Advances in High Throughput Screening of Biomass Recalcitrance

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Method Development

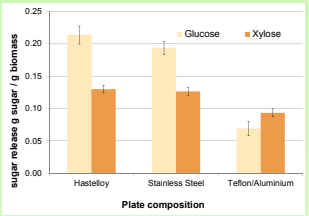
Advances on previous high throughput screening of biomass recalcitrance methods have resulted in improved conversion and replicate precision. Changes in plate reactor metallurgy, improved preparation of control biomass, species-specific pretreatment conditions, and enzymatic hydrolysis parameters have reduced overall coefficients of variation to an average of 6% for sample replicates. These method changes have improved plate-to-plate variation of control biomass recalcitrance and improved confidence in sugar release differences between samples. With smaller errors plant researchers can have a higher degree of assurance more low recalcitrance candidates can be identified.

Method Development

Effects of plate metallurgy on sugar release: Sugar release is highly dependent on plate materials-of-construction. Initial plate reactors were made of aluminum with various corrosion-resistant coatings. The severe pretreatment conditions resulted in coating failure through cracks and peeling, resulting in aluminum leaching into the reaction mix during pretreatment.

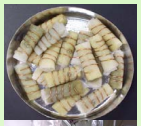
The combined effects of inhibited sugar release, degradation of final products, and limitation of cellulase activity by metal ions resulted in ~3-fold lower sugar release for aluminum reactor plates versus Hastelloy, a high nickel alloy.

Populus sugar release for differing plate metallurgy



Method Conditions:

- Plates pretreated (hydrothermal) at 180°C for 40 minutes
- Digested using Spezyme and Novo 188 (70 and 3 mg enzyme / g biomass respectively)
- Incubated at 40°C for 70 hours
- Analyzed using GOPOD/XDH assays



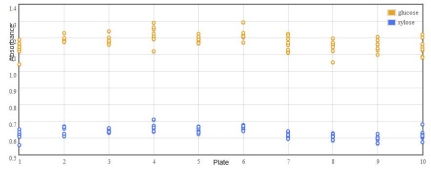
Sample/Control Preparation:

- Collect and mill biomass by hand
  - –Very labor intensive
- Load into "teabags" by hand
  - –Very labor intensive
  - –Label each teabag
- De-starch/extract "teabags" in bulk
  - –Wash extensively, ethanol extract in Soxhlet
    - •Removes extractives and amylase/glucose
  - –Results in dry, de-starched, extracted biomass
    - •Structural polysaccharides dominate
- Solids- Symyx MTM Powdernium
  - –Dispense/track quantities
    - •Automated, 0.1 mg precision
  - –Multiple substrate capacity
    - •144 or 200 samples capacity
    - •15 plate capacity

Removal of starch and extractives: Eight biomass controls account for plate-plate variation and used to normalize.

- -Starch removal and extraction reduces replicate variability
- -Decreases plate-to-plate sugar release variability
- -Allows for reliable normalization

Control monitoring



Enzyme Cocktail Effects: Enzyme age and composition was shown to be critical to effective and reproducible hydrolysis of cellulose in the pretreated samples. An older batch of SpezymeCP (Genencor) was compared to a new CTec2 preparation (Novozymes).

Sugar yield under Standard conditions

Enzyme	average g glc/g BM - 70 hours	SD	CV
Spezyme	0.192	0.016	8.18
CTEC 2	0.308	0.016	5.18
Enzyme	**average g xyl/g BM - 70 hours**	**SD**	**CV**
Spezyme	0.146	0.024	16.78
CTEC 2	0.149	0.009	5.90

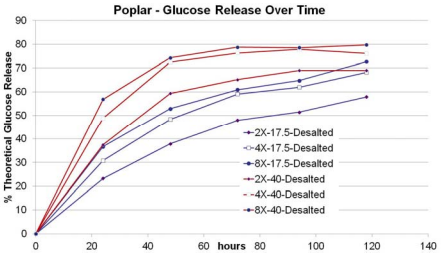
→Yield up 50%
→Variation down as much as 3 fold

Effect of Pretreatment Time and Enzyme Desalting: CTec2 was desalted in a 10 kDa MWCO centrifugal concentrator at 3 different degrees of low molecular weight reduction.

Target ~70% glucose release on poplar after 180°C pretreatment using low (x2), medium (x4) and high (x8) enzyme loading:

- -17.5 mins (lower slower enzymatic hydrolysis)
- -40 mins

Enzyme Comparison

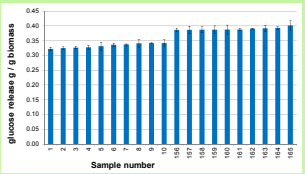


Other Improvements:

- Increased incubation temperature from 40°C to 50°C
- Added 0.02% sodium azide to enzyme solution to prevent microbial growth during hydrolysis

***Populus* Examples:** With many sets the variation between the highest and lowest sugar release values is small e.g. population mapping or QTL studies. In these cases a reduction in error is critical. Now with lower variation differences can be seen that were not visible before.

Below is a *populus* example were there would have been no significant difference between the highest and lowest samples using the old method. The new method reveals obvious differences between the 10 highest and 10 lowest glucose release values.



Left: Glucose release of an example *Populus* set, 10 highest and 10 lowest sugar release values

Biomass-specific pretreatment conditions: Using the poplar method as a template, pretreatment conditions were modified to achieve the desired level of sugar release for switchgrass.

-Target 70% glucose release
-Preferably keep either time or temperature the same as the *populus* method

Different pretreatment time and temperatures were tested, resulting in a range of combined pretreatment/enzyme hydrolysis glucose conversion ranging from 13-83%. Pretreatment at 180°C for 17.5 min was chosen, as the glucose yield was well above "no pretreatment/low severity pretreatment" but was not so high as to limit our ability to detect increased conversion levels for improved variants. The first set large set of switchgrass (~1450) is in progress.

Severity assessment for switchgrass

Temp (°C)	Time (min)	Severity (log R_0)	Avg g/g glucose	%Conversion
120	20	1.89	0.0484	13.1
140	10	2.18	0.0590	16.0
140	20	2.48	0.0671	18.2
160	10	2.77	0.0817	22.2
180	17.5	3.60	0.2478	67.3
180	20	3.66	0.2619	71.2
180	40	3.96	0.3051	82.9

Summary

Significant changes in plate reactor, control biomass preparation, pretreatment conditions and enzyme have significantly reduced sample and control replicate variability.

- Reactor plate metallurgy significantly impacts sugar release
  - Aluminum leaching into reaction during pretreatment degrades sugars and inhibits enzyme activity
- Removal of starch and extractives significantly decreases control biomass variability
- New enzyme formulations give more consistent and higher conversion levels
  - However required re-optimization for switchgrass
- Pretreatment time and temperature (severity) should be adjusted to specific biomass types *i.e.* woody vs. herbaceous
- Desalting of enzyme preps to remove low molecular weight stabilizers and improved conversion levels
  - Likely due to water activity impacts on enzyme structure and substrate interactions
  - Not attempted here due to need to continually desalt and validate precise enzyme concentration and activity

References: 1) Selig, M. J., M. P. Tucker, R. W. Sykes, K. L. Reichel, R. Brunecky, M. E. Himmel, M. F. Davis, and S. R. Decker. (2010) "Biomass Recalcitrance Screening by Integrated High Throughput Hydrothermal Pretreatment and Enzymatic Saccharification". Ind. Biotechnol. 6:104-111 2) Decker, S. R., R. Brunecky, M. P. Tucker, M. E. Himmel, and M. J. Selig. (2009). "High Throughput Screening Techniques for Biomass Conversion". Bioenergy Research. 2:79-192. 3) Studer, et al. Lignin content in natural *Populus* variants affects sugar release *PNAS 2011, 108 (15) 6300-6305.*

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