Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol

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Abstract

Wheat straw consists of 48.57 ± 0.30% cellulose and 27.70 ± 0.12% hemicellulose on dry solid (DS) basis and has the potential to serve as a low cost feedstock for production of ethanol. Dilute acid pretreatment at varied temperature and enzymatic saccharification were evaluated for conversion of wheat straw cellulose and hemicellulose to monomeric sugars. The maximum yield of monomeric sugars from wheat straw (7.83%, w/v, DS) by dilute H2SO4 (0.75%, v/v) pretreatment and enzymatic saccharification (45°C, pH 5.0, 72 h) using cellulase, β-glucosidase, xylanase and esterase was 565 ± 10 mg/g. Under this condition, no measurable quantities of furfural and hydroxymethyl furfural were produced. The yield of ethanol (per litre) from acid pretreated enzyme saccharified wheat straw (78.3 g) hydrolyzate by recombinant Escherichia coli strain FBR5 was 19 ± 1 g with a yield of 0.24 g/g DS. Detoxification of the acid and enzyme treated wheat straw hydrolyzate by overliming reduced the fermentation time from 118 to 39 h in the case of separate hydrolysis and fermentation (35°C, pH 6.5), and increased the ethanol yield from 13 ± 2 to 17 ± 0 g/l and decreased the fermentation time from 136 to 112 h in the case of simultaneous saccharification and fermentation (35°C, pH 6.0).

Keywords: Wheat straw; Fuel ethanol; Dilute acid pretreatment; Enzymatic saccharification; Separate hydrolysis and fermentation; Simultaneous saccharification and fermentation

1. Introduction

Ethanol is a renewable, bio-based oxygenated fuel. In the USA, the production of fuel ethanol from corn starch reached about 2.81 billion gallons in 2003. Developing ethanol as fuel, beyond its current role as fuel oxygenate, will require developing lignocellulosic biomass as a feedstock because of its abundance and low cost. Previously, we targeted corn fiber (obtained from corn wet-milling industries) as a model substrate for use as lignocellulosic biomass because of its high carbohydrate content (70%) containing 20% residual starch, 15% cellulose and 35% hemicellulose, and low lignin content (>8%) [1]. Corn fiber can be enzymatically saccharified to fermentable sugars with a yield of 85–100% after pretreatment with dilute acid at a moderate temperature [2]. Corn fiber hydrolyzates containing a mixture of sugars (glucose, xylose, arabinose and galactose) were successfully fermented to fuel ethanol by mixed sugar utilizing, ethanologenic recombinant bacteria and yeast [3]. Based on these results, a pilot scale process can be developed for conversion of corn fiber to fuel ethanol.

In many countries, including the USA, wheat straw is an abundant by-product from wheat production. The average yield of wheat straw is 1.3–1.4 lb per lb of wheat grain [4]. Based on the data from FAO, 63.5 million metric tonnes of wheat were produced in the USA in 2003 (world production, 556.3 million metric tonnes) [5]. Wheat straw contains 35–45% cellulose, 20–30% hemicellulose and 8-
15% lignin and can also serve as a low cost attractive feedstock for production of fuel alcohol. Research has been done on the separation of cellulose, hemicellulose and lignin components from wheat straw and structural characterization of the hemicellulose fraction [6-10]. Also, a few reports are available on the production of ethanol from wheat straw hydrolyzates [11-15]. To our knowledge, no research paper is available on ethanol production from dilute acid pretreated enzyme saccharified wheat straw hydrolyzates by the mixed sugar utilizing ethanologenic recombinant microorganisms. In the present study, conditions for obtaining a high sugar yield from wheat straw using dilute H2SO4 as pretreatment option and enzymatic saccharification were examined.

The utilization of both cellulose and hemicellulosic sugars present in typical lignocellulosic biomass hydrolyzate is essential for the economical production of ethanol. The conventional ethanol fermenting yeast (Saccharomyces cerevisiae) or bacterium (Zymomonas mobilis) cannot ferment multiple sugar substrates to ethanol [3]. Escherichia coli metabolizes a wide variety of sugars. Our research unit has developed a recombinant E. coli (strain FBR5) that can ferment mixed multiple sugars to ethanol [16]. The strain carries the plasmid pL1297, which contains the genes from Z. mobilis necessary for efficiently converting pyruvate into ethanol. It selectively maintained the plasmid when grown anaerobically. In the present paper, the results of alcohol production by this recombinant bacterium from dilute acid pretreated and enzyme saccharified wheat straw hydrolyzates containing glucose, xylose, arabinose and galactose are presented. Fuel ethanol was produced from dilute acid pretreated wheat straw using a simultaneous saccharification and fermentation (SSF) approach. The effect of detoxification of acid pretreated hydrolyzates using lime is also presented.

2. Materials and methods

2.1. Materials

Wheat straw was purchased from a local farmer. It was dried in a forced-air oven at 55 °C for 24 h and milled in a hammer mill to pass through a 1.27 mm screen. The milled wheat straw was stored at room temperature. Celluclast 1.5 L, Novozyme 188, laccase from Trametes versicolor, lipase from Candida rugosa, glucose, xylose, arabinose, furfural, hydroxymethyl furfural (HMF) and levulinic acid were purchased from Sigma Chemical Co., St. Louis, MO. Viscostar 150 L and Pectinase solids were supplied by Dyadic Corp., Jupiter, FL. Aminex HPX 87P column (300 mm × 7.8 mm), Aminex HPX 87H column (300 mm × 7.8 mm), Carbo-P micro-guard cartridge (30 mm × 4.6 mm) and Cation H micro-guard cartridge (30 mm × 4.6 mm) were purchased from Bio-Rad Laboratories Inc., Hercules, CA.

2.2. Dilute acid pretreatment of wheat straw

Milled wheat straw was slurried in water or dilute H2SO4 (7.83% on dry solid basis, DS, w/v, unless otherwise stated) and pretreated in an autoclave at 121 °C for 1 h or sand bath at a desired temperature (140, 160 and 180 °C) for 15 min. The pretreated wheat straw was adjusted to pH 5.0 with 10 M NaOH or Ca(OH)2 before enzymatic saccharification.

For two stage pretreatment, wheat straw slurried in 0.5% H2SO4 (v/v) was placed in the sand bath at 140 °C for 15 min and cooled. The liquid was collected by centrifugation (15,000 × g, 10 min). The solid residue after washing once with water was slurried with 0.5% H2SO4 (v/v) and then treated at 190 °C for 10 min.

2.3. Enzyme assays

Carboxymethyl cellulase (CMCase) and xylanase activities were assayed in a reaction mixture (0.5 ml) containing 1% (w/v) carboxymethyl cellulose and 1% (w/v) oat spelt xylan, respectively, 50 mM acetate buffer, pH 5.0 and appropriately diluted enzyme solutions. After 30 min incubation at 50 °C, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid (DNS) method [17]. One unit (U) of each enzyme activity is defined as the amount of enzyme, which produces 1 μmol reducing sugar as glucose (xylose in the case of xylanase) in the reaction mixture per minute under the above-specified conditions.

β-Glucosidase, β-xylosidase and α-D-arabinofuranosidase activities were assayed in the reaction mixture (1 ml) containing 4 mM p-nitrophenyl β-D-glucoside, 2 mM p-nitrophenyl β-D-xyloside or 1 mM p-nitrophenyl-α-D-arabinofuranoside, respectively, 50 mM acetate buffer, pH 5.0 and appropriately diluted enzyme solutions. After incubation at 50 °C for 30 min, the reaction was stopped by adding 1 ml of ice-cold 0.5 M Na2CO3, and the color that developed as a result of p-nitrophenol liberation was measured at 405 nm. One unit (U) of each enzyme activity is defined as the amount of enzyme, which releases 1 μmol p-nitrophenol per minute in the reaction mixture under these assay conditions.

2.4. Enzymatic saccharification

The enzymatic saccharification of the diluted acid pretreated wheat straw was performed by shaking slowly (100 rpm) at 45 °C after adjusting the pH to 5.0 with NaOH and adding enzymes at each enzyme dose of 2 ml/100 g DS of wheat straw, unless otherwise stated. For pectinase solids, the enzyme dose was 1 g/100 g DS. Samples (1 ml) were withdrawn and kept at −20 °C before processing for analysis.

2.5. Overliming with calcium hydroxide

Calcium hydroxide was added to the dilute acid pretreated (0.75%, v/v, 121 °C, 1 h) and enzyme saccharified (45 °C, pH 5.0, 72 h) wheat straw hydrolyzate to increase the
pH to 10.5. The whole mixture was stirred for 30 min at 90 °C, allowed to cool slowly to room temperature and then adjusted back to pH 6.5 with HCl. It was then centrifuged (15,000 × g, 30 min) to remove any precipitate formed before using as substrate for fermentation.

2.6. Bacterial strain and preparation of inoculum

Recombinant *E. coli* strain FBR5 was used [16]. The strain was maintained in glycerol vials at −20 °C for use as a working stock. It was plated on Luria broth (10 g tryptone, 5 g yeast extract and 5 g NaCl) containing 20 g xylose and 40 mg tetracycline solidified with 15 g/l agar. Plates were incubated at 35 °C. Cells from a single well-isolated colony were inoculated into a 500 ml flask containing 200 ml of Luria broth with 50 g/l xylose. Cultures were incubated for 24 h at 35 °C and 130 rpm and used as seed culture for fermentation experiments.

2.7. Fermentation experiments

Batch culture experiments were carried out in pH-controlled 500 ml flasks with a working volume of 350 ml under semiaerobic conditions essentially as described by Bothast et al. [18]. A picture of the pH-controlled flasker fermentation system set-up has recently been published [19]. Luria broth containing wheat straw hydrolyzate was used as the substrate. It is prepared by dissolving 10 g tryptone, 5 g yeast extract and 5 g NaCl in the hydrolyzate (per litre) and autoclaving at 121 °C for 15 min. A 4 M KOH solution was used for pH control. Samples were withdrawn periodically to determine cell mass, ethanol content and residual sugars and stored at −20 °C prior to analysis. Base consumption and pH were also recorded. For SHF experiments, the fermentation was performed at pH 6.5 and 35 °C using the liquid portion of the hydrolyzate after separating it from the solids. For SSF experiments, 21 fermenters (Biostat B, B. Braun Biotech International, Allentown, PA) with working volumes of 1.5 l were used at pH 6.0 and 35 °C at the agitation rate of 150 rpm. The dilute acid pretreated whole-wheat straw hydrolyzate was added to the fermenter as a substrate after adjusting the pH to 6.0 with 4N NaOH before adding enzymes and inoculum. Inoculum size was 10% (v/v) in both cases.

2.8. Analytical procedures

The composition of milled wheat straw with respect to crude protein, crude fat, crude fiber, ash, moisture, neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose and lignin content was determined by the University of Missouri Agriculture Experiment Station Chemical Laboratories, Columbia, MO. Hemicellulose content of wheat straw was determined as NDF minus ADF using the van Soest method [20]. Sugars, furfural, HMF, levulinic acid, acetic acid, ethanol and succinic acid were analyzed by high-pressure liquid chromatography (HPLC) [2]. The separation system consisted of a solvent delivery system (P2000 pump, Spectra-Physics, San Jose, CA) equipped with an autosampler (717, Waters Chromatography Division, Millipore Corp., Milford, MA), a refractive index detector (410 differential refractometer, Waters), a dual A absorbance detector (2487, Waters) and a computer software based integration system (Chromquest 4.0, Spectra-Physics). Two ion moderated partition chromatography columns (Aminex HPX-87P with a Carbo-P micro-guard cartridge, Aminex HPX 87H with Cation H micro-guard cartridge) were used. The Aminex HPX-87P column was maintained at 85 °C, and the sugars were eluted with Milli-Q filtered water at a flow rate of 0.6 ml/min. The Aminex HPX-87H column was maintained at 65 °C, and the sugars, organic acids, furfural and HMF and ethanol were eluted with 10 mM HNO₃ prepared using Milli-Q filtered water at a flow rate of 0.6 ml/min. Peaks were detected by refractive index or UV absorption (277 nm) and were identified and quantified by comparison to retention times of authentic standards (glucose, xylose, galactose, arabinose, furfural, HMF, acetic acid, levulinic acid, succinic acid and ethanol). For comparative purposes, total-reducing sugars present in the hydrolyzate was also estimated by using DNS method [17]. Cell growth of the bacterium was monitored by measuring the optical density of the appropriately diluted culture broth at 660 nm in the case of SHF experiments.

3. Results

3.1. Dilute acid pretreatment and enzymatic saccharification

3.1.1. Effect of acid dose

Wheat straw used in this investigation contained 48.57 ± 0.30% cellulose and 27.70 ± 0.12% hemicellulose which make up the total carbohydrate content of 76.27 ± 0.42% on dry solid basis (Table 1). Table 2 shows the commercial enzyme preparations used for saccharification of pretreated wheat straw and the activity level of each assayed enzyme present in these preparations.

<table>
<thead>
<tr>
<th>Table 1 Composition of wheat straw on dry solid basis</th>
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<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>Crude protein</td>
</tr>
<tr>
<td>Crude fat</td>
</tr>
<tr>
<td>Crude fiber</td>
</tr>
<tr>
<td>Ash</td>
</tr>
<tr>
<td>Cellulose</td>
</tr>
<tr>
<td>Hemicellulose</td>
</tr>
<tr>
<td>Lignin</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
</tr>
</tbody>
</table>

The data presented are averages of two independent analyses.

* a Calculated value.
Initially, we have evaluated dilute acid pretreatment of wheat straw at 121 °C. The effects of acid dose (0.0-1.0%, v/v) on dilute H₂SO₄ pretreatment at 121 °C for 1 h and enzymatic saccharification using cellulase (Celluclast) and β-glucosidase (Novozyme 188) at 45 °C and pH 5.0 for 72 h of wheat straw are shown in Table 3. With 7.83% (w/v, DS) wheat straw, an acid dose of 0.75% (v/v) gave maximum yield (485 ± 22 mg/g, 64% of total carbohydrates) of fermentable sugars. About 92% of hemicellulose was converted to sugars (255 ± 13 mg/g DS) and only 47% cellulose (230 ± 9 mg/g DS) was converted to glucose. No detectable quantities (>0.001%, w/v) of furfural and HMF were present in these hydrolyzates. The effect of the increase of H₂SO₄ dose (up to 4%, v/v) on the pretreatment and enzymatic saccharification of wheat straw was also studied. Total sugars present in the hydrolyzates were 455 ± 3 and 400 ± 1 mg/g DS at the acid doses of 2 and 4% (v/v), respectively (data not shown).

The addition of ethylene carbonate (1%, w/v) with dilute H₂SO₄ (0.75%, v/v) pretreatment at 121 °C for 1 h and enzymatic saccharification (45 °C, pH 5.0, 72 h) by cellulase (Celluclast) and β-glucosidase (Novozyme 188) increased the sugar yield to 563 ± 17 mg/g DS (glucose, 272 ± 5 mg; xylose/galactose, 242 ± 9 mg and arabinose, 49 ± 3 mg) (74% yield). The addition of metals (FeSO₄, Al₂(SO₄)₃, and MgSO₄, each at 0.2 mM) with 0.75% H₂SO₄ (v/v) did not enhance the enzymatic saccharification yield of wheat straw (data not shown).

The addition of laccase, lipase or both preparations with cellulase and β-glucosidase preparations did not enhance the saccharification yield of 0.75% H₂SO₄ (v/v) pretreated wheat straw (data not shown). The addition of Tween 20 (2.5 g/l) increased the enzymatic (Celluclast, Novozyme 188 and Viscostar 150 L) saccharification yield of H₂SO₄ pretreated (0.75%, v/v, 1 h) wheat straw (7.83%, w/v, DS) from 488 ± 3 to 520 ± 4 mg/g DS. The addition of pectinase preparation to the three enzyme combination (Celluclast, Novozyme 188 and Viscostar 150 L) increased the saccharification yield of wheat straw to 518 ± 1 mg (glucose, 261 ± 1 mg). In combination with Tween 20 (2.5 g/l), this four enzyme cocktail gave 565 ± 10 mg/g total sugars (glucose, 297 ± 6 mg; xylose/galactose, 240 ± 4 mg and arabinose, 27 ± 0 mg) DS of wheat straw which gives a yield of 74% of the total carbohydrate content.

Wheat straw was treated with concentrated H₂SO₄ (2:1, w/w) at 25 °C for 3 h, neutralized and the sugar composition was determined. The yield of total sugars was only 373 ± 6 mg/g DS (glucose, 284 ± 1 mg, xylose/galactose, 72 ± 4 mg and arabinose, 16 ± 1 mg, 49% of total carbohydrate content). Under this condition, 24.6 ± 2.0 mg acetic acid, 9.4 ± 0.5 mg furfural and 1.3 ± 0.0 mg HMF were produced per gram DS of wheat straw.
3.1.2. Effect of temperature

The effects of temperature (140, 160 and 180 °C) on dilute H₂SO₄ (0.00, 0.25 and 0.50%, v/v) pretreatment of wheat straw for 15 min and subsequent enzymatic saccharification (45 °C, pH 5.0, 72 h) were evaluated. Dilute acid (0.5%, v/v) pretreatment at 180 °C for 15 min and subsequent enzymatic saccharification using the commercial cellulase and β-glucosidase preparation generated 576 mg total sugars per gram DS of wheat straw (75% yield, Fig. 1A–C). Acetic acid formation is also shown in Fig. 1D. The acetate formation increased with both temperature and acid dose. No furfural was detected in the wheat straw hydrolyzates pretreated at 140 and 160 °C. But at 180 °C, 11 and 32 mg furfural were produced per gram DS of wheat straw at acid dose of 0.25 and 0.5% (v/v), respectively. No HMF was detected in any of the hydrolyzates.

The effect of pretreatment time (15, 30 and 60 min) on dilute H₂SO₄ (0.5%, v/v) pretreatment of wheat straw at 140 °C was also studied (Table 4). The overall saccharification yield did not increase with the increase of treatment time as a result of decrease in hemicellulose conversion even though the cellulose saccharification was enhanced.

Table 4
Effect of pretreatment time on dilute acid pretreatment at 140 °C and enzymatic saccharification of wheat straw

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Enzymatic saccharification</th>
<th>Sugar yield (mg/g DS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 ± 0</td>
<td>116 ± 9</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>269 ± 11</td>
<td>195 ± 11</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>12 ± 0</td>
<td>117 ± 0</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>307 ± 5</td>
<td>208 ± 3</td>
<td>23 ± 0</td>
</tr>
<tr>
<td>16 ± 5</td>
<td>109 ± 22</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>313 ± 8</td>
<td>198 ± 11</td>
<td>25 ± 3</td>
</tr>
</tbody>
</table>

Wheat straw (7.83%, w/v, DS) was treated with dilute H₂SO₄ (0.5%, v/v) at 140 °C. Enzymatic saccharification was performed using cellulase (Celluclast), β-glucosidase (Novozyme 188) and xylanase (Viscostar 150 L) at 45 °C and pH 5.0 for 72 h. Enzymes used, 2 ml/100 g DS of wheat straw. The data presented are averages of two separate experiments.
Table 5
Ethanol production from wheat straw hydrolyzate by recombinant Escherichia coli strain FBR5

<table>
<thead>
<tr>
<th>Hydrolyzate</th>
<th>Fermentation time (h)</th>
<th>Sugar (g/l)</th>
<th>Ethanol (g/l)</th>
<th>Ethanol (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separate hydrolysis and fermentation</td>
<td>Acid, enzyme</td>
<td>118</td>
<td>46 ± 1</td>
<td>19 ± 1</td>
</tr>
<tr>
<td></td>
<td>Acid, enzyme, overliming</td>
<td>39</td>
<td>48 ± 0</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Simultaneous saccharification and fermentation</td>
<td>Acid, enzyme</td>
<td>136</td>
<td>-</td>
<td>13 ± 2</td>
</tr>
<tr>
<td></td>
<td>Acid, overliming, enzyme</td>
<td>112</td>
<td>-</td>
<td>17 ± 0</td>
</tr>
</tbody>
</table>

For pretreatment, wheat straw (7.83%, w/v, DS) was treated with 0.75% H₂SO₄ (v/v) at 121 °C for 1 h. Enzymatic saccharification was performed using cellulase (Celluclast), β-glucosidase (Novozyme 188) and xylanase (Viscostar 150 L) at 45 °C and pH 5.0 for 72 h. Enzymes used, 2 ml/100 g DS of wheat straw. Overliming (pH 10.5) was done at 90 °C for 30 min. Fermentation experiments were performed at 35 °C and pH 6.5 for separate hydrolysis and fermentation or at pH 6.0 for simultaneous saccharification and fermentation. The data presented are average of two separate experiments.

* Time of maximum alcohol production.

3.1.3. Two stage dilute acid pretreatment

The two stage dilute H₂SO₄ pretreatment of wheat straw was carried out with the aim of first depolymerizing hemicellulose at a lower temperature (140 °C, 15 min) in order to avoid the formation of furan compounds and carboxylic acids, and then the second stage was performed at a higher temperature (190 °C, 10 min) to make cellulose much more accessible to enzymatic hydrolysis. The liquid phase containing the soluble materials was removed between the treatments, thereby avoiding further degradation of monosaccharides formed. The hydrolyzate contained only 140 ± 13 mg glucose, 165 ± 6 mg xylose, 13 ± 4 mg galactose and 62 ± 2 mg arabinose (total sugars, 382 ± 18 mg/g DS) of wheat straw. Moreover, it contained 45.1 ± 4 mg furfural and 10.3 ± 0.3 mg HMF in addition to 30.8 ± 1.7 mg acetic acid per gram DS.

3.2. Fermentation of wheat straw hydrolyzates

The results of fermentation of dilute H₂SO₄ pretreated and enzyme saccharified wheat straw by the recombinant E. coli strain FBR5 are summarized in Table 5. It is clear that separate hydrolysis and fermentation (SHF) approach worked better than simultaneous saccharification and fermentation method. Overliming (pH 10.5, 90 °C, 30 min, and then adjusting the pH to 5.0) helped to reduce the fermentation time dramatically from 118 to 39 h in the case of SHF. For SSF, overliming helped to increase the yield of ethanol from 13 ± 2 to 17 ± 0 g/l and also decreased the fermentation time from 136 to 112 h (Table 5). The maximum yield of ethanol (per litre) from wheat straw (78.3 g) hydrolyzate by recombinant E. coli strain FBR5 was 19 ± 2 g with a yield of 0.24 g/g DS (0.32 g/g theoretical sugars present in wheat straw on DS basis) by SHF. The average cell densities (A₆₆₀ nm) were 2.3 at 118 h in the case of SHF without overliming and 1.4 at 39 h in the case of SHF with overliming, respectively. The time courses of ethanol production by the recombinant E. coli strain from wheat straw hydrolyzate is shown in Fig. 2. It is evident that the ethanol production rate was much higher at the initial stage of the fermentation. At 22 h, the ethanol yields were 11.3, 14.4, 9.1 and 10.9 g/l for SHF, SHF with overliming, SSF, SSF with overliming, respectively.

4. Discussion

Wheat straw, an abundant low value by-product of wheat production world wide, is an attractive feedstock for conversion to fuel ethanol. Pretreatment of any lignocellulosic biomass is crucial before enzymatic saccharification [21]. Dilute acid pretreatment has become a state of the art technology for pretreating any lignocellulosic biomass [22,23]. It has the advantage of not only solubilizing hemicellulose but also converting solubilized hemicellulose to fermentable sugars [2]. The dilute acid pretreatment, thus, eliminates or reduces the need for use of hemicellulase enzyme mixtures. However, depending on the temperature, the pretreatment usually produces sugar degradation products, such as furfural and HMF, which are inhibitory to the fermentative microorganisms [24]. The objective of this study is to perform the dilute acid pretreatment at a lower temperature, and thus avoid the formation of inhibitory sugar degradation products.
This study indicates that 0.75% (v/v) H₂SO₄ gave good yields of sugars after enzymatic saccharification without forming sugar degradation products (furfural and HMF). We have been able to obtain a saccharification yield of 74% by using 0.75% (v/v) acid pretreatment at 121°C for 1 h and saccharifying with a cocktail of four commercial enzyme preparations. Also, most of the hemicellulose was solubilized and degraded to component monosaccharides (xylose, arabinose and galactose) without further degradation to furfural and HMF. Previous research at our Center on wheat straw pretreatment using 1N trifluoroacetic acid (TFA) for 7 h at reflux temperature (99–100°C) gave a xylose yield of 80% based on the xylan content of hemicellulose and the residue after enzymatic saccharification with cellulase gave 70–75% conversion of cellulose to glucose [25]. Delgenes et al. [15] reported efficient hydrolysis of the hemicellulose fraction by treating wheat straw with 2% (w/v) H₂SO₄ for 23 h at 91.5°C under reflux using 20 ml acid per gram wheat straw chips.

Concentrated acid treatment offers advantage of not using any enzymes for saccharification [26]. Delgenes et al. [15] treated wheat straw with 72% (w/v) H₂SO₄ for 30 min at 30°C using 15 ml acid per gram wheat straw and obtained 11.1 g monosaccharides from 18.8 g wheat straw on DS basis. In our study, treatment of wheat straw with concentrated H₂SO₄ under the conditions used gave only 49% conversion of total carbohydrates to sugars even though the method did not generate much furfural and HMF. Research needs to be performed to find an optimized condition for generating sugars from wheat straw by concentrated acid treatment.

The data in Fig. 1 indicates that the sugar and acetic acid yield are dependent upon the H₂SO₄ dose as well as temperature of pretreatment. The maximum yield of sugars was 76% after enzymatic saccharification of 0.5% H₂SO₄ (v/v) pretreated wheat straw for 15 min at 180°C. However, at this temperature, 32 ± 4 mg furfural and 27 ± 0 mg acetic acid, but no HMF were produced per gram DS of wheat straw. This clearly indicates that pentose sugars (xylose and arabinose) derived from hemicellulose were further degraded. The two stage dilute acid (0.5%, v/v) pretreatment (140°C, 15 min; 190°C, 10 min) did not give good yields of sugars from wheat straw. One reason is that the cellulose degradation was not enhanced at all under the conditions used. The quantity of total sugars present in each hydrolyzate determined by HPLC analysis of sugars was found to be correlated well with the total sugars estimated by DNS method (data not shown).

A critical problem in the fermentation of dilute acid hydrolyzates has been the inability of the fermentative microorganism to withstand inhibitory compounds produced during pretreatment and usually a detoxification step is needed to improve fermentability [27,28]. Amarteer et al. [29] reported that overliming helped the fermentation of sugars derived by acid hydrolysis of the hemicellulose fraction of wheat straw to ethanol by *Bacillus steaothermophilus* T-13, a lactate dehydrogenase deficient mutant. The results of our study clearly indicate that it is possible to obtain a good yield of fermentable sugars from wheat straw using conventional dilute H₂SO₄ pretreatment at a moderate temperature and commercially available enzymes. Even though mild acid and moderate temperature were used for dilute acid pretreatment and the hydrolyzate did not contain any detectable quantities (0.001%, w/v) of furfural and HMF, it still needs to be conditioned further by overliming (Table 5). Otherwise, the fermentation of the hydrolyzate by the recombinant ethanologenic *E. coli* takes much longer time in the case of SHF. In the case of SSF, the yield is much better and production rate was also improved by overliming (Table 5). It is thought that apart from furans formed by sugar degradation, some unknown aromatic lignin degradation products were formed during acid pretreatment [24]. These compounds are likely to be responsible for the inhibition or delay in fermentation activity of the recombinant *E. coli* used in this investigation. These inhibitory compounds get precipitated during overliming [30]. However, the overliming method of detoxification has a drawback in the form of sugar loss [31].

We are now concentrating our effort to pretreat and saccharify wheat straw in such a way as not to produce any inhibitors or minimize the formation of these fermentation inhibitors. The maximum ethanol yield from fermentation of wheat straw hydrolyzate was 19 ± 2 g/l from 78.3 g/l of wheat straw on DS basis. Even though SSF is preferred with respect to process integration and simplification, it did not perform well in comparison with SHF. One main reason is that the SSF was performed at a compromised pH 6.0 and temperature 35°C instead of enzyme optimal activity at pH 4.5–5.0 and 45–50°C. This made the SSF process much slower. For this reason, Szczodrak used thermotolerant *Kluveromyces fragilis* for SSF of pretreated wheat straw to ethanol at 43°C and obtained 3% (w/v) ethanol from 10% (w/v, DS) of chemically treated straw in 24 h [32]. Moreover, the acid hydrolyzate of wheat straw used as such after neutralization for SSF added much insoluble substrate to the reactor (fermenter) that required very good agitation, which might have denatured some enzyme activities. SHF was performed at their respective optimal temperature and pH and it also offered much easier mixing during fermentation because of the separation of the insoluble materials from the hydrolyzate before fermentation. The recombinant *E. coli* strain FBR5 can produce 42 g ethanol from 95 g xylose per litre at 35°C and pH 6.5 [16]. Attempts will be made to increase the sugar concentration in the hydrolyzate so as to obtain more ethanol in the fermentation broth. We are exploring other pretreatment options, such as alkali and alkaline peroxide, for wheat straw.

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References


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