DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

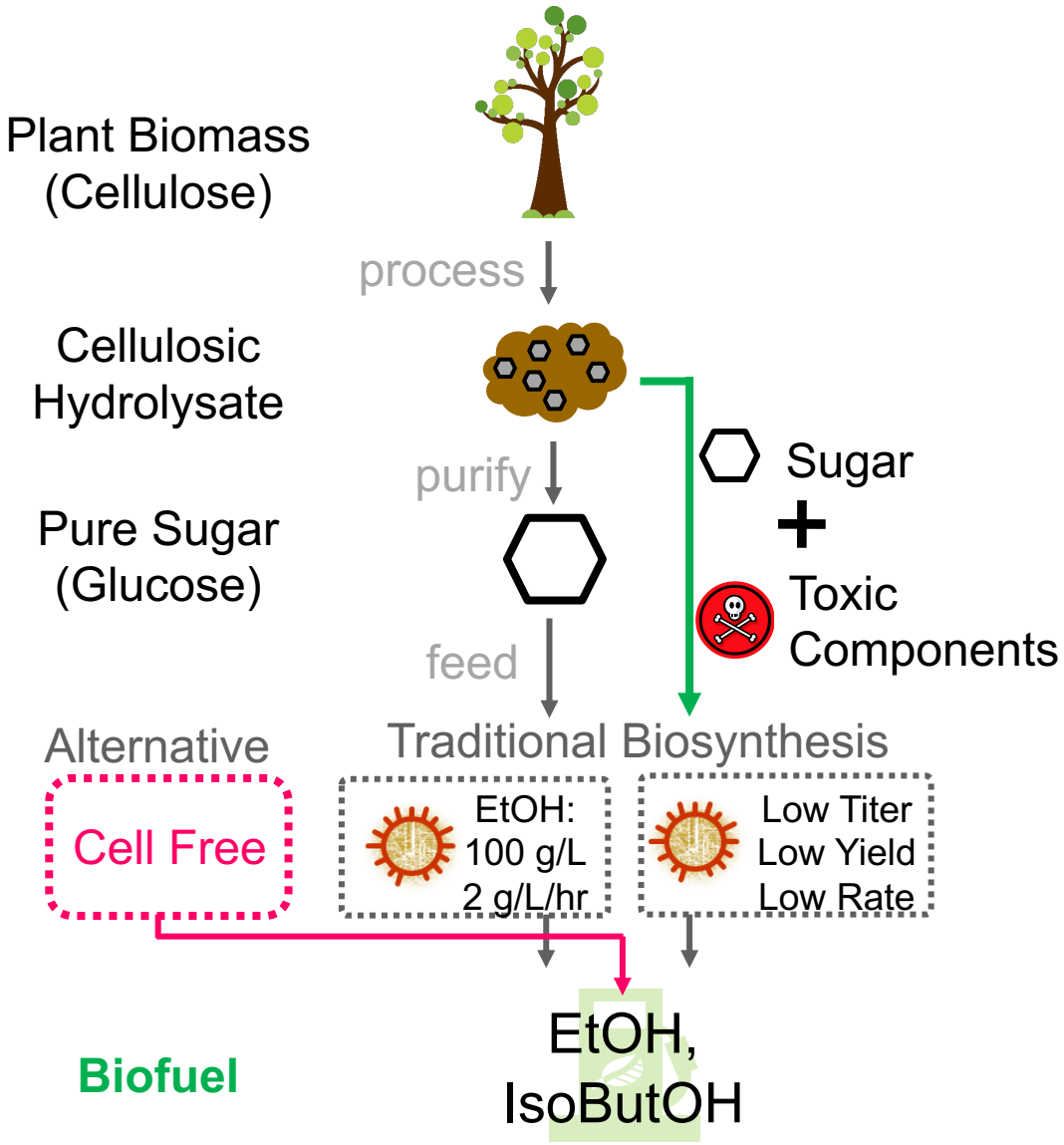
Towards Economical Cell-free Isobutanol Production
&
Cell-Free Biochemical Production of Terpenoid Chemical Astaxanthin using Crude Cofactor Lysates

Date: April 5th, 2023
Technology Area Session: Biochemical Conversion and Lignin Utilization

Principal Investigator: Paul Opgenorth, PhD. & Tyler Korman, PhD
Organization: Invizyne Technologies, Inc.

Project Overview

Biocommodity and Biofuel Production from Sugar



- Biomass derived sugars can be used by engineered microbes to make fuels and chemicals
  - Works well for pure glucose which is more costly and less flexible
  - Cellulosic sugars can come from any biomass but toxic components limit use by microbes

Alternative methods should be explored to improve cellulosic utilization and conversion

- Using only enzymes (cell-free) instead of the whole microbes may improve conversion of cellulosic sugars into useful chemicals
  - Benefits include higher titer, yields, and productivity compared to cells
  - Cell-free is an emerging technology and must be validated at scale and with cellulosic feedstocks

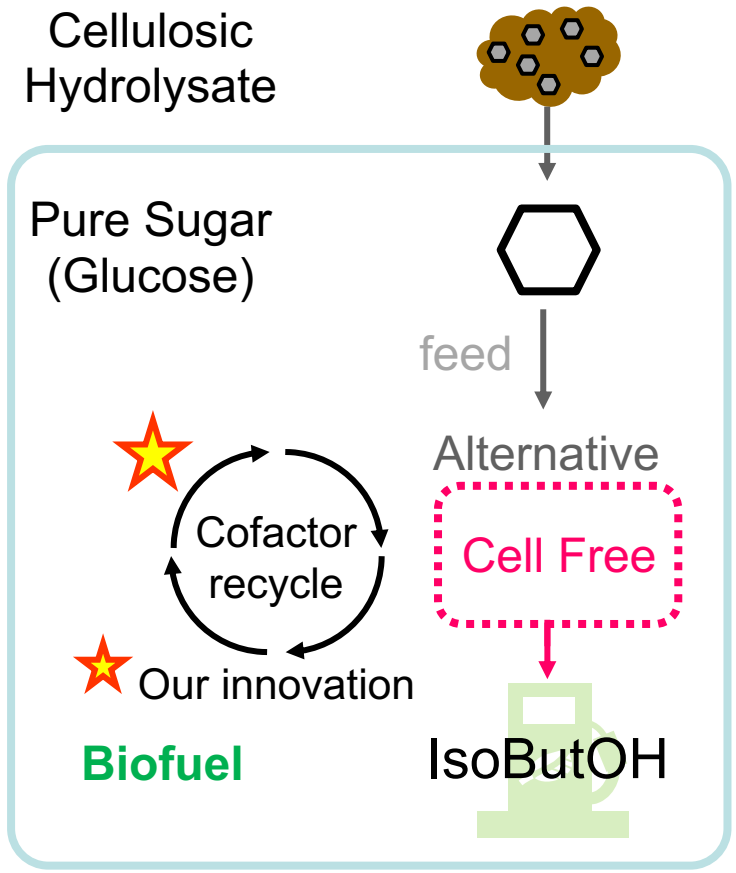
End of Project Milestone: 500 g/L isobutanol

- **20x higher than cell-based methods**

In 2018 BETO recognized that cell-free biological systems show promise to advance the bioeconomy warranting further investment to de-risk this important technology

Project Overview

Biocommodity and Biofuel Production from Sugar



Past work – Baseline system
(already >10x higher titer than cell-based systems)

Because we start at higher titers than cells, even a 25-50% increase in titer would be significant!

- We want to build on our previous high impact work:

Highlights of Past Work

1. New ATP and Redox balancing systems that enable cell-free catalysis over many days to reach high titers
2. Incorporation of stable enzymes enables extremely high titers (*already* 10x cell-based)
3. Ability to quickly make numerous products from low-cost inputs (e.g. glucose) at titers 10 to 100x higher than any reported in living cells

Overall Goals of Current Work

1. Increase overall titers to 500 g/L
2. Convert all sugars from cellulosic feedstock
3. Decrease cost by using less/more stable/more active protein
4. ***Decrease cost by using cheaper cofactors***

Effect of Cofactor Choice on Isobutanol Cost			
TEA assumption: 300,000 L @ 4g/L/hr for 4 days with 10 g/L enzyme			
Cofactor	**$/kg**	**Isobutanol ($/kg)**	**% of total cost**
NADP	$9000	$22	85%
NAD	$200	**$3.57**	**13 %**

Quad Chart Overview Project #1

Timeline

- 10/01/2019
- 03/30/23

	FY20 Costed	Total Award
DOE Funding	(10/01/2019 – 9/30/2020) $302,278	$2,078,605
Project Cost Share	$91,756	$563,204

Project Goal

The goal of this project is to develop a novel route to advanced biofuels from cellulosic sugars by developing cell-free enzymatic routes that have the potential to be cost effective processes.

End of Project Milestones

12b. Produce isobutanol from pure glucose at 10 g/L/h productivity reaching a titer of 500 g/L by 5 days at >90% yield at a 150 mL scale.

13b. System that produces isobutanol from glucose-rich cellulosic hydrolysate at 2 g/L/h productivity reaching a titer of 40 g/L by 5 days at >90% yield at a 150 mL scale.

Funding Mechanism

DE-FOA-0002029, AOI 7a: Advanced Bioprocessing, 2019

TRL at Project Start: 2
TRL at Project End: 4

Project Partners*

- UCLA (Dr. James U. Bowie)

*Only fill out if applicable.

Quad Chart Overview Project #2

Timeline

- 08/01/22
- 07/30/24

	FY22 Costed	Total Award
DOE Funding	(08/01/22 – 01/31/23) $173,467.63	$1,073,125
Project Cost Share	NA	NA

Project Goal

The goal of this project is to develop a novel route to enrich cell lysates for cofactors, namely ATP. Additionally, we will demonstrate that these lysates can support cell free enzymatic pathways for the production of long chain terpenes

End of Project Milestones

12b. Establish a method for producing 5g/L of C40 terpene with cofactors from a microbial lysate

13b. Scale this system to 10L with the help of ABPDU.

Funding Mechanism

DE-FOA-002572, Topic 8a: BIOENERGY, 2022

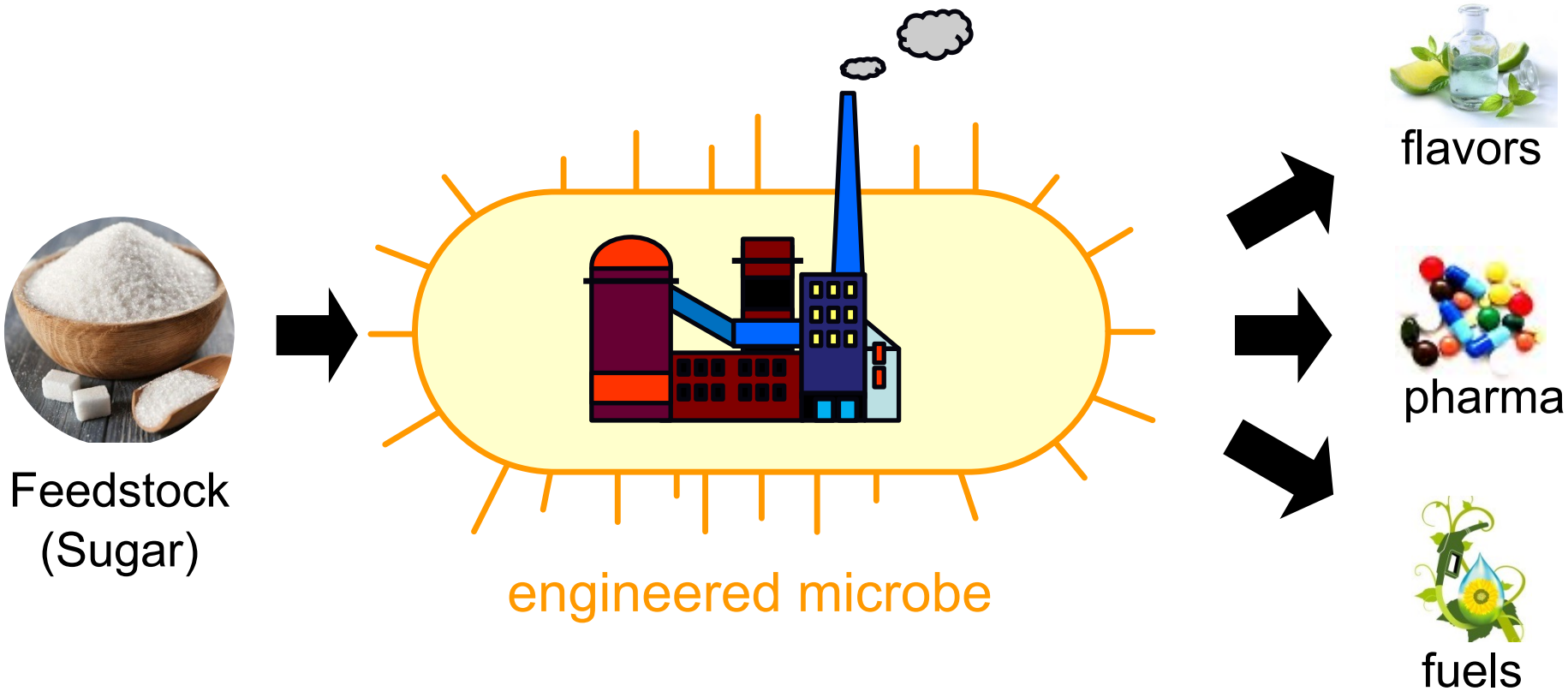
TRL at Project Start: 1
TRL at Project End: 3

Project Partners*

ABPDU (James Gardner)

*Only fill out if applicable.

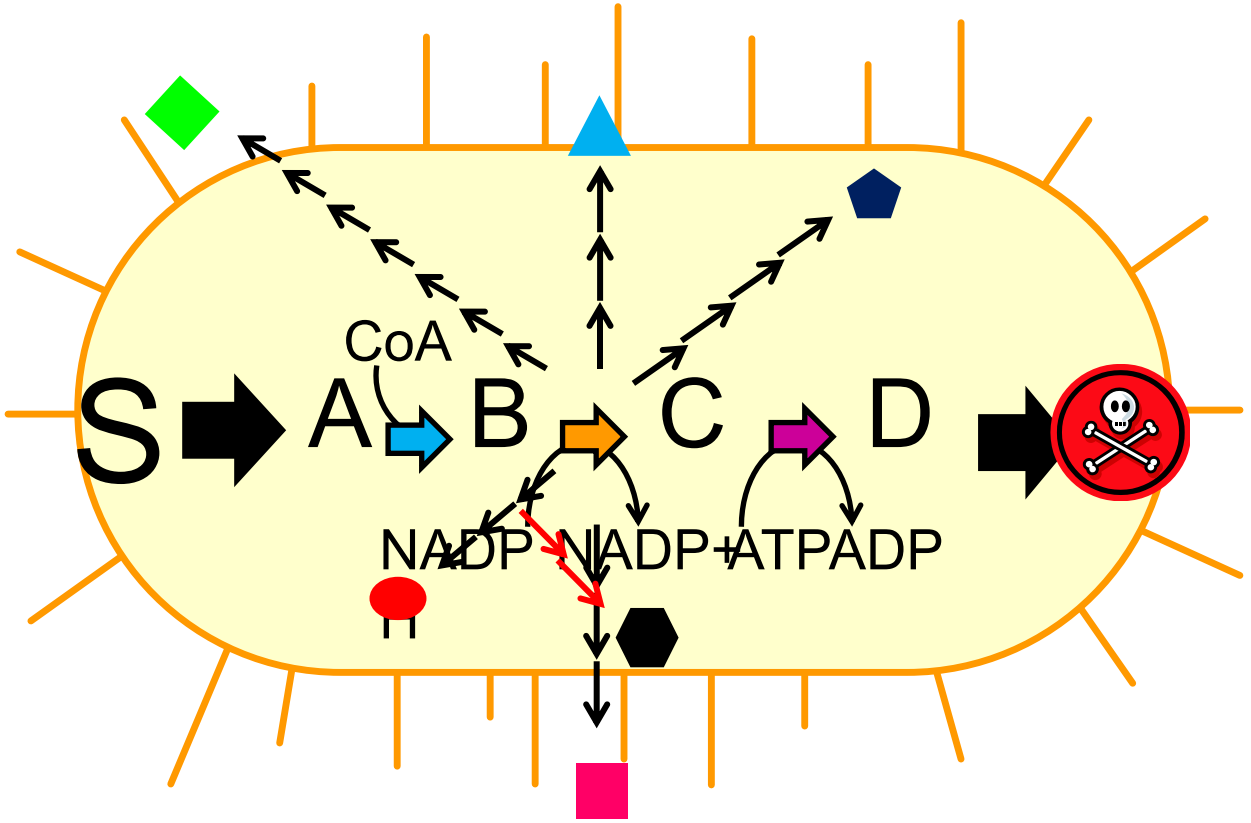
1st Generation Synthetic Biology: Cells as a Factory



Challenges with Living Cells*

The Problem with Cells

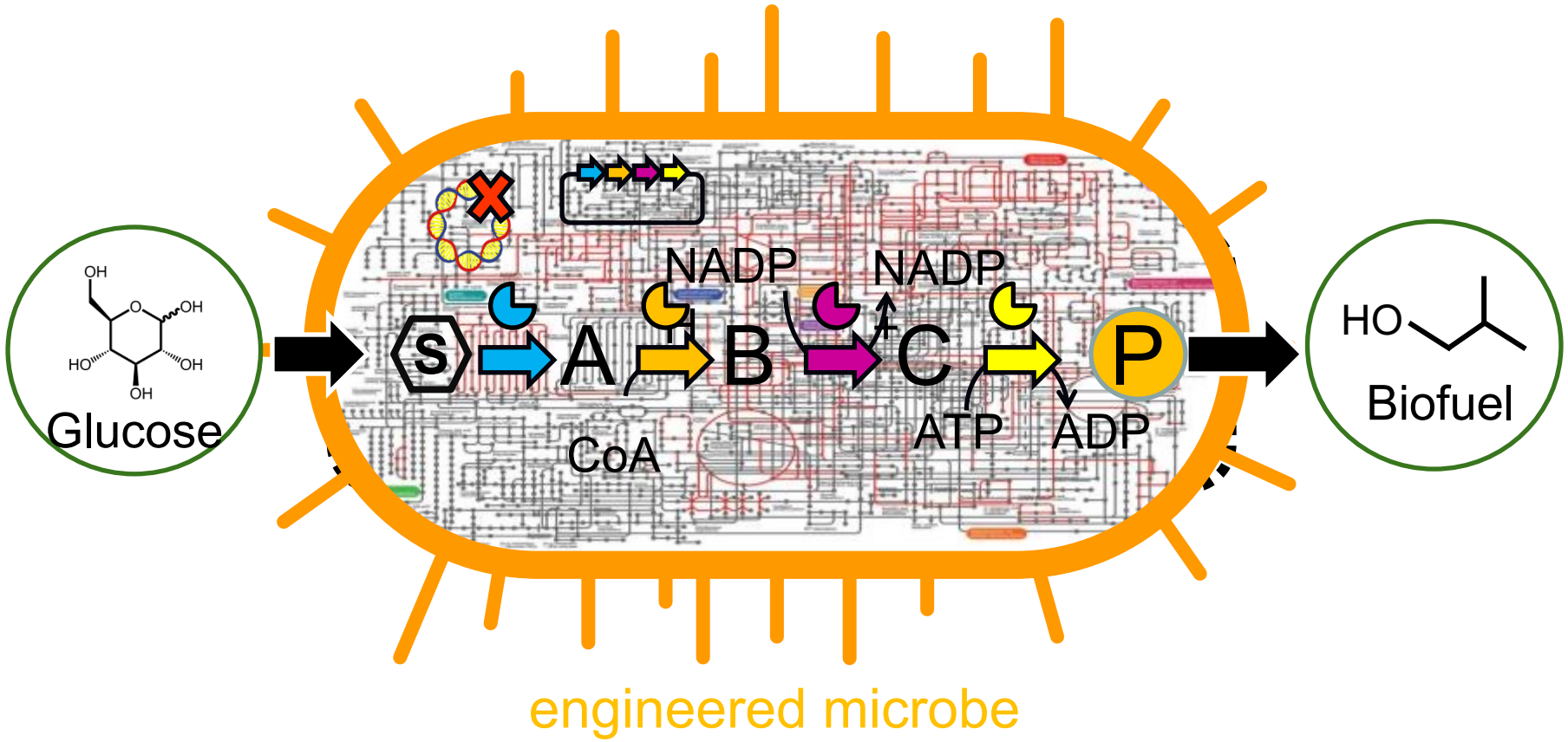
- Background metabolism lowers yield and titers
- Toxic products and Intermediates
- Unwanted side-effects



*

Unknown/unwanted outcomes and long DBT cycles makes cell engineering difficult, slow, and costly

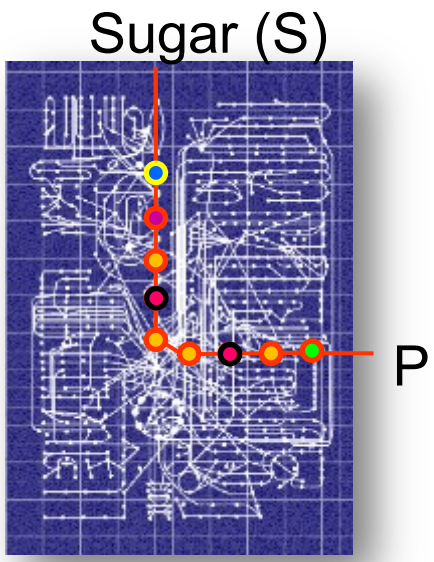
Invizyne's Approach: Simplify Biocatalysis



The Solution:

Get rid of the cells.

Invizyne's *in vitro* strategy



Only add elements necessary for biotransformation and regulation

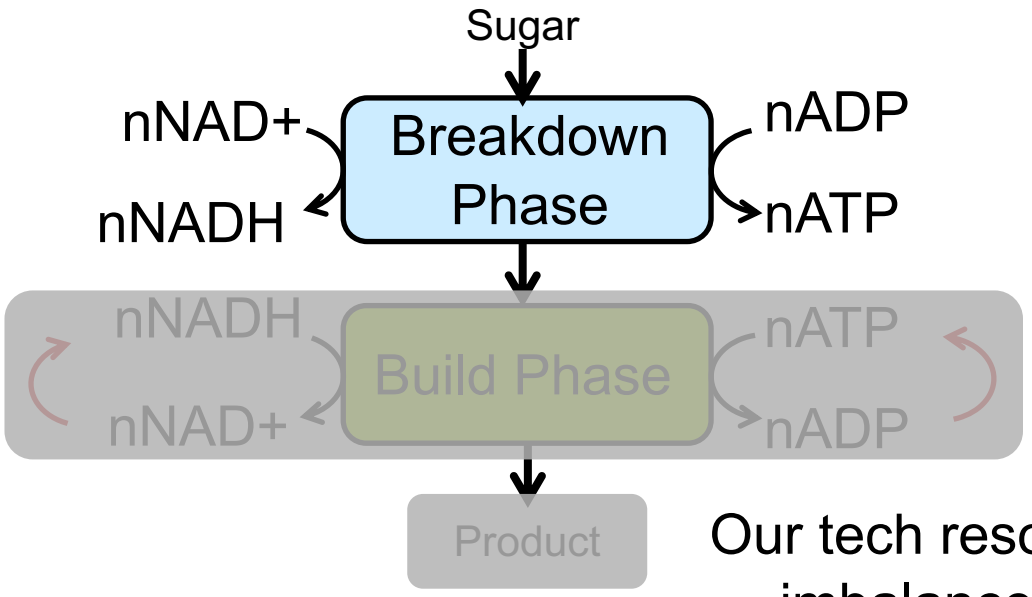
Don't need to worry about background metabolism

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

Synthetic Biochemistry

Main Advantages

- High yields
- Easy optimization/Total Control
- Rapid Design-Build-Test Cycles
- Great flexibility in pathway design
- No toxicity headaches
- Easier product purification
- Potential for much higher productivity



Our tech resolves imbalances!

Main Challenges

- Enzyme Cost
- Enzyme Stability
- Cofactor cost
- Cofactor Recycling and Maintenance

Project Specific

- Ease of Scaling
  - Is it linear like a chemical reaction?
- Cellulosic Sugar Effects
  - Are compound present inhibitory to enzymes?
- Cofactor Use and Cost
  - Can cheaper cofactors be produced and used?

Effect of Cofactor Choice on Isobutanol Cost			
TEA assumption: 300,000 L @ 4g/L/hr for 4 days with 10 g/L enzyme			
Cofactor	$/kg	Isobutanol ($/kg)	% of total cost
NADP	$9000	$22	85%
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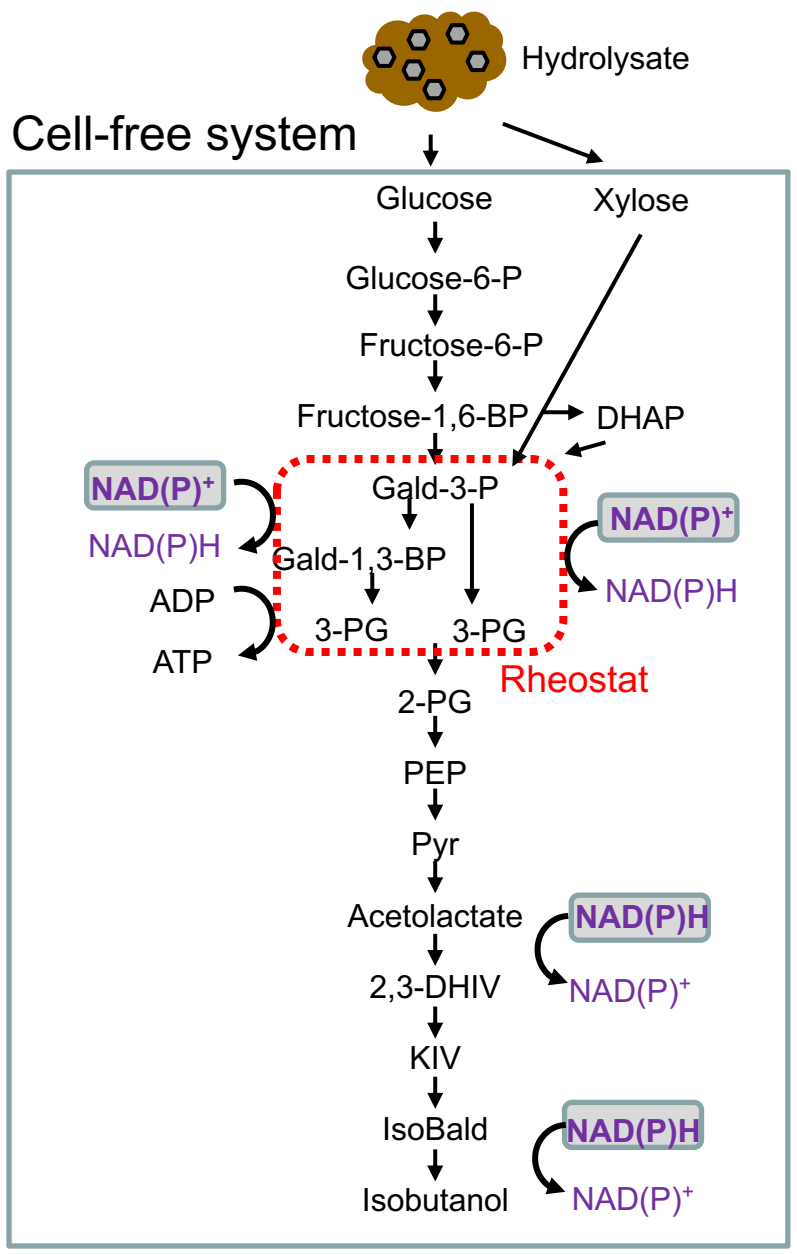
2 – Approach-EERE



Isobutanol from Cellulosic Hydrolysate

Approach 1: Pure Glucose

- Optimize rates and loads
- Demonstrate high productivity
- Demonstrate high titer
- Use lessons learned with pure glucose to enable high titer production with cellulosic

Approach 2: Cellulosic

- Optimize conversion
- Increase titer
- Increase productivity
- Determine factors that contribute to differences compared to pure glucose

Go/No Go Criteria 2: Reach meaningful metrics for isobutanol production using cellulosic hydrolysate with a scaled system

- Establishes validity of process and demonstrates impact

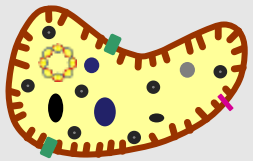
Final Metric: Scaled system(s) for production isobutanol from:

1. Pure glucose at high rate (10 g/L/h) and titer (>500 g/L)
2. Cellulosic hydrolysate at 2 g/L/h, >40 g/L, and >75% yield

Technical metrics measured by productivity (g/L/hr and titer (g/L)

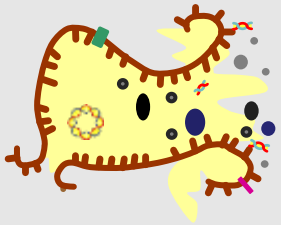
3 – Impact

Increasing Simplicity and Flexibility

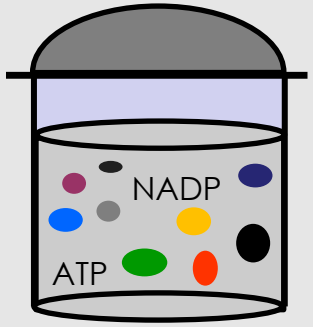


Cell-based (*in vivo*)

Standard in the field
Often costly and difficult
Limited substrate/product



Cell-lysate



Cell-free (*in vitro*)

Flexible
Direct Prototype to Product
Toxic Product OK
Simple Purification
Expanded Chemical Space

Potential for ***high impact*** by allowing bioconversions to perform more like chemical reactions and lower cost of production

Demonstrated High Impact

- Publication in high impact journals
  - Nature Communications
  - Nature Chemical Biology
- Interest from large industrial companies (chemicals, proteins, etc)
  - Talks ongoing

4 – Progress and Outcomes: Cell-free Isobutanol Production

Task Breakdown

Invizyne
Cellulosic Hydrolysate Tasks (9,12,13)
Enzyme Rate/Cofactor Utilization Tasks (4)
Protein Production Tasks (10)

UCLA
Pure Glucose Tasks (8,11)
Enzyme Rate/Stability Tasks (4)
Protein Production Tasks (10)

FY19-20	Task	Description	Planned	Actual
Go/No-Go 2	**8**	Scaled Pure Glucose to Isobutanol System	100%	100%
Go/No-Go 2	**9**	Improved and Scaled Cellulosic Isobutanol System	100%	100%
	4	Improve Rate of Slowest Enzymes	100%	100%
	10	Enzyme Production for Scale	100%	100%
	11	Optimize Parameters to Reach Metrics for Final System	100%	90%
Final	12	Scaled Improved Pure Glucose to Isobutanol System	100%	90%
Final	13	Scaled Improved Cellulosic Isobutanol System	100%	100%

All Tasks on Track to Reach or Exceed Important Milestones on Schedule

4 – Progress and Outcomes: Cell-free Isobutanol Production

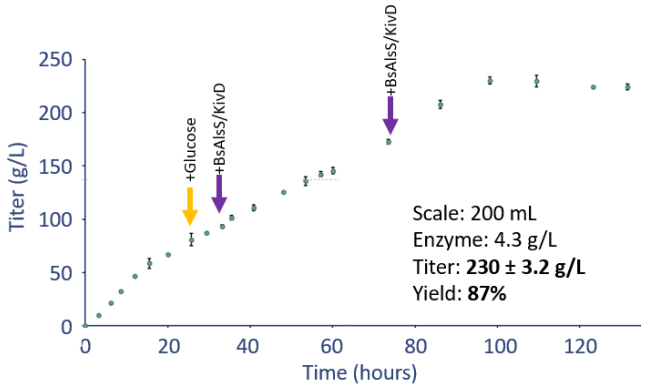
- **Task 8: Scale pure glucose to isobutanol system**
  - Completed. Reached production metrics 4 g/L/h max rate, titer >250 g/L, >95% yield, 200 mL scale
- **Task 9: Scaled system for cellulosic glucose to isobutanol**
  - Exceeded metrics. >2 g/L/h max rate, >60 g/L titer, >95% yield (all sugars, C5+C6). Scaling ongoing.
- **Task 4: Improve rate of specific enzymes**
  - More active variants found and implemented for multiple steps in the pathway
  - A 2x faster variant of IlvC identified and implemented for one of the two remaining slow steps
- **Task 10: Produce sufficient enzyme for scaling efforts**
  - Enough enzyme produced an on-hand to complete Tasks 12 and 13 and reach Final Milestones
- **Task 11: Optimize system to produce isobutanol with improved metrics compared to Task 8/9**
  - Metrics improved for rate of production with some issues that currently limit overall titer
- **Task 12: Scaled system for pure glucose to isobutanol**
  - Met some production metrics. Scaling work ongoing
- **Task 13: Scaled system for cellulosic glucose to isobutanol**
  - Exceeded all production metrics. Scaling work ongoing

4 – Progress and Outcomes: Cell-free Isobutanol Production

Task 8

Scalable system

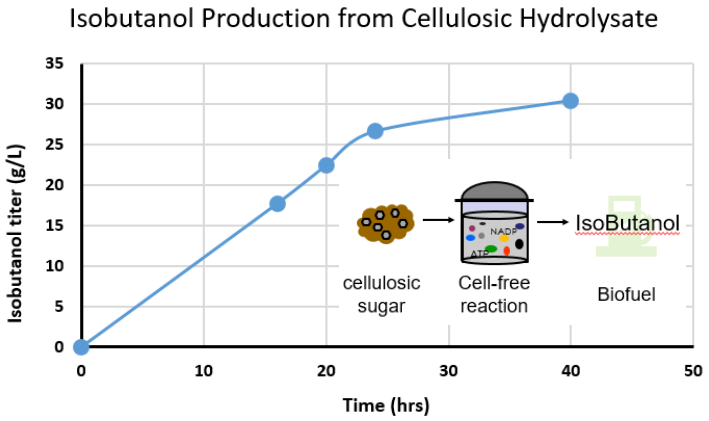
Pure Glucose – 200 mL



Task 9

Optimized Production

Hydrolysate

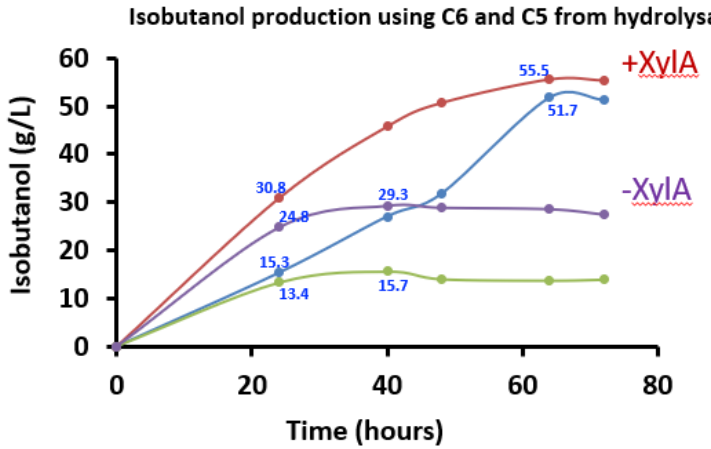


to

Task 13

Increased Titer and Scale

Hydrolysate

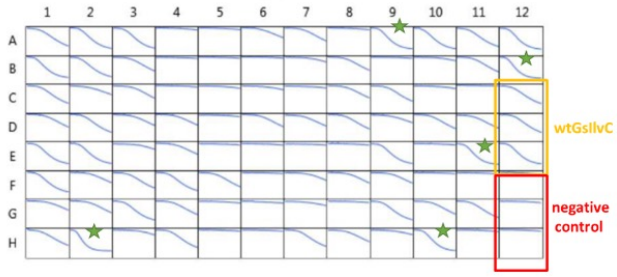


High titer!
C5+C6 Sugars!

In Progress:
Scaled Cellulosic System

Task 4

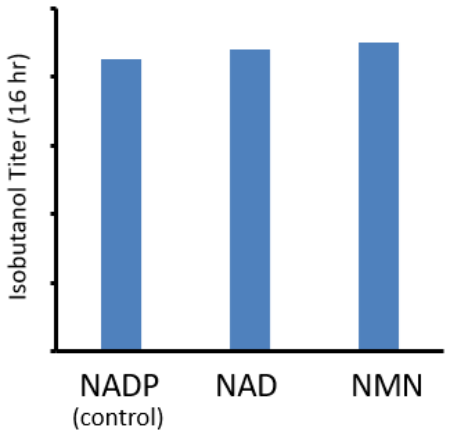
Library Screen - IlvC



IlvC Mutant	ID	%Activity (compared to WT)
WT	WT	1
1	5-A9	1.6
2	5-E5	1.4
3	5-F7	1
5	combined 1	1.5
6	combined 2	2

Increased Activity Lowers Loading and Cost!

Task 11



Cheap Cofactors!

- ✓ Hydrolysate to isobutanol optimized
- ✓ Cofactor and protein loads optimized
- ✓ Cofactor preference of 3 of 4 enzymes
- ✓ Reaction scaling
- ✓ Enzyme engineering
- ✓ Reaching final goals on track

DE-EE0008925

Summary: Cell-free Isobutanol Production

- *Multi-step enzymatic cell-free bio-transformations are real alternatives to microbial conversions*

- *Glucose from a cellulosic hydrolysate can be used as efficiently as pure glucose without toxicity problems but with some dilution issues*

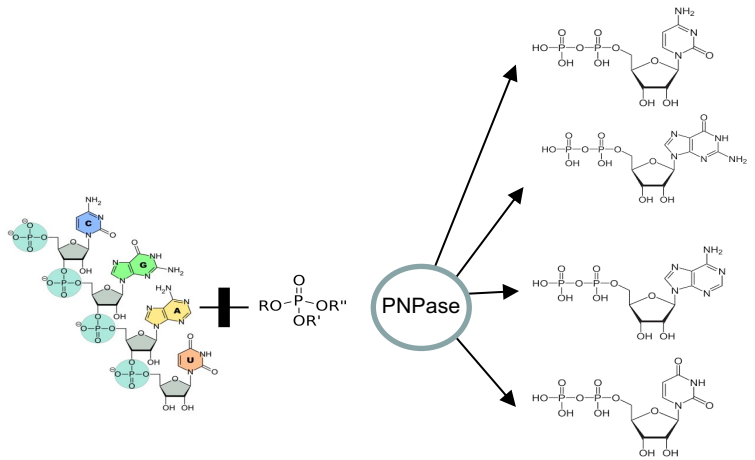
- *Enzyme engineering can be used efficiently to lower costs by enabling the use of cheaper cofactors and lower protein loads*

- *By the end of the Project we established a cell-free system that outperforms any previous microbial system for the conversion of a biofuel from cellulosic feedstock*

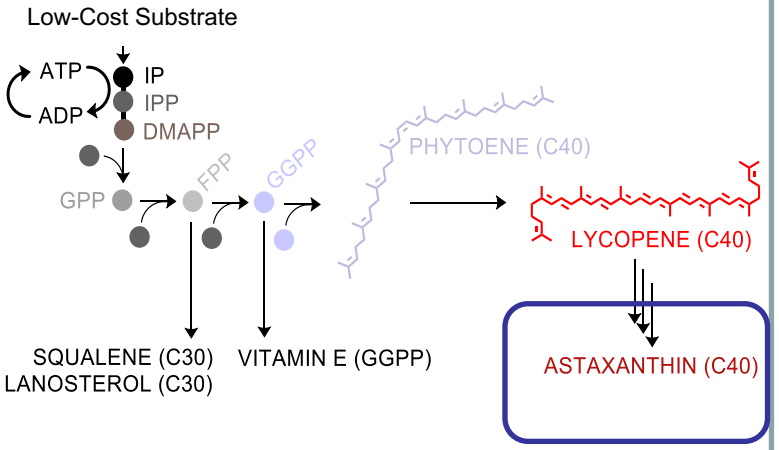
2 – Approach - SBIR

Cell-free system - Figure

Aim 1:Cofactor production



Aim 2: Astaxanthin production



Cofactor Generation and Astaxanthin

Approach 1: Cheaper NTPs

- Cofactors are a significant additional cost associated with cell free biocatalysis.
- Optimize cell lysates for cofactor (ATP) concentration
- Depolymerized RNA is a significant source of NTP cofactors

Approach 2: Astaxanthin

- Long chain terpenes are a large family of compounds that are bioactive
- Astaxanthin is the main cost driver in aquaculture feed
- Optimizing production of long chain terpenes

AIM 1 Criteria: Depolymerization of RNA/DNA

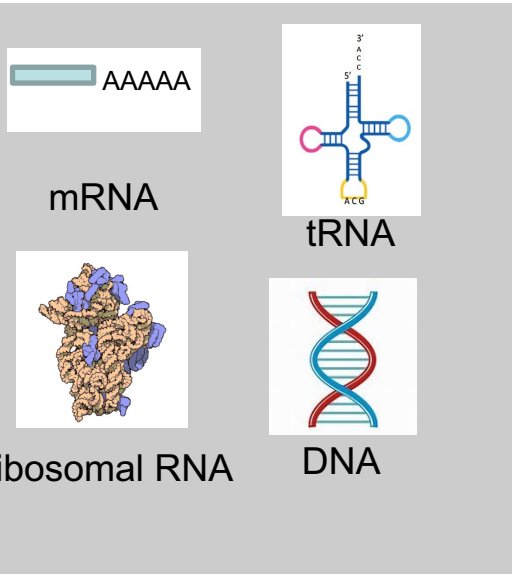
- Increase ATP concentration by depolymerization RNA that can be converted into the nucleotide triphosphate
- Optimize ATP concentration in a lysate by selectively depolymerizing RNA from microbial waste steams

AIM 2 Criteria: Production of c40 Terpenes

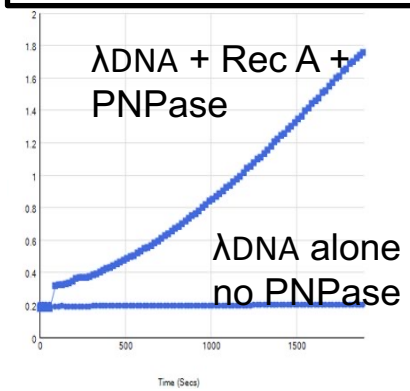
- Identify, express, and assemble a cell free pathway for Astaxanthin production
- Produce and scale a cell free system to produce 10g/L of a long chain terpene from an enriched cofactor lysate

4 – Progress and Outcomes: Lysates for Cofactors and Terpenes

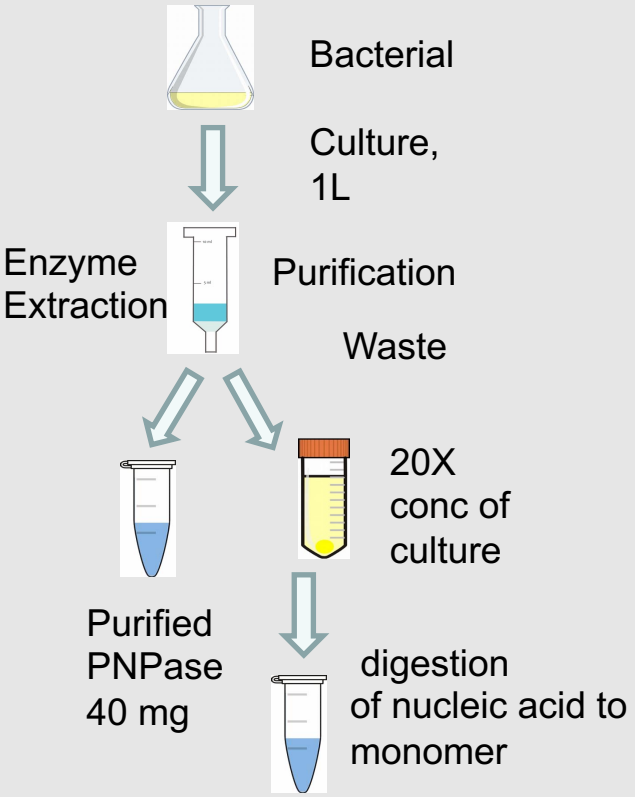
Optimization of Depolymerization

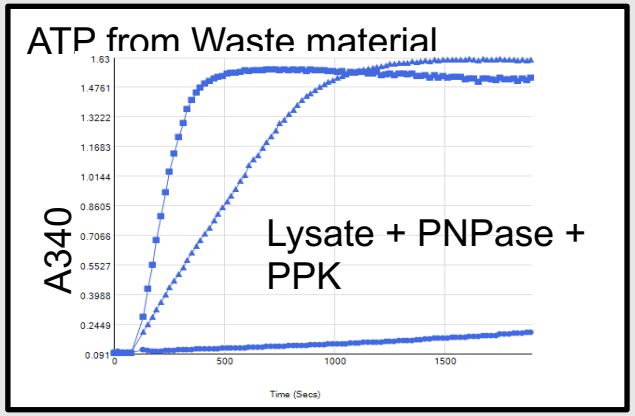


Generation of 1 mM ATP solution from control DNA

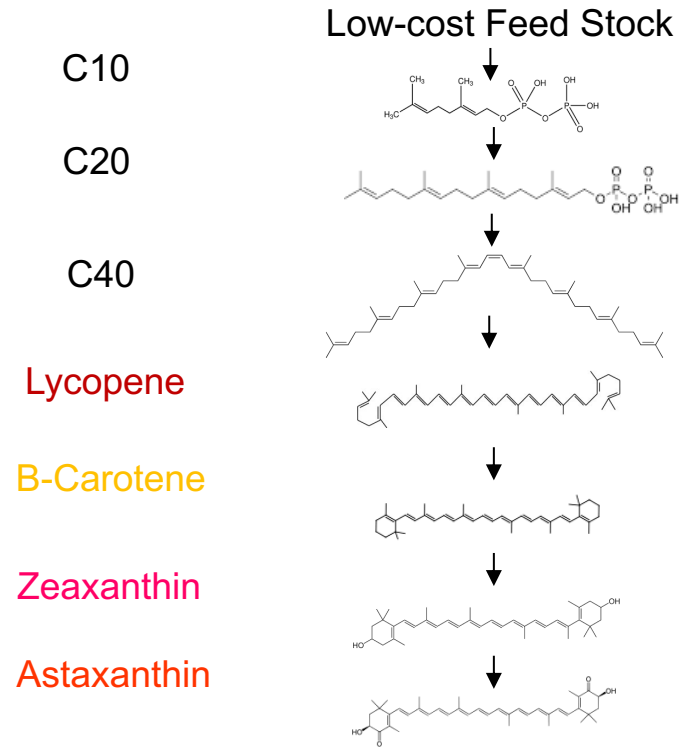


Production in lysate

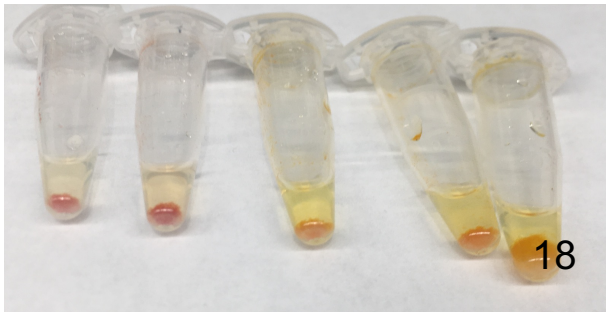




C40 Terpene production



Lycopene to Beta carotene (~1g/L)



Summary: Lysates for Cofactors and Terpenes

- *Bringing down the overall cost of cell free systems is important for wide-spread usage, especially for commodity chemicals*
- *Nucleotide bases from DNA and RNA are a potential source for NTP cofactors to power cell free systems*
- *Microbial waste streams can be repurposed as a low-cost source of cofactors*
- *Valuable long chain c40 terpenes can be produced in cell free systems*

Thank You!
Questions?

Invizyne Team
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Saken Sherkhanov, PhD

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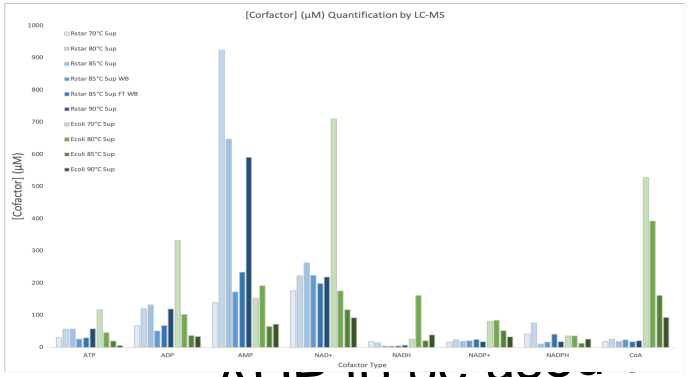
BIOENERGY TECHNOLOGIES OFFICE

Additional Slides

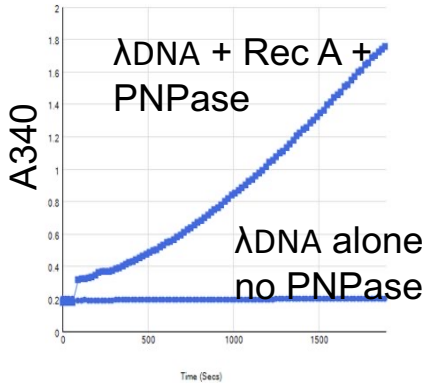
Component	Concentration
RNA	75-120 mg/mL
DNA	11-18 mg/mL
ATP	0.76 mg/mL
ADP	.35 mg/mL
AMP	.1 mg/mL

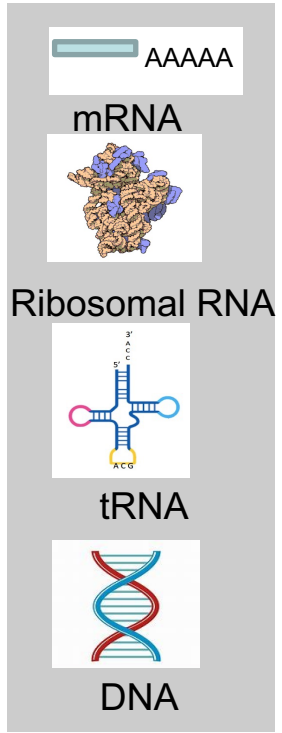


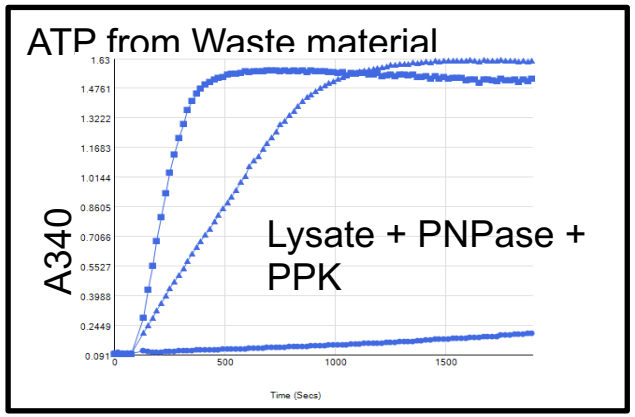
cofactor

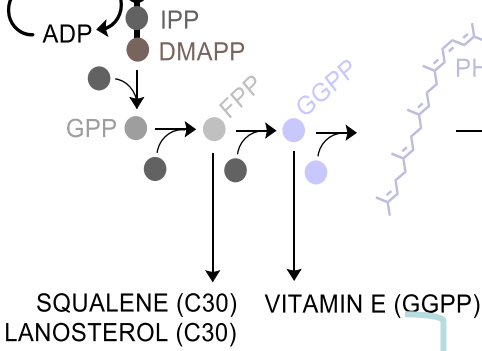
ADP to be used

Generation of 1 mM ATP solution from control DNA









1 – Management (Project #1)

Logistics

Invizyne and UCLA (Bowie Lab) will perform research tasks independently and share important results in a collaborative approach to reach all milestones. Materials and protocols will be shared to the extent possible. This is especially important for tasks to reach Go/No-Go and Final Milestones.

Task Breakdown

Invizyne

Cellulosic Hydrolysate Tasks (1,9,12,13)
Enzyme Rate/Cofactor Utilization Tasks (4,6)
Protein Production Tasks (7,10)

UCLA

Pure Glucose Tasks (2,8,11)
Enzyme Rate/Stability Tasks (3,4,5)
Protein Production Tasks (7,10)

Risks

1. **Enzyme stability/cost**: Enzyme engineering for faster/more stable enzymes lowers load and cost
2. **Cofactor stability/cost**: System engineering to use NAD(H) significantly lowers cost
3. **Feedstock usage**: Focus on different feedstocks ensure problems with one will inform the other
4. **Product separation**: Separation method will be key for economic success long-term

Team Communication

internal

- Invizyne and UCLA PIs have a strong working relationship over 10 years. The two groups have weekly virtual meetings to discuss results, challenges, and future directions to ensure all milestones are met.

external

- Quarterly reports and virtual quarterly update meetings with the Program Manager/Monitor help identify risks and relay strategies to mitigate problems with the awarding agency.
- Invizyne has a strong relationship with team at NREL (Bomble Lab) on different but related BETO projects. The PIs discuss monthly progress and challenges, especially related to enzymes, that effect both projects. Collaboration will also help normalize results from different groups using different but similar feedstocks.