BETO 2021 Peer Review: Cell Free and Immobilization Technologies (CFIT)

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NREL
Biochemical Conversion & Lignin Utilization Session
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Project Overview

Context:

- **Several factors can negatively impact the production of biochemicals:** End-product or intermediate toxicity, diversion of carbon to biomass formation, undesired byproducts.
- An **alternative is to operate metabolic pathways without the cells** to circumvent these problems.
- There are still risks involved in developing cell free approaches but getting these technologies to a mature stage would **dramatically change the landscape** of biochemical production.

Project goals:

- Develop new science and technologies guided by TEA to **derisk cell free based bioprocesses**.
- Demonstrate the viability of cell free based approaches by producing 2,3 BDO at 40 g/L (productivity >1 g/L/h) from process hydrolysates using cell free metabolic pathways with cofactor recycling.

Heilmeier Catechism :

- **What:** Developing broadly enabling cell free approaches by **leveraging enzyme tethering, enzyme immobilization and entrapment, enzyme engineering, cofactor recycling strategies.**
- **Today:** Recent work shows cell free biocatalysis can become a viable technology. However, breakthroughs are needed to **reduce overall biocatalysts cost and improve cofactor management.**
- **Importance:** Need to develop technologies to **efficiently produce toxic products and handle toxic feed streams with limited CO_2 evolution.**
- **Risks:** Instability of the system, but new results show that these can be overcome.

Quad Chart Overview (for AOP Projects)

Timeline

- Project start date - 2018
- Project end date 2021

	FY20	Active Project
DOE Funding	(10/01/2019 – 9/30/2020) $900,000	$2,700,000 (FY19-FY21)

Project Partners*

Prof. Kane Jennings (Vanderbilt University)

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.**

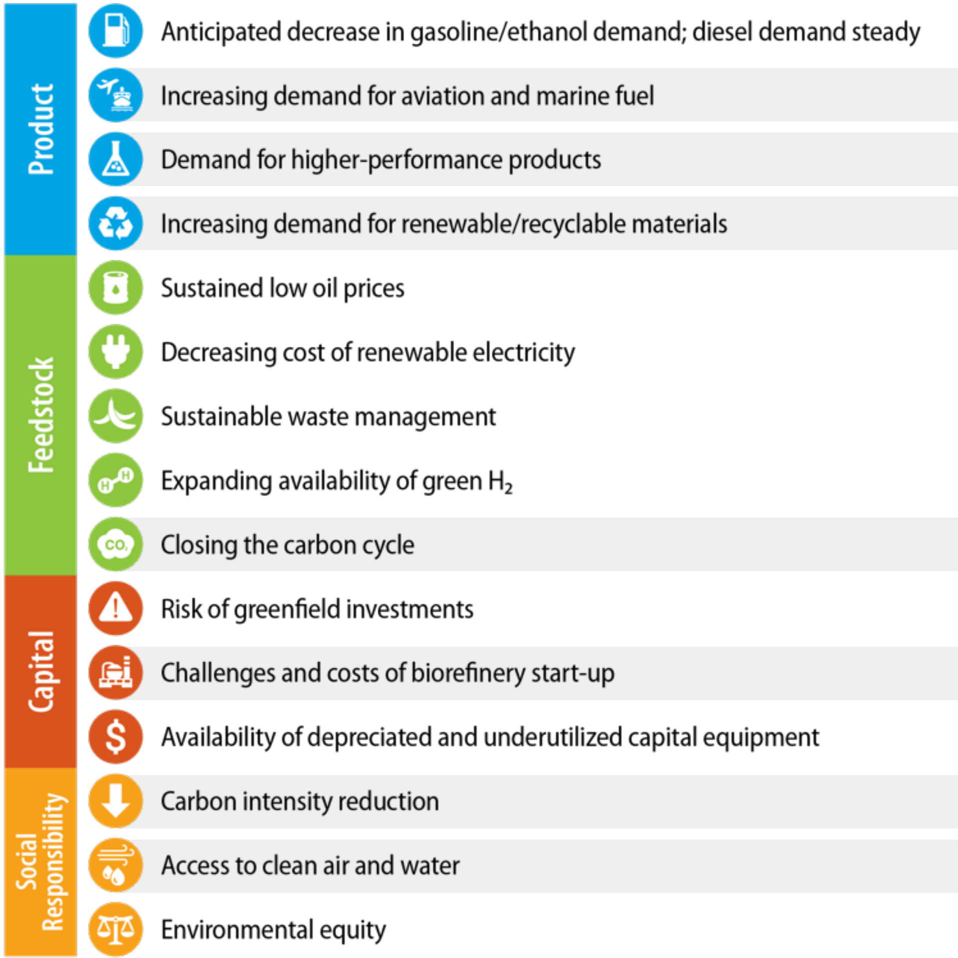
End of Project Milestone

Produce 2,3 BDO at 40 g/L with a productivity of at least 1 g/L/h from process relevant hydrolysates using cell free metabolic pathways combined with either sacrificial cheap biomimetic cofactors, conductive porous bead-based, or enzymatic based cofactor recycling.

Funding Mechanism

AOP as WBS# - 2.5.4.101

Market Trends



Category	Trend
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

- The new bioeconomy needs robust and selective processes for the production of toxic jet fuels and other value added products.
- Supports industry in its goal to convert a variety of feedstocks with high carbon efficiency.

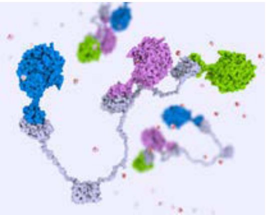
Key Differentiators

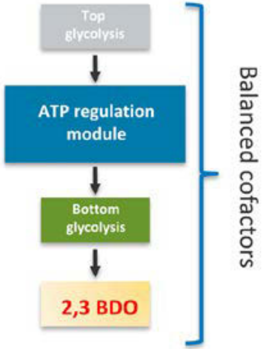
- Processes with very high yield, titers, and productivity even for toxic feedstocks and products.
- We are identifying approaches to lower the cost of biocatalysts and lower the cost of system upkeep leveraging principles from nature and using enzyme engineering.

1. Management

The project is **divided in two complementary tasks**. Milestone objectives are shared between these tasks. Each task is responsible for relevance, AOP, milestones, quarterly reporting according to the guidance of BETO, communication with other projects, tracking go/no-go activities, budget management.

Task 1. Pathway and Enzyme Engineering for Cell Free Technologies (750K): Product selection, enzyme and pathway engineering for applications in cell free systems.





Top glycolysis

ATP regulation module

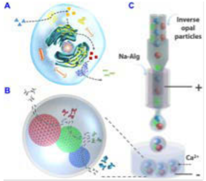
Bottom glycolysis

2,3 BDO

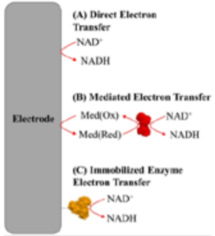
Balanced cofactors

Enzyme characterization
Pathway engineering
Enzyme design
Techno economic analysis

Task 2. Immobilization and electrochemistry for Cell Free Technologies (150K): Increasing stability, operating lifetime, and efficiency of the enzymes by immobilization. Developing approaches for efficient cofactors recycling.



Biopolymers
Electrochemistry



Subcontract with G. Kane Jennings (Vanderbilt University) (80K/Year): immobilization on smart biohybrid surfaces for optimizing electron transfers for cofactor recycling (Monthly calls and reports).

1. Management

We have built a **multidisciplinary team** to address challenges associated with our approach. We are also **taking advantage of the expertise in other BETO projects and collaborators to overcome these challenges (risks: cofactor cost and management, lack of enzyme stability and processing cost).**

Current Team Members

Yannick Bomble (Biophysics), Alahuhta Petri (Enzyme engineering), Qi Xu (Molecular Biology), Neal Hengge (Protein Production), Rida Noor (biochemistry), Michael Himmel (biochemistry), Andrea Buchholz (biochemistry), Sam Mallinson (Enzyme engineering), Ashutosh Mittal (Chemistry), Kane Jennings (Electrochemistry), Kody Wolfe (Electrochemistry)

Interactions with other projects within BETO

- **BPMS** (modeling/theory): Enzyme engineering, kinetic modeling, non natural pathways
- **EEO** (Enzyme engineering): Enzyme engineering, cofactor utilization, biochemical assays
- **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

External Collaborations with academia, national labs, and industry

Yongqin Jiao, Group Leader, **LLNL** (Advanced materials for encapsulation), **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), **Rob Paton**, Professor, **CSU,** (hydride transfer in redox enzymes), **Sophie Barbe,** Group Leader, **INRAE**, (cofactor specificity), **Tyler Korman**, Director of R&D, **INvizyne Technologies**

2. Approach

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

- As a demonstration we are focusing on the **production of 2,3 BDO from hydrolysates using a cell free approach (proof of concept)**.
- Work with our **TEA team** to assess the promise of **cell free biocatalysis as a viable alternative to fermentative processes** for selected biochemicals and polymer precursors and **set research priorities**.
- **Develop broadly enabling cell free tools such as cofactor recycling systems** to address one of the main challenges associated with cell free approaches.
- Ultimately, our main goal is **to access a product space not available with traditional microbial routes.**

2. Approach

Challenges with cell free systems

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: **Lowers cost of the overall process by eliminating the need for cofactor addition.**

Challenges	Solutions
Enzyme expression issues	• Replacement enzymes from natural diversity. • Other expression hosts including cell free protein synthesis.
Lack of long term enzyme stability	• Encapsulation and immobilization. • Natural diversity. • Enzyme engineering.
Cofactor cost, lack of efficiency of cofactor recycling, or loss of cofactors	• Rebalance pathways. • Better product separation technologies. • Cofactor recycling on porous conductive surfaces. • Immobilization of cofactors. • Use biomimetic cofactors. • Direct electron injection to run redox enzymes.

Green: Being actively pursued Orange: Considering now Red: Not considering at the moment

2. Approach

Task 1. Pathway and Enzyme Engineering for Cell Free Technologies:

Product selection, enzyme and pathway engineering for applications in cell free systems.

- Engineer balanced pathways for upgrading hydrolysates and byproducts.
- **Construct fusion enzyme as needed for our protein scaffold tethering approach.**
- Use enzyme engineering to increase stability of some enzymes and **enable the use of synthetic cofactors**.
- **Utilize techno economic analysis (TEA) to provide the sensitivities of the process to enzyme loading, activity, cofactor recycling**, pH, reactor volumes, and target the most relevant biochemicals.

Task 2. Immobilization and electrochemistry for Cell Free Technologies:

Increasing stability, operating lifetime and efficiency of the enzymes by immobilization. Developing approaches for efficient cofactors recycling.

- **Immobilize pathway enzymes or combinations of enzymes** on several different conducting polymers **to increase overall stability required for industrial conditions.**
- **Enable cofactor recycling at these interfaces** with or without mediators for electron transfer.

3. Impact

Our approach can help address several conversion barriers by:

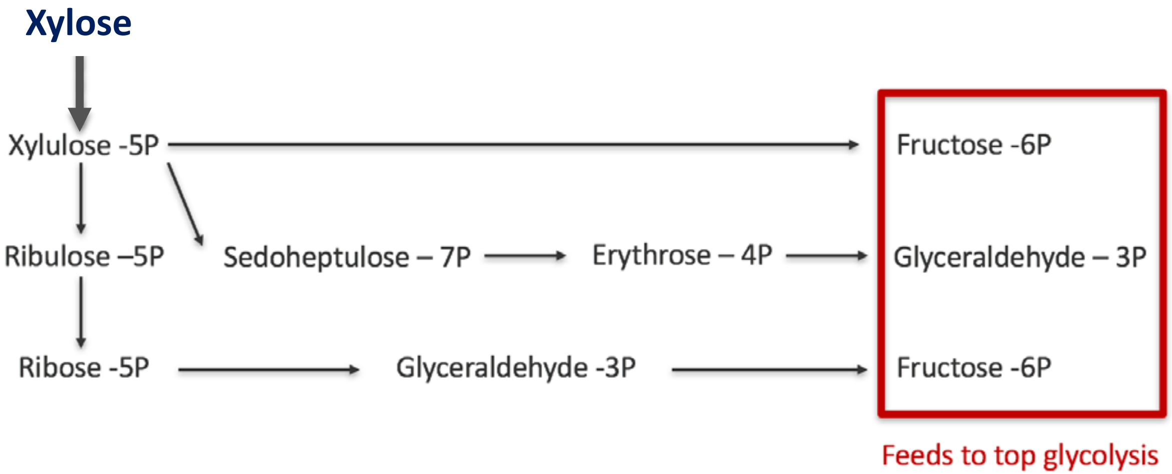
- Increasing titers, yields, and productivity of toxic products due to **higher toxicity thresholds** and **more carbon efficient conversion**.
- **Reducing the cost of separation** due to the absence of microbial cells and media in these processes.
- Offering more flexibility as it is **more resilient 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.

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

Publications, presentations, and IP: A **patent was recently awarded** on this technology notably the used of protein scaffolds for multiple applications. A **provisional patent** will be filled in March on the design of new metabolic pathway with **no CO_2 evolution**. Other IP items are also in preparation on enzyme engineering. Published several manuscripts but as we are in the first funding cycle most of our publications are in preparations.

Progress and Outcomes

Demonstrated Xylose assimilation in Top Glycolysis


Xylose

Xylulose -5P

Ribulose –5P

Ribose -5P

Sedoheptulose – 7P

Erythrose – 4P

Glyceraldehyde -3P

Fructose -6P

Glyceraldehyde – 3P

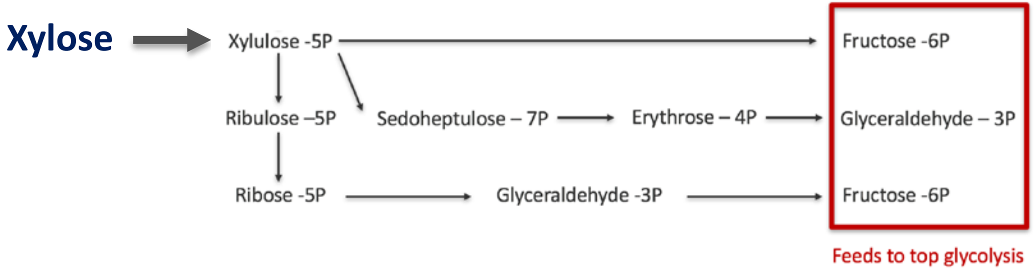
Fructose -6P

Feeds to top glycolysis

Preliminary results mock hydrolysate produced **~1.75 g/L 2,3-BDO from a mock** hydrolysate **and ~3 g/L from xylose (6g/L** of xylose or hydrolysate).

Progress and Outcomes

Complete pathway for the conversion of C5/C6 to 2,3 BDO


Xylose

Xylulose -5P

Ribulose –5P

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Erythrose – 4P

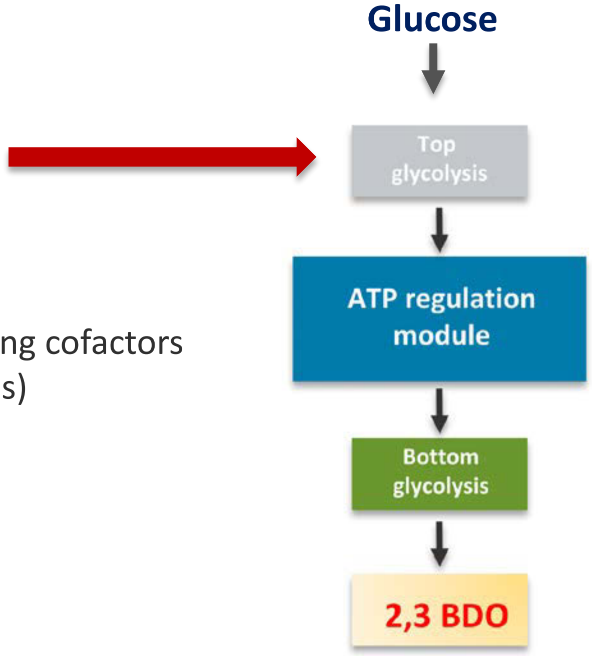
Glyceraldehyde -3P

Fructose -6P

Glyceraldehyde – 3P

Fructose -6P

Feeds to top glycolysis


Glucose

Top glycolysis

ATP regulation module

Bottom glycolysis

2,3 BDO

Production of 2,3 BDO (**23 enzymes for 12h with cofactor recycling**, starting cofactors (once) – **1mM ATP, 1mM NADH** with modified lysate and purified enzymes)

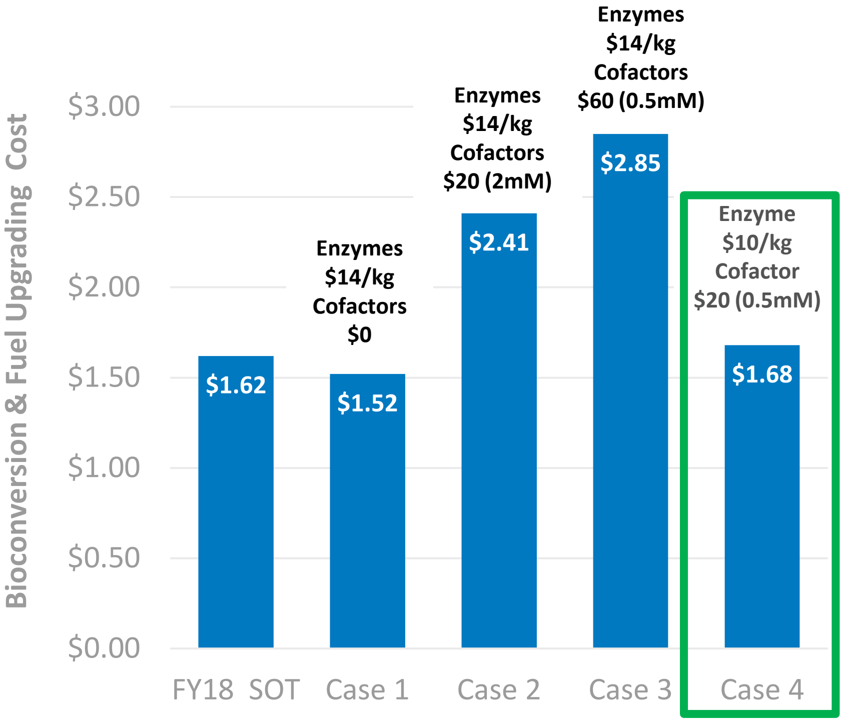
Feedstock	2,3-BDO titer	Productivity	Yield
Glucose	**29.5 ± 2.1 g/L**	2.5 g/L/h	67%
Mock Hydrolysate	20.1 ± 1.9 g/L	1.7 g/L/h	64%
Hydrolysate	**16.2 ± 1.4 g/L**	1.4 g/L/h	59%

Identified enzymes that need prospecting and engineering to increase their operating lifetime and increase overall yields (**yields with pure enzymes is 93% with 16g/L titers at 1.3 g/L/h**) (ALS, GAPN, Pyk, GAPDH, PGK)

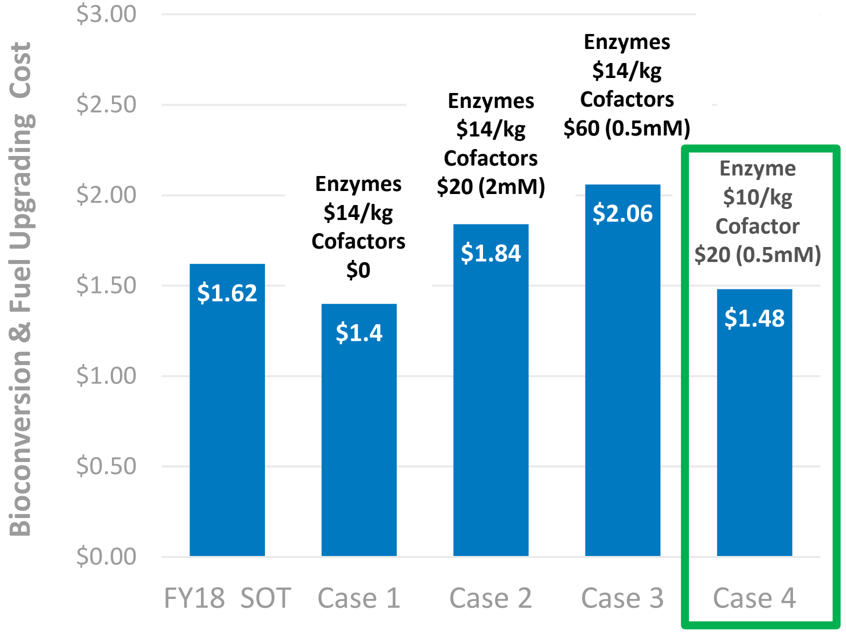
Progress and Outcomes

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



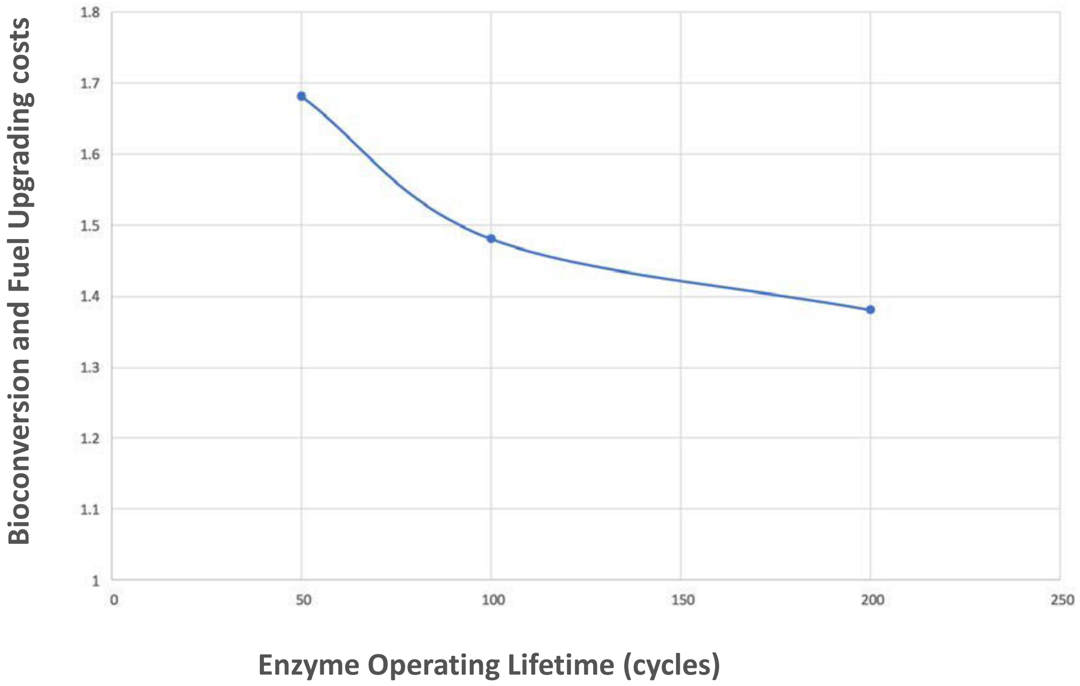
Enzyme operating lifetime: 100 cycles

Progress and Outcomes

Cofactor cost followed by enzyme cost drive the cost of production

Diminishing return after some time, but this shows how far you need to push the cycle

Effect of enzyme operating lifetime on conversion costs



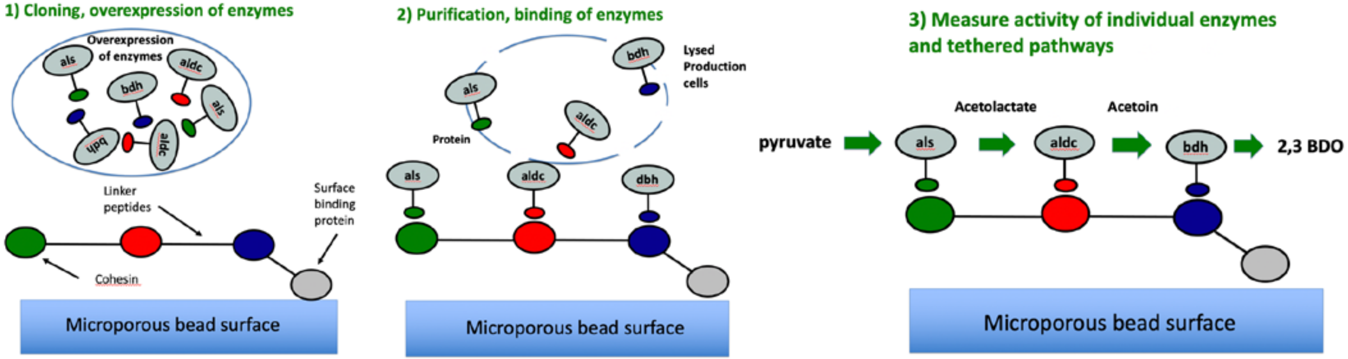
Can be achieved with:

- Enzyme Engineering
- Immobilization
- Encapsulation
- Reactor design
- Enzyme Tethering and fusions
- Operating conditions

Progress and Outcomes

Consolidated CF production is possible: Process advantages of our protein tethering platform

Conversion of pyruvate to 2,3 BDO (4 enzymes with cofactor recycling, no additional cofactors needed)
>90g/L (avg 2g/L/h, max 11.7g/L/h) of 2,3BDO from pyruvate without optimization in <48 hours with 1g/L of enzyme.



- Requires no purification.
- One step purification for co-purification of poorly soluble proteins.
- Helps with enzyme stability.
- Conducted in compact bead beds with a more limited footprints.
- Cell free pathway assembly could achieve higher productivity.

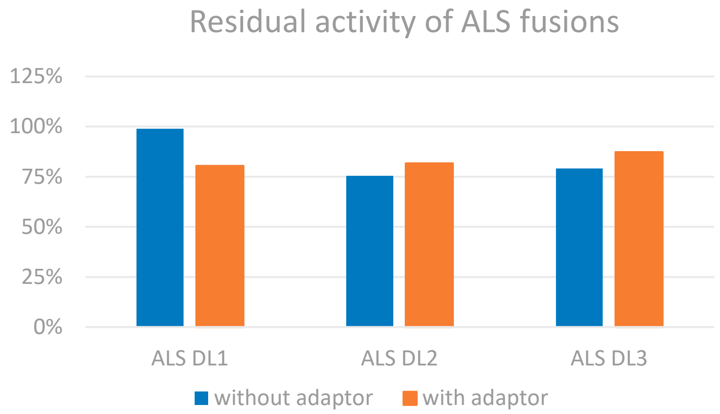
Starting cofactors (once) –1mM NADH, formate dosed in for recycling using a formate dehydrogenase for demonstration purposes only.

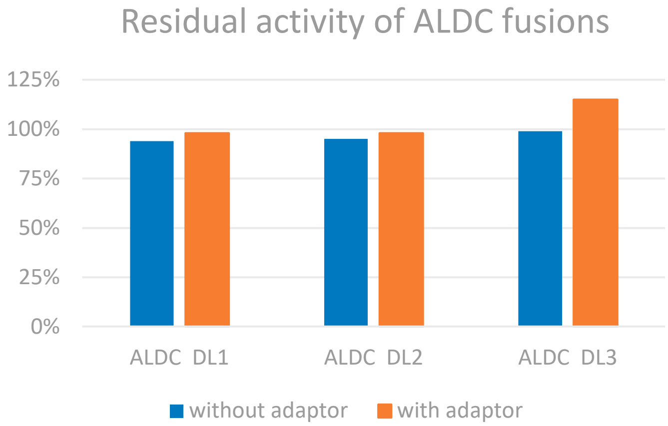
We can produce **17g/L (1.1g/L/h) of 2,3BDO** from pyruvate without optimization in **<16 hours**. (Milestone was 10 g/L at 0.5 g/L/h). Some enzymes (BDH) lose more ~60% activity in fusion constructs, free enzyme titers would be >40g/L. **Can fusion constructs be optimized focusing on linker lengths and composition?**

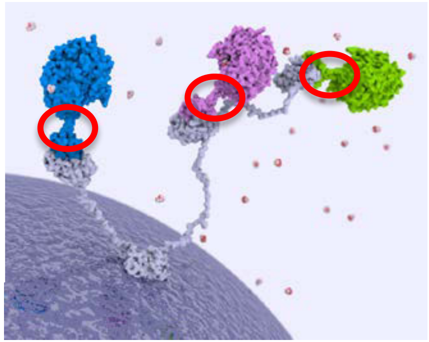
4. Progress and Outcomes

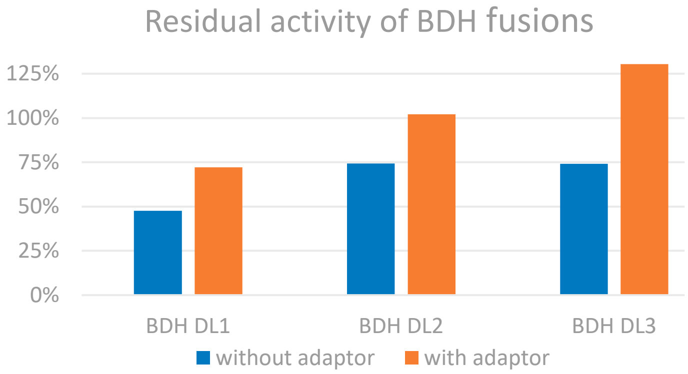
Linker length can be optimized to recover wild type activity for consolidated CF production

(L1: 5 Amino Acids, L2: 65 AAs, L3: 113 AAs)


Residual activity of ALS fusions


Residual activity of ALDC fusions




Residual activity of BDH fusions

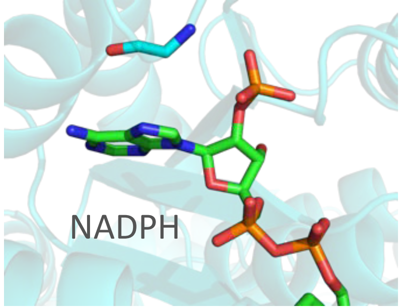
- In the process of testing the full pathway.
- In some cases, the binding to the adaptor helps recover specific activity as observed before for glycerol conversion.
- Better understanding of the classes of enzymes better suited or more adversely impact by fusion constructs.

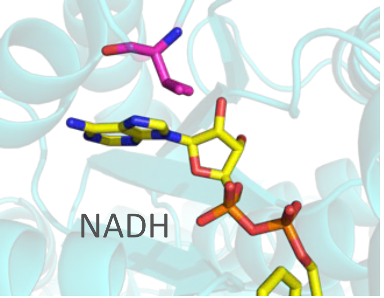
Progress and Outcomes

Prospecting and engineering for better cofactor management

Switching cofactor specificity in an essential enzyme: Glyceraldehyde-3-phosphate dehydrogenase (GAPN)

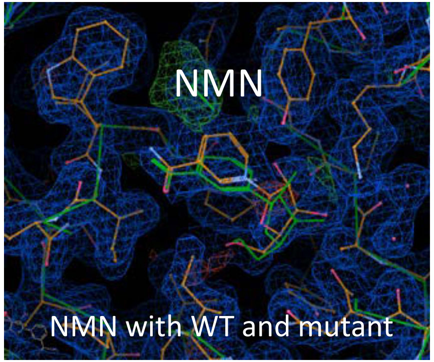
- NADH dependent GAPNs are essential but don’t exist natively.
- Using machine learning approaches and structure guided mutagenesis to generate mutant candidates.
- Generated mutants with a 10- fold improvement in NADH utilization over **WT to about 20% of NADPH utilization.**

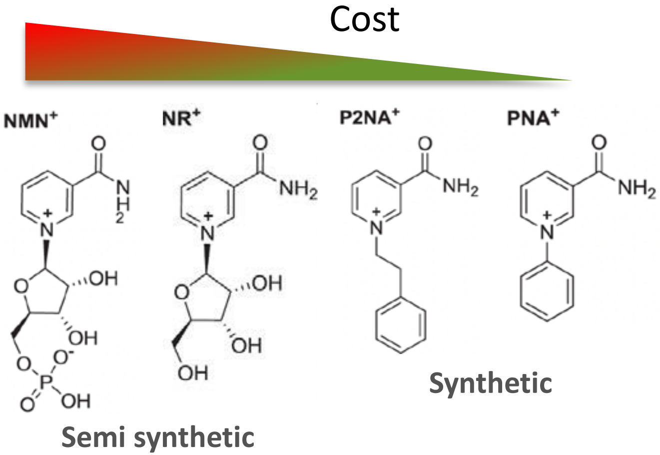




Enabling the utilization of biomimetic cofactors

- **Engineered BDHs are able to use the biomimetic cofactor NMN.**
- Using structural biology **to derive design principles that can be used** in other redox enzymes and for other cofactors.
- **Engineering promising water forming oxidase that have some native ability for NMN (25%).**



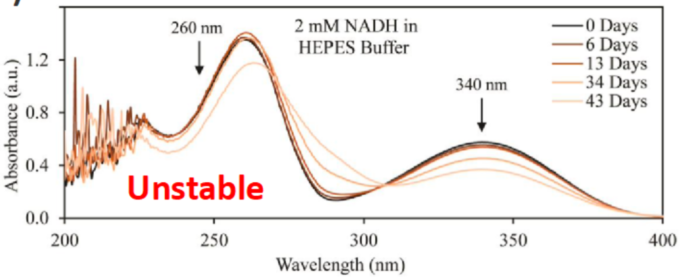


Progress and Outcomes

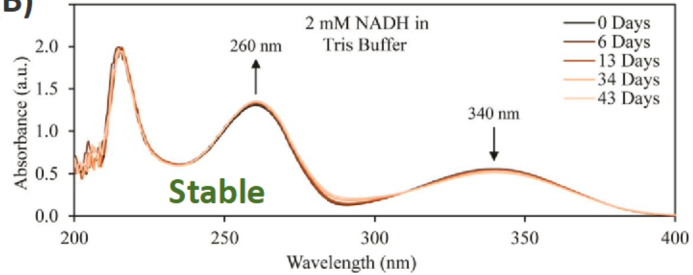
Cofactors are stable for weeks in certain buffers and can be recycle at electrodes with new designs

Cofactors are stable for more than a month in certain buffers

(A)



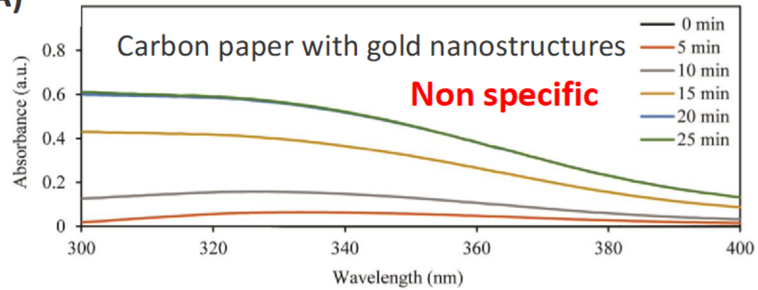
(B)



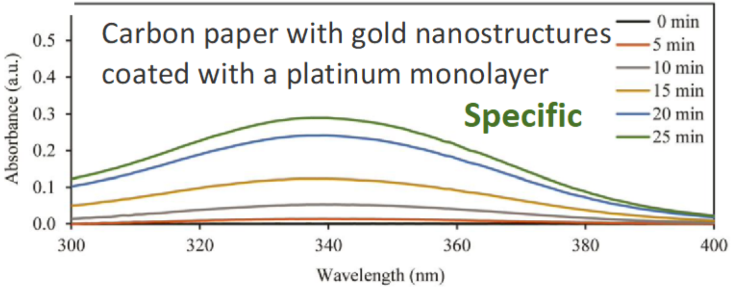
NADH is very unstable in some buffers **(A)** but stable in others **(B)** for close to 1.5 months. We also know that NADH is efficiently enzymatically recycled for more than 7 days without losses. **These results bode well for extending cofactor lifetime to reduce overall cost.**

New electrodes can be designed to efficiently recycle NADH

(A)



(B)



The strong increase in absorbance **(A)** at lower wavelengths is most likely due to the production of different forms of NADH. The Gaussian distribution **(B)** shows selective production of NADH from NAD+.

Progress and Outcomes

Most relevant progress to date

Conversion of pyruvate to 2,3 BDO (4 enzymes with cofactor recycling, no additional cofactors needed)

>90g/L (2g/L/h) of 2,3BDO from pyruvate without optimization in <48 hours.

Developed the assimilation of xylose in our cell free system

We have confirmed the assimilation of xylose in top glycolysis towards the production of 2,3BDO.

Conversion of glucose and hydrolysate to 2,3 BDO (with cofactor recycling, no additional cofactors needed)

~30g/L (2.5g/L/h) of 2,3BDO from glucose and **~16g/L (1.4g/L/h)** of 2,3BDO from hydrolysate.

Conversion of glycerol to 1,3 PDO and 3HP

Developed a promising cell free approach to convert glycerol based on enzyme tethering to protein scaffolds.

Designed new pathways for the conversion of hydrolysates to diacids with no CO2 evolution

Using a combination of a new C5/C6 non oxidative glycolysis and reverse beta oxidation.

Demonstrated cofactor recycling at electrodes and assessed long term cofactor stability

We have shown the conversion of NAD+ to NADH at electrodes and shown that **cofactors are stable for more than a month in some buffers**.

Identified and engineering enzymes for better cofactor management

Several enzymes show promise for use of different cofactors including the utilization of biomimetic cofactors.

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 products from hydrolysates with no cofactor addition.
- Demonstrated cofactor recycling at electrodes and assessed long term cofactor stability.
- Engineered enzymes to enable better cofactor management
- Demonstrated that enzyme tethering is a viable approach to assemble enzyme with optimization

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
Andrea Buchholz
Neal Hengge
Patrick Hewitt
Michael Himmel
Kane Jennings – Vanderbilt
Sam Mallinson
Ashutosh Mittal
Rida Noor
Kody Wolfe – Vanderbilt
Qi Xu

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

Biochemical Process Modeling and Simulation (Bomble)
Enzyme Engineering and Optimization (Himmel)

Collaborators

Min Zhang – NREL (TMD)
Ling Tao - NREL (BPA)
Jim Bowie – UCLA
Yongqin Jiao - LLNL
Rob Paton – CSU
Han Li - UCI
Sophie Barbe – (TBI-France)
Tyler Korman - (INvizyne Technologies)

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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**)

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