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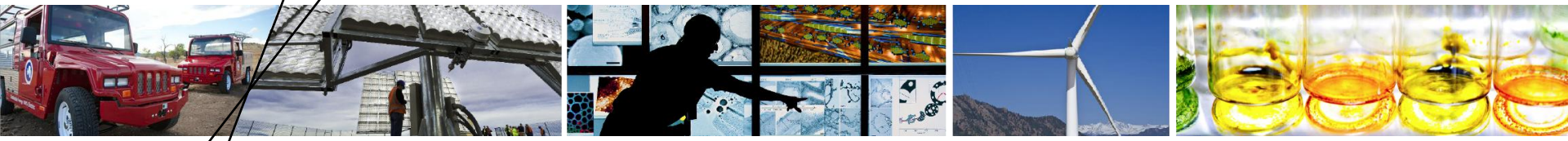
Enzyme Synergy and Cooperation in Biomass Conversion



Pacific Rim Summit on Industrial Biotechnology & Bioenergy

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11 October 2012

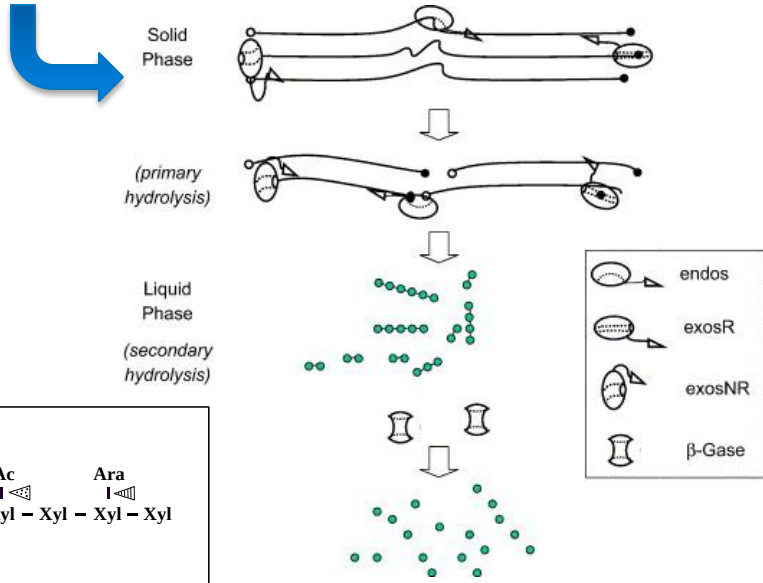
Problems in Biomass Enzyme Synergy

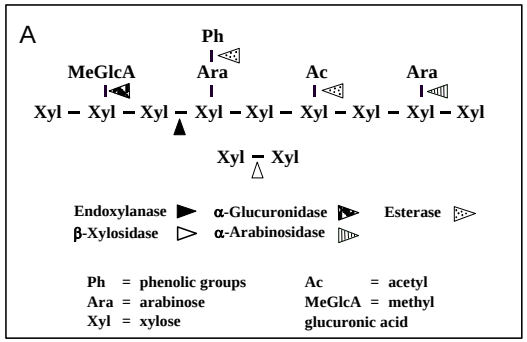
- **"Real" substrates are highly variable**
  - Different feedstocks, pretreatment chemistries and severities
    - Highly heterogenous
    - Variable particle size, surface area:volume ratios
- **Model substrates are also highly variable**
  - Cellulose
    - different levels of crystalline, paracrystalline, and amorphous content
      - Different crystalline isoforms
    - lignin and hemicellulose residuals
    - degree of polymerization
    - processing changes, etc.
  - "native" hemicelluloses are likely soluble
    - Most available xylans have been de-esterified
    - Hemicellulose varies greatly in structure and chemical composition
- **Complex enzyme systems**
  - Endo/exo/b-glucosidase/PMOs for cellulases
    - Multiple variants of each
  - More than a dozen activities involved in xylan hydrolysis
    - Endos, exos, debranching, accessory enzymes
- **Conflicts in literature**
  - No standardized assay methods, variable substrate composition determination, wide range of enzyme purities and sources
  - ***Synergy in biomass conversion has few hard and fast rules; studies should be evaluated primarily as stand-alone results***

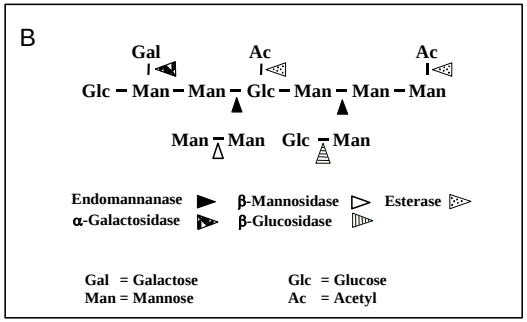
Types of Synergy

- **Recent Review**
  - Kostylev and Wilson. 2012. Synergistic interactions in cellulose hydrolysis. Biofuels. 3(1), 61-70
- **Cellulase-cellulase**
  - Endo-exo synergy
  - Exo-exo synergy
  - Cellulase-disruptor proteins
  - Exo-β-Glucosidase
- **Hemicellulase-accessory enzymes**
  - Solids
  - Soluble oligomers
- **Cellulase-Hemicellulase**
  - Cellulase-xylanase mixes
- **Enzyme-Pretreatment**
  - Targeted enzyme cocktails for pretreatment/feedstock combination
  - *in planta* enzyme expression
- **Cellulase-cellulase redux**
  - Fungal cellulase-cellulosomes

Zhang, Y-H. P., M. E. Himmel, J. R. Mielenz. 2006. Outlook for cellulase improvement: Screening and selection strategies. Biotech. Adv. 24 (5), pp. 452–481



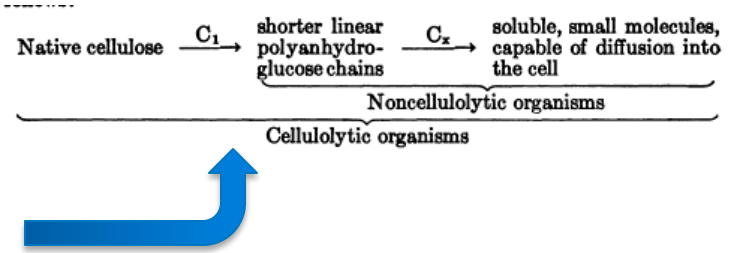


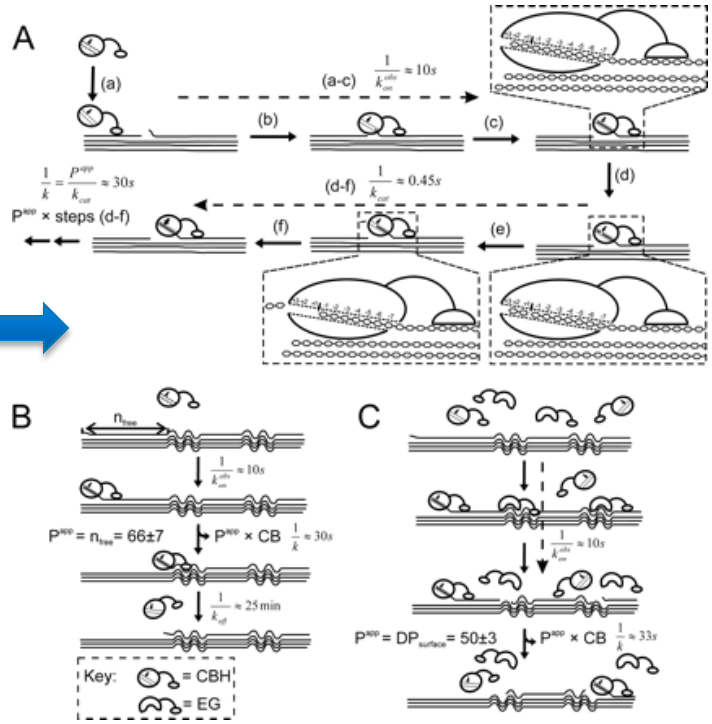


Decker, S. R., M. Siika-aho, and L. Viikari. 2008. Enzymatic Depolymerization of Plant Cell Wall Hemicelluloses". In: M. E. Himmel (ed). Biomass Recalcitrance. Blackwell Publishing, Oxford, UK.

Classical Cellulase Synergy

- **Endo-Exocellulase synergy**
  - Hinted at by Elwyn Reese et al. 60+ years ago
    - Reese E. T., Siu R. G., Levinson H. S. (1950) The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59, 485–497
    - C_1/C_x model: C_1 releases substrates for C_x
  - Classical synergy model- Wood and McCrae
    - T. M. Wood and S. I McCrae. 1979. Synergism between enzymes involved in the solubilization of native cellulose. Advances in chemistry. 181:181-209.
      - Endos (C_x)create new chain ends for exos
        - » # chain ends is rate-limiting
      - Exos (C_1)disrupt crystalline regions, exposing more sites for endos
  - Recently revisted by Jürgen Jalak et al.
    - J. Jalak, M. Kurasin, H. Teugjas, P. Väljamäe. 2012. Endo-exo Synergism in Cellulose Hydrolysis Revisited. J Biol Chem. 17:287:28802-15
    - Endocellulases increase exocellulase average processivity
      - k_{off} for cel7A is rate- limiting
      - Removes amorphous regions which stall exos
      - Remove single chains from surface





Exo-Exo Cellulase Synergy

- **Different exocellulases attack different ends**
  - Proposed by Fagerstam and Pettersson in 1980
    - Fagerstam, L. and L. Pettersson. 1980. The 1,4-b-glucan cellobiohydrolases of Trichoderma reesei QM9414. An new type of cellulolytic synergism. FEBS Lett. 119(1), 97-100.
  - Demonstrated by Barr *et al* in 1996
    - Barr, B. K., Y. L. Hsieh, B. Ganem, D. B. Wilson. 1996. Identification of two functionally different classes of exocellulases. Biochem. 35(2), 586-592
    - Reducing vs. non-reducing ends
      - GH7s are reducing end specific, GH6s are non-reducing end specific
  - Insight from ultra-high speed AFM
    - Igarashi, K., T. Uchihashi, A. Koivula, M. Wada, S. Kimura, T. Okamoto, M. Penttilä, T. Ando, and M. Samejima. 2011. Traffic Jams Reduce Hydrolytic Efficiency of Cellulase on Cellulose Surface. Science 333 (6047) pp. 1279-1282
    - Demonstrated cel7A is processive and peels layers
      - Gets stuck and stalls on surface
    - Cel6A frees stuck cel7A
      - Supports Jalak *et al* model

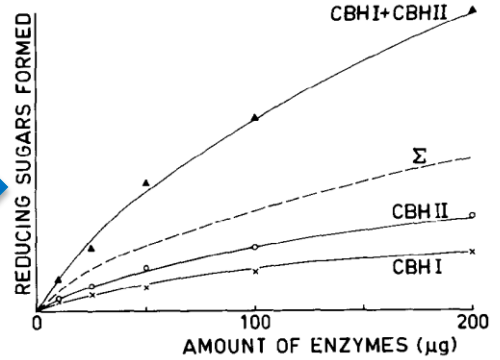
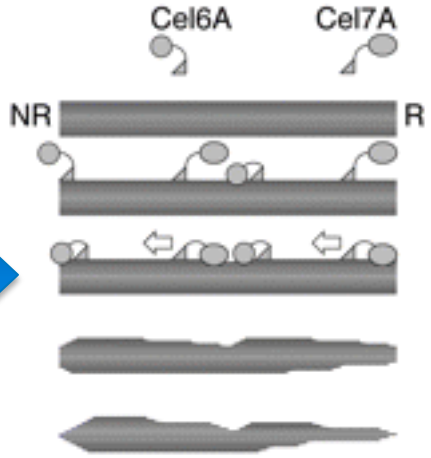
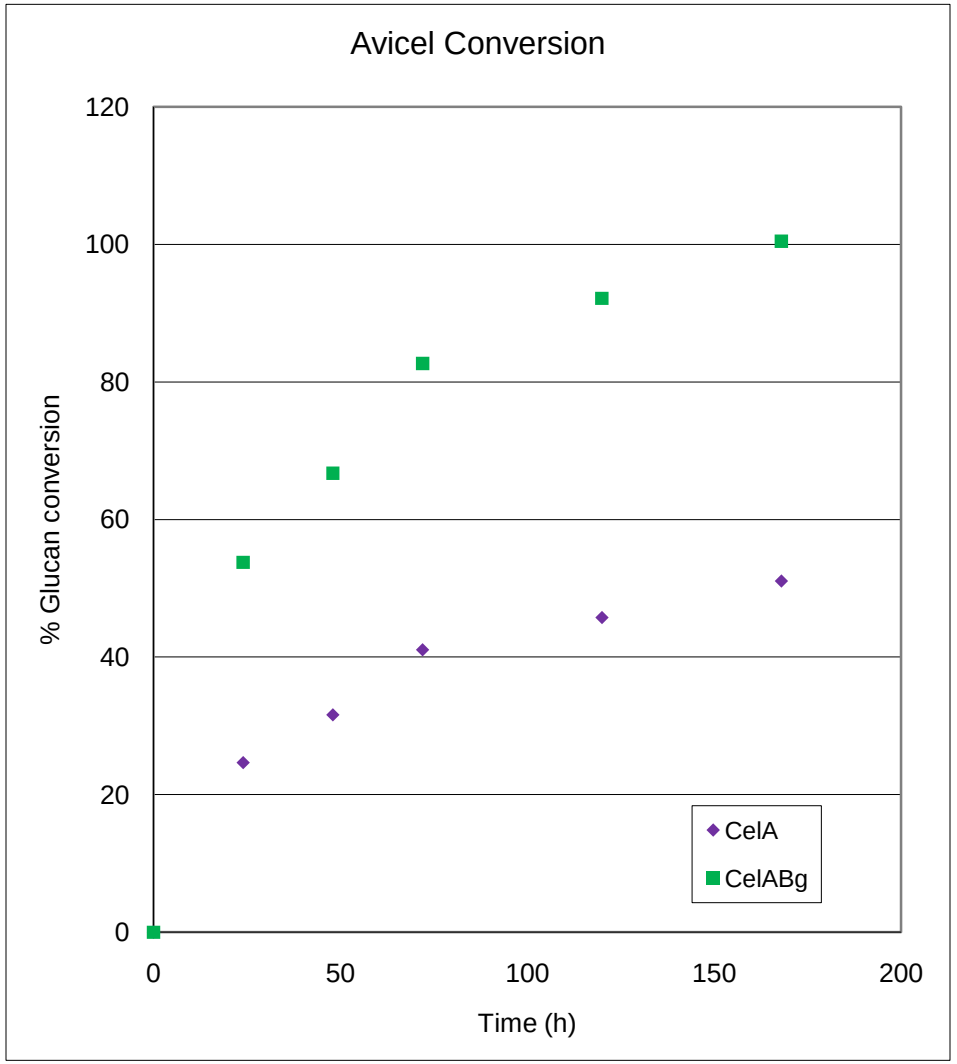


Fig.2. Synergism towards crystalline cellulose. CBH I and CBH II were incubated alone and in combination with 1% Avicel as substrate. The samples were incubated for 1 h at 40°C in 50 mM sodium acetate buffer (pH 5.0). The non-synergistic sum of the activities of CBH I and CBH II is indicated by Σ.



Cellulase-β-glucosidase Synergy

- **Generally thought to be due to end-product (cellobiose)inhibition of cellulase**
  - Usually cel7A or bacterial analog
- **Typical experiment shows increased digestion**
  - CelA from *Caldicellulosiruptor bescii*
    - 15 mg/g cellulose
  - β-G from *Thermotoga maritima*
    - 1 mg/g cellulose
  - Similar to many other cellulase/β-G combinations
  - 1.5% avicel

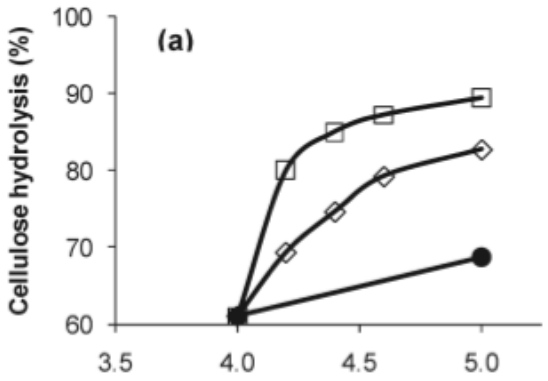


A New Cellulose Hydrolysis Mechanism

Biochemistry 2010, 49, 3305–3316

Stimulation of Lignocellulosic Biomass Hydrolysis by Proteins of Glycoside Hydrolase Family 61: Structure and Function of a Large, Enigmatic Family†

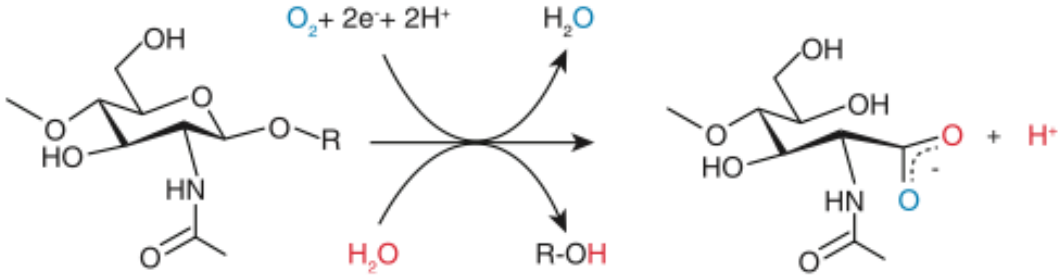
Paul V. Harris,*,‡,// Ditte Welner,§,// K. C. McFarland,‡ Edward Re,‡ Jens-Christian Navarro Poulsen,§ Kimberly Brown,‡ Rune Salbo,§ Hanshu Ding,‡ Elena Vlasenko,‡ Sandy Merino,‡ Feng Xu,‡ Joel Cherry,‡ Sine Larsen,§ and Leila Lo Leggio§

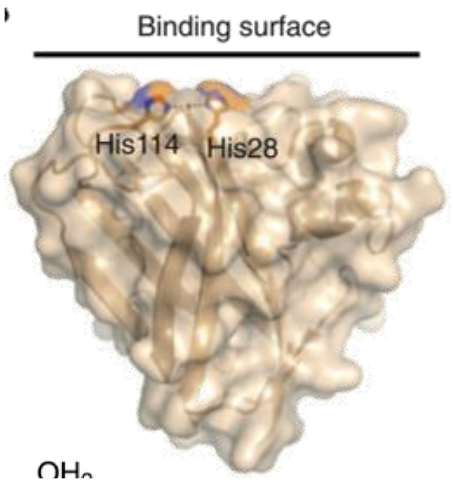


7 months later:

An Oxidative Enzyme Boosting the Enzymatic Conversion of Recalcitrant Polysaccharides

Gustav Vaaje-Kolstad, Bjørge Westereng, Svein J. Horn, Zhanliang Liu, Hong Zhai, Morten Sørlie, Vincent G. H. Eijsink*

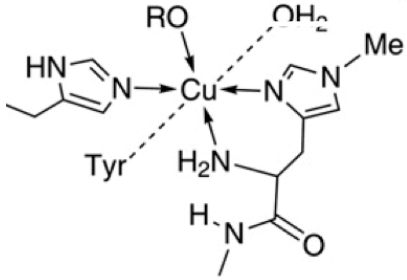




10 months later:

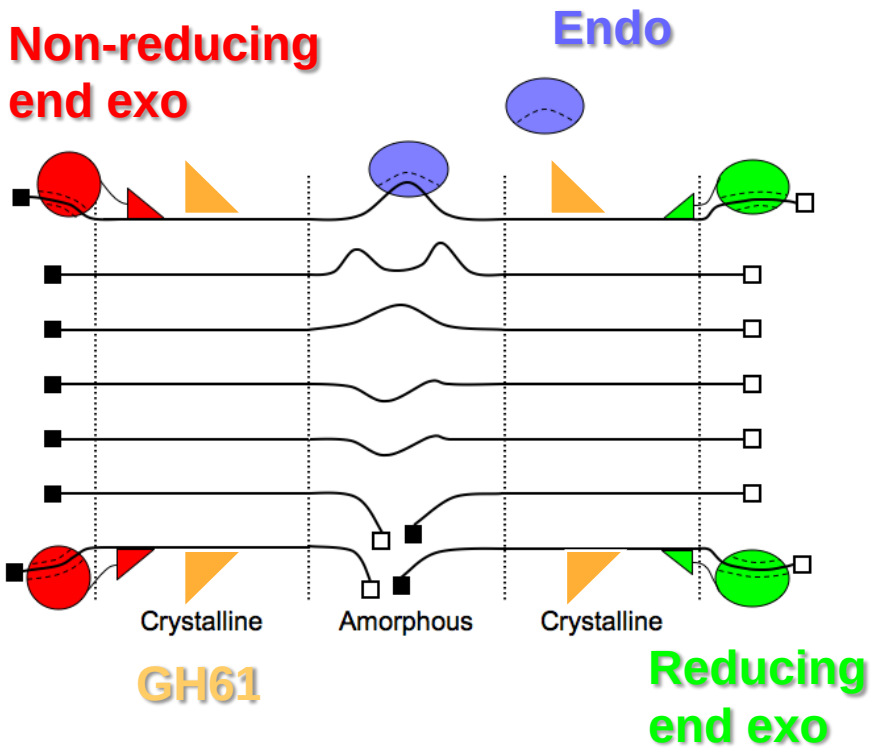
Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components

R. Jason Quinlan[a,1], Matt D. Sweeney[a,1], Leila Lo Leggio[b], Harm Otten[b], Jens-Christian N. Poulsen[b], Katja Salomon Johansen[c,2], Kristian B. R. M. Krogh[c], Christian Isak Jørgensen[c], Morten Tovborg[c], Annika Anthonsen[c], Theodora Tryfona[d], Clive P. Walter[c], Paul Dupree[d], Feng Xu[a], Gideon J. Davies[e], and Paul H. Walton[e]



Where does GH61 fit in Cellulose Hydrolysis?

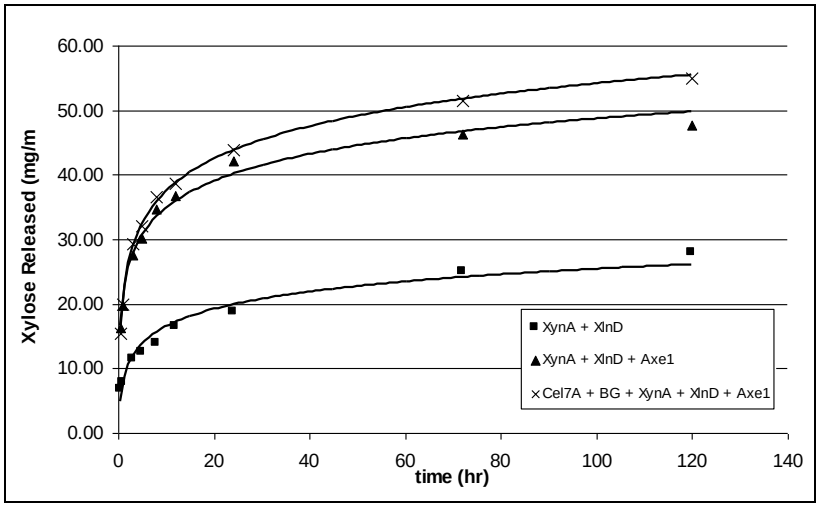
- **GH61 likely acts to remove cellulose surface aberrations**
  - Analogues to endos or cel6A
  - May initiate new binding sites for exos
- **Many questions remain including:**
  - What is the mechanism of GH61 action?
  - What residues are important for specificity?
  - What other substrates can GH61s act on?
  - How do GH61s behave on cellulose?
  - What is the structural diversity of GH61s?

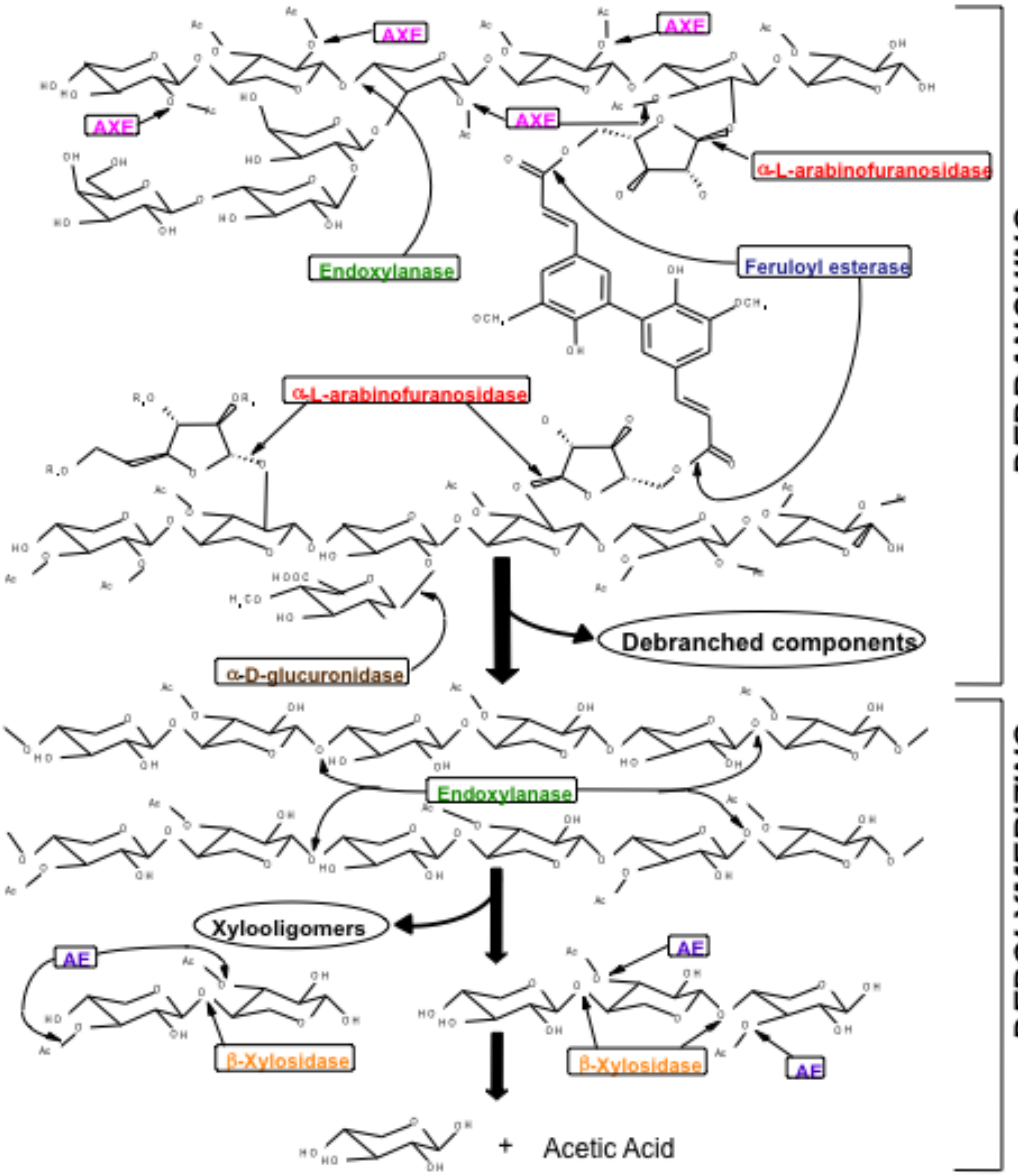


M. Wu, G.T. Beckham, A.S. Larsson, S. Horn, B. Westereng, S. Kim, M.F. Crowley, M.E. Himmel, C.M. Payne, V.G.H. Eijsink, J. Ståhlberg, M. Sandgren

Synergy in Hemicellulose Hydrolysis

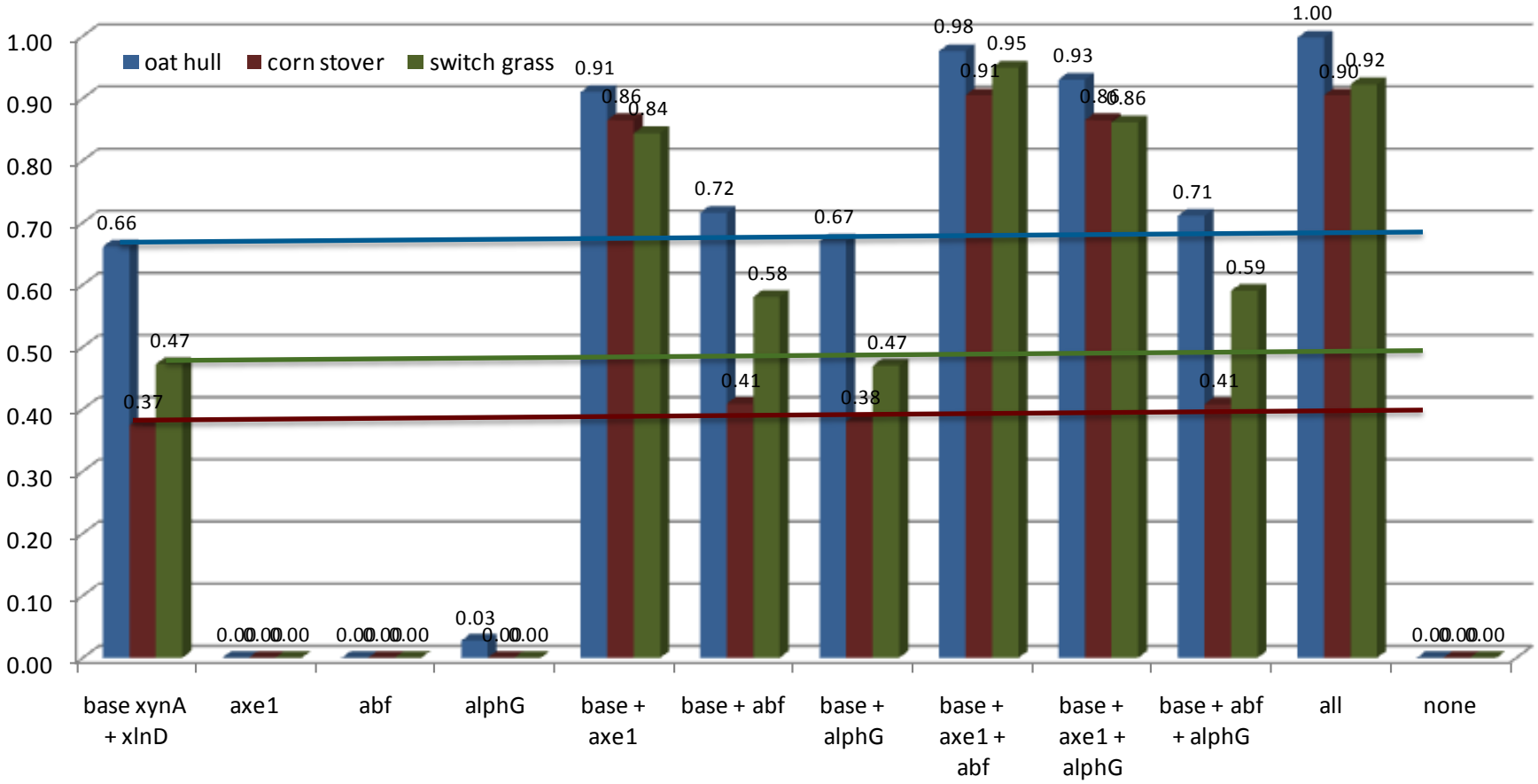
- **Hemicellulose complexity requires a variety of enzyme classes**
  - Hardwoods vs. softwoods vs. herbaceous
- **Native hemicellulose is thought to be "soluble"**
  - Extracted with ester-links intact, it is
  - Side chains prevent intermolecular association and retain water
  - Debranching leads to insoluble backbone
    - Slower enzyme binding kinetics
- **Not really synergy, as enzymes release different products**
  - Cooperativity is closer





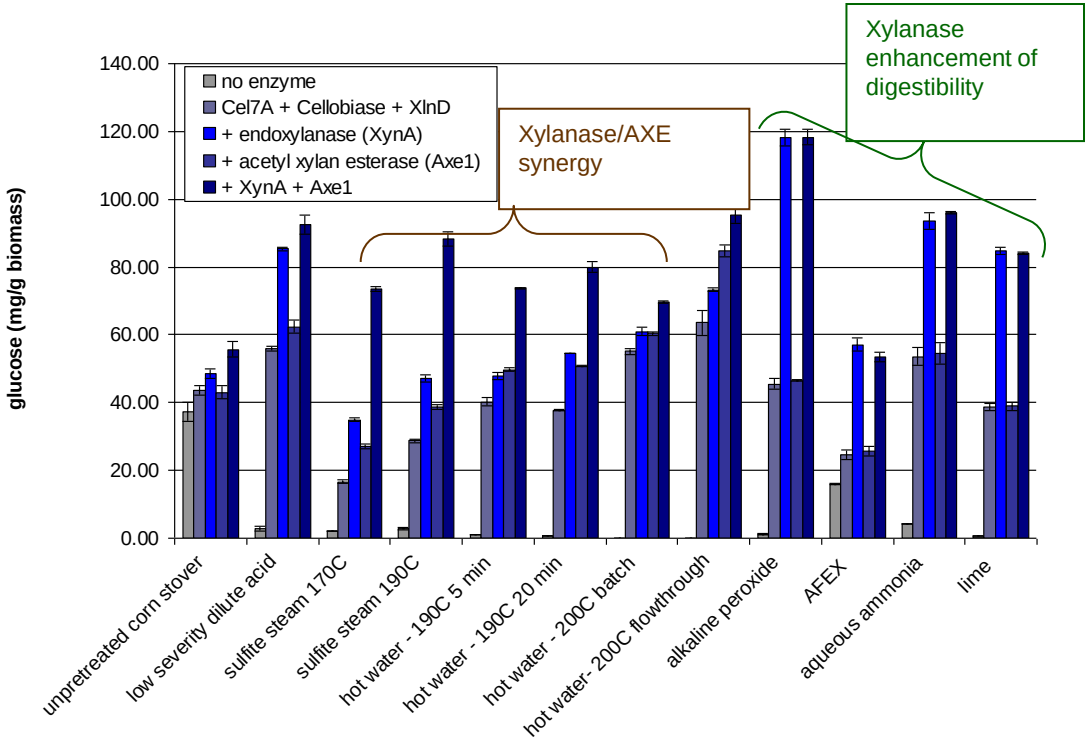
Digestion of Natively Extracted (DMSO) Xylans

- **Cooperativity of accessory enzymes mixes on purified xylans**
  - xynA/xlnD + abfB, glrA, axe1



Enzyme Synergy in Pretreatment Solids

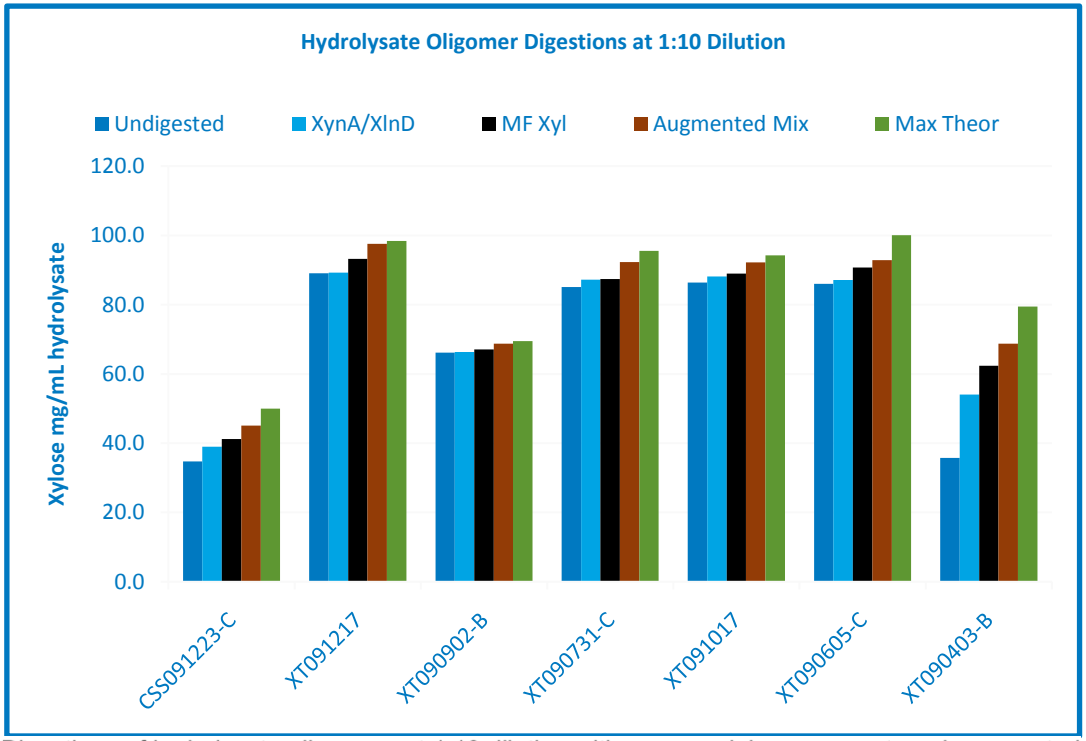
- **Xylanase enhances glucose release**
  - Xylanase/AXE synergy on SSE/low severity HW
  - No AXE synergy- alkaline de-esterification
- **Cellulase enhances xylose release**
  - Sulfite steam exploded corn stover-low severity
- **Improvements in xylan and glucan conversion continue over long digestions**
  - Synergism improves rates
  - Synergism improves extent of conversion
  - Improved accessibility to target structures
- **Can lower enzyme loading for given severity**
  - DA-180°C, 40 →15 mg prt/g cellulose



Selig, M. J., W. S. Adney, M. E. Himmel and S. R. Decker. (2009). The Impact of Cell Wall Acetylation on Corn Stover Hydrolysis by Cellulolytic and Xylanolytic Enzymes. Cellulose. 16:711-722

Enzyme Synergy on Hydrolysate Oligomers

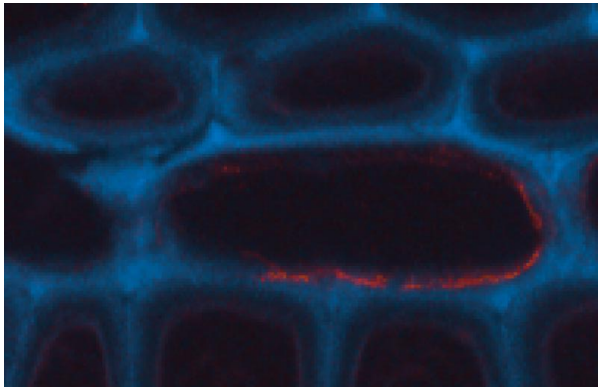
- **End product inhibition is significant**
  - enzymes are not capable of digesting all of the oligomers and solids to completion in SSF.
- **Commercial xylanases lack critical activity(s)**
  - □-xylosidase, AXE, □-glucuronidase, and/or □-L-arabinofuranosidase.
  - Even high-xylose yield pretreatments can benefit from additional enzyme activities
- **Endoxylanase and □-xylosidase can be as effective as a commercial enzyme prep.**
  - Some xylanases are deficient in debranching/accessory enzymes

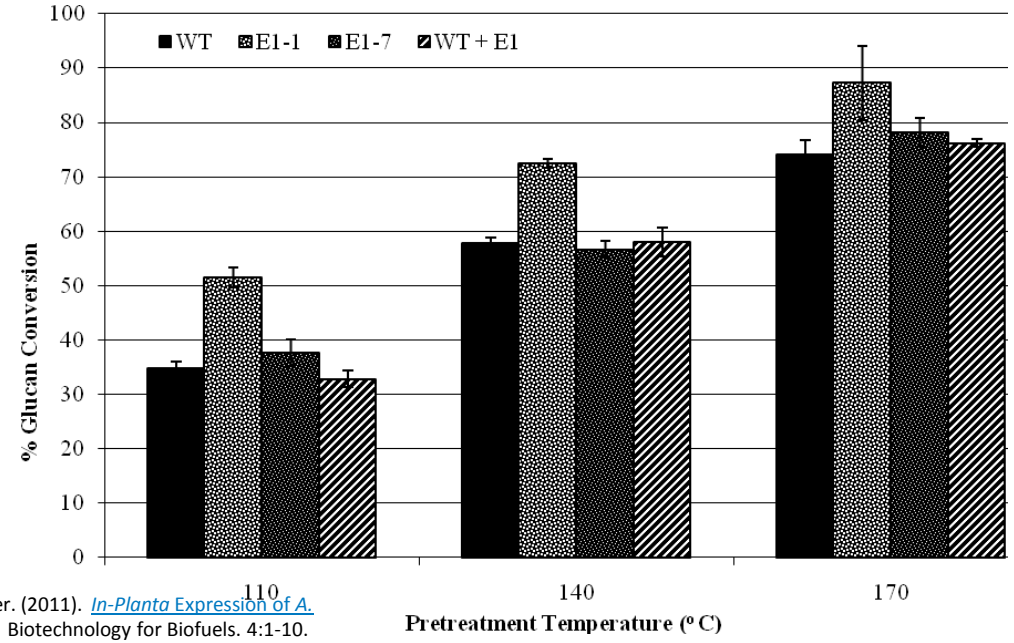


Digestions of hydrolysate oligomers at 1:10 dilution with commercial, component, and augmented commercial xylanases. XynA/XlnD was a mix of XynA (10 mg per g oligomer) and XylD (1 mg per g oligomer). MFXyl was Multifect Xylanase loaded at 10 mg per g oligomer. The Augmented Mix The augmented mix consisted of MF Xylanase (20 mg/g oligomer), AXE (5 mg/g), abf (5 mg/g), α-glucuronidase (2.5 mg/g), and β-xylosidase (1 mg/g). Conversion is corrected to undiluted sample.

“Other” Enzyme Enhancements

- **Plant expression of cellulase**
  - Old idea
    - enzyme production
    - Post-harvest digestion
  - New idea
    - Specifically weaken cell wall to pretreatment
    - Lower pretreatment severity and/or decrease enzyme loading
  - Expressed cel5A in corn
    - Pretreated with dilute acid at several temperatures
    - E1-stover was consistently more digestible than WT





Brunecky, R., M. J. Selig, T. B. Vinzant, M. E. Himmel, D. Lee, M. J. Blaylock and S. R. Decker. (2011). *In-Planta* Expression of *A. cellulolyticus* Cel5A Endocellulase Reduces Cell Wall Recalcitrance in Tobacco and Maize. Biotechnology for Biofuels. 4:1-10.

Summary

- **Endo-exo and exo-exo cellulase synergies are likely due to prevention of cel7A stalling**
  - GH61 may act similarly
- **Cellulase - β-glucosidase synergy is likely due to end-product inhibition relief**
  - Similar effect in xylanase - β-xylosidase
- **Hemicellulase – accessory enzyme synergy is in part a function of maintaining backbone chain solubility**
  - Debranching can lead to insoluble chains
  - Synergy in oligomer conversion can enhance yield
- **Free cellulases and cellulosomes are synergistic**
  - Only on cellulose, not biomass
  - Mechanism is not understood
- ***Enzyme synergy in biomass conversion is complicated***

Acknowledgements

- Mike Himmel- our fearless leader
- John Baker- enzyme kinetics
- Mike Selig- hemicellulase assayer, temporary Canadian
- Mike Resch- cellulosome and GH61 biochemistry
- Bryon Donohoe- TEM imaging
- Todd Vinzant- pretreatment
- Gregg Beckham- GH61 research coordinator
- Roman Brunecky- bacterial enzyme kinetics