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

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

Project Overview

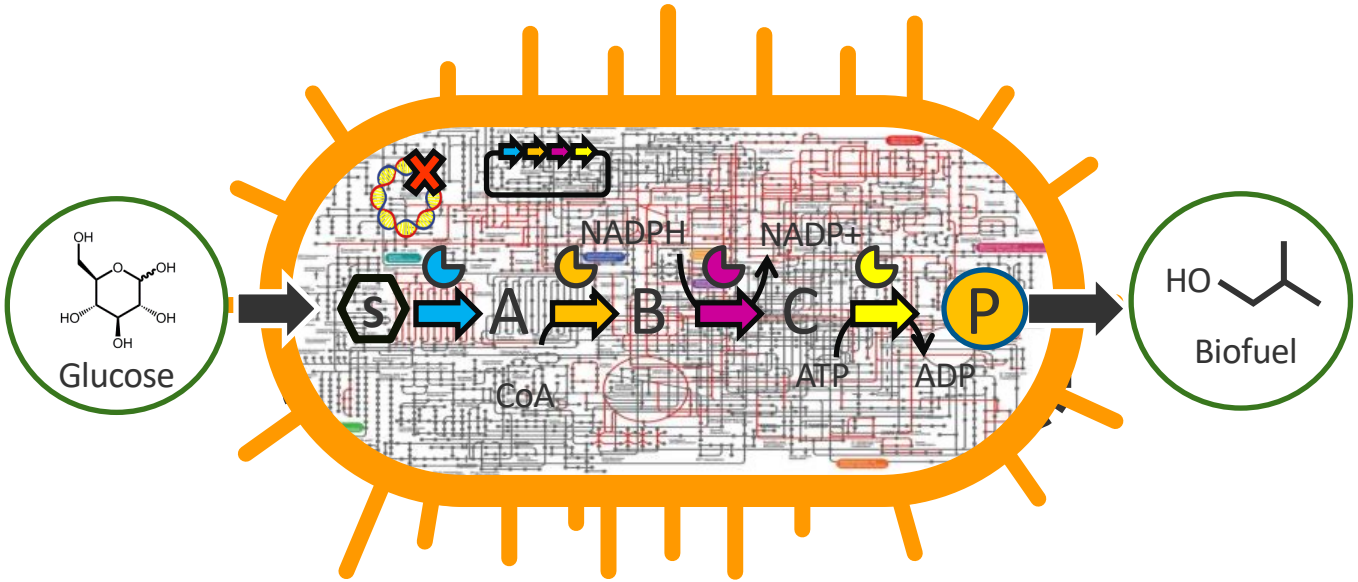
Context:

- **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 and use optimized enzyme cascades *in vitro* instead.
- 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 2050 goals of 60B gallons of renewable hydrocarbon fuels and >450 million ton reduction of CO_2 annually.**

Cell-Free Biocatalysis Background



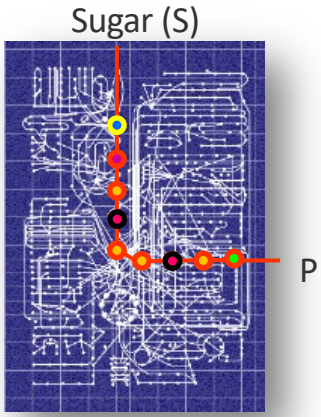
The Solution:

Simplify biocatalysis. Get rid of the cells.

- Unwanted side-effects of DBT cycles
- Substrate and products transport

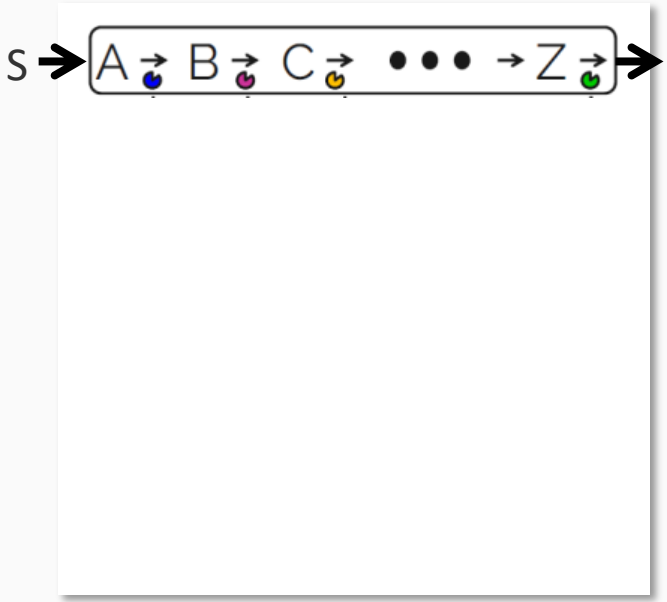
Courtesy of Tyler Korman (Invizyne)

Cell-Free Biocatalysis Background



Don't need to worry about background metabolism

Only add elements necessary for biotransformation and regulation



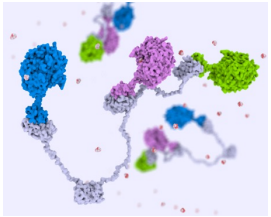
1. Design Pathway
2. Clone/Express
3. Isolate Enzymes
4. Mix Enzymes and Cofactors
5. Run Bioreactor

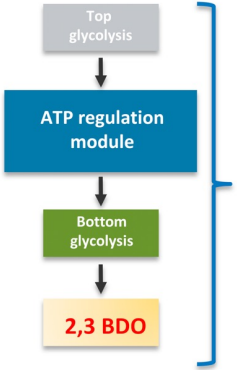
Courtesy of Tyler Korman (Invizyne)

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.

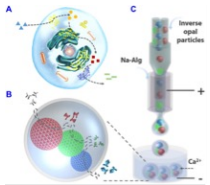




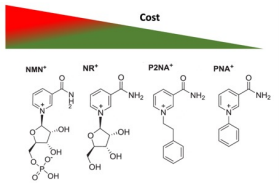
Enzyme characterization
Pathway engineering
Enzyme design
Techno economic analysis

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 the cost of cofactors.



Biopolymers Synthesis



Subcontracts: Yongqin Jiao (LLNL) ($200K/Year): enzyme immobilization. **Rob Paton (CSU) ($60K/Year):** computational engineering of synthetic cofactors (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, reducing 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:**

- We focus on the **production of various terpenes as SAF and diesel intermediates or as direct replacements using a cell free approach** (First funding cycle-2,3 BDO as proof of concept).
- Work with our **TEA team** to make cell free biocatalysis 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.**

DEI Plan:

- Work with all our partners to make sure they have a strong DEI program which includes All Hands Training.
- Working with our university, national lab, and university partners recruit and train researchers from underrepresented communities in STEM.
- Initiate collaborations with MSIs to complement our research which will also help in our recruiting efforts.

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 the cost of the overall process by eliminating the need for addition of expensive cofactors.**

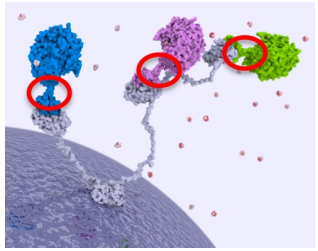
Risks/Challenges	Mitigation strategies
Enzyme expression issues	• Replacement enzymes from natural diversity. • Other expression hosts to increase enzyme production titers (TCF).
Lack of long term enzyme stability	• Immobilization. • Natural diversity. • Enzyme engineering.
Cofactor cost, lack of efficiency 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 of biomimetic cofactors.

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

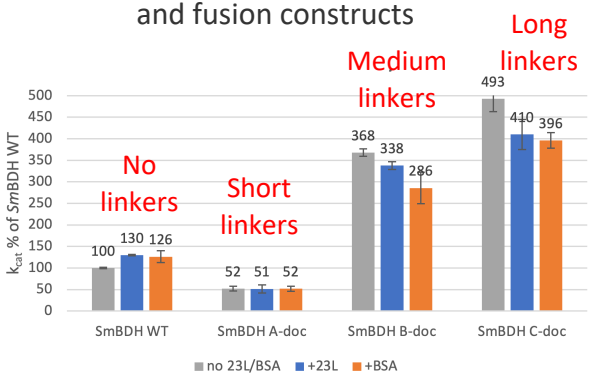
2- Progress and Outcomes: Demonstrated consolidated cell free production

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

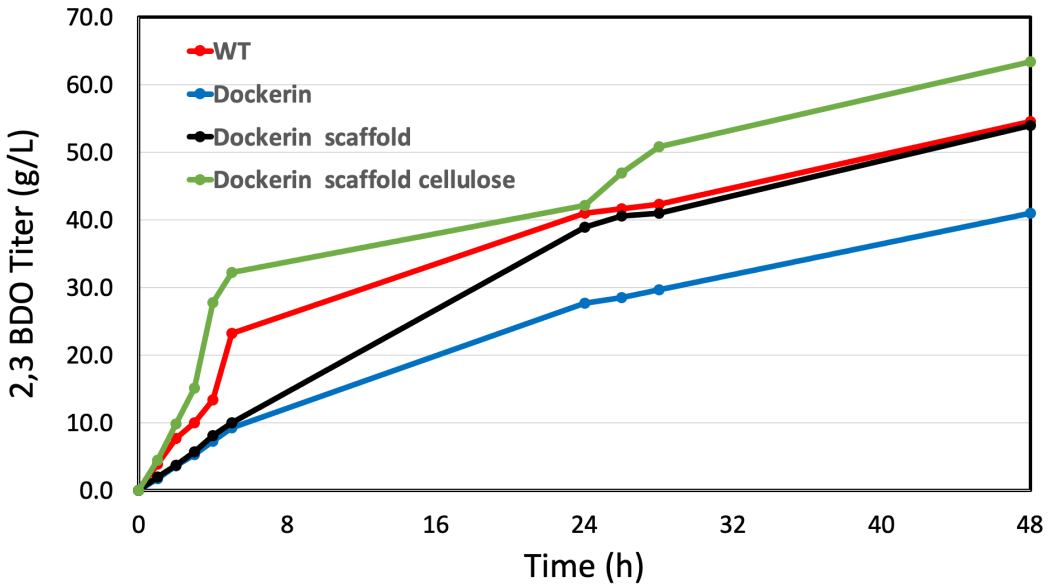
Leveraging natural tethering strategies to array enzymes automatically when co-expressed



Optimization of linker lengths and fusion constructs



The pathway with dockerin constructs reaches the same 2,3 BDO titer when immobilized and exceeds it when bound to cellulose.

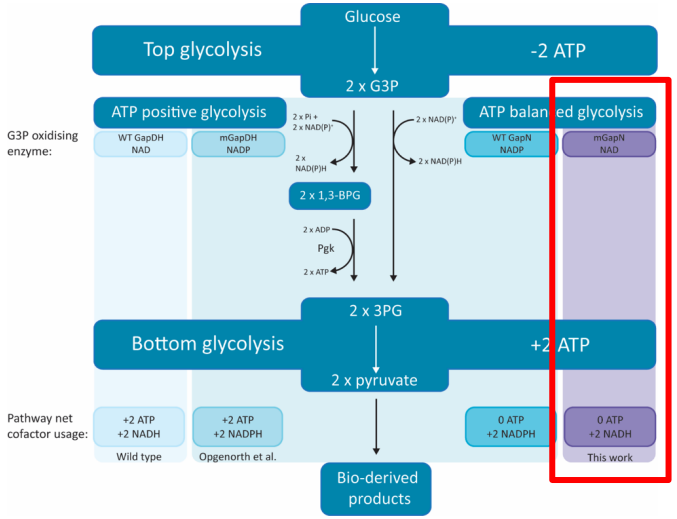


In review at Frontiers in Bioengineering and Biotechnology (2023)

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 but unusual enzyme for ATP neutral glycolysis with NADH.

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

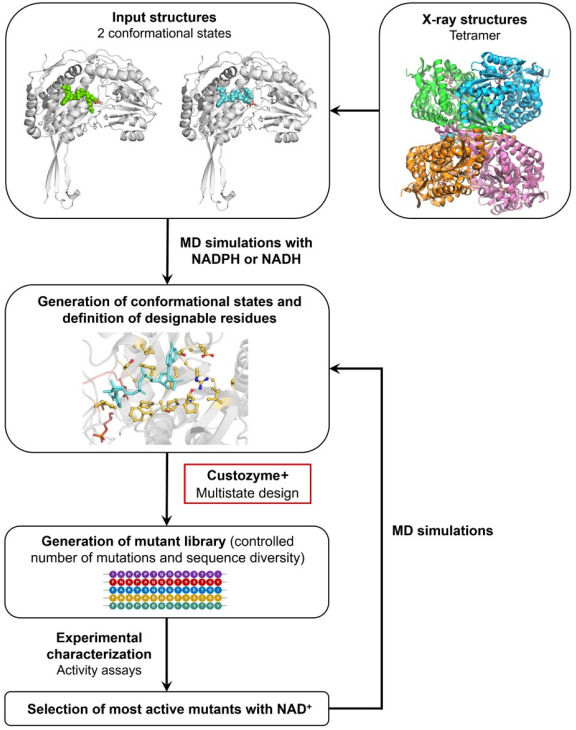


- Efficient NADH dependent GAPNs are essential but do not exist natively.
- Used atomistic modeling, machine learning, and structure guided mutagenesis to generate mutant candidates.

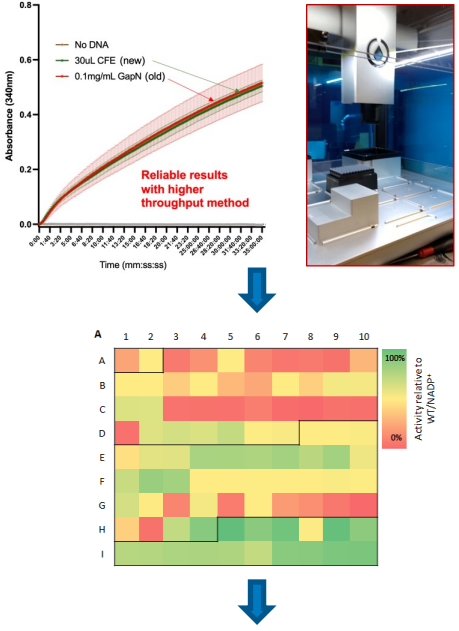
Collaboration with Sophie Barbe and BETO-BPMS

In review at ACS catalysis (2023)

Computational variant prediction

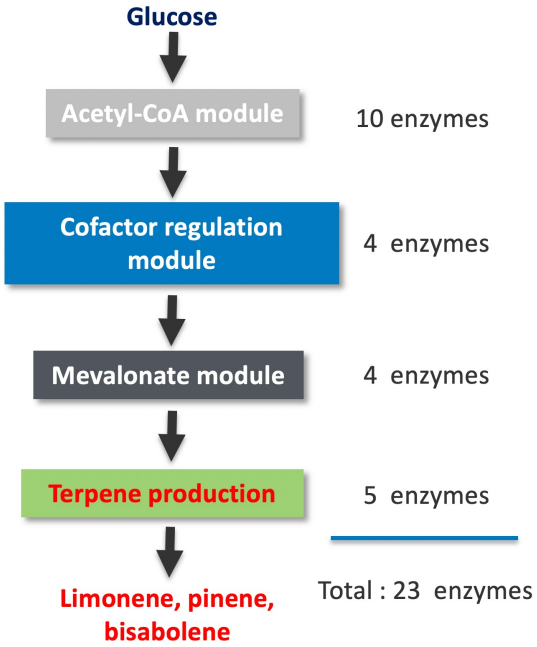


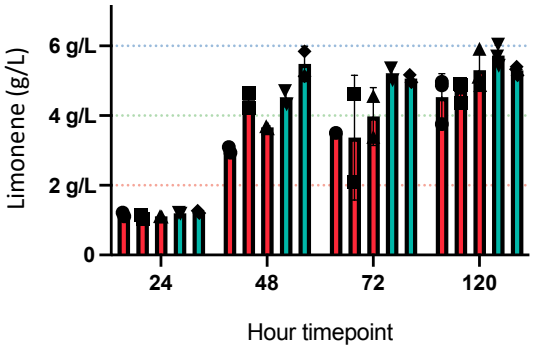
Variant screening



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

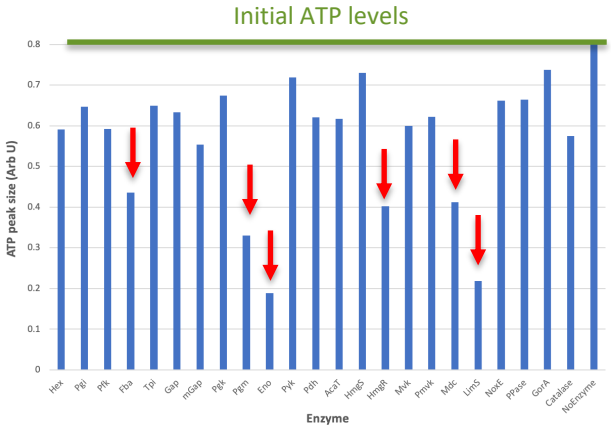
2- Progress and Outcomes: Enabling terpene production from glucose with minimal protein purification

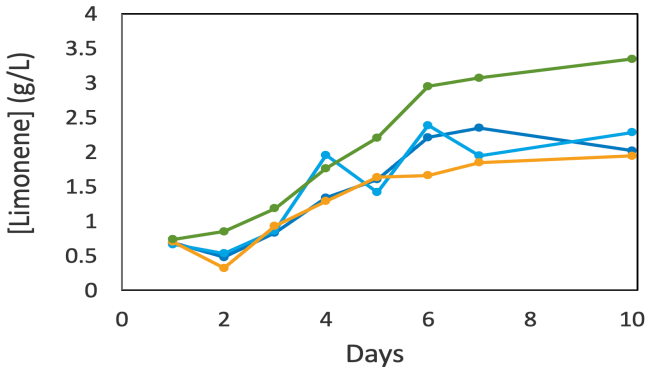




- Demonstrated the production of mevalonate from glucose at >10g/L (>90% yield).
- Demonstrated the production of limonene from mevalonate at >6g/L (>95% yield) with crude purifications and stable proteins.

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





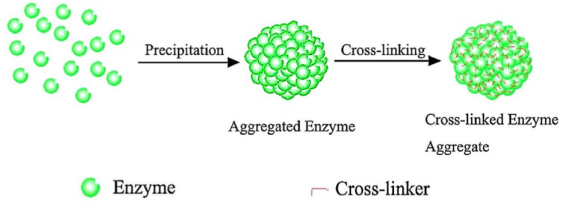
- The postprocessing of enzyme preparations is a major bottleneck.
- Working with crude enzyme preparations to simplify and reduce the cost of cell free terpene production.

Collaboration with Jim Bowie (UCLA).

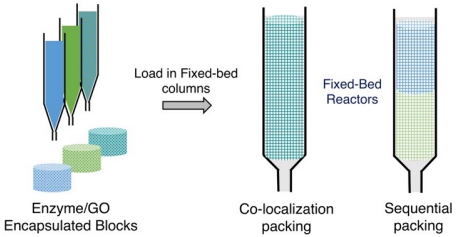
2- Progress and Outcomes: Initiated systematic enzyme immobilization campaigns

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.

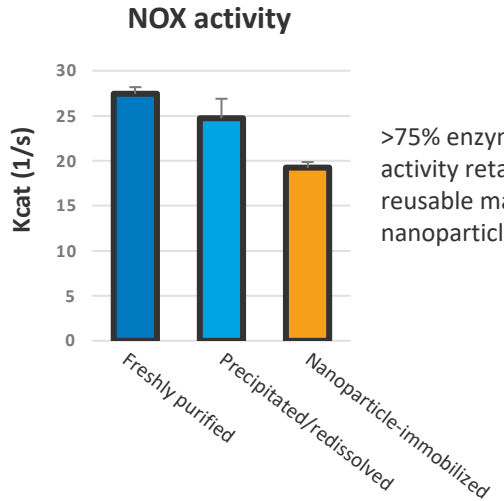
Explore different enzyme immobilization strategies



Explore different packed bed reactor designs (BETO-BPMS)



- Successfully generated active cross-linked aggregates (CLEAs) of several enzymes under development for cell-free biosynthesis:
  - NOX, a NADH water forming oxidase
  - GapN, the G3P dehydrogenase component of the glycolysis module
- Generated active NOX-linked magnetic nanoparticles for efficient enzyme recovery and reuse.
- Demonstrated activity retention of ~99% for immobilized GapN following recycling.
- Combining immobilization approaches to maximize stability and reusability, and applying to additional enzymes in the biosynthetic pathway.



>75% enzyme activity retained on reusable magnetic nanoparticles

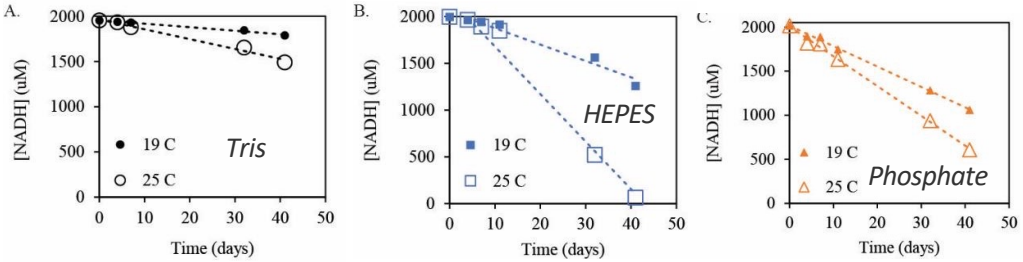
Initial enzyme immobilization attempts with rather low-tech solutions seem promising with some enzymes. Constructs with other enzymes showed a varying degree of success due to enzyme inactivation. Risk mitigation strategy: Now exploring more targeted and generalizable immobilization approaches to avoid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

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

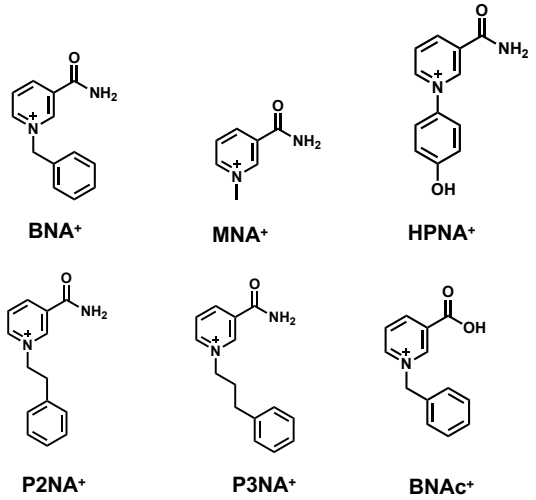
2- Progress and Outcomes: Enabling the utilization of biomimetic cofactors

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

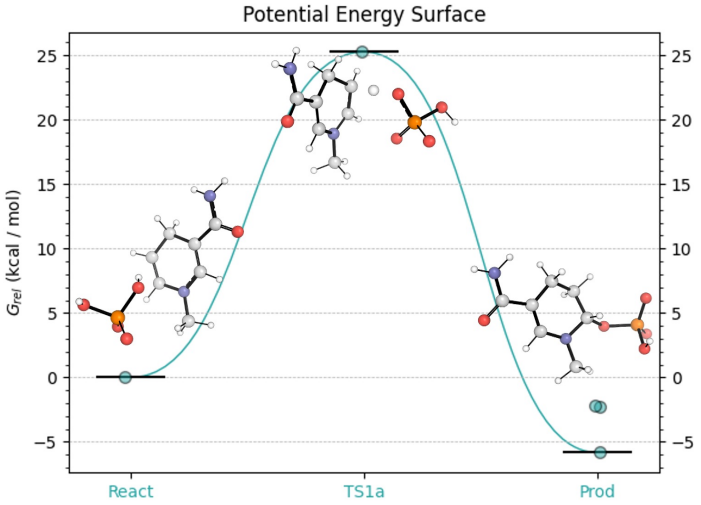
Demonstrated that natural cofactors become unstable as temperature increases

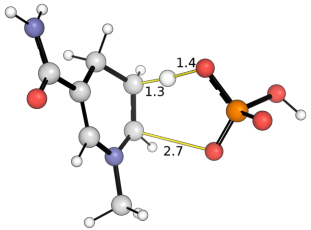


Synthesized cofactors from cheap starting materials at high yields (75-90%) and purity (>95%). Demonstrated activity with several of our redox enzymes. Engineering underway to further improve these enzymes.



Developing approaches to predict the characteristics of cofactors including stability and redox potentials e.g., decomposition mechanism in potassium phosphate buffer.





Optimization of transition state

Work conducted at CSU: Heidi Klem and Rob Paton

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 >20g/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.
- **De-risk cell free based bioprocesses for greater adoption in industry** and to access a product space not available with traditional microbial routes.

Overall, our project will enable the production of SAF to contribute to BETO's 2050 goals of 60B gallons of renewable hydrocarbon fuels and >450 million ton reduction of CO_2 annually. These technologies can also enable the production of renewable chemicals contributing to the 40B pounds 2050 BETO goal.

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 for *Zymomonas mobilis*.
- New TCF project awarded on reducing the cost of protein production for cell free systems with an industrial partner (Invizyne).
- Share engineered enzymes with 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 (ROI-22-46).
- Pegboard protein purification plate for high throughput protein characterization (Provisional Patent Application No. 63/359,649).

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 synthetic 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**

Progress and Outcomes:

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

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.

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 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 2050 goals of 60B gallons of renewable hydrocarbon fuels and >450 million ton reduction of CO_2 annually by developing processes with increased carbon efficiency and limited CO_2 evolution.

Funding Mechanism

AOP as WBS# - 2.5.4.101

Acknowledgments

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  - BETO TM: Beau Hoffman
  - NREL LPM and Platform Lead: Zia Abdullah, Mike Guarnieri

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Rui Katahira
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Qi Xu

LLNL

Ziye Dong
Yongqin Jiao
Dante Ricci
Xun Wang

INvizyne Technologies

Tyler Korman

CSU

Heidi Klem
Rob Paton

Projects with joint or collaborative milestones Focused on cofactor management with biomimetics and the design of new cell free reactors

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)

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Responses to Previous Reviewers' Comments

Q: The metric used is "bioconversion and fuel productivity cost," although it is not clear what this metric is measuring (is it $/GGE of biofuel, or something else?). This project will be able to provide valuable insights into whether cell-free systems with a large number of enzymes could be competitive with microbial conversion processes or whether the opportunities for cell-free systems would be better focused on specialized short pathways to be used in combination with other microbial and/or chemical processes.

A: The cost targets in our TEA are indeed in $/GGE biofuels. Here we focused on the "bioconversion and fuels" component of the total fuel cost, as this is where most of the benefits of cell-free production will be seen. With enzymes operating for 100 production cycles, we can see a 10% benefit with cell-free systems over traditional fermentations, even for a nontoxic product such as 2,3-BDO. We expect this comparison to be more advantageous for cell-free production with toxic products.

Q: Electrochemical processes, here suggested for the recycling of cofactors, will be considered exotic within industry. It would be useful if the PIs could point to examples of large-scale electrochemical conversions of the type planned in this work, other than long-established approaches (chlor/alkali, hydrodimerization of acrylonitrile).

A: Regarding the use of electrochemical processes for cofactor recycling, we agree that these approaches could be a priori considered challenging in industry. However, electrochemical conversions are already widely used in a host of industrial-scale processes, including the production of many value-added organic and inorganic products. In the bioelectrochemical area, microbial fuel cells, in which electrons are transferred from bacteria to an anode, have found commercial applications in wastewater treatment. In the past year, researchers have efficiently scaled up an electrochemical cofactor recycling system from 4 mL to 500 mL and also showed that 5-L reactors can be readily achieved with larger electrodes. This work shows that scale-up could later be achieved, especially given the smaller reactor volumes associated with cell-free processes. However, we have decided to discontinue these efforts as they are more long term and focus instead on more short term targets.

Responses to Previous Reviewers' Comments

Q: A suggestion for this project is to also evaluate its feasibility using LCA. For instance, while it may be the case that some cell-free systems operate with no CO2 evolution, the production of enzymes and cofactors may have a non-negligible CO_2 footprint that needs to be considered. This project, with its connections to other BETO projects, seems to be the proper place to perform such analysis and this work is now underway and will be finished by the end of FY23.

A: The consideration of LCA is indeed a really good suggestion. We do anticipate that cell-free processes can be designed to increase carbon efficiency but also limit or reuse any CO2 emission evolved during the production process. Additionally, cell-free processes will have much higher volumetric rates, which will help lower water treatment needs. It is also correct to point out that enzyme production has a CO_2 footprint that has to be carefully considered. In this regard, increasing the stability and operating lifetime of these enzymes is crucial to reduce overall CO_2 emission originating from their production.

Q: The only potential weakness is the choice of product: 2,3-butanediol is relatively inert, and most microorganisms can tolerate high concentrations. Perhaps the team should consider producing a toxic molecule, thus taking advantage of one of the primary strengths of cell-free systems.

A: Regarding the selection of 2,3-BDO, as our project was new to BETO, we decided to select a product that would be aligned with other projects that relied on fermentations for 2,3-BDO production to directly compare our titers, yields, and productivities and demonstrate that cell-free systems can be cost-competitive even when considering a product that can be produced by microbial fermentations at high titers and that has low toxicity. We have now moved to more toxic and valuable products to fully demonstrate the benefits of cell-free biocatalysis over fermentative processes for these types of products.

Responses to Previous Reviewers' Comments

Q: There are significant concerns whether cell-free systems can scale due to the costs of the enzymes and cofactors. Some believe that these components can be made sufficiently cheap, especially when the enzymes can be recycled and cofactors regenerated. Others do not. As a consequence, the impact is unclear.

A: We do agree that there are significant obstacles that need to be overcome for cell-free biocatalysis to become a reality at the industrial scale. However, enabling this technology at that scale could be a game-changer for the production of biochemicals and bioproducts leading to reduced production cost and less carbon-intensive processes. One of these obstacles is cofactor management, but we and others are demonstrating that cofactors can be recycled and are stable for multiple production cycles, thus leading to reduced cost. This cost can also be further reduced by enabling the use of cheaper and more stable synthetic cofactors.

Regarding enzyme production and costs, we are confident that scale-up production can be achieved using dedicated production strains. Some enzymes will need to be expressed intracellularly, but would only require crude purifications such as heat precipitation when thermophilic enzymes are used. We have now shown in a new TCF project that several of these enzymes can be expressed at more than 3g/L of enzyme titers in bacteria and some can also be secreted in yeast. This bodes well for scale up and for measures needed to reduce production costs. The contribution of enzyme cost to the overall process can also be mitigated by increasing their operating lifetime, which we are trying to achieve using a combination of enzyme prospecting, engineering, and immobilization/encapsulation. We already know that some enzymes remain active for days in solvents without immobilization/encapsulation, which bodes well for the prospects of enzymes operating for 30+ days once stabilization approaches are put in place to enable continuous operation. The same strategies used to increase enzyme operating lifetime can be used to increase the enzyme tolerance to toxic compounds encountered during production. Some enzymes will always be more susceptible to degradation, and new process strategies such as modular reactors have to be developed in the future to be able to selectively swap these enzymes for smooth and continuous operation

Publications and IP Since Last Peer Review

Publications:

- Sam Mallinson, Delphine Dessaux, Sophie Barbe, Yannick Bomble, "Rational redesign of redox enzyme cofactor specificity", (in review at ***ACS catalysis***)
- Markus Alahuhta, Nadia Ganjoloo, Qi Xu, Neal Hengge, Michael E. Himmel, and Yannick J. Bomble, "Optimization of a 2,3 BDO metabolon for consolidated cell free production", (in review at ***Frontiers in Bioengineering and Biotechnology***)
- Kody D. Wolfe, Markus Alahuhta, Michael E. Himmel, David E. Cliffel, Yannick J. Bomble, and G. Kane Jennings, "Long-Term Stability of NAD^+/NADH in Aqueous Buffers" (in review at ***Catalysts***)
- Gerald M. Carroll, G. Matthew A. Gebbie, Shannon S. Stahl, Mathew R. Johnson, Oana R. Luca, O. R., Haley A. Petersen, Yannick J. Bomble, Nathan R. Neale, Randy D. Cortright, "Alternative Energy Carriers: Unique Interfaces for Electrochemical Hydrogenic Transformations", ***Adv. Energy Mater,*** DOI: 10.1002/aenm.202203751 (**March 2023**)
- Yannick J. Bomble and Michael C. Jewett, "Cell Free Biocatalysis for the Production of Bioproducts", ***Frontiers in Energy Research,*** DOI: 10.3389/fenrg.2021.781552 (**Oct 2021**)
- Qi Xu, Markus Alahuhta, 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", ***Frontiers in Energy Research,*** DOI: 10.3389/fenrg.2021.680313 (**July 2021**)

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 (ROI-22-46).
- Pegboard protein purification plate for high throughput protein characterization (Provisional Patent Application No. 63/359,649).