Saccharification of bamboo by dilute acid pretreatment and enzymatic hydrolysis for cellulosic ethanol production

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Abstract: In this research, dilute sulphuric acid pretreatment for enzyme saccharification using herbaceous lignocellulose, giant bamboo, was investigated for the purpose of studying its potential as feedstock for ethanol production. Bamboos are giant woody, tree-like, perennial evergreen C4 grasses with more than 70 genera and about 1000 species. Bamboos grow naturally in tropical, subtropical, and temperate regions around the world. The central composition design was employed to optimize the pretreatment parameters, pretreated temperature range from 178 to 205 ºC, residence time range from 1 to 15 minutes and acid concentration range from 0.14 to 0.44 g/ 100 g material, at reactor loading of 30%w/v (solid weight/liquid volume) in the small scale tubular reactors. The pretreated solids were enzymatically hydrolysed using enzyme loading 15 FPU/g WIS (water insoluble solid) at 50 ºC for 72 h. Up to 50% glucose and 83% xylose were released from bamboo by pretreatment and enzymatic hydrolysis.

INTRODUCTION

Cellulosic ethanol from renewable lignocellulosic biomass will result in a new industrial revolution from a fossil fuel-based economy to a sustainable carbohydrate economy. Cost effective production of fermentable sugars from lignocellulosic biomass remains the largest obstacle to emerging cellulosic ethanol industry (Lee et al. 2008). Since the world energy consumption predicted to increase 54% between 2001 and 2015, considerable effort has being directed towards the development of sustainable and carbon neutral energy sources to meet future needs. Biofuels such as bioethanol, obtained from biomass, represent one of the few alternatives for short-term diversification in the transportation sector as they are the only renewable products that can be easily integrated into the current distribution systems. At present, industrial bioethanol is mainly produced from sucrose or starchy biomasses, which are designated to the food market. Using cellulose-rich materials to produced so-called second generation ethanol is considered a promising choice in order to meet the future increase in biofuels demand without compromising food security. The cultivation of energy crops such as bamboo can play an important role in this capacity. Bamboo is a group of plants classified as true perennial grasses and form part of the subfamily Bambusoideae (McClure 1966; Aoyama et al. 1999; Loretta et al. 2007). The species are fast growing plants and can reach maturity within five years and get heights from 3-30 m within months (Scurlock et al. 2000; Lin et al. 2001). Its promise of high productivity together with desirable fuel characteristics has made bamboo a promising raw material for bioprocesses producing bioethanol using enzymatic hydrolysis (Scurlock et al. 2000). The biochemical conversion of cellulosic biomass to ethanol involves three main steps: (1) pretreatment to open up the lignocellulose structure making the cellulose more accessible to enzymes, (2) hydrolysis with enzyme preparations to break down the cellulose to sugars and (3) fermentation of these sugars to ethanol. Dilute acid pretreatment at elevated temperatures is an effective method to disrupt the lignocellulose structure for subsequent enzymatic hydrolysis. It is a thermochemical method that focuses on removing the hemicellulose as both monomeric and oligomeric sugars (Lavarrack et al. 2002). Hemicellulose removal is influenced by acid concentration, residence time, temperature and solids concentration (Larsson et al. 1999; Lloyd et al. 2005; Li et al. 2005; Karimi et al. 2006; Ruiz et al. 2008). The feedstocks usually used to study dilute acid pretreatment have been softwoods (Stenberg et al. 1998; Nguyen et al. 2000; Tengborg et al. 2001), hardwoods, agricultural residues (Lloyd et al. 2005), grasses and bamboo (Yang et al. 2009; Leenakul et al. 2010). The main aim of this work is to evaluate the potential of Bamboo for bioethanol production by means of dilute acid pretreatment and enzymatic hydrolysis. The influence of pretreatment parameters (temperature, acid concentration and residence time) on total recovery of fermentable sugars, mainly as xylose and glucose, and the enzymatic hydrolysis yield was studied in order to optimize the use the bamboo
in a biomass to ethanol process. To evaluate the effects of these parameters, central composite and surface response technologies were employed for experimental design.

**MATERIAL AND METHODS**

**Raw Material**

Bambusa balcooa was collected from Nelspruit in South Africa. Mature culms were harvested and a representative sample of the bamboo was chipped and immediately airdried for three weeks to equilibrium moisture content. A garden shredder (Viking, GE 103, Stihl, UK), an impact mill (Condux LV15M, Netzsch-Condux GmbH, Hanau, Germany) and an ultra centrifugal mill (Retch ZM 200, Monitoring and Control Laboratories, Parkhurst, RSA) with a 2 mm screen was used to reduce the particle size according to National Renewable Energy Laboratory’s (NREL) Preparation of Samples for Compositional Analysis (Sluiter et al. 2005). The fraction collected between 40 and 60 mesh (250-420μm) was used in all experiments. The biomass was stored at room temperature until used for chemical composition analysis or pretreatment. The composition of the raw material was determined using the standard Laboratory Analytical Procedures for biomass analysis provided by the National Renewable Energy Laboratory (NREL) (Colorado, USA, www.nrel.gov/biomass/analytical_procedures.html). The starch content of the raw material was determined by the hydrolysis of the starch using the Megazyme kit (K-TSTA Lot no. 70302-2, Wicklow, Ireland).

**Dilute Acid Pretreatment**

Dilute acid batch pretreatment was carried out in tubular reactors as described by Lloyd and Wyman (2005) at the University of California, Riverside. 1.5 grams of biomass will be soaked overnight in the required acid concentration at a solids concentration of 5% (w/v). After soaking, the biomass was filtered to remove the excess moisture to obtain a solid content of 30% (w/v). Each reactor was loaded with approximately 6.5 grams of wet biomass. Each pretreatment was run in triplicate to produce enough pretreated biomass for analysis of the water insoluble solid (WIS) fraction and for enzymatic hydrolysis. After cooling, the pretreated material was filtered into a WIS fraction and a liquid fraction or prehydrolyzate. The WIS fractions were dried for 24 h at 40 °C and analyzed for structural components (Lloyd et al. 2005). These solids were used as substrate in the enzymatic hydrolysis experiments. The liquid fraction after pretreatment was analyzed for sugars as described analytical methods below.

**Experimental Design**

The diluted acid pretreatment was performed using a central composite design (CCD) to optimize and evaluate the parameters that have significant effects on the overall sugar yield. The parameters studied were reaction temperature (178-205 °C), residence time (1-15 minutes) and acid concentration (0.14-0.44 g/ 100 g material)

This experimental design was developed in the commercial software STATISTICA 7.1 (Statsoft Inc., Tulsa, USA) and is based on a three-level, 3 factor (33) central composite design. This will include 6 axial points and 4 central points. The experimental error was determined at the centre point and the significance of effects was estimated by ANOVA.

**Enzymatic Hydrolysis**

The washed WIS after pretreatment of bamboo was enzymatically hydrolyzed to determine the effect of the different pretreatment conditions in the enzyme accessibility. Enzymatic hydrolysis was performed in 24 ml glass tubes, each containing 10 ml of 0.05 M sodium citrate buffer (pH 4.8) at 2% (w/v) dry pretreated substrate loading at 50 °C for 72 h. Sodium azide at 0.02% (w/v) was used to prevent microbial contamination. Cellulose-hydrolysing enzymes, Spezyme CP (Genencor, Leiden, Netherlands) with an activity of 65 FPU/ml, and Novozym 188 (Novozymes A/S, Denmark) with a β-glucosidase activity of 590 IU/ml, were used in all experiments. Enzyme loading of 60 FPU /g of dry pretreated substrate of Spezyme CP and 60 IU β-glucosidase/g dry pretreated substrate of Novozym188 was employed. Samples were withdrawn from the hydrolysis media after 24, 48 and 72 hours and sugar content was analysed by HPLC as described below.
Analytical Method

Enzyme preparations were subjected to standardized tests to determine main enzymes activities relevant in the conversion of lignocellulose: cellulase and cellobiase activities. Cellulase and β-glucosidase activities were measured according to methods described by Ghose (Ghose et al.1987).

The liquid fractions from the pretreated material were analysed for their monomeric soluble oligomeric sugar content. The oligosaccharide concentration was defined as the difference in monomer sugar concentration before and after complete acid hydrolysis of oligosaccharide sugars. The hydrolysates were centrifuged and filtered through a 0.2 μm filter before analysis for sugars (glucose, cellobiose and xylose) by high performance anionic exchange chromatography on a Dionex Ultimate® 3000 system equipped with a CarboPac PA1 column (4x250 mm). Likewise, cellobiose, glucose and xylose concentrations after completion of enzymatic hydrolysis tests were also measured. By-products (acetic acid, hydroximethylfurfural and furfural) were analyzed with an Aminex HPX-87H Ion Exclusion Column equipped with a Cation-H cartridge (Biorad, Johannesburg, RSA) with a UV detector at 220 and 280 nm (Waters 2487, Microsep, Johannesburg, RSA). All analytical determinations were performed in duplicate and average results are shown.

RESULTS AND DISCUSSION

Raw material composition

Chips of giant bamboo were initially characterized with regard to their chemical composition (Table 1). Giant bamboo showed to contain 47.1% of glucose (1.5% coming from starch), 15.8% of hemicellulose (xylan), 22.1% of acid insoluble lignin and 15.9% of extractives, which added up to 100.9% in a dry weight basis. These were the data used to estimate all of the pretreatment recovery yields described below.

Table 1: Chemical Compositional Analysis.

<table>
<thead>
<tr>
<th>Components</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>15.8 ± 1.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>47.1 ± 5.4</td>
</tr>
<tr>
<td>Hot Water Extractives</td>
<td>13.9 ± 1.2</td>
</tr>
<tr>
<td>Ethanol Extractives</td>
<td>2.04 ± 1.2</td>
</tr>
<tr>
<td>Lignins</td>
<td>22.1 ± 2.6</td>
</tr>
</tbody>
</table>

*The yield is the percentage of the initial oven dry mass
bLignin calculated on a acid insoluble basis

Dilute acid pretreatment

Effects of pretreatment on the composition of bamboo

Results of solid recovery yield, WIS composition on dry weight basis (dwb) and sugar recovery in prehydrolyzate are shown in Table 2. Solid recoveries ranged from 53.6% to 84.5%. Higher solubilization was obtained under harsher conditions, showing a notable influence of residence time, temperature and catalyst concentration on solids recovery. The solubilization of water-soluble biomass components into prehydrolyzate resulted in a cellulose enriched-WIS fraction in relation to the untreated raw material. As can be observed, glucose proportion of the solid residues (37% to 84.5%, average 63.8%) increased in relation to untreated material (47.1%) depending on pretreatment conditions.

The glucose yields in the liquid fractions from pretreatment corresponded up to around 6% of the glucan recovered as glucose and gluco-oligosaccharides. Since starch hydrolysis is possible under the conditions applied in the experiments, it can be concluded that glucose found in the prehydrolysate was originated from the hydrolysis of the starch and amorphous cellulose contained in the raw material.

The hemicellulose remaining in WIS after pretreatment exclusively consisted of xylan. As temperature and time increased, xylan content in WIS decreased as a result of a greater release into the liquid fraction. The lowest xylose content (2.2%) was found in the material treated at 212 °C for 8 minutes with a catalyst concentration of 0.29 g/100 g raw material. However, the release of xylose from the raw material during pretreatment does not always derive into a recovery in prehydrolyzate, due to the sugar degradation at high temperatures turning these...
components into lower molecular weight products. In order to maximize the utilization of all sugars present in raw material, it is essential to optimize the recovery of sugars dissolved in prehydrolyzate. Therefore, sugars recovery is one of the parameters chosen in this work as response variable to the variation of pretreatment conditions. A maximum xylose recovery value of 98% was reached in the liquid fraction at 195 °C for 8 minutes at 0.44% catalyst concentration.

Table 2: Solid Recovery (%), Carbohydrate Compositional Analysis and Digestibility (%) of the solid and liquid fractions obtained under the different pretreatment conditions

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Solid Recovery a (%)</th>
<th>Carbohydrates</th>
<th>Digestibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>Temp (ºC)</td>
<td>Time (min)</td>
<td>H₂SO₄ % (w/v)</td>
</tr>
<tr>
<td>1</td>
<td>185</td>
<td>3</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>185</td>
<td>13</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>185</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>185</td>
<td>13</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>205</td>
<td>3</td>
<td>0.18</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>205</td>
<td>3</td>
<td>0.4</td>
</tr>
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<td>8</td>
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<td>13</td>
<td>0.4</td>
</tr>
<tr>
<td>9</td>
<td>178</td>
<td>8</td>
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</tr>
<tr>
<td>10</td>
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<td>8</td>
<td>0.29</td>
</tr>
<tr>
<td>11</td>
<td>195</td>
<td>8</td>
<td>0.14</td>
</tr>
<tr>
<td>12</td>
<td>195</td>
<td>8</td>
<td>0.44</td>
</tr>
<tr>
<td>13</td>
<td>195</td>
<td>1</td>
<td>0.29</td>
</tr>
<tr>
<td>14</td>
<td>195</td>
<td>15</td>
<td>0.29</td>
</tr>
<tr>
<td>15-18</td>
<td>195</td>
<td>8</td>
<td>0.29</td>
</tr>
</tbody>
</table>

This good adjustment of the mathematical model to the experimental data was reflected in the contour graph/surface response prepared for the xylose recovery in prehydrolyzate (Fig. 1). A clear tendency towards the optimal value for this variable around experimental condition of 210°C for 12 minutes can be deduced from Fig. 1. The optimal value predicted from mathematical model will be 212°C for 10 minutes, resulting in almost a complete recovery of xylose.

Effects of dilute acid pretreatment on cellulose digestibility.

In view of the ethanol-production not only the hemicelluloses hydrolyzed by pretreatment is important, but also the effect of this release of the enzymatic degradability of the cellulose and hemicellulose. Therefore, enzymatic hydrolysis tests were performed to assess the effect of different pretreatment conditions tested on the digestibility of pretreated bamboo (WIS fraction). Glucan and xylan digestibility’s, expressed as percentage of glucose/xylose released during hydrolysis at 72 h in relation to potential glucose/xylose in WIS fraction, are summarized in Table 1. Dilute acid pretreatment significantly improved the cellulose conversion during enzyme hydrolysis. The untreated bamboo had an observed cellulose conversion of 2% (data not shown). Dilute acid pretreatment produced material with 11.9–77.1% theoretical cellulose conversion compared with the untreated bamboo of 2%. Elevated temperature and increased time, up to some extent, increased the enzymatic hydrolysis of cellulose to glucose. Pretreatment at 195 °C for 15 min with a catalyst concentration of 0.29% (w/w) resulted in the highest cellulose conversion (77.1%). Harder pretreatment conditions did not provide an enhancement in cellulose digestibility. This negative impact on cellulose accessibility could be due to the re-deposition of lignin mobilized under such high temperatures and low pH (Selig et al. 2007) but further studies would be required to elucidate this trend.
Overall sugar yield

An effective pretreatment should disrupt the lignocellulose structure to increase the accessibility of the enzymes and therefore the enzymatic hydrolysis yield. However, in establishing optimum conditions it is also valuable to recover a high concentration of sugars, considering also the sugars solubilised during pretreatment. Results of the glucose and xylose yields in both steps are represented in Figure 2. The highest yield of glucose in the enzymatic hydrolysis step, 19 g/100 g raw material, was obtained after pretreatment at 195 °C, 0.29% catalyst for 15 minutes. At these conditions, the overall glucose yield was also maximum, 23.7 g/100 g raw material. The overall glucose yield decreased to 7.1 g/100 g raw material when the pretreatment was carried out at 185 °C, 0.18% sulphuric acid for 3 minutes.
Since the xylose is the major component in the hemicellulose structure of giant bamboo, its recovery could contribute positively in the final ethanol yield when an industrial pentose fermenting microorganism is developed. Figure 3B showed the yield of xylose during pretreatment and enzymatic hydrolysis. Pretreatment performed at 195 °C, 0.44% (w/v) acid concentration for 8 minutes provided the highest overall xylose yield (15.7 g/100 g raw material), mainly from the liquid fraction.

CONCLUSIONS

In summary, diluted acid pretreatment of bamboo produced a material with enhanced digestibility compared to the raw material. The highest overall glucose yield obtained was 23 g per 100 g dry bamboo (50% of the theoretical), which was achieved after pretreatment at 195°C for 15 minutes with 0.29% catalyst concentration. A high yield of xylose was also obtained, 13 g per 100 g dry bamboo (82% of the theoretical) at these pretreatment conditions. Taking into account the overall glucose yield, it is possible to obtain a theoretical yield of 117.3 kg of ethanol per dry ton of bamboo. This yield could be increased up to 151.1 kg of ethanol per dry ton of bamboo considering the pentose sugars.

REFERENCES

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