BETO 2023 Peer Review
Cell Free and Immobilization Technologies (CFIT)

April 7th, 2023
Biochemical Conversion & Lignin Utilization Session
Yannick Bomble
NREL

This presentation does not contain any proprietary, confidential, or otherwise restricted information

Project Overview

Context:

- **Several factors can negatively impact the production of biochemicals:** End-product or intermediate toxicity, diversion of carbon to biomass formation and cell survival, and undesired byproducts.
- An **alternative is to operate metabolic pathways without the cells** to circumvent these problems.
- There are risks involved in developing cell free approaches but getting them to a mature stage would **dramatically change the landscape** of biomanufacturing.
- Current terpene titers from microbial conversations <1g/L (except for farnesene). There is a need to enable the production of a multitude of terpenes for use in SAF.
- This project is in its 2nd funding cycle. Current budget $1.345M per year, this funding cycle started in FY22

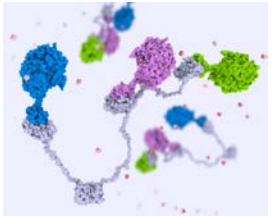
Project goals:

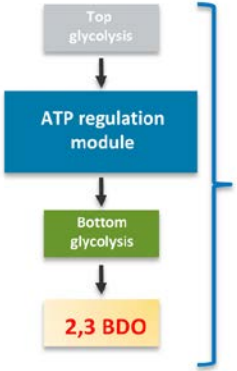
- Develop new science and technologies guided by TEA to **derisk cell free based bioprocesses for greater adoption in industry** and to **access a product space not available with traditional microbial routes**.
- Demonstrate the viability of cell free based approaches by producing **terpenes at > 20g/L (>90% yield) to enable the production of SAF to contribute to BETO's 2040 goals of 17M gallons of commercial production**.

1. Approach: Management

The project is **divided in two complementary tasks**. Milestone objectives are shared between these tasks

Task 1. Pathway and Enzyme Engineering for Cell Free Technologies ($900K): Product selection, enzyme, cofactors recycling, and pathway engineering for applications in cell free systems.

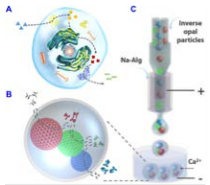




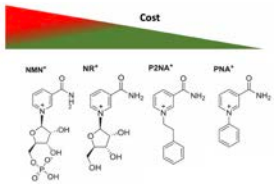
Enzyme characterization
Pathway engineering
Enzyme design
Techno economic analysis

Subcontract with Invizyne ($50K/Year): Consultant on terpene production in cell free systems and scale up (bimonthly calls)

Task 2. Immobilization and cofactor engineering for Cell Free Technologies ($400K): Increasing stability, operating lifetime, and efficiency of the enzymes by immobilization. Developing approaches to reduce cofactors cost.



Biopolymers Synthesis



Subcontracts: Yongqin Jiao (LLNL) ($200K/Year): enzyme immobilization. **Rob Paton (CSU) ($60K/Year):** computational engineering of synthetic cofactor (Monthly calls and quarterly reports).

1. Approach: Management

We have built a **multidisciplinary team** to address risks associated with our approach. We are also **taking advantage of the expertise in other BETO projects and collaborators to overcome these challenges.**

Key Personnel

Yannick Bomble (NREL), Yongqin Jiao (LLNL), Rob Paton (CSU), and Tyler Korman (INvizyne Technologies).

Active management: reassess approaches, focus, and partners to maximize impact *e.g.* discontinued subcontract on cofactor recycling at electrodes (too long term), placed new subcontracts to address gaps in the team.

Interactions with other projects within BETO

- **BPMS** (modeling/theory): Enzyme engineering, kinetic modeling, non natural pathways
- **TMD** (microbial 2,3 BDO production): *In vivo* titer comparisons, enzyme prospecting and testing
- **BPA** (Technoeconomic analysis): TEA of cell free platforms, setting priorities, product selection
- **SepCon** (separation): Separation of terpenes, retention of enzymes and cofactors, design of unit operations
- **TCF** (Enzyme production): scaling up protein production, optimizing storage, reduce cost of production

External Collaborations with academia, national labs, and industry

Jim Bowie, Professor, **UCLA** (cell free production of biochemicals), **Han Li**, Professor, **UCI** (biomimetic cofactor utilization), **Zachary Sun**, CEO, **Tierra Biosciences**, (Cell free protein synthesis), **Sophie Barbe,** Group Leader, **INRAE**, (cofactor specificity).

1. Approach: Setting priorities and goals

Our approach taken as a whole, will go **beyond conventional cell free technologies** and will judiciously combine, on a case by case basis, enzyme immobilization, enzyme engineering, cofactor recycling strategies, and pathway redesign strategies.

- We focus on the **production of various terpenes as SAF and diesel intermediates or as direct replacements using a cell free approach** (First cycle-2,3 BDO as proof of concept).
- Work with our **TEA team** to make cell free biocatalysis as a viable alternative to fermentative processes for SAF and diesel molecules and **set research priorities**.
- **Develop broadly enabling cell free tools such as new and cheaper cofactors** (synthetic with improved characteristics) to address one of the main challenges associated with cell free approaches.
- Use enzyme engineering to **increase stability** of some enzymes and **enable the use of synthetic cofactors**.
- Immobilize pathway enzymes or combinations of enzymes on several materials to **increase overall stability required for industrial conditions.**

1. Approach: Identifying risks and mitigation strategies

Cell free approaches have the potential to revolutionize biochemicals production but major challenges remain.

Success factors

- Long term enzyme stability: **offsets the cost of enzyme production**, guarantees the smooth operation and the **reliability of the process**.
- Efficient cofactor management and cheaper cofactors: **Lowers cost of the overall process by eliminating the need for cofactor addition.**

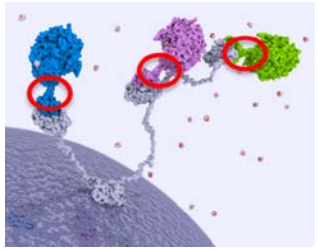
Risks/Challenges	Mitigation strategies
Enzyme expression issues	• Replacement enzymes from natural diversity. • Other expression hosts to increase enzyme production titers (TCF).
Lack of long term enzyme stability	• Immobilization. • Natural diversity. • Enzyme engineering.
Cofactor cost, lack of efficiency of cofactor recycling, or loss of cofactors	• Better product/cofactor separation technologies (with SepCon). • Cofactor recycling on porous conductive surfaces (previous effort). • Immobilization of cofactors (LDRD). • Use biomimetic cofactors.

Green: actively pursued Red: Not pursued directly in this project

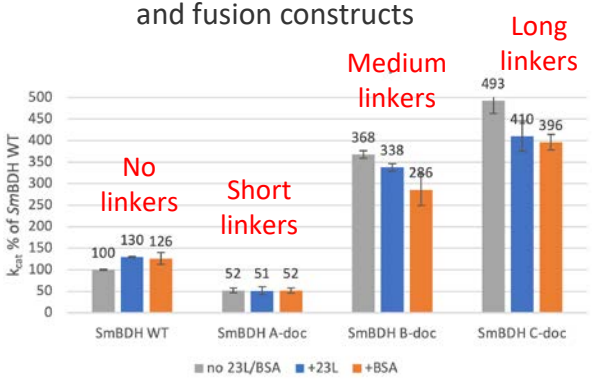
2- Progress and Outcomes: Demonstrated consolidated cell free production

Leveraged and optimized naturally occurring enzyme tethering strategies to facilitate enzyme purification and immobilization to produce 2,3 BDO at high titers from pyruvate.

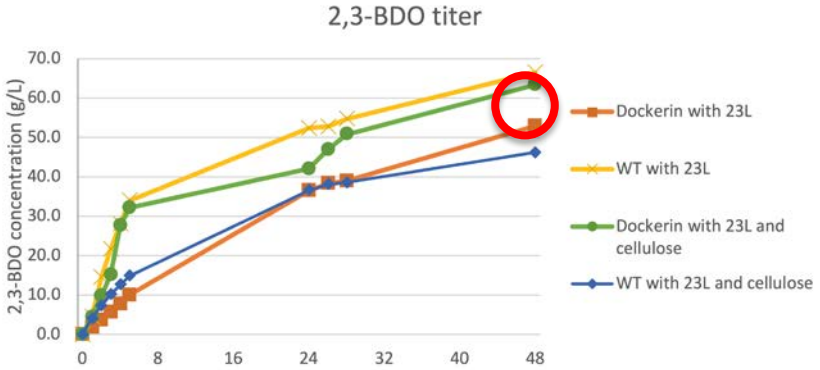
Leveraging natural tethering strategies to array enzymes automatically when co-expressed



Optimization of linker lengths and fusion constructs



Linker optimization recovers the same titer as WT enzymes when immobilized



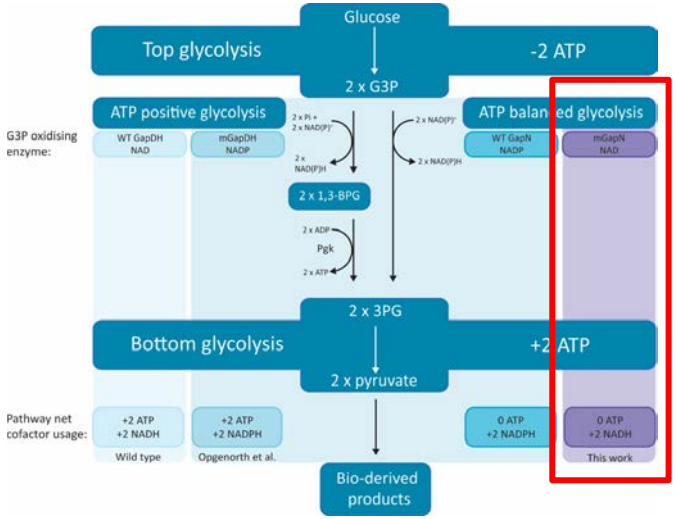
Initial constructs produce ~20% less 2,3-BDO

*In review at **Frontiers in Bioengineering and Biotechnology (2023)***

2- Progress and Outcomes: Enabling more routes for cell free glycolysis

Computational modeling and HTP screening leads to successful switch in cofactor specificity in an essential enzyme for ATP neutral glycolysis with NADH.

(Risk mitigation strategy for NADH linked pathways with ATP neutral glycolysis)

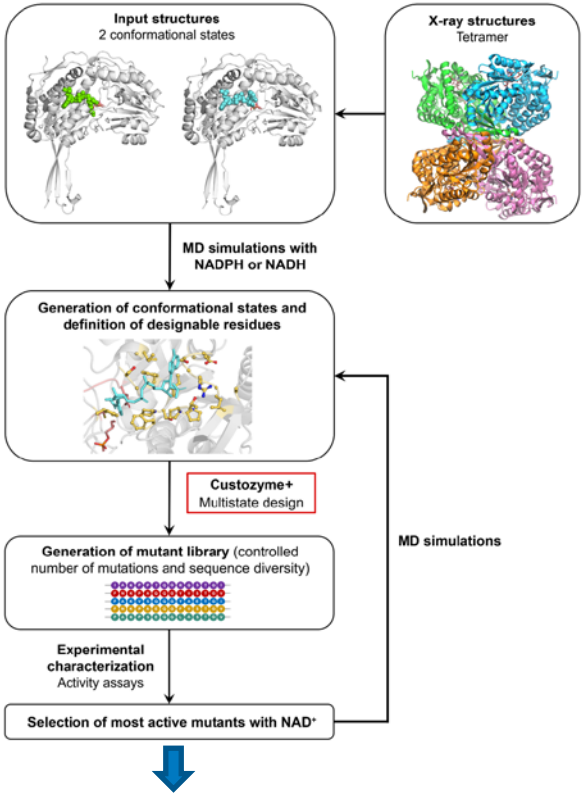


- NADH dependent GAPNs are essential but do not exist natively.
- Using machine learning approaches and structure guided mutagenesis to generate mutant candidates.

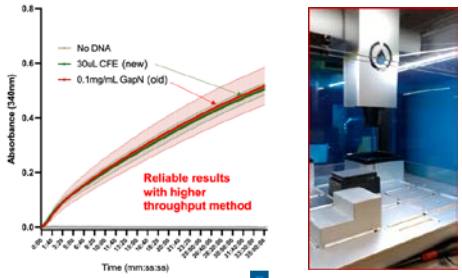
Collaboration with Sophie Barbe and BETO-BPMS

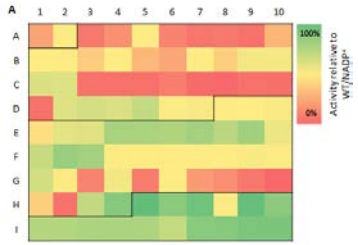
In review at ACS catalysis (2023)

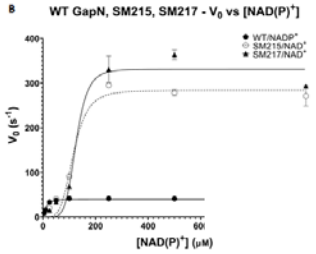
Computational variant prediction



Variant screening





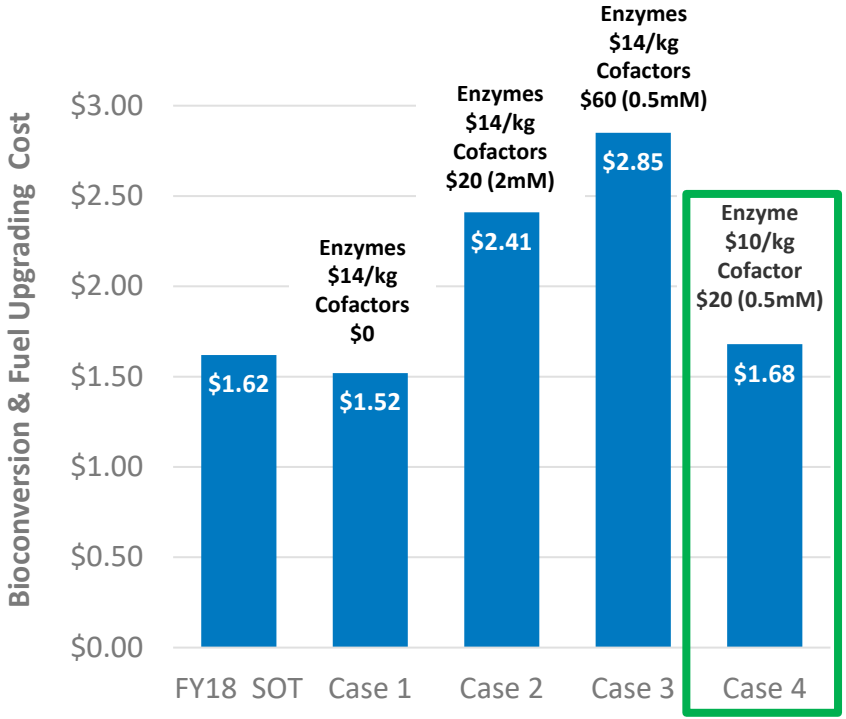


Generated mutants with a 10,000- fold improvement in NADH utilization over WT to more than 80% of NADPH utilization.

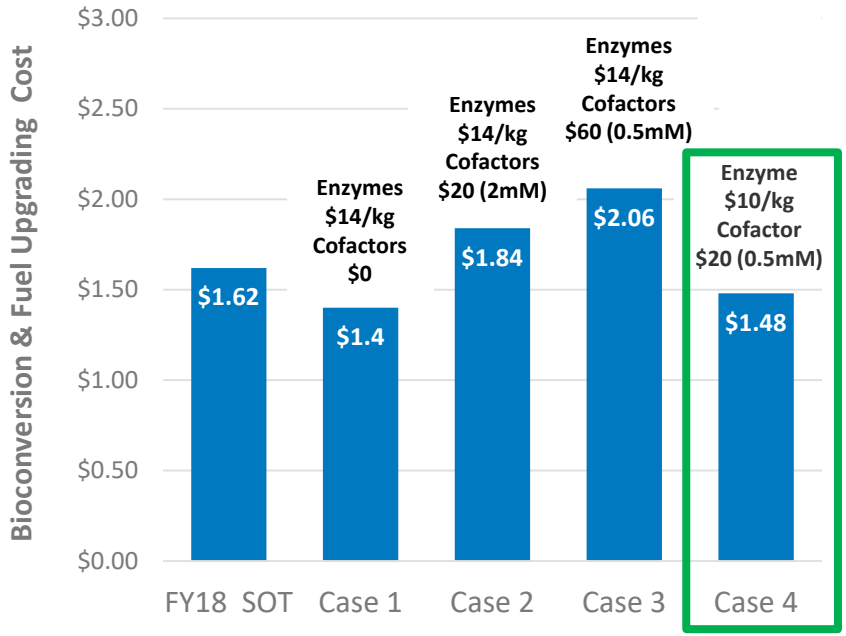
2- Progress and Outcomes

Cofactor cost and enzyme cost drive the cost of production

- Using TEA as a tool to run scenarios where you can **identify the cost drivers and set research priorities**.
- Costs will be much different **when considering toxic products** or toxic feedstock streams.

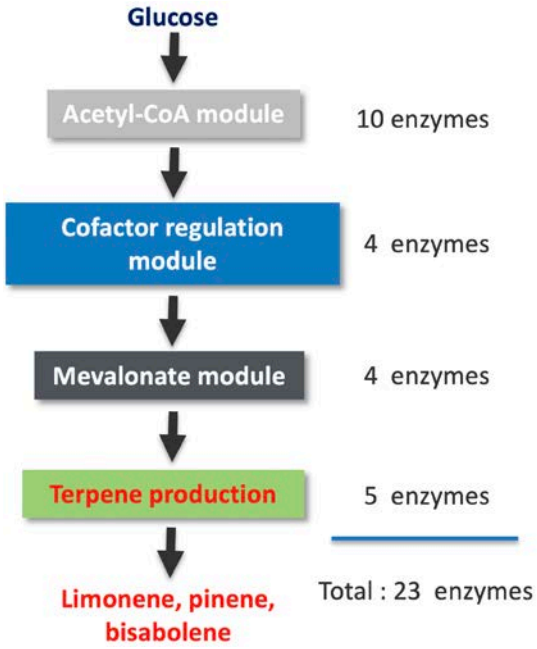


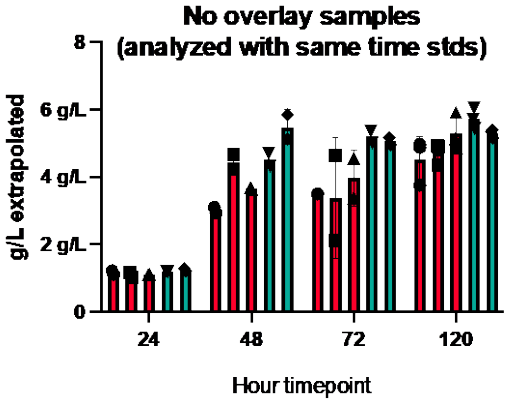
Enzyme operating lifetime: 50 cycles



Enzyme operating lifetime: 100 cycles

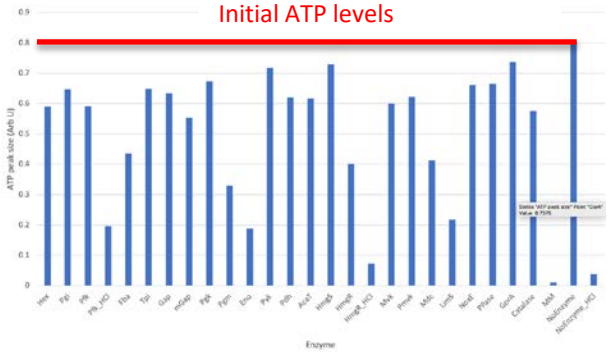
2- Progress and Outcomes: Enabling terpene production from glucose with minimal protein purification

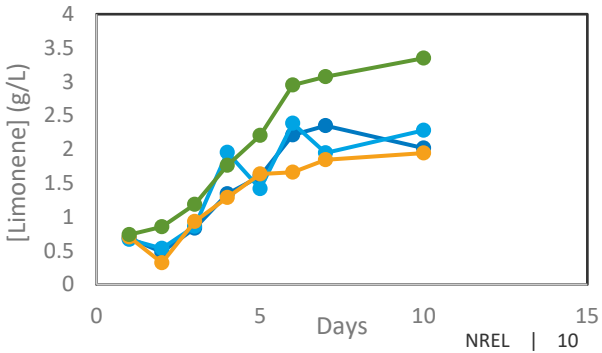




- Demonstrated the production of mevalonate from glucose at >10g/L (>90% yield).
- Demonstrated the production of limonene from mevalonate at >6g/L (>95% yield) with crude purifications and stable proteins.

Initial limonene production directly from glucose was low due to ATPase contamination but but heat treatment helped mitigate ATPase contamination and increase limonene titers with crude preparations.





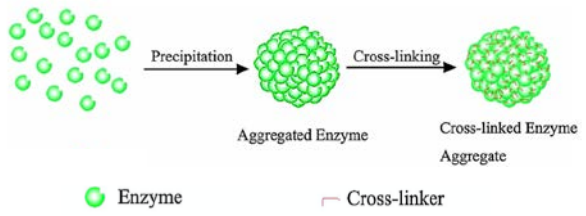
- The postprocessing of enzyme preparations is a major bottleneck.
- Working with crude enzyme preparations to simplify and reduce the cost of cell free terpene production.

Collaboration with Jim Bowie (UCLA)

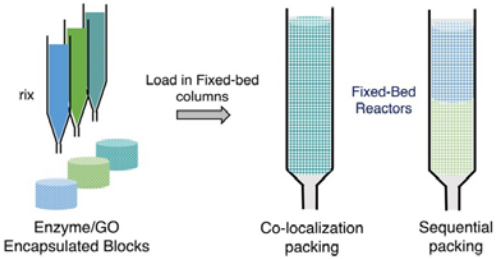
2- Progress and Outcomes: Initiated systematic enzyme immobilization campaigns

Enzyme immobilization will be necessary to increase enzyme stability, extend enzyme operating lifetime, and to enable enzyme recycling and product separation in fixed-bed reactors.

Explore different enzyme immobilization strategies

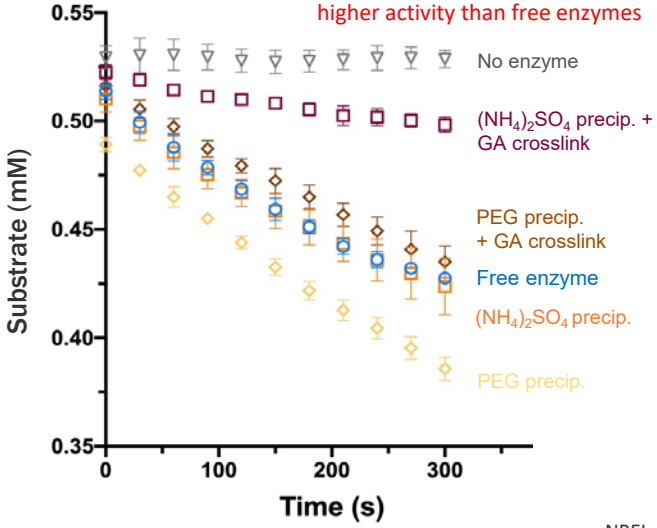


Explore different packed bed reactor designs (BETO-BPMS)



- Successfully generated fully active cross-linked aggregates (CLEAs) of NOX, a cofactor-regenerating oxidase that will be used in cell-free terpene biosynthesis.
- Generated active NOX-linked magnetic nanoparticles for efficient enzyme recovery and reuse
- Compared alternative approaches to identify the most efficient path(s) to optimization
- Combining immobilization approaches to maximize stability and reusability, and applying to additional enzymes in the biosynthetic pathway

NOX (water-forming oxidase) CLEAs showing higher activity than free enzymes



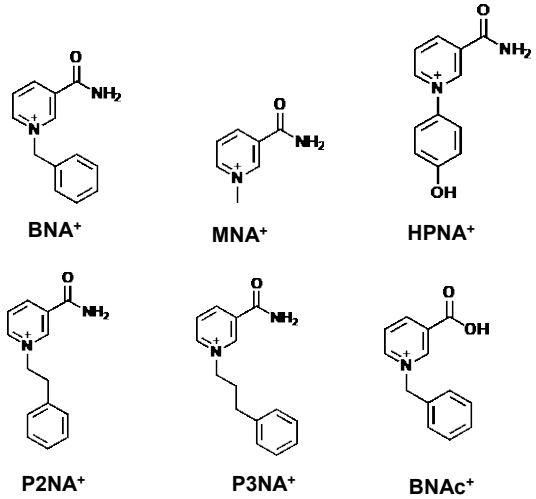
Initial enzyme immobilization attempts with rather low-tech solutions seems promising with some enzymes. Constructs with other enzymes showed a varying degree of success due to enzyme inactivation. Risk mitigation strategy: Now exploring more targeted and generalizable immobilization approaches to avoid the risk of inactivating enzymes.

Work conducted at LLNL: Dante Ricci, Ziye Dong, Yongqin Jiao

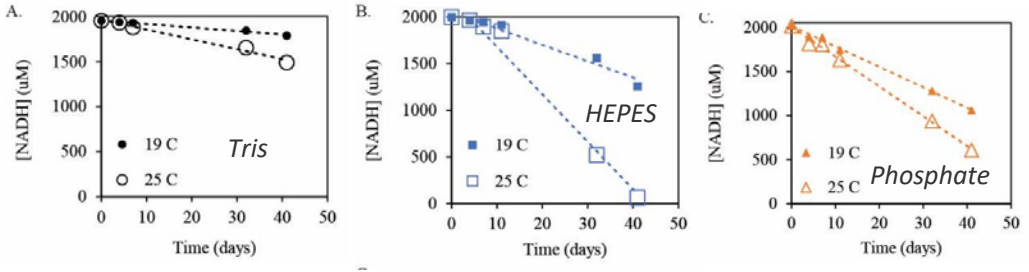
2- Progress and Outcomes: Enabling the utilization of biomimetic cofactors

- Several synthetic cofactors have the potential to be more than 200x cheaper than natural cofactors. (natural >$500/kg, semi synthetic >$100/Kg, synthetic < $10/kg)
- They can also be more stable at elevated temperature and might be more amenable to electrochemical recycling.

Synthesized cofactors from cheap starting materials at high yields (75-90%) and purity (>95%).



Demonstrated that natural cofactors become unstable as temperature increases



Developed approaches to predict the characteristics of cofactors including stability and redox potentials

3. Impact

Our approach can help address several conversion barriers by:

- Increasing titers, yields, and productivity of toxic products due to **higher toxicity thresholds** and **more carbon efficient conversion**. (end of project goal 25g/L of different terpenes for SAF and diesel knowing that cytotoxicity is ~1g/L)
- **Reducing the cost of separation** due to the absence of microbial cells and media in these processes.
- Offering more flexibility as it is **more resilient with respect to inhibitors** released during pretreatment or enzymatic hydrolysis.
- **Reducing capital cost and de-risk scale up of biorefineries** due to much **greater process intensity** and volumetric rates of conversion.
- **Enable the production of SAF to contribute to BETO's 2040 goals of 17M gallons of commercial production**

3. Impact

Interactions with industry: We have had discussions with potential industrial partners in addition to **Invizyne** who realize the potential of these technologies including **BASF,** and **Novozymes who are very supportive of this effort.**

Impact on other projects:

- Knowledge and expertise was used to prototype 2,3 BDO pathways in *Zymomonas mobilis*.
- Awarded a TCF project on reducing the cost of protein production for cell free system with an industrial partner (Invizyne).

IP:

- Carbon negative production of diacids and other biochemicals using cell free biocatalysis (Patent application U.S. 2022/0315955 A1)
- An engineered non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase for utilizing NAD and NADP cofactors (Provisional Patent)
- Pegboard protein purification plate for high throughput protein characterization (Provisional Patent)

Summary

Management:

- Built a **multidisciplinary team** to address challenges associated with our approach.
- **Leveraging the expertise in other BETO projects and collaborators to overcome these challenges.**

Approach:

- Develop broadly enabling cell free tools such as cofactor recycling systems.
- **Using TEA to guide efforts to maximize impact**.
- **Focus on challenges for cell free systems: long term enzyme stability and efficient cofactor management**

Impact:

- Approach can lead to **more carbon efficient conversions to toxic products** and be **more resilient to inhibitors.** Can **reduce the cost of separation** due to the absence of microbial cells in these processes.
- Gathering support from industry and generating new concepts in the form of IP and publications.

Progress and Outcomes:

- Demonstrated process relevant titers of terpenes with no cofactor addition.
- Demonstrated synthesis and utilization of some synthetic cofactors
- Engineered enzymes to enable better cofactor management
- Demonstrated that enzyme tethering is a viable approach to assemble enzyme with optimization
- Demonstrated enzyme stabilization using immobilization strategies

Quad Chart Overview

Timeline

- Project start date – October 1st 2021
- Project end date September 30th 2024

	FY23	Active Project
DOE Funding	(10/01/2022 – 9/30/2023) $1.345M	$4.035M (FY22-FY24)

Project Partners*

Dr. Yongqin Jiao (LLNL)
Prof. Rob Paton (CSU)
Dr. Tyler Korman (INvizyne Technologies)

Barriers addressed

Ct-H. Efficient Catalytic Upgrading of Sugars/Aromatics, Gaseous and Bio-Oil Intermediates to Fuels & Chemicals.

Im-E: Cost of Production

Project Goal: Develop new science and technologies guided by TEA to derisk cell free based bioprocesses for the production of fuels and chemicals and to access a product space not available with traditional microbial routes.

End of Project Milestone: Produce pinene or limonene and bisabolene, at 25g/L from hydrolysate, glucose, ethanol, or mevalonate (>90% yield) with complete cofactor recycling. Combine newly engineered enzymes, kinetic modeling, and immobilization to achieve these goals (with BPMS/Sepcon).

FY22 Q2 Go/NoGo: Produce pinene or limonene, and bisabolene, at 10g/L from hydrolysate or ethanol, or 10g/L f from mevalonate with more than 90% yield with complete cofactor recycling.

Decarbonization Pillars and EERE Emphasis Areas: This project will focus on R&D to enable the production of SAF to achieve BETO's 2040 goals of 17M gallons of commercial production by developing processes with increased carbon efficiency and limited CO_2 evolution.

Funding Mechanism

AOP as WBS# - 2.5.4.101

Acknowledgments

Funding

- U.S. DOE EERE Bioenergy Technologies Office
  - BETO TM: Beau Hoffman
  - NREL LPM and Platform Lead: Zia Abdullah, Rick Elander

NREL Project Members

Markus Alahuhta
Neal Hengge
Sam Mallinson
Yongqin Jiao – LLNL
Tyler Korman - (INvizyne Technologies)
Rob Paton – CSU
Qi Xu

Projects with joint or collaborative milestones Focused on cofactor management with biomimetics

Biochemical Process Modeling and Simulation (Bomble)

Collaborators

Min Zhang – NREL (TMD)
Michael Himmel - NREL
Ling Tao - NREL (BPA)
Jim Bowie – UCLA
Han Li - UCI
Sophie Barbe – (TBI-France)

Thank You

www.nrel.gov

NREL/PR-2700-85592

This work was authored by the National Renewable Energy Laboratory, operated by Alliance for Sustainable Energy, LLC, for the U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. Funding provided by U.S. Department of Energy Office of Energy Efficiency and Renewable Energy, Bioenergy Technologies Office. The views expressed in the article do not necessarily represent the views of the DOE or the U.S. Government. The U.S. Government retains and the publisher, by accepting the article for publication, acknowledges that the U.S. Government retains a nonexclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this work, or allow others to do so, for U.S. Government purposes.

Responses to Previous Reviewers' Comments

Q: One factor that needs to be considered is the challenges and costs associated with scaling a cell free system. Should focus on protein stability and cofactor recycling, as these two issues will determine the feasibility of the process.

A: We are now indeed focusing on enzyme stability (prospecting, engineering, tethering, immobilization) and cofactor recycling as these are the most important challenges. Now that we have completed the pathway we are able to assess the problematic enzymatic steps as well as the cofactor recycling efficiency. We do know that the glycolysis pathway can achieve close to 100% recycling efficiency for several days. Additionally, we have taken steps to replace natural cofactors with biomimetic cofactors which will be much cheaper leading to reduced production costs.

Q: The challenges of scaling up cell free processes is not considered. This could be more difficult than the separations scale-up which they are intending to replace.

A: We do anticipate that there could be challenges scaling up these processes. However, we do expect that these challenges can be overcome with R&D. Indeed, these processes should be more resilient than current microbial biocatalysts based processes. We are currently evaluating how cost of enzymes and cofactors can be minimized. Also, we have had discussion with the separation consortium about the best designs for separation and how chemical looping could be used for cofactor recycling.

Publications and IP Since Last Peer Review

- Ziegler, S. J., Mallinson, S. J. B., St. John, P. C., & Bomble, Y. J. Advances in integrative structural biology: Towards understanding protein complexes in their cellular context. Computational and Structural Biotechnology Journal, 19, 214–225. doi:10.1016/j.csbj.2020.11.052 (**2021**).

- Qi Xu, Alahuhta Petri, Patrick Hewitt, Nicholas S. Sarai, Neal Hengge, Michael E. Himmel, and Yannick J. Bomble, “Self-assembling metabolon enables the cell free conversion of glycerol to 1,3 PDO” (in review at *Frontiers in Energy Research)* (**2021***)*

- Yannick J Bomble, Michael E Himmel, Jeffrey Linger, Roman Brunecky, John Aikens “ENZYME SCAFFOLDS AND METHODS OF USE” awarded patent with the *U.S. Patent Office*, receiving patent number U.S20170275653A1 (**2020**)

- Markus Alahuhta, Sam Mallinson, Michael E. Himmel, Yannick J. Bomble “NREL ROI-21-34 Carbon Negative Production of Diacids and Other Biochemicals Using Cell Free Biocatalysis” Provisional patent to be submitted in March **2021**.