DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Direct Photosynthetic Production of Biodiesel by Growth-Decoupled Cyanobacteria (Bioenergy Technology Incubator 2 Project)

March 6, 2019

Advanced Algal Systems

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Arizona State University

From the Bioenergy Technology Incubator 2 FOA:

"BETO recognizes that there may be very novel and potentially disruptive ideas that do not necessarily satisfy the requirement of specific FOAs yet still meet BETO's goals and mission. The Bioenergy Incubator Program is intended to identify these potentially impactful ideas that are not meaningfully addressed in BETO's strategic plan or project portfolio. It is NOT intended to fund projects that are incremental improvements to current products or processes or for established work in BETO's strategic plan or current portfolio.

BETO is issuing this Incubator 2 FOA to support innovative technologies and solutions that could help meet existing goals but are not currently represented in a significant way in the BETO's MYPP and current project portfolio."

Goals

- Directly produce excreted, ‘drop in’ ready biofuel (ethyl or methyl laurate) by cyanobacteria using CO_2, water, and light as the main inputs
- Uncouple growth of the culture (biomass production) from production of the biofuel to increase the lauroyl ester yield
- Further increase lauroyl ester production by (1) boosting metabolic flux through the fatty acid biosynthesis pathway and (2) reducing the production of exopolysaccharides

Outcomes:

- Successful production and excretion of methyl laurate
- Improved biofuel yields (current laurate production is at about 20 mg/L/day)

Relevance:

- Direct production of excreted biofuel from CO_2, water and light
- Scalable with limited processing needs

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Quad Chart Overview

Timeline

- Start: September 1, 2016
- End: December 31, 2018
- 100% complete

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	2,394	712,993	963,588	139,025
Project Cost Share*	0	137,238	115,050	0

Partner: Algenol (providing ethanologenic plasmids); no cost to DOE

Barriers addressed

Aft-B. Sustainable Algae Production

Aft-C. Biomass Genetics/Development

Objective

Develop cyanobacteria that excrete photosynthetically produced biodiesel and that invest an increased amount of fixed carbon in biodiesel production

End of Project Goals

- Improve fatty acid production
- Demonstrate excreted biodiesel (lauroyl (m)ethyl ester) production by cyanobacteria
- Investigate whether growth arrest can lead to increased partition of fixed carbon to the biofuel product
- Decrease the fixed-carbon demand of cells by removing some of the exopolysaccharides
- Perform a TEA on lauroyl ester production in cyanobacteria

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1 – Project Overview

Project Tasks

- **Task 1:** Increase Laurate Production *(make more free fatty acid by increasing the flux through the pathway)*
- **Task 2:** Engineer Lauroyl Ester Biosynthesis *(introduce genes for ethanol biosynthesis and for making acyl esters).* **Was changed mid-project from producing ethyl laurate to making methyl laurate via introduction of a methyl transferase.**
- **Task 3:** Decouple *Synechocystis* Growth and Lauroyl Ester Production *(downregulate critical transcripts to stop growth and invest photosynthate in making fatty acids instead)*
- **Task 4:** Reduce Exopolysaccharide Production *(resulting in less food for contaminants and more photosynthate for fatty acids)*
- Demonstrate lauroyl ester production at the 55-L photobioreactor scale
- **Task 5:** Perform a Techno-Economic Analysis on Lauroyl Ester Production in Cyanobacteria *(e.g., determine value of product vs. biomass)*

2 – Approach (Management)

Team:

- Wim Vermaas (PI; ASU, School of Life Sciences)
  - Fatty acid production improvement; methyl laurate; exopolysaccharide reduction
- David Nielsen (Co-PI; ASU, School of Engineering of Materials, Transport and Energy)
  - Growth arrest by metabolic engineering
- Xuan Wang (Co-PI; ASU, School of Life Sciences)
  - Molecular biology; overexpression of fatty acid biosynthesis pathway genes; ethyl laurate balance
- Rob Stirling (ASU School of Sustainable Engineering and the Built Environment)
  - Lauroyl (m)ethyl ester TEA
- Anna Keilty (ASU School of Life Sciences)
  - Project coordinator
- Three postdocs, four graduate students, one laboratory staff member
- Biweekly meetings of the team

2 – Approach (Technical)

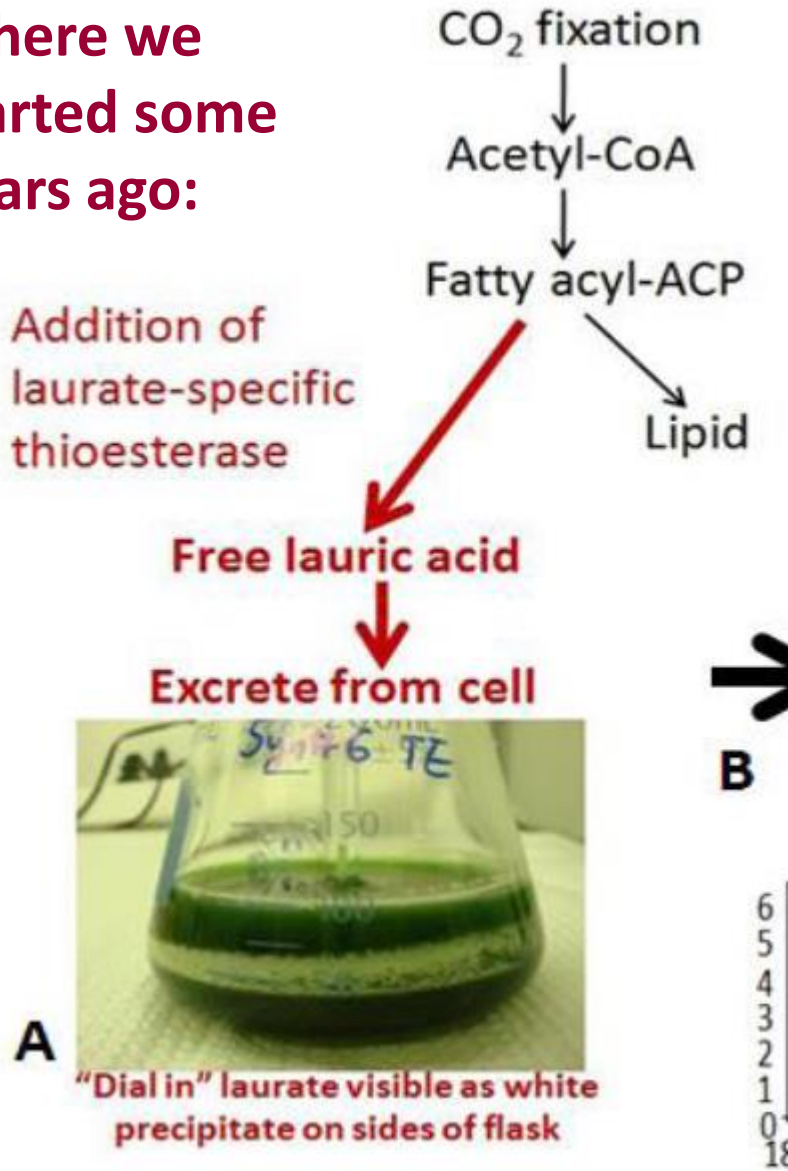
Tasks:

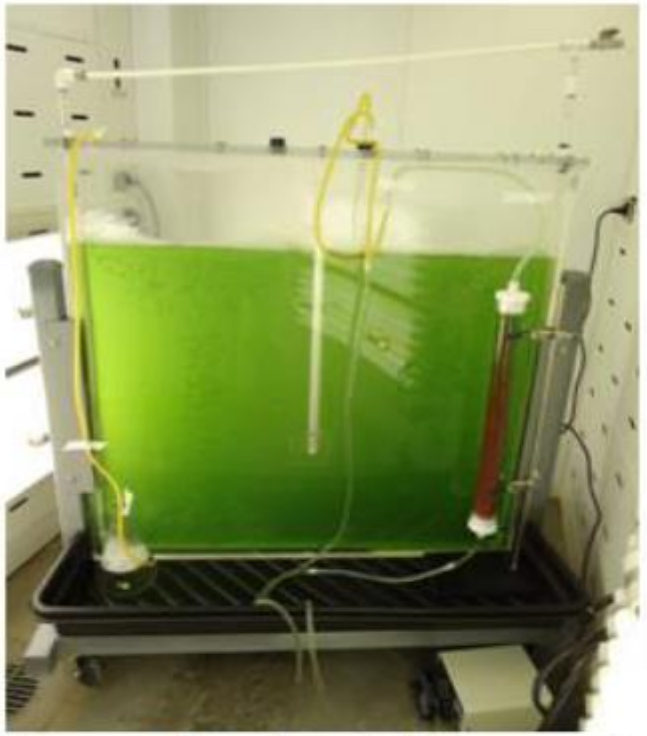
1. Increase laurate production (more laurate means more (m)ethyl laurate)
   - Metabolic engineering (fatty acid biosynthesis regulation, overexpression)
   - Was successful: increased free fatty acid production
2. Engineer lauroyl (m)ethyl ester biosynthesis
   - Introduce ethanol production; convert laurate to lauryl-CoA; convert lauryl-CoA and ethanol to ethyl laurate
   - Go/No-Go milestone on this met, but strain was not stable
   - *Challenge overcome: produce methyl laurate rather than ethyl laurate: successfully produced methyl laurate at >0.1 g/L scale*
3. Decouple growth and ethyl laurate production
   - Inducible (e.g., Ni^{2+}-inducible) growth arrest (CRISPR-dCas9); remains a challenge
4. Reduce exopolysaccharide production
   - Delete genes involved in exopolysaccharide biosynthesis
   - Demonstrated reduced exopolysaccharide content of cells

   Demonstrate biofuel production and growth at the 55-L photobioreactor scale
5. Techno-economic analysis of methyl laurate production in cyanobacteria

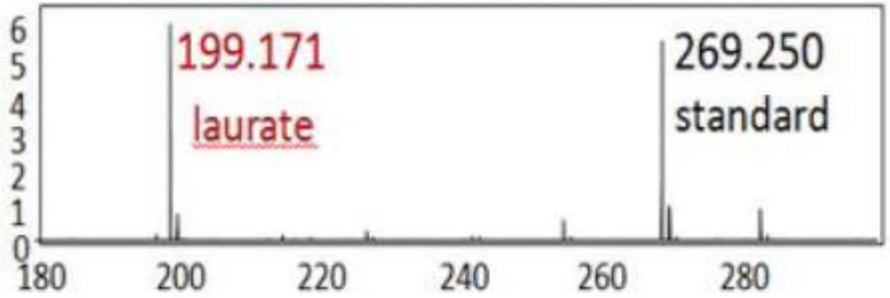
3 – Technical Accomplishments/Progress/Results

Where we started some years ago:



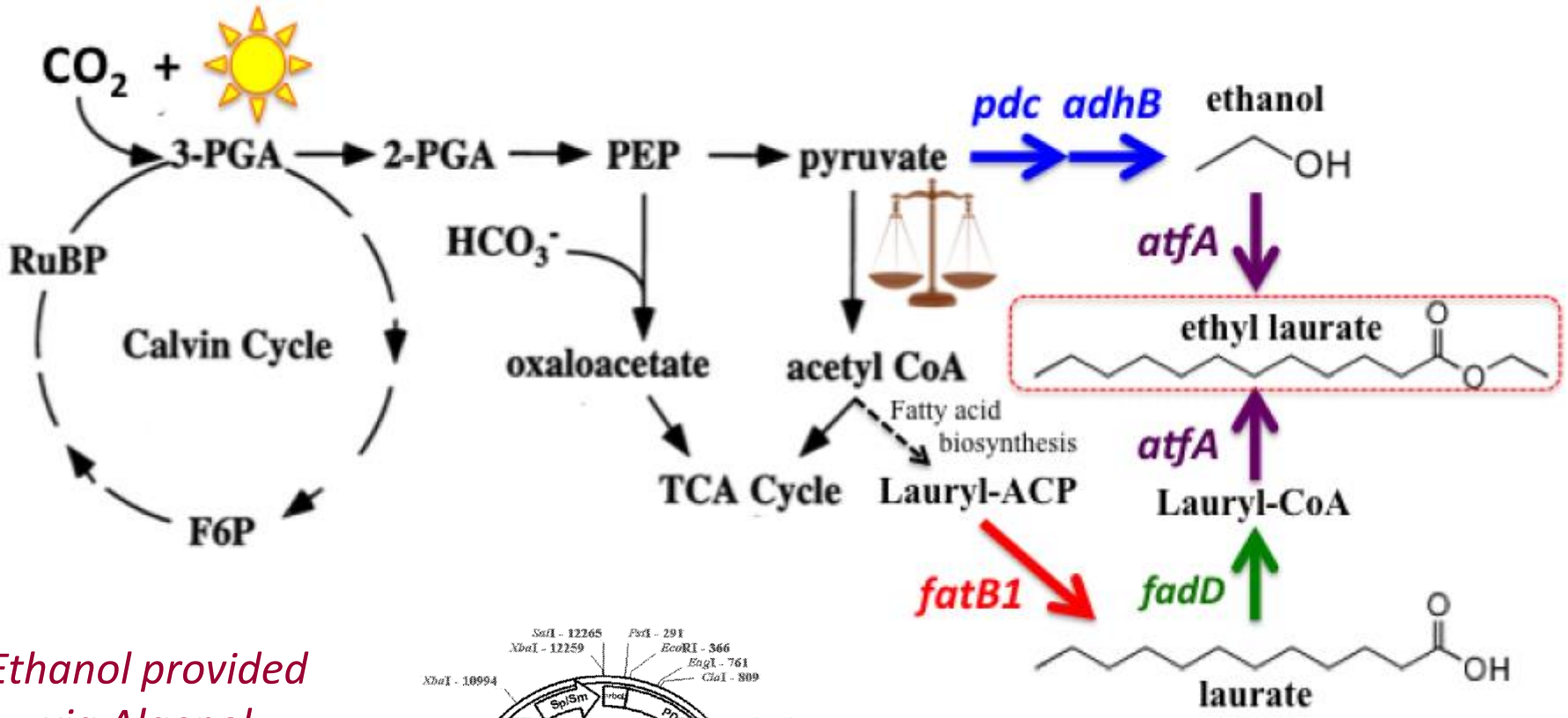


B 55L-scale photobioreactor with resin harvest column

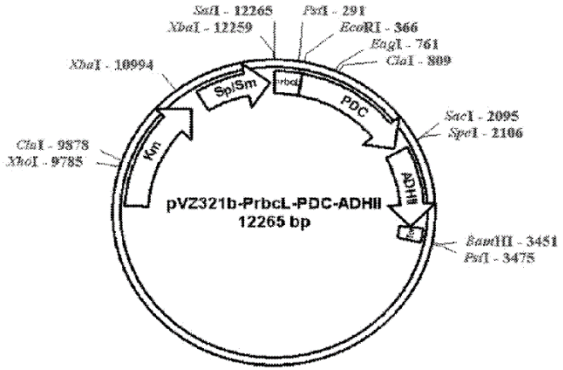


3 – Technical Accomplishments/Progress/Results

The original idea of this project was to modify the laurate-producing strain to make ethyl laurate, and make as much as possible



Ethanol provided via Algenol plasmids/constructs or by our own constructs



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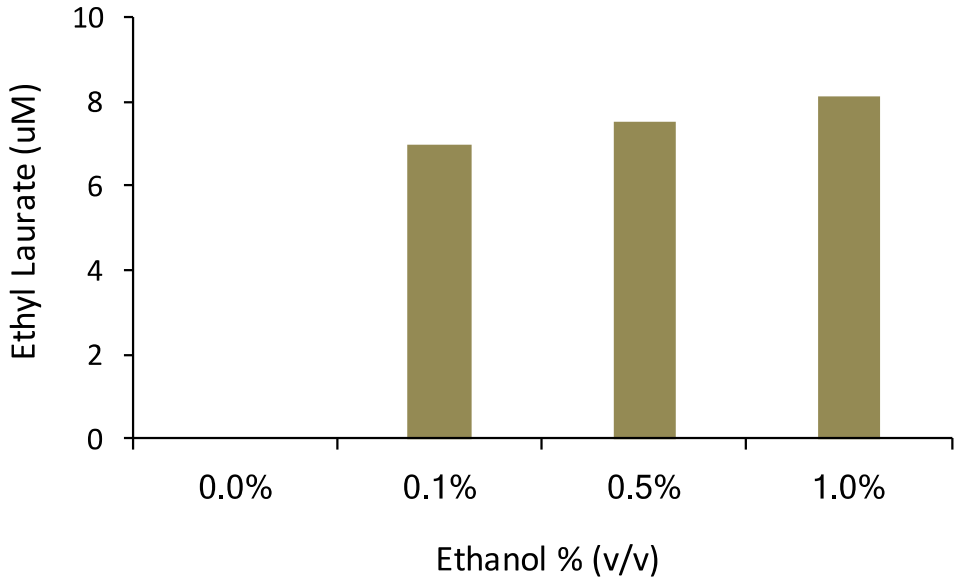
3 – Technical Accomplishments/Progress/Results

While we met the Go/No-Go decision point (produce ethyl laurate in the presence of added ethanol), the productivity was disappointing



Ethyl laurate production by the TE/Δ*slr1609/fadD/atfA* strain as detected in the 10% (v/v) dodecane overlay when grown in the presence of 0.1, 0.5, or 1% exogenous ethanol for three days. The ethyl laurate concentration has been calculated back to the volume of the culture (aqueous phase).

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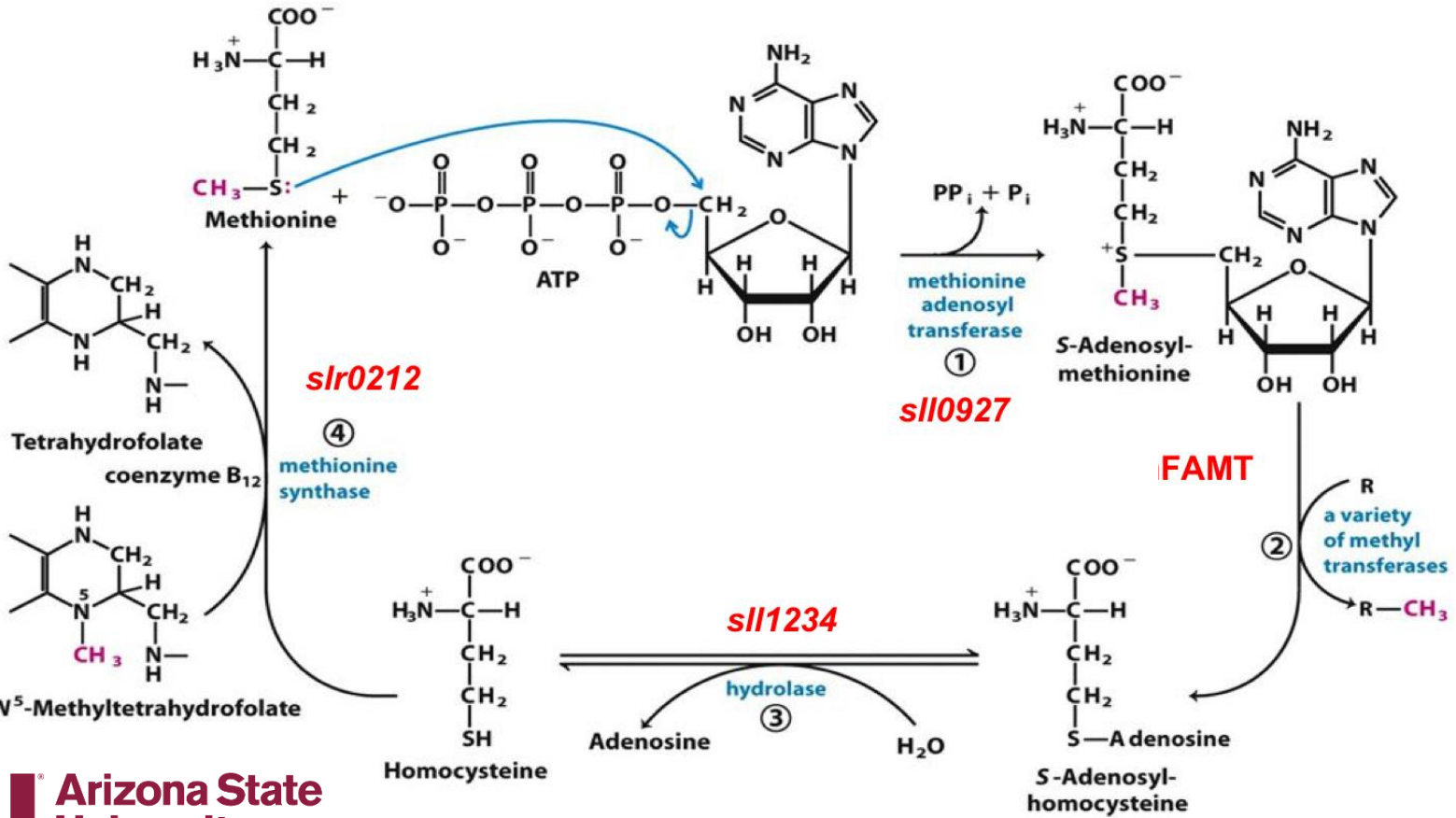
The fadD gene (or lauryl-CoA) appeared to be toxic to the strain, and no stable cyanobacterial strains expressing fadD were obtained.

3 – Technical Accomplishments/Progress/Results

Plan B: Methyl Laurate

As stable ethyl laurate production was elusive, we considered alternatives. We selected using a fatty acid methyl transferase (FAMT) with activity toward laurate.



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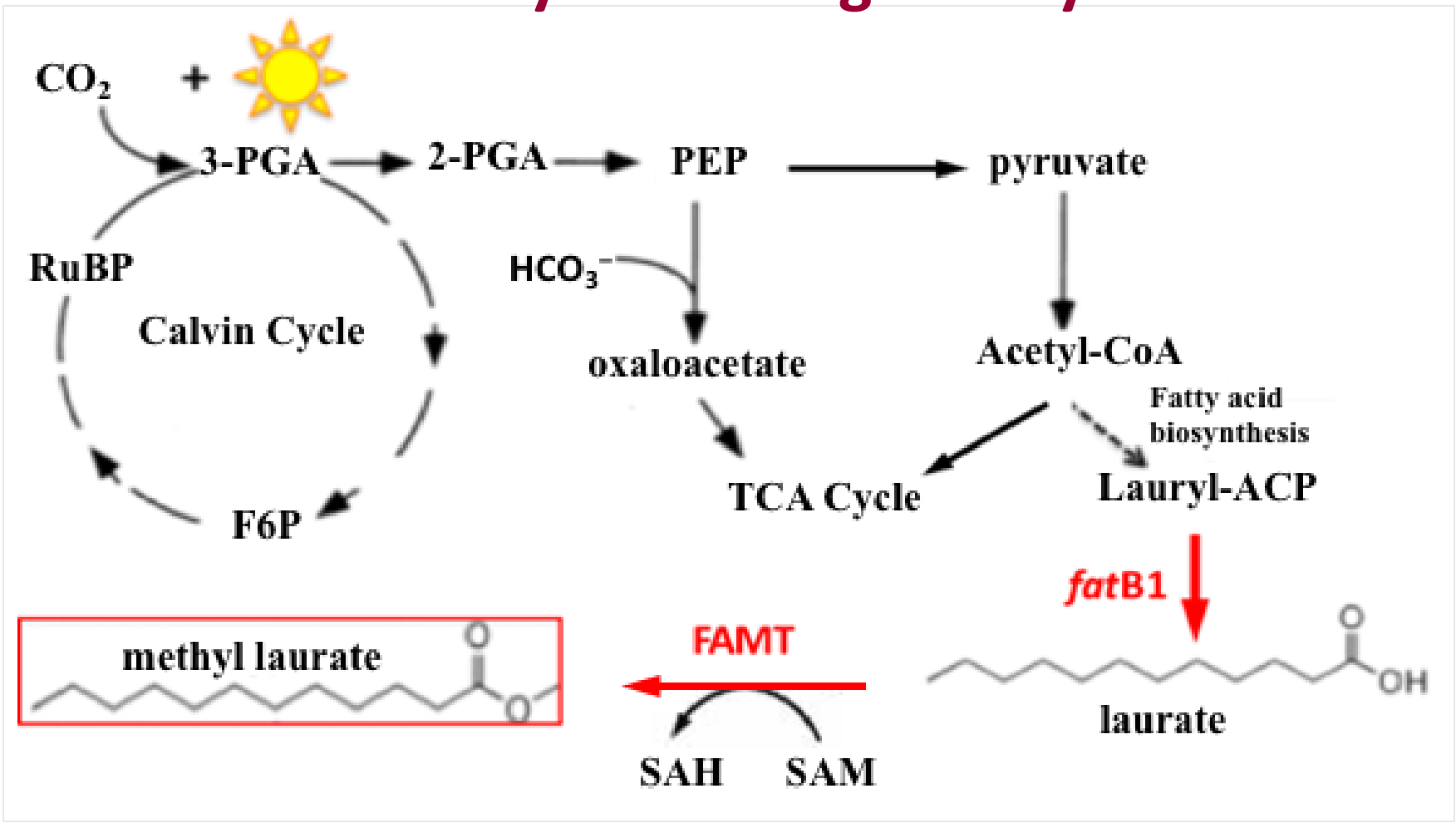
3 – Technical Accomplishments/Progress/Results

Our New Way of Making Lauroyl Esters



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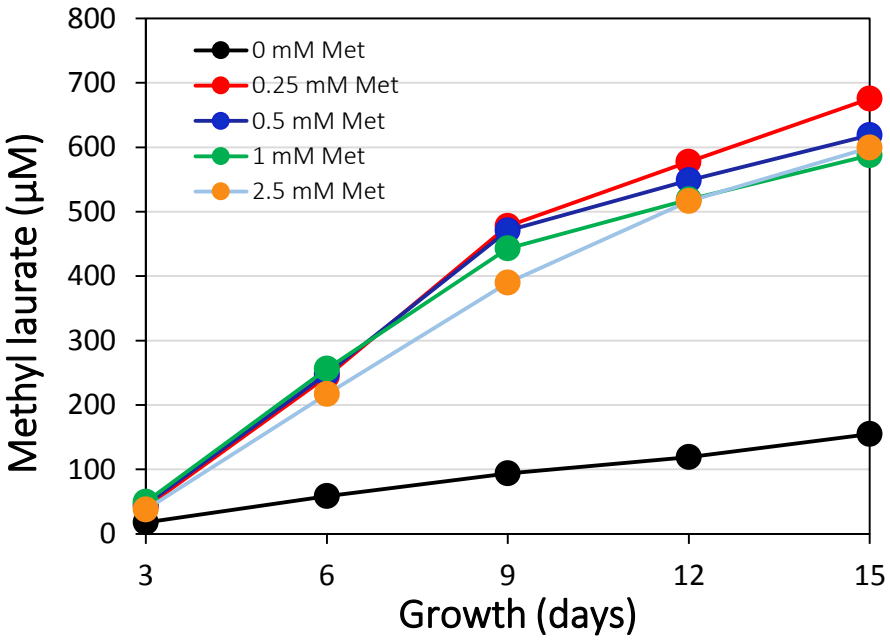
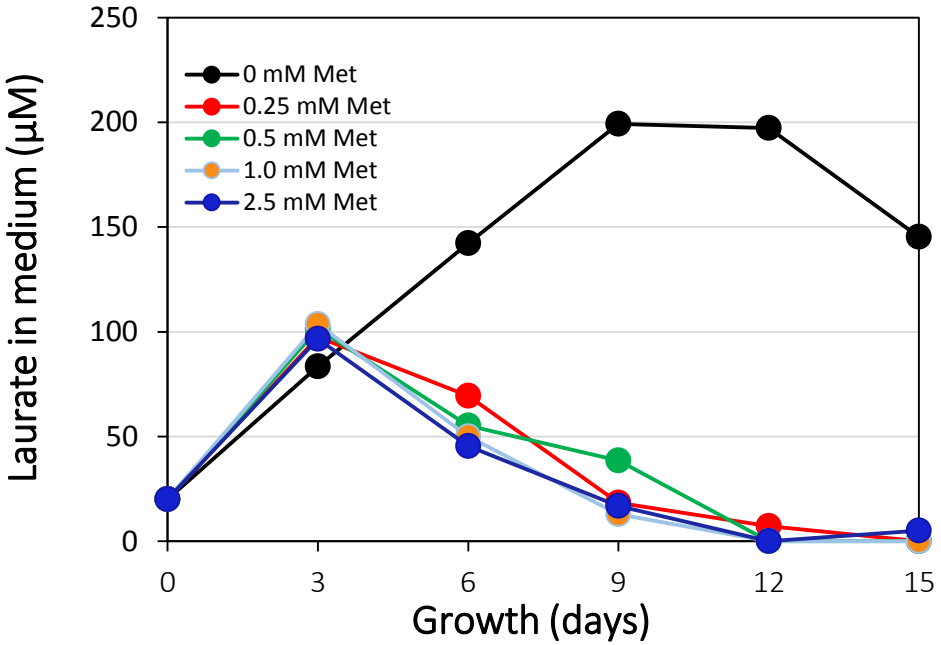
Other advantage: This eliminates the requirement for an appropriate stoichiometry of produced ethanol and laurate.

3 – Technical Accomplishments/Progress/Results

Some Methionine Is Required to "Prime the Pump"





- Note that laurate levels are depleted when methyl laurate accumulates
- We are now overexpressing enzymes in the SAM (S-adenosyl methionine) regeneration cycle, in the hope that no methionine needs to be added anymore for good productivity

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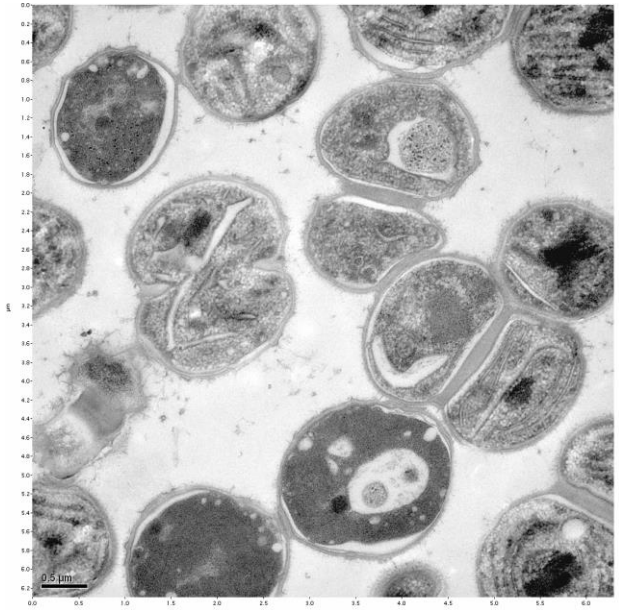
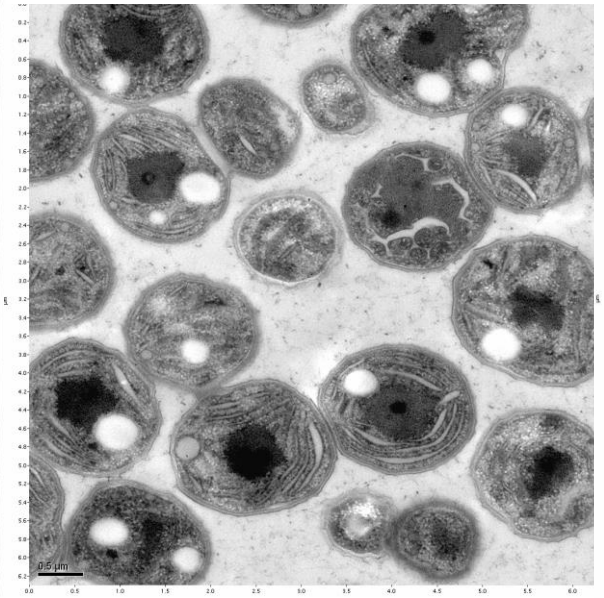
3 – Technical Accomplishments/Progress/Results

Methyl Laurate Also Appears to Accumulate in Cells

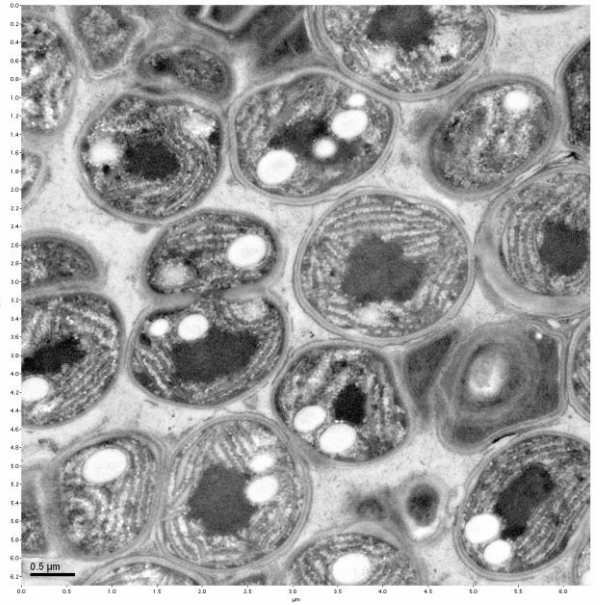


10 mM bicarbonate
+ dodecane

(negative control)



1 mM methionine



1 mM methionine

(large white inclusions most likely are from methyl laurate)

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3 – Technical Accomplishments/Progress/Results

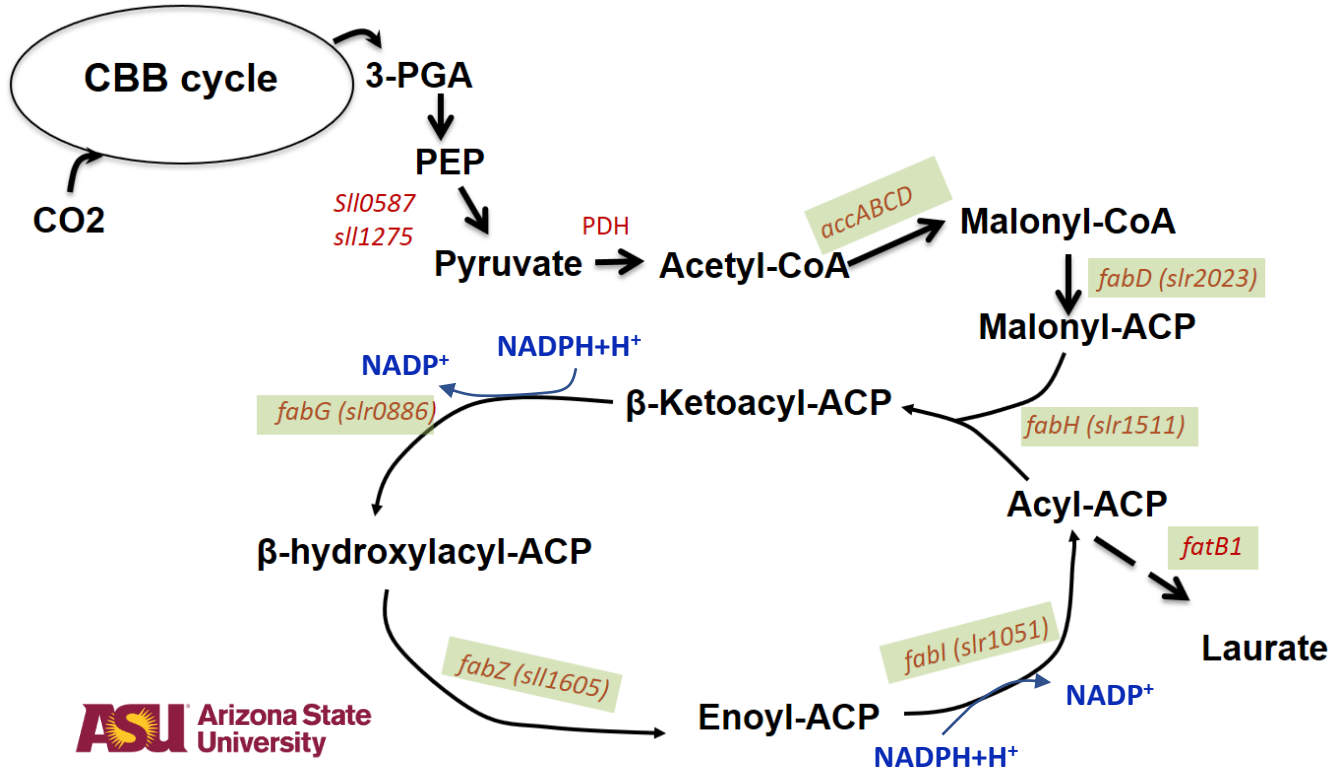
Bottom Line on Lauroyl Ester Production

- We have succeeded in demonstrating ample lauroyl ester production
- Methyl laurate production suppresses laurate accumulation
- Methyl laurate production requires addition of “catalytic” (0.25 mM or so) amounts of methionine
- We are in the process of overexpressing SAM regeneration enzymes to see whether this obviates the need for methionine addition
- In any case, we have developed a strain that efficiently converts free fatty acids to methyl esters

Task 1. Increasing laurate production from fixed CO_2

The fatty acid biosynthesis pathway is heavily regulated: requires a lot of energy and reducing equivalents. Regulation via, for example:

- GlnB (PII protein): Inhibition of ACC (acetyl-CoA carboxylase) by GlnB at low 2-OG and with non-phosphorylated PII protein **Inactivation of *glnB***
- Allosteric inhibition of fatty acid biosynthesis enzymes? (demonstrated in *E. coli*) **Expression of *Synechocystis* and *E. coli fab* genes**
- Feedback inhibition upon fatty acyl-ACP accumulation? **Overexpression of ACP**, **Expression of *tesA***



Combination of multiple positive genetic modifications may further boost laurate production

For good measure, also:

Increase NADPH availability

Overexpress *acc* genes

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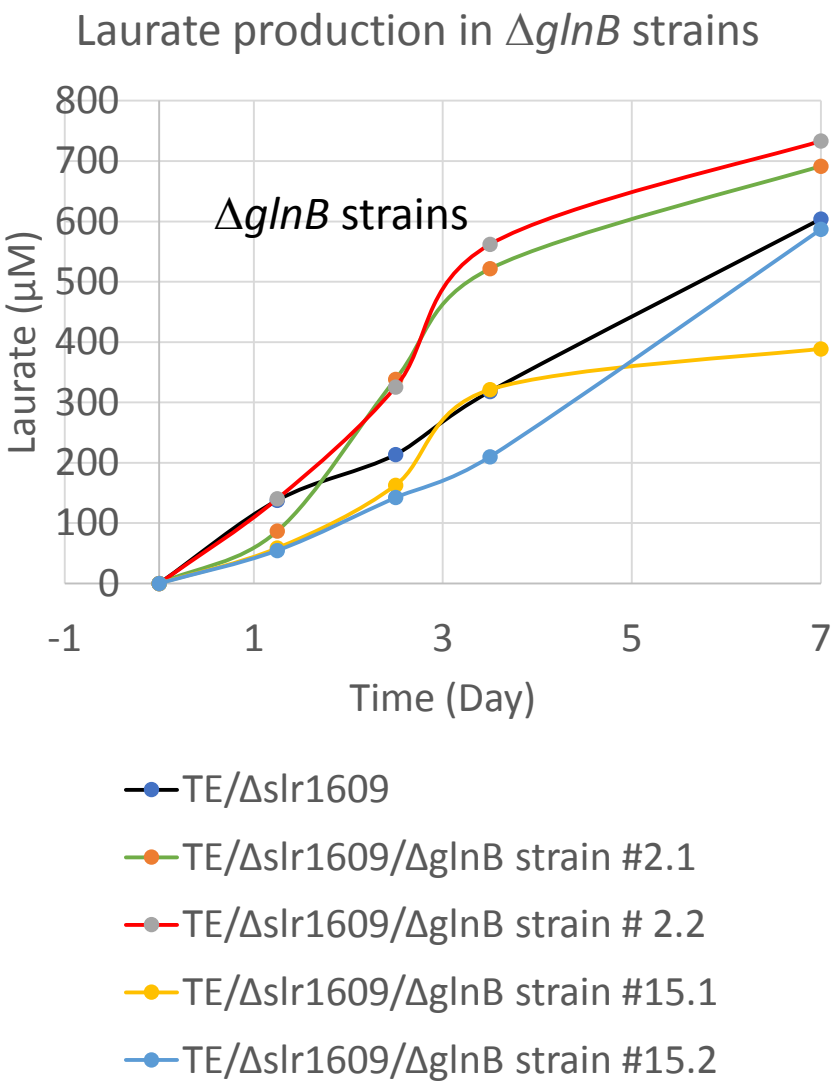
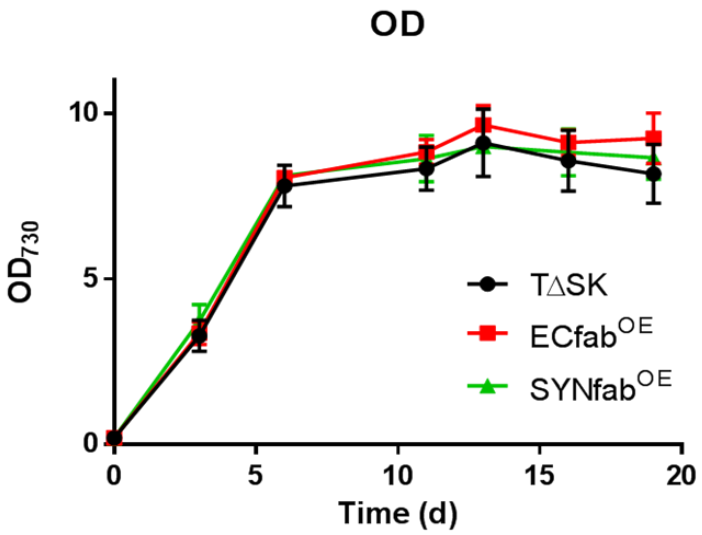
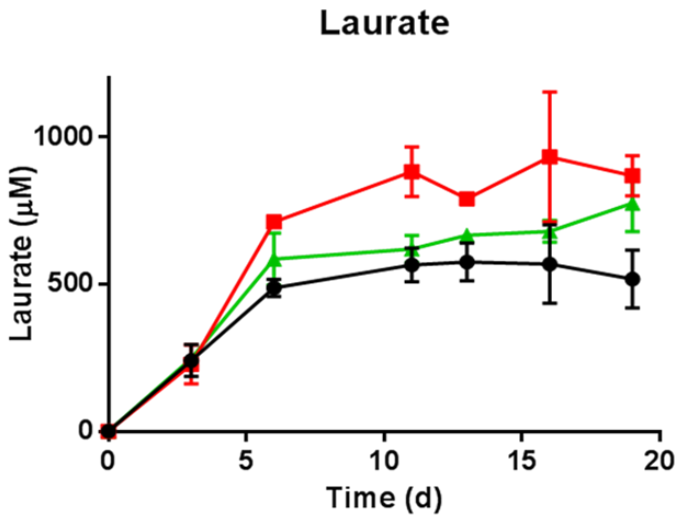
Task 1. Increasing laurate production from fixed CO_2

Indeed, significant increases in laurate production can be achieved upon deletion of *glnB* or overexpression of foreign or native fatty acid biosynthesis genes

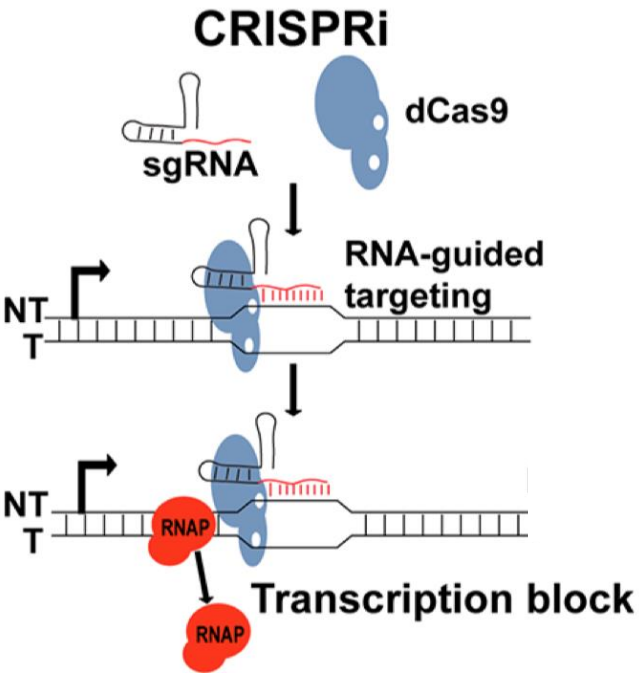






Task 3: Decouple *Synechocystis* Growth and Ethyl Laurate Production

Mechanism



Control strategy

- Ni^{2+}-inducible P_{nrsB} promoter
- Both *dcas9* and gRNA

Targets

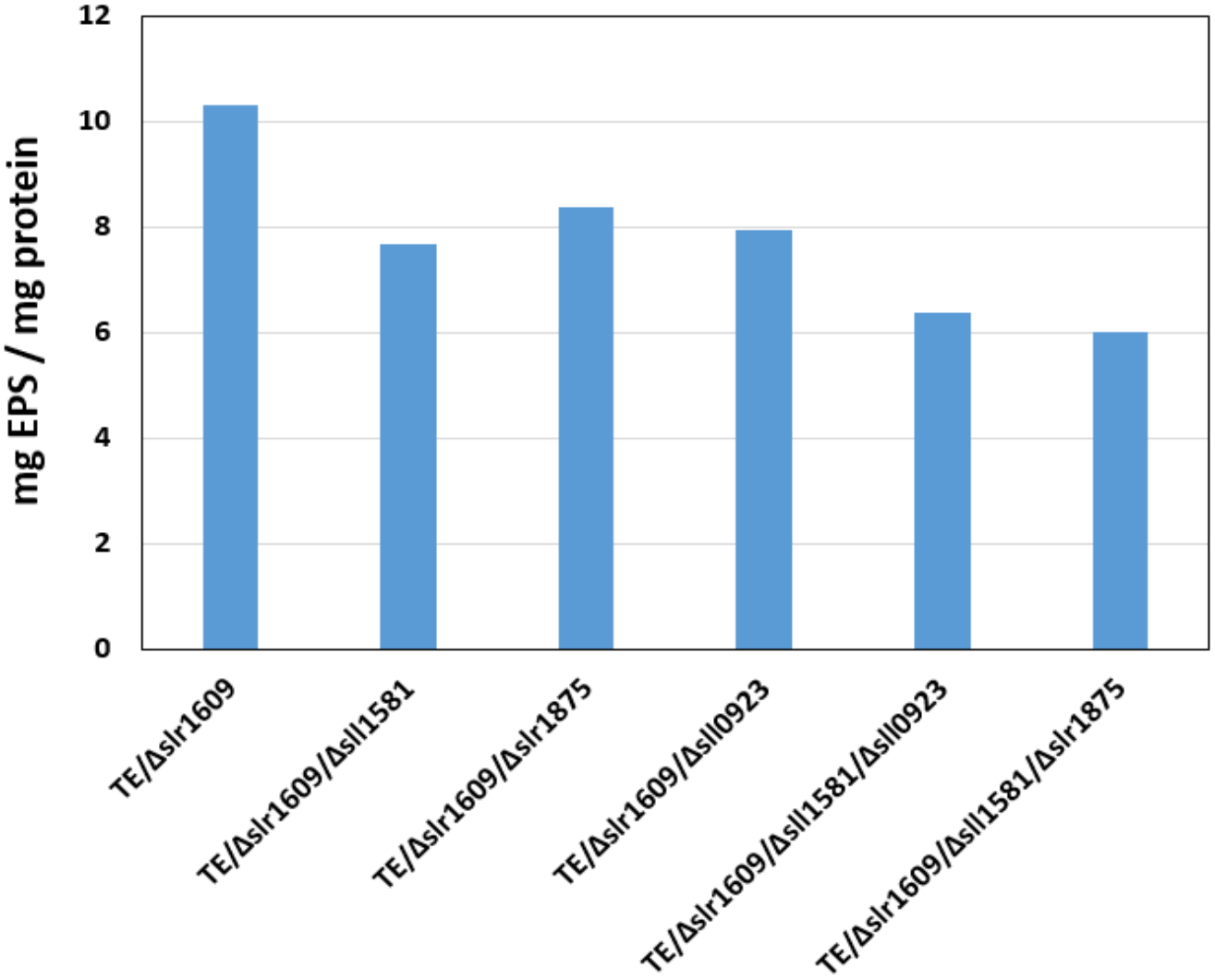
	Target	Function
1	*pyrf* (*sll0838*)	Pyrimidine biosynthesis
2	*thyX* (*sll1635*)	Pyrimidine biosynthesis
3	*ftsZ* (*sll1633*)	Cell division
4	*plsX* (*slr1510*)	Phospholipid biosynthesis
5	*plsC* (*sll1848*)	Phospholipid biosynthesis
6	*gltA* (*sll0401*)	2-oxoglutarate biosynthesis
7	*murA (slr0017)*	peptidoglycan biosynthesis
8	*gfp*	GFP positive control

Building and optimize CRISPRi system:

- *Screen efficacy of gRNAs*
- *Optimize expression conditions*
  - *Inducer level, timing*

However, still difficult in practice, in part because *dcas9* is somewhat toxic to *Synechocystis*

Task 4: Reduce Exopolysaccharide Production



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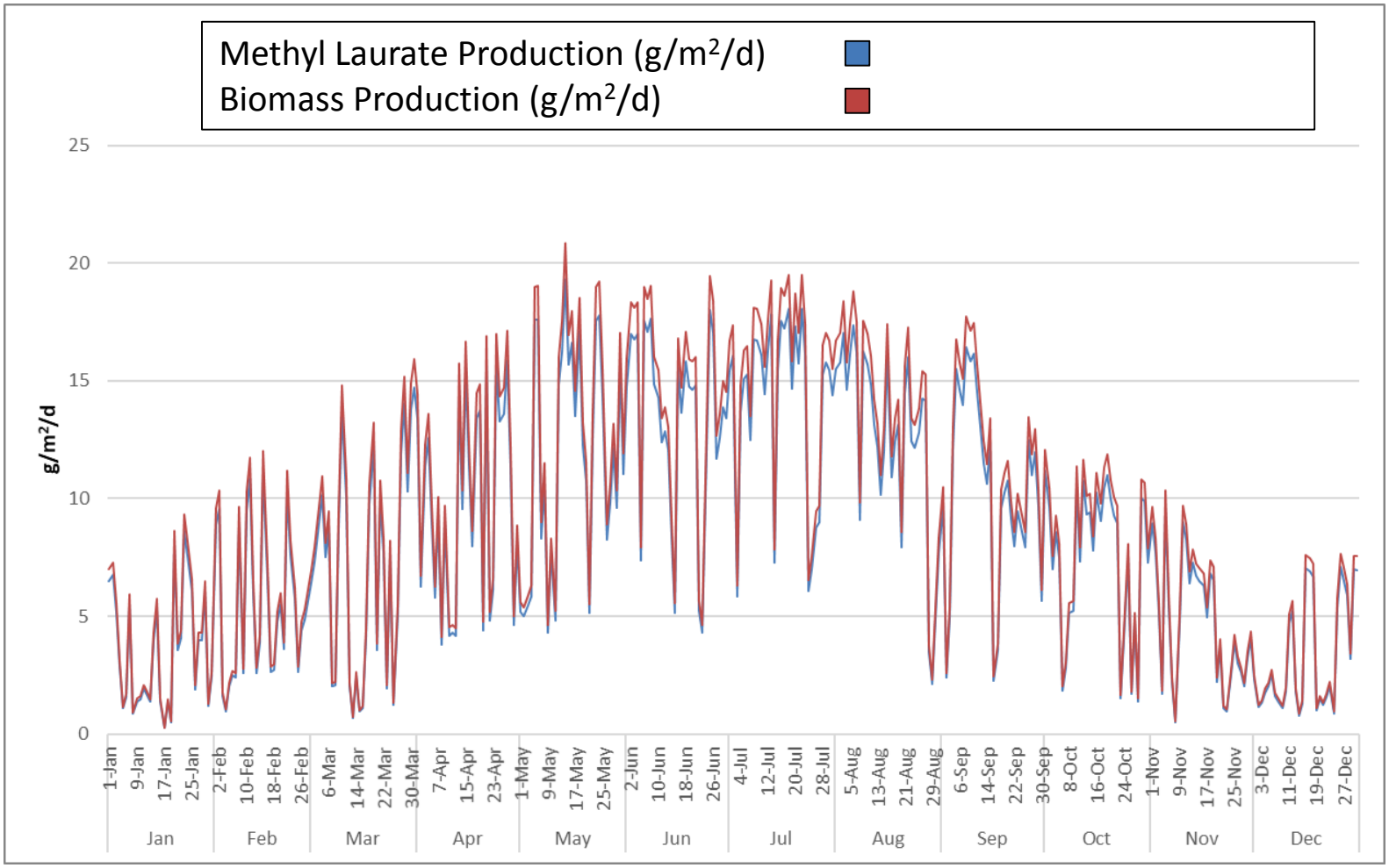
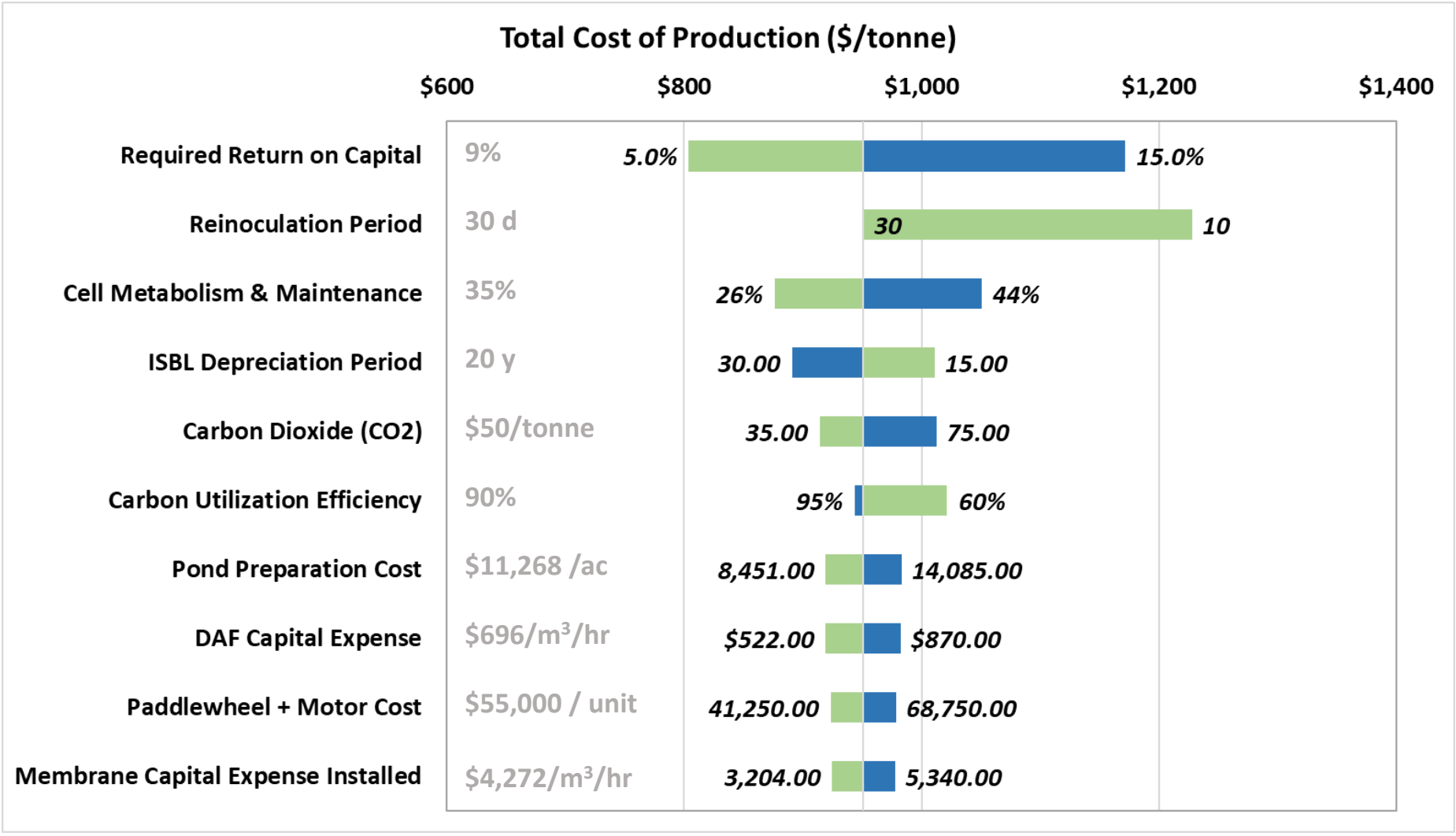
The amount of EPS indeed can be reduced

Task 5. Techno-economic analysis of methyl laurate production



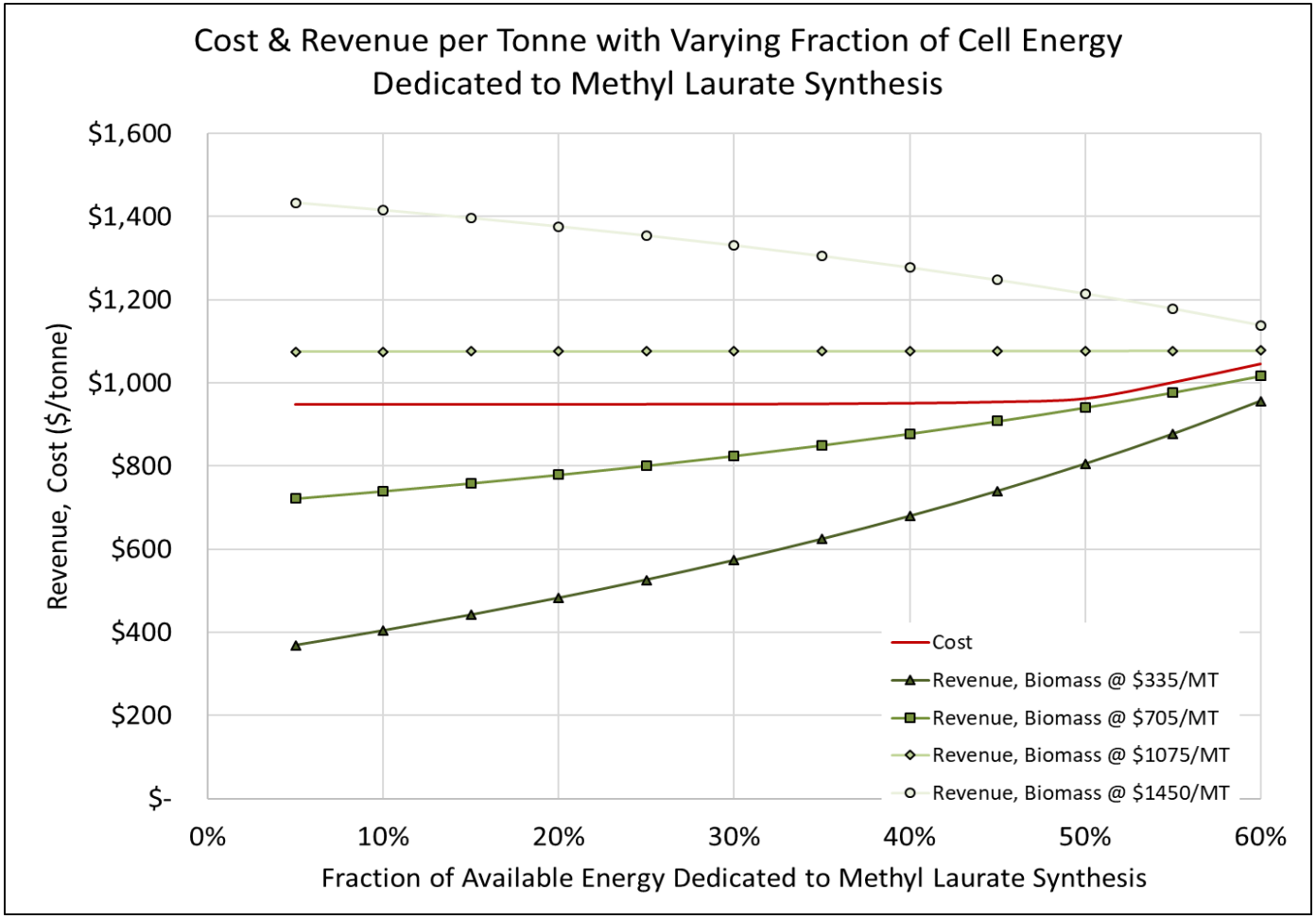
Annual production simulation. About equal productivity of biomass and methyl laurate is assumed. Production of biomass and methyl laurate is hourly estimated as a fraction of this energy and dependent on pond biomass density (g/L). Peak productivity corresponds to roughly 38 g/m^2/d of only biomass. Location: Corpus Christi, TX

Task 5. Techno-economic analysis of methyl laurate production



These are the Top 10 “Most Influential” assumptions related to production cost. Baseline values are in gray. Cell Metabolism and Maintenance is a proxy for all forms of non-productive energy. The more lost energy, the lower the productivity of biomass and methyl laurate (in $g/m^2/d$).

Task 5. Techno-economic analysis of methyl laurate production



Revenue and cost as the fraction of available energy dedicated to methyl laurate synthesis is varied. Methyl laurate is conservatively valued at $1,078/MT; high-purity methyl laurate is several-fold more valuable.

Bottom line: Cyanobacterial methyl laurate production is economically feasible, assuming a sufficiently large fraction of fixed carbon going to methyl laurate.

4 – Relevance

- BTI2 projects aim to add novel, promising components to BETO's portfolio
- Breakthrough concept in this project: Direct production of excreted biofuel (methyl laurate) from CO_2, water and light
  - Lauroyl esters are nearly immiscible with H_2O and are stable, forming a layer on top of the culture
  - Methyl laurate is an immediate biodiesel equivalent
  - Scalable and reduced processing costs
  - No need for biomass extraction; biomass remains unaltered
  - Biomass may yield valuable co-products, and may be used as feedstock
  - TEA results are promising
- Helps reaching BETO's 2030 $2.50/gasoline gal equivalent goal
- Strains and knowledge resulting from this project are of use in a current project aimed at increasing carbon utilization efficiency (presented in a poster session earlier this week)

Summary

- Demonstrated successful photosynthetic production of methyl laurate at competitive yields in a cyanobacterium, with concomitant reduction in laurate levels
- This demonstrates the capability of transformable cyanobacterial systems to produce excreted drop-in biodiesel from CO_2, water and light
- TEA analysis supports the viability of this approach, with increasing methyl laurate titers being an important parameter
- Success in the great majority of tasks, yielding increased flux through the fatty acid biosynthesis pathway, production of a "new" biofuel compound (methyl laurate), reduction in exopolysaccharide levels, and successful growth and production in 55-L photobioreactors
- From our perspective, this is a viable and promising addition to BETO's portfolio, and it will contribute to yield and cost goals of BETO's MYPP

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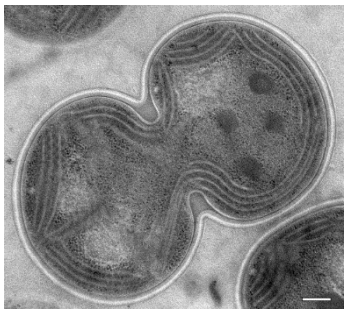

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Additional Slides

Response to Previous Reviewers' Comments

- The project was viewed to be high-risk. We concur, but within the two years of the project we have demonstrated very good production of methyl laurate with concomitant decrease in free fatty acid levels. One aspect did not pan out all that well yet, and that is increased biofuel production upon inducing a cessation of growth, but that aspect is icing on the cake and not critical to the overall success of the project.
- Some reviewers thought that harvesting would be difficult. Currently we successfully are using an organic, biocompatible top layer that does not mix with water (dodecane, isopropyl myristate, etc.) to enhance separation, but at higher productivity methyl laurate will separate from the aqueous phase even in the absence of an added organic top layer and harvesting may simply be a matter of settling and scooping off.
- Some reviewers commented regarding scaling. Whereas we have not demonstrated production beyond the 55-L photobioreactor scale, methyl laurate is much more biologically stable than laurate, and therefore more suitable for large-scale production by photosynthetic microbes.
- Indeed, we agree with the reviewers that this project has added valuable diversity to BETO's portfolio.
- We thank one of the reviewers for the suggestion of thinking in the near term about laurate esters as industrial chemicals that could help with deployment; indeed, pure methyl laurate is several-fold more expensive than biodiesel.

Publications, Patents, Presentations, Awards, and Commercialization

- No publications on this work are out yet. Several are in preparation. Note that this was a two-year project that finished recently.
- A patent application is being prepared for methyl laurate production in cyanobacteria.
- Aspects of this work have been presented at various conferences, including the Western Photosynthesis Conference in 2018 and 2019.
- Whereas commercialization is not expected for a Bioenergy Technology Incubator 2 project, strains developed in this project are now prepared for being used in another EERE/BETO-supported project on improving carbon utilization.