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Transforming ENERGY

# BETO 2021 Peer Review
# Targeted Microbial Development (TMD)
# WBS 2.4.3.102

March 9, 2021

Biochemical Conversion Session

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National Renewable Energy Laboratory

# Project Overview

***What** are you trying to do**?***

Overall Goal: Develop microbial pathways capable of producing high carbon efficiency intermediates for catalytic upgrading to hydrocarbon fuels and chemicals with reduced cost

- *Engineer Zymomonas mobilis* for 2,3 butanediol (2,3-BDO) production at high titer, yield and productivity from biomass sugars

***How** is it done today?*

- 2,3-Butanediol production using *Klebsiella oxytoca* and *Bacillus polymyxa* and other engineered organisms; have significant conversion of carbon source into cell mass and ethanol, glycerol and various organic acids as by-products

***Why** is it important?*

Provide cost competitive fuels and chemical product conversion process to utilize biomass

***What** are the risks?*

Technical: low conversion rate for xylose and arabinose, robust fermentation process
Commercialization: conversion costs, feedstocks logistics, product market and policy

# Quad Chart Overview

## Timeline
- Project start date: Oct 2018
- Project end date: Sept 2021

| | FY20 | Active Project |
|---|---|---|
| **DOE Funding** | $ 1.05 M | $ 2.9 M |

## Project Partners*
- Partner 1
- Partner 2

## Barriers addressed:
**Ct-D. Advanced Bioprocess Development**

## Project Goal
Develop microbial pathways capable of producing high carbon efficiency intermediates for catalytic upgrading to hydrocarbon fuels with reduced cost.

## End of Project Milestone
Engineer *Zymomonas mobilis* with improved pentose utilization rate(s) to produce 2,3-BDO from glucose, xylose and arabinose at 95%, 85%, and 50% yield in batch fermentation in mixed sugar fermentation containing 8% glucose, 4% xylose and 0.5% arabinose in 40 hours (in collaboration with BSRD)

## Funding Mechanism
AOP via Merit Review Process

**Only fill out if applicable.*

# Market Trends

**Product**

- Anticipated decrease in gasoline/ethanol demand; diesel demand steady
- Increasing demand for aviation and marine fuel
- Demand for higher-performance products
- Increasing demand for renewable/recyclable materials

**Feedstock**

- Sustained low oil prices
- Decreasing cost of renewable electricity
- Sustainable waste management
- Expanding availability of green $H_2$
- Closing the carbon cycle

**Capital**

- Risk of greenfield investments
- Challenges and costs of biorefinery start-up
- Availability of depreciated and underutilized capital equipment

**Social Responsibility**

- Carbon intensity reduction
- Access to clean air and water
- Environmental equity

# NREL's Bioenergy Program Is Enabling a Sustainable Energy Future by Responding to Key Market Needs

## Value Proposition

- Efficient conversion of biomass sugars to 2,3 BDO which is a versatile chemical building block for **producing fuels** and chemicals such SAF,  MEK and butadiene.

## Key Differentiators

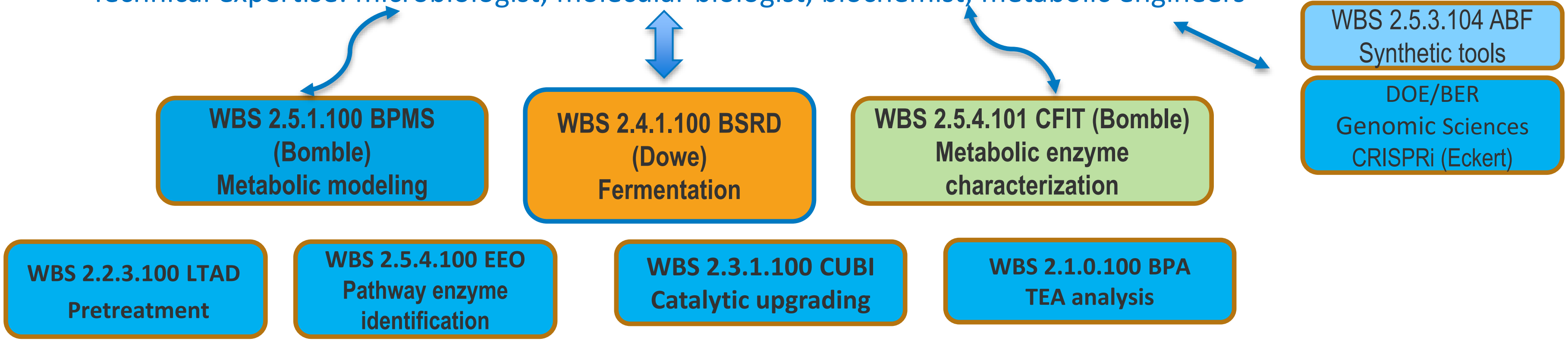
- *Zymomonas* offers fast sugar (glucose) utilization rate and high yield to ethanol (native product) at high titer.
- Biomass sugar to ethanol conversion process using engineered *Zymomonas* capable of xylose and arabinose utilization is demonstrated in NREL PDU as well as DuPont's cellulosic ethanol plant.

# 1. Management-Project structure

## TMD Project Structure and Team Members Responsibilities

**Task 1. Balance the redox for anaerobic process (Hui, Chou and Lunin)**
**Task 2. Engineering pentose utilization to include arabinose (Chou)**
**Task 3. Reduce byproduct formation (Franden, Hui)**

Technical expertise: microbiologist, molecular biologist, biochemist, metabolic engineers




- **Biweekly project meetings**
- **Quarterly reports**
- **Project update in BETO lab calls**

# 1. **Management**-Risk mitigation and strategies

**Process robustness**:

- 2,3 BDO's unique fermentation pathway requires low level of oxygen, how do we manage this fermentation process?
  - ➢ Can we balance the pathway through heterologous pathways with co-products
  - ➢ Process optimization-easy of maintaining high yield and less byproduct formation including cell mass

**Pentose Utilization:**

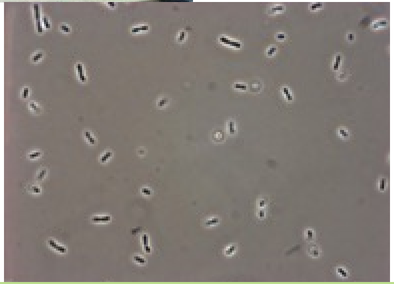
- Low yield and lower rate (less preferred sugar compared with glucose) sugar fermentation process.
  - ➢ Pentose transport
  - ➢ Pentose metabolism
  - ➢ Energetics
- Efficient utilization of xylose and arabinose - critical success factors for a cost competitive conversion process from biomass sugars!

# 2. Approach - *Zymomonas mobilis – The Ethanologen*

**Advantages**

- High ethanol yield from glucose (98%)
- High sugar uptake rate -3 x of yeast
- High specific ethanol productivity – 3X that of yeast
- High ethanol tolerance (13%w/v)
- Less cell mass formation
- No oxygen requirement
- GRAS

**Glucose**
**Fructose**
**Sucrose**
**Xylose**
**Arabinose**



**Ethanol**

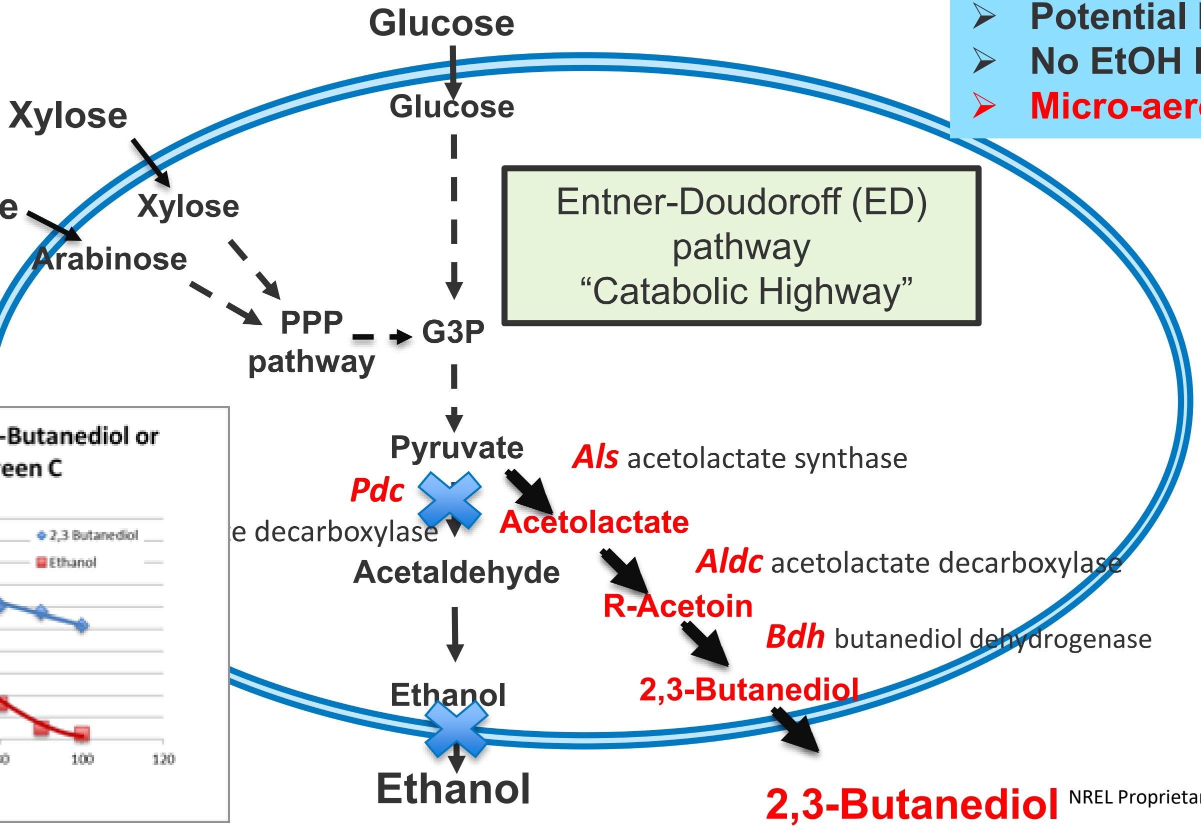

**NREL developed strain that ferments biomass hydrolysates/slurries to high ethanol titers which was used to demonstrate DOE 2012 EtOH cost target at the 9000L pilot scale.**


- Rogers PL, Lee KJ, Tribe DE (1979). *Biotechnology lett.* vol.1:165-170.
- Zhang, M. C. Eddy, K. Deanda, M. Finkelstein and S. Picataggio (1995) *Science, 267,* 240-243, 1995

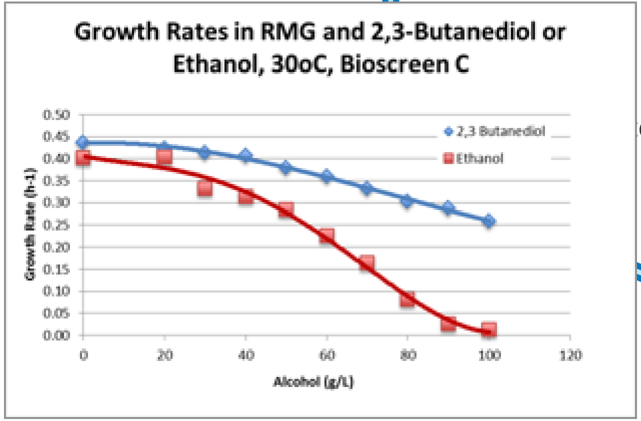
# 2. Approach -*Zymomonas mobilis* – The 2,3-BDO Strain

Slurry after DMR pretreatment and enzymatic hydrolysis containing mixed C6/C5 sugars

- Potential high TRY
- No EtOH Production
- Micro-aerophilic



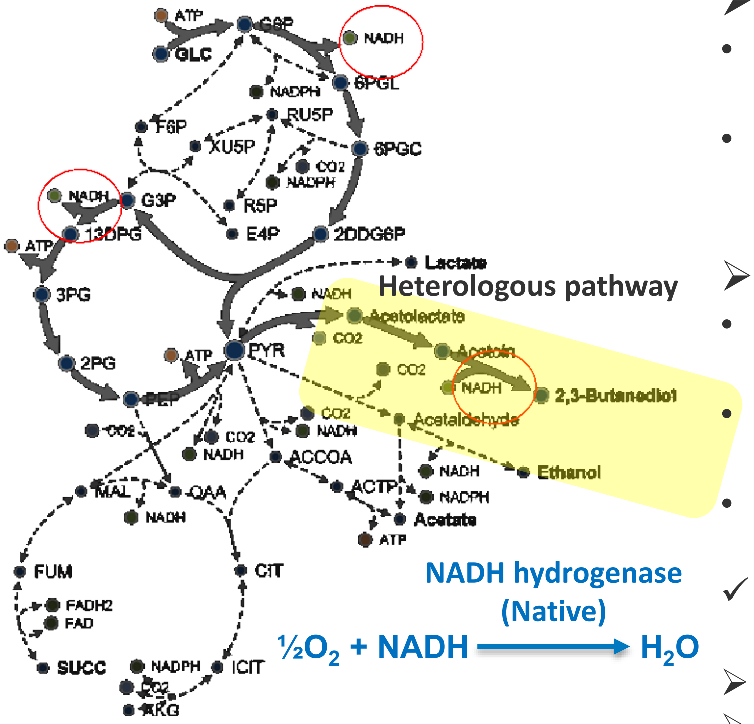

## BDO is less toxic




NREL Proprietary for discussions with POET

# 2. Approach-Strategy

**Challenges:** BDO fermentation leads to redox imbalance which need low level of oxygen (air) to balance the excessive redox (1 mol NADH/mol glucose).




NADH hydrogenase (Native)

$\frac{1}{2}O_2$ + NADH → $H_2O$

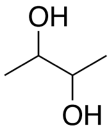
Bomble & St. John (BPMS)

- ➢ **Balance the redox – Biological solution (TMD)**
  - $H_2$ production: BDO yield from sugars remain same, H2 generated can be used for upgrading.
  - Co-production of succinate: less BDO produced but with succinate as co-product can potentially achieve overall >30% higher carbon yield.
- ➢ **Microaerobic fermentation- Process solution (BSRD)**
  - Too much oxygen: Resulting in low BDO and high acetoin concentrations (potential acetoin toxicity).
  - Less oxygen: glycerol formation-loss of carbon to byproduct, low BDO yield and contaminant for catalytic conversion.
  - Cost and process complexity.

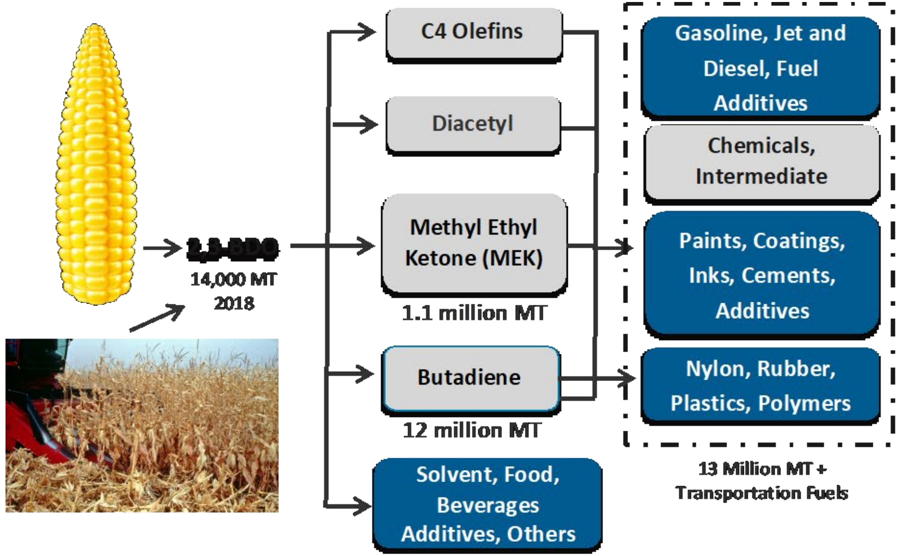
✓ **Go-No-Go decision: Process vs Biological solution**

- ➢ **Arabinose utilization for BDO production**
- ➢ **Improve BDO productivity from pentose utilization as well as its less preferred utilization vs glucose**

# 3. Impact- Platform Chemicals




## 2,3 BDO : versatile chemical building block for producing jet fuels and fuel additives, solvents, polymers (butadiene) and other chemicals




**Key Takeaways:** Existing 2,3 BDO market is small, ~14,000 MT per year, with forecasted growth of ~3%. However, BDO derivative markets are a promising opportunity to grow into novel chemicals, polymers, solvents, fuel additives

(Z. Abdullah, L. Tao 2020)

# 3. **Impact**- Technology

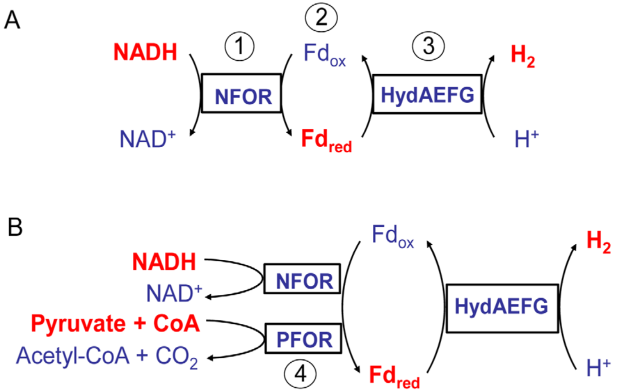
- Successfully **engineered *Zymomonas* strains** to **produce industrial relevant titers of BDO from hydrolysate liquor.**
- Provide an **efficient and robustness biomass conversion process** (as demonstrated by BSRD) that can be readily **adapted using glucose-rich feedstocks** by industrial partners; **Near term opportunity in Gen 1** corn ethanol of diversifying ethanol plants to produce an alternative to ethanol and that *Zymononas* is already highly effective on glucose
- Industry inquiries on using ***Zymomonas*-BDO technology for chemical production** market from biomass sugars
- Enables *Zymomonas* as an **effective and familiar microbial platform for fuels and chemicals** for industry applications
- **Directly support BETO's mission**: "Develop and transform our renewable biomass resources into commercially viable high performance biofuels"
- One patent application was filed and published several peer-reviewed articles

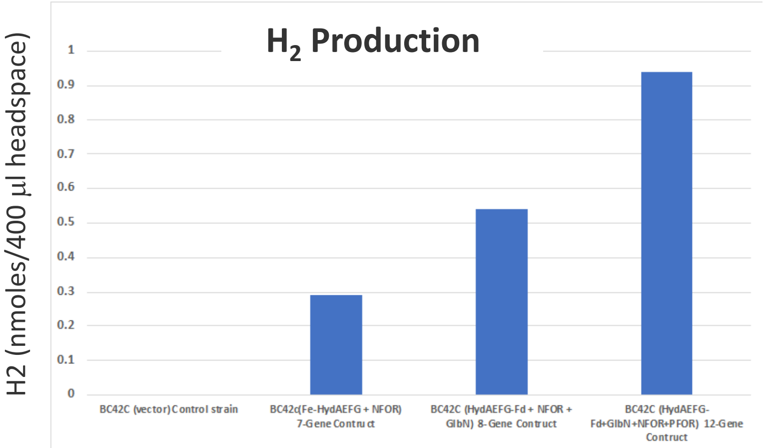
# 4. Progress and Outcomes – $H_2$ production


Hui Wei

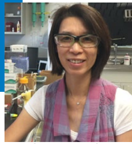
## Investigating heterologous pathways to balance to redox with H2 production:

- ➢ H2 production: BDO yield from sugars remains same, H2 generated can be used for upgrading
- Introducing **NADH dependent hydrogenase system (7 genes)** by co-expressed NFOR (NADH-dependent ferredoxin oxidoreductase), Fd (ferredoxin) and hydAEEG (hydrogenase hydA, maturases hydEFG). *In vivo* **hydrogen production is observed**
- **H2 production level increased** by co-expressing with hemoglobin-like protein (GlbN)
- **H2 production** further improved by co-expressing with PFOR (pyruvate:ferredoxin oxidoreductase).
- Observed **changes of the NADH and $NAD^+$ pools** but **anaerobic growth is limited**
- **In summary, pathway optimization efforts increased $H_2$ production by near 3x but still too low to enable anaerobic fermentation**



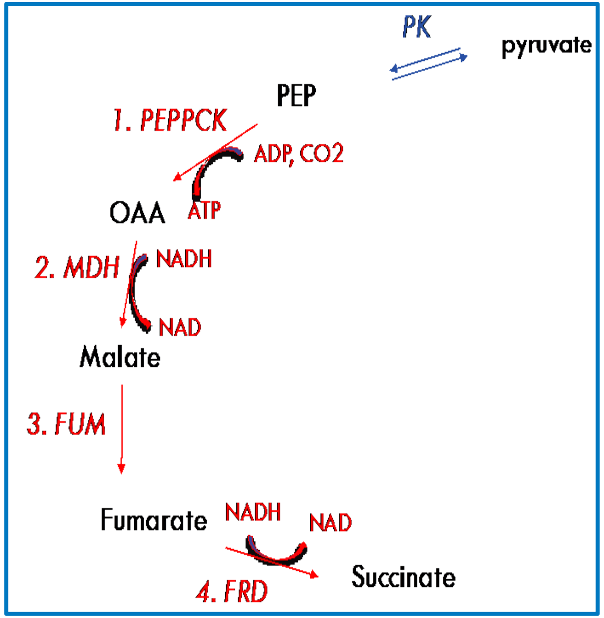



# 4. Progress and Outcomes –Succinate pathway




Investigating co-production of succinate:

- Succinate co-production can improve overall higher carbon yield (>30% improvement)




- Introduced succinate pathway encoding **PCK, MDH, FUM and FRD**
- Confirmed active expression of first 3 gene products: **PCK, MDH, FUM** by activity measurement
- Investigated two sources of **FRD** (*Actinobacillus succinogenes and Trypanosoma brucei*); Observed gene expression (by RT-PCR) but the No enzymatic activities! His-tagged FRD was detected but significant degradation was observed.
- No succinate production in flask fermentation evaluation.

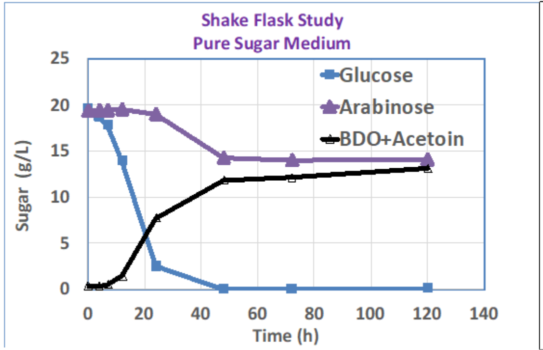
- Go/No Go: As these two redox balance pathways presented significant challenges to meet an anaerobic BDO fermentation, and BSRD was successful in identifying a fermentation condition with overlay air, decision is made not to further pursue these strategies.

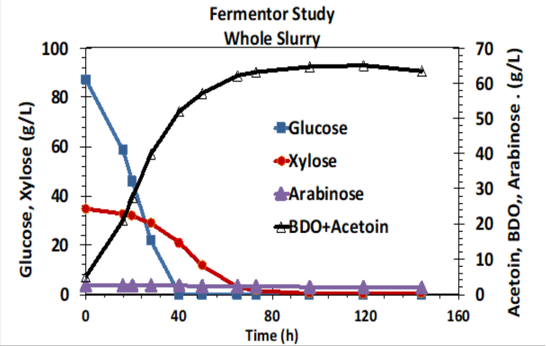
# 4. Progress and Outcomes –Arabinose utilization

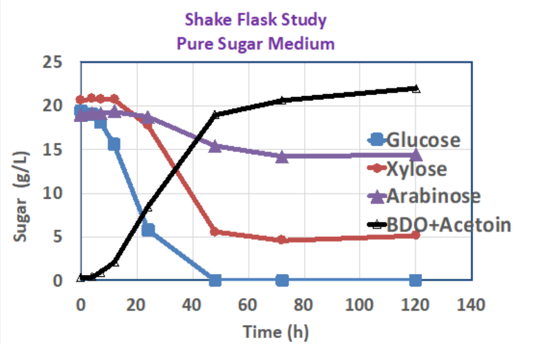
Yat-Chen Chou

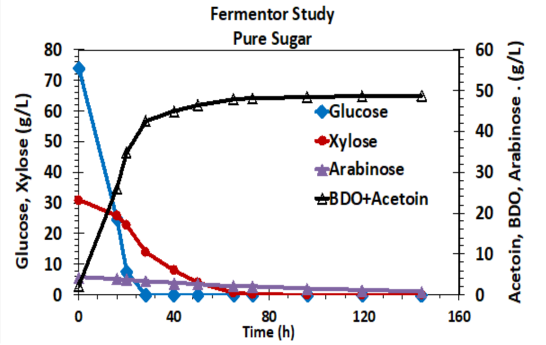
- L-arabinose is significant in hemicellulose(~10-20%) in corn stover.
    - Any unutilized sugars will be problematic in downstream catalytic upgrading.
- About 40-44% of the total pentoses in corn fiber (Gen 1.5 feedstock)

➢ Introduce the arabinose pathway genes into the BDO producing *Zymomonas* strain.



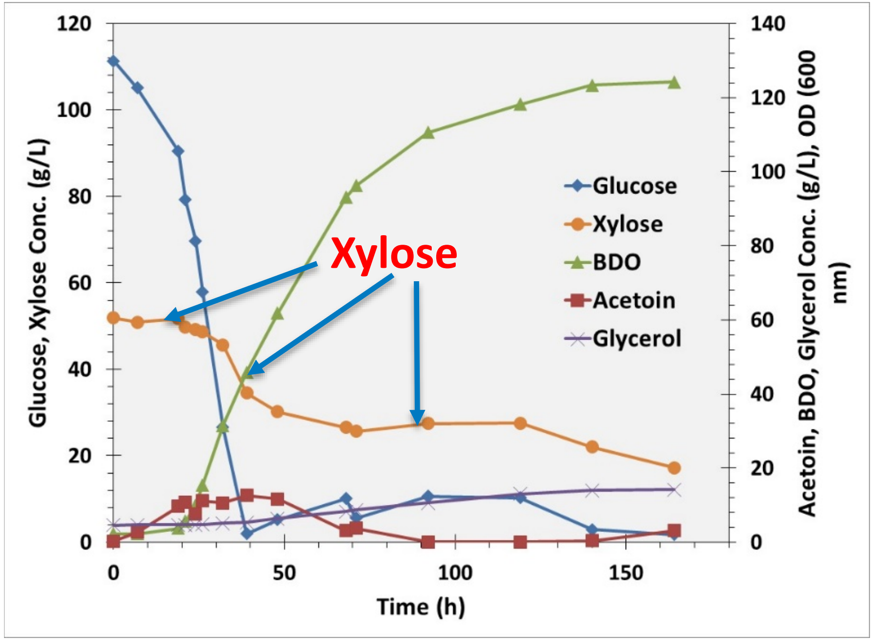




- **Achieved 50% arabinose utilization in 60h on pure sugar -met milestone target**
- **Arabinose utilization is very slow (at least 10 x slower compared with glucose)**
- **Minimum arabinose utilization in whole slurry:**
    1. **Arabinose transport?**
    2. **Slow arabinose pathway flux?**
    3. **Inhibitor in DMR hydrolysate?**

➢ **Further investigation needed**

# 4. Progress and Outcomes –Xylose utilization

- **In batch fermentations, both glucose and xylose utilization are > 95% and high BDO yield**
- **However, slow and incomplete xylose utilization were observed in BSRD's FY20 Q4 fed-batch fermentation which demonstrated 125 g/L BDO titer on biomass sugars**



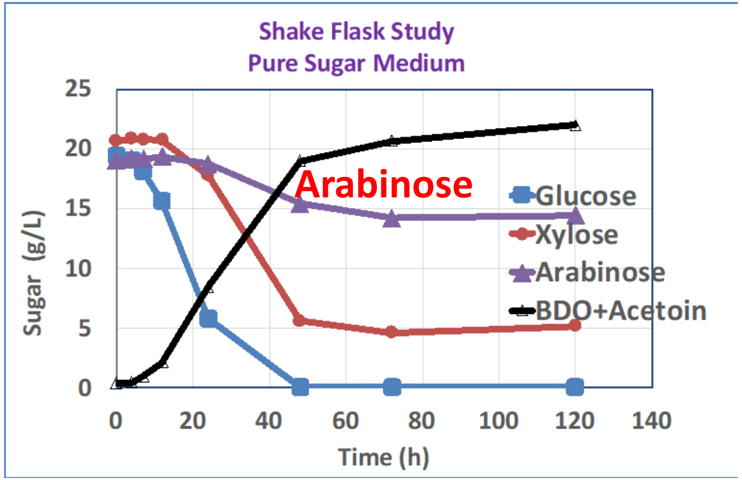

- **Xylose uptake is inhibited by glucose**
- **Xylose utilization rate is ~ 3 x slower compared with glucose**
  - **Buildup of xylose in fed-batch fermentation when pushing high BDO titers**
- **Xylose utilization incomplete**

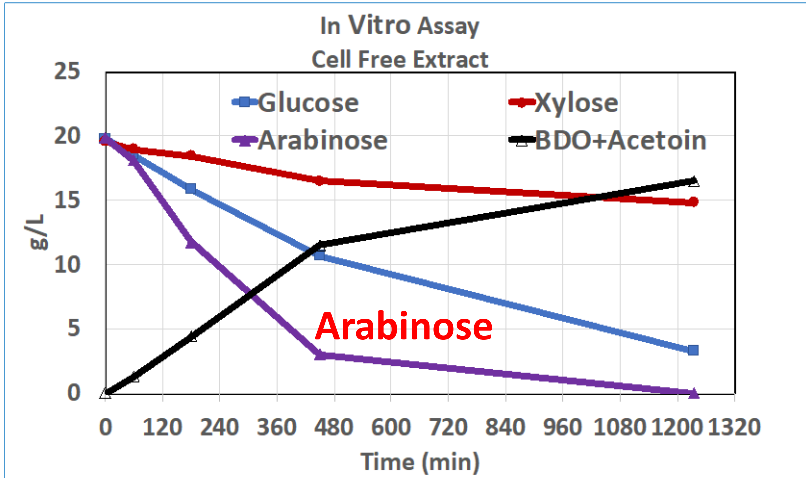
➢ ***Slow xylose utilization and incomplete!***

# 4. Progress and Outcomes - In Vitro cell-free extract assay


- Setting up In Vitro cell-free metabolic conversion experiments to compare conversion rates of glucose, xylose and arabinose to BDO and compare to in vivo fermentation rates to determine the impact of the pentose transporters vs metabolic enzymes

➢ Identify key limiting steps and engineer to improve the conversion rate **FY21 Q4 milestone**



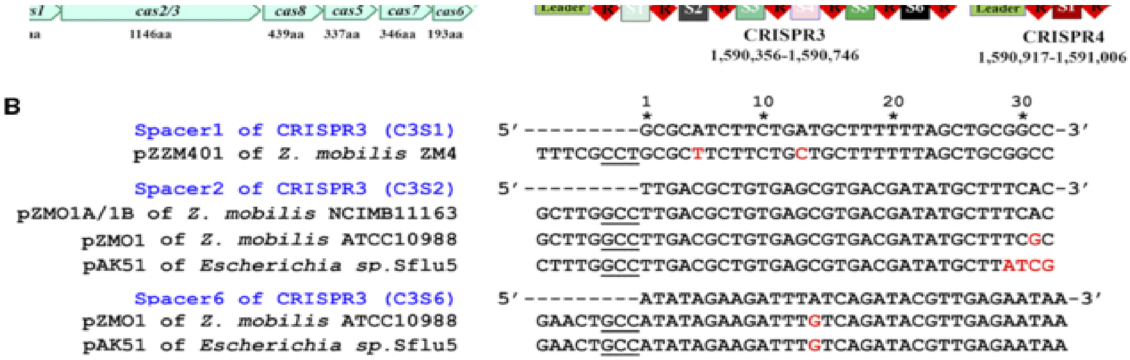




➢ In Vitro CFE assay suggest that the slower arabinose utilization is mostly limited by sugar transport.

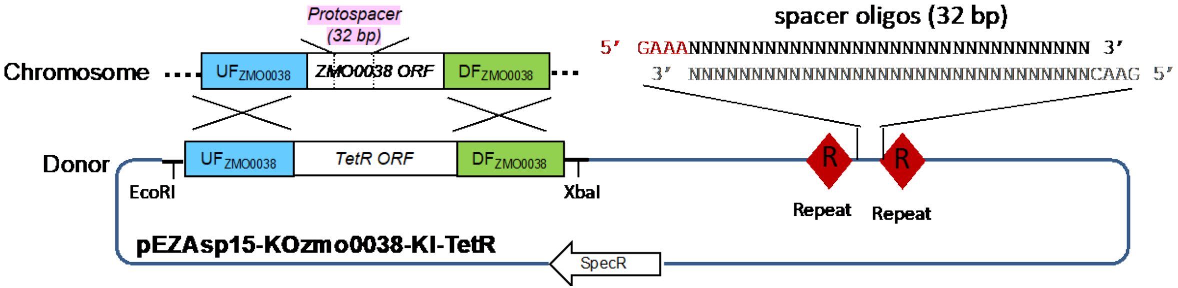
➢ Xylose utilization may be limited by both transport and metabolism.

# 4. Progress and Outcomes –CRISPR

- **Effective Pathway Engineering Tools with CRISPR Systems**
  - ***Zymomonas'* native CRISPR - Self-defense mechanism against phage infection**
    - **Recently demonstrated working as genome editing tool** (Zheng et al. 2019)




- **Our ongoing work to knock out and knock in genes (FY21 Q2)**




➢ **CRISPR can enhance our ability to conduct pathway engineering effectively!**

# Summary

- **Management**
  - Focusing on improving pentose utilization as the biological approach of balancing redox met limited success. Fermentation with overlay air demonstrated achieving high BDO yield (BSRD)
- **Approach**
  - Introducing heterologous pathways to balance the surplus NADH
  - Improve pentose utilization as it is critical factor in cost reduction for biomass sugar conversion process
- **Impact**
  - 2,3 BDO is a versatile platform chemical for producing fuels and chemicals such SAF,  MEK and butadiene
  - Successfully engineered *Zymomonas* strains to produce industrial relevant titers of BDO from hydrolysate liquor.
- **Progress and Outcomes**
  - Demonstrated $H_2$ production but low not sufficient to balance the redox
  - Enabled arabinose utilization but further improvement in both xylose & arabinose utilization
  - Identified initial targets for xylose and arabinose as well as efficient tools CRISPR

- *Funding*
- U.S. DOE EERE Bioenergy Technology Office (BETO)

- NREL:
  - Min Zhang
  - Yat-Chen Chou
  - Hui Wei
  - Mary Ann Franden
  - Vladimir Lunin
  - Michael Himmel


# Thank you!

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Specially Thanks to

- Nancy Dowe (PI for BSRD)
- Yannick Bumble (BMBP)

- HQ: TM: Ian Rowe
- NREL LPM and Platform Lead: Zia Abdullah, Rick Elander


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# Additional Slides

**(Not a template slide – for information purposes only)**

*The following slides are to be included in your submission for evaluation purposes, but <u>will **not** be part of your oral presentation</u> –*
*You may refer to them during the Q&A period if they are helpful to you in explaining certain points.*

# Responses to Previous Reviewers’ Comments

**Overall Impressions from Reviewers:**

"The team has successfully engineered Zymomonas strains to produce industrial relevant titers of BDO from hydrolysate liquor, increasing titer from 20g/L-80 g/L"

"The strain development and fermentation groups need to be congratulated on a good effort. They have demonstrated over the past three years that they can improvement titers and strain performance."

"The team has focused on solving the key issues of ethanol byproduct and redox balancing, which will reduce manufacturing cost due to easier separations and lower aeration requirement, respectively. The team works closely with Bench Scale Integration to evaluate their strains in fermentation. Through a combination of strain engineering and process development, significant progress has been made toward reaching commercial relevant metrics.  Little work has been done on improving pentose and arabinose utilization, but this work is planned for the coming year".

**Responses to Reviewers:**

We thank the reviewers for the positive comments about the progress the project has made over the past three years with regard to achieving the high 2,3 BDO titers from hydrolysate liquors using engineered Zymomonas strains.  We appreciate the reviewers' supportive feedback on our future research plan to improve pentose sugar utilization and addressing issues associated with redox balancing.

# Responses to Previous Reviewers' Comments

- **Why was 2,3 BDO not 1,4-BDO?**
  – The simplicity of the pathway and versatility of the product were the basis for this product choice. 1,4 BDO is a great product for consideration; however, its production by pathway engineering involves a complex metabolic pathway which would necessitate more extensive resources than were available.

- **Why *Zymomonas* is used for 2,3 BDO production?**
  – As described in the first slide, there are many native and engineered microorganisms, including yeast, that are capable of producing high titers of 2,3 -BDO from glucose. However, many of these strains do not ferment xylose well, including the engineered yeast strains. In addition, to reach high productivity, these organisms usually grow under highly aerobic conditions to produce cell mass in the first stage, feedstock carbon is therefore used for cell mass growth rather product formation, resulting in overall lower yields. *Zymomonas* has a 3-fold higher specific sugar uptake rate compared to yeast. Also, *Zymomonas* does not have an active TCA cycle; therefore, carbon is mostly directed to the product formation which supports high production rates. *Zymomonas* also have low cell mass formation.

# Publications, Patents, Presentations, Awards, and Commercialization

1 “Phylogenetics-based identification and characterization of a superior 2, 3-butanediol dehydrogenase for Zymomonas mobilis expression” V Subramanian, VV Lunin, SJ Farmer, M Alahuhta, KT Moore, A Ho, Yogesh B Chaudhari, Min Zhang, Michael E Himmel, Stephen R Decker. Biotechnology for Biofuels 13 (1), 1-20. (2020)

2 “Metabolic engineering of Zymomonas mobilis for anaerobic isobutanol production” M Qiu, W Shen, X Yan, Q He, D Cai, S Chen, H Wei, EP Knoshaug, Min Zhang, Michael E Himmel, Shihui Yang. Biotechnology for biofuels 13 (1), 15 (2020)

3 “High titer fatty alcohol production in Lipomyces starkeyi by fed-batch fermentation” Wei Wang, Eric P Knoshaug, Hui Wei, Stefanie Van Wychen, Chien-Yuan Lin, Todd Vander Wall, Qi Xu, Michael E Himmel, Min Zhang. Current Research in Biotechnology 2, 83-87 (2020)

4 “Transcriptomic profiles of Zymomonas mobilis 8b to furfural acute and long-term stress in both glucose and xylose conditions” S Yang, MA Franden, X Wang, YC Chou, Y Hu, SD Brown, PT Pienkos, M Zhang. Frontiers in microbiology 11, 13 (2020)

5 Methods for Metabolic Engineering of a Filamentous Trichoderma reesei. YC Chou, A Singh, Q Xu, ME Himmel, M Zhang. Metabolic Pathway Engineering, 45-50

6 Ferrous and Ferric Ion-Facilitated Dilute Acid Pretreatment of Lignocellulosic Biomass under Anaerobic or Aerobic Conditions: Observations of Fe Valence Interchange and the Role of Fenton Reaction. H Wei, W Wang, PN Ciesielski, BS Donohoe, M Zhang, ME Himmel, Xiaowen Chen, Melvin P Tucker. Molecules 25 (6), 1427 (2020)

7 Prediction and characterization of promoters and ribosomal binding sites of Zymomonas mobilis in system biology era, Y. Yang, W. Shen, J., Runxia Li, Y. Xiao, H. Wei, Y.Chou, M. Zhang, M.E Himmel, S. Chen, L. Yi, L. Ma, S. Yang. Biotechnology for biofuels, 12 (1), 52, 2019

8 “Solvent-free spectroscopic method for high-throughput, quantitative screening of fatty acids in yeast biomass”. Lieve ML Laurens, Eric P Knoshaug, Holly Rohrer, Stefanie Van Wychen, Nancy Dowe, Min Zhang. Analytical methods 11(1), 58 2019

# Publications, Patents, Presentations, Awards, and Commercialization

9. “Xylose utilizing oleaginous yeast” EP Knoshaug, M Zhang, A Singh, MT Guarnieri, US Patent 10,533,196
10. “Engineered Zymomonas for the production of 2, 3-butanediol”. Inventors, Min Zhang, Yat-Chen Chou, Mary Ann Franden, Michael E Himmel. US patent application 16173910 on 2019/5/23
11. “Complete genome sequence and the expression pattern of plasmids of the model ethanologen Zymomonas mobilis ZM4 and its xylose-utilizing derivatives 8b and 2032”. S Yang, JM Vera, J Grass, G Savvakis, OV Moskvin, Y Yang, SJ McIlwain, Y Lyu, I Zinonos, AS Hebert, JJ Coon, DM Bates, TK Sato, SD Brown, ME Himmel, M Zhang, R Landick, KM Pappas, Y Zhang. Biotechnology for biofuels 11 (1), 125, 2018
12. “Identification of inhibitors in lignocellulosic slurries and Determination of Their effect on hydrocarbon-Producing Microorganisms”. S Yang, MA Franden, Q Yang, YC Chou, M Zhang, PT Pienkos. Frontiers in bioengineering and biotechnology 6, 23, 2018
13. “Lipid accumulation from glucose and xylose in an engineered, naturally oleaginous strain of Saccharomyces cerevisiae”. EP Knoshaug, S Wychen, S Arjun, M Zhang. Biofuel Research Journal 5 (2), 800-805, 2018
14. Expression of an endoglucanase–cellobiohydrolase fusion protein in Saccharomyces cerevisiae, Yarrowia lipolytica, and Lipomyces starkeyi Biotechnology for biofuels, 11 (1), 322. 2018
15. Engineering Zymomonas mobilis as a platform organism for biomass conversion: advances and challenges. Zhang, M., Chou, Y.C., Franden, M.A., Wei, H., Lunin, V., Spiller, R., Rohrer, H., Dowe, N., St John, P., Bomble, Y.J. and Himmel, M., 2019, April. In 41st Symposium on Biotechnology for Fuels and Chemicals. SIMB
16. “Redirecting Carbon Metabolism in Zymomonas mobilis: A Promising New Pathway to Fuels and Chemicals from Biomass” at the 40th Symposium on Biotechnology for Fuels and Chemicals in Clearwater Beach, FL
17. “Down Regulating the Ethanol Pathway in Zymomonas” by Yat-Chen Chou*, Ali Mohagheghi, Mary Ann Franden, Nancy Dowe, Ryan Spiller, Michael E. Himmel and Min Zhang at the the 40th Symposium on Biotechnology for Fuels and Chemicals in Clearwater Beach, FL

# Publications, Patents, Presentations, Awards, and Commercialization

18. "Enabling Zymomonas mobilis using Systems Biology: An Effective Platform for Production of Fuels and Chemicals from Biomass Sugars" at the 12th Metabolic Engineering Conference in Munich, Germany
19. "Redirecting Carbon Metabolism in Zymomonas mobilis: A Promising New Pathway to Fuels and Chemicals from Biomass" Yat-Chen Chou, Mary Ann Franden, Mike E Himmel, Yannick Bomble, Nancy Dowe, Zhang et al. manuscript in preparation