academicJournals

Vol. 12(31), pp. 4956-4965, 31 July, 2013
DOI: 10.5897/AJB2013.12461
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Alkaline pretreatment of Mexican pine residues for bioethanol production

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Accepted 17 July, 2013

The locally sourced residue samples of *Pinus arizonica, Pinus cooperi,* and *Pinus durangensis* from the state of Durango in Mexico were analyzed for optimal yield of ethanol production. The samples were mixed at an equal proportion using a particle size of 0.59 mm. Each individual mixture was pretreated with either NaOH or Ca $(OH)_2$ (at 0.5, 1.0 and 1.5% w/v) for periods of 30, 60, and 90 min at 60, 90, and 120°C. The pretreated blending was subjected to enzymatic hydrolysis for 130 h at 80 rpm and 50°C with an enzymatic load of 25 filter paper units (FPU) and 50 IU β-glucosidase per gramme of cellulose to obtain a maximum yield of reducing sugars (RS) with NaOH subject at 120°C for 90 min. The results show that the hydrolysis yield depends on temperature and alkali concentration particularly (NaOH), which increased from 2.0 to 3.5% w/v. The best yield of glucose (41.33% w/w) was obtained using a pretreatment of 2.5% NaOH for 90 min, 120°C, and a hydrolysis residence time of 130 h. The removal of lignin and hemicellulose acetylation was observed to have influence on the enzymatic digestibility of cellulose. This process could theoretically produce a maximum yield of 90.19% of ethanol / substrate (glucose) and about 80 L of bioethanol per dry ton of woody biomass from pine residues.

Key words: Lignocellulosic biomass, alkaline pretreatment, enzymatic hydrolysis, fermentable sugars, fermentation.

INTRODUCTION

Energy dependence on fossil fuels can lead to depletion, environmental degradation, and economic instability. The effect of fossil fuel dependency has increased the number of research-oriented generations of alternative energy sources that are sustainable and renewable. These alternative sources include biodiesel, bioethanol, and biogas listed in the Kyoto Protocol (Kyoto, 2007). Bioethanol, just like other biofuels, contributes to the reduction of pollutant emissions and prevents the greenhouse effect due to the traditional process of its production from sucrose (sugar cane and sugar beet) and starch (corn and wheat). The derivation of the bioethanol from these food sources competes with food availability for human consumption, and, in most cases, lead to an increase in food prices and derived products (Hill et al., 2006). A sustainable solution for the use of biofuels would be its production from lignocellulosic waste (agricultural residues, grasses, sawdust, wood chips, etc) because they are abundant, renewable, and relatively inexpensive (Elshafei et al., 1991).

Lignocellulosic materials mainly contain lignin and a carbohydrate matrix (holocellulose) which, consists of

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cellulose and hemicelluloses. These materials are connected by chains of ether or ester bonds (lignin and hemicellulose), hydrogen bridges (cellulose and hemicellulose), and other removable and inorganic materials in minor proportions (Olofsson et al., 2008). Lignin is a polymer of phenolic compounds (p-coumaric acid, coniferyl, and sinapyl) which contribute to the rigidity of the plant cell wall, while the cellulose, the primary component of the plant cell wall, is a linear homopolysaccharide consisting of chains of 2,000 to 10,000 glucose units bound together by β-glycosidic bonds (1 to 4). Lignin and cellulose form crystalline ordered and disordered as well as amorphous regions. Hemicellulose, the second largest component of lignocellulosic biomass, is a complex polymer of branched structure (similar to lignin) formed by joining units of different types of sugar such as pentoses (xylose, L-arabinose), hexoses (D-glucose, D- mannose, and Galactose), and acids as the D-glucuronic and D-galactorinic (Sjöström, 1981).

The waste of forest biomass is an important provision of raw material which may also contribute to the economy of many rural communities. Nevertheless, the use of biomass to satisfy the local and regional fuel needs must be based on the conservation of the biodiversity and on the sustainability of ecosystems (Zhao et al., 2008). The lignocellulosic biomass can be an important raw material for the production of bioethanol because it is harvested throughout the year, eliminating the need of long-term storage. The high density and low content of ashes eliminates the dead load which reduces the costs of transportation and processing (Zhu and Pan, 2010). It is also known that the collection of forest residues usually reduces the risk of forest fires and generates economic revenue by creating jobs. However, an intensive extraction of forest biomass reduces the accumulation of organic matter, diminishes the capacity of the soil to retain water, and eventually reduces forest productiveness (Wyman, 1999).

The state of Durango is considered as one of the largest reserves of pine forests in Mexico. It has a total area of 12.3 million hectares, of which 40% is dedicated to forest production. In this area, about 2.7 million m^3 (stand tree) are harvested annually representing approximately 30% of the timber production in Mexico. Softwoods occupy almost 75% of the authorized volume while the remaining 25% corresponds to hardwoods (SEMARNAT, 2010). Due to the characteristics of the harvesting methods, a considerable amount of biomass (between 5% and 10% of the harvested volume) in the form of branches and tree tops are left on the field. Part of this residue is cut into smaller pieces to prevent forest fire hazards and soil erosion, which favors incorporation into the organic matter. However, much of this material (particularly, sawdust and industrial by-products) is simply piled and burned to reduce the cost of a proper disposal, which again, leads to more pollution. Several authors have reported floristic, ecological inventories, and analysis of the number of arboreal species indicating the existence of wide range of pine species in the state of Durango, with *Pinus arizonica, P. cooperi and P. durangensis* constituting the predominant species (González et al., 2007; Valenzuela and Granados, 2009; Silva–Arredondo and Návar–Cháidez, 2009).

All biological processes used for the industrial conversion of lignocellulosic materials and production of bioethanol are performed in multiple stages. The process normally begins with a pretreatment stage which can be physical, chemical, and/or biological followed by a hydrolysis of the cellulose in monomers of sugars ending with a fermentation stage to transform sugars into alcohol. The primary obstacle using biomass lignocellulosic for ethanol conversion is the physical protection that the lignin exerts on the cellulose; for this reason, the stage of pretreatment is the most important stage as it secures a good yield of fermentable sugars. Therefore, an effective pretreatment stage aims to obtain a proper deslignification of the biomass and defragmentation of the matrix of polymers in order to reduce the cellulose at grade crystalline and facilitate the enzymatic attack of the cellulases (Ruggeri and Sassi, 2003).

The stage of pretreatment has been recognized as a necessary step to remove the recalcitrant biomass that blocks the enzymatic and biological processes during the production of bioethanol. A physical pretreatment is the reduction of the size of the particles to increase the superficial area of the material and the accessibility of the cellulolytic enzymes (Zhu and Pan, 2010). In addition, chemical pretreatment uses chemical products to modify the composition and structure of the materials to facilitate the enzymatic saccharification of cellulose contained in the biomass. At this stage, the components that suffer the most important modifications are hemicelluloses and lignin.

The alkaline pretreatment is widely used for lignocellulosic materials to facilitate the enzymatic attack of cellulose chains. This pretreatment is based on the chemical reactions between the alkali and material produces an effect of swelling and an increase of the internal surface of the cellulose as well as a decrease in the grade of polymerization and crystallinity, disintegrating the lignin-carbohydrate linkage. The lignin takes an untidy structure continued by the saponification of the intermolecular ester linkage which interlaces the hemicellulose and other components. This favors the increase in the porosity of the lignocellulosic biomass which allows its accessibility and enzymatic attack (Heinze and Koschella, 2005). When compared with other pretreatments, the alkaline pretreatment includes soft pressure and certain temperature conditions that cause less degradation of sugars and allow recovery of some of the used alkalis. The usual alkaline compounds are hydroxides of sodium, potassium, calcium, and

ammonium; and the recommended size of material is a particle size less than 10 mm (Elshafei et al., 1991).

In the present paper, we investigated the effects of the alkaline concentrations (NaOH and $Ca(OH)_2$), under different temperatures and pretreatment residence times, on the glucose yield from enzymatic hydrolysis. The underline aim is to identify an efficient method of alkaline pretreatment that allows the best production of bioethanol from waste materials of the three most abundant pine species in the northern state of Durango in Mexico.

MATERIALS AND METHODS

Biomass samples

The samples were collected from branches (diameter over 5 cm) and tree tops (diameter less than 16 cm) of pine trees. The samples of *Pinus durangensis* and *P. cooperi* were collected in the village of El Brillante, 23°46' 50"N, 105°20' 40" W, located on the Sierra Madre range mountain. Other samples of *P. arizonica* were collected in the village of Altares, 24°59'13"N, 105°54'46"W, and also on the slopes of the Sierra Madre. The samples were dried for 24 h at 100°C and ground until a particle size of about 0.590 mm using a mesh net type 30. The samples of each tree were prepared to evaluate their moisture (TAPPI Standard Method T 258 om-89), extractives (TAPPI Standard Method T 204 om-88), ashes (TAPPI Standard Method T 211 om-85), and lignin (TAPPI Standard Method T 222 om-88) contents.

Total carbohydrates

Extractive-free samples (0.3 g) were placed in a test tube and given a hydrolysis acid treatment using 72% sulphuric acid for 1 h. The samples were spilled in 250 ml flasks and 84 ml of deionized water was added to each flask. The hydrolysis was reinforced for 1 h at 15 psia. The mixture was then cooled and neutralized to a pH of 5.5 using hydroxide of barium (Coello, 2006). Filtered samples were analyzed by high performance liquid chromatography (HPLC) method. A standard calibration curve was used for xylose, glucose, mannose, fructose, sucrose, and maltose for total carbohydrate quantification.

The samples of the three pine species were mixed at an equal proportion. Three 5-g sub-samples were extracted and mixed with 50 ml of an alkaline solution (NaOH or Ca $(OH)_2$) to a concentration of 0.5, 1.0 or 1.5% w/v in a 250 ml flask. They were heated at 60, 90 or 120°C for 30, 60 or 90 min (López et al., 2008) and filtered using a nylon cloth. The solid residues were washed with 400 ml of deionized water to eliminate the excess alkalis and other by-products that could inhibit the enzymatic activity. The pretreated samples were dried at room temperature for 24 h and later at 100°C for 5 h. The samples were stored in plastic, hermetically-sealed bags for their conservation and enzymatic hydrolysis. The liquors obtained were centrifuged at 2,500 rpm for 5 min. Three 100 µL samples of the supernatants were taken to determine the content of RS (Miller, 1959) using Equation 1, considering that solely hemicellulose is hydrolyzed.

$$\text{Chemical saccharification } (\%) = \frac{\text{Amount of polysaccharides hidrolisates after chemical saccharification} \cdot 0.89 \cdot 100}{\text{Amount of polysaccharides in hemicellulose}} \quad (1)$$

Taking into account that every unit of mannan, xylan, and galactans produces a molecule of mannose, xylose, galactose, and arabinose, due to the addition of a water molecule, the average relation was 0.89. This number was used in the calculation of the yield of the conversion hydrolytic of the hemicellulose (Zhao et al., 2008).

Enzymatic saccharification

The enzymatic hydrolysis of the pretreated biomass was carried out in 30 ml plastic containers. 1 g of the pretreated sample was mixed with enzymatic concentrate to give 25 FPU (Celluclats 1.5 L of NOVOZYMES, Bagsvaerd, Denmark) per g of cellulose. During the hydrolysis, 2 ml of sodium azida and acetates buffer (0.1M and pH of 4.7) were added (0.1% w/v) to obtain a final reaction volume of 10 mL (López et al., 2008). The samples were incubated for 130 h with shaking at 80 rpm and 50°C and then filtered. The supernatant was then centrifuged at 2,500 r.p.m. for 5 min. 0.1 mL aliquot was taken to determine the content of RS (Miller, 1959). Considering that glucose was not degraded in chemical saccharification, the yield of the sugars in the stage of enzymatic pretreatment was estimated using Equation 2.

$$\text{Enzymaticsaccharification of glucan } (\%) = \frac{\text{Amount of glucose produced after enzymatic hydrolysis} \cdot 0.9 \cdot 100}{\text{Amount of glucan in the pretreated sawdust}} \quad (2)$$

Assuming that every unit of glucans produces a molecule of glucose, and due to the injection of a water molecule, the average relation was estimated to be 0.9. This constant was used in the calculation of the yield of the hydrolytic conversion of the hemicellulose (Zhao et al., 2008).

Statistical analysis

To investigate the effect of temperature, alkali concentration, residence time of the pretreatment, and their interaction on the yield of enzymatic hydrolysis, a 3^3 factorial experimental design with three replicates was employed. The null hypothesis was that there were no significant differences in the concentration of polysaccharides for the independent and combined action of each of the aforementioned variables.

The mean and standard deviation of the yield of enzymatic hydrolysis of each treatment was determined. The experiments were analyzed by one-way ANOVA with Fisher homogeneous groups (α=0.05). The Fisher's least significant difference (LSD) test was employed to compare treatment group means if the ANOVA F-test null hypothesis of equal means were rejected. The statistical tests were performed using the Statistical software (StatSoft, version 7.0, Tulsa, OK, United States of America).

Fermentation

After the optimum conditions of the hydrolysis process were established, the fermentation of sugars was recovered for the production of bioethanol using the fed-batch method of fermentation with the *Saccharomyces cerevisiae* strain ITD00185 (Paez et al., 2011). This strain was isolated and used to initiate the fermentation process. Prior to fermentation, the enzymatic hydrolyzate (EH) was sterilized in an autoclave at 15 psia for 15 min. The nitrogen content of the hydrolyzate was determined with the appropriate ratio of carbon / nitrogen (C/N = 80) according to Aerny (1996). The yeast was activated in YDP medium containing 2% casein peptone, 1% yeast extract, and 2% glucose at 28°C, 80 rpm and after 12 h. The

Table 1. Chemical analysis (Arithmetic average ± Standard Deviation) of wood forest samples of *P. arizonica, P. cooperi* and *P. durangensis* in Durango, Mexico.

Lignocellulosic biomass	Extractives (%)	Lignin K. (%)	Ashes (%)	Xylans (%)	Glucans (%)	Hemicellulose[a] (%)
P. arizonica	15.81 ± 0.63	32.81 ± 0.16	0.44 ± 0.08	21.05 ± 1.02	34.71 ± 0.50	16.19 ± 0.56
P. cooperi	6.25 ± 0.22	33.02 ± 0.19	0.35 ± 0.03	19.78 ± 1.47	32.97 ± 1.37	27.41 ± 1.37
P. durangensis	5.64 ± 0.40	33.64 ± 0.42	0.47 ± 0.03	20.54 ± 1.44	33.17 ± 0.36	27.08 ± 0.36
Average	9.23 ± 5.70	33.15 ± 0.43	0.42 ± 0.06	20.45 ± 0.63	33.62 ± 0.93	25.55 6.37

Hemicellulose[a] contents were determined as: Hemicellulose (%) = 100 - (extractives + lignin + ash + glucans).

fermentation was carried out in 15 mL glass tubes, 10 ml of EH was added and additional nutrients such as $(NH_4)_2SO_4$, yeast extract, and casein peptone were added as necessary. The raw juice was inoculated with an amount of 10^6 cells mL^{-1}. The reaction tubes were hermetically sealed and incubated at 28°C without stirring for 37 h. Samples were taken periodically to determine the content of glucose, ethanol, and microbial biomass. Ethanol production and substrate consumption were measured by HPLC. The HPLC equipment (Agilent Technologies 1260 infinity No. JP02679164, Palo Alto, CA, United States of America) includes a refractive index detector and a column NP Carbonix H 7.8 × 300 mm. The temperatures of the detector and column were set at 50 and 80°C, respectively. The deionized and filtered water with flow rate of 0.400 ml min^{-1} was used in the mobile phase.

The kinetics of microbial growth was determined by direct count using a Neubauer chamber. Calculation of theoretical yield of bioethanol was based on the relationship between yield product and substrate relative to the theoretical yield of 0.51% (w/w) (Mirahmadi et al., 2010).

RESULTS AND DISCUSSION

Chemical analysis of biomass

The chemical analysis of the samples included an estimation of the content of glucans, xylans, lignin, ashes, and extractives. The content of cellulose is represented by the glucans content of the biomass (Table 1). However, the content of galactose, and arabinose could not be determined given that we used a specific column in the HPLC. Sung and Chen (2002) and Söjström (1981) suggested a concentration range of 5 to 50, 25 to 35, and 25 to 35%, for cellulose, hemicellulose, and lignin in softwood, respectively. Other studies have also considered the content of carbohydrates present in *Pinus lodgepole, P. ponderosa, P. loblolly,* and *P. red,* reported a concentration range of 41 to 45 and 3 to 9%, for glucose and xylose respectively (Youngblood et al., 2009; Wang et al., 2009). For extractives, a concentration range of 2 and 10% for *Pinus* has been reported (Sjöström, 1981). Earlier reports had also shown variation in the concentration of various components in the tree trunk and branches. For example, in conifers, it is between 5 and 8% whilst in "*latifoliadas*" (broadleaf), it is between 2 and 4% (Otero, 1988).

Chemical and enzymatic saccharification

The understanding of the variables for the proper digestibility of the cellulose during the pretreatment stage is challenging in the development of economically viable technologies for the production of ethanol of second generation. The results of chemical and enzymatic saccharification under different pretreatment conditions and for the two types of alkalis are shown in Figure 1. A lower yield of chemical saccharification of 9.73% (treatment 1) was observed in samples pretreated with NaOH at 60 °C, a ratio of 0.5% w/v, and a residence time of 30 min. For the same reactant, a higher yield of 43.54% (treatment 21) was obtained from a pretreatment at 120°C, a ratio of 0.5% w/v, and a residence time of 90 min. As for the samples pretreated with $Ca(OH)_2$, a yield range of 0.38% (treatment 12) and 7.87% (treatment 27) was obtained for the material pretreated at 90°C and 120°C with 0.5 and 1,5% w/v and 90 minutes of pretreatment, respectively. For both pretreatments, it was observed that the chemical hydrolysis yields are higher when higher temperatures, alkaline concentration, and residence times were used.

For samples pretreated with NaOH, a yield of enzymatic saccharification in the range of 17.07% (treatment 20) and 30.68% (treatment 27) for samples subjected to 120°C for 60 and 90 min, with 0.5 and 1.5% w/v, respectively was obtained. For samples pretreated with $Ca(OH)_2$, a yield range of 6.58% (treatment 10) and 19.18% (treatment 5) for pretreatment at 90 and 60°C for 30 and 60 min with 0.5, 1.0% w/v, respectively was also obtained.

Pretreatment variables

A factorial analysis of variance was used to evaluate the effects of the interacting variables (temperature, alkaline concentration, and residence time in the pretreatment) on the yield of enzymatic hydrolysis using either NaOH or $Ca(OH)_2$(Table 2). According to the Fisher´s exact test for homogeneous groups, the treatments that have the most significant differences (P= 0.04) are those that were

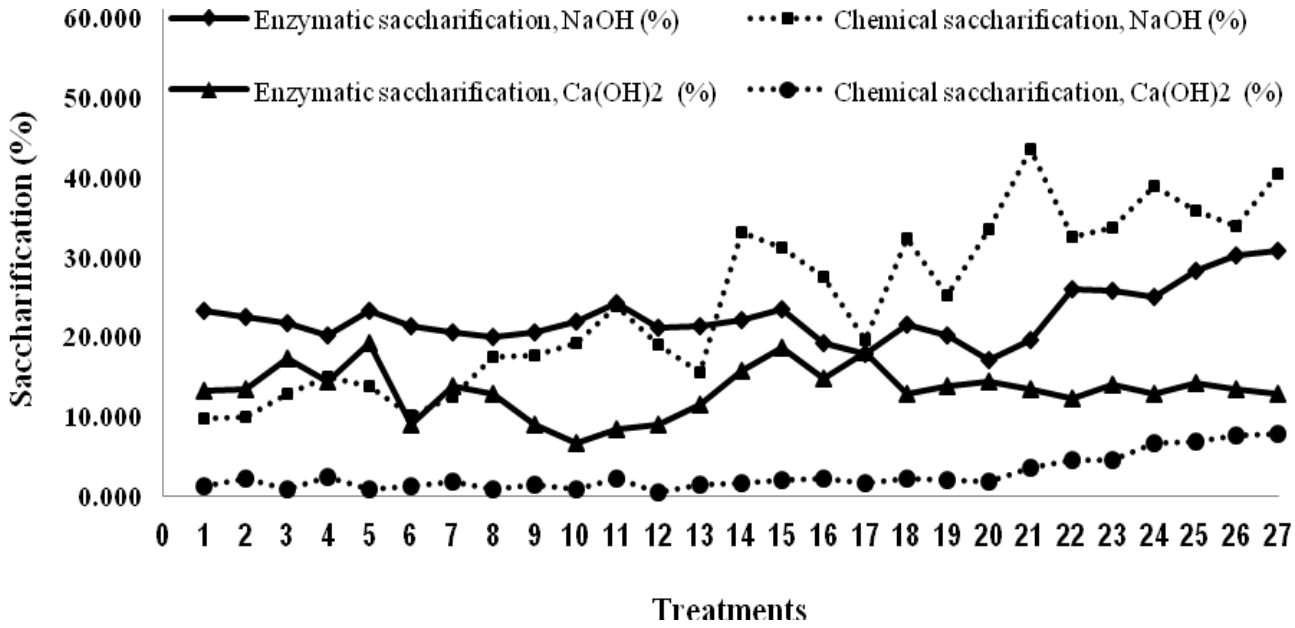

Figure 1. Chemical and enzymatic saccharification (%) obtained for different pretreatments (1 to 27) conditions when two alkalis are used in pine species mixture.

Table 2. Factorial analysis of variance of chemical and enzymatic saccharification on pine residues.

Variable	NaOH				$Ca(OH)_2$			
	CS		ES		CS		ES	
	F	p	F	p	F	P	F	p
Intercept	20604.98	0.00	16494.29	0.00	8733.83	0.00	17722.97	0.00
Temperature	1411.51	0.00	40.65	0.00	1672.01	0.00	4.96	0.01
Concentration	60.73	0.00	13.28	0.00	382.43	0.00	35.8	0.00
Time	100.51	0.00	0.67	0.52	13.08	0.00	30.67	0.00
Temperature*concentration	7.95	0.00	53.77	0.00	238.31	0.00	94.87	0.00
Temperature*time	24.1	0.00	0.96	0.43	49.70	0.00	17.95	0.00
Concentration*time	17.67	0.00	2.44	0.05	23.04	0.00	26.41	0.00
Temperature*concentration*time	39.7	0.00	2.79	0.01	16.36	0.00	26.34	0.00

CS, Chemical saccharification; ES, enzymatic saccharification.

subjected to 120°C using any of the two alkalis (treatments 22-27).

The temperature is associated with energy required to break the covalent and hydrogen bonds present in the binding of cell wall polymers. Watanabe (2003) pointed out that these links are of types (I)-benzyl ether and (II) glycosidic ether, which are between the p-hydroxyl group and the anomeric carbon aromatic (III) benzyl ester (IV) acetal groups found in lignin-carbohydrate complexes of softwood and hardwood; freeing during alkaline treatment and ferulic p-coumaric acids from carbohydrates remained as residual lignin fraction-bound carbohydrates. A high concentration of hydroxide ions facilitates the rapid dissolution of the cellulose. The concentration of sodium ions has an effect on the ionic strength of the cooking liquor given that the sodium ion concentration has been shown to significantly affect the delignification rate producing a decrease in the concentration of carbohydrates caused by loss of hemicellulose (Teder and Olm, 1981).

Kinetic performance of the enzymatic hydrolysis

We performed enzymatic hydrolysis kinetic for the pretreatment conditions that showed the highest

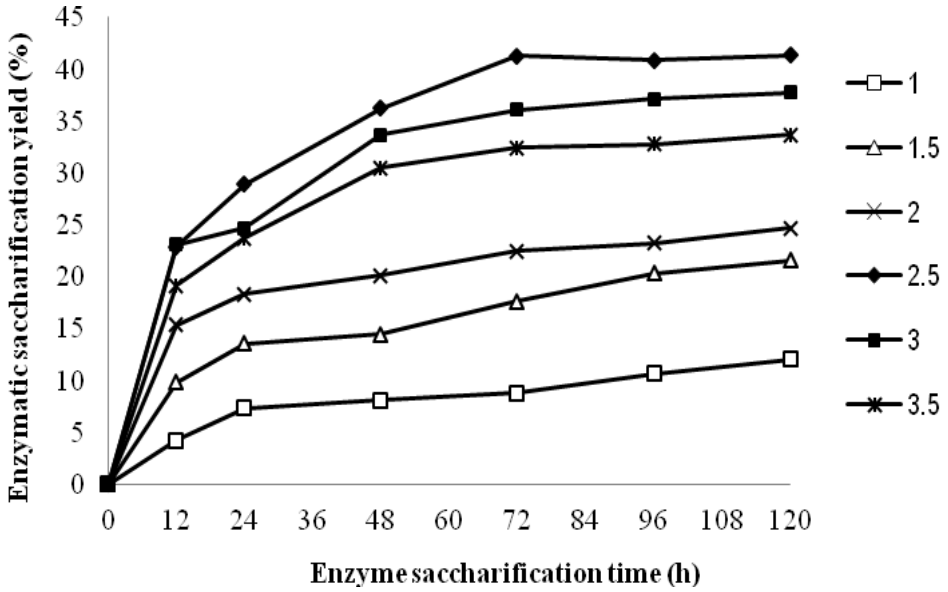

Figure 2. Kinetics of the enzymatic hydrolysis of the lignocellulosic material pretreated with different concentrations of NaOH.

significant difference (F = 408.08; P< 0.05). A maximum RS yield was obtained for the treatments 24 and 27 with NaOH subject at 120°C for 90 min. Thus, the concentration of NaOH was increased in order to determine the optimal enzymatic hydrolysis time required to obtain the highest yield of glucose produced by enzymatic hydrolysis (Figure 2).

Figure 2 also shows the gradual increase in the production of glucose from cellulose for all conditions tested in this study. At 120 h, all concentrations of NaOH produced the best significant enzymatic hydrolysis yields. The efficiency of enzymatic hydrolysis increased notably for NaOH concentrations from 2.5 to 3.5% in comparison with the concentrations from 0.5 to 2.0%. However, no improvement was noted when the concentration was increased from 3.0 to 3.5%. Enzymatic saccharification yields showed that a maximum percentage of 41.33% was obtained for materials pretreated with 2.5% w/v NaOH at 120°C for 90 min.

Compared with the initial biomass, namely biomass untreated, there was significant variation in the content of extractives (P< 0.05), lignin (P=0.002), and ash (P< 0.05) in the samples assayed (Table 3). A 14% reduction (P> 0.05) was observed in lignin content of the sample pretreated with 2.5% NaOH. Kim and Holtzapple (2006) indicated that an effective pretreatment removes the acetyl groups and reduce lignin content of more than 10% in comparison to the pre-treated biomass. However, contradictory results have been obtained with respect to the degree of delignification influence on enzymatic saccharification of cellulose. Ramos et al. (1992) and Schwald et al. (1989) reported that after the removal of hemicellulose and lignin in wood pretreated by steam, there was no significant increase in explosion and the rate of enzymatic hydrolysis was very low. The authors further posited that the short residence times and the use of sodium chlorite and potassium hydroxide are sufficient to remove most of the lignin and hemicelluloses, respectively, and to produce hydrolysis of cellulose. Experimentally, it has been shown that the unspecific adsorption of enzymes also depends on the chemical structure of lignin. Certain types of lignin have strong affinity for the enzymes. The findings of Pan et al. (2005) demonstrated that when the lignin in *Pseudotsuga* (Douglas-fir) was reduced from 43% to 36% by alkaline extraction, an enzymatic digestibility of 32% was obtained. These results showed that the alkaline pretreatment broke ester, ether, and hydrogen bonds which are more easily broken by an alkali rather than by an acid.

Results of the depolymerization of polysaccharides up to 11.10%, represented by the solubilization or degradation of the hemicellulose present in the black liquor, are also shown in Table 3. Himmel et al. (2007) reported that it is possible to obtain approximately 20 to 40% w / w of biomass due to its low degree of polymerization and its amorphous state. Sojstrom (1981) pointed out that the content of monosaccharides in the black liquor has a higher xylans and glucomannans concentration which are easily extracted by solvents such as NaOH neutral and it can be hydrolyzed or fermented. However, Himmel et al. (2007) noted that the detoxification of black liquor, required for its use, would represent additional costs to the process because their

Table 3. Chemical composition of the solids from pretreated and hydrolyzed biomass and black and white liquors obtained in the pretreatment during the enzymatic hydrolysis stages. Results are based on 100-g samples of untreated biomass.

NaOH	Alkaline pretreatment					Enzymatic hydrolysis	
	Black liquor	Pretreated biomass				White liquor	Hydrolyzed biomass
	Hemicellulose[a]	Holocelllulloce[b]	Lignin	Extractive	Ash	Glucose	Holocellulose[c]
0.5	10.29 ± 0.53	43.79 ± 0.53	29.07 ± 0.86	8.56 ± 0.16	0.38 ± 0.02	3.70 ± 0.22	40.08 ± 0.22
1	10.01 ± 0.21	44.06 ± 0.21	29.74 ± 0.23	5.91 ± 0.33	0.35 ± 0.38	4.50 ± 0.19	39.28 ± 0.19
1.5	10.08 ± 0.22	44.00 ± 0.22	29.84 ± 1.08	7.95 ± 0.54	0.34 ± 0.00	8.03 ± 0.49	35.72 ± 0.49
2	10.37 ± 0.20	43.70 ± 0.20	31.16 ± 0.40	9.25 ± .53	0.32 ± 0.01	9.23 ± 0.13	34.55 ± 0.13
2.5	10.62 ±0.19	43.45 ± 0.19	30.97 ± 2.03	4.55 ± 0.62	0.36 ± 0.03	15.43 ± 0.22	28.35 ± 0.22
3	10.86 ± 0.19	43.21 ± 0.19	28.24 ± 1.65	8.47 ± 0.72	0.33 ± 0.00	14.10 ± 0.52	29.68 ± 0.52
3.5	11.10 ± 0.20	42.97 ± 0.20	29.84 ± 0.81	5.35 ± 0.50	0.34 ± 0.00	12.58 ± 0.63	31.19 ± 0.63

Values are arithmetic average ± Standard Deviation. The sum of the average of glucans and xylans was considered as the total carbohydrates in biomass (54.07%); a. The total monosaccharides present in the black liquor are considered to come from the decomposition of hemicelluloses; b. Holocellulose (hemicellulose and cellulose) content in the pretreated biomass was calculated as the difference between the total monosaccharides and monosaccharides initiates sample present in the black liquor; c. Holocellulose content in the hydrolyzed biomass was calculated as the difference between the total monosaccharides and monosaccharides pretreated biomass contained in the hydrolyzate (white liquor).

residual content of lignin, extractives, and some carboxylic acids which are toxic to the cellulase enzymes and yeasts.

These results indicate that increasing the concentration of NaOH during the hydrolysis, it can produce higher yields. However, in various studies using softwoods, no significant improvements were observed (18%) when the dose of sodium hydroxide was increased beyond 7% for 2 h at 60°C.The combination of urea with sodium hydroxide would further enhance the enzymatic hydrolysis of softwood biomass (Zhao et al., 2008). However, Lopez et al. (2008) demonstrated that the pine sawdust subjected to various pretreatments (alkaline, acid, and steam explosion) produced higher yields of RS during enzymatic hydrolysis than alkaline pretreatment of 33.26 % (1.30 h at 121°C; NaOH 3%).

Mirahmadi et al. (2010) used alkaline pretreatment of lignocellulosic biomass under atmospheric pressure and moderate temperature (2.0 h at 100°C; NaOH al 7 %) and obtained enzyme yields ranging from 16.6 to 24.0%. These authors concluded that alkaline pretreatment is a viable alternative for softwoods and hardwoods, which both are reliable sources of raw material for bioethanol and biogas conversion. They also noted that by using such processes, there was no destruction of lignin and that the alkaline solution can be reused. Park et al. (2010) obtained a high enzymatic yield (93%) by subjecting the lignocellulosic biomass in organo-solvent pretreatment using various catalysts (0 min at 180°C and a mixture of 50% ethanol and 1% sulfuric acid). However, they highlighted that high energy consumption are required to achieve these yields (Table 4).

Fermentation

The problem of bioethanol production is to obtain not only the higher sugar extraction, but to achieve the highest fermentability of the liquor produced during the hydrolysis. In the fermentation step, it is necessary that the pretreated biomass is adequately flushed to prevent the presence of toxic agents that can inhibit enzymatic hydrolysis of the cellulose and thus fermentation yields.

Figure 3 shows the kinetics for the production of bioethanol by fermentation from pinewood pretreated using optimum conditions. As noted,

Table 4. Production of bioethanol under different pretreatment conditions as reported in the literature.

Substrate and medium	Sugar concentration (g L^{-1})	Dilution Rate (h^{-1})	Ethanol (g L^{-1})	Ethanol yield (g g^{-1})	Theoretical yield (%)	Ethanol productivity (g L^{-1} h^{-1})	Reference
Alkaline							
Spruce	N.D.	N.D	N.D	N.D.	26.05	N.D.	Mirahmadi et al. (2010)
Birch	N.D.	N.D.	N.D.	N.D.	54.76	N.D.	
Dilute acid	Total sugars: 42.3	N.D	20	0.50	92.71	N.D	Sreenath and Jeffries (2000)
Steam exploted oak chips	Glucose:180	0.22	77	0.43	83.87	16.9	Lee et al. (2000)
Sugar cane bagasse pretreated with NaOH	Glucose:90	0.13	31	0.19	67.00	4.1	Ghose and Tyagi (1979)
Alkaline: Pine residues	Glucose: 16	N.D.	7.18	0.41	90.19	N.D.	The present study

N.D. No data.

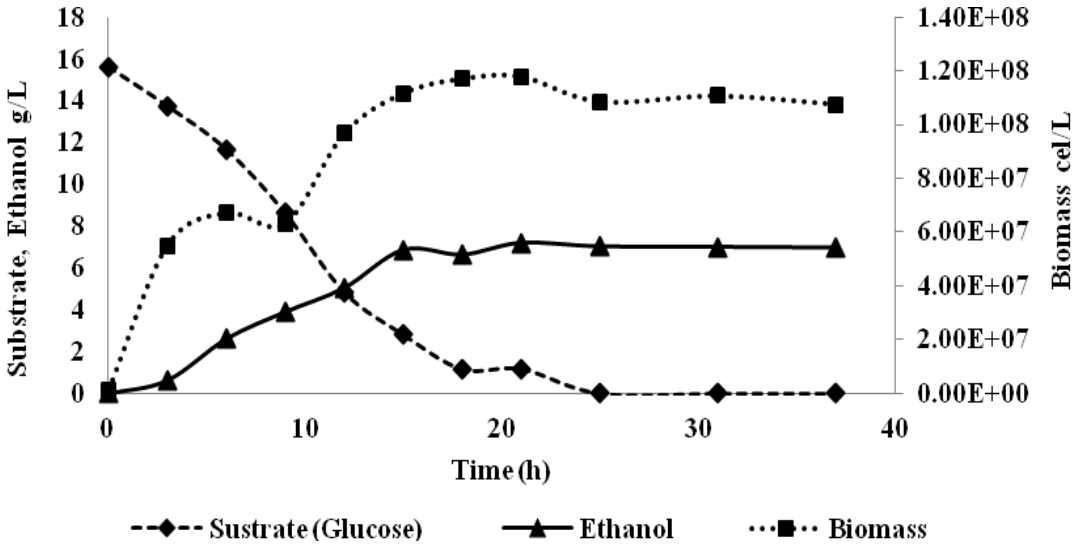

Figure 3. Kinetic comportment of the sugar fermentation during the bioethanol production process using the enzymatic hydrolyzate of a pretreated pine mixture.

after 21 hours of fermentation, glucose was totally consumed by obtaining a maximum concentration of bioethanol of 0.46 ± 0.03 g g^{-1} substrate corresponding to a maximum yield of 90.38% ± 7.34 relative to the theoretical yield. This represents the optimal amounts of nutrients used for fermentation in a medium with a pH of 5.0 and with absence of inhibitors.

However, these yields are within the range set for lignocellulosic biomass. According to Vázquez and Dacosta (2007), under experimental performance, it varies between 90% and 95% from theoretical values, that is, between 0.469 and 0.485 g g^{-1}, respectively. Thus, the returns in the industry vary between 87 and 93% from theoretical metrics. This performance is high when compared with the results presented in Table 4. Mirahmadi et al. (2010) used the *Saccharomyces cerevisiae* strain CCUG 53310 (culture collection of the University of Gothenburg, Sweden) at 30°C and 24 h of fermentation from glucose using as substrate Spruce and Birch. Similarly, Sreenath and Jeffries (2000) worked with a mixture of branches from softwood and hardwood subjected to diluted acid pretreatment. The hydrolysates were fermented with the strain *P. stipitis* FPL-Y-606 or *C. shehatae* FPL-Y-049 at 100 rpm and a temperature range of 25 to 27°C for 3 to 5 days using as a source of substrate for both yeasts: xylose, glucose, mannose, and galactose. Lee et al. (2000) employed Oak wood lignocellulosic biomass which was subjected to a steam explosion and enzymatic hydrolysis and fermented by the *Saccharomyces cerevisiae* strain provided by the Seoyoung Ethanol Industry of Korea. The authors used a continuous culture process of fermentation, where the inhibitor concentration formed in the sterilization stage affected the theoretical yield of ethanol production. Ghose and Tyagi (1979) used sugar cane bagasse as lignocellulosic biomass under

alkaline pretreatment and enzymatic hydrolysis with *T. reesei* (cellulases). These researchers applied a continuous fermentation process and used, under anaerobic conditions, the yeast *Saccharomyces cerevisiae* at 30°C with recirculation of the yeast.

Conclusions

The results of this study have demonstrated that the enzymatic digestibility of the pretreated cellulose depends upon the removal of lignin and hemicelluloses, and the acetylation of hemicellulose. However, it is necessary to use optimal temperatures and high concentrations of alkali to reduce the content of hemicellulose and lignin, because they significantly affect hydrolysis yields. Based on the glucose content obtained using the maximum yield for enzymatic saccharification through this biotechnological process, the percentage of bioethanol obtained as 90.19% (converted to theoretical values) indicates possibility of producing 80 L of bioethanol per dry ton of woody biomass. This result lies within the range obtained for wood (28 to 120 L ethanol/ton woody biomass) as reported by various authors using similar pretreatment technologies: dilute acid, alkali, and steam explosion (Mirahmadi et al., 2010; Mohsenzadeh et al., 2012). The pretreatment of lignocellulosic biomass is a determinant stage in the synthesis of biofuels, thus further studies should explore the various pretreatment processes in order to choose the one that best fit the structure of the substrate.

ACKNOWLEDGEMENTS

This work was performed in the Industrial Biotechnology Laboratory of the Instituto Tecnológico de Durango under the supervision of Dr. Javier López Miranda. We are indebted to Dr. J. Honorato Amador Salazar from the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) in San Martinito Puebla for his participation in the development of the chemical analysis of the *Pinus* samples. This research was funded by the Environment Network (REMA) and SIP project 20100317 of the Instituto Politécnico Nacional. Mario A. Rodríguez-Pérez holds a scholarship from Comisión de Operación y Fomento de Actividades Académicas / Instituto Politécnico Nacional.

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