DOE Bioenergy Technologies Office (BETO)
2019 Project Peer Review

Targeted Microbial Development (TMD)
WBS 2.4.3.102

Session: Biochemical Conversion

March 4, 2019

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National Renewable Energy Laboratory

March 4-8, 2019

Denver, CO

Goal Statement

Goal

- To develop microbial pathways capable of producing high carbon efficiency intermediates amenable to economic bioconversion, separation, and catalytic upgrading to hydrocarbon fuels and chemicals to substantially contribute towards achieving **BETO's 2022 cost target of $3.00/GGE (2.50/GGE in 2030)**.

Outcome

- Provide leading technologies for the production of **high carbon efficiency intermediates suitable for catalytic upgrading to HC fuels with reduced cost**.
  - Engineer *Zymomonas* for 2,3 butanediol (2,3 BDO) production
  - 2,3 BDO can be chemically upgraded to fuels and other chemicals as coproducts
- Identify **future sugar upgrading technologies; as well as a critical knowledge base** for the bioenergy industry and research community to further R&D working towards production of impactful HC biofuels from biomass.

Relevance

- This project develops and improves metabolic pathways and strains that result in cost competitive, robust biological conversion routes to intermediates.

Quad Chart Overview

Timeline

- Project start date: 2015
- Project end date: 2021
- Percent complete: 58%

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$1.7M	$1.7M	$800K	$3.5M
Project Cost Share*				

- **Partners: If multiple DOE recipients are involved in the project, please list level of involvement, expressed as percentages of project funding from FY 17-18. [(i.e. NREL (70%); INL (30%)]**
- In collaboration with BSRD and BPMS

Barriers addressed:

Ct-D. Advanced bioprocess development: Increasing titer, rates, and yields of bioproducts through metabolic engineering and fermentation processing improvements is critical to lowering the costs of fuels and chemicals produced from biomass.

Objective

Engineer a *Zymomonas mobilis* strain producing 2,3-BDO from biomass sugars including glucose, xylose, and arabinose at high yield, titer and rate.

End of Project Goal

- Engineer *Z. mobilis* to produce 2,3-BDO from glucose, xylose, and arabinose at 95%, 90%, and 85% yield in batch fermentation or equivalent 95%, 90%, and 85% total carbon/mass yield (if co-products are made).
- Use whole slurry hydrolysate at 20% TS or higher under anaerobic condition in 54 hours.

1—Project Overview

Focused on three upgrading strategies (FY16-17):

- Anaerobic HC Intermediates from *Zymomonas mobilis* – producing 2,3 BDO *(*Task 1)
- Aerobic Aromatic Catabolism/lignin upgrading to muconic acid - *Pseudomona*s (Task 2)
- Advanced Concepts for Producing HCs (low TRL) (Task 3)
  - Aerobic yeast fermentation for secreted fatty alcohol (FA) production
  - Aerobic yeast fermentation for consolidated bioprocessing (CBP)

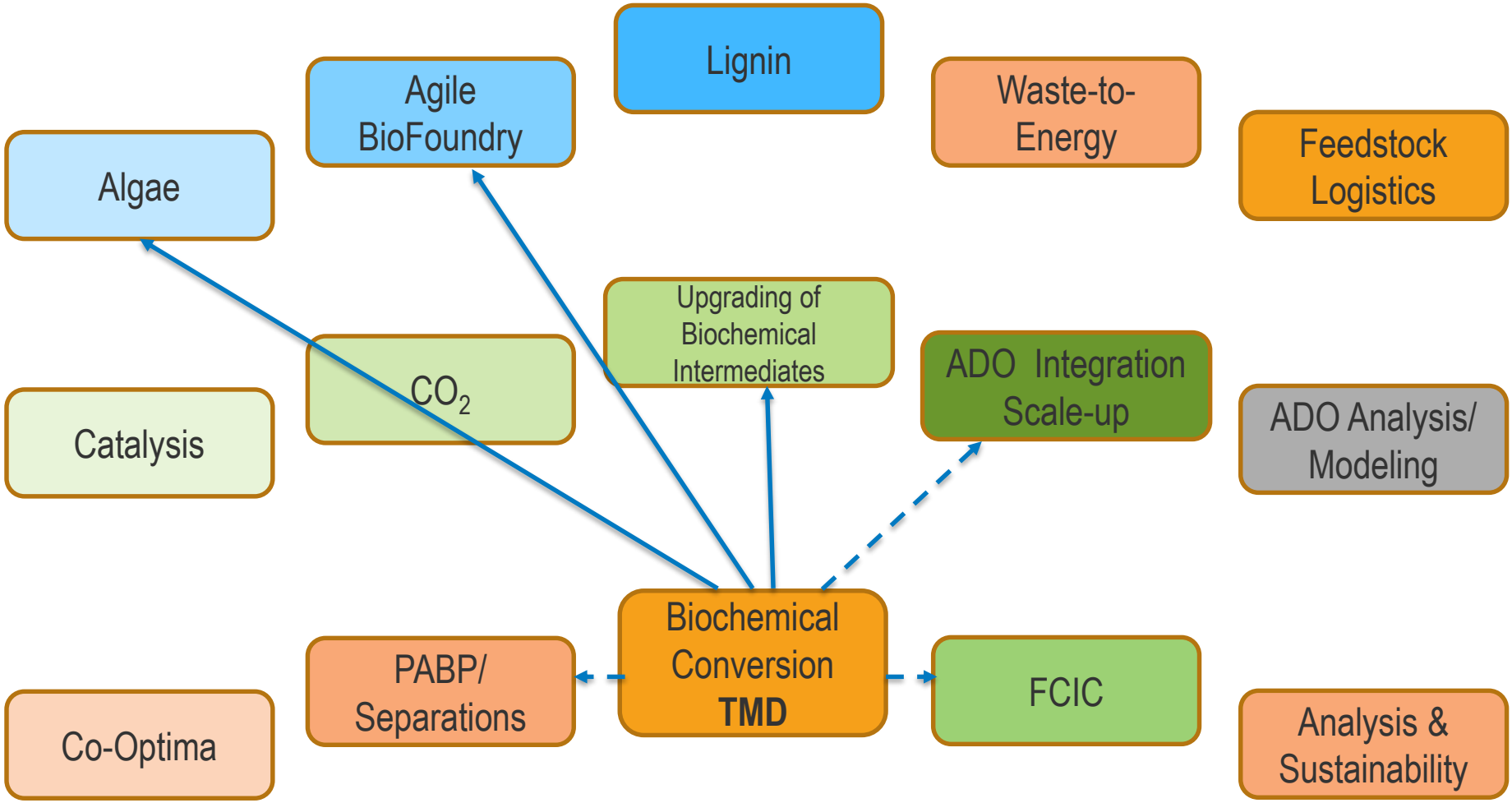
Down selected to one pathway in FY18

- Successfully demonstrated transition from mixed alcohol/diol production to diol only; therefore reducing process complexity and processing cost.
- Focus on engineering *Z. mobilis* for producing 2,3 BDO as intermediate for further catalytic upgrading to hydrocarbon fuels and chemicals from biomass sugars.
- Further improving the product yield, rate and titer (broader substrate utilization, metabolic engineering for redox balancing to eliminate O_2 requirement, increasing carbon flux).

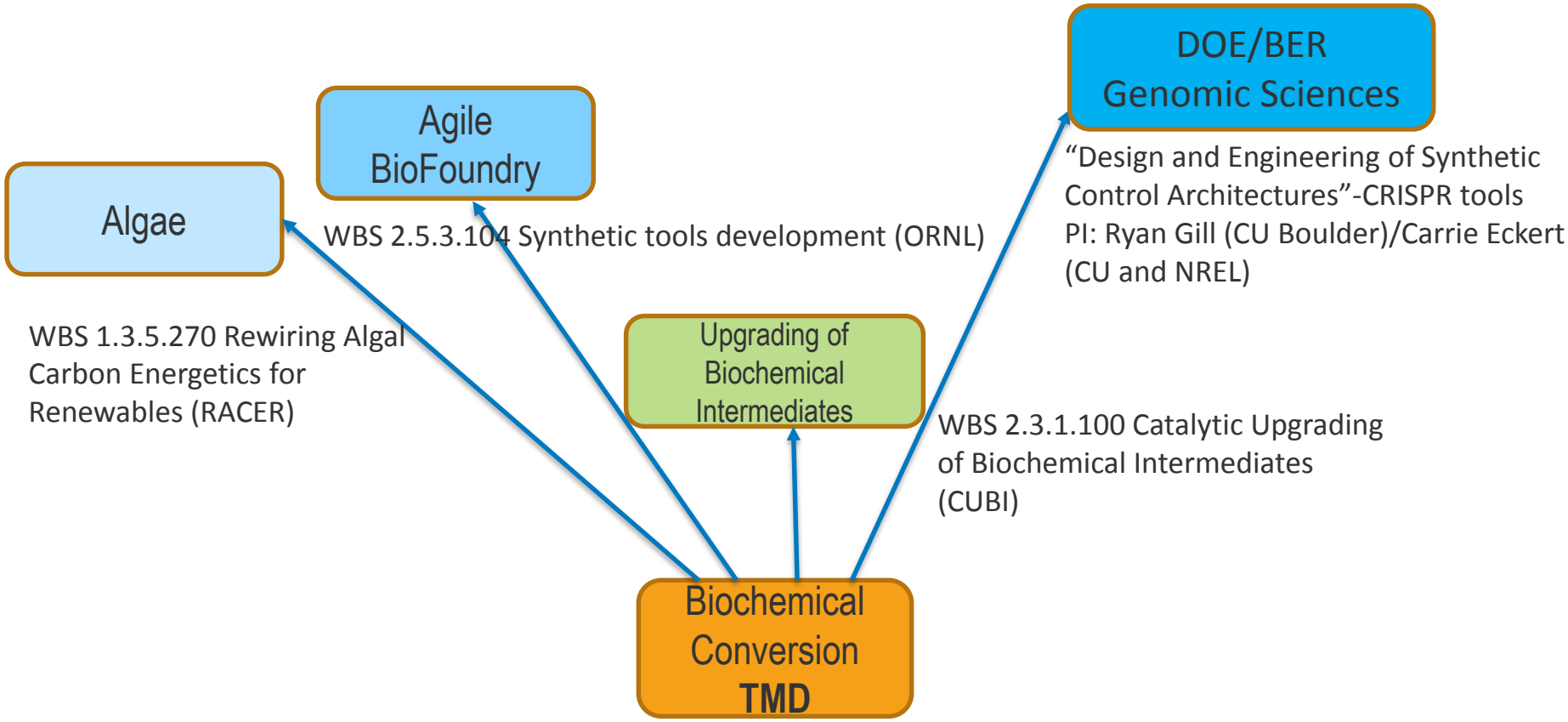
The BDO pathway advantages

- Identified by the Biochemical Platform Analysis (BPA) team.
- Robust fermentation with high sugar utilization (>90% glucose and xylose).
- Demonstrated at bench scale using both model and real hydrolysates.
- Whole-slurry hydrolysate fermentation – simple process and high sugar yield.
- *Z. mobilis* tolerance to high BDO titers enables high TS% and fed-batch fermentation.
- High overall hydrocarbon yield via aqueous catalytic conversion shown at bench scale.
- A range of potential chemical coproducts may be readily derived from BDO intermediate including MEK or 1,3-butadiene.

Project Integration



Project Integration



"Design and Engineering of Synthetic Control Architectures"-CRISPR tools PI: Ryan Gill (CU Boulder)/Carrie Eckert (CU and NREL)

WBS 2.5.3.104 Synthetic tools development (ORNL)

WBS 1.3.5.270 Rewiring Algal Carbon Energetics for Renewables (RACER)

WBS 2.3.1.100 Catalytic Upgrading of Biochemical Intermediates (CUBI)

- WBS 2.1.0.100 Biochemical Platform Analysis (BPA)
- WBS 2.2.3.100 Low Temperature Advanced Deconstruction (LTAD)
- WBS 2.4.1.100 Bench Scale R&D (BSRD)
- WBS 2.5.1.100 Biochemical Process Modeling and Simulation (BPMS)
- WBS 2.5.4.100 Enzyme Engineering & Optimization (EEO)
- WBS 2.5.4.101 Cell Free & Immobilization Technologies (CFIT)

2—Approach (Management)

Project Structure and Team Members Responsibilities

FY16-FY18

Targeted Microbial Development
Michael Himmel

FY19-FY21

Targeted Microbial Development
Min Zhang

Task 1. Engineering *Zymomonas* Producing 2,3-BDO
- **M. Zhang > Technical breakthrough-focus efforts**

Task 2. Lignin Upgrading to Muconic Acid by *Pseudomonas*
- **G. Beckham > Moved to Lignin in FY18**

Task 3. Advanced Concepts for Producing HCs
- **M. Zhang > Low TRL discontinued in FY18**

Task 1. Engineering *Zymomonas producing 2,3-BDO*
- M. Zhang

- **Balance the redox for anaerobic process**
- **Achieve high yields from all pentose**
- **Enhance the pentose utilization rate**

2—Approach (Management)

- **Utilize diverse skill sets**: microbiology, molecular biology, biochemistry/enzymology, metabolic engineers, and fermentation engineers.
- **TEA analysis to help down select sugar upgrading strategies and prioritize research needs** - Biochemical Platform Analysis (BPA).
- Provide engineered strains for fermentation strain evaluation by Bench Scale R&D team (BSRD)); provide fermentation data to the Biochemical Process Modeling and Simulation (BPMS) team for evaluation/feedback for further strain improvement.
- **Provide improved strains to BSRD Team for fermentation process development and integration and SOT demonstration.**
- Collaborate with multiple teams to meet research needs in advanced tool development–including Agile BioFoundry, BPMS for pathway modeling, Enzyme Engineering & Optimization (EEO) for improved metabolic enzymes, Low Temperature Advanced Deconstruction (LTAD) for toxicity analysis, Cell Free & Immobilization Technologies (CFIT) for pathway optimization, Catalytic Upgrading of Biochemical Intermediates (CUBI) for catalytic upgrading, and the BER Genome Sciences project supporting CRISPR development.
- Provide support for pathway engineering; i.e., mannose to 2,3 BDO (Algae/RACER).
- Submit BETO-approved Regular (Smart) milestones and Go NoGo decisions.
- Publish significant findings in peer reviewed journals.

2—Approach (Technical)

Explain the technical approach to achieving your goal(s)

- Apply **metabolic engineering and synthetic biology tools** to engineer microorganisms for efficient utilizing biomass sugars to HC intermediates for high yield, rate and titer.
- Identify and eliminate unwanted pathways to **redirect carbon flow** for high product yield.
- Recruit novel pathways to **balance redox** to achieve anaerobic fermentation
- **Enhance carbon flux** though pathway optimization and protein engineering to achieve high rate of pentose utilization.

Explain the top 2 to 3 potential challenges. . . .

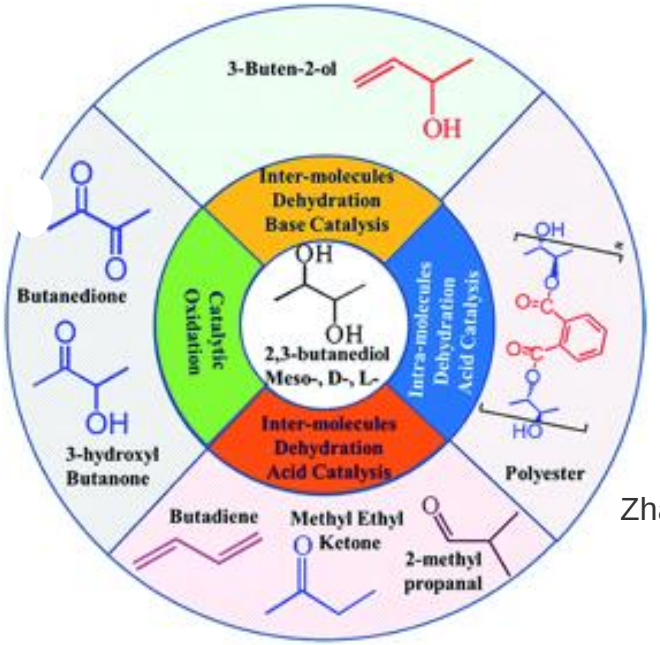
- Achieving redox balance for the fully anaerobic 2,3 BDO fermentation using the currently identified approaches.
- Complete utilization of sugar feedstocks (i.e., pentoses).

Describe critical success factors. . . .

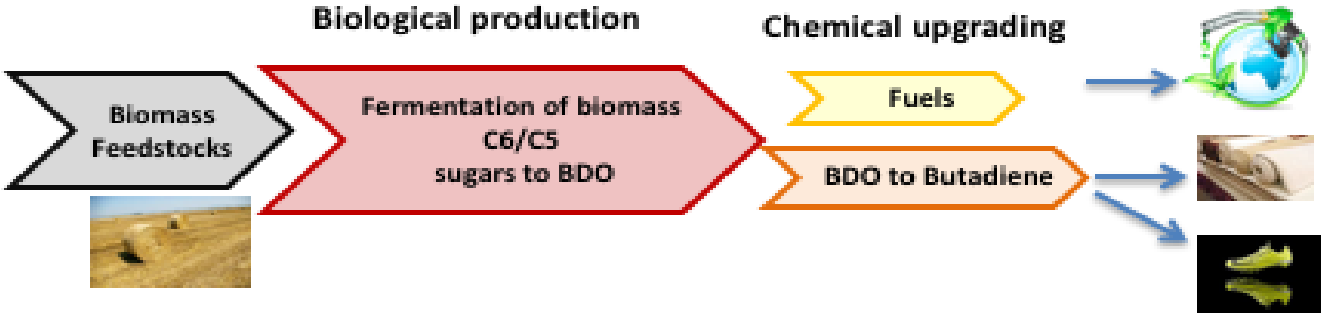
- Demonstrate effective production of HC intermediates and chemicals in relevant yields, rates and titers under anaerobic conditions (for example: >100 g/L BDO, 1 g/L/h, and 0.47 g/g).
- Demonstrate high yield, titer, and productivity for a cost effective process.
- Robustness and scalability of strains in a process relevant, commercial context.

2—Approach (Technical)

2,3-Butanediol (BDO): Versatile Chemical Building Block for Producing Solvents, Chemicals, Gasoline, Diesel and Jet Fuels



Zhao et al. RSC Adv., 2016, 6, 16988-16995



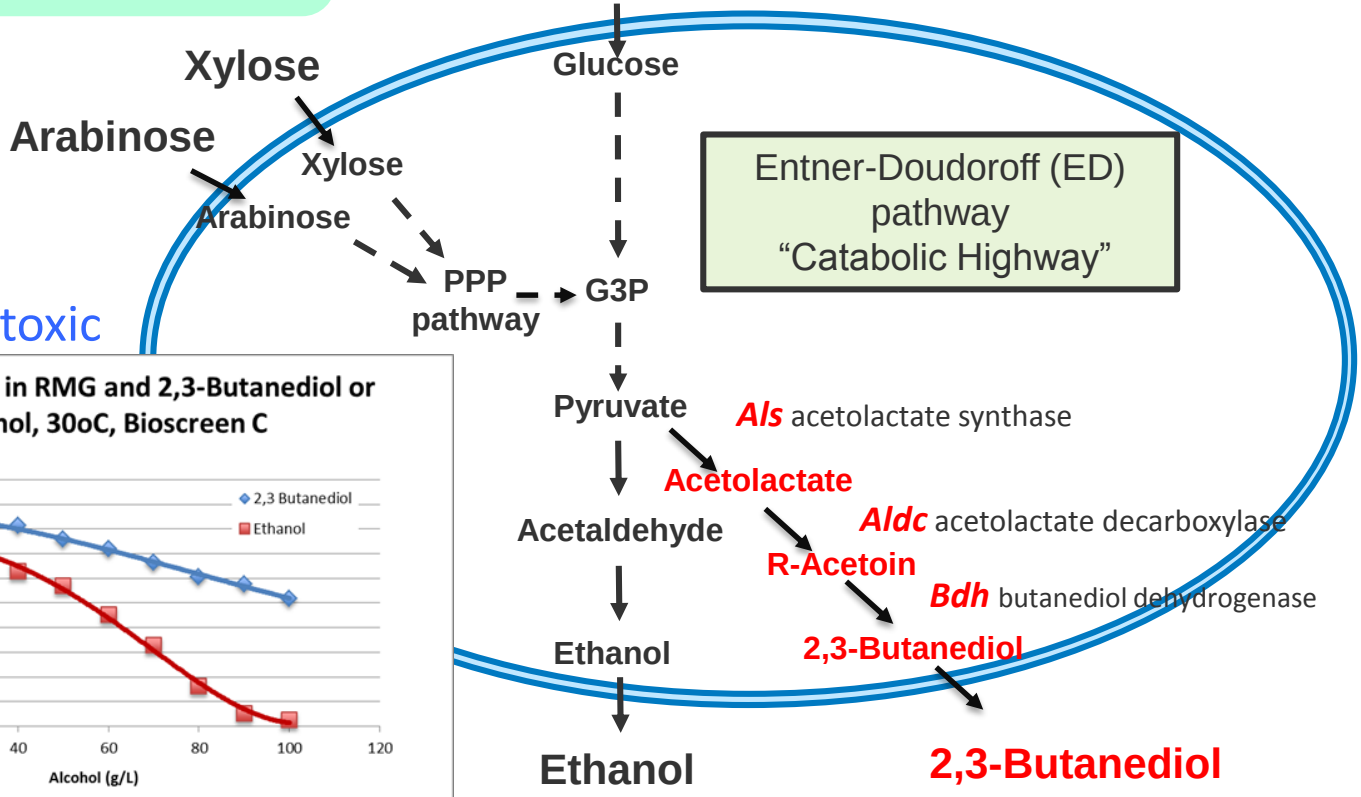
3—Technical Accomplishments/ Progress/Results

Rewiring the Carbon Metabolism *Z. mobilis for* Producing 2,3 BDO

DMR pretreated biomass and enzymatic hydrolysis containing mixed C6/C5 sugars

Technical Progress (up to FY16/FY17)

- **Initial titer: 2-5 g/L BDO with high ethanol**
- **Pathway optimization improved to 10 g/L BDO**
- **Process optimization reached to ~20 g/L BDO**



BDO is less toxic

3—Technical Accomplishments/ Progress/Results

Challenges in Rewiring Carbon Metabolism *Z. mobilis for* Producing 2,3 BDO

- Mixed products: ethanol and 2,3 BDO, **increased separation costs** because of two separate purification and catalytic upgrading trains.
- **Process oxygen dependent: potential scale up challenges.**

✓ ***Redirect the carbon flow to only 2,3 BDO and no ethanol -*** *pdc* Gene Knockout
✓ ***Process oxygen dependent: recruiting heterologous pathways instead of oxygen to balance the redox (and potentially increase carbon or energy output).***

Historic Challenges in *pdc* Gene Knockout in *Zymomonas*

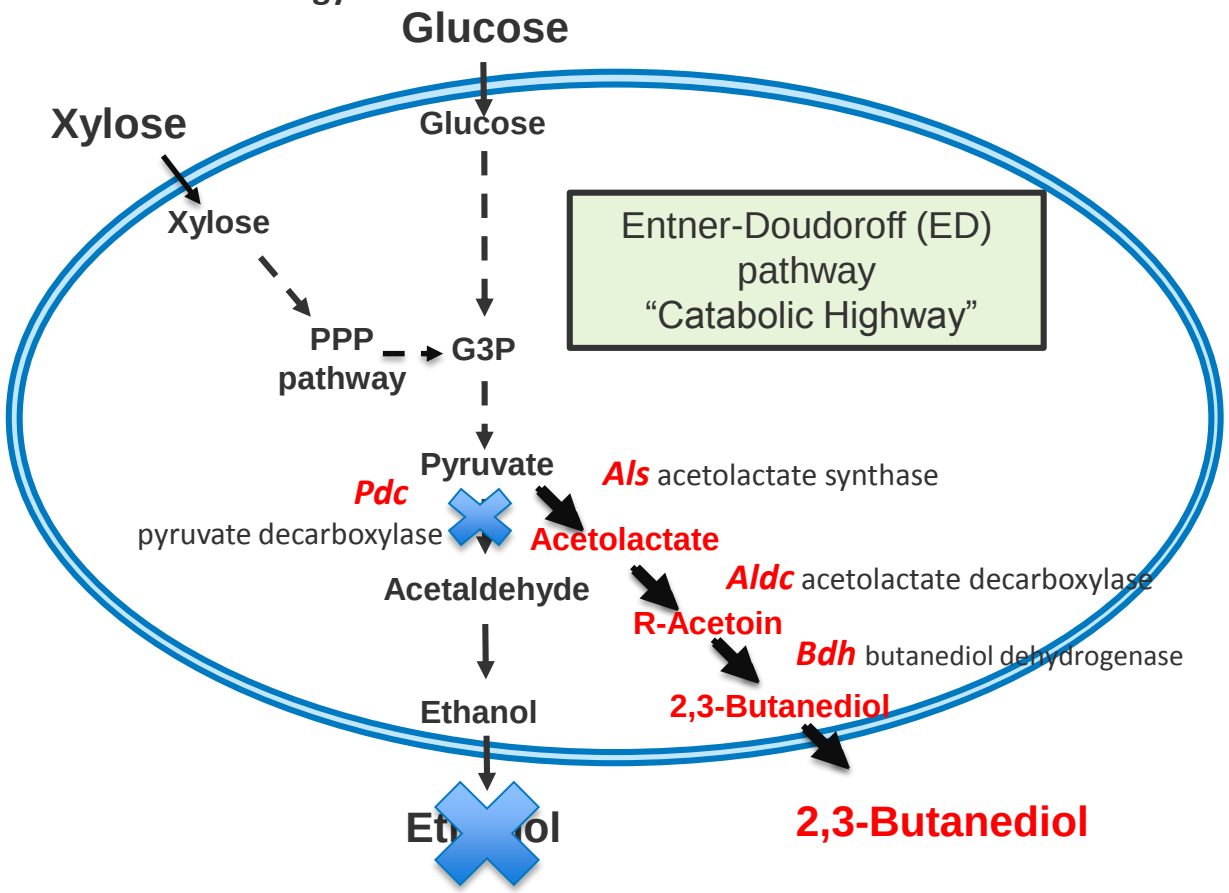
- Seo *et al.* US2009/0162919 A1 claimed obtained a *pdc* gene knockout mutant; however, the strain is lost due to its instability (personal communication).
- Zhao (2011) reported **unsuccessful *pdc* gene knockout efforts** (detection of both WT *pdc* and mutant *pdc fragments* in the mutant strain).
- Shui *et al.,* 2015 reported unsuccessful *pdc* gene knockout (detection of both WT *pdc* and mutant *pdc* fragments in the mutant strain)**.**
- ***A Study by Berkeley group (Skerker et al. 2013) using mutant library Indicates…. Is Zymomonas polypoid?***

3—Technical Accomplishments/ Progress/Results

Demonstrated Success in *pdc* Gene Knockout in *Zymomonas*, Created Healthy BDO-Producing Strains

Three critical steps in > two years of sustained effort:

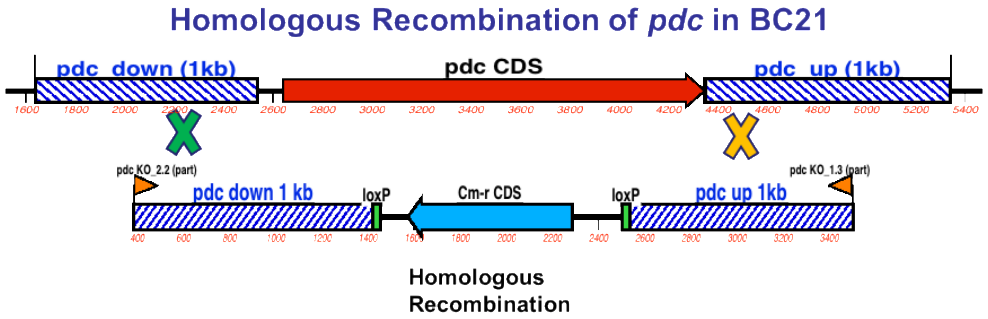
- ✓ Heterologous pathway
- ✓ DNA design
- ✓ Selection strategy



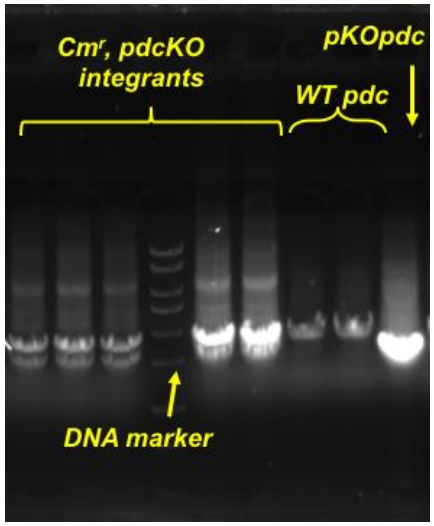
3—Technical Accomplishments/ Progress/Results

pdc Gene Knockout

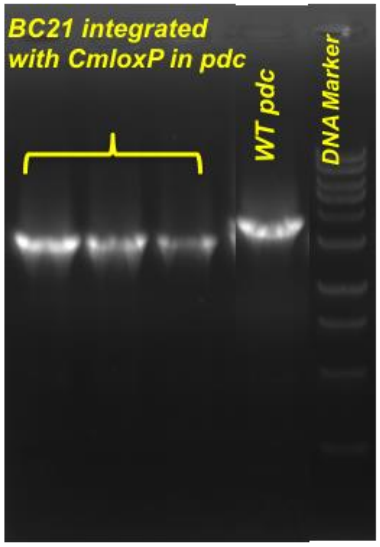
- Construction of a non-replicative plasmid pdcKO in BDO-producing *Z. mobilis* strain BC21.



- PCR analysis of initial Cm^r transformants: Double bands indicate single crossover recombination; Single band indicates double crossover and complete knockout of *pdc*.



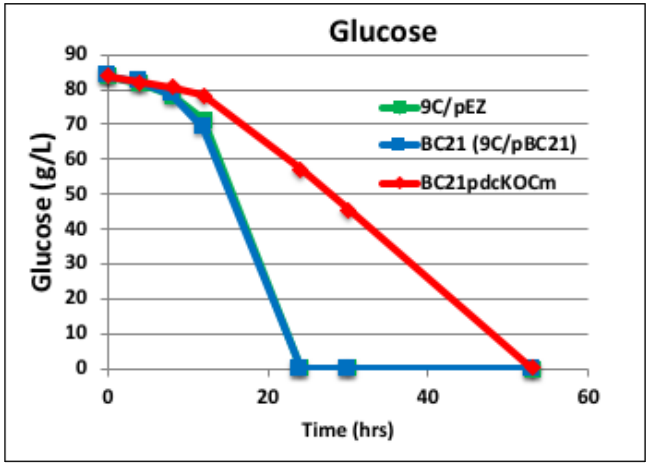
Successive selection by subculturing and isolation

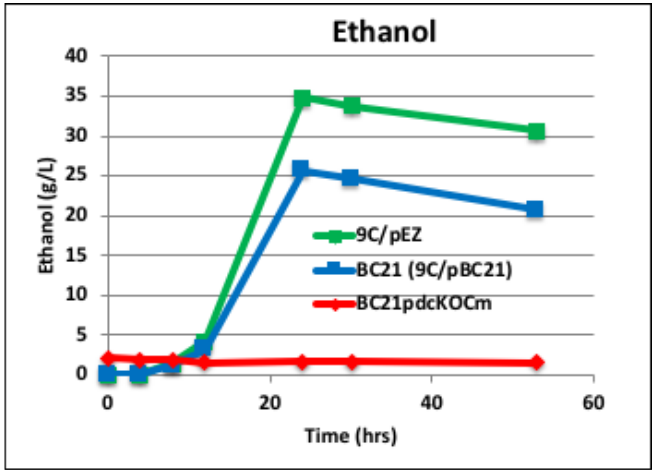


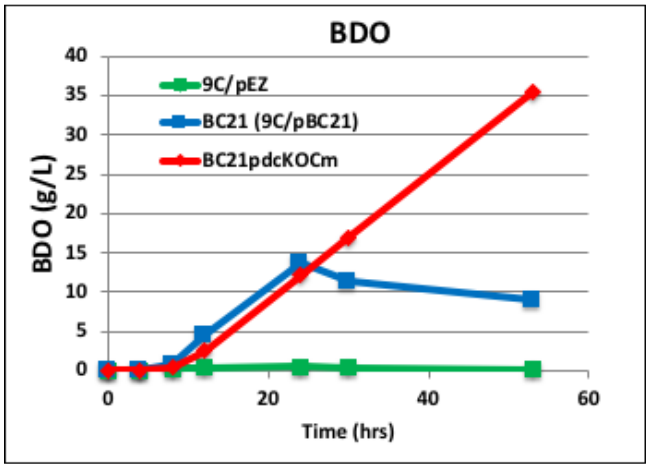
Zhang et al. Engineered Zymomonas for the Production of 2,3-Butanediol. US patent Application No. 16/173,910

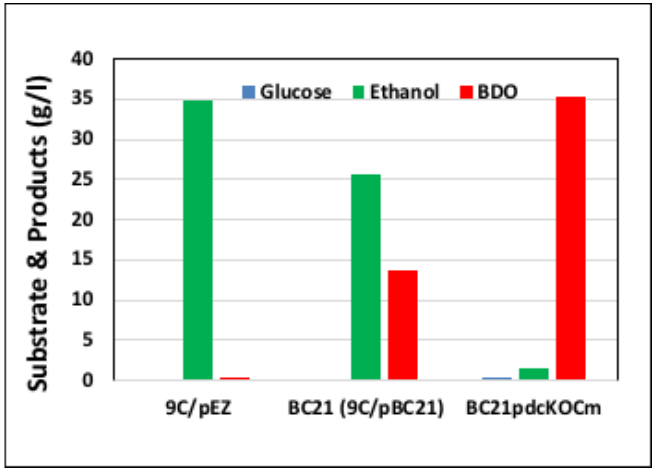
3—Technical Accomplishments/ Progress/Results

Fermentation Evaluation of pdcKO, BDO-producing *Z. mobilis*









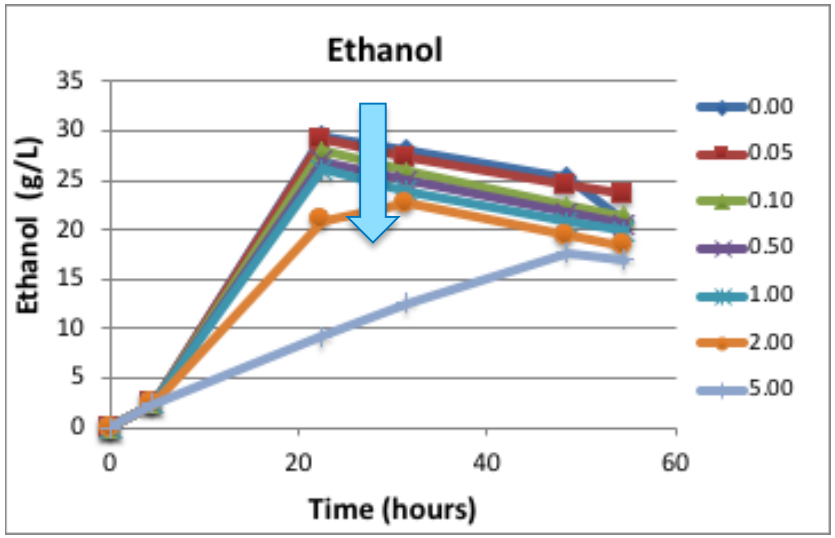
✓ Strains developed in TMD provided to BSRD (led by Nancy Dowe) for fermentation process development and are showing excellent results (titer, productivity, etc.)

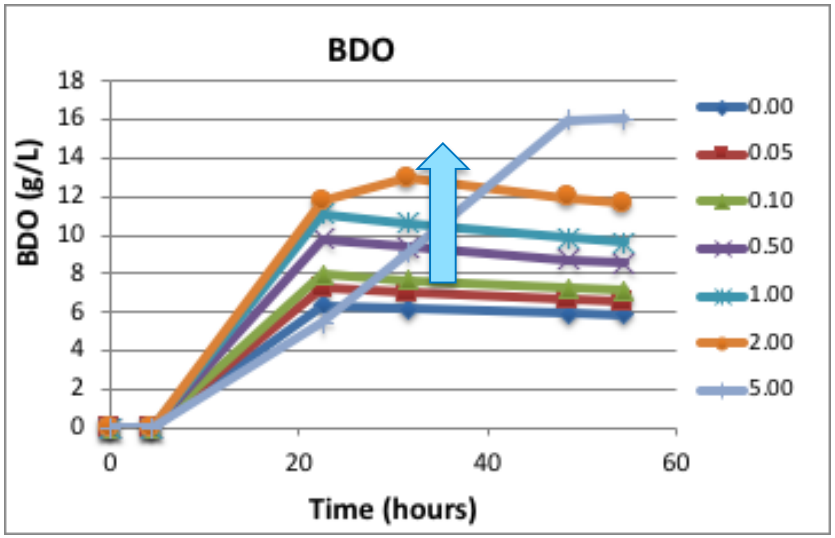
3—Technical Accomplishments/ Progress/Results

Construction of Genome Integrated BDO Producing *Zymomonas* strains

- Integrated the BDO pathway genes into the genome, creating intBC21 strains.
- Constructed strain with a weak and inducible promoter P_{tet} for BDO pathway.

ZHC129-8 (intBC21) Induced @ various Tc concentrations





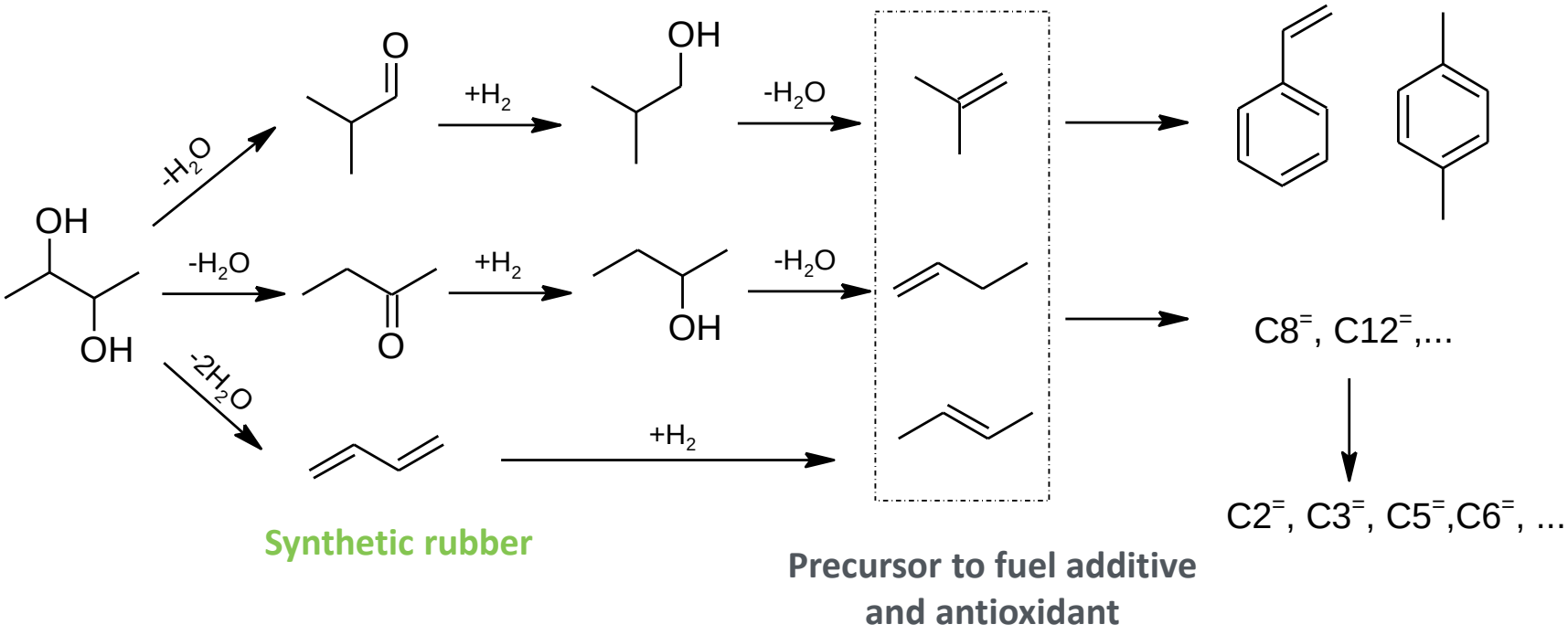
- Conducted *pdc* knock out strategy similar to the plasmid bearing strain, further removed the antibiotic marker, generating strains 41C and 42C.

➢ Provide to BSRD team for evaluation

  - Stable fermentation at 100L and produced >80 g/L BDO in fed-batch hydrolysate liquor. The integrated strain has a slightly reduced growth rate, need to optimize the flux.

3—Technical Accomplishments/ Progress/Results

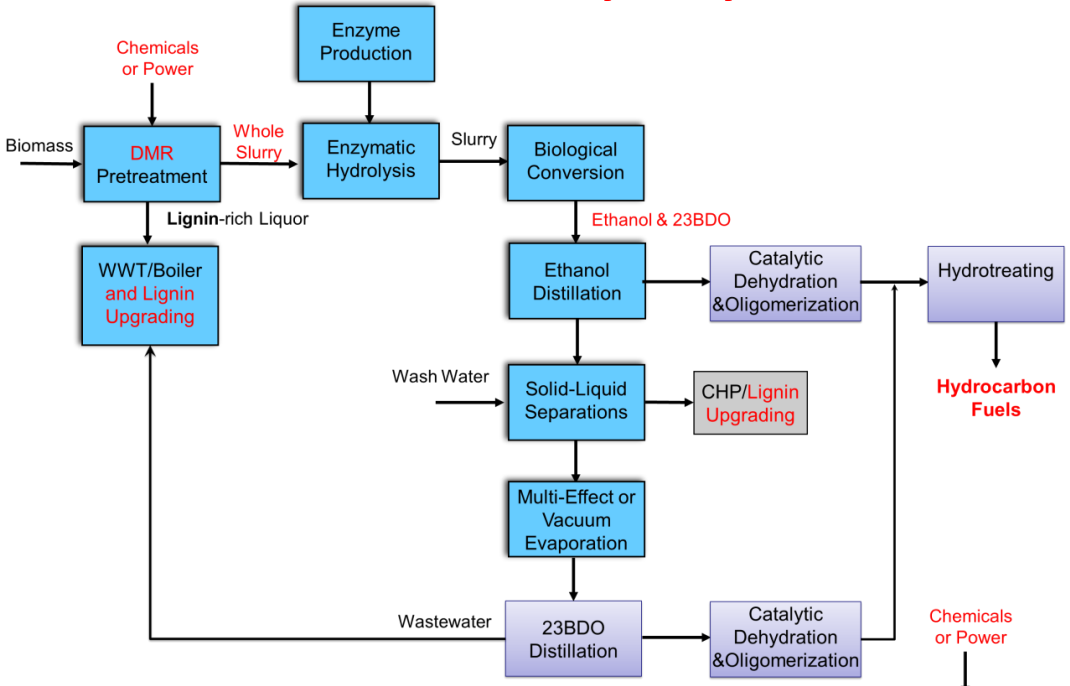
Upgrading of 2,3 BDO by Catalytic Upgrading of Biochemical Intermediates (CUBI) Producing Solvents, Chemicals, Gasoline, Diesel and Jet Fuels



- ORNL (Z. Li), NREL (D. Vardon) and PNNL under CUBI are working on the upgrading strategies.
- Great potential for reducing fuel production cost to achieve BETO 2030 goal of $2.5/GGE by producing valuable co-products.

3—Technical Accomplishments/ Progress/Results

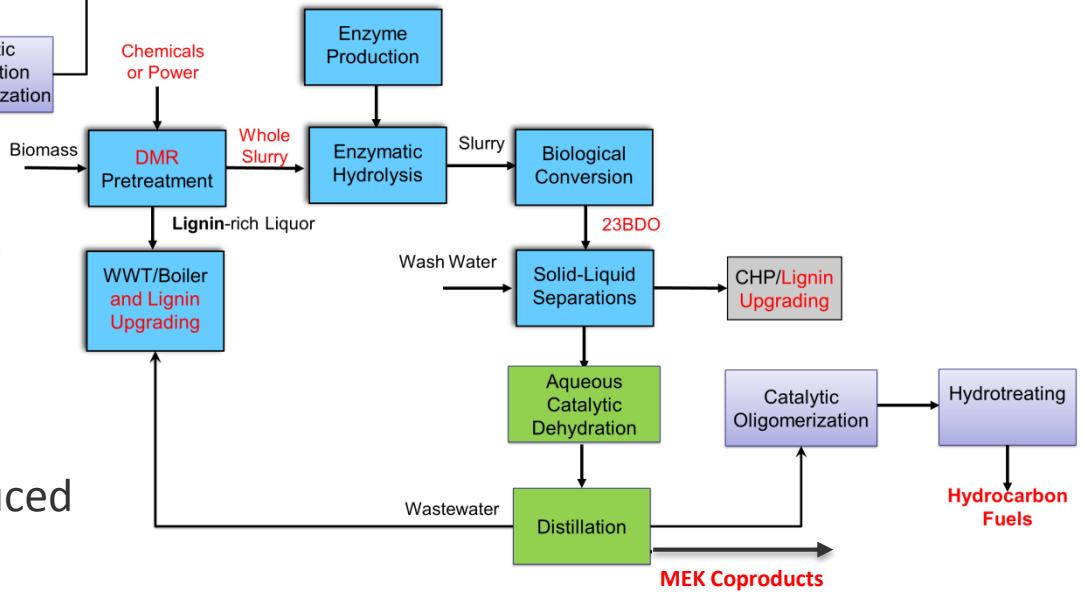
Technoeconomic Analysis by Biochemical Platform Analysis (BPA) Project



Major Proposed Changes:

- Biological conversion only produces 2,3 BDO, without ethanol.
- 2,3 BDO is catalytically upgraded in the aqueous phase, avoiding the complex 2,3 BDO purification steps.

Cost savings (~$0.50/GGE)



- Additional savings can be made if methyl ethyl ketone (MEK) is produced as coproduct (only representative).

3—Technical Accomplishments/ Progress/Results

Identify and implement strategies to reduce microaerobic requirements and improve redox balance in modified *Z. mobilis* strains by introducing beneficial heterologous pathways or redirecting metabolic flux.

- Investigating several strategies in which the pathways/reactions can utilize the surplus NAD(P)H.
- Building on the existing stoichiometric metabolic model (Biochemical Process Modeling and Simulation team), we are determining strategies for avoiding the redox imbalance posed by the anaerobic production of 2,3 BDO in *Z. mobilis*.
- Focusing on the conversion of the excess NADH through H_2 generation (which can be utilized in catalytic upgrading) by introducing NADH dependent hydrogenase system.
  - ***In vivo* hydrogen production** is observed at low levels in *Zymomonas*.
  - Working on **optimization** of the pathway.
- Working on recruiting necessary enzymes to complete the NADH demanding succinic pathway in *Zymomonas*.

4—Relevance

- Developing microbial pathways capable of producing high carbon efficiency intermediates amenable to economic bioconversion, separation, and catalytic upgrading to hydrocarbon fuels and chemicals to substantially contribute towards achieving BETO's 2022 cost target of $3.00/GGE (2.50/GGE in 2030).

- Directly contribute to cost savings as NREL TEA shows that significant cost savings for advanced fuels are: product yield, titer and rate; secreted products; anaerobic culture.

- Relevance to Industry and Tech transfer:
  - The **BDO intermediate has several process advantages and leads to numerous options for upgrading to HC gasoline, diesel, jet fuels and important chemical co-products.**
  - ROIs and patent applications ongoing.
  - 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."

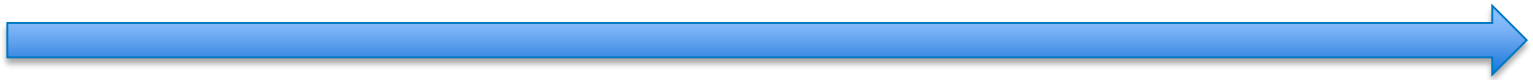
- Project fulfills MYPP plan: "Efficient, highly active, selective, durable biological catalysts; Pathways that tolerate feedstock variability and inhibitors."

5—Future Work

- *Explain what you plan to do through the end of the project (9-30-2020)*
  - Improve Redox Balance – continue to optimize strategies to mitigate microaerobic requirements for BDO fermentation in *Zymomonas* by introducing beneficial heterologous pathways or redirecting metabolic flux to balance redox.
  - Improve Pentose Utilization for Higher BDO Yield - utilization of arabinose and elimination of byproducts.
  - Enhance Xylose Utilization Rate and BDO productivity - identify metabolic limiting steps and improve substrate channeling to enhance carbon flux for fast xylose utilization and high BDO productivity, thus enabling alternative fermentation options.
- *Highlight upcoming key milestones*
  - QPM2 (3/30/2019). Engineering arabinose utilization pathway Task 2. Engineering arabinose utilization pathway into the glucose, xylose-utilizing 2,3 BDO-producing *Zymomonas* strain to achieve 50% of the arabinose utilization.
  - QPM3 (6/30/2019). Verify expression of intracellular tethered enzymes for BDO pathway in *Zymomonas.*
  - Annual SMART (9/30/2019) - Engineer *Zymomonas* to produce 2,3-BDO from glucose, xylose, and arabinose at 90%, 80%, and 50% yield in batch fermentation on whole slurry hydrolysate at 20% TS in 60 hours (in collaboration with BSRD).
  - Annual SMART. (9/30/2020). Engineer *Zymomonas* to produce 2,3-BDO from glucose, xylose and arabinose at 95%, 85%, and 50% yield in batch fermentation on whole slurry hydrolysate at 20% TS in 54 hours (in collaboration with BSRD).

5—Future Work

TMD Progress to Date (FY15-FY18) and Proposed Future Research



Timeline	FY15	FY16	FY17	FY18/FY19-FY21	2022
Technical progress/ accomplishments	Demonstrate BDO production; BC11	Improved BDO strain BC21; BSI testing in fermenters with oxygen optimization; 10% DMR and DDA hydrolysates	BC32; Major breakthrough- PDC knockout, no ethanol production; BSI testing in fermenters with oxygen optimization; 20% DMR hydrolysate	Task 1. Mitigation O_2 requirement and improve BDO yield Task 2. Arabinose utilization; High yield, titer at 20% or higher whole slurry Task 3. Enhance the pentose utilization rate	Strain fine-turning; support BSI for process integration; Pilot scale testing; Whole slurry (20% or higher)
BDO titers	5 g/L	10-20 g/L	54 g/L	FY18: 75 g/L (BSRD) >80% yield (TMD)	~100 g/L

5—Future Work

- *Address how you will deal with any decision points during that time (Go/No-Go Points)*
- Go/No-Go (3/30/2020). Achieve high 2,3 BDO yield from glucose, xylose and arabinose (90%, 80%, and 50% yield) in hydrolysates using improved *Zymomonas* strains by incorporating NADH utilizing pathway(s), such as co-production of hydrogen or succinate acid under **reduced aeration conditions**.

  - ✓ A GO decision indicates continuation as planned to further optimize the NADH utilizing pathway.
  - ✓ A NOGO decision can take different directions, pursuant to discussion with DOE:
    1) Investigate additional NADH utilizing pathway with co-production of useful intermediates for upgrading to fuel molecules or **chemicals (evaluate with TEA and BPMS).**
    2) Investigate limiting steps (omics etc.) of current hydrogenase and succinate pathways for further improvement.
    3) Abandon the research to balance redox using complimentary pathways and rely on the process optimization by BSRD.

6—Summary

Overview

The goal is to develop microbial pathways capable of producing high carbon efficiency intermediates amenable to economic bioconversion, separation, and catalytic upgrading to hydrocarbon fuels and chemicals to substantially contribute towards achieving BETO's 2022 cost target of $3.00/GGE (2.50/GGE in 2030)

Approach

Apply metabolic engineering and synthetic biology tools to engineer microorganisms for efficient utilizing biomass sugars to HC intermediates for high yield, rate and titer; Addressing technical barriers using TEA as guidance to driving the production cost.

Technical Accomplishments/Progress/Results:

Demonstrated successful *pdc* gene knockout and created novel *Zymomonas* strain produced 2.3-BDO without ethanol.

- Simplified process with reduced cost (TEA)
- Increased BDO tier to >80g/L in fed-batch fermentation from hydrolysate liquor
- Created stable strain capable of working at 100L scale
- Investigating several strategies in which the pathways/reactions can utilize the surplus NAD(P)H and demonstrated hydrogen co-production

Relevance

The BDO intermediate has several process advantages and leads to numerous options for upgrading to HC gasoline, diesel, jet fuels and important chemical co-products. Improving production yield, rate and titer directly contribute to cost savings for economic bioconversion of biomass to fuels.

Future work

Focus on developing anaerobic fermentation process with coproduction of hydrogen or succinic acid; as well as achieving higher yield from biomass sugars at high rates especially from xylose and arabinose.

Acknowledgments

Funding

- U.S. DOE EERE Bioenergy Technology Office (BETO)
  - HQ: Jonathan Male, Kevin Craig, Ian Rowe
  - NREL LPM and Platform Lead: Zia Abdullah, Rick Elander

NREL Project Members

Yat Chen Chou

Hui Wei

Mary Ann Franden

Thank You

www.nrel.gov

Publication Number

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Responses to Reviewers' Comments (2017)

Overall Impressions

- "The project has identified what seem to be relevant target pathways for co-products in order to improve overall economics. The metabolic engineering strategies selected are generally reasonable and redirect the expertise present at NREL in metabolic engineering. However, in the context of the current SOT with regard to synthetic biology in both industry and academia, the scope of the metabolic engineering strategies laid out here seem very limited. It is hard to think that progress towards both the TRY for each task and the understanding gained would not be faster and a more efficient use of resources with high-throughput strain generation approaches."
- "One of the NREL strengths is developing a clear understanding of how biochemical systems work and then linking that understanding to processes that meet BETO's strategic direction. This knowledge-based approach is proving itself through the identification of systems that support BETO's goals, for example, the discovery of organisms that can attain nearly theoretical yields of muconate from lignin derivatives. This capability will be critical for current projects and ongoing efforts to engineer organisms that can selectively and rapidly convert both lignin and carbohydrates to high-value products.. "
- "Three pathways to upgrading or co-products are being pursued in this project. The mixed ethanol/diol product is the most advanced, and BDO titers have dramatically improved with metabolic engineering and fermentation optimization. This is a nice, original, co-product proof of concept. The team has done a good job of recognizing the complexity of the separation and fermentation scale-up, and they are considering options. Lignin upgrading to fatty alcohols and muconate are successfully demonstrated concepts. They are further from their target titers. Two of the tasks are considering scope changes due to their understanding of the economics and robustness of scalability. Doing a good job of using TEA to make decisions."
- "This is a good team with ambitious targets, which are relevant in aiding and expediting metabolic engineering of various hosts and are aligned with the MYPP goals. The overall TEA of fatty alcohols should be evaluated as this might be too challenging of a target for the oleaginous yeast program. How- ever, thorough benchmarking of progress toward the TEA goal will likely keep things in spec, and the team, together with Hal Alper's collaboration, has the skill set to tackle the technical challenges."

Responses to Reviewers' Comments (2017)

PI Response to Reviewer Comments

We thank the Review Panel for the supportive comments and helpful feedback. In terms of high-throughput synthetic biology–based strain engineering, we are attempting to actively deploy a rapid genome-scale editing tool in *P. putida* KT2440 currently in collaboration with a world-leading synthetic biology group. In addition, we are leveraging modern systems biology tools (proteomics, transcriptomics, and metabolomics) to identify bottlenecks and adaptive laboratory evolution to improve both exogenous and endogenous pathways. If these approaches are successful, we will be able to very rapidly modify and improve flux through aromatic-catabolic pathways in this robust host.

We are also attempting to deploy in *Zymomonas* a rapid genome-scale editing tool, such as CRISPR, which will enable us to be more efficient in high-throughput strain generation. We are also leveraging modern systems biology tools (proteomics, transcriptomics, and metabolomics) to identify bottlenecks. As explained in the presentation, the CBP work is our lowest TRL effort and is thus treated as exploratory and low priority. Regarding expression of Cel7A cellulases, these proteins are known to be difficult to fold in yeast. We, and others, assume that enzymes from GH families 5, 10, and 11 will be less of a problem. With that said, we agree that there are remaining challenges in pathway engineering for the three metabolic routes.

It is nice to hear that we are doing a good job of using TEA to guide our research. For example, we are working to develop anaerobic pathways, which will scale more effectively than microaerophilic or aerobic fermentations. With guidance from NREL's TEA analysis, we consider 2,3 BDO as precursor to butadiene as an alternative strategy, possibly for products valorization of a fuels production process. Our primary focus is maximizing fuel yields in our base case. In fact, we considered a route that takes both ethanol and BDO to hydrocarbon fuels.

Outreach & Communication

Publications and Patents

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Outreach & Communication

Publications and Patents-cont'd

9. "Expression and secretion of fungal endoglucanase II and chimeric cellobiohydrolase I in the oleaginous yeast *Lipomyces starkeyi*" (2017) Q Xu, EP Knoshaug, W Wang, M Alahuhta, JO Baker, S Yang, T Wall, SR Decker, ME Himmel, M Zhang, H Wei, *Microbial cell factories* 16 (1), 126
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