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Pretratamiento soda-etanol de pino y su influencia en la hidrólisis enzimática

Pretreatment soda-ethanol of pine and its influence on enzymatic hydrolysis

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Resumen

La obtención de bioetanol de segunda generación a partir de aserrín de pino incluye: pretratamiento, hidrólisis enzimática (HE) y fermentación. Uno de los factores más influyentes en la hidrólisis es la composición química del sustrato, directamente relacionada con las condiciones y tipo de pretratamiento al cual es sometido el material. El objetivo fue estudiar la digestibilidad enzimática del aserrín de pino sometido a un pretratamiento etanol-soda. En el experimento se varió la carga alcalina y el tiempo a temperatura máxima. Para la caracterización del material, la actividad enzimática y la hidrólisis se usaron los Procedimientos Analíticos de Laboratorio (LAPs, NREL). Los materiales pretratados presentaron diferente composición química. Solo el efecto de la carga alcalina sobre el rendimiento de la HE fue significativo. Etanol y soda exhibieron una sinergia positiva con respecto al rendimiento. Los resultados sugieren una correlación negativa entre el rendimiento de la HE y el contenido de lignina.

Palabras clave: Deslignificación; Hidrólisis Enzimática; Aserrín de pino; Pretratamiento organosolv; Bioetanol.

Abstract

The production of second-generation bioethanol using pine sawdust involves pretreatment, enzymatic hydrolysis (EH) and fermentation. One of the most influencing factor in the cellulose hydrolysis is the chemical composition of the substrate, which is directly linked with the kind and conditions of the applied pretreatment to biomass. The aim of this work was to study the enzymatic digestibility of pine sawdust submitted to alkaline - ethanol pretreatments. In the experiments, we varied alkaline charges and time at maximum temperature. Laboratory Analytical Procedures (LAPs,NREL) were used for the chemical characterization of the material, the enzymatic activity and the hydrolysis. The pretreated materials presented different chemical compositions. Only the effect of alkaline charge on yield was significant. Ethanol and soda exhibited a positive synergy effect on yield. Results suggest the existence of a negative correlation between EH yield and lignin content.

Keywords: Delignification; Enzymatic Hydrolysis; Pine Sawdust; Organosolv Pretreatment; Bioethanol.

Introduction

Pine *(elliottii and taeda)* sawdust is one of the main residues of the primary industrialization of wood in the Northeast of Argentine (NEA Region). About 50% of industrially processed wood is become in waste, generating 1.5 million of dry ton wood wastes per year, which are not properly availed. A typical composition of *Pinus elliottii* from Misiones, Argentina is about 41–44% of cellulose, 28–31% of lignin, 27–33% of hemicelluloses, and 2–4% of extractives. Wastes from conifers sawmills constitute an attractive biomass for the production of bioethanol due to their high hexoses content and broad availability. Second generation bioethanol obtained from cellulose involves three stages: pretreatment/fractionation, hydrolysis and fermentation. Nevertheless, resinous pines have not been exhaustive studied, mainly because their high cellulose crystallinity, high lignin content, and specially, their high extractives content [1]. For this reason, a pretreatment step is necessary to enhance cellulose digestibility.

In lignocellulose, cell wall polysaccharides are embedded in a complex lignin matrix that hinders the enzymes from accessing polysaccharides that can be converted to fermentable sugars [2]. Pretreatment choice depends on the raw material and has a significant influence in the following stages to bioethanol conversion [3, 4]. Pretreatments aim to extract hemicelluloses and lignin, and to increase the accessibility of the material to enzymatic hydrolysis, avoiding the formation of hydrolysis and/or fermentation inhibitors [5].

Most promising method to produce bioethanol from lignocellulosic materials is based on enzymatic hydrolysis and fermentation [6]. Enzymatic hydrolysis (EH) is friendlier with the environment than chemical hydrolysis.

Endoglucanases, exoglucanases and β-glucosidases or cellobiases form an enzymatic complex that acts synergically to degrade cellulose to glucose [6]. Enzymes and substrate are the main factors influencing the enzymatic hydrolysis; the last one is directly related with the pretreatment to which the material has been subjected [6].

The organosolv process was developed initially for pulp production from woody biomass and has demonstrated to by an effective pretreatment method for high-lignin lignocellulosic biomass because it. This treatment breaks down internal lignin and hemicelluloses bonds and thus, removes the portion of lignin from biomass [7]. The most common relation ethanol/water applied is 50/50, but there are works with 65/35 or 75/25. Most common catalysis uses sulfuric acid, but it has its disadvantages, it is toxic, corrosive, hazardous, and inhibitory characteristics [8]. Time at maximum temperature goes from 10 to 60 minutes generally. Temperatures used for loblolly pine and similar feed stocks go from 170º C to 190º C [8].

Alkali such as sodium hydroxide has been used for lignocellulosic biomass pretreatment since it is effective for lignin removal. Delignification improves the accessibility of the material and avoids lignin combination with enzymes. These effects also depend on the enzymatic charge [3]. The application of alkaline catalyst also increases surface area through alkaline swelling and causes defiberization, which can improve enzyme accessibility.

With the hypothesis that resultant chemical composition of the material is an important factor on enzymatic saccharification, the aim of this work was to evaluate the effect of the chemical characteristics of pine sawdust (a mixture of *Pinus elliottii* and *Pinus taeda)* subjected to an ethanol and sodium hydroxide pretreatment on its enzymatic digestibility. Therefore, different conditions of alkaline charge and time were applied to evaluate the influence of the resultant chemical composition on saccharification.

Materials and methods

Raw material

Pine sawdust (mixture of *Pinus elliottii* and *Pinus taeda*) was provided by a local sawmill (Forestal AM, Misiones). The sawdust was air-dried, screened, and maintained in closed plastic bags. The fraction passing 3 mm^2 screen was used.

Organosolv pretreatment

Organosolv pretreatment was carried out in a 200 mL stainless steel reactor (into a glycerin bath) loaded with 20 g of wood sawdust (dry weight base) and 100 mL of a 35:65% (v/v) ethanol: water mixture containing sodium hydroxide. Liquor to wood ratio inside the reactor was 5:1 (w/w). The effect of alkaline charge (15 and 25 % w/w) and time (60 and 90 min) were evaluated. These experiences were performed by duplicate. Three additional experiences were performed to evaluated effect of reagents separately; two without ethanol (experiences 5 and 6), and one without NaOH (experience 7). Table 1 summarizes conditions of each experience. The temperature was maintained at 170º C.

After the process ended, the reactor was cooled in a water–ice bath. The liquor and solid were separated by filtration. The solid was washed with water and with a mixture of water and ethanol, filtered through paper filter, and then stored in plastic bags at 4º C. The solid obtained in this process was named as "pretreated material".

Table 1: Pretreatments conditions applied to pine sawdust.

Experience Nº	Time at Tmax[(a)] (min)	NaOH (%w/W)	Ethanol/water (v/v)
1	60	15	35/65
2	60	25	35/65
3	90	15	35/65
4	90	25	35/65
5	90	15	0/100
6	90	25	0/100
7	90	0	35/65

[(a)]Heating time was 20 minutes.

Substrates characterization

Raw material was characterized according to NREL-LAP standards, including: total solid and moisture (NREL/TP-510-42621), extractives in water and in ethyl alcohol (NREL/TP-510-42619), and structural carbohydrates, glucans, xylans and arabinans, acetyl groups, lignin soluble and insoluble in acid (NREL/TP-510-42618).

The pretreated solids were characterized in the same way, excluding the determination of soluble substances in water and ethanol. The quantification of sugars, organic acids and degradation products was carried out by liquid chromatography HPLC (Waters Corp. Massachusetts, USA), using a column AMINEX-HPX97H (BIO-RAD) with the following chromatographic conditions: eluent: H_2SO_4 4mM, flow: 0.6 mL/min, temperature: 35º C, detector: refraction index and diode array.

The quantification of homopolymers (glucans, xylans, arabinans) in the solid was carried out multiplying sugars by the hydrolysis stoichiometric factors of 0.88 (or 132/150) for sugars with five carbons (xylose and arabinose) and 0.90 (162/180) for sugars with six carbons (glucose).

Enzymatic hydrolysis (EH)

Commercial Enzymes: cellulases from *Trichoderma Reesei* and celobiases from *Aspergillus niger* (commercial enzymes provided by Sigma-Aldrich).

Enzyme activity assays: cellulose activity was done in

terms of "filter paper units" (FPU) according to NREL/TP-510-42628 standard. Activity of β-glucosidase was determined by its capacity to hydrolyze 4-nitrophenol β-D-glucopyranoside (p-NPG) to 4-nitrophenol (p-NP). This method consists in adding 0.5mL of different enzyme dilutions to 2 mL of 1mmol/L of p-NPG solution, incubating 30min at 50º C, and then stopping the reaction with 2.5 mL Na_2CO_3. Finally, the measure of absorbance is made at 400 nm and expressed in IU [9].

Enzymatic treatment: The never-dried solid material from the pretreatment stage was submitted to saccharification with cellulases and celobiases according to NREL-LAP standards (NREL/TP- 510-42629) [10], with a few modifications. Enzymatic hydrolysis was performed in 50mL Erlenmeyer flask using 1 g dry weight of pretreated material suspended in 50mL 0.05M sodium citrate (pH 4.8), at 130 rpm and 50º C for 72 h. The enzyme dose was 20 FPU per gram of glucans (cellulases) [11] and 40 IU per grams glucans (cellobiose).

Glucose content in the resulting hydrolysates was determined by HPLC (Waters Corp. Massachusetts, USA), with an AMINEX-HPX87H column (conditions: 4mM of H_2SO_4 as eluent, 0.6 ml/min, 35º C), and refractive index detector.

Hydrolysis yield (digestibility) was calculated according to equation 1.

$$EH\ yield\ (\%) = \frac{\text{glucose x } 0{,}9}{glucans\ in\ the\ material} x100 \qquad (1)$$

EH yield (%): digestibility
Glucose: grams
Glucans in the material: grams
0.9: stoichiometric factor

The Statgraphics software was used to accomplish the statistical analysis.

Results and discussion

The raw material was composed (%od) of 39.85 % glucans (almost cellulose), 24.89% other sugar polymers (hemicelluloses), 31.41% lignin and 3.52% extractives. The digestibility of the raw material was very low (5.4% at 72h).

Table 2 shown chemical composition and yield of the solid materials after the application of the different pretreatments, as well as, the yields obtained after the EH of pretreated solid materials.

Table 2: Chemical composition and yields after different treatments

Experience number	Yield of pretreated material (%)[a]	Chemical composition (% odm)			EH yield (%)
		Glucans	Xilans-galactans-manans-arabinans	Lignin	72 h
1	59.18 ± 1.22	57.91	14.04	19.49	27.41 ± 1.03
2	47.31 ± 1.20	68.45	15.10	6.41	91.08 ± 3.18
3	57.35 ± 0.76	59.99	13.62	17.90	29.47 ± 0.82
4	46.15 ± 1.49	71.83	15.54	5.28	89.22 ± 2.64
5	66.97	52.03	11.08	27.64	14.18 ± 1.15
6	49.94	64.49	12.18	16.05	40.40 ± 1.42
7	72.92	50.64	11.41	30.35	8.70 ± 2.92

a: Mass (g) of pretreated material obtained from 100 g of dry sawdust.

The yields of pretreated materials with 25% NaOH (experiences 2, 4 and 6) were lower than 50%, mainly because their high delignification (Table 2). Nevertheless, the degradation of glucans did not exhibit significant change (3- 8%).

The Analysis of Variance determined that the effect of time was not significant (p-value<0.05), so 60 minutes of treatment are sufficient to reach the desired results. On the other hand, the alkaline charge affected the EH in a significant way (p-value<<0.05).

The enzymatic hydrolysis yields at 72 h and lignin contents for different pretreatment conditions (all at 90 minutes at maximum temperature) are shown in Figure 1. It is clear that the applied chemicals present a synergistic effect since the hydrolysis yields of the materials submitted to only one reagent (ethanol or sodium hydroxide) are much lower than those of the materials submitted simultaneously to both reagents.

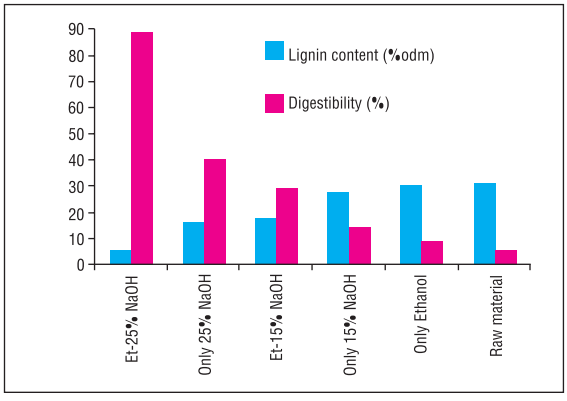

Figure 1: Enzymatic hydrolysis yields at 72 hours and lignin content for different pretreatment conditions (all at 90 minutes at maximum temperature).

The increased digestibility after delignification has also been confirmed by this study. Furthermore, there is a strong negative correlation between lignin content and hydrolysis yield (r= -0.96; p-value << 0.05). The explanation could be that lignin tends to irreversibly bind the enzymes through

hydrophobic interactions causing a loss in cellulases activity [12].

The comparison with results obtained by other authors treating similar raw materials (pine) is shown in Table 3. Results can vary appreciably if a different raw material is used because of differences in reactivity, structure, and distribution of lignin.

Table 3: Reported enzymatic saccharification of pines submitted to similar pretreatments.

Raw material	Pretreatment and EH conditions	Maximum enzymatic digestibility	Reference
Loblolly pine (*Pinus taeda*)	170º C, ethanol/water: 65/35, 1.1% (odm) sulfuric acid, 60 min. EH: 50º C, 8 FPU/g cellulose (Celluclast) and 16 IU/g cellulose (Novozym 188), 80h	≈70%	Sannigrahi *et al* 2010 [13]
Pitch pine (*Pinus rigida*)	a. 190º C, ethanol/water: 50/50, 20 min, 2% (w/v) NaOH. b. 210º C, ethanol/water: 50/50, 2% (w/v) NaOH, 20min. EH: 50º C, 60 FPU/g cellulose (Celluclast) and 64pNPGU/g cellulose (NS-50010, Novozym), 72h	a.58% and b.85.4%	Park *et al* 2010 [8]
Loblolly pine (*Pinus taeda*)	170º C, 75% ethanol, 1% (w/w) sulfuric acid overnight, 60min, SLR: 7:1. EH: 50º C, 10 FPU/ g glucan (Novozym 22C), 72 h	41.2%	Lai *et al* 2014 [14]
Pine	1. Autohydrolysis. Solid/liquid ratio 1:10; 180 °C; 60 min. 2. Ethanol-water delignification in two stages. a. First stage: 75% (v/v) aqueous ethanol solution + 1% w/w sulfuric acid; 10–15 min; b. Second stage: 180 ± 1 °C; 60 min. EH: 45º C, 25 FPU per g dry weight of pretreated biomass, 72 h	1. + 2. a. <10% b. 23%	Amiri *et al* 2016 [15]
Pinus taeda L.	Ethanol-water delignification: 170°C; 60 min; 1% dilute sulfuric acid; ethanol concentration 55%. EH: 55º C, 60 FPU/ g glucan, 48h	75% yield	Heringer *et al* 2016 [16]
Pinus radiata	189°C, ethanol/ water (v/v): 50:50; liquid/solid ratio (LSR): 6:1; 8 min and 1.1% w/w H_2SO_4. EH: 50º C, 0.044 g of enzyme per g of dry pretreated material, 96 h	94% (72h)	Valenzuela *et al* 2016 [17]
Mixture of *Pinus elliottii* and *Pinus taeda*	170º C, ethanol/water: 35/65, 25% (odm) NaOH, 60 min. EH: 50º C, 20 FPU/g glucans and 40 IU/g glucans, 72h	91%	this work

As shown in Table 3, the most frequent ethanol/water ratio used was 50/50, although it was reported that the use of low ethanol concentrations (about 30% v/v) is favorable for Organosolv processes [18].

Compared to Park et al [8], who worked with softwood and organosolv – NaOH process, higher EH yields were obtained in this work at lower temperatures and lower enzyme load, presumably due to differences in the ethanol-soda ratio or/and to the time at maximum temperature. The same conclusions can be reached when comparing with most of the acid organosolv treatments.

Ethanol-soda pretreatment has demonstrated to provide high EH yield at moderate temperature. Ethanol catalyzed with sulfuric acid has been the most applied organosolv process despite the fact that alkali has been recognized as one of the most effective agents for swelling the biomass and the degree of swelling is an important property that affects enzymatic hydrolysis [7].

Conclusions

The soda-ethanol pretreatment and its conditions affect enzymatic hydrolysis.

Chemical composition, specifically lignin content is crucial on enzymatic hydrolysis yield.

Time effect resulted not significant between 60 and 90 minutes whereas alkaline charge affected delignification and EH yield.

Ethanol-soda pretreatment has demonstrated to provide high enzymatic hydrolysis yield at moderate temperature.

Abbreviations

NREL: National Renewable Energy Laboratory
LAP: Laboratory Analytical Procedure
EH: Enzymatic hydrolysis
HPLC: high performance liquid chromatography
odm: on oven dry material
v/v: Volume / Volume
w/v: Weight / Volume
w/w: Weight / Weight

References

1. **Stoffel, R.B.; Felissia, F.E.; Silva Curvelo, A.A.; Gassa, L.M. and Area, M.C.** *Optimization of sequential alkaline-acid fractionation of pine sawdust for a biorefinery, Industrial Crops and Products*, 61: p. 160-168. 2014.
2. **Kafle, K.; Lee, C.M.; Shin, H.; Zoppe, J.; Johnson, D.K.; Kim S. H. and Park, S.** *Effects of delignification on crystalline cellulose in lignocellulose biomass characterized by vibrational sum frequency generation spectroscopy and X-ray diffraction*, Bio Energy Research, 8 (4): p. 1750-1758. 2015.
3. **Yu, Z.; Jamee, lH.; Chang, H. and Park, S.** *The effect of delignification of forest biomass on enzymatic hydrolysis,* Bioresource Technology, 102: 9083-9089. 2011.
4. **Araque, E.; Parra, C.; Freer, J.; Contreras, D.; Rodríguez, J.; Mendonc, R. and Baeza, J.** *Evaluation of organosolv pretreatment for the conversion of Pinus radiata D. Don to ethanol*., Enzyme and Microbial Technology, 43: p. 214-219. 2008.
5. **Luo, L.; Voet, E. V. D. and Huppes, G.** *Biorefining of lignocellulosic feedstock – technical, economic and environmental considerations, Bioresource Technology*, 101: p. 5023-5032. 2011.
6. **Balat, M.** *Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review*, Energy Conversion and Management, 52: p. 858-875. 2011.
7. **Koo, B.; Kim, H.; Park, N.; Lee, S.; Yeo, H. and Choi, I.** *Organosolv*

pretreatment of Liriodendron tulipifera and simultaneous saccharification and fermentation for bioethanol production, Biomass and bioenergy, 35: p. 1833-1840. 2011.

8. **Park, N.; Kim, H.; Koo, B.; Yeo, H. and Choi, I.** *Organosolv pretreatment with various catalysts for enhancing enzymatic hydrolysis of pitch pine (Pinus rigida)*, Bioresource Technology, 101: p. 7046-7053. 2010.
9. **Matsuura, M.; Sasaki, J. and Murao, S.** *Studies on β-glucosidases from soybeans that hydrolyzed aidzyn and genistin: isolation and characterization of an isozyme*, Bioscience, Biotechnology and Biochemistry, 59: p. 1623-1627. 1995.
10. **Selig, M.; Weiss, N.; Ji, Y.** *Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure, National Renewable Energy Laboratory*, NREL/TP-510- 42629. 2008.
11. **Stephen, J. D.; Mabee, W. E. and Saddler, J. N.** *Will second-generation ethanol be able to compete with first-generation ethanol? Opportunities for cost reduction*, Biofuels, Bioproducts and Biorefining, 6: p. 59-176. 2012.
12. **Araya, F.; Troncoso, E.; Mendonça, R. T.; Freer, J.; Rencoret, J. and Del Río, J. C.** *Structural characteristics and distribution of lignin in eucalyptus globulus pulps obtained by a combined autohydrolsis/alkaline extraction process for enzymatic saccharification of cellulose*, J. Chil. Chem. Soc., 60 (Nº 2): p. 2954-2960. 2015.
13. **Sannigrahi, P.; Miller, S. J. and Ragauskas, A. J.** *Effects of organosolv pretreatment and enzymatic hydrolysis on cellulose structure and crystallinity in Loblolly pine,* Carbohydrate Research, 345: p. 950-970. 2010.
14. **Lai, C.; Tu, M.; Shi, Z.; Zheng, K.; Olmos, L. G. and Yu, S.** *Contrasting effects of hardwood and softwood organosolv lignins on enzymatic hydrolysis of lignocelluloses Bioresource Technology*, 163: p. 320–327. 2014.
15. **Amiri, H. and Karimi, K.** *Integration of autohydrolysis and organosolv delignification for efficient acetone, butanol, and ethanol production and lignin recovery*, Ind. Eng. Chem. Res., 55 (17): p. 4836–4845. 2016.
16. **Heringer, N. B.** *Minimizing ethanol concentration in organosolv pretreatment for the saccharification of loblolly pine*, Biological and Agricultural Engineering Undergraduate Honors Theses. Paper 41. 2016.
17. **Valenzuela, R.; Priebe, X.; Troncoso, E., Ortega, I.; Parra, C. and Freer and J.** *Fiber modifications by organosolv catalyzed with H2SO4 improves the SSF of Pinus radiate*, Industrial Crops and Products, 86: p. 79–86. 2016.
18. **Mesa, L.; González, E.; Cara, C.; González, M.; Castro, E. and Mussatto, S.I.** *The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse,* Chemical Engineering Journal, 168: p. 1157-1162. 2011.

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