Conversion of Lower Lignin Mutants of *Sorghum bicolor* to Ethanol

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Fermentation Biotechnology Research

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Growing use of corn for ethanol

*Increases in ethanol balanced by increases in crop yields.*

*Only 1% of global arable land used for biofuels crops (2006).*

**1990/1991 Crop Year**

- Feed Use/Residual: 60%
- Exports: 20%
- Fuel Alcohol: 14%
- Food/Seed Industrial (excludes fuel alcohol): 6%

**2006/2007 Crop Year**

- Feed Use/Residual: 50%
- Exports: 19%
- Fuel Alcohol: 19%
- Food/Seed Industrial (excludes fuel alcohol): 12%

**1 B gal ethanol**

**6.5 B gal ethanol**
## What Grain Alcohol Can Do

<table>
<thead>
<tr>
<th>Year</th>
<th>Ethanol</th>
<th>Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>5 B</td>
<td>19%</td>
</tr>
<tr>
<td>2015</td>
<td>15 B</td>
<td>38%</td>
</tr>
</tbody>
</table>

Data: NCGA & CFA

U.S. Gasoline Demand
140 B gal
Herbaceous energy crops can be part of the solution

- Enough energy crops can be grown in US to produce 35+ billion gal/yr of ethanol
- Can be cultivated on marginal farming land, so, no conflict with food production
- Equal to corn ethanol in substituting for oil and uses less natural gas (0.08 BTU/BTU oil & 002 BTU/BTU gas)
- More effective at reducing emissions of green house gases (12% vs. 83%)
- Perennials may add to soil quality and serve as wildlife refuges
Cellulosic Biomass to Fermentable Sugars

- **Grind**
- **Pretreatment to Open up Cell Wall**
- **Enzymatic Breakdown of Polysaccharides**
  - **Residual Solids**
  - **Electricity & Processing Heat**
  - **Fermentation**
  - **Product Recovery**

Sugars

Courtesy of Hans Jung
Grass secondary cell wall model

Lignin

Hemicellulose

Cellulose
Ethanol yield largely determined by accessibility of cellulases to cellulose

(adapted from Mosier)
Lignin is particularly hard to remove as a barrier because it is hydrophobic, highly networked, and has ether links & aromatics.

Source of reduced lignin biomass for this study

Registration of Seven Forage Sorghum Genetic Stocks Near-Isogenic for the Brown Midrib Genes bmr-6 and bmr-12

Seven forage sorghum [Sorghum bicolor (L.) Moench] genetic stocks, N592 to N598, (Reg. no. GS-121–GS-127, PI639702–PI639708) near-isogenic to their wild-type counterparts for the brown midrib genes bmr-6 and bmr-12 were developed jointly by the USDA-ARS and the Agricultural Research Division, Institute of Agriculture and Natural Resources, University of Nebraska, and were released in January 2005.

J.F. Pedersen,* D.L. Funnell, J.J. Toy, A.L. Oliver, and R.J. Grant
Lignin Synthesis Pathway in Plants

BMR6 = reduced cinnamyl alcohol dehydrogenase (CAD)
BMR12 = reduced caffeate O-methyltransferase (COMT)

(Chen and Dixon, 2007)
## Comparison of lignin contents (%w/w, db)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Klason Lignin</th>
<th>ADL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sorghum with grain removed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bmr-12/bmr-6</td>
<td>10.6</td>
<td>1.09</td>
</tr>
<tr>
<td>bmr-6</td>
<td>12.4</td>
<td>2.16</td>
</tr>
<tr>
<td>bmr-12</td>
<td>12.7</td>
<td>2.03</td>
</tr>
<tr>
<td>Wild type</td>
<td>14.6</td>
<td>2.92</td>
</tr>
<tr>
<td><strong>Whole sorghum plant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bmr-12/bmr-6</td>
<td>9.24</td>
<td>0.79</td>
</tr>
<tr>
<td>bmr-6</td>
<td>11.2</td>
<td>1.89</td>
</tr>
<tr>
<td>bmr-12</td>
<td>11.8</td>
<td>1.89</td>
</tr>
<tr>
<td>Wild type</td>
<td>13.3</td>
<td>2.80</td>
</tr>
</tbody>
</table>
Differences in lignin are significant

![Bar chart showing lignin content across different genotypes.](chart.png)
Genotype does not influence carbohydrate contents

<table>
<thead>
<tr>
<th>Sorghum Genotype/Maturity</th>
<th>Carbohydrate Content (g/kg, db)</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/ Grain</td>
<td></td>
</tr>
<tr>
<td>wild-type</td>
<td></td>
</tr>
<tr>
<td>bmr-12</td>
<td></td>
</tr>
<tr>
<td>bmr-6</td>
<td></td>
</tr>
<tr>
<td>stacked</td>
<td></td>
</tr>
<tr>
<td>No Grain</td>
<td></td>
</tr>
<tr>
<td>wild-type</td>
<td></td>
</tr>
<tr>
<td>bmr-12</td>
<td></td>
</tr>
<tr>
<td>bmr-6</td>
<td></td>
</tr>
<tr>
<td>stacked</td>
<td></td>
</tr>
</tbody>
</table>
### Theoretical Ethanol Yield

<table>
<thead>
<tr>
<th>Sorghum/Genotype</th>
<th>Glucans</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>bmr-6/bmr-12</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>bmr-12</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>bmr-6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>wild-type</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Grain</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>bmr-6/bmr-12</td>
<td>80</td>
<td>40</td>
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<tr>
<td>bmr-6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>wild-type</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

The diagram illustrates the theoretical ethanol yield (gal/ton, db) for different sorghum genotypes, categorizing the yield into glucans and others.
**Digestion Assay**

**Pretreatment**
- pH = 1
- Temp = 121ºC
- Time = 1 hr

**Digestion Assay**
- Cellulase: 50 FPU/g glucan + 40 U of beta-glucosidase/g glucan
- Temperature = 50ºC, pH = 4.8, & 100 rpm
- Time = 72 hr

**Analysis**
- Glucose
- Other sugars (e.g. Arabinose, galactose, fructose, and xylose)
Lignin does not effect acid hydrolysis of xylan
Negative effect of lignin on glucose yield from cellulose

![Graph showing the negative effect of lignin on glucose yield from cellulose](image)

- No Grain, $R = -0.971$
- Whole Plant, $R = -0.880$
Ethanol Fermentation Assay

Pretreatment conditions
pH = 1
Temp = 121°C
Time = 60 min
Neutralization: Calcium hydroxide until pH 5.0

Simultaneous Saccharification & Fermentation
Biocatalyst = *Saccharomyces cerevisiae*
Cellulase: 5 FPU/g (db) + 12 U/g of beta-glucosidase
Temperature = 35°C & pH = 4.5
Time = 72 hr

Analysis
Ethanol
Nonfermentable Sugars (e.g. arabinose and xylose)
Lignin reduces ethanol yield from cellulose

- No Grain, $R = -0.943$
- Whole Plant, $R = -0.849$
Ammonium hydroxide pretreatment

**Pretreatment conditions**
- Ammonium hydroxide: 4%
- Temp = 170°C
- Time = 20 min
- Ammonia Removal: evaporated 48 hr at ambient temp

**Simultaneous Saccharification & Fermentation**
- Biocatalyst = *Saccharomyces cerevisiae* D5A
- Cellulase: 5 FPU/g (db) + 12 U/g of beta-glucosidase
- Temperature = 35°C & pH = 4.5
- Time = 72 hr

**Analysis**
- Ethanol
- Nonfermentable Sugars (e.g. arabinose and xylose)
SSF of ammonium hydroxide pretreated stacked & wild-type sorghum samples

![Bar chart](chart.png)

- **Ethanol Efficiency (% max)**
  - 60
  - 70
  - 80
  - 90

- **Klason lignin (%w/w)**
  - 10.0
  - 11.2
  - 14.3
  - 14.9

- **Labels**
  - **bmr stacked**
  - **wild-type**
# Comparison of ethanol and feed yields

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lignin (%wt/)</th>
<th>Ethanol Eff(^1) (% of max)</th>
<th>IVDMD(^2) (% of max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacked</td>
<td>10.6</td>
<td>54</td>
<td>86</td>
</tr>
<tr>
<td>bmr-6</td>
<td>12.4</td>
<td>44</td>
<td>78</td>
</tr>
<tr>
<td>bmr-12</td>
<td>12.7</td>
<td>43</td>
<td>82</td>
</tr>
<tr>
<td>Wild-type</td>
<td>14.6</td>
<td>37</td>
<td>75</td>
</tr>
</tbody>
</table>

\(^1\)B.S. Dien, G.S. Sarath, J.F. Pedersen, D.L. Funnell, S. Sattler, J.J. Toy, & N.N. Nichols

\(^2\)H. M. Dann, A. M. DiCerbo, J. F. Pedersen, and R. J. Grant (estimated from graph)
Prior reported results

- Determined that two bmr mutants in sweet sorghum improved enzymatic saccharification by 30-60%. AI Saballos, W Vermerris, and G Ejeta. (2007). Development of Brown Midrib Sweet Sorghum as a Dual-Source Feedstock for Ethanol Production. Abstract


- Numerous studies detail advantages of bmr mutations for increasing forage digestibility & bmr sorghum seed is produced commercially. One example: AL Oliver, JF Pedersen, RJ Grant, & TJ Klopfenstein. (2005) Comparative Effects of the Sorghum bmr-6 and bmr-12 Genes: I. Forage Sorghum Yield and Quality.
Chemical plant composition for near-isogenic sorghum lines carrying \textit{bmr} were similar, except for lignin content.

Glucose and ethanol yields for sorghum biomass samples pretreated with low severity dilute-acid were negatively correlated with Klason lignin content and differences in ethanol conversion efficiencies ranged over 20%.

Lower lignin mutants also showed improved ethanol conversion efficiencies when pretreated using a higher temperature ammonium hydroxide pretreatment – the maximum efficiency for glucan conversion was 77%.
Acknowledgments

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