CELLULOSE CHEMISTRY AND TECHNOLOGY

LOW TEMPERATURE ACID HYDROLYSIS OF GRASS-DERIVED LIGNOCELLULOSE FOR FERMENTABLE SUGARS PRODUCTION

REMY M. K. VALA and L. TICHAGWA

Department of Chemistry, University of Fort Hare, Alice, South Africa

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Fermentable sugars from food crops have been used for ethanol biofuel production. However, a major food security concern has led to an increase in the research of non-food crops as biomass for biofuels. Lignocellulosic materials (biopolymers) have been considered as biomass, but their use involves challenging production processes to release fermentable sugars. Several approaches using high temperatures and pressures have been used to facilitate the breakdown of lignocellulose. In this study, Kikuyu grass was investigated as a low-cost source of fermentable sugars. The lignocellulosic material from grass was subjected to pretreatment and then hydrolysed by sulphuric acid at comparatively low temperatures of below 100 °C at atmospheric pressures to produce fermentable sugars. A simple and low-cost method for the removal of some inhibitors (by-products produced during hydrolysis) using wood shavings packed in a chromatographic column was developed. The yield of fermentable sugars obtained after hydrolysis was 22.26 g/L (38.4% w/w).

Keywords: lignocellulose, biomass, acid hydrolysis, bioethanol, hydrolysate, fermentable sugars

INTRODUCTION

Increasing costs and growing concerns over the environmental impact of fossil fuels and their inevitable depletion have led to intense research on the development of alternative energy sources. Lignocellulosic biomass has been investigated as a possible renewable resource for bioethanol production. Pretreatment is needed to break down the lignocellulosic structure and utilize cellulose as a source of sugar for subsequent fermentation. The effect of pretreatment of lignocellulosic materials has been studied and reported widely.[1] Physical pretreatment requires reduction in biomass particulate size and other processes, such as steam explosion, hydrothermolysis, vibratory ball milling, compression milling and heat at times,[2-4] whereas chemical pretreatment usually involves the use of hot water, acids or bases. A combination of both physical and chemical pretreatment is sometimes needed in order to achieve higher efficiency. The purpose of the pretreatment is to remove lignin and hemicelluloses, as well as reduce cellulose crystallinity in order to increase the porosity of the materials.[5] From the fermentable sugars obtained from cellulose, it is possible to use enzymes in fermentation to produce bioethanol.[6] In the chemical route, mineral acids, organosolv, Lewis acids and wet oxidation methods have been utilized to disrupt cellulose structure and promote hydrolysis, as well as to dissolve hemicelluloses in the production of fermentable sugars.[7]

In this work, sulphuric acid was used for the low temperature (<100 °C) hydrolysis of grass biomass. The experiments allowed the determination of optimal operational conditions for the biopolymer breakdown. By-products of hydrolysis (which included inhibitors of fermentation) were first removed, before the amount of fermentation sugars was determined and further processed.

EXPERIMENTAL

Grass (*Pennisetum clandestinum* or Kikuyu grass) was collected during autumn (the grass cellulose content is at its maximum during this period).[8] Pure cellulose was used as reference material and was supplied by Accordis, Germany.

Grass was pretreated before hydrolysis to disrupt the complex structure of lignocellulose. The solid residues collected after pretreatment were then hydrolysed with sulphuric acid.

Biomass pretreatment

Grass was manually cut into pieces of about 1-2 cm, washed and dried. The plant material was then milled into powder, using a domestic grinder to allow passage through a 0.4 mm U.S. standard sieve in order to increase the surface area exposed to the hydrolysis agent. Extraction was carried out in hot water (98 °C) until the filtrate became colorless and the solid residue was oven dried to constant mass at 105 °C overnight.[9-10] The resultant powder was stored in a glass bottle and kept in the dark at room temperature until used for hydrolysis.

Several parameters, such as sulphuric acid concentration, temperature and hydrolysis time, were varied so as to try to determine the optimum conditions for breaking down the lignocellulosic material to obtain fermentable sugars. Pure cellulose was used as a control and was not subjected to the pretreatment procedure described for grass.

Dilute acid hydrolysis

The hydrolysis of lignocellulosic materials in dilute acid solution was adapted from the methods used by Shibazaki *et al.*,[11] Curreli *et al.*[12] and Hong and Qiu,[13] as follows: biomass (5 g of pretreated grass powder or pure cellulose) was suspended in 50 mL of 0.5, 1, 1.5 and 2% (w/v) H_2SO_4 solution, stirred for one hour at room temperature and then heated (or under reflux) in a 250 mL round flask at different temperatures and times, as shown in Table 1. By-products were removed from the obtained fermentable sugars before further processing.

Table 1
Refluxing conditions for hydrolyzates in dilute acid

	Dilute H_2SO_4							
% H_2SO_4 (w/v)	0.5	0.5	1.0	1.0	1.5	1.5	2.0	2.0
Temperature (°C)	65	97.5	65	97.5	65	97.5	65	97.5
Time (hours)	5	5	5	5	5	5	5	5

Table 2
Refluxing conditions of hydrolyzates in dilute acid

	Concentrated H_2SO_4											
% H_2SO_4 (w/v)	20	20	24	24	28	28	50	50	60	60	70	70
6% $ZnCl_2$ (ml)	0	10	0	10	0	10	0	10	0	10	0	10
Time (h)	4	4	4	4	4	4	3	3	3	3	3	3
Temperature	65	65	65	65	65	65	Rt	Rt	Rt	Rt	Rt	Rt
(ºC)	97.5	97.5	97.5	97.5	97.5	97.5	65	65	65	65	65	65

Rt = Room temperature (~22 °C)

Concentrated acid hydrolysis

The method for hydrolysis in concentrated acid solution was adapted from the work of Xiang[14] and Romeo *et al.*[15] Varying concentrations of H_2SO_4 (30 mL aliquots) were added to 3 g of biomass in 250 mL flask and the mixture was heated (or under reflux). To some of the runs, 10 mL of 6% $ZnCl_2$ was added with the sulphuric acid to study its effect on the solubility of the biomass (Table 2). The hydrolysate was then used to determine the amount of fermentable sugars.

Hydrolysate treatment

Biomass hydrolysis produces by-products that inhibit the fermentation process. Thus, hydrolysates were treated to remove the by-products that inhibit fermentation. A simple method was developed in this study to remove some of the inhibitors (such as furfural). A chromatography column packed with wood shavings treated with 1 M H_2SO_4 and 1 M NaOH was used to separate by-products from fermentable sugars at pH 5.5-6.0. Distilled water was used as eluent at about 10 drops per minute.

Removal of by-products

Thin-layer chromatography (TLC) and Fourier Transform Infrared spectroscopy (FT-IR) were used to investigate the effectiveness of the removal of by-products from the hydrolysates. TLC chromatograms and FTIR spectra of water washed wood shavings, acid and alkali-treated wood shavings, as well as used wood shavings, were obtained.

Thin Layer Chromatography (TLC) procedure

TLC was performed using TLC aluminium sheet silica gel plates with specifications 60 F254 (20 x 7 cm, layer thickness 0.2 mm) supplied by Merck. Solutions of the samples before and after by-products removal were spotted on the plate. Acetonitrile-water (85:15 v/v) was used as solvent for the thin-layer chromatography.[16-17] D-glucose and D-fructose supplied by Merck were used as standards. TLC spots on the dried plate were examined under a UV-lamp at 366 nm. The TLC plate is shown in Figure 1.

FT-IR spectroscopy

IR spectra were obtained using Perkin-Elmer 2000 FT-IR spectrophotometer. The by-products from hydrolysis were first oven dried at 35 °C for 72 hours, before being mixed with KBr and pressed into pellets. This was done to study the effect of wood shavings (as solid phase of column chromatography) in the removal of by-products (inhibitors of fermentation). Figure 2 shows IR spectra of untreated wood, wood treated with 1 M NaOH/1 M H_2SO_4 and treated wood that had been used as the solid phase for chromatography.

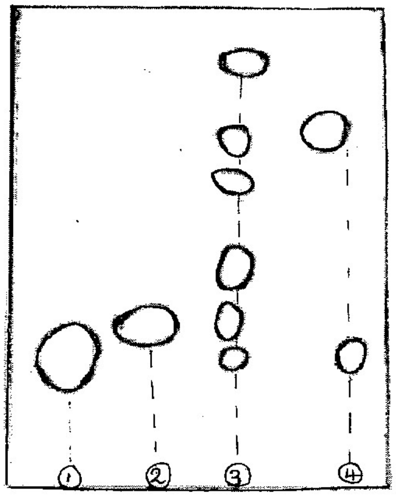

Figure 1: TLC before and after removal of by-products: 1: Glucose; 2: Fructose; 3: Hydrolyzate before removal of inhibitors, and 4: Hydrolyzate after removal of inhibitors (by wood shavings)

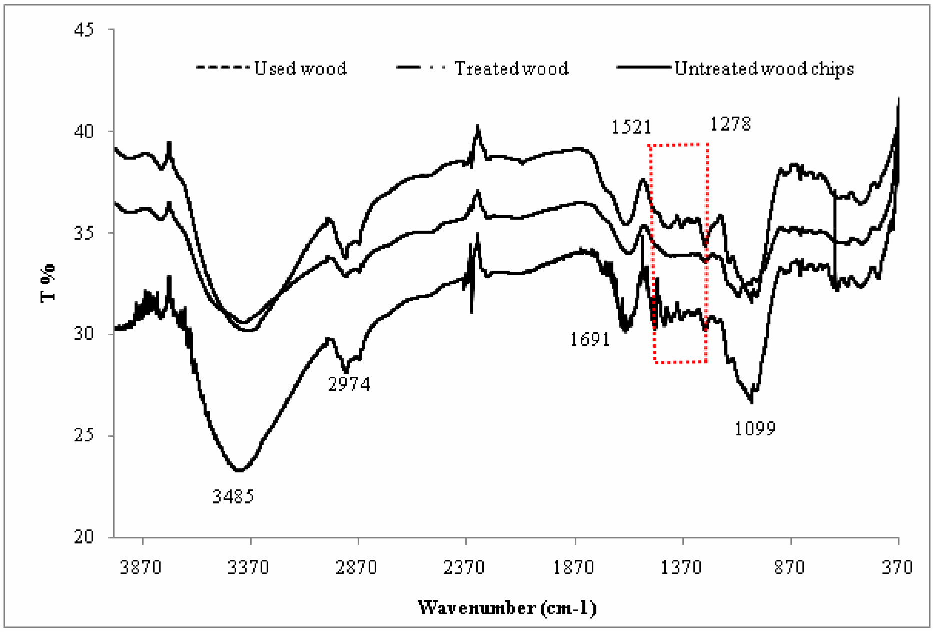

Figure 2: FTIR spectra of treated wood (before column) and used wood (after column) shavings with grass hydrolyzate from 2% (w/v) H_2SO_4

Determination of fermentable sugars

The amounts of fermentable sugars obtained from grass hydrolysis were determined by using the 3,5-dinitrosalicylic acid method.[18-20] Concentrations were calculated using a calibration curve (R^2 = 0.9997), which was obtained with standard solutions of glucose (0.00, 0.18, 0.54, 0.90 and 1.26 g/L).

RESULTS AND DISCUSSION

Sulphuric acid hydrolysis of grass materials produced yellowish hydrolysates, suggesting the presence of by-products, with colours varying from light to dark yellow as the acid concentration increased.

The assignment of IR peaks is given in Table 3. The untreated, treated and used wood shavings showed common absorptions around 3485, 2974, 1691, 1521, 1278 and 1099 cm^{-1}. The reduction in the intensity of peaks at 1521 and 1278 cm^{-1} and the shifting of peak 1099 cm^{-1} on the IR spectrum of the used shavings can probably be attributed to the removal of inhibitors.

Table 3
Assignment of FT-IR peaks from analysis of wood shavings

Wavenumber range (cm^{-1})	Assignment
3485	O-H
2974	C-H
1691	C=O
1521	C-C
1396	CH_3
1099	C-O

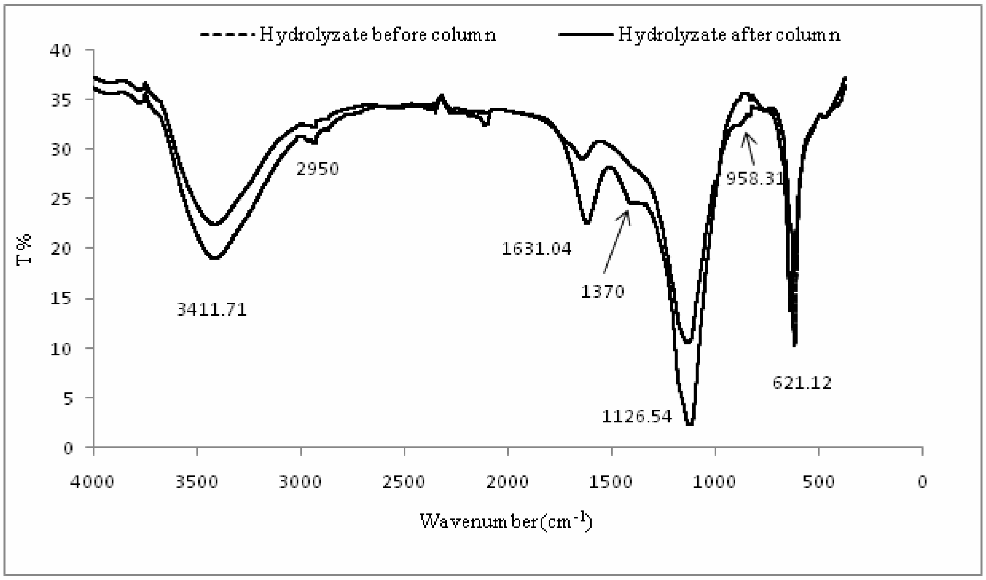

Figure 3: FTIR of hydrolyzates

In Figure 3, the IR spectrum of the hydrolysate from the treated wood shavings did not show the peaks at 1370 cm^{-1}, implying a change in wood composition. However, the reduction of the peak at 1631.04 cm^{-1} gave rise to a possible reduction of functional group (C=C) assigned to that particular wavelength. No explanation could be found for the appearance of a peak at 958.31 cm^{-1}. Nonetheless, the change in color, as well as the disappearance of the IR peaks, clearly demonstrates the effectiveness of the treated wood shavings in removing inhibitors of alcoholic fermentation.

TLC chromatography

The TLC chromatogram shows that wood shavings removed 4 components from the 6 components found in untreated hydrolysates (Figure 1). This confirms the efficiency of the method for by-products removal. The components removed by wood shavings are clearly different from sugars such as D-glucose (1) and fructose (2) used as references.

However, the spot representing fructose (as standard) was also removed by the wood shavings in the hydrolysate.

Table 4
Assignment of FT-IR peaks from analysis of hydrolyzates

Wavenumber range (cm^{-1})	Assignment
3411.71	O-H
2950	C-H
1631.04	C=O
1370	CH_3
1126.54	C-O
958.31	C-H
621.12	C-H

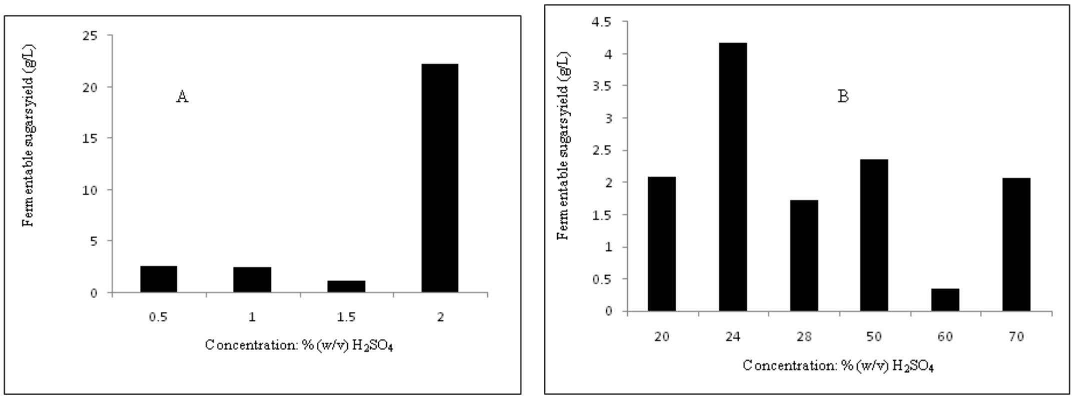

Figure 4: Effect of acid concentration on fermentable sugars yield at different temperatures:
A) dilute acid at 97.8 °C; B) concentrated acid at 65 °C

Yield of fermentable sugars

Several experiments were carried out to determine optimum conditions for grass hydrolysis to produce fermentable sugars. Different yields were deduced from the calibration curve obtained with standard solutions. The different parameters (sulphuric acid concentration, zinc chloride concentration and temperature) that were studied had variable impact on the yield of fermentable sugars. Hydrolysis conditions at 97.5 °C under reflux with 2% (w/v) H_2SO_4 yielded the highest concentration of fermentable sugars. The effects of each of the diffent parameters are discussed below.

Effect of acid concentration

The use of 2% sulphuric acid yielded the highest concentration of fermentable sugars from grass at 22.26 g/L. Figure 4 shows the differences between the yields of fermentable sugar from dilute and concentrated sulphuric acids. Soluble pure cellulose yielded 33.34 g/L under the same conditions. Similar results (from 2.3 g/L to 35.9 g/L of fermentable sugars) were reported by Larsson *et al.*[21] and Hong and Qiu[13] using spruce (*Picea abies*) and konjac powder, respectively, as biomass with 1.5% sulphuric acid. Concentrated sulphuric acid solutions (from 20 to 70%) could yield significantly high amounts of fermentable sugars, however it has been reported that harsh conditions (high concentrations and temperatures) promote conversion of sugars to furfural.[22-24]

This will probably explain the low conversion efficiency of concentrated acid solutions in the production of fermentable sugars. Conversion of sugar to other chemicals under the refluxing process is one of the major causes of low yield.[23-25]

Effect of zinc chloride

Zinc chloride (10 mL, 6% concentration) in sulphuric acid (from 20 to 70%) was used in parallel runs to facilitate the dissolution of biomass. The 2% sulphuric acid concentration was not enough to provide a medium for $ZnCl_2$ to dissolve the biomass. Such aqueous chloride solutions are reported in the literature as capable of dissolving cellulose,[25-26] which explains why grass hydrolysates from zinc chloride/sulphuric appeared to be nearly totally dissolved, compared to those without zinc chloride. Figure 5 shows the effect of $ZnCl_2$ on the yield of fermentable sugars at 65 °C. It was found that zinc chloride (with 20% sulphuric acid) increased the concentration of fermentable sugars at temperatures of 65 °C and 97.5 °C. The presence of zinc chloride in the medium could have prevented the sugar being converted to by-products. The decrease in the yield of sugar in the high sulphuric acid concentration (>24%) can be attributed to the combined effects of concentration and temperature. However, in acid concentration of 60 and 70% (w/v), the influence of zinc chloride on recovered sugars was not significant. The hydrolysates from low acid concentrations (without zinc chloride) yielded significant amounts of fermentable sugars, compared to those from the mixture of sulphuric acid/zinc chloride.

Effect of temperature

The effect of temperature on hydrolysis was reported by Clausen and Gaddy,[27] where they noted low yields resulting from high temperature hydrolysis at long times in the hydrolysis of lignocellulose.

Figure 6 (A: at room temperature, B: at 65 °C, C: at 97.5 °C) shows the effect of temperature on the yield of fermentable sugars. A comparison of the amount of fermentable sugars obtained at different temperatures with the same acid concentration showed that only 50% (w/v) H_2SO_4 at 65 °C yielded a relatively high amount, compared to that obtained at room temperature. All the other concentrations, either 65 °C versus room temperature, or 65 °C versus 97.5 °C, yielded amounts aligned with the results reported by Clausen and Gaddy.[27]

This might be explained by the fact that the breaking down of cellulose into fermentable sugars by high acid concentrations resulted most likely in the conversion of hydrolysates to by-products.

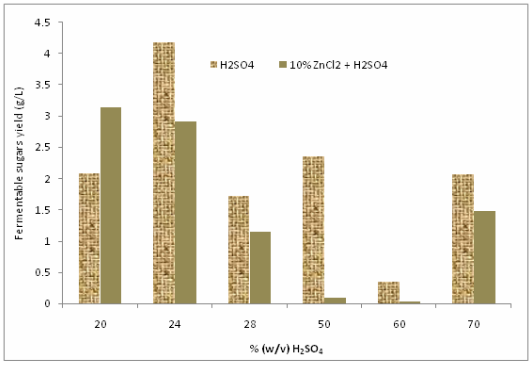

Figure 5: Effect of $ZnCl_2$ on fermentable sugars yield at 65 °C

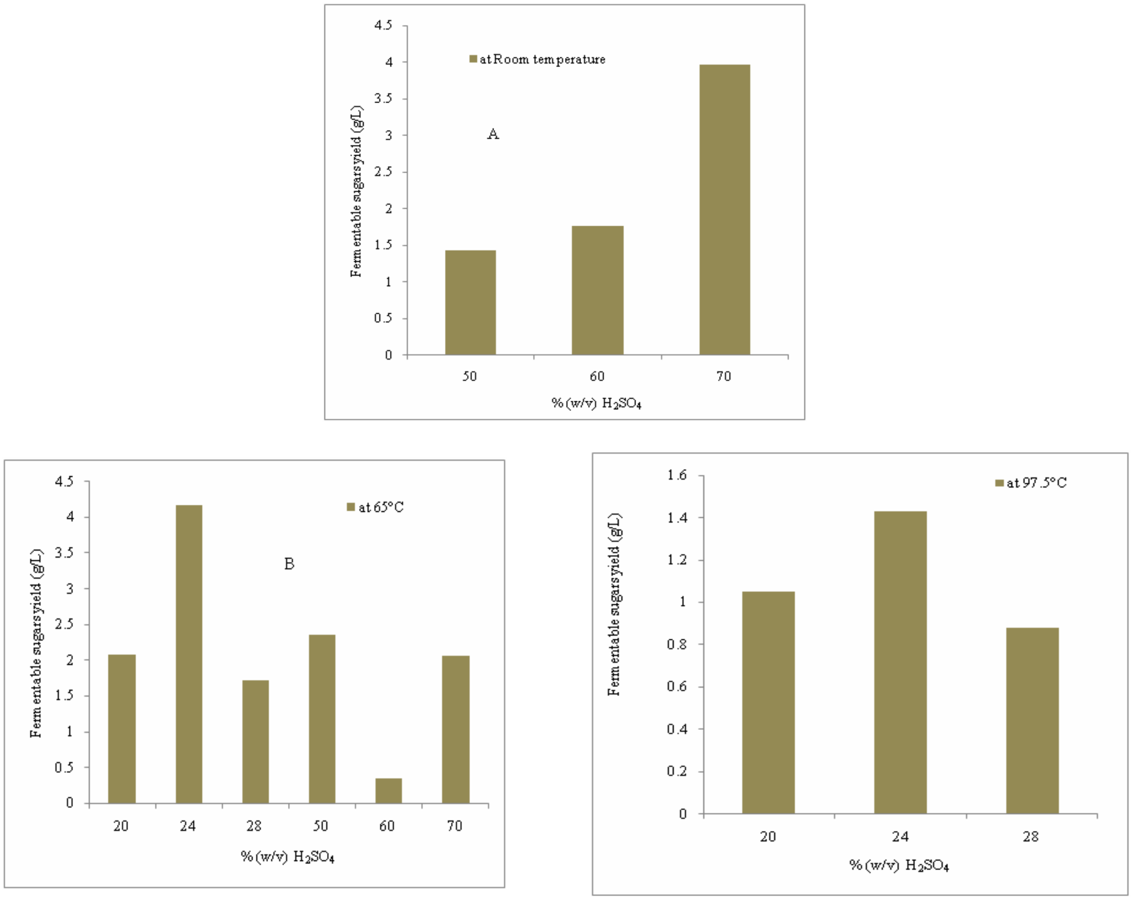

Figure 6: Comparison of temperature effect on fermentable sugars yield:
A) Room temperature, B) at 65 °C and C) at 97.5 °C

CONCLUSION

This study showed that it was possible to use grass as biomass in the production of fermentable sugars at low temperature conditions (≤100 °C) using a chemical route. In this work, the optimum conditions for grass hydrolysis could be obtained by refluxing in 2% (w/v) sulphuric acid for 5 hours at 97.5 °C and normal pressure. The findings could be used in small-scale bioethanol production processes, which are usually carried out at high temperatures and pressures, as this reduces power costs significantly.

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