Effects of Lignin and Phenolic Inhibitors
On Enzyme Loading

Leading Pretreatment Technologies for Production of Fuels, Chemicals, and Feed (Charles Wyman, Chair)

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BIO Pacific Rim Summit, San Diego, CA
December 8, 2014
Acknowledgements

Organizing Committee, BIO Pacific Rim Summit

Purdue University
Colleges of Agriculture and Engineering

US Department of Energy
Cooperative Agreement GO18103,
GO17059-16649,
0012846 DE-SC0000997

Indiana Corn Marketing Council

EMBRAPA: Dr. Cristiane Farinas
Aqueous based (Acid, Alkaline, or Neutral), microbial / protein catalysts, mild conditions. Major cost is due to enzymes. Yields < 10 to 20% in the absence of pretreatment

Different Pretreatments result in different levels of inhibition of yeast and enzymes

Degree of Inhibition

Pretreatment Type

Acid  Neutral  Alkaline

Focus on Enzymes

Yeast  Enzyme

Novo, 2009; Sao Carlos, 2013
Enzyme Costs

Based on

Loadings
Specific Activity
Yield

Cost of production (facility dependent, i.e., capital costs, + consumables, labor, raw materials).

Models for calculating enzyme costs are available but published industrial cost data is not available.
Magnitude of Order Estimate of Enzyme (Protein) Costs for Pretreated Ligno-cellulose (Corn Stover)  
(based on Klein-Marcuchamer, Blanch, et al, 2012)

<table>
<thead>
<tr>
<th>Enzyme (Protein) Loading / g pretreated solids (assume 50% cellulose)</th>
<th>Assumed Protein Cost / $ / kg</th>
<th>Type of Protein</th>
<th>For specified ethanol yields in gal / ton</th>
<th>Est. enzyme cost in $ / gal ethanol produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPU / g</td>
<td>mg / g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td>$1.13</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td></td>
<td></td>
<td>$0.55</td>
</tr>
<tr>
<td>none</td>
<td>10</td>
<td>1.25</td>
<td>Soy</td>
<td>$0.14</td>
</tr>
<tr>
<td>none</td>
<td>5</td>
<td>1.25</td>
<td>Soy</td>
<td>$0.07</td>
</tr>
</tbody>
</table>

Base case, 20% pretreated corn stover solids, in 5 day fermentation with 70% yield
Pretreatment enables high cellulose hydrolysis yields by making substrate accessible and susceptible to active site of enzyme.
Aqueous pretreatments:

Steam Explosion and Liquid Hot Water

Both use water to open up the plant cell wall structure. High severity refers to high temperature, longer time.

Steam explosion
may add acid (to hydrolyze xylan)
releases pressure through explosive decompression

Liquid hot water (LHW) cooking (pressurized)
no chemicals added
pH at 4 to 7; lignocelluloses self-buffer to this pH
temperatures between 160 and 215 C
carried out under pressure (heat up to cool down)
pressure conditions keep water in liquid phase
LHW Pretreatment (Minimize Hydrolysis and Inhibitors)

Four step process:
1. add water to Biomass,
2. heat to between 160 to 210 °C,
3. hold for at temperature for 10 to 40 min
4. cool and recover heat and biomass

\[ C \xrightarrow{k_1} C* \xleftarrow{k_2} K \xrightarrow{k_3} \text{Degradation Products} \xleftarrow{k_4} C \]

pretreatment (a physical change)

C = native cellulose
C* = hydrated cellulose
Enzyme Hydrolysis of LHW Treated Biomass

1. Prepare material for addition to fermenters
2. Add enzyme and yeast
3. Hydrolyze and ferment for 3 to 5 days

\[ C \xrightarrow{k_1} G_n \xrightarrow{k_3} G \]

\[ k_2 > k_1 \]

C = native cellulose remaining after pretreatment
C* = hydrated cellulose
G_n = glucans (oligosaccharides)
G = glucose (monomer)
Pretreatment and Cost Effective Enzymes are Key

Pretreatment increases accessibility of both lignin (undesirable) and cellulose (desirable), but also releases enzyme inhibitors:

- xylo-oligosaccharides
- phenols
- tannic acids

And may form fermentation inhibitors:

- acetic acid (from hemicellulose)
- aldehydes (fufural)

Washing of pretreated material removes soluble inhibitors.
Soluble Enzyme Inhibitors: Phenolics and Xylo-oligosaccharides

Kim et al, 2011

Hydrolysis of Solka Floc

% glucose yield

Time (hr)

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

Less Inhibitors
Tannic Acid Deactivates Cellulases at Hydrolysis Conditions

Deactivation of FPAase and Endoglucanase activities by Tannic acid over time

Ximenes et al, 2010
Product and Lignin Derived Inhibitors Affect Enzymes


But if Inhibitors Washed Away, Inhibition Persists
SEM of Untreated and Pretreated Hardwood

Untreated surfaces are smooth

Pretreated

Lignin droplets formed from cell wall

Ko et al, 2014
High Enzyme Loadings = High Yields at High Severity

Ko et al, 2014
Addition of BSA to Enzyme
High Yield at Lower Enzyme Loading and High Severity

Cellic Ctec2 of 5 FPU (8 mg protein)/g glucan, pH 4.8, in 50 mM citrate buffer, 50°C, 200 rpm for 168 hrs. Equivalent to 3.5 mg/g total solids prior to pretreatment.

Kim et al, 2014
Diluting Enzyme with Non Catalytic Protein Increases Yield

As specific activity decreases, conversion increases.

Cellulase loading fixed at 1.8 FPU / g glucan, equivalent to 1.3 FPU / g pretreated solids.

\[ y = -0.192 \ln(x) + 0.1593 \]

\[ R^2 = 0.9701 \]
Pretreatment Conundrum

Pretreatment exposes both cellulose and lignin. Although yield goes up, more enzyme is needed to achieve the yield due to adsorption of cellulase (circles) onto lignin (purple lines). Addition of non-catalytic protein reverses this effect.
Summary

Low cost production processes will define cellulose ethanol

Lignin derived inhibitors are the next target to reducing cost.

Bio-processing routes are attractive
  a. Less harsh conditions
  b. More selective
  c. Compatible with biorefinery concept
  d. Low cost technologies are possible