DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Production of High Performance Lubricants from Cellulosic Sugar

March 6, 2019
Biochemical Conversion Session

Tom McMullin
Cargill, Inc

Goal Statement

- Enable the production caprylic acid derived lubricants from cellulosic sugars
- Utilizing a novel bioprocess that converts lignocellulosic hydrolysate sugars to caprylate methyl ester
- Which will lay the foundation for this product to be used more extensively in current applications and open the market for new applications

Quad Chart Overview

Timeline

- Project start date; January 1, 2016
- Project end date: December 31, 2107
- Percent complete: 100%

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$1,011,801	$896,796	$82, 160	$1,990,758
Project Cost Share*	$252,949	$224,199	$20,540	$497,689

•**Partners:** **None**

Barriers addressed

Ct-H. Efficient Catalytic Upgrading of Sugars/Aromatics, Gaseous and Bio-Oil Intermediates to Fuels and Chemicals

Objective

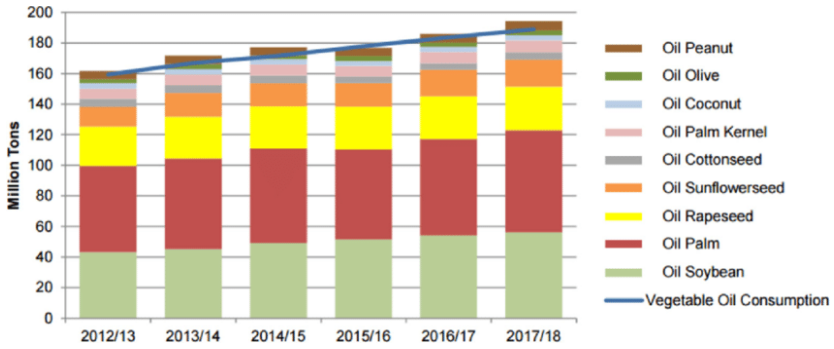
Enable the production caprylic acid derived lubricants from cellulosic sugars.

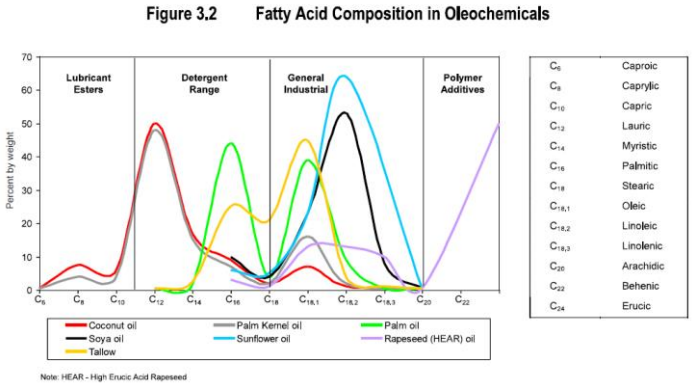
End of Project Goal

Develop a robust microbial strain along with a proprietary fatty acid production pathway that is capable of using cellulosic hydrolysate as feedstock.to produce caprylic methy ester at titer, rate, and specificity metrics that meet project final milestones

1 - Project Overview

- Global demand for bio-based lubricants is growing at an estimated 5-10% per year
- Certain classes of Fatty Acids and Fatty Acid Methyl Esters (FAMEs) have commercial value as raw material that can be converted to high performance lubricants
- Their broader use in lubricants and other applications has been limited by the high cost and limited availability
- A fermentation process for the production of FAME from cellulosic sugars contributes to BETO's mission to displace all fractions of a barrel of crude oil.





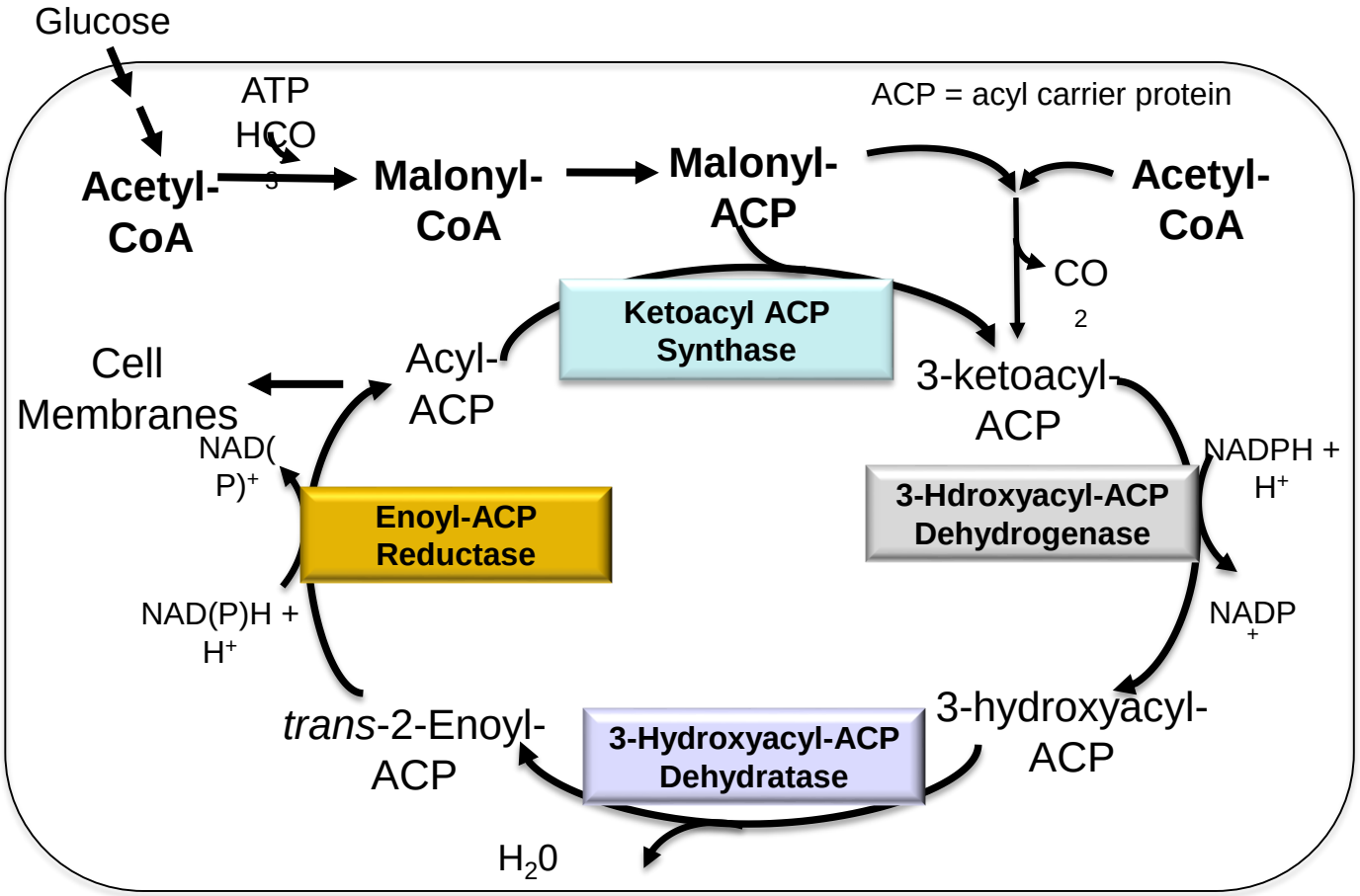
Foundational Technology for Bio-Based Fatty Acids

- OPXBIO developed a proprietary metabolic pathway in E. coli that produces fatty acids using coenzyme A (CoA)-bound pathway intermediates rather than intermediates carried on an acyl carrier protein
- Utilizes an acyl-CoA molecule as the fatty acid chain-elongation primer and a malonyl-CoA molecule as the chain extender.
- Operates independently of the production of lipids for cellular membranes (acyl-ACP pathway)
- OPXBIO previously demonstrated a proprietary approach to increasing the metabolic pool and flux of malonyl-CoA when developing 3-HP technology
- Cargill obtained OPXBIO technologies in 2015, including the project described here. Project work took place at Cargill from Fall 2015 to December 31, 2017

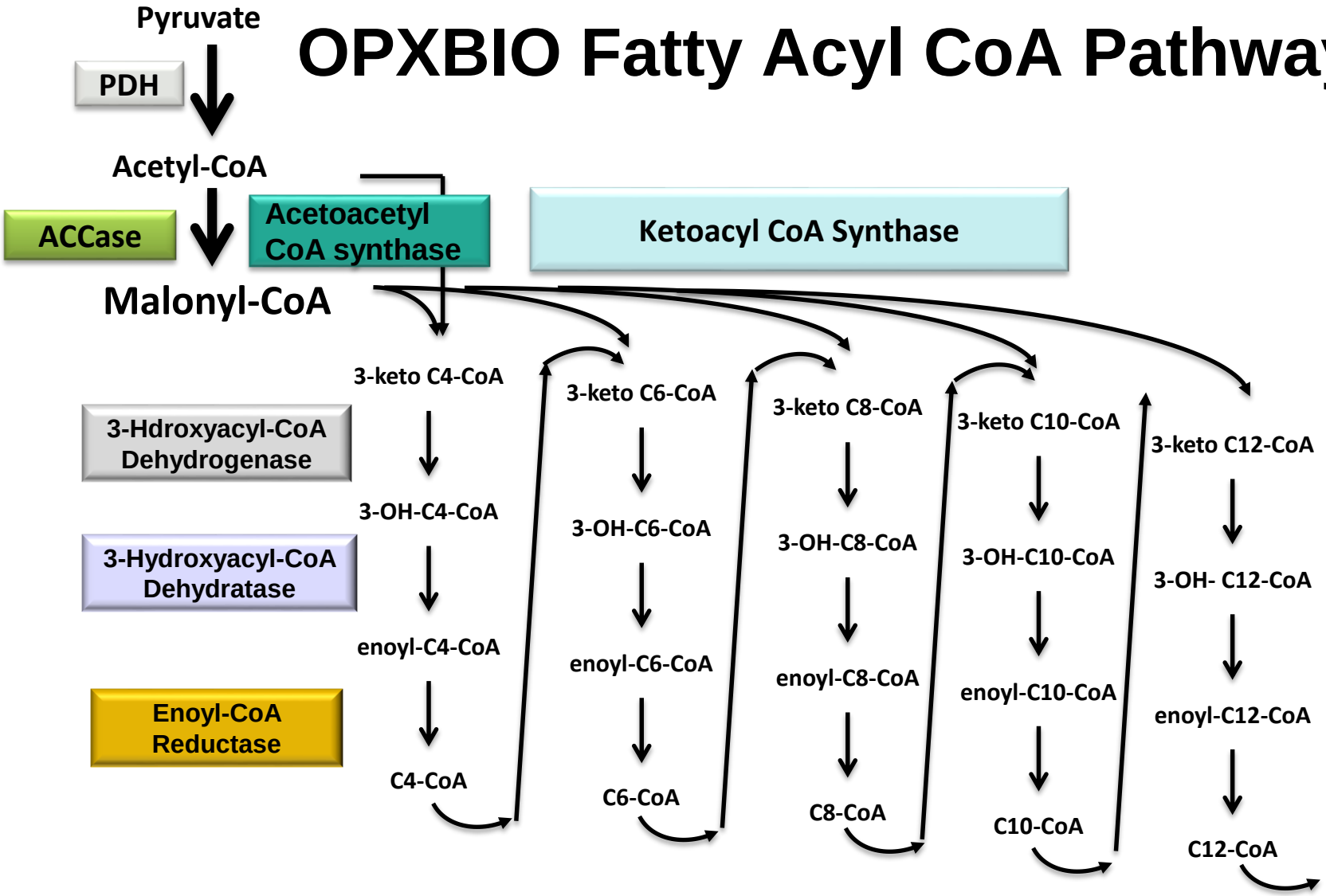
opxbio good chemistry.

Cargill®

Metabolic Pathway for Fatty Acid Biosynthesis



OPXBIO Fatty Acyl CoA Pathway



Goal Statement

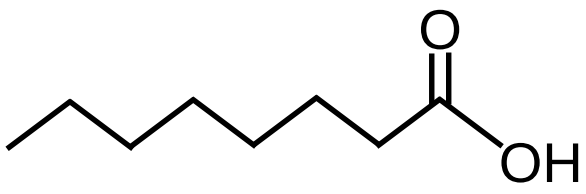
- Goal of this project was to enable the production caprylic acid (C8) derived lubricants from cellulosic sugars

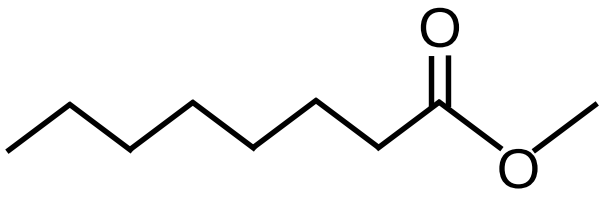
- This involved developing a novel metabolic pathway, a host microorganism and bioprocess that converts lignocellulosic hydrolysate sugars to caprylate methyl ester at rate, titer, and specificity metrics which advance the technology toward commercial viability

- Success in this program will lay the foundation for this product to be used more extensively in current applications and open the market for new applications through stable cost and supply from renewable cellulosic feedstocks

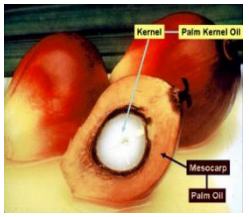
Why was this project funded? What is its creative advantage?

- A novel and potentially disruptive technology that is supportive of BETO's mission to displace all fractions of a barrel of crude oil.
- Impact: Broader use of polyolesters in motor oils and other lubricant applications, improving product performance and substituting for petroleum-based oils used today.
- Enable commercialization of bio-derived products that are currently supply-limited.

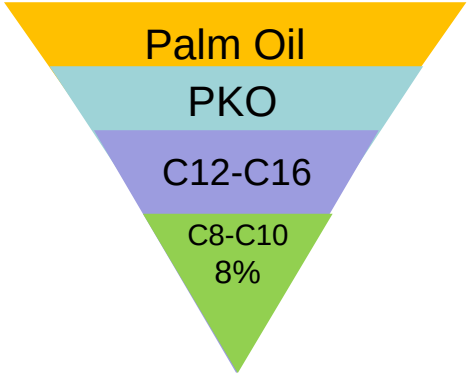




1 - Project Overview



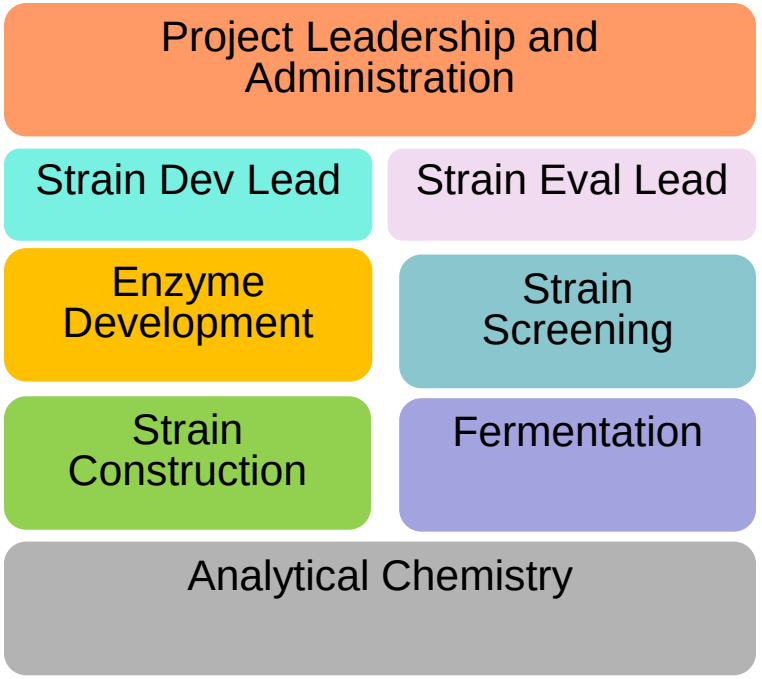
- Caprylic (C8) fatty acid is used in a variety of applications
  - Synthetic lubricants, hydraulic fluids and refrigerant lubricant.
  - Esters for perfumes and in dyes
  - Production of various amides and plasticizers
  - Activator/accelerator in the rubber industry
  - Antimicrobial for food processing equipment
  - Surfactant In soaps and detergents



- Sources of caprylic acid are primarily cuts from coconut and palm oils
  - Current sources (palm and coconut) limit the supply of caprylic acid
  - Current market is characterized by price and supply volatility
  - A fermentation derived source of caprylic acid has potential to open new or expand existing markets

2 – Approach (Management)

- **Team Structure**
  - Project Management
  - Strain Development Lead
    - Enzyme Development
    - Strain Construction
  - Strain Evaluation Lead
    - Strain Screening (small volume)
    - Bench Scale Fermentation (2L)
  - Analytical Chemistry



- Cross functional team that met weekly to discuss results and track progress
- Project leadership met monthly with DOE to update on progress
- Strain Development Leads managed the enzyme development and strain construction staff and their tasks
- Strain Evaluation Lead managed the strain screening and bench scale fermentation staff and their tasks
- Other roles involved intellectual property, project finances

2 – Approach (Technical)

- **High level approach involved four categories**
  1. Identify and improve the catalytic properties of two key pathway enzymes
  2. Improve the robustness of the host strain (sugar consumption rate, product tolerance)
  3. Combine the improved enzymes with host strain, and make further strain improvements (modulating expression of key genes)
  4. Develop a fermentation process that utilizes lignocellulosic hydrolysate and methanol as feedstocks, and evaluate strains for key performance targets

2 – Approach (Technical)

- **Top technical challenges**
  1. Development of the two key pathway enzymes such that they have sufficient substrate specificity and catalytic throughput necessary to achieve product specifications (titer, rate, and FAME specificity)

  2. Development of a fermentation process that overcomes challenges of feeding both cellulosic hydrolysate and methanol, and the insolubility of the final product

Key Performance Targets to Track Progress Against Milestone

Key Performance Targets (KPT)
• 3-Ketoacyl-CoA Synthase in vitro activity (U/mg)
• Wax Ester Synthase in vitro activity (U/mg)
• Cellulosic sugar uptake rate (g/L-hr)
• Caprylic ester titer (g/L)
• Caprylic acid or ester specificity (%)
• Caprylic acid or ester production rate (g/L-hr)

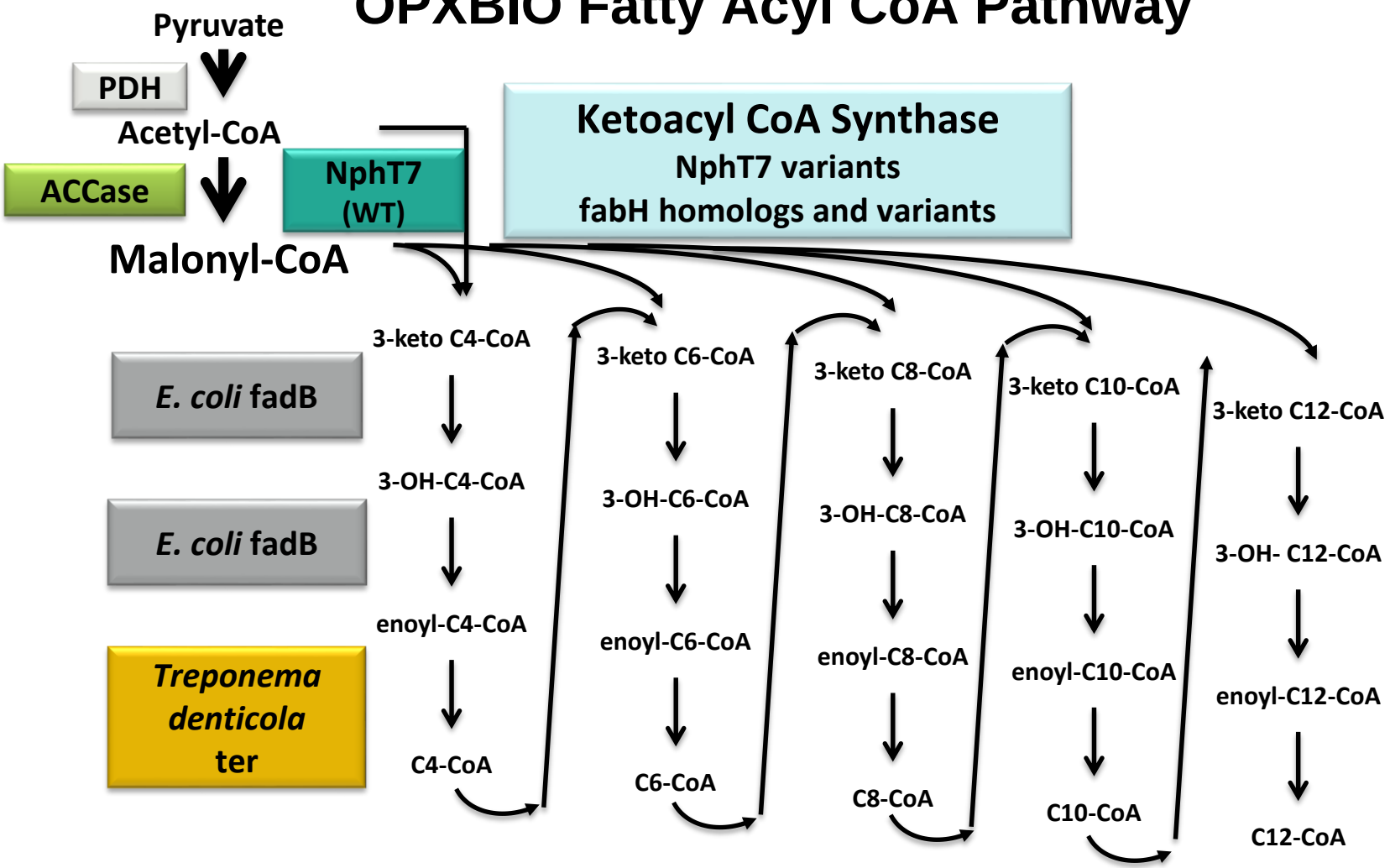
Work Breakdown Structure

Milestone and Go/NoGo Gates

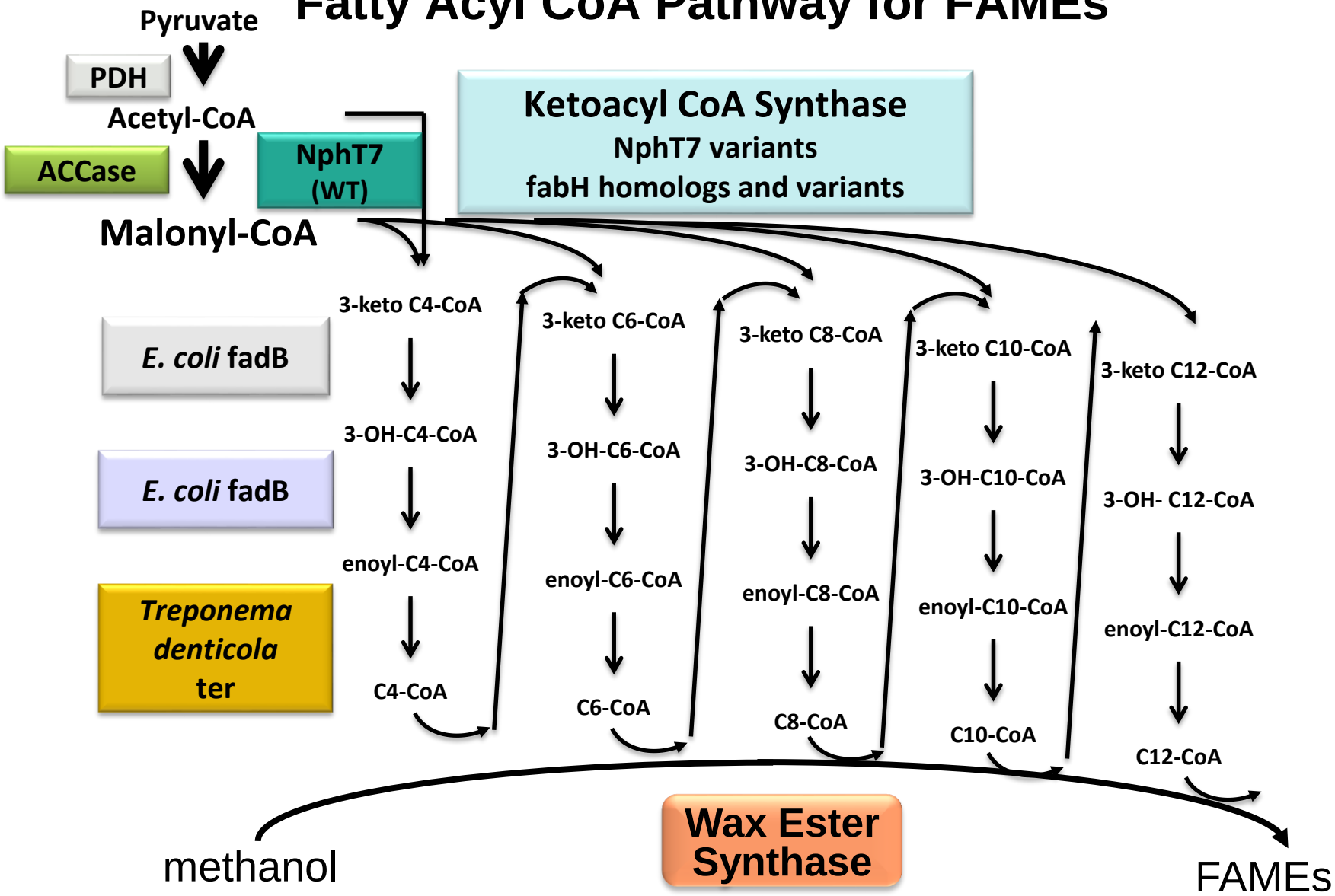
Task	Year 1				Year 2			
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8
Task 1: Production Pathway Engineering								
1.1 3-Ketoacyl-CoA Synthase Engineering		♦		●				
1.2 Ester Synthase Engineering		♦		●				
1.3 Pathway enabled in Base Host Strain to meet KPTs		♦		●				
Task 2: Production Host Strain Engineering								
2.1 Improved Cellulosic Sugar Utilization						♦		
Task 3: Strain Construction								
3.1 Assembly of C8 Pathway in Production Host Strain					♦		♦	
Task 4: Bioprocess Development								
4.1 Process Development for C8 Ester Production and Cellulosic Sugar Feedstocks						♦		
4.2 Integrated Process Development								●

Diamond symbols (♦) indicate milestones; Circle symbols (●) indicate go/no go decision points.

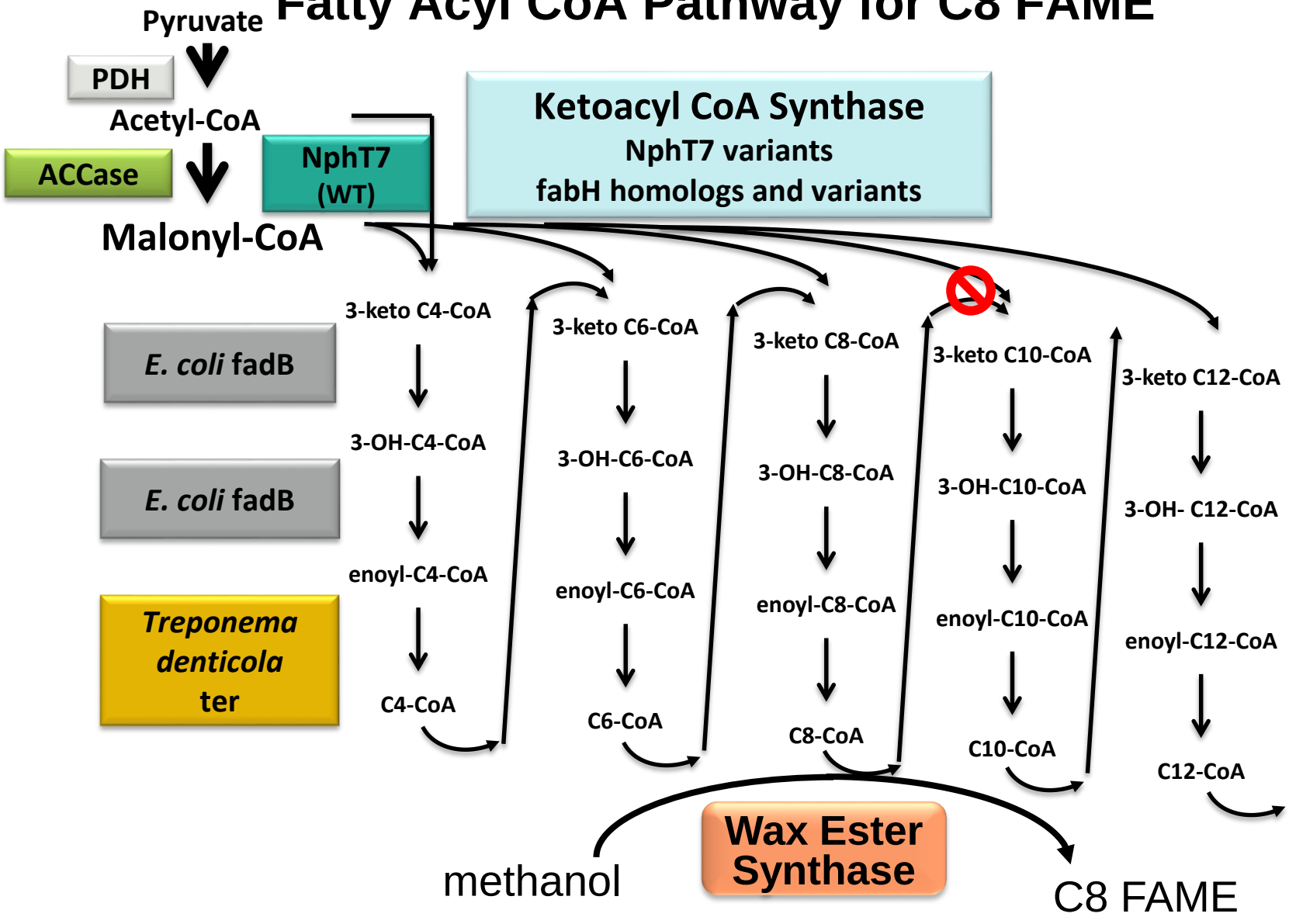
OPXBIO Fatty Acyl CoA Pathway



Fatty Acyl CoA Pathway for FAMEs



Fatty Acyl CoA Pathway for C8 FAME



Improving Ketoacyl CoA Synthases

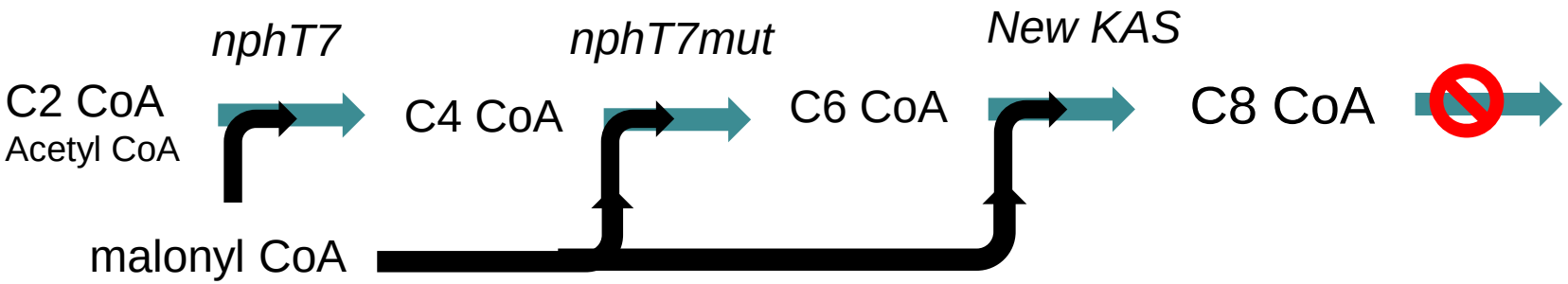
Two distinct types in the bioengineered pathway

nphT7

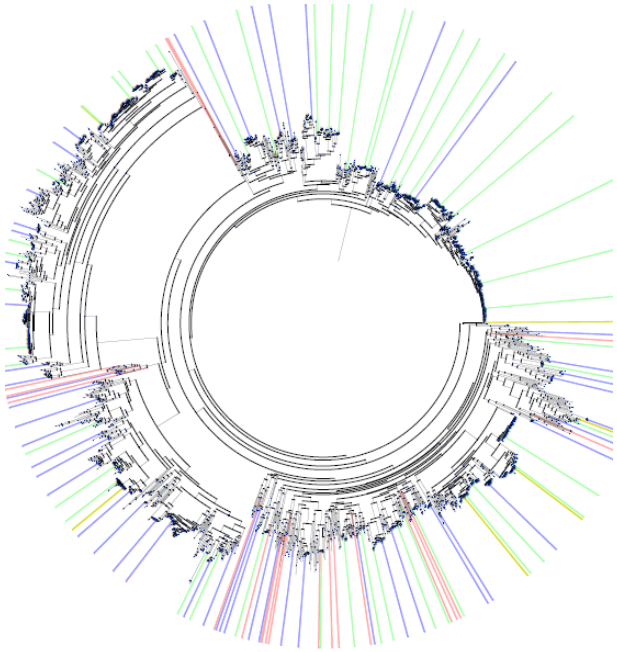
- Streptomyces, mevalonate pathway
- WT is only active on C2-CoA
- Mutants with activity on C4-CoA substrate were available

BACTERIAL fabH

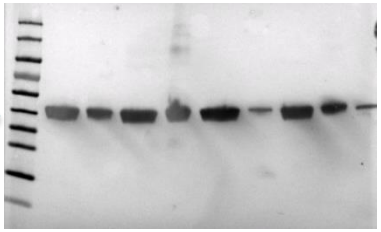
- Homologs identified and synthesized
- Expressed, purified, assayed on C2-CoA – C12-CoA
- Best candidates mutated and screened for increased activity and/or chain length specificity



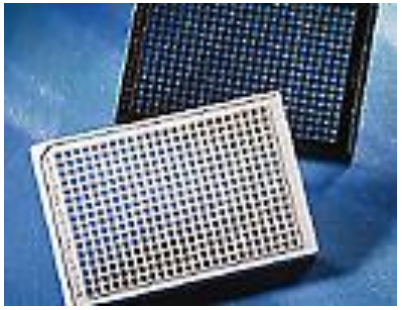
Diversity Screens for Acyl CoA Synthase Homologs



100's of fabH homologs identified and synthesized



Expressed in E. coli and purified

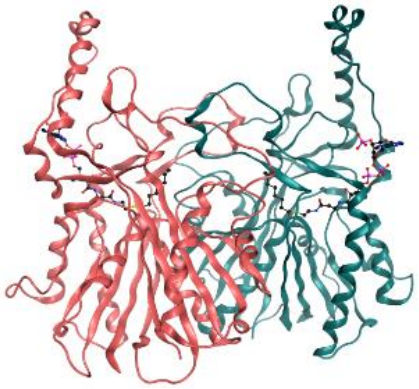


Evaluated *in vitro* for specific activity using various chain length substrates

Enzymes Improvement Approaches

Machine
Learning

Rational
Engineering



Random
Mutagenesis

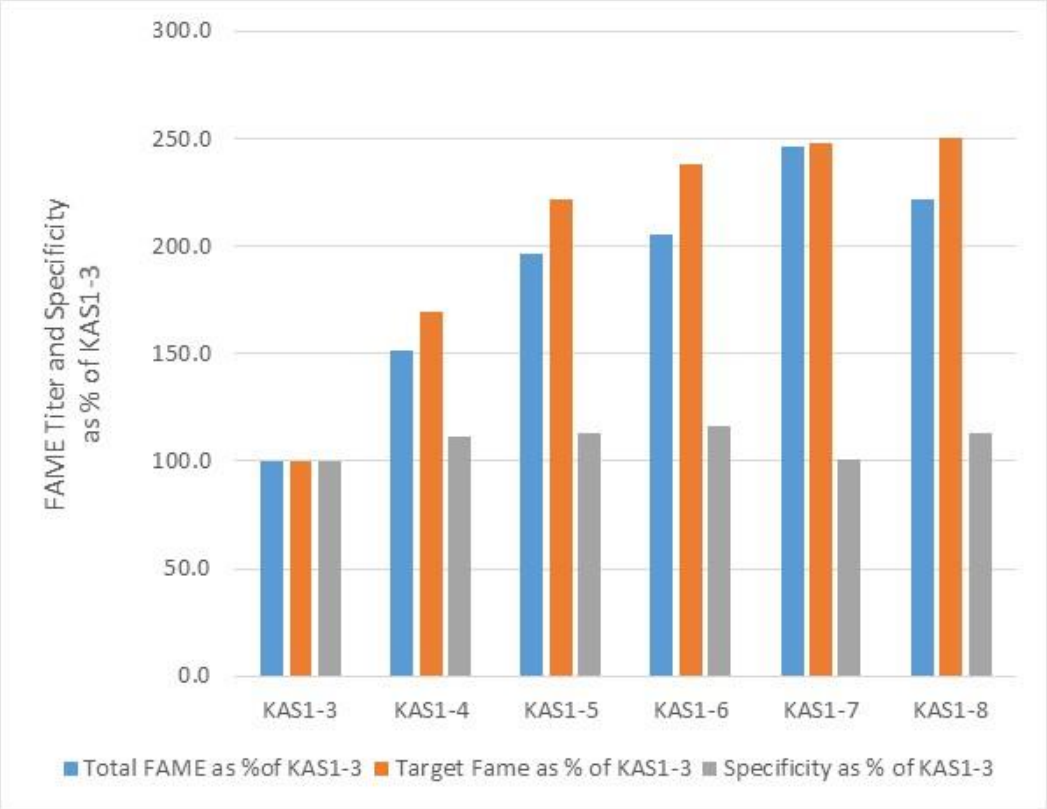
**Improved
Enzymes**

Generating and Testing Mutants of a Single KAS Homolog

- Early rounds focused on mutations within the active site
- Later rounds applied random mutagenesis to the KAS homolog gene
- Improved random mutants were sequenced and their mutations combined with others to look for synergistic effects
- Screening moved from in vitro enzyme activity measurements to in vivo product formation asssays in 1 ml microtiterplate cultures

		Lysate specific activity (U/mg)	
KAS	# of Variations	C6-CoA	C8-CoA
KAS1-5	5 mutations	0.10	0.23
KAS1-6	6 mutations	0.15	0.55
KAS1-7	6 mutations	0.10	0.05

FAME Production and Specificity of Improved KAS Mutants



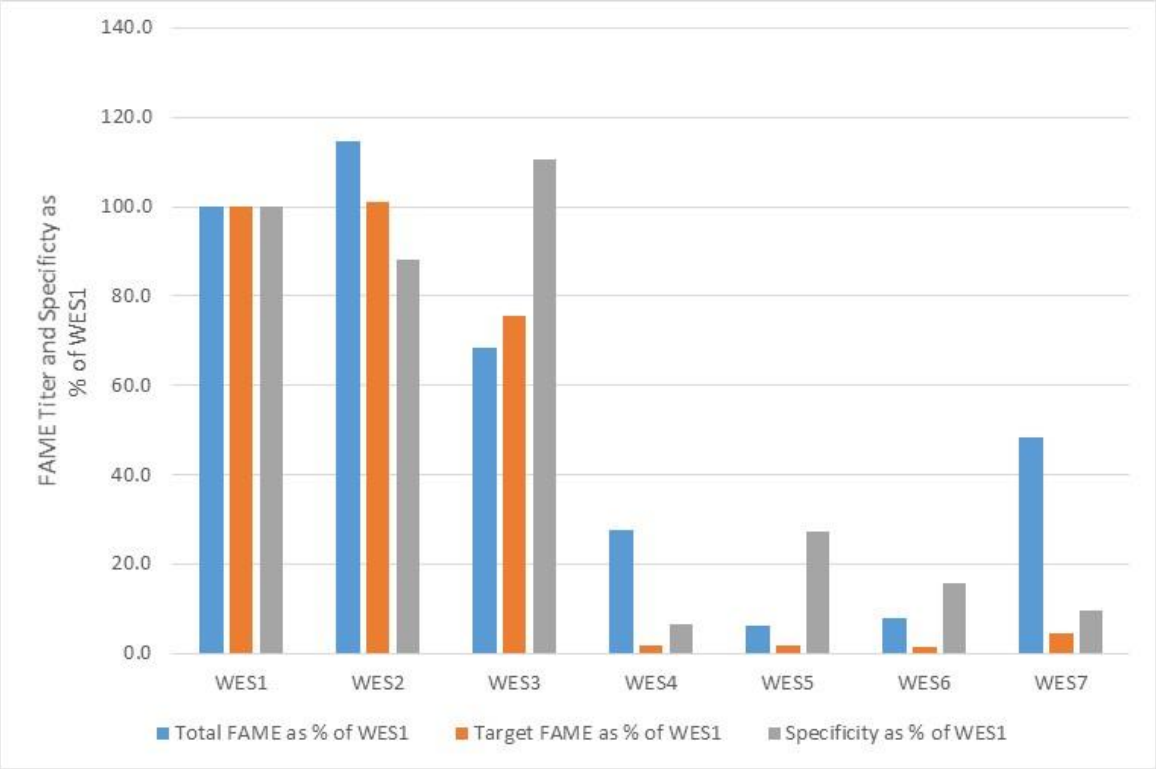
- KAS enzyme variants (single and combinations) were evaluated in small volume cultures to screen for improvements in FAME titer and specificity

Performance Targets for KAS Enzymes

Enzyme Metrics	Q2	Q4	Q8
3-Ketoacyl-CoA Synthase in vitro activity	0.06 U/mg for C6-CoA	0.08 U/mg for C6-CoA	0.1 U/mg for C6-CoA
	0.003 U/mg for C8-CoA	0.0015 U/mg for C8-CoA	0.0005 U/mg for C8-CoA

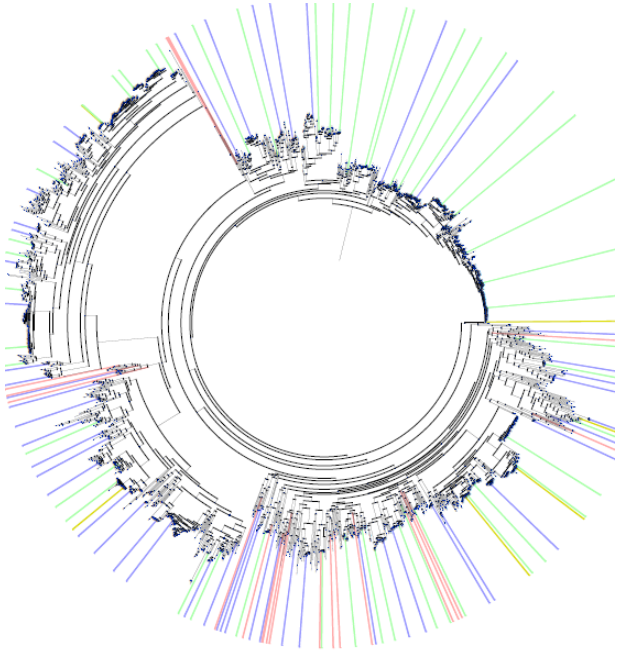
- Specific activity increases for C6-CoA were met for each Q milestone. Measured activity as high as 0.148 U/mg
- Specific activity for C8-CoA was not met, but activity was lowered significantly from 0.23 U/mg to as low as 0.05 U/mg
- Despite this, *in vivo* product specificity increased throughout the project, and did not necessarily correlate with in vitro enzyme activity

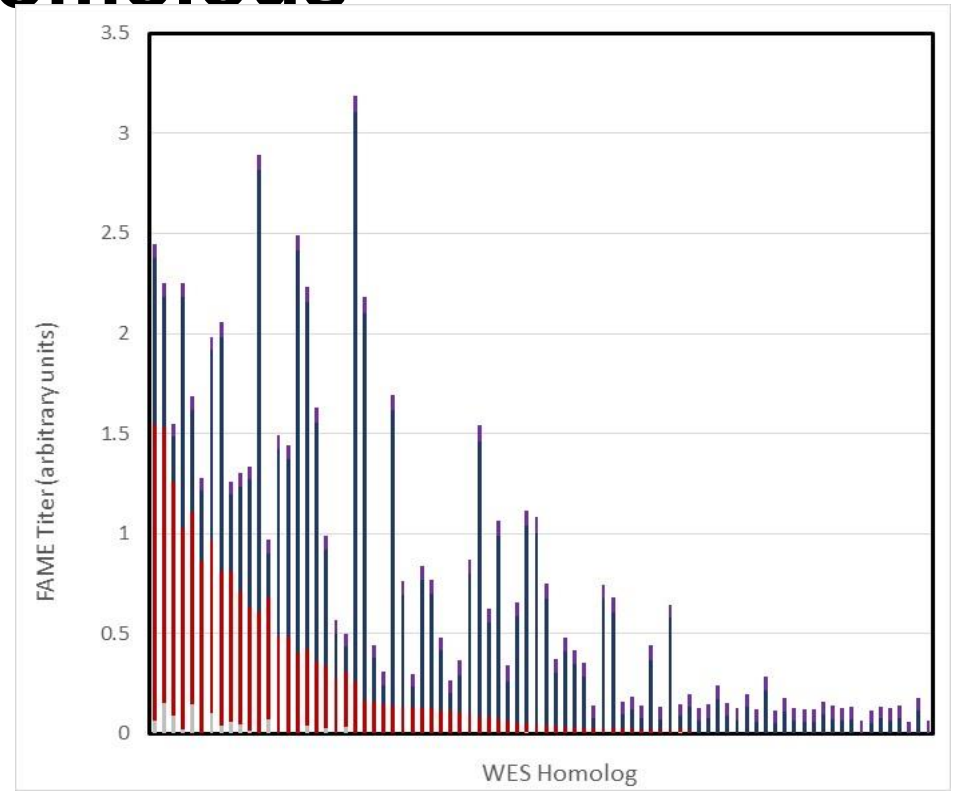
FAME Production by Strains Expressing WES Candidates



- Clear differences in FAME production and specificity among the candidates
- Some were higher in total FAME production, but lower in specificity
- Some were higher in specificity, but production was lower
- Several were low in all metrics

FAME Production by Strains Expressing WES Homologs



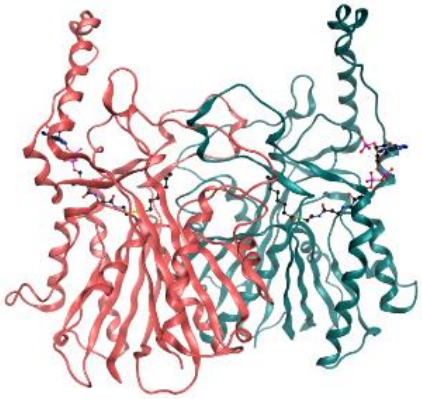


- Additional diversity screening based on homology to the leading WES candidate
- Selected best overall candidate based on FAME production and specificity for additional enzyme improvement efforts

Enzymes Improvement Approaches

Machine Learning

Rational Engineering



Random Mutagenesis

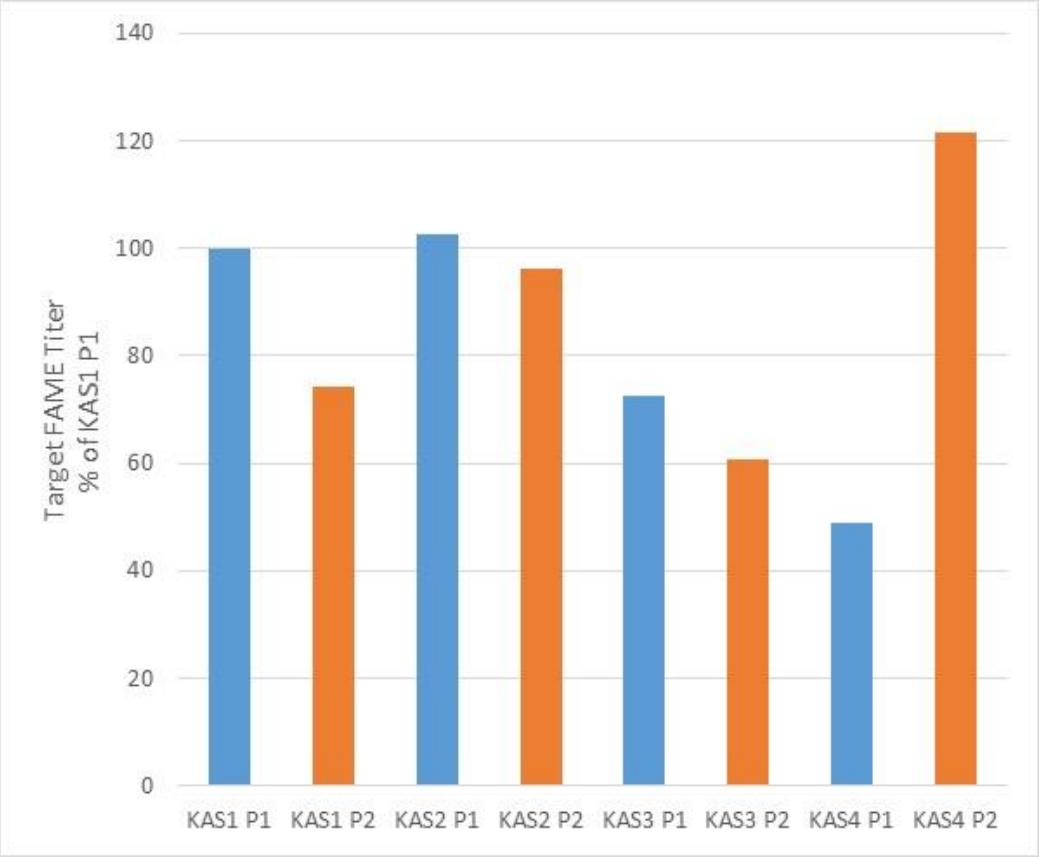
Improved Enzymes

Performance Targets for WES Enzymes

Enzyme Metrics	Q2	Q4	Q8
Wax Ester Synthase lysate in vitro activity	1.2x10-4 U/mg	4.6x10-4 U/mg	4.6x10-3 U/mg

- Final milestone for WES activity was achieved ahead of schedule
- Vast majority of the WES activity was found in the particulate fraction
- Activity varied greatly depending on the alcohol chain length, with longer alcohols reacting preferentially
- The assays reported above were conducted using methanol

Strains Expressing KAS variants Driven by Different Promoters



Sugar Utilization Rate-Round 1

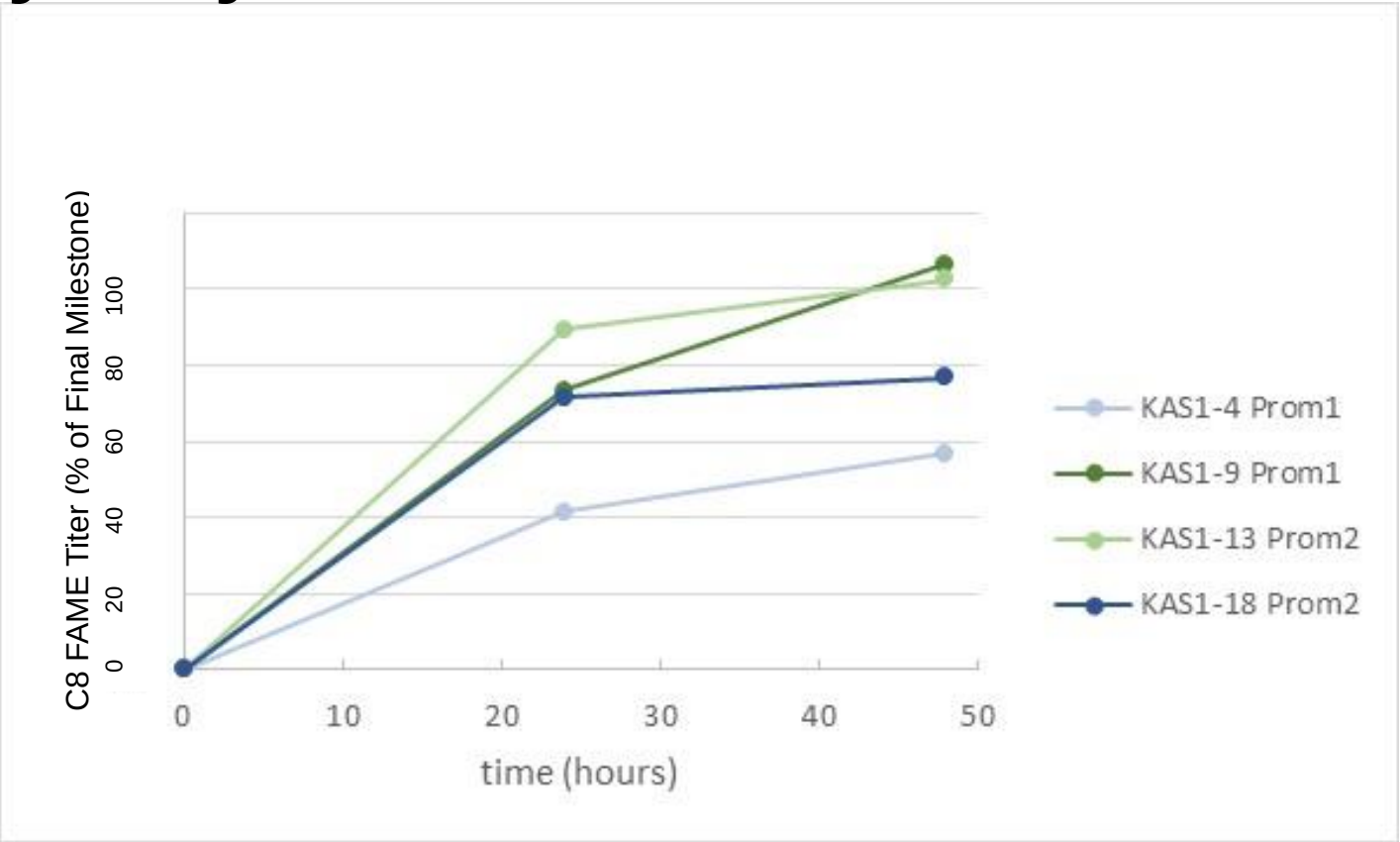
	Volumetric Sugar Consumption Rate (Glucose+Xylose) % of Final Milestone Target
Control strain, E. coli host	170%
Pathway enabled E. coli	180%

- Tested sugar consumption rate using a synthetic hydrolysate (glucose, xylose, acetate at levels found in Renmatix hydrolysate)
- Found that the host E. coli consumed the sugars very well under theses conditions

Renmatix Hydrolysate

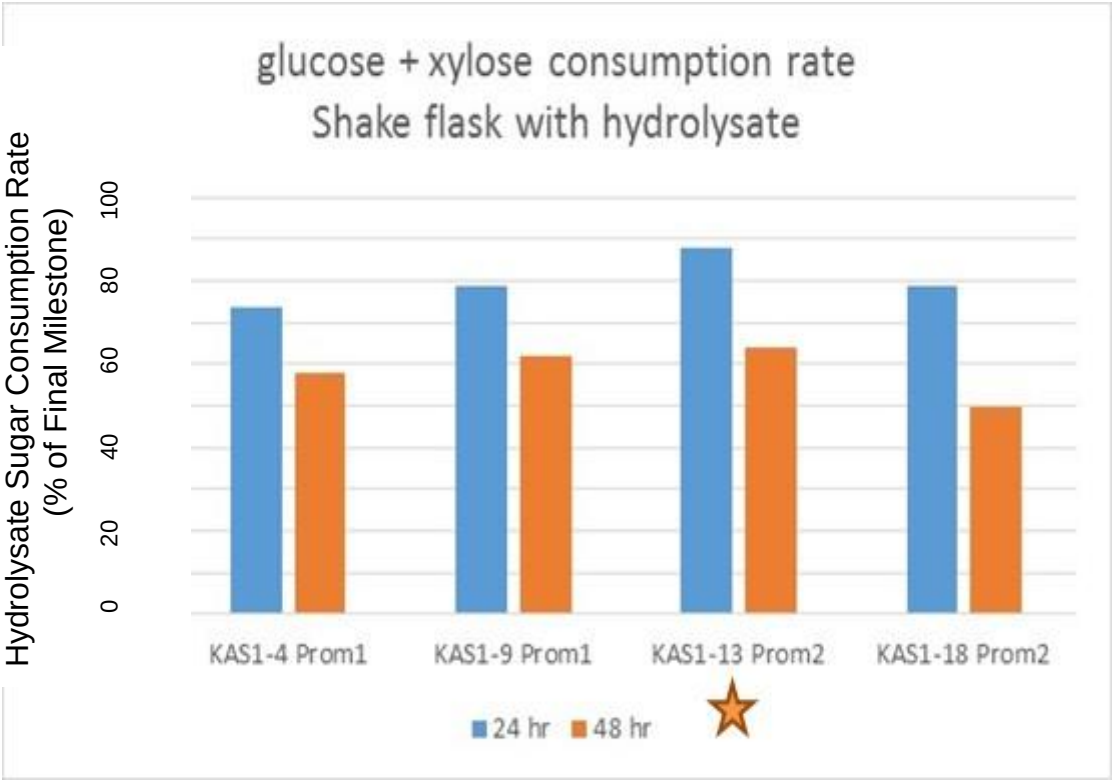
- Renmatix sugars for Cargill were produced from hardwood feedstock processes via the Renmatix Plantrose process.
- The Plantrose process utilizes water at elevated temperature and pressure to solubilize sugar and sugar oligomers without enzymes, while leaving a highly active lignin residue.
- The soluble carbohydrates were then refined into two fermentable sugar streams, one primarily C6 and one primarily C5 sugars. C6 sugar was provided for this project.
- The project utilizes the base refining scheme; however, another key advantage of the Plantrose process is flexibility in the refining scheme to meet the specification and cost requirements of the specific needs of the technology utilizing the sugars.

C8 FAME Production
Hydrolysate Medium in Shake Flasks



- C8 FAME titers reached the target for the final titer milestone
- Rate declined over time, probably due to pH drop
- Strain containing KAS1-9Prom2 was chosen for scale-up into 2L fermenters

Sugar Consumption Rate in Hydrolysate Medium



- In shake flasks using real hydrolysate medium, strains did well, but did not quite meet the final milestone for sugar consumption rate
- Factors such as pH, aeration, and osmolarity may have had negative impact

Process Development for FAME Production and Cellulosic Sugar Feedstocks

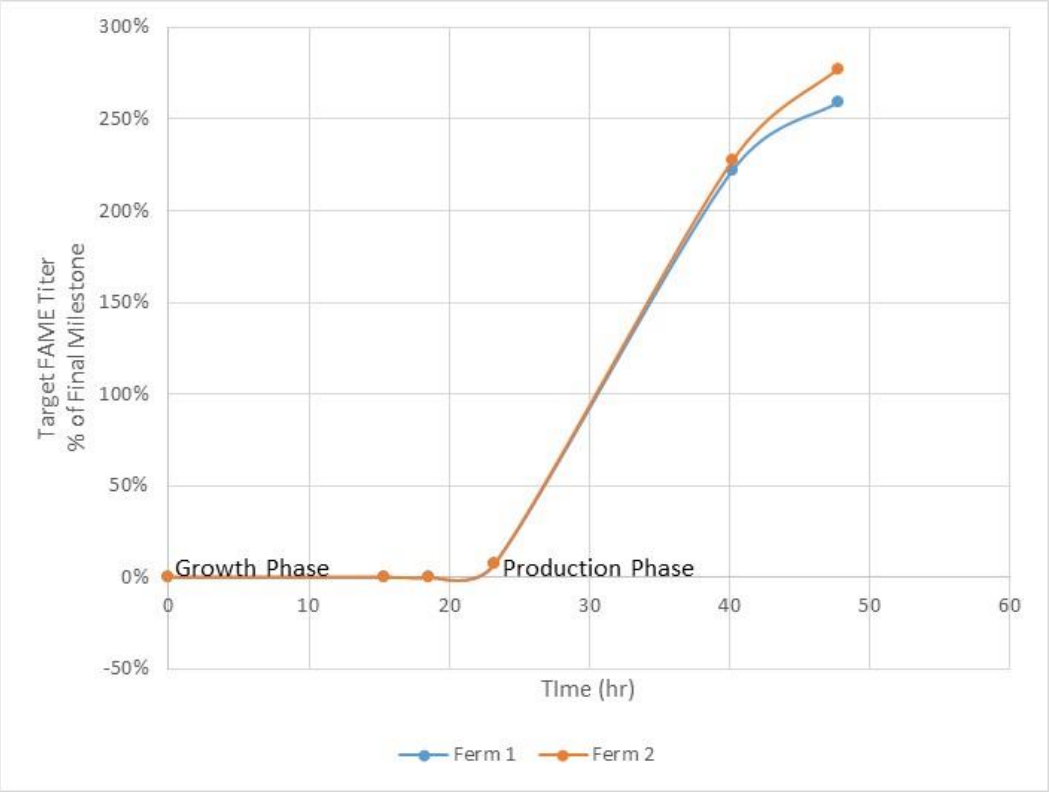
- Elements of the process that needed to be explored and optimized included fermentation agitation and aeration, pH, temperature, feeding schedules for carbon source and methanol, and concentrations of certain key nutrients.
- In addition, timing of pathway induction needed to be worked out as well as sampling methodology.
- Concentrations of medium components were adjusted to compensate for dilution due to hydrolysate and methanol feeding during the fermentation.

Benchtop Fermentations Conditions and Calculations

- Chemically defined medium.
- The first 18 hours are a biomass growth phase; no FAME production, all carbon sources depleted
- Next 6 hours are a transition phase. FAME pathway is induced.
- Production phase initiated when hydrolysate and methanol feeds are started

- FAME specificity was calculated as the C8 FAME titer divided by the sum of all FAME produced
- The production rate was calculated from the start of production phase to the end of fermentation
- Cellulosic sugar uptake was calculated from the start of production phase to the end of fermentation
- Cellulosic sugar uptake = total sugars consumed/production time

Production of Target FAME Using Renmatix Cellulosic Hydrolysate as Feedstock



- Of the cellulosic hydrolysate sugars fed, ~98% of the glucose and ~65% of the xylose were consumed during the production phase.
- It is likely that more of the xylose would have been consumed if the fermentations had been allowed to continue.

Performance vs. Project Final Milestones

Key Performance Targets	% of Final Milestone Target Using Cellulosic Hydrolysate
3-Ketoacyl-CoA Synthase in vitro activity (U/mg)	**74%***
Ester Synthase in vitro activity (U/mg)	**3250%**
Cellulosic sugar uptake rate (g/L-hr) weight of dextrose+xylose consumed/unit time	**260%**
FAME titer (g/L) weight of target FAME/volume	**269%**
FAME specificity (%) (weight of target FAME/weight of total FAME)*100	**100%**
FAME production rate (g/L-hr) weight of target FAME produced/volume*time	**460%**

*Strains earlier in the lineage were shown to have 3-ketoacyl-CoA synthase activity that met the final milestone target; however, the more advanced strains in terms of FAME production had lower activity.

4 – Relevance

- Bioenergy Technology Office mission is to engage in activities to transform renewable biomass resources into commercially viable, high-performance biofuels and bioproducts
- Goal of this project was to enable the production caprylic acid derived lubricants from cellulosic sugars
- A biological pathway for production of low-cost caprylic acid and/or ester from cellulosic sugar is a novel and potentially disruptive technology that is supportive of BETO's mission to displace all fractions of a barrel of crude oil
- A stable supply of caprylic acid at a commercially attractive price point
  - May result in a broader use of polyolesters in lubricants and oils, improving product performance and replacing the petroleum-based oils used today.
  - Will enable the development of additional market opportunities that are particularly price sensitive, such as industrial cleaners, detergents, plasticizers, food products and/or agrochemicals

5 – Future Work

- DOE funded project completed on December 31, 2017, final report submitted
- Two patent applications have been filed on technology developed during this project, prosecution of these continues
- Further internal development work is on hold due to changes in the market for capyrlic acid and priorities of the Cargill business units

Summary

1. Overview
    1. Caprylic (C8) fatty acid and FAME is used in a variety of applications
    2. Potential to be used more extensively in current applications and open the market for new applications through stable cost and supply from renewable cellulosic feedstocks
2. Approach
    - Identify and improve the catalytic properties of two key pathway enzymes
    - Improve the robustness of the host strain
    - Combine and make further strain improvements
    - Develop a fermentation process
3. Technical Accomplishments/Progress/Results
    1. Improved two key enzymes in the novel acylCoA fatty acid pathway
    2. Developed strains and fermentation process that produced C8 FAME a
    3. Met or exceeded project milestones for titer, rate, and specificity using cellulosic hydrolysate sugars to produce C8 FAME

Summary

1. Relevance
   - Transforms renewable biomass resources into commercially viable, high-performance bioproducts, especially in the lubricants area
   - Supportive of BETO's mission to displace all fractions of a barrel of crude oil
2. Future work
   - Project completed on December 31, 2017
   - Further internal development work is on hold

Summary

Thank you

Questions?

Additional Slides

Responses to Previous Reviewers' Comments

- This project has not undergone a previous peer review
- Passed the Go/No-Go Review at the end of Yr1

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Publications, Patents, Presentations, Awards, and Commercialization

- Two patent applications have been filed based on work from this project
- There have been no technology transfer or commercialization efforts

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Activity of Purified 3-Ketoacyl-CoA Synthase Homologs

3-ketoacyl-CoA synthase	Measured Activity on Substrate (U/mg)				
	C2-CoA	C4-CoA	C6-CoA	C8-CoA	C10-CoA
Homolog 1	0.09	0.55	1.00	1.14	0.00
Homolog 2	0.01	0.26	0.64	1.35	0.00
Homolog 3	0.07	0.58	0.58	0.93	0.00
Homolog 4	0.01	0.39	0.57	0.80	0.00
Homolog 5	0.04	0.28	0.53	1.81	0.02
Homolog 6	0.05	0.43	0.45	1.39	0.06
Homolog 7	0.02	0.33	0.39	1.76	0.03
Homolog 8	0.02	0.21	0.38	0.69	0.00
Homolog 9	0.02	0.24	0.37	1.16	0.02
Homolog 10	0.00	0.13	0.32	0.47	0.00

- Several of the most promising homologs were expressed in the pathway enabled E. coli host and screened for FAME production
- The best of these was taken forward for further improvement using both directed active site mutations as well as random mutagenesis

Specific Activities of Purified Ester Synthases on Acyl-CoA Substrates with Methanol

	Measured Activity on Substrate (U/mg x10^3)		
Enzyme	**C6-CoA**	**C8-CoA**	**C10-CoA**
Ester synthase 1	5.30	4.36	3.65
Ester synthase 2	2.00	2.21	1.27
Ester synthase 3	0.00	2.09	1.27
Ester synthase 4	0.00	1.39	1.27
Ester synthase 5	1.84	1.26	0.85
Ester synthase 6	0.46	1.14	1.16
Ester synthase 7	0.00	1.07	0.53
Ester synthase 8	0.00	0.95	0.85
Ester synthase 9	0.00	0.88	0.32
Ester synthase 10	0.00	0.57	0.48
Ester synthase 11	0.46	0.38	0.32
Ester synthase 12	0.00	0.38	2.91
Ester synthase 13	0.00	0.38	1.11
Ester synthase 14	0.46	0.38	1.22
Ester synthase 15	0.00	0.38	0.48

Mutants Generated from WES1

- Identified several mutants that improved FAME specificity
- Did not find any that improved productivity (titer)

Impact of Two-Plasmid vs. One Plasmid Pathway Expression System