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Evaluation of pretreatment methods for lignocellulosic ethanol production from energy cane variety L 79-1002

V. Sri Harjati Suhardi [1], Bijeta Prasai [2], David Samaha, Raj Boopathy*

Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA

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ABSTRACT

Approximately half of the 80 billion tons of crop produced annually around the world remains as residue that could serve as a renewable resource to produce valuable products such as ethanol and butanol. Ethanol produced from lignocellulosic biomass is a promising renewable alternative to diminishing oil and gas liquid fuels. Sugarcane is an important industry in Louisiana. The recently released variety of "energy cane" has great potential to sustain a competitive sugarcane industry. It has been demonstrated that fuel-grade ethanol can be produced from post harvest sugarcane residue in the past, but optimized ethanol production was not achieved. Optimization of the fermentation process requires efficient pretreatment to release cellulose and hemicellulose from lignocellulosic complex of plant fiber. Determining optimal pretreatment techniques for fermentation is essential for the success of lignocellulosic ethanol production process. The purpose of this study was to evaluate three pretreatment methods for the energy cane variety L 79-1002 for maximum lignocellulosic ethanol production. The pretreatments include alkaline pretreatment, dilute acid hydrolysis, and solid-state fungal pretreatment process using brown rot and white rot fungi. Pretreated biomass was enzymatically saccharified and subjected to fermentation using a recombinant *Escherichia coli* FBR5. The results revealed that all pretreatment processes produced ethanol. However, the best result was observed in dilute acid hydrolysis followed by alkaline pretreatment and solid-state fungal pretreatment.

1. Introduction

Concerns over the United States' dependency on other countries for fuel and the negative influence that modern day fuels have on environmental issues such as global warming have sparked interests in finding a more efficient and cleaner way to produce fuel (Jeffries, 2006). A potential solution is the production of ethanol from cellulosic and hemicellulosic waste products. These agricultural residues are composed of high-energy bonds and could be used to make value added products such as ethanol and butanol, but instead they are commonly disposed by open air burning (Dawson and Boopathy, 2007).

The U.S Government's Advanced Energy Initiative began an effort to reduce America's dependence on foreign oil by establishing domestic renewable alternatives to liquid fuels. Lignocellulosic biomass is a promising alternative source of energy because of a national abundance of renewable and sustainable feedstocks (U.S. DOE, 2006; U.S. DOE, 2009). Biofuels produced from lignocellulosic biomass will, not only enhance national security, but also stimulate the economy, create jobs, and reduce global climate change. Biomass refers to grasses, agricultural and woody residues and wastes that can be converted to fuels, chemicals, and electricity (U.S. DOE, 2009). Sugarcane is one of the most efficient crops in converting sunlight energy to chemical energy for fuel (Tew and Cobill, 2008). Brazil uses sugarcane as an important energy crop, converting the raw sugar into ethanol. Sugarcane is Louisiana's leading agricultural row crop, worth over $600 million in 2008 (Salassi et al., 2009). The introduction of energy cane varieties to Louisiana sugarcane farmers could be the forefront of a competitive edge of the sugarcane industry.

The new energy cane varieties are a promising development for cellulosic ethanol production. Energy cane produces large amounts of biomass that can be easily transported, and production does not compete with food supply and prices (Cobill, 2007) because energy cane can be grown on marginal land instead of land for food crops.

* Corresponding author. Tel.: +1 985 448 4716.
E-mail address: Ramaraj.Boopathy@nicholls.edu (R. Boopathy).
[1] Current address: School of Life Sciences, Bandung Institute of Technology, Jl Ganesha 10, Bandung 40132, Indonesia.
[2] Current address: Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA.

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In 2007, three energy cane varieties were released, namely, L 79-1002 (Tew et al., 2007c), HoCP 91-552 (Tew et al., 2007b), and Ho 00-961 (Tew et al., 2007a).

Lignocellulosic biomass consists of a network of cellulose and hemicellulose bound by lignin. The process of converting biomass to ethanol involves pretreatment to remove lignin and free sugars followed by enzymatic saccharification and fermentation. The lignin sheath as well as the crystallinity of cellulose presents major challenges to these pre-treatment techniques (Cowling and Kirk, 1976). However, alkaline (Gould, 1984, 1985; Gould and Freer, 1984; Dawson and Boopathy, 2007, 2008) and weak acid solutions (Knappert et al., 1981; Grohmann et al., 1986; Dawson and Boopathy, 2007, 2008) can effectively remove lignin and reduce cellulose crystallinity. Determining the optimal pretreatment for energy cane is necessary to develop efficient fermentation for ethanol production.

The release of cellulose and hemicellulose allows for post-treatment enzymatic saccharification of these carbohydrates to simple sugars for fermentation. The more effective the pretreatment is at loosening the crystallinity of lignocellulosic biomass, more carbohydrates will be available for enzymatic saccharification, thereby increasing ethanol yield from fermentation (Krishna and Chowdry, 2000; Chapple et al., 2007). The purpose of this study was to evaluate three pretreatment methods, namely, dilute acid hydrolysis, alkaline pretreatment, and fungal pretreatment for energy cane variety L 79-1002 for lignocellulosic ethanol production. The results showed that dilute acid hydrolysis is the best pretreatment method for maximum ethanol yield for the energy cane variety L 79-1002.

2. Materials and methods

2.1. Materials

Leaves of energy cane varieties L 79-1002 was collected in May and June of 2010 from the United States Department of Agriculture (USDA) sugarcane research unit in Houma, LA. Leaf tops were cut in 2–5 cm pieces and stored in muck buckets in the laboratory. A recombinant *Escherichia coli* FBR 5 was kindly provided by Dr. Mike Cotta of National Center for Agricultural Utilization Research of USDA, Peoria, IL, USA. This recombinant *E. coli* is known to ferment both glucose and xylosic sugars from cellulose and hemicellulose of wheat hydrolysate (Saha and Cotta, 2011). Brown rot and white rot fungi, namely, *Cerioporiopsis pannocinta* (ATCC 9409) and *Phanerochaete chrysosporium* (ATCC 32629) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All chemicals used in the study were of reagent grade. *E. coli* was maintained in LB broth medium and the fungi were maintained in potato dextrose agar (PDA) medium. Cellulase, β-glucanase, and endo-1,4-β-xylanase enzymes were from Sigma chemicals, St. Louis, MO.

2.2. Alkaline pretreatment

Previous study showed that 2% hydrogen peroxide at alkaline pH removed lignin from commercial sugarcane biomass (Dawson and Boopathy, 2007, 2008). Energy cane variety L 79-1002 was treated with 2% hydrogen peroxide solution at various alkaline pHs of 8, 10, 12, and 13. Deionized (DI) water was used as control. Potassium hydroxide stock solution was added to 2% hydrogen peroxide solution to increase the pH to 8, 10, 12, and 13.

Energy cane L 79-1002 was cut into 2–5 cm pieces and dried in an oven at 100 °C to remove any moisture. Ten grams of the dry energy cane were placed into each labeled flask. Two percent hydrogen peroxide solution with different pHs was added so that the energy cane was submerged (150 ml). After 24 h of soaking, the alkaline peroxide solutions were removed through cheesecloth to retain the biomass. The treated mass was then triple rinsed with DI water for a total of 30 min to remove alkaline traces. The washed sample was then placed in 250 ml reactor for saccharification and fermentation as described in Section 2.5.

2.3. Dilute acid hydrolysis

Dilute acid pretreatments at moderate temperatures free hemicellulose and cellulose (Knappert et al., 1981) and disrupt lignin, thereby releasing cellulose for enzymatic reactions (Yang and Wyman, 2004). In this study 0, 1, 2, 3, and 4% H_2SO_4 solutions were used for pretreatment of energy cane biomass.

Energy cane L 79-1002 was cut into 2–5 cm pieces and dried in an oven at 100 °C to remove any moisture. Ten grams of the dry energy cane were placed into each labeled flask. Different concentrations of H_2SO_4 solution were added so that the energy cane was submerged (150 ml). All acid treatments were done in triplicate as well as the control, which used DI water. Each sample was soaked for 24 h in respective concentrations of H_2SO_4 and then autoclaved at 121 °C for 20 min. The H_2SO_4 solution was removed, and each sample was triple rinsed with DI water for a total of 3 h (one rinse per hour).

2.4. Fungal pretreatment

The fungal pretreatment was performed in solid state using a sterile Ziploc bag filled with 10 g of energy cane cut into 2–5 cm pieces as described in detail by Lynn et al. (2010). Fungal treatment includes individual fungus alone, namely, *Cerioporiopsis pannocinta* (ATCC 9409) and *Phanerochaete chrysosporium* (ATCC 32629) and combination of both fungi together with a total of three treatments and each treatment had triplicates. Pre-grown fungi were inoculated into the Ziploc bags as an agar plug grown on PDA for three days with 100% coverage of mycelium on the agar surface. A 5% (W/W) agar plug was used as inoculum. The bags were maintained with 70% moisture and incubated for 10 days at room temperature (20–22 °C) to simulate the biomass storage conditions prior to processing for biofuel in a large-scale production unit. A control was maintained in triplicate without any addition of fungus.

2.5. Enzymatic saccharification and fermentation

The pretreated biomass from alkaline, dilute acid and fungal pretreatments were subjected to simultaneous saccharification and fermentation (SSF). Pretreated samples underwent SSF with enzymatic saccharification for 18 h at 30 °C with the addition of cellulase enzymes (Sigma C9748), β-glucanase (Sigma G4423), and hemicellulose enzyme 1,4-β-xylanase (Sigma X2629) at 10% protein of enzyme dosing of each enzyme as described by Shields and Boopathy (2011). After 18 h of enzyme reaction, a 5% recombinant *E. coli* FBR 5 pregrown in LB medium with the optical density of 1.2 at 600 nm was introduced into individual fermentor to start the fermentation. The fermentation medium was basic mineral salt medium with the volume of 150 ml in 250 ml fermentor as described by Shields and Boopathy (2011). The initial pH of the medium was 6.0 and the fermentation temperature was 30 °C. Samples were periodically drawn for ethanol analysis. The fermentation lasted for six days.

2.6. Sugar and ethanol analysis

Prior to fermentation, the pretreated hydrolysates were analyzed for glucose and xylose using the same method described below for ethanol. The organic acid column used in the analysis was able to separate all sugars as well as ethanol. All fermentation

samples were analyzed for ethanol production using high performance liquid chromatography (HPLC) as described by Dawson and Boopathy (2007) and Shields and Boopathy (2011). A Varian Pro Star Autosampler Model 410 liquid chromatograph equipped with two solvent pumps and Infinity UV and diode array detector with a data module, and a model 320 system controller were used. 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. An ethanol standard was used for quantification of ethanol in the sample. Glucose and xylose sugars were used as standards for sugar quantification.

2.7. Statistical analysis

Analysis of variance (ANOVA), followed by a Tukey *post-hoc* range test ($p < 0.05$; Neter et al., 1990), was used to analyze sugar and ethanol production data.

3. Results and discussion

3.1. Effect of pretreatment on release of free sugars

The biomass of energy cane was subjected to three different pretreatment methods as described in method section. After the pretreatment, the hydrolysate underwent enzymatic saccharification step. Cellulose in the biomass was broken down to glucose by cellulase and β-glucanase and the hemicellulose was broken down to various pentose and hexose sugars namely, glucose, arabinose, glucuronic acid, mannose, and xylose by the enzyme endo-1,4-β-xylanase. The total free sugar released after enzymatic saccharification was given in Table 1. The saccharification step depends on the availability of cellulose and hemicellulose for enzyme reaction and this availability further depends on the effectiveness of the chemical and biological pretreatments used in this study. Among the various alkaline pretreatments, maximum glucose of 2002 mg/l and xylose of 901 mg/l was obtained in pH 13 followed by pH 12, 10, and 8. There was no statistical difference in sugar release between pH 12 and 13. These two pHs yielded almost similar amount of glucose and xylose. Based on this result, the lower pH of 12 is recommended for pretreatment of energy cane.

Table 1
Effect of pretreatments on release of free sugars after enzymatic saccharification.

Treatment	Glucose (mg/l)	Xylose (mg/l)
Control (no pretreatment)	5 ± 0.23	3.4 ± 0.11
Alkaline pretreatment:		
pH 8	199 + 12.4	102 + 9.7
pH 10	1276 + 21.9^{A}	678 + 5.7^{A}
pH 12	1998 + 33.1^{B}	895 + 11.8^{B}
pH 13	2002 + 42.3^{B}	901 + 23.6^{B}
Dilute acid hydrolysis:		
1% sulfuric acid	1324 + 22.3^{A}	543 + 10.1
2% sulfuric acid	2147 + 34.2^{B}	998 + 9.8^{A}
3% sulfuric acid	3786 + 29.9^{C}	1198 + 18.7^{A}
4% sulfuric acid	3987 + 31.9^{C}	1234 + 13.3^{A}
Fungal Pretreatment:		
Cerioporiopsis alone	1055 + 16.8^{A}	608 + 5.6^{A}
Phanerochaete alone	1119 + 29.6^{A}	639 + 8.9^{A}
Cerioporiopsis + *Phanerochaete*	1636 + 11.4^{B}	799 + 12.5^{B}

Results are average of triplicates in each treatment with S.D. Data with similar letters (A, B) are not significantly different from each other under each treatment condition for two different sugars based on ANOVA.
All pretreated biomass were treated with a cocktail of cellulases and xylonase enzymes as described in the method section.
Xylose sugars include the sum of the following sugars: mannose, arabinose, xylose, and glucuronic acid.

The sugar release among various dilute acid hydrolysis varied among the acid concentrations. The maximum sugar release was observed in the dilute acid concentrations of 3% and 4%. Even though the 4% acid produced slightly higher sugar concentration than 3% dilute acid, the statistical analysis showed no difference in these two treatments. The lower dilutions of 1 and 2% produced significantly lesser sugar than 3 and 4% acid treated biomass (Table 1). This result showed 3% dilute acid pretreatment could be economical and may be used in ethanol production from energy cane L 79-1002.

The fungal pretreated biomass also released sugar and the best fungal treatment was the combination of both *Cerioporiopsis* and *Phanerochaete*, which produced 1636 mg/l glucose and 799 mg/l xylose (Table 1). The individual fungal treatments produced sugars, but they were significantly lower than the combined treatment. Among the individual treatments, there was no statistical significance, both fungi yielded almost similar sugar concentration. This result suggested the use of combined *Cerioporiopsis* and *Phanerochaete* treatment for higher sugar yield for ethanol production from the energy cane.

3.2. Ethanol production in alkaline pretreated energy cane

Results from previous studies demonstrated that the sugarcane residue treated with 2% hydrogen peroxide under alkaline pH removed lignin and released cellulose and hemicellulose for enzymatic reaction (Dawson and Boopathy, 2007, 2008; Shields and Boopathy, 2011). In this study, an attempt was made to find the optimum alkaline pH for 2% hydrogen peroxide solution to enhance the liberation of cellulose and hemicellulose from energy cane biomass for enzymatic reaction. The results suggested that the elevated pH of 12 and 13 produced maximum ethanol of 1455 and 1475 mg/l respectively. There was no statistical difference between these two pHs in terms of ethanol yield. However, the ethanol production was significantly less in pH 8 and 10 (Fig. 1A). The Recombinant *E. coli FBR 5* used in this study is known to produce ethanol from both pentose and hexosic sugars of cellulose and hemicellulose (Dien et al., 1998, 2000; Saha and Cotta, 2011). The mass balance of sugar to ethanol showed close to theoretical yield of ethanol, which is 0.51 g of ethanol per gram of sugar (Dien et al., 2000; Saha and Cotta, 2011). The available sugar from alkaline pretreatment was 2002 mg/glucose and 901 mg/l xylose in pH 13, which is added up to a total sugar of 2903 mg/l available for ethanol fermentation (Table 1). From this sugar, maximum ethanol yield obtained was 1455 mg/l in pH 12 and 13 (Fig. 1A). This study showed that the for the energy cane L 79-1002 variety, alkaline pretreatment at pH 12 will be the optimum alkaline treatment for maximum ethanol production. Because lignin is the primary site of alkaline peroxide reaction (Gould, 1985), alkaline pretreatment can remove lignin, making sugars more available for enzymatic saccharification and fermentation (Dawson and Boopathy, 2007, 2008). Gould (1984) determined that pH 11.5 pretreatment could remove half of the total lignin in agricultural residues after 24 h of soaking at room temperature. Alkaline peroxide treatments can effectively remove enough lignin so that enzymes convert almost 100% of cellulose to glucose (Gould, 1984). An advantage of alkaline peroxide pretreatment is that the byproducts released during lignin degradation by alkaline peroxide pretreatment are not inhibitory or toxic to *S. cerevisiae* (Gould and Freer, 1984) unlike the toxic byproducts released during acid pretreatment.

3.3. Ethanol production in dilute acid pretreated energy cane

Fig. 1B shows ethanol production from dilute acid pretreated energy cane biomass. The result showed maximum ethanol production in 3 and 4% sulfuric acid treated biomass. A maximum

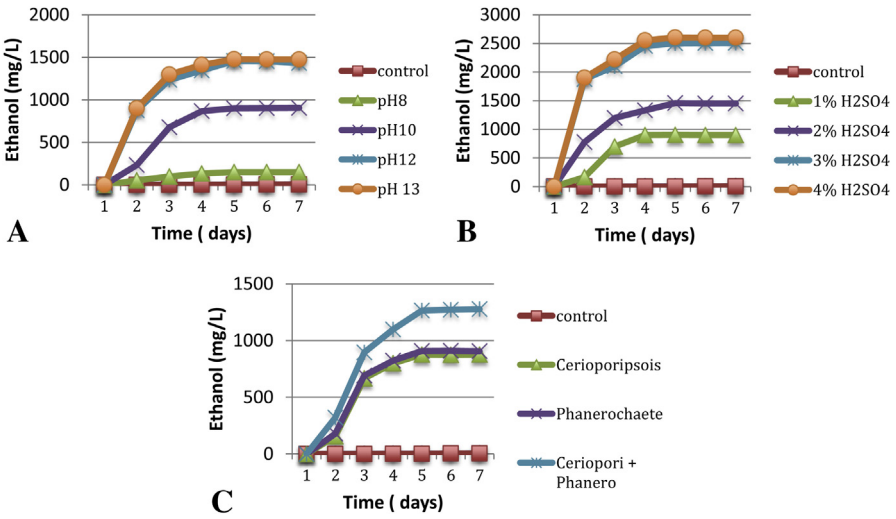

Fig. 1. Effect of pretreatments (A. alkaline, B. dilute acid, and C. fungal) of energy cane L 79-1002 biomass on ethanol production. Data represent mean of triplicates in each treatment.

ethanol yield of 2601 mg/l was observed in 4% sulfuric acid treatment. The ethanol yield in 3 and 4% sulfuric acid treatment showed no difference in statistical significance. However, there was lower ethanol yield in 1 and 2% sulfuric acid treatments. Comparing the sugar production in dilute acid treatments (Table 1) and ethanol yield shown in Fig. 1B demonstrated that the recombinant *E. coil FBR 5* produced maximum possible theoretical yield of ethanol from total free sugars available after enzymatic saccharification of dilute acid pretreated biomass of energy cane L 79-1002. The pretreatment method using acid hydrolysis and enzymatic catalysis proved effective in increasing the ethanol yield using both cellulose and pentose-sugar fermenting recombinant *E. coli.* It has been shown that recombinant plasmids can be used to produce strains of *Saccharomyces* that are capable of fermenting sugars. This process involves the use of three xylose-metabolizing genes, xylose reductase, xylitol dehydrogenase, and xylulokinase to convert xylose to xylitol, xylitol to xylulose, and xylulose to xylulose-5-phosphate, respectively (Ho et al., 1998). Once xylose is converted to xylulose-5-phosphate, it is readily accessible by many bacteria and fungi for metabolism using the non-oxidative phase of the pentose phosphate pathway (Jeffries, 2006). In addition to providing the enzymatic capability to proceed in the first step of xylose fermentation, xylose reductase has also been shown to aid *S. cerevisiae* in the reduction of inhibitory furaldehyde compounds released during acid hydrolysis (Almeida et al., 2008). Compared to available literature on lignocellulosic ethanol production, the recombinant *E. coli FBR 5* used in this study effectively produced ethanol from both cellulosic and hemicellulosic sugars and the yield was close to theoretical maximum. Even though the 4% dilute acid treatment produced higher sugar content than 3% dilute acid treatment, the ethanol yields in these two treatments were almost similar. This may be due to the production of inhibitory compounds such as furfural and 5-hydroxymethyl furfural in higher acid concentration as reported by Almeida et al., (2008) and Boopathy, 2009.

3.4. Ethanol production from fungal pretreated energy cane

Fungal Pretreatment of energy cane L 79-1002 yielded significant amount of total free sugar (Table 1) and when this sugar was subjected to fermentation by recombinant *E. coli FBR 5*, the ethanol yield was close to theoretical maximum (Fig. 1C). Maximum ethanol was obtained in the combined pretreatment of both fungi, *Cerioporiopsis* and *Phanerochaete,* which produced 1299 mg/l ethanol in six days of fermentation, which is statistically significant compared to individual fungal pretreatment (ethanol yield of around 900 mg/l). In natural systems, fungi especially, the brown rot and white rot fungi are known to decompose fallen leaves from trees and other plants to humic and water soluble compounds (Lynn et al., 2010). These fungi produce various enzymes such as lignin peroxidase, phenol oxidase, manganese peroxidase, and laccase (Kuhad et al., 1997; Lenowicsz et al., 1999; Howard et al., 2003). These enzymes can be produced both under submerged fermentation (SmF) and solid-state fermentation (SSF) (Osma et al., 2007). In this study, the SSF pretreatment showed effective removal of lignin, which resulted in significantly higher ethanol production in the fungal pretreated energy cane compared to control.

3.5. Comparison of all pretreatments

The best conditions under each of the pretreatment studied were compared and the result is presented in Fig. 2. The best pretreatment of energy cane L 79-1002 is 3% sulfuric acid, which is statistically significant compared to pH 12 alkaline hydroxide pretreatment and combined fungal pretreatment of *Cerioporiopsis* and *Phanerochaete.* Among the fungal and alkaline pretreatments, the ethanol production showed no difference in significance.

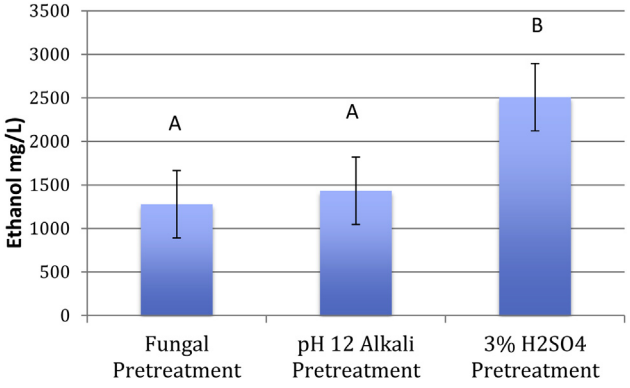

Fig. 2. Comparison of best results from three pretreatments in ethanol production after six days of fermentation. Data represent mean of triplicates with S.D. Letters above samples represent Tukey groupings based ANOVA results. Data with similar letters are not significantly different from each other.

Pretreatment of lignocellulosic biomass is a costly step (Lynd et al., 1996), but is essential for high ethanol yields on a commercial level. Efficient pretreatment can affect downstream process costs by reducing the use of enzymes or fermentation time (Lynd et al., 1996). In our previous studies, we reported acid pretreatment was better than alkaline pretreatment in removing lignin from commercial sugarcane residues such as leaf and bagasse (Dawson and Boopathy, 2007, 2008; Shields and Boopathy, 2011). In the current study, based on the results obtained from three different pretreatments, dilute acid pretreatment with 3% sulfuric acid could be used as an effective pretreatment method for energy cane L 79-1002. Further experiments should be carried out to combine the dilute acid pretreatment with fungal pretreatment in order to reduce the use of acid, which will be a big cost factor in large scale biofuel production systems. Combining the fungal treatment with dilute acid treatment could significantly lower the volume of acid that is needed for pretreatment of energy cane for ethanol production. This combined pretreatment makes practical sense as the biomass can be treated with fungi during storage period prior to biomass processing. Biofuels are a potential sustainable solution to the global fuel crisis that is depleting natural resources as it contributes to climate change. The development of energy cane varieties for ethanol production has both environmental and economic significance. For Louisiana, the advent of new sugarcane varieties could help sustain the sugarcane industry while providing a new niche of jobs and capital. The advantage of producing an efficient source of ethanol could lead to greater net benefit with regard to carbon dioxide emissions as well as a smaller ecological footprint.

4. Conclusions

This study showed the results of three pretreatments methods, namely, alkaline, dilute acid, and biological in removing lignin from the lignocellulosic materials of energy cane. The results indicated lignin was removed and cellulose and hemicellulose were released by all pretreatment methods. The best pretreatment in terms of ethanol yield was acid pretreatment followed by alkaline, and fungal pretreatment. Among the various acid pretreatment conditions, the best result was achieved in 3% sulfuric acid pretreated biomass. Combining the fungal treatment with dilute acid treatment could significantly lower the volume of acid that is needed for pretreatment of energy cane for ethanol production. This combined pretreatment makes practical sense as the biomass can be treated with fungi during storage period prior to biomass processing.

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