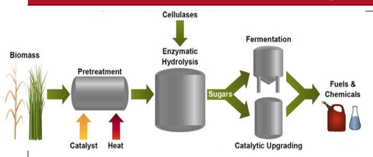
Novel Plant Biomass Pretreatments For Enhancing Biofuel Production

Mr. Shashwat Gupta, Mr. Ketley Alves, Ms. Marissa Berger
Prof. Shishir Chundawat (Faculty Advisor)
Glycans, Glycoconjugates, and Glycan Active Proteins/Enzymes Engineering Laboratory (G^3EL), Department of Chemical and Biochemical Engineering, Rutgers University

Abstract

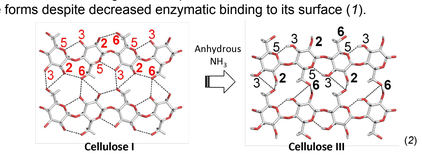
Plants are a renewable resource suited for energy production and fuel needs; however, the conversion of lignocellulosic biomass to fuels is hindered by plant recalcitrance to sugar extraction. Cellulose is the most abundant organic molecule in plant cell walls that, in its natural cellulose-I form, remains resistant to enzymatic hydrolysis, the breaking down of sugars. Transforming cellulose-I to cellulose-III through ammonia-based treatment decreases hydrolytic resistance, yet the amount of enzymes needed to achieve high sugar yields remains costly. By analyzing and comparing the yields of the hydrolysis of untreated and treated cellulose-I and III and corn stover samples through DNS assay, one can determine the most cost-efficient pretreatment. Results from the assays at various loadings of C.Tec2 enzyme confirm the increased hydrolytic activity of C-III versus C-I and of the pretreated Avicel samples versus C-I. Surprisingly, these data indicate a lower recalcitrance of the Avicel 25-AT (treated with ammonium thiocyanate at 25°C) samples versus the Avicel 50-AT (treated at 50°C). Corn stover responds similarly, but overall yields for CS are lower than for cellulose. With this data, there is a potential for more cost- and energy-efficient recalcitrance treatment of plant biomass by optimizing the treatments for lower protein loadings.

Background



Overview to integrated cellulosic biomass pretreatment, biochemical and catalytic conversion of cellulosic biomass to fuels and chemicals. Figure adapted from Payne et al, Fungal Cellulases. Chem Rev 2015, 115 (3):1308-1448

- Cellulose-I: cellulose Iβ and cellulose Iα are the predominant forms of cellulose in higher plants and primitive microorganisms respectively. The cellulose Iβ in plants can be transformed into other allomorphic forms through thermochemical treatment.
- Cellulose-III: cellulose III is a nonnative form of cellulose formed through treatment with amines or ammonia. It demonstrates significant improvement in recalcitrance versus cellulose Iβ and other nonnative forms despite decreased enzymatic binding to its surface (*1*).



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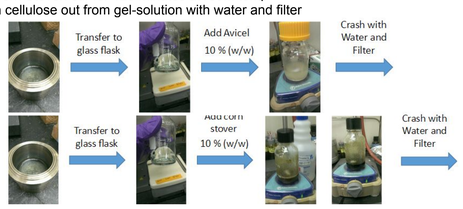
- C.Tec2: Cellic C.Tec2 is an enzyme cocktail composed of various cellulases, hemicellulases, and β-glucosidases that specializes in the breakdown of cellulose into fermentable sugars. The enzyme is advantageous in that less can be used for higher sugar yields, and it operates optimally around a pH of 5. (*3*)
- Corn stover: 38% (of dry mass) cellulose, 26% hemicellulose, 19% lignin, 4% acid detergent lignin, 5% crude protein, 6% ash (*4*)
- Novel Ammonia-Ammonium Thiocyanate Pretreatment (*5*)
  - Prepare Pretreatment Solvent Mix (72.1 %(w/w) NH_4SCN, 26.5% (w/w) NH_3, 1.4% (w/w) H_2O)
  - Transfer to a glass flask and add 10% Avicel or corn stover (dry weight basis)
    - Cellulose: stir for 60 minutes at room temp
    - Corn Stover: stir for 120 minutes at room temp
  - Crash cellulose out from gel-solution with water and filter



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Hypothesis: Optimally varying biomass pretreatment conditions can produce a pretreated substrate suited towards efficient hydrolysis by commercially available cellulases at low protein loadings.

Methods and Materials (Part 1)

X-Ray Diffraction

Philips XPert Powder Diffractometer

15 Sample Magazine

2-200 mg Powder Samples

Si Sample

*Si sample used for calibration

$$n\lambda = 2d\sin(\theta)$$

*d = spacing between layers of atoms
*n = integer
*λ = wavelength of X-Ray
* θ = angle of incidence

$$B(2\theta) = K\lambda/(L\cos\theta)$$

*K = shape factor
*B = Peak Width
*λ = wavelength of X-Ray
*L = crystallite size
* θ = angle of diffraction

References

1. Gao, D, Chundawat, SPS, Sethi, A, Balan, V, Gnanakaran, S, Dale, BE (2013). Increased enzyme binding to substrate is not necessary for more efficient cellulose hydrolysis. *Proc Natl Acad Sci U S A* 110:10922-10927.
2. Chundawat SPS, Bellesia G, Uppugundla N et al (2011). Restructuring the crystalline cellulose hydrogen bond network enhances is depolymerization rate. *J Am Chem Soc* 133:11163-11174.
3. SHEET, A. Cellic® CTec2 and HTec2-Enzymes for hydrolysis of lignocellulosic materials.
4. Lee, D., Owens, V., Boe, A., & Jeranyama, P. (2007). Composition of Lignocellulosic Biomass Feedstock Resources. In *Composition of herbaceous biomass feedstocks*. Brookings, South Dakota: North Central Sun Grant Center, South Dakota
5. Holcomb, L. *Ammonium Thiocyanate Experiment* [PowerPoint Slides]. Retrieved from https://docs.google.com/presentation/d/1nJ15T6yXXOv28Ikzi_9Rk9d4S2Y-WaJLCPQxQkrvVN4/edit#slide=id.p3.

Methods and Materials (Part 2)

Determination of Total Solids in Biomass

0.5 g Biomass	
Aluminum Weighing Pan	
Analytical Balance	
Oven set to 105°C ± 1°C	

*% total solids of the biomass is determined for dry weight conversion

Biomass Enzymatic Hydrolysis

Biomass, dry weight basis	1M Sodium Citrate Buffer
Weighing Paper	20 g/L Sodium Azide
Analytical Balance	Water
20 mL glass vials	C.Tec2 Enzyme diluted in 50 mM Sodium Citrate
Incubator set at 50°C ± 1°C	

*sodium citrate (pH 4.5) prevents large pH changes
*sodium azide deters microbial contamination
*C.Tec2 breaks down the carbohydrate polymers into sugars like glucose

DNS Reducing Sugar Assay

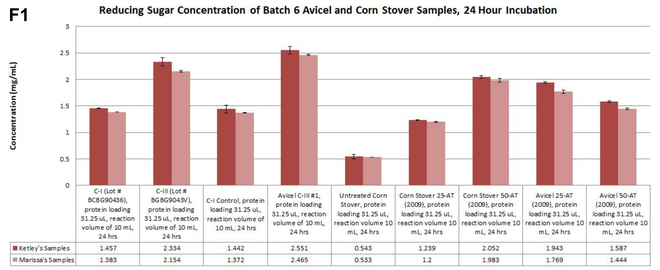
PCR tubes	PCR Thermal Cycler, incubation of 95°C for 5 min., 10°C for 10 min.
96-well PCR plate	
96-well microplate	Centrifuge
Dinitrosalicylic acid (DNS)	Spectrophotometer, reading at 540 nm

*DNS turns the product red in the presence of reducing sugars
*Samples read against standard curve to determine their concentrations

Enzymatic Hydrolysis Results

Figure 1 shows the measured reducing sugar concentrations of treated and untreated cellulose and corn stover samples after 24 hours shaking incubation at 50°C ± 1°C. Sugar concentrations were obtained through DNS assay and samples through hydrolysis. **Both cellulose and corn stover show higher concentrations for treated samples but reverse trends for the 25-AT versus 50-AT treatments. Cellulose samples yield higher concentrations of reducing sugars than the corn stover samples at all treatment levels with the exception of the 50-AT.** All samples were spun down before DNS assay, and only the supernatant was taken. The untreated CS, C-I, and C-III samples acted as positive controls.

F1



	C-I (Lot # BCBG9046J), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-III (Lot # BCBG9043V), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-I Control, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	Avicel C-III #1, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	Untreated Corn Stover, protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Corn Stover 25-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Corn Stover 50-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 25-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 50-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs
Ketley's Samples	1.457	2.334	1.442	2.551	0.543	1.239	2.052	1.943	1.587
Marissa's Samples	1.383	2.154	1.372	2.465	0.533	1.2	1.983	1.769	1.444

Figure 2 shows the percent glucan conversion to glucose of treated and untreated cellulose and corn stover samples after 24 hours shaking incubation at 50°C ± 1°C. Percentages are based on the assumption that the samples are 100% glucan, though the corn stover samples are in actuality less and therefore results are not accurate. Samples were measured based on dry weight; however, the % total solids of all the treated Avicel and corn stover samples were estimated to be similar to those of C-III and the untreated CS respectively and not actually measured. **Avicel 25-AT is seen to be the closest in % glucan conversion to the C-III.**

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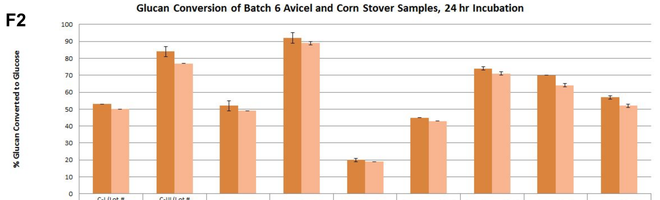
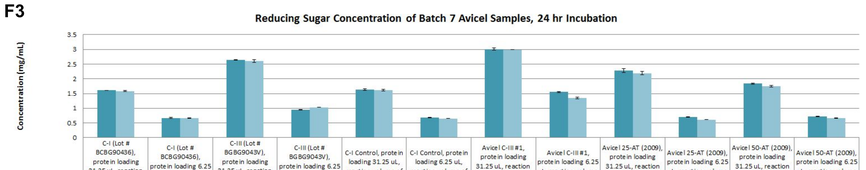


	C-I (Lot # BCBG9046J), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-III (Lot # BCBG9043V), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-I Control, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	Avicel C-III #1, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	Untreated Corn Stover, protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Corn Stover 25-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Corn Stover 50-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 25-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 50-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs
Ketley's Samples	53	84	52	92	20	45	74	70	57
Marissa's Samples	50	77	49	89	19	43	71	64	52

F3



	C-I (Lot # BCBG9046J), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-I (Lot # BCBG9046J), protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	C-III (Lot # BCBG9043V), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-III (Lot # BCBG9043V), protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	C-I Control, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-I Control, protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	Avicel C-III #1, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	Avicel C-III #1, protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	Avicel 25-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 25-AT (2009), protein loading 6.25 uL, reaction volume 10 mL, 24 hrs	Avicel 50-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 50-AT (2009), protein loading 6.25 uL, reaction volume 10 mL, 24 hrs
Ketley's Samples	1.608	0.671	2.638	0.947	1.642	0.677	3.009	1.55	2.277	0.701	1.834	0.719
Marissa's Samples	1.586	0.658	2.612	1.034	1.608	0.654	2.993	1.354	2.191	0.616	1.743	0.664

Figure 3 shows the measured concentrations of treated and untreated cellulose samples at protein loadings of 31.25 µL and 6.25 µL after 24 hours shaking incubation at 50°C ± 1° C. **All samples show lower final concentrations for the lower protein loading versus the higher protein loading.** All vials held the same total volume (10 ml) and dry weight (25 mg) for each sample.

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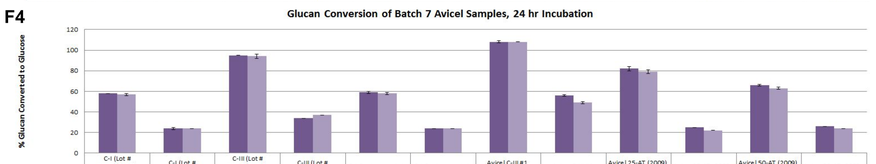

	C-I (Lot # BCBG9046J), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-I (Lot # BCBG9046J), protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	C-III (Lot # BCBG9043V), protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-III (Lot # BCBG9043V), protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	C-I Control, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	C-I Control, protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	Avicel C-III #1, protein loading 31.25 uL, reaction volume of 10 mL, 24 hrs	Avicel C-III #1, protein loading 6.25 uL, reaction volume of 10 mL, 24 hrs	Avicel 25-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 25-AT (2009), protein loading 6.25 uL, reaction volume 10 mL, 24 hrs	Avicel 50-AT (2009), protein loading 31.25 uL, reaction volume 10 mL, 24 hrs	Avicel 50-AT (2009), protein loading 6.25 uL, reaction volume 10 mL, 24 hrs
Ketley's Samples	58	24	95	34	59	24	108	56	82	25	66	26
Marissa's Samples	57	24	94	37	58	24	108	49	79	22	63	24

Figure 4 shows the percent glucan conversion to glucose of treated and untreated cellulose samples at protein loadings of 31.25 µL and 6.25 µL after 24 hours shaking incubation at 50° C ± 1°C. **The Avicel 25-AT remains the closest to achieving the same percent conversion as C-III and is higher in this percent than the Avicel 50-AT.**

X-Ray Diffraction Results

Figure 5 shows the X-Ray Diffraction (XRD) patterns for untreated corn stover, 25-AT treated corn stover, 50-AT treated corn stover, and cellulose-I. **Both the 25-AT (purple) and 50-AT (blue) treated CS are more crystalline in structure than the untreated CS (green), with the 25-AT being the most crystalline. All corn stover samples are less crystalline than cellulose-I (red).**

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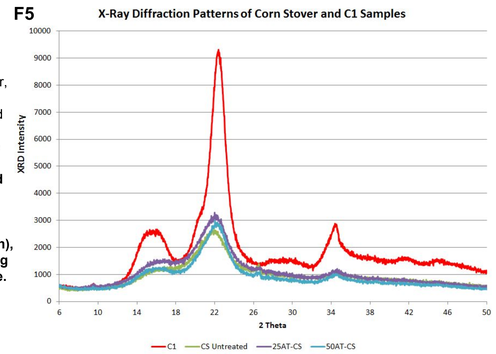
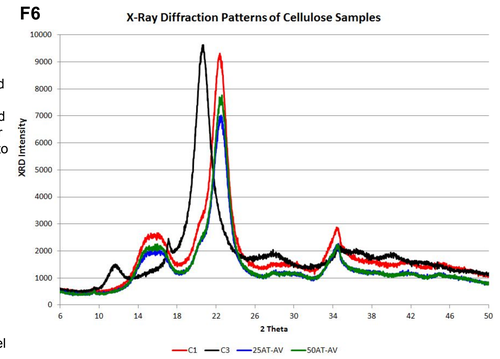


Figure 6 shows the XRD data for cellulose-I, cellulose-III, 25-AT treated cellulose, and 50-AT treated cellulose. The treated samples have similar atom arrangements to the C-I (red), but all three differ from the C-III (black) structurally. **The treated Avicel samples appear to have lowered crystallinities as compared to C-I.** Avicel 50-AT (blue) remains more crystalline than Avicel 25-AT (purple).

F6



Conclusions

- **Isolated cellulose is less resistant to enzymatic hydrolysis than corn stover and is a more optimal biomass for sugar production.**
  - At high protein loadings of 31.25 uL, cellulose-I and cellulose-III produce higher concentrations of reducing sugars than corn stover with the exception of the Avicel 50-AT versus the CS 50-AT.
  - Higher percentages of the glucan in cellulose were converted to glucose than in corn stover.
- **Pretreating biomass with novel ammonia-ammonium thiocyanate solvent mixture led to higher sugar yields**
- Higher protein loadings yielded higher sugar production/concentrations for both treated and untreated cellulose
- **Increasing hydrolysis yields were obtained for the samples tested in the following order; C-I<50-AT<25-AT<C-III**
  - The 25-AT treatment is closest to achieving sugar yields near that of cellulose-III and suggests future optimization of pretreatment conditions is necessary.

Future Direction

- Further investigation of ammonium thiocyanate pretreatment as an alternative to the more dangerous and expensive ammonia-based conversion of cellulose-I to cellulose-III
- Hydrolysis analysis of treated and untreated lignocellulosic samples where the biomass consists of cellulose and lignin. Studies into this will determine the effectiveness of the treatments on more complex structures.
- Hydrolysis of treated and untreated samples at other varying protein loads to find the optimal protein loading for large sugar production at minimal cost
- Engineering of proteins to deconstruct biomass more cheaply than complex protein cocktails

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