CELLULOSIC ETHANOL PRODUCTION FROM SUGARCANE BAGASSE WITHOUT ENZYMATIC SACCHARIFICATION

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Sugarcane processing generates a large volume of bagasse. Disposal of bagasse is critical for both agricultural profitability and environmental protection. Sugarcane bagasse is a renewable resource that can be used to produce ethanol and many other value-added products. In this study, we demonstrate that cane processed bagasse could be used to produce fuel grade ethanol without saccharification. A chemical pre-treatment process using alkaline peroxide and acid hydrolysis was applied to remove lignin, which acts as physical barrier to cellulolytic enzymes. Yeast *Saccharomyces cerevisiae* ATCC, strain 765, was used in the experiment. The pre-treatment process effectively removed lignin. Ethanol production in the culture sample was monitored using high performance liquid chromatography. The results indicate that ethanol can be made from the sugarcane bagasse.

Keywords: Bagasse; Ethanol: Lignin; Acid hydrolysis; Alkaline hydrolysis; Yeast

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INTRODUCTION

The sugarcane stalk consists of two parts, an inner pith containing most of the sucrose and an outer rind with lignocellulosic fibers. During sugar processing, the sugarcane stalk is crushed to extract sucrose (Boopathy 2004). This procedure produces a large volume of residue, the bagasse, which contains both crushed rind and pith fibers. In Louisiana there are 16 sugar mills that produce approximately five million tons of bagasse every year (LSU Ag Center 2003). Most of this bagasse (approximately 75%) is used as in-house fuel for power generation or as raw material for producing low-value products such as mulch or ceiling tiles (Boopathy 2004). The remaining 25% is considered as waste that goes to landfill or is allowed to decay (LSU Ag Center 2003). This 25% accounts for 1.25 million tons of bagasse, which could be used as a raw material for cellulosic ethanol production. Ethanol is a clean-burning renewable resource that can be produced from fermented cellulosic biomass (Yu and Zhang 2004). In many parts of the world, demand for ethanol as an alternative fuel source has steadily increased (Sheoran et al. 1998) due to dwindling fossil fuel resources and increased gasoline prices. The growing demand for gas and oil was not been met domestically in the U.S. Recently the U.S government showed serious concern for developing renewable energy sources in an effort to ease the severity of unexpected energy shortages. One possibility is the conversion of agricultural wastes such as sugarcane bagasse into liquid and gaseous fuels.

Currently, the U.S produces approximately three billion gallons of ethanol from corn annually (Potera 2004).

There are major limitations to efficient ethanol production from agricultural residues (Krishna and Chowdary 2000; Martin et al. 2002). These limitations include the close physical and chemical associations between lignin and plant cell wall polysaccharides, together with cellulose crystallinity (Gould 1984). Lignin forms a protective shield around cellulose and hemicellulose, protecting the polysaccharides from enzymatic degradation. To convert the biomass into ethanol, the cellulose must be readily available for cellulase enzymes (Krishna and Choudary 2000). Thus, by removing the lignin, the cellulose becomes vulnerable to enzymes and allows the yeast to covert the glucose into ethanol during fermentation (Wyman 1996). Therefore, a pretreatment must be applied to remove lignin in the bagasse, decrease cellulose crystallinity, and increase the surface area for enzymatic activity. Most reports on lignocellulosic ethanol production involve acid hydrolysis followed by enzymatic saccharification using cellulase enzyme (Krishna and Choudary 2000; Gould 1984; Martin et al. 2002). The enzymatic saccharification step is cost-prohibitive because of the high cost of the commercial cellulase enzyme. Ethanol has been produced without enzymatic sachhari-fication using recombinant and mutant bacteria and yeast (Ho et al. 1998; Dien et al. 2003; Kim et al. 2007; van Maris et al. 2006).

The present study was conducted to determine the optimal pretreatment conditions for high efficiency ethanol production from sugarcane bagasse. The major components of sugarcane bagasse are lignin (22%), cellulose (30%), and hemicellulose (23%) (Dawson 2005). The bagasse was subjected to alkaline hydrogen peroxide pretreatment and sulfuric acid pretreatments, followed by three weeks of fermentation using ATCC yeast, *Saccharomyces cerevisiae*, strain 765. We did not use the enzymatic saccharification step in this study. The results indicated that ethanol can be made from sugarcane bagasse, but the ethanol yield was eight times less than the theoretical yield.

MATERIALS AND METHODS

Microbial Source

The yeast *Saccharamyces cerevisiae*, strain 765, was obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. This strain is known to produce ethanol from cellulosic material, specifically, from the bark of trees (Boopathy 2005). The role of this yeast strain producing ethanol without saccharification using sugarcane leaf was reported earlier by Dawson and Boopathy (2007). *S. cerevisiae* culture was maintained in tryptic soy broth (TSB) and a fresh one week old culture grown on TSB was used in the fermentation experiment.

Alkaline Pretreatment

The purpose of the alkaline pretreatment was delignification. The removal of lignin is necessary for cellulose to become readily available for the enzymes, which permit the yeast to convert the glucose into ethanol (Wyman 1996). The amount of weight lost following chemical pretreatment of residue was due to lignin removal

(Wyman 1996). Greater weight loss indicates more lignin loss. The percent weight lost was used to compare pretreatment effects on lignin removal. Delignification was tested by soaking bagasse in various concentrations (0, 1, 2, and 5%) of household hydrogen peroxide at various pHs (8, 11.5, and 13), for various time intervals (8, 24, and 48 hours).

Initially, dry sugarcane bagasse (supplied by the United States Department of Agriculture office in Houma, Louisiana) was weighed to approximately 3 g and placed in flasks. Then, three 1% H_2O_2 solutions were made. The pH of a 1% H_2O_2 solution was adjusted to 8, 11.5, or 13 by adding NaOH tablets. The treatment solution was added to individual flasks to submerge the sugarcane residue and allowed to soak for 8, 24, or 48 hours. Each flask was placed in a shaker at 100 rpm to ensure that all of the bagasse came into contact with the treatment solution. This experiment was repeated for H_2O_2 concentrations of 0, 2, and 5%. DI water was substituted for H_2O_2 for the 0% H_2O_2 treatment level. In addition, a DI water control without the pH adjustment was used in the experiment. Each H_2O_2, pH, and time treatment combination was repeated four times. After the required time for soaking, the residue was removed from the solutions by filtering through cheesecloth and then triple-rinsed for thirty minutes in DI water. The residue was then dried in an oven at 100°C for approximately 12 hours. Finally, the residue was re-weighed. The weight difference was equated to the amount of lignin removed. Upon conclusion of the alkaline pretreatments, analysis of variance was used to determine the pretreatment conditions that removed the most lignin. The best pretreatment was then used for further fermentation experiments. Fermentation experiments were conducted using the yeast *S. cerevisiae*, strain 765.

The pretreatment with 2% H_2O_2, pH 11.5, and soaking for 48 hours removed the most lignin most efficiently (Table 1) and was chosen for the fermentation experiment. In this experiment, 2 g of the pretreated bagasse residue was placed into anaerobic bottles that contained 100 mL of sterile tap water and 5% (v/v) of the yeast *S. cerevisiae*, strain 765. This experiment was run in triplicates along with triplicate controls. Samples were taken on days 0, 6, 12, 18, and 21 using a 5 mL syringe and needle. Samples were microcentrifuged at 10,000 rpm for 6 minutes. The supernatant, transferred to HPLC vials, was used to monitor ethanol production using HPLC analysis as described below.

Table 1. Alkaline Pretreatments of Sugarcane Bagasse Shown to Remove the Most Weight. (Treatments denoted by the same letters are not significantly different from each other)

H_2O_2 Concentration	Time (hours)	pH	% Weight Loss
2%	48	11.5	60.45^{A}
5%	48	13	56.04^{AB}
1%	48	13	55.70^{AB}
2%	48	13	51.45^{B}
1%	24	13	51.32^{B}

Acid Pretreatment

The purpose of acid hydrolysis was to break up lignin and make cellulose available for yeast fermentation. Dilute sulfuric acid (H_2SO_4) concentrations (0.0, 0.2, 0.4, and 0.8M) were used in this pretreatment. For the acid hydrolysis pretreatment, approximately 3 g of bagasse residue was placed into anaerobic bottles containing 100 mL of DI water and 0.2 M H_2SO_4 and allowed to soak for 24 hours, based on our previous study (Dawson 2005). The bottles were subsequently autoclaved and allowed to cool before 5% (v/v) of the yeast *S. cerevisiae*, strain 765, was added. Samples were taken on days 0, 6, 12, 18, and 21 using a 5 mL syringe, microcentrifuged at 10,000 rpm for 6 minutes, and transferred to HPLC vials. Ethanol production was monitored using HPLC analysis as described below. This experiment was repeated using 0.0, 0.4, and 0.8 M H_2SO_4, and each treatment had three replicates.

Analytical Techniques

Ethanol production was analyzed by high performance liquid chromatography (HPLC) on a Varian Pro Star Autosampler Model 410 liquid chromatograph equipped with two solvent pumps, a model 210 programmable multiwavelength refractive index detector set at 210 nm, a data module, and a model 320 system controller. The mobile phase was 0.0025 N H_2SO_4. Aliquots of 10 µL were injected into an organic acid column (Varian organic acid column, Cat # SN 035061) at 22°C. The flow rate of the mobile phase was 0.6 mL/min, and the analysis was done under isocratic mode. Quantification of ethanol was done by using standard ethanol.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) followed by a Tukey *post-hoc* range test ($p \leq 0.05$; Neter et al. 1990).

RESULTS AND DISCUSSION

Alkaline Pretreatment

The alkaline pretreatment with 2% H_2O_2 (pH 11.5) and soaking for 48 hours removed the most lignin in bagasse, compared to other treatment combinations (Table 1). Therefore, this treatment was chosen for the fermentation experiments. Results of treatment combinations consisting of pH 8 soaking for 48 hours did not show significant effects ($p = 0.9$) (data not shown).

Lignocellulosic biomass cannot be saccharified by enzymes to high yields without a pretreatment, mainly because the lignin in plant cell walls forms a barrier against enzymatic attack (Sewalt *et al.* 1997). An ideal pretreatment would reduce the lignin content and crystallinity of the cellulose and increase the surface area (Krishna and Chowdary 2000). Lignin is degraded in nature by various organisms, but the mechanism of natural degradation is largely unknown. It is thought that oxidants such as H_2O_2 may play an important role. Under certain conditions, H_2O_2 is known to react with lignin and has been widely used to bleach high-lignin pulps (Gould and Freer 1984). Gould (1984) recently reported that under suitable conditions H_2O_2 will delignify wheat straw and other

crop residues to a point where the cellulose can be enzymatically converted to glucose with near-quantitative yield. According to Gould and Freer (1984), H_2O_2-treated lignocellulosic materials can be rapidly fermented to ethanol with greater than 90% efficiency in the presence of cellulase. In the present study, sugarcane bagasse residue pretreated with 2% H_2O_2 at a pH of 11.5 and soaked for 48 hours (Table 1) removed 60.45% of the total weight of the sample; therefore, this treatment removed more lignin than any other pretreatment option evaluated.

These results were similar to those concluded from other research. Maximum delignification of wheat straw occurred at a pH of 11.5 or higher and the increase in saccharification efficiency was nearly complete after 8 hours at room temperature (Gould 1984). Krishna and Chowdary (2000) concluded that alkaline peroxide pretreatments were effective in providing fractionation of the hemicellulose and lignin components and resulted in efficient hydrolysis in linn leaves.

In another study by Gould and Freer (1984), wheat straw treated for several hours at room temperature with 1% H_2O_2 at a pH of 11.5 released slightly more than one-half of its lignin as water-soluble degradation products. They found that increased concentrations of H_2O_2, more alkaline pH, or repeated H_2O_2 treatments did not alter the total amount of lignin solubilized. However, based upon the present research, increased pH levels did remove more lignin than lower pHs. Furthermore, Gould and Freer (1984) concluded that in the absence of H_2O_2 only a very small fraction of the lignin present in the straw was released. Boopathy (2005) also obtained similar results in research conducted on sugarcane leaf.

Sugarcane bagasse pretreated in 2% H_2O_2 (pH 11.5) for 48 hours was subjected to fermentation. Sugarcane bagasse was fermented for 21 days and sampled periodically. Optimum fermentation condition for pretreated bagasse was determined to occur on day 21, producing a mean of 140.5 mg/L ethanol (Fig. 1).

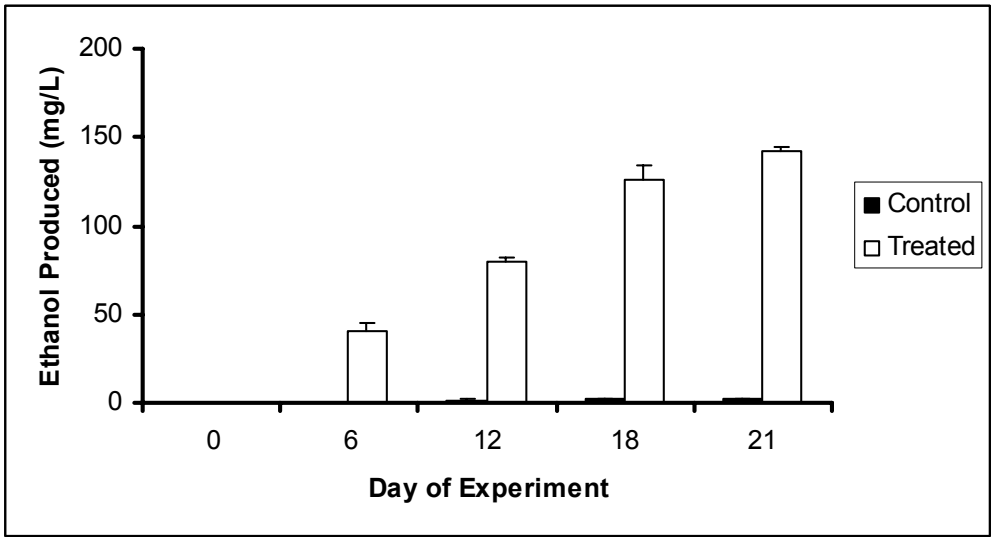

Figure 1. Mean (± standard deviation) ethanol production from alkaline pretreated sugarcane bagasse residue, 2% H_2O_2 at a pH of 11.5 for 48 hours, after fermentation over time.

Results from this research were slightly higher than those reported by Boopathy (2005), where ethanol production was 118 mg/L. However, Gould and Freer (1984) reported that alkaline pretreated corn cobs, corn husks, and corn stalks produced ethanol with an overall 90% efficiency, while kenaf and oak shavings produced enhanced ethanol yields, although significantly below the theoretical maximum. It must be noted that Gould and Freer (1984) added cellulase enzyme prior to fermentation.

Acid Hydrolysis

The sugarcane bagasse acid treatment of 0.8 M H_2SO_4, with fermenting for 18 days, produced more ethanol than any other treatment combination up to day 18. Fermentation for more than 18 days did not increase ethanol production (Fig. 2). Fermentation of bagasse showed the most efficient acid hydrolysis treatment with 0.8 M H_2SO_4, producing 395.5 mg/L ethanol (Fig. 3). In acid hydrolyzed experiments of waste cotton conducted by Yu and Zhang (2004), 0.2 mol/L H_2SO_4 was the optimal acid treatment, producing 14.2 g/L of ethanol in 24 hours.

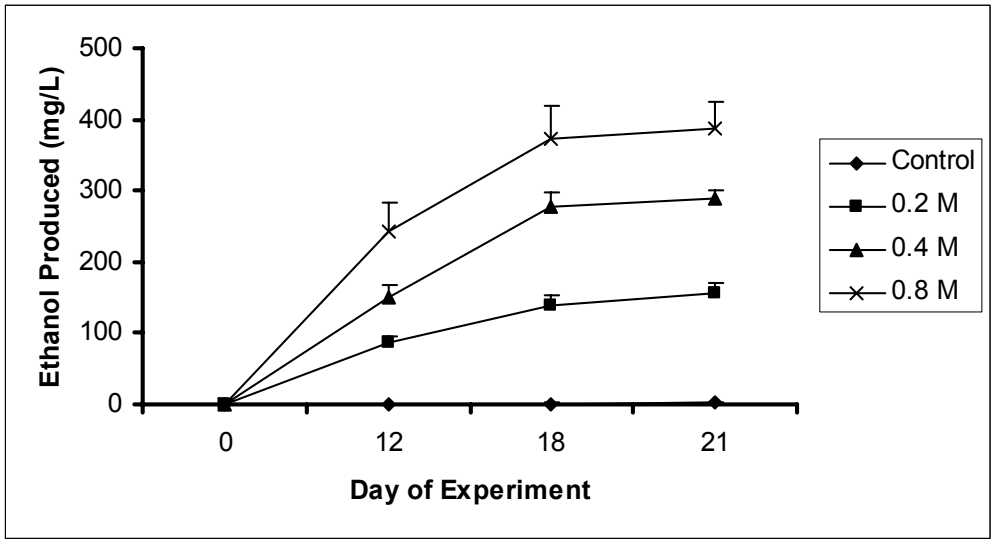

Figure 2. Mean (± standard deviation) ethanol production (mg/L) over time from sugarcane bagasse residue soaking in different acid hydrolysis treatments

After comparing alkaline H_2O_2 and H_2SO_4 acid treatments, it was shown that acid hydrolysis produced the most ethanol from the residue. More ethanol was produced from bagasse treated with 0.8M H_2SO_4 for 18 days, compared to alkaline pretreated residue at 2% H_2O_2 (pH 11.5) for 48 hours and fermented for 21 days (Fig. 3).

This preliminary study showed that ethanol production from post-harvest sugarcane residue such as bagasse is possible without the addition of cellulase enzyme. The ethanol yield in our study was eight times lower than the theoretical yield as per National Renewable Energy Laboratory (NREL) calculation, which can be seen at (http://www1.eere.energy.gov/biomass/ethanol_yield_calculator.html).

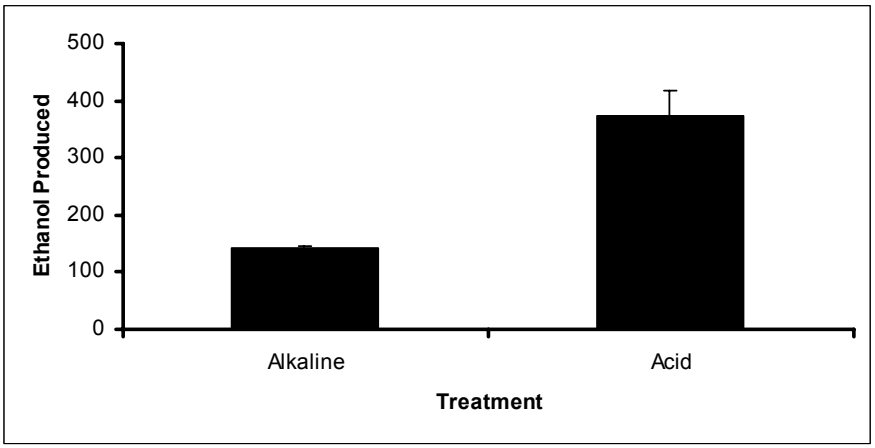

Figure 3. Mean (± standard deviation) ethanol production from the alkaline pretreated bagasse residue, 2% H_2O_2 (pH 11.5) 48 hours, and the acid treated residue, 0.8 H_2SO_4, 18 days. The asterisk denotes a significant difference at p = 0.05

Lignin prevents the degradation of cellulose mainly by acting as a physical barrier between the cellulolytic enzyme and its substrate. Consequently, the rate and extent of enzymatic cellulose degradation in lignocellulosic materials is inversely related to the lignin content (Gould 1984) with maximum cellulose degradation occurring only after 50% or more of the lignin has been removed. In the present study we did not see any inhibition from potential inhibitors such as acetic acid and furfural. This may be due to the more dilute acid used in this study compared to other reports (Klinke et al. 2004; Palmarola-Adrados et al. 2005; Hahn-Hagerdal et al. 2007). In this study, we achieved a significant removal of lignin from the bagasse, which resulted in higher production of ethanol. Further research is needed to optimize the conditions for maximum production of ethanol from bagasse.

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