DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Cell Free and Immobilization Technologies

WBS is 2.5.4.101

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Biochemical Platform Review

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Denver, CO

Goal Statement

- **Goal 1 -** Develop Cell Free/Immobilization Technology (CFIT) processes to **decrease production and separation costs of biochemicals**
- **Goal 2 –** Integrate existing technologies, enzyme and **pathway engineering with novel tethered enzymatic systems** and reactor/immobilization designs **to enable new cell free approaches**
- **Outcome 1 – Processes with higher yields, productivity, and titers** from C5/C6 sugars with **no fermentation byproducts**.
- **Outcome 2 –** A comprehensive **technoeconomic analysis of cell free approaches** for the production of biochemicals.
- **Relevance**
  - Production of biochemicals is still costly due to reliance on microorganisms.
  - Non natural metabolic pathways are still difficult to implement *in vivo.*
  - We can achieve higher concentrations of biochemicals that would otherwise be toxic to microorganisms.
  - Cell free technologies for the production of biochemicals have the potential **to reduce OPEX and CAPEX significantly.**

Quad Chart Overview

Timeline

This research started in Q2 of FY18 as a seed in **2.5.4.100 Enzyme Engineering and Optimization (EEO) (Himmel)**

- Project Start Date: Oct 1st 2018
- Project End Date: Sept 30th 2021
- Percent Complete: 14%

Budget

	Total Costs Pre FY 17	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date
DOE Funded	$0	$0	$0	$2.7M
Project Cost Share (Comp.)	0	0	0	0

Partners: Internal: EEO, TMD, BPMS, BSI, PSI, SDA, CUBI
Subcontractors: Kane Jennings (Vanderbilt University)
Academic Collaborators: Jim Bowie (UCLA), Sonja Salmon (NCSU).
National Lab/Industry Collaborators: Yongqin Jiao (LLNL), Alex Berlin (Arbiom), Zachary Sun (Synvitrobio).

Barriers

- Ct-D. Advanced bioprocess development: Enzymes immobilized in reactors **can function for extended periods of time and can increase process intensity**. Cell free approaches **can increase yield by avoiding the production of byproducts and cell upkeep**. Real time monitoring of intermediates is easier
- Ct-K. Developing methods for co-product production: Cell free pathways can be designed to **avoid the production of byproducts but instead producing desired co-products** leading **to higher product purity** and reduction in **separation costs**.

Objective

Enable the development of Cell Free/Immobilization Technology (CFIT) based processes to **reduce production cost** of biochemicals. **Reduce risks of production scale ups.**

End of Project Goal (9/30/2021)

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 appropriate cofactor recycling as needed. Compare the yields with microbial routes.

1—Project Overview

Context:

- ***Several factors negatively impact the production of biochemicals:*** End-product or intermediate toxicity, diversion of carbon to biomass formation, undesired byproducts.
- *An* ***attractive alternative is to operate metabolic pathways without the cells*** *to circumvent problems with microbial biocatalysts but these approaches still suffer from low productivities due to:* Free diffusion of intermediates, cofactor cost, inefficient recycling rates, cost of enzyme production/purification, long term enzyme stability.
- *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.*
- *There already exists* ***mature and promising applications relying on cell free approaches****:* Biodiesel production using immobilized lipases, high fructose corn syrup production, as well as L-aspartate. More recently the production of isobutanol using a (>10 enzyme pathway) from glucose.

New approach:

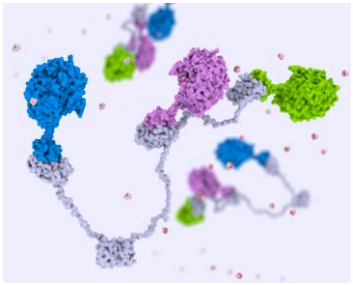
- *This project represents a new effort to* ***develop innovative and cost competitive routes*** *to producing biochemicals from a variety of feedstocks using cell free approaches combined with tethered or immobilized pathway enzymes. This project will* **reduce the current risk and cost associated with classical cell free production** and help address the conversion barriers in multiple areas in the MYPP.

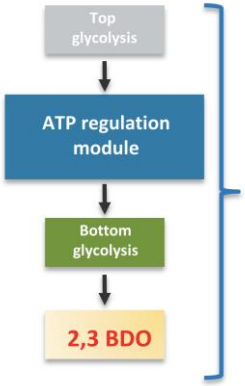
2—Approach (Management)

FY19—Cell Free and Immobilization Technologies

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 (including TMD, EEO, BPMS), tracking go/no-go activities, budget management.

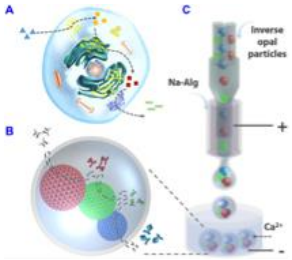
FY19 Task 1. Pathway and Enzyme Engineering for Cell Free Technologies (750K): Product selection, enzyme and pathway engineering for applications in cell free systems.





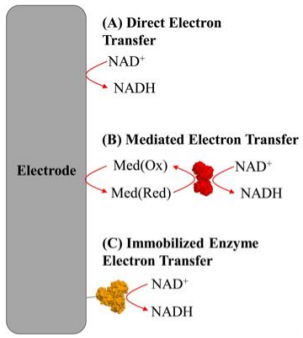
Enzyme characterization
Pathway engineering
Enzyme design
Techno economic analysis

FY19 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.



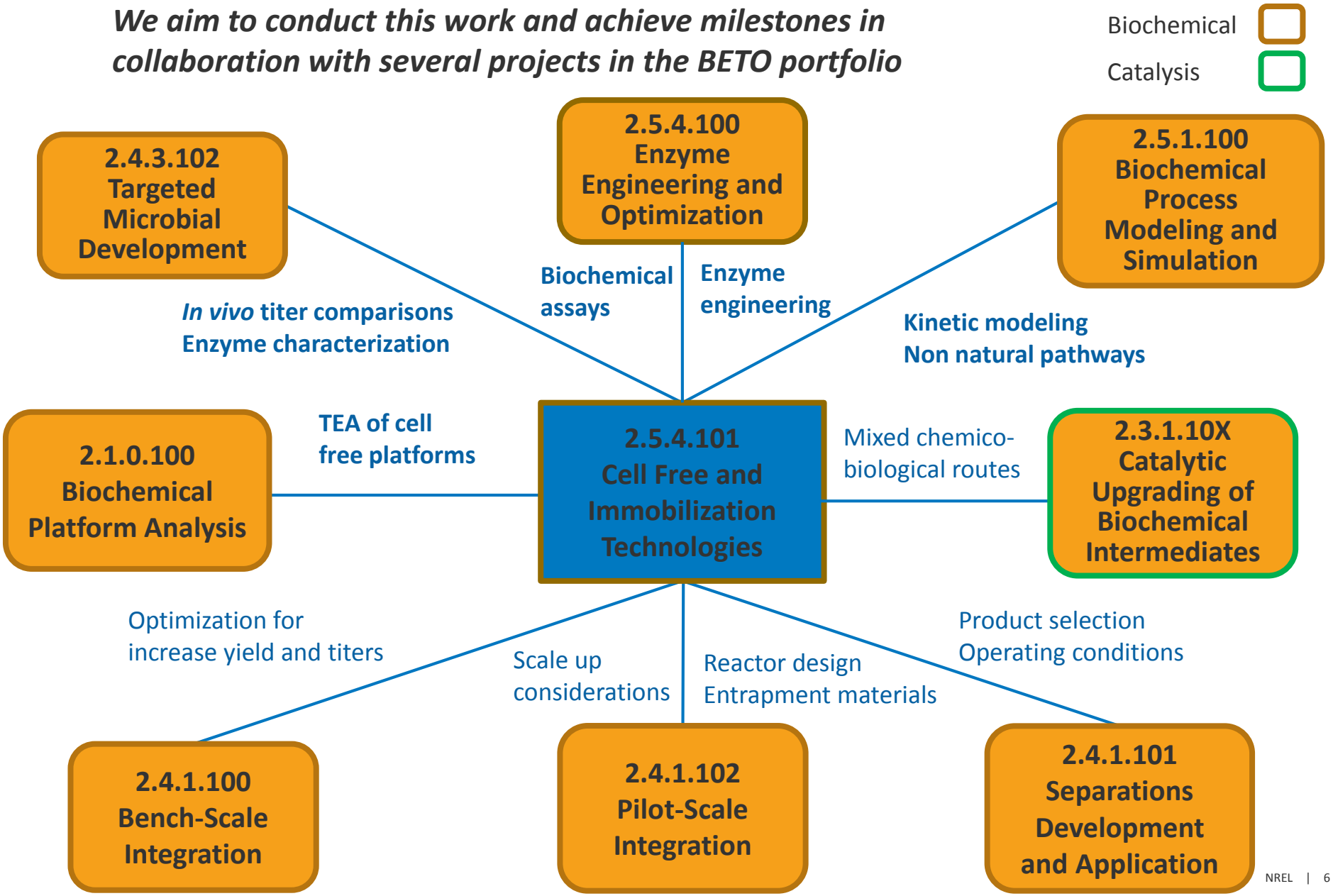
Biopolymers
Electrochemistry

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



2—Approach (Management)

We aim to conduct this work and achieve milestones in collaboration with several projects in the BETO portfolio



2—Approach (Technical)

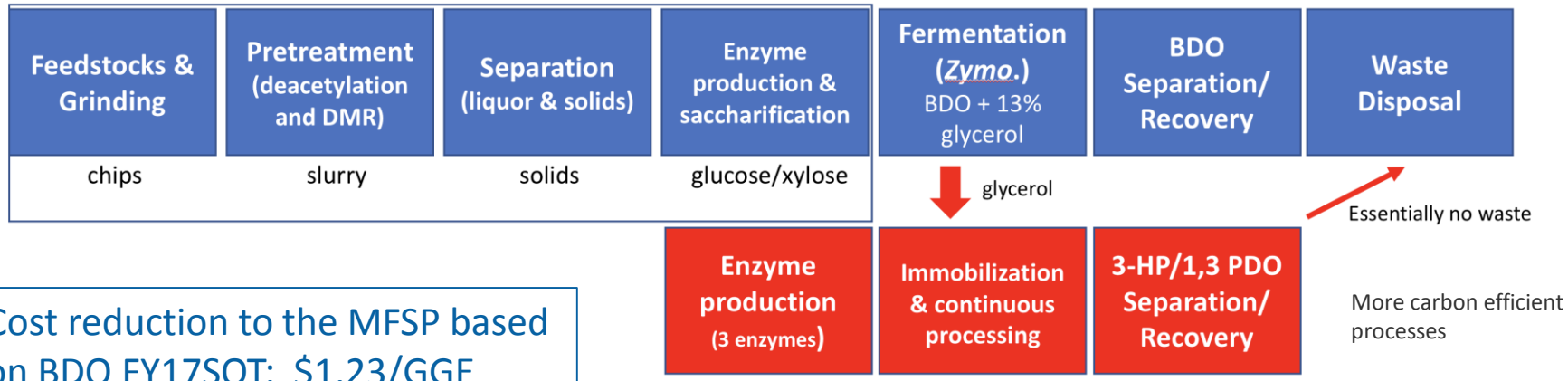
Our approach taken as a whole, will go **beyond conventional cell free technologies** and will judiciously combine, on a case by case basis, **enzyme tethering, enzyme immobilization and entrapment**, enzyme engineering, cofactor recycling strategies, and pathway redesign strategies.

- Our main target will be to **produce 2,3 BDO from hydrolysates using a cell free approach**. (Q4 FY21).
- Determine the feasibility of using **cell free approaches for the conversion of fermentation byproducts** such as glycerol (Q1 FY19 milestone).
- Work with our **TEA team** to assess the promise of **CFIT as a viable alternative to fermentative processes** for selected biochemicals and polymer precursors. (Q1 FY19 milestone).
- Develop broadly enabling cell free tools such as cofactor recycling systems to address one of the main challenges associated with cell free approaches.
- Closely work with other projects to develop a comprehensive strategy based on cell free technologies to **efficiently produce other biochemicals from C5/C6 sugars, lignin, or byproduct streams**.

2—Approach (Technical)

Conversion of byproducts to biochemicals using cell free approaches

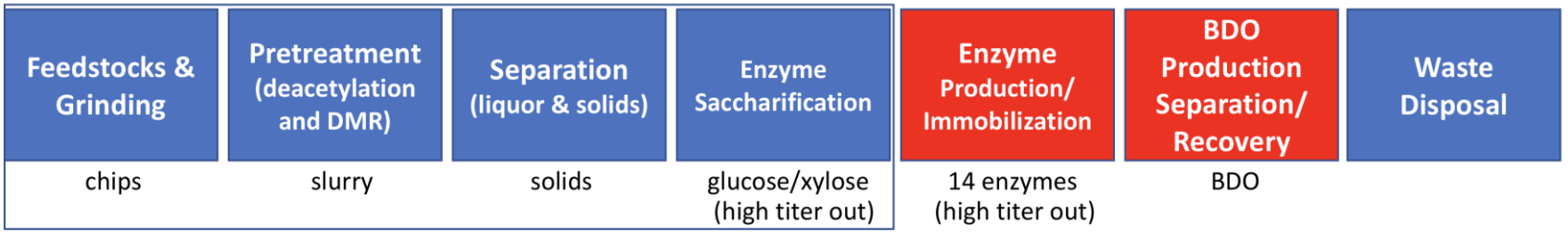
Cell Free/Immobilization (BDO + 3-HP/1,3 PDO)



Cost reduction to the MFSP based on BDO FY17SOT: $1.23/GGE

Direct conversion of sugars to biochemicals using cell free approaches

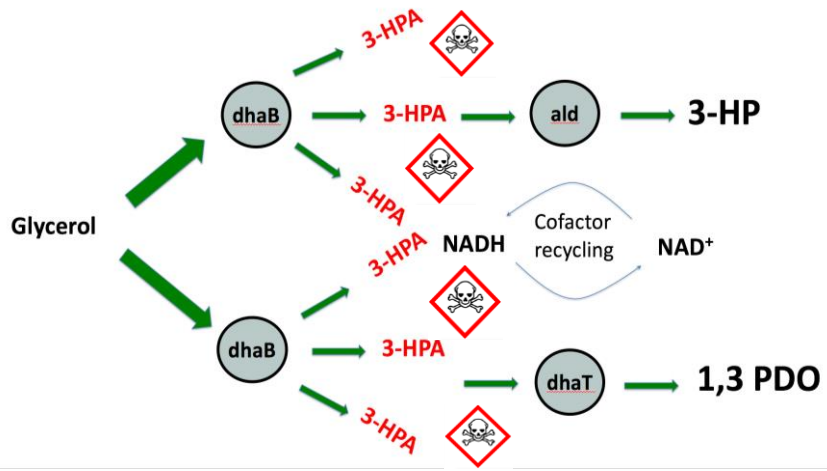
Cell Free/Immobilization (BDO)



Potential cost reduction: $2/GGE

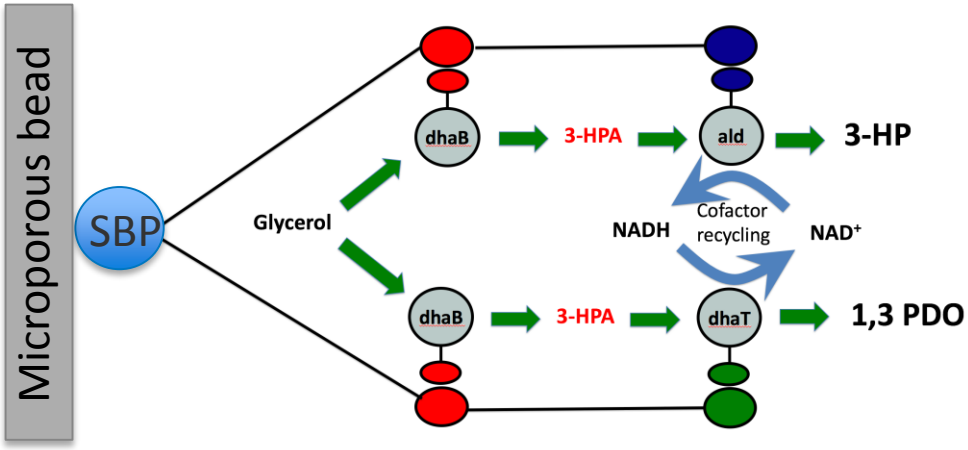
2—Approach (Technical)

Traditional Cell Free Approach: Free Metabolic Pathways



- Loss of metabolites due to free diffusion
- Lack of enzyme stability/recycling
- Accumulation of toxic intermediates
- Inefficient cofactor recycling
- Need for purification

New Cell Free Approach: Tethered Metabolic Pathways



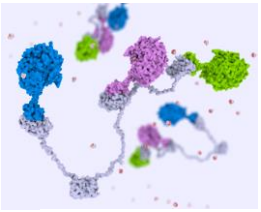
- Channeling of intermediate between enzymes
- Increased stability
- Better cofactor recycling
- Cheaper purification
- Easier product separation

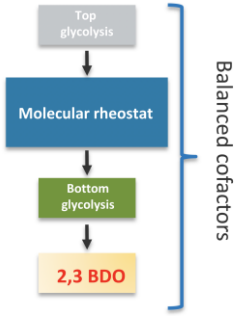
2—Approach (Technical)

FY19 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 problematic enzymes.
- Utilize techno economic analysis (TEA) to provide the sensitivities of the process to enzyme loading, activity, pH, reactor volumes, cofactor recycling and target the most relevant biochemicals.

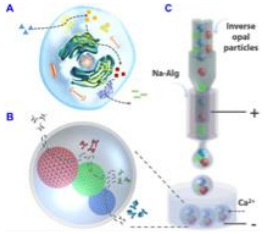




FY19 Task 2. Immobilization and electrochemistry for Cell Free Technologies:

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 using mediators for electron transfer.



2—Approach (Technical)

Main Challenges

- Expression issues with a subset of these enzymes: Identify replacement enzymes from several natural sources with differing phylogenetic origin.
- Lack of efficiency of cofactor recycling or loss of cofactors: Initiate immobilization of cofactors, accelerate development of electron delivery via enzyme collocated on porous conductive surfaces, and rebalance pathways with the use of molecular rheostats to limit cofactor use.
- Lack of long term enzyme stability: Increase enzyme engineering efforts accordingly and leverage the know-how at NREL in protein engineering to conduct this task (EEO, BPMS).

Success factors

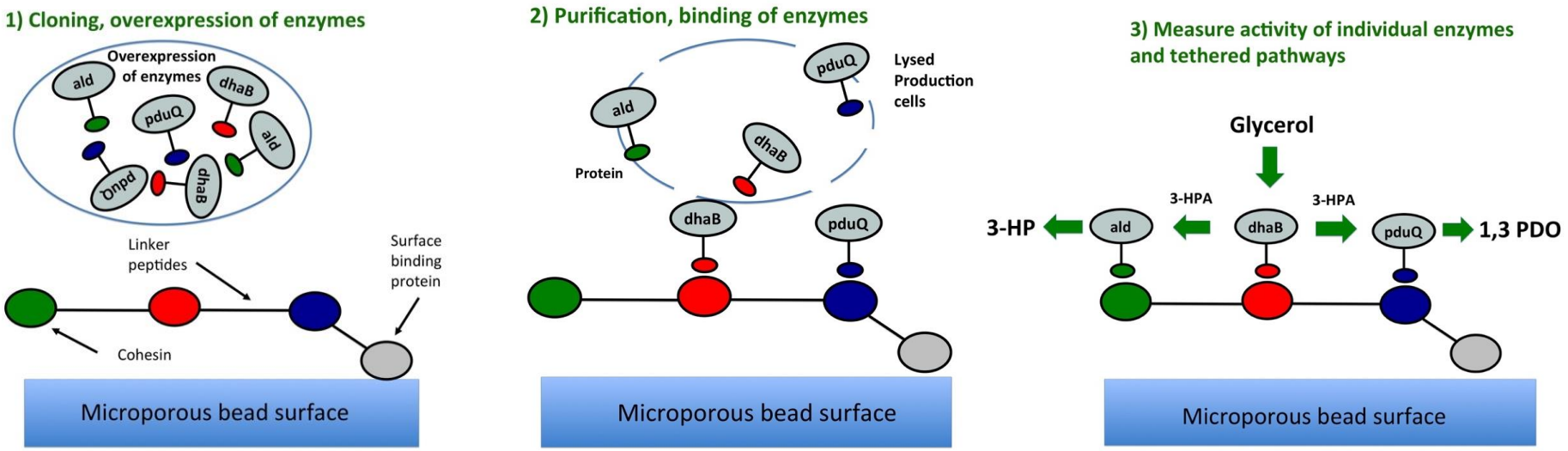
- Long term enzyme stability: **offsets the cost of enzyme production**, guarantees the smooth operation and the **reliability of the process**.
- Efficient cofactor management: **Lower the cost of the overall process by limiting/eliminating the need for cofactor supplementation.**

Collaborators with diverse expertise from academia, industry, and other national laboratories to **increase our chances for success and reduce risks**:

Sonja Salmon, Associate Professor, **NCSU** (Enzyme encapsulation), **Yongqin Jiao**, Group Leader, **LLNL** (Advanced materials for encapsulation), **Jim Bowie**, Professor, **UCLA** (cell free production of biochemicals and protein folding), **Alex Berlin**, CTO, **Arbiom**, (Generation of new materials from 2G sugars producing plant), **Zachary Sun**, CEO, **Synvitrobio**, (Cell free protein synthesis), **CUBI,** NREL,ORNL, LANL,PNNL (catalytic upgrading of sugars/related Intermediates into hydrocarbon fuels and co-products)

3—Progress – Validation of our approach

We first needed to assess the viability of our approach for different metabolic enzymes including some with problematic properties

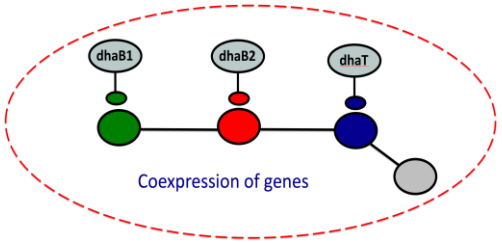


- Total of 26 enzymes cloned and expressed in 4 protein classes: **70% success rate from cloning to activity,** one enzyme (dhaB) is problematic and is not soluble with or without a binding platform without the scaffold present.

- **We have confirmed that all binding platforms are functional.**

3— Progress – One step CFT is possible

- **Cell free pathway assembly achieves higher productivity.**
- **Requires no purification.**
- **One step purification that allows co-purification of poorly soluble proteins.**
- Can be conducted in compact bead beds with a much more limited footprints.

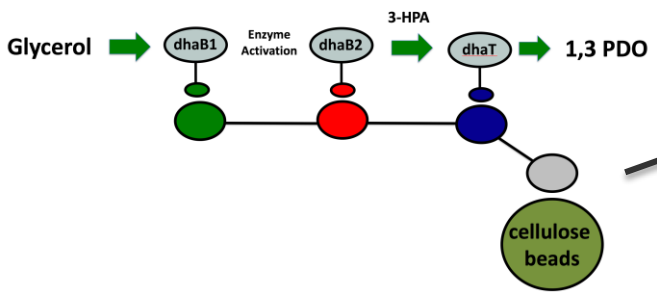


E. coli cell

Cellulose beads
Cell lysis
Rinse

50mM Glycerol

21 mM 1,3 PDO
3 days *in vivo*



14.2 mM 1,3 PDO
12 hours *in vitro*
and similar for 3-HP

1,3PDO

4—Relevance

Decreasing conversion cost using new technologies

Overarching Goals

Develop Cell Free/Immobilization Technology (CFIT) based processes to reduce production cost of biochemicals. Reduce risks of production scale ups.

Our approach can help address several conversion barriers by:

- increasing titers, yields, and productivity of toxic products and polymer precursors 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.

Relevance to BETO and the Biorefinery Industry

BETO's 2022 and 2030 goals rely on the cost effective production of value added products to achieve a MFSP of $3 and $2.5/gge.

5—Future Work – Key milestones

Key milestones

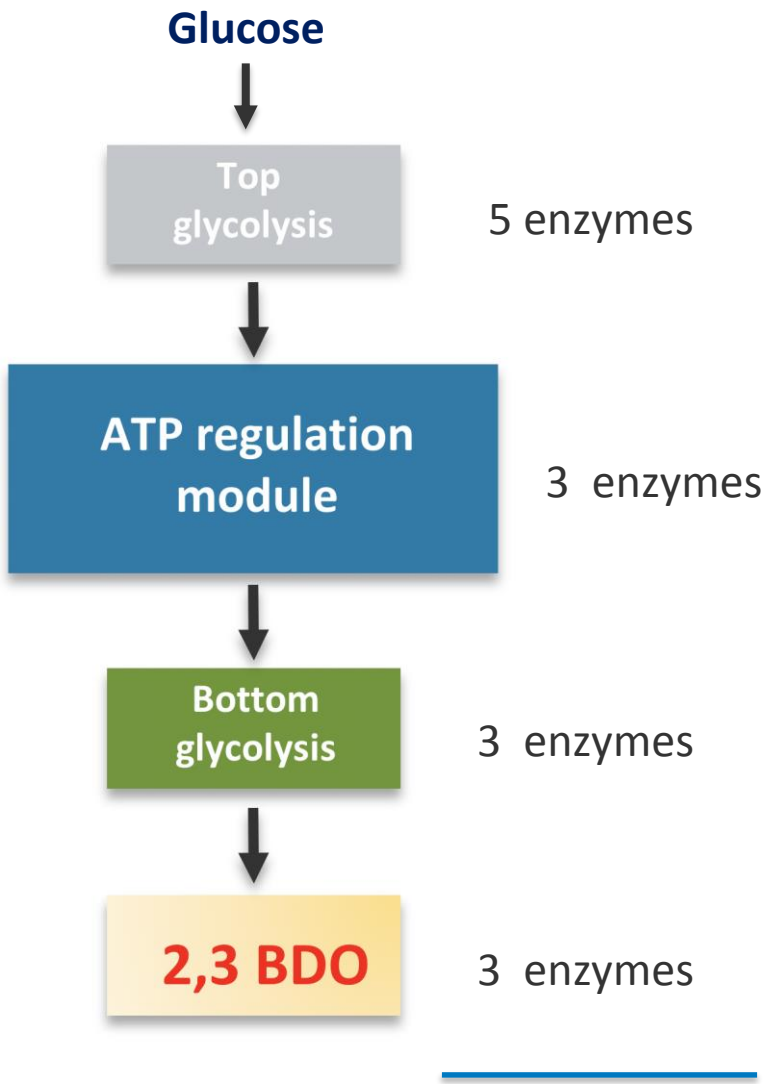
- Provide a full TEA analysis of a complete CFT process from a C5/C6 sugar streams to 2,3 BDO. (**3/31/2019)**
- Produce **2,3 BDO at 10 g/L** with a **productivity of at least 0.5g/L/h from pyruvate** using cell free metabolic pathways immobilized on conductive polymers. Compare the yields with microbial routes. (**9/30/2019)**
- Produce **2,3 BDO at 20 g/L** with a **productivity of at least 0.75g/L/h from a mock C5/C6 hydrolysates** using immobilized cell free metabolic pathways. Contrast cofactor recycling using conductive polymers or biomimetic cofactors with engineered enzymes. Compare the yields with microbial routes. (**9/30/2020)**
- 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 appropriate cofactor recycling as needed. Compare the yields with microbial routes. (**9/30/2021)**

Go/NoGo (3/31/2020)

- Produce 2,3 BDO from hydrolysate using immobilized cell free metabolic pathways 10 g/L from hydrolysates.
- A GO decision indicates continuation as planned.
- A NOGO decision can take different directions, pursuant to discussion with DOE. One direction could be **to rewire the pathway using different metabolic enzymes** or select another **more promising route or abandon the 2,3 BDO target**.

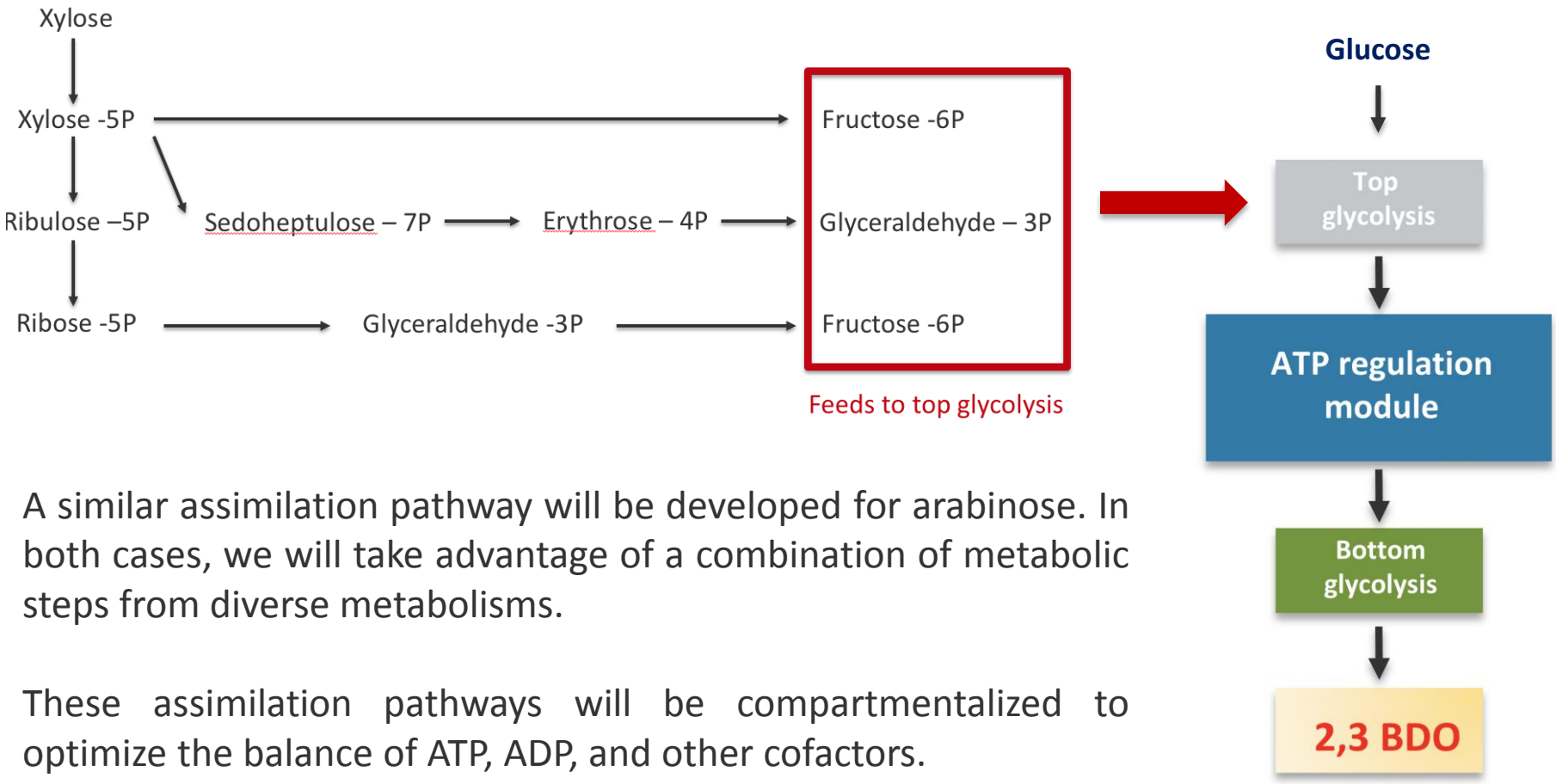
5— Future Work– Glucose to 2,3 BDO can be achieved

- We have confirmed that all enzymes in this pathway express and are active in their native forms.
- We will finish the construction of tethered metabolic pathways to convert C5/C6 hydrolysates to 2,3 BDO and determine the optimal compartmentalization of the different metabolic steps.
- Determine optimal combinations of enzymes to construct the most efficient cell free pathway (from natural diversity).
- Use ATP and other regulation modules to control the level of cofactors.
- Incorporation of xylose and arabinose is needed to achieve our end project goal.



5—Future Work - Incorporation of xylose and arabinose

Develop xylose assimilation pathways –We will first rely on the pentose phosphate pathways to generate Fructose -6P and Glyceraldehyde – 3P that can feed back directly into top glycolysis.

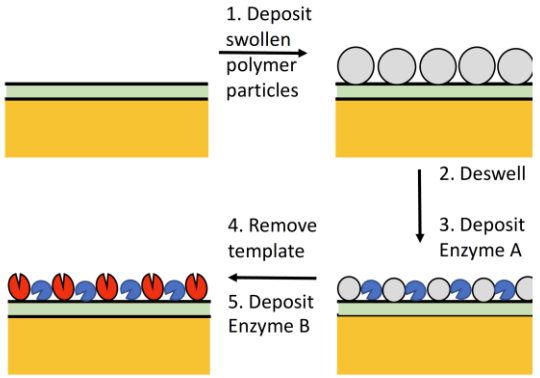


A similar assimilation pathway will be developed for arabinose. In both cases, we will take advantage of a combination of metabolic steps from diverse metabolisms.

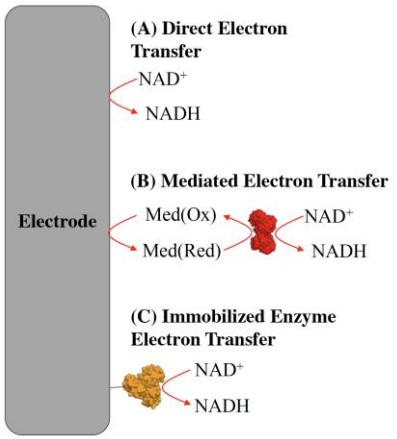
These assimilation pathways will be compartmentalized to optimize the balance of ATP, ADP, and other cofactors.

5—Future Work – TEA, Immobilization/cofactor recycling

- Determine the advantages of encapsulation of individual enzymes or metabolic cascades – Encapsulation can be pursued in complement or instead of tethering for some enzymatic steps when most beneficial. We will report on the increase in enzyme longevity for different encapsulation approaches.



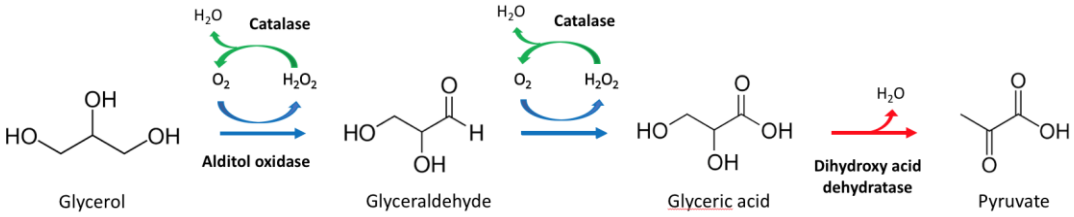
- Develop new immobilization strategies specifically designed for cofactor recycling in cell free applications - take advantage of materials providing high accessible surface areas (such as self-assembled monolayers of conductive polymers) and the ability to transfer available renewable electrons for low cost and efficient cofactor recycling.



- Work towards a full TEA analysis of a complete cell free production of 2,3 BDO (and other targets) from C5/C6 hydrolysates - provide the sensitivities of the process to enzyme loading, activity, pH, reactor volumes, cofactor recycling, encapsulation materials. These analyses will guide our efforts and help us refocus our research to have the most impact for meeting BETO's goals.

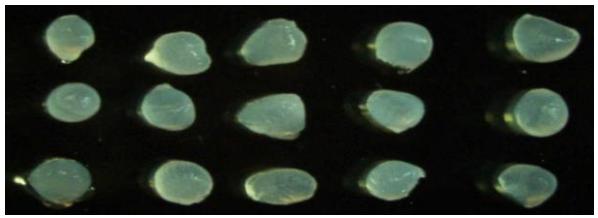
5— Future Work – CFIT allows the use of non natural pathways

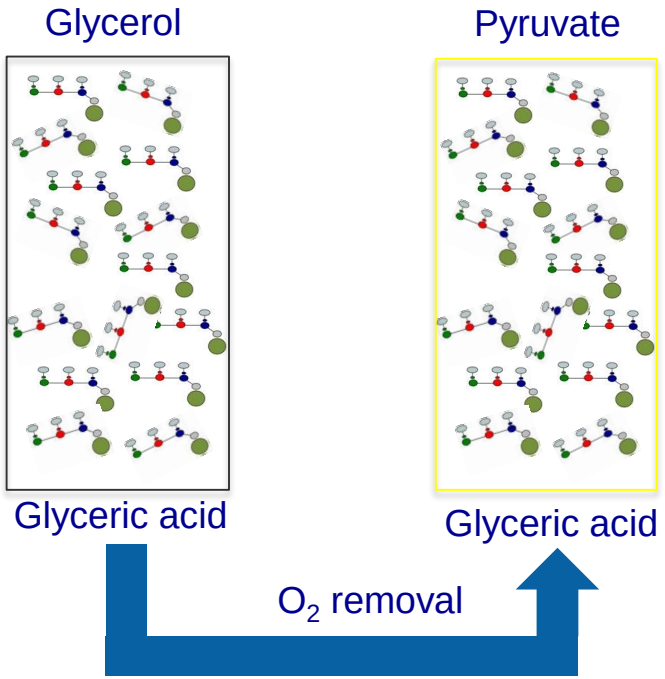
- Cell free can benefit from non natural pathways that **would not be amenable to microbial biocatalysts**. Some of these could be designed to **limit the need for cofactors**.
- Due to the much smaller size of the fermentation vessels, **oxygen can be provided to the system without being cost prohibitive**.



We have confirmed that this pathway is promising, **we are now generating the fusion enzymes needed to increase efficiency and stability.**

- Within this pathway, DHAD, shows oxygen sensitivity, which can be addressed by compartmentalizing the pathway.
- **This enzyme will be encapsulated in alginate beads including oxygen scavengers.**





6—Summary

Overview

The main goal is to **enable the production 2,3 BDO and other biochemicals at high titers and productivity relevant for industrial applications** from process relevant hydrolysates using cell free based approaches.

Approach

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

Technical Accomplishments/Progress/Results

Reported on a new cell free approach based on enzyme tethering to protein scaffolds.
Glycerol could be upgraded to 1,3 PDO and 3HP or pyruvate using our cell free approach.
Converting glucose to 2,3 BDO using a long metabolic pathway is possible but optimization is required.

Relevance

Our approach **aims to address several conversion barriers by increasing titers, yields, productivity of toxic products and polymer precursors due to higher toxicity threshold and more carbon efficient conversion processes.** There is industrial interest in these approaches (letters of interests and NDAs are forthcoming with industrial partners).

Future work

Finish the construction of tethered metabolic pathways to convert C5/C6 hydrolysates to 2,3 BDO. Develop new immobilization strategies specifically designed for cofactor recycling in cell free applications. **Enzyme selection will be key to achieving our goals but it is also anticipated that several enzymes will have to be engineered for stability.**

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  - NREL LPM and Platform Lead: Zia Abdullah, Rick Elander

NREL Project Members

(Most between 50 and 100% effort)

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Min Zhang

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Jim Bowie – UCLA
Ed Bayer - Weizmann Institute
Zachary Sun - Synvitrobio, Inc
Alex Berlin - Arbiom, Inc
Sonja Salmon - NCSU

Thank You

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