2013 DOE Bioenergy Technologies Office (BETO) Project Peer Review

Targeted Conversion Research

Date: 5/20-5/24 2013

Technology Area Review: Biochemical Conversion

Principal Investigator: Michael E. Himmel
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 (2011-2013)

Project Objectives

- Provide key technology fundamentals regarding enzyme saccharification required to meet the 2012 xylan & glucan conversion performance goals.
- Provide key technology fundamentals regarding biomass pretreatment required to meet the 2012 xylan conversion performance goals (xylan to xylose and xylose to furfural).
- Provide key technology fundamentals regarding biochemical conversion (sugar production) processes to meet the DOE's 2017 advanced fuels goals.
- Provide key technology fundamentals regarding development of new consolidated microbial bioprocesses to meet the DOE's 2017 advanced fuels goals.

Relevance of project to BETO

- The near term correlative development of more active cellulases, improved pretreatment processes, and efficient sugar conversion to products ensures attainment of DOE's 2012 biofuels goals.
- Lay the foundations for science knowledge needed by industry to reduce risk and meet new targets for DOE's 2017 goals for advanced biofuels.
- To enable the dramatically new technologies providing 36 billion gallons of renewable fuels by 2022 (EISA), considerable improvement in understanding of biomass recalcitrance must be achieved.

From DOE MYPP:

- Through RDD&D, make cellulosic biofuels competitive with petroleum-based fuels at a modeled cost of mature technology of $3/gallon gasoline equivalent ($2011), based on EIA projected wholesale prices in 2017.
- Help create an environment conducive to maximizing the sustainable production and use of biofuels by 2022.

Quad Chart Overview

Timeline

- Project start date = 2001
- Project end date = 2017
- Percent complete = 70%

Barriers

2012 MYPP Barriers addressed

- Bt.C Biomass Recalcitrance
- Bt.D/E Pretreatment Chemistry/Cost
- Bt.G Cellulase Enzyme Loadings
- Bt.J Catalyst Development

Budget

Funding for FY11: $6,500K / 0

(DOE / Cost share) $700 K subcontracts

Funding for FY12: $5,000K / 0

(DOE / Cost share) $200 K subcontracts

Funding for FY13: $4,750K / 0

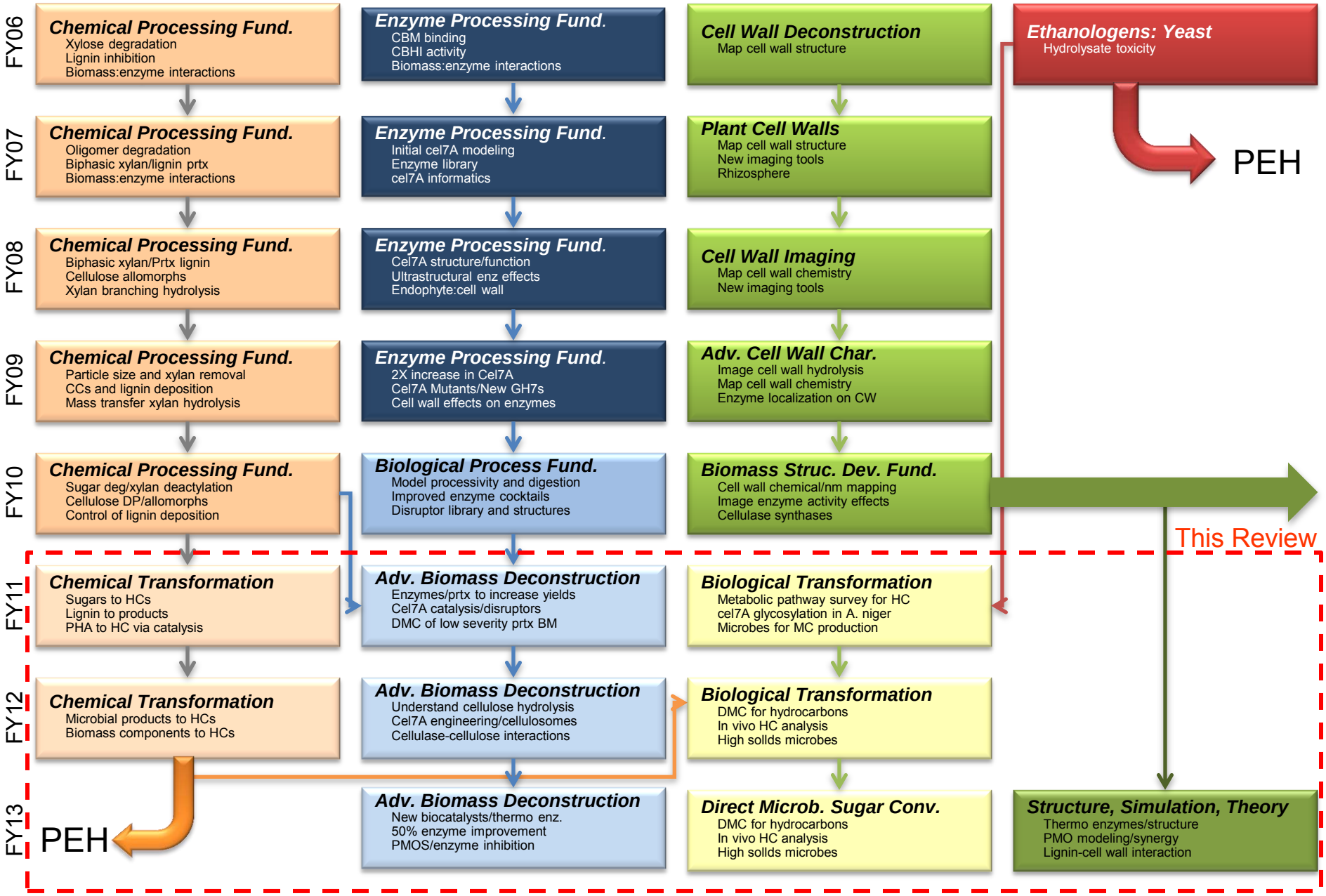
(DOE / Cost share) $200 K subcontracts

Funded since 2001 with an estimated average annual funding of $4,400K

Partners

- **CRADAs**: Genencor/Danisco, Edenspace
- **Subcontracts**: Colorado State University, NIST, Cornell University, Vanderbilt University, Weizmann Institute of Science, UC Berkeley
- **Collaborations:** Rajai Atalla (FPL), Simo Sarkanen (UMin), Baron Peters (UCSB), Norm Lewis (WSU), Charles Brooks III (Univ of Michigan), Lee Makowski (ANL), Paul Langan (LANL), Sunney Xie (Harvard), Lee Woodcock (Univ So. Florida), Will York (CCRC), Alex MacKerell (Univ Maryland), Scott Baker (PNNL), Adrian Tsang (Concordia Univ), Bruce Dale (MSU), Simon Cragg (Portsmouth Univ, UK); Simon McQueen Mason (York Univ, UK)

Evolution of TCR Subtasks



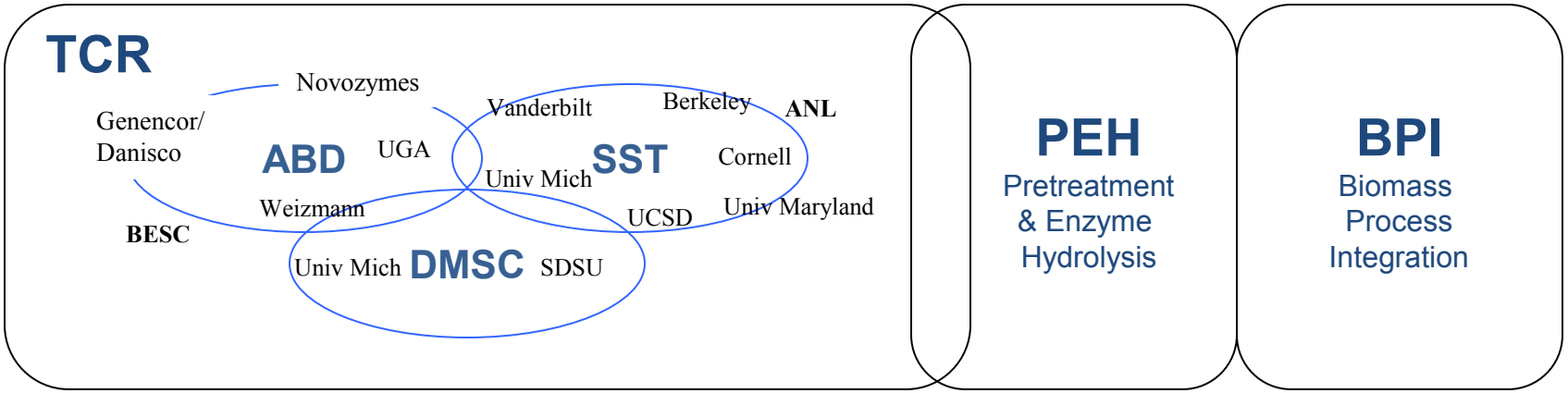
Approach

Detailed description of TCR tasks for 2013

- **Structure, Simulation, and Theory (SST).** To enhance our understanding of the mechanisms of biomass-degrading enzymes at the molecular level in order to improve their functionality. Maintain a strong interface with the experimental efforts conducted in ABD which tests these concepts. Tools such as computer modeling, thermodynamic theory, x-ray crystallography, and bioinformatics will be used to achieve this goal. ***Mike Crowley***

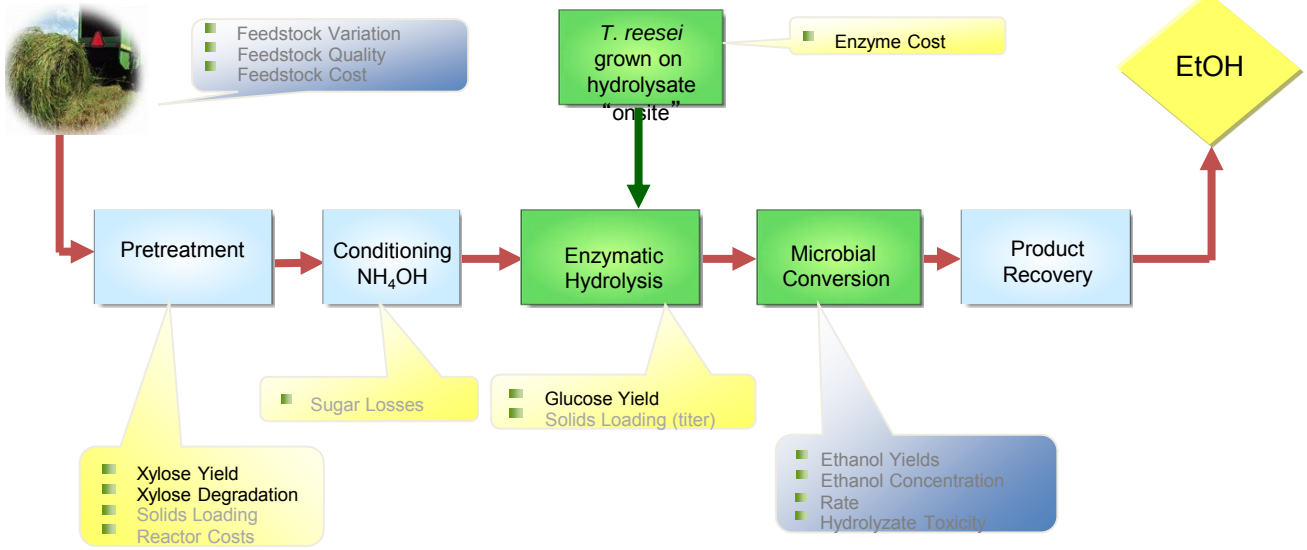
- **Advanced Biomass Deconstruction (ABD).** To improve key biomass degrading enzymes in order to enable a reduction in pretreatment severity; as well as maximizing the glucose and xylose yields after saccharification. A key technical barrier to commercializing fuels and chemicals from biomass by the sugar platform route is the high cost and relative inefficiency of producing fermentable sugars from lignocellulosic biomass. This is critical to the generation of cheap and acceptable sugar streams regardless of whether the downstream process produces ethanol, hydrocarbons, or chemicals. ***Steve Decker***

- **Direct Microbial Sugar Conversion (DMSC).** To engineer new host strains that are excellent biomass degraders and have high potential hydrocarbon-producing capabilities. Our work will support industry's interest in developing microorganisms that produce drop-in fuels or high-energy intermediates using a direct microbial conversion platform. The goal is to obtain and share fundamental knowledge regarding the challenging metabolic and enzymatic barriers to enabling this technology. ***Min Zhang***

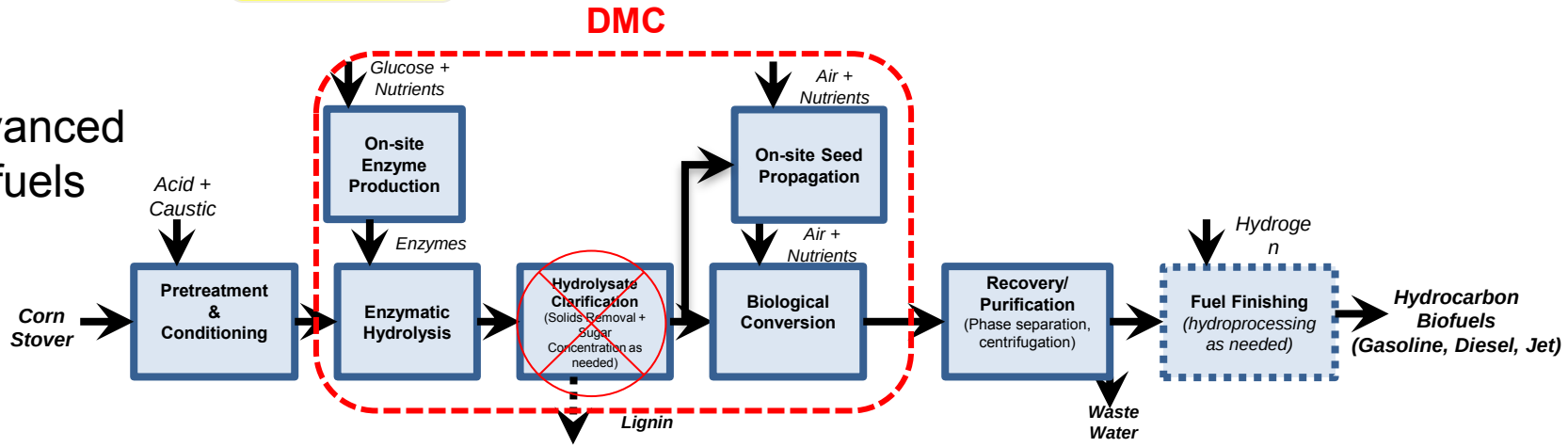


TCR Overview: SOT Cases

Bioethanol



Advanced Biofuels



ABD Subtask Overview – S. Decker

Rationale

- A key technical challenge to commercializing fuels and chemicals from biomass by the sugar platform route is the high cost and relative inefficiency of producing fermentable sugars from lignocellulosic biomass.
- This focus drives towards maximizing cellulose conversion extent, efficiency, and yield; thus reducing the overall cost of sugars from biomass.

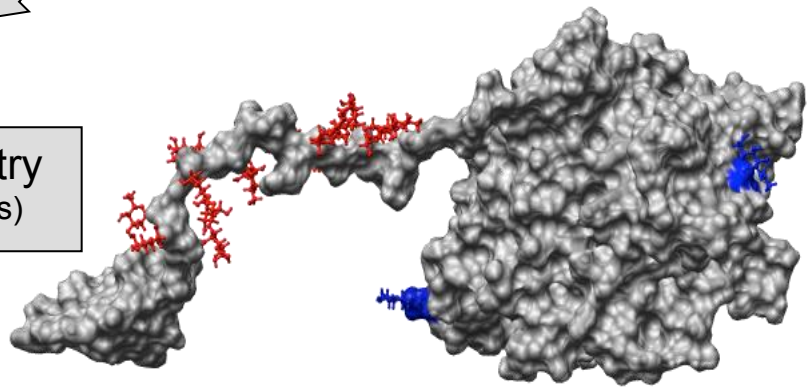
Outcome and Relevance

- Production of Cellulosic Sugars and Carbohydrate Derivatives- Pretreatment and Enzymatic Saccharification. Barrier Area 3: Enzyme Science & Biotechnology
  - The mechanistic basis underlying the action of most hydrolytic enzymes are still largely misunderstood
  - Poor categorization and understanding of the natural diversity in hydrolytic enzymes
  - The advantages and disadvantages of using lignolytic enzyme systems have received relatively little R&D focus
  - The specific activity of hydrolytic enzymes are typically low
  - End products (sugars and degradation products) inhibit enzymes, preventing high sugar concentrations
  - Lignin derived species hinder enzyme efficiencies
  - Cellulase enzyme loading with high solids (>20% w/w) is cost prohibitive

Key Cellulases: Make Them More Effective

Molecular Structure
(experimental parameters)

X-ray crystallography
Structure diversity (genomics)
Homology modeling



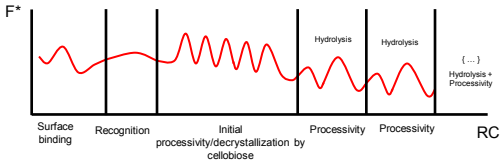
Physical Biochemistry
(experimental parameters)

Protein purification
Physical chemical analyses
MS and spectro. analyses
Special and HTP activity testing

Numerical Models
(subsets to entire system)

Molecular dynamics
QM/MM
Multi-scale modeling
Code development
Force fields
Supercomputers

Mechanistic Model
(kinetic and thermodynamic)



Not All Cel7As Are Created Equal

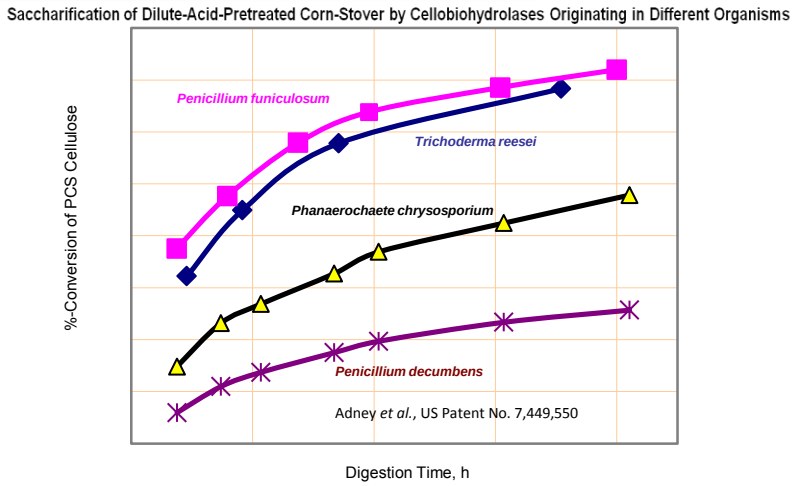
Relevance: Increase enzyme activity to decrease loading and cost

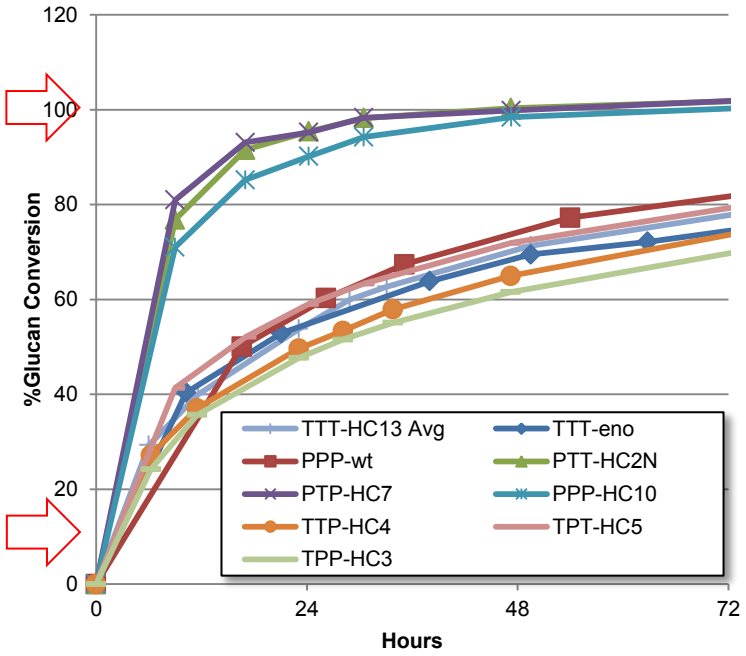
Rationale

- Fungal Cel7A enzymes vary in activity and properties
- Can we design better Cel7A enzymes than Nature?
  - ➢ Combine best traits from various versions

Outcome

- Combining modeling with experimental validation
- Learning from natural enzyme diversity
  - ➢ GH7 family diversity, fungi, protists, arthropods
- Cel7A is most critical cellulase enzyme; mechanism is not known.
  - ➢ What is the biochemical relationship between structure and function?
  - ➢ What are the experimental strategies for improving Cel7A?
- Genetic engineering of Cel7A
  - ➢ Domain swapping (chimeras), site-directed, loop engineering
  - ➢ *Penicillium funiculosum* Cel7A is superior to *T. reesei*
    - Becomes even better when expressed in *T. reesei*
    - Catalytic domain of *P. funiculosum* appears to be critical part





Improving the Catalytic Domain (CD)

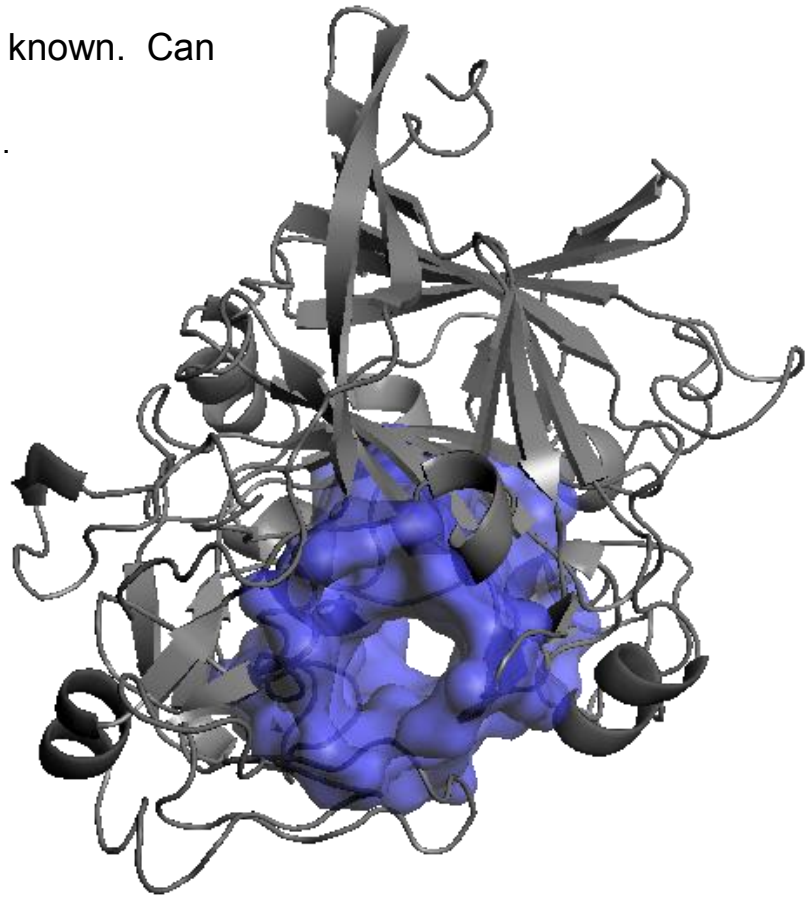
Relevance: D Milestone: CBH I with 2-3x activity of *T. reesei* CBH I

Rationale

- The processive mechanism for the CD of Cel7A is not known. Can it be improved?
  - Considerable natural diversity is a key to the mechanism.

Outcome

- Probe binding tunnel for Cel7s to determine which residues participate in cellulose and oligomer degradation
- Models of the active site tunnels of key CDs have revealed differences in surface area, volume, and architecture.
- Experimental approach
  - Computational: determine quantitative processivity mechanism for cellodextrin chain
    - Demonstrate which residues play a significant role in processivity
  - Experimental: mutate tunnel binding residues (W > A) and measure digestion on crystalline/amorphous/soluble substrates



Trichoderma reesei Enzyme Expression

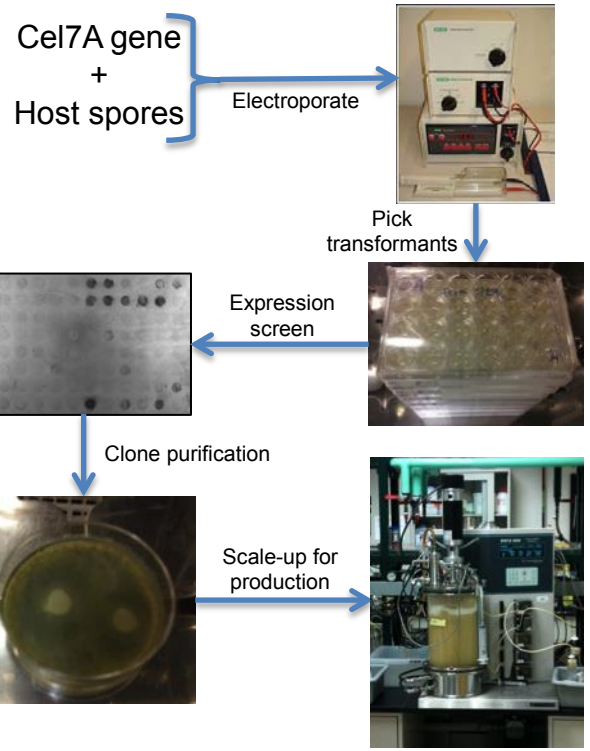
Relevance: "Industrial" production host to evaluate real effects of enzyme engineering

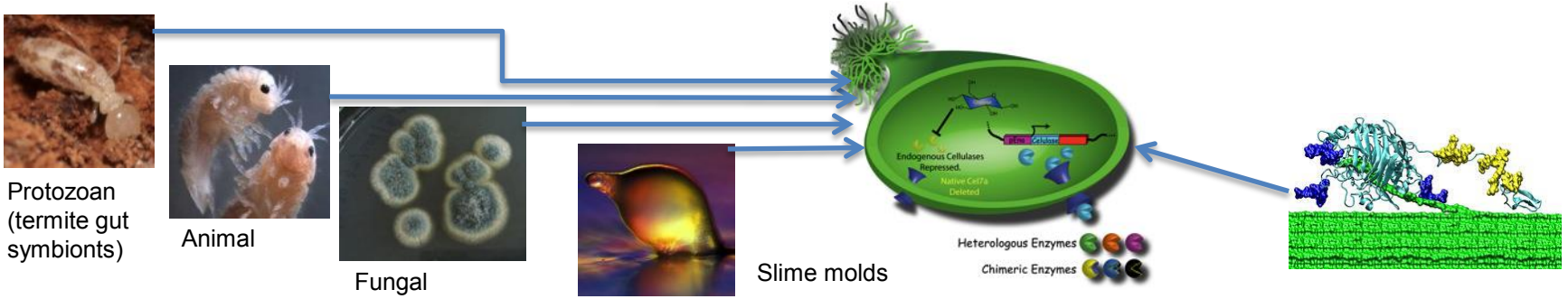
Rationale

- Cel7A (primary fungal cellulase) is susceptible to many post-translational modifications and translation issues
  - Expression host dependent
  - Makes comparison of engineered variants very difficult

Outcome

- *T. reesei* is the dominant cellulase producer world-wide
  - **Expression in yeast, *Aspergillus*, or bacteria results in non-native properties of fungal enzymes**
  - Necessitated the development of a new *T. reesei* expression system for *cel7a*
    - ✓ Generate *cel7a* delete strain w/ cellulase system repression
    - ✓ Developed new vectors, transformation, and selection protocols
    - ✓ Resulted in high transformation efficiency and rapid selection for high-producing strains with native-like Cel7A properties
- System is applicable to *cel7a* genes from other organisms





Cellulase/Cellulosome Synergy*

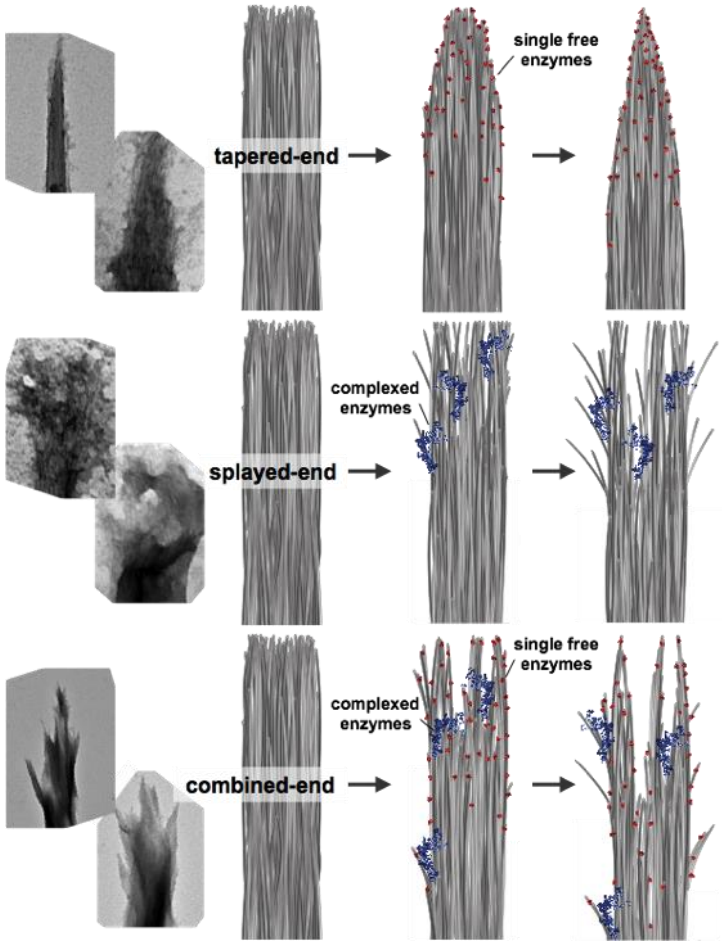
Relevance: Understand enzyme function to increase activity and decrease enzyme cost

Rationale

- Cellulases and complexed cellulosomes are two very different systems evolved to address the same problem
  - Efficacy of each system is dependent on substrate and varies by pretreatment and feedstock

Outcome

- Free enzymes are more active on pretreated biomass, cellulosomes are more active on purified cellulose.
  - When combined, these systems display dramatic synergistic enzyme activity on cellulose, but not biomass
- High-resolution TEM offers insights into the mechanisms of synergistic deconstruction.
  - TEM indicates different mechanisms of cellulose deconstruction by free enzymes and cellulosomes.
    - Free enzymes employ an ablative, fibril-sharpening mechanism
    - Cellulosomes physically separate individual cellulose microfibrils from larger particles, enhancing access to cellulose surfaces.
- Insight into the mechanisms underlying these two different cell wall deconstruction paradigms will enable new strategies for enzyme engineering to overcome biomass recalcitrance.



Resch MG, Donohoe BS, Baker JO, Decker SR, Bayer EA, Beckham GT, Himmel ME. Fungal cellulases and complexed cellulosomal enzymes exhibit synergistic mechanisms in cellulose deconstruction. Energy & Environmental Science (2013). doi:10.1039/c3ee00019b

*BESC Collaboration

High-Temperature Hold Hydrolysis

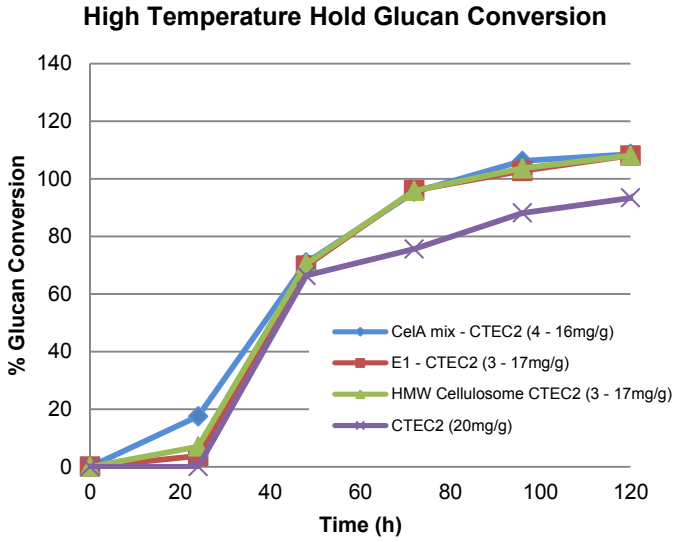
Relevance: Decrease pretreatment cost/severity, increase yield, decrease enzyme cost

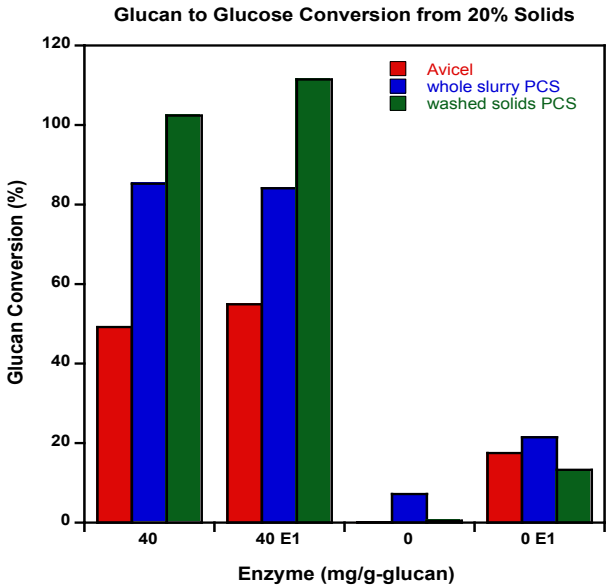
Rationale

- Take advantage of thermal energy in pretreatment slurries thermotolerant enzymes
  - Faster reaction kinetics
  - Pre-hydrolyze biomass/oligomers for mesophilic hydrolysis

Outcome

- Thermophilic enzymes are inherently faster than mesophilic, however highly thermostable Cel7s do not exist
- Heat from pretreatment can be utilized in part by pre-hydrolyzing the slurries while at elevated temperature
  - SHF/SSF at reduced temps for cel7/microbes
  - Total protein loadings (thermal hold + SHF/SSF) remain constant
- Currently evaluating *A. cellulolyticus* E1cd, *Cl. thermocellum* cellulosomes, and *Ca. bescii* CelA
  - In the context of CTec2 SHF
  - Collaborative work with BESC
  - ~10-15% improvement over CTEC2 alone
- High solids digestions with E1cd show improvements on washed solids





Mechanical Disruption Effects Pretreatment

Relevance: Reduce ptrx severity to decrease cost and degradation products

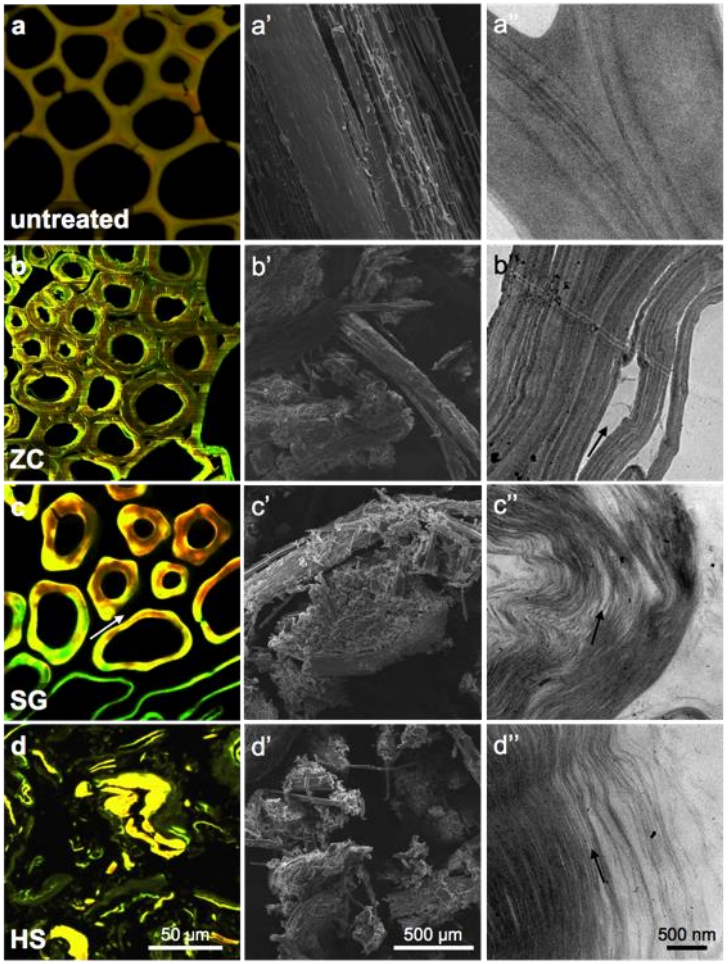
Rationale

- Cell wall deconstruction is highly dependent on accessibility of catalysts (chemical and enzymatic) to their substrates
  - Physical disruption of cell wall ultrastructure allows faster and more complete access, resulting in lower pretreatment severity

Outcome

- This mechano-physical energy helps disrupt cross-linked matrices and results in a high degree of cell wall nanofibrillation and explains the increased xylan removal
  - ➢ Increased dislocation, surface roughness, delamination, and nanofibrillation revealed by direct observation of the micro- and nanoscale change in accessibility explains the superior performance of the Steam Gun (SG) and Horizontal (HS) reactors compared to the Zipperclave (ZC)
- Reactor designs that augment pretreatment with physical energy better overcome biomass recalcitrance

reactors	composition of pretreated (2% H_2SO_4, 160°C, 5 min) samples			% glucan released
	% glucan	% xylan	% lignin	
ZC	54.0	8.8	24.2	68.7
SG	60.2	4.8	25.0	88.0
HS	57.4	3.2	24.8	95.2



Ciesielski PN, Wang W, Chen X, Vinzant TB, Tucker MP, Johnson DK, Decker SR, Himmel ME, and Donohoe BS. Effect of Mechanical Disruption on the Effectiveness of Three Reactors Used for Dilute Acid Pretreatment of Corn Stover Part 2: Morphological and structural substrate analysis. Submitted.

GH61-Polysaccharide MonoOxygenase

Relevance: Decrease enzyme loading/cost by using new activities

Rationale

- In 2010, a new class of cellulose-degrading enzymes was "discovered"
  - Oxidative, not hydrolytic, synergistic with cellulases
  - Possibly decreased yields due to oxidized products

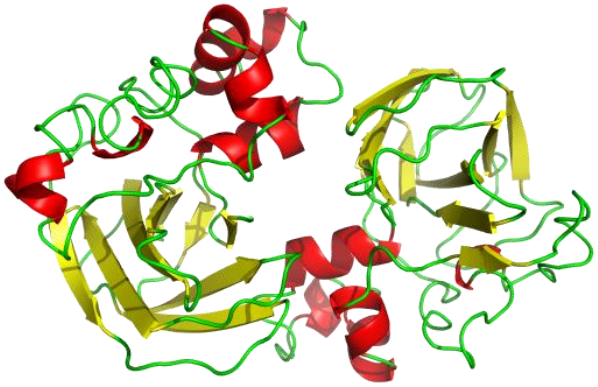
Outcome

- GH61s are Polysaccharide Mono-Oxygenases (PMOs)
- Reported to be formulated into "new" biomass-targeted commercial cellulases

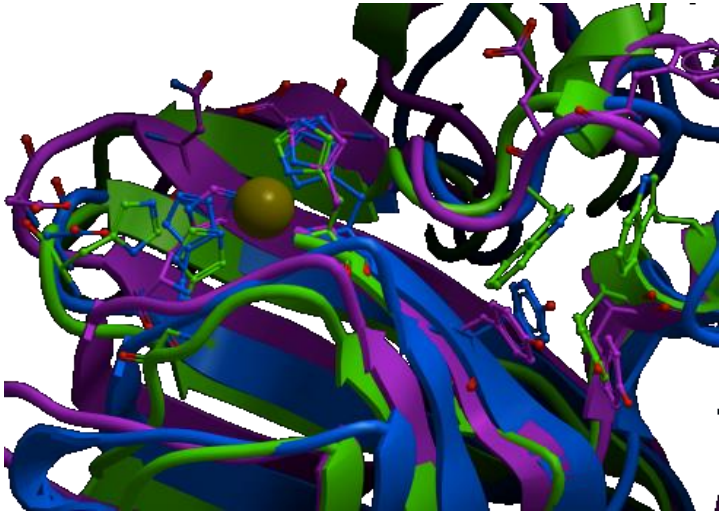
✓ Solved the structure of E7, a bacterial PMO from *Thermobifida fusca*
  - Thermophile an be used in high temperature enzyme cocktails
  - First bacterial PMO structure to show Cu-binding in active site
  - Maps to other GH61 structures

✓ Modeling GH61 mode-of-action
  - Binding to surface may dictate which oxidized product is formed

✓ Studying effects of oxidized products of GH61 action on glycoside hydrolases (are they inhibitory?)



T. fusca E7
(asymmetric dimer)



Cellulase Interaction w/ Pretreated Lignin

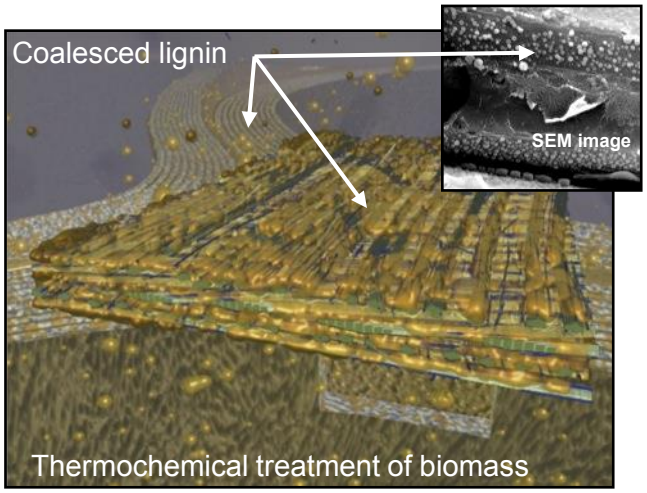
Relevance: Understand lignin-enzyme and lignin-cell wall interactions to increase conversion

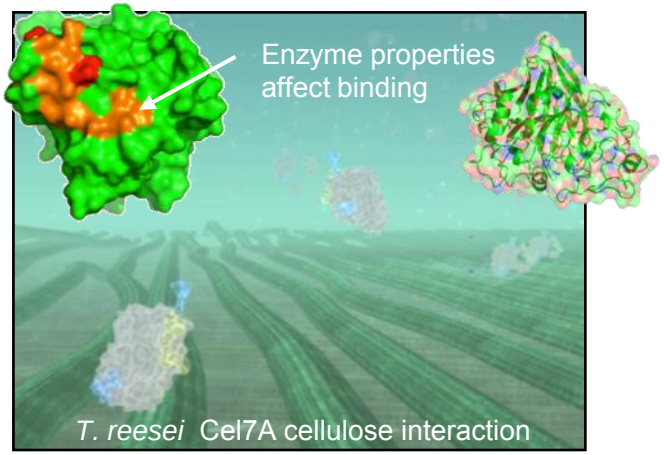
Rationale

- Thermochemical pretreatment changes the structure and chemistry of lignin and exposes it to cellulases
  - Binding of cellulase to lignin can cause loss of enzyme activity, decreased conversion yields

Outcome

- The fungal secretome has many glycoside hydrolases that depolymerize cellulose and hemicellulose.
  - Enzymes work synergistically
  - Productivity is lost in the presence of lignin
- To investigate the interactions of these enzymes in the presence of lignin
  - Elucidate the mechanism(s) of non-productive binding of enzymes to lignin
  - Examine if binding induces protein denaturation
  - Evaluate steric interference of lignin on polysaccharide substrates
- Identify which enzymes are affected and develop strategies to overcome this process impact
  - General phenomenon or enzyme-specific?





Measuring Lignin-Protein Interactions

Relevance: Test changes in enzymes for increased activity from decreased binding

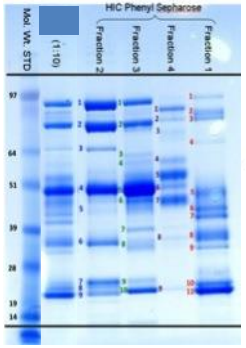
Rationale

- Post-pretreatment protein binding to lignin is a potential issue
  - ➢ Loss of activity due to non-productive binding or denaturation
  - ➢ Impacts required protein loading = higher enzyme cost

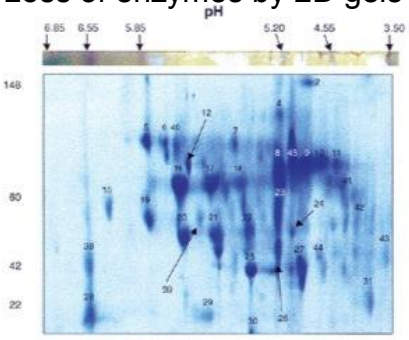
Outcome

- Determination of which enzyme components are affected
  - ➢ Use chromatography and 1D/2D electrophoresis to ID proteins before and after binding
- Measurement of bound protein is complicated by lignin properties
  - ➢ Interference with std protein concentration assays
  - ➢ Desorption of low molecular weight lignin components cause significant drift
  - ➢ Separating the lignin from carbohydrate is very difficult
    - Required to differentiate lignin & cellulose binding
- Method of choice is Quartz Crystal Microbalance
  - ➢ Measures change in mass of a coated surface, ng resolution
  - ➢ Dynamic method, real-time data
  - ➢ Demonstrated stable lignin substrate for QCM

HIC to rank enzymes

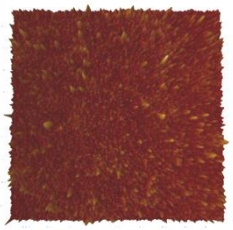


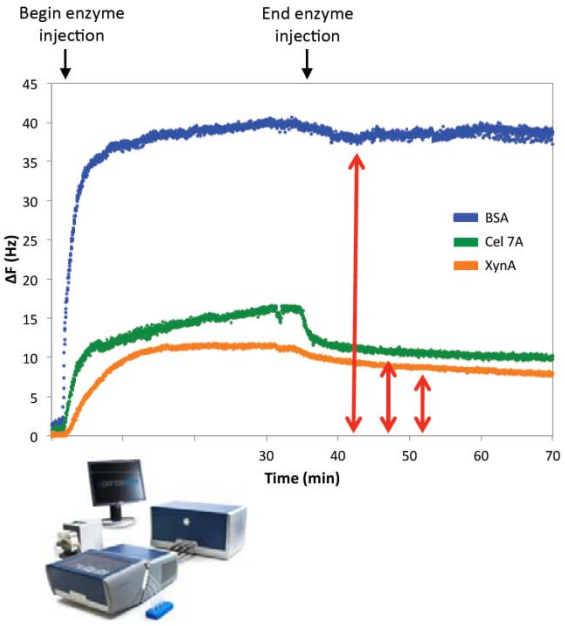
Loss of enzymes by 2D gels



cellulose

lignin





SST Subtask Overview – M. Crowley

Rationale

- Uncover molecular mechanisms of enzymes on biomass using theory, simulation (MD, QMMM), bioinformatics, and crystallography with the aim of decreasing enzyme loadings by increasing enzyme efficiency.
- Predict molecular modifications to proteins, methods, and conditions to enhance biomass conversion.
- Develop advanced computational tools that illuminate mechanisms and predict useful modifications (CHARMM, AMBER, etc).

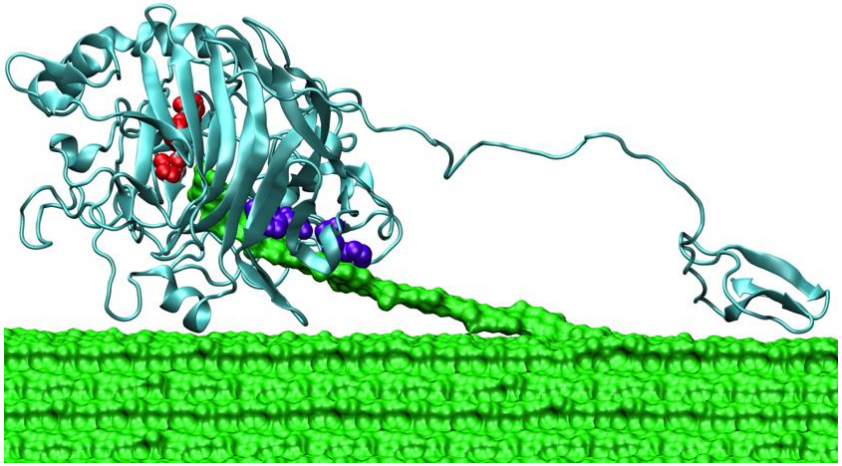
Outcome and Relevance

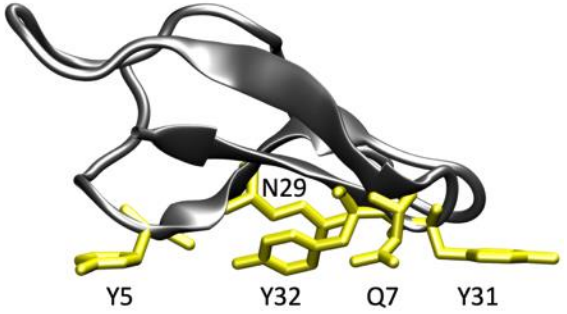
- Production of Cellulosic Sugars and Carbohydrate Derivatives- Pretreatment and Enzymatic Saccharification. Barrier Area 3: Enzyme Science & Biotechnology
  - The mechanistic basis underlying the action of most hydrolytic enzymes are still largely misunderstood; understanding is essential to improving efficiency.
  - Poor categorization and understanding of the natural diversity in hydrolytic enzymes
  - The advantages and disadvantages of using lignolytic enzyme systems have received relatively little R&D focus
  - The specific activity of hydrolytic enzymes are typically low
  - End products (sugars and degradation products) inhibit enzymes, preventing high sugar concentrations
  - Lignin derived species hinder enzyme efficiencies
  - Cellulase enzyme loading with high solids (>20% w/w) is cost prohibitive

Improving Cel7A Through Enhanced Understanding

Questions

- Processivity: role of aromatics and polar residues in CD from tunnel entrance to exit
- CBM binding affinity to cellulose: aromatic and polar residues on the CBM
- CD binding affinity to cellulose: construct Cel7A mutants to thread, but not react

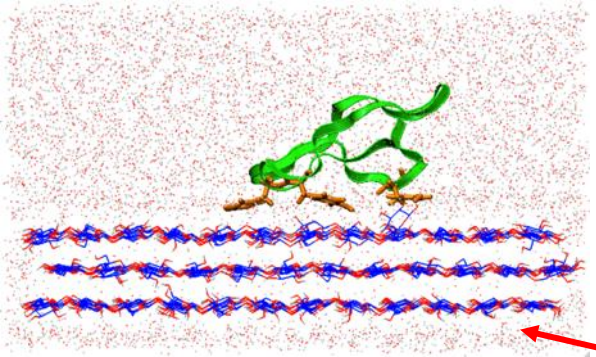




Outcome and Relevance

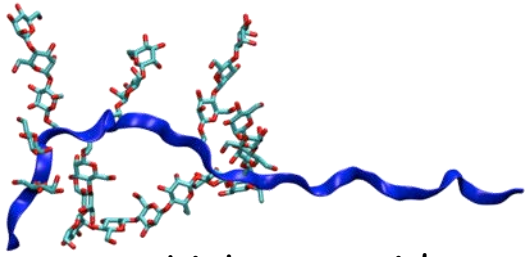
- Thermodynamics of elementary steps to determine the targets for improvement in efficiency
- Quantify thermodynamic reasons for increased activity in enzyme chimeras
- Recommend an enzymatic assay for cellulose reactivity (pure crystalline cellulose?)

Understanding Cel7A: MD Simulation & Mutation



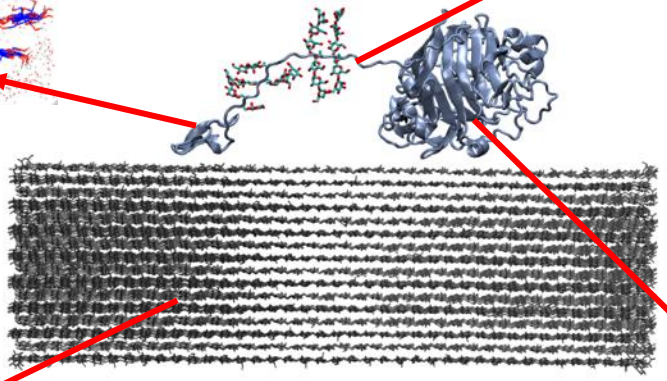
Binding Domain

- Adsorption, binding energy
- Mobility on cellulose surface
- Interaction with broken strands

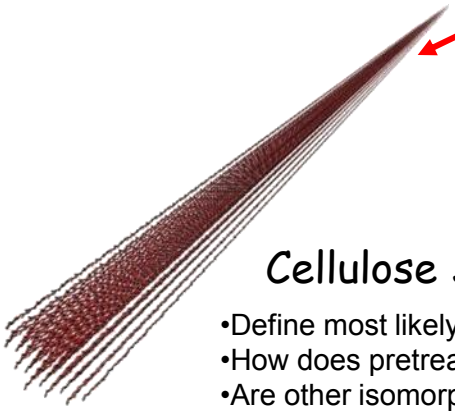


Linker peptide

- Define function and functionality
- Spring action
- Interactions with substrate and water

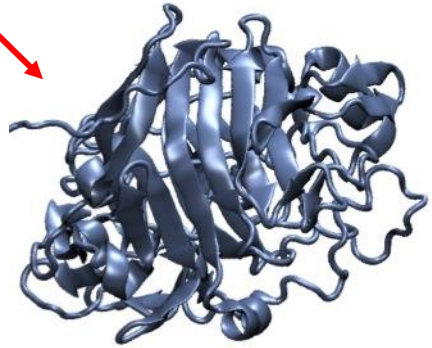


800,000 atoms



Cellulose Substrate

- Define most likely form of cell wall cellulose
- How does pretreatment change it?
- Are other isomorphs better substrates?



Catalytic Domain

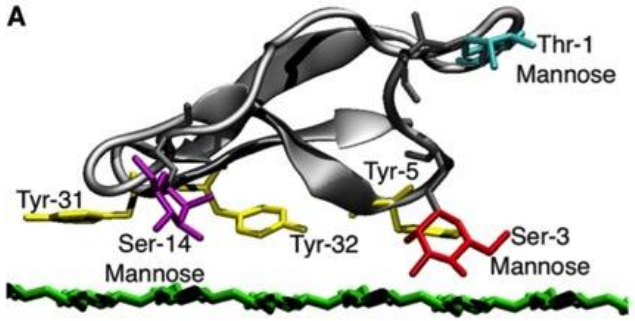
- Free energy of motion of cellodextrin in tunnel
- Exiting of cellobiose
- QM/MM of reaction and structural changes

CBM Binding Affinity Glycosylation Effect

Relevance: D Milestone: Provide detailed mechanistic models for the processive cellulases and preliminary models for the oxidative cellulases.

Rationale

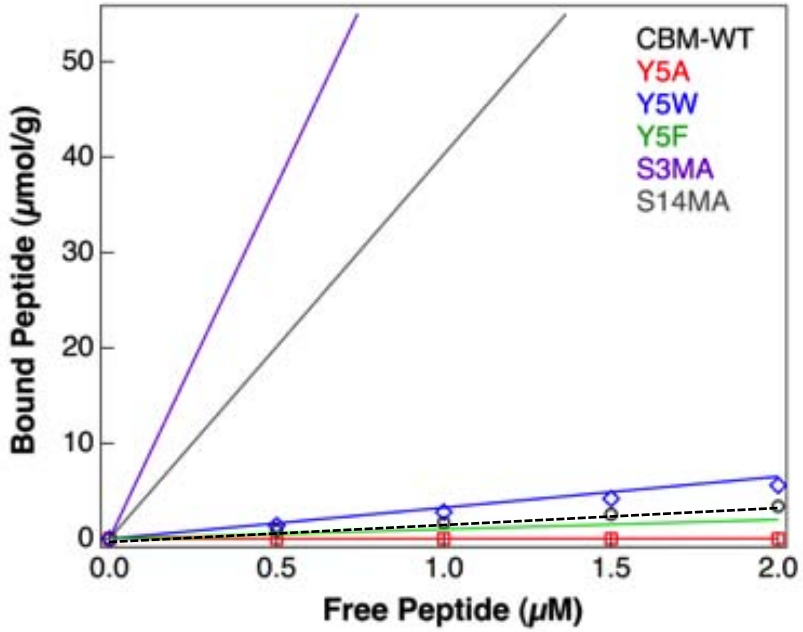
- Thermodynamic Integration methods allow in-silico mutations within the sampling methods to determine relative thermodynamic properties.
  - Relative binding properties of CBMs with glycosylation.



Outcome

- Mutation of "flat-face" aromatic residues.
  - Binding affinity: Y5W > Y5F > Y5A
  - Results congruent with experimental results
  - Y5A – F5A = Y5F validating simulation protocol
- Addition of a single mannose found to improve binding affinity resulting in a 20 - 40 fold increase in Kb.
  - Position of glycan does not have impact
  - **Experimental validation of results underway**

		K_M/K_W
Y5A	2.62 ± 0.62	0.01
Y5F	0.32 ± 0.18	0.59
Y5W	-0.40 ± 0.64	1.92
S14MA	-1.91 ± 1.35	**23.70**
S3MA	-2.29 ± 1.61	**43.64**



"Computational investigation of glycosylation effects on a Family 1 carbohydrate-binding module"
CB Taylor, MF Talib, C McCabe, L Bu, WS Adney, ME Himmel, MF Crowley, GT Beckham, *Journal of Biological Chemistry,* **2012**, 287(5), 3147-3155.

CBM Binding to Cellulose Faces

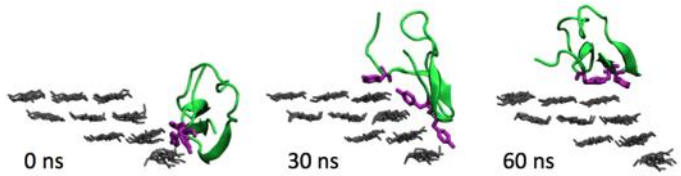
Relevance: D Milestone: Elucidate new structure-function relationships in GH7 enzymes via molecular simulations and bioinformatics studies for protein engineering purposes

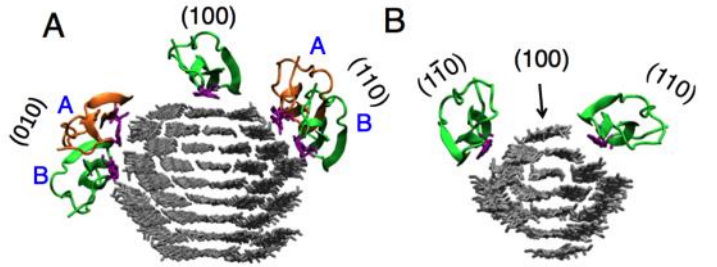
Rationale

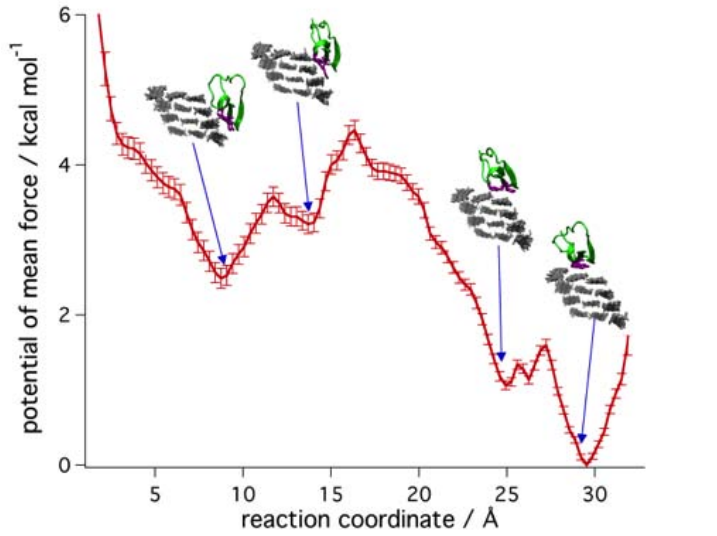
- Test the hypothesis that CBM1 preferentially binds to hydrophobic faces.
  - ➢ Molecular dynamics and Free Energy sampling can determine the validity of the hypothesis.

Outcome

- CBMs may be key in binding cellulases to the optimal faces of cellulose microfibrils for enzyme activity.
- Understanding the preferences helps to design better enzymes and substrates for optimal conversion rates.
- CBM1 shows a strong preference for binding to the hydrophobic face of cellulose







"Binding preferences, surface attachment, diffusivity, and orientation of a family 1 carbohydrate-binding module on cellulose", MR Nimlos, GT Beckham, JF Matthews, L Bu, ME Himmel, MF Crowley, *Journal of Biological Chemistry*, **287**, 20603-20612.

Role of Linker Domain in Cellulases

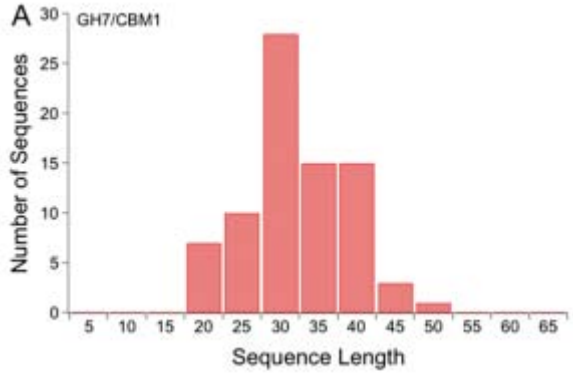
Relevance: D Milestone: Elucidate new structure-function relationships in GH7 enzymes via molecular simulations and bioinformatics studies for protein engineering purposes.

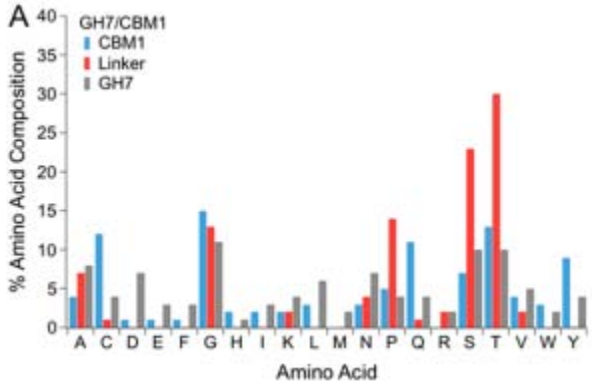
Rationale

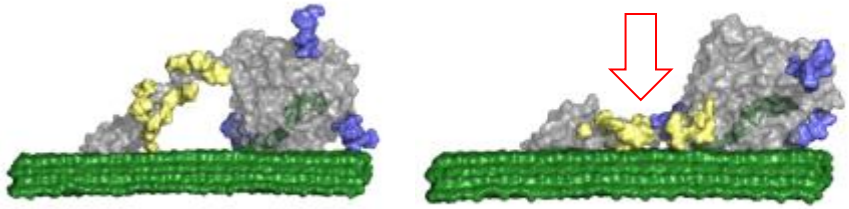
- Bound enzyme simulations predict linker binding.
  - Analysis reveals linker characteristics are conserved.
  - Experimental measurements will validate the theoretical finding that the linker adds significant binding affinity to the enzyme.

Outcome

- Enzyme activity is highly dependent on binding
  - Linker has strong binding characteristics.
  - Large increase in binding when linker is added to CBM.
- Design enzymes tuned for binding to industrially relevant substrates through linker design and glycosylation.
  - Linkers are confirmed to be disordered proteins.
  - Changes in linker length, composition, and glycosylation can be used to change binding characteristics and activity of selected enzymes for optimal biomass conversion.







"Cellulase linkers are optimized based on domain type and function: Insights from sequence analysis, biophysical measurements, and molecular simulation" DW Sammond, CM Payne, R Brunecky, ME Himmel, MF Crowley, GT Beckham, *PLoS ONE*, 2012

pH Dependence in GH7 enzymes

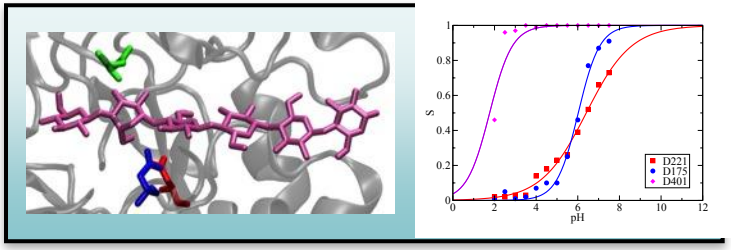
Relevance: D Milestone: Elucidate new structure-function relationships in GH7 enzymes via molecular simulations and bioinformatics studies for protein engineering purposes.

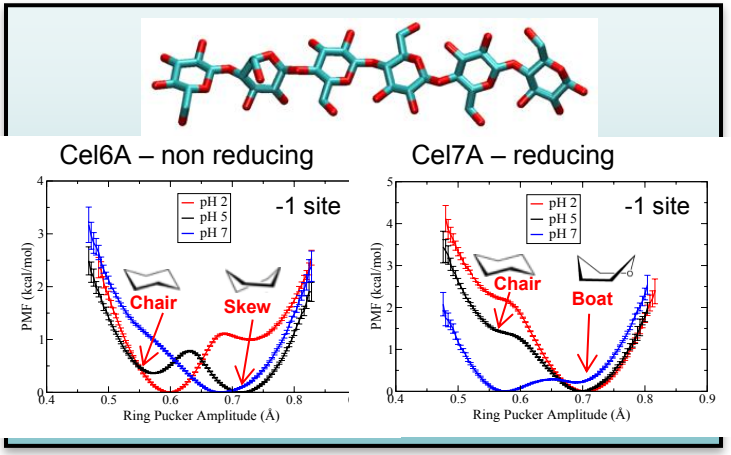
Rationale

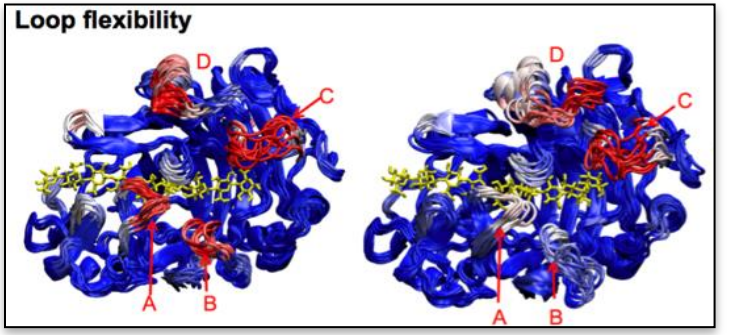
- Modeling pH dependence of protein properties can lead to design of better industrial enzymes (Constant pH MD).
  - Understanding the effect of pH on enzymes and substrates helps define best operating conditions.

Outcome

- Enzyme activity is highly pH dependent
  - Active site protonation states (pKa)
  - Conformational changes (glucosyl pucker)
  - Loop flexibility and motion
- Design enzymes to perform at optimal industrial pH
  - Active site acids and bases must have pKas that allow them to function at an optimal pH. The pKa can be adjusted by changes in the protein environment of the catalytic residues.
  - Modeling and simulation can predict changes to adjust pKas and initially test them before experimentally verifying activity.







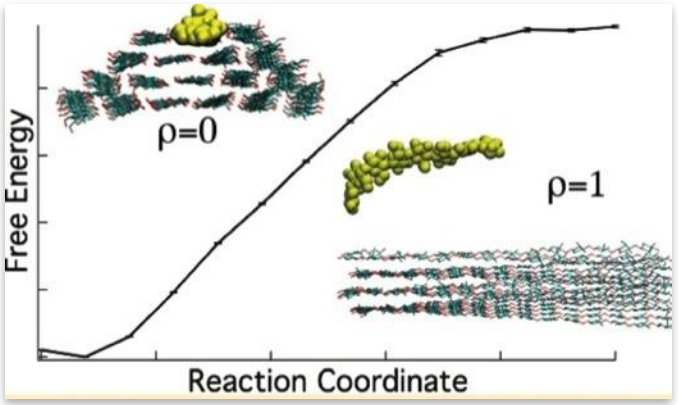
Computational investigation of pH dependence on loop flexibility and catalytic function in glycoside hydrolases
L Bu, MF Crowley, ME Himmel, GT Beckham, *Journal of Biological Chemistry*, **2013**, *doi:10.1074/jbc.M113.462465.*

Cellulose Thermodynamics

Relevance: D Milestone: Elucidate new structure-function relationships in GH7 enzymes via molecular simulations and bioinformatics studies for protein engineering purposes

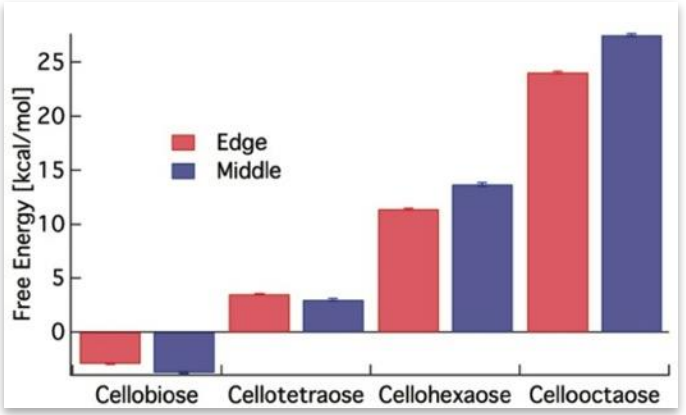
Rationale

- Cellulose structure and properties can be studied in models and simulations in much more detail than current experiments allow



Outcome

- Solubility of short chains is not well characterized.
- Determine the probability of release of oligosaccharides from endoglucanase activity.
  - Only 2-mer and 4-mer cellodextrins spontaneously release into solution
  - Cellohexaose and larger are thermodynamically more stable adsorbed and will recrystalize into the surface of cellulose.
- Determine whether cellobiose and cellotetraose re-adsorb to cellulose crystals and cause inhibition of cellulases.
  - NO, cellobiose will not stably readsorb to the surface of cellulose.
  - This theory can be dismissed: cellobiose readsorbtion to cellulose inhibits cellulose hydrolysis.



"Molecular-level origins of biomass recalcitrance: Decrystallization free energy of four common cellulose polymorphs", GT Beckham, JF Matthews, YJ Bomble, B Peters, ME Himmel, MF Crowley, *Journal of Physical Chemistry B*, **115**, 4118-4127.

"Decrystallization of Oligosaccharides from the Cellulose Iβ Surface with Molecular Simulation", CM Payne, ME Himmel, MF Crowley, GT Beckham, *The Journal of Physical Chemistry Letters*, **2011**, 2,1546-1550

Cellulase Processivity

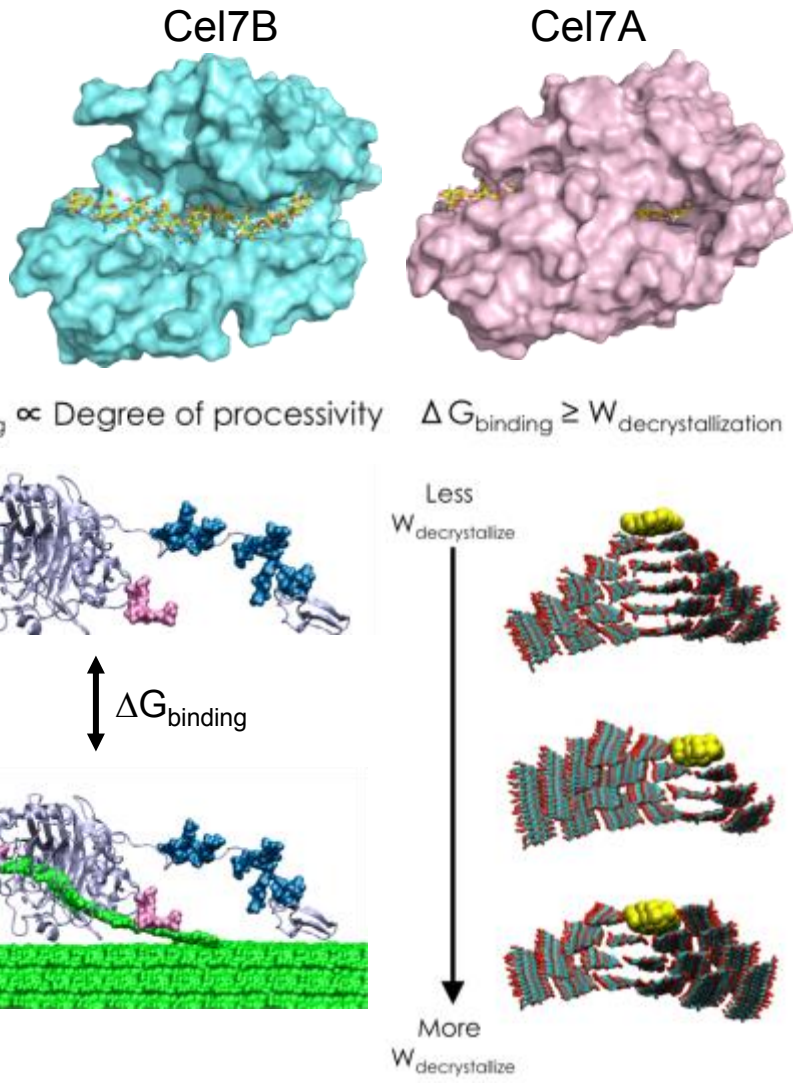
Relevance: D Milestone: Elucidate new structure-function relationships in GH7 enzymes via molecular simulations and bioinformatics studies for protein engineering purposes

Rationale

- Understand the molecular-level basis for processivity
  - Used molecular simulation, free energy calculations, and structural studies in concert
  - Work with well-characterized systems from bacteria first

Outcome

- Defined hallmarks of processivity in glycoside hydrolases
- Applying this work now to GH7 enzymes
- Will compare that to decrystallization work required to depolymerize cellulose chains
- Enable enzyme design for key GH7 cellobiohydrolases and will enable development of structure-activity relationships



"Molecular-level origins of biomass recalcitrance: Decrystallization free energy of four common cellulose polymorphs", GT Beckham, JF Matthews, YJ Bomble, B Peters, ME Himmel, MF Crowley, *Journal of Physical Chemistry B*, **115**, 4118-4127.

"Hallmarks of processivity in glycoside hydrolases from crystallographic and computational studies of the *Serratia marcescens* chitinases", CM Payne, J Baban, SJ Horn, PH Backe, AS Arvai, B Dalhus, M Bjorås, VGH Eijsink, M Sørlie, GT Beckham, G Vaaje-Kolstad, *Journal of Biological Chemistry*, **287**, 36322-36330.

Enzyme Crystallography

Relevance: Understanding of enzyme structure/function to increase activity, decrease cost

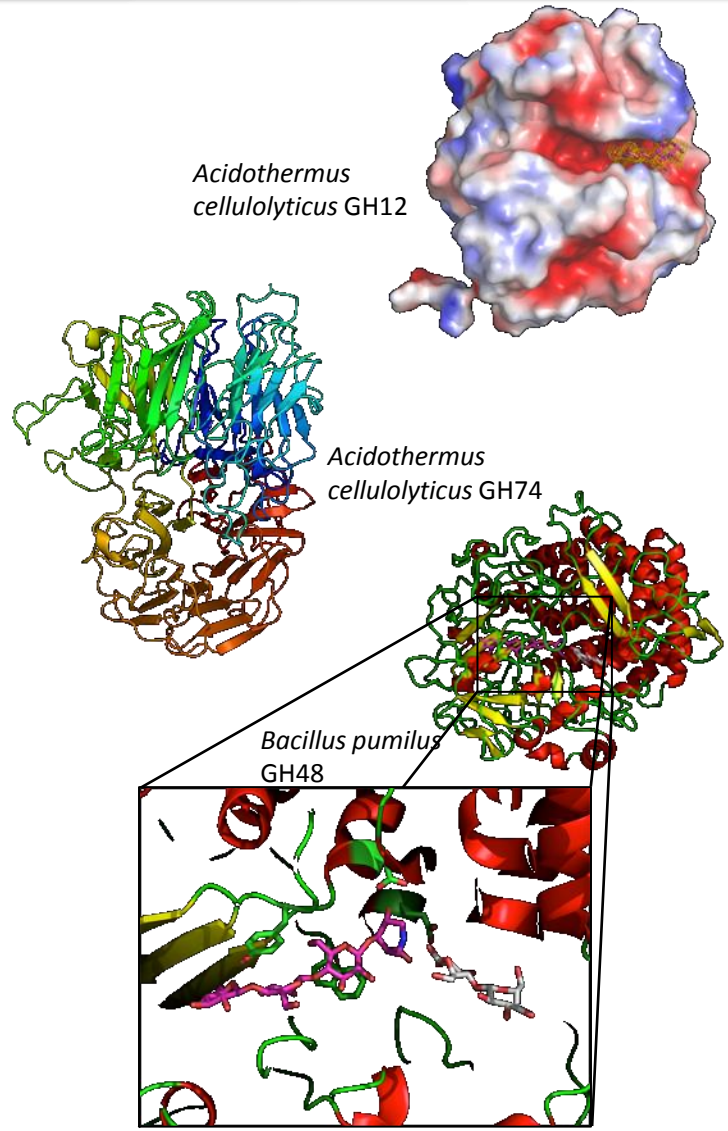
*BESC Collaboration

Rationale

- Use enzyme structural information to understand function and properties
  - Understanding leads to rational design improvements
  - Glycosyl hydrolases pose additional crystallization challenges

Outcome

- Thermal stability of enzymes allows for higher operating temperatures
  - Faster reaction kinetics
  - *Acidothermus cellulolyticus* GH12 – a model enzyme for studying thermal stability improvement through mutations
- Enzyme specificity determines substrate utilization
  - Broader activity, understanding of how to improve activity
  - *Acidothermus cellulolyticus* GH74 – a thermal stable xyloglucanase with broad substrate specificity
- Inhibitor interaction in active/binding site leads to
  - Better understanding of reaction details
  - Redesign to relieve substrate/product inhibition
  - Understanding of pretreatment-generated inhibitor effects
  - The structure of *Bacillus pumilis* GH48 in complex with cellobiose and isofagomine-derived inhibitor reveals the interactions of the active site residues with the substrate



Predicting Lignin-Protein Interactions

Relevance: Predict enzyme mutations to decrease lignin interaction and increase activity

Rationale

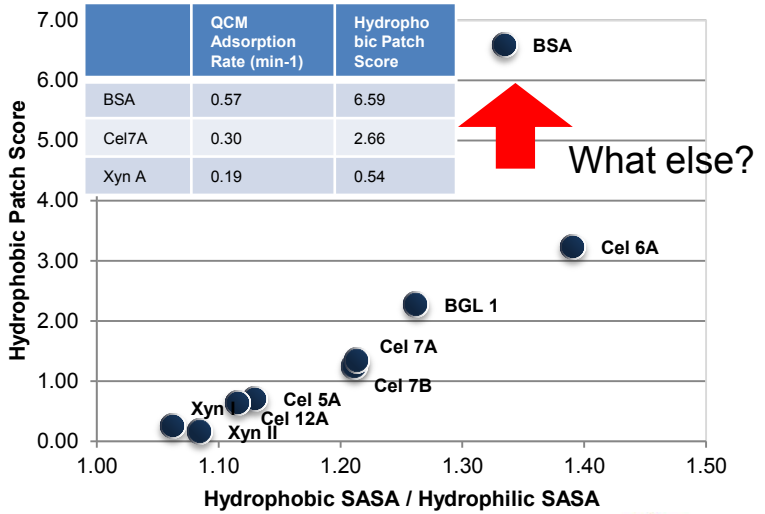
- A primary candidate for protein-lignin binding is hydrophobicity
  - Evaluating protein surface hydrophobicity and lignin interaction will help design more effective cellulases

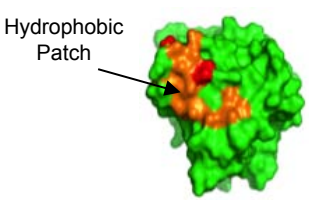
Outcome

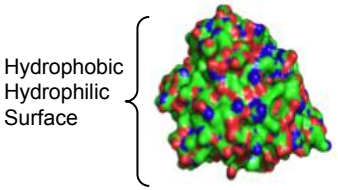
- Non-productive Binding mechanism
  - hydrophobic interaction
  - electrostatic interactions
- Some proteins have distinct hydrophobic patches evolved to bind other molecules
  - BSA has known hydrophobic binding regions, resulting in a higher "Hydrophobic Patch Score" compared to its "Hydrophobic SASA/Hydrophilic SASA" ratio.
- Importantly, surface regions remote from the active site or other conserved functional region can be ideal spots for protein engineering efforts
  - Here we have identified such regions in two important cellulase enzyme domains

Estimating Surface Hydrophobicity

	QCM Adsorption Rate (min-1)	Hydrophobic Patch Score
BSA	0.57	6.59
Cel7A	0.30	2.66
Xyn A	0.19	0.54







	Full-length Protein				Catalytic Domain		Accessory Domain	
T. reesei enzymes	predicted pI	MW	Domain Architecture		$Hphob_{SASA}/Hphil_{SASA}$	hpatch score		hpatch score
Cel 7B	4.73	48.2	GH7	CBM1	1.21	1.25	CBM1	1.44
Cel 5A	4.97	44.2	CBM1	GH5	1.13	0.70	CBM1	4.16
Cel 12A	6.69	24.9	GH12		1.12	0.64		
Cel 61A	5.29	35.5	GH61	CBM1			CBM1	3.04
Cel 7A	4.65	54.07	GH7	CBM1	1.21	1.34	CBM1	6.56
Cel 6A	5.11	49.7	CBM1	GH6	1.39	3.23	CBM1	4
XYN I	5	24.6	GH11		1.06	0.26		
XYN II	7.87	24.1	GH11		1.08	0.16		
Cel 74A	5.42	87.1	GH74	CBM1			CBM1	3.68
BGL I	6.38	78.4	GH3	fn3-like	1.26	2.27	fn3-like	
BSA (control)					1.33	6.59		

DMSC Subtask Overview – M. Zhang

Rationale

- Identify, understand and overcome the critical barriers for conversion of lignocellulosic feedstocks to hydrocarbons
  - similar sensitivities to bioethanol process (biomass conversion cost and less than theoretical microbial product formation)
- Direct microbial conversion (Consolidated BioProcessing) platform
  - Reducing process cost
  - Eliminate enzyme costs and reduce process complexity

Outcome and Relevance

- Biological Conversion of Sugars and Carbohydrate Derivatives- Barrier Area 1: Carbon Utilization
  - C5 utilization for biomass feedstocks
  - Redox balance in microbes producing a fuel
- Direct Microbial Conversion to Fuels from Unconventional Sources- Barrier Area 3: Consolidated Bioprocessing
  - Integrate deconstruction methodologies
  - Incorporate physical deconstruction process
  - Develop analytical tools for complex systems
  - Microbes growing on dense biomass

Research Approach

Rationale

- Identify, understand and overcome the critical barriers for conversion of lignocellulosic feedstocks to hydrocarbons
- Stay pre-commercial – solve technology "pinch points"

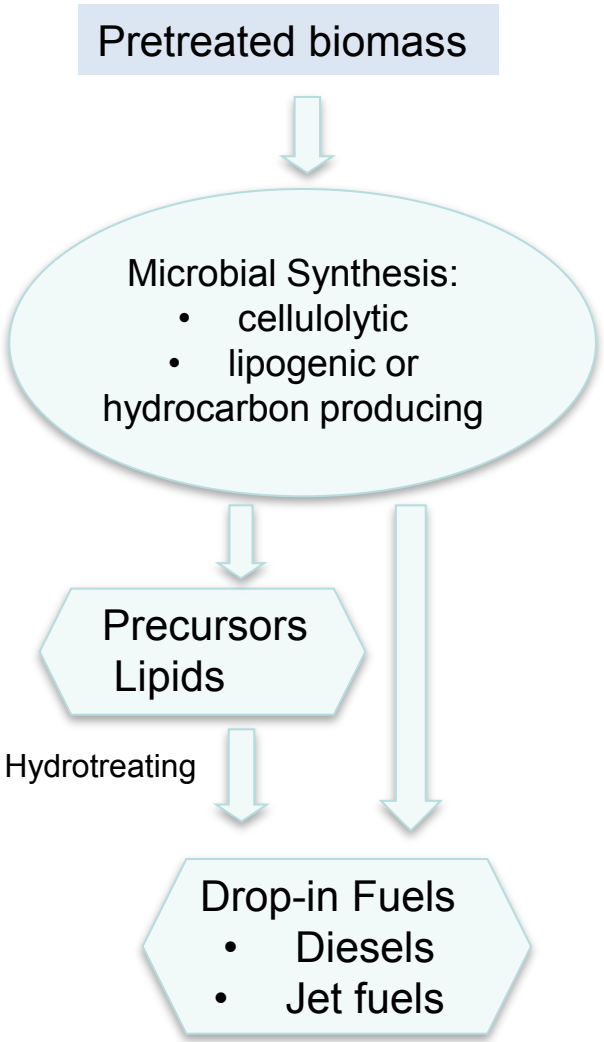
Outcome

- The strain should be an excellent biomass degrader or have extraordinary potential hydrocarbon-producing capabilities
  - Obtain fundamental knowledge regarding the microorganisms' cellulolytic capability; as well as ability to produce hydrocarbons
  - Understand the potential technical barriers and then devising strategies for improvement.

✓ Lipogenic DMC Yeast

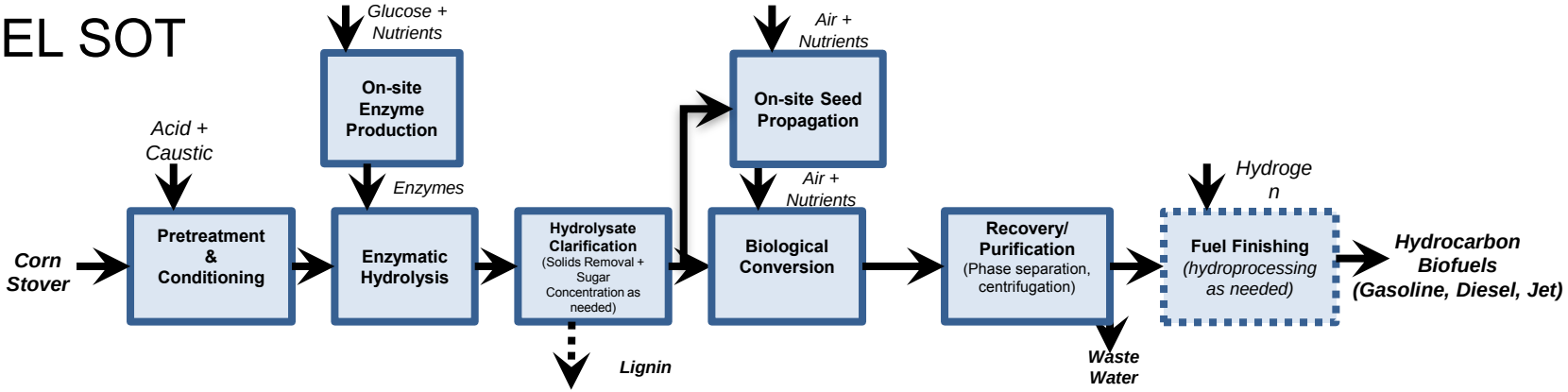
✓ Cellulolytic fungi producing hydrocarbons

- Hydrocarbon synthesis pathways and enzymes
- Synthetic biology tools

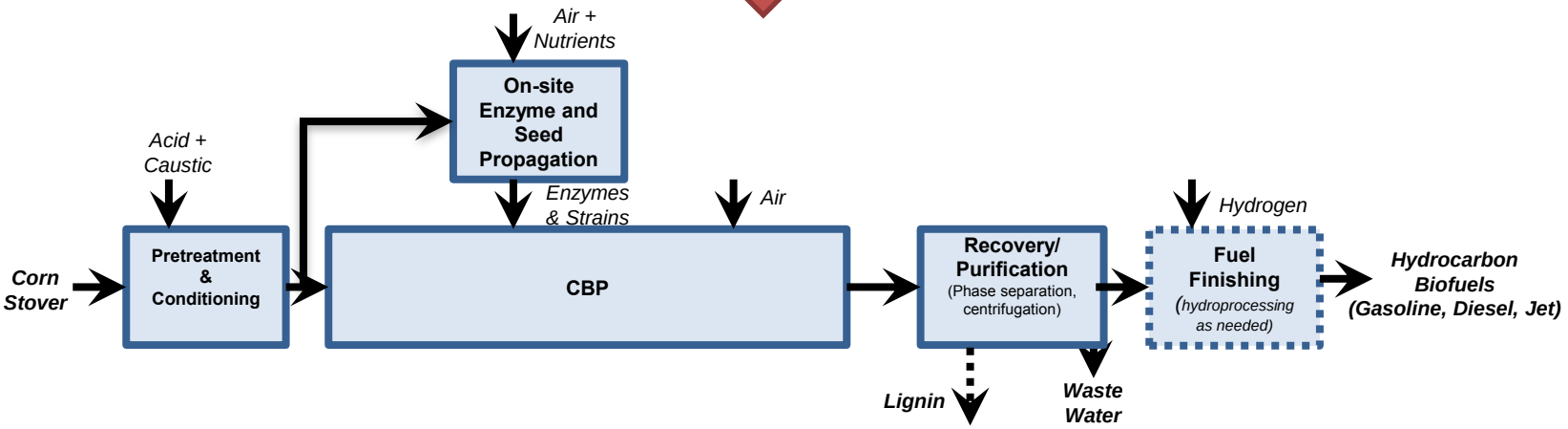


Preliminary Techno-Economic Analysis

NREL SOT



Adopting CBP* concept



*TEA "strawman" process design

Cellulase Expression in Oleaginous Yeast: *Yarrowia*

Relevance: E Milestone: Incorporate new and improved glycoside hydrolases provided by the ABD subtask into lipogenic yeast. D Milestone: Demonstrate expression of hemicellulase and accessory enzymes in lipogenic yeast

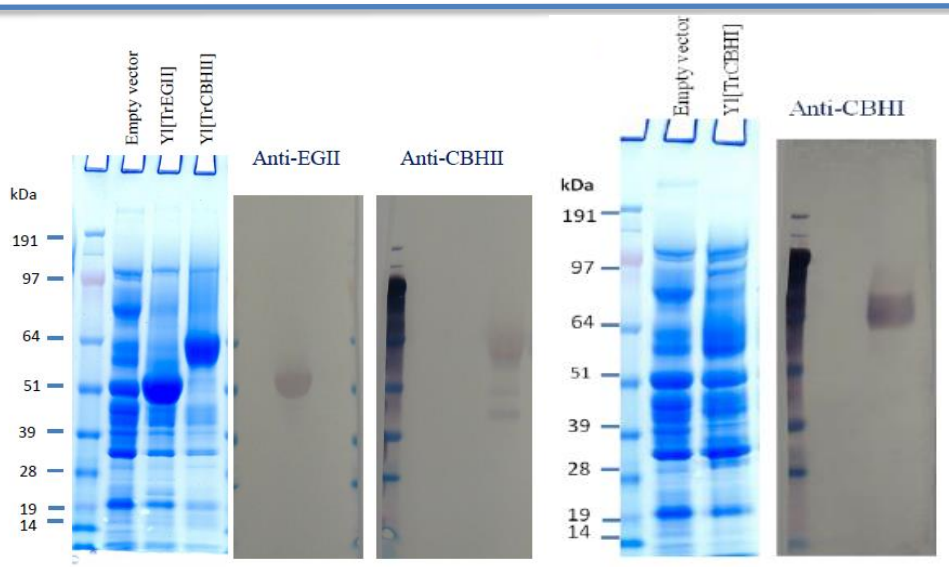
Rationale

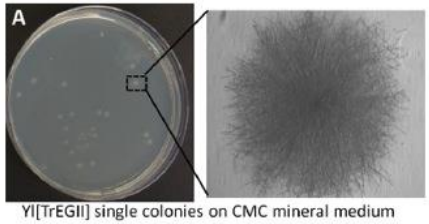
- *Yarrowia*: model oleaginous yeast with genome available
  - Genetic transformation system available
  - Known to secrete industrial enzymes

Outcome

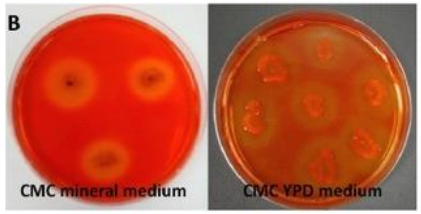
- Three types of cellulases were successfully expressed
  - β-glucosidase
  - Endoglucanases: EGII
  - Exoglucanases: Cel7A*
- Explore the synergistic action of the enzymes in utilizing cellulose
  - Avicel utilization by co-culture of Cel7A + EGII transformants
- Cloned hemicellulase genes and characterization will continue
- Next: show FAME production growing on cellulose

*Following the work of van Zyl (2011)

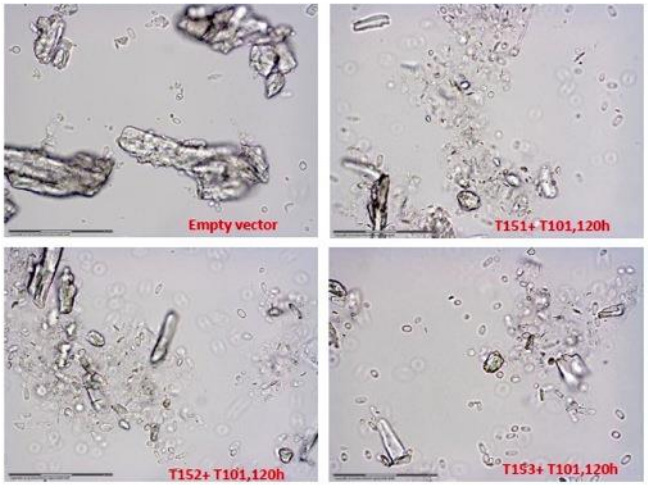




Yl[TrEGII] single colonies on CMC mineral medium



Yl[TrEGII] growth on CMC-containing media followed by Congo red staining



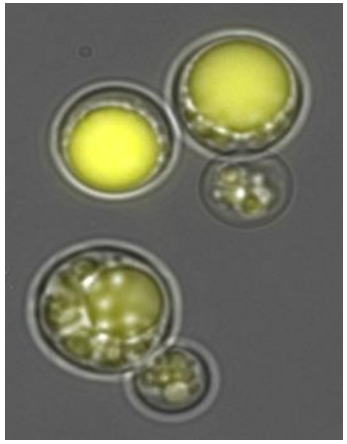
- **Avicel utilization by transformants Cel7A + EG II. Scale bar = 150 um.**

Promising Oleaginous Yeast

Relevance: Identify efficient lipid producers

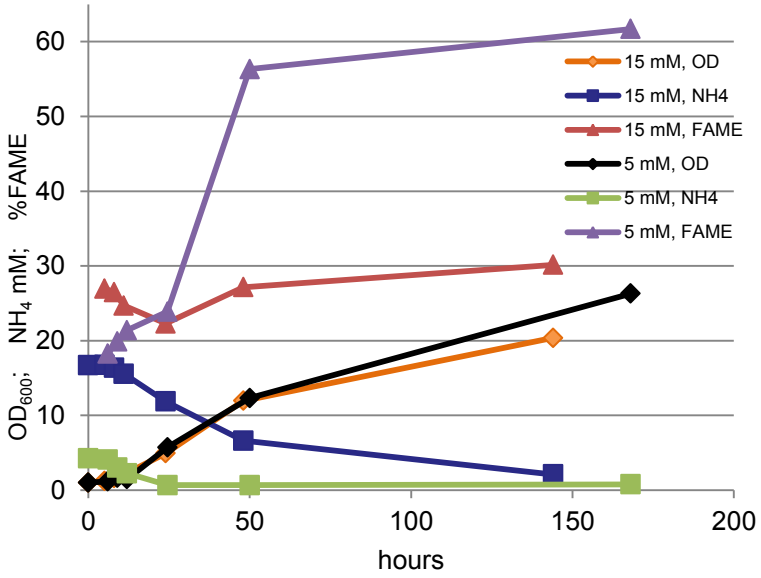
Rationale

- Oily yeasts have the potential to provide fuel precursors for advanced biofuels in the form of lipids
  - Capable of accumulating ~ 60% lipid on dry cell weight basis



Outcome

- Screened and evaluated oleaginous yeasts
  - *Cryptococcus curvatus* (21% FAME in 48 hr)
  - *Lipomyces starkeyi* (56% FAME in 48 hr)
- Genetic manipulation through transformation
  - *C. curvatus*
    - Achieved transformation with antibiotic selective marker
  - *L. starkeyi*
    - Genetic tools for transformation are being developed
- Nitrogen starvation induced lipid production
  - High sugar conversion yield 0.24 g lipid/g glucose
  - Produced 13 g/L at 0.1 g/L/h
- Can be potentially engineered to produce cellulolytic enzymes to degrade cellulose directly



Lipogenic fungi: *Mucor*

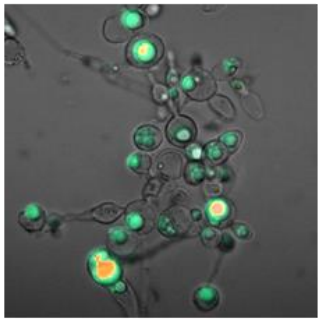
Relevance: D Milestone: Examine the biomass degrading systems of lipogetic fungi and determine which glyoside hydrolases they lack for efficient conversation.

Rationale

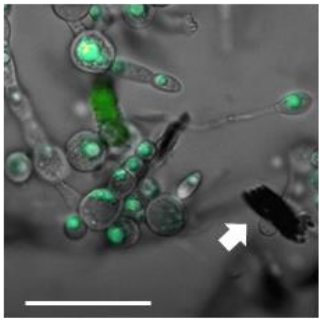
- Oleaginous fungi are reported to produce endoglucanases
- Utilize glucose, xylose but not cellulose
  - Assess its cellulase system -supplement with the cellulases it lacks, it could be developed as a CBP strain

Outcome

- We discovered *Mucor* has EG and β-G but lacks only exoglucanase (bioinformatics)
- Mucor can use pre-saccharified Avicel and dilute acid-pretreated corn stover
- Significant shift to short chain fatty acids when grown on Avicel (good for jet fuels)
- *Mucor* grown on glucose or Avicel + Cel7A showed lipid formation by Nile red staining
  - *Mucor* growing on Avicel achieved 30% of FAME (fatty acid methyl ester) content of that on glucose
  - Lipid yield (based sugar utilized) on Avicel is 80% of that of glucose



Glucose



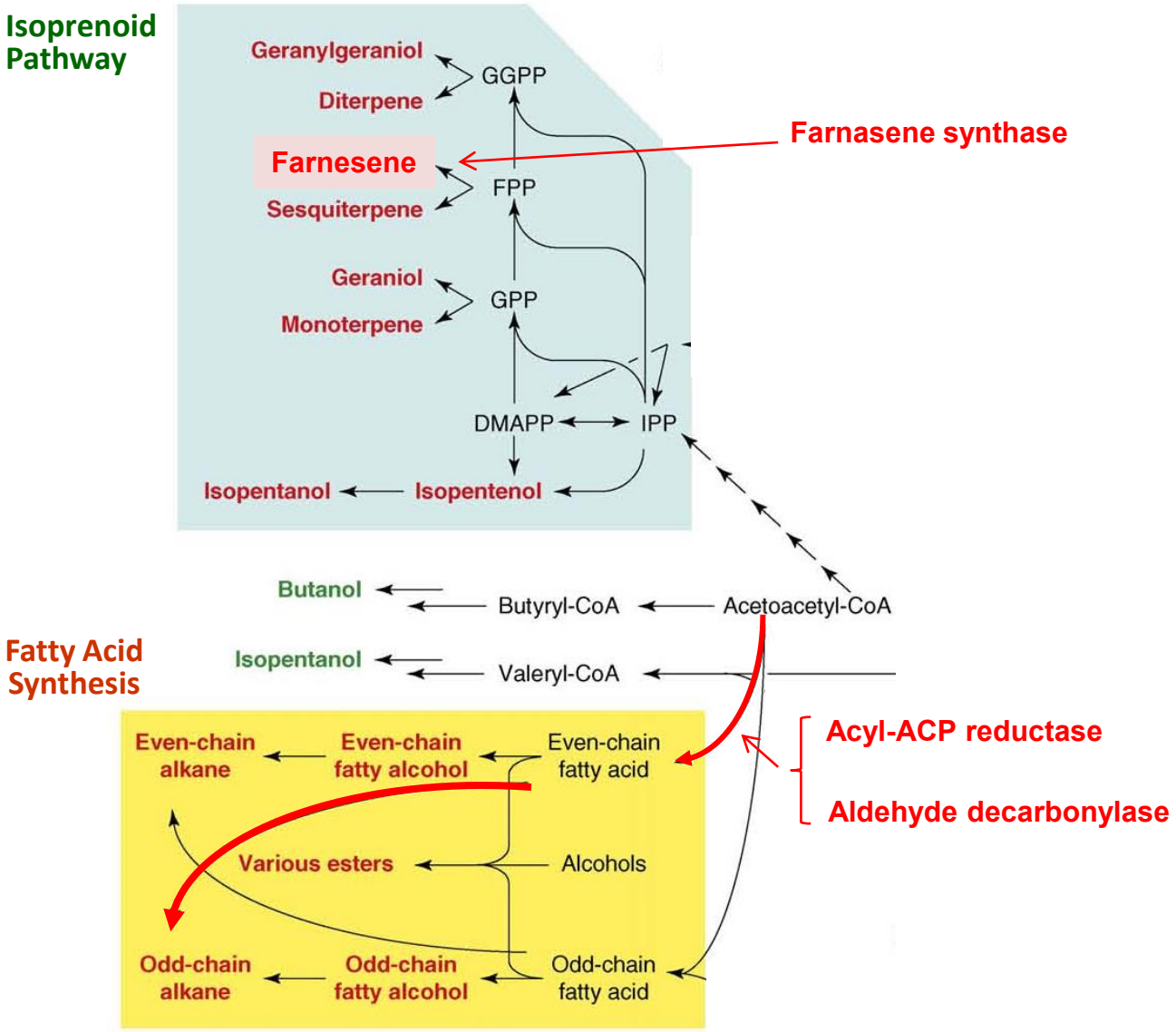
Avicel + CBHI

http://genome.jgi-psf.org

Type of enzymes	Enzyme activity	Genomic analysis (secretory prot)	Secretomic analysis (abundant prot)	Transcript analysis (less abundant prot)
Endoglucanases	+++	7 prot	2 prot	5 prot
Exoglucanases	-	-	-	-
BGLs	+++	2 prot.	-	2

Anabolic vs. Catabolic Product Pathways

C5, C10, C15 Compounds



C13 to C17 Compounds

Source: Fortman *et al*. 2008. Trends in Biotech. 26(7): 375-381

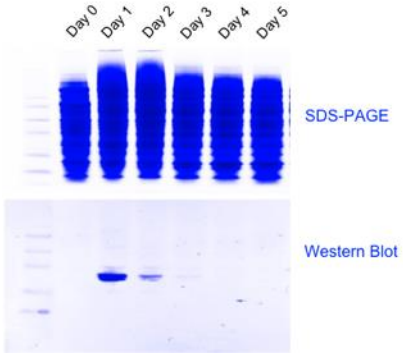
Hydrocarbons from *Trichoderma reesei*

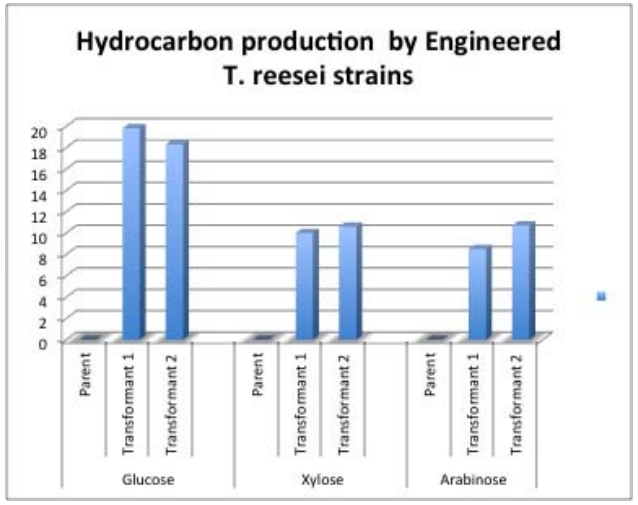
Direct Microbial Sugar Conversion

Relevance: E Milestone: Demonstrate that the heterologous enzymes in the hydrocarbon production pathway in *T. reesei* are active. D Milestone: Demonstrate production of 0.1-1% hydrocarbon production of *T. reesei* strain growing on Avicel.

Rationale

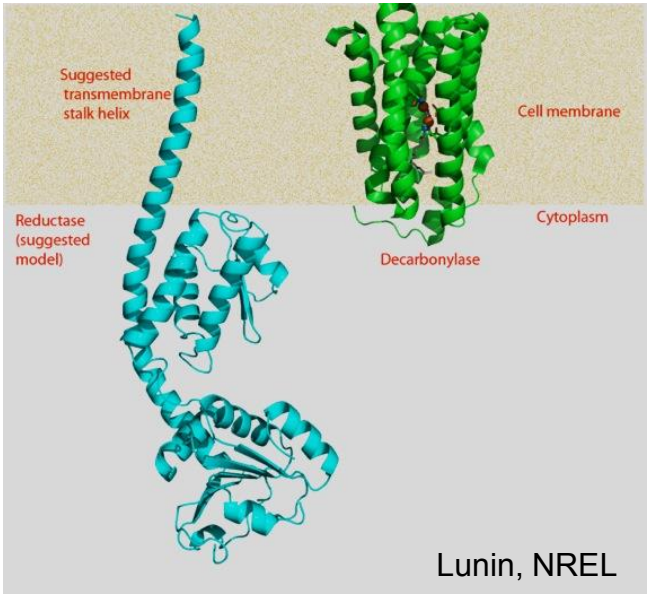
- Excellent cellulase producer
- Industrially important microorganism
- Utilize xylose and arabinose





Outcome

- ✓ Introduced hydrocarbon synthesis genes into *T. reesei* for production of hydrocarbons from either isoprenoid pathway or fatty acid pathway
- ✓ Expression of hydrocarbon synthesis enzyme in *T. reesei*
- ✓ Demonstrated hydrocarbon production from biomass sugars
- ✓ Predict the models for hydrocarbon synthesis enzymes
  - ➢ Likely to be the membrane protein
  - ➢ Need to improve the activities
  - ➢ Solving the x-ray structure of the aldehyde reductase
- ✓ Process cost advantage
  - ➢ Typical cost savings for CBP expected to apply
  - ➢ No separate enzyme production and process simplification



Relevance

Describe how project accomplishments contribute to meeting the platform goals and objectives of the Biomass Program Multi-Year Program Plan (updated November, 2012)

- This project provides fundamental and applied science strategies to enable the process engineering targets listed by the Multi-Year Program Plan to achieve cost-competitive cellulosic ethanol on corn stover.
- This overarching goal is: "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 of the expected outputs

- Increase confidence (reduce risk) for process implementation by demonstrating that the process unit operations are underpinned by considerable technical "know how"; in some cases extending to the molecular level.
- We consider that our publications and presentations will be used by industry to design bench and pilot scale improvements to biomass pretreatment, enzyme production & saccharification, and microbial conversion of sugars to advanced biofuels.

Your objectives should be clear regarding the relevance of your project to the Biomass Program, the market, and alignment with MYPP goals

- 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.
- Bt-D/E. Pretreatment Chemistry/Cost: 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.
- 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.
- Bt.J. Conversion Development: Reducing the uncertainties in key rate limiting steps in the conversion of sugars in biomass hydrolysate streams to advanced biofuels using the microbial consolidated bioprocessing approach.

Testimony from Novozymes

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

novozymes® Rethink Tomorrow

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 *Thielavia 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:

Selig, M. J., T. B. Vinzant, M. E. Himmel, and S. R. Decker. 2009. "The Effect of Lignin Removal by Alkaline Peroxide Pretreatment on the Susceptibility of Corn Stover to Purified Cellulolytic and Xylanolytic Enzymes *Appl. Biochem. Biotechnol*. 155:397-406.

Decker, S. R., M. Siika-aho, and L. Viikari. 2008. Enzymatic Depolymerization of Plant Cell Wall Hemicelluloses. In: M. E. Himmel (ed*). Biomass Recalcitrance.* Blackwell Publishing, Oxford, UK.

Selig, M. J., E. P. Knoshaug, W. S. Adney, M. E. Himmel, and S. R. Decker. 2008. "Synergistic Enhancement of Cellobiohydrolase Performance on Pretreated Corn Stover by Addition of Xylanase and Esterase *"Bioresource Technol*. 99:4997-5005.

Selig, M. J., S. R. Decker, E. P. Knoshaug, J. O. Baker, M.E. Himmel and W. S. Adney 2008. "Heterologous Expression of *Aspergillus niger* β-d-Xylosidase (XlnD): Characterization on Lignocellulosic Substrates *Appl. Biochem. Biotechnol*. 146:57-68.

Knoshaug, E. P., M. J. Selig, J. O. Baker, S. R. Decker, M. E. Himmel, and W. S. Adney. 2008. "Heterologous Expression of Two Ferulic Acid Esterases from *Penicillium funiculosum Appl. Biochem. Biotechnol*. 146:79-87.

2-D gel fingerprinting:

Vinzant, T.B., W. S. Adney, S. R. Decker, J. O. Baker, M. T. Kinter, N. E. Sherman, J. W. Fox, and M. E. Himmel. 2001. "Fingerprinting *Trichoderma reesei* Hydrolases in a Commercial Cellulase Preparation*" Appl. Biochem. Biotechnol*. 91-93:99-107.

Glycosylation:

Jeoh, T., Michener, W., Himmel, M. E., Decker, S. R., & Adney, W. S. 2008. Implications of cellobiohydrolase glycosylation for use in biomass conversion. *Biotechnol Biofuels*, 1:10.

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Critical Success Factors

Describe critical success factors (technical, market, business) which will define technical and commercial viability

- Meeting the DOE OBP 2012 technology technical targets for cellulose conversion to glucose, glucose conversion to ethanol, xylan conversion to xylose, xylose conversion to ethanol, cellulase cost, fermentation time, and solids concentrations for pretreatment.
- Meet near term and future technical targets for DOE's 2017 goals for advanced biofuels. To some extent, these targets are still not fully defined.

Explain the top 2-3 potential challenges (technical and non-technical) to be overcome for achieving successful project results

- 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 (EFRC-C3Bio, BRC-BESC, BRC-JBEI, and BRC-GLBRC).

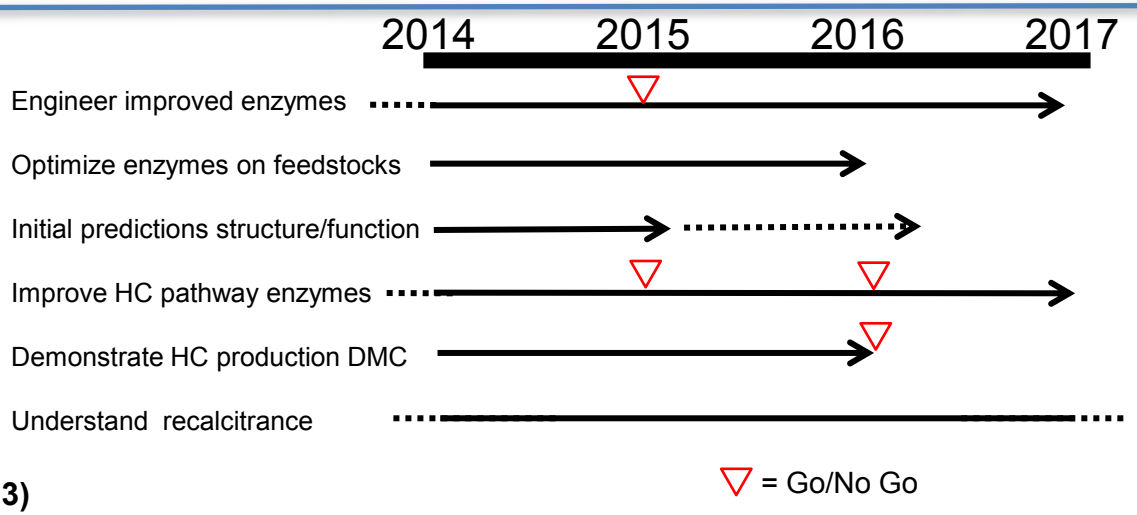
Demonstrate that the successful project will advance the state of technology and positively impact the commercial viability of biomass and /or biofuels

- The production of cheaper enzymes and pretreatments has been demonstrated to reduce the overall cost of converting biomass. We report new cellulase enzymes ready for formulation into leading industrial preparations; as well as promising new microbial candidates for advanced biofuels production directly from biomass using CBP.
- The success of commercial operations is based on risk reduction (real and perceived) as well as on hard technical accomplishments. Our work (publications and patents) reduces risk by sharing factual accomplishments based in knowledge and understanding. *i.e.*, knowledge is fully transferrable and timeless.

Future Work

Explain what it is you plan to do by 2017

- We plan to enable post-2012 technologies defined by DOE by developing new understandings in the production of advanced hydrocarbon biofuels, while maintaining cutting-edge science for basic understanding of biomass recalcitrance.
- To this end, TCR has been redefined: 1) Structure, Simulations, and Theory, 2) Advanced Biomass Deconstruction, and 3) Direct Microbial Sugar Conversion.



Highlight upcoming key D milestones (2013)

- Determine product inhibition effects of PMO-derived products in *T. reesei* Cel7A.
- Examine surface charge mutations of cellulases to prevent lignin binding .
- Elucidate new structure-function relationships in GH7 enzymes via molecular simulations and bioinformatics.
- Demonstrate biomass degrading enzyme preparations with performance improved by 50% using enzyme engineering, enzyme synergy, new enzymes, or thermal-hold enzymes.
- Determine the mechanism for lignin binding to cellulases using biochemical and advanced imaging approaches; test mutations which should reduce this affect.
- Demonstrate production of 0.1-1% hydrocarbon production of *T. reesei* strain growing on Avicel.
- Demonstrate expression of hemicellulase and accessory enzymes in lipogenic yeast.

Address how you will deal with any decision points during that time and any remaining issues

- Introduction of new processing steps (deacetylation and disk refining) and feedstock blends in 2012 will require new base line studies regarding optimizing biomass saccharification and fermentation. Resource reallocation will respond change.
- Output from other DOE biomass conversion programs (BRCs, EFRCs, SciDAC), as well as programs in NSF and USDA, could affect the specific directionality of TCR and thus both awareness of progress made and plans to compensate.
- The discovery of a new class of cell wall degrading enzymes (polysaccharide monooxygenases) in 2011-12 suggests that new understanding of these catalysts is critical for formulation of improved enzyme cocktails.

Summary

Relevance

- This project focuses on the conversion science underpinning the corn stover to ethanol process as defined by DOE's 2012 goal; focusing on biomass pretreatment and saccharification. This project also initiates new fields of study supporting the advanced biofuels goals for 2017 and beyond.

Approach

- Understand the scientific basis for biomass recalcitrance at the level of the (pretreated) plant cell wall and the molecular mechanisms of cellulase enzymes.
- Understand the key technical hurdles important for efficient conversion of sugars to fuels.

Technical Accomplishments

- Evaluated the likely mechanisms of biomass recalcitrance to dilute acid pretreatment and recommended focus on the reduction in xylan content and cellulose morphology. Defined the chemical mechanisms for parasitic reactions of sugars in pretreatment. Improved cellulase action using protein engineering inspired by mechanistic knowledge

Success Factors and Challenges

- Worked to understand the mechanism of CBH enzymes at a level sufficient to demonstrate to industry that a 3x in performance is possible. It has been proposed that cellulases could not be improved beyond the natural baseline. We have made progress on all the milestone related project goals since the 2011 OBP Review. Most notable are the formulation of testable hypotheses for the molecular mechanisms of action for CBH I and the application of CBP to production of advanced biofuels. Historically, we have contributed to the attainment of the MESP ($2.40 in 2009 and now $1.98 in 2010); the xylan to xylose yield (80% in 2009 and now 85% in 2010); and the enzyme cost reduction ($0.35 in 2009 and $0.17 in 2012).

Technology Transfer and Future Work

- Published ~46 peer reviewed manuscripts in two years (see slide set). Our future work will address new enzymatic routes for biomass conversion; as well as new metabolic pathways for production of advanced fuels.


Questions?

TCR: 2011-2013 Publications

1. "In planta expression of *A. cellulolyticus* Cel5A endocellulase reduces cell wall recalcitrance in tobacco and maize", R. Brunecky, M. Selig, T. Vinzant, M.E. Himmel, D. Lee, M. Blaylock, S.R. Decker, Biotechnol.for Biofuels 2011, 4:1
2. "Molecular-level Origins of Biomass Recalcitrance: Decrystallization Free Energies for Four Common Cellulose Polymorphs," Gregg T. Beckham, James F. Matthews, Baron Peters, Yannick J. Bomble, Michael E. Himmel, Michael F. Crowley, J. Phys. Chem. B 115(14), 4118–4127 (2011).
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6. "In Situ Imaging of Single Carbohydrate-Binding Modules on Cellulose Microfibrils" Dagel, Daryl; Liu, Yu-San; Zhong, Lanlan; Luo, Yonghua; Himmel, Mike; Xu, Qi; Zeng, Yining; Ding, Shi-You; Smith, Steve, J. Phys. Chem. B 115(4), 635-41 (2011).
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8. "The High Temperature Behavior of Cellulose I," James F. Matthews, Malin Bergenstråhle, Gregg T. Beckham, Michael E. Himmel, Mark R. Nimlos, John W. Brady, Michael F. Crowley, J. Phys. Chem. B 115(10), 2155–2166 (2011).
9. "Probing Carbohydrate Product Expulsion from a Processive Cellulase with Multiple Absolute Binding Free Energy Methods," Lintao Bu, Gregg T. Beckham, Michael R. Shirts, Mark R. Nimlos, William S. Adney, Michael E. Himmel, Michael F. Crowley, J. Biol Chem. 286(20), 18161-9 (2011).
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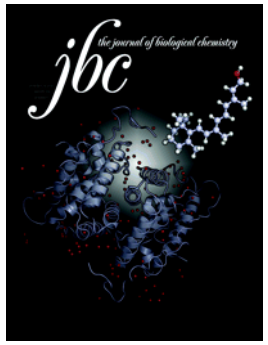

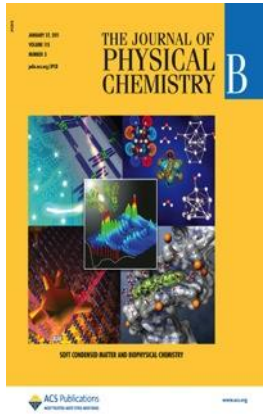

TCR: 2011-2013 Publications

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19. "A computational investigation of the impact of glycosylation on the Family 1 carbohydrate binding module," Courtney B. Taylor, Clare McCabe, Lintao Bu, William S. Adney, Michael E. Himmel, Michael F. Crowley, Gregg T. Beckham, J. Biol. Chem. 287(5):3147-55 (2012).
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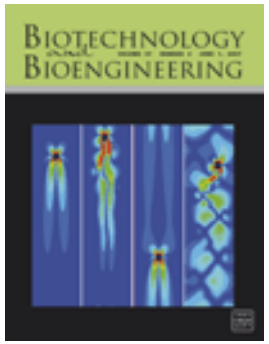

TCR: 2011-2013 Publications

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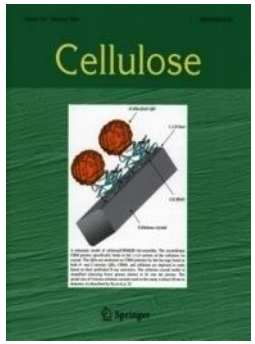

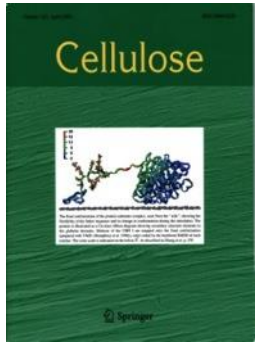

TCR: 2011-2013 Publications

30. “Improved ethanol yield and reduced minimum ethanol selling price (MESP) by modifying low severity dilute acid pretreatment with deacetylation and mechanical refining: 2) Techno-economic analysis,” Ling Tao, Xiaowen Chen, Andy Aden, Eric Kuhn, Michael E Himmel, Melvin Tucker, Mary Ann A Franden, Min Zhang, David K Johnson, Nancy Dowe and Richard T Elander, Biotechnol. for Biofuels 5:69 (2012) doi:10.1186/1754-6834-5-69.
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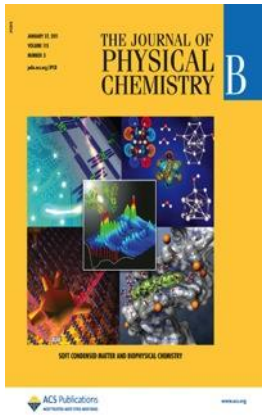
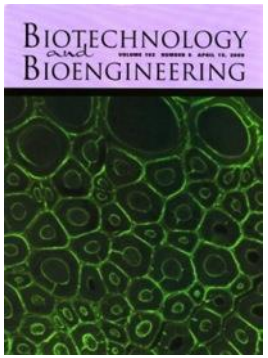
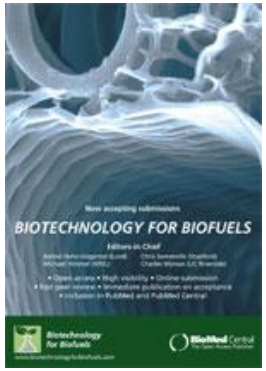
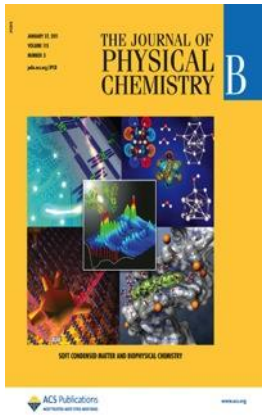


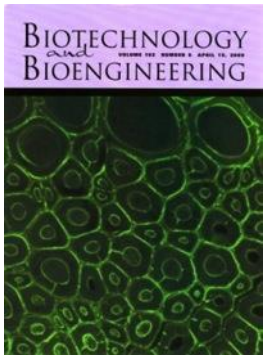


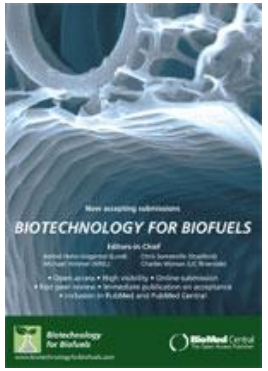


TCR: 2011-2013 Publications

40. “Fungal Cellulases and Complexed Cellulosomal Enzymes Exhibit Synergistic Mechanisms In Cellulose Deconstruction,” Michael G. Resch, Bryon S. Donohoe, John O. Baker, Stephen R. Decker, Edward A. Bayer, Gregg T. Beckham, Michael E. Himmel, Energy and Environmental Science , 2013, In Press.
41. “Crystal structure and computational characterization of the lytic polysaccharide monooxygenase GH61D from the basidiomycota fungus *Phanerochaete chrysosporium*,” Miao Wu, Gregg T. Beckham, Anna M. Larsson, Takuya Ishida, Seonah Kim, Christina M. Payne, Michael E. Himmel, Michael F. Crowley, Svein J. Horn, Bjorge Westereng, Kiyohiko Igarashi, Masahiro Samejima, Jerry Stahlberg, Vincent G. H. Eijsink, and Mats Sandgren, J. Biol. Chem., 288: 12828-12839 (2013).
42. "Computational Investigation of pH Dependence on Loop Flexibility and Catalytic Function in Glycoside Hydrolases," Lintao Bu, Michael F. Crowley, Michael E. Himmel, and Gregg T. Beckham, J. Biol. Chem., 288: 12175-12186 (2013).
43. “Binding Site Dynamics and Aromatic-Carbohydrate Interactions in Processive and Non-Processive Family 7 Glycoside Hydrolases," Courtney Taylor, Christina Payne, Michael E. Himmel, Michael F.Crowley, Clare McCabe, and Gregg T. Beckham, J. Physical Chem. B. 2013, In Press.
44. “Cellulose Polymorphism: Exocyclic CH_2OH Conformation and Chain Orientation Determined by Sum-Frequency-Generation (SFG) Vibration Spectroscopy,” Christopher M. Lee, Ashutosh Mittal, Anna L. Barnette, Kabindra Kafle, Yong Bum Park, Heenae Shin, David K. Johnson, Sunkyu Park, and Seong H. Kim, Angewandte Chemie International Edition, Cellulose, 2013, In Press.
45. “Structural characterization of the first marine animal Family 7 cellobiohydrolase suggests a mechanism of cellulase salt tolerance," Marcelo Kern, John E. McGeehan, Simon D Streeter, Richard N. A. Martin, Katrin Besser, Luisa Elias, William Eborall, Graham P. Malyon, Christina M. Payne, Michael E. Himmel, Kirk Schnorr, Gregg T. Beckham, Simon M Cragg, Neil C Bruce, Simon J McQueen-Mason, PNAS, 2013 Submitted.
46. “Irreversible Transformations of Native Celluloses Upon Isolation at Elevated Temperatures Carbohydrate Polymers,” Rajai Atalla, Rowan Atalla, Michael Himmel, Michael Crowley, Special Issue: Carbohydrate Polymers, Energy and Environmental Science, 2013 In Press.







Reviewers Comments from 2011

Technical Progress and Accomplishments

- Reviewer A*: "A lot of money spent to know what was already known through years of pretreatment research, that being that xylose and acetyl removal as well as small particle size are needed for more efficient cellulase hydrolysis. *(They)* Did find new insights into cellulose structure. The question is, is this what DOE wants? Interesting understanding of the micromolecular interaction of substrate and enzymes. How can this translate to increased process economics in a timely manner?"
  - Reviewer B: "The project identified biomass recalcitrance (inability to access the last 15% of sugars present in cellulosic substrates) as the long-term target of this work. This is a key aspect in providing a high yield process."
  - Reviewer C: "The research contributes to understanding the critical processes of pretreatment and saccharification and hopefully leads to improvements in efficiency that meets or exceed biochemical platform goals."
  - Reviewer D: "The objective of the project is to take leadership in providing the critical underpinning science upon which the biomass conversion operations are based. This is an essential component of the program in that the application of this type of information greatly accelerates systematic improvements in the various unit operations".
  - Reviewer E: "A better understanding of the molecular mechanism of the cellulase enzyme supports the platforms overall objectives by producing data that can be used by genetic engineering of cellulase enzyme."

* Reviewers were given random ids here, not the same for each case.

Reviewers Comments from 2011

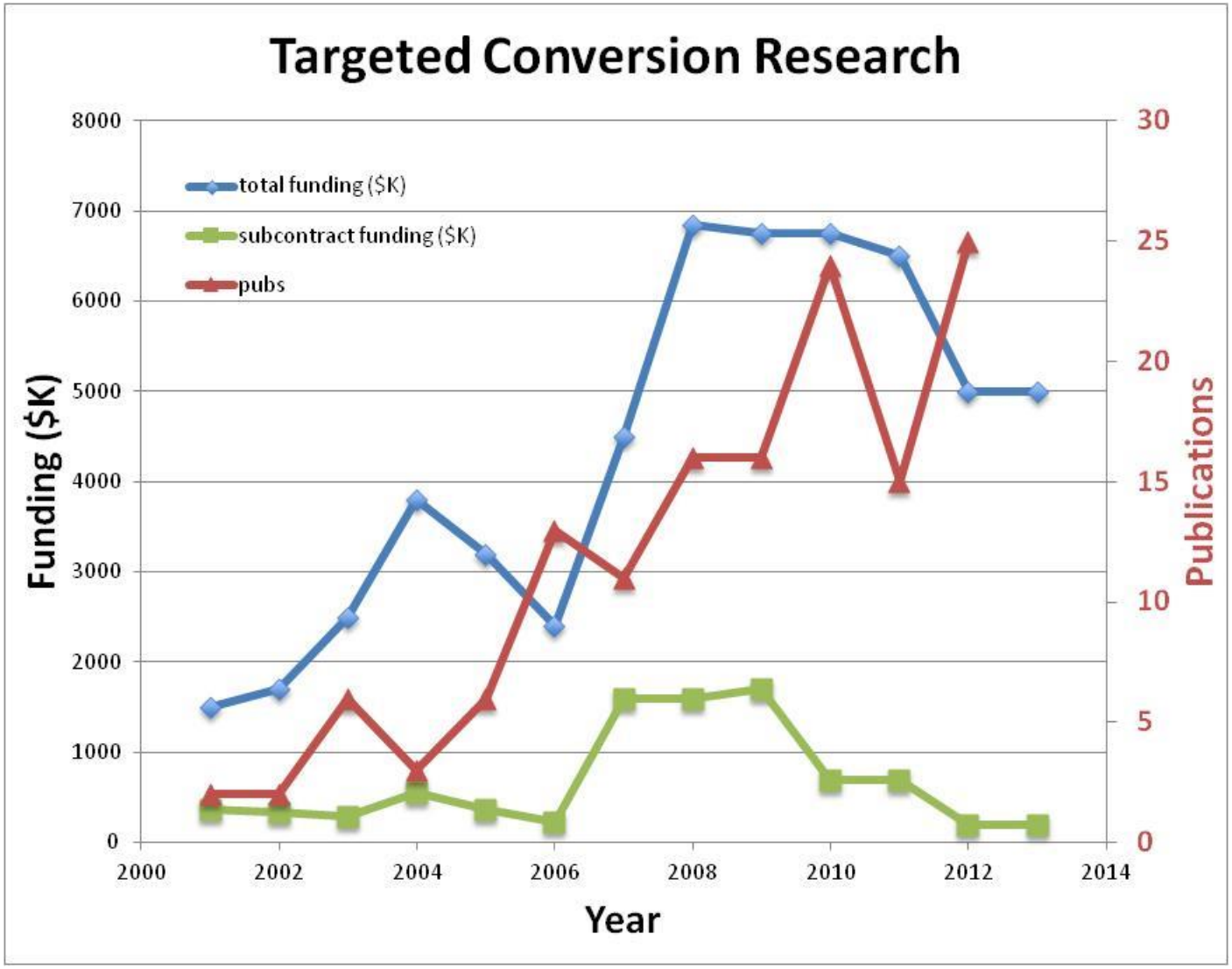
Critical Success Factors

- Reviewer A*: "Commercial success will be dependent upon the ability to translate the understanding these processes into application"
  - Reviewer B: "The major success factor is that of acquiring the appropriate information, and enough of it, to provide sufficient understanding upon which to make technological improvements. This is the nature of this project. The project has tremendous potential for advancing the state of technology; in fact, it seems an essential component of the Biochemical Platform if one expects to make systematic improvements based in gained knowledge."
  - Himmel: We have maintained close working relationships with the enzyme production companies as shown by an R&D 100 award with both Genencor and Novozymes in 2004. See the recent slide testimony from Novozymes in 2013 attached.

Overall Impressions

- Reviewer A: "The project brings recognition to the biomass program by demonstrating cutting edge analytical tools as they are applied to the program. What economic contributions it makes to the program are still to be seen."
  - Reviewer B: "This is a unique project within the Biochemical Platform in that its primary goal is to generate knowledge, rather than to improve a technology. However, the knowledge sought is to be directly applicable to biomass conversion technology improvements. This type of project is important to the program because it provides a means of addressing technical issues - that being through a better understanding of the fundamentals. The project is well managed and it is widely respected by those in the biomass conversion field. My impression is that many of the improvements that have resulted from Biochemical Platform efforts have been supported by this project."

General TCR Statistics



Cellulase Structure: SAXS and SANS

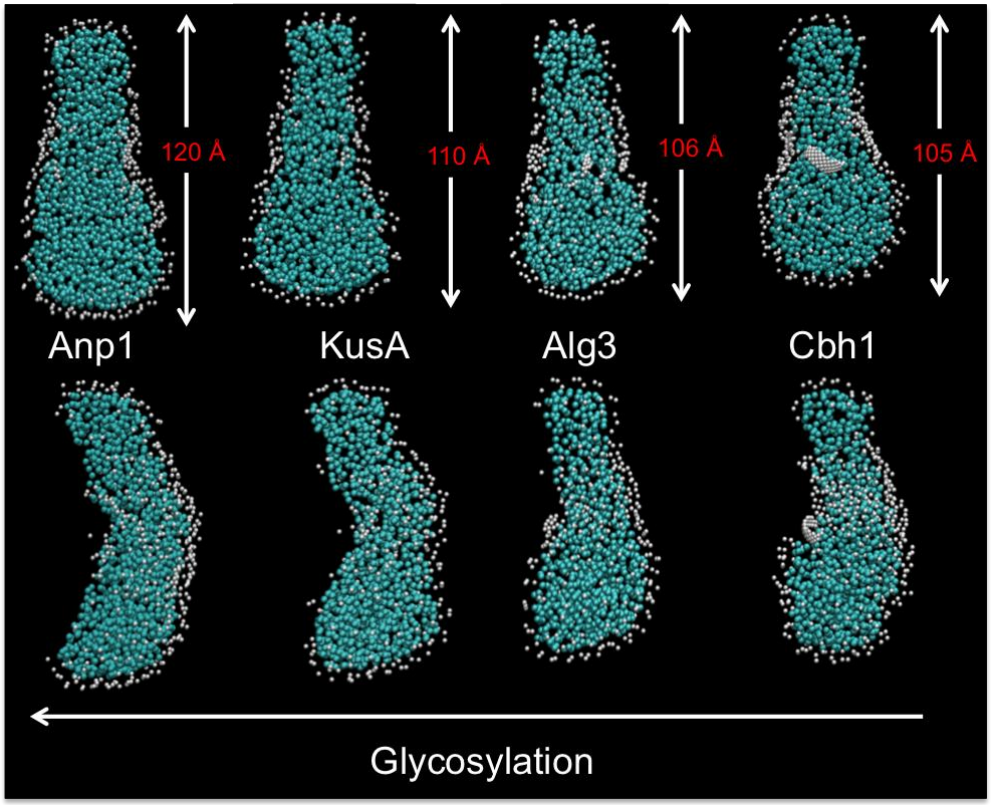
Relevance: D Milestone: Elucidate new structure-function relationships in GH7 enzymes via molecular simulations and bioinformatics studies for protein engineering purposes

Rationale

- Analyze the experimental results from SAXS and SANS with more accurate simulations than currently used in this field by improving molecular dynamics codes such as CHARMM.

Outcome

- Gain more insight on CBH I (Cel7A) mechanism, CBM/CD relationship, and effect of glycosylation.
- Complete these studies using small angle neutron scattering at ORNL allowing us to look at cellulases bound and digesting cellulose.



Sample Preparation- Multi-scale Microscopy*

Relevance: Real understanding of pretreatment and enzyme effects on biomass

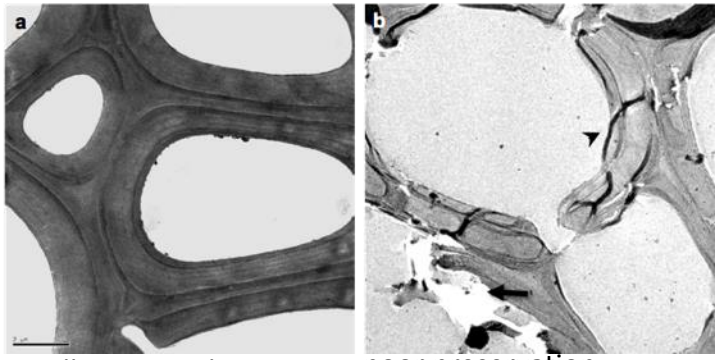
Rationale

- Understanding the structural changes caused by pretreatment, enzyme hydrolysis, or other processes can lead to better process design
  - Better sample preservation = better detailed understanding

Outcome

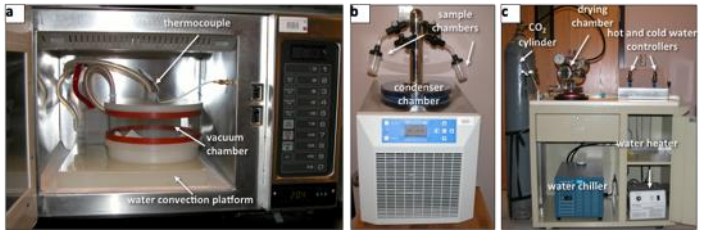
- Biomass requires size reduction such as dissection and microtomy to reveal features of interest for any microscopic and nanoscopic analyses.
  - Sectioning and microtomy are complicated by the inherent porosity of plant tissue that necessitates embedding in a supporting matrix to preserve structural integrity and prevent the introduction of artifacts.
  - The moisture content of biomass is dramatically reduced compared to living tissue, yet samples must still be properly dehydrated for microscopic techniques such as SEM.
- We compiled five years of research into a single methods chapter to serve as reference for the field to enable more effective and accurate multi-scale microscopic analysis.

*C3Bio Collaboration

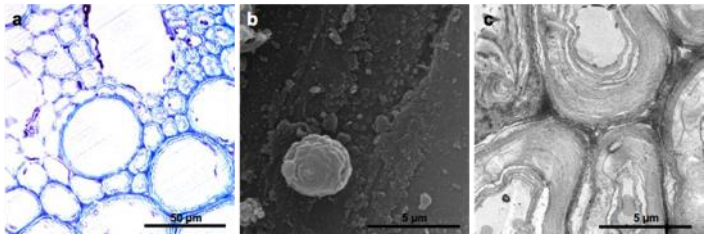


well preserved ultrastructure

poor preservation



biomass specific sample preparation protocols



accurate multi-scale microscopic analysis

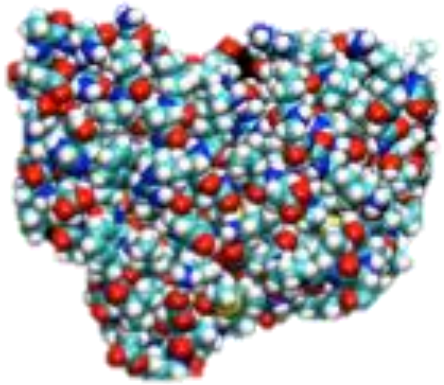
Donohoe, B. S., Ciesielski, P. N. & Vinzant, T. B. Preservation and Preparation of Lignocellulosic Biomass Samples for Multi-scale Microscopy Analysis. Biomass Conversion. 31–47 (Humana Press, 2012).

CHARMM Performance

Relevance: E Milestone: Determine the high resolution structures of three new glycoside hydrolases

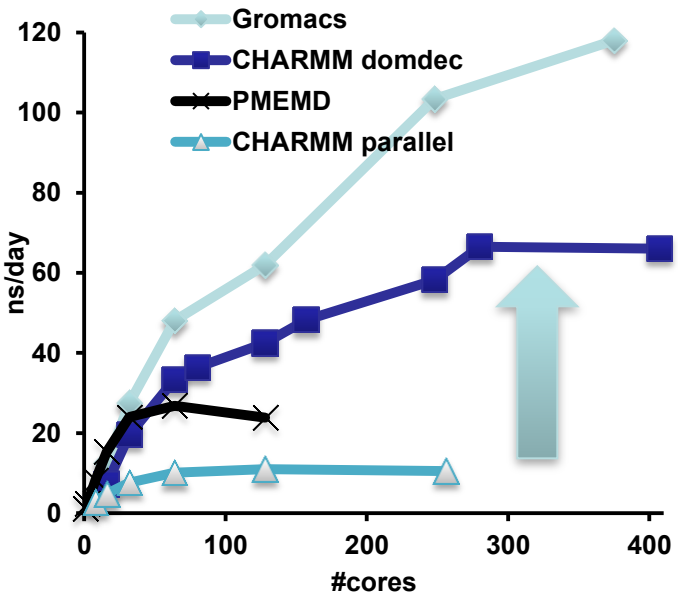
Rationale

- Use full power of HPC by improving performance of CHARMM and Amber.
- Add new algorithms and methods to existing toolkits.



Outcome

- Current parallel performance limits simulations to less than 10 ns per day on any supercomputer no matter how large.
- Improvements to underlying computational methods and parallel performance boosts output nearly 10-fold.
- Allows the power of methods only in CHARMM to be used with 10-fold more output, meaning 10 times the number of proteins that can be studied and 10 times the sampling for more accurate and converged thermodynamic quantities.



"New faster CHARMM Molecular Dynamics Engine" Antti-Pekka Hynninen, Michael Garrahan, Charles Brooks, and Michael F. Crowley, in preparation.

Enzyme and Substrate Modeling - Summary

Relevance: Develop predictive models to engineering more effect enzymes

Rationale

- Molecular and Quantum Mechanics modeling can be used to gain mechanistic understanding of biomass hydrolysis
- Experimental testing conducted in ABD

Outcome

- Determining *T. reesei* Cel7A and Cel6A mechanisms for processivity and hydrolysis
  - Recognition, binding, hydrolysis, expulsion, procession
  - Active site tunnel architecture
- Protein structure determination
- Enzyme-substrate interactions
- Substrate (cellulose) modeling
- Enzyme *O*- and *N*-linked glycosylation
- Product inhibition and substrate specificity