Pacific Northwest
NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

DOE Bioenergy Technologies Office (BETO) 2015 Project Peer Review

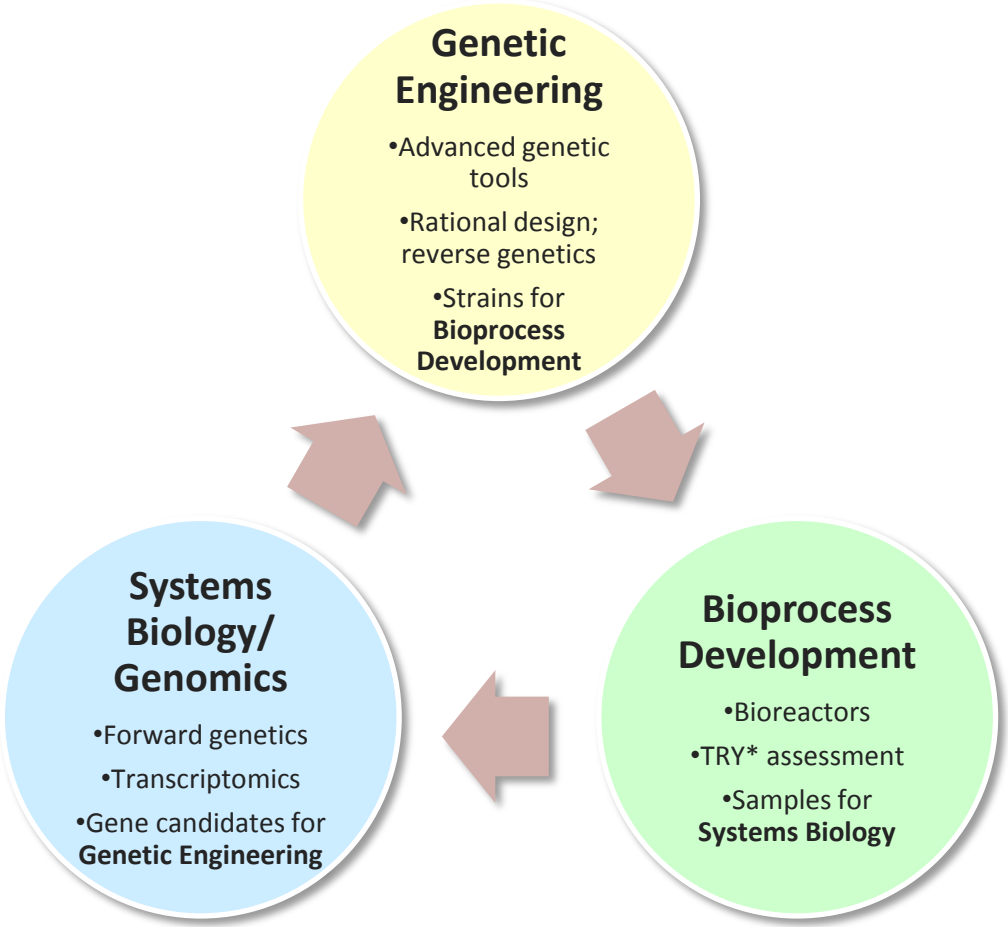
2.3.2.103 Fungal Genomics

March 25, 2015
Biochemical Technology Area Review

Jon Magnuson
PNNL

Goal Statement

- **Position:** **Biotechnology will continue to be a key component of the US energy future and fungi are the *key* players in this industry.**
- **Goal:** **We are focused on developing efficient and robust biocatalysts & bioprocesses and advanced tools to accelerate development.**



*** TRY** = Titer, Rate & Yield

Quad Chart Overview

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Timeline

- AOP project start date: 10/1/05
- Current scope start: 10/1/12
- Project end date: 9/30/18
- Percent complete: 40

Barriers

- Barriers addressed
  - **Bt-J. Catalyst Development**
  - Bt-L. Biochemical/Thermochemical Interface

Budget

	Total Costs FY 10 – FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
DOE Funded	7.25	**1.5**	**1.6**	**1.75**
Project Cost Share (Comp.)*	0	0	0	0

Partners

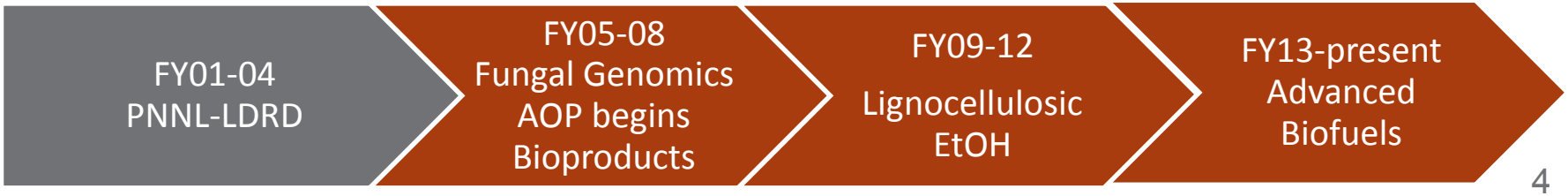
- Partners
  - No funded partners
- Collaborators
  - University of Kansas (Berl Oakley)
  - NREL, JBEI, JGI
- **Industrial Advisory Panel**
  - Novozymes*
  - POET Research*
  - BP Biofuels
  - Mycosynthetix
  - Dyadic*
  - ***Cost share or funds-in partners on past projects**

1 - Project Overview

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

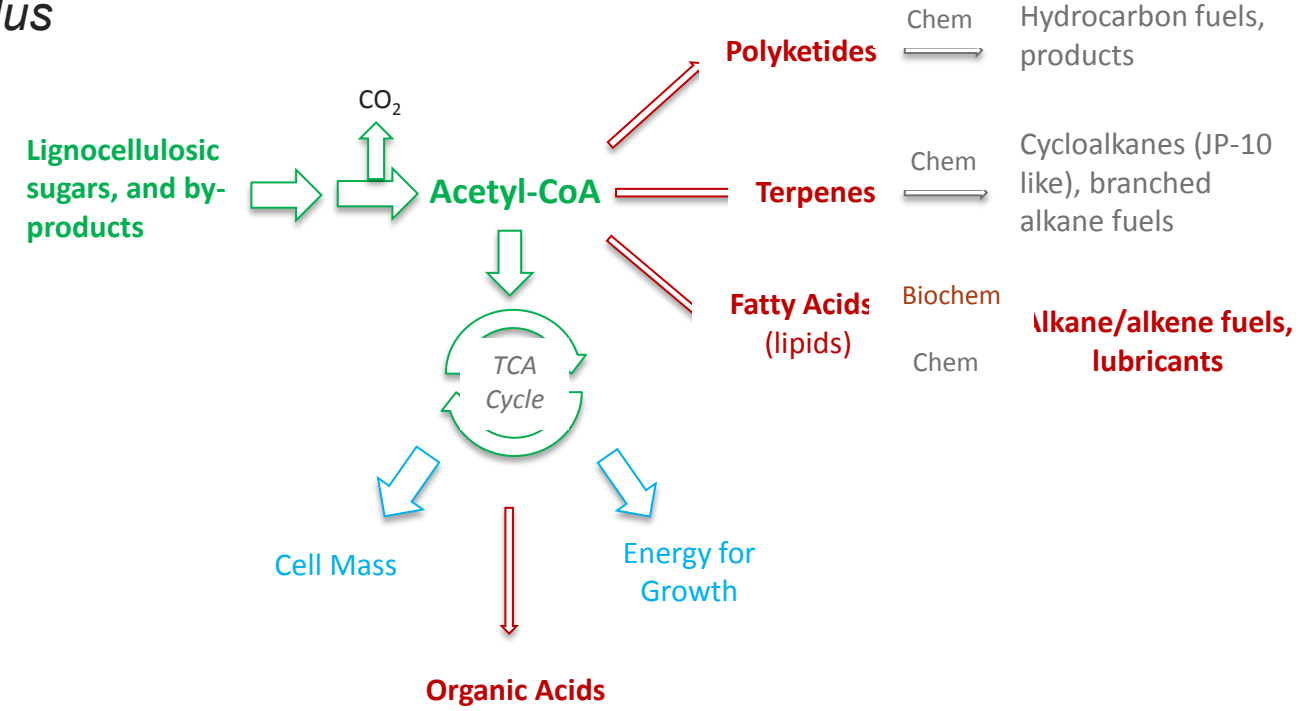
Historical Perspective

- PNNL invested in building a fungal biotechnology capability through the Laboratory Directed Research and Development (**LDRD**) program
- The **Fungal Genomics AOP** project was started to take advantage of the breakthroughs in modern genomics and build broadly applicable tools and capabilities in organisms & bioprocesses of relevance to the bioenergy industry
- Hence the emphasis from the start of having industrial involvement in the form of a **Industrial Advisory Panel**
- Initially the focus was on bioproducts, such as, organic acids. Transitioned to emphasis on lignocellullosic biomass conversion and ethanol production
- FY13 witnessed a major shift in the project with a sharp focus on a new system and target: **oleaginous yeast and lipids** for hydrocarbon fuels
- **The expertise residing at PNNL in fungal genetics, genomics and bioprocess development has been the constant throughout the project(s)**



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*



  - Unifying feature of targeted biofuels and bioproducts is their derivation from the central metabolite **Acetyl-CoA**

2 – Management Approach

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Management Approach

- Annual **project management plan** (PMP) and statement of work (SOW) tied to DOE-BETO goals
- Quarterly **milestones** to direct, focus and measure progress
- Quarterly project reporting to BETO
- Weekly **team meetings** to maintain focus and tackle technical challenges
- Co-PI (Butcher) and sub-task **technical leads** (Bruno, Collett) to utilize strengths of team* members and maintain 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, BP Biofuels, Mycosynthetix, Dyadic
- Input from a variety of internal and external **reviews**

***Scientific Team**

Ken Bruno	Mark Butcher
Jim Collett	Dave Culley
Ziyu Dai	Shuang Deng
Beth Hofstad	Sue Karagiosis
Ellen Panisko	

2 – Technical Approach

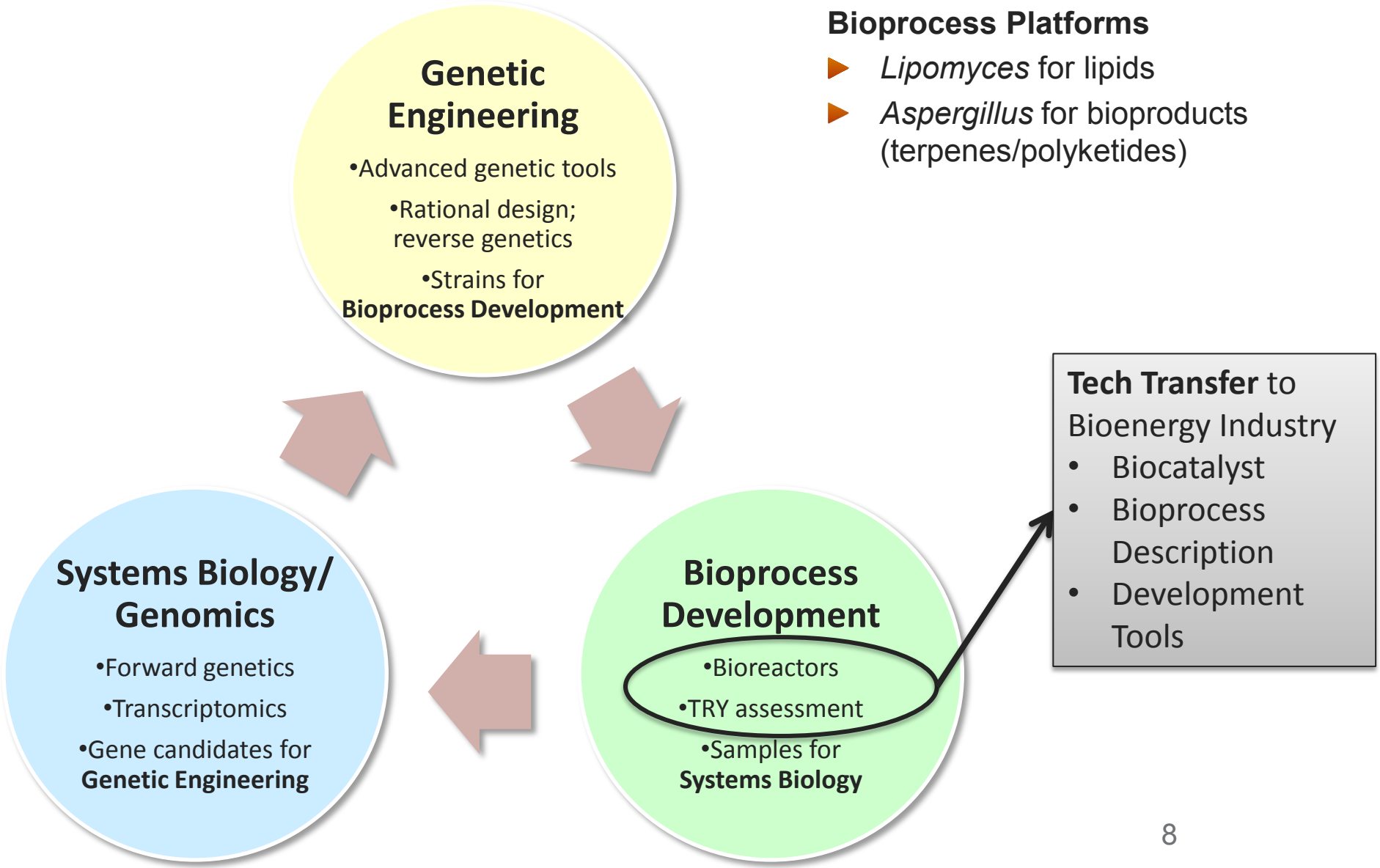
Purpose: Develop and utilize modern fungal genetics, genomics and bioprocess technology capabilities to produce robust, efficient biocatalysts and bioprocesses for biofuels and bioproducts

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 biomass hydrolysates containing inhibitors and mixed sugars
- Development of **reproducible**, robust and efficient **bioprocesses**

2 – Technical Approach: Biocatalyst Development; Design and Test Cycle

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965



Bioprocess Platforms

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

Tech Transfer to Bioenergy Industry

- Biocatalyst
- Bioprocess Description
- Development Tools

3 – Why is Lipomyces a relevant choice for an industrial biocatalyst?

Attributes

- Highly productive oleaginous yeast: >60% 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 and mannose
  - Good on **xylose** and **cellobiose**
  - Fair on L-arabinose
- Appropriate for conventional aerobic bioreactors

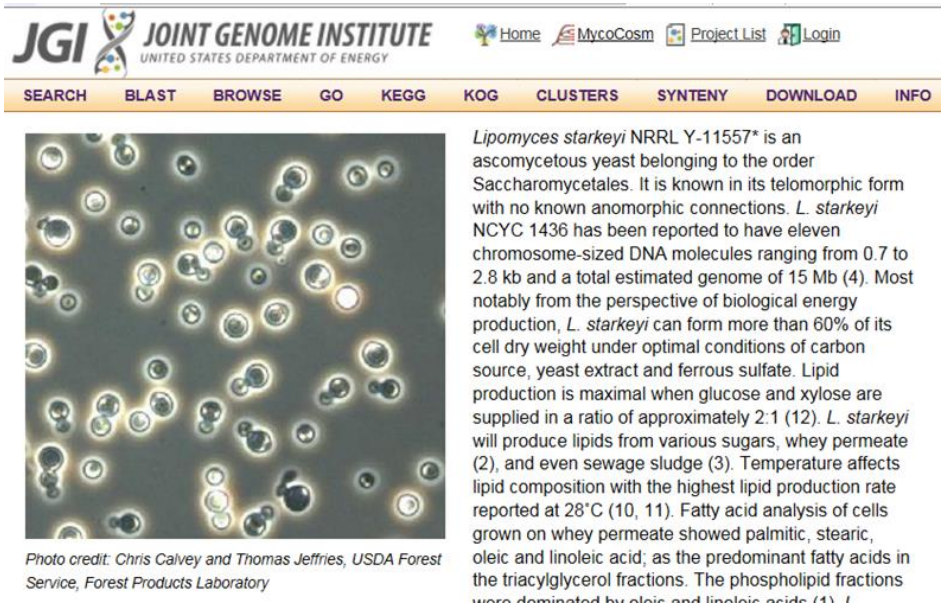
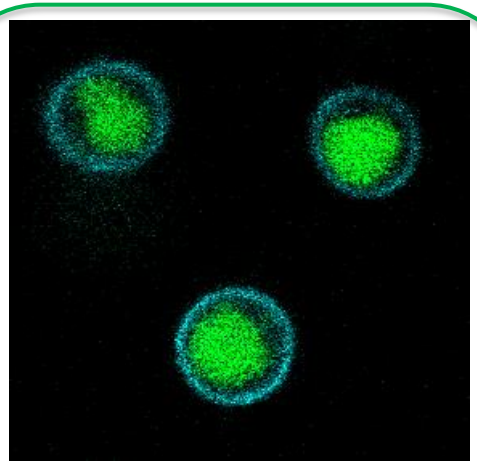


Photo credit: Chris Calvey and Thomas Jeffries, USDA Forest Service, Forest Products Laboratory



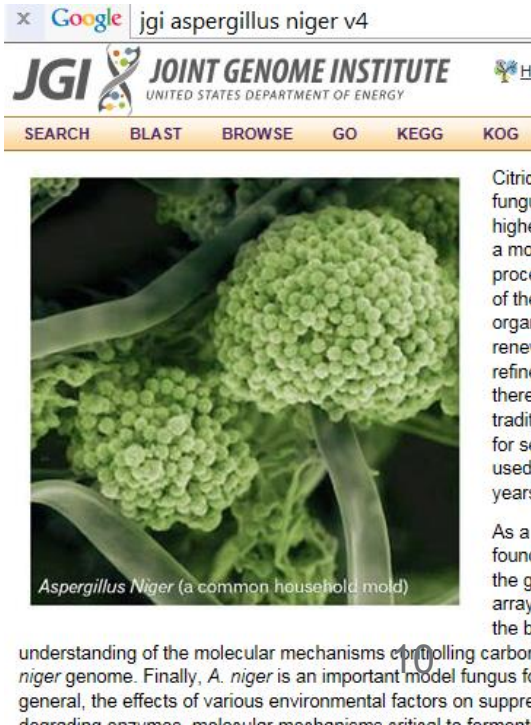
Nile red (lipid, green), Calcofluor (cell wall, blue)

3 – Why is Aspergillus a relevant choice for an industrial biocatalyst?

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Attributes

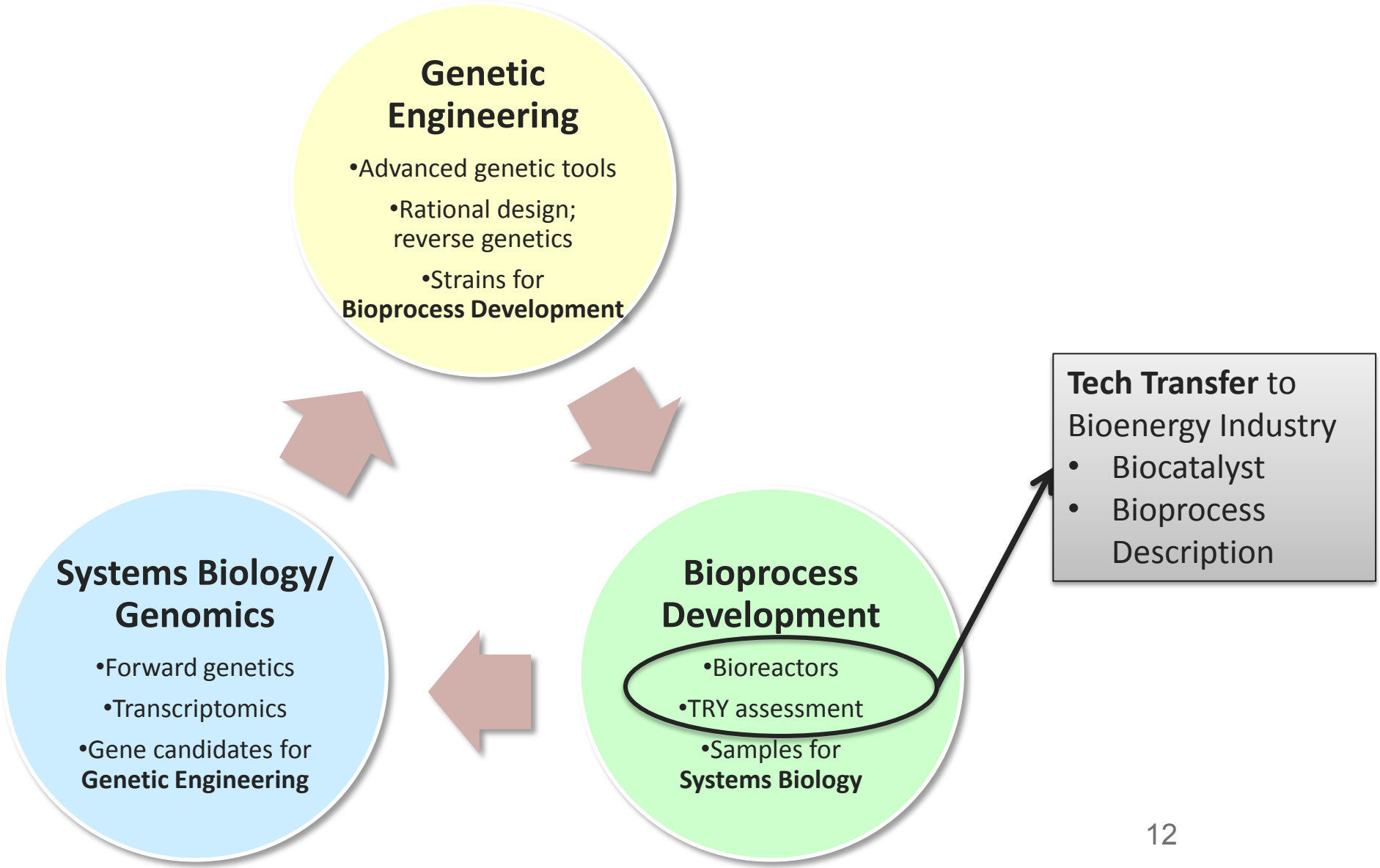
- Highly productive fungus for **enzymes and biochemicals**, including generally recognized as safe (GRAS) products
- Has multiple **genome** sequences: ATCC 1015, 11414
- Excellent **genetic** tools
- Grows and produces bioproducts on **lignocellullose** relevant sugars
- Produces **enzymes** to deconstruct lignocellulose
- Utilizes **simple inorganic nutrients**
- **Acid tolerant** (pH1.5): minimizes sterility requirements, and advantageous for organic acid production
- Appropriate for conventional aerobic bioreactors



3 – Status Summary from 2013 Peer Review

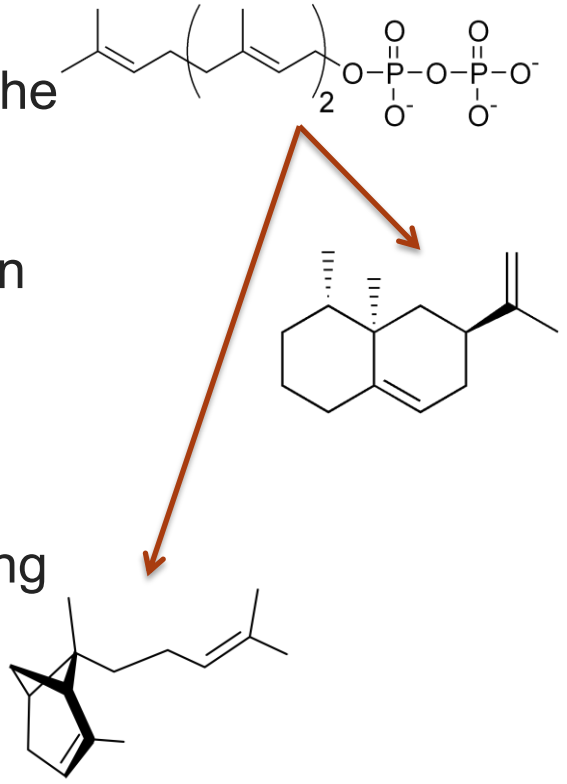
- Oleaginous yeast
  - Started with an attractive but challenging strain L. starkeyi NRRL Y-11557-- produced a highly viscous broth that presented bioprocess development challenges: aeration, harvesting, carbon sink
  - We were just beginning to develop the **required** genetic tools needed to improve the process
  - In May 2013 our best **TRY** was:
    - **T**iter: 5.49 g/L lipids (as FAMEs)
    - **R**ate: 0.069 g/L/hr (80 hr avg.); 0.174 g/L/hr during the lipid production phase
    - **Y**ield: 0.092 g lipid per g glucose; 9.2%

3 – Technical Accomplishments
Biocatalyst Design and Test Cycle



3 – Genetic Engineering *Aspergillus* for Terpene Bioproducts

- Examining biosynthesis of **sesquiterpenes** (15 carbons) in FY15 as potential biofuels/bioproducts
- Branched chain hydrocarbons have desirable fuel properties
- Fungi have good flux through the sesquiterpene precursor farnesyl pyrophosphate, a precursor of the essential cell membrane component ergosterol

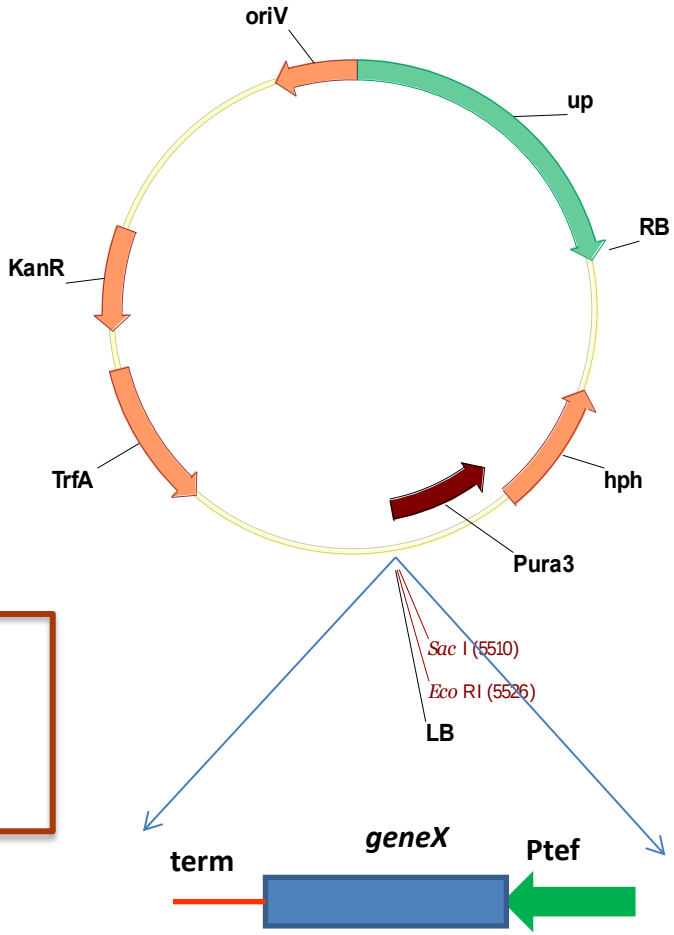


- Have an **aristolochene** synthase gene construct in *Aspergillus*
- Collaboration with Berl Oakley of University of Kansas

- Vector with **bergamotene** synthase gene also being constructed

3 – Technical Needs for Improving the Lipomyces System -- Genetics

- Issue: Need a transformation system to engineer the organism to eliminate carbon sinks, increase inhibitor resistance, etc. but…
- *L. starkeyi* didn't have any transformations systems
- It does have a genome to facilitate vector construction
- Investigated a number of methods
- *Agrobacterium tumefaciens* mediated **transformation successful!**



3 –Systems Biology/Genomics Lipomyces "Dry Mutant"

- NRRL Y-11557 is **wet/mucoid** due to secreted acidic polysaccharide
  - Causes **viscosity problems** in the bioreactor
  - Acts as a **sink for carbon**; not going to lipid

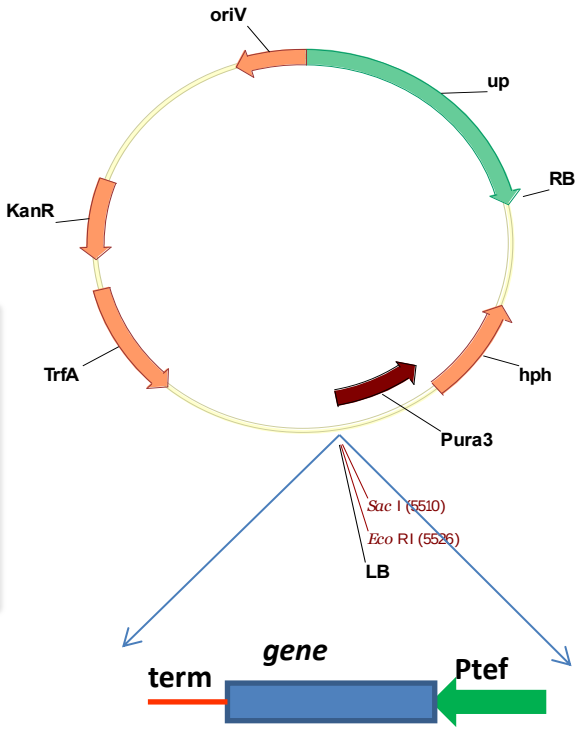
- Identified a strain, NRRL Y-11558, in the literature described as a "**dry mutant**"
- Leveraged our participation in JBEI to resequence the genome of the dry mutant at JGI
- Long list of genes with mutations
- Good candidate gene could be involved in polysaccharide production
- Transformed *gh#* gene into the dry mutant to determine if the gene is necessary and sufficient to restore a "wet" phenotype

- **Candidate gene to knock-down in the wild type and produce a *clean* genetically engineered strain**

3 – Systems Biology/Genetics

- Selected 8 genes based on biochemical reasoning, or precedence
- 5 assessed: **3 good candidates** worth pursuing further
- 3 more assessed and results being analyzed

- **PMM milestone in Sept 2014: 10% improvement in lipid production RATE**
- **2 genes gave 10-33% improvement**

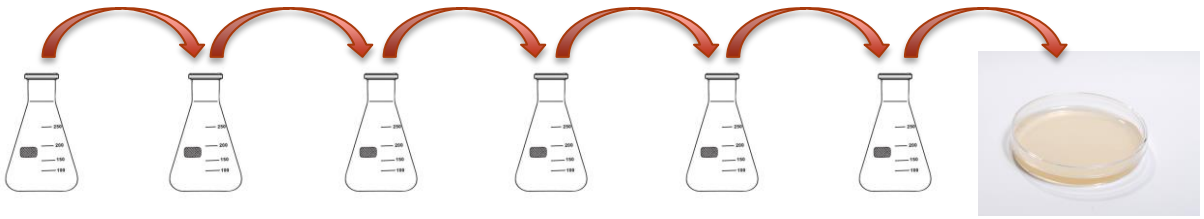


Strain	Lipid Accumulation Rate: g FAME/g dry mass/L as % of parental strain
Y-11558 (control, parent strain)	100%
abc-1	110%
abc-5	128%
def-2	133%
def-4	115%

3 – Systems Biology/Genetics Selection on PCS

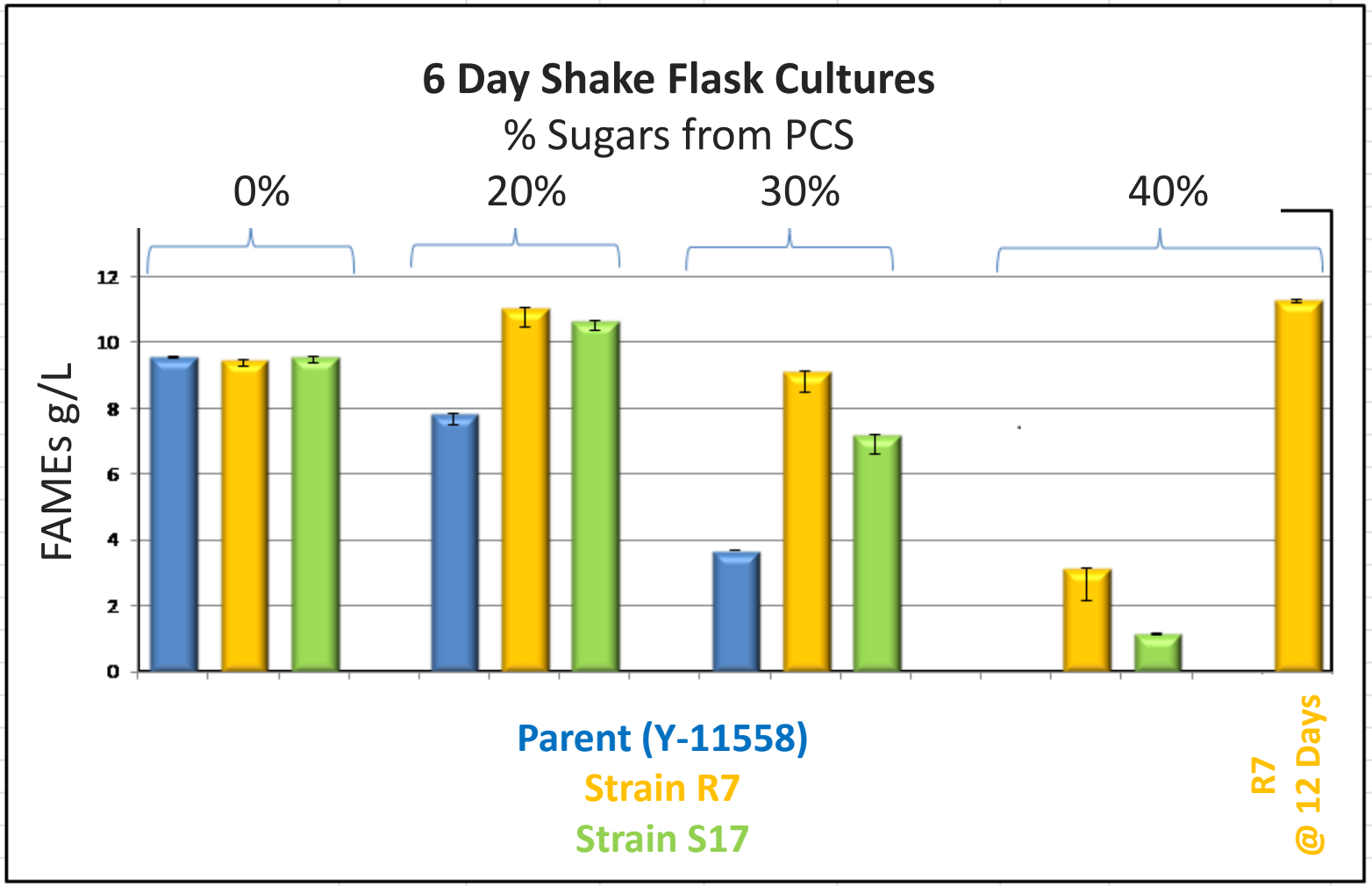
- **Background:** *L. starkeyi* grows on low concentrations of saccharified pre-treated corn stover (S-PCS)
- **Objective**: obtain a more robust strain with higher inhibitor resistance to enable use of lower quality **lignocellulosic** feedstocks—increase TRY

- Performed 20 generation serial transfer and selection experiment on increasing amounts of PCS
- Kept sugars at 80 g/L to avoid selecting against high sugar tolerance
- Plated and obtained a large number of single cell isolates (strains)
- Tested 12 strains in shake flasks with two distinct colony morphologies: *rough (R)* and *smooth (S)*



3 – Technical Accomplishments Selection on PCS

- R7 and S17 performed far better than the parent strain Y-11558 on PCS
- Parent strain doesn't grow at 40%
- Next: transcriptomics analysis of R7 vs. Y-11558



3 – Bioprocess Development Improvements

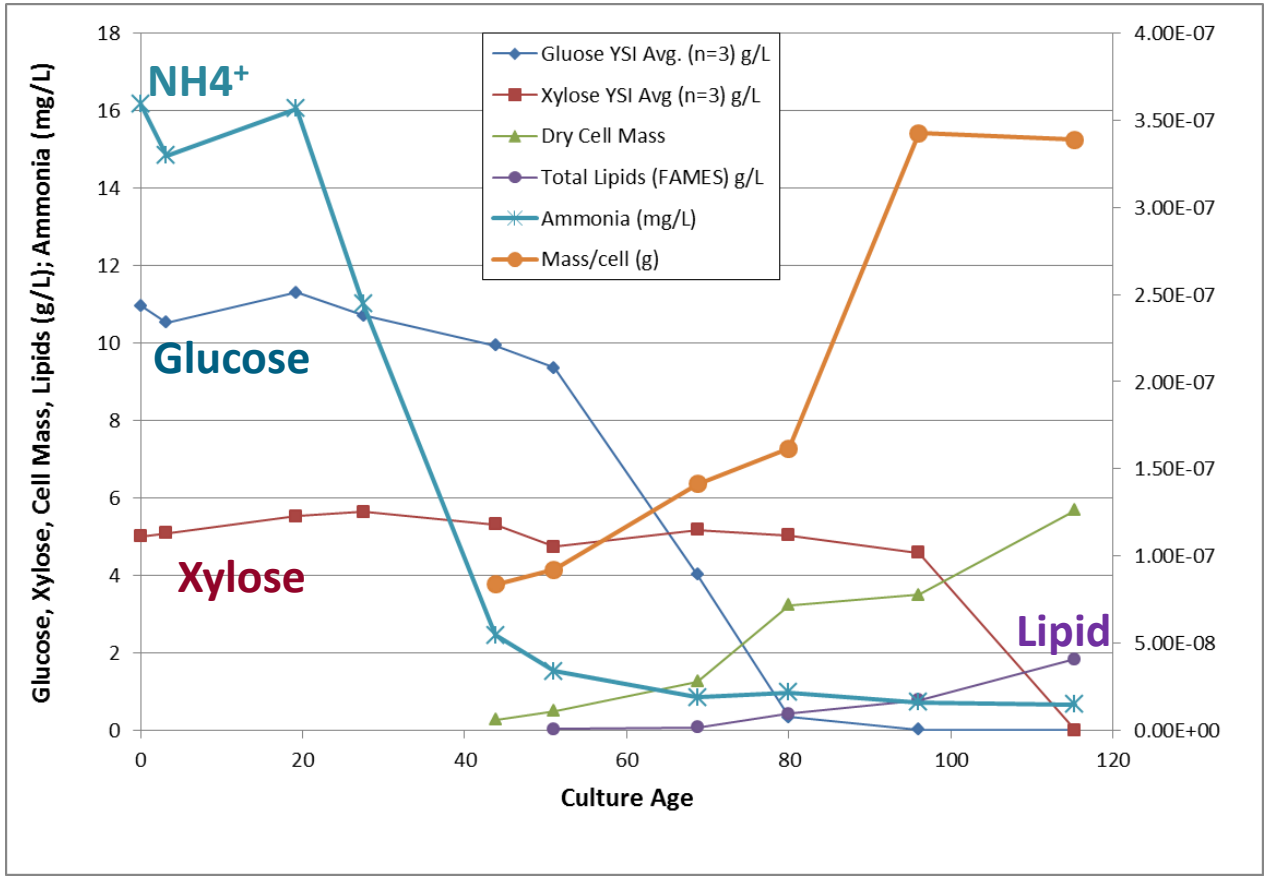
- Fed-batch growth on clean sugars: have achieved 66% lipid content
- Growth on enzymatically saccharified PCS -- **no** added nutrients and **no** inhibitor "mitigation"

Bioreactor Run	*L. starkeyi* Strain	Medium	Feeding Regimen	Sugar Consumed (Glc + Xyl)	Cell Mass (g/L)	**TITER** FAMEs (g/L)	Overall Process **RATE** FAMEs g/L/h	Peak **RATE** FAMEs g/L/h	**YIELD** (g FAMEs / g Sugars)	Lipid as % of Cell Mass
FY13: 3	Y-11557	MM	Batch	60 (Glc)	26	5.5	0.069	0.174	9.2%	21
7	Y-11557	MM	Batch	80	26	7.7	0.076	0.522	11%	30
9	Y-11558	MM	Batch	80	25	8.8	0.089	0.416	11%	35
11	Y-11558	PCS	Batch	16	5.7	1.9	0.031	0.187	12%	34
12	Y-11558	MM	Fed Batch	47	20	**13**	**0.142**	**0.899**	**28%**	**66**

- Parameters improved: aeration regimen, C:N ratio optimization, strain
- We've challenged the system AND made significant improvements in TRY in the last 22 months
  - Titer: **2.3x** improvement
  - Rate, overall: **2.1x** improvement. Max rate: 5x2x improvement
  - Yield: **3x** improvement

3 – Technical Accomplishments
Bioprocess: Objectives for Improvements

- Growth on enzymatically **saccharified PCS** in 20L bioreactor
  - **no** added nutrients
  - **no** inhibitor mitigation



- Lipid production on PCS indicates **bioprocess relevance of *Lipomyces***
- **Future**: room for improvement on TRY, especially in PCS
  PCS resistant strain R7 may help

4 – Relevance

BETO Program Relevance

- "By 2017, achieve an nth plant modeled conversion cost of $3.30/GGE utilizing blended formatted biomass via a biochemical conversion pathway"
- The BETO cost targets are very challenging goals for biofuels.
- Increase **TRY of lipids for biofuels** to decrease bioprocess costs
- **Bioproducts** will be needed to enable achievement of this goal, hence increased scope in this area

- **MYPP**: "The strategic **goal** of Conversion R&D is to develop commercially viable technologies for converting biomass feedstocks into energy dense, fungible liquid transportation fuels, as well as bioproducts... "
- Our research supports the Conversion R&D area by developing **industrially relevant biocatalysts** and the **tools** for manipulating them to produce a variety of biofuels and bioproducts

4 – Relevance

BETO Program Relevance

- **MYPP**: "Reaching 2022 volumetric **goals** will require technologies that can convert biomass to fuels at **high efficiencies** or specificities and/or accept lower quality, but potentially lower cost biomass, thereby allowing access to a larger biomass supply."
- Our focus on improvements in TRY is specifically aimed at **efficiency** in utilization of biomass and decreased energy/water usage
- Our focus on utilizing **real world substrates**, identifying strains able to use a variety of sugars and exhibit resistance to inhibitors addresses the need to utilize lower quality biomass

4 – Relevance

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Industrial Relevance

- Our focus on development of the biocatalysts and bioprocesses in **bioreactors** and use of **real world feedstocks** maximizes their potential usefulness for industrial bioenergy and bioproducts
- Industry is currently **producing lipids** for fuel and animal feed applications, so 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 manipulate the organism

5 – Future Work

FY15 Remaining Milestones Explained

- Transcriptomic experiments from bioreactor time courses: inhibitor resistant strain vs. *L. starkeyi* parent strain
- **Objective**: allow the organism to tell us about additional gene candidates for biocatalyst and bioprocess improvement
- Obtain proof of principle quantities of a sesquiterpene

Due Date	Upcoming FY15 Milestones
March 31	Express a sesquiterpene synthase in Aspergillus sp. to make a terpene with excellent fuel/product precursor traits
June 30	Perform a transcriptomics experiment on a PCS to lipid bioprocess to ID genes responsible for inhibitor robustness and improved lipid TRY
Sept. 30	Demonstrate a bioprocess producing 50 mg/L of a desirable sesquiterpene from an Aspergillus sp.

5 – Future Work

FY16

- Batch and fed-batch experiments with sugar mixtures and **PCS** pre-treated corn stover with the objective to: raise **Titer** of lipid, increase **Rate**, obtain consistently high **Yield**
- Isolating genomic DNA from R7 for genome resequencing to identify inhibitor resistance genes
- Understand **lipid production** and **inhibitor resistance** through transcriptomics studies and use genetic engineering to increase it
- With respect to TRY, identify **gene candidates** for rate improvement, greater flux through productive metabolic pathways
- Incorporate known highly reduced **polyketide** gene candidates into *Aspergillus* for bioproducts/biofuels
- Work with **catalytic chemists** to investigate the bioproduct potential of various terpenes and polyketides given the variety of hydrocarbons fungi can produce from the central metabolite Acetyl-CoA

Summary

1. **Overview**: fungi are an excellent **platform** for fuels and bioproducts
2. **Approach**: genetic engineering, functional genomics and bioprocess development tools drive faster development and improvement of **industrially relevant biocatalysts/bioprocesses**
3. **Technical Accomplishments**:
   - Solid genetic, genomic and bioprocess development tool box for two fungi:
     the well understood lipid producer *Lipomyces*,
     and the workhorse bioproduct platform *Aspergillus*
   - **>2 fold improvements in TRY** of lipids 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** and building organism robustness, expansion in regard to bioproducts made

Additional Slides

(Not a template slide – for information purposes only)

- *The following slides are to be included in your submission for Peer Evaluation purposes, but will **not** be part of your oral presentation –*
- *You may refer to them during the Q&A period if they are helpful to you in explaining certain points.*

Responses to Previous Reviewers' Comments

- 6. Please comment on the degree to which the project coordinates with other institutions and projects to provide additional benefits to both BETO and the industry.
  Reviewer comment: "Did not discuss much in this area."
  - Our work within/with JBEI/JGI is enabling genomics investigations of our oleaginous yeast strains.
  - Sharing information and some personnel time with Scott Baker at EMSL with his BER funded work on the oleaginous yeast *Yarrowia lipolytica*
  - Sharing experience and yeast strains with Nancy Dowe's team at NREL who is working on bioprocess scale-up
  - Leveraging established collaborations with Berl Oakley of KU for Aspergillus secondary metabolite genes/constructs/strains
  - Paul Harris of Novozymes (Partner's Review Board member) suggested the selection experiment for developing increased feedstock inhibitor resistance in Lipomyces
  - Previous projects with POET Research (1:1 funds-in, matched by technology maturation funding) and Novozymes (BETO funded CRADA) drive towards technology transfer

Publications, Patents, Presentations, Awards, and Commercialization, 2013-15

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Meetings:

Bruno, K., Session Chair, 2015-Synthetic Biology in Filamentous Fungi, 28th Fungal Genetics Conference

Ziyu Dai, Shuang Deng, David E. Culley, Kenneth S. Bruno, Jon K. Magnuson 2015. " Agrobacterium tumefaciens-mediated transformation of the oleaginous yeast genus Lipomyces" Accepted for poster presentation at 37th Symposium on Biotechnology for Fuels and Chemicals, San Diego, CA on April 27, 2015.

Collett JR, PA Meyer, Y Zhu, ER Hawley, Z Dai, MG Butcher, and JK Magnuson. 2015. "Bioreactor performance data and preliminary biorefinery techno-economics for the production of distillate fuels via bioconversion of pretreated corn stover by Lipomyces starkeyi." Accepted for poster presentation at 37th Symposium on Biotechnology for Fuels and Chemicals, San Diego, CA on April 27, 2015. PNNL-SA-107273.

Intellectual Property:

Agrobacterium tumefaciens mediated transformation of oleaginous (oil-producting) yeast Lipomyces 30396-E 23-91636-01 (2014) Ziyu Dai, Jon K. Magnuson, Shuang Deng, Kenneth S. Bruno and David E. Cully.

Publications:

Increasing intracellular fatty acid levels of the filamentous fungus Aspergillus carbonarius ITEM5010 by regulating fatty acid catabolism Submitted to Biotechnology for Biofuels February 2015

Publications, Patents, Presentations, Awards, and Commercialization , 2013-15

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Publications:

Increasing intracellular fatty acid levels of the filamentous fungus Aspergillus carbonarius ITEM5010 by regulating fatty acid catabolism Submitted to Biotechnology for Biofuels February 2015

Melanin and the Secondary Metabolite Aspulvinones Share a Common Precursor Generated by Two Distinct Genes Accepted with minor revisions requested from Proceedings of the National Academy of Sciences of the United States of America February 2015

Tamano, K., Bruno, K.S., Koike, H., Ishii, T., Miura, A., Umemura, M., Baker, S.E., Machida, M. (2014). Increased Production of Free Fatty Acids in Aspergillus oryzae by the Combination of Disruption of a predicted Acyl-CoA Synthetase Gene, Complementation of a Nitrate Reductase Gene, and Enhancement of Nitrate Supplementation. (Submitted)

Guo, C.J., Sun, W.-w., Bruno, K.S., and Wang, C.C. (2014). Molecular Genetic Characterization of Terreic Acid Pathway in Aspergillus terreus. Organic letters. (In Press)

Gallo, Antonia , Benjamin P. Knox, Kenneth S. Bruno, Michele Solfrizzo, Scott. E. Baker, Giancarlo Perrone. (2014). Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in Aspergillus carbonarius.. Journal of Food Microbiolog. 179, 10-17

Publications, Patents, Presentations, Awards, and Commercialization , 2013-15

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Publications:

Simmon BA, and JK Magnuson (2013) Interview: A Conversation with Blake Simmons, VP Deconstruction Division, and Jon Magnuson, Director Fungal Biotechnology. Industrial Biotechnology Vol. 9 No. 3, pp. 108-112

Guo, C.-J., Knox, B.P., Sanchez, J.F., Chiang, Y.-M., Bruno, K.S., and Wang, C.C. (2013). Application of an Efficient Gene Targeting System Linking Secondary Metabolites to their Biosynthetic Genes in Aspergillus terreus. Organic letters. 15 (14), pp 3562–3565 [CoCorrespondingAuthor]

Guo, C.-J., Yeh, H.-H., Chiang, Y.-M., Sanchez, J.F., Chang, S.-L., Bruno, K.S., and Wang, C.C.C. (2013). Biosynthetic Pathway for the Epipolythiodioxopiperazine Acetylaranotin in Aspergillus terreus Revealed by Genome-Based Deletion Analysis. Journal of the American Chemical Society 135, 7205-7213.

Yeh, H.H., Chang, S.L., Chiang, Y.M., Bruno, K.S., Oakley, B.R., Wu, T.K., and Wang, C.C.C. (2013). Engineering Fungal Nonreducing Polyketide Synthase by Heterologous Expression and Domain Swapping. Organic Letters 15, 756-759.

Tamano, K., Bruno, K.S., Karagiosis, S.A., Culley, D.E., Deng, S., Collett, J.R., Umemura, M., Koike, H., Baker, S.E., and Machida, M. (2013). Increased production of fatty acids and triglycerides in Aspergillus oryzae by enhancing expressions of fatty acid synthesis-related genes. Applied Microbiology and Biotechnology 97, 269-281.

Collett, J., A. Meyer and S. Jones (2014). Preliminary Economics for Hydrocarbon Fuel Production from

Publications, Patents, Presentations, Awards, and Commercialization , 2013-15

Pacific Northwest NATIONAL LABORATORY
Proudly Operated by Battelle Since 1965

Publications:

Collett, J., A. Meyer and S. Jones (2014). Preliminary Economics for Hydrocarbon Fuel Production from Cellulosic Sugars, Pacific Northwest National Laboratory. PNNL-23374.

J Anderson, Lindsey N.; Culley, David E.; Hofstad, Beth A.; Chauvigne-Hines, Lacie M.; Zink, Erika M.; Purvine, Samuel O.; Smith, Richard D.; Callister, Stephen J.; Magnuson, Jon M.; Wright, Aaron (2013) Activity-based protein profiling of secreted cellulolytic enzyme activity dynamics in Trichoderma reesei QM6a, NG14, and RUT-C30 T.MOLECULAR BIOSYSTEMS Volume 9, issue 12, year 2013, pp. 2992 – 3000

Tamano K, KS Bruno, SA Karagiosis, DE Culley, S Deng, JR Collett, M Umemura, H Koike, SE Baker, and M Machida. 2013. "Increased Production of Fatty Acids and Triglycerides in Aspergillus oryzae by Enhancing Expressions of Fatty Acid Synthesis-Related Genes." Applied Microbiology and Biotechnology 97(1):269-281.