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DOE Bioenergy Technologies Office (BETO) 2015 Project Peer Review

2.3.2.103 Fungal Genomics

March 8, 2017
Biochemical Conversion Area Review

Jon Magnuson
PNNL

Goal Statement

- **Goal:** Development of efficient and **robust fungal** biocatalysts and **bioprocesses** that utilize **lignocellulose** feedstocks to produce advanced biofuels and bioproducts at lower cost.

Quad Chart Overview

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Timeline

- AOP project start date: 10/1/15
- Project end date: 9/30/18

Budget

	FY 15 Costs	FY 16 Costs	FY 17 Costs	Total Planned Funding (FY 15-17Project End Date
DOE Funded	**1.5**	**1.5**	**1.5**	**4.5**
Project Cost Share (Comp.)*	0	0	0	0

Barriers

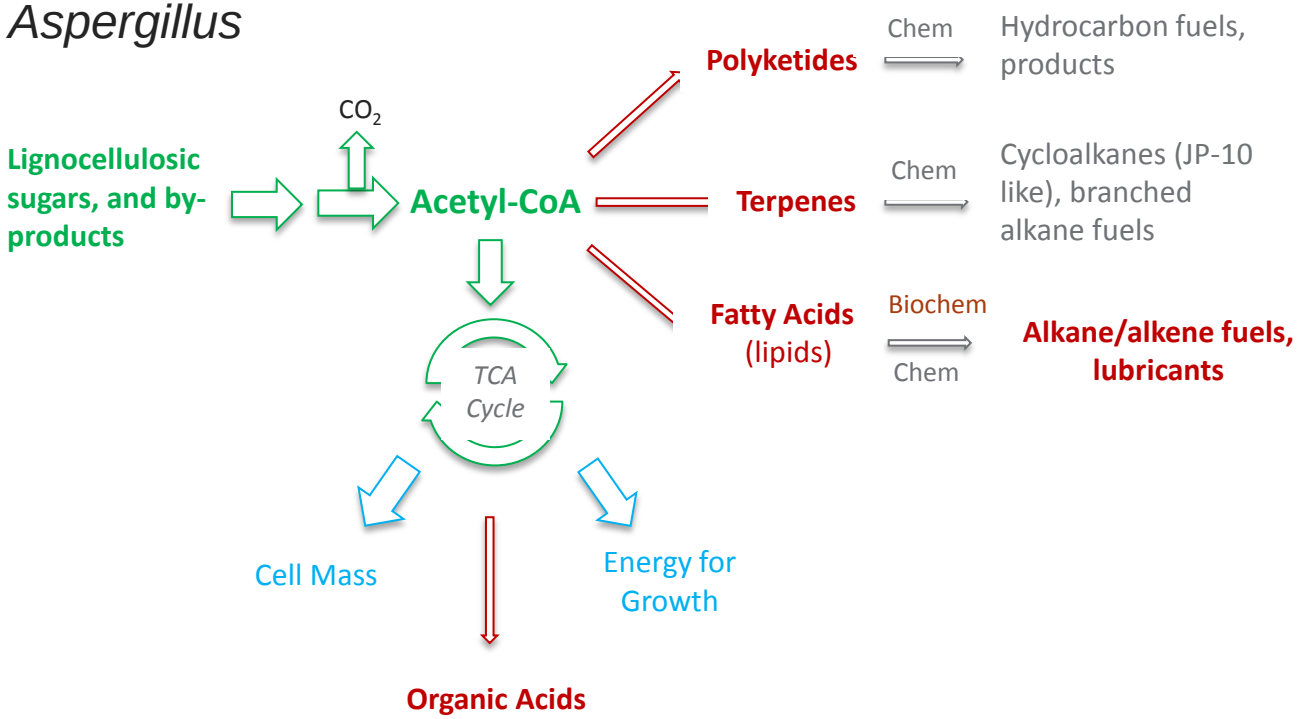
- Barriers addressed
  - Bt-J. Catalyst Development
    - Process robustness and productivity to lower cost.
    - Higher value bioproducts to support economical biofuel production
  - Bt-L. Biochemical/Thermochemical Interface
    - Lipids for catalytic upgrading to fuel
    - Fungal cell mass for HTL
    - Bioproduct precursors for catalytic processing to various chemicals.

Partners

- **Funded Partners**
  - U. of Kansas, **Berl Oakley**, 55K
- Collaborators
  - NREL, JBEI, JGI
- **Industrial Advisory Panel**
  - Novozymes*
  - POET Research*
  - Mycosynthetix
  - ***Cost share or funds-in partners on past projects***

1 - Project Overview

- Development of industrially* relevant **fungal** biocatalysts & bioprocesses for fuels and bioproducts (chemicals)
  - **Hydrocarbon fuels and bioproducts**: triacylglycerides (TAGs), terpenes, polyketides
  - Develop and utilize modern genetic, genomic and bioprocess tools to produce the hydrocarbons and maximize TRY in two platform fungi: *Lipomyces* and *Aspergillus*



  - * **Robust** growth and production in the presence of inhibitors, efficient utilization of different sugars, **scalable** bioprocess

2 – Management Approach

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Management Approach

- Annual operating plan (**AOP**) tied to DOE-BETO goals
- PNNL has an internal project management plan (**PMP**)
- Quarterly **milestones** & **Go/No-Go** decisions to direct, focus and measure progress
- Quarterly written reporting and conversations with **BETO TM**
- Weekly **team meetings** to maintain focus and tackle technical challenges
- Co-PI (Butcher) to coordinate efforts and maintain team focus
- Work with **chemical catalysis** experts to identify biomolecules of interest for products/fuels
- Tap into **industrial perspective** through quarterly meetings with Industrial Advisory Panel: Novozymes, POET, Mycosynthetix
- Input from a variety of internal and external **reviews**

***Scientific Team**

Mark Butcher
Jim Collett
Dave Culley
Ziyu Dai
Shuang Deng
Beth Hofstad
Ellen Panisko
Kyle Pomraning
Swarnendu Tripathi

2 – Technical Approach

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Approach: Develop and ***utilize*** modern fungal genetic engineering, integrated genomics and bioprocess engineering capabilities to produce robust, efficient organisms (biocatalysts) and bioprocesses for biofuels and bioproducts

Critical Success Factors

- Maximize **TRY**:
  - high **Titer** for downstream processing efficiency
  - high **Rate** to minimize CAPEX/OPEX
  - high **Yield** to maximize use of costly biomass feedstocks
- **Robust organisms** for conversion of challenging biomass hydrolysates containing **inhibitors** and **mixed sugars**
- Development of reproducible, robust and **efficient bioprocesses** that will **scale**

2 – Technical Approach: Complementary Biocatalyst and Bioprocess Development

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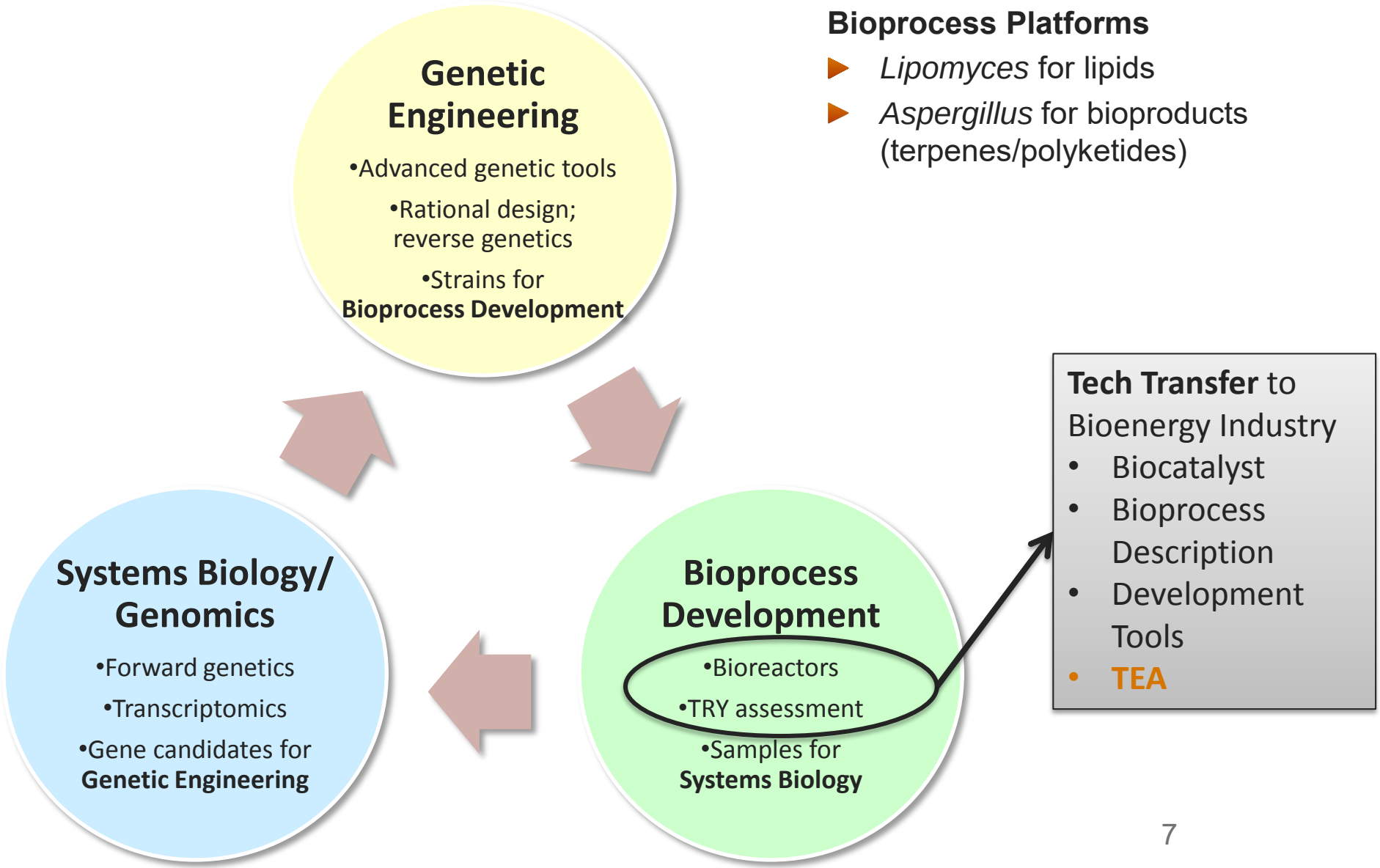
Bioprocess Platforms

- *Lipomyces* for lipids
- *Aspergillus* for bioproducts (terpenes/polyketides)



3 – Project Accomplishments

Why is Lipomyces an excellent choice for an industrial biocatalyst?

Attributes

- Highly productive oleaginous yeast: **~65%** of cell mass as lipid
- Has a genome sequence from JGI: NRRL Y-11557
- Grows and produces lipids on lignocellullose relevant sugars:
  - Excellent on **glucose**, galactose, mannose, and **xylose**
  - Also utilizes **cellobiose** and L-arabinose
- We've developed genetic tools
- Behaves well in conventional aerobic bioreactors

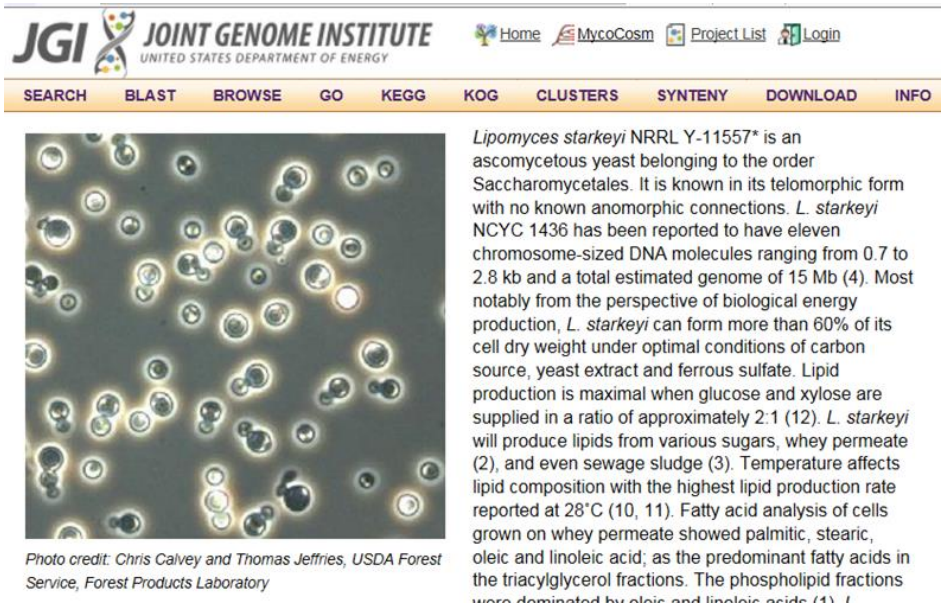
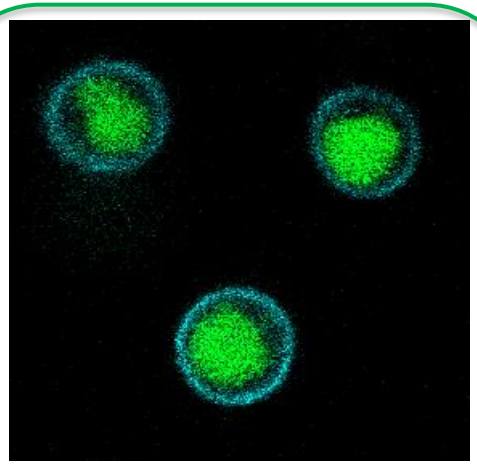


Photo credit: Chris Calvey and Thomas Jeffries, USDA Forest Service, Forest Products Laboratory



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

3 – Project Accomplishments

Why is Aspergillus an excellent choice for an industrial biocatalyst?

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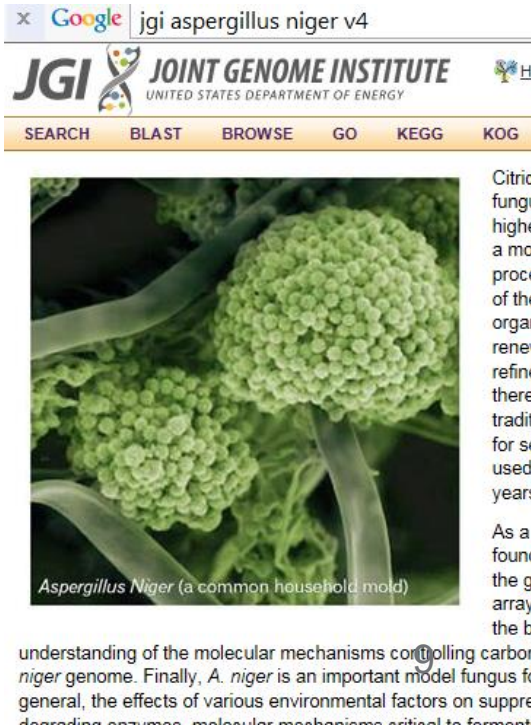
Attributes

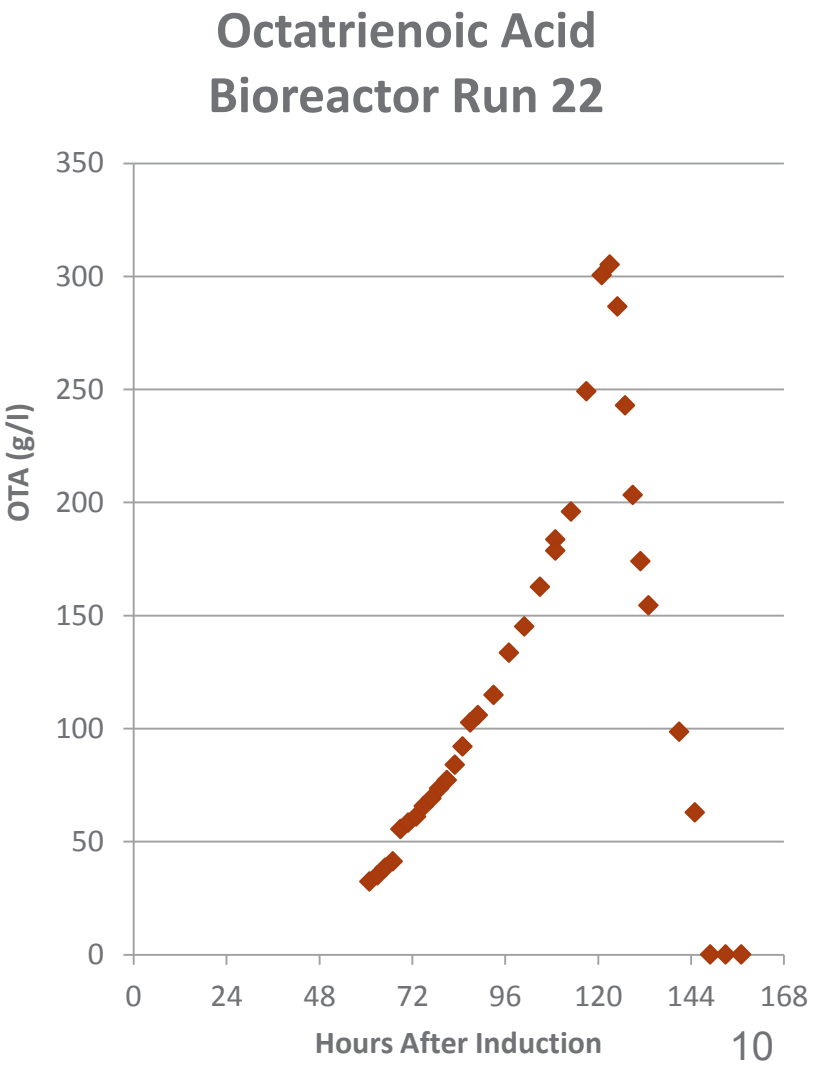
- Highly productive fungus for **enzymes** and **biochemicals**, including generally recognized as safe (GRAS) products
- Has multiple **genome** sequences: ATCC 1015, 11414
- Great **genetic** tools
- Grows and produces bioproducts on **lignocellullose** relevant sugars
- Produces enzymes to deconstruct lignocellulose
- Utilizes simple **inorganic nutrients**
- Highly **acid tolerant** (pH 1): minimizes sterility requirements, and advantageous for organic acid production
- Appropriate for conventional aerobic bioreactors



3 – Project Accomplishments
Aspergillus for Polyketides

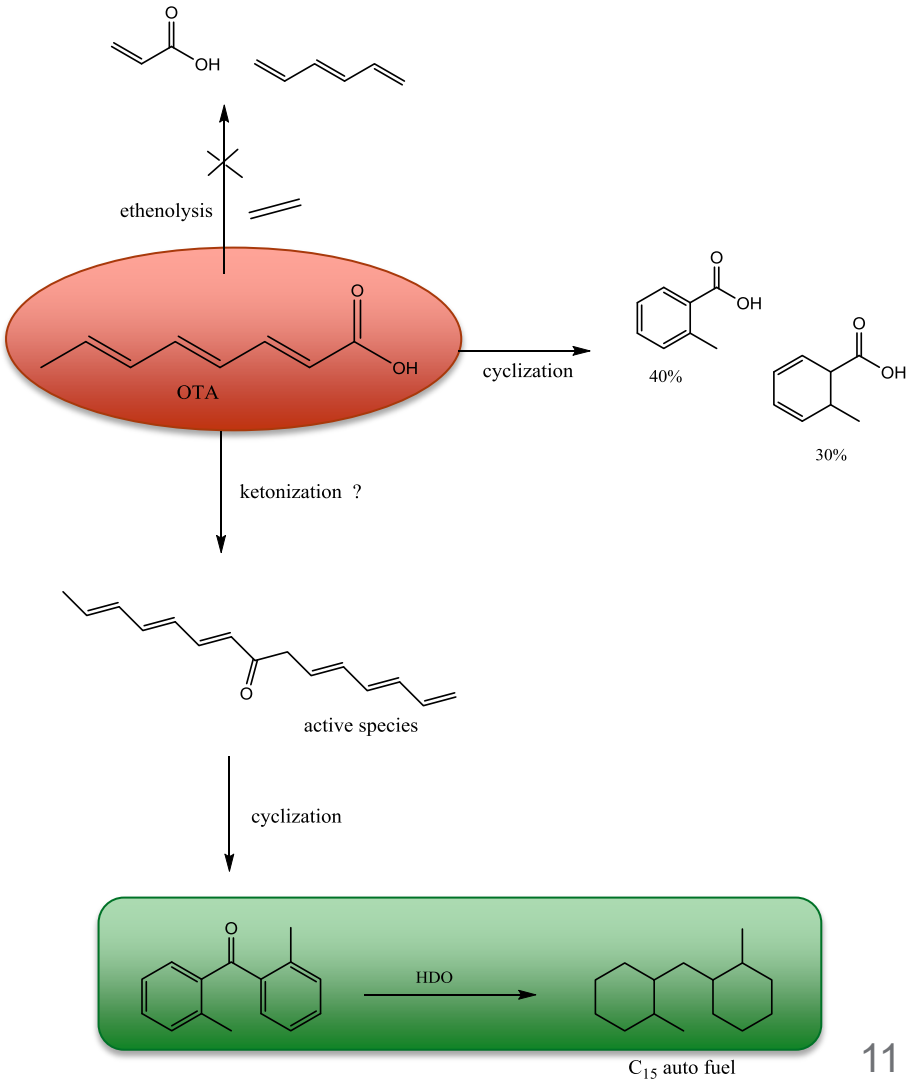
- There are many (est. >100K) different polyketides made by fungi
- We are focused on **highly reduced polyketides** as potential biofuels and bioproducts precursors
- We have produced 0.7 and **0.5 g/L** of two polyketides: asperbenzaldehyde and **octatrienoic acid**
- 9/30/17 Milestone and a mid FY Go/No-Go dictate concentrating on one for further optimization

- **Selected** the secreted compound octatrienoic acid for further development as an interesting **intermediate** to biofuels/bioproducts



3 – Project Accomplishments
Chemical conversion of OTA from *Aspergillus*

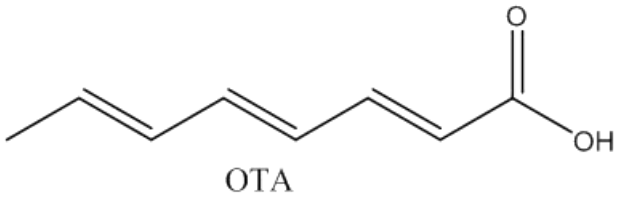
- **Octatrienoic acid** is a potential precursor for biofuels or bioproducts
- Ethenolysis/metathesis did not yield the desired acrylic acid and olefin products
- Ketonization will be examined as a possible route to a **C15 cycloparaffin**
- Cyclization is another potential route to aromatics and cyclic chemicals



3 – Project Accomplishments
Genetic Engineering of *Aspergillus* for Polyketide TRY

- Maximizing TRY can involve: **increasing** production and/or **decreasing** degradation

- OTA-PKS under control of a strong MEK-inducible promoter, which provided **0.5 g/L** titers in shake flasks
- Product degradation occurred after a sharp maximum OTA titer in the bioreactor
- **Goal**: utilize a different promoter that does not require a volatile or expensive inducer…more practical for a real bioprocess
- Examining two other promoters:
  - Constitutive *gpdA* promoter
  - Nutrient inducible *nmt1* promoter

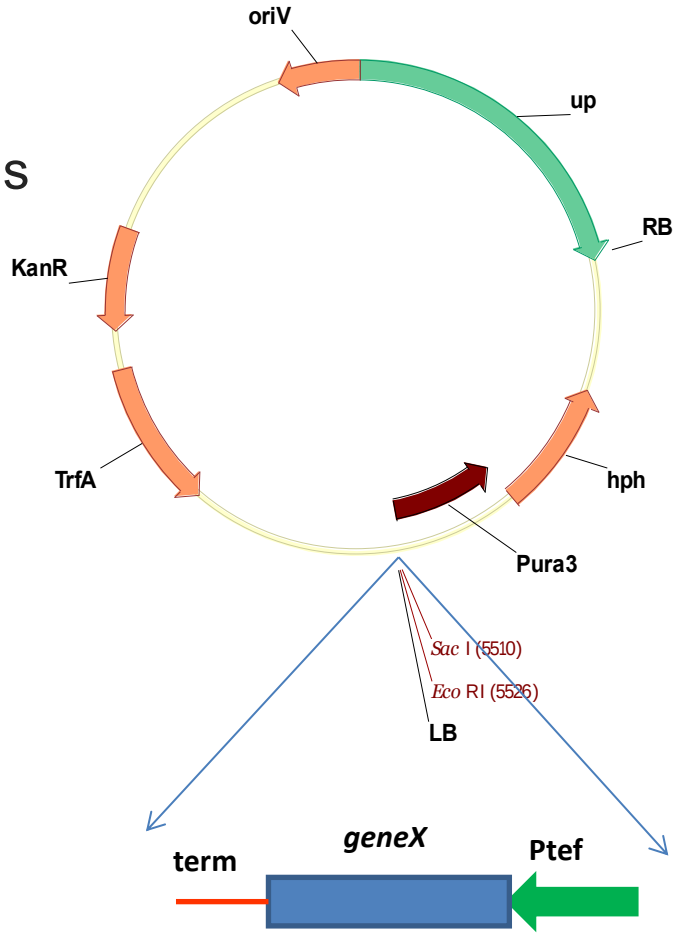


OTA

- **Goal**: **prevent product degradation** or modification
- Targeting likely catabolic pathways
  - Beta-oxidation
  - Alpha-oxidation

3 – Project Accomplishments
Improving Lipomyces– Homologous Transformation

- Challenge: Need a transformation system for **gene deletion-**- to eliminate carbon sinks, increase inhibitor resistance, etc. but…
- *L. starkeyi* didn't have any transformations systems
- We previously reported *Agrobacterium tumefaciens* mediated transformation
- Have since succeeded in using a lithium acetate and electroporation method for gene deletion
- Have disrupted ***ku70*** to obtain **homologous recombination!**



3 – Project Accomplishments
Improving *Lipomyces*– CRISPR

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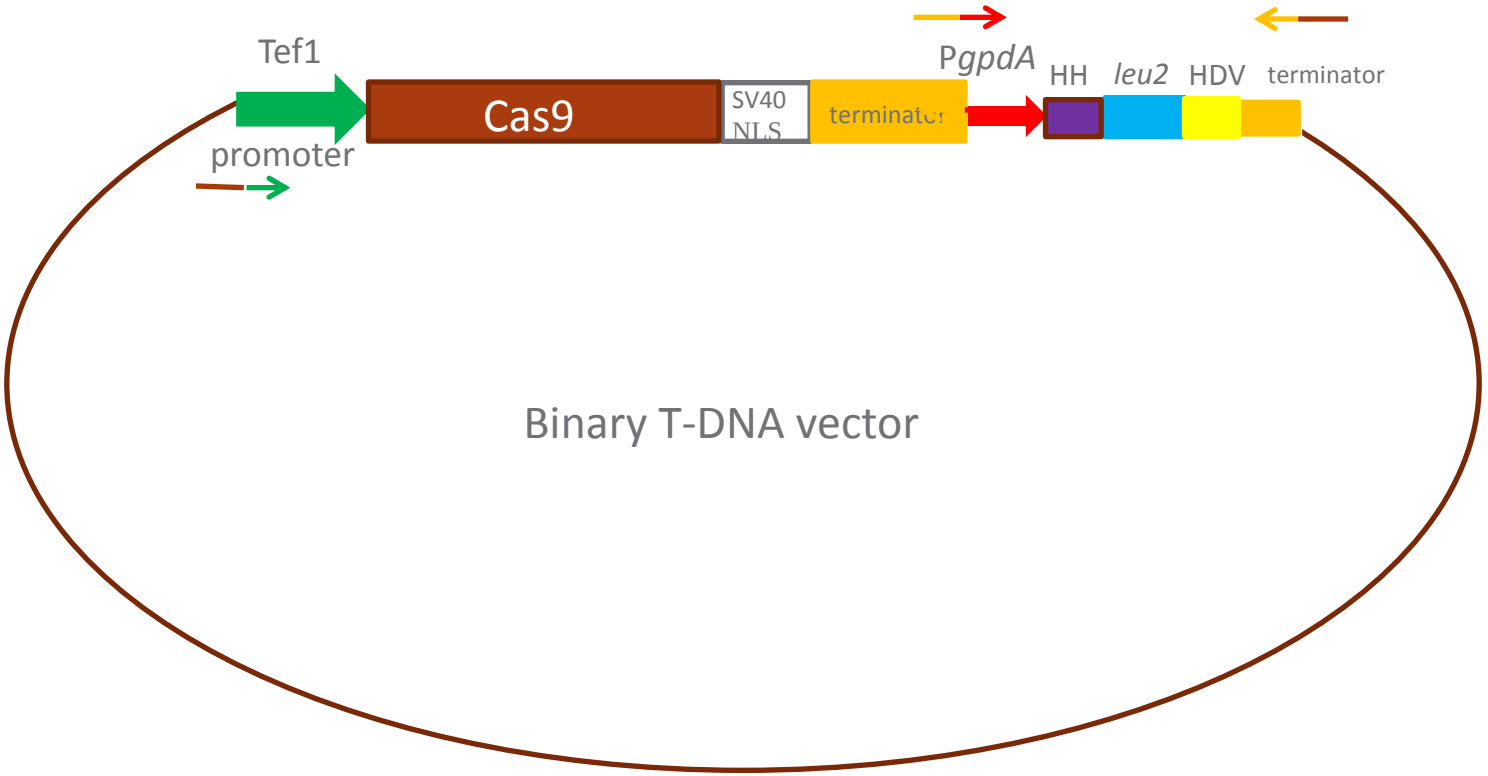
- **Goal**: faster genome editing to **accelerate** targeted genetic improvements
- Have succeeded in instituting CRISPR/cas9
- Used CRISPR to generate a *leu2* knockout = leucine auxotroph
- **Increasing efficiency of CRISPR**—examining alternate promoter



3 – Project Accomplishments
Lipomyces Metabolic Modeling

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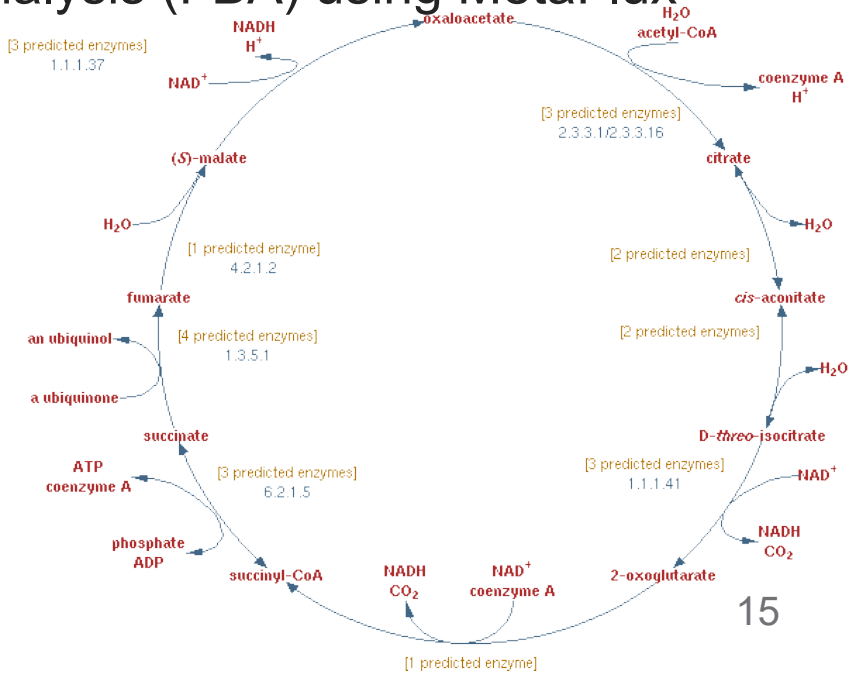
- Used **Pathway Tools** and the **JGI** genome data of *L. starkeyi* to establish a Pathway/Genome Database (PGDB)
- Using our RNA-Seq data and Pathway-Hole-Filler we closed 107 pathway holes
- Ran the Transport-Inference-Parser to annotate 65 unique transport reactions catalyzed by 333 unique transporters
- Currently creating a metabolic model within the Pathway Tools environment to perform Flux Balance Analysis (FBA) using MetaFlux
- Provides a framework for:
  - **Understanding** the system
  - Making **predictions** about genes utilizing omics and bioprocess data



3 – Project Accomplishments
Comparative transcriptomics for gene target ID

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- Two bioreactor runs for transcriptomics samples:
  1. Saccharified Pre-treated Corn Stover (PCS)
  2. Clean Sugars, 2:1 Glucose:Xylose
- Purposes:
  - Time course analysis to examine **lipid production, degradation** and other **C sinks**
  - Comparative analysis between runs to **identify inhibitor responses**

Run	Medium	Final Dry Cell Mass (g/L)	Specific growth rate (hr^{-1})	Yield of Cell Mass from Sugar (g/g)	Lipid **Titer** (g/L)	Lipid Synthesis Rate (g/L/h)	Peak **Rate** (g/L/h)	**Yield** of Lipids from Sugar (wt%)	Lipids as % of Dry Cell Mass
18. PCS	**PCS/MM;** 2.1% Total Sugars	18.6	0.11	**0.43**	**6.7**	0.05	**0.21**	**22%**	36%
19. Glc/Xyl	2.7% 2:1 Glc/Xyl/MM	22.0	0.14	**0.43**	**9.7**	0.09	**0.29**	**19%**	44%

Constants: defined minimal medium components (MM), 2:1 Glc:Xyl ratio, cell inoculum, temperature, aeration, pH

3 – Project Accomplishments
Comparative transcriptomics for gene target ID

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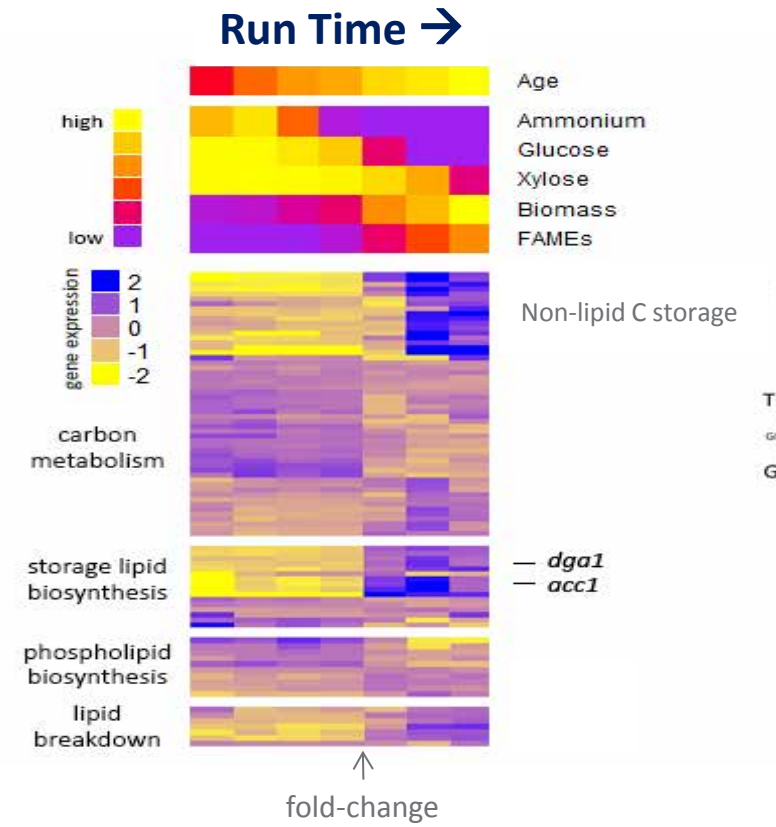
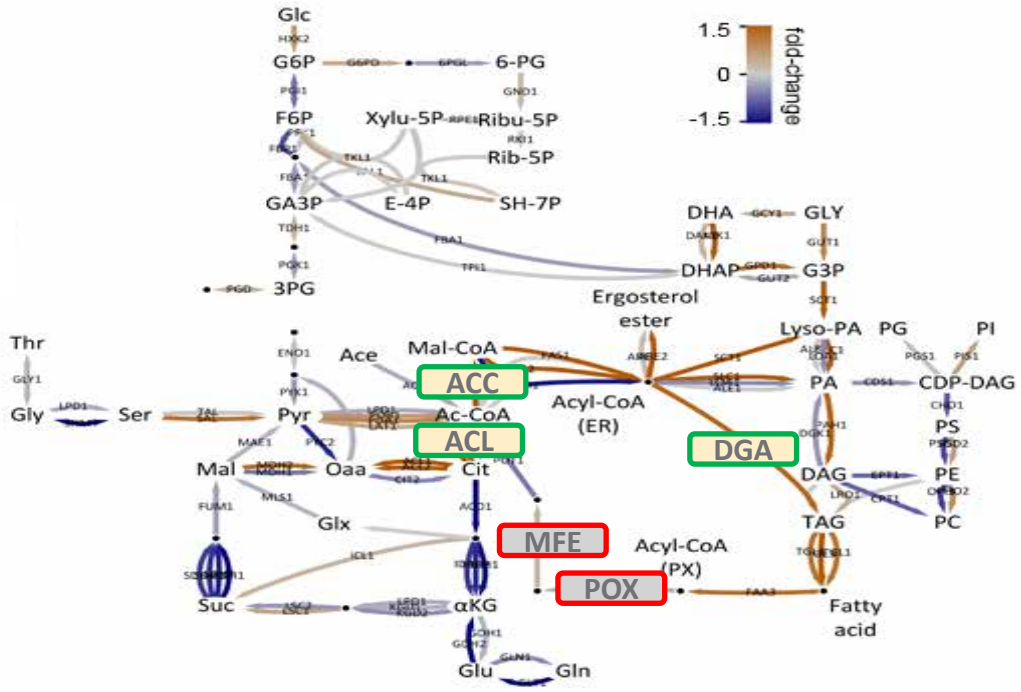
- Nitrogen depletion & lipid biosynthesis conditions
- Gene Targets
  - Overexpression of enzymes for triglyceride biosynthesis
  - Deletion of beta-oxidation or upstream enzymes to increase net productivity
  - ID'd other enzymes in triacylglyceride degradation that may be even **better targets**
  - ID'd interesting alternative C sink to eliminate
- Tools: new promoter motifs identified for expressing multiple genes





3 – Project Accomplishments
Initial Genetic Engineering Targets

- Over-expressed ***acc1*** for acetyl CoA carboxylase and previously reported promising results in shake flasks
- Did not see an improvement in a 30L bioreactor run
- ***pex10*** deletion to disrupt the peroxisome in general, and therefore β-oxidation
- Did not appear effective in *Lipomyces* under the conditions examined but may manifest an effect under more stressful conditions

- **Currently**
- Targeting **multiple gene up-regulation** for triglyceride biosynthesis…
- …plus some new upstream targets in triglyceride degradation from the comparative transcriptomics analysis

TEA and Design Case Considerations

- Higher Rate
- Higher Total Mass
- Separation of solids by low cost belt filter
- …and/or utilization of those solids downstream

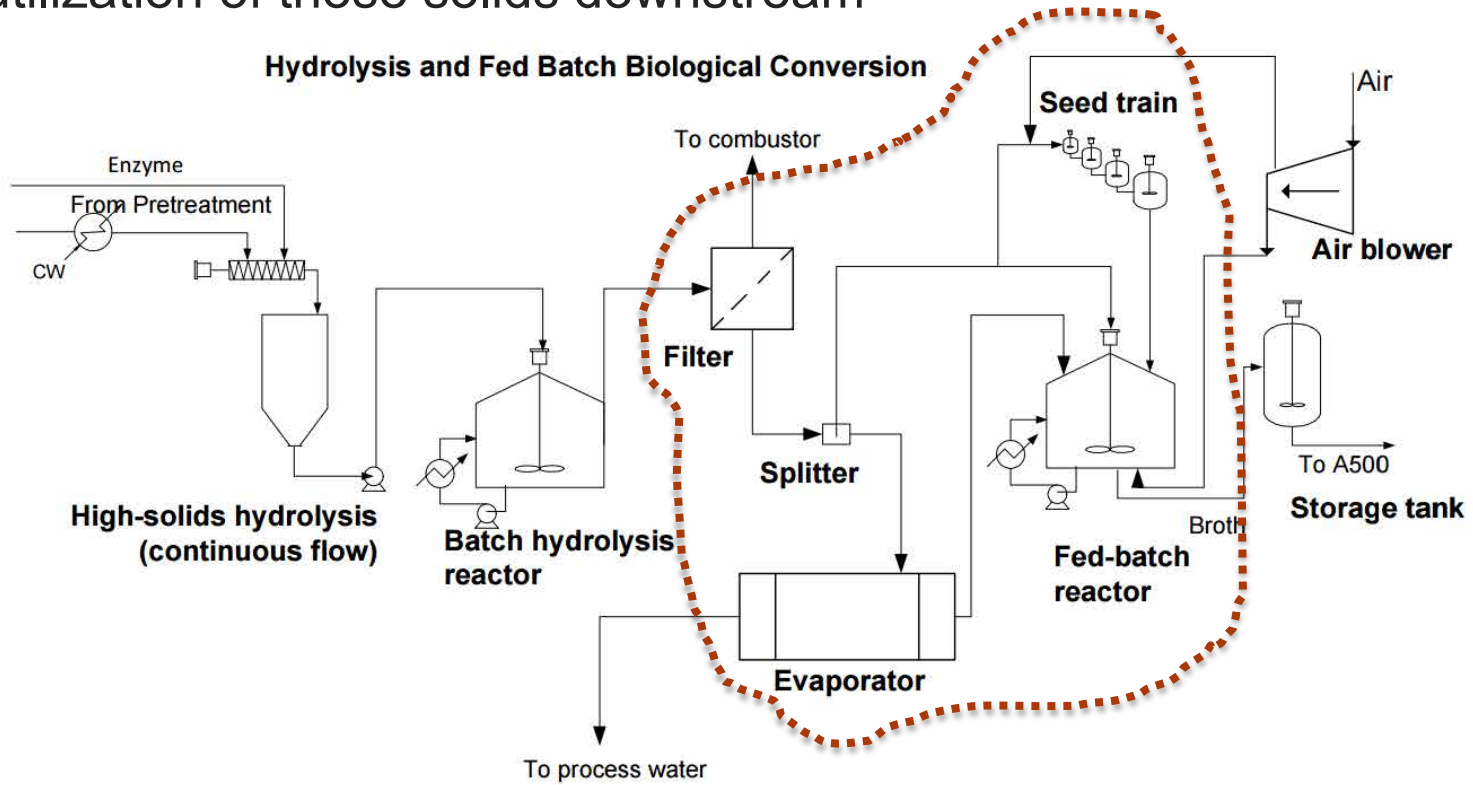


Figure 9. Simplified flow diagram of the enzymatic hydrolysis, hydrolysate conditioning, and bioconversion process

3 – Technical Accomplishments
Objectives for Improvements of the Bioprocess

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- **Real World Substrates**: dilute acid pre-treated corn stover (PCS)
- Fed-batch to increase total sugar utilized but **reduce inhibitor load**
- **Maximize cell mass** for hybrid conversion approach: HTL

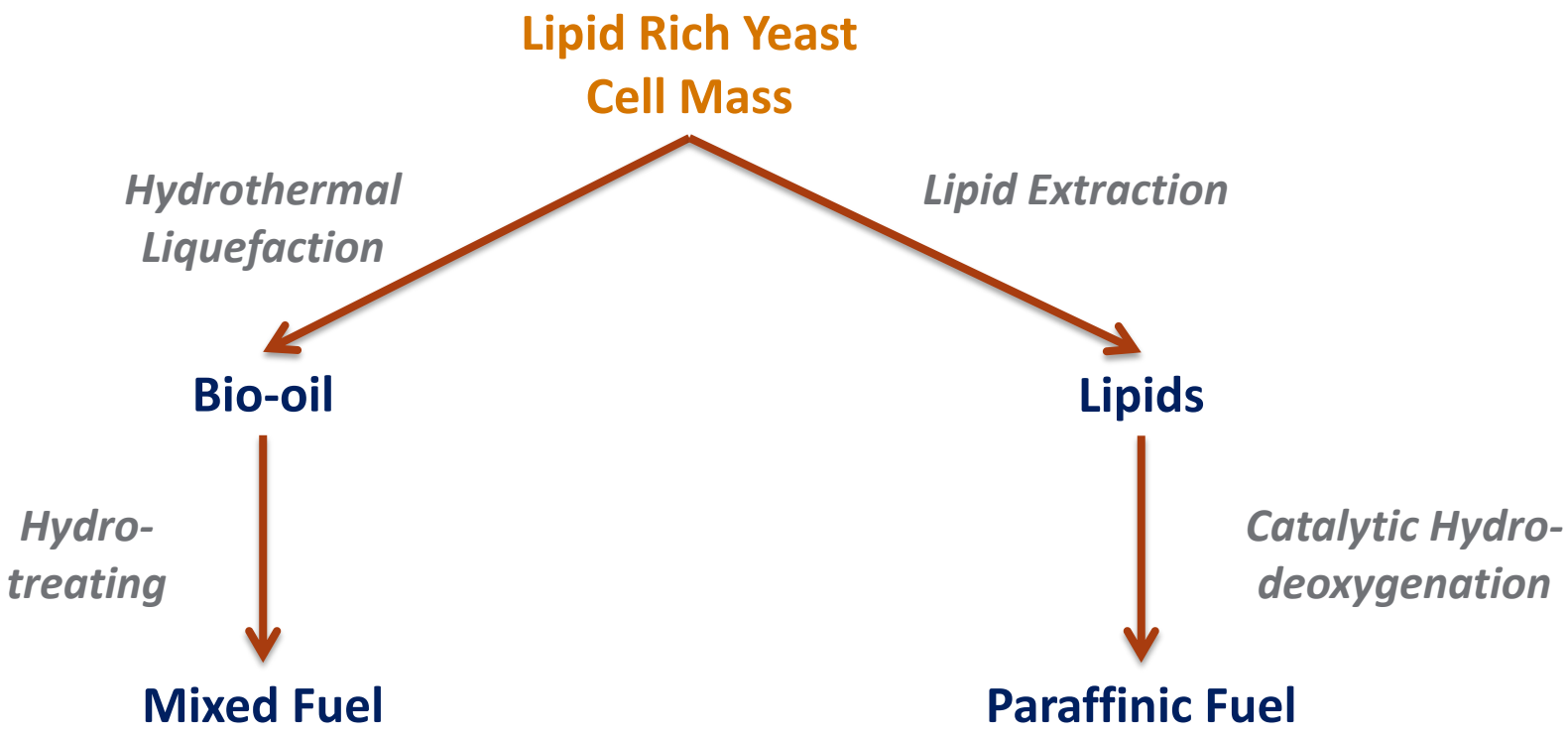
	CONDITIONS			CELLS			LIPIDS				
Expt.	Medium	Inoculum (cells/ml)	Feeding Regimen	DCW (g/L)	Specific growth rate (hr^{-1})	Yield: DCW/Sugar (wt%)	**Titer** (g/L)	Overall Rate (g/L/h)	Peak **Rate** (g/L/h)	**Yield:** Lipids from Sugar (wt%)	Lipids as % of DCW
12	**CLEAN SUGAR** 2.5% 2:1 Glucose, Xylose MM*	1.9E+07	Manual Fed Batch - 46.5% Glucose	**20.0**	0.08	39%	**13.0**	0.14	**0.90**	**26%**	**65%**
17	**PCS** only, 2.3% sugars equivalent	1.0E+06	Batch	7.0	0.10	28%	3.1	0.03	0.07	12%	44%
18	**PCS** MM*, 2.1% sugars equivalent	1.0E+06	Batch	18.6	0.11	43%	6.7	0.05	0.21	22%	36%
24	CLEAN SUGAR 6% Glucose MM*	1.2E+06	Manual Fed Batch 100% Glucose, then 76% Xylose	**155.7**	**0.12**	**38%**	**56.7**	0.46	**1.05**	**14%**	**36%**

− N
+ N

* MM = minimal media

3 – Technical Accomplishments
Oleaginous yeast production alternatives

- **HTL** (hydrothermal liquefaction): whole cell mass
- Extraction of lipids and catalytic conversion of triglycerides to alkanes



3 – Technical Accomplishments
Improvements of the Bioprocess: Maximizing Cell Mass

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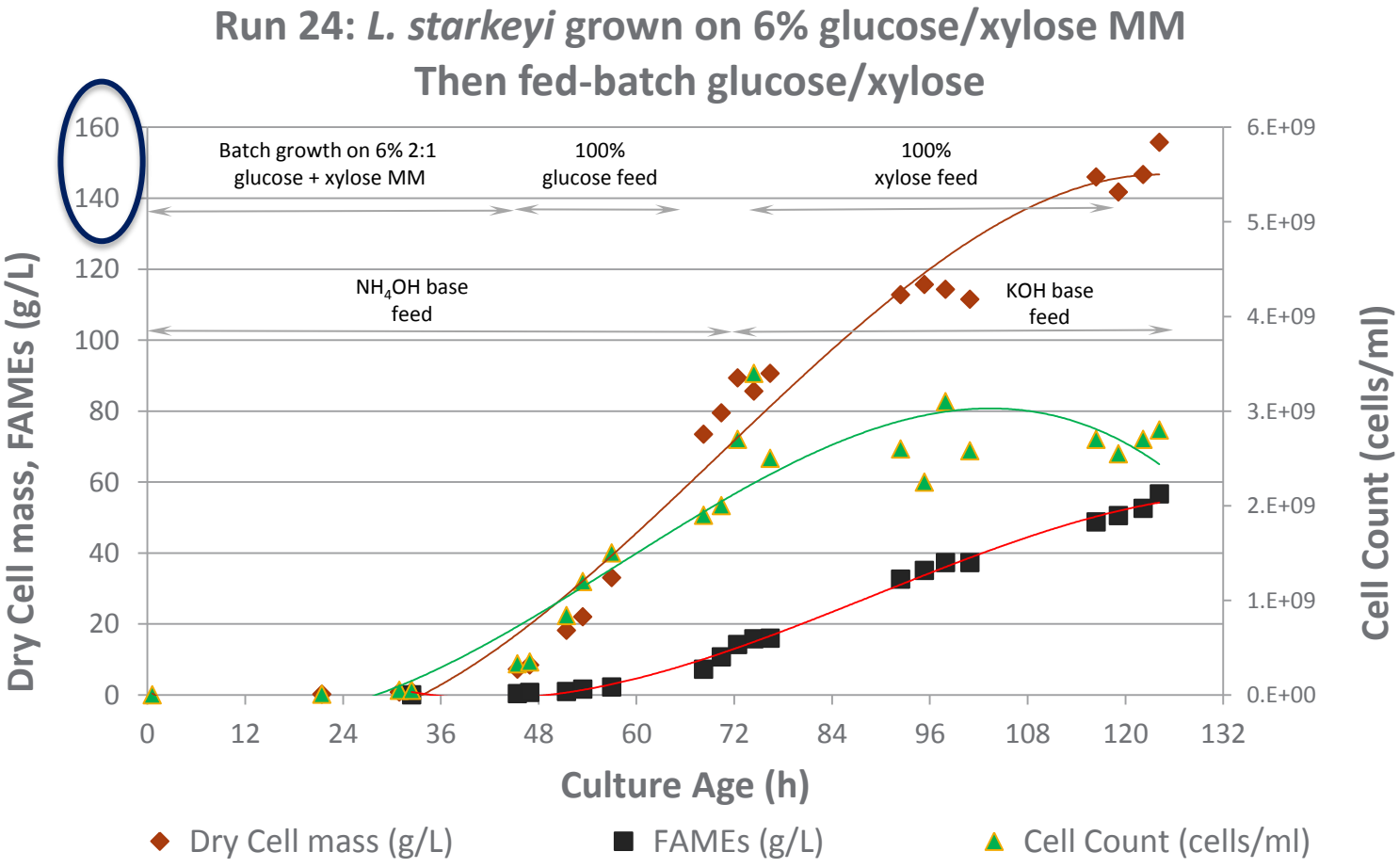
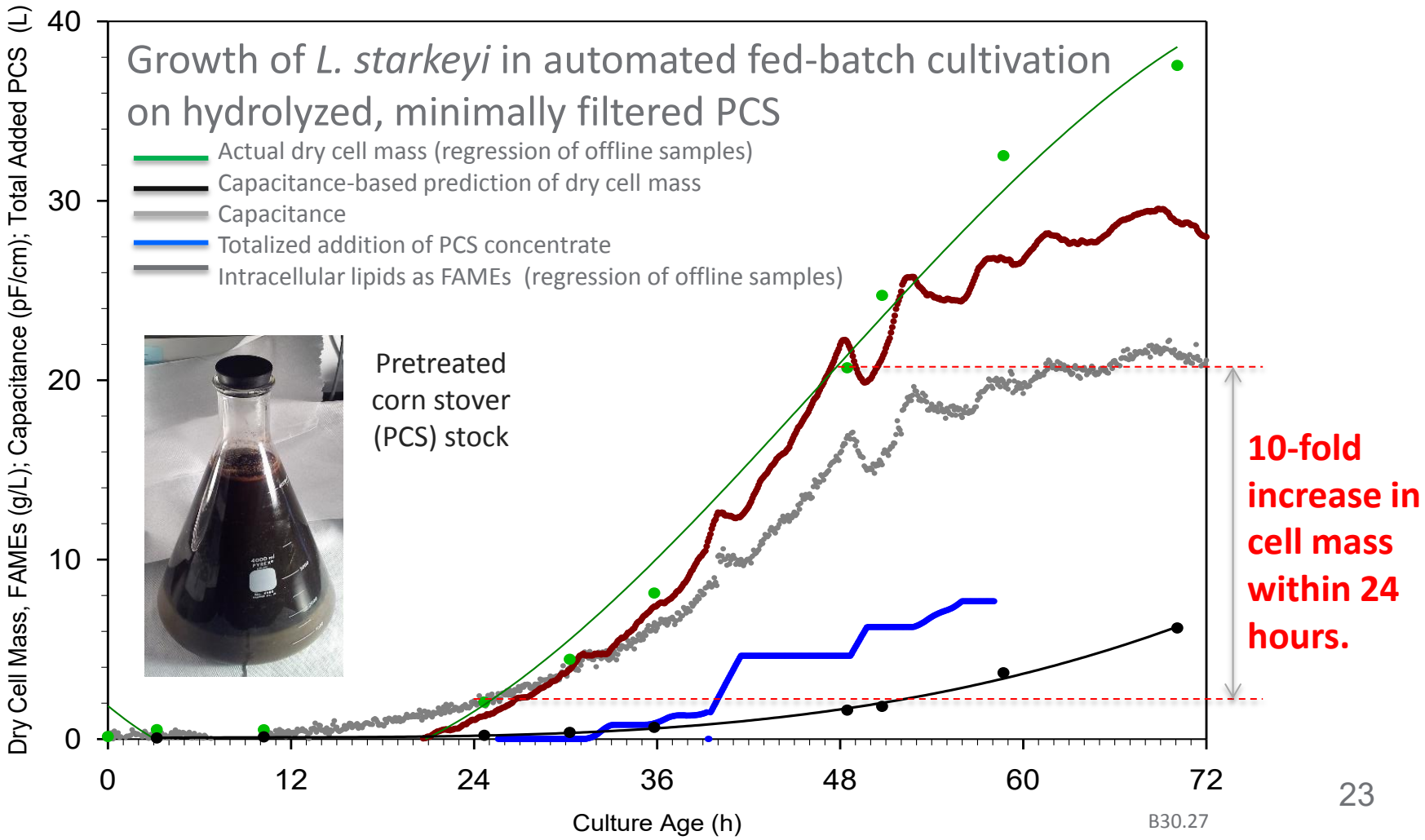
- High cell mass important for **HTL** (hydrothermal liquefaction)
- Also important for lipid conversion to alkanes process
- Need to drive up lipid content for the latter



B30 24_Dry wts_FAMEs_mgb_jrc.xlsx

3 – Technical Accomplishments
Improvements of the Bioprocess: Rate of Growth for Seed

- Examining the **seed train**
- Design case: requires **10 x increase** in cells per day in the seed bioreactor
- Have **achieved that mark**



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4 – Relevance

- **Goal: Development of efficient and robust fungal biocatalysts and bioprocesses that utilize lignocellulose feedstocks to produce advanced biofuels and bioproducts at lower cost.**

Why?

- Fungi, *Saccharomyces* and *Aspergillus*, are already the key players in the 1st generation biofuel/bioproduct **industry—biofuels, bioproducts, enzymes**
- Pre-competitive R&D to generate robust industrially-relevant fungal platforms for utilization of real world biomass substrates is our emphasis to meet BETO programmatic and 2nd generation biorefinery industry needs
- Parallel & iterative development of the organisms & the bioprocesses in an environment that can scale to biorefineries is emphasized to encourage tech to market

4 – Relevance

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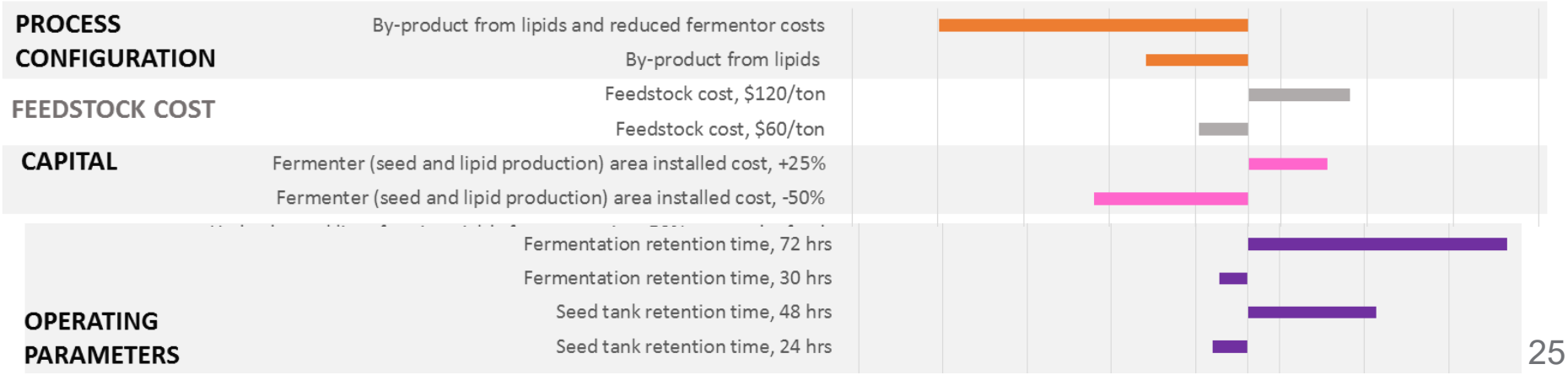
BETO Program Relevance

- BETO cost target of MFSP = $3 per GGE is challenging for advanced biofuels in general
- We are contributing pathways for biofuels and bioproducts to **decrease risk**
- In ***Lipomyces*** need to increase **TRY of lipids** and/or **cell mass for biofuels** to decrease bioprocess costs
- **TEA** indicates that **Rate** is very important…effects on process time and capital cost
- **Bioproducts** needed to enable achievement of this goal…and ***Aspergillus*** is a good platform in this area…coupled with upgrading chemistry
- **Integration** with BETO Projects: 2.5.3.104-12 ABF (9 NLs), 2.5.1.102 SCADA, 2.1.0.301 Anal. & Sustain. Interface, 2.2.3.102-3 "Thermoascus" (LBNL), 2.4.1.102 Process Scale Integration (NREL)



4 – Relevance

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Industrial Relevance

- Our focus on development of biocatalysts and bioprocesses in **bioreactors** and use of **authentic feedstocks** maximizes their potential usefulness for industrial bioenergy and bioproducts
- Industry is currently **producing lipids** for fuel and animal feed applications—a fungal system that produces triglycerides with a fatty acid profile similar to plant derived lipids (akin to palm oil) could allow integration into the same product stream
- 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 needs**: cheap, fast, easy
  - produce fuels and bioproducts of value
  - at high TRY
  - with reproducible bioprocess characteristics
  - able to utilize different pre-treated biomass sources
  - tolerant of **inhibitors**
  - tools available to genetically manipulate the organism

4 – Future Work

FY18

- A focus on automated fed-batch and seed train mimic runs with **PCS**: increasing **Rate**, with simultaneous optimization of **Titer** and **Yield** of lipid
- Use our understanding of **lipid production** and **inhibitor resistance** from transcriptomics and mutagenesis studies to obtain improved strains through genetic engineering and/or selection
- Focus on improving TRY from PCS of a highly reduced **polyketide** produced by *Aspergillus* for bioproducts/biofuels
- **Integrate** with **catalytic chemists** to investigate the bioproduct potential of the polyketide
- Continue to communicate our fungal engineering lipid, cell mass and bioproduct advances to our **IAP** and others with **T2M** goal

Summary

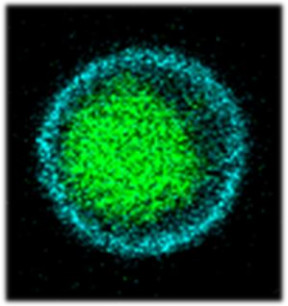
1. **Overview**: fungi are **proven platforms** for biofuels and bioproducts to meet the needs of BETO and the biorefinery industry
2. **Approach**: parallel fungal genetic & bioprocess engineering drive faster development and improvement of **industrially relevant biocatalysts/bioprocesses**
3. **Technical Accomplishments**:
   - Improved genetic, genomic and bioprocess development tool box for two fungi:
     the well understood lipid producer *Lipomyces*,
     and the workhorse bioproduct platform *Aspergillus*
   - Bioprocess development has realized large **Rate and Titer improvement** in cell mass from PCS using *Lipomyces*
4. **Relevance**: engagement with **Industrial Advisory Panel** to guide our research and produce relevant biocatalysts and bioprocesses
5. **Future work**: maintain **focus on improving TRY on lignocellulose** and building organism robustness, expansion in regard to bioproducts made

Additional Slides

NEEDS REVISION/INPUT

3 – Status Summary from 2015 Peer Review

- We had made significant improvements in TRY over the previous two years
  - Titer: **2.3x** improvement to 5.5 → 13 g/L lipids
  - Rate, **5x** improvement in *peak* rate 0.18 to 0.90 g/L/h
  - Yield: **3x** improvement 9 to 27% (100% of theoretical)



- Had observed theoretical **Yield**. Had observed high **Titers**
- Room to improve on **Rate** and **simultaneous** high TRY
- **TEA** indicates rate has a major impact on MFSP
- Had just begun work on biomass hydrolysates so most of our emphasis since has been on hydrolysates

- We had developed the **minimum required** genetic tools needed to express genes in the organism

- Still needed improvements in genetic tools, especially **gene deletion** capability

4 – Future Work

FY17 Milestones

- **Go/No-Go** to determine if promising titers can be obtained of a polyketide product (3/31)
- **Demonstrate** engineering of *Lipomyces* for improved lipid TRY and/or inhibitor tolerance (6/30)
- **Demonstrate** engineering a significant advance in titers of a polyketide product (9/30)

Due Date	Remaining FY17 Milestones
March 31	Over-express or delete four (4) gene targets implicated by transcriptomics or other results as improving lipid TRY, or enhancing robustness to inhibitor rich feedstocks in *L. starkeyi*
March 31	Obtain at least 1 g/L of 1 of the 2 target polyketides/terpenes selected in M1. GO/NO-GO
June 30	Measure TRY for each of the genetic modifications obtained in M2 and demonstrate a 20% improvement in either titer, rate, or yield vs. the wild type strain of L. starkeyi in lignocellulosic hydrolysates
Sept. 30	Obtain 2.5 g/L of a polyketide or terpene in a scaled (20L) bioprocess

Publications, Patents, Presentations, Awards, and Commercialization, 2015-16

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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.
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