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# Optimization of Pretreatment and Enzymatic Saccharification of Cogon Grass Prior Ethanol Production

Jhalique Jane R. Fojas and Ernesto J. Del Rosario



***Abstract*—**The dilute acid pretreatment and enzymatic saccharification of lignocellulosic substrate, cogon grass (*Imperata cylindrical, L.*) was optimized prior ethanol fermentation using simultaneous saccharification and fermentation (SSF) method. The optimum pretreatment conditions, temperature, sulfuric acid concentration, and reaction time were evaluated by determining the maximum sugar yield at constant enzyme loading. Cogon grass, at 10% w/v substrate loading, has optimum pretreatment conditions of 126°C, 0.6% v/v $H_2SO_4$, and 20min reaction time. These pretreatment conditions were used to optimize enzymatic saccharification using different enzyme combinations. The maximum saccharification yield of 36.68mg/mL (71.29% reducing sugar) was obtained using 25FPU/g-cellulose cellulase complex combined with 1.1% w/w of cellobiase, ß-glucosidase, and 0.225% w/w of hemicellulase complex, after 96 hours of saccharification. Using the optimum pretreatment and saccharification conditions, SSF of treated substrates was done at 37°C for 120 hours using industrial yeast strain HBY3, *Saccharomyces cerevisiae*. The ethanol yield for cogon grass at 4% w/w loading was 9.11g/L with 5.74mg/mL total residual sugar.

***Keywords*—**Acid pretreatment, bioethanol, biomass, cogon grass, fermentation, lignocellylose, SSF.

## I. Introduction

ALTERNATIVE energy source has become a necessity nowadays, not only because of the crisis in the world's energy supply but mainly due to the environmental constraints resulting from its production and use [1]. These forced the market to shift its interest on renewable energy sources such as biofuels, and convert oil-based refinery to biomass-based processing [2]. The use of biomass is of significant interest to countries like the Philippines which produces million tons of agricultural by-products annually. These lignocellulosic wastes which include municipal solid wastes and many agricultural wastes like corn cobs, rice stalks and weeds can be sources for low-cost biofuel such as ethanol [3].

Cogon grass has been an agricultural problem in the Philippines. It is a perennial, rhizomatous grass that grows from 2 to over 4 feet in height and is considered as ecological threat due to its inhibitory effect, making other plants nearly impossible to coexist [4]. It is a fast-growing weed which requires minimal water and grows even in an unfertile soil. Cogon grass' viability as a substrate for ethanol production has not been determined yet. The composition of the cogon grass can be approximated as that of the many other grasses which contain cellulose and hemicellulose, and is therefore a viable ethanol source. Since it is considered as pest to many upland crops because it consumes a large amount of pesticide thereby increasing the inputs needed for the land [5], utilizing it as raw material could lead to the decrease in the amount of pesticide consumption, at the same time, minimize the use of food and feed grade crops as substrates for bioethanol production.

The conversion of lignocellulosic materials to ethanol has two main processes, one is the pretreatment and hydrolysis wherein starch and cellulose are hydrolyzed to fermentable sugars and the other is the fermentation of sugars to ethanol [6]. Pretreatment is usually employed since the biodegradation of untreated lignocellulose has low extent of conversion. The most common pretreatment method uses dilute acid-high temperature process [7]. Although this pretreatment method has a very low cost, prehydrolysis conditions should be optimized to provide the highest yield of fermentable sugars and avoid degradation by-products. After pretreatment, cellulose components can be converted to constituent monosaccharides via acid hydrolysis or enzymatic saccharification. Acid hydrolysis is found to require a shorter time than enzymatic saccharification and pretreatment becomes unnecessary, however, neutralization and waste disposal increase the cost of production. In addition, furfural and other degradation products are found to inhibit fermentation process if untreated [8]. Enzymatic saccharification on the other hand produces sugars of high purity due to its specificity, and has higher fermentable sugar yield given optimum pretreatment conditions. In this study, optimization of both pretreatment parameters and enzymatic saccharification conditions were done to maximize ethanol yield from lignocellulose. Parametric relations among pretreatment temperature, acid concentration and reaction time, and enzyme loading and combinations were done to give the optimum conditions for both prehydrolysis and hydrolysis process prior to ethanol conversion. Simultaneous saccharification and fermentation (SSF) process was used for the ethanol production.

J. J. R. Fojas was with the Institute of Chemistry, University of the Philippines Los Baños. She is now with the Harvard Medical School-Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115 USA (phone: 857-222-1696; e-mail: jfojas@partners.org).

E. J. Del Rosario is with the Institute of Chemistry, University of the Philippines Los Baños, College, Laguna, 4031 Philippines (e-mail: ejdros@yahoo.com).

## II. Materials and Methods

### A. Sample Preparation and Compositional Analysis

Cogon grass stalks and leaves were harvested, washed and sun dried for 10 hours prior grinding. The dried samples were then milled to 0.1mm size and were further dried in oven at 40°C for 2 hours. Compositional analysis of the samples was done to determine the total solids and initial cellulose content of the samples. The total solids were obtained by constant drying at 105°C and the percent total solids in dry weight basis were calculated as the percentage of the ratio of the final weight and initial weight of dried samples. The total solids were used to determine the proportion of substrate to dilute acid solution. The cellulose content was determined by Updegraff method using acetic-nitric solution in Anthrone reagent.

### B. Prehydrolysis Using Dilute Sulfuric Acid

Optimization of different parameters, temperature, acid concentration and reaction time was done by subjecting cogon grass samples of 10%w/v substrate loading to different prehydrolysis conditions. Three batches of samples were prepared and soaked to different acid concentrations, 0.2%, 0.4%, and 0.6%v/v $H_2SO_4$. Each batch of samples was autoclave at different temperatures, 115°C, 121°C, and 127°C and allowed to react at different length of time, 10min, 20min, and 30min. Neutralization of the pretreated samples were done using excess $Ca(OH)_2$ solution and suction filtration was done to separate the pretreated fibers from the supernatant solution. The residue and filtrates were collected for sugar analysis to determine both the amount of hydrolysable sugar after pretreatment and the amount of substrate hydrolyzed during the pretreatment process prior the enzymatic saccharification. The total sugar and reducing sugar for each samples at different operating conditions were determined using Phenol-sulfuric acid and 3,5-dinitrosalicylic acids (DNS) methods, and the absorbance were obtained using a UV-Vis spectrometer. The sugar concentrations were calculated from the absorbance obtained using the calibration curve of glucose standards. The optimum conditions, temperature, acid concentration and reaction time were determined based on the pretreated sample with the highest sugar yield.

### C. Enzymatic Saccharification

Enzyme assay using filter paper assay was done to determine the enzyme activity prior use. The total cellulase activity was described by filter paper units (FPU) with the Whatman No. 1 filter paper strip, 1.0x6.0cm (approximately 50mg) as a substrate. The xylanase activity in the cellulase was assayed using 1% (w/w) oat spelts xylan as substrate. The total reducing sugars were estimated by DNS method. Enzyme activity was expressed in international units (U) as the amount of enzyme required to release 1μmol of either glucose (FPU/ml) or xylose (U/ml) per minute under the assay conditions of pH 4.8 and 50°C.

The ideal pretreatment parameters obtained were used to optimize the hydrolysis process at various enzyme loading, 15FPU, 20FPU, and 25FPU cellulase, enzyme combinations using Novozymes Biomass kit, 700EGU/g NS50013-Cellulase complex, 250CbU/g NS50010-Cellobiase, and 100FBG/g NS50012-Hemicellulase complex, and saccharification time of up to 96 hours. The optimum parameters were chosen based on the maximum total sugar and reducing sugar after enzymatic hydrolysis.

### D. Ethanol Production Using SSF Method

Using the optimum pretreatment and enzymatic saccharification, ethanol fermentation by simultaneous saccharification and fermentation was done for 5 days. The recommended substrate loading for SSF is 3-6%w/w TS to facilitate mixing and wetting of the samples. The total cogon grass substrate loading used was 4%w/w TS and the SSF experiments was done at 37°C under constant mixing. The ethanol content was measured using gas chromatography (GC).

## III. Results and Discussions

### A. Dilute Acid Pretreatment

The cogon grass samples containing 94.53%w/w total solids and 31.41% hydrolysable sugar from cellulose were pretreated at various conditions for a substrate loading of 10%w/v. The pretreatment using dilute $H_2SO_4$ acid at high temperature condition was done to solubilize some of the lignin and expose the cellulose and hemicellulose to subsequent hydrolysis while minimizing its degradation. The main purpose of optimizing the pretreatment was the conversion of crystalline cellulose to an amorphous type of cellulose and liberation of the cellulose and hemicelluloses component upon disrupting the lignin during the pretreatment process. However, liberation of the cellulose alone does not guarantee the effectiveness of the pretreatment process. The production of the possible inhibitory by-products such as furfural and hydroxymethylfurfural, regarded as the most toxic inhibitors present in lignocellulosic hydrolyzate, need to be minimized to reduce its inhibitory effects on the subsequent hydrolysis and fermentation. Thus, the pretreatment conditions were evaluated base on both the severity of the process and saccharification efficiency of the pretreated sample.

The amount of total sugar obtained in the filtrate after the pretreatment indicated the amount of substrate degraded in the process. This amount of sugar loss prior enzymatic saccharification was determined for the evaluation of the severity of the pretreatment process. It was observed that at constant acid concentration the amount of substrate hydrolyzed increased with increasing reaction time and temperature, with almost linear relations at concentrations 0.2%v/v and 0.4%v/v $H_2SO_4$. In general, the degree of hydrolysis of the cellulosic component of substrates was directly proportional to the severity of the pretreatment conditions, temperature, acid concentration and reaction time. The most severe pretreatment condition was at 0.6% v/v $H_2SO_4$, 127°C at 30 minute-reaction-time (Fig. 1) yielding the highest total sugar loss of 14.60mg/mL. The amount of sugar in the filtrate after neutralization quantified the hydrolysis of

the lignocelluloses especially the hemicellulose which was found to be more sensitive and less compact as compared to its cellulose component.

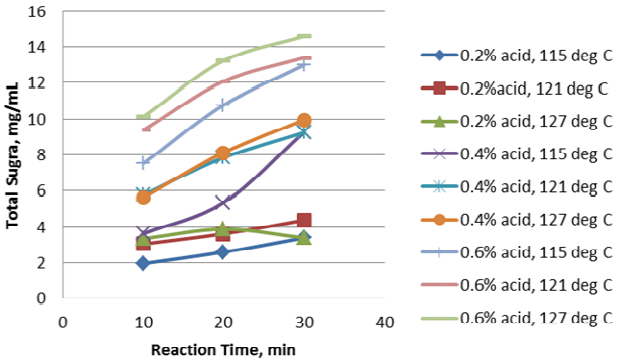



Fig. 1 Total sugar concentration (mg/mL) in the filtrate after dilute acid pretreatment

On the other hand the amount of sugar obtained from the pretreated fibers after enzymatic saccharification using 20FPU/g-cellulose NS50013-Cellulase complex, 1.1% w/w TS 250CbU/g NS50010-Cellobiase and 0.225% w/w TS of 100FBG/g NS50012-Hemicellulase complex for 72 hours indicated the remaining hydrolysable sugars after pretreatment. The optimum pretreatment conditions yielding the maximum sugar of 38.98mg/mL after saccharification were at 127°C, 0.6% v/v $H_2SO_4$, subjected at 20 minute-reaction-time (Fig. 2).

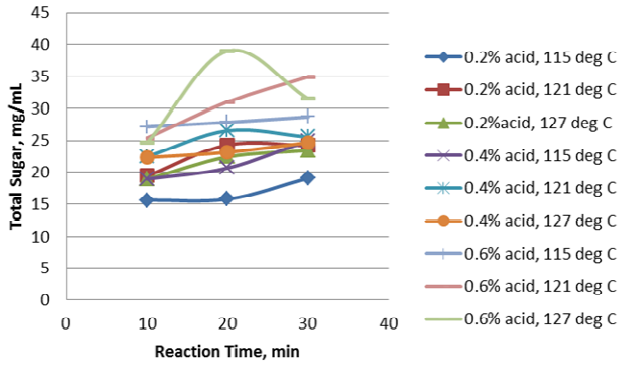
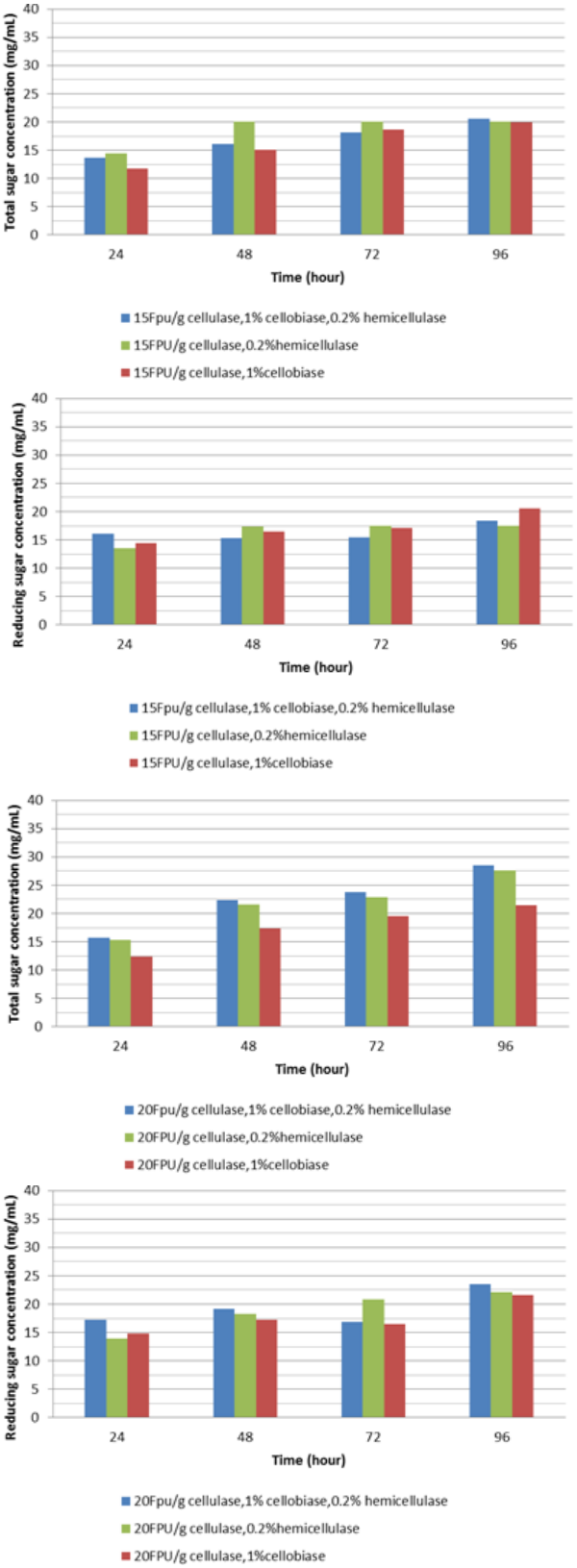



Fig. 2 Total sugar (mg/mL) of pretreated fibers after 72 hours of enzyme saccharification

*B. Enzymatic Saccharification*

It was observed that for all different enzyme loading, 15FPU, 20FPU, and 25FPU cellulase, the highest total sugar yield was achieved at an enzyme combination of cellulase-cellubiase-hemicellulase (Fig. 3). Also, the yield increases linearly with saccharification time for all enzyme loading and combinations. The conditions yielding maximum sugar for each enzyme loading and combination were determined to be at 25FPU/g Cellulase loading with enzyme combination of 1.1% w/w Cellobiase and 0.225% w/w Hemicellulase. The optimum conditions obtained were verified statistically using two-way ANOVA.



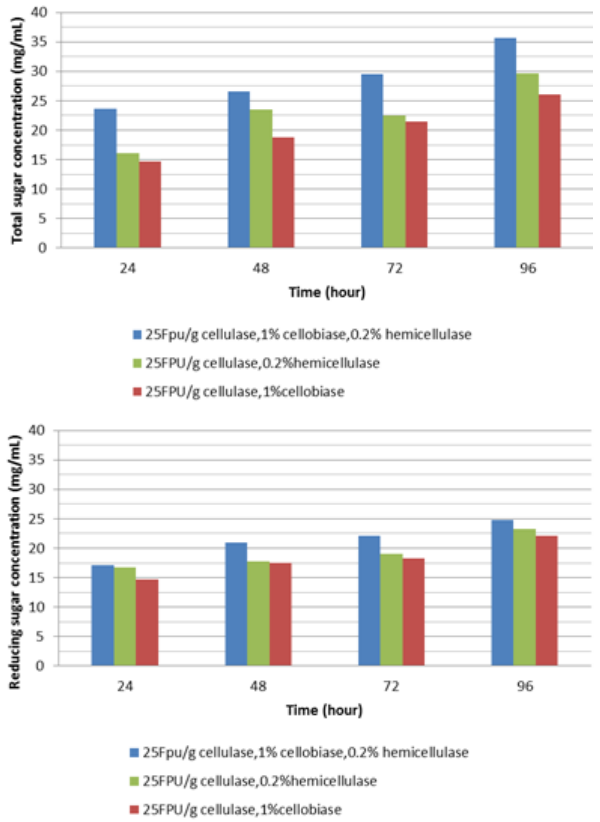



Fig. 3 Total sugar and reducing sugar yield at different enzyme loading and various combinations

### C. Fermentation

Ethanol and sugar concentration were monitored for 5 days. It was observed that the ethanol concentration increases with time while the sugar concentration reached its maximum at 24hours and gradually decreases with increasing ethanol concentration (Fig. 4). These can be explained by the simultaneous action of yeast HBY3 after enzymes have hydrolyzed the cellulose and hemicellulose content of the substrate.

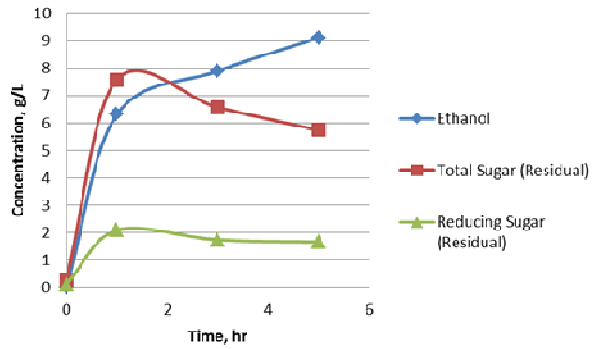



Fig. 4 Ethanol and sugar profile

The highest ethanol yield of 9.11g/L was obtained on day 5 of saccharification and fermentation process. The residual total sugar was 5.74g/L with 1.66g/L of which was reducing sugar.

## IV. Summary

The optimum pretreatment conditions prior enzymatic saccharification of cogon grass at 10% substrate loading was achieved at 127°C, an acid concentration of 0.6% v/v $H_2SO_4$ and at a reaction time of 20 minutes. While the maximum saccharification (based on both total and reducing sugar yield) was obtained at 25FPU/g cellulose NS50013-Cellulase complex loading combined with 1.1% w/w TS dosage of NS50010-Cellobiase, β-glucosidase, and 0.225% w/w TS dosage of NS50012-Hemicellulase complex at 96 hour saccharification time. The maximum total sugar yield was 36.68mg/mL, 71.29% of which is reducing sugar. Although the saccharification efficiency was very high it was observed that the maximum ethanol yield after 5 days was low with high residual sugars in the solution. This relatively large amount of unfermented sugar was due to the inability of HBY3 to ferment sugars from hemicelluloses hydrolysis. Lignocellulosic substrates such as cogon grass have almost 50-50% cellulose and hemicellulose. The total sugar produced was basically from both hemicelluloses and cellulose. The hemicellulose hydrolysis yields large amount of D-pentoses such as xylose, arabinose, mannose, galactose, and rhamnose which cannot be fermented using the available yeast strains and only glucose from the hydrolysis of cellulose was converted to ethanol.

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