Pacific Northwest
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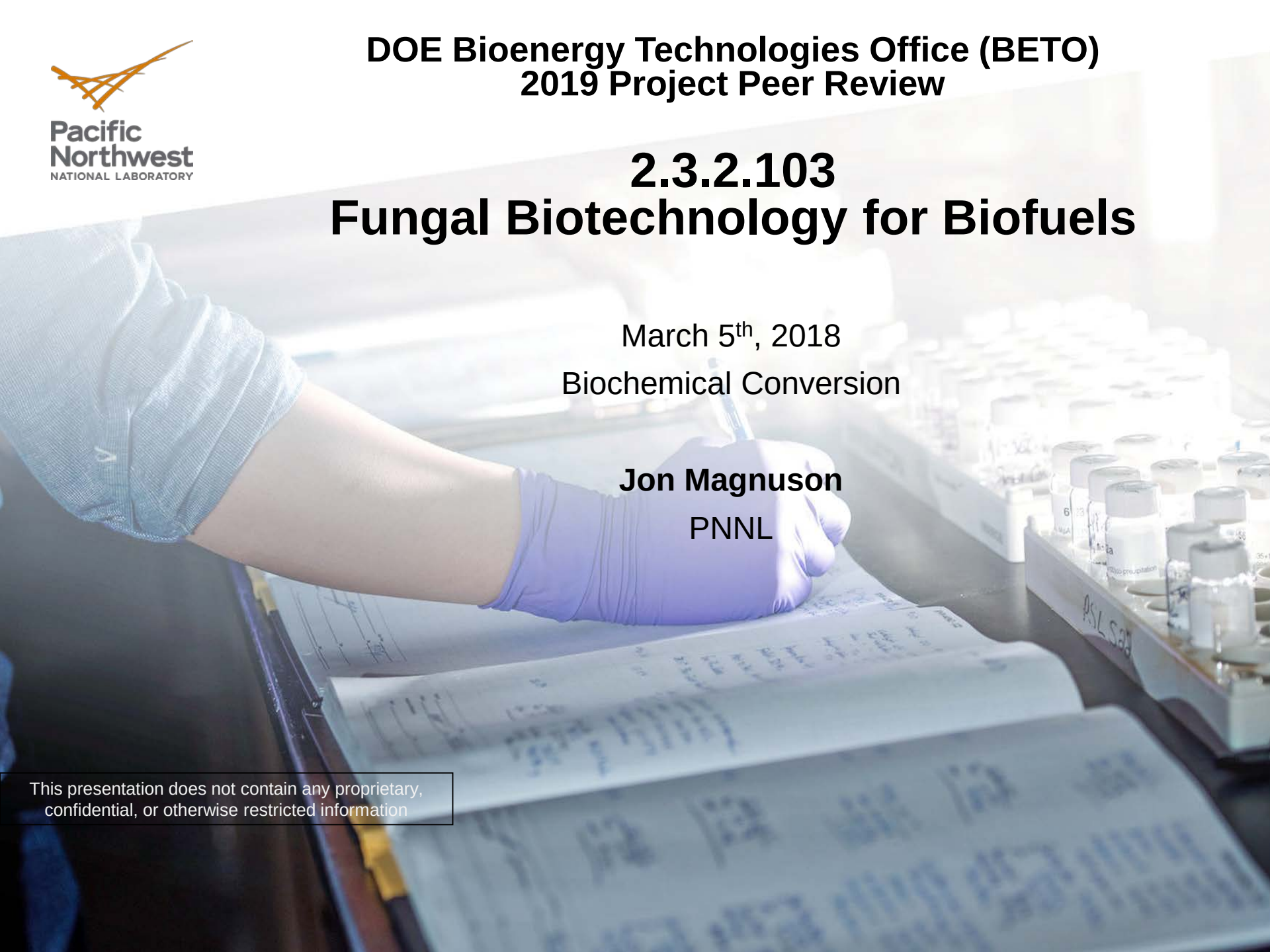
DOE Bioenergy Technologies Office (BETO)
2019 Project Peer Review

2.3.2.103
Fungal Biotechnology for Biofuels

March 5th, 2018

Biochemical Conversion

Jon Magnuson
PNNL



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Challenge Statement

- **Biological conversion routes from carbohydrates to biofuels are inherently *carbon inefficient*, since oxygen must be removed as carbon dioxide (and water, or both).**

- **Conversion to *hydrocarbon biofuels* is even more challenging.**

- **Potential Solution: *carbon fixing* reactions incorporated into the pathway for a valuable bioproduct to increase overall carbon efficiency of the biofuel & bioproduct process**

Goal Statement

Challenge to address

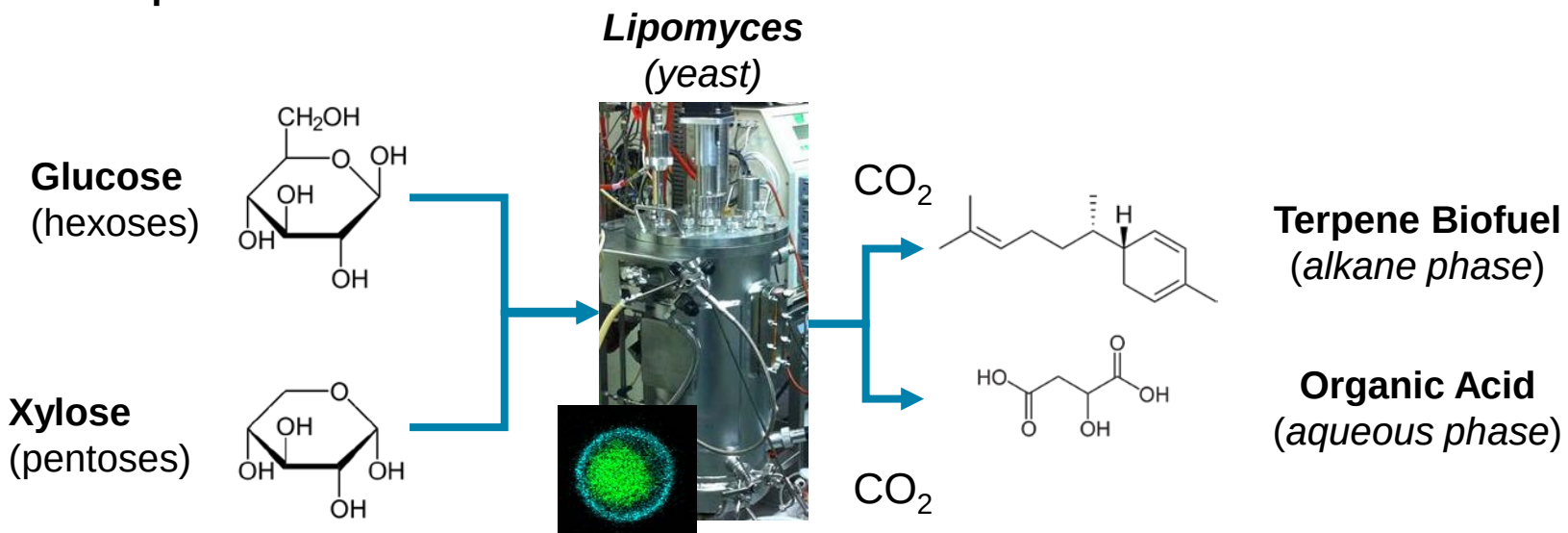
- **Conversion routes from carbohydrates to hydrocarbon biofuels are inherently carbon inefficient.**

Project Goal

- **Engineer a *Lipomyces* strain that maximizes carbon efficiency by conversion of biomass sugars to phase separated biofuels and bioproducts in parallel.**

Project Outcome

- **Produce at least 5 g/L of both a sesquiterpene biofuel and a phase-separated organic acid co-product that incorporates CO_2 on lignocellulosic hydrolysates by the end of the project (36 months) as a significant advancement towards a techno-economically viable bioprocess**.



Quad Chart Overview

Timeline
- Project start date: 10/1/2018
- Project end date: 9/30/2021
- Percent complete: 11%

	Total Costs Pre FY17**	FY 19 Costs	FY 20 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded		650K	650K	1950K
Project Cost Share*				

•Partners: Non-funded industrial advisory panel

Barriers addressed

Ct-F. Increasing the Yield from Catalytic Processes

Ct-D. Advanced Bioprocess Development

Objective

Engineer a *Lipomyces* strain that maximizes carbon efficiency by conversion of biomass sugars to phase separated biofuels and bioproducts in parallel.

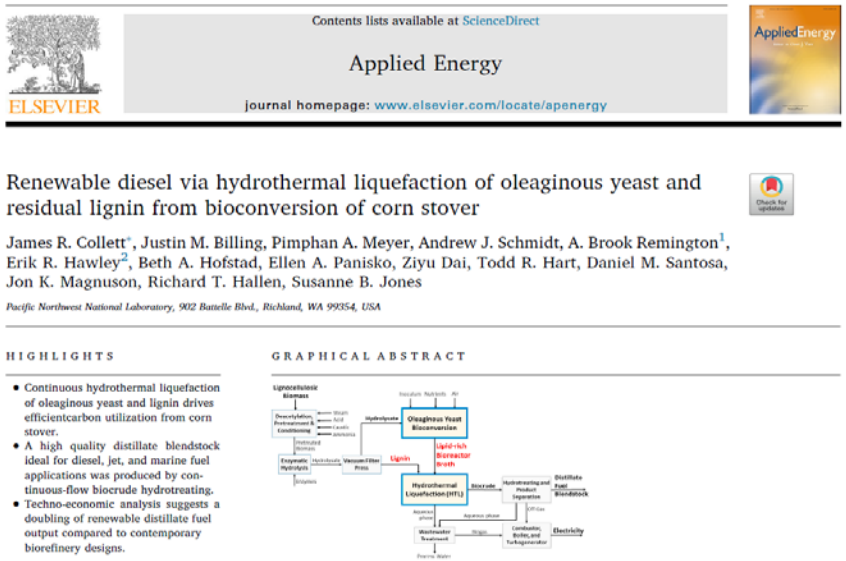
End of Project Goal

Produce 5 g/L of both a terpene hydrocarbon fuel precursor and a valuable organic acid co-product, with a TEA analysis to provide a clear assessment of the technology and to identify the most impactful areas of future R&D focus.

1 - Project Overview

History

- Our team is experienced in developing fungal **genetic engineering tools for industrially relevant fungi** (filamentous and yeasts) and applying them to development of biofuel and bioproduct **bioprocesses** in stirred tank reactors
- Previously **developed IP** regarding a genetic toolbox for the oleaginous yeast, ***Lipomyces starkeyi ;*** **USP 9,914,932** ***Agrobacterium mediated transformation of Lipomyces***
- Developed integrated conversion processes. High lipid productivity bioprocesses for hydrocarbon fuel precursors and high cell mass to use in hydrothermal liquefaction for fuels



Contents lists available at ScienceDirect

Applied Energy

journal homepage: www.elsevier.com/locate/apenergy

Renewable diesel via hydrothermal liquefaction of oleaginous yeast and residual lignin from bioconversion of corn stover

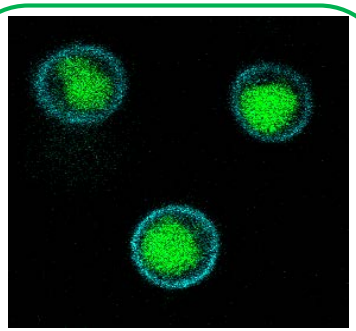
James R. Collett*, Justin M. Billing, Pimphan A. Meyer, Andrew J. Schmidt, A. Brook Remington[1], Erik R. Hawley[2], Beth A. Hofstad, Ellen A. Panisko, Ziyu Dai, Todd R. Hart, Daniel M. Santosa, Jon K. Magnuson, Richard T. Hallen, Susanne B. Jones

Pacific Northwest National Laboratory, 902 Battelle Blvd., Richland, WA 99354, USA

HIGHLIGHTS

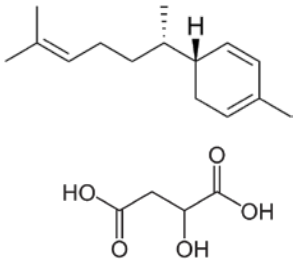
- Continuous hydrothermal liquefaction of oleaginous yeast and lignin drives efficientcarbon utilization from corn stover.
- A high quality distillate blendstock ideal for diesel, jet, and marine fuel applications was produced by continuous-flow biocrude hydrotreating.
- Techno-economic analysis suggests a doubling of renewable distillate fuel output compared to contemporary biorefinery designs.

GRAPHICAL ABSTRACT

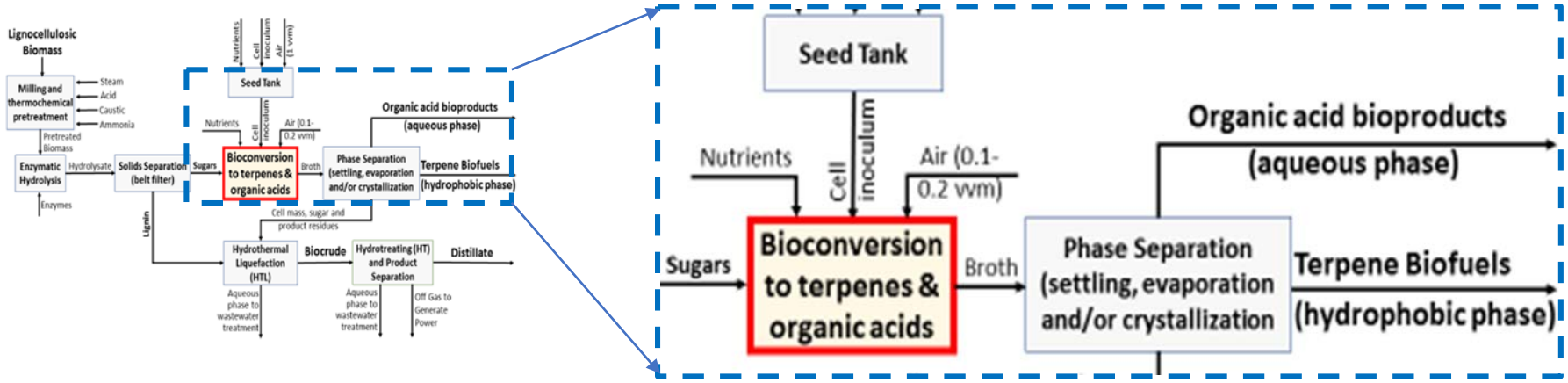


Nile red (lipid, green), Calcofluor (cell wall, blue)

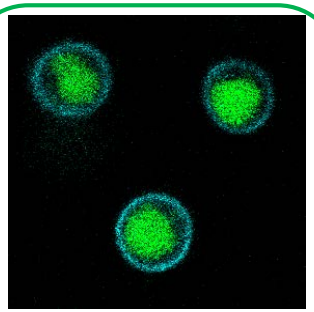
1 - Project Overview



- **Goal:** Engineer a *Lipomyces* strain that **maximizes carbon efficiency** by conversion of biomass sugars to **phase separated** biofuels and bioproducts in parallel.
- Develop an integrated organism to produce **hydrocarbon (terpene) biofuels** in a hydrophobic phase (**Task 1**) and **organic acid products in a carbon dioxide fixing pathway** in the aqueous phase (**Task 2**).
- Optimize the bioprocess in bioreactors (**Task 3**) and provide data for TEA to guide our work
- Address issues suggested by TEA and LCA regarding gas handling, by identifying yeast species that grow even better than *L. starkeyi* under **microaerobic** conditions and developing genetic tools (**Task 4**)
- Continue to think outside the **box** (conversion process) with respect to upstream & downstream processes



1 - Project Overview



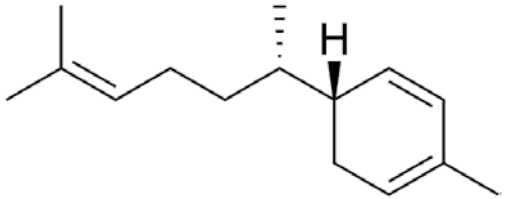
Lipomyces starkeyi

Why this organism?

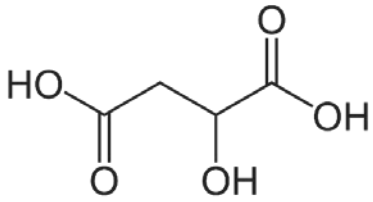
- We have genetic tools
- ***Lipomyces starkeyi*** grows on hexoses, pentoses, cellobiose = well suited for lignocellulose hydrolysates
- **Oleaginous** yeast: high metabolic flux through **Acetyl-CoA** to lipids or **terpenes**
- Tolerant to **low pH, produces the organic acid**, not the salt

Why these molecules?

- **Zingiberene**
  - 15 carbons, hydrogenated form is suitable for diesel
  - Valuable flavor product
  - Precursor for other possible products



- **Malic acid**
  - One of a family of valuable four carbon dicarboxylic acids (succinic, fumaric)
  - Acidulant in food industry, etc.



2 – Approach (Management)
Communication with BETO and Industry

- Annual operating plan (**AOP**) tied to DOE-BETO goals
- Internal project management plan (**PMP**) and **project controls expert** assigned to the project to manage personnel resources and spending
- Quarterly **milestones** & **Go/No-Go** decisions to direct, focus and measure progress
- Quarterly written reporting and phone conference conversation with **BETO TM**
- Weekly **team meetings** to keep everyone engaged and tackle technical challenges together
- Work with **fuel chemistry** experts to identify biomolecules of interest for fuels/products. Co-optima, PNNL chemists
- Work with **TEA** team to assess the technology and identify most impactful issues
- **Communication** with PIs and staff on: 2.1.0.301 “Analysis project”, 2.5.1.102 “PATCO”
- Tap into **industrial perspective** through quarterly meetings with Industrial Advisory Panel: Novozymes, POET, Mycosynthetix
- Input from a variety of internal and external **reviews**

2 – Approach (Management)

Internal Leadership Structure

- **PI, Magnuson, co-PI, Hofstad**
- **Task 1. Terpene biofuel production in *Lipomyces starkeyi* (Task lead: Ziyu Dai):** *Lipomyces starkeyi* will be genetically engineered to produce terpene biofuels in a hydrophobic phase. **M1 and M4**
- **Task 2. Phase separated co-products (Task lead: Ziyu Dai):** engineer *L. starkeyi* to produce L-malic acid in the aqueous phase during the production of the terpene biofuels to increase carbon efficiency of the overall process. **M3**
- **Task 3. Bioprocess engineering (Task lead: Jim Collett):** provide data at bioreactor scale for TEA and samples to enable systems biology experiments to identify additional gene targets for improving TRY of Task 1 and 2 targets.
- **Task 4. Alternative anaerobic yeasts (Task lead: Kyle Pomraning):** characterize 2-6 facultative anaerobic yeast strains as microaerophilic alternatives to *L. starkeyi* or adjunct process organisms with unique properties. **M2**
- **Task Leads** Subject matter experts in their tasks to **lead and conduct** that specific research and provide valuable guidance on overall **project directions**
- **Co-PI Beth Hofstad** coordinates daily efforts and maintains team focus

***Scientific Team**

Jim Collett
Ziyu Dai
Shuang Deng
Beth Hofstad
Joonhoon Kim
Ellen Panisko
Kyle Pomraning
Marie Swita

2 – Approach (Technical)

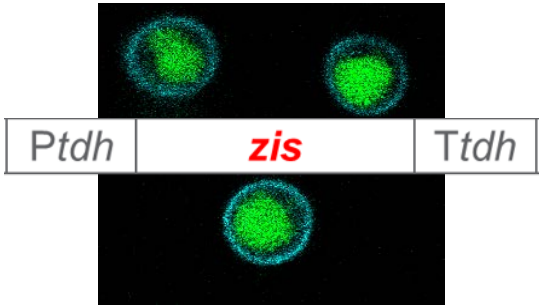
Genetic & bioprocess engineering

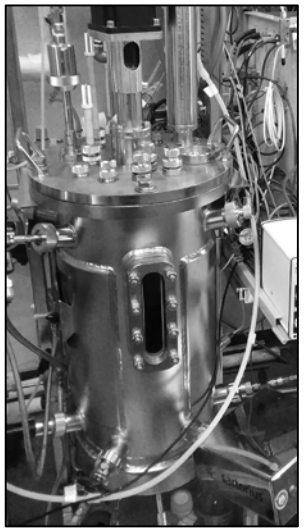
Approach: Utilize modern fungal genetic engineering and bioprocess engineering capabilities to generate a carbon efficient organism for terpene biofuels and organic acid bioproducts

Critical Success Factors

- Demonstrate pathways to both fuels and products at promising titers of 5 g/L, in separate phases
- **Increase efficient carbon capture from feedstock sugars by co-producing reduced terpene fuels and oxidized product acids**
- **Robust organisms** for conversion of challenging biomass hydrolysates containing **inhibitors** and **mixed sugars**
- Development of reproducible, robust and **efficient bioprocesses;** bioreactor optimization improves TRY and provides data to TEA team to focus research directions on impactful factors

Lipomyces starkeyi

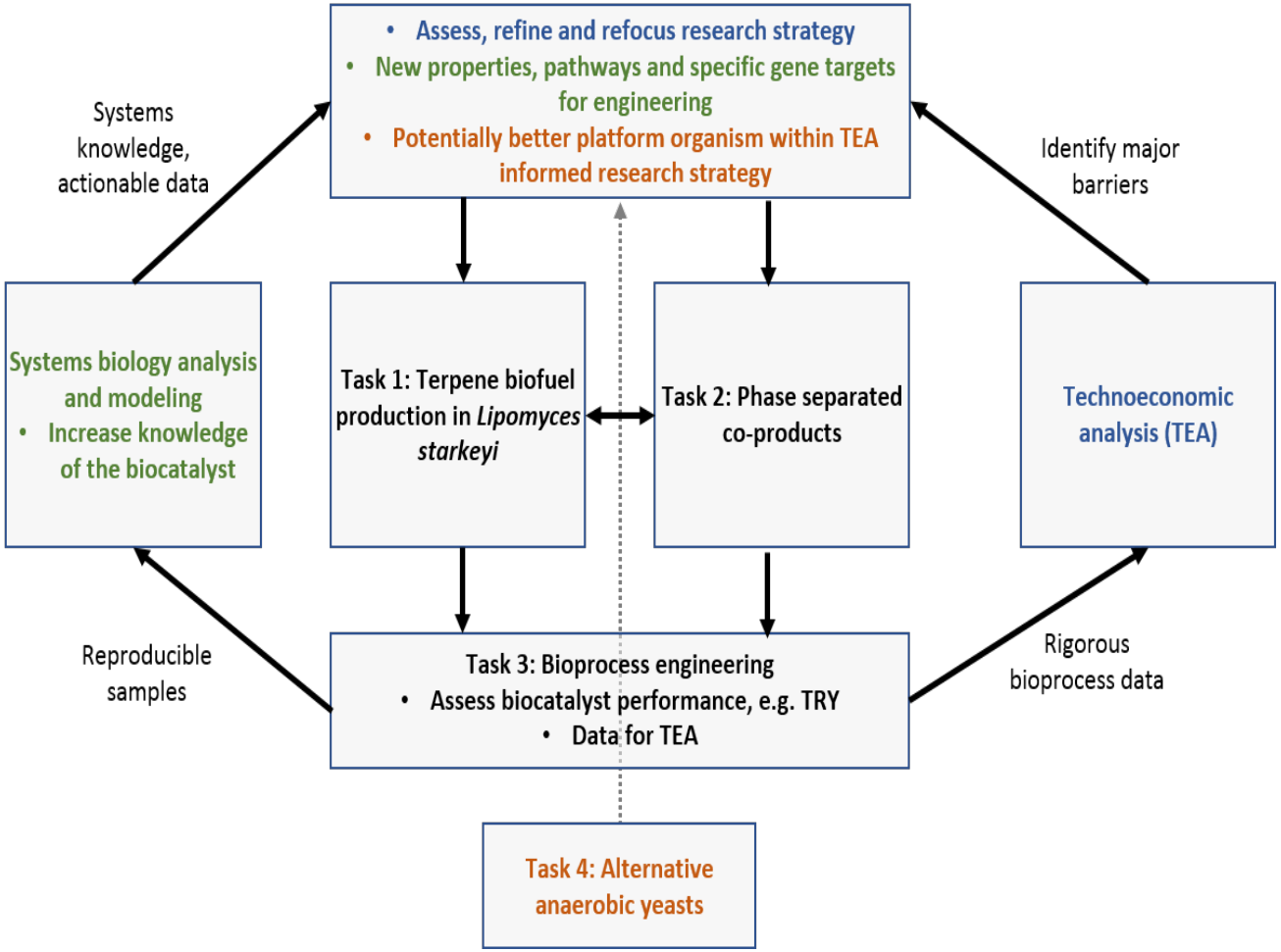




2 – Approach (Technical)

Integrated Task Structure

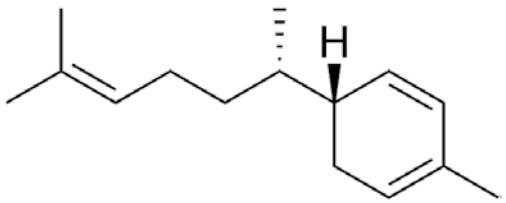
- **Task 1 and 2** are the **majority** of the effort and closely linked
- **Task 3** is the organism **bioprocess** test bed and data generator
- **Task 4** is focused on **microaerobic non-model** yeasts
- Project **leverages** ties to four other BETO projects, especially on TEA and bioprocess development



3 – Technical Accomplishments
FY19 Progress To Date. Task 1

- **Milestone 1**, Dec 31, 2018: **"Achieve gene integration for biofuels"**
- Critical to the success of this biofuel focused project, build both the very large **zingiberene synthase** (*zis*), and ZIS:FPP synthase dual gene expression constructs and obtain transformants of at least one construct in *L. starkeyi* in order to work toward our Annual Milestone"

- **Criteria**
  - Obtain a transformant with the *zis* gene integrated into the *L. starkeyi* chromosome.

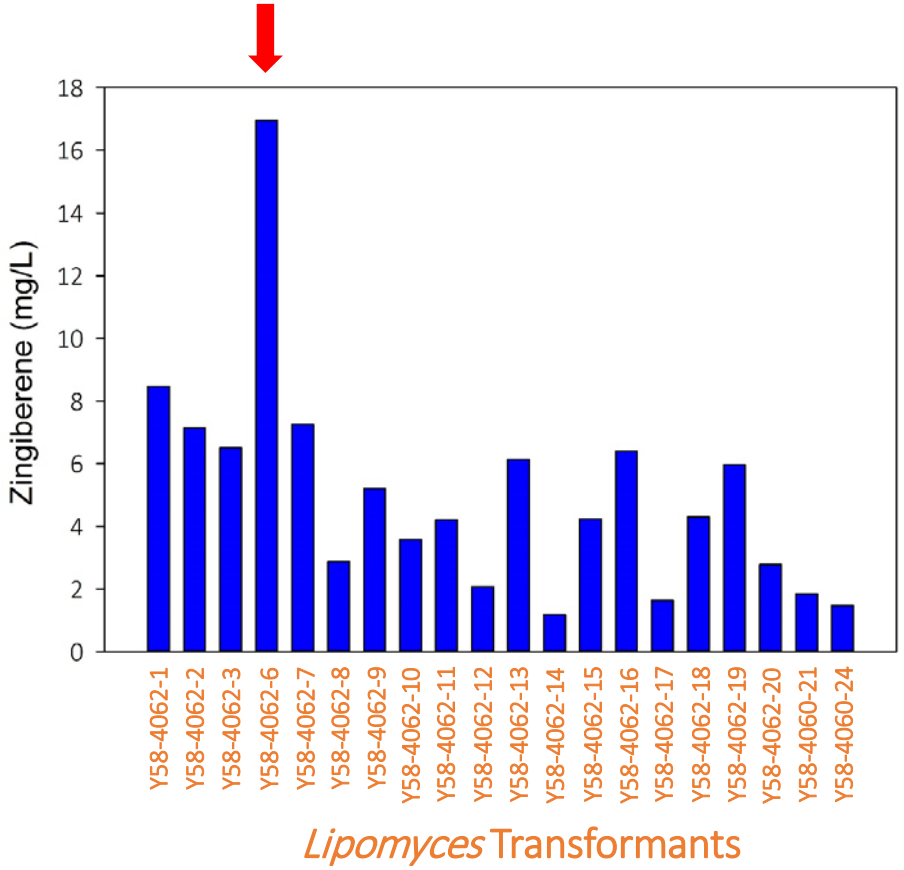


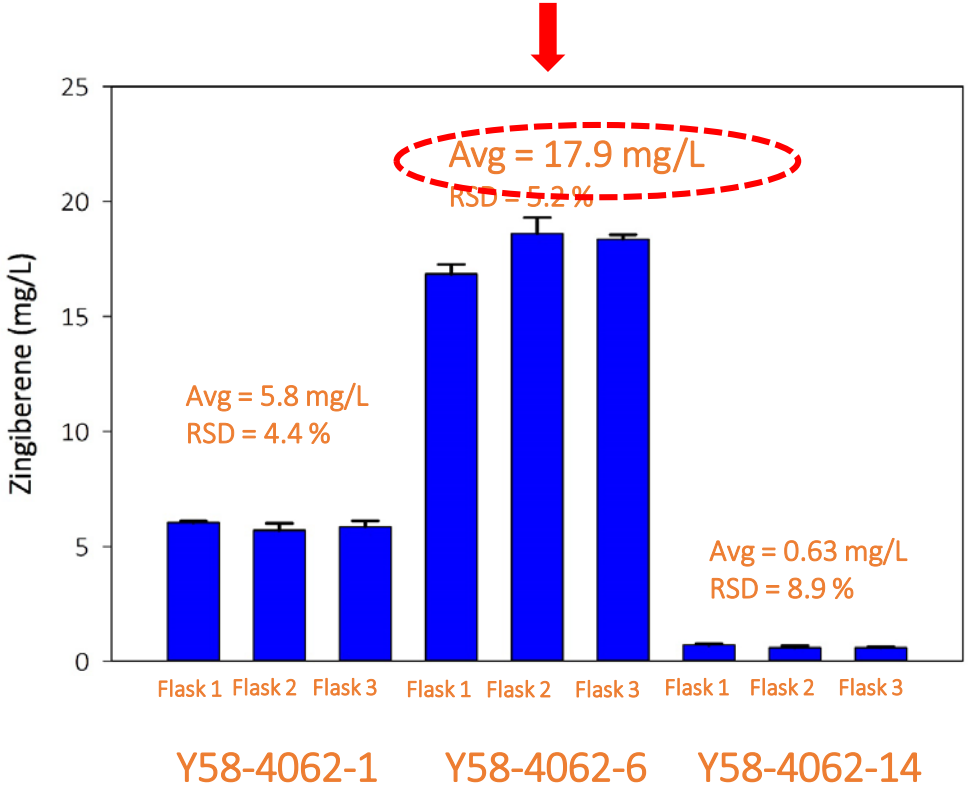
Zingiberene
- Sesquiterpene; $C_{15}H_{24}$
- Hydrogenated product = bisabolane, diesel

Task 1: Zingiberene Production in *Lipomyces*

5'-ku70	P*tdh*	***zis***	T*tdh*	*hph*	3'-ku70

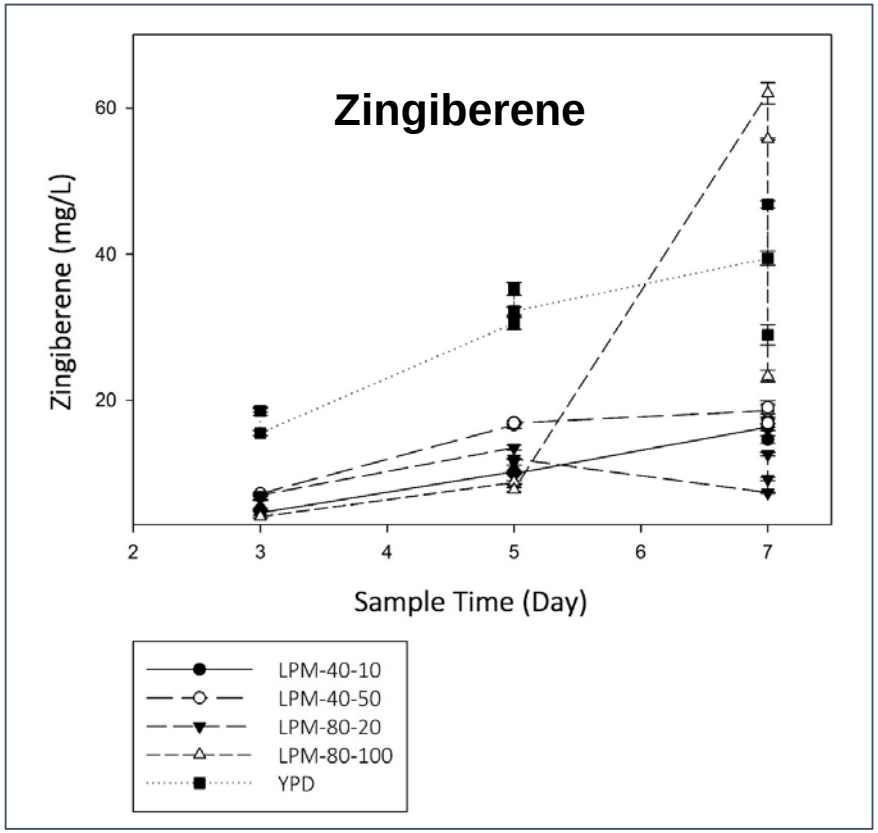
- Zingiberene synthase (*zis*) codon optimized for ***Lipomyces starkeyi***
- Transformation by ATMT (*Agrobacterium*)
- Day 4 sampling of dodecane overlay





Expression construct was successful; zingiberene production achieved.

Task 1: First Round of Media Optimization



LPM-80-100,
Avg = 44 mg/L

KEY

e.g., LPM-80-100

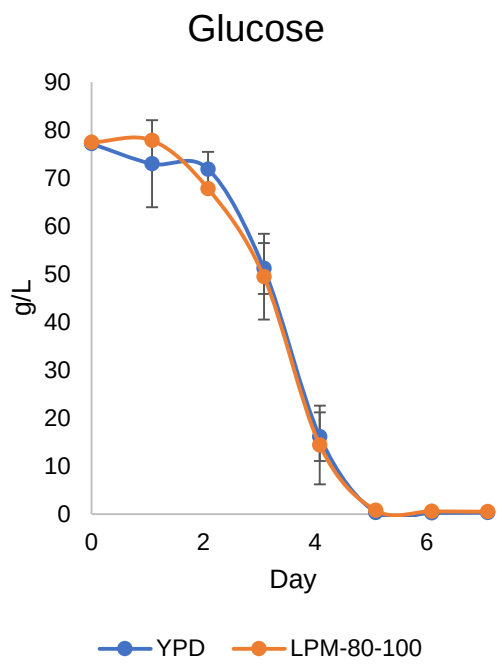
- *LPM*: Lipid Production Medium
- *80*: 80 g/L glucose
- *100*: C:N ratio = 100

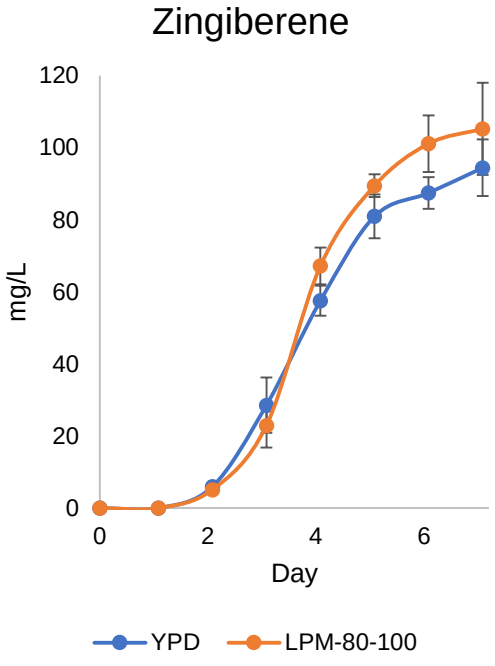
- YPD: Yeast Potato Dextrose @ 8% glucose

Media Optimization

- **Minimal medium**: Lipid production medium at different glucose concentrations and C:N ratios
- **Rich medium**: YPD (Yeast/Peptone/Dextrrose), for comparison

Task 1: Second Round: Aeration





LPM-80-100,
Avg = 105 mg/L

KEY

e.g., LPM-80-100

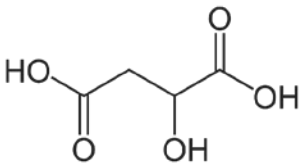
- *LPM*: Lipid Production Medium
- *80*: 80 g/L glucose
- *100*: C:N ratio = 100

- YPD: Yeast Peptone Dextrose @ 8% glucose

3 months of team met Milestone 1:

- Built constructs, transformed *L. starkeyi*
- Produced 18 mg/L **zingiberene**
- Increased initial titer 6x to **105 mg/L**
- **A lot of carbon likely still going to cell mass and triglycerides**
- **Future: genetic improvements to increase TRY**

Task 2: Phase Separated Co-products



- **Task 2.**

Engineer *L. starkeyi* to produce **L-malic acid** in the **aqueous phase** during the production of the terpene biofuel zingiberene

- **Rationale is to improve carbon efficiency of the whole bioprocess by:**
  - **Retaining maximum amount of carbon from the sugars in either biofuel or bioproduct**
  - **Phase separation: producing terpene fuels in the hydrophobic phase and organic acid products in the aqueous phase**

- Stoichiometry, energy and redox balance of alternative pathways examined in detail (**Section 5: Future Work**) but introduced briefly next…

3 – Technical Accomplishments

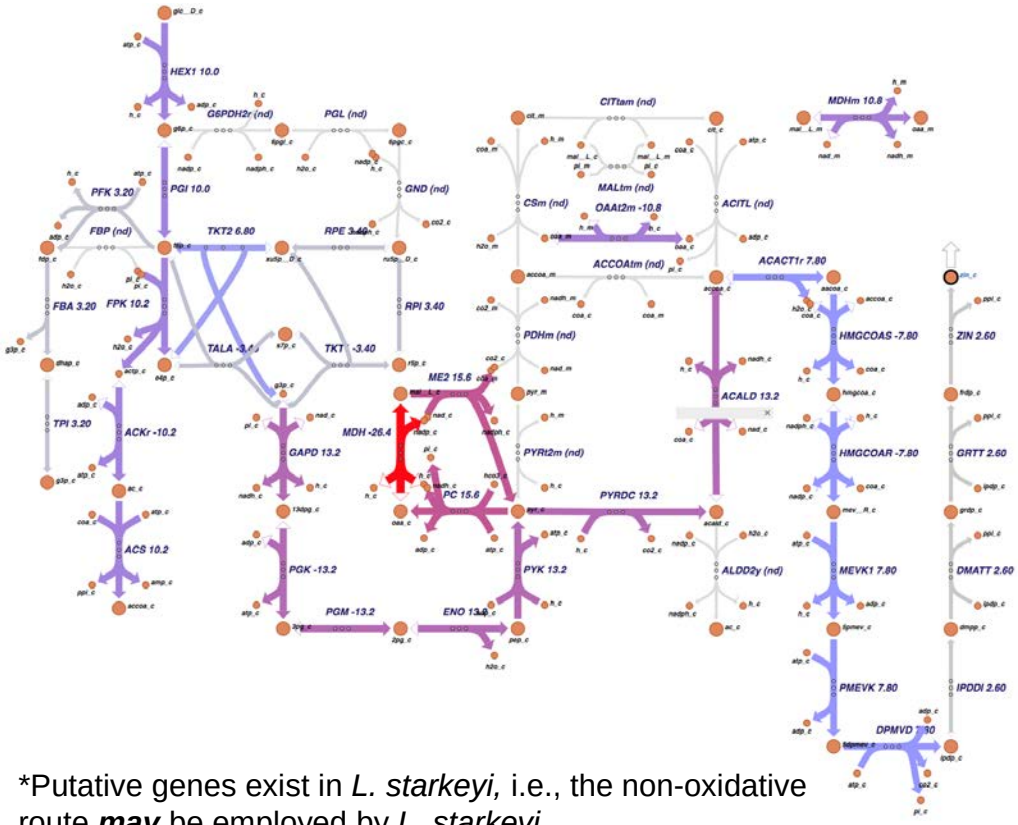
Task 1 & 2. Pathway Assessment in Genome Model Maximum yield, energy and redox balance

Zingiberene	ATP requirement	NADPH requirement	NADH generation	Zingiberene yield (mol/mol glucose)
Glycolysis	1.80	1.20 from PP pathway	3.60	0.200
Glycolysis	3.33	1.33 from malic enzyme	2.33	0.222
Non-oxidative glycolysis*	4.23	1.52 from PP pathway	1.34	0.254
Non-oxidative glycolysis*	3.60	1.56 from malic enzyme	1.08	0.260

Malic acid	ATP requirement	CO_2 requirement	NADH generation	L-malic acid yield (mol/mol glucose)
Glycolysis	0 (balanced)	2 for pyruvate carboxylase	0 (balanced)	2

- Energy and **redox cofactor (NADPH/NADH) balance** will be crucial
- Malic acid production is well balanced for all factors
- ***Challenge*****:** *Can efficient co-production be achieved?*
- ***Payoff***: Increased carbon efficiency if two CO_2 from each glucose used for terpene production can be incorporated into two molecules of Malic Acid

Task 1 & 2: Assessing metabolic energy cost and yield of co-producing zingiberene and malic acid



*Putative genes exist in *L. starkeyi,* i.e., the non-oxidative route ***may*** be employed by *L. starkeyi*

Highest Yield

- Non-oxidative glycolysis*
- NADPH from malic enzyme
- Pyruvate carboxylase

Need
3.6 ATP
1.56 NADPH

Excess
1.08 NADH

Max: 2.6 zingiberene per 10 glucose

Max: 2 malic acid per 1 glucose

FY19 Progress To Date

Milestone 2, 3/31/19 pertains to **Task 4**

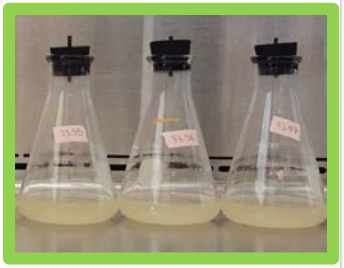
- *Select 2 non-model yeast strains with 25% higher microaerobic growth rate (cell mass accumulation rate) to L. starkeyi for further development*.

- Other criteria of importance:
  - More than one antibiotic sensitivity
  - Broad sugar substrate range
  - Tolerance to low pH
  - Tolerance to terpenes

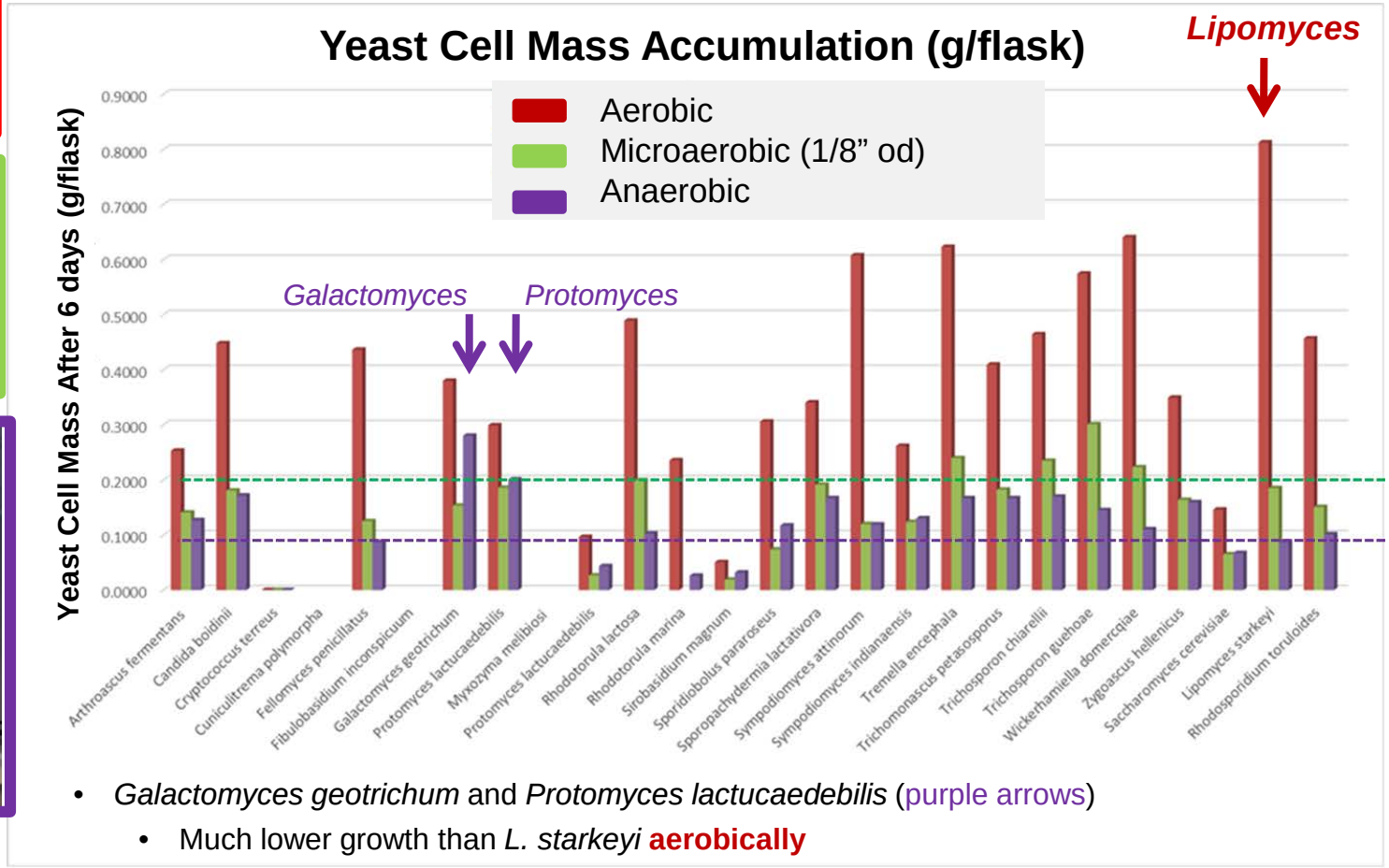
- Final selection of two species will be clear by 3/31/19

- The **aim** is to find a microaerobic strain that grows faster than *L. starkeyi* under low aeration, and for which genetic tools can be rapidly developed and has the other criteria discussed above. **Go/No-Go at 18 months**.
- This would represent a non-model organism worthy of further development.
- Test its potential by inserting the best terpene pathway identified at that later date in the project

Task 4: Alternative Anaerobic Yeasts



- **Why?** Low air/gas handling microaerobic processes have LCA advantages
- **What?** 25 diverse "yeast" species from across the Ascomycota and Basidiomycota tested for aerobic to anaerobic growth vs. *Lipomyces*



- *Galactomyces geotrichum* and *Protomyces lactucaedebilis* (purple arrows)
  - Much lower growth than *L. starkeyi* **aerobically**
  - Similar under **microaerobic** conditions
  - Better under **anaerobic** conditions

Task 4: Alternative Anaerobic Yeasts

- 25 down-selected to:
- 16 yeast species for further testing then
- **10 promising robust facultative anaerobic yeasts** chosen for further development based on:
  - Broad **substrate range** and **pH tolerance**
  - Potential for **genetic modification**

Substrate utilization | Acidic pH tolerance

	NH_3	NH_4	Glucose	Xylose	Arabinose	Galactose	Mannose	Cellobiose	DMR-EH	pH 2.16	pH 2.86	pH 3.56	pH 4.26
Lipomyces starkeyi	+	+	+	+	+	+	+	+	+	+	+	+	+
Candida boidinii	+	+	+	+	+	+	+	-	+	+	+	+	+
Zygoascus hellenicus	+	+	+	+	+	+	+	+	+	+	+	+	+
Trichomonascus petasosporus	+	+	+	+	+	+	+	+	+	+	+	+	+
Sympodiomyces indianaensis	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactomyces geotrichum	+	+	+	+	+	+	+	-	+	-	+	+	+
Trichosporon chiarellii	+	+	+	+	+	+	+	+	+	+/-	+	+	+
Tremella encephala	+	+	+	+	+	+	+	+	+	-	+	+	+
Fibulobasidium inconspicuum	+	+	+	+	+	+	+	+	+	-	+	+	+
Brettanomyces bruxellensis	+	+	+	+	+	+	+	+	+	*acetic acid and furfural resistant			

Antibiotic sensitivity (ug/mL) | Product tolerance in 10% Dodecane

	Hygromycin B				G418			Nourseothricin			Limonene (%)				Linalool (%)		
	0	15	60	240	15	60	240	15	60	240	0	0.33%	1%	3%	0.33%	1%	3%
Lipomyces starkeyi	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Candida boidinii	+	+	+	-	+	-	-	-	-	-	+	+	-	-	+	+	-
Zygoascus hellenicus	+	+	+	-	+	+	+	+	+	-	+	+	-	-	+	+	-
Trichomonascus petasosporus	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+/-	-
Sympodiomyces indianaensis	+	+	+	-	+	+	+	+	+	-	+	+	+	-	-	-	-
Galactomyces geotrichum	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+/-	-
Trichosporon chiarellii	+	+	-	-	+	+	+	+	-	-	+	+	+	-	+	+/-	-
Tremella encephala	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-
Fibulobasidium inconspicuum	+	+	-	-	+	-	-	+	+	-	+	-	-	-	+	-	-
Brettanomyces bruxellensis					*transformable												

+ indicates growth, so - indicates sensitivity to antibiotic

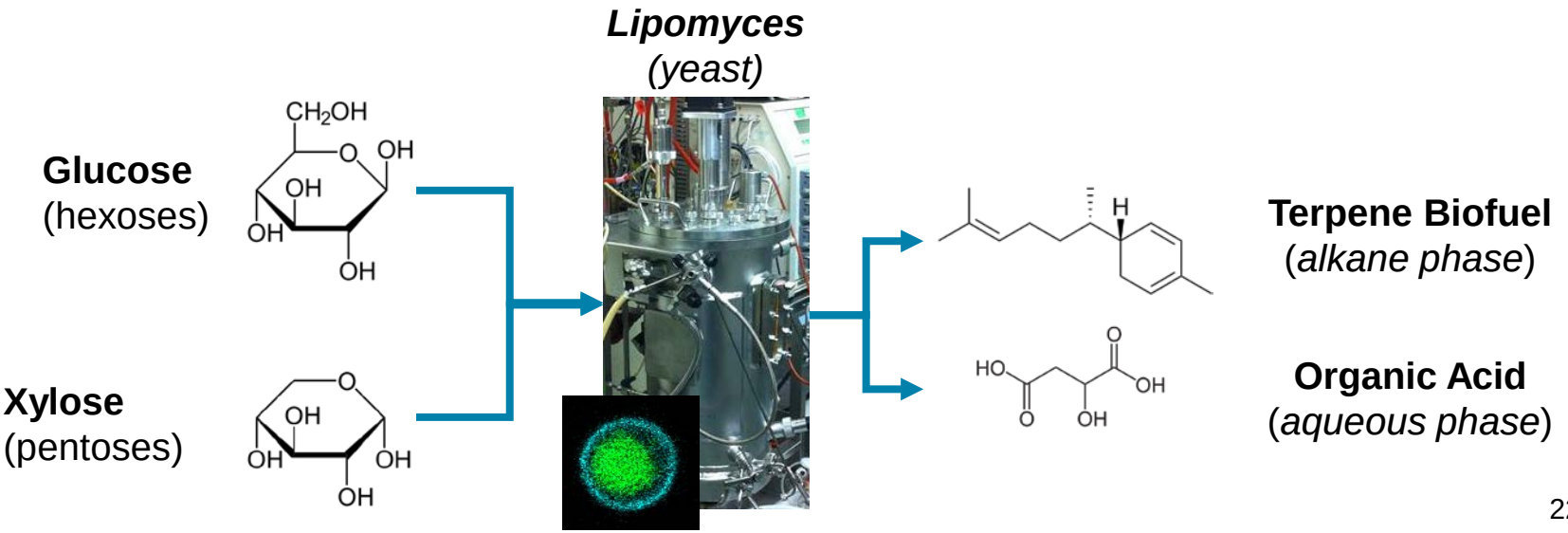
4 – Relevance: Project Goal

Project Goal

- **Engineer a *Lipomyces* strain that maximizes carbon efficiency by conversion of biomass sugars to phase separated biofuels and bioproducts in parallel.**

Project Outcome

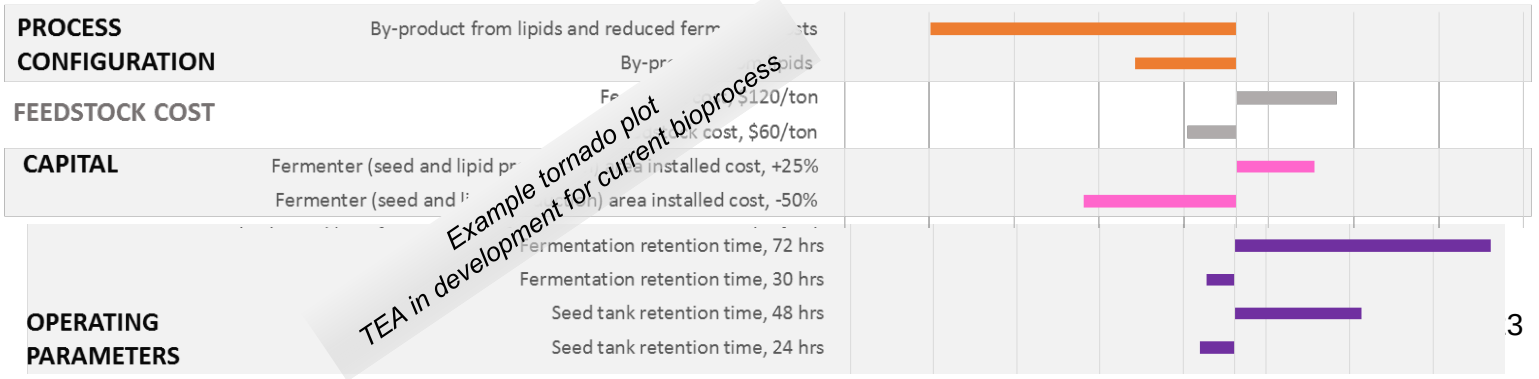
- **Produce at least 5 g/L of both a sesquiterpene biofuel and a phase-separated organic acid co-product on lignocellulosic hydrolysates by the end of the project (36 months) as a significant advancement towards a techno-economically viable bioprocess**.



4 – Relevance

BETO Program Relevance

- Challenge: biological conversion to hydrocarbon fuels is inefficient
- Innovation: building more **carbon efficient** processes with easily phase separated biofuels and bioproducts for improved process economics
- Developing towards low gas handling–**micoaerobic**—processes, to address **LCA** issue with regard to power for gas compressors
- We are building coordinated metabolic pathways for biofuels and bioproducts
- **TEA** indicates that **rate** is very important, affecting process time and capital cost
- **TEA** indicates that **yield** is very important, efficient utilization of costly feedstock
- **Bioproducts** needed to enable achievement of this goal
- **Integration** with BETO Projects: 2.5.3.104-111 Agile BioFoundry (8 NLs), 2.5.1.102 PAT, 2.1.0.301 Anal. & Sustain. Interface, 2.#.#.# “hybrid processing”



4 – Relevance

Industrial Relevance

- Development of fungal biocatalysts and bioprocesses (Tasks 1,2, 4) in **bioreactors** (Task 3)
- This **integrated development** effort on the biocatalyst and the bioprocess maximizes potential for tech transfer to the biofuels and bioproducts industry
- Active **communication** with **industrial partners** in the bioenergy marketplace helps us test the relevance of our research and provides potential tech transfer points
- Industry's **biocatalyst development needs**: lower cost, robust, reproducible, malleable
  - Produce fuels and bioproducts of value
  - Pushing toward higher TRY
  - Minimal ancillary costs: low cost nutrients, minimal pH control chemicals, resistant to contamination, cognizant of separations and waste streams
  - Reproducible bioprocesses = low failure rate, low down time
  - Biologically well understood organisms with genetic tools available to redesign the organism for other fuels/products

5 – Future Work

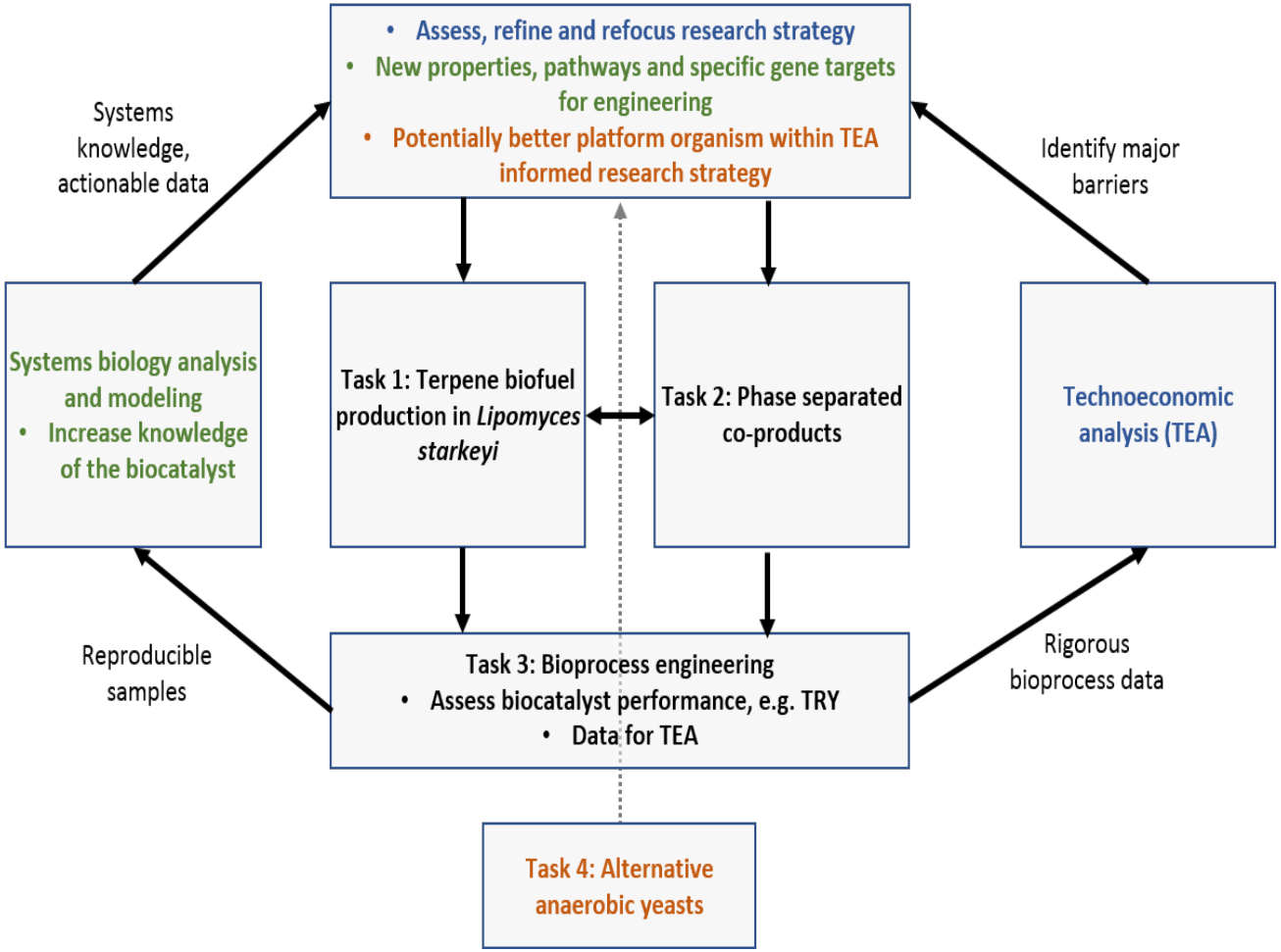
Milestones and Go/No-Go for remainder of FY19 and FY20

Milestone Name/Description	Criteria	End Date
Achieve gene integration for co-products.	Obtain a transformant containing at least the two genes for malate synthase and a carbon fixing gene expressed as active enzymes in L. starkeyi.	6/30/2019
Demonstrate a titer of at least of 0.3 g/L of zingiberene in an engineered strain.	Obtain a titer of 0.3 g/L of zingiberene in an engineered strain of L. starkeyi.	9/30/2019
Go/No-Go Can novel yeast be transformed? Is their microaerobic growth vastly superior?	Microaerophilic growth characteristics (>1 g/L/d dry cell mass). Ability to transform a selectable marker (antibiotic resistance gene) into the chosen strain.	3/31/2020
Demonstrate a titer of at least of 1.2 g/L of zingiberene and 1.2 g/L of L-malic acid in different engineered strains.	Obtain 1.2 g/L of zingiberene in one strain of L. starkeyi and 1.2 g/L of L-malic acid in another strain of L. starkeyi. Stretch: demonstrate the same titers in a single strain producing both molecules at the same 1.2 g/L titers.	9/30/2020

- Milestones tied to specific tasks and task leader
- Big push on the fuel titer (Task 1, Annual milestone) and the co-product (Task 2, M3 in June)
- Go/No-Go focused on the need to develop genetic tools in alternative yeast in order to make it useful

5 – Future Work
Integrated Task Structure

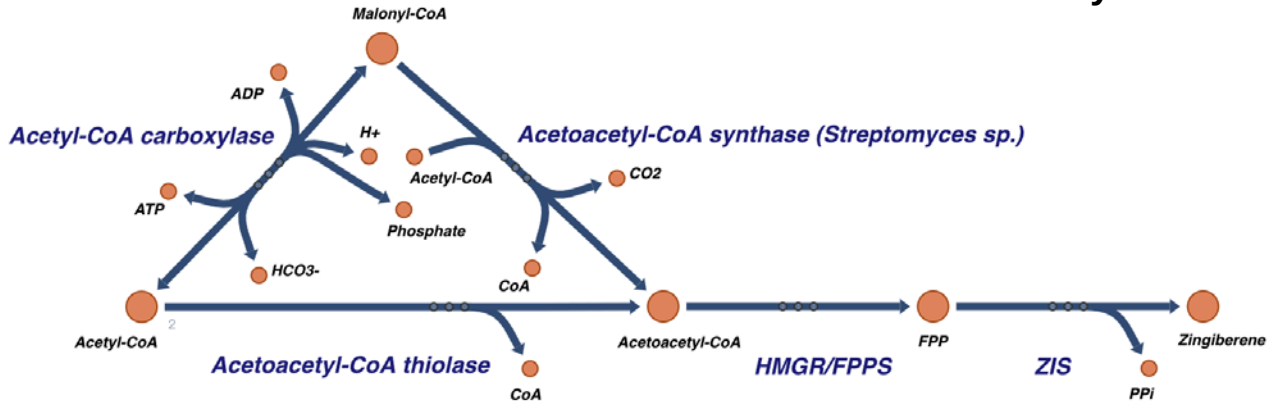
- Task 1 and 2 are the majority of the effort and closely linked
- Task 3 is the organism bioprocess test bed and data generator
- Task 4 is focused on microaerobic non-model yeasts
- Project leverages ties to four other BETO projects, especially on TEA and bioprocess development



5 – Future Work

Task 1: Constructs and Transformation

- **Milestone**: Improvement of titer from the current **105 mg/L** to **300 mg/L** by 9/30/19
- A significant challenge!
- **4 of 6 second round constructs built. Transformation underway!**



Done 5’-dga1 nptII

Done 5’-dga1 nptII Ppdc aat 3’-dga1

Done 5’-dga1 nptII Peno fpps 3’-dga1

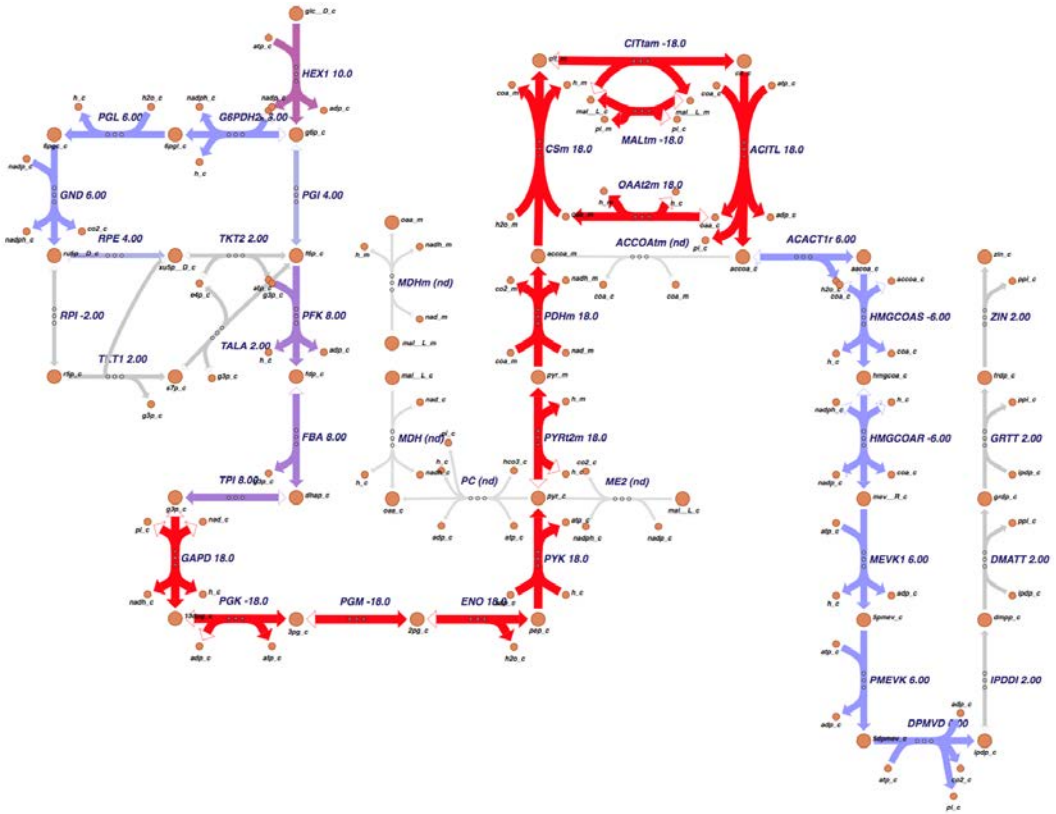
Done 5’-dga1 nptII Ptef1 hmgr 3’-dga1

5’-dga1 nptII Ppgk1 hmgs 3’-dga1

5’-dga1 fpps nptII hmgs aat hmgr 3’-dga1

5 – Future Work

Task 1&2: Zingiberene production metabolic energy cost and yield



Lowest Yield

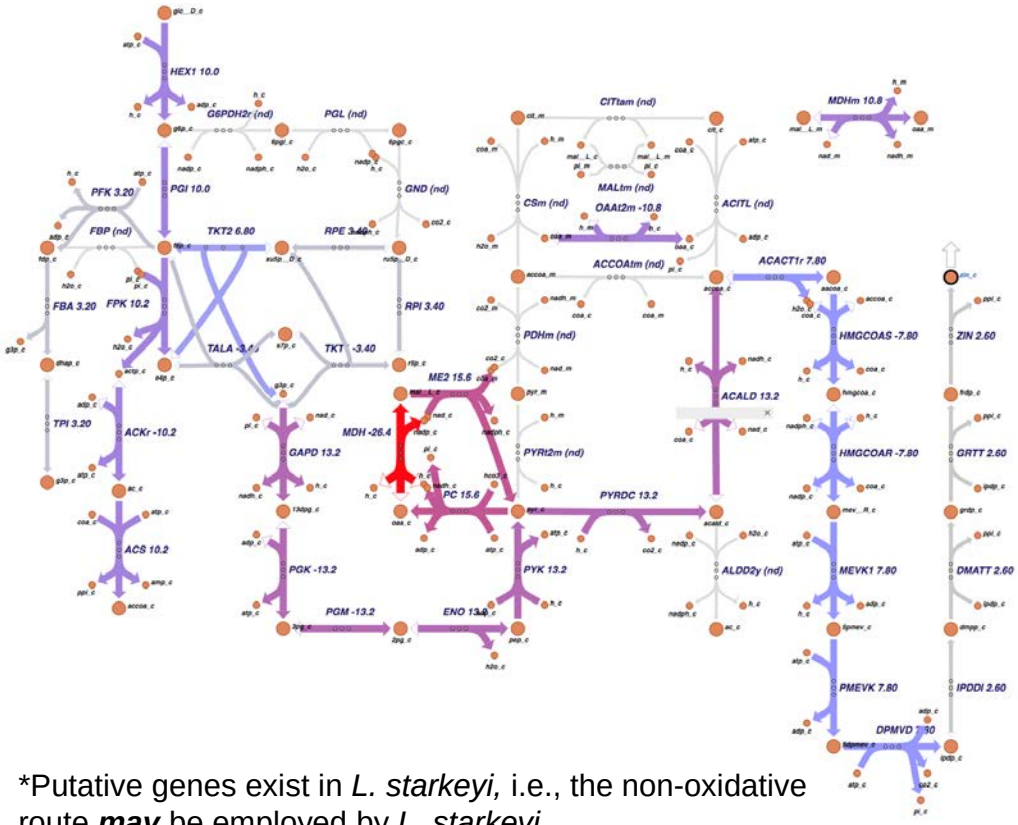
- Glycolysis
- NADPH from PP pathway
- Citrate synthase
- ATP-citrate lyase

Need
1.8 ATP
1.2 NADPH

Excess
3.6 NADH

2.0 Zingiberene per 10 glucose

Task 1 & 2: Zingiberene and malic acid production metabolic energy cost and yield



*Putative genes exist in *L. starkeyi,* i.e., the non-oxidative route ***may*** be employed by *L. starkeyi*

Highest Yield

- Non-oxidative glycolysis*
- NADPH from malic enzyme
- Pyruvate carboxylase

Need
3.6 ATP
1.56 NADPH

Excess
1.08 NADH

Max: 2.6 zingiberene per 10 glucose

Max: 2 malic acid per 1 glucose

Task 1 & 2. Pathway Assessment in Genome Model Maximum yield, energy and redox balance

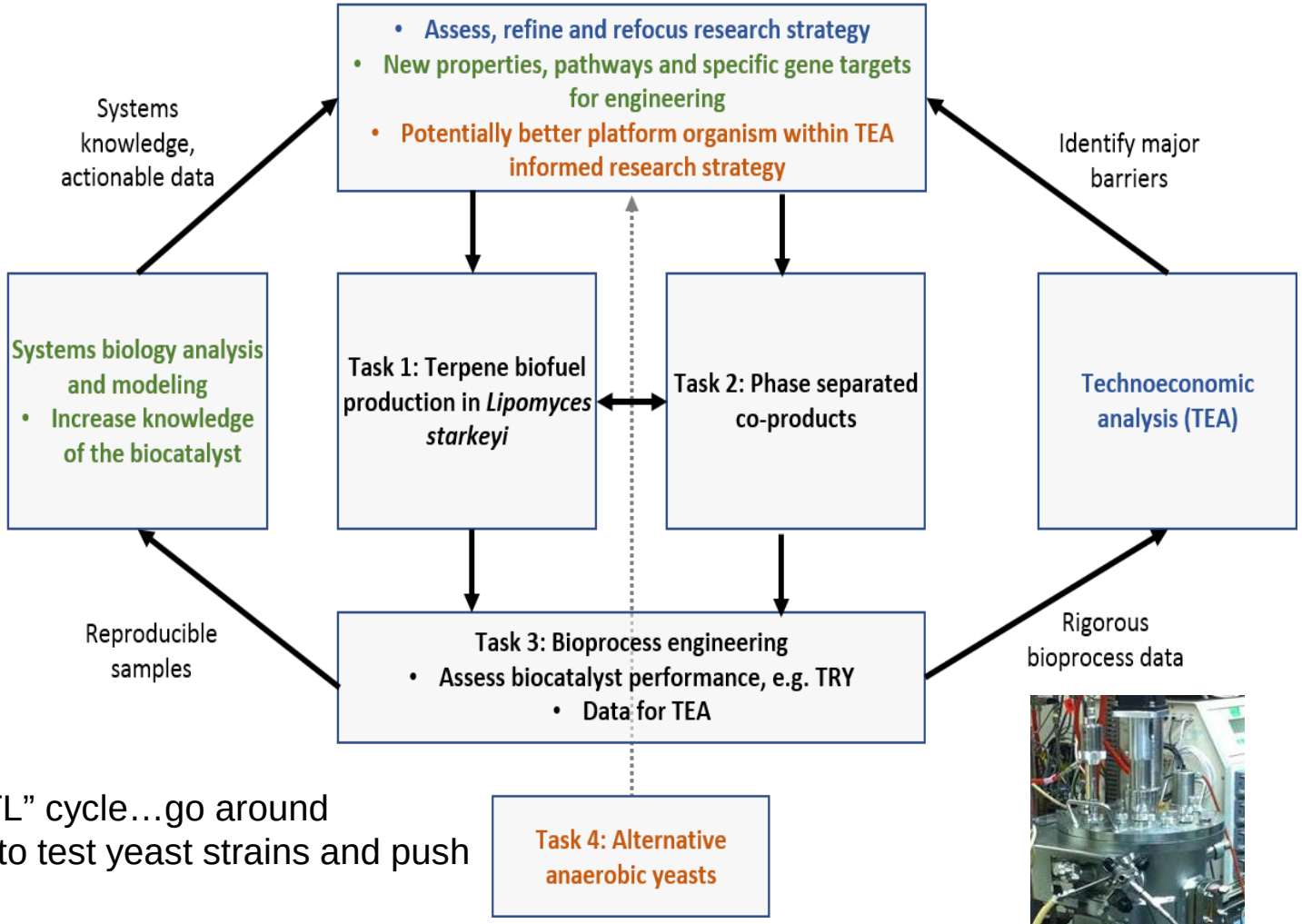
Summary Table

Zingiberene	ATP requirement	NADPH requirement	NADH generation	Zingiberene yield (mol/mol glucose)
Glycolysis	1.80	1.20 from PP pathway	3.60	0.200
Glycolysis	3.33	1.33 from malic enzyme	2.33	0.222
Non-oxidative glycolysis*	4.23	1.52 from PP pathway	1.34	0.254
Non-oxidative glycolysis*	3.60	1.56 from malic enzyme	1.08	0.260

Malic acid	ATP requirement	CO_2 requirement	NADH generation	L-malic acid yield (mol/mol glucose)
Glycolysis	0 (balanced)	2 for pyruvate carboxylase	0 (balanced)	2

- Energy and **redox cofactor (NADPH/NADH) balance** will be crucial
- Malic acid production is well balanced for all factors
- ***Challenge*****:** *Can efficient co-production be achieved?*
- ***Payoff***: Increased **carbon efficiency** if two CO_2 from each glucose used for terpene production can be incorporated into two molecules of Malic Acid

Task 3: Bioprocess Engineering



Key to making the "DBTL" cycle…go around

- **Scaling** bioprocess to test yeast strains and push Titer Rate Yield
- **Improve** bioprocess parameters
- Provide data for **TEA**
- Provide samples for **Testing** the strains

Task 4: Alternative Yeasts

Go/No-Go		
Description	**Criteria**	**End Date**
Go/No-Go Can novel yeast be transformed? Is their microaerobic growth vastly superior?	Microaerophilic growth characteristics (>1 g/L/d dry cell mass). Ability to transform a selectable marker (antibiotic resistance gene) into the chosen strain.	3/31/2020

Challenge of the Task

- Identify yeast strains from across the fungal kingdom with superior microaerobic growth properties
- Assess substrate range, pH tolerance, terpene tolerance, antibiotic susceptibility
- **Payoff**: strains that grow faster, under reduced aeration, have nascent genetic tools AND produce biofuels/bioproducts more efficiently
- Down select to two strains with the best characteristics in regard to the criteria
- Develop genetic tools, transformation systems
- If transformation cannot be developed in the 18 month time frame, assess probability of near term progress and make a decision on termination of the task

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Summary

1. **Overview**: Developing carbon efficient conversion of biomass to Biofuels & Bioproducts

2. Approach: incorporate pathways for two products that phase separate for downstream ease of separation

3. Technical Accomplishments/Progress/Results: Have demonstrated 105 mg/L zingiberene production already

4. Relevance: represents a strategy for economical production of biofuels by co-producing valuable products in a carbon efficient manner from costly feedstock. Examining microaerobic processes and strains to decrease problematic LCA concerns with energy for aeration

5. Future work: 1. 15 carbon terpene biofuel, 2. protonated malic acid bioproduct, Incorporate both pathways in one strain for carbon efficient conversion of biomass sugars and test at scale, 4. Identify the most promising microaerobic yeast species as future biocatalyst platforms with potential to reduce life cycle impacts

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Thank you

Additional Slides

- Future Work: Milestones and G/N-G
- Publications, Patents, Presentations

5 – Future Work: Milestones and G/N-G

Milestone Name/Description	Criteria	End Date
Achieve gene integration for biofuels.	Obtain a transformant with the zis gene integrated into the L. starkeyi chromosome.	12/31/2018
Complete identification of 2 non-model yeast strains.	Select 2 non-model yeast strains with 25% higher microaerobic growth rate (cell mass accumulation rate) to L. starkeyi for further development.	3/31/2019
Achieve gene integration for co-products.	Obtain a transformant containing at least the two genes for malate synthase and a carbon fixing gene expressed as active enzymes in L. starkeyi.	6/30/2019
Demonstrate a titer of at least of 0.3 g/L of zingiberene in an engineered strain.	Obtain a titer of 0.3 g/L of zingiberene in an engineered strain of L. starkeyi.	9/30/2019
Demonstrate a titer of at least of 1.2 g/L of zingiberene and 1.2 g/L of L-malic acid in different engineered strains.	Obtain 1.2 g/L of zingiberene in one strain of L. starkeyi and 1.2 g/L of L-malic acid in another strain of L. starkeyi. Stretch: demonstrate the same titers in a single strain producing both molecules at the same 1.2 g/L titers.	9/30/2020
Demonstrate a titer of at least of 5 g/L of zingiberene and 5 g/L of L-malic acid in the combined engineered strains.	Produce 5 g/L of zingiberene and 5 g/L of L-malic acid from lignocellulose-derived sugars.	9/30/2021

Go/No-Go	**Date: 3/31/2020**	
Description	**Decision**	**Measurable Criteria**
Assessment of the spectrum of yeast for substrate range and growth properties will be conducted in FY19 and genetic tool development will commence on no more than 2 of the most promising strains if they have desirable microaerophilic growth characteristics.	If neither of the two most promising strains can be transformed by the mid-point then make a decision in consultation with BETO on whether the effort to do so is of sufficient value or whether to focus exclusively on the existing biofuel production host *L. starkeyi*.	Microaerophilic growth characteristics (>1 g/L/d dry cell mass). Ability to transform a selectable marker (antibiotic resistance gene) into the chosen strain.

Publications, Patents, Presentations

Selected for team background in yeasts, relevant capabilities and fuels from prior projects

PUBLICATIONS

Bredeweg EL, Pomraning KR, Dai Z, Nielsen J, Kerkhoven EJ, Baker SE. A molecular genetic toolbox for Yarrowia lipolytica. Biotechnol Biofuels 2017 Jan 3; 10:2.

Butcher MG, PA Meyer, RT Hallen, KO Albrecht, CK Clayton, E Polikarpov, KG Rappe, SB Jones, and JK Magnuson. 2018. "Fungal Metabolites as Precursors to Renewable Transportation Fuels." Fuel 215:123-141. doi:10.1016/j.fuel.2017.10.052

Dai Z, KR Pomraning, S Deng, BA Hofstad, EA Panisko, D Rodriguez, MG Butcher, DE Culley, and JK Magnuson (2017) Deletion of the ku70 homologue facilitates gene dissection and synthetic biology for fuel and chemical productions in Lipomyces starkeyi strain NRRL Y-11558. Curr Genet. 2018 Aug 18

Dai Z, S Deng, DE Culley, KS Bruno, and JK Magnuson (2017) Agrobacterium tumefaciens-mediated transformation of oleaginous yeast Lipomyces species. Appl. Microbiol. Biotechnol.101(15): 6099-6110

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Yaegashi J, Kirby J, Ito M, Sun J, Dutta T, Mirsiaghi M, Sundstrom ER, Rodriguez A, Baidoo E, Tanjore D et al: Rhodosporidium toruloides: a new platform organism for conversion of lignocellulose into terpene biofuels and bioproducts. Biotechnology for biofuels 2017, 10:241.

Publications, Patents, Presentations
Continued

PATENTS

Agrobacterium Mediated Transformation of *Lipomyces*: Patent Number, 9,914,932

PRESENTATIONS

Characterization of Useful Promoters to Expand the Genetic Versatility of the Oleaginous Yeast Lipomyces starkeyi. 39th Symposium on Biotechnology for Fuels and Chemicals (SBFC). San Francisco, CA, May 1-4, 2017

Deletion Analysis of the Itaconic Acid Production Gene Cluster Components in Aspergillus pseudoterreus ATCC32359. April 29-May 2, Clearwater, FL, 2018, 40th Symposium on Biotechnology for Fuels and Chemicals.

“Multi-omics analysis of Rhodosporidium toruloides producing biofuels from depolymerized polysaccharides and lignin,” Pathway Tools Workshop, Menlo Park, CA, Feb 2018.

“Genomic and transcriptomic analysis of Rhodosporidium toruloides producing biofuels from depolymerized polysaccharides and lignin,” American Chemical Society (ACS) National Meeting, San Francisco, CA, Apr 2017.

“Characterizing in vivo isopentenyl diphosphate (IPP) toxicity in isoprenol-producing E. coli,” American Chemical Society (ACS) National Meeting, San Francisco, CA, Apr 2017.

“Genomic and transcriptomic analysis of Aspergillus niger producing thermophilic bacterial cellulases,” Fungal Genetics Conference, Pacific Grove, CA, Mar 2017.