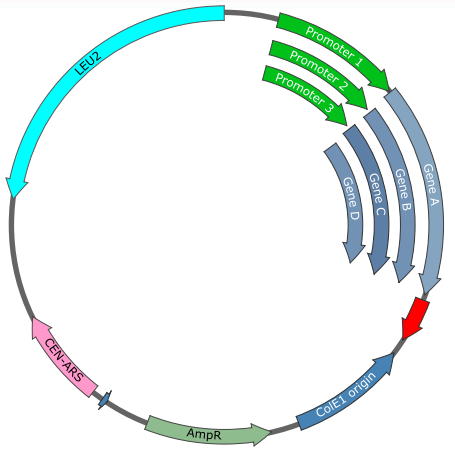


2 – Approach

II. Engineer total cellulosic hydrolysate tolerance

→ Screen **detoxification genes** targeting inhibitors
→ Screen **multidrug efflux pumps** targeting inhibitors
→ **Mutagenesis & selection** for superior gene variants
→ Assess production metrics in benchmark hydrolysate ± **membrane potential strengthening adjustments** (from prior work)
→ Assess in **genuine cellulosic feedstocks** (per 2019 Peer Review)



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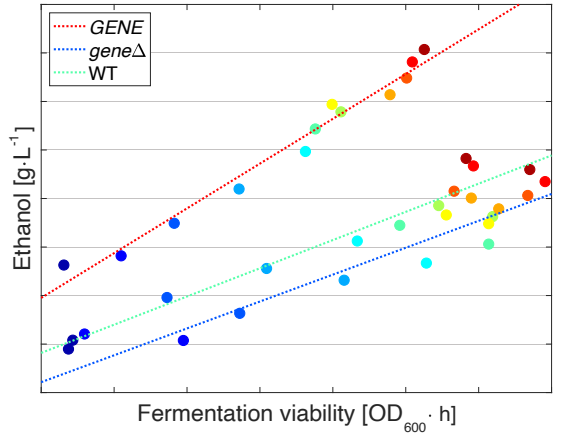


Challenges:
→ Candidate genes insufficiently specific to inhibitors; side effects
→ Need to mitigate total toxicity
→ Tolerance only works in small subset of real feedstocks

Go/No-Go Decision Points:
→ Genetic + fermentation specifications enabling **cellulosic** EtOH **comparable** to **1st generation** EtOH (e.g., >100 g/L in 2-3 days)

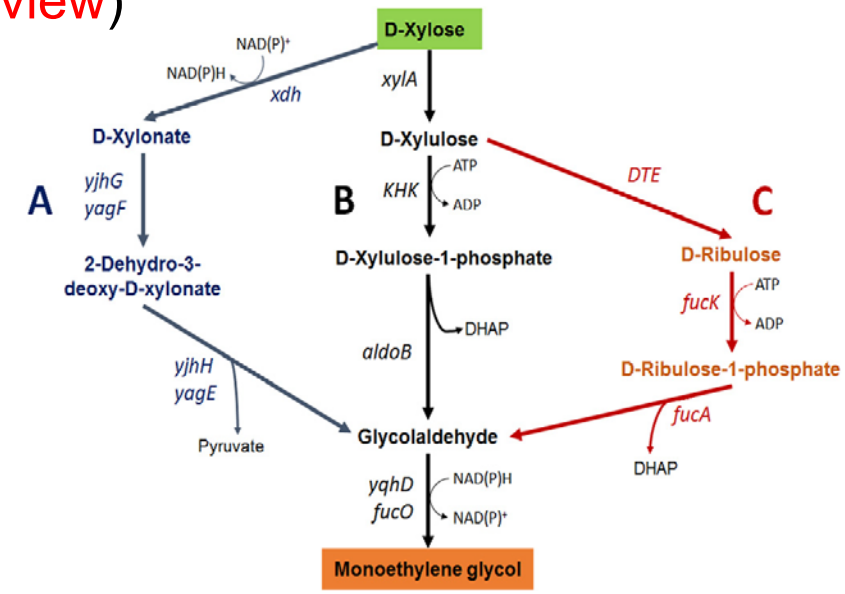
2 – Approach

III. Transfer tolerance to non-native end-product

→ Prototype **xylose** → **MEG** (monoethylene glycol) pathway
→ Metabolic engineering to **delete competing fluxes**
→ Metabolic engineering to **eliminate** / **decrease EtOH byproduct**
→ Combine with tolerance gene + fermentation conditions (Aim II) to enable **cellulosic MEG**
→ Assess in **genuine cellulosic feedstocks** (per 2019 Peer Review)

Challenges:
→ MEG pathway unachievable
→ Low yields from EtOH persistence
→ Cellulosic MEG unachievable

Go/No-Go Decision Points:
→ 1–10 g/L MEG from (clean) xylose
→ Technology demonstration (any titer) of cellulosic MEG



3 – Impact

- Lignocellulose: **most abundant**, **renewable** terrestrial resource → sustainable **liquid fuels**, non-fuel **commodities** via microbial **fermentation**
- **Adoption remains nascent** due to **utilization challenges**: detoxification / complex pretreatments / refinements add **costs**, **scalability hurdles**
- **More aggressive** / **simpler** pretreatments yield **inhibitory sugars** → utilizable only if biocatalyst can withstand toxicity

➔ Engineer **high microbial tolerance** to **full hydrolysate toxicity**

➔ Maximum potential requires **production-parity** with clean-sugar equivalents, **portability beyond EtOH** to other metabolic chassis strains

Dissemination:

- Publication in wide readership, high impact journals (e.g., *Nat Biotech*, *Science*)
- IP protection, seek industrial partnerships for biomass / production / manufacturing

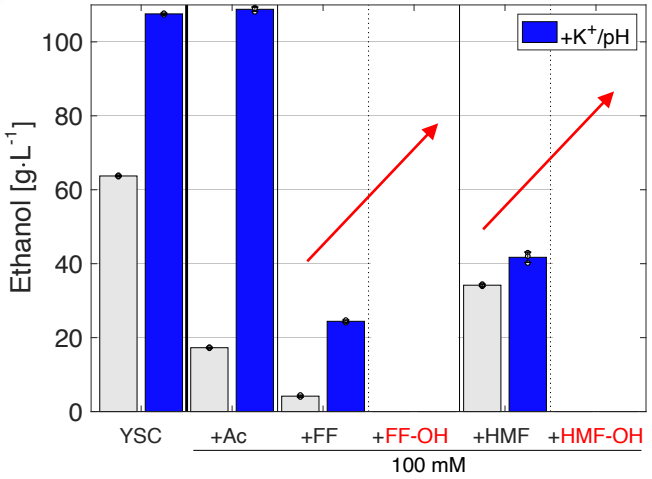
4 – Progress and Outcomes

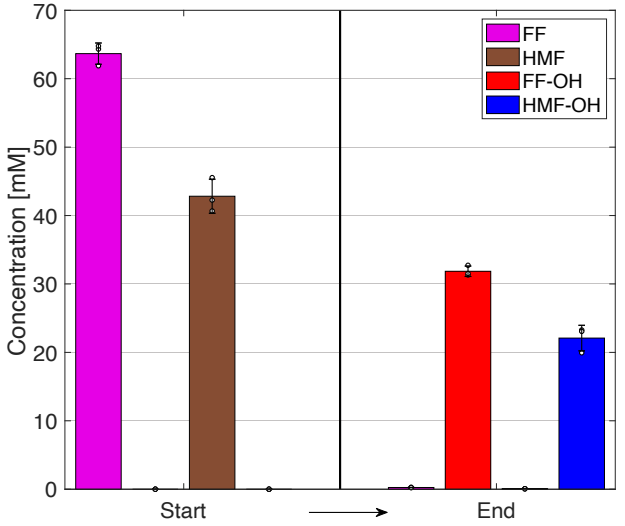
I. Systematically characterize yeast hydrolysate toxicity

Inhibitors AC (Acetic acid), FF (Furfural), HMF (5-hydroxy-methylfurfural):

- Universal / dominant: derive directly from hemicellulose, C6, C5 sugar pretreatment
- EtOH fermentations **enhanced** by extracellular **elevated K^+/pH**:
  - ➢ Ac fully neutralized > pH 4.8 ✓
  - ➢ Yeast slowly detoxify FF, HMF to alcohols FF-OH, HMF-OH
    - ➞ K^+/pH then protect specifically against alcohols ✓

➔ Strategy: engineer yeast to **boost** FF-OH, HMF-OH → ferment with elevated **K^+/pH** conditions for total tolerance



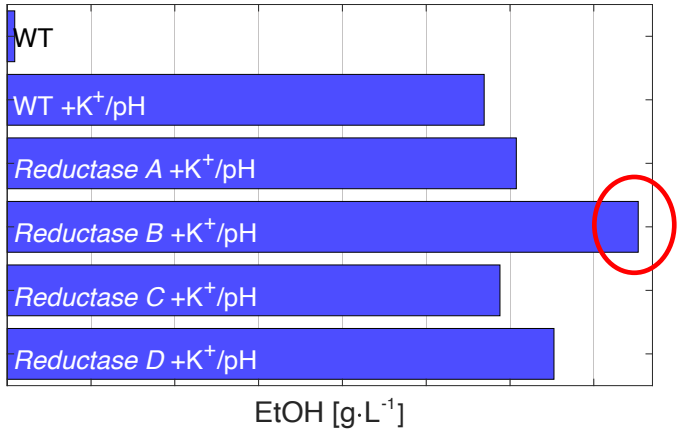


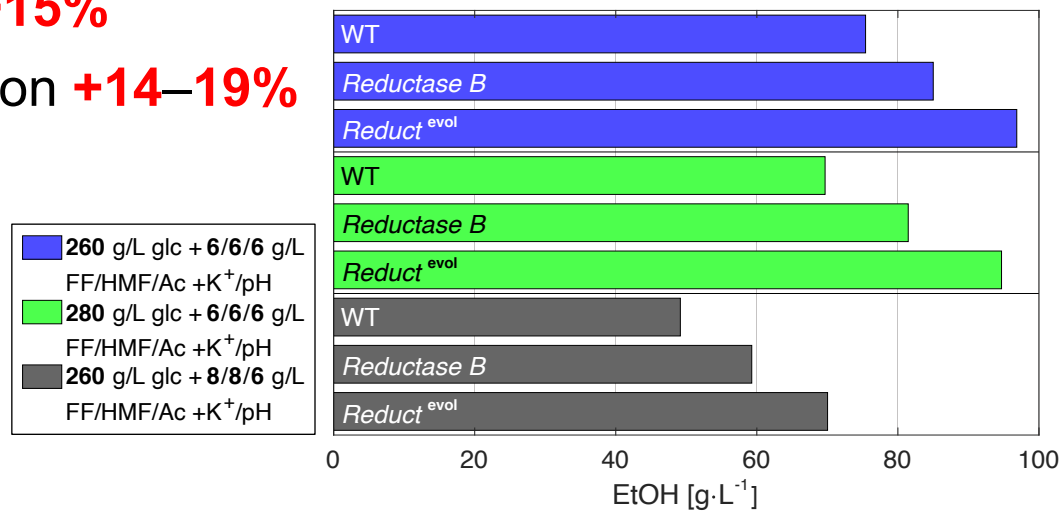
4 – Progress and Outcomes

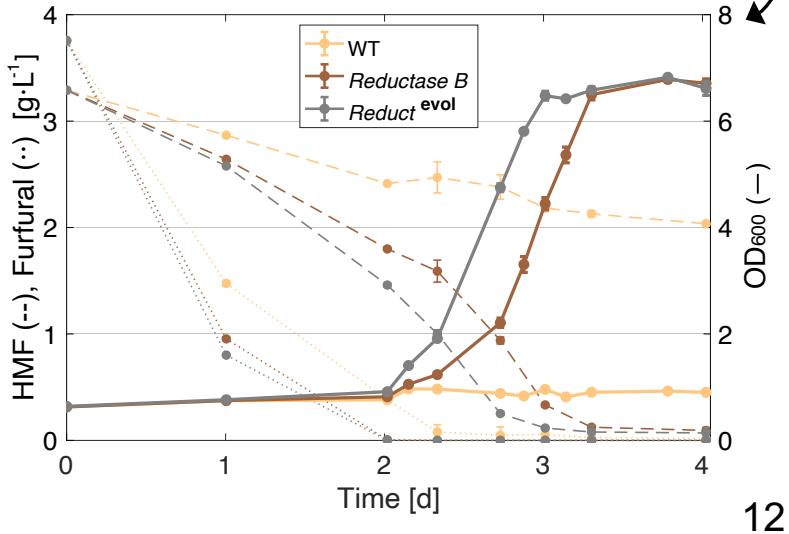
II. Engineer total cellulosic hydrolysate tolerance

- Screened **reductases** for high FF-OH, HMF-OH activity
- Top reductase **improved full toxicity** EtOH fermentation by **25–35%**

- **Evolved reductase** under increasing FF + HMF + Ac load
- Vs. unevolved *Reduct B*, superior *Reduct* ^{evol} improved:
  - ➢ FF + HMF detoxification **+29%**
  - ➢ Growth fitness **+15%**
  - ➢ EtOH fermentation **+14–19%**







4 – Progress and Outcomes

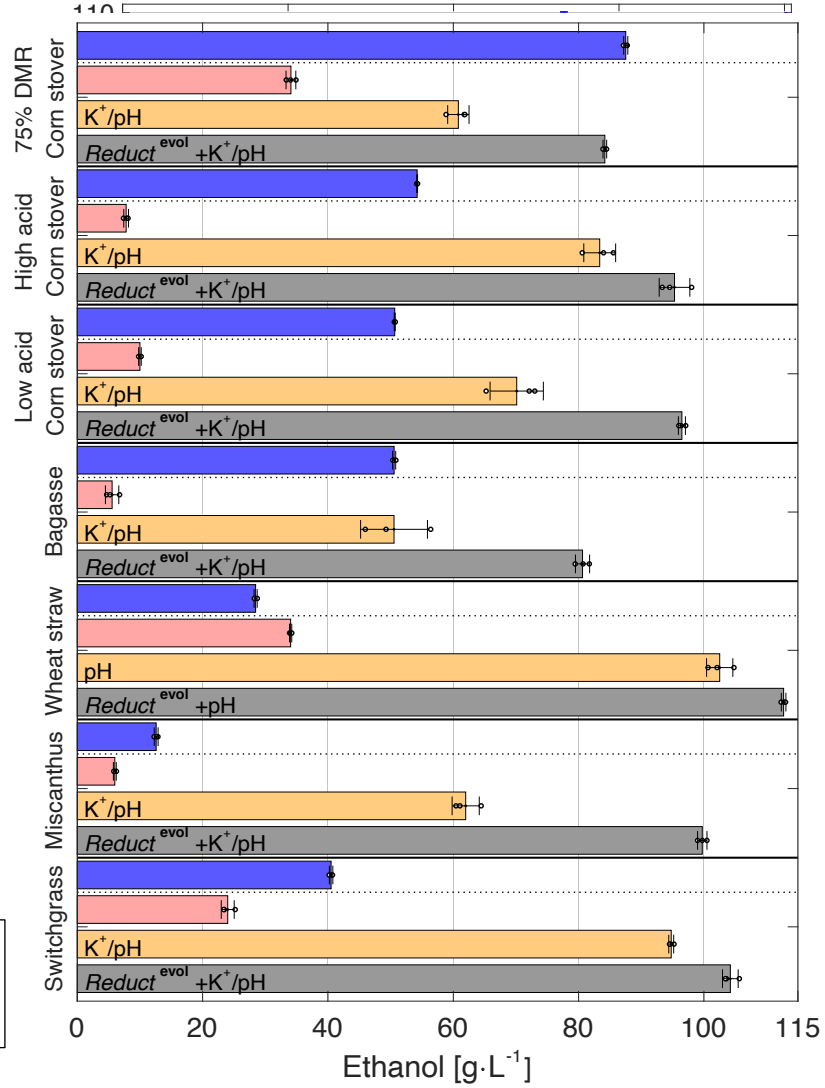
II. **Engineer total cellulosic hydrolysate tolerance** (cont.)

- Vs. clean-sugar 1G EtOH, *Reduct*^{evol} achieved **97% production** under **full toxicity** (synthetic lab hydrolysate)

- Per 2019 Peer Review, sourced and tested **7 real hydrolysates**:
  - Corn stover, bagasse, wheat straw, miscanthus, switchgrass
  - Dilute sulfuric acid, mechanically refined, hot water treatments

  - ➢ Achieved **>100 g/L cellulosic EtOH** where sugar permitted
  - ➢ Mean gain of **802%** (gray) over toxified equivalent (red bars)
  - ➢ **Single strain** handled **full diversity** of **feedstocks**

➔ Full tolerance: a) express *Reduct*^{evol} b) elevate feedstock **K⁺/pH**



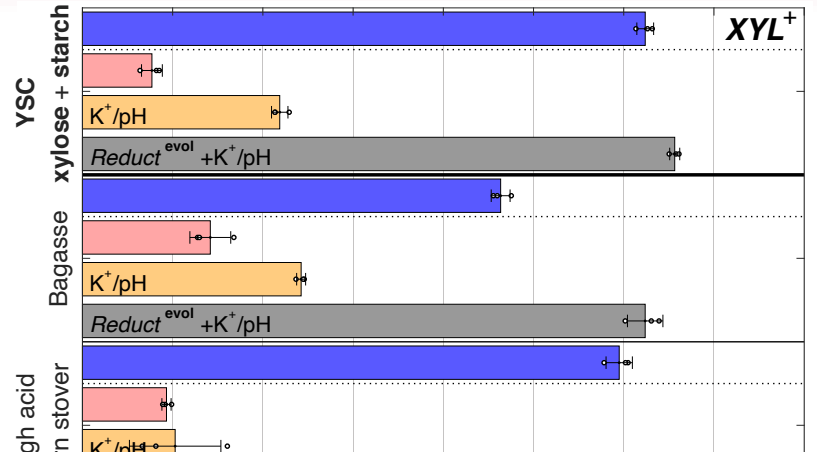
4 – Progress and Outcomes

III. Transfer tolerance to non-native end-product

- **Portable** atop pre-existing **xylose** → **EtOH** strain (dominant C5 cellulosic sugar)
- Transformed in *Reduct*^{evol} : single gene, no further engineering

➢ Single **cellulosic xylose** strain: **full tolerance**, **multi-feedstock** robustness transferred

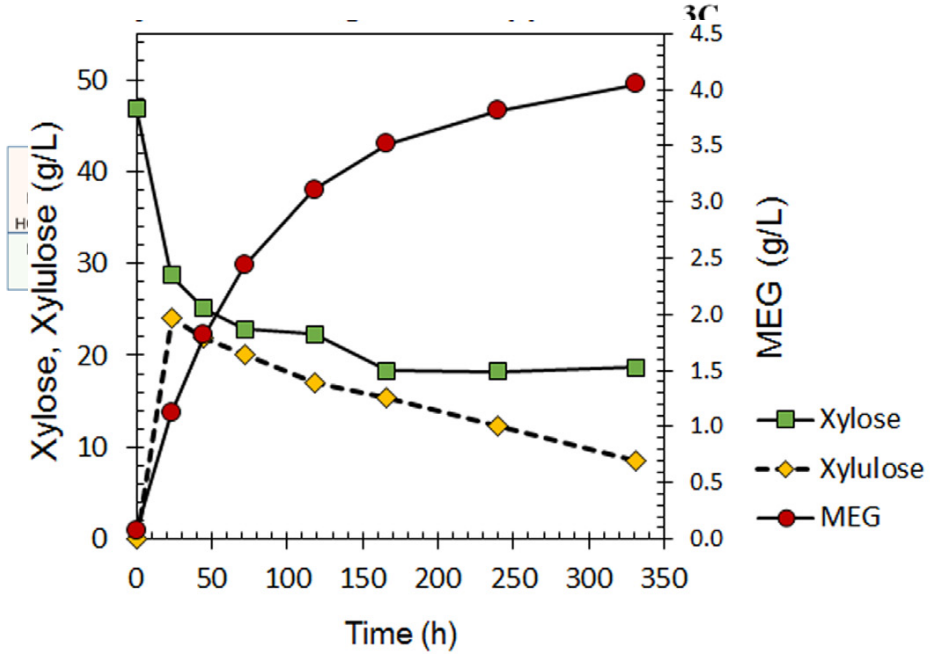
➢ Mean gain of **676%** (gray) over toxified equivalent (red)



- Engineered (clean) **xylose** → **MEG** (monoethylene glycol) strain

➢ Achieved **4 g/L MEG**, highest to-date from yeast (Uranukul *et al.*, *Metab Eng*. 2018)

→ ~~Started **EtOH-reduced**, hydrolysate-tolerant **cellulosic MEG** strain~~ **COVID**: lost 6 months



4 – Progress and Outcomes

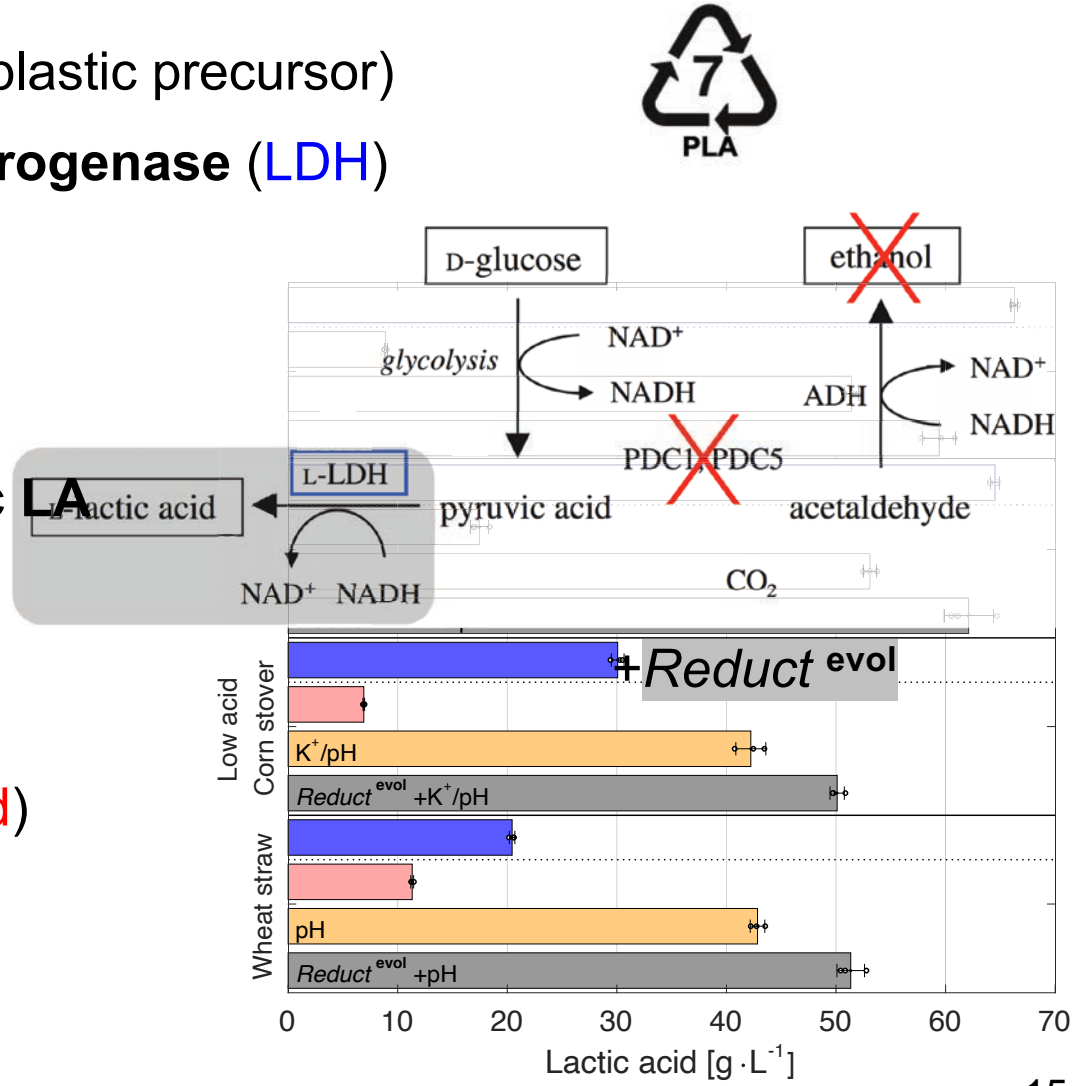
III. **Transfer tolerance to non-native end-product** (cont.)

- Pivoted to **glucose** → **LA** (lactic acid: biodegradable plastic precursor)
- Pre-existing **EtOH-reduced** strain with **lactate dehydrogenase** (LDH)
- Transformed in *Reduct* **evol** for hydrolysate-tolerant **cellulosic LA**. No further engineering

➢ Elevated **K⁺/pH** in feedstocks → **50–60 g/L cellulosic LA** from toxic **real hydrolysates**

➢ Single strain: **full tolerance**, **multi-feedstock** robustness, clean-sugar **performance preserved**

➢ Mean gain of **450%** (gray) over toxified equivalent (red)



Summary

- Elevated extracellular **K^+/pH** confer partial tolerance in yeast to hydrolysate inhibitors:
  - Acetic acid (**Ac**): fully neutralized at pH **> 4.8**
  - Furfural (**FF**), 5-hydroxymethylfurfural (**HMF**): yeast slowly detoxify to less toxic FF-OH, HMF-OH → **K^+/pH** then specifically **protect against alcohols**
- Identified and evolved **reductase** boosting FF-OH, HMF-OH activity
- Elevated **K^+/pH** + *Reduct*^{evol} confer tolerance to full toxicity:
  - Achieved **97%** of equivalent **1G EtOH** (clean-sugar) fermentation
  - **Single strain** fermented **full range** of real hydrolysates (corn stover, miscanthus, etc.)
  - **>100 g/L cellulosic EtOH** from **toxic** feedstocks where sugar permitted
- Elevated **K^+/pH** + *Reduct*^{evol} constitute modular, drop-in hydrolysate capability:
  - **Pre-existing** xylose, lactic acid strains → **cellulosic xylose**, **LA** strains
  - Single *Reduct*^{evol} transformation — no further engineering required
  - **Full tolerance**, **multi-feedstock robustness**, clean-sugar **production specs preserved**

Additional Slides

Responses to 2019 BETO Peer Review Comments

*"The project would be strengthened by the **inclusion of competitive benchmarks**, as similar work has been done already by many other groups."*

➔ We agree that the lack of benchmarks, particularly against commercial strains, was a weakness. However, it is one that remains difficult to address given the proprietary nature of such strains, their performance data, as well as competitive interests. Therefore, production numbers **available in the literature** (e.g., 72 g/L cellulosic EtOH from MYPP, 3/2016) continued to serve as our default metric. Furthermore, we have opted to use clean-sugar **1G EtOH** as a benchmark: fermentation performance in full toxicity feedstocks should be comparable if cellulosic processes are to be economically attractive.

Responses to 2019 BETO Peer Review Comments

*"The team also needs to use **real hydrolysates** rather than synthetic ones, which they readily acknowledge. Finally, they also need to consider **hydrolysate variability**, which would improve the impact of the work."*

➔ We agree that assessing our tolerance strategy (engineered strains + K^+/pH feedstock modifications) on real industrial hydrolysates would provide significant validation. Spurred on by these suggestions, as well as solicitations to the 2019 Peer Review audience, we were able to source and test a total of **7 genuine feedstock** samples (EtOH and lactic acid results shown in Progress & Outcomes). Furthermore, that these represented **5 different crop sources** and **7 different pretreatments** singularly enabled us to assess variability. Without such range, we would have been unable to observe the high degree of robustness our strategy exhibited across feedstocks — a result that has indeed tremendously improved the impact of our work.

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)

F. Lam, B. Turanli-Yildiz, D. Liu, G.R. Fink, G. Stephanopoulos. Engineered tolerance in yeast enables efficient production from toxic lignocellulosic feedstocks. *Sci. Adv.* (2021) *Under review*

Patents (in preparation):

F. Lam, G.R. Fink, G. Stephanopoulos. Combined genetic and extracellular modulations for fermentation of toxic lignocellulosic feedstocks in yeast. (2021)