Effects of Liquid Hot Water Pretreatment on Enzyme Loading and Hydrolysis of Hardwood

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Classical Cellulose to Ethanol Conversion

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

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>$ / kg</td>
<td>67 gal / ton</td>
<td>89 gal / ton</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>10</td>
<td>Enzyme</td>
<td>$ 1.13</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>10</td>
<td>Enzyme</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

\[ \text{ pretreatment (a physical change) } \]

\[ C = \text{ native cellulose} \]

\[ C^* = \text{ hydrated cellulose} \]

Ladisch and Dale, 2008
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

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

- \( C \): native cellulose remaining after pretreatment
- \( C^* \): hydrated cellulose
- \( G_n \): glucans (oligosaccharides)
- \( G \): glucose (monomer)

Ladisch and Dale, 2008
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 (furfural)

Washing of pretreated material removes soluble inhibitors.
Hardwood Lignin

Hardwood lignin (Nimz, 1973)

Hydroxyphenyl : Guaiacyl : Syringyl

0-8% : 25-50% : 45-75%
Liquid hot water (LHW) pretreatment

Increases enzymatic hydrolysis yield of cellulose by
- solubilizing xylan
- decreasing particle size
- increasing porosity

However...
More lignins are exposed to cellulases
At low cellulase loading: Inhibitory role of lignin more noticeable

Research objectives

Understand the underlying inhibitory mechanism of lignin on enzymatic hydrolysis of LHW pretreated hardwood

- How does the pretreatment modify the lignin structure?

- How does lignin inhibit enzymatic hydrolysis of cellulose?
**Cellulase Enzyme (Protein)**

**Cellic Ctec2**

Commercial cellulase cocktail (from Novozyme)

Derived from *Trichoderma reesei*

Cellulase activity: 118 FPU/mL  
Protein amount: 190 mg/mL

Mixture of cellulases (cellobiohydrolase, endo-glucanase)  
and β-glucosidases needed for efficient hydrolysis
High Enzyme Loadings = High Yields at High Severity

Ko et al, 2015
SEM of Untreated and Pretreated Hardwood

Untreated

Lignin droplets formed from cell wall

Ko et al, 2014
Enhancement in enzymatic hydrolysis of hardwood

Enzyme: Ctec2
5 FPU (8mg)/g-glucan at pH 4.8 for 72hrs

Pretreated hardwood:
Glucan 58%
Lignin 40%

Glucose yield (%)

<table>
<thead>
<tr>
<th>CT</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreated HW</td>
<td></td>
<td></td>
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</tbody>
</table>

BSA blocking

Add β-G
pH 5.5
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, 2015
Diluting Enzyme with Non Catalytic Protein Increases Yield

As specific activity decreases, conversion increases

\[ y = -0.192 \ln(x) + 0.1593 \]
\[ R^2 = 0.9701 \]

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

Kim et al, 2015
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.

Ladisch et al, 2015
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
Conclusions

Surface area is made accessible and exposed by pretreatment

Lignin shields cellulose from hydrolysis and interferes with enzyme action both before and after pretreatment

Inhibition / deactivation varies with pretreatment severity.

Major reductions in amount of enzyme needed for cellulose hydrolysis are possible by blocking effects of lignin