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.

- **Genetic Engineering**
  - Advanced genetic tools
  - Rational design; reverse genetics
  - Strains for Bioprocess Development

- **Bioprocess Development**
  - Bioreactors
  - TRY* assessment
  - Samples for Systems Biology

- **Systems Biology/Genomics**
  - Forward genetics
  - Transcriptomics
  - Gene candidates for Genetic Engineering

*TRY = Titer, Rate & Yield*
Quad Chart Overview

**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**

<table>
<thead>
<tr>
<th></th>
<th>Total Costs FY 10 – FY 12</th>
<th>FY 13 Costs</th>
<th>FY 14 Costs</th>
<th>Total Planned Funding (FY 15-Project End Date)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DOE Funded</strong></td>
<td>7.25</td>
<td>1.5</td>
<td>1.6</td>
<td>1.75</td>
</tr>
<tr>
<td><strong>Project Cost Share (Comp.)</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**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

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 an **Industrial Advisory Panel**.
- Initially the focus was on **bioproducts**, such as, organic acids. Transitioned to emphasis on lignocellulosic 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).

<table>
<thead>
<tr>
<th>FY01-04</th>
<th>FY05-08</th>
<th>FY09-12</th>
<th>FY13-present</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNNL-LDRD</td>
<td>Fungal Genomics AOP begins Bioproducts</td>
<td>Lignocellulosic EtOH</td>
<td>Advanced Biofuels</td>
</tr>
</tbody>
</table>
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**
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

Genetic Engineering
- Advanced genetic tools
  - Rational design; reverse genetics
- Strains for Bioprocess Development

Bioprocess Platforms
- Lipomyces for lipids
- Aspergillus for bioproducts (terpenes/polyketides)

Systems Biology/Genomics
- Forward genetics
- Transcriptomics
- Gene candidates for Genetic Engineering

Bioprocess Development
- Bioreactors
- TRY assessment
- Samples for Systems Biology

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 lignocellulose relevant sugars:
  - Excellent on glucose, galactose and mannose
  - Good on xylose and cellobiose
  - Fair on L-arabinose
- Appropriate for conventional aerobic bioreactors

Nile red (lipid, green), Calcofluor (cell wall, blue)
3 – Why is Aspergillus a relevant choice for an industrial biocatalyst?

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 **lignocellulose** relevant sugars
- Produces **enzymes** to deconstruct lignocellulose
- Utilizes **simple inorganic nutrients**
- **Acid tolerant** (pH 1.5): minimizes sterility requirements, and advantageous for organic acid production
- Appropriate for conventional aerobic bioreactors
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:
  - Titer: 5.49 g/L lipids (as FAMEs)
  - Rate: 0.069 g/L/hr (80 hr avg.); 0.174 g/L/hr during the lipid production phase
  - Yield: 0.092 g lipid per g glucose; 9.2%
3 – Technical Accomplishments
Biocatalyst Design and Test Cycle

Genetic Engineering
- Advanced genetic tools
  - Rational design; reverse genetics
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Tech Transfer to Bioenergy Industry
- Biocatalyst
- Bioprocess Description
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
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!
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**
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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lipid Accumulation Rate: g FAME/g dry mass/L as % of parental strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-11558</td>
<td>100%</td>
</tr>
<tr>
<td>abc-1</td>
<td>110%</td>
</tr>
<tr>
<td>abc-5</td>
<td>128%</td>
</tr>
<tr>
<td>def-2</td>
<td>133%</td>
</tr>
<tr>
<td>def-4</td>
<td>115%</td>
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</table>
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**

### 6 Day Shake Flask Cultures

<table>
<thead>
<tr>
<th>% Sugars from PCS</th>
<th>Parent (Y-11558)</th>
<th>Strain R7</th>
<th>Strain S17</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>10</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>20%</td>
<td></td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>30%</td>
<td></td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>40%</td>
<td></td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

**FAMES g/L**

- Parent (Y-11558) @ 12 Days
- Strain R7 @ 12 Days
- Strain S17 @ 12 Days
Fed-batch growth on clean sugars: have achieved 66% lipid content

Growth on enzymatically saccharified PCS -- no added nutrients and no inhibitor “mitigation”

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

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
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
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.
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
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

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

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


Intellectual Property:


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

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


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


Publications:

