NREL
NATIONAL RENEWABLE ENERGY LABORATORY





Biological Upgrading of Sugars

WBS 2.3.2.105

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Organization: National Renewable Energy Laboratory

2017 DOE Bioenergy Technologies Office (BETO) Project Peer Review

Date: March 7, 2017

Technology Area Review: Biochemical Conversion

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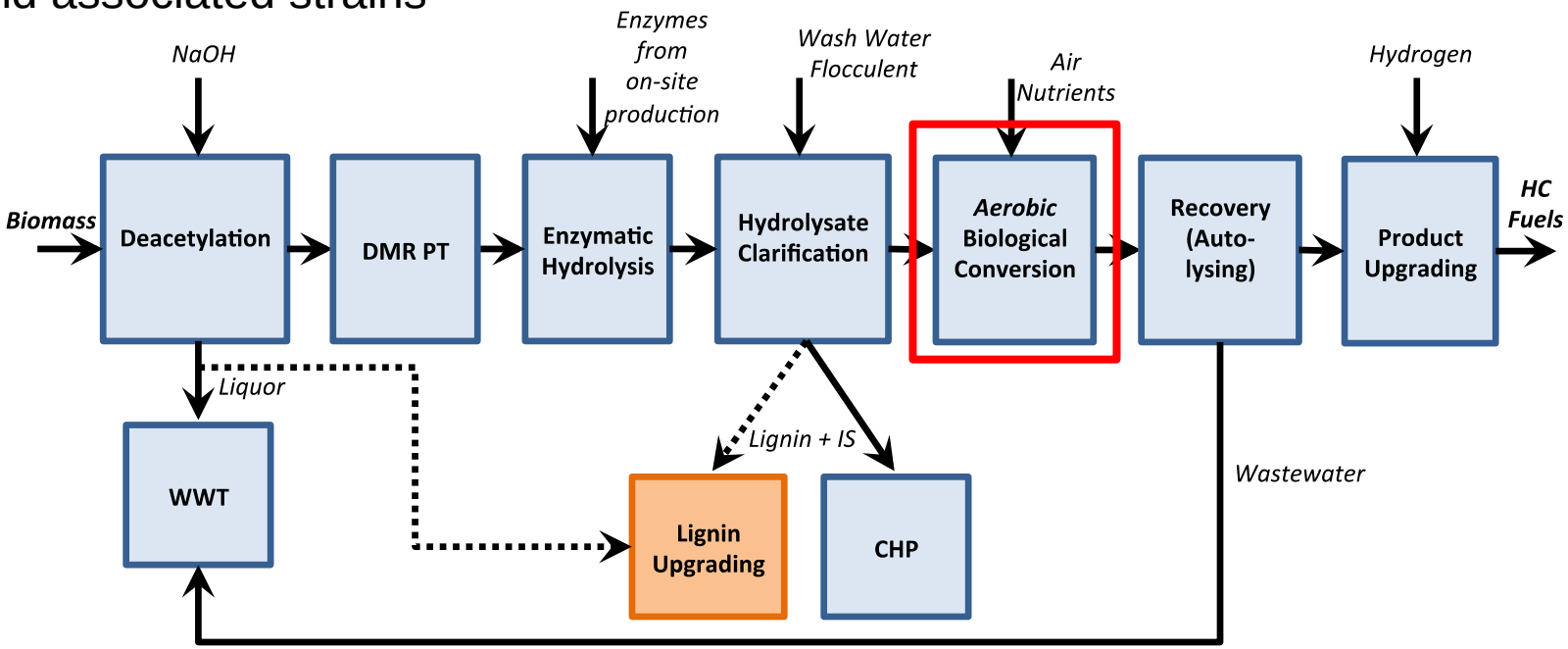
Goal Statement

Goal: Develop industrially-relevant strains to meet titer, rate, and yield targets for fuel precursors for the 2022 BC Platform cost target goals of $3/GGE

- Focus on **aerobic fatty acids** and **anaerobic, secreted C2-C6 carboxylic acids**

Relevance: Success could enable eventual commercial adoption of these routes and associated strains



HC fuels will be a major benefit to the U.S. biorefinery infrastructure.

- Conduct TEA/LCA to identify cost drivers and data gaps and to refine process options.
- Collaborate with national labs, academics, and industry for strain development and process demonstration.
- **Outcome:** Robust strains to meet **titer, rate, and yield targets** for producing HC fuels precursors.

Quad Chart

Timeline

- Start date: 10/14
- End date: 09/17
- Percent complete: 83%

Barriers

- Ct-H: Efficient Cat. Upgrading of Sugars
  - Strain development for titer, rate, yield targets
- Ct-J: Process Integration
  - Employing hydrolysate in bioreactors and working with upstream and downstream partners
- Ct-G: Efficient Intermediate Cleanup

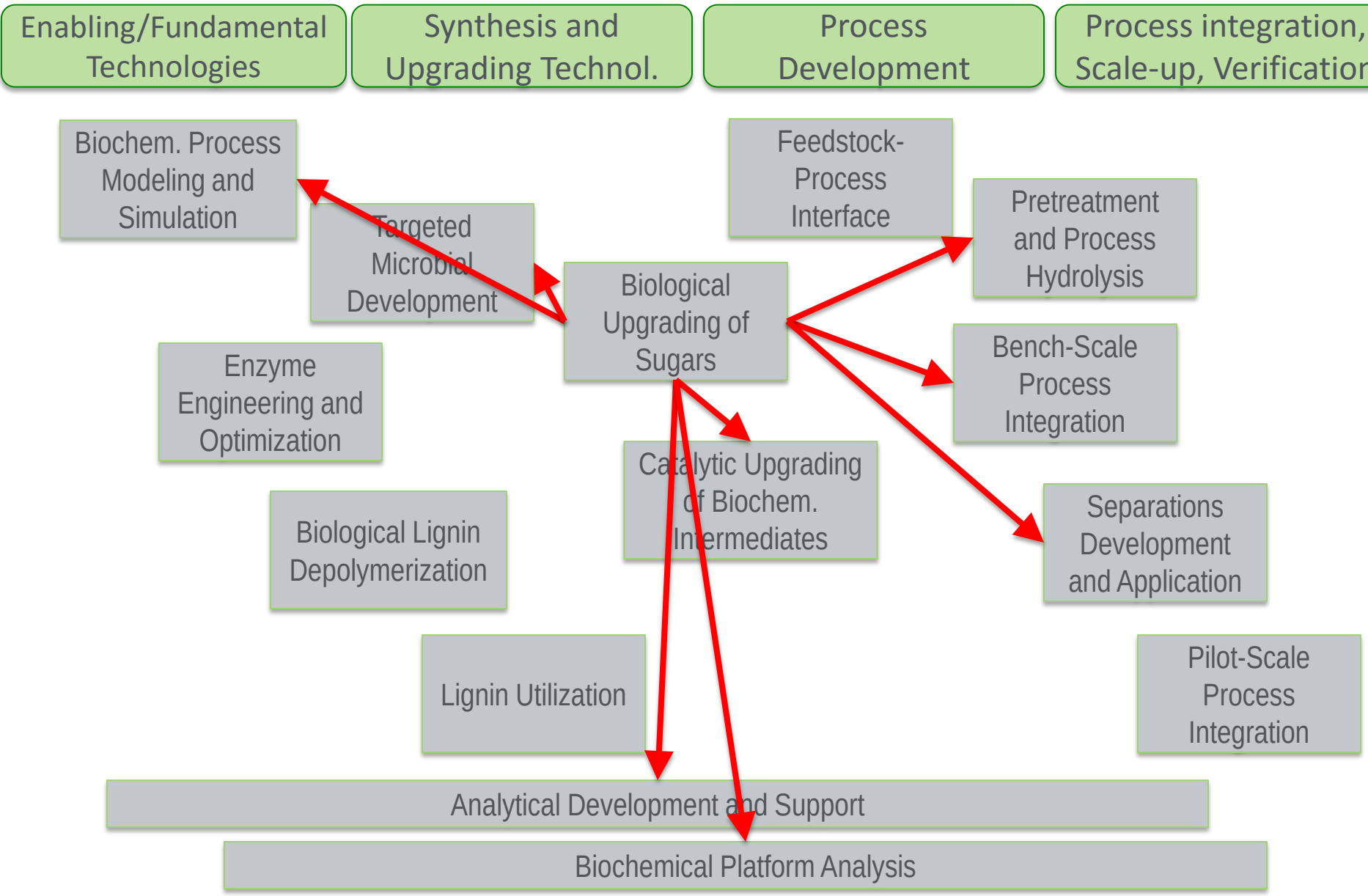
Budget

	FY15	FY16	Total Planned Funding (FY17)
DOE-funded	$1,800K	$1,800K	$1,800K

Partners and Collaborators

- **NREL BETO projects:** Biochemical Platform Analysis; Bench-Scale Integration; Separations Development and Application; Catalytic Upgrading of Sugars; Pretreatment and Process Hydrolysis; Pilot-Scale Integration; Biochemical Process Modeling and Simulation; Strategic Analysis Platform
- **BETO-funded national lab projects:** Agile BioFoundry, Separations Consortium, Catalytic Upgrading of Biochemical Intermediates
- **Industrial collaborators:** Fondevila NeolBio, Earth Energy Renewables
- **Academic collaborators:** Colorado State University; University of Pretoria; MIT; Penn State University; Temasek Life Sciences Laboratory; Università degli Studi di Milano-Bicocca

Interactions with other BC Conversion Projects



Project Overview

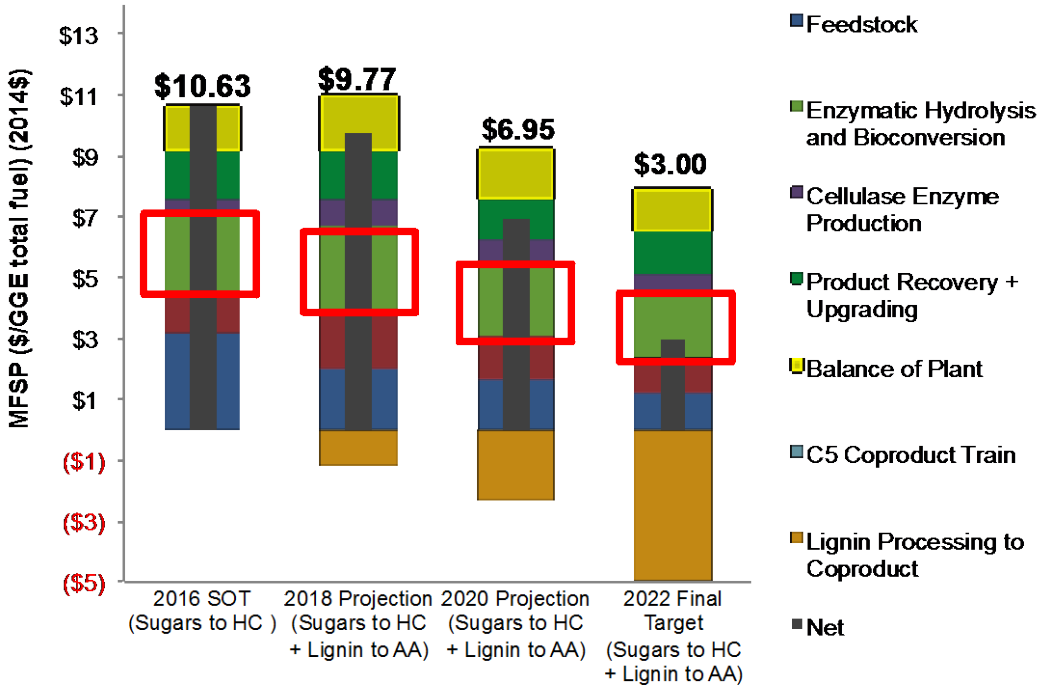
Davis et al., NREL Design Report, 2014

History: HC fuel R&D primarily began at NREL in the Nat'l Adv. Biofuels Consortium

- TEA suggests coproducts essential to cost-effective HC production
- NREL developing plans after 2012 to meet 2022 targets for $3/GGE HC fuels
- BUS project began in FY15

Context: "Beyond ethanol" to produce a portfolio of biofuels

- Produce direct replacements or blendstocks for **diesel** and **jet fuel**
- Shifted to intermediates that can be upgraded to diesel and jet fuel



Project Objectives:

- Develop industrially relevant strains for fatty acids and short-chain carboxylic acids to meet 2022 cost targets.
- Focus efforts toward titer, rate, and yield targets set by TEA/LCA modeling.
- Rapidly test strains with Bench-Scale Integration to identify and solve problems in scaling.

Technical Approach

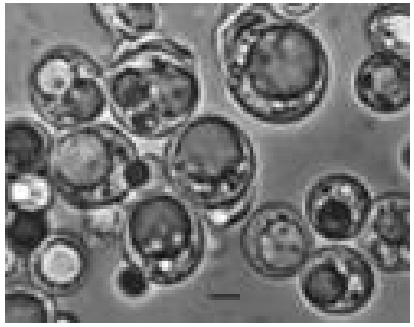
Aim 1: Develop a robust oleaginous strain

Approach:

- Target: 1 g/L/h rate, 60% lipid content, and a 0.27 g/g yield on hydrolysates
- Screen naturally oleaginous strains.
- Engineer strains for secretion or easier lysis.

Primary challenges and success factors:

- High yield and productivity of lipids
- Availability of genetic tools in strains for metabolic engineering
- High cost for cell lysis
- High cost for aerobic processing



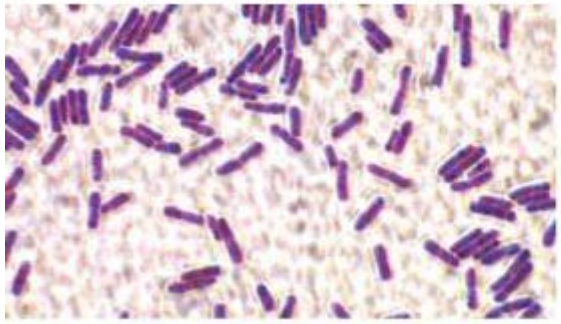
Aim 2: Develop robust C2 and C4 carboxylate strains

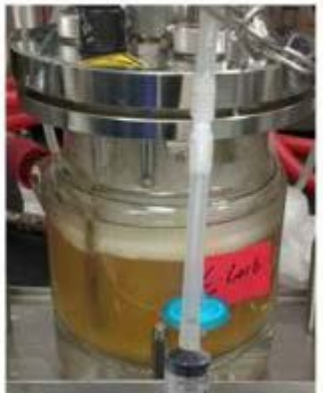
Approach:

- Target: >95% sugar utilization, ≥2 g/L/h rate, ≥80% of theoretical yield on hydrolysates
- Screen yeast and bacteria strains on hydrolysates.
- Engineer a strain for higher yields.

Primary challenges and success factors:

- Overcoming hydrolysate toxicity.
- Increasing carbon flux to target acids over side products (in bacteria).
- Incomplete sugar utilization (in yeast)





Management Approach

Experienced task leads in fermentation, microbiology, and metabolic engineering

Biological Upgrading of Sugars

Milestones prioritized to down-select single strains for 2022 deployment

Yeast Strain Evaluation (**Violeta Sànchez**)

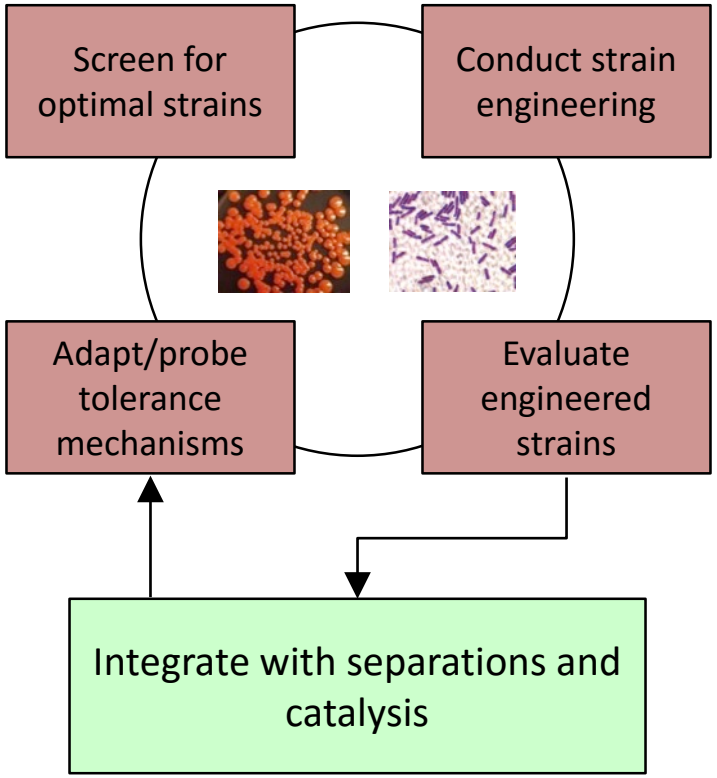
Yeast Strain Engineering (**Jeffrey Linger**)

Bacterial Strain Evaluation (**Davinia Salvachua**)

Bacterial Strain Engineering (**Michael Guarnieri**)

Approach:

- Define optimal strain attributes based on performance in bioreactors with hydrolysate
- Down-select optimal strains (e.g., oleaginous, low pH, or effective carboxylate producer)
- Develop genetic tools (if none exist)
- Define targets in collaboration with TEA, LCA, and downstream processing (separations and catalysis)
- Improve other strain attributes (e.g., knockout by-products, increase sugar utilization capacity, target a different product than native metabolism)
- Evaluate engineered strains in bioreactors on hydrolysate

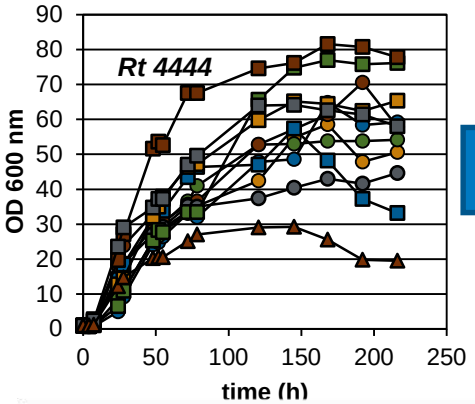


Systematic screen of 32 oleaginous yeast strains

Shake flasks

32 strains

Hydrolysate
100 g/L sugars



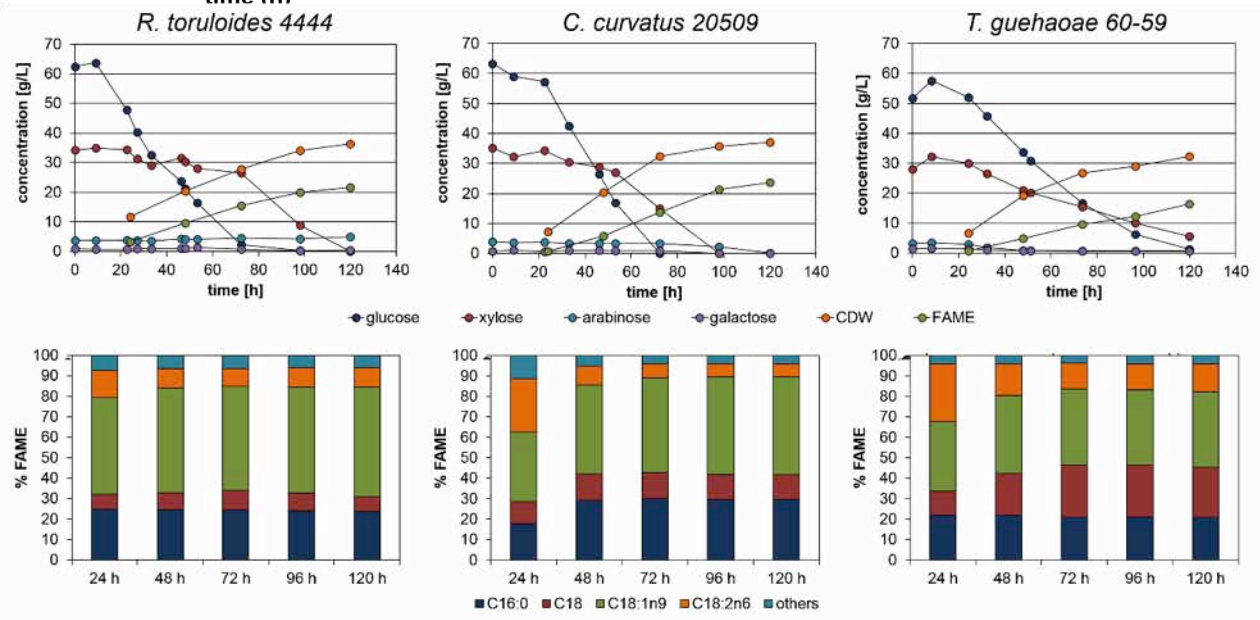
FAME: %, Q and titer

Top performers

Rhodosporidium toruloides
Cryptococcus curvatus
Trichosporon guehoae

Bioreactor performance on hydrolysate

- Titer, rate, yield
- Lipid classing
- Oil extraction
- Detoxification mechanisms
- Oil upgrading

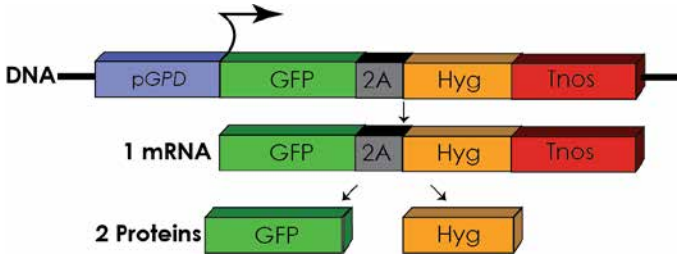


Outcome: Down-selected *R. toruloides* 4444 as a robust oleaginous strain in hydrolysate that is also genetically tractable; fed-batch optimization ongoing

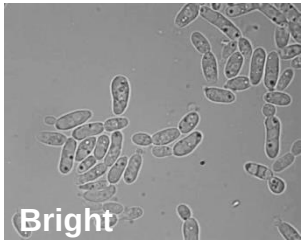
Engineering *R. toruloides*

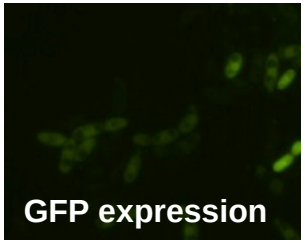
Generated expression plasmids

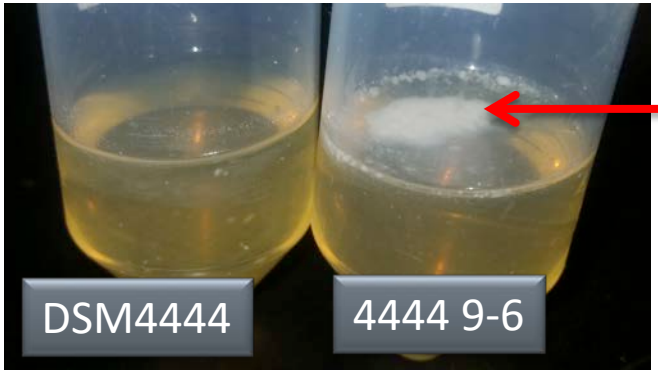
Transformations via conjugation and electroporation



Polycistronic expression constructs





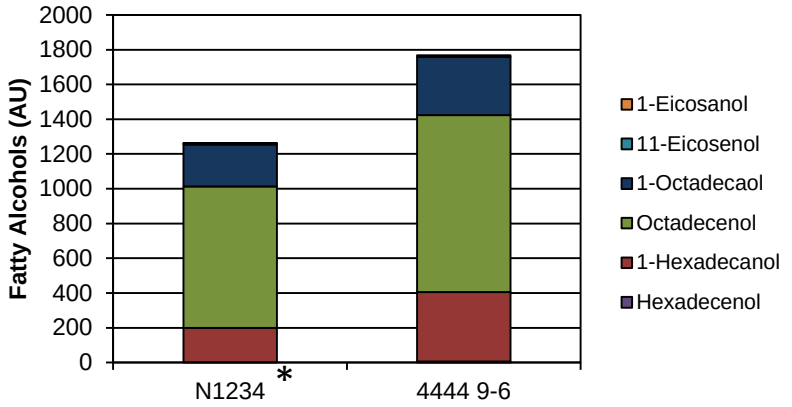


Fatty Alcohols

In partnership with:

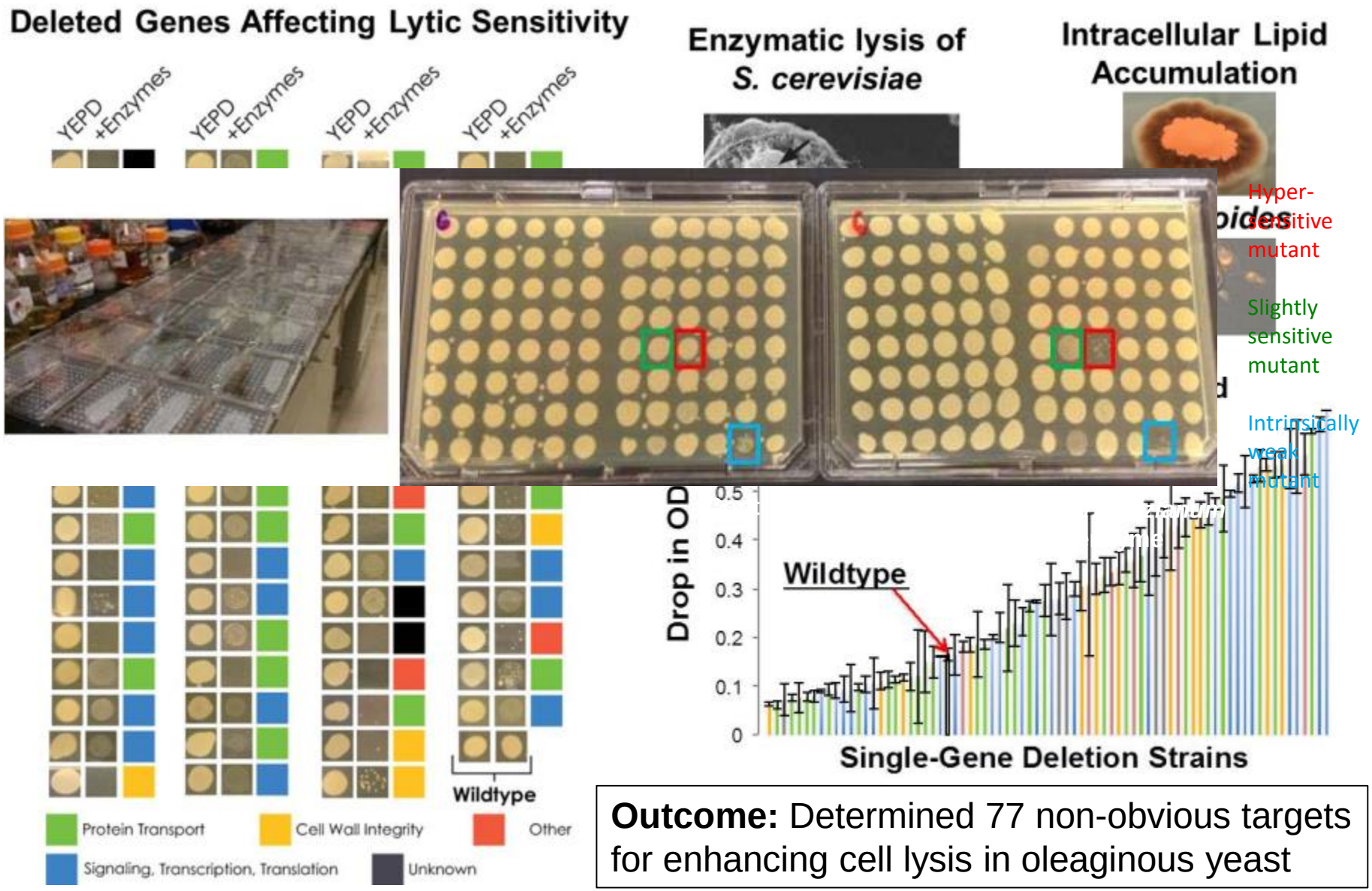
NEOLBIO

Fatty alcohols present in *Rhodosporidium* strains



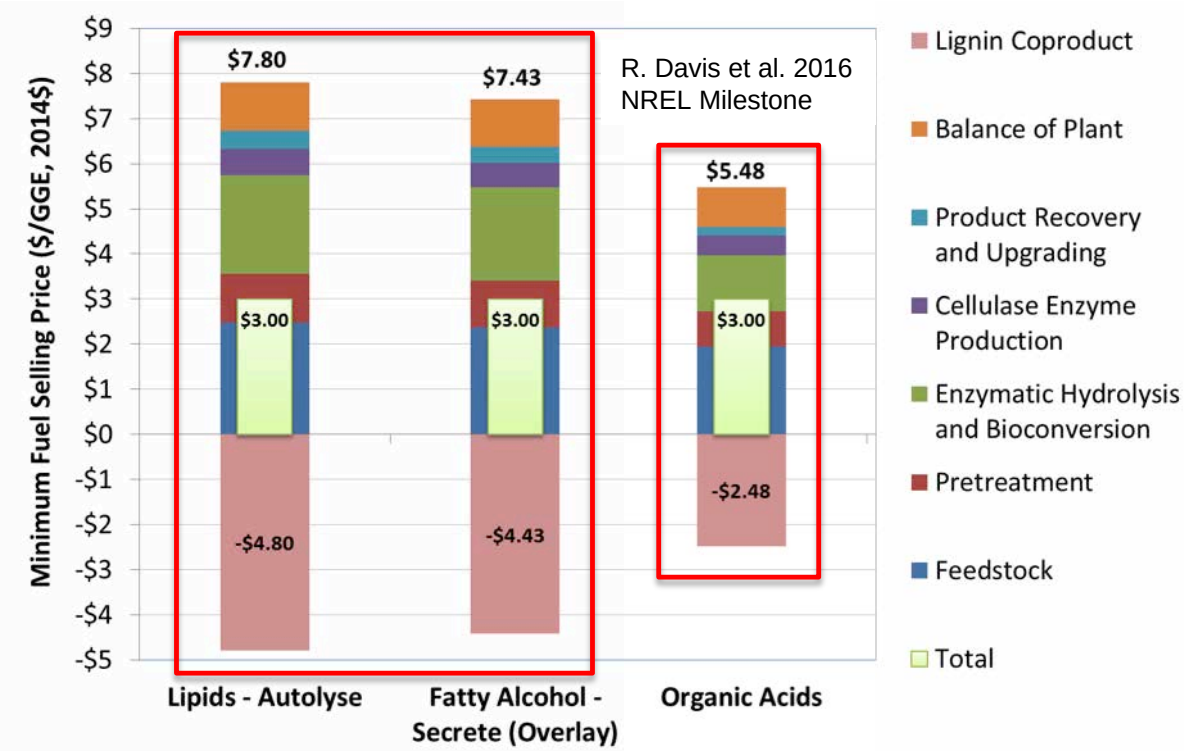
*NSL134: 8 g/L fatty alcohols (Fillet *et al*. 2015)

Enhancing cell lysis



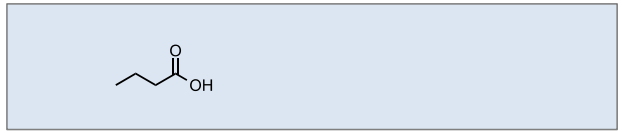
Outcome: Determined 77 non-obvious targets for enhancing cell lysis in oleaginous yeast

Shifting priorities to nearer term pathways



C2-C4 acids are ideal fuel precursors

- Same theoretical yield as EtOH
- Can produce branched fuels



C8-C12 Branched Alkanes

C9-C15 Branched Cycloparaffins

C5-C3 C5-C5-C5

Oleaginous pathways at $3/GGE:

- 1 g/L/h, 70% lipids, 0.27 g lipids/g total sugars, 100% glucose, 85% C5 utilization
- **$5/GGE contribution from lignin**
- 95% lipid recovery via auto-lysis
- **Scale** of aerobic << anaerobic
- Produces straight-chain HCs

Anaerobic acid pathway at $3/GGE:

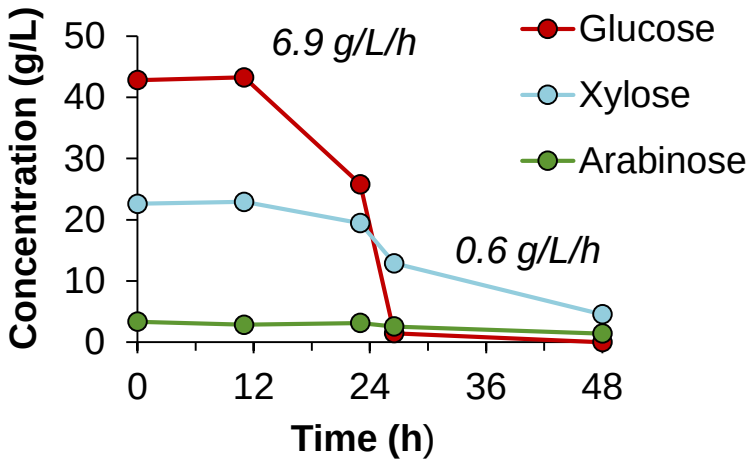
- 100% glucose utilization, 85% C5 utilization, 1.5-day fermentation
- **$2.50/GGE contribution from lignin**
- Either extractive or low-pH fermentation
- **Can produce branched diesel and jet fuel-range HCs**

Bacterial screening for carboxylate production

Strains selection based on

- C5/C6 sugar utilization
- Anaerobic production of C2-C4 acids
- Tolerant of inhibitors in hydrolysate
- Genetically tractable
- Screened multiple anaerobic bacteria

***C. butyricum* on hydrolysate**



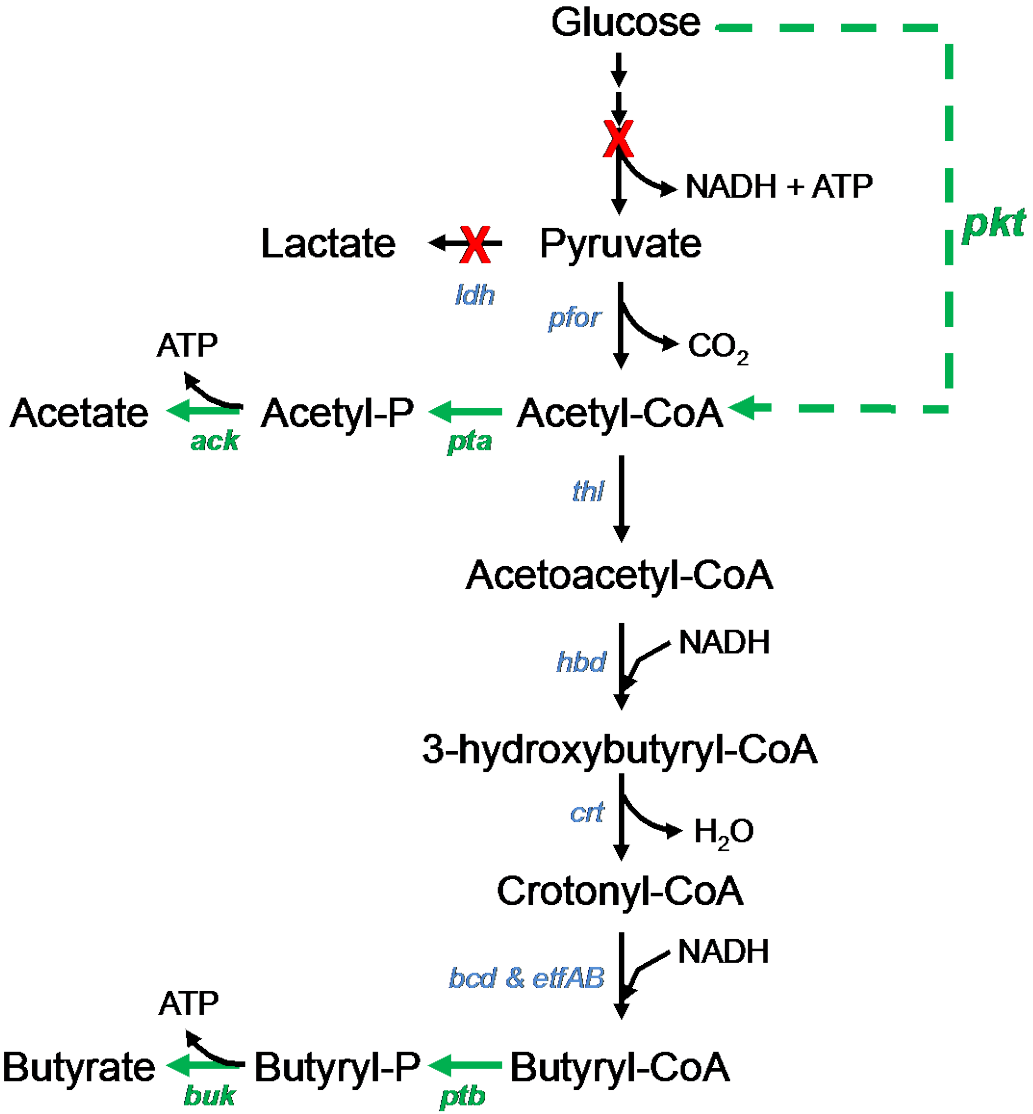
	C4	C2
Titer (g/L)	20.7	10.5
Yield (g/g)	0.3	0.15
Max. productivity (g/L/h)	1.6	0.8

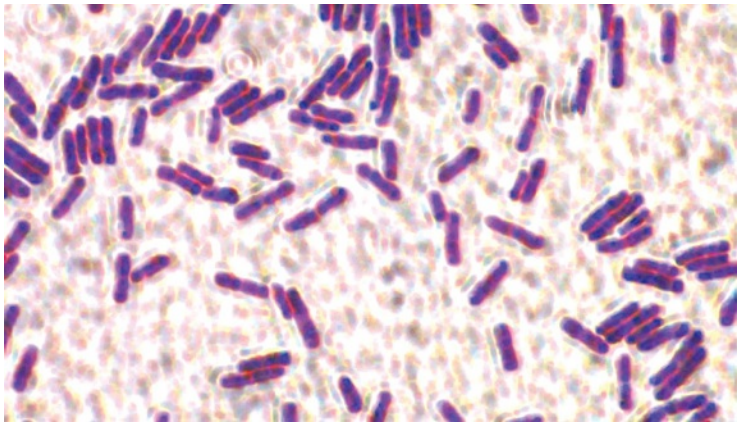
✓ ***Clostridium butyricum:*** genetically tractable, grows in biomass hydrolysate, produces C2 and C4 acids, utilizes all sugars efficiently

C. tyrobutyricum: genetically tractable, grows in biomass hydrolysate, produces AA and BA, poor xylose and arabinose utilization

C. tyrobutyricum ***C. butyricum***

Clostridium butyricum metabolic engineering





Target increased flux to acetate and butyrate with enhanced carbon efficiency

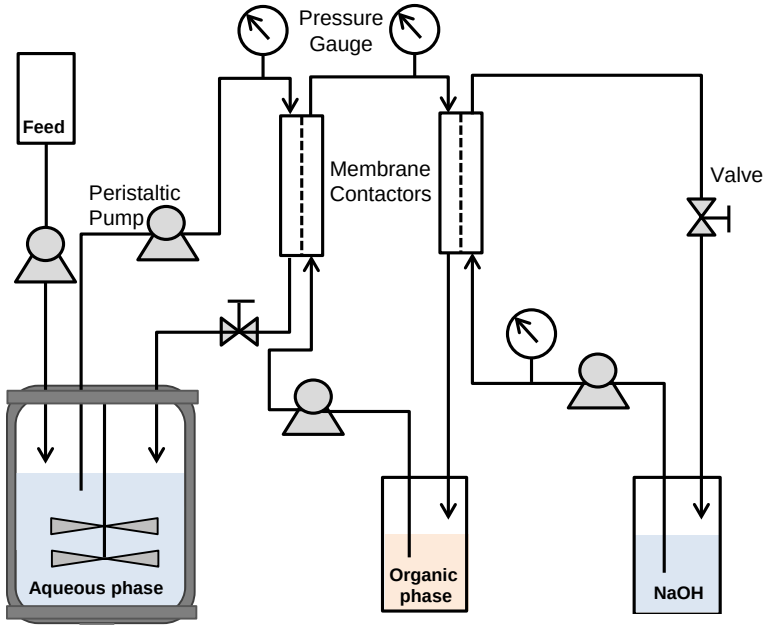
- Overexpression of C2 and C4 acid biosynthetic machinery
- Knockout of competitive carbon pathways (lactate, formate, EtOH)
- Bypass of pyruvate decarboxylation via homologous overexpression of native phosphoketolase pathway

Adapted from Yu et al. 2011. *Metabolic Engineering* 13(4), 373–382.

Demonstrating extractive fermentation

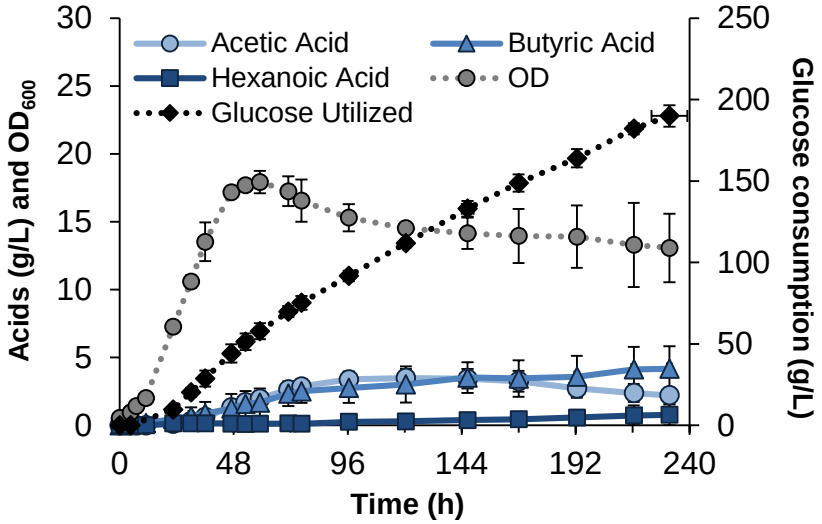
Baseline extraction

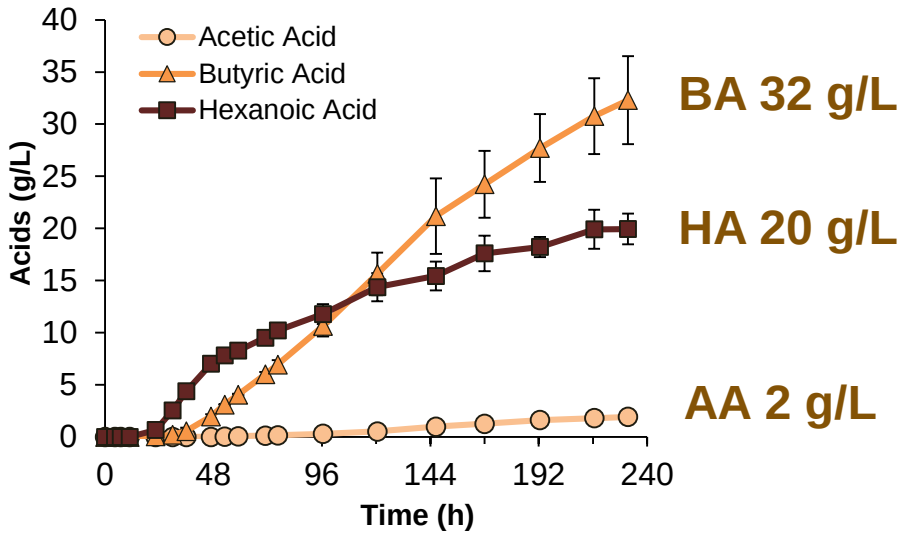
- Used a C4 and C6 acid-producing strain “off-the-shelf” to develop baseline extraction
- Working with the **Sep. Consortium to develop extraction methods to recover acids**



Extractive fermentation

- **No pH control**
- Process intensification
- Decreased product toxicity
- Increased C4 extraction efficiency
- Continuous feeding





Yeast could enable low-pH carboxylic acid production

Zygosaccharomyces bailii:

- Acid tolerant (pH 2, high concentration of organic acids)
- Osmotolerant (can grow up to 90% sugars)

Pichia kudriavzevii:

- Engineered for malonic, lactic, and succinic acids, along with xylonate
- Acid tolerant (pH 2–3)

Candida glabrata:

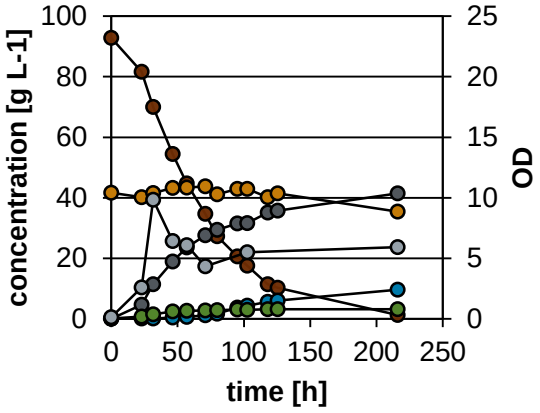
- Effective pyruvic acid producer
- CRISPR-Cas9 tools developed
- Acid tolerant (can grow at pH 2)

Anaerobic hydrolysate performance (3 strains):

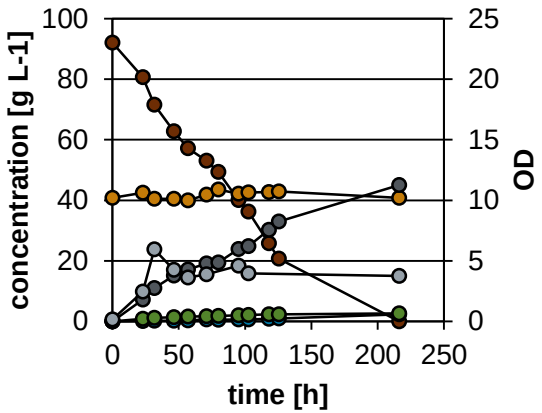
- High metabolic EtOH yields
- Able to ferment hydrolysate w/o extra nutrients
- Engineering planned for C5 sugar utilization

	Zb	***Pk***	***Cg***
Titer (g/L)	41.9	45.0	38.5
Yield (g/g)	0.42	0.49	0.45
Max. productivity (g/L/h)	0.36	0.27	0.31

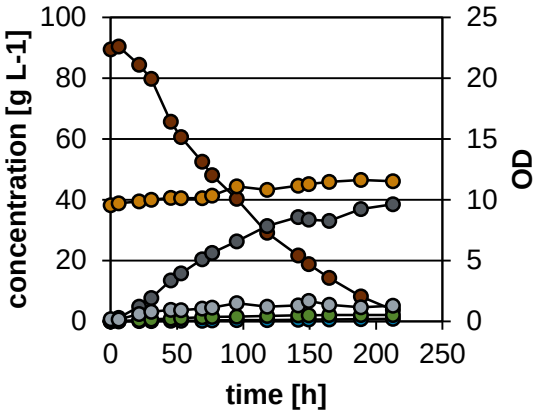
Z. bailii

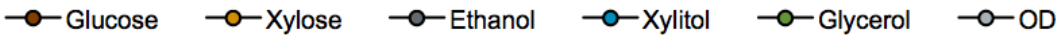


P. kudriavzevii

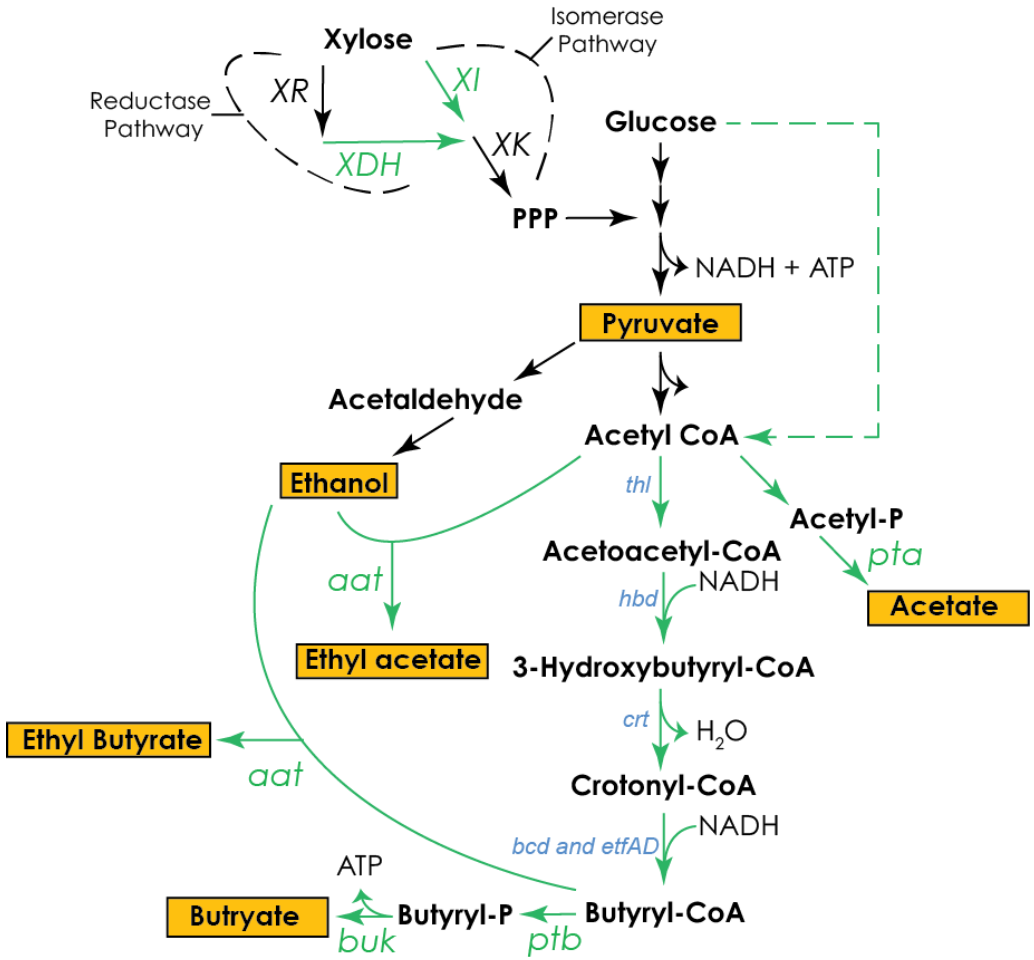


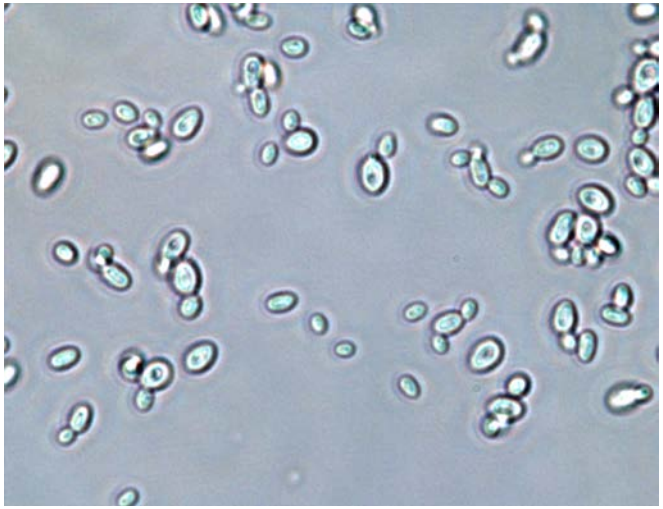
C. glabrata





Low-pH yeast metabolic engineering targets





Leverage existing genetic tools to:

- Heterologously express C2 and C4 acid biosynthetic machinery.
- Bypass pyruvate decarboxylation to increase carbon efficiency by overexpression of the phosphoketolase (*pkt*) pathway.
- Integrate xylose assimilation pathway.
- **Heterologously express** alcohol acetyl transferases (AATs) to generate ethyl acetate and ethyl butyrate, in non-*pkt*-overexpressed strains.

Adapted from Yu et al. 2011. *Metabolic Engineering* 13(4), 373–382.

Relevance

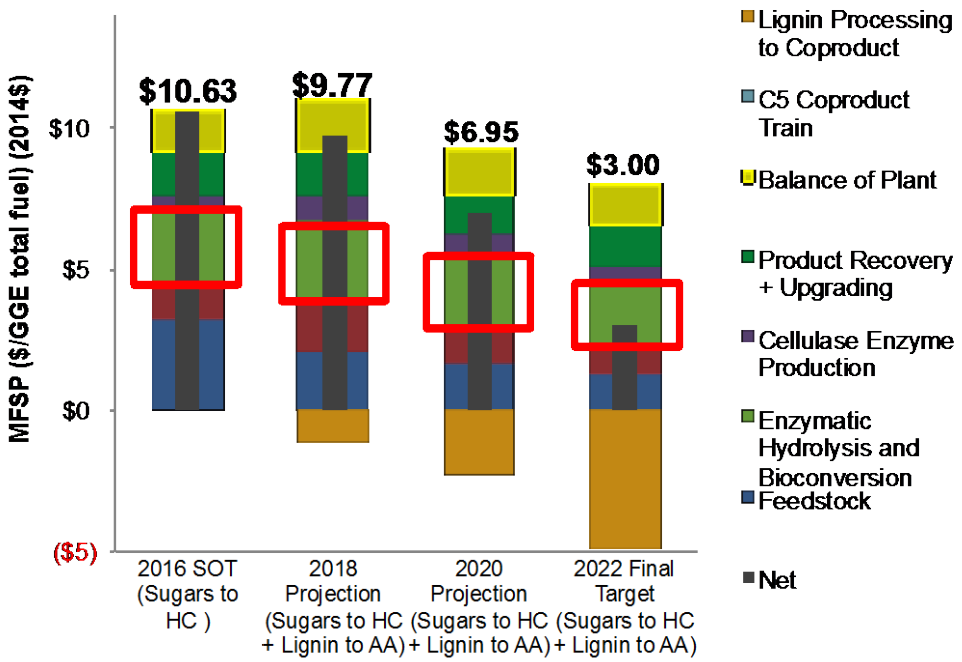
Key MYPP areas for the Biological Upgrading of Sugars project:

Efficient Upgrading of Sugars
- Developing efficient biocatalysts to produce advanced fuels and fuel precursors
- Improving titer, rate, yield key to economic viability

Biochemical Conversion Process Integration
- Coupling process considerations with organism development
- Working with BSI task to iterate on fermentation needs and organism modifications/evolution

Efficient Clean-up and Conditioning
- Elucidating inhibitor effects on biocatalysts and downstream processing

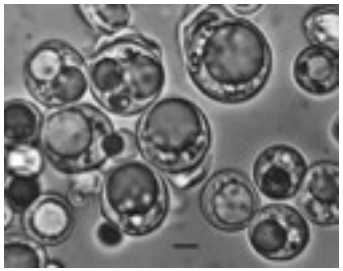
Key Stakeholders and Impacts
- **Research focused on carbohydrate utilization in HC fuel production from biomass**
- **Portfolio of potential oleo- and carboxylate-based fuels and chemicals will diversify and accelerate the biomass value chain**
- Methods to upgrade sugars to organics acids can be leveraged well beyond fuel precursors



Future Work

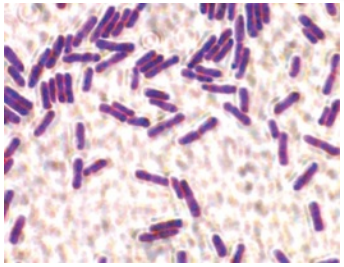
Oleaginous yeast:

- Transition away from this work because of high process cost for producing diesel
- Hand off strains and tools to longer term strain development in the Targeted Microbial Development project



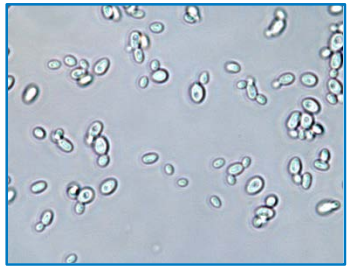
Anaerobic bacteria platform:

- Down-selected to *C. butyricum*
- Engineer improved flux to C2 and C4 products
- Integrate with separations and catalysis



Anaerobic low-pH yeast platform:

- Performing down-selection now
- Improve C5 sugar utilization
- Target C2 and C4 acids requiring no online extraction (low-pH fermentation)



Metabolomics, transcriptomics, and proteomics to baseline performance

Future Work

Strain engineering will require a $1.25/GGE improvement from the current SOT

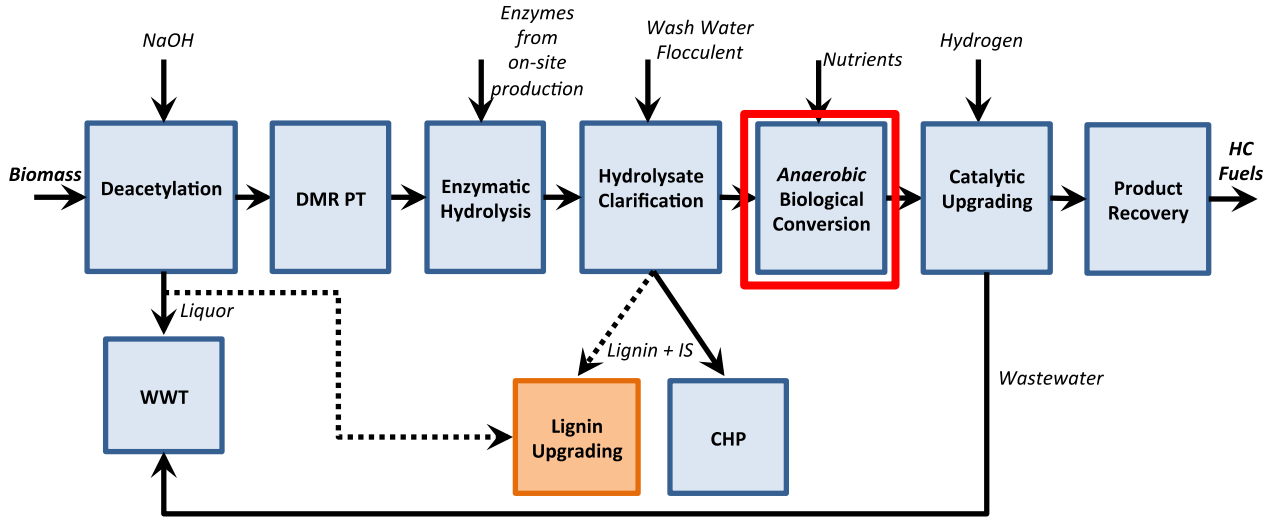
Carboxylates are efficient intermediates that can enable production of jet fuel and diesel

***Clostridium butyricum*:**

- Intensify fermentation and separations for higher titer, rate, yield toward targets (1.5-day fermentation, >95% sugar utilization, no pH control extraction)
  - 2-day fermentation, ~90% sugar utilization, extraction with other microbes successful so far
- Maximize carbon flux to C2 and C4 acids or easily separable products (e.g., esters)

Low-pH yeast:

- Expand sugar utilization to include pentose sugars
- Divert more carbon to fuels through more efficient strains



Aim: Single strain and associated process by end of FY19 for scaling

Summary

1) Approach:
   - Develop oleaginous yeast for lipid production for renewable diesel blends
   - Develop carboxylate fuel precursor strains for C2/C4 acids to produce branched jet/diesel blendstocks
2) Technical accomplishments
   - Screened a large collection of oleaginous yeast, down-selected *R. toruloides*
   - Identified multiple mechanisms of inhibitor tolerance in aerobic oleaginous yeast
   - Identified 77 gene targets for enhancing cell lysis in oleaginous yeast strains
   - Developed fatty alcohol strains of *R. toruloides*
   - Demonstrated **near-theoretical yields** of C2/C4 acids in *C. butyricum* and evaluated low pH yeast strains for anaerobic growth, both on biomass hydrolysates
   - Developed bench-scale extractive fermentation capabilities
   - Metabolic engineering underway for bacteria (*C. butyricum*) and will start soon in low pH yeast
3) Relevance
   - Directly **impacts the 2022 HC fuel cost target demonstrations** through strain development
   - Addresses Whole Barrel of Oil Initiative and bolsters the biomass value chain
4) Critical success factors and challenges
   - **Economic** *and* **sustainable** production of fuel precursors, rapid tool development and integration with downstream processes
5) Future work:
   - Continue towards down-selection of a carboxylate strain for 2022 demonstration
6) Technology transfer:
   - Working with **industry** to build commercialization path for oleaginous yeast pathways, developing a broad IP portfolio on engineered carboxylate strains

Acknowledgments

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- Michael Bradfield
- Adam Bratis
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External collaborators

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Publications

Publications in press:

1. J.D. McMillan and G.T. Beckham, "Thinking big: Towards ideal strains and processes for large-scale aerobic biofuels production", in press at *Microbial Biotech.*, invited Crystal Ball Feature.
2. D. Salvachúa, H. Smith, P.C. St. John, A. Mohagheghi, D.J. Peterson, B.A. Black, N. Dowe, G.T. Beckham, "Succinic acid production from lignocellulosic hydrolysate by *Basfia succiniciproducens*", *Biores. Tech*. (2016) 214, pp. 558-566
3. M.J. Biddy, R. Davis, D. Humbird, L. Tao, N. Dowe, M.T. Guarnieri, J.G. Linger, E.M. Karp, D. Salvachúa, D.R. Vardon, G.T. Beckham, "The techno-economic basis for coproduct manufacturing to enable hydrocarbon fuel production from lignocellulosic biomass", *ACS Sust. Chem. Eng*. (2016) 4(6), pp. 3196-3211.
4. D. Salvachúa, A. Mohagheghi, H. Smith, M.F.A. Bradfield, B. Black, W. Nicol, M.J. Biddy, N. Dowe, G.T. Beckham, "Succinic acid production on xylose-enriched biorefinery streams by *Actinobacillus succinogenes* in batch fermentation", *Biotech. Biofuels*. (2016) 9, 28.
5. M.F.A. Bradfield, A. Mohagheghi, D. Salvachua, H. Smith, B.A. Black, N. Dowe, G.T. Beckham, W. Nicol, "Continuous succinic acid production by *Actinobacillus succinogenes* on xylose-enriched hydrolysate", *Biotech. Biofuels*. (2015) 8, 181.

Publications in review

1. R. Nelson, D. Peterson, E.M. Karp, G.T. Beckham, D. Salvachúa, "Mixed carboxylic acid production by Megasphaera elsdenii from glucose and lignocellulosic hydrolysate", in review as an invited article at *Fermentation*.
2. M.T. Guarnieri, Y.C. Chou, D. Salvachua, A. Mohagheghi, P. St. John, Y.J. Bomble, G.T. Beckham, "Metabolic engineering of *Actinobacillus succinogenes* for enhanced succinic acid production", in review at *AEM*.

Publications in preparation:

1. J.G. Linger *et al.*, "Enhanced cell lysis for microbial lipid production"
2. V. Sànchez i Nogué, B.A. Black, J.S. Kruger *et al.*, "Microbial lipid production, separations, and catalytic upgrading to renewable diesel"
3. E.M. Karp *et al.*, "Separation of high purity succinic and propionic acids from hydrolysate fermentation broth"
4. D.R. Vardon, E.M. Karp, D. Salvachúa, N.A. Rorrer, *et al.* "Integrated production of succinic acid, 1,4-butanediol, and polybutylene succinate from lignocellulosic biomass"

Previous Reviewer Comments

Project overview

- The project performers have communicated the projects history, the context in which the project fits into the portfolio, and its high level objectives.
- Biological upgrading of sugars; C5 and C6 derived products. two separate streams "going beyond ethanol" to produce a broad portfolio of biofuels and biobased chemicals
  TEA suggests co-products are required for hydrocarbon production
- Good project overview, although it's starting to look like every project is utilizing the alkaline-pretreated material in favor of the more commonly seen (commercially) acid-pretreated material. While the project clearly fits into some aspects of the BETO portfolio, it would be good to see the project history and background show a drive to improve economics/viability of technologies closer to commercialization.
- Good overview, looking for strains for C5 --> chemical, C6 --> fuel.
- HC fuels and coproducts strains. Great context and overview and rational for project
- Last october start - goal 2017 produce $5 gge. Alkaline - acid – hydrolysis.
- Lipids and succinic acid.
- Coproduct focus is a good focus.
- Collaboration with other national labs and academics.
- Strains for fatty acid and co-products.

Previous Reviewer Comments

Project approach

- The project performers have implemented technically sound research, development, and deployment approaches, and have demonstrated the results needed to meet their targets.
- The project performers have identified a project management plan that includes well-defined milestones and adequate methods for addressing potential risks.
- The project performers have clearly describe critical success factors which will define technical and commercial viability, and that they have explained and understand the challenges they must overcome to achieve success.
- Oleaginous yeast - fuel (lipids) and succinic acid from C5 stream.
- Bench scale integration.
- Combination of an oleaginous yeast to produce a hydrocarbon (lipid) and succinic acid as a value-add coproduct from the C5 stream.
- Good approach. Clearly demonstrated benefit of driving C5 coproduct generation. Very clear, quantified goals.
- Looking for natural strains, evolved strains and engineering of strains, good approach.
- Oleaginous yeast strain- clear targets and overview of approach
- SA strain - clear targets and overview of approach
- Task and task leaders identified.
- Nice flow chart to visualize relation of tasks and approach.
- targets are quantifiable, challenges outlined, develop 2 strains for the two products/fuel.
- Schedule driven to hit 2017.
- Risk mitigation considered in strain selection and development C5 and C6 effective strains.

Previous Reviewer Comments

Technical Progress and Accomplishments

- The project performers have made progress in reaching their objectives based on their project management plan.
- The project performers have described their most important accomplishments in achieving milestones, reaching technical targets, and overcoming technical barriers.
- The project performers have clearly described the progress since the period of the last review.
- Many strains being tested; *Lipomyces* is an example, high lipid content, also consumes C5 sugars
  Cereulenin mutagenesis
  targets in oleaginous yeast pathways also working in *Saccharomyces* as a model - rapid screening tools for increasing fatty acid biosynthesis
- Steel in the ground to make starch based succinic, a number of strains evaluated for C5 sugars, two anaerobes and CO2 fixers, achieving high titers already
- Very good progress is being made to date.
- Although this is a new project, just getting off the ground, significant progress has been made. Clear explanation of why work done to date has been done, with down-selection justified clearly, as well. Excellent use of the Death Star in presentation. Clearly in the market for collaborators/etc to help with moving forward, this matches with other highly-integrated work and enhances probability of success.
- Good results to date on biomass sugars.
- Good performance of oleaginous yeast on C6 sugar hydrolysates
  Discovery and engineering of oleaginous yeasts - good approaches and delivering results
- SA coproduct - logical selection of strains
- FA results
  .20 g/g sugar to product yield from hydrolyzate sugars looks promising
- C to FA synthesis pathway focus
- Using S cerevisiae is really understood and seems like a good place to focus the research.
- Succinic acid C5 development using lignocellulosic C5's as feed.
- Literature search initial selection of strains - 2 strains selected

Previous Reviewer Comments

Project relevance

- The project performers have describes how the project contributes to meeting Program/Technology Area goals and objectives and the Bioenergy Technologies Office, as cited in the MYPP.
- The project performers have considered applications of their expected outputs.
- The project performers have presented the relevancy of this project and how successful completion of the project will advance the state of technology and impact the viability of commercial bioenergy applications.
- Just getting started; essential to hit $5/gge target
- Very closely linked to other efforts
- Coordinated with cleanup and separation
- Co-products required for meeting economics
- Very relevant program since it is well accepted that value add co-products are required to meet fuel economics for hydrocarbon production.
- Project clearly works toward 2017 hydrocarbon fuel cost targets. Catalyst efficiency, process integration, and separations are target areas, supporting portfolio objectives. The project has clearly considered applications of their expected outputs. Success will have a significant impact on the state of the technology.
- Given the program targets for 2017/2022 this is very important to understand organisms that can work in the pretreated hydrolyzates and enzyme sugars to make succinic and lipids.
- High impact and alignment with BETO goals. Good linkage and coordination with other activities.
- Catalyst efficiency, biochemical integration, and separation all part of program goals.

Previous Reviewer Comments

Future work

- The project performers have outlined adequate plans for future work, including key milestones and go/no go decision points through September 30, 2016.
- The project performers have communicated key planned milestones and addressed how they plan to deal with upcoming decision points and any remaining issues.
- Down select to smaller number of strains in the future.
  will need to develop routes to multiple chemicals in the future since the market sizes are vastly different
- Future work is clearly described, relevant, and includes SMART goals. Clear explanation of how future work will support move away from use of lignin in CHP. Impacts "whole barrel" initiative.
- Good milestones and plans for succinic and lipid fermenting organisms.
- Clear plan for selecting and improving strains. Quantitative targets for performance.
- Down select strains to focus on.
- Evaluate a different kind of pretreatment chemicals/mechanical?

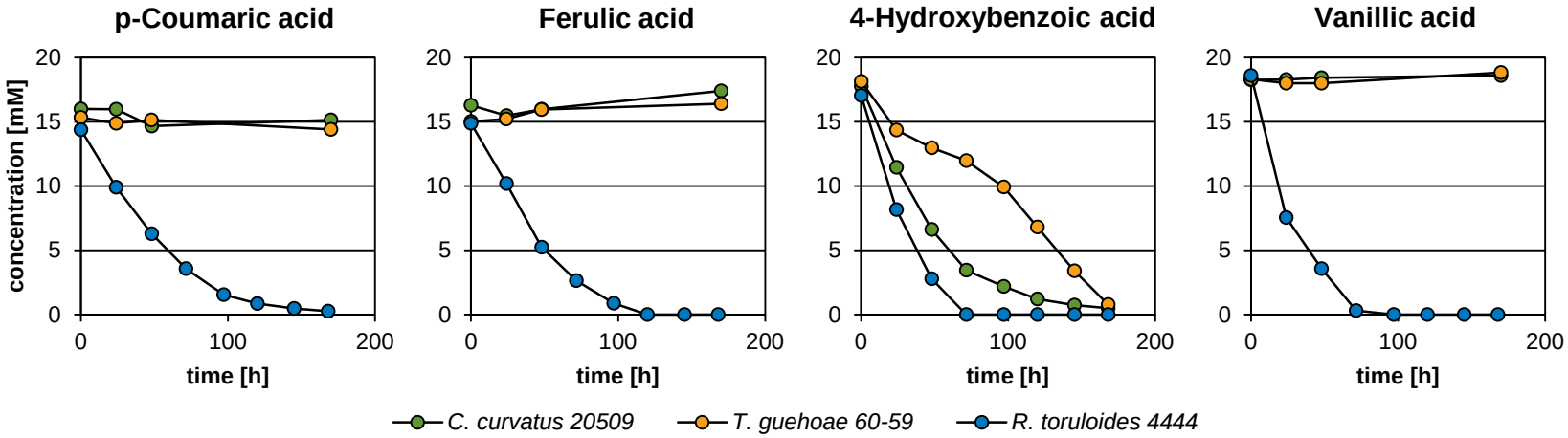
Previous Reviewer Comments

Overall Impressions (Not Scored)

- This is a very relevant program due to the poor theoretical yields of hydrocarbons from sugars (resulting in poor economics). The co-production of a value added chemical will aid in the overall economics, however there are potential market size issues. The market size of succinic acid is far smaller than hydrocarbons, therefore one may saturate the chemicals market quickly which may limit the size of the hydrocarbon end of the process. Thus this may require multiple co-products be made instead of one. Secondly the capital and operating costs of a "split stream" biomass conversion process may be more than combined approach. This should be monitored.
- This is a good project, but almost seems to be two parallel projects working toward a common goal. Utilization of C5 for succinic acid and C6 for lipid production by a different organism raises questions as to commercial viability, as it's moving down a path long-travelled by unsuccessful projects. Separation of the streams increase capital costs, footprint, and complexity, but the inclusion of value-added products in the process (such as those produced from this project) may offset those issues. Focus should be on making chemical precursors in lieu of products that have the potential to flood the market immediately upon commercial scale application, since the scale economics may well be driven by C6 utilization.
- With the program process set as it is and succinic and lipids selected as the products of choice, this is an excellent task.
- Very well conceived and planned project for converting biomass sugars into hydrocarbons. Selection of strains and target molecules is clear and methodical. Some of the strains and approaches are innovative and promise to deliver some new options for hydrocarbon production.
- Interesting research, the fuel/chemical combination key.

Evaluation of oleaginous yeasts

- **Aromatics as C source**



- **Oil upgrading (separations and catalysis)**