DOE Bioenergy Technologies Office (BETO) 2015 Project Peer Review

Advanced Enzyme Deconstruction/Enzyme Engineering & Optimization

Date: March 23-27
Technology Area Review: Biochemical Platform

Principal Investigator: Michael E. Himmel
Task Leader: Steve Decker
Organization: NREL

*On many slides, the slide notes section has important additional information*

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

• Present the goal of this project and describe how the project fits with the goals/objectives of the Technology Area and ultimately the goals/objectives of the DOE Bioenergy Technologies Office, and the overall bioenergy industry.
  – The goal of this project is to ensure that cellulase enzyme loadings can be reduced to the target of 10 mg/g cellulose on DA corn stover using modern enzyme engineering and formulation optimization strategies. The attainment of this overall enzyme cost is consistent with BETO’s technoeconomic evaluations for meeting the 2017 biomass conversion target. Given that cost effective biomass conversion to sugars is a common theme for many conversion processes, the overall bioenergy industry also benefits from this work.

• Explicitly state relevance and tangible outcomes for the United States.
  – It is important that the U.S. shows leadership in the alternative fuels arena. This work will permit new science and technology to be developed in the U.S. which will benefit U.S. industry, as well as academic researchers seeking longer term solutions.
  – Ensure U.S. jobs and energy infrastructure
Quad Chart Overview

Timeline
• Project start date - 2001
• Project end date - 2017
• Percent complete - 82%

Barriers
• Barriers addressed
  – Bt.C Biomass Recalcitrance
  – Bt.D Pretreatment Chemistry
  – Bt.G Cellulase Enzyme Loadings

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>DOE Funded*</td>
<td>$3.5M</td>
<td>$3.3M</td>
<td>$1.9M</td>
<td>$4.8M ($1.6M fy15)</td>
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<tr>
<td>Project Cost</td>
<td>$3.4M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Share (Comp.)*</td>
<td>$3.0M</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Partners

- Subcontractors: Colorado State University, NIST, Cornell University, Vanderbilt University, Weizmann Institute of Science, UC Berkeley
- Collaborators: Zhongping Tan (CU), Jhih-Wei Chu (Berkeley), Charles Brooks III (Univ Michigan), Lee Makowski (ANL), Paul Langan (LANL), Sunney Xie (Harvard), Parastoo Azadi (UGA CCRC), Scott Baker (PNNL), Simon Craig (Univ York, UK), Jerry Stahlberg (Swedish Univ Ag Sci), John McGeehan (Univ Portsmouth, UK), Joel Kaar (CU-Boulder), Vincent Eijsink (NMBU), Kiyo Igarashi (U Tokyo), Michelle O'Malley (UCSB)
- DOE FOA to Genencor, subcontracted to NREL under CRADA ($150,000/3 years)
1 - Project Overview

• For the past decade, we have used enzyme engineering approaches for improving enzymes and formulations critical to BETO’s cost goals.
  – Our strategy is to employ a knowledge based approach which permits linking protein structure to performance.
  – To this end, we have pioneered the close coordination of computational analysis and experimental validation for cellulases.

• We will supply improved enzymes for the near term advanced biofuels platform and provide real-time coupling to feedstock and pretreatment efforts.
  – In contrast to other DOE supported work on cell wall degrading enzymes, our work will be targeted specifically to the needs of the BETO processes where ~one third the fuel cost is due to cellulases.
  – Our work will reduce the enzyme loading from the 2014 SOT of 14 mg/g cellulose to the target of 10 mg/g cellulose for 2017.

• To meet these aggressive goals, we have established working relationships with the enzyme companies (Genencor-DuPont, DSM, and Novozymes)

• To support BETO’s longer term goals and mission, we will also work to ensure that the cost and protein loading targets for 2022 are met.
  – We will target reformulation of enzyme cocktails to respond to changes in feedstocks (new genetically engineered crops from BER, BES, ARPA-E), pretreatment chemistries and severities, and targeted products/co-products.
2 – Approach for FY15 (Technical)

• *Describe critical success factors* ....
  – Meeting BETO’s 2017 technology targets for cellulose conversion to glucose, cellulase cost, and solids concentrations for pretreatment.
  – Meet near term and future technical targets for BETO’s 2022 goals for advanced biofuels. To some extent, these targets are still not fully defined, but reduced conversion cost is likely to be critical.

• *Explain the top 2-3 potential challenges* ....
  – To show that current commercial enzyme cocktails can be reformulated for improved performance and reduced cost.
  – The *introduction of new processing steps in 2014* (DDR, deacetylation and disk refining and lignin centric pretreatments) may require new baseline studies on optimizing biomass saccharification and fermentation.
  – To show that the sciences of biomass pretreatment, enzyme digestion, and microbial transformation to fuels can be understood sufficiently to enable industry to be successful in the near term.
  – To integrate properly the **new fast pace of information** coming from the DOE centers charged with conducting biomass conversion research (DOE EFRCs and BRCs).
2 – Approach (Technical)

2017 Biochemical Pathway – Biological Conversion

- Lignin utilization – (2022)
- Product versatility
- Boost to economics
- Avoids C5 utilization
- Oleaginous yeast

### Minimum Fuel Selling Price ($/GGE, 2011$)

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minimum Fuel Selling Price ($/GGE, 2011$)</strong></td>
<td>$5.10</td>
<td>$12.97</td>
<td>$10.14</td>
<td>$7.43</td>
<td>$5.03</td>
</tr>
<tr>
<td>Feedstock Contribution ($/GGE, 2011$)</td>
<td>$1.76</td>
<td>$3.88</td>
<td>$3.20</td>
<td>$2.47</td>
<td>$1.87</td>
</tr>
<tr>
<td>Conversion Contribution ($/GGE, 2011$)</td>
<td>$3.33</td>
<td>$9.09</td>
<td>$6.93</td>
<td>$4.97</td>
<td>$3.16</td>
</tr>
<tr>
<td>RDB Fuel Yield (GGE/dry ton)</td>
<td>45</td>
<td>19</td>
<td>20</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Succinic Acid Yield (lb/dry ton)</td>
<td>NA</td>
<td>197</td>
<td>206</td>
<td>232</td>
<td>270</td>
</tr>
</tbody>
</table>

### Pretreatment/ Separation

<table>
<thead>
<tr>
<th>Pretreatment/ Separation</th>
<th>2014</th>
<th>2015</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solids Loading (wt%)</td>
<td>30%</td>
<td>30%</td>
<td>30%</td>
<td>30%</td>
</tr>
<tr>
<td>Xylan to Xylose (including conversion in C5 train)</td>
<td>&gt;73%</td>
<td>73%</td>
<td>75%</td>
<td>78%</td>
</tr>
<tr>
<td>Hydrolysate solid-liquid separation</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Xylose Sugar Loss (into C6 stream after acid PT separation)</td>
<td>NA</td>
<td>5%</td>
<td>4%</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

### Enzymes

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>2014</th>
<th>2015</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Loading (mg/g cellulose)</td>
<td>10</td>
<td>14</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Enzymatic Hydrolysis &amp; Bioconversion – C6 Train</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Solids Loading to Hydrolysis (wt%)</td>
<td>20%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
</tr>
<tr>
<td>Enzymatic Hydrolysis Time (d)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Hydrolysis Glucan to Glucose</td>
<td>90%</td>
<td>77%</td>
<td>85%</td>
<td>85%</td>
</tr>
</tbody>
</table>

### Enzyme Loading vs Onsite Cellulase Production Cost

<table>
<thead>
<tr>
<th>Enzyme Loading</th>
<th>Onsite Cellulase Production Cost</th>
<th>Total MFSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/gram cellulose</td>
<td>$.82/GGE</td>
<td>$5.03/GGE</td>
</tr>
<tr>
<td>20 mg/gram cellulose</td>
<td>$1.54/GGE</td>
<td>$5.78/GGE</td>
</tr>
</tbody>
</table>
Overview: Technical Objectives for 2015

- **Multi-fold improvement in Cel7 activity** for formulation in highly active commercial cellulases is needed for biomass conversion (targeting reduction in cellulase loading to 10 mg/gram cellulose by 2017).
- Understanding the role glycosylation plays in cellulase functionality and stability and the ability to manipulate/control these glycosylation profiles to improve biomass conversion.
- Identify and evaluate new enzymes for an ability to enhance the performance of *T. reesei* cocktails (non commercial).
- Evaluate **new process scheme scenarios** for enhanced saccharification of biomass cellulose
- Development of a yeast high throughput screening system for identification of superior Cel7s.
Cel7A is being used industrially now

~5,000 tons of enzyme/plant/year with a 2,000 ton/day plant
2 – Approach (Management)

• **Describe critical success factors ....**
  – Meeting BETO’s 2017 technology targets for cellulose conversion to glucose, cellulase cost, and solids concentrations for pretreatment.
  – Disseminating technical achievements in a timely manner (publications/presentations)
  – Meeting near term and future technical targets for BETO’s 2022 goals for advanced biofuels. To some extent, these targets are still not fully defined, but reduced conversion cost is likely to be critical.

• **Explain the top 2-3 potential challenges ....**
  – For the 2017 cost goals, good communication with the enzyme industry is important and thus we maintain relationships with DSM, DuPont, and Novozymes.
  – The current TEA analysis of the impact of cellulase cost is based on onsite production of a leading commercial formulation – a publicly available strain and enzyme formulation would be more robust !

• **Emphasize the structure of your approach....**
  – Our approach is to use a basic RACI internal management plan which assigns work to researchers based on the milestone structure.
  – Milestones are Regular, Quarterly, and Annual, with several SMART milestones identified. One Go/No-Go decision is planned for FY2016.
2 – Approach (Management)
There were three major focus areas for the ABD Task in FY14

**T. reesei Cel7 Subdomain Engineering (informed by natural variants)**
Improving and understanding Cel7 function remains critical. We are examining multiple aspects of Cel7 structure/function relationships including glycosylation, diverse evolutionary sources, point mutations, and subdomain swapping between diverse Cel7s. This rational approach will give us insight into understanding how this critical enzyme functions.

**Evolutionary Engineering of Cellulases**
Random evolution is a powerful force in enzyme improvement, however it requires an effective screen in order to harness its potential. Until now, there has not been an effective means of rapidly evaluating improvements in the tens of thousands of variants generated by these methods. We are developing such a screen in order to capture the potential of random evolution as a tool for the improvement of Cel7.

**Multi-domain/Accessory Cellulases Expressed in Fungi**
Bacterial and fungal biomass hydrolyzing machinery are very different; however, they are being shown to have highly synergistic behavior in degrading biomass. We are exploring the potential to functionally express these complex bacterial enzymes in fungi in order to develop a single enzyme production system with higher activity than either parent system.
2014 ABD Engineering the Key Cellulase - Cel7A

2014 key milestones

<table>
<thead>
<tr>
<th>Q1</th>
<th>12/31/2013</th>
<th>Regular</th>
<th>Structural Design Mutations for Improving Cel7 Cellulases (Joint with Structure, Simulation &amp; Theory Project)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q4</td>
<td>9/30/2014</td>
<td>Regular</td>
<td>Improved Cellulases for Biomass Conversion</td>
</tr>
</tbody>
</table>

- Understand structure/function of catalytic domain
- Understand structure/function of CBM and linker
- Natural variants of GH7 cellobiohydrolases exhibit different properties
  - pH tolerance
  - thermal stability
  - binding affinities
  - activity on biomass
  - glycosylation patterns

✓ Requires a rigorous purification and testing scheme
Cellulases are typically multi-modular glycoproteins (Cel7A)

Catalytic domain with N-glycosylation

Linker domain with O-glycosylation

Carbohydrate-binding module (CBM)

Single most important enzyme in industrial lignocellulosic biomass conversion

Approach: separate the problem into components to study sub-domains and elementary steps

Overall aim: understand function to aid protein engineering efforts
3 – Technical Accomplishments/ Progress/Results

Function(s) of the carbohydrate-binding module
What are the functions of CBMs and how can we engineer them?

- Increases proximity of enzyme to substrate
- Targets specific cellulose faces
- Higher CBM binding affinity leads to higher activity
- Many of the residues are highly conserved

J. Baker, NREL

Purified enzymes 1% Avicel

Graph showing conversion rates for Catalytic Domain and Intact CBH1.

Sequence alignment with conserved residues highlighted.

16
CBM simulations predict improved binding by 2 to 140 fold over non glycosylated form.

One O-glycan can alter the CBM binding affinity quite dramatically.

from simulation
Exper. Data from M. Linder et al 1995

3 – Technical Accomplishments/ Progress/Results

2014 - CBM engineering improves binding affinity by a factor of 8

<table>
<thead>
<tr>
<th></th>
<th>Thr-1</th>
<th>Ser-3</th>
<th>Ser-14</th>
<th>T_m (°C)</th>
<th>K_A (μM⁻¹)</th>
<th>Half Life (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>H</td>
<td>H</td>
<td></td>
<td>62</td>
<td>0.095</td>
<td>0.18</td>
</tr>
<tr>
<td>Man</td>
<td>Man</td>
<td>H</td>
<td></td>
<td>72</td>
<td>0.21</td>
<td>3.31</td>
</tr>
<tr>
<td>Man</td>
<td>Man</td>
<td>Man</td>
<td></td>
<td>74</td>
<td>0.75</td>
<td>3.12</td>
</tr>
</tbody>
</table>

- T_m increase of 10-12 °C
- Binding affinity increase of 2 to 8-fold
- Proteolytic half life increase of 17-fold

CBMs made by chemical synthesis (Tan, CU Boulder)

What does the linker do?
What does the linker do when the enzyme is complexed on cellulose?

A: It deforms and binds strongly to the surface!

MD simulations using Kracken

C.M. Payne et al., PNAS, 2013
Experimental binding affinity measurements
Linker imparts significant binding affinity improvements over CBM alone

\[ [B] = \frac{B_{\text{max}} [F]}{K_{\text{ads}}^{-1} + [F]} \]

<table>
<thead>
<tr>
<th>System</th>
<th>( K_{\text{ads}} ) [( \mu M )](^{-1} )</th>
<th>( B_{\text{max}} ) [( \mu mol/g ) cellulose]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBM</td>
<td>0.08 ( \pm ) 0.02</td>
<td>15.4 ( \pm ) 2.9</td>
</tr>
<tr>
<td>CBM-Linker</td>
<td>0.80 ( \pm ) 0.07</td>
<td>7.7 ( \pm ) 0.3</td>
</tr>
</tbody>
</table>
**2 – Approach (Management)**

2015 – Enzyme Engineering and Optimization (EEO)

**Task 1. Enzyme Improvement**

- Use enzyme engineering approaches for improving cellulases based on our significant record of published studies of fungal cellulase (Cel6A, Cel7A, Cel7B, GH61) kinetics and action at the molecular scale.
- Move toward a combination of rational design, random evolution, and gene-shuffling techniques with selective screening of candidate enzymes.

**Task 2. Formulation and Application**

- Screen and develop enzyme cocktails targeted to specific feedstock/pretreatment combinations by combinatorial mixing of single-activity expressing *T. reesei* strains with successful formulations being introduced into a multi-enzyme *T. reesei* strain for pilot testing.
- New and improved enzymes will be mixed to identify synergistic properties in order to minimize protein loading while maximizing activity on selected feedstocks for 2017 targets. Some of this will work will include cellulosomes and multi-domain protein (i.e., CelA) synergy with free fungal enzyme systems; however, expansion of this new enzyme paradigm discovery has been curtailed due to reduced resources.
- Enzyme synergy, feedstock-enzyme matching, high-temp hold, high-solids inhibition relief, and product inhibition relief studies will be conducted at NREL (1 to 500 mL scale), with scale-up by industry or NREL pilot-plant (est. 1 to 100L).
3 – Technical Accomplishments/ Progress/Results

Task 1 2015 EEO Engineering the Key Cellulase - Cel7A

Task 1. Generate >12 *T. reesei* strains expressing phylogenetically diverse or mutant cellulases. (Joint with the BC Process Modeling & Simulation Project)  

<table>
<thead>
<tr>
<th>Task</th>
<th>Description</th>
<th>Due Date</th>
<th>Milestone Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Task 1</td>
<td>Generate &gt;12 <em>T. reesei</em> strains expressing phylogenetically diverse or mutant cellulases. (Joint with the BC Process Modeling &amp; Simulation Project)</td>
<td>6/30/2015</td>
<td>Quarterly Progress Measure (Regular)</td>
</tr>
<tr>
<td>Task 2</td>
<td>Report the predicted partial attainment of a one-quarter reduction in enzyme cost (~$0.13 g/g cellulose loading) based on the combined approaches taken by the project. Share results with enzyme companies and publish. SMART milestone.</td>
<td>9/30/2015</td>
<td>Annual Milestone (Regular)</td>
</tr>
</tbody>
</table>

- Evaluate variants and combine properties
  - Engineer subdomain swaps
  - Engineer mutants with reduced glycosylation
  - Examine natural sources - Other fungi, *Daphnia* spp. and social amoebae
  - Note: Challenges of microheterogeneity - differences in protein glycosylation produced in a single strain

- Correlate structural differences with observed properties

- Requires a robust and native-like expression system
  - Began developing *T. reesei* expression systems in 2013
  - NREL constitutive promoter system demonstrated in 2014
    - This system is now published and shared within the R&D community
We discovered Pfun Cel7A has higher $T^\circ_{\text{opt}}$ /higher specific activity on PCS than *T. reesei*

- Structure determined at NREL
- Differences are found in several regions of the protein (active site and subdomains)
- Amenable to industrial productivity
  - Swapping 12 *P. funiculosom* subdomains into *T. reesei* and vice-versa
  - Testing *P. funiculosom* Cel7A in Cel7A delete strains of *T. reesei*
3 – Technical Accomplishments/ Progress/Results

Overall structure comparison with other key GH7s

Green = TrCel7A (4C4C)
Cyan = PcCel7D (1GPI)
Pale green = TrCel7B

Active site cleft increasingly open

Orange = PfCel7A

Distinctly different loop configuration over tunnel

4C4C ligand shown (cellononaose)
3 – Technical Accomplishments/ Progress/Results

A1 loop - compared to \( Tr\text{Cel7A} \)

‘Top’ loop - compared to \( Tr\text{Cel7A} \)

Product sites (+1/+2) compared

\( Pf\text{Cel7A} \) and \( Pc\text{Cel7D} \) have an ‘exo’ loop

4C4C ligand shown (cellononaose)
Can we capture the design features of the *P. fun* enzymes? *(P. fun subdomains into *T. reesei* Cel7A)*

![Graph showing enzymatic activity](image)
Yes, we can capture the design features of the P. fun enzymes

3 – Technical Accomplishments/ Progress/Results

• Preliminary single-subdomain swaps (P. funiculosum into T. reesei) have given us two swaps that significantly enhance activity of T. reesei Cel7A.

• Follow-up efforts (currently underway)
  – We have to date examined 3 of 12 swaps identified from structure
  – Determine whether combining the two productive swaps results in effects that are additive (or even synergistic).
  – Express and evaluate the analogous “T. reesei to P. funiculosum” swaps.
Does glycosylation affect cellulase performance?

• Post-translational processing leads to differences in protein glycosylation
  – Affects substrate interaction, activity, pH and Topt parameters, protease susceptibility, etc.
  – Can be separated based on charge or hydrophobicity
  – Long HIC or AEX gradients reveal distinct activity subpopulations

• Structural analysis in partnership with UGA-CCRC

• Test with a SDM program targeting N and O-linked glycans
3 – Technical Accomplishments/ Progress/Results

Activity of AEX fractions also suggest less glycosylated species are more active

- AEX is ion exchange but also mixed partition chromatography
  - Three distinct fractions generated
  - Late eluting fraction is most active
  - Late eluting fraction is lowest MW (SDS gels not shown)
- Dilemma: no fraction equals the loading sample
3 – Technical Accomplishments/ Progress/Results
Engineering Cel7A CD glycosylation

Build a Cel7A glycan mutation data base

• N (asparagine) on catalytic domain
  - Point N→A mutations on cat
  - 9 mutants tested to date
• O (serine) on linker and CBM
  - Block O→A on linker
  - Experiments still underway
• Single mutations result in 1.5 fold improvement on Avicel
• N>A mutant don’t appear to be additive
“Other” Cel7A Enzymes

- **Daphnia pulex** - the water flea
  - Collaboration between NREL and U. Portsmouth
  - Genomic GH7 Cellobiohydrolase
    - higher $K_m$, lower $K_{cat}$, lower $T_{opt}$, higher $pH_{opt}$ compared to *T. reesei*
    - No native linker or CBM

- **Social amoebae cellobiohydrolases**
  - Slime molds- *Dictyostelium discoideum* and *D. purpureum*
  - Equivalent activity to *T. reesei* on PCS
  - Reduced sensitivity to end product inhibition
    - Potentially better on high solids
3 – Technical Accomplishments/Progress/Results

*L. quadripunctata* Cel7B surface is highly negatively charged

Surface features may impart benefits to biomass digestion
Challenges with Potential Host Organisms

**E. coli**
- Instability, unglycosylated, difficulty with disulfide bonds, proteolysis

**S. cerevisiae:**
- Productivity and efficient secretion challenging
- Hyperglycosylation
- Lower enzyme stability and activity, folding issues

**Pichia pastoris**
- Productivity
- Hyperglycosylation

**Aspergillus**
- Different glycosylation
- Proteolysis
Critical Tool Development - Building a *T. reesei* expression system

- **Began in *Aspergillus awamori***
  - Industrial analog strain
  - Did not produce “native” protein conformations
    - Wrong glycosylation/N-terminal processing
    - Unstable/susceptible to proteases
- **Moved to *Trichoderma reesei***
  - Several iterations required to get good productivity and correct folding
    - Started with QM6a (base-strain, totally native)
  - Current system is good productivity, constitutive production, correct PTM
    - Expression levels appear to be source dependent
- **Next step is increased production host strain (QM9414)**
3 – Technical Accomplishments/ Progress/Results

**T. reesei as an Expression Host**

**Pros**
- Native producer of a powerful cellulolytic suite
- A proven industrial organism
- High levels of production and secretion
- Native glycosylation patterns

**Cons**
- Lower throughput strain engineering than many microbes
- Cellulase suite is co-induced making individual enzyme purification challenging

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**T. reesei Expression**

**NREL System V 2.0**

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J. Linger, NREL
3 – Technical Accomplishments/ Progress/Results

Constitutive Expression in the Absence of “Contaminating” Cellulases

Catabolite repression serves as a surrogate for a comprehensive cellulase deletion strain.
3 – Technical Accomplishments/ Progress/Results

Purification of pTrEno-Driven Cel7A

HIC1

AEX

HIC2

SEC

Typical purification outcome

Wildtype Cel7A
pTrEno-Cel7A

64 kDa-
51 kDa-
3 – Technical Accomplishments/ Progress/Results

Digestion of Pretreated Corn Stover by Purified *T. reesei* and *P. funiculorum* Cel7As Added Back to Cel7A-Delete *T. reesei* Broth

1.25 fold improvement
2 – Approach (Management)
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Use yeast or filamentous fungi to express multi-domain cellulases (MDCs) that are functional in hydrolyzing cellulose and/or biomass.

MDCs are bacterial in origin and comprise either multiple catalytic and/or binding domains joined together by linker peptides (single gene product) or through a separate "connector" protein (scaffoldin) in cellulosomes.

Current attempts in this area have had very limited success; however, the advantages of these enzymes, including high thermal tolerance, high specific activity, and multi-substrate hydrolysis, make pursuing this work worthwhile.

In FY2015, focus is on *C. bescii* CelA – the most active single cellulase we have encountered.

Optimize cellulase addition strategies – SHF in high temp hold mode
3 – Technical Accomplishments/ Progress/Results

Commercially relevant expression host for *C. bescii* CelA

- Design commercially relevant expression system for multi-domain glycoside hydrolases
- Focus on *C. bescii* CelA initially
  - Cannot be expressed with full activity in *E. coli*
  - Cannot be expressed with full activity in *T. reesei*
  - Developing strategies for *T. reesei* in FY2015-16
3 – Technical Accomplishments/Progress/Results

High temperature pre-digestion of corn stover biomass for improved product yields

Low solids digestion curves

High solids endpoint conversions (5d)

Biomass coming from pretreatment reactors take time or energy to cool.

By utilizing a small loading of thermostable enzymes, 15% of total loading, earlier in the process, we can pre-digest the biomass to enable higher extents of conversion which impacts process economics.

Brunecky et al BfB, 2014

<table>
<thead>
<tr>
<th>MESP ($/gal)</th>
<th>3day</th>
<th>4day</th>
<th>5day</th>
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<tbody>
<tr>
<td>CelA-mix</td>
<td>$2.50</td>
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<td>E1-mix</td>
<td>$2.52</td>
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<tr>
<td>Ctec2</td>
<td>nd</td>
<td>$2.44</td>
<td>$2.30</td>
</tr>
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</table>
• Reported the first activity improvement of Cel7A through enzyme engineering
  – 1.6 fold improvement using ternary systems on biomass (HIC fractions - low glycan forms)
  – 1.5 fold improvement using ternary systems on biomass (N-linked glycan mutants)
  – 1.25 fold improvement using fungal broth on biomass (P fun add back to delete QM6a broth)
• Discovery of the critical nature of CBM glycosylation and binding
  – 2-140 fold improvement in binding for single and double O-linked mutations (computational)
  – 8 fold improved binding for Thr1 oligosaccharide O-linked mutations (experimentally verified)
  – 10 fold improved binding of CBM + linker mutants (experimentally verified)
• Developed a constitutive promoter driven *Trichoderma reesei* expression system to enable enzyme engineering in a native/industrially relevant host.
• Methods for hydrophobic surface area developed and predictions made for lignin binding – not shown.
• Demonstrated utility of a high temperature hold process step using *C. bescii* CelA and *A. cellulolyticus* E1.
4 – Relevance

• *Describe how project accomplishments contribute to*….
  
o  This project *provides fundamental and applied science strategies to enable the process engineering targets* listed by the Multi-Year Program Plan to achieve advanced biofuels.
  
o  This overarching goal: “Enable the production of biofuels nationwide and reduce dependence on oil through the creation of a new domestic bioenergy industry supporting the EISA goal of 36 bgy of renewable transportation fuels by 2022”.

• *Demonstrate how the project considers applications* ….
  
o  Increase confidence (reduce risk) regarding process implementation by demonstrating that the major process unit operations are underpinned by considerable technical “know how”; in some cases even to extending the molecular level.  

> Knowledge is Forever

  
o  We consider that our publications and presentations will be used by industry to design bench and pilot scale improvements to biomass handling, pretreatment, and enzyme saccharification.

• *Your objectives should be clear regarding the relevance of your project* …
  
o  Bt-C. Biomass Recalcitrance: We are working to understand the biomass recalcitrance problem at the range of length scales and chemistries necessary to reduce processing costs.
  
o  Bt-D. Pretreatment Chemistry: Pretreatment is necessary to render biomass more susceptible to hydrolysis by cellulase enzymes and we are working to better define the critical process parameters most likely to reduce costs and increase yield (see slide 24).
  
o  Bt-G. Cellulase Enzyme Loading: Reducing the cost of enzymatic hydrolysis depends on identifying more efficient enzymes and we are working to improve the specific performance of cellulases using rational design strategies, based on informatics and mechanistic models.
4 – Relevance

• **Demonstrate that the successful project will advance the**…..
  
  o Both the near term (2017) and longer term (2022) BETO goals for advanced biofuels require continued reduction in conversion (pretreatment, cellulase production, saccharification)
  
  o New feedstocks, pretreatments, process schemes, and products are likely to require reformulation of enzyme cocktails used in the process
  
  o The knowledge based approach we are taking to improving enzyme cellulase/hemicellulase performance will provide the portfolio of enzymes needed for these goals.

• **Tech transfer/marketability**
  
  o NREL ref. no. 12-28 U.S. patent application 13/941,754 “Enzymes for Improved Biomass Conversion.”
  
  o Material Transfer Agreements (MTA) executed with:
    
    o Novozymes – see following letter (2015)
    
    o DuPont
    
    o Dyadic
    
    o DSM
  
  o Technical Services Agreement (TSA) executed with California Safe Soil
  
  o Negotiating CRADAs with enzyme companies
4 – Relevance

“It is common to distinguish between first generation (1G) biofuels made from feedstock also suitable for human food production and second generation (2G) biofuels made from cellulosic material not useable as a food source.” Energies 2014, 7, 4430-4445; doi:10.3390/en7074430
4 – Relevance

“It is common to distinguish between first generation (1G) biofuels made from feedstock also suitable for human food production and second generation (2G) biofuels made from cellulosic material not useable as a food source.” *Energies* 2014, 7, 4430-4445; doi:10.3390/en7074430
5 – Future Work

• Future work to the end of the project
  – We will stack the successful N>A mutations in Cel7A with the subdomain swaps
  – We will also stack the above mutations with those for O-glycan deletions (available this spring)
  – We will design and generate a targeted gene insertion T. reesei strain for in vivo testing.
  – We will test all successful Cel7A mutations for performance in vivo using both add back and transformed T. reesei strains.
  – Demonstrate high throughput Cel7A screening system using S. cerevisiae host

• Highlight upcoming key milestones

<table>
<thead>
<tr>
<th>Go/No Go Name</th>
<th>Description</th>
<th>Criteria</th>
<th>Date</th>
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<tbody>
<tr>
<td>Determine value of single-enzyme production strains of T. reesei and potential need for additional single enzyme strains</td>
<td>(3/30/2016) Standard Trichoderma cellulase production is induced by a variety of compounds, each of which induces a different set of proteins, some required, some extraneous. By decoupling induction, we can produce single enzyme activities that can be mixed and matched to a given pretreated feedstock. By eliminating unneeded proteins, the total protein loading can be reduced and protein production is more efficient. If these criteria for continuing have not been met, we will multi-enzyme systems and/or new enzyme discovery options.</td>
<td>Three goals must be met in order to continue work: 1) a single enzyme production system with high production (&gt;5 mg/L of heterologous protein) and minimal background protein (&lt;20% of total secreted protein) needs to be established. 2) At least 12 heterologous proteins must have been expressed and assayed on at least 3 biomass feedstocks, and 3) a combinatorial screen capable of screening complex enzyme mixes (up to 5 activity components) must have been established and demonstrated to quantify biomass conversion on a % yield basis for glucan and xylan. Successful establishment of these three goals along with at least one mix demonstrating activity greater than Ctec2 (on a sugar yield/g protein basis) on pretreated biomass.</td>
<td>3/31/2016</td>
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<td>Task 1. Reduction in enzyme cost using improved or new enzymes or synergistic combinations (~$0.10/g cellulose loading). SMART milestone.</td>
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<td>10/1/2017 (Regular)</td>
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<td>Task 2. Report the predicted partial attainment of a one-quarter reduction in enzyme cost (~$0.13 g/g cellulose loading) based on the combined approaches taken by the project. Share results with enzyme companies and publish. SMART milestone.</td>
<td></td>
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Go/No-Go Points in next three years
Summary

Overview
The goal of this project is to ensure that cellulase enzyme loadings can be reduced to the DOE 2017 target of 10 mg/g cellulose using modern enzyme engineering and formulation optimization strategies. Publications, presentations, and patents are stressed to disseminate progress to industry.

Approach
To employ a knowledge based approach which permits linking protein structure to performance. We have pioneered the close coordination of computational analysis and experimental validation for cellulase improvement and achieved positive results.

Technical Accomplishments/Progress/Results
We have successfully demonstrated the ability to model cellulase action, design structural features likely to impact performance, build targeted mutations, and show improved performance on pretreated biomass. We have also demonstrated the utility of the high temperature hold biomass conversion scheme using caldiphilic bacterial cellulases.

Relevance
The attainment of this overall enzyme cost is consistent with BETO’s technoeconomic evaluations for meeting the 2017 biomass conversion target. Given that cost effective biomass conversion to sugars is a common theme for many conversion processes, the overall bioenergy industry also benefits from this work.

Future work
The early stages of selection of glycosylation and P fun subdomain mutations suggested for future work in FY2013 are being accomplished in FY2015. Active site mutations and mutations suggested by newly initiated HTP yeast screening will be accomplished in FY2015-16. Work to express newly discovered, highly active bacterial cellulases in T. reesei will be accomplished in FY2015-17. If Out-Years are funded, the newly discovered LPMOs should be subjected to enzyme engineering studies to improve their native performance under process conditions.
Acknowledgements

• Funding
  • U.S. DOE EERE Office of the Biomass Program
    • HQ: Jonathan Male, Valerie Sarisky-Reed, Leslie Pezzullo, Bryna Guriel
    • NREL LPM and Platform Lead: Adam Bratis and Rick Elander

• NREL Project Members (all between 10 and 50% FTE)
  Markus Alahuhta
  John Baker
  Gregg Beckham
  Yannick Bomble
  Roman Brunecky
  Mike Crowley
  Stephen Decker (Task Lead)
  Bryon Donohoe
  Sarah Hobdey
  Brandon Knott (PD)
  Jeff Linger
  Vladimir Lunin
  Kara Podkaminer (PD)
  Mike Resch
  Logan Schuster (student)
  Larry Taylor II
  Todd Vanderwall
  Todd Vinzant
  Qi Xu
  John Yarbrough

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<tr>
<td>J Phys Chem B</td>
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<td>2</td>
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Additional Slides
Responses to Previous Reviewers’ Comments

• **2013 Peer Review Report – Reviewers comments**
  
  – “Extensive amount of research going on in this project. It seems like there are numerous tasks and findings all going on simultaneously. The cycle back and forth between enzyme development and pretreatment is important. Several publications generated from this research to date are significant.”
  
  – “The inclusion of basic, fundamental research in the portfolio is a good investment in the long term viability of BETO’s mission. The output of this work may not be immediately viable for commercialization, but the knowledge that is created may prove to be invaluable down the road. The government is uniquely positioned to fund this type of work and it should be maintained.”
  
  – “This is very fundamental research to get a better understanding of what’s happening at the molecular level, which will result in improved enzyme and decreased costs.”
  
  – This is a very impressive body of work demonstrating how fundamental knowledge may benefit applied project goals. This reviewer just wished there had been more highlights demonstrating technology impact.” Me too (MEH)
  
  – This is not a single unified project, but rather an evolving group of projects. It has a strong track record and will certainly continue to be productive. The advanced biomass deconstruction aspect is the strongest. The structure component (both x ray and bioinformatics) of the structure, simulation and theory aspect is strong, but this reviewer has reservations about the practical knowledge arising from application of simulation and theory to these complicated systems. The direct microbial sugar conversion aspect will be very challenging, but it is important to gain this fundamental knowledge despite the extreme technical difficulty.” Several important points introduced by this reviewer require comment. We also agreed that this Project was becoming too large, so the Simulation and Theory and Biochemical Transformations tasks became new Projects in FY2014 (MEH).
  
  – M. Himmel: As one can see from the diverse comments above, the applied fundamental (and especially Theory) aspects of this work were both heralded and questioned in terms of near term value to BETO. However, we believe that a well planned near term technology program should have some element of longer term R&D in its portfolio - all reviewers seem to agree to this point.

• **Also provide highlights from any Go/No-Go Reviews.** None
Publications, Patents, Presentations, Awards, and Commercialization

• Publications:
  – 20 publications from 2013 with impact factors above 6.0

• Patents
  – 2014 NREL ROI: “In planta enzymes for reduced recalcitrance traits.”
  – 2014 NREL ROI: “Enzymatic pre-conditioning for improved sugar release from biomass”

• Technology transfer or commercialization efforts
  – CRADA with Genencor-DuPont: completed in 2013
  – CRADA with DSM: ongoing
  – CRADA with Novozymes: under negotiation

• Awards
  – 2014 Colorado State University, Department of Biochemistry & Molecular Biology, Distinguished Alumnus
  – 2013 Battelle Memorial Institute Inventor of the Year

- “Glycoside Hydrolase Processivity is Directly Related to Oligosaccharide Binding Free Energy” Payne, Christina; Jiang, Wei; Shirts, Michael; Himmel, Michael; Crowley, Michael; Beckham, Gregg. *JACS*, 135(50), 18831-18839 (2013).
ABD and EEO*: 2013-2015 Publications

• “ Revealing Nature's Cellulase Diversity: The hyperactive CelA from *Caldicellulosiruptor bescii,*” Roman Brunecky, Markus Alahuhta, Qi Xu, Bryon S. Donohoe, Michael F. Crowley, Irina A. Kataeva, Sung-Jae Yang, Michael W. W. Adams, Vladimir V. Lunin, Michael E. Himmel, and Yannick J. Bomble, *Science*, 342(6165), 1513-6 (2013). *Attributed to BESC funding but key to on going work for BETO.


- “Clean Fractionation Pretreatment Reduces Enzyme Loadings for Biomass Saccharification and Reveals the Mechanism of Free and Cellulosomal Enzyme Synergy,” Resch, Michael; Donohoe, Bryon; Ciesielski, Peter; Nill, Jennifer; Magnusson, Lauren; Himmel, Michael; Mittal, Ashutosh; Katahira, Rui; Biddy, Mary; Beckham, Gregg, *ACS Sustainable Chemistry & Engineering*, (2014) In Press.


• “Investigating The Role of Lignin on Biphasic Xylan Hydrolysis During Dilute Acid and Organosolv Pretreatment of Corn Stover,” Ashutosh Mittal, Todd B. Vinzant, Roman Brunecky, Stuart K. Black, Michael E. Himmel and David K. Johnson, Green Chemistry (2014), In Press.


Additional accomplishments
3 – Technical Accomplishments/ Progress/Results

Our hypothesized action of a processive enzyme on cellulose

See BC Process Modeling and Simulation (Crowley)
3 – Technical Accomplishments/ Progress/Results

Task 1 2015 EEO Yeast Expression of GH7 Cellobiohydrolase

| Task 1. Develop a strain of *S. cerevisiae* or *Yarrowia* spp. for HTP cellulase expression and optimization | 3/30/2015 | Quarterly Progress Measure |
| Task 1. Test 5000 rCel7A expressing yeast colonies using the new HTP yeast screening method for enhanced activity or biochemical properties. Select top 10-20 strains for further analysis. | 9/30/2015 | Annual Milestone (Regular) |

• Advantages of yeast expression of GH7s
  – Powerful expression tools
  – HTP screening capable, allow for random evolution and screening of Cel7A.
• Disadvantages
  – Poor processing of TrCel7A, low yields
  – No growth on cellobiose- *Saccharomyces*.
• Overcoming barriers
  – *Talaromyces* and *Humicola* GH7s express better than *Trichoderma*.
  – +β-G grow on cellobiose, +EG to grow on cellulose.
  – Add in glutaminyl cyclase to allow correct processing.
• Progress
  – B-G is expressing → growth on cellobiose
    • Limited growth on cellulose but better than native
    • We have expressed several GH7s in *Saccharomyces* and *Yarrowia*; however, titers are very low.
  – Glutaminyl cyclase gene identified and cloned.

![Table 1](test# 1 2 3 4 5 6 7 8 9
carbon source | D | - | CB | GA | CB | CB | GA | D | GA | -
enzyme | bG | bG | bG | bG | bG
result | + | - | - | - | + | - | - | + | -

D- Dextrose, CB = cellobiose, GA = gluconic acid, bG = beta-glucosidase expressed in yeast

**Conclusions:**

• *S. cerevisiae* cannot grow on cellobiose (col. 3)
• bG expression allows growth on cellobiose (col. 5)
• GA inhibits bG activity on cellobiose (col. 6)
• GA is not a suitable carbon source for growth (col. 7)
Conclusions

• Glycosyl hydrolases are adsorbing to lignin under process conditions
• Temperature, pH and ionic conditions influence this phenomena
• The predominate enzymes affected are β-glycosidase and xylanases
• The bulk of β-glycosidase activity remains functional when bound to lignin
• Mechanisms of enzyme adsorption to lignin appear to be hydrophobic and electrostatic in nature
<table>
<thead>
<tr>
<th>Qtr</th>
<th>Due Date</th>
<th>Type</th>
<th>Milestones, Deliverables, or Go/No-Go Decision</th>
<th>Decision Criteria</th>
</tr>
</thead>
</table>
| Q1  | 12/31/2013 | Regular | **Structural Design Mutations for Improving Cel7 Cellulases**  
  Design phase of mutations to improve cel7 cellulase. Use homology and molecular mechanics modeling to generate in silico mutations that are predicted to alter one or more biophysical properties of Cel7A from T. reesei, P. funiculosom, or other GH7 exocellulase. Twelve or more variants will be designed to improve processivity, kon/koff parameters, lignin binding, and/or temperature stability. |                   |
| Q2  | 3/31/2014  | Regular | **High Throughput Mutation and Functional Expression Screening for Cellulase Improvement**  
  Success is to develop new transformation/expression methodology in yeast to functionally screen tens of thousands of random mutations in cel7A from Talaromyces emersonii, T. reesei, or other fungal source. The critical development is the screening methodology whereby non-active or low-activity variants are not propagated and variants with higher-than-baseline activity can be differentiated and ranked by activity. The screen must be stringent enough to reject the lowest 90%+ of the variants based on functional expression. It is expected that the host yeast will need to be engineered to functionally express Cel7 enzymes. The addition or deletion of post-translational modification genes such as glutaminyl cyclase, glycosyl transferases, chaperonins, or disulfide bond facilitators may be required for host yeast development. |                   |
| Q3  | 6/30/2014  | Stretch | **Demonstrate Functional Expression of Multi-component Cellulases in Fungal Host**  
  Use yeast or filamentous fungi (T. reesei or A. awamori) to express multi-domain cellulases (MDCs) that are functional in hydrolyzing cellulose and/or biomass. At high level, functionality is defined by performance similar to the enzyme from its native host. MDCs are bacterial in origin and comprise either multiple catalytic and/or binding domains joined together by linker peptides (single gene product) or through a separate "connector" protein (scaffoldin) in cellulosomes. We will examine at least two examples. In this case, functionality of the expressed cellulase is determined by hydrolysis of cellulose and/or cellulosic biomass (depends on which catalytic domains are present) at levels that are meaningful, i.e. >60% conversion at 120 h with protein loadings below 50 mg protein/g substrate. Current attempts in this area have had very limited success; however the advantages of these enzymes, including high thermostolerance, high specific activity, and multi-substrate hydrolysis, make pursuing this work worthwhile. |                   |
| Q4  | 9/30/2014  | Regular | **Improved Cellulases for Biomass Conversion**  
  Cellulase improvement is incremental, as we continue to explore and engineer diverse enzyme systems. Random and rational-design Cel7A engineering, HTP functional screening in yeast, and multi-component cellulase expression all hold promise for improved activity, defined here as a meaningful increase in specific activity (>10%), thermostolerance (>50 C), decreased protein loading requirements (10% or more less protein/g substrate for same conversion level), increased initial rate (>10% reduction in time-to-target of 80% conversion), increased extent of conversion (>2% increase in conversion yield of glucose or xylose), or increased activity by engineering decreased non-specific lignin binding. |                   |
## Detailed Milestone chart for 2015

<table>
<thead>
<tr>
<th>Milestone Name/Description</th>
<th>End Date</th>
<th>Type</th>
</tr>
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<tbody>
<tr>
<td>Task 1. Develop a strain of <em>S. cerevisiae</em> or <em>Yarrowia</em> spp. for HTP cellulase expression and optimization</td>
<td>3/30/2015</td>
<td>Quarterly Progress Measure (Regular)</td>
</tr>
<tr>
<td>Task 1. Generate &gt;12 <em>T. reesei</em> strains expressing phylogenetically diverse or mutant cellulases.</td>
<td>6/30/2015</td>
<td>Quarterly Progress Measure (Regular)</td>
</tr>
<tr>
<td>Task 1. Test 5000 rCel7A expressing yeast colonies using the new HTP yeast screening method for enhanced activity or biochemical properties. Select top 10-20 strains for further analysis.</td>
<td>9/30/2015</td>
<td>Annual Milestone (Regular)</td>
</tr>
<tr>
<td>Task 1. Reduction in enzyme cost using improved or new enzymes or synergistic combinations (~$0.10 /g cellulose loading). SMART milestone.</td>
<td>10/1/2017</td>
<td>Annual Milestone (Regular)</td>
</tr>
<tr>
<td>Task 2. Assemble library of pretreated substrates. Evaluate with base fungal cocktail to identify needed activities and publish the results.</td>
<td>12/30/2014</td>
<td>Quarterly Progress Measure (Regular)</td>
</tr>
<tr>
<td>Task 2. Report analysis of novel enzyme addition schemes to reduce protein loading.</td>
<td>6/30/2015</td>
<td>Quarterly Progress Measure (Regular)</td>
</tr>
<tr>
<td>Task 2. Report the predicted partial attainment of a one-quarter reduction in enzyme cost (~$0.13 g/g cellulose loading) based on the combined approaches taken by the project. Share results with enzyme companies and publish. SMART milestone.</td>
<td>9/30/2015</td>
<td>Annual Milestone (Regular)</td>
</tr>
<tr>
<td>Task 2. Report improved high-solids digestion activity of a) 25% increase in rate of hydrolysis with 20% solids, b) 10% increase in final yield of sugar at 20% solids, or c) maintain rate and yield of 1% solids with 10% or higher solids loadings. SMART milestone.</td>
<td>9/30/2016</td>
<td>Annual Milestone (Regular)</td>
</tr>
<tr>
<td>Task 1. Publish new HTP yeast screening method and share results with industrial collaborators.</td>
<td>12/30/2015</td>
<td>Quarterly Progress Measure (Regular)</td>
</tr>
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4 – Relevance

2014 NREL’s Accomplishments Attracting Attention from Biofuels Companies

R&D100 Committee
To Whom It May Concern,

I recently read with great interest the article in Science (Fimbercy et al. 2013) regarding NREL’s characterization of Cel7A from the fungus Colletotrichum lagenarium. This enzyme was engineered for use in biofuels production, and it has several desirable characteristics: high activity, stability at high temperatures, and ability to degrade cellulose. The development of such enzymes is crucial for the biofuels industry, as they can help in the efficient conversion of biomass into biofuels.

For these reasons, I am interested in staying current with NREL’s Cel7A research and would like to support their application for the R&D100 Award based on this technology.

Best regards,

[Signature]

ABENGOA RESEARCH

Seville, Spain 3-10-2014

R&D100 Committee
To Whom It May Concern,

We read with great interest the recent article in Science (Fimbercy et al., 2013) regarding NREL’s discovery of the very high performance of the new biofuel cellulase, Cel7A from Colletotrichum lagenarium. Because Cel7A appears to work synergistically with other, more traditional cellulases, this enzyme looks attractive for formulating systems designed for biofuel production. Several desirable characteristics of Cel7A are: 1) very high specific activity, 2) stability at high temperatures, and 3) a novel digestion mechanism. Furthermore, NREL’s transmission electron microscopy studies suggest that Cel7A is capable of not only the common surface abrasion mechanism to break down cellulose, but also of extruding extensive cavities down into the surface of the plant cell wall. We also note that during digestion experiments, Cel7A achieved 60% conversion of cellulose, a component of plant materials, demonstrating its potential for industrial processes that use mild or no pretreatment.

For these reasons, we are interested in staying current with NREL’s Cel7A research and fully support their application for an R&D100 Award based on this technology.

Yours sincerely,

[Signature]

ABENGOA RESEARCH

Juan L. Ramos
Head of the Biotechnology Unit of Abengoa
Testimony from Novozymes in 2013

Examples illustrating how TCR task’s fundamental discoveries impact commercial enzyme development

Key TCR Discoveries

“Accessory components” might be limiting in T. reesei secretome:
- Extensive work published 2008-2009 indicated that overall catalytic performance of T. reesei cocktails could be improved by addition of non-cellulolytic components
- These findings inspired further work among enzyme development companies, including Novozymes

2-D gel fingerprinting - powerful enzyme discovery tool:
- Very early work on 2-D fingerprinting of T. reesei proteins was influential in the development of Novozymes’ secretome analysis capabilities
- Use of the tool ultimately led to a number of important discoveries including the abundance and diversity of GH61 proteins in Theliavia terrestris.

Glycosylation is important factor impacting the specific activity of heterologous cellulases
- Increased awareness of role of glycosylation, and impacted selection of expression hosts in discovery pipeline

Relevant TCR Publications

Accessory components:


2-D gel fingerprinting:


Glycosylation:
The goal of this project is to ensure that cellulase enzyme loadings can be reduced to the DOE 2017 target of 10 mg/g cellulose using modern enzyme engineering and cocktail formulation optimization strategies. Given that cost effective biomass conversion to sugars is a common theme for many conversion processes, the overall bioenergy industry also benefits from this work. We are using a knowledge based approach, which links protein structure to performance. We have pioneered the close coordination of computational analysis and experimental validation for cellulase improvement and have achieved positive results (1.25 to 1.6 fold improvement in Cel7A performance). We have successfully demonstrated the ability to model cellulase action, design structural features likely to impact performance, build targeted mutations, and show improved performance on pretreated biomass. We have also demonstrated the utility of the high temperature hold biomass conversion scheme using cocktails of caldiphilic bacterial cellulases. The early stages of selection of glycosylation and *P. funiculosum* subdomain mutations in *T. reesei* Cel7A suggested for future work in FY2013 are being accomplished in FY2015, with successful outcomes. Active site mutations and mutations suggested by newly initiated HTP yeast screening will be accomplished in FY2015-16. Work to express newly discovered, highly active bacterial cellulases in *T. reesei* will be accomplished in FY2015-17.