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Research Article

Study of Chemical and Enzymatic Hydrolysis of Cellulosic Material to Obtain Fermentable Sugars

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The objective of this study was to evaluate the chemical and enzymatic hydrolysis using a factorial experimental design (2^3) in order to obtain fermentable sugars from cellulose-based material (CBM) usually used as pet litter. In assessing chemical hydrolysis, we studied the effect of temperature, in addition to H_2SO_4 concentration and reaction time, on the production of total sugars, reducing sugars, soluble lignin, carbohydrate profile, furfural (F), and hydroxymethyl furfural (HMF). We performed a response surface analysis and found that, at 100°C, 1% acid concentration, and 60 min reaction time, the yields of 0.0033 g reducing sugar/g biomass and 0.0852 g total sugars/g biomass were obtained. Under the above conditions, F is not generated, while HMF is generated in such a concentration that does not inhibit fermentation. We pretreated the CBM with H_2SO_4, NaOH, CaO, or ozonolysis, in order to evaluate the effectiveness of the enzymatic hydrolysis from the pretreated biomass, using an enzymatic cocktail. Results showed that CBM with acid was susceptible to enzymatic attack, obtaining a concentration of 0.1570 g reducing sugars/g biomass and 0.3798 g total sugars/g biomass. We concluded that acid pretreatment was the best to obtain fermentable sugars from CBM.

1. Introduction

Ethanol production from lignocellulosic material is a topic of interest because it does not undermine crops used for human consumption but it uses various sources of lignocellulosic biomass such as agricultural and municipal solid wastes [1]. In the energy sector, bioethanol has been recently used as an additive, because when mixed with gasoline bioethanol may reduce CO_2 emissions and replace the use of toxic oxygenate agents such as methyl *tert*-butyl ether (MTBE) [2].

Converting lignocellulosic biomass to ethanol involves four stages: pretreatment, hydrolysis, fermentation, and ethanol recovery by distillation [3]. Due to the complex and recalcitrant structure of lignocellulosic biomass, various pretreatment processes are required for carbohydrate hydrolysis, depending on the biomass. Pretreatment increases biomass digestibility for efficient fermentable sugar production, which reduces the cost of bioethanol production [4]. Various pretreatment methods have been suggested, depending on the purpose of removing hemicellulose [5] or lignin from the biomass [6, 7]. Dilute acid pretreatment is a promising pretreatment capable of high solubilization of hemicellulose [8]. This process degrades most of the hydrogen bonds in hemicelluloses and partially degrades cellulose and lignin [9]. In addition, acid pretreatment permits hemicellulose hydrolysis of pentoses and hexoses, removes some of the lignin, and makes the cellulose structure more accessible, so that a fraction can be converted to glucose enzymatically. Pretreatment is a necessary step to alter some structural characteristics of lignocellulose, increasing glucan and xylan accessibility to the enzymatic attack. The structural modifications of the lignocellulose are highly dependent on the type of pretreatment employed and have a great effect on the enzymatic hydrolysis [9] and subsequent steps. The choice

of pretreatment technology for a particular raw material depends on several factors, some of them directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate in addition to the pretreatment conditions has a great influence on biomass digestibility [10].

This paper proposes the evaluation of a cellulose-based material (lignocellulosic waste: pet litter), to produce bioethanol. The optimization of chemical hydrolysis is described, using an experimental design to determine the best conditions for obtaining high yields of fermentable sugars. Enzymatic hydrolysis of the pretreated biomass with the enzymatic cocktail called Celluzyme XB™ is also described, applying pretreatments such as ozonolysis and alkali and chemical pretreatment.

2. Experimental Methodology

2.1. Characterization of Cellulose-Based Material. Lignocellulosic biomass, known as "cellulose absorbent" or cellulose-based material (CBM), biodegradable or litter for pet bed, was obtained from a commercial distributor and was characterized in order to know the biomass chemical composition. A total of 250 g of CBM was ground in a Retsch mill. Then, the ground material was sieved to obtain a 60–80-mesh size. The sample was washed with water at 80°C, filtered under vacuum, and dried at 50°C for 48 h in a convection oven, Felisa model FE02292A, in order to remove components brought with the cellulose and to avoid any interference. For a proximal chemical composition analysis, determination of ash and moisture was performed. The ash content was determined in a muffle furnace, Lindberg/Blue, BF51866A-1 model, at 500°C ± 25°C for 24 hours in order to burn the organic compounds from the biomass. The ash content is reported as percent dry basis, using the formula described by Sluiter et al. [11]. The moisture content was determined in an MB 35 OHAUS thermobalance.

Carbohydrates content, in addition to soluble and acid-insoluble lignin, is obtained through two stages of hydrolysis with H_2SO_4. In the first stage, 3 g cellulose with a particle size of 60–80 reacted with 3 mL of 72% H_2SO_4 (w/w) at 30°C for 1 h in a shaking water bath. Subsequently, the hydrolyzate was transferred to an Erlenmeyer flask and 54 mL of distilled water was added to obtain a final sulfuric acid concentration of 4% (w/w), for a second hydrolysis step in which was carried out the reaction, performed on a SE510 Yamato autoclave at 120°C for 1 h. The hydrolysate was filtered under vacuum and the liquid was used for carbohydrates and lignin determination. The solid was washed with distilled water to remove any excess acid present, since the amount of insoluble solid lignin was determined [12].

Total sugars (TS) were determined by the phenol-sulfuric acid method [13], for which glucose was used as a standard and reducing sugars using the method of 3,5-dinitro-salicylic acid (DNS) [14]. The degree of polymerization (DP) of the syrup was obtained in order to evaluate the average monosaccharide units existing, that is, to determine the number of times the repeating monomer unit exits from a chain [15].

The qualitative and quantitative determination of sugars generated was conducted by high-performance liquid chromatography (HPLC). This analysis was performed using a stationary phase with isocratic elution (acetonitrile) and a refractive index detector (RID), or a detector for evaporative light scattering (ELSD), using the Pinnacle II amino column to analyze the monosaccharides and disaccharides, having a detection limit of 100 pg/mL. The evaporative light scattering detector (ELSD) has a flow of 1 mL/min at 80°C, with an injection volume of 10 uL, and the mobile phase used is 18% water and 82% acetonitrile. The concentration of carbohydrates was calculated by Sluiter et al.'s equation [12].

2.2. Chemical Pretreatments. Pretreatments were carried out under conditions set by our group (see Aragon et al. [16] as an example), except for dilute acid pretreatment, for which conditions were set by an experimental design for optimizing chemical hydrolysis.

2.2.1. Acid Pretreatment. The CBM was suspended in 10 mL of 1% H_2SO_4 (dry basis, 10% of total solids) in 50 mL vials. The reaction was carried out for 1 h in an evaporator at 100°C (set conditions in experimental design). After the elapsed reaction time, the reaction vessel was quenched in an ice bath; samples were filtered under vacuum, obtaining a solid and a liquid. From the liquid, we obtained soluble lignin concentration, and the remaining liquid was adjusted to pH 7 with $CaCO_3$ and subsequently centrifuged at 10,000 rpm for 15 min at 25°C to remove excess $CaCO_3$. Liquid was obtained to determinate total and reducing sugars (TS and RS, resp.). The pretreated solid obtained was washed with citrate buffer until the solid had pH 4.8 for subsequent use of enzymatic hydrolysis and for performing the same analysis by infrared (IR) spectrophotometry to analyze the change undergone by functional groups contained in the lignin. Likewise, the washed solid was stored for later use in enzymatic hydrolysis, for which moisture content ($H = 73.60\% \pm 0.7$) was determined to obtain data on a dry weight basis.

To determine the optimum conditions of pretreatment, we conducted a 2^3 factorial experimental design, that is, three factors with two levels; to determine experimental error, 5 central points were established. During chemical hydrolysis, different H_2SO_4 concentrations were used (0.5, 1.0, and 1.5%), in addition to temperature (80, 100, and 120°C) and time (30, 60, and 90 min). Constant variables were the amount of biomass (1 g), H_2SO_4 volume (10 mL), and mesh size (60–80). Table 1 shows the factorial design matrix with combinations made according to the established model.

An analysis of variance (ANOVA) was performed using Statistical Product and Service Solutions (SPSS) version 2010, in addition to a principal component analysis (PCA) in Statistica software, version 98. In these analyses, the following dependent variables were considered: total sugars (TS), reducing sugars (RS), degree of polymerization (DP), glucose (GLUC), fructose (FRUC), xylose (XIL), sucrose (SUC), furfural (F), and hydroxymethyl furfural (HMF). These variables can be explained by three factors: time (min), temperature (°C), and acid concentration (%). A surface response model was subsequently used to analyze the results

Table 1: Factorial experimental design (2^3), including independent variables (temperature, H_2SO_4 concentration, and time).

Treatment	Codified variables			Real variables		
	T (°C)	[H_2SO_4]	Time (min)	T (°C)	[H_2SO_4]	Time (min)
1	–1	–	–	80	0.5	30
2	+	–	–	80	1.5	30
3	–	+	–	80	0.5	90
4	+	+	–	80	1.5	90
5	–	–	+	120	0.5	30
6	+	–	+	120	1.5	30
7	–	+	+	120	0.5	90
8	+	+	+	120	1.5	90
9	0	0	0	100	1	60
10	0	0	0	100	1	60
11	0	0	0	100	1	60
12	0	0	0	100	1	60
13	0	0	0	100	1	60

obtained by the 2^3 factorial design [17] in Statistica software, version 98, taking into account the independent variables that were more significant (obtained from ANOVA and PCA), that is, temperature and acid concentration ($p < 0.05$). Time was standardized as 60 minutes, because it was not a significant variable ($p < 0.05$), and after 60 min no changes occurred in the response variables.

2.2.2. Basic-Oxidant Pretreatment. A total of 0.05 g of CBM was placed in a 250 mL Erlenmeyer flask with a cap, to which 0.3 mL of a solution of 9% H_2O_2 was added, and then the pH was adjusted to 11.5 with NaOH 2.5 M. The reaction was conducted at 35°C and 60 rpm for 26 h on a shaking water bath, whereafter the sample was filtered to produce a liquid and a solid. The liquid was centrifuged at 10,000 rpm for 15 min at room temperature, and then determination of soluble lignin, TS (phenol-sulfuric acid), and reducing sugar (DNS) was performed. The solid was washed with citrate buffer (pH = 4.8), until the solid remained at this pH, to avoid affecting the enzymatic hydrolysis. For the dry basis weight of material used for the subsequent enzymatic hydrolysis, the moisture content was determined (H = 71.73% ± 0.8).

2.2.3. Basic Pretreatment with NaOH. A total of 2 g of CBM, 60–80-mesh size, was placed in an Erlenmeyer flask with a cap and suspended in 20 mL of 1% NaOH; the sample was incubated in an autoclave at 121°C for 60 min so that the reaction could take place. The solid was washed with citrate buffer (pH 4.8). Solid moisture was 74.23% ± 0.6. Soluble lignin (SL), total sugars (phenol-sulfuric acid), and reducing sugars (DNS) were determined in the remaining liquid.

2.2.4. Basic Pretreatment with CaO. A total of 1 g of CBM was mixed with 10 mL 1% CaO. The reaction took place in a 75°C water bath with stirring for 60 min. Then, a sample was filtered to obtain a liquid from which the amount of soluble lignin was measured, in addition to total sugars (phenol-sulfuric acid) and reducing sugars (DNS). The solid obtained was washed with citrate buffer (pH 4.8) and the moisture content (65.99% ± 0.8) was determined, to obtain a dry base weight for enzymatic hydrolysis.

2.2.5. Ozonolysis. Ozonolysis was performed in a fixed bed reactor under environmental conditions. To the reactor (a glass column of 75 ml volume, 7 cm height, and 4.6 cm diameter), 1 g of cellulose was added, which was wetted with 1 ml of water for each test. The material was exposed to ozone for 1 h, and during the reaction ozone was wetted with potassium iodide 0.05 M phosphate buffer (pH 7) to prevent dehydration and to determine the amount of ozone that reacted with the sample. Flow rate entrance was 46.6 ± 5 L/h and ozone concentration, which interacted with the sample, was 0.056 ± 0.03% (v/v). A pretreated solid with ozone was obtained and washed with citrate buffer to obtain TS, RS, and soluble lignin. The solid was washed with citrate buffer and the moisture content was determined (66.24% ± 0.8) to obtain 0.1 g of the material pretreated in dry weight for the enzymatic hydrolysis.

2.3. F and HMF Determination. During chemical hydrolysis, some carbohydrates are formed, which generated products such as F and HMF, which inhibit bioethanol production [18]. These compounds were analyzed by high-performance liquid chromatography (HPLC) with a UV-Vis detector. The sample was filtered with a nylon syringe filter of 0.25 um and 1 mL was placed in a vial with 1.5 mL capacity for HPLC F and HMF determination during a period of 15 min, using the calibration curves.

2.4. Enzymatic Activity Determination. The activity of "Celluzyme XB" cocktail from Novozyme was determined through a series of enzyme dilutions. Strips of Whatman filter paper No. 1 were used as a substrate. Paper was placed in a capped vial with 1 mL of 0.05 M citrate buffer (pH 4.8). 0.5 mL of each dilution of the enzyme was added to a Syncore evaporator and incubated at 50°C and 180 rpm for 60 min.

Table 2: Chemical composition of the cellulose-based material.

Parameter	Values
Soluble lignin in acid (%)	10.160 ± 0.10
Nonsoluble lignin in acid (%)	25.968 ± 0.10
Total lignin (%)	36.13
Total sugars (g sugars/g biomass)	0.639 ± 0.03
Reducing sugars (g sugars/g biomass)	0.538 ± 0.05
Degree of polymerization	1.197 ± 0.07
TS/TL ratio	1.77
Ash (%)	0.490 ± 0.06
Moisture (%)	4.410 ± 0.05

Once the reaction was completed, the enzyme was inactivated by immersing the tubes in water at boiling temperature, and then the sample was filtered through 0.45 μm.

Subsequently, the amount of sugars was measured with a glucose calibration curve on a Thermo Spectronic UV-Visible spectrophotometer at 540 nm. The amount of released protein was determined by the Bradford method. To use the calibration curves, the amount of sugars present in the target enzyme was subtracted. Total filter paper units (FPU) were determined by the equation described by Sluiter et al. [12].

2.4.1. Enzymatic Hydrolysis. The pretreated samples of CBM were washed with 200 mM citrate buffer (pH 4.8) in a centrifuge model 5810-R to 10,000 rpm at 25°C, until the material reached pH in the range 4-5. A total of 0.1 g of material was weighed and added to 2 mL of citrate buffer (pH 4.8) and 0.04 mL of enzyme cocktail "Celluzyme XB" and incubated at 50°C for 1 h. Samples were filtered using a nylon filter of 0.45 μm. Carbohydrates in the syrup were determined as described above.

3. Results and Discussion

3.1. Characterization of Cellulose-Based Material. The main composition of the material is shown in Table 2. We found a total sugar (TS) and lignin (TL) content of 63.9 and 36.13% w/w, respectively, with a TS/LT ratio of 1.77. In accordance with our experience, such TS/LT value is typical for forest residues.

3.2. Chemical Hydrolysis from a Design of Experiments. Conditions for hydrolysis with H_2SO_4 were analyzed using an experimental design (Figure 1). Results showed that, by increasing acid concentration, the release of total sugars increases during the chemical hydrolysis, reaching a maximum concentration of 0.274 g total sugars/g biomass at a H_2SO_4 concentration of 1.5% at 80°C, that is, significantly increasing the solubilization of cellulose for obtaining total sugars, generated in a process in which 1 g of CBM was treated with H_2SO_4 concentration ranging from 0.5 to 1.5% (v/v) and at temperature between 80 and 120°C, the latter being the determining factor in the total sugars released. The results obtained with regard to the generation of total sugars could be because cellulosic biomass is partially hydrolyzed, increasing

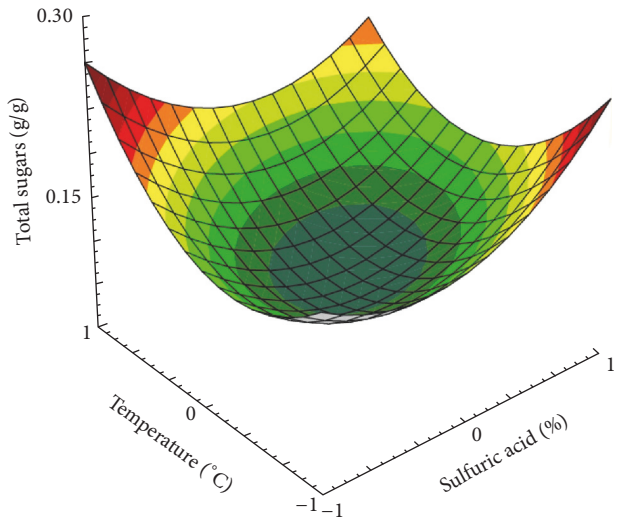

Figure 1: Influence of temperature (°C) and diluted sulfuric acid concentration (%) on the generation of total sugars.

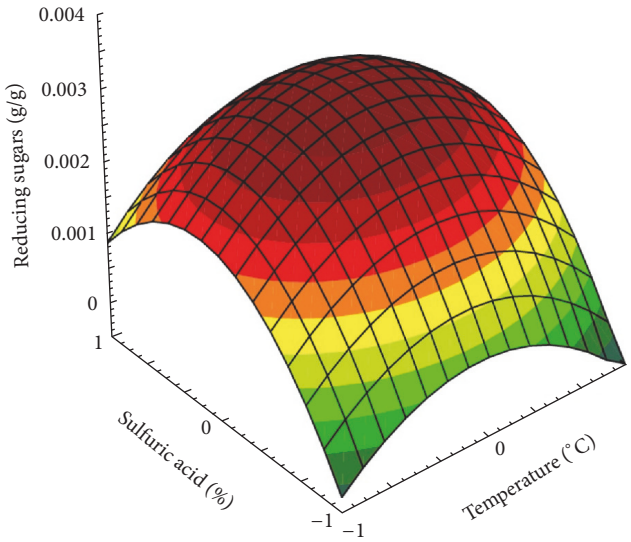

Figure 2: Influence of temperature (°C) and sulfuric acid concentration (%) on the generation of reducing sugars.

the surface and exposure of the cellulose fibers [19], that is, significantly increasing the solubilization of cellulose for obtaining total sugars, generated in a process in which CBM is treated. Time reaction was also studied; however, there was not any significant difference ($p < 0.05$); therefore, total sugars generation consistently increases at minute 60.

There is a maximum in the production of reducing sugars (Figure 2) at elevated temperatures and the highest dilution of acid. Chemical hydrolysis showed a reducing sugar concentration of 0.0033 ± 0.0004 g reducing sugars/g biomass, establishing that favorable experimental conditions for sugars released are 100°C and a concentration of 1% H_2SO_4. These factors have a positive effect on the amount of hydrolyzed sugars, playing an important role in separating the cellulose-hemicellulose complex [20].

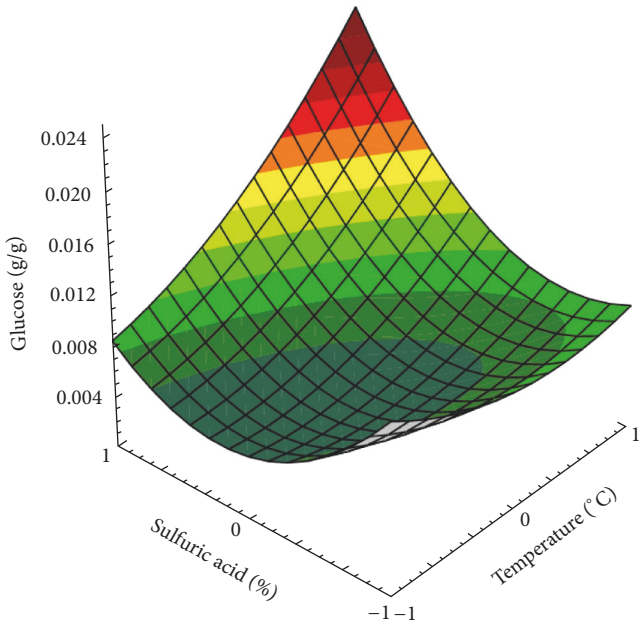

Figure 3: Relationship between temperature (°C) and sulfuric acid concentration (%) with respect to glucose formation.

The polymerization degree was analyzed in order to assess the average number of units of monosaccharides existing in the extracted polysaccharides [14]. A good efficiency of chemical pretreatment increases the accessibility of glycan to saccharification and therefore obtaining a value of polymerization degree close to one, so that they can be converted to ethanol [21]. According to the results, a degree of polymerization of 29.640 was obtained at 100°C and an acid concentration of 1%, which is proven to be the least of the above conditions.

3.2.1. Generation of Toxic Compounds Derived from Furfural. During chemical hydrolysis, F is generated from pentoses and HMF from hexoses [22]. In experiments, degradation products increase in concentration as temperature and H_2SO_4 concentration increase, reaching a maximum concentration of 2.8962% F and 3.62% HMF at 120°C and 1.5% acid concentration. With respect to toxic compounds during ethanol production [23], what is sought is to have the optimum conditions in which there is no generation of these compounds, or at least not existing in relevant concentrations, by which it was proposed to work at 100°C and 1% acid concentrations, where F is not generated and HMF is generated below 2.98%. It has been shown that F concentrations above 3 g/L of and 3.2 g/L HMF inhibit bioethanol fermentation by 20 and 50%, respectively [24]. Therefore, it can be said that 2.98% 5-HMF does not affect the next steps in the fermentation process for bioethanol production.

3.2.2. Carbohydrate Profile. After applying the chemical hydrolysis to the CBM, liberated cellulose is degraded to glucose by the action of dilute H_2SO_4. Figure 3 shows that, at a temperature of 100°C and acid concentrations of 1.5%, a maximum concentration of 0.016 g glucose/g biomass is generated. Low concentration of glucose generally indicates that cellulose degradation suffered poor or incomplete hydrolysis; hence, it follows that the chemical hydrolysis is quite selective since the chemical hydrolysis conditions are not so severe as to cause cellulose solubilization, because its crystal structure makes it difficult to complete the hydrolysis [25].

During fructose generation after CBM is hydrolyzed, the increased generation of fructose is conducted at temperatures of 100°C and at 1.5% sulfuric acid concentrations, giving a maximum concentration of 0.00674 g fructose/g biomass. Fructose concentration is not significant when compared with five-carbon sugars such as xylose, yet it is capable of being fermented in smaller molecules and more easily because they are sugars of six carbon atoms, and they are those with greater availability.

Sucrose shows a different behavior to glucose, since it increases at a 1% sulfuric acid concentration and as temperature increases, thereby obtaining a concentration of 0.006 g sucrose/g biomass at 120°C and H_2SO_4 concentration of 0.5%.

Xylose formation is increased with increasing temperature and acid concentration, thus creating a maximum concentration of 0.18342 g xylose/g biomass. Chains of hemicellulose suffered disruption, generating all possible monosaccharides present in chains, and one of them is xylose, from which a yield of 18% (w/w) was obtained. The direct application of chemical hydrolysis to CBM also reduces aggregation between cellulose microfibrils, as well as making direct hydrolysis obtain good yields (0.008–0.275 g total sugars/g biomass), depending on the experimental conditions to which the biomass is submitted. Chemical hydrolysis may be used then as a pretreatment that generates soluble sugars but also facilitates further enzymatic hydrolysis as seen by a higher yield (0.379 g total sugars/g biomass) and is discussed below.

3.2.3. Statistical Analysis. The results from ANOVA showed statistically significant differences ($p < 0.05$) between the treatments used. Factors included in the analysis of main components explain 81.4% of the total variance. The temperature favors the generation of F, HMF, glucose, and xylose; the acid concentration increases the generation of reducing sugars, lignin solubilization, and increased sucrose.

The factors relevant to production of soluble sugars are temperature and acid concentration followed by time. However, we set a reaction time of 60 minutes since we observed maximum generation of soluble sugars. Acid hydrolysis is favored at conditions of 100°C, 1% sulfuric acid, and a reaction time of 60 min.

Results of the experimental design show that acid concentration was only significant for fructose, glucose, and HMF generation. Time is significant in reducing sugars' generation and consequently the degree of polymerization. The statistical study, based on the p values, shows that only temperature has a statistically significant effect on the chemical hydrolysis, while the other two factors are significant but less significant than temperature.

From response surfaces, PCA, and ANOVA, it was concluded that the optimum conditions to obtain fermentable

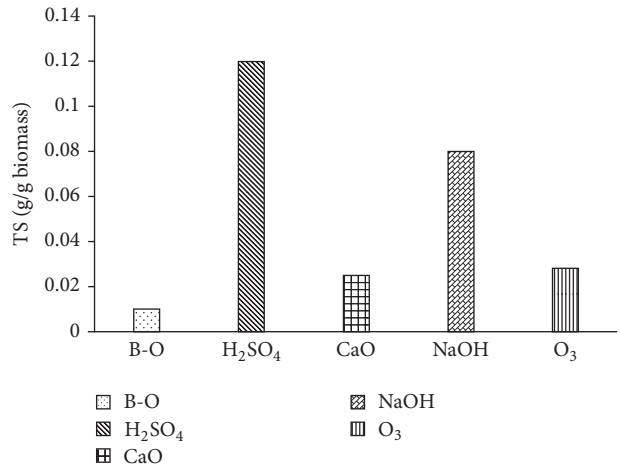

Figure 4: Effect of different pretreatments on the generation of total sugars.

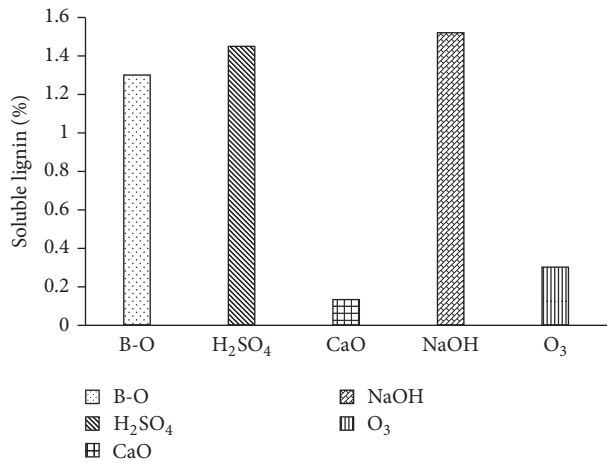

Figure 5: Concentration of soluble lignin based on the different pretreatments made to cellulose-based material.

sugars from CBM by chemical hydrolysis are 100°C for a reaction time of 60 min and 1% acid concentration.

3.3. Enzymatic Hydrolysis. Enzymatic hydrolysis was performed on solids obtained from the pretreatment, in order to evaluate the effect of each pretreatment with respect to the generation of soluble sugars, so that the effect of different pretreatments on hydrolysis with enzyme cocktail "Celluzyme XB" is studied.

3.3.1. Pretreatments Applied to CBM. Figure 4 shows the effect that different pretreatments had on the CBM with respect to the generation of total sugars. Acid pretreatment resulted in a higher concentration of total sugars having a concentration of 0.115 g ± 0.003 g total sugars/g biomass for 10% solids. In the other pretreatments, sugars release is also promoted, however in smaller amounts.

Reducing sugars measured by the DNS technique showed that dilute acid pretreatment presented a concentration of 0.0045 ± 0.004 g reducing sugars/g biomass, being the best treatment in separating the cellulose-hemicellulose complex. Possibly, the failure in finding reducing sugars from alkaline pretreatments was mainly due to how they produce swelling of cellulose, but not necessarily reaching hydrolysis of reducing sugars present or their chemical composition changes [26]. Although the applications of basic pretreatment with CaO, basic pretreatment with NaOH, oxidizing basic pretreatment, and ozonolysis are effective because they improve the opening of cellulosic fibers [10], they do not degrade sugars at this stage, that is, only making the material susceptible to enzymatic attack.

Efficiency of different pretreatments with respect to lignin solubilization (Figure 5) showed that basic-oxidant pretreatment solubilized 1.53% lignin, being the best pretreatment. This behavior was probably because treatment with diluted NaOH produces a swelling in the biomass, leading to an increased internal surface area and a decrease in crystallinity and structural separation joints between lignin and carbohydrates, causing a break in the structure of lignin. The mechanism of the alkaline hydrolysis of biomass seems to be based on the saponification of intermolecular ester bonds linking the xylan of the hemicellulose and other components [27]. It is noteworthy that the effectiveness of the pretreatment depends on the lignin content of the material to be pretreated.

Dilute sulfuric acid pretreatment with 1.45% solubilized lignin is the second best pretreatment to solubilize lignin, and such pretreatment already has been reported because of its high reaction rates and its effective hydrolysis of cellulose. However, lignin does not dissolve as well as other pretreatments but rather increases yields of enzymatic hydrolysis [12].

3.3.2. Effect of Various Pretreatments on Polysaccharides Chemical Structure Using Infrared Spectrophotometry. Infrared spectra of the five pretreatments (dilute acidic pretreatment, basic-oxidant pretreatment, basic pretreatment with sodium hydroxide, basic pretreatment with calcium oxide, and ozonolysis) performed on solids (insoluble lignin) were analyzed in the region of 400–4000 cm^{-1} wavenumber (Figure 6). These spectra show structural differences in nonpretreated solid and treated solids. The absorption peaks observed in the infrared spectrum were attributed to vibrations of stretching peaks through structural changes with respect to the hydroxyl groups that are in a region between 3550 and 3300 cm^{-1}. Comparison between spectra from pretreated and nonpretreated biomass shows similar patterns; however, bands from treated material have a stretching vibration in their OH bands; that is, the area is augmented. In the region between 1500 and 1400 cm^{-1}, there are peaks that are very similar to those identified by Pearl [28] and Singh et al. [29], representing vibrations associated with guaiacyl and syringyl units (monomers of lignin), which are the same as shown in the spectra obtained, however with greater intensity of guayacil bands. Another important value is at approximately 1160 cm^{-1} which represents in-plane deformation of CO bonds in aliphatic secondary alcohols and ethers. At 1265 cm^{-1}, there is stretching of the CO bonds

Table 3: Enzymatic activity of the enzyme cocktail Celluzyme XB.

Enzyme	Protein (mg/mL)	Cellulolytic activity (FPU/mL)	Specific activity (FPU/mg protein)
Celluzyme XB	77.11	217.65	2.82

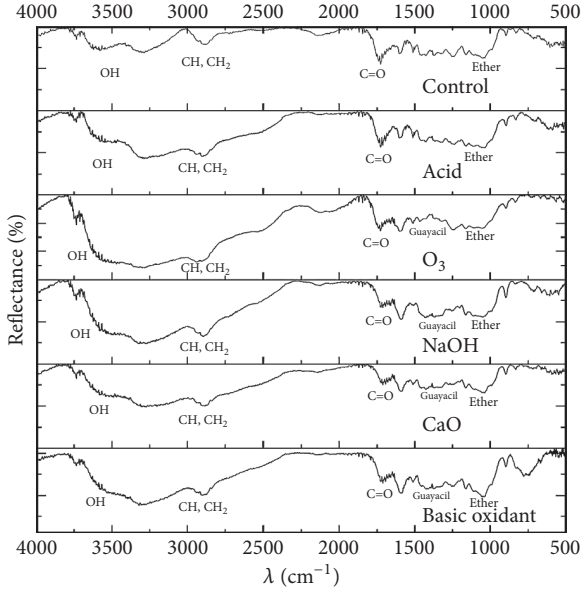

Figure 6: Spectrum of different pretreatments analyzed by infrared spectroscopy.

representing vibration in the rings of the guayacil with more units of carbon-oxygen bonds [30].

The OH group initiates substitution reactions on the solid pretreated with dilute acid. This condition causes the transformation of the hydroxyl group to an allyl ether and ultimately the ether is substituted with an acid group. The benefit of this reaction is that the presence of the acid group in the molecule causes the lignin polymer to be soluble in water [30].

The ester linkage containing glucose monomers in a polymer chain is the most important lignin link. Therefore, ether bond cleavage can lead to separation of lignin from the polysaccharide matrix and degradation of the polymers of monomeric sugars and lignin fragments. The cleavage of this bond occurs through solvolytic reactions, which may take place under acidic or alkaline conditions or by different mechanisms. In the case of lignin, under acidic conditions, the ether bond is converted to OH and then converted to carbonyl or carboxyl before being fragmented into molecules of C3 and C2. Under alkaline conditions, the mechanism is different and the end result is not a fragmented side chain, but the separation of the aromatic rings. In the case of cellulose cleavage of ether bonds, this may be contained either in acidic or in alkaline media. When acidic media are used, the acid acts as a catalyst for the protonation of the oxygen atom. The charged group leaves the polymer chain and is replaced by the hydroxyl group of water. The reaction that occurs is a first-order reaction.

In order to determine which pretreatment is more efficient for delignified cellulosic material, a comparative analysis was performed before and after pretreatment. In the CBM, nonpretreated well-defined peaks are shown (Figure 6) with vibration of OH groups, in addition to their CH, CH_2, carboxylic acids, phenolic ethers, aromatic groups, and characteristic guaiacyl lignin groups. However, treatment with dilute H_2SO_4 shows similarity in their spectra with respect to the original sample, but functional groups are present in stretching vibrational peaks due to the effect of the pretreatment that was applied.

For basic pretreatments, likewise, a change in the size of their peaks occurred, vibrational stretching was present, and lignin signals remained apparent via, as mentioned above, characteristic groups of lignin (aromatic compounds, phenol groups, ethers present, and carbonyl groups). Notwithstanding, in the spectral range 1700–1160 cm^{-1}, it is indicated that there is the presence of monolignans such as guayacil, as inferred by the shape of the wing and the functional groups which occur nearby [30].

With ozonolysis, some lignin is degraded causing a variety of compounds, among which are aromatic groups, phenols, alcohols, ethers, and carbonyl groups.

3.4. Determination of Enzyme Activity. Table 3 shows the results of enzymatic activity of Celluzyme XB, expressed as hydrolyzed filter paper units (FPU) per milligram of enzyme. An FPU is defined as the amount of enzyme necessary to produce a mole of reducing sugars per minute per gram of substrate [31].

3.5. Enzymatic Hydrolysis of Pretreated Cellulosic Material. The material being pretreated with the oxidizing basic treatment, for which a concentration of 0.5642 g reducing sugars/g biomass was obtained, favored the obtaining of reducing sugars. This was due to applying the basic oxidizing pretreatment, whereby the materials increased the size of their pores, giving greater access penetration of enzymes and therefore increased enzymatic breakdown of carbohydrates. The significant increase in the generation of reducing sugars was due to an enzymatic cocktail of cellulases and hemicellulases (217.65 FPU/mL).

The generation of total sugars was favored in all pretreatments but significantly in the basic-oxidant (0.5642 g sugar/g biomass), basic CaO (0.4961 g sugar/g biomass), and dilute acid (0.3798 g sugars/g biomass) pretreatments. This difference could be due to a modification of the crystalline state of the cellulose, due to the implementation of basic pretreatments with NaOH and CaO, having the effect of increasing the size of the pores of the cellulosic material, which stimulates more accessibility and susceptibility to attack by an amorphously structured enzyme. As the acid pretreatment degraded during the pretreatment process, many

of the hemicellulose to cellulose bonds were held together with lignin, thus becoming more susceptible to the enzymatic attack of biomass. In general, before applying the CBM, the pretreatment increased the solubility of lignin, freeing the cellulose and thus making it available for enzymatic hydrolysis [10], highlighting the efficiency of pretreatments in obtaining acceptable levels of total sugars.

In order to understand the degradation of total and reducing sugars, the degree of polymerization (on average) of different pretreatments was calculated. Basic pretreatment with sodium hydroxide had the lowest degree of polymerization. That is, treatment promotes smaller carbohydrates, which would be more accessible to the microorganism during fermentation. Among the chemical pretreatments applied to the lignocellulosic materials is the basic pretreatment with dilute NaOH, which increases the surface area and decreases the degree of polymerization and crystallinity by removing links between lignin and carbohydrates [12]. The value obtained depended on the cellulosic material used and the pretreatment applied to the fibrous material; hence, with the basic pretreatment using NaOH (GP = 5.7) and CaO (GP = 6.6), sugars are obtained with a lower degree of polymerization; that is, they are easier to degrade.

For carbohydrates, qualitative and quantitative analyses were performed via HPLC. The pretreatment that had the greater effect for the transformation of cellulosic material to the accessible enzyme was saccharification of dilute acid, a pretreatment that generates sugars such as xylose (0.07069 g xylose/g biomass) and cellobiose (0.02946 g cellobiose/g biomass). Although the saccharification of pretreated cellulose with dilute H_2SO_4 showed good yields (0.3798 g total sugar/g biomass), an important concentration of cellobiose was generated, indicating that the enzymatic hydrolysis was incomplete [12]. This could be explained in terms of a poor cellobiase activity inside the enzymatic cocktail.

Regarding glucose generation, the pretreatment that generates a greater concentration was the basic pretreatment with NaOH, yielding a concentration of 0.02011 g glucose/g biomass, and the second pretreatment that was mostly predisposed, via the CBM, to enzymatic attack oxidant was a basic pretreatment, obtaining thus a glucose concentration of 0.01668 g/g biomass. This was due to an increased internal surface of cellulosic biomass and lower crystallinity, and due to the effect of the alkali treatment, the enzyme cocktail had greater access to the material and therefore it degraded more easily the cellulose into smaller units such as with the glucose.

Among the various pretreatments applied in this work, dilute acid pretreatment is the one that has been largely studied and significantly improves the enzymatic hydrolysis [32, 33]. Generally, this pretreatment significantly improved the obtaining of fermentable sugars after enzymatic hydrolysis was applied to the CBM using diluted acid, made with the optimal conditions obtained via experimental design.

4. Conclusions

CBM is a good source of fermentable sugars. The production of ethanol from this cellulose can be an alternative product of this waste recovery. Pretreatment with acid did not generate F, while HMF concentration did not inhibit fermentation.

The evaluation of enzymatic hydrolysis of pretreated biomass showed that basic-oxidant pretreatment largely promoted enzymatic attack of the cellulose which was concluded from the observation of sugars obtained from the saccharification process that was previously pretreated. In addition, the analysis by infrared spectroscopy helped to analyze the effectiveness of each pretreatment.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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