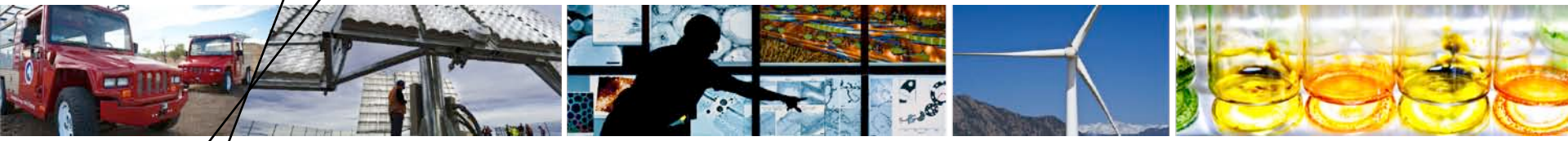
Biological Upgrading of Sugars
WBS 2.3.2.105



2015 DOE BioEnergy Technologies Office (BETO) Project Peer Review

Date: March 25th, 2015

Technology Area Review: Biochemical Conversion

Principal Investigator: Gregg T. Beckham

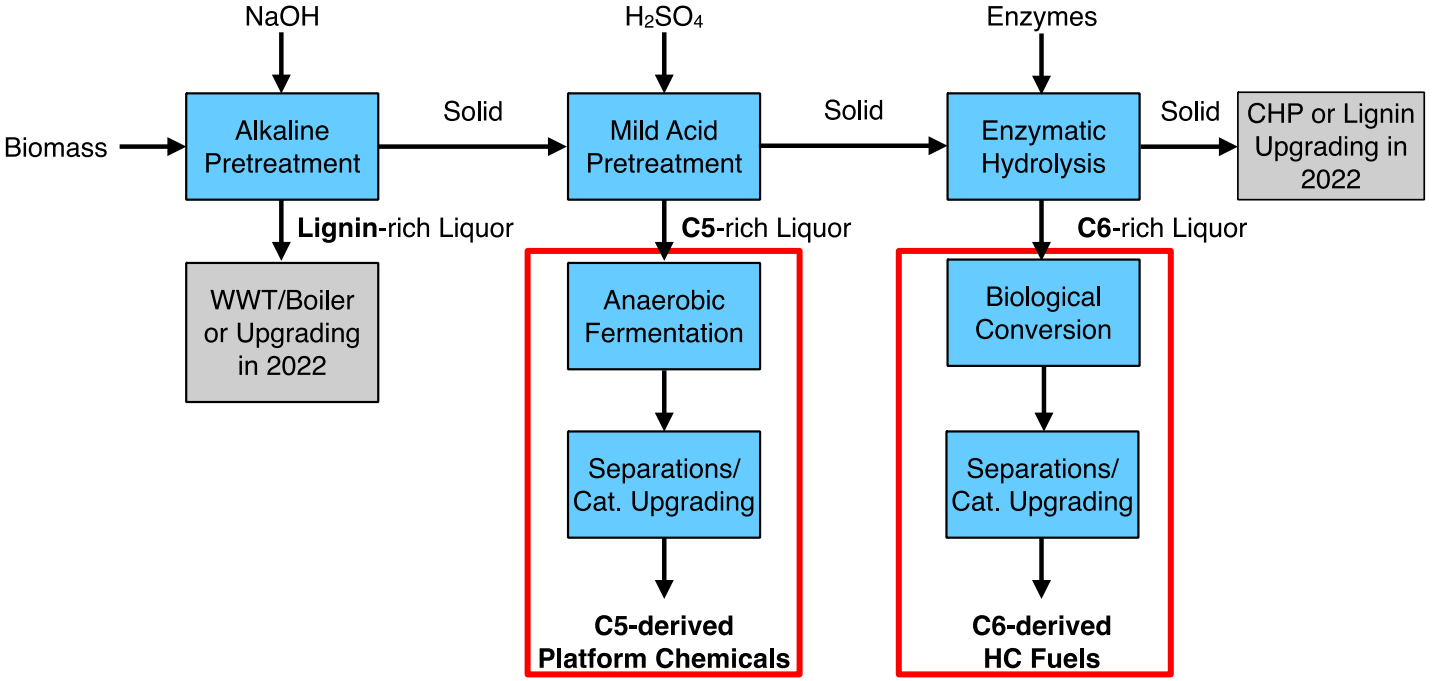
Organization: National Renewable Energy Laboratory

This presentation does not contain any proprietary, confidential, or otherwise restricted information

Goal Statement

Goal: develop strains to produce fuels and co-products for the 2017 and 2022 Biochemical Conversion Platform cost target goals of $5/gge and $3/gge

- Fatty acids as fuel precursors, succinic acid as an *example* product, both aligned with TEA targets
- "Bioproducts are on the Critical Path" – DOE BETO



HC fuels alongside co-products will be a major benefit to the US biorefinery infrastructure

- Conduct TEA/LCA to identify cost drivers and data gaps and to refine process options
- Collaborate with industry and academics for joint development of strains and process demonstrations
- **Outcome:** demonstrated, robust strains for producing HC fuels and co-products in the biorefinery

Quad Chart

Timeline

- **New Project**
- Start date: October 2014
- End date: September 2017
- Percent complete: 10%

Barriers

- Bt-I Catalyst Efficiency
- Bt-J Biochemical Conversion Process Integration
- Bt-H Cleanup/Separation

Budget

	FY15	Total Planned Funding (FY16-Project End Date)
DOE funded	$1,800,000	$4,200,000

Partners and Collaborators

- **Industry partners:** in talks with industrial entities regarding collaborations around both HC and co-product development
- **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:** Ongoing discussions with PNNL efforts in strain development
- **Academic collaborators:** University of Pretoria, MIT, UC Davis Phaff Yeast Culture Collection, currently in talks with other groups for collaborations around both HC and co-product development

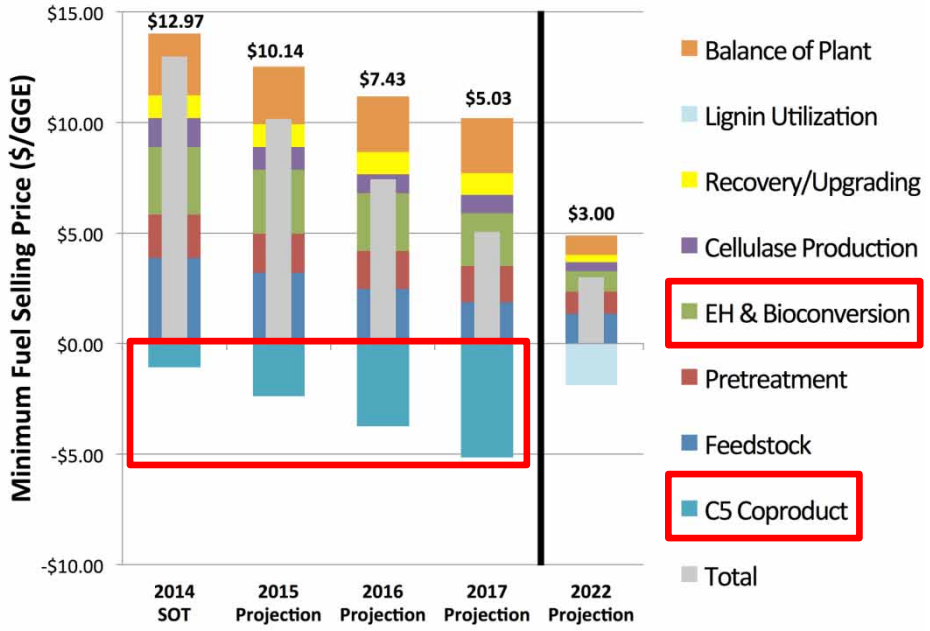
Project Overview

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

- TEA suggests chemicals are essential to cost-effective HC production
- NREL began developing plans after the 2012 ethanol demonstration to meet 2017 and 2022 cost targets for HC fuels at $5/gge and $3/gge

Context: Going "beyond ethanol" to produce a broad portfolio of biofuels

- Produce direct replacements or blendstocks for gasoline, diesel, jet fuel markets
- Move closer to petroleum refinery models with fuel and chemicals production together
- De-risk capital investments in fuels via co-product manufacturing



Project Objectives:

- *Develop industrially-relevant strains for fatty acids and an example co-product to meet 2017 and 2022 cost targets*
- Focus efforts towards titer, rate, and yield targets set by TEA/LCA modeling
- Rapidly test strains with Bench-Scale Integration Project to identify and solve problems in scaling and integration

Davis *et al.*, NREL Design Report, 2014

Technical Approach

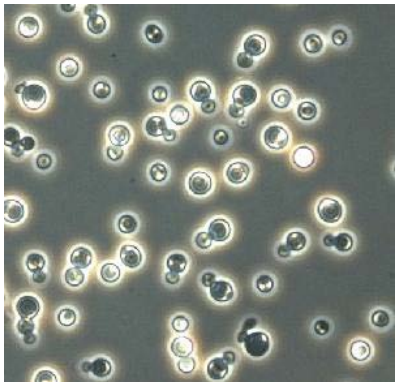
Aim 1: Develop a robust oleaginous strain

Approach:

- Target: 0.4 g/L/hr rate, 60% lipid content, and a 0.27 g/g yield on C6-enriched sugars
- Screen natural oleaginous yeast strains
- Evolve strains to increase lipid yields
- Engineer select strains for high lipid yields

Primary challenges and success factors:

- High yield and productivity of lipids
- Availability of genetic tools in strains for metabolic engineering



Aim 2: Develop robust succinic-acid strain

Approach:

- Target: 2.0 g/L/hr rate, 0.795 g/g yield on C5-enriched sugars
- Evaluate natural strains on C5-hydrolyzates
- Adapt strains to tolerate pretreatment inhibitors
- Engineer a strain for higher SA yields

Primary challenges and success factors:

- Overcoming hydrolyzate toxicity
- Increasing carbon flux to SA over side products

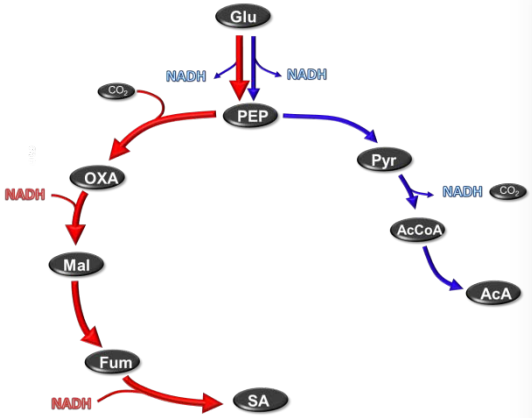
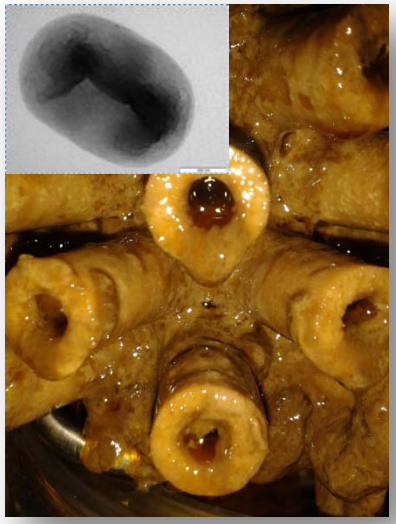


Image from W. Nicol



Management Approach and Outline

Experienced task leads in fermentation, microbiology, and metabolic engineering

Biological Upgrading of Sugars

Milestones prioritized to down-select single fuel and co-product strains for 2017 deployment

Fuel Precursor Strain Evaluation (**Nancy Dowe**)

Fuel Precursor Strain Development (**Jeffrey Linger**)

Co-Product Strain Evaluation (**Davinia Salvachua**)

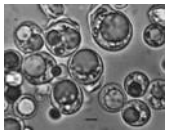
Co-Product Strain Development (**Michael Guarnieri**)

Fuel Precursors: 3-pronged strategy to mitigate risk

Natural Strains

Evolved Strains

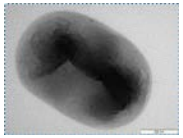
Engineered Strains



Bench-Scale Integration Project for Advanced Fermentation Testing

Develop/improve genetic tools

Apply genetic tools for strain engineering



Adapt/probe tolerance mechanisms

Evaluate strains on C5-hydrolyzate

Bench-Scale Integration Project for Advanced Fermentation Testing

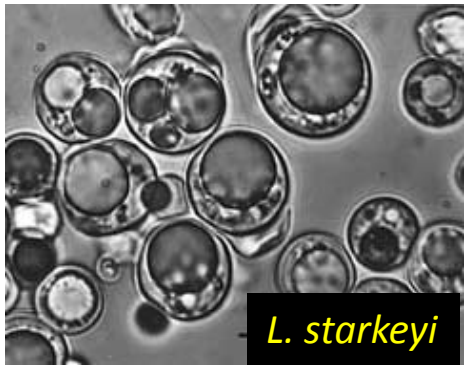
Self-consistent screening of oleaginous yeast

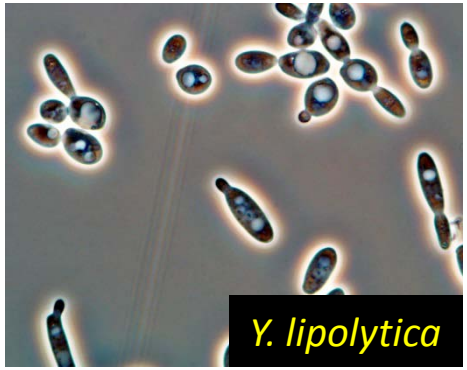
Natural Strains

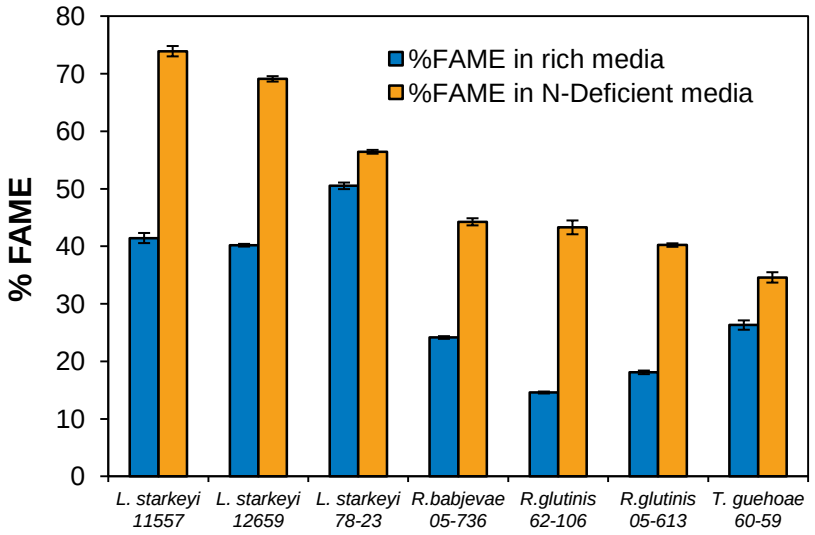
- Obtained oleaginous yeast collection
- Pursuing **self-consistent screening results**

Species being screened :

- *Cryptococcus curvatus*
- *Cryptococcus wieringae*
- *Kurtzmaniella cleridarum*
- *Leucosporidiella creatinavora*
- *Lipomyces starkeyi (3)*
- *Rhodosporidium babjevae* (4)
- *Rhodosporidium dibovatum*
- *Rhodosporidium paludigenum*
- *Rhodosporidium sphaerocarpum*
- *Rhodosporidium toruloidies* (6)
- *Rhodotorula glutinis (2)*
- *Rhodotorula glutinis "like"*
- *Sporopachydermia opuntiana*
- *Tremella encepala*
- *Trichosporon guehoae*
- *Yarrowia lipolytica* (10)





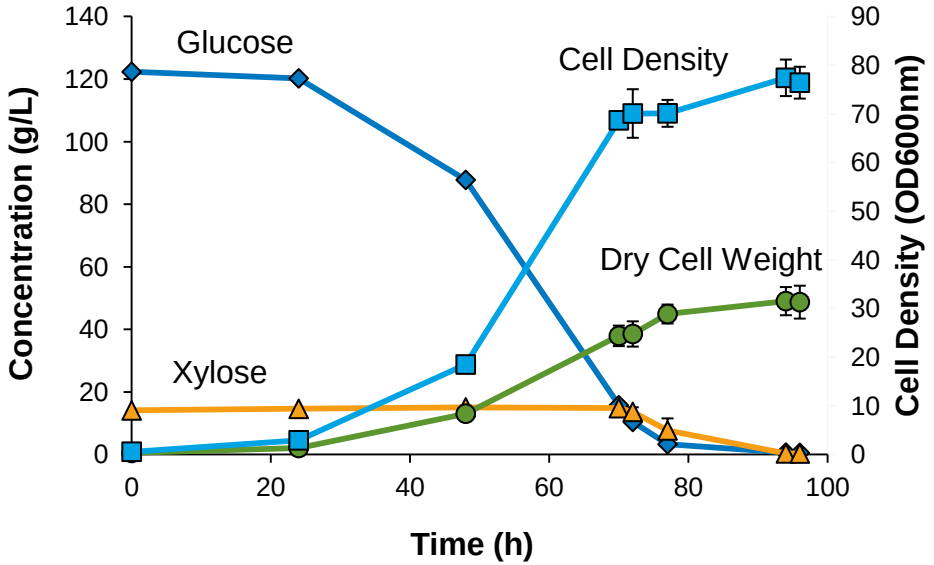


Evaluation of oleaginous yeast

Natural Strains

Lipid production by *L. starkeyi* in shake flasks

Lipid production by *L. starkeyi* in small fermentors – BSI Early Work



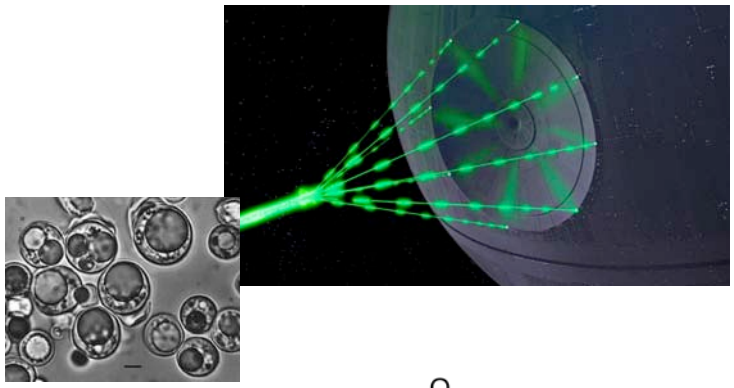
Metric	Pure Sugar - Flasks	Pure Sugar - Fermentor	C6 Biomass Sugars from Enz Hyd.- Fermentor
Glucose utilization (total)	98%	80%	100%
Lipid content	59%	60%	57%
Volumetric productivity (g/L-hr) at 72 h	0.05 (batch culture)	0.18 (batch culture)	0.29 (batch culture)
Lipid process yield (total sugar-to-product, g/g)	0.07	0.13	0.20

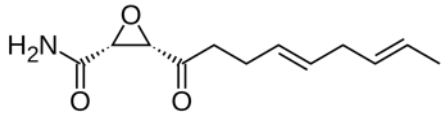
Strain evolution efforts

Evolved Strains

- β-ketoacyl-acyl carrier protein synthases (KS) regulate FA synthesis and are inhibited by **cerulenin**
- Cells can overcome this inhibition by increasing FA synthase production

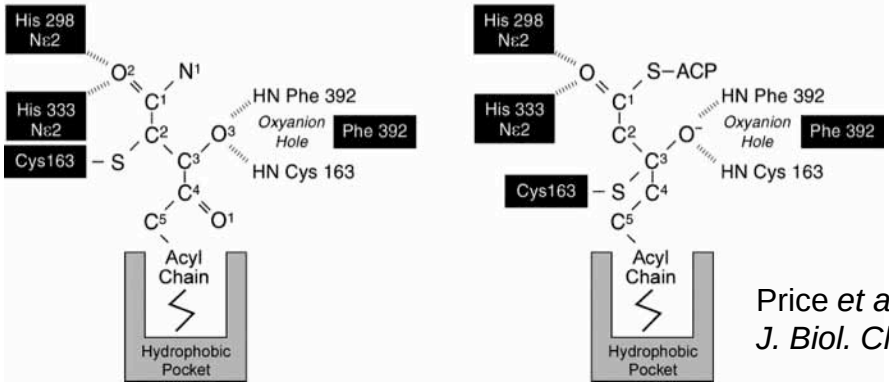
Tapia *et al.* 2012, *AMB Express*



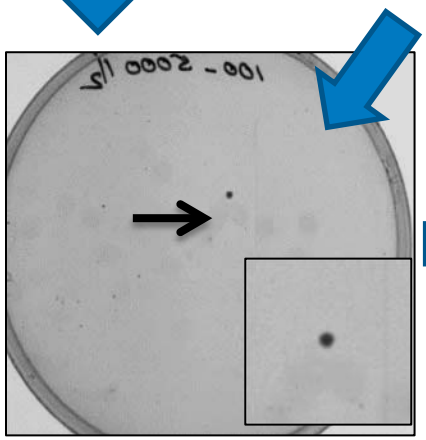


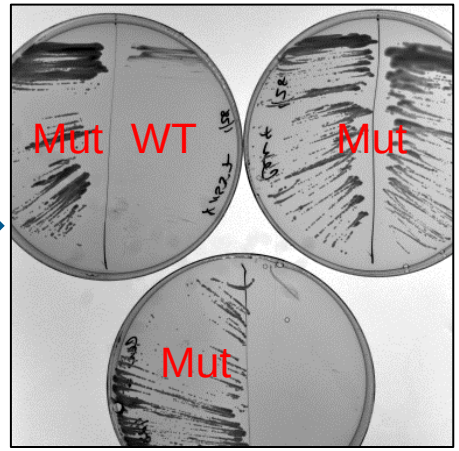
Cerulenin

Condensation Transition State



Price *et al.* 2001, *J. Biol. Chem.*





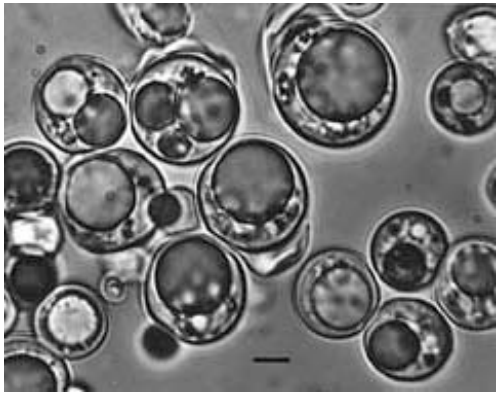
- Approach rapidly led to cerulenin-resistant mutants
- Testing these mutants for enhanced lipid production currently
- Will work with JGI to pinpoint genetic changes if positive hits are found (to make changes permanent)

Engineering increased lipids

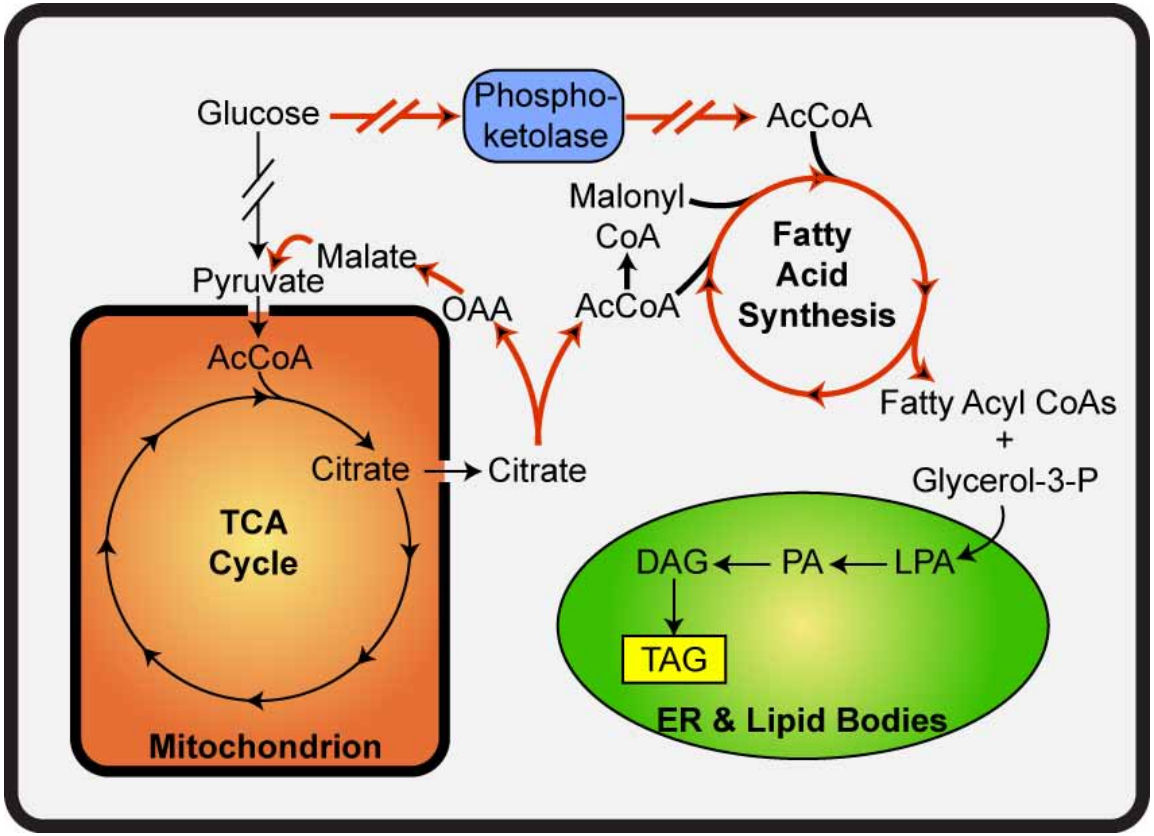
Engineered Strains

Chose *L. starkeyi* as initial strain for engineering

- Very high lipid productivities and titers
- Strain NRRL Y-11557 genome sequenced (Tom Jeffries/JGI)
- DNA Transformation established (Calvey *et al*. 2014)



Simplified metabolism for triacylglycerol production

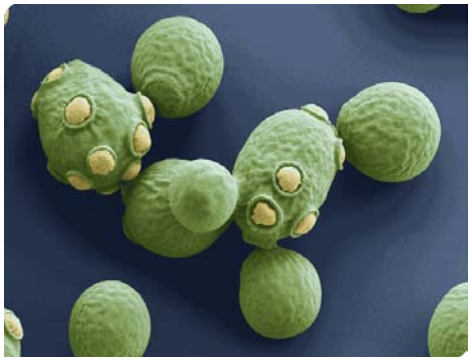


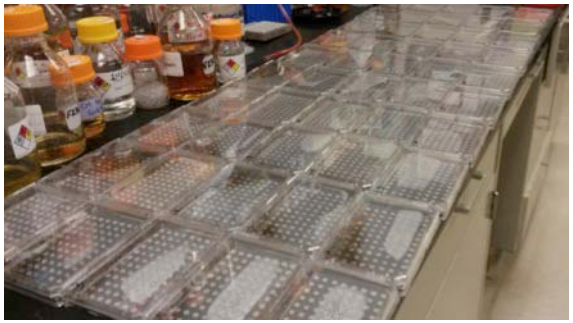
RED lines represent initial focal points for engineering

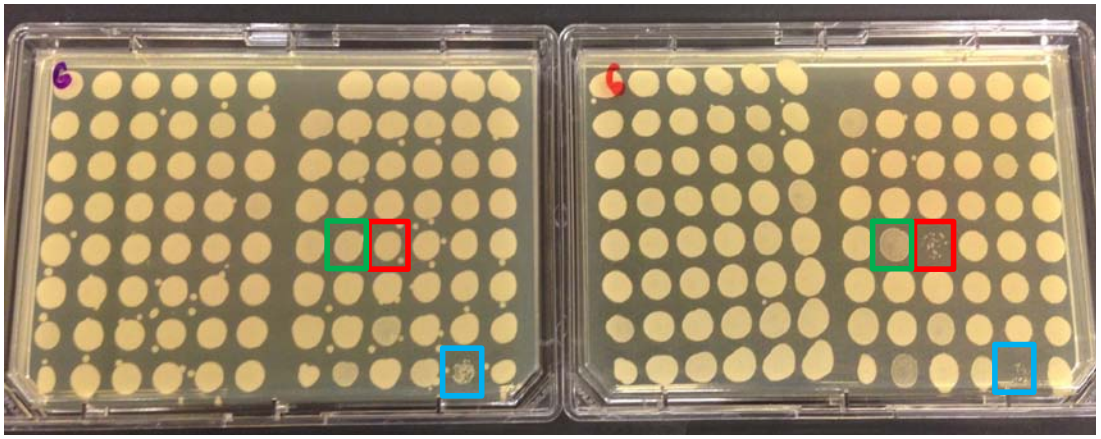
Overexpression of native biosynthetic genes and heterologous expression of a phosphoketolase to increase acetyl-CoA (AcCoA) pools

Leveraging *S. cerevisae* for rapid gene identification

Engineered Strains







Hyper-sensitive mutant

Slightly sensitive mutant

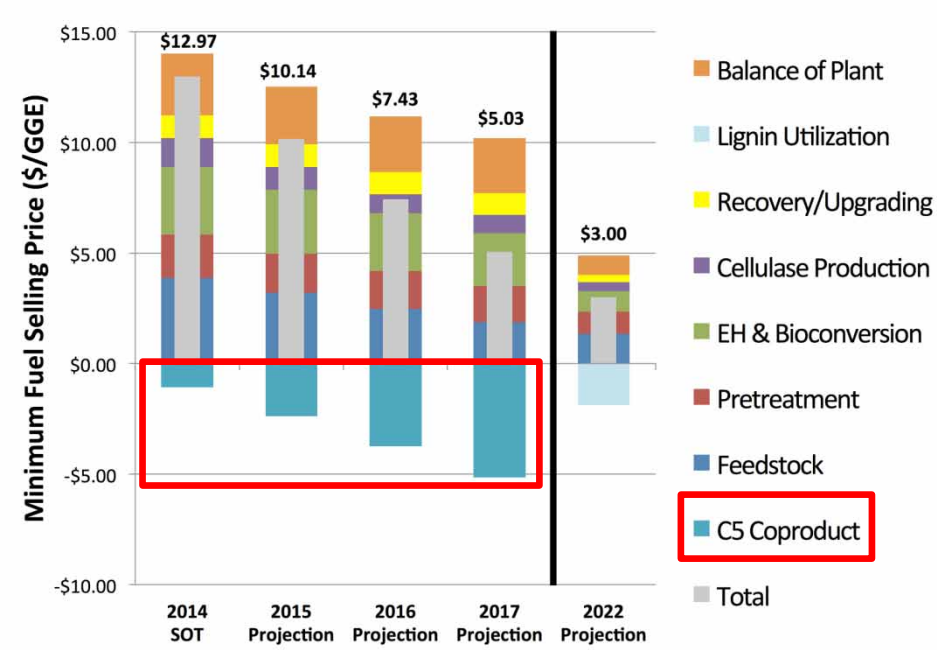
Intrinsically weak mutant

me

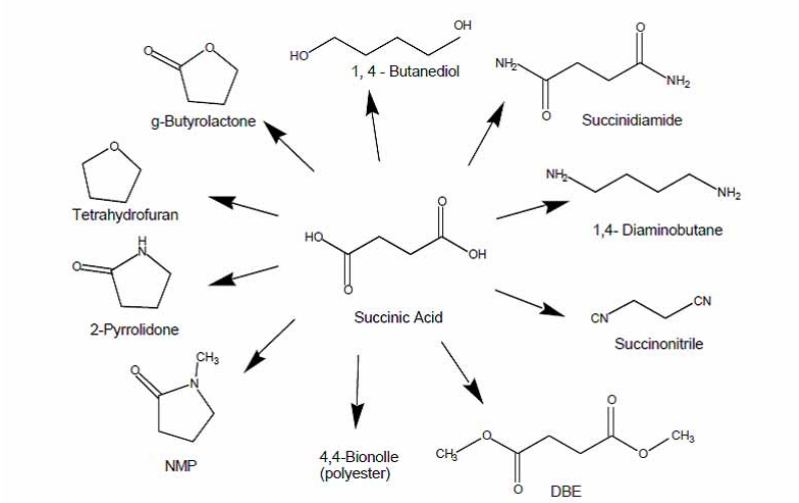
Hypersensitive mutants	77
Sensitive mutants	63
Intrinsically weak mutants	23

- Leverage single gene deletion and single gene overexpression collections developed in *S. cerevisiae*
- Developed, validated HTP method to screen for enhanced lipid production
- Currently screening ~5,000 single gene deletion strains and ~5,000 single gene overexpression strains to identify genes whose alteration increases lipid production
- Leverage these results to apply to more process-relevant but less genetically malleable strains, e.g., *L. starkeyi*

Why a C5-derived co-product? Why succinic acid?



Significant industrial interest already in this molecule



Top-Ten Value Added Chemicals from Biomass, Vol. 1, 2004

Direct and **functional replacement** markets for SA

- Potential for 4 MM tons/year (Top Ten Report)

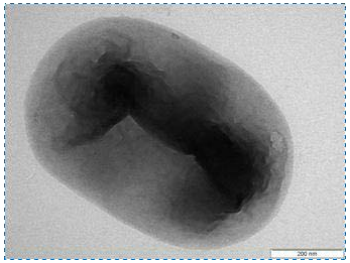
Disseminated results will aid industrial transition from starch to lignocellulosic sugars

- Similar to track record with ethanol demonstration

Acid functionality common to products of interest

- Broadly applicable insights in **integrated process**

Robust strains exist, enabling an aggressive timeline to an integrated 2017 demonstration



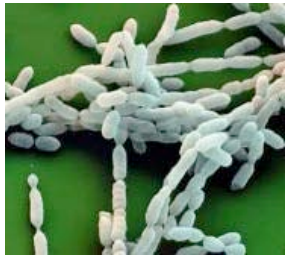


Image from BASF

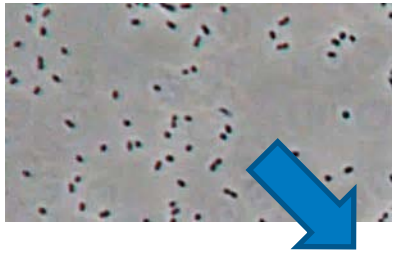
Strain down-selection

Evaluate strains on C5-hydrolyzate

Species examined from the literature

- *Anaerobiospirillum succiniciproducens*
- *Bacteroides fragilis*
- *Enterococcus faecalis* RKY1
- *Succinivibrio dextrinosolvens*
- *Fibrobacter succinogenes*
- *Mannheimia succiniciproducens*
- *Actinobacillus succinogenes*
- *Basfia succiniciproducens*

A. succinogenes



B. succiniciproducens

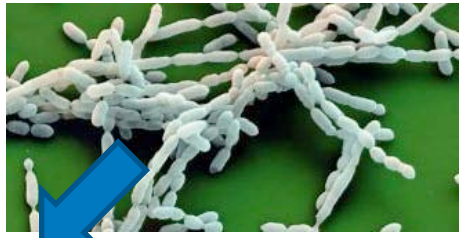


Image from BASF

0.5 L fermentors

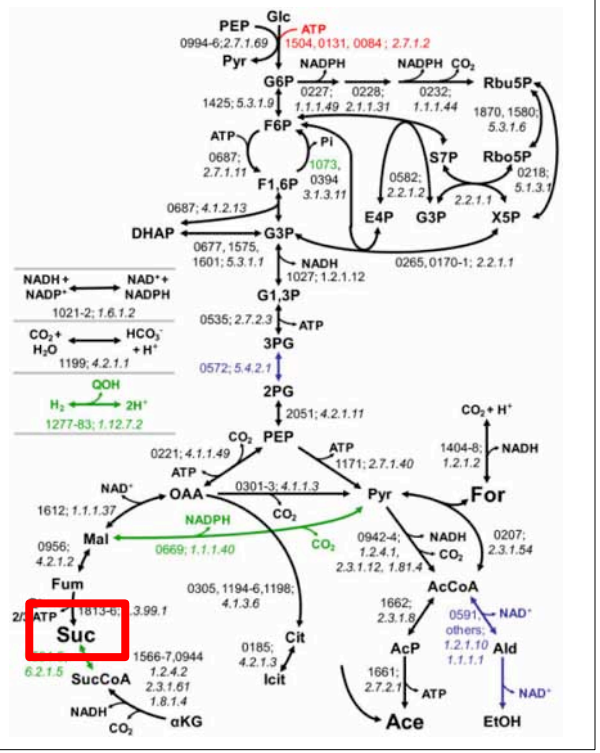
	Lignin	Monomeric sugars					Acetic acid	HMF	Furfural
All in g/L		Cellobiose	Glucose	Xylose	Galactose	Arabinose			
DCS-hydrolyzate	7.6	1.7	13.1	93.4	6.5	15.8	3.8	0.26	1.8

- Three strains were Biosafety Level 2 (in blue), two strains did not consume xylose (in red), and *M. succiniciproducens* is not publically available
- Rapidly down-selected to *B. succiniciproducens* and *A. succinogenes*
- Initially screening strains in batch reactors on C5-rich hydrolyzates

Two leading strains for SA production

Evaluate strains on C5-hydrolyzate

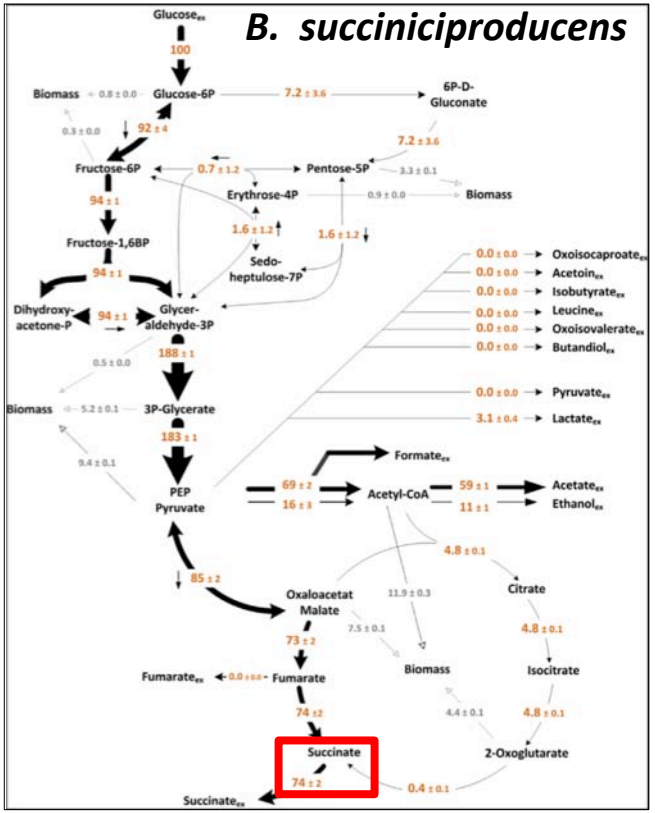
A. *succinogenes*



McKinlay JB, et al. (2010) BMC Genomics

- Facultative anaerobe, CO_2 fixer
- Produces formate, acetate, ethanol
- Does not have oxidative TCA cycle branch
- Forms biofilm
- Extensive information

B. *succiniciproducens*



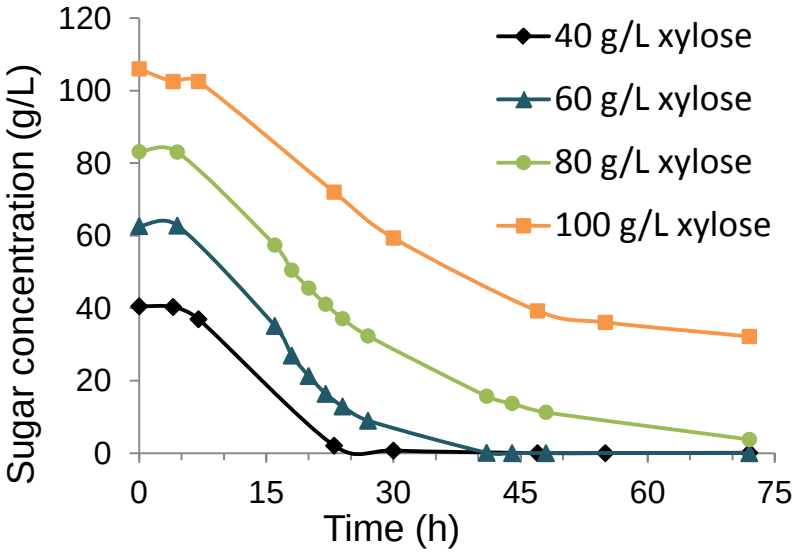
Becker, J. et al (2013) Biotechnol Bioeng.

- Facultative anaerobe, CO_2 fixer
- Produces formic, acetic, ethanol, lactate
- Produce SA via oxidative TCA cycle branch
- Does not form biofilm
- Limited information about this bacterium

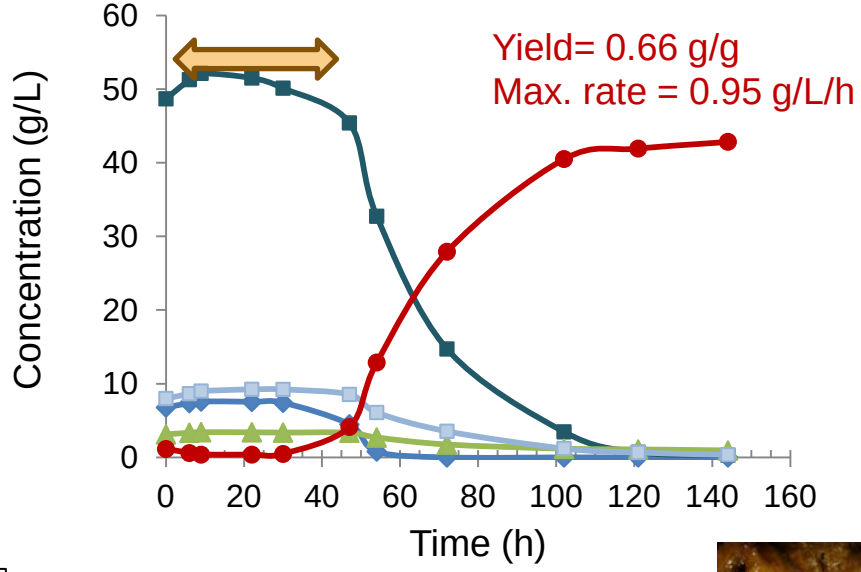
Testing inhibition in *A. succinogenes*

Adapt/probe tolerance mechanisms

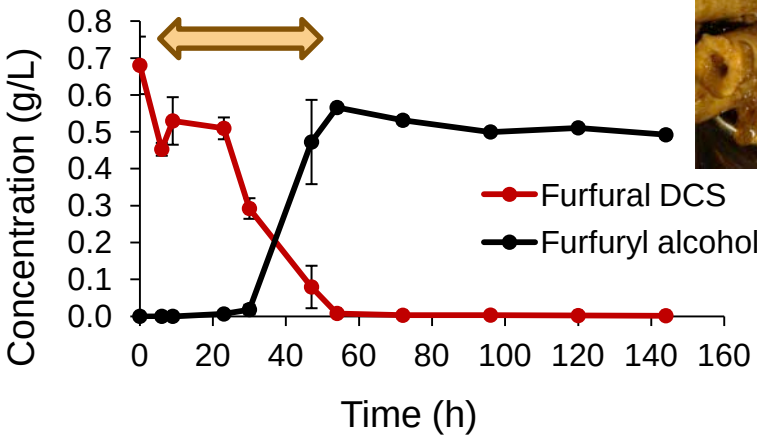
Tolerates sugar concentrations up to 80 g/L



Reasonable yield and rate on C5-rich hydrolyzate with significant lag phase



- Early work in BSI in FY14
- Furfural and HMF reduction correspond to lag
- Transferred to BSI Project for **continuous fermentation to obtain high yield and rate**
- Cleanup ongoing to overcome rate limitations
- Ongoing: **transcriptomics, proteomics, metabolomics, metabolic flux analysis**
- Similar work ongoing in *B. succiniciproducens*



Metabolic Engineering for Improved SA Biosynthesis

Apply genetic tools for strain engineering

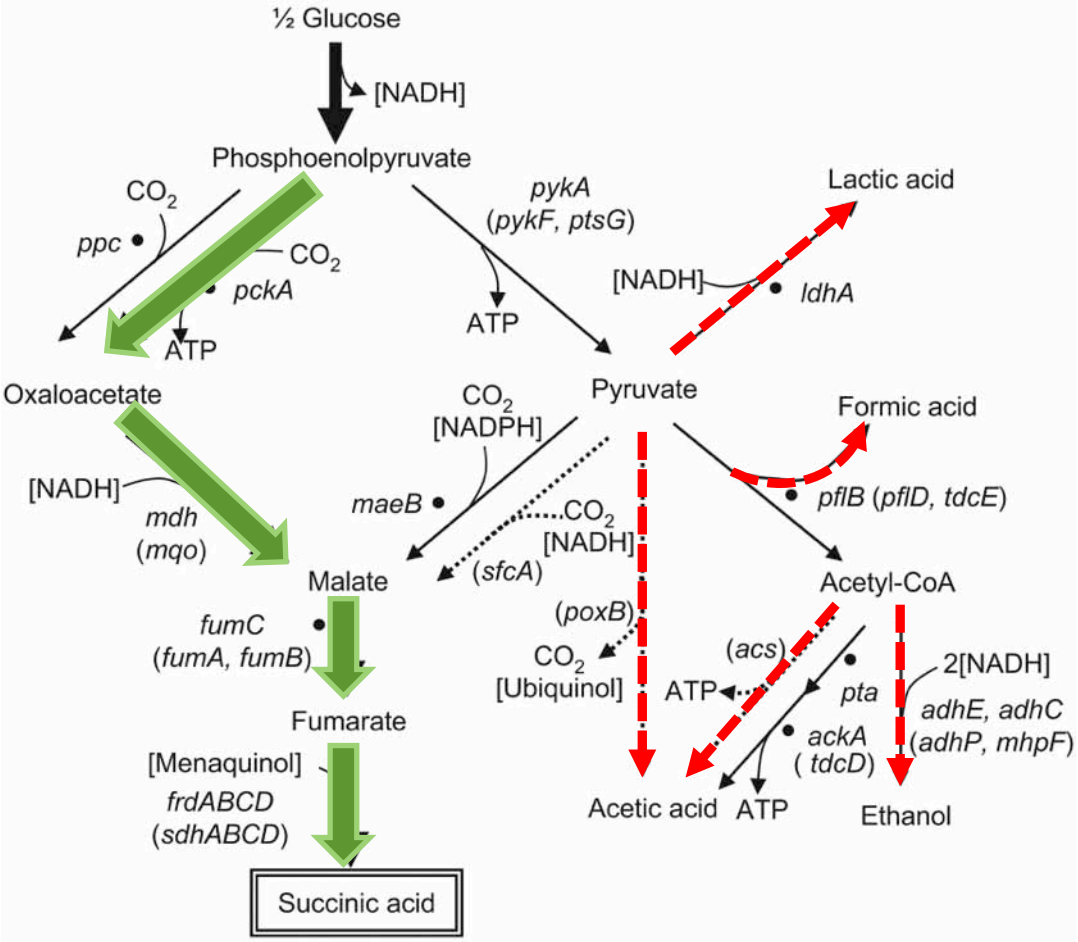


Image from S. Vaswani, 2010

Genetic tools will enable two parallel approaches to enhance flux to succinate:

- Overexpression of succinate biosynthetic components (*green arrows*)
- Down-regulation and/or knockout of competitive fermentation pathways: lactate, acetate, formate, and ethanol (*red arrows*)

Relevance

This project is essential for 2017 HC fuel cost targets of $5/gge

Key MYPP areas targeted by the Biological Upgrading of Sugars Project:

Catalyst Efficiency
- Developing efficient bio-catalysts to produce advanced fuels and chemicals
- Improvement in 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

Cleanup/Separation
- Elucidating inhibitor effects on biocatalysts and downstream processing

Key Stakeholders and Impacts:

- **Industrial and academic research focused on carbohydrate utilization in both HC fuel production and co-product manufacturing including chemical and polymer precursors from biomass**
- Will enable demonstration of C5-rich stream to chemicals in a scalable manner
- Co-products impact the "**Whole Barrel of Oil**"
- **Portfolio of chemicals will diversify and accelerate development of the biomass value chain**
- Significant amounts of peer-reviewed science and IP will be generated from this work
- Methods to upgrade sugars to organics acids can be leveraged well beyond succinic acid

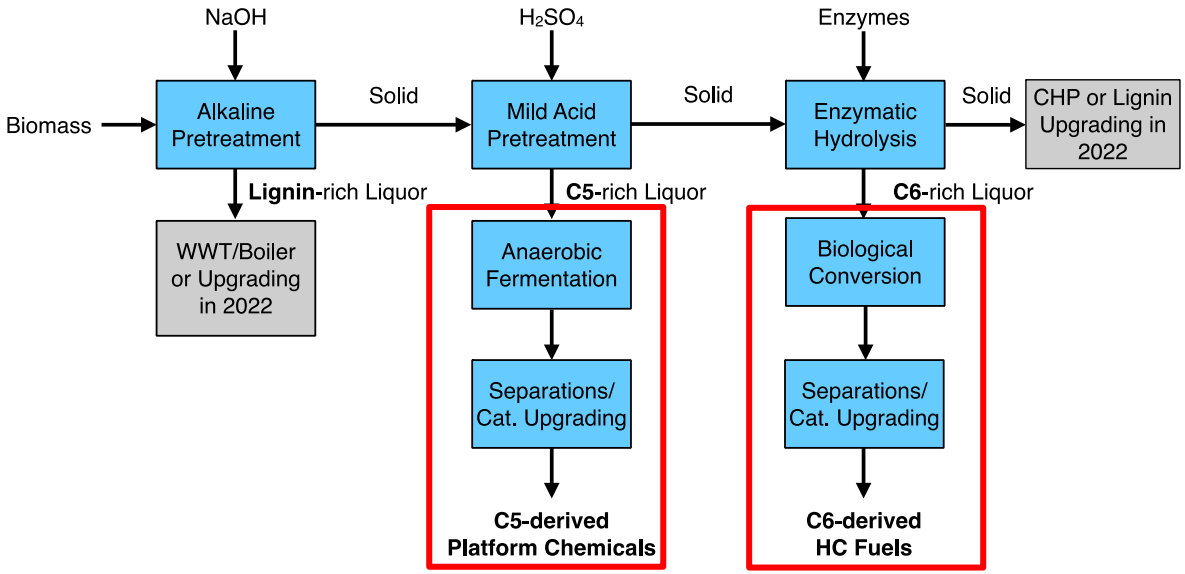
Future Work

Fatty Acid Production

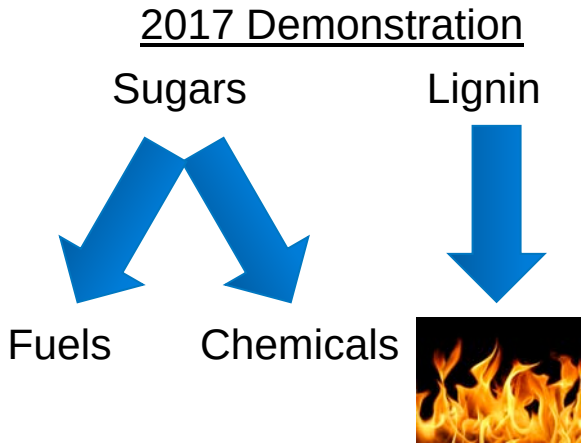
- Define 2-3 strains by end of FY15 with BSI
- Target a “final” strain by end of FY16
- 0.4 g/L/hr rate, 60% lipid content, and a 0.27 g/g yield on C6-enriched sugars

Succinic Acid Production

- Down-select strain by end of FY15 with BSI
- Target a “final” strain by end of FY16
- Target: 2 g/L/hr, 0.795 g/g yield on C5-enriched sugars



2017 Demonstration



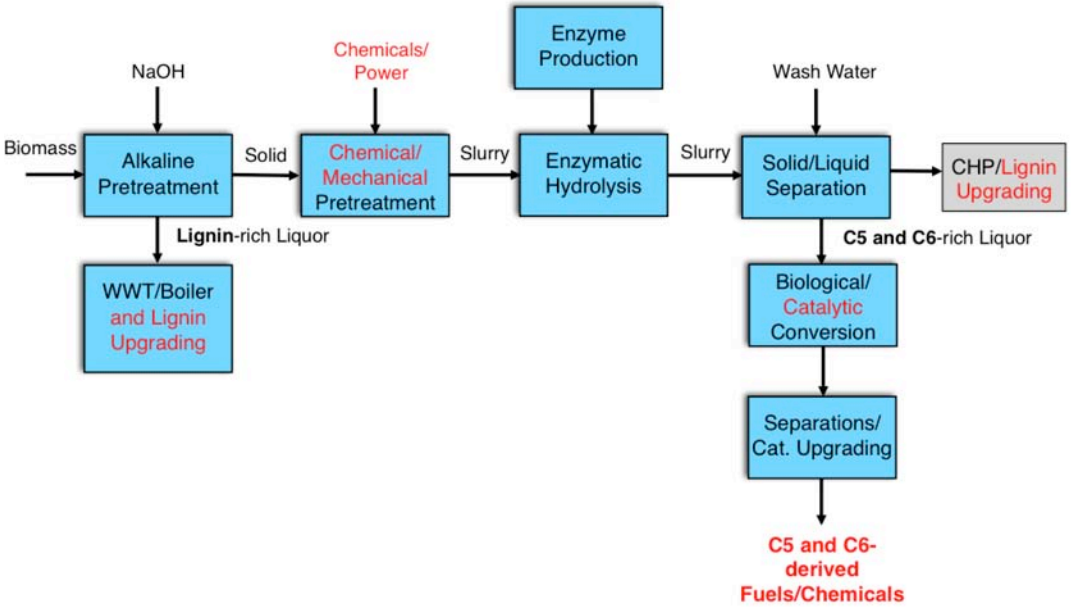
Future Work

Fatty Acid Production

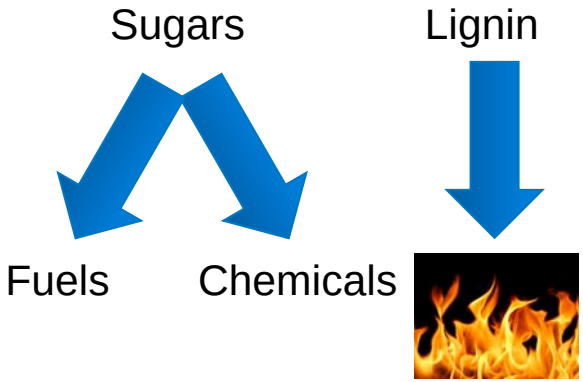
- Define 2-3 strains by end of FY15 with BSI
- Target a “final” strain by end of FY16
- 0.4 g/L/hr rate, 60% lipid content, and a 0.27 g/g yield on C6-enriched sugars

Succinic Acid Production

- Down-select strain by end of FY15 with BSI
- Target a “final” strain by end of FY16
- Target: 2 g/L/hr, 0.795 g/g yield on C5-enriched sugars



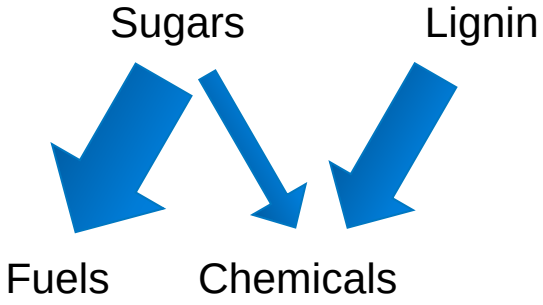
2017 Demonstration



Towards 2022 demonstration

- Emphasize step changes in lipid recovery through cell wall engineering and improved carbon flux
- Explore fuel precursors with higher C-efficiency pathways
- Divert more carbon to fuels through more efficient strains

Towards 2022



Summary

1) Approach:
   - Develop oleaginous yeast for lipid production for renewable diesel blends from C6-rich streams
   - Develop example co-product train (succinic acid) from C5-rich streams from dilute-acid pretreatment
2) Technical accomplishments (4 months of work thus far)
   - Screening large collection of oleaginous yeast in a self-consistent manner
   - Demonstrated ability to rapidly evolve *L. starkeyi* strains towards higher lipid production
   - Developed a HTP method for screening for gene candidates for lipid production in a model system
   - Demonstrated **high yields** of succinic acid on process-relevant hydrolysate
   - Identified multiple inhibitors that cause a lag phase in *A. succinogenes* growth and SA production
   - Metabolic engineering in progress for both FA and SA industrial production hosts
3) Relevance
   - Directly **impacts the 2017 and 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 co-products, high yields of FAs and products needed
5) Future work:
   - Continue all fronts towards down-selection of strains for 2017 demonstration, partial transition of efforts to 2022 targets in mid- to late-FY16
6) Technology transfer:
   - Initiating contact with **industry** to build commercialization path for both fuel and co-product trains

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External collaborators

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- School of Chemical Engineering Practice, MIT
- Kyria Boundy-Mills, UC Davis Phaff Yeast Culture Collection

Acronyms

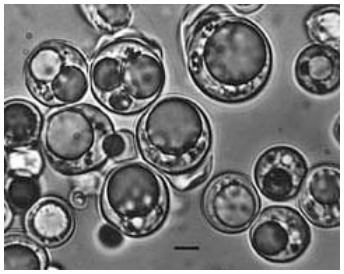
- FA: Fatty Acid
- LCA: Life-Cycle Analysis
- NHEJ: Non Homologous End Joining
- SA: Succinic Acid
- TEA: Techno-Economic Analysis

L. starkeyi: Initial strain for engineering

Engineered Strains

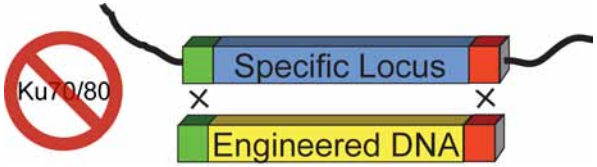
Strain Highlights :

- Very high lipid productivities and titers
- Strain NRRL Y-11557 genome sequenced (Tom Jeffries/JGI)
- DNA Transformation established (Calvey et al. 2014)

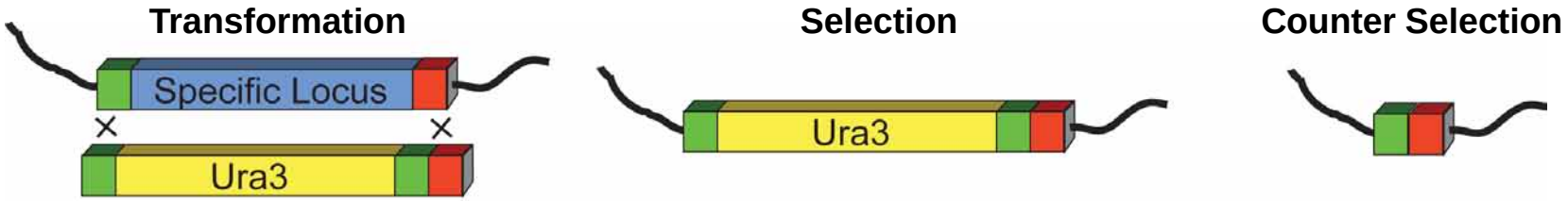


Initial Genetic Tool Development goals:

- **Increasing the genetic engineer-ability:** Disruption of Ku70/80 genes should increase the efficiency of gene targeting by eliminating NHEJ for DNA repair
- Currently screening hundreds of transformants to identify *ku70* deletion mutants



Reusable selectable/counter-selectable marker: Generated random mutant *ura3* auxotrophic strains and are screening transformants for “clean” deletions to enable marker recycling:



Genetic Tool Development in *A. succinogenes*

Develop/improve genetic tools

Replicative plasmid (pLGZ920) obtained for facile gene expression

- Complete plasmid re-sequenced to facilitate construct design

Efficient electroporation transformation method developed

- 9 x 10^4 cfu/µg plasmid
- Sufficient for plasmid transformation and good starting point for linear DNA transformation (for gene knockout)

Similar tools in place for *B. succiniciproducens*

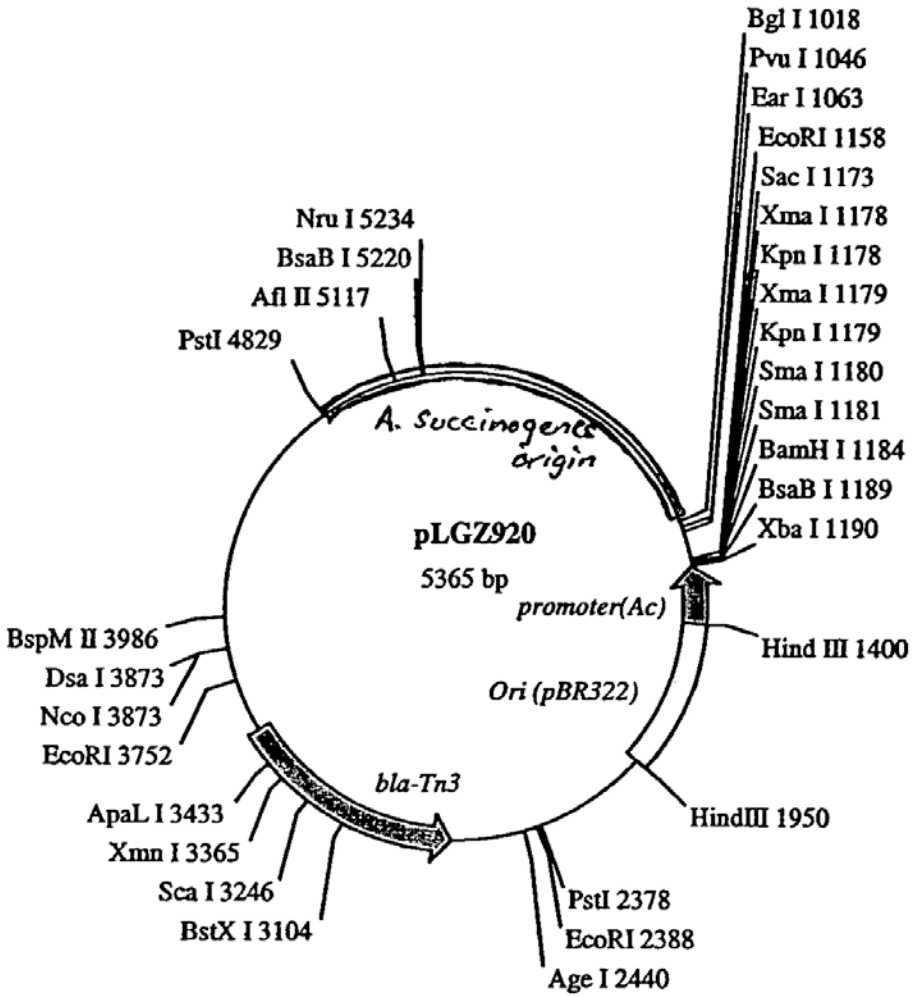


Image from JG Zeikus *et al.*

A. succinogenes -omic Analyses

Develop/improve genetic tools

Identify promoters across an array of expression levels

- Facilitate fined-tuned expression of strain-engineering targets

Comparative analyses between solution state and biofilm (production) state

- Identify novel targets for induction of biofilm formation and temporal regulation of succinate biosynthesis