SO$_2$-Catalyzed Steam Explosion of Corn Fiber for Ethanol Production

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Abstract

Corn fiber, a by-product of the corn wet-milling industry, represents a renewable resource that is readily available in significant quantities and could potentially serve as a low-cost feedstock for the production of fuel-grade alcohol. In this study, we used a batch reactor to steam explode corn fiber at various degrees of severity to evaluate the potential of using this feedstock in the bioconversion process. The results indicated that maximum sugar yields (soluble and following enzymatic hydrolysis) were recovered from corn fiber that was pretreated at 190°C for 5 min with 6% SO$_2$. Sequential SO$_2$-catalyzed steam explosion and enzymatic hydrolysis resulted in very high conversion (81%) of all polysaccharides in the corn fiber to monomeric sugars. Subsequently, *Saccharomyces cerevisiae* was able to convert the resultant corn fiber hydrolysates to ethanol very efficiently, yielding 90–96% of theoretical conversion during the fermentation process.

Index Entries: Corn fiber; steam pretreatment; enzymatic hydrolysis; fermentation; ethanol.

Introduction

The bioconversion of lignocellulosic substrates to ethanol can provide an environmentally friendly alternative fuel to gasoline, and thereby reduce our dependence on nonrenewable fossil fuel sources and concurrently reduce greenhouse gas emissions. Various lignocellulosic biomass feed-

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stocks, including agricultural residues, food-processing waste, wood residues, municipal solid wastes, herbaceous energy crops, and pulp and paper industry wastes, have the potential to serve as low-cost, abundant feedstocks for the production of fuel ethanol *(1–4)*.

Corn fiber represents a renewable resource that is readily available in significant quantities from the corn wet-milling industry, and could potentially serve as a low-cost feedstock for the production of fuel-grade alcohol. Currently, in Canada, more than 9 billion kilograms of corn are processed annually by the corn wet-milling industry in the production of syrups, starch, oil, and alcohol fuel. Although substantial, this is only a fraction (4%) of that processed in the United States, which consumes ~229 billion kilograms of corn annually *(5)*. Corn fiber is a mixture of corn hulls and residual starch not extracted during the milling process, and comprises up to 11% of the dry weight of the corn kernel *(6)*. Therefore, corn fiber is a very attractive lignocellulosic substrate for the bioconversion to ethanol process, because it has very high carbohydrate content, is present in large quantities in most ethanol- and starch-processing plants, has few competing users, and is inexpensive. Presently, 238 million liters of ethanol is produced annually in Canada, of which approx 73% is derived from corn *(5)*. It has been predicted that the utilization of the corn fiber fraction in the conversion process could potentially increase the overall ethanol yield by approximately 13% *(2)*. Although various feedstocks, including hardwoods, softwoods, and agricultural residues, have been evaluated for their potential in this process, and consequently, several common process variables in the overall lignocellulose-to-ethanol operation (i.e., pretreatment, enzymatic hydrolysis, fermentation) have been established, significant technical and economical challenges still remain. Basically, the success of a bioconversion process depends largely on the development of environmentally friendly pretreatment procedures, highly effective enzyme systems for the conversion of pretreated residual biomass to fermentable sugars, and microorganisms that can efficiently convert multiple sugars to ethanol. Although several pretreatment options for corn fiber have been evaluated for their efficacy in fractionating, solubilizing, hydrolyzing, and separating the major compositional constituents, to date no single process has been proven truly superior.

Osborn and Chen *(7)* evaluated the use of acid hydrolysis at various temperatures for its potential in converting corn fiber, and demonstrated that partial hydrolysis could be readily accomplished by treatment with dilute sulfuric acid at 135°C. Employing a similar process, it was also shown that corn fiber could effectively be converted when treated with dilute sulfuric acid at temperatures ranging from 140 to 160°C, detoxified with calcium hydroxide, and the solubilized sugar fermented to ethanol using recombinant *Escherichia coli* KO11 *(8)*. However, neither group achieved complete hydrolysis of the polysaccharides in the water-insoluble fraction of the corn fiber. Significant inhibitor formation was observed, even under relatively mild conditions.

Grohmann and Bothast *(2)* investigated the saccharification of polysaccharides in corn fiber by sequential treatment with dilute sulfuric acid at 100–160°C followed by partial neutralization and enzymatic hydrolysis with mixed cellulase and amyloglucosidase enzymes at 45°C. Although this process showed that enzymatic hydrolysis achieved an 85% conversion of all polysaccharides in the corn fiber, it was also apparent that significant inhibitor formation occurred at all pretreatment conditions tested between 140 and 160°C *(2)*.

An alternative method has also been developed in attempts to overcome the toxicity effects of pretreatment and neutralization problems of dilute-acid treatments. Moniruzzaman et al. *(9)* established and determined the best conditions for ammonia fiber explosion treatment (AFEX) of corn fiber. While this research indicated that the AFEX process circumvented inhibitory the formation of by-products, it pointed to a need for better xylan-degrading enzymes, because the hemicellulose remained in the polymeric form after processing.

Although many corn fiber pretreatment methods have been proposed (enzymatic, acid hydrolysis, and AFEX), none have been proven to be effective. Therefore, in an attempt to establish and optimize the bioconversion of corn fiber to ethanol, an alternate pretreatment method, SO_2-catalyzed steam explosion, was evaluated.

Previous work has shown that SO_2-catalysed steam explosion can successfully pretreat softwood *(10–14)* and hardwood residues *(3,15,16)* during the bioconversion process. It has also been suggested that steam explosion is one the most cost-effective pretreatment methods for lignocellulosic residues prior to enzymatic saccharification *(10,17)*. Additionally, SO_2 impregnation has been shown to facilitate lower reaction temperatures and shorter reaction times and, consequently, reduce the formation of degradation products *(3)*. The results clearly demonstrate increased enzymatic accessibility to cellulose, and enhanced recovery of the hemicellulose-derived sugars *(14,15,18,19)*. It has also been shown that the combinations of steam explosion with acid-catalyzed hydrolysis increases pore volume, and therefore, enhances enzyme accessibility *(20)*, reduces particle size *(14)*, and increases available surface area *(21)*.

The aim of the present study was to evaluate the influence of steam explosion pretreatment parameters (time, temperature, and pH) on the subsequent enzymatic saccharification of corn fiber, in an attempt to establish optimum pretreatment conditions for the feedstock (i.e., those that facilitated complete enzymatic hydrolysis of the polysaccharides while concurrently generating high-yield hydrolysates of hemicellulose-derived monomeric sugars).

Materials and Methods

Pretreatment of Substrate

Corn fiber (60.3% moisture content) was obtained from the National Center for Agricultural Utilization Research (Peoria, IL) and stored at –20°C

until use. Samples of 300 g (dry weight) of corn fiber were impregnated overnight with anhydrous SO_2 in plastic bags (total weight of biomass monitored and adjusted appropriately). The uptake of SO_2, expressed as a percentage of the oven-dried corn fiber, was measured by weighing the corn fiber before and after the addition of SO_2. The samples were then loaded in 50 g batches into a preheated 2 L Stake Tech II batch reactor (Stake Tech-Norvall, Ontario, Canada) and exploded at different severities (temperature ranging from 170 to 200°C, time ranging from 1.5 to 5 min, and SO_2 concentration ranging from 0 to 6% weight/oven-dried weight of fiber). The severity of the steam explosion pretreatment was represented by the severity factor as defined by Overend and Chornet *(22)*. This severity factor (R_o) combines the effects of time and temperature, as follows:

$$R_o = t \times e^{(T_r - T_b)/14.75}$$

in which t is the residence time (min), T_r is the reaction temperature (°C), and T_b is a reference temperature (100°C) *(22)*. It was established that during steam explosion, the applied SO_2 was converted to sulfuric acid by either oxidation or disproportionation, or both *(23)*.

Following steam explosion, the concentration of sugars in the water-soluble fraction was quantified by high-pressure liquid chromatography (HPLC), while the water-insoluble fraction was collected and adjusted to 2% (w/w) dry matter content and subsequently used for enzymatic hydrolysis (without further water washing). Shot yields (%) were determined by dividing the dry weight of the steam-exploded sample by the dry weight of the non-pretreated sample (300 g). The oven-dried weight was determined by overnight drying at 105°C.

Characterization of Substrate

The chemical composition of the original starting material and steam-exploded solids was determined using a modified Klason lignin method derived from the TAPPI Standard method T222 om-88 *(24)*. Briefly, 0.2 g of sample was incubated at 20°C with 3 mL of 72% H_2SO_4 for 2 h with mixing every 10 min. The reaction was then diluted with 112 mL of deionized water (final acid concentration of 4% H_2SO_4) and transferred to a serum bottle. The solution was then subject to autoclaving at 121°C for 1 h and filtered through a medium coarseness sintered-glass filter for the gravimetric determination of acid-insoluble lignin. Each experiment was run in triplicate. The concentration of sugars both in the filtrate and in the steam-pretreated hydolysate, as well as inhibitors such as 5-hydroxymethylfurfurals (5-HMFs), were determined using HPLC analysis. The HPLC system (Dionex DX-500) was equipped with an ion-exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a Spectra AS 3500 autoinjector (Spectra-Physics). Prior to injection, samples were filtered through 0.45-mm HV filters (Millipore, Bedford, MA) and a volume of 20 μL was loaded. The column was equilibrated with 250 m*M* NaOH and eluted with deionized water at a flow rate of 1.0 mL/min.

Ethanol and furfural concentration were determined using a Hewlett-Packard 5890 gas chromatograph equipped with a 6890 autoinjector, splitless injector system, and FID detector. Components in the prehydrolysate were separated using a 30 m Stabilwax-DA column supplemented with a 5 m deactivated guard column (Restek). During the analysis, the following parameters were used: 90°C injector temperature, 250°C detector temperature, helium as a carrier gas at a flow rate of 1 mL/min. The column temperature was controlled as follows: isocratic at 45°C for 6 min, ramped to 230°C at a rate of 20°C/min, and held for 16 min. Prior to injection, diluted samples were filtered through 0.45 μm filters and injected at a volume of 2 μL.

Enzymes

A complete *Trichoderma reesei* cellulase system (Celluclast®, Novo-Nordisk, Denmark) was used in combination with a commercial β-glucosidase (Novozym 188®, Novo-Nordisk) for cellulose degradation, while glucoamylase (200 U/mL) and α-amylase (300 U/mL) (Sigma, St. Louis, MO) were used to ensure complete hydrolysis of the starch. The Celluclast preparation contained 49 mg of protein/mL as measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and the following hydrolytic activities: 80 filter paper units (FPU)/mL, 52 IU/mL of carboxymethylcellulase (CMCase), 226 IU/mL of xylanase, and 50 IU/mL of β-glucosidase. The protein content and activities of Novozym 188 were as follows: 44 mg/mL, 792 cellobioase units (CBU)/mL, 5 FPU/mL, 34 IU/mL of CMCase, 94 IU/mL of xylanase, and 500 IU/mL of β-glucosidase. The enzyme activities were measured as described by Ghose *(25)*.

Enzymatic Hydrolysis

Hydrolysis experiments were carried out at 2% (w/v) solid concentration in 50 m*M* sodium acetate buffer (pH 4.8), supplemented with 40 μg/mL of tetracycline and 30 μg/mL of cycloheximide (total volume 50 mL) at 45°C with continuous agitation (200 rpm). The entire pretreated solids feedstock including the water-soluble sugars obtained during steam explosion was used during hydrolysis experiments. Each flask was inoculated with enzyme based on FPU of cellulase and CBU (Novozym 188) at a CBU:FPU ratio of 4:1, with an excess of glucoamylase and α-amylase as described by Grohmann and Bothast *(2)*. Aliquots of 200 μL were aseptically taken at various time intervals for analysis and boiled for 5 min to inactivate the enzymes. The sugar concentration was then determined by HPLC. Each experiment was run in duplicate.

Fermentation of Microorganisms

A spent sulfite liquor (SSL)–adapted strain of *S. cerevisiae* was generously provided by Tembec, Quebec, Canada, and used for all fermentations. The yeast was maintained at 4°C with periodic transfer to fresh GMYP

agar slant (1.0% glucose, 0.5% malt extract, 0.3% yeast extract, and 0.5% peptone). *S. cerevisiae* was pre-grown in 500 mL of GMYP medium (1% glucose, 1% yeast extract, and 1% peptone) at 30°C for 3 d, then harvested after 24 h, and resuspended in fresh GMYP medium. After a triple wash, the inoculum cell concentration was adjusted with sterile deionized water to provide a final cell concentration of 6 g/L (oven-dried cell weight).

Fermentation of Corn Fiber Hydrolysate

The water-soluble fractions generated from steam explosion treatments were preadjusted to pH 7.0 with calcium hydroxide. Fermentations were conducted in magnetically stirred 500 mL covered beakers (Fleaker®, Corning, Corning, NY) equipped with pH, temperature, and agitation controls. Each 500 mL Fleaker culture vessel contained 270 mL of the water-soluble hydrolysate generated during steam explosion pretreatment, supplemented with 15 mL of GMYP medium without malt extract and 15 mL of *S. cerevisiae* inoculum. The fermentation vessels were run at 30°C and stirred magnetically at 300 rpm. Sugars, ethanol, 5-HMF, and furfurals were determined periodically from the aliquot culture samples during the course of the hydrolysis. Each experiment was run in duplicate.

Detoxification of Inhibitors

Overliming with the addition of sodium sulfite was used to detoxify steam-exploded corn fiber prior to fermentation *(26)*. In short, the recovered hydrolysate (after steam explosion) was adjusted to pH 10.0 by the addition of calcium hydroxide (4 g/L), reduced by adding sodium sulfite (1 g/L), incubated at 90°C for 30 min, and finally neutralized with concentrated sulfuric acid to pH 7.0. Following neutralization, the resulting precipitates were removed by centrifugation (6000 g for 10 min). As a control treatment, the hydrolysate was neutralized to pH 7.0 by adding calcium hydroxide (4 g/L) and centrifuged for 10 min at 6000 g for 10 min *(26)*. The concentration of toxic compounds and ethanol in the hydrolysates was measured before and after overliming using HPLC and gas chromotography, as described previously. Each experiment was run in duplicate.

Results and Discussion
Composition of Corn Fiber

We initially determined the carbohydrates, Klason lignin, and ash composition of the original untreated corn fiber (Table 1). The total polysaccharides content proved to be quite high (~76.2%), as observed by other investigators *(2)*, making this agricultural residue an attractive material for saccharification and fermentation processes. The remaining components of the original corn fiber feedstock that were not quantified during these analyses included protein and crude fat *(2)*. Glucose followed by xylose and arabinose were shown to be the most abundant components of the corn fiber as determined by secondary acid hydrolysis of constituent polysac-

charides. It has been suggested that arabinoxylan is the major hemicellulose found in corn fiber (corn fiber gum) with branches containing xylose, arabinose, galactose, and glucuronic acid units in descending order of abundance *(27)*. Acid-insoluble lignin (Klason lignin) content was determined to be ~8.7%, which concurs with previous findings using similar quantification techniques *(2)*.

Monomer Sugar Yield

We evaluated the effect of temperature (170–200°C), time (1–5 min), and SO_2 concentration (0–6%) on the efficacy of hemicellulose recovery and the efficiency of subsequent enzymatic saccharification of recovered polysaccharide from corn fiber. Based on the resultant sugar yields (soluble and following enzymatic hydrolysis), the five best pretreatment conditions (Table 2) demonstrating the highest concentration of recovered sugars were chosen for subsequent evaluation. It was apparent that in each of the five pretreatment conditions, a large percentage of hemicellulose-derived sugars (except glucose) was recovered in monomeric form in the water-soluble fraction (Fig. 1A). It was also clearly shown that the concentration of polysaccharide in the ensuing hydrolysate increased with pretreatment severity, reaching the highest recovery (50%) of hemisugars (except glucose) at pretreatment conditions of 190°C, 5 min, and 6% SO_2 (Fig. 1A). Monomeric arabinose was liberated in the greatest quantities at all severities, followed by xylose and galactose, as has been previously shown by other researchers *(2)*. It has been suggested that the high susceptibility of arabinosyl linkages to hydrolysis may be in part responsible for fragmentation and solubilization of cell wall components in the corn fiber *(28,29)*. It was apparent that at an explosion temperature of 190°C, longer time and higher SO_2 concentration led to a better monomer recovery. This trend was also observed when softwood residues were steam exploded at 175°C *(19)*. Previous research has indicated that hemicellulose-derived sugars are less amenable to inhibitory by-product generation at temperatures ≤200°C, for softwood and hardwood residues *(3,13)*. Carrasco et al. *(13)* found better recoveries of hemicellulose sugars at 190°C for *Pinus pinaster*, while Excoffier et al. *(3)* showed a progressive decrease in xylose recovery from poplar wood with increasing treatment severity.

The pretreatment severity had a strong effect on total recovery of solids after steam explosion. This was clearly demonstrated, since the shot yields ranged from 86 to 94%, depending on the pretreatment conditions used (Table 2). It was apparent that increased pretreatment severity reduced recovery of the original corn fiber. Similar results have been reported during SO_2-catalyzed steam explosion of *Pinus radiata (10)*.

Enzymatic Hydrolysis

Recovered pretreated corn fiber solids were subject to enzymatic hydrolysis for 24 h with a combination of cellulases and amylases supplemented with excess β-glucosidase. The results indicated that the

Table 1
Composition of Corn Fiber (% weight)

Ara	Gal	Glu	Xyl	Man	Total sugars	Klasonlignin	Ash
12.11 ± 0.31	2.70 ± 0.16	42.75 ± 1.08	18.03 ± 0.56	0.58 ± 0.03	76.17 ± 2.08	8.72 ± 0.21	0.65 ± 0.02

Table 2
Conditions for SO_2-Catalyzed Steam Pretreatment of Corn Fiber and Shot Yields
Expressed as a Percentage of the Original Oven Dried Corn Fiber After
Steam Explosion over a Range of Temperatures, Time and SO_2 Concentrations

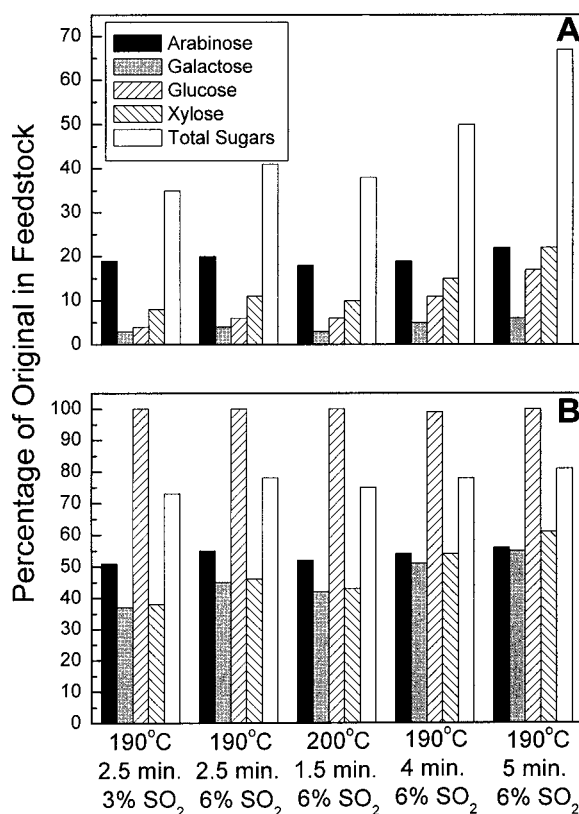| Sample	Temperature (°C)	Time (min)	SO_2 (%)	Shot yield (% original)
cf1	190	2.5	3.0	94
cf2	190	2.5	6.0	92
cf3	200	1.5	6.0	90
cf4	190	4	6.0	87
cf5	190	5	6.0	86

Fig. 1. Percentage of monomeric sugars in **(A)** water-soluble fraction of steam-exploded corn fiber following different pretreatment conditions, and **(B)** hydrolysates (unwashed solids = solids + water-extracted sugars) generated following sequential steam explosion and enzymatic hydrolysis.

hydrolyzability of the solids improved as the pretreatment conditions became more severe (Fig. 1B). This trend has also been previously observed in softwood residues, in which optimal enzymatic hydrolysis of the cellulosic component occurred at higher pretreatment severities *(19)*. The sequential steam explosion pretreatment followed by enzymatic hydrolysis of corn fiber in the current study showed an 81% conversion of all original polysaccharides to monomeric sugars, which is comparable with the work of Grohmann and Bothast *(2)* in which 85% conversion was achieved using dilute-acid treatment in combination with enzymatic hydrolysis *(2)*.

It was also apparent that only glucose was released in large quantities from the pretreated corn fiber, showing 100% conversion by enzymatic hydrolysis at all severities (Fig. 1B). It has been suggested that the glucose is released by the enzymatic hydrolysis of gelatinized and solubilized starch *(7)*, which has clearly been made quite accessible by the steamexplosion pretreatment. Furthermore, the residual starch remaining after the milling process seems to be susceptible to enzymatic hydrolysis without the need

for significant hydrolysis of xylosyl linkage created by very severe steam explosion conditions. Although SO_2-catalyzed steam pretreatment is necessary prior to enzymatic hydrolysis, near-complete glucose conversion is attainable by subsequent enzymatic hydrolysis stages at all tested severities. This clearly suggests that the mild pretreatment conditions are optimal for this lignocellulosic feedstock. The recovery yields of galactose, arabinose, and xylose of the solids obtained from the most severe treatment (190°C, 5 min, and 6% SO_2) ranged from 55 to 61%. These findings support the conclusions of Grohmann and Bothast *(2)*, who indicated that using diluted sulfuric acid pretreatment following enzymatic hydrolysis-optimal conditions for enzymatic hydrolysis-requires approx 60% conversion of xylosyl units to xylose by pretreatment. At this point, arabinose is released from arabinosyl units in 80–85% yield, and galactose from galactosyl units in 56–64% yield, both by acid treatment alone *(2)*. Since only 40, 60, and 73% of the original xylose, arabinose, and galactose, respectively, was released during the most severe pretreatment in the present investigation, there seems to be incomplete enzymatic hydrolysis of the pretreated solids.

These findings suggest that the current conditions do not liberate all the hemicellulose-derived sugars, and other approaches may be required in order to release a greater proportion of the available polysaccharide. The observed incomplete enzymatic hydrolysis may be influenced by the formation of degradation products such as sugar-lignin complexes and protein-linked polysaccharides inhibiting enzymatic digestibility; however, this requires further investigation.

Fermentation of Corn Fiber Hydrolysate

It is well established that as steam explosion severity increases, so does the degradation of monomeric sugars, by dehydration and condensation reactions that occur at higher temperatures and longer cooking times *(11)*. As previously indicated (Fig. 1), maximum sugar yields (soluble and following enzymatic hydrolysis) were recovered from corn fiber pretreated at 190°C for 5 min with 6% SO_2. Therefore, the water-soluble fraction obtained from these pretreatment conditions was assessed for its feasibility as a medium for effective fermentation to ethanol. As expected, only the hexose sugars, glucose and mannose, liberated in the corn fiber hydrolysate were effectively used by *S. cerevisiae* during the fermentation process. Conversions to ethanol with yields of 90 to 96% of theoretical were attained (Table 3). These results were comparable with those of Dien et al., *(30)* who indicated 90–94% conversion rates for corn fiber hydrolysates fermented by *E. coli* FBR 3.

Strategies for inhibitor abatement released during pretreatment were also assessed in this study. Previously, it has been suggested that overliming and the addition of sulfite had beneficial effects on the fermentation *(26,30,31)*. Consequently, in an attempt to detoxify the hydrolysates gener-
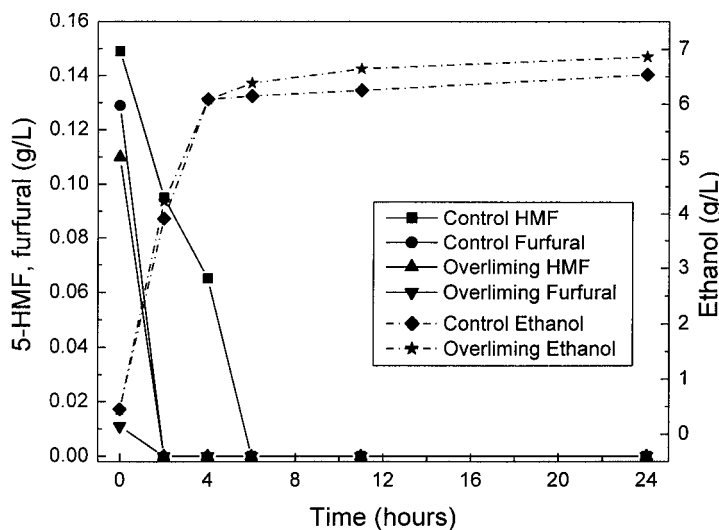
Fig. 2. Consumption of furfural and 5-HMF and generation of ethanol during fermentation of hydrolysates prepared by neutralizing and overliming with sulfite addition.

ated during the steam explosion pretreatment at different severities, overliming with the addition of sulfite was compared to the standard neutralization protocol. Our results indicate that overliming prior to fermentation increased the ethanol yields by 6% and the average volumetric ethanol productivity by 5%, when compared to the standard neutralizing method (Table 3). During the fermentations, the sugar-derived degradation products formed primarily from hexose and pentose sugars, such as 5-HMF and furfurals, respectively, were quantified. Neutralization of the hydrolysate by the addition of calcium hydroxide reduced the original 5-HMF (0.23 g/L) and furfural (0.83 g/L) concentrations by 35 and 85%, respectively, while overliming decreased the amount of original 5-HMF and furfural by 50 and 99%, respectively (Table 3). Larsson et al., *(32)* also reported the beneficial effect of overliming with the addition of sodium sulfite, indicating reductions in the amount of 5-HMF and furfural generated during the steam explosion of Norway spruce (*Picea abies*) by 50%.

It was apparent that *S. cerevisiae* was able to utilize both 5-HMF and furfurals in the overlimed hydrolysate within 2 h of fermentation (Fig. 2). A low concentration of inhibitors produced during the pretreatment and very high conversion rates of sugars to ethanol suggest that 190°C, 5 min and 6% SO_2 conditions were optimal for an efficient fermentation process.

Conclusion

The results show that a two-stage treatment for corn fiber processing comprising of SO_2-catalyzed steam explosion and posthydrolysis by a

Table 3
Influence of Hydrolysate Detoxification Methods on Fermentations.

Method	Sugar utilized (%)	Maximum ethanol (g/L)	Ethanol yield (g/g)	5-HMF (g/L)	Furfural (g/L)
Neutralization	48.0 ± 3.56	6.63 ± 0.08	0.46 ± 0.01	0.15 ± 0.01	0.13 ± 0.02
Overliming	48.0 ± 3.96	6.89 ± 0.09	0.49 ± 0.01	0.11 ± 0.02	0.01 ± 0.00

mixture of cellulolytic and amylolytic enzymes is a very effective method (81% conversion) for converting the polysaccharides in the residue to monomeric sugars. Maximum sugar yields (soluble and following enzymatic hydrolysis) were recovered from corn fiber pretreated at 190°C for 5 min with 6% SO_2. The results obtained also demonstrate that *S. cerevisiae* was able to efficiently convert corn fiber hydrolysate to ethanol with yields ranging from 90 to 96% of theoretical during the fermentation process. Furthermore, neutralization of the resultant hydrolysates by overliming with the addition of sulfite improved ethanol productivity and yield during fermentation.

Acknowledgments

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