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: 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

Timeline

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

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

Budget

<table>
<thead>
<tr>
<th></th>
<th>FY 15 Costs</th>
<th>FY 16 Costs</th>
<th>FY 17 Costs</th>
<th>Total Planned Funding (FY 15-17 Project End Date)</th>
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</thead>
<tbody>
<tr>
<td><strong>DOE Funded</strong></td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>4.5</td>
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<td><strong>Project Cost Share (Comp.)</strong>*</td>
<td>0</td>
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</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*

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

**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
  - TEA
Why is Lipomyces an excellent choice for an industrial biocatalyst?

Attributes

- Highly productive oleaginous yeast: \( \sim 65\% \) 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, mannose, and xylose
  - Also utilizes cellobiose and L-arabinose
- We’ve developed genetic tools
- Behaves well in conventional aerobic bioreactors
Why is Aspergillus an excellent 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
- Great genetic tools
- Grows and produces bioproducts on lignocellulose 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
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.
**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.

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```
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 *ntm1* promoter

**Goal**: **prevent product degradation** or modification

Targeting likely catabolic pathways
- Beta-oxidation
- Alpha-oxidation
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**!
- **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

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**Binary T-DNA vector**

- **Tef1** promoter
- **Cas9**
- **SV40 NLS** terminator
- **PgdA**
- **HH**
- **leu2**
- **HDV** terminator

Lipomyces – CRISPR
3 – Project Accomplishments

**Lipomyces Metabolic Modeling**

- 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
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**

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

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

**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

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Conditions</th>
<th>Cells</th>
<th>Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculum (cells/ml)</td>
<td>Feeding Regimen</td>
<td>DCW (g/L)</td>
</tr>
<tr>
<td>12</td>
<td>CLEAN SUGAR 2.5% 2:1 Glucose, Xylose MM*</td>
<td>Manual Fed Batch - 46.5% Glucose</td>
<td>20.0</td>
</tr>
<tr>
<td>17</td>
<td>PCS only, 2.3% sugars equivalent</td>
<td>Batch</td>
<td>7.0</td>
</tr>
<tr>
<td>18</td>
<td>PCS MM*, 2.1% sugars equivalent</td>
<td>Batch</td>
<td>18.6</td>
</tr>
<tr>
<td>24</td>
<td>CLEAN SUGAR 6% Glucose MM*</td>
<td>Manual Fed Batch 100% Glucose, then 76% Xylose</td>
<td>155.7</td>
</tr>
</tbody>
</table>

* 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

![Diagram of lipid extraction process]

- **Lipid Rich Yeast Cell Mass**
  - Hydrothermal Liquefaction
  - Lipid Extraction

  ![Sub-diagrams]

  - **Bio-oil**
    - Hydro-treating
    - Mixed Fuel
  - **Lipids**
    - Catalytic Hydro-deoxygenation
    - Paraffinic Fuel
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

Run 24: *L. starkeyi* grown on 6% glucose/xylose MM
Then fed-batch glucose/xylose

![Graph showing cell growth and FAME production](image.png)
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**

Growth of *L. starkeyi* in automated fed-batch cultivation on hydrolyzed, minimally filtered PCS

- Actual dry cell mass (regression of offline samples)
- Capacitance-based prediction of dry cell mass
- Capacitance
- Totalized addition of PCS concentrate
- Intracellular lipids as FAMEs (regression of offline samples)

**10-fold increase in cell mass within 24 hours.**

Pretreated corn stover (PCS) stock

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**10-fold increase in cell mass within 24 hours.**

Pretreated corn stover (PCS) stock
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
**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)

<table>
<thead>
<tr>
<th>PROCESS CONFIGURATION</th>
<th>By-product from lipids and reduced fermentor costs</th>
<th>By-product from lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEEDSTOCK COST</td>
<td>Feedstock cost, $120/ton</td>
<td>Feedstock cost, $60/ton</td>
</tr>
<tr>
<td>CAPITAL</td>
<td>Fermenter (seed and lipid production) area installed cost, +25%</td>
<td>Fermenter (seed and lipid production) area installed cost, -50%</td>
</tr>
<tr>
<td>OPERATING PARAMETERS</td>
<td>Fermentation retention time, 72 hrs</td>
<td>Fermentation retention time, 30 hrs</td>
</tr>
<tr>
<td></td>
<td>Seed tank retention time, 48 hrs</td>
<td>Seed tank retention time, 24 hrs</td>
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</table>
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
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
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)

<table>
<thead>
<tr>
<th>Due Date</th>
<th>Remaining FY17 Milestones</th>
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<tbody>
<tr>
<td>March 31</td>
<td>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</td>
</tr>
<tr>
<td>March 31</td>
<td>Obtain at least 1 g/L of 1 of the 2 target polyketides/terpenes selected in M1. <strong>GO/NO-GO</strong></td>
</tr>
<tr>
<td>June 30</td>
<td>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</td>
</tr>
<tr>
<td>Sept. 30</td>
<td>Obtain 2.5 g/L of a polyketide or terpene in a scaled (20L) bioprocess</td>
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Publications


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

Publications (cont.)


Intellectual Property


Presentations


Presentations (cont.)


Presentations (cont.)
