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Combined biological and chemical pretreatment method for lignocellulosic ethanol production from energy cane

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Abstract

The process of converting lignocellulosic biomass to ethanol involves pretreatment to disrupt the complex of lignin, cellulose, and hemicellulose, freeing cellulose and hemicellulose for enzymatic saccharification and fermentation. Determining optimal pretreatment techniques for fermentation is essential for the success of lignocellulosic energy production process. The purpose of this study was to evaluate energy cane for lignocellulosic ethanol production. Various pretreatment processes for energy cane variety L 79-1002 (type II) were evaluated including different concentrations of dilute acid hydrolysis and solid-state fungal pretreatment process using brown rot and white rot fungi. Pretreated biomass was enzymatically saccharified and fermented using a recombinant *Escherichia coli*. The results revealed that all pretreatment processes that were subjected to enzymatic saccharification and fermentation produced ethanol. However, the best result was observed in dilute acid hydrolysis of 3% sulfuric acid. Combination of fungal pretreatment with dilute acid hydrolysis reduced the acid requirement from 3% to 1% and this combined process could be more economical in a large-scale production system.

Keywords: Dilute acid hydrolysis, energy cane, cellulose, hemicelluose, lignin, ethanol, fermentation

Introduction

Environmental impacts of petroleum exploration, as well as the increasing price of oil and gas, necessitate an alternative energy solution [1]. Lignocellulosic biomass is a promising alternative source of energy because of a national abundance of renewable and sustainable feedstocks [2,3]. Biofuels produced from lignocellulosic biomass will enhance national security and 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 [2]. Sugarcane is one of the most efficient crops in converting sunlight energy to chemical energy for fuel [4]. 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 [5]. 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. In 2007, three energy cane varieties were released by the United States Department of Agriculture (USDA), namely, L 79-1002, HoCP 91-552, and Ho 00-961 [4]. A current commercial variety of sugarcane is HoCP 96-540. Energy cane can withstand freeze and it is a 12 month crop compared to commercial sugarcane which is a nine month crop and it has to be harvested before freeze set in every year. Energy cane also can grow in poor agricultural soil and it does not compete with food crop [5]. The energy cane variety L 79-1002 is more suitable for ethanol production compared to other varieties of energy cane because it contains significantly higher cellulose and hemicellulose content than any other sugar cane [6].

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. However, alkaline [7-10] and dilute acid solutions [8-12] can effectively remove lignin and reduce cellulose crystallinity. Determining the optimal pre-treatment for energy cane varieties 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 cyrstallinity of lignocellulosic biomass the more carbohydrates are available for enzymatic saccharification, thereby increasing

ethanol yield from fermentation [13,14]. In this study the biomass used was sugarcane leaf from the energy cane. Every year after sugarcane is harvested, farmers typically reduce residue by open air burning. This is a cost-effective way to remove the fibrous content that would otherwise significantly reduce milling efficiency and decrease profits, as well as to clear residue from the field that hinders farming [9]. The open air burning practice not only affects the quality of air but also the quality of life to those who live in the area. One alternative to open air burning is the production of ethanol from sugarcane residue. Ethanol is a clean burning, renewable resource that can be produced from cellulosic biomass. The main purpose of this study was to find an economical and best pretreatment method for a particular energy cane variety L 79-1002 for lignocellulosic ethanol production.

Materials and methods

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 three to five centimeter 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 [15]. 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.

Pre-treatment

Dilute acid pretreatments at moderate temperatures free hemicellulose and cellulose [11] and disrupt lignin, thereby releasing cellulose for enzymatic reactions [16]. In this study 0, 1, 2, 3, and 4% H_2SO_4 solutions were used for pretreatment of lignocellulosic biomass.

Energy cane L 79-1002 was cut into 2-5 cm pieces and dried in an oven at 100°C for six hours to remove any moisture. Ten grams of the dry energy cane were placed into each labeled anaerobic bottle. Different concentrations of H_2SO_4 solution were added so that the energy cane was submerged (150 mL). The volumetric ratio of biomass to dilute acid was 1:15 (10 g biomass and 150 mL dilute acid). All acid treatments were done in triplicate as well as the control, which used DI water. Each sample was soaked for 24 hours in respective concentrations of H_2SO_4 and then autoclaved at 121°C for 20 minutes at 15 psi . The H_2SO_4 solution was removed, and each sample was triple rinsed with DI water for a total of three hours (one rinse per hour).

The fungal pretreatment was performed in solid state fermentation (SSF) using a sterile Ziploc bag filled with 10 gram of dry energy cane cut into 2-5 cm pieces as described in detail by Lyn *et al.,* [17]. Fungal treatment includes individual fungus alone and combination of both fungi 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 (analyzed using a moisture analyzer, Fisher Scientific, St. Louis, MO) 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.

Combination of fungal and acid pre-treatment

An experiment was conducted with a fungal pretreated biomass with both fungi as described in pre-treatment section. The fungal pretreated biomass was subjected to dilute acid pretreatment with low concentrations of acids, namely, 0.25, 0.5, 1, 1.5, and 2% sulfuric acid as described above. These various combined pretreated biomasses underwent enzymatic saccharification and fermentation as described in enzymatic saccharification and fermentation sections.

Enzymatic saccharification and fermentation

The pre-treated biomass from dilute acid, fungal, and combined pretreatments were subjected to separate hydrolysis and fermentation (SHF). Pretreated samples underwent SSF with enzymatic saccahrification for 18 hours at 30°C with the addition of cellulase enzymes (Sigma C9748), β-glucanase (Sigma G4423), and hemicellulose enzyme endo-1,4-β-xylanase (Sigma X2629) at 10% protein of enzyme dosing of each enzyme as described by Shields and Boopathy [6]. The enzyme activity was equal to three filter paper units (FPU) per 1% protein of the enzyme. After 18 hours of enzyme reaction, a 5% recombinant *E.coli* FBR 5 pregrown in LB medium with the optical density of 1.2 at 600nm was introduced into individual treated bottles to start the fermentation. The fermentation medium was basic mineral salt medium with the volume of 150 mL in 250 mL anaerobic bottle as described by Shields and Boopathy [6]. The fermentation was carried out anaerobically without shaking in the anaerobic bottles. The initial oxygen in the headspace was rapidly consumed within six hours of incubation. 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.

Ethanol analysis

All fermentation samples were analyzed for ethanol

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production using high performance liquid chromatography (HPLC) as described by Dawson and Boopathy [9] and Shields and Boopathy [6]. 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.

Statistical analysis

Analysis of variance (ANOVA), followed by a Tukey *post-hoc* range test ($p < 0.05$) as described by Neter *et al.,* [18] was used to analyze ethanol production.

Results and Discussion

Two sets of pretreatment were studied in detail: one with different concentrations of dilute sulfuric acid and another with two kinds of fungi under individual and combined fungal treatment conditions. The results are presented in **Table 1**. The dilute acid pretreatment showed an increase in ethanol yield as the acid concentration increased from 1% to 3%. Further increase in acid concentration to 4% showed a decrease in ethanol yield compared to 3% acid treatment. This may be due to the presence of inhibitory compounds such as furfural and 5-hydroxy methylfurfural. Several reports indicate the presence of these inhibitory compounds at higher acid concentrations [6,9]. The maximum ethanol concentration of 3021 mg/L was observed in 3% sulfuric acid treatment, which was significant compared to other concentrations used in the study and also other studies reported in the literature including Dawson and Boopathy [9,10] and Shields and Boopathy [6].

Table 1 also shows ethanol yield among various fungal pretreatments. The best result was achieved in a combined pretreatment of *Cerioporiopsis* (brown rot fungus) and *Phanerochaete* (white rot fungus). The ethanol yield in this condition was 1345 mg/L, which was significantly less than dilute acid pretreatment. Individual fungal pretreatment produced lower ethanol yield. 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 [17]. These fungi produce various enzymes such as lignin peroxidase, phenol oxidase, manganese peroxidase, and laccase [19-21]. These enzymes can be produced both under submerged fermentation (SmF) and solid-state fermentation (SSF) [22]. In this study, the SSF pretreatment showed effective release of cellulose and hemicellulose, which resulted in significantly higher ethanol production in the fungal pretreated energy cane compared to control.

Table 1. Effect of Various Pretreatments on Ethanol Production after Six Days of Fermentation.

Pretreatment	Ethanol Production (mg/L)
Dilute acid pretreatment: Control (no acid treatment)	15 ± 0.6
1% sulfuric acid	877 ± 21.5^{A}
2% sulfuric acid	1725 ± 32.1^{A}
3% sulfuric acid	3021 ± 27.8^{AB}
4% sulfuric acid	2876 ± 39.5^{AB}
Fungal pretreatment: Control (no fungus)	2 ± 0.7
Cerioporiopsis alone	687 ± 7.5^{C}
Phanerochaete alone	766 ± 5.6^{C}
Cerioporiopsis+Phanerochaete	1345 ± 14.9^{CD}

Results are average of triplicates in each treatment with S.D. Data with similar letters are not significantly different from each other under two different sets of pretreatment conditions. The pretreated energy cane was subjected to enzymatic saccharification and fermentation with recombinant *E.coli* FBR 5 as detailed in methods section.

Based on the results obtained from two different pretreatments, further experiments were 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. Energy cane was subjected to a pretreatment condition with *Cerioporiopsis* and *Phanerochaete* together, which yielded higher ethanol yield among various fungal pretreatments (**Table 1**) as detailed in method section. Following ten days of fungal pretreatment, the energy cane was pretreated with various low concentrations of sulfuric acid (0, 0.25, 0.5, 1, 1.5, and 2%). The pretreated biomass was enzymatically saccharified and subjected to fermentation using recombinant *E.coli* FBR 5. The results from this study are given in **Table 2**. The energy cane with 0% sulfuric acid after 10 days of fungal treatment produced ethanol concentration of 1266 mg/L compared to 2% sulfuric acid treatment of fungal pretreated biomass, which produced 3055 mg/L of ethanol (p value = 0.01). However, the lower dilution of 1 and 1.5% produced equally good amount of ethanol, namely, 2876 and 2956 mg/L, respectively. Statistical analysis showed no significant difference among 1, 1.5, and 2% dilute acid treatment of fungal pretreated energy cane with a p value of 0.32.

Pretreatment of lignocellulosic biomass is a costly step [23], 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 [23]. 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 [6,9,10]. In the current study, acid pretreatment with fungal treatment of biomass was chosen as the best pretreatment

Table 2. Effect of Fungal Pretreatment on Dilute Acid Pretreatments in Ethanol Production after Six Days of Fermentation.

Treatment	Ethanol Production(mg/L)
0% sulfuric acid	1266 ± 11.5^{A}
0.25% sulfuric acid	1325 ± 22.7^{A}
0.5% sulfuric acid	1971 ± 29.5^{A}
1% sulfuric acid	2876 ± 39.2^{AB}
1.5% sulfuric acid	2956 ± 41.2^{AB}
2% sulfuric acid	3055 + 25.3^{AB}

Results are average of triplicates in each treatment with S.D. Data with similar letters are not significantly different from each other. Energy cane was pretreated with Cerioporiopsis and Phanerochaete for 10 days followed by various dilute acid treatments before the hydrolysate was subjected to enzymatic saccharification and fermentation with recombinant *E.coli* FBR 5 as detailed in methods section.

to release cellulose and hemicelluose from lignin in the leaf biomass of energy cane L79-1002. The results from the two experiments showed that the combination fungal pretreatment with very dilute sulfuric acid (1%) of energy cane produced 2876 mg/L of ethanol and the ethanol production was 3021 mg/L in the treatment that received 3% sulfuric acid without fungal treatment (**Tables 1** and **2**). The ethanol production in these two treatments are almost similar. Thus combining the fungal treatment with dilute acid treatment could save a significant volume of acid that is needed for pretreatment of energy cane for ethanol production. This difference of 2% acid volume is significant and could be practical in the large scale bioprocessing of lignocelluosic materials for biofuel production as the biomass can be treated with fungi during storage period prior to biomass processing. Further research is needed in scaling up the process, which will help us to do economical analysis for biofuel industry.

Conclusions

This study shows that dilute acid pretreatment released cellulose and hemicellulose, which are available for enzymatic saccharification and fermentation. The best dilute acid pretreatment was 3% sulfuric acid.

The use of fungal pretreatment enhanced ethanol production. Brown rot and white rot fungi produced almost similar ethanol yield. The combined treatment of brown rot and white rot fungi together produced significantly higher ethanol yield compared to control, however, produced less ethanol compared to 3% dilute sulfuric acid pretreatment.

The combination of fungal pretreatment with lower dilute acid pretreatment produced the best result of this study. A 10 day fungal pretreated energy cane with both brown rot and white rot fungi together treated with 1% sulfuric acid showed ethanol production of 2876 mg/L, which is comparable to ethanol production in 3% dilute acid treatment without fungal pretreatment and thus combining the fungal pretreatment with acid pretreatment makes practical sense.

Competing interests

The authors declare that they have no competing interests.

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