Developing Herbaceous Energy Crops

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Fermentation Biotechnology Research
Current Paradigm

- Wheat
- Corn
- Sugarcane
Potential of corn to replace oil for U.S. market

(RFA & NCGA, 2006)

% U.S. Corn Harvest Going to Ethanol

% Auto Fuel Replaced by Ethanol

Calendar Year

(RFA & NCGA, 2006)
Sources of Biomass

- Cottonwoods
- Paper
- Switchgrass
- Wood chips
- Corn stover
- Bagasse
Long-term Goals of ARS Energy Crops Working Group

- Develop new cultivars of herbaceous perennials that give superior ethanol yields through breeding.

- Develop a better understanding of the interactions among species, maturity, and cell wall structure and the response to pretreatment and ethanol fermentation.
### What can cellulosics do for us?

<table>
<thead>
<tr>
<th>Feedstocks</th>
<th>Million dry ton per yr</th>
<th>Billion gal.s of ethanol per yr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agricultural Land (selected)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Stover</td>
<td>75</td>
<td>4.50</td>
</tr>
<tr>
<td>Wheat Straw</td>
<td>11</td>
<td>0.66</td>
</tr>
<tr>
<td>CRP Biomass</td>
<td>18</td>
<td>1.08</td>
</tr>
<tr>
<td>Perennial Crops</td>
<td>156</td>
<td>9.36</td>
</tr>
<tr>
<td><strong>Forestlands (selected)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Logging &amp; Processing residues</td>
<td>134</td>
<td>8.04</td>
</tr>
<tr>
<td>Total:</td>
<td>4,894</td>
<td>23.6</td>
</tr>
</tbody>
</table>

*This is 17% of our total oil needs.*

Notes: (1) 60 gal/ton ethanol yield; (2) source: http://feedstockreview.ornl.gov/pdf/billion_ton_vision.pdf
Hypothesis: species (and plant type) and intra-species cell wall differences can influence enzymatic digestibility.

Approach:

- Vary species (plant type): **alfalfa** (legume, dicot), **reed canarygrass** (C3 cool season grass), and **switchgrass** (C4 warm season grass)

- Vary cell wall structure: by evaluating different maturities for each species
<table>
<thead>
<tr>
<th>Species</th>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alfalfa</strong> (<em>Medicago sativa</em> L.)</td>
<td></td>
</tr>
<tr>
<td>Bud (A1)</td>
<td>Stems, flower buds present, no open flowers</td>
</tr>
<tr>
<td>Full Flower (A2)</td>
<td>Stems, open flowers on all stem shoots</td>
</tr>
<tr>
<td><strong>Reed canarygrass</strong> (<em>Phalaris arundinacea</em> L.)</td>
<td></td>
</tr>
<tr>
<td>Vegetative (CG1)</td>
<td>Leaf blades and sheaths, no stem elongation</td>
</tr>
<tr>
<td>Ripe Seed (CG2)</td>
<td>Whole herbage, ripe seed</td>
</tr>
<tr>
<td><strong>Switchgrass</strong> (<em>Panicum virgatum</em> L.)</td>
<td></td>
</tr>
<tr>
<td>Pre-boot (SG1)</td>
<td>Leaf blades and sheaths, elongated stems</td>
</tr>
<tr>
<td>Anthesis (SG2)</td>
<td>Whole herbage, flower panicle on stems open</td>
</tr>
<tr>
<td>Post-Frost (SG3)</td>
<td>Whole herbage, ripe seed, senescent, post-frost</td>
</tr>
</tbody>
</table>
Samples were analyzed as follows

- Complete chemical composition using the Uppsala fiber analysis system (and detergent fiber system)
- Fiber digestibility by treating with dilute acid pretreatment and cellulase enzymes (using modified methods developed by NREL, DOE)
- Ethanol yields with *S. cerevisiae*, currently only for switchgrass
## Overall composition of biomass (g/kg, DM)

<table>
<thead>
<tr>
<th>Species/Maturity</th>
<th>Ether Extracts</th>
<th>Crude Protein</th>
<th>Total Carbo</th>
<th>Klason Lignin</th>
<th>Ash</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alfalfa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bud</td>
<td>9</td>
<td>127</td>
<td>563</td>
<td>158</td>
<td>81</td>
<td>970</td>
</tr>
<tr>
<td>Full Flower</td>
<td>7</td>
<td>88</td>
<td>598</td>
<td>175</td>
<td>58</td>
<td>950</td>
</tr>
<tr>
<td><strong>Reed Canarygrass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetative</td>
<td>22</td>
<td>88</td>
<td>518</td>
<td>109</td>
<td>128</td>
<td>889</td>
</tr>
<tr>
<td>Ripe Seed</td>
<td>13</td>
<td>45</td>
<td>597</td>
<td>148</td>
<td>95</td>
<td>908</td>
</tr>
<tr>
<td><strong>Switchgrass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Boot</td>
<td>10</td>
<td>65</td>
<td>569</td>
<td>133</td>
<td>89</td>
<td>875</td>
</tr>
<tr>
<td>Anthesis</td>
<td>10</td>
<td>32</td>
<td>655</td>
<td>154</td>
<td>57</td>
<td>917</td>
</tr>
<tr>
<td>Post-Frost</td>
<td>16</td>
<td>30</td>
<td>650</td>
<td>173</td>
<td>57</td>
<td>915</td>
</tr>
</tbody>
</table>
# Break-down of carbohydrates

<table>
<thead>
<tr>
<th>Species/Stage</th>
<th>Soluble</th>
<th>Storage</th>
<th>Cellulose</th>
<th>Xylan</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td><strong>Alfalfa Stems</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bud</td>
<td>55</td>
<td>3</td>
<td>275</td>
<td>148</td>
<td>481</td>
</tr>
<tr>
<td>Full Flower</td>
<td>49</td>
<td>2</td>
<td>306</td>
<td>165</td>
<td>522</td>
</tr>
<tr>
<td><strong>Reed Canarygrass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetative</td>
<td>81</td>
<td>35</td>
<td>209</td>
<td>171</td>
<td>496</td>
</tr>
<tr>
<td>Ripe Seed</td>
<td>45</td>
<td>54</td>
<td>265</td>
<td>212</td>
<td>576</td>
</tr>
<tr>
<td><strong>Switchgrass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Boot</td>
<td>40</td>
<td>5</td>
<td>273</td>
<td>231</td>
<td>549</td>
</tr>
<tr>
<td>Anthesis</td>
<td>76</td>
<td>39</td>
<td>283</td>
<td>238</td>
<td>636</td>
</tr>
<tr>
<td>Post-Frost</td>
<td>27</td>
<td>7</td>
<td>322</td>
<td>273</td>
<td>629</td>
</tr>
</tbody>
</table>
Theoretical ethanol yields broken down by carbohydrates

<table>
<thead>
<tr>
<th>Species/Stage</th>
<th>Soluble</th>
<th>Storage</th>
<th>Cellulose</th>
<th>Xylan</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alfalfa Stems</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bud</td>
<td>10</td>
<td>1</td>
<td>48</td>
<td>26</td>
<td>84</td>
</tr>
<tr>
<td>Full Flower</td>
<td>8</td>
<td>0</td>
<td>53</td>
<td>29</td>
<td>91</td>
</tr>
<tr>
<td><strong>Reed Canarygrass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetative</td>
<td>14</td>
<td>6</td>
<td>36</td>
<td>30</td>
<td>86</td>
</tr>
<tr>
<td>Ripe Seed</td>
<td>8</td>
<td>9</td>
<td>46</td>
<td>38</td>
<td>100</td>
</tr>
<tr>
<td><strong>Switchgrass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Boot</td>
<td>7</td>
<td>1</td>
<td>47</td>
<td>41</td>
<td>96</td>
</tr>
<tr>
<td>Anthesis</td>
<td>13</td>
<td>7</td>
<td>49</td>
<td>42</td>
<td>111</td>
</tr>
<tr>
<td>Post-Frost</td>
<td>5</td>
<td>1</td>
<td>56</td>
<td>48</td>
<td>110</td>
</tr>
</tbody>
</table>

Corn stover = 113 gal/ton
### Comparison of Upsalla & Detergent Fiber systems

(Selected data)

<table>
<thead>
<tr>
<th>Species</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>Glucose</td>
<td>ADF-ADL</td>
<td>Sugars</td>
</tr>
<tr>
<td>Alfalfa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flower</td>
<td>306</td>
<td>444</td>
<td>122</td>
</tr>
<tr>
<td>Reed Canarygrass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ripe Seed</td>
<td>265</td>
<td>356</td>
<td>218</td>
</tr>
<tr>
<td>Switchgrass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthesis</td>
<td>283</td>
<td>340</td>
<td>245</td>
</tr>
</tbody>
</table>

**DFS overestimates cellulose, hemicellulose and underestimates lignin**
Measuring recoverable sugar yields

Biomass

Milling/drying

Dilute-acid pretreatment

Cellulase Treatment

Products

Glucose

Other Sugars

Sugars

Other

Glucose

Products

Glucose
Effect of Pretreatment

Lignin

Amorphous Region

Cellulose

Crystalline Region

Hemicellulose

Pretreatment
Severity of Dilute Acid Pretreatment of Cellulose for Enzymatic Digestion

Combined Severity Factor (CSF):

\[
CSF = \log\left[ t \times \exp\left( \frac{T - 100}{14.75} \right) \right] - pH
\]

Factors for pretreatment:
- Time (at temperature)
- pH (or Acidity)
- Temperature (exponential effect!)

The lower the severity the lower the cost of pretreatment, the higher the recovery of xylan associated sugars, and more fermentable the product.
Amount of acid that needs to be added to reach similar pH’s

- Alfalfa
- Reed Canarygrass
- Switchgrass

Target pH
Detailed Protocol for Measuring Sugar Yields

Milled Biomass

- Dilute sulfuric acid treatment at pH 1, 150°C for 20 min

- Cellulase treatment with 50 FPU/g cellulose at pH 4.5, 50°C for 72 hr
  (note: Celluclast + Novo188 (Novozymes, Inc.))

- Measured released glucose and non-glucose sugars by HPLC

- Calculate sugar yields (g/g dry biomass) & % recovery as free sugars
Changes in glucose yields with maturity and species

Crop & Maturity

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A2</th>
<th>CG1</th>
<th>CG2</th>
<th>SG1</th>
<th>SG2</th>
<th>SG3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Yields (mg sugar / g biomass, db)</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>250</td>
<td>300</td>
<td>350</td>
<td></td>
</tr>
</tbody>
</table>

Alfalfa RCG Switchgrass
Changes in Cellulose Digestion with Species and Maturity

Crop & Maturity

A1  A2  CG1  CG2  SG1  SG2  SG3

% Glucose Recovery

50  60  70  80  90  100

Error bars = 95% CI

CRG  Switchgrass  Alfalfa
Lignin vs. glucose conversion efficiencies

Glucose Release (%)

Klason Lignin (g kg$^{-1}$ DM)
Increasing severity for alfalfa stems

Cellulose Digestibility

stage of harvest

A1 A2

150C 165C 180C
Recovery of other sugars

Crop & Maturity

% Non-glucose Recovery

Alfalfa
CRG
Switchgrass

A1 A2 CG1 CG2 SG1 SG2 SG3

Crop & Maturity
Pretreating Biomass at 121°C vs. 150°C

Crop & Maturity

% Recovery of Sugars

A1 A2 CG1 CG2 SG1 SG2 SG3

121°C 150°C
Role of fructose in reducing yields

![Graph showing the relationship between fructose (g/kg, DM) and reduced non-glucose yield (g/kg, DM). The graph illustrates a linear relationship with a positive slope.]

Fructose (g/kg, DM) vs. Reduced Non-Glucose Yield (g/kg, DM)
Inhibitors formed during hydrolysis

- **Hemicellulose**
  - Pentoses (Ara, Xyl)
  - Galactose

- **Cellulose**
  - Glucose

- **Lignin**
  - Phenolics
  - Formic
  - Furfural
  - HMF
  - Acetic
  - Ferulic
  - Levulinic
Treatment of sugars at pH 1, 121°C for 1 hr

- Pretreated Sugar: Arabinose, Fructose, Galactose, Glucose, Xylose
- Sugar Recovery (%)
- HMF or Furfural (mM)
- Recovery %
- HMF/Furfural (mM)
Future work

- Developing a screening method capable of evaluating hundreds of cultivars for *relative* cellulose fermentation efficiencies that will allow us to select the best for further development (discussed today)

Selecting other pretreatment methods for treating forage type material (not discussed today)
Proposed SSF screening method

Milled Biomass

Dilute sulfuric acid treatment at 121°C for 1hr & Neutralize to pH 4.8 with Ca(OH)₂

Ferment w/ S. cerevisiae in presence of cellulase (5 FPU/g, DM) at 35°C for 72 hr

Measure ethanol and non-glucose sugars by HPLC

Calculate Ethanol yields (g/g dry biomass) & % of theo. yield
Effect of Maturity of Switchgrass on Ethanol Yield

![Bar chart showing ethanol yield for different maturity stages of switchgrass (SG1, SG2, SG3)].

- **Ethanol Yield (g ethanol/kg SG, db)**
- **Maturity Stage**
- **Error bars = 95% CI**
Effect of Maturity of Switchgrass on Glucan Conversion Efficiency to Ethanol

![Graph showing the effect of maturity on glucan conversion efficiency to ethanol. The x-axis represents the maturity stages (SG1, SG2, SG3), and the y-axis represents the glucan conversion efficiency (% of max). Error bars indicate 95% CI.](image-url)
Conclusions

♦ Recoverable sugars (& ethanol yields) varies with species and maturity w/ in species

♦ Available glucose varied inversely with maturity and lignin content. However, total glucose yield increased with maturity due to higher cellulose contents.

♦ Cell wall polysaccharides, as determined by the widely applied detergent fiber system are inaccurate. Overestimates cellulose and hemicellulose and underestimates lignin.

♦ Soluble sugar content can be significant and may be problematic for dilute acid pretreatment, especially fructose
What’s next?

♦ Expand scope of samples to include additional cultivars.

♦ Conduct actual fermentations using conventional yeast as well as recombinant yeasts and bacteria capable of fermenting pentoses as well as hexoses.

♦ Develop screening tools to handle greater throughput evaluations.
Acknowledgements

Peoria, Illinois
Bruce S. Dien
Loren Iten

Lincoln, Nebraska
Kenneth P. Vogel
Robert B. Mitchell
Gautum Sarath

St. Paul, Minnesota
Hans-Joachim G. Jung
JoAnn F. S. Lamb

Madison, Wisconsin
Michael D. Casler