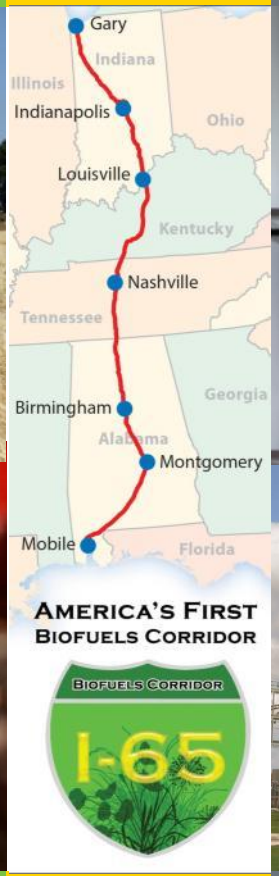
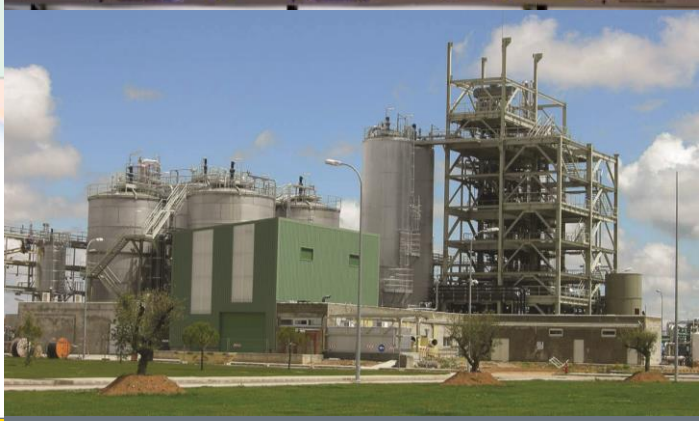
2013 DOE Bioenergy Technologies Office (BETO) Project Peer Review

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy





Fungal Genomics

aka Fungal Biotechnology

May 21, 2013 Principal Investigator: Jon Magnuson

Technology Area Review: Biochemical Conversion

Organization: PNNL

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

Goal Statement

- Our **goal** is enabling accelerated development of fungal bioprocesses using fungi that are industrially relevant.
- Use systems biology, genetic engineering and bioreactors to understand, manipulate and assess the bioprocess organisms.
- We are **focused** on developing biochemical conversion processes for producing **biofuels** or biofuel precursors (e.g., lipids), and increasing their efficiency with respect to techno-economics and sustainability in support of BETO objectives and cost targets.
- We aspire to have our fungi -- or organisms developed with our enabling tools and approaches -- used in one or more biorefineries.

Quad Chart Overview

Timeline

- 10/2004
- 09/2017
- Percent complete: 65%

Barriers

- Barriers addressed
  (*for current project scope*)
  - Bt-J. Catalyst Development

Budget

- Funding for FY11: 2.5M
- Funding for FY12: 2.25M
- Funding for FY13: 1.5M
- Funded since 2005
- Avg. annual funding 1.9M

Partners

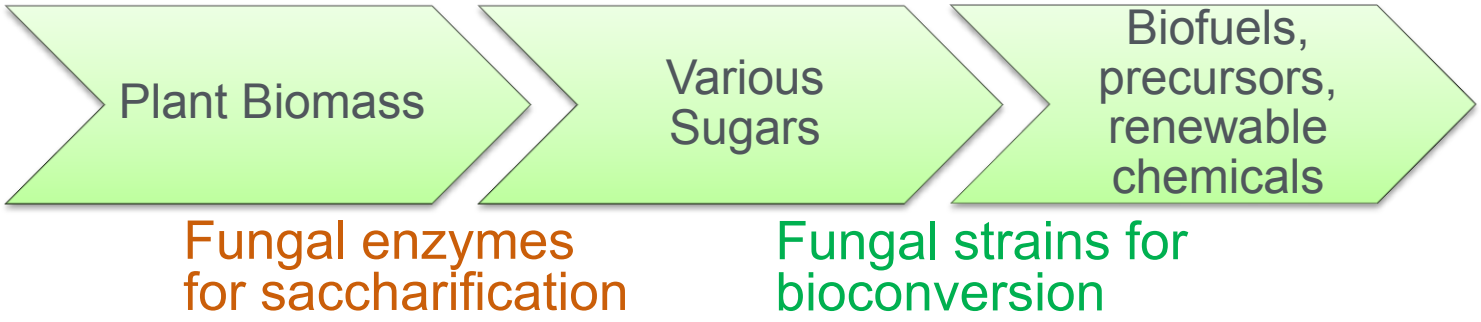
- Partners Review Board: Novozymes, POET, BP Biofuels, Dyadic & Mycosynthetix
- US Universities: USC, U. Wisc., U. Kansas
- DOE NL/Inst.: NREL, JGI, JBEI
- European & Japanese collaborators: DTU, Wien U. of Tech., AIST

Project Overview

- History
  - Fungal Biotechnology Team at PNNL was built through an LDRD (Laboratory Directed R&D) investment
  - BETO funding started in 2005 with a focus on filamentous fungal biprocesses research for understanding and maximizing productivity of bioproducts (organic acids)
  - Evolved into the increasing use of genomics and functional genomics tools for enabling a wide variety of fungal research relevant to the BETO portfolio in biomass saccharification and sugar conversion through FY12
  - **For FY13 we had a significant technology shift—incorporating oleaginous yeast in alignment with BETO's increased focus on hydrocarbon fuels**
- We have sharpened our **focus on production of hydrocarbon biofuels or precursors--lipids**
- A broad objective of establishing and maintaining a core capability in fungal biotechnology R&D to develop modern tools and approaches for accelerating fungal bioprocess development is a constant

1 – Technical Approach: Previous

FY11-12 Fungal Biotechnology



Tasks

- Genomics
- Proteomics
- Strain development
- Hyperproductivity
- Pentose utilization
- Advanced biofuels
- Process informatics

Organisms of Interest

- *Aspergillus niger*
- *Aspergillus oryzae*
- *Aspergillus terreus**
- *Aspergillus carbonarius**
- *Hormoconis resinae*
- *Trichoderma reesei*
- *Trichoderma atroviride**
- *transformation systems developed at PNNL

- Developed broadly enabling fungal technologies
- Used them for investigating biofuels processes and saccharification enzymes

1 – Technical Approach: Current

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FY13 Fungal Biotechnology

PI changed: Baker to Magnuson

Significant change in focus

Fungal strains for bioconversion

Tasks

- Genetics of fungal hydrocarbons
- Metabolic systems

Organisms of Interest

- *Aspergillus niger*
- *Lipomyces starkeyi**
- *Hormoconis resinae*

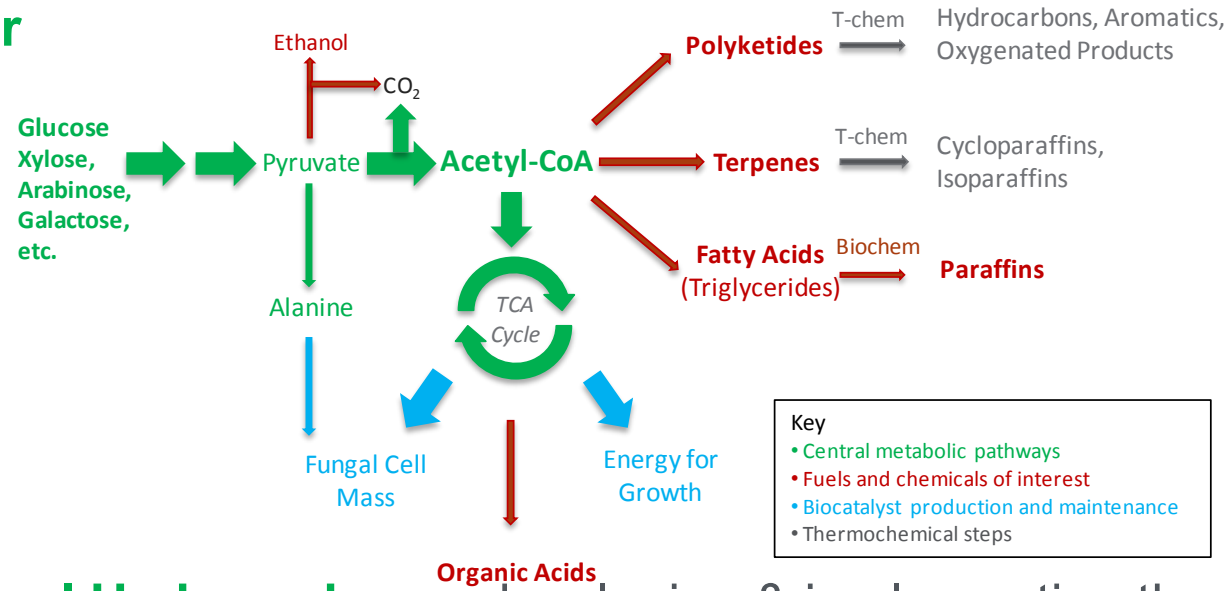
Constant: Strong team with expertise in fungal molecular biology, systems biology, metabolic modeling, biochemistry, bioprocessing, and multi-disciplinary interfaces

Management Approach

- Develop project management plan (PMP)
- Statement of work and how it relates to DOE goals
- Quarterly milestones to direct linear progress
- Go/No Go decision point FY15 Q3: techno-economic evaluation of fungal lipid production as a viable route progressing towards the BETO FY17 cost targets of $5/gge
- Frequent project communications

1 – Technical Approach: Current

Fungal strains for bioconversion

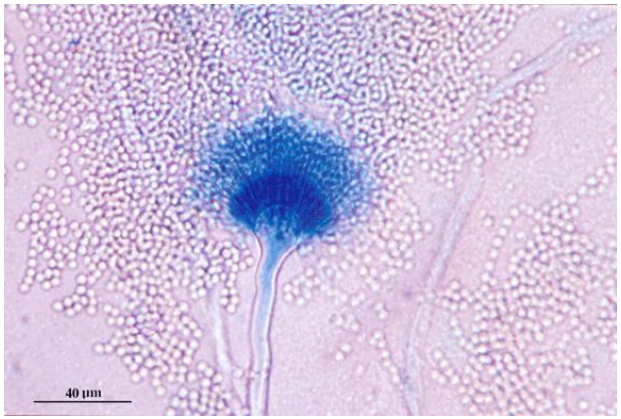


Tasks

- **Genetics of Fungal Hydrocarbons**: developing & implementing the genetic tools to engineer improved strains
- **Metabolic Systems**: using systems biology (omics analysis and modeling) coupled to fermentations to find rational gene targets for improving the organism and/or bioprocess parameters to maximize TRY (titer, rate, yield)
- Challenge: finding the optimal balance between carbon flux to biocatalyst and lipid production
- Address **cost** barriers by: maximizing utilization of carbon (feedstock cost) to lipid in the minimal amount of time (biorefinery capital cost)

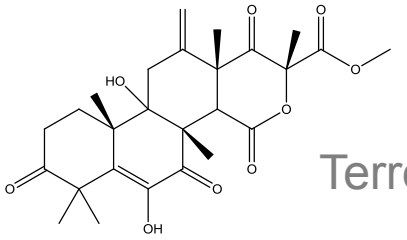
1 - Technical Approach
Why Fungi?

- **Industrially relevant platform organisms** for production of small organic molecules and enzymes
- Filamentous fungi can utilize multiple types of polysaccharides
- Utilize multiple types of sugars: hexoses and pentoses
- Grow at acidic pH
- Grow on inorganic nutrients
- Many genomes available
- Genetic tools are available and we have an excellent team for developing new tools



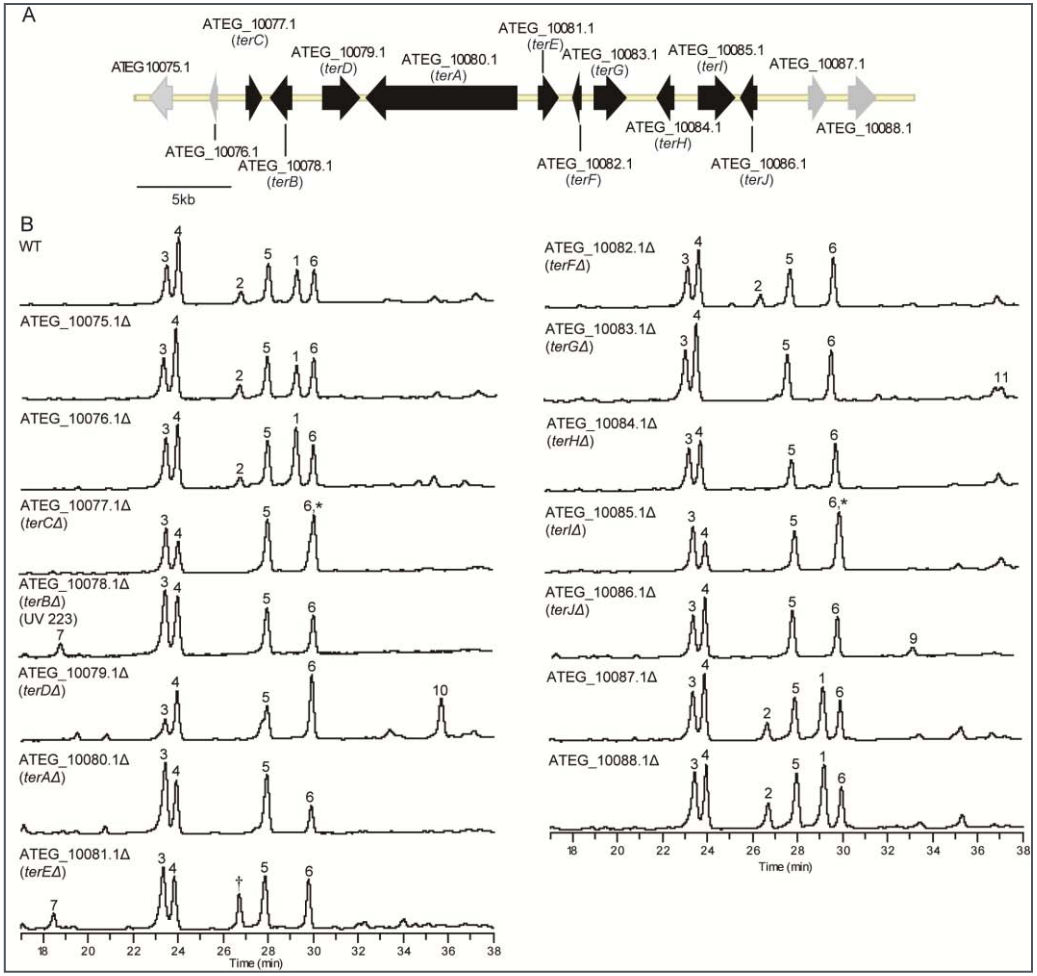
Alternative hydrocarbon fuel precursor pathways: **Polyketides**

- Polyketide synthesis exists in gene clusters in fungi
- Have identified the function of each gene in the terretonin cluster
- Collaboration with USC chemist Clay Wang to analyze products of cluster



Terretonin

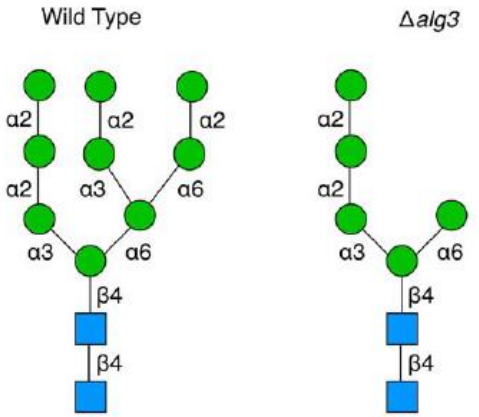
- Competitively awarded SynBio project (Bruno) now under way examining smaller, highly reduced polyketides

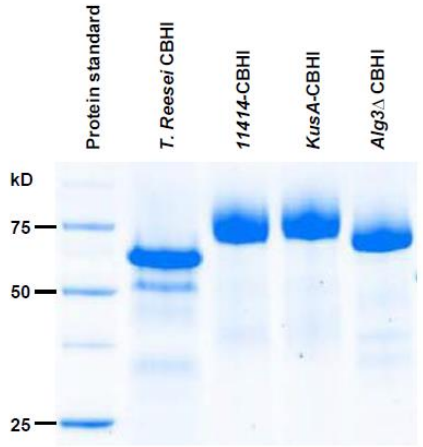


2 - Technical Accomplishments FY 11-12

Understanding enzymes used for biomass saccharification and the fungi that produce them

- Constructed *alg3*Δ strain in *A. niger*
- Expressed *T. reesei* cellobiohydrolase (CBHI) in wt and *alg3*Δ strains





- **Potential impact**: expressing promising prokaryotic saccharification enzymes where hyper-glycosylation could negatively affect activity
- Collaboration with NREL (Himmel et al.). Manuscript submitted to Molecular Microbiology

Understanding enzymes used for biomass saccharification and the fungi that produce them

- *T. reesei* toolbox paper: various promoters, integration and construct techniques to accelerate manipulation of *T. reesei*

Schuster *et al. Biotechnology for Biofuels* 2012, **5**:1
http://www.biotechnologyforbiofuels.com/content/5/1/1

Biotechnology for Biofuels

RESEARCH Open Access

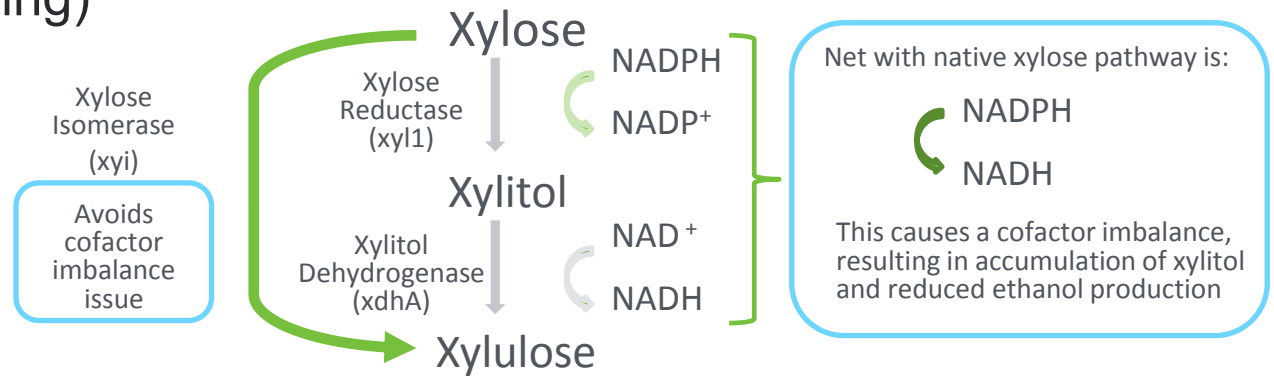
A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*

André Schuster[1,2], Kenneth S Bruno[1], James R Collett[1], Scott E Baker[1], Bernhard Seiboth[2], Christian P Kubicek[2*] and Monika Schmoll[2]

- PNNL is now a member of the Joint BioEnergy Institute (FY13-17). Developing a heterologous protein expression platform in *A. niger*
- Expressing newly discovered, more effective prokaryotic glycoside hydrolases at higher titers to decrease saccharification costs

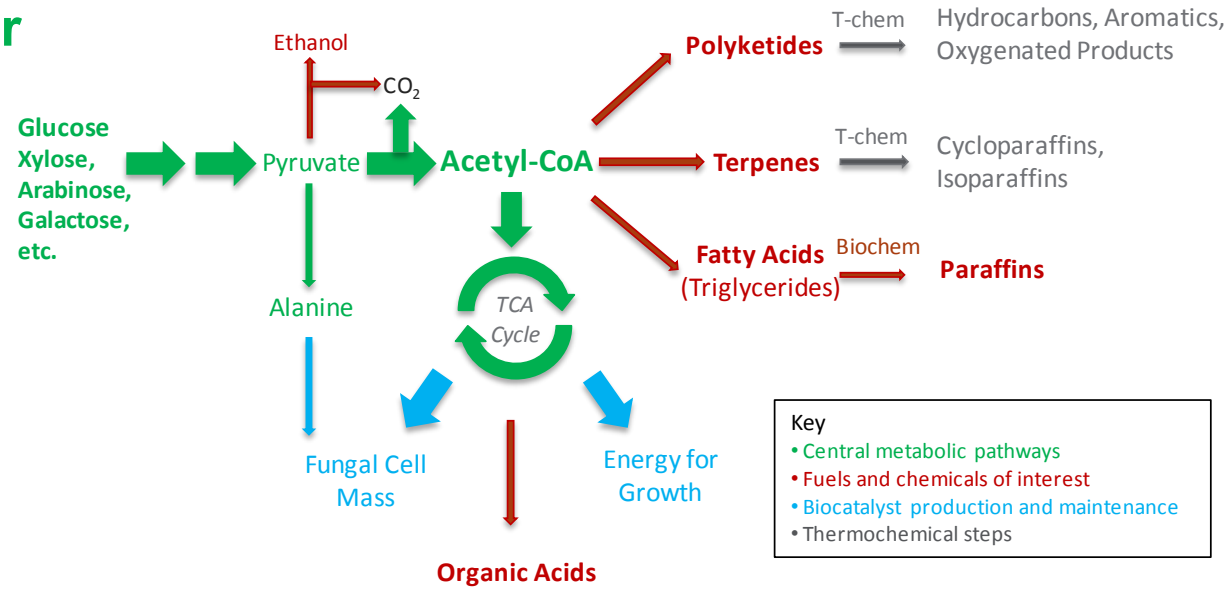
2 - Technical Accomplishment:

Pentose utilization is an important consideration for any lignocellulosic biomass to biofuels scenario and an example of pathway engineering to address alternative substrate utilization, increased product biosynthesis, i.e., flux (pushing or pulling)



- Pathway engineering is challenging (full of unintended consequences) but useful for increasing substrate utilization and flux to products
- Metabolic models have limits and need to be tested and refined by incorporating experimental data
- **We are using these tools, and the lessons learned, specifically for pentose utilization and generally for metabolic modeling and engineering approaches in our current work**

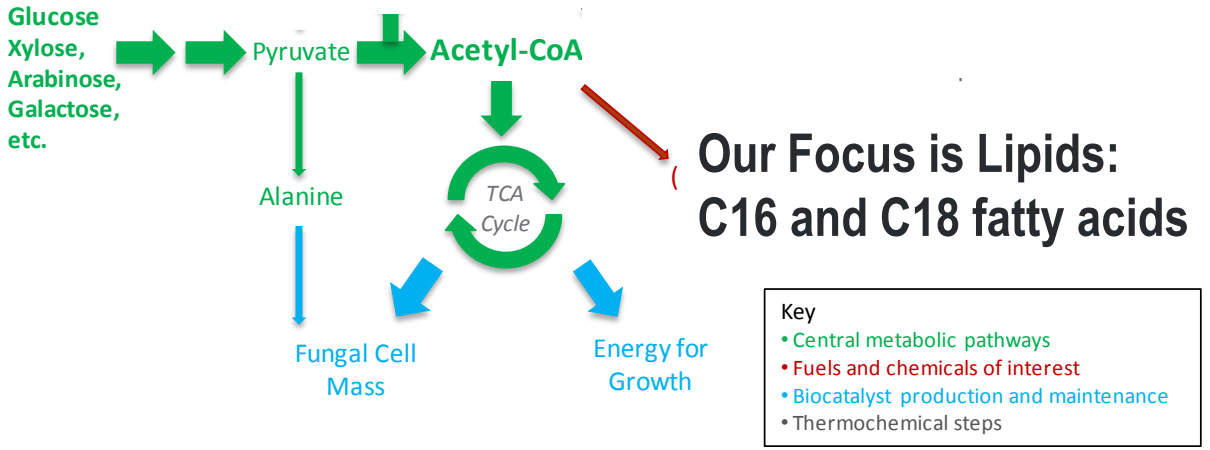
Fungal strains for bioconversion



Tasks

- Genetics of Fungal Hydrocarbons
- Metabolic Systems

Fungal strains for bioconversion

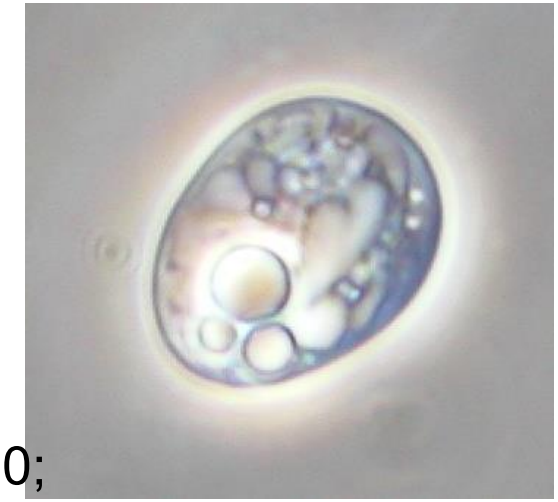


Tasks

- **Genetics of Fungal Hydrocarbons**
- **Metabolic Systems**

- **Central intermediates**--e.g., **Acetyl-CoA**--are **branch points** leading to multiple biofuels/biofuel precursors of interest
- **Increasing flux** to and from central intermediates is key…applicable to virtually all bioprocesses
- Optimizing TRY (titer, rate, yield): finding the ideal **balance** between **biocatalyst** generation and maintenance **vs. product** synthesis

2 - Technical Accomplishments FY13



October 2012: Began work with a new organism; the **oleaginous yeast, *Lipomyces starkeyi***

- Became adept at handling (storage, culturing)
- Developed defined media that supports good growth
- Instituted analytical methods for sugars, FAMEs, etc.
- Have surveyed hexose/pentose/disaccharide and mixed sugar utilization in shake flasks
- Examined **carbon:nitrogen molar ratios** from 30-100; glucose concentrations from 2-12%
- Have run 30 L fermentors +/- pH control; 6% glucose, C:N = 60
- Rapidly acidifies medium
- pH control **increased** growth rate and **lipid TRY**
- Produces prodigious amounts of exogenous polysaccharide (glucuronogalactomannan)
  - Viscosity increase presents bioprocess issues with O_2 transfer
  - Carbon sink: need to identify and delete glycosyl transferase

- **Project team has made great progress on fungal lipid production**

2 - Technical Accomplishments FY13

Examined C:N ratios and glucose concentrations from 6-12%

Glucose (%, w/v)	C:N Ratio	Dry wt. cells (g/L)	Glucose consumed (g/L)	g lipid / g dry wt. cells	FAMEs (g/L)	grams FAMEs per gram glucose consumed
6	60	22	57	25%	5.5	0.10
6	80	26	55	28%	7.5	0.14
6	100	28	41	25%	7.2	0.18
8	60	24	66	25%	6.0	0.09
8	80	24	70	27%	6.5	0.09
8	100	29	72	28%	8.1	0.11
10	60	24	76	24%	5.7	0.08
10	80	25	60	26%	6.5	0.11
10	100	28	68	27%	7.6	0.11
12	60	24	43	26%	6.2	0.15
12	80	25	45	27%	6.8	0.15
12	100	27	45	25%	6.8	0.15
12	120	29	39	24%	7.1	0.18

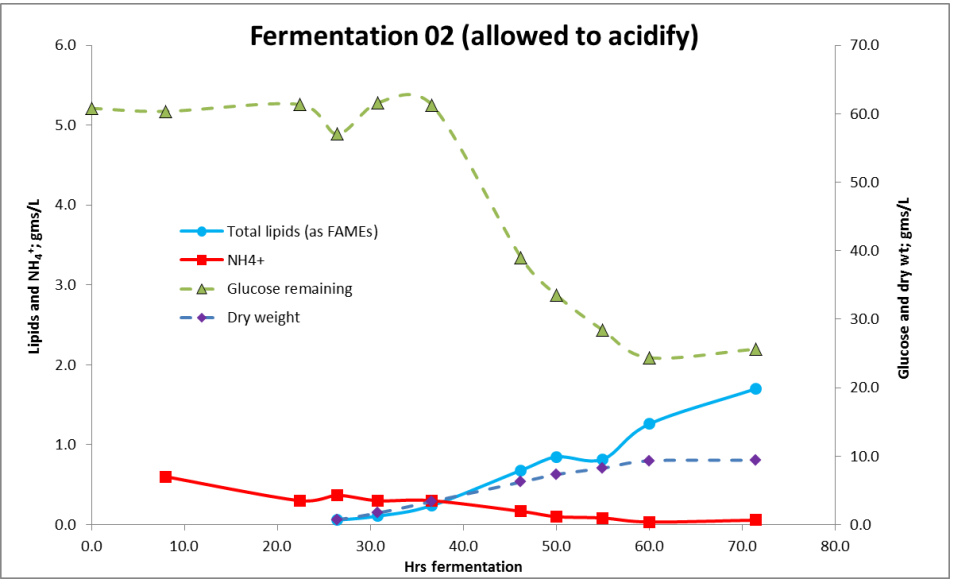
Defining parameters

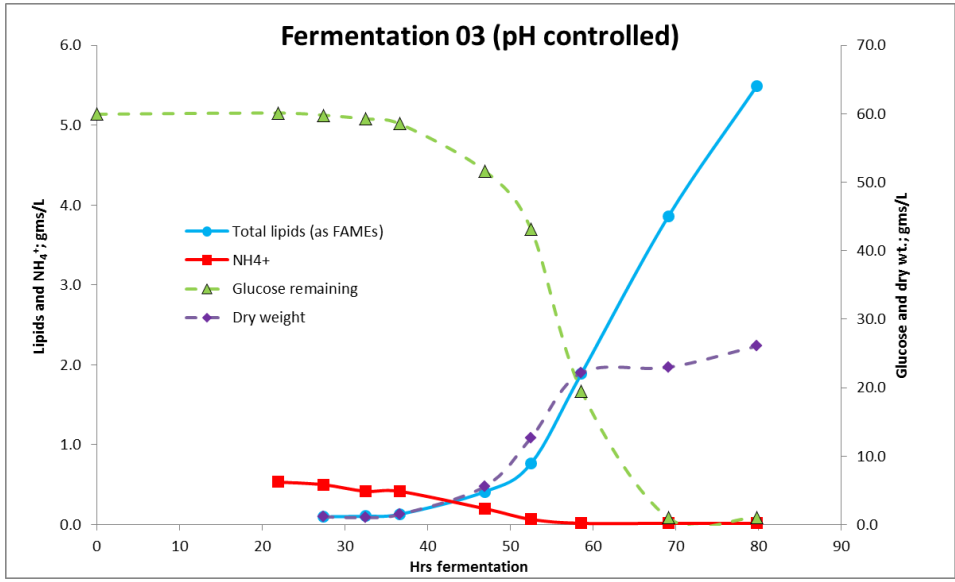
Higher C:N ratios (at a given glucose concentration) increase titer rate and yield

2 - Technical Accomplishments FY13

Fermentors: 6% glucose, C:N = 60, starting pH = 6

- Ferm 2: pH not controlled (decreased to 1.7)
- Ferm 3: pH controlled at 5.5
  - **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%**

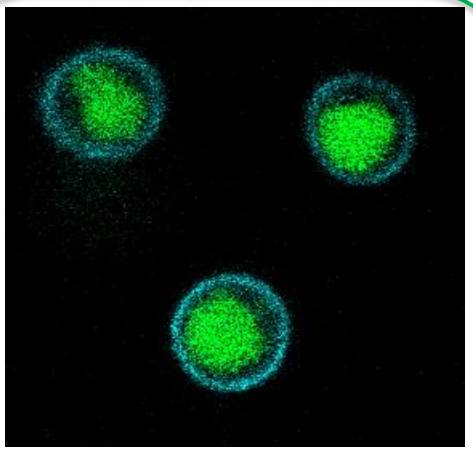




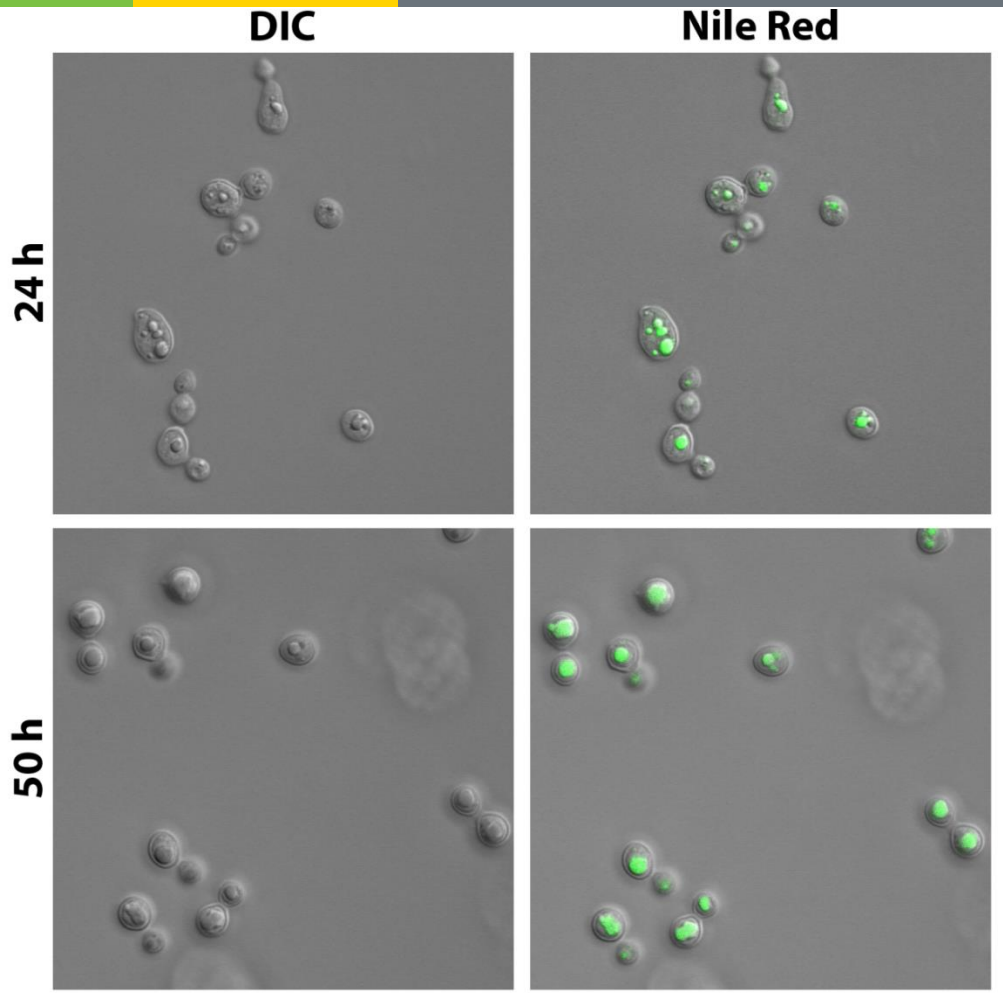
- Ferm 3, controlled pH: **carbon 100% utilized**, **lipid titer and rate improved**
- **SMART milestone** due 9/30/13: **0.1%** w/w lipid yield

2 - Technical Accomplishments FY13

Differential Interference Contrast (DIC) & Confocal fluorescence micrographs of *Lipomyces starkeyi* at 24 hours, before N is depleted, and 50 hours, during peak lipid production phase.



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



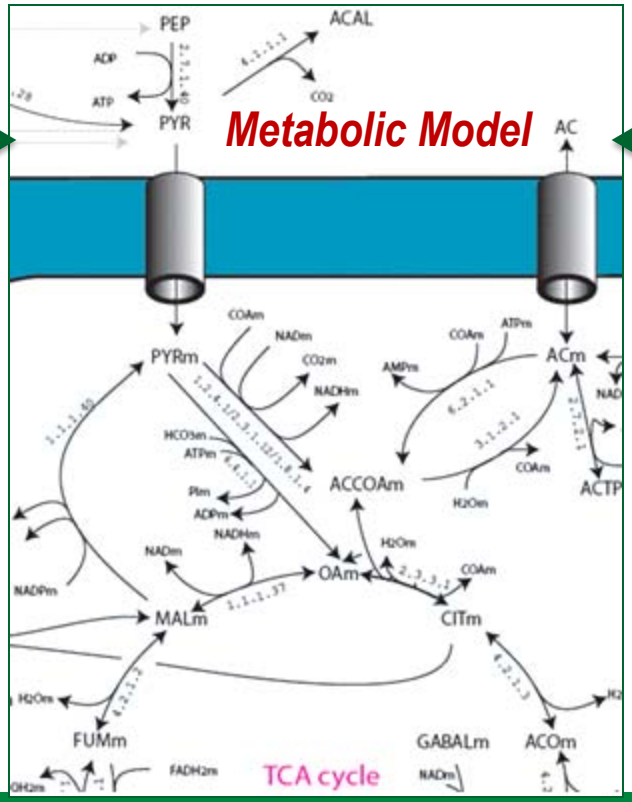
Post N depletion: lipid body size increases rapidly; cell count and dry weight data show cell numbers plateau but cell weight increases

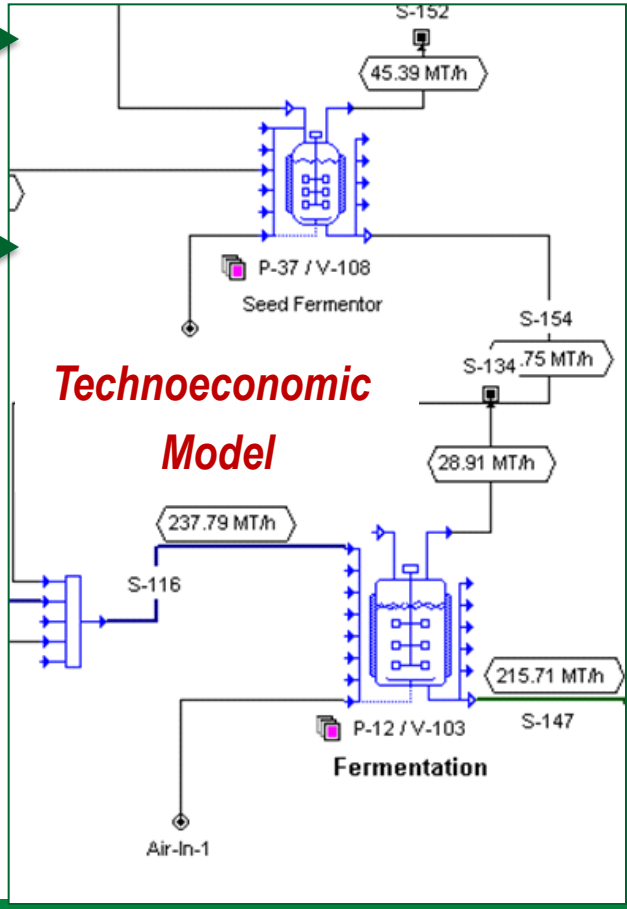
2 - Technical Accomplishments FY13

- Genetic Engineering Tools for *L. starkeyi*
  - Determined antibiotic sensitivity: tested 6 antibiotics and selection agents at range of concentrations; sensitive to 3 of them, includes hygromycin
  - Developed the **1st successful transformation** system for this yeast
  - Developed expression constructs
  - Continuing to refine and develop additional tools: additional promoters, gene deletion tools
  - Succeeded in formation of spheroplasts
  - Continuing to work on chemical transformation of the spheroplasts…a method preferred for targeted integration and gene deletions
  - Have a prioritized list of **gene targets** involved in precursor synthesis and redox cofactor balance associated with FA synthesis
  - The transformation system and gene candidates provide the first leg of our iterative strategy of **design-model-test**

Oleaginous Yeast Metabolic Model

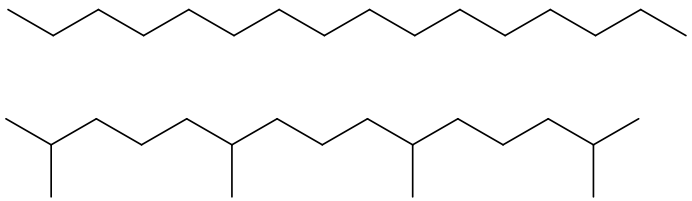
- Goals: To understand practical **yield limits** and to guide **metabolic engineering**
- Leverages published yeast models to reconstruct *L. starkeyi* metabolism and model
- 30-liter bioreactor data is informing both metabolic models and TE models (2.6.1.2)



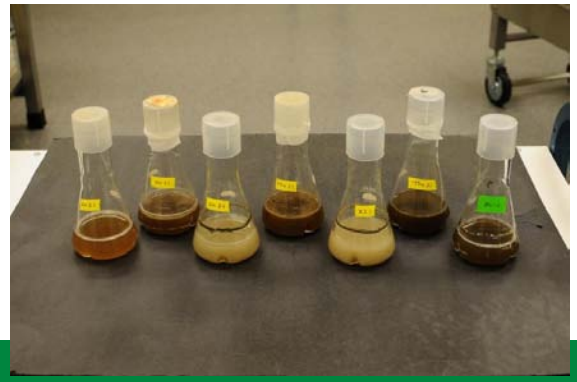


2 - Technical Accomplishments FY13

- Fatty acid biosynthesis in a proven platform fungus – *A. niger*
  - Advantages:
    - *A. niger* is robust, industrially relevant, grows on defined inorganic media
    - An organism that we are adept at manipulating genetically
  - Higher risk aspect: not an "oleaginous" fungus…a good platform for improving lipid biosynthesis
  - We are constructing fatty acid oxidation deletion strains and over-expression strains for fatty acid biosynthesis or precursor biosynthesis—improving net lipid productivity
- *Hormoconis resinae* (the kerosene fungus): source of novel biofuel synthesis genes
  - Transcriptomics study in progress in collaboration with JGI and JBEI
  - Goal is to identify genes involved in alkane and pristane biosynthesis
  - RNA samples from multiple culture conditions submitted to JGI for RNA-Seq



Main products on glucose
Walker, 1973



Milestones/Metrics and Progress:

Title/Description	Due Date	Completed
Develop SOP for transformation of *Lipomyces starkeyi*	Dec-12	✓
L. starkeyi baseline cultivation for lipid production and metabolic mass balances	Mar-13	✓
Amorphotheca resinae transcriptome	Jul-13	In progress
Baseline pentose utilization by *L. starkeyi*	Sep-13	In progress
SMART Milestone: Demonstrate 0.1% w/w lipid yield in *L. starkeyi* Baseline pentose utilization by *L. starkeyi*	Sep-13	✓

3 - Relevance

- We are focused on barrier area "**Bt-J Catalyst Development**" -- Fungal Biocatalysts
- Developing enabling technologies for faster development of biocatalysts for conversion of sugars to biofuels or biofuel precursors
- Primary **focus** is on one biofuel precursor class: **lipids**/fatty acids
- **Technology** for understanding, manipulating and testing the biocatalyst is **broadly applicable** to other processes and classes of biofuels
- We are developing our fungal biocatalyst in coordination with techno-economic analysts in order to focus our R&D on areas with the most impact on driving down the cost of the biofuel precursor and hence the biofuel…meeting BETO cost targets
- Our **goal** is to build robust fungal organisms that use lignocellulosic hydrolysates in a biorefinery (scaling to a 30 L fermentor is critical to scaling further to biorefinery volumes)

4 - Critical Success Factors

- **Maximize TRY** (titer, rate, yield):
  - Titer to minimize processing costs
  - Rate to decrease capital costs
  - Yield to minimize feedstock costs (utilize what we get)
- Relevant Issues:
  - Utilize all sugars from the relatively expensive feedstock. Understand the effects of authentic hydrolysates on the efficiency of our bioprocesses and how to mitigate any negative impacts
  - Energy costs for air compression, fermentor stirring…optimize our bioprocess to minimize these costs
  - Nutrient costs. Identify and eliminate auxotrophies, if possible.
  - Our primary focus is on developing a process for a biofuel *precursor* (lipid), hence we are dependent on those who would process the precursor to biofuel…fortunately that is existing technology
  - *Need to produce a "clean product"; clean with regard to inorganic catalysts for converting TAGs to diesel or jet. Low ash, low S, etc. Positively related to "nutrient costs" since minimizing nutrient inputs can produce a cleaner product stream
- Our ability to **genetically manipulate *L. starkeyi*** is an advancement in the state of technology that enables our approaches to further biocatalyst improvement

5. Future Work

ML or DL	Description	FY13 Q3	FY13 Q4	FY14 Q1	FY14 Q2	FY14 Q3	FY14 Q4
DL	*Amorphotheca resinae* transcriptome						
ML	**SMART** Milestone: Demonstrate 0.1% w/w lipid yield in *L. starkeyi*						
DL	Development of SOP for transformation of *A. resinae*						
ML	Baseline *A. niger* and *L. starkeyi* performance on lignocellulosic hydrolysates						
DL	Comparative transcriptomics of *L. starkeyi* to identify additional genes						
ML	Genetically engineer L. starkeyi to decrease or eliminate a carbon sink						

- Continue to **improve transformation systems** for *L. starkeyi*
- Transformation system for *A. resinae* to identify function of alkane/pristane biosynthesis genes identified by RNA-Seq
- Genetically engineer *L. starkeyi* and *A. niger* for improved TRY of lipid—**knockout carbon sinks**, improve flux to product
- Baseline oleaginous systems with **hydrolysates**
- Improve performance on hydrolysates; maximize carbon utilization **efficiency**
- **Go/No-Go**, Q3 FY15: techno-economic evaluation of fungal lipid production as a viable route progressing towards meeting the BETO FY17 cost targets of $5/gge

Summary

1) Approach:
   a) We have a sharpened focus on biofuel precursors for hydrocarbon biofuels aligned with current BETO goals.
   b) Tight management reflects tight focus.
2) Technical accomplishments:
   a) Developed 1st transformation system for *Lipomyces starkeyi*
   b) Demonstrated proficiency with culturing *L. starkeyi* from flasks to fermentors
   c) Exceeded most milestone due dates and metrics -- including SMART milestone on lipid yield from sugar due at the end of FY13
   d) Published many genome papers on bioprocess relevant fungi
   e) Understanding and manipulating polyketide biosynthesis in Aspergilli
   f) Developed genetic tools for the major cellulase production organism *T. reesei*
   g) Past R&D has led to **opportunities** in areas of biofuels research that are relevant to BETO goals and leverages their investments: JBEI, Synthetic Biology award
3) Relevance:
   a) We are utilizing our expertise to directly address improvement of bioprocesses for bioconversion of sugars to hydrocarbon biofuel precursors -- lipids
   b) The methods and approaches we develop will be widely applicable to other biofuel precursors or biofuels

Summary

4) Critical Success factors and challenges:
   a) Demonstrate improvements over the baseline and pay attention to most critical areas for decreasing costs as informed by TEA
5) Future Work
   a) Eliminating carbon sinks
   b) Transcriptomics of *L. starkeyi* fermentation for gene targets to increase TRY of lipids from sugars & lignocellulosic hydrolyzates. EMSL or JGI/JBEI collaboration
   c) Optimizing bioprocess conditions (nutrients, aeration) to decrease costs
6) Technology transfer
   a) *Lipomyces starkeyi* transformation technology
   b) Bioprocess organism improvements through genetic changes
   c) Alkane fuel producing genes/enzymes
   d) Continued consultations with Industrial Partners

Acknowledgements

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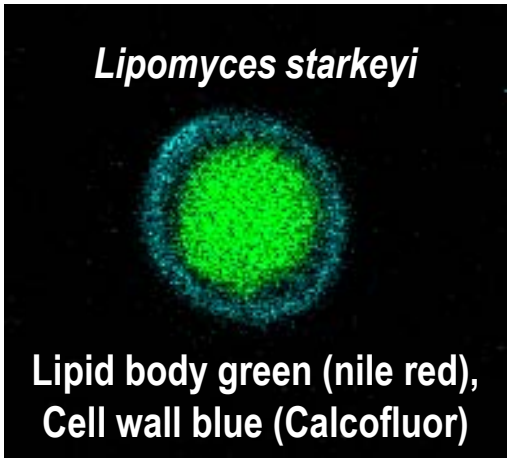
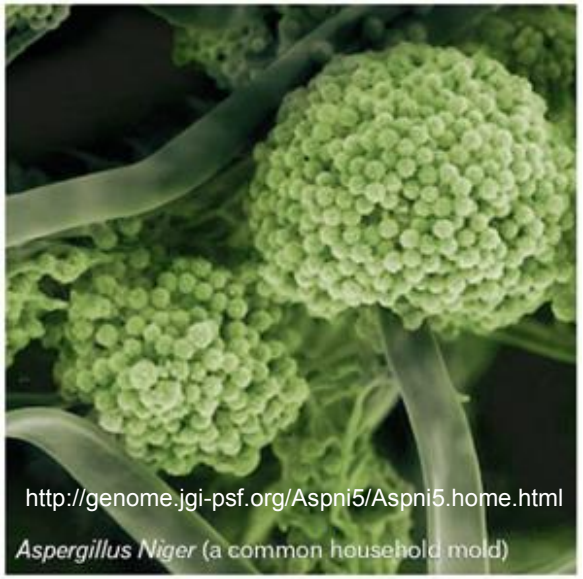
  - Paul Harris (Novozymes)
  - Jamie Ryding (BP Biofuels)
  - Steve Lewis (POET Research)
  - Cedric Pearce (Mycosynthetix)
- B ER: JGI, JBEI





Additional Slides

Responses to Previous Reviewers' Comments

- Reviewer comment: "In this project, the major 'success factor' is essential to the success of the project - in fact, it appears to be a non-stated goal of the project. That being the ability to manipulate fungi as necessary for the targeted outcome -- and each of those outcomes."
- The genome enabled genetic manipulation of fungi is a strength of our team. Hopefully we've articulated that more clearly at this review.

- Reviewer comment: "The research addresses some of the barriers to biochemical conversion of biomass in a general way"
- We hope our new scope is more focused and clearly addressing one defined barrier area.

- Reviewer comment: "The project has an excellent publication record and good relations with industrial partners. Basic advances are appropriately available for each industrial partner to adapt to its own use."
- Thank-you for your positive comments.

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- Brown, D.W., Butchko, R.A.E., **Baker, S.E.**, Proctor, R.H. 2012. Phylogenomic and functional domain analysis of polyketide synthases in *Fusarium.* Fung. Biol. *In press.*
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- Schuster, A., **Bruno, K.S., Collett, J.R., Baker, S.E.**, Seiboth, B., Kubicek, C.P., Schmoll, M. 2012. A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. Biotechnol. Biofuels. 5(1):1.
- **Baker, S.E.**, Perrone, G., Richardson, N.M., Gallo, A., Kubicek, C.P. 2012. Phylogenomic analysis of polyketide synthase encoding genes in *Trichoderma*. Microbiol. 158(1):147-54.
- Ryu, J.S., Shary, S., **Panisko, E.A.,** Korripally, P., St. John, F.J., Crooks, C., Siika-Aho, M. **Magnuson, J.K.,** Hammel, K.E. Proteomic and functional analysis of the cellulase expression system by *Postia placenta* during brown rot of solid wood. 2011 Appl Environ Microbiol. 77:7933-7941.
- Wang, L. Aryal, U.K, **Dai, Z.**, Mason, A.C., Monroe, M.E., Tian, Z-X., Zhou, J-Y., Su, D., Weitz, K.K., Liu, T., Camp, D.G., Smith, R.D., **Baker, S.E.**, Wei-Jun Qian, W-J. 2011. Mapping N-linked glycosylation sites in the secretome and whole cells of *Aspergillus niger* using hydrazide chemistry and mass spectrometry. J. Prot. Res. 11(1):143-56.
- Yang, B., **Dai, Z.,** Ding, S.-Y.,Wyman C.E. 2011. Enzymatic hydrolysis of cellulosic biomass. Biofuels 2(4): 421-450.

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- Grigoriev, I.V., Cullen, D., Hibbett, D., Goodwin, S.B., Jeffries, T.W., Kubicek, C.P., Kuske, C., **Magnuson, J**., Martin, F., Spatafora, J.W., **Baker, S.E.** 2011. Fueling the future with fungal genomics. Mycology, 2(3):192-209.
- Andersen, M.R., Salazar, M.P., Schaap, P.J., van de Vondervoort, P.J., **Culley, D.**, Thykaer, J., Frisvad, J.C., Nielsen, K.F., Albang, R., Albermann, K., Berka, R.M., Braus, G.H., Braus-Stromeyer, S.A., Corrochano, L.M., **Dai Z.**, van Dijck, P.W., Hofmann, G., Lasure, L.L., **Magnuson, J.K.**, Menke, H., Meijer, M., Meijer, S.L., Nielsen, J.B., Nielsen, M.L., van Ooyen, A.J., Pel, H.J., Poulsen, L., Samson, R.A., Stam, H., Tsang, A., van den Brink, J.M., Atkins, A., Aerts, A., Shapiro, H., Pangilinan, J., Salamov, A., Lou, Y., Lindquist, E., Lucas, S., Grimwood, J., Grigoriev, I.V., Kubicek, C.P., Martinez, D., van Peij, N.N., Roubos, J.A., Nielsen, J., **Baker, S.E.** 2011. Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res. 21(6):885-97.
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- Seidl-Seiboth, V., Gruber, S., Sezerman, U., Schwecke, T., Albayrak, A., Neuhof, T., von Döhren, H., **Baker, S.E.**, Kubicek, C.P. 2011. Novel Hydrophobins from *Trichoderma* Define a New Hydrophobin Subclass: Protein Properties, Evolution, Regulation and Processing. J Mol Evol. 72(4):339-51.
- **Baker, S.E, Panisko, E.A.** Proteome Studies of Filamentous Fungi. 2011. In *Fungal Genomics*, Methods in Molecular Biology, Vol. 722. Ed. J.R. Xu and B. H. Bluhm, Springer.
- Martin, F., Cullen, D., Hibbett, D., Pisabarro, A., Spatafora, J.W., **Baker, S.E.,** Grigoriev, I.V. Sequencing the Fungal Tree of Life. 2011 New Phytologist. 190(4):818-821.
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- Chiang, Y.M., Meyer, K.M., Praseuth, M., **Baker, S.E.**, Bruno, K.S., Wang, C.C.C. Characterization of a polyketide synthase in Aspergillus niger whose product is a precursor for both dihydroxynaphthalene (DHN) melanin and naphtho-γ-pyrone. 2011 Fungal Genet Biol. 48: 430-7.

Commercialization

- Continuing to develop and refine transformation system for *L. starkeyi* and periodically reviewing progress with commercialization manager at PNNL

Additional Slides

The following slides contain more details on certain topics that were trimmed from the main presentation in the interest of time

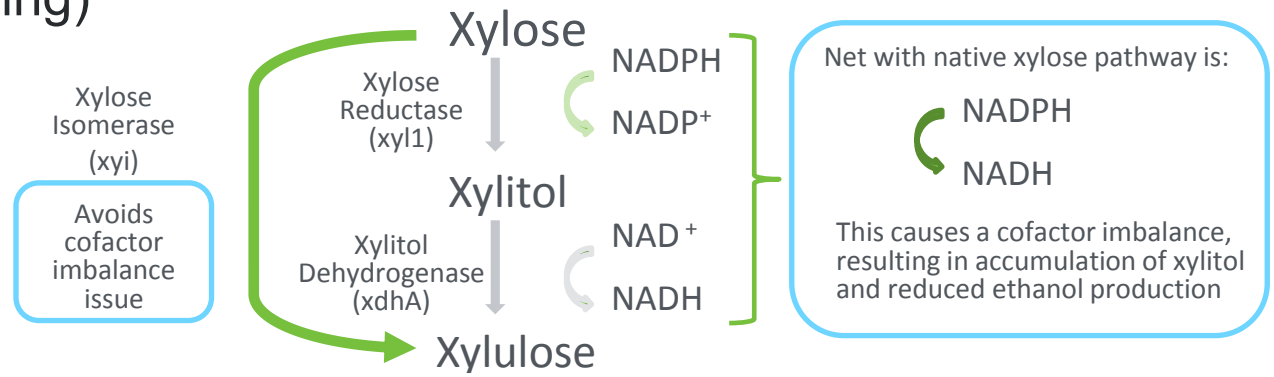
1 –Approach: Management

- Prepare a project management plan (PMP) annually with **milestones** and **deliverables** approved by BETO
- Biochemical Conversion Technology Area phone conference calls
- Quarterly reporting of progress against milestones to BETO

- Quarterly phone calls with industrial partners to solicit external input on project progress and direction
- Weekly internal project meetings to facilitate communication and efficiently focus our R&D efforts towards meeting objectives
- Monthly one-on-one meetings with team members
- Communicate with PNNL TEA team to understand the principal techno-economic challenges

2 - Technical Accomplishment:

Pentose utilization is an important consideration for any lignocellulosic biomass to biofuels scenario and an example of pathway engineering to address alternative substrate utilization, increased product biosynthesis, i.e., flux (pushing or pulling)

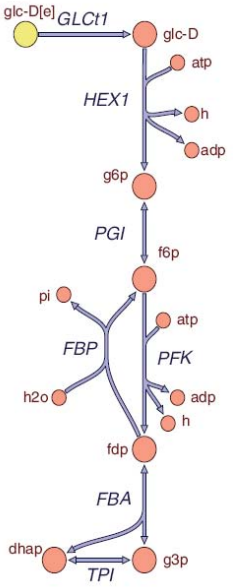


- Strains, deletions: *xdhA*, *xyl1*, *ladA* (arabitol dehydrogenase); *xdhA/xyl1*, *xdhA/ladA*; insertion *xyi*
- Double deletion of *xdhA/xyl1* decreased but did not eliminate growth on xylose
- *xdhA* partially compensated by *ladA*. Double deletion of *xdhA/ladA* severely decreased but did not eliminate growth. Inserted xylose isomerase in the wild type background and it appeared to decrease growth
- Pathway engineering is challenging (full of unintended consequences) but useful for increasing substrate utilization and flux to products
- Metabolic models have limits and need to be tested and refined by incorporating experimental data
- We are using lessons learned specifically for pentose utilization and generally for metabolic modeling and engineering approaches in our current work

Metabolic Network Modeling Methods

- Constraint-based reconstruction and analysis (COBRA) approach is being used.
- COBRA integrates network stoichiometry into a system of linear equations.
- The COBRA Toolbox provides a MATLAB-based simulation environment for modeling distributions of mass fluxes that maximize an objective function (typically, growth and/or specific metabolite production). The design of the stoichiometric matrix and *constraints* on nutrient uptakes and metabolite secretions limit the output to a reasonably searchable solution space.

Network → **Reactions and Genes** → **Stoichiometric matrix**



atp[c] + D-glucose[c] -> h[c] + g6p[c] + adp[c]
(YALI0B22308g or YALI0E15488g or YALI0E20207g)

g6p[c] <=> f6p[c]
YALI0F07711g

atp[c] + f6p[c] -> h[c] + fdp[c] + adp[c]
YALI0D16357g

fdp[c] <=> g3p[c] + dhap[c]
YALI0E26004g

Enzymes(genes)

Metabolites	GLCt1	HEX1	PGI	PFK	FBP	FBA	TPI	EX_glc
glc-D[e]	−1	0	0	0	0	0	0	−1
glc-D	1	−1	0	0	0	0	0	0
atp	0	−1	0	−1	0	0	0	0
H	0	1	0	1	0	0	0	0
adp	0	1	0	1	0	0	0	0
g6p	0	1	−1	0	0	0	0	0
f6p	0	0	1	−1	1	0	0	0
fdp	0	0	0	1	−1	−1	0	0
pi	0	0	0	0	1	0	0	0
h2o	0	0	0	0	−1	0	0	0
g3p	0	0	0	0	0	1	1	0
dhap	0	0	0	0	0	1	−1	0

= S

UB = (∞ ∞ ∞ ∞ ∞ ∞ ∞ ∞)

LB = (−∞ 0 −∞ 0 0 −∞ −∞ −2)

- Becker SA, Feist AM, Mo ML, Hannum G, Palsson BØ, Herrgard MJ. Nature Protocols 2007;2(3):727-38.
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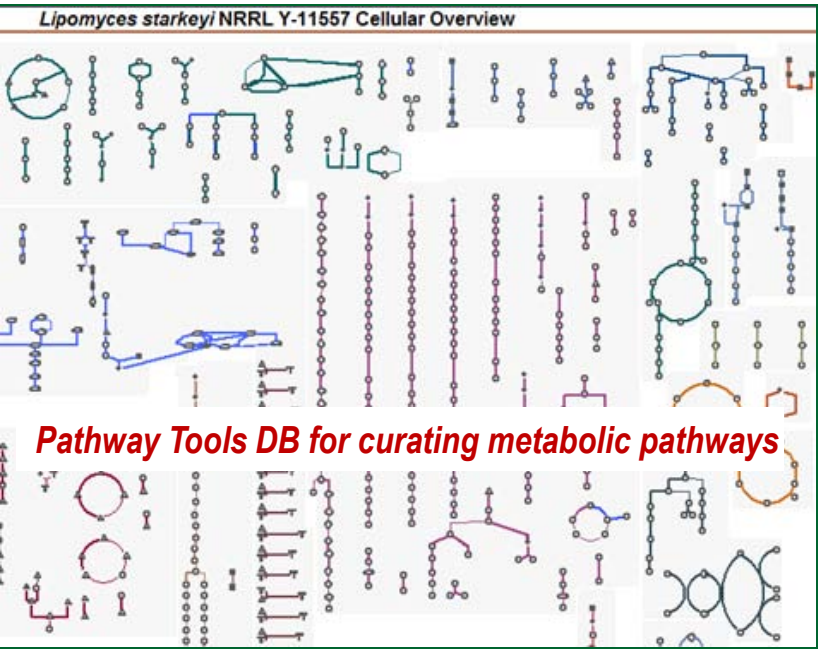
L. starkeyi Metabolic Modeling Progress

- Pathway Tools used to build a pathway/genome database (PGDB) from JGI's *L. starkeyi* genome annotations…establishing the metabolic network.
- Orthologous Markov Clustering (OrthoMCL) used to identify *L. starkeyi* DNA sequences that match genes in published COBRA models for the yeasts *Yarrowia lipolytica, S. cerevisiae, S. stipitis,* and *P. pastoris.*
- The highest average ortholog score was between the oleaginous yeasts, *L. starkeyi* and *Y. lipolytica*.



- 1122 enzymatic reactions in the *Y. lipolytica* model; 984 mapped to *L. starkeyi* genes; remainder had partial (2/3) or no (1/3) matches
- Manually checking the *L. starkeyi* PGDB to find the missing genes or to confirm that *L. starkeyi* does not have those genes
- All expected *L. starkeyi* genes for energy generation in central metabolism have been identified
- Working on identification of *L. starkeyi* lipid catabolic/anabolic pathway genes
- Working on integrating the genome-scale network and new bioreactor experimental data into a COBRA model for flux balance analysis simulations.

Methods based on: Thiele I, Palsson BØ., Nat Protoc. 2010 Jan;5(1):93-121.
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