Cellulase Inhibitors/Deactivators in Lignocellulosic Biomass

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  LORRE group
Effect of Pretreatment

- Liquid hot water (neutral pH, no acids)
- Dilute sulfuric acid
- AFEX
- Lime
- Soaking in ammonia, Ammonia recycled percolation

Mosier et al., 2005
Liquid Hot Water Pretreatment (LHW) - No Acids added

- pH maintained at 4-7 by adding base or by buffering effect from biomass itself

- Solubilizes 25-45% of initial dry mass

- Solubilizes mainly xylan fraction of the lignocellulose

- Majority of the solubilized components are sugar-oligomers, monomeric sugars, phenolic compounds, acetic acid

*Kim et al., 2009, Methods in Molecular Biology: Biofuels (ed. By Mielenz, J.R.)*
Composition of Untreated and LHW Pretreated Maple and Corn Stover Solids

- Pretreatment Conditions:
  - **Maple**: 200°C, 20 min, 23% w/w dry solids
  - **Corn Stover**: 190°C, 20 min, 15% w/w dry solids
Inhibitory Effect of Pretreatment Liquid on Enzymatic Hydrolysis of Pretreated Biomass

(1% glucan loading, 15 FPU cellulase/g glucan, 50°C, pH 4.8, 168 hr hydrolysis)
Objectives

- To identify potential cellulase inhibition compounds derived from lignocellulose
- To assess the extent of inhibition
- To study the effect of removal of inhibitors on cellulose hydrolysis
Model Substrate: Solka Floc

• A highly purified and finely divided wood cellulose.

• A mixture of crystalline and amorphous cellulose produced by hammer-milling sulfite wood pulp.

• Minimal or negligible lignin content.

• 80% Glucan and 20% Xylan as measured.
# Composition of Maple and Corn Stover LHW Pretreatment Liquid

- **Pretreatment Conditions:**
  - **Maple:** 200°C, 20 min, 23% w/w dry solids
  - **Corn Stover:** 190°C, 20 min, 15% w/w dry solids

<table>
<thead>
<tr>
<th>g/L</th>
<th>Maple</th>
<th>Corn Stover</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sugar Oligomers</strong></td>
<td>12.7</td>
<td>13.5</td>
</tr>
<tr>
<td>Gluco-oligomers</td>
<td>(1.5)</td>
<td>(3.5)</td>
</tr>
<tr>
<td>Xylo-oligomers</td>
<td>(11.2)</td>
<td>(10)</td>
</tr>
<tr>
<td><strong>Monomeric sugars</strong></td>
<td>9.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>(0.6)</td>
<td>(0.1)</td>
</tr>
<tr>
<td>Xylose</td>
<td>(9.2)</td>
<td>(3.8)</td>
</tr>
<tr>
<td>Organic acids (mainly acetic acid)</td>
<td>13.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Sugar degradation products</td>
<td>4.1</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Phenolic compounds</strong></td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Others (glycerol, butanediol)</td>
<td>1</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Inhibition of Cellulose Hydrolysis by Maple Pretreatment Liquid

- Substrate: Solka Floc 300FCC
- Source of inhibitors: Pretreatment liquid from red maple

A. Solka Floc + Buffer (No inhibitors)
B. Solka Floc + Maple PL (Xylo-oligomers, Xylose, Phenolics)

Hydrolysis of cellulose: 0.1 g Solka Floc + 10 mL of A or B + enzymes

Enzymes: 15 FPU Spezyme CP/40 CBU Novo 188 per g cellulose

![Graph showing glucose yield over time for different treatments]

- Slower initial rate
- Hydrolysis stops
Effect of Removing Inhibitors on Cellulose Hydrolysis – Inhibitors from Maple

- Substrate: Solka Floc
- Source of inhibitors: Pretreatment liquid of maple pretreated at 200 °C, 20 min, 23% w/w dry solids. Pretreatment liquid obtained by filtration of the pretreated maple slurry using No 41 Whatman filter paper

A. No inhibitors:
   pH 4.8 citrate buffer

B. High monomeric sugars
   pH 4.8 citrate buffer + Reagent grade sugars

C. High monomeric sugars + Phenolics removed
   Phenolics removal from maple pretreatment hydrolysate by 10% w/w activated carbon
   Incubate at 25 °C for 10 min followed by 5 min centrifugation at 15000 rpm.

D. High monomeric sugars + High Phenolics (6 mg phenolics/mg protein)
   Hydrolyzed pretreatment liquid of maple.

E. Low monomeric sugars + High phenolics (6 mg phenolics/mg protein) + Oligomeric sugars
   Pretreatment liquid of maple as is.
Inhibitor Concentrations in the Test Solutions

Concentration (g/L)

- sugar oligomers
- monomeric sugars
- phenolic compounds

Buffer, Buffer + X, PL (X), PL (X, phenolics), PL (XO, X, phenolics)

Low inhibitors: A, B, C, D
High inhibitors: E
Effect of Removing Inhibitors on Cellulose Hydrolysis – Inhibitors from Maple

(A) No Inhibitors (Buffer only)
(B) Xylose (reagent grade)
(C) Xylose (phenolics removed by AC)
(D) Xylose+Phenolics
(E) Xylose+Xylo-oligomers+Phenolics

% glucose yield vs Time (hr)
Phenolic Compounds in Plants

• Play an important role in growth, reproduction and defence mechanisms in plants

• Phenolic acids: simple low-molecular-weight phenolics
  
  * hydroxybenzoic acid (gallic, p-hydroxybenzoic, vannilic, syringic acid)
  * hydroxycinnamic acid (caffeic, ferulic, p-coumaric, sinapic acid)

• Tannins: water soluble, high-molecular-weight polyhydroxyl phenolic compounds

• Lignin degradation products
# Total Phenolic and Protein-Precipitable Phenolic Content for Maple and Corn Stover Before and After LHW Pretreatment

<table>
<thead>
<tr>
<th></th>
<th>Total phenolics (extracted by 70% acetone) % by dry solids</th>
<th>Protein-Precipitable phenolics to total phenolics (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maple, Untreated</td>
<td>0.70%</td>
<td>50%</td>
</tr>
<tr>
<td>Corn Stover, Untreated</td>
<td>0.70%</td>
<td>50%</td>
</tr>
<tr>
<td>Pretreated Maple Solids</td>
<td>4.20%</td>
<td>47%</td>
</tr>
<tr>
<td>Pretreated Corn Stover Solids</td>
<td>2.40%</td>
<td>25%</td>
</tr>
</tbody>
</table>

(Phenolics measured by Folin Ciocalteu method. Total phenolics expressed as tannic acid equivalent)
Calculated Ratio of Phenolics to Proteins at Various Cellulase Loadings for Maple and Corn Stover
(Spezyme CP contains 82 mg protein/mL)
Phenolic Compounds Released During Enzymatic Hydrolysis of Pretreated Maple and Corn Stover

Hydrolysis: 2% dry solids, 50 C, 200 rpm, pH 4.8, 0.3 mg protein/mL hydrolysate

- Maple, with cellulase
- Corn Stover, with cellulase
- Maple, no cellulase (Sub. Blank)
- Corn Stover, no cellulase (Sub. Blank)
- Buffer and cellulase (Enz. Blank)
Effect of Phenolic Compounds on Cellulose Hydrolysis at Various Ratio of Phenolics to Protein

- Source of Phenolics: Maple pretreated at 200°C/20 min/12% dry solids hot-water washed (54% glucan, 5% xylan)

- Extraction of Phenolics: Used 70% acetone
  Extract was concentrated further via evaporation and re-dissolved in 70% acetone

- Ratio of Phenolics to Protein (mg phenolics/mg cellulase protein) Tested:
  0 (buffer only), 0.02, 0.1, 0.2, 0.5

- Hydrolysis: Solka Floc at 1% glucan, 50°C, 250 rpm, pH 4.8
  0.3 mg protein/mL hydrolysate with varying ratio of phenolics to cellulase (Spezyme CP) enzyme

- Controls: Buffer only hydrolysis
  Hydrolysis of Solka-floc with varying concentrations of acetone
% Glucose Yields Reduction at Various Ratio of Phenolics to Cellulase as Measured at 168 hr Hydrolysis
% Remaining Activity of Cellulase

% Remaining Filter Paper Activity of Spezyme CP

Phenolics from Maple

Phenolics from Corn Stover

mg phenolics/mg protein
Conclusions

1. Liquid hot water (LHW) pretreatment and enzymatic hydrolysis solubilize compounds that are inhibitory to cellulase enzymes.

2. Soluble inhibitors in the LHW pretreatment liquid of cellulosic biomass are present in the following order ranked by concentration:

   Sugar oligomers > Monomeric sugars >>>>> Phenolics

3. Due to their high concentrations, sugar oligomers and monomeric sugars in the LHW pretreatment liquid inhibit the digestibility of the biomass significantly.

4. Despite their low level in the pretreatment liquid and hydrolysates, the phenolic compounds are strong cellulase inhibitors/deactivators.
Thank You!