

# INFLUENCE OF PRETREATMENTS ON CRYSTALLINITY AND ENZYMATIC HYDROLYSIS IN SUGAR CANE RESIDUES

Luz M. Flórez Pardo<sup>1</sup>, Jairo G. Salcedo Mendoza<sup>2\*</sup> and Jorge E. López Galán<sup>3</sup>

<sup>1</sup> Universidad Autónoma de Occidente, Departamento de Energética y Mecánica, Cali, Colombia. E-mail: lmflorez@uao.edu.co, ORCID: 0000-0001-8779-8120

<sup>2</sup> Universidad del Valle, Escuela de Ingeniería Química, Cali, Colombia. E-mail: jorge.lopez@correounivalle.edu.co, ORCID: 0000-0002-5829-1360

<sup>3</sup> Universidad de Sucre, Sincelejo, Colombia. E-mail: jairo.salcedo@unisucra.edu.co, ORCID: 0000-0002-9901-9793

(Submitted: March 4, 2018 ; Revised: June 12, 2018 ; Accepted: August 14, 2018)

**Abstract** - This research evaluated the effect of different delignification pretreatments (enzymatic and organosolv), on the crystallinity and enzymatic hydrolysis of harvested sugar cane residues. The Crystallinity Index (CrI), the Relative Number of Intensity (Ir), the degree of cellulose mercerization (IIC-%), and the Global Index of Saccharification (GIS) were used as measurement parameters for six different substrates obtained from sugar cane residues (tops and leaves) by different processes. In this characterization, the spectroscopic techniques of Fourier Transform infrared spectroscopy (FTIR), X-ray diffraction and scanning electron microscopy (SEM) were used. Substrates to which only organosolv pretreatment was applied, without any further treatment, presented good behavior for the enzymatic hydrolysis and a high CrI, possibly due to the increase of the crystallinity by elimination of amorphous material.

**Keywords:** Crystallinity index; Sugar cane; Organosolv pretreatment; Enzymatic hydrolysis.

## INTRODUCTION

Lignocellulose biomass is available everywhere in the world, and because of the carbon content in its structure, it is a material that could contribute to energy security in nations, especially the ones that are dependent on oil. It is estimated that the annual production in the world of this biomass is around 10-50 billion tons (Claassen et al., 1999) and biomass energy accounts for about 14% of the world's total primary energy supply (International Energy Agency, 2015), most of it considered waste, but that might be a potential raw material for bioethanol production (Sánchez et al., 2010), pharmaceuticals, chemicals, cosmetics and food, among others, under the concept of biorefinery. Among these products, bioethanol is increasingly more important due to the accelerated

population growth and demand for fuels, especially for fossil-based replacements. Its use allows reducing consumption of these fuels and greenhouse gas emissions.

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin (Collard and Blin, 2014). These biomasses can be converted to ethanol in three stages: pretreatment, hydrolysis and fermentation (Kumar et al., 2008). However, these stages in the current production of ethanol are expensive (Wen et al., 2009) and therefore require significant technical progress in various fields in order to reduce production costs to a competitive level with gasoline (International Energy Agency, 2010). The recalcitrant nature of the cell wall of the lignocellulose material is a challenge in that field. The major component of the cell wall is cellulose, which is protected by a

\* Corresponding author: Jairo G. Salcedo Mendoza - E-mail: jairo.salcedo@unisucra.edu.co

matrix of hemicellulose (the second most abundant component), and lignin, which confers resistance to microorganisms, enzymes and chemical attacks (Aro et al., 2005; Sharma and Qin, 2017). Cellulose is a linear homopolymer, high molecular weight, composed of units of D-glucopyranose, forming a crystalline and amorphous material (Bian, et al., 2013; Laureano-Perez et al., 2005). The crystalline cellulose exists in the form of microfibrils, which are paracrystalline assemblies of some dozens of (1.4)  $\beta$ -D-glucan chains, united to each other by hydrogen bonds along their length (Laureano-Perez et al, 2005; Mittal et al., 2011). Four different crystalline polymorphs have been identified by their X-ray diffraction pattern: cellulose I, II, III and IV (Mittal et al., 2011). Native cellulose I is the most abundant in nature, and consists of parallel chains that form flat sheets, aligned side-by-side by hydrogen bonds (Bian et al., 2013). It is a mixture of two distinct crystalline allomorphs, cellulose I $\alpha$  (one-chain triclinic structure) and cellulose I $\beta$  (two-chain monoclinic structure) that is the predominant form found in higher plants. Cellulose II and III can be prepared by different routes: as mercerization (treatments with alkali), and regeneration (solubilization and subsequent recrystallization) (Mittal et al., 2011; Sun et al., 2004). Cellulose II is believed to consist of a two-chain P21 monoclinic unit cell in which cellulose chains are stacked with opposite polarity, a so-called antiparallel structure (Langan et al., 2001; Wada et al., 2004). Cellulose III can be formed from cellulose I and II through treatment with liquid ammonia. Cellulose IV may be obtained by heating cellulose III (Atalla and Vanderhart, 1984). Scanning electronic microscopy does not differentiate between amorphous and crystalline regions in a single cellulose fiber, but examining the morphology before and after pretreatment, can allow one to differentiate these two regions. It is supposed that cellulose that has a high content of amorphous regions is usually more easily digested by enzymes (Zhao et al., 2006). However, this claim is not clear. Other factors besides the crystallinity, such as the lignin/hemicellulose relationship, its content and distribution, as well as porosity and particle size, can affect this digestibility (Park et al., 2010), aside from the insoluble nature of cellulose and its degree of polymerization (Hall et al., 2011). Thus, it is necessary to pre-treat the material to delignify it, since lignin is thought to be a critical factor that inhibits enzymatic hydrolysis by irreversible adsorption on cellulose, which is believed to reduce the amount of available enzyme for enzymatic hydrolysis (Zhu et al., 2008). An ideal pretreatment is that in which the lignocellulosic biomass is susceptible to quick hydrolysis with increased yields of monomeric sugars and limited formation of inhibitory compounds and minimized energy demands and operational cost

requirements (Gupta and Verma, 2015). Within the methods used to delignify biomass, the organosolv method actually removes the lignin and favors the recovery of glucose (Sannigrahi et al., 2010), and is effective in the pretreatment of sugarcane harvest residues (Salcedo et al., 2011).

In Valle del Cauca (Colombia) sugar cane is grown. This crop generates abundant residues of lignocellulose materials- around nine million tons per year (Cenicaña, 2010), consisting mainly of leaves and tops, which can be used in the production of second generation fuels. In the global context, if nearly  $5.4 \times 10^8$  tons of dry sugar cane per year are produced (Cardona et al., 2010), there might be around  $1.4 \times 10^8$  tons of dried leaves and tops available. Understanding this scenario, it is interesting to find technological alternatives that will allow the conversion to fermentable sugars of agricultural residues. For this reason, and in order to improve the process of saccharification, the effects of different pretreatments on the crystalline structure and enzymatic hydrolysis of lignocellulosic material obtained from sugar cane harvest residues (leaves and tops) were evaluated in this research. Several techniques were used to achieve this purpose such as Fourier Transform infrared spectroscopy (FTIR), X-ray diffraction and scanning electron microscopy (SEM).

## EXPERIMENTAL METHODS

### Materials

All of the chemical reagents used in this study were of analytical quality, purchased from different companies. Harvest residues, mainly composed of leaves and tops of sugarcane varieties CC8475 and CC8592 (CC: Cenicaña-Colombia), were kindly provided by the Centre for Cane Research (Cenicaña, Florida-Colombia), and are the widest cultivated in the region. The residues were washed with water at 70° C, dried at 40° C until constant weight and mashed. The material utilized had a particle diameter between 0.25 and 0.42 mm. A homogenized sample was used for all the tests and analyses, which were triplicate.

### Pretreatment

Six different substrates of sugar cane harvest residues were treated by different delignification methods, whose characteristics can be observed in Table 1. The residues obtained from the harvest, without prior treatment, were taken as the baseline (S1); all residues were dried up to 3.9% (w.b.). Subsequently, they were reduced in size until 94.5% passed 10 mesh and 22% above 60 mesh. In the delignification treatment with the suberosa enzyme (S2), a vanillin mediator was used at a concentration of 5 mg/g of dry solid. The enzyme dosage was 0.33 mL/g of dry material, and the laccase enzyme activity was 0.83 UI/mL. In

**Table 1.** Pretreatment to delignified residues (leaves and tops) from the harvest of sugar cane.

Substrate	Pretreatment	T (°C)	Relation NaOH (w/v)	Time (min)	Reference
S1	Without pretreatment				
S2	Enzyme Suberosa®	40		1380	Salcedo et al. (2011); Bourbonnais et al. (1995); Silva et al. (2002); Jong-Rok et al. (2008)
S3	Organosolv	160	0.15	120	Salcedo et al. (2011), Mutis (2010)
S5	Organosolv	160	0.50	120	Salcedo et al. (2011), Mutis (2010)
S6	Organosolv	160	3	120	Salcedo et al. (2011), Mutis (2010)
S7	Organosolv	130	3	5	Salcedo et al. (2011), Mutis (2010)
S8	Organosolv	120	3	20	Salcedo et al. (2011), Mutis (2010)

treatment S3, after applying an organosolv treatment with ethanol as a solvent, the material was treated at 80°C with 4% (w/w) sodium hydroxide, in a 4:1 liquid/solid relationship (v/w), for three hours. For substrate S6, 1N sulfuric acid was added until pH of five after treatment with solvent, keeping a solid/liquid ratio of 85:15 (w/v). Then, EDTA was added in ratio of 1/1000 (w/w) in relation to the solid material. The mixture was stirred at 1000 rpm for 10 minutes. Afterwards, the solid was filtered and washed with abundant water. This solid was treated with 1N sodium hydroxide until the mixture reached a pH of 10.8, with a solid/liquid ratio of 85:15 (w/v). The content of lignin, cellulose and hemicellulose in the delignificated material was determined by the method described by Van Soest (1983), and the Kappa number by the Tappi standard T-236-cm-85.

### Enzymatic Hydrolysis

In the enzymatic hydrolysis of sugarcane harvest residues, a mixture of commercial enzymes was used (Salcedo et al., 2011), with the following enzymatic activities: 52.75 UI / ml of hemicellulases, 27.53 FPU/ml of cellulases and 550 pNPG/ml of betaglucosidase. Progress curves for the hydrolysis reaction were obtained from the enzyme/substrate ratios (E/S): 0.05, 0.1, 0.2, 0.3 (v/w) for S1, S2, S3, S5, S6, S7 and S8 substrates. The reaction took place in acetate buffer solutions at pH 4.8 with a solid/liquid ratio of 6:100 (s/v), at 40°C, and a 150 rpm agitation speed. Samples were taken at reaction times of 0, 2, 6, 9, 24 and 28 hours. Reducing sugars were determined in supernatants by the method of DNS (Miller, 1959). Progress curves were constructed based on the production of reducing sugars, as a function of time and the kinetic models were reported by Salcedo et al. (2012). The calculation of the percentage of saccharification was carried out according to Equation 1 (Ghose, 1987).

$$\%S = \frac{\text{Reducing sugars (mg/ml)} \times 0.9}{\text{Substrate concentration (mg/ml)}} \times 100 \quad (1)$$

In addition, for the Saccharification Index (IS) and the Global Index of Hydrolysis (IGH), equations 2 and 3 were assessed, respectively (Salcedo, 2011).

$$IS = \frac{\Delta\%S}{\Delta \log \left( \frac{E}{S} \right)} \quad (2)$$

$$IGS = \overline{\%S} \times \overline{IS} \quad (3)$$

where:

$$\overline{\%S} = \frac{\sum_{i=1}^4 \%S_{E/S_i}}{4}$$

$$\overline{IS} = \frac{\sum_{i=1}^5 IS_i}{4}$$

E/S: enzyme/substrate ratio

### Fourier Transform Infrared Spectroscopy (FTIR)

The samples were analyzed using attenuated total reflectance techniques. The spectra was recorded (10 scans with 4 cm<sup>-1</sup> resolution) in a Shimadzu Spectrophotometer, model IR Prestige-21. The method used in the preparation of samples was dispersion in KBr to produce tablets.

### X-Ray Diffraction

Diffraction data was collected in the range of 2θ from 10° - 40° at a speed of 2°/min. X-ray powder diffraction patterns were measured using a Bruker diffractometer (Madison, USA) model D8 with a Cu Kα radiation source (λ = 0.154 nm). Crystallinity index (CrI) was calculated by the method proposed by Segal et al. (1959), Ir number by models developed by Zhang et al., (1993) and degree of mercerization (IIC-%) by Mansikkamaki et al. (2007).

### Scanning Electron Microscopy (SEM)

Quanta 200F model with a resolution of 1.2 nm, low vacuum, with field emission gun was used. Elemental analyses were performed with an EDX detector brand EDAX adapted to the SEM. The conditions of the equipment were: voltage of electronic acceleration of 4000-30000 Kv, with a pressure in the range of 40.5318 Pa.

## RESULTS AND DISCUSSION

### Substrates Characterization

The compositions of the different substrates used in the present study are presented in Table 2.

It can be seen that substrates S5 and S6, which had the highest content of cellulose, increased its concentration by approximately 120% with respect to the value found in the substrate S1, without any treatment. Compared to other substrates, there was a variation of composition of holocellulose (cellulose + hemicellulose) from 72% to S1, up to 90% for

S6. These seven substrates present a wide range of compositions of cellulose, hemicellulose and lignin, which permitted the evaluation of different hydrolytic enzymes.

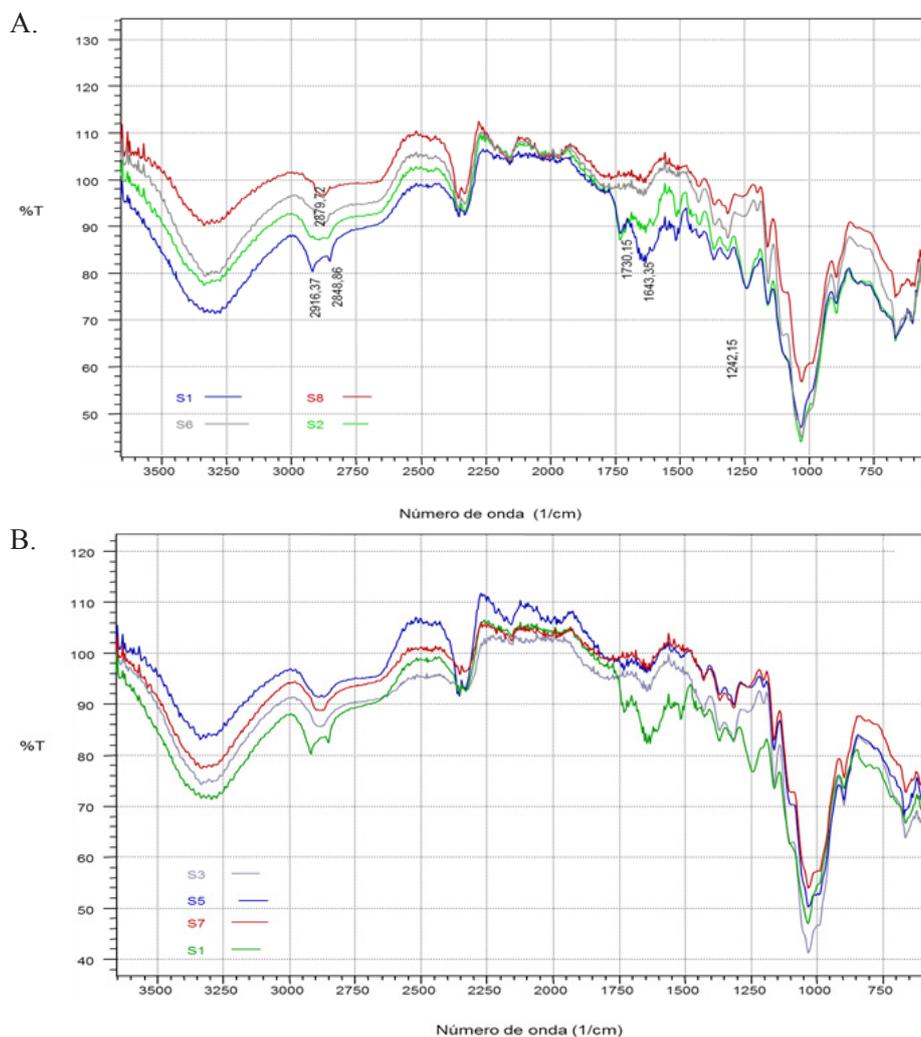
### FTIR Analysis

It is practical to use FTIR for the identification of pure substances. However, biological systems are a mixture of complex substances, so FTIR gives a complex spectral profile in which it is possible to identify the presence of only a few compounds or kinds of compounds (Rose, 2003). Figures 1a and 1b, allow

**Table 2.** Compositional analysis (% w.b) of the different pretreated substrates.

	S1	S2	S3	S5	S6	S7	S8
Cellulose	37.4±1.4	44.5±0.9	80.5±0.2	82.4±0.4	82.6±0.8	71.9±0.4	77.4±0.2
Hemicellulose	34.6±0.8	37.3±0.4	13.4±0.6	3.0±0.05	7.4±0.06	10.9±0.08	10.4±0.2
Total lignin	21.2±0.04	12.5±1.58	1.4±0.04	7.9±0.25	4.8±0.5	10.8±0.4	6.6±0.3
Extractives	3.9±0.03	5.1±0.06	4.2±0.01	9.0±0.2	5.1±0.01	4.7±0.02	6.5±0.04
Humidity (b.h)	3.9±0.8	5.1±0.7	4.2±0.4	8.9±0.02	5.1±0.10	6.4±0.1	4.7±0.07
Ash	1.1±0.04	0.7±0.05	0.1±0.03	1.4±0.02	0.2±0.01	1.6±0.04	0.54±0.02
Residual lignin	7.1±0.1	7.2±0.06	0.9±0.1	1.9±0.18	1.8±0.15	2.8±0.14	2.2±0.11
No Kappa*	47±0.3	38.6±0.5	5.9±0.3	27.4±0.3	6.2±0.2	16.8±0.4	13.2±0.4

\* Not included in the percentage analysis.



**Figure 1.** Infrared spectra of (a) substrates S1, S2, S6 and S8 and (b) of substrates S1, S3, S6 and S7.

comparison of the profile of the infrared spectrum of the substrate S1, which corresponds to the material collected from the field without any pretreatment. This substrate is taken as a baseline to compare the effects of pretreatments on the infrared spectrum of the pretreated substrates S2, S3, S5, S6, S7 and S8. In general, functional groups of the substrate S1 present greater intensity of absorbance in the range of 600 to 3600  $\text{cm}^{-1}$ .

The bands from 3020  $\text{cm}^{-1}$  to 3600  $\text{cm}^{-1}$  are representative of the OH-vibration (Alves et al., 2012; Colom et al., 2003; Viera et al., 2007). In this case, the absorption intensities from greatest to lowest, were as follows: S1, S3 (S2, S6), S5 and S7. The change is caused by the variation of binding energy of hydrogen in the system of internal and intermolecular interactions. After the pretreatments, bands are closer to 3400  $\text{cm}^{-1}$ . This proves that the changes of hydrogen bonds in the pretreatment are mainly due to the loss of these bonds in the lignin structure and hemicellulose. The absorption band at 3010  $\text{cm}^{-1}$  represents the vibration = CH of the aromatic ring, to 2936  $\text{cm}^{-1}$  the asymmetric vibration -CH<sub>2</sub> (guaiacil, syringyl), and to 2840  $\text{cm}^{-1}$  symmetric vibration band of guaiacil - syringyl -CH<sub>2</sub> (Carballo et al., 2004). The major intensity of absorption of these groups linked to lignin occurs in the substrate S1 followed by S2, S3, S7, S6, S5 and S8 substrates. This information is an indicator of the loss of lignin during pretreatments, due to the removal of the aromatic structure of lignin. The peak at 1730  $\text{cm}^{-1}$  represents the carboxyl group stretching C = O not conjugated to an aromatic ring. This occurs with great intensity in the S1 and S2 substrates, with insignificant values for S6 and S8, and low values for S3, S5 and S7. The band at 1646  $\text{cm}^{-1}$  refers to the bending vibration of the absorbed water. The peaks at 1246  $\text{cm}^{-1}$  and 1201  $\text{cm}^{-1}$  are found within the band range 1250  $\text{cm}^{-1}$ -1050  $\text{cm}^{-1}$ , and part of the C-O bonds of the syringyl ring in the structure of lignin (Carballo et al., 2004). A decrease is shown in the intensity of absorbance of this band from substrates S1 and S2,

followed by the substrates S3 to S8, which has the lowest value. The decrease of this band also represents lignin elimination.

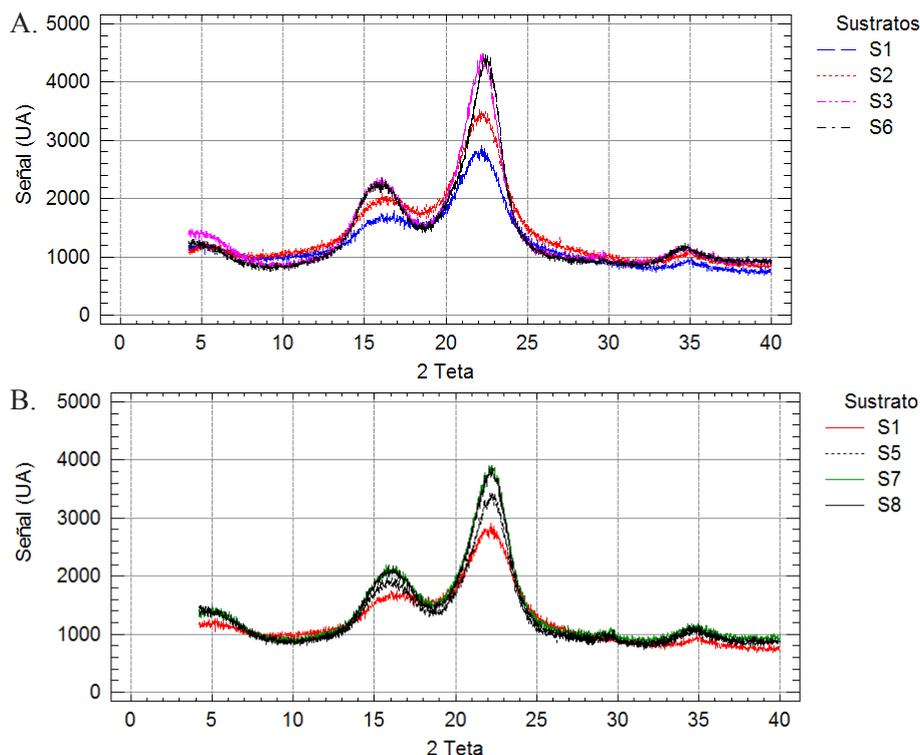
That bands 1030  $\text{cm}^{-1}$ , 603  $\text{cm}^{-1}$ , 666  $\text{cm}^{-1}$ , 1102  $\text{cm}^{-1}$  and 1161  $\text{cm}^{-1}$  are related to asymmetric vibrations of bonds C-O -C of anhydroglucose rings and tension C-OR and C-O vibrations of the skeleton of the piranose (Poletto et al., 2011). The order of absorbance intensity from the greatest value to the lowest, is as follows: S3, S2, S1, S6, and in a lower range, S8, S7 and S5. In this case, the intensities are directly related to the polymeric structure of cellulose. Table 3 shows the most relevant peaks that differ in the infrared spectrum between the substrate S1 and pretreated substrates of Figure 1a. A decrease of the functional groups that make up the lignin can be seen. In addition, S1 and S2 substrates have similar values of absorbance intensity at 1730  $\text{cm}^{-1}$  and 1242  $\text{cm}^{-1}$ , related to the aromatic structure of lignin. It is the opposite in the S3, S5, S6 and S7 substrates, which present low levels of absorbance intensity at these peaks.

These results suggest that lignin polymers associated with the hemicellulose or cellulose are closely linked. The use of NaOH produces a higher level of lignin removal from the raw material. This is consistent with Mesa et al. (2010). In addition, the organosolv pretreatment attacks mainly components of hemicellulose and lignin. X-ray diffraction was another criterion that was used in this research. Figures 2a and 2b present the diffraction profiles for different substrates and this information was used to determine the Index of Crystallinity. Also, the effect of different pretreatments on the progress of the crystallinity was evaluated based on the conversion of cellulose (CI) to cellulose (CII), as was the number of relative intensity and the degree of cellulose mercerization.

The Crystallinity Index was evaluated through the model proposed by Segal et al. (1959), Equation 4. To calculate the Crystallinity Index, data averages of intensity were taken from the two proposed ranges  $I_{200}$  and  $I_{am}$ .

**Table 3.** Description of the absorbance intensity of the most relevant peaks in the infrared spectrum, for the different substrates obtained from the sugar cane harvest residues.

Intensity Absorbance $\text{cm}^{-1}$	S1	S2	S3	S5	S6	S7	S8
Absorbance at 1730 $\text{cm}^{-1}$ : (C = O) stretching C = O not conjugated to the aromatic ring	0.054	0.054	0.021	0.013	0	0.034	0
Absorbance at 1427 $\text{cm}^{-1}$ : C-H . CH <sub>2</sub> Vibrations	0.058	0.041	0.047	0.00069	0.02	0.00069	0.076
Absorbance at 1327 $\text{cm}^{-1}$ : C-H <sub>2</sub> . CH syringyl ring emissions	0.082	0.068	0.074	0.037	0.044	0.0418	0.024
Absorbance at 1315 $\text{cm}^{-1}$ : C-H <sub>2</sub> . CH vibrations	0.081	0.071	0.08	0.044	0.058	0.048	0.032
Absorbance at 2916 $\text{cm}^{-1}$ : Stretching - CH methyl and methylene groups	0.096	0.057	0.057	0.032	0.028	0.039	0.0025
Absorbance at 2848 $\text{cm}^{-1}$ : saturated OH bonds	0.084	0.054	0.058	0.036	0.035	0.041	0.0098
Absorbance at 1242 $\text{cm}^{-1}$ : C-O syringyl ring bonds	0.115	0.115	0.049	0.029	0.036	0.026	0.0149



**Figure 2.** X-ray diffraction profiles for (a) S1, S2, S3 and S6 substrates; (b) S1, S5, S7 and S8 substrates.

$$\text{CrI} = \left( \frac{I_{200} - I_{\text{am}}}{I_{200}} \right) \times 100 \quad (4)$$

where CrI is the percentage of Crystallinity Index;  $I_{200}$  is the maximum intensity of diffraction for  $2\theta$  between  $22$  and  $23^\circ$ , and  $I_{\text{am}}$  is the intensity of diffraction for  $2\theta$  between  $18$  and  $19^\circ$  for cellulose I.

Based on reports of X-ray diffraction, derived methods were used that evaluate with precision the conversion of cellulose I (CI) to cellulose II (CII), which are used to study the effects of different alkali treatments on cellulose crystals (Mansikkamaki et al., 2007). These have been adapted in this work, with the aim of evaluating the changes of the crystalline forms of different substrates. Among these methods, the Number of Relative Intensity ( $I_r$ ) or the conversion to CII (equation 5) is reported. In this case, the zero value corresponds to pure native cellulose, value 1 for completely mercerized cellulose, and intermediate values indicate partial mercerizing (Ranby, 1952; Zhang et al., 1993).

$$I_r = C_{\text{II}} = \frac{2I_{\text{II}}}{I_{\text{I}} + 2I_{\text{II}}} \quad (5)$$

where  $I_{\text{I}} = I_{\text{t}}$  is the sum of intensities  $I_{14.7} + I_{16.1}$ , and  $I_{\text{II}} = I_{12}$ ;  $I_r$  = Number of Relative Intensity: is an indicator of conversion of cellulose II (CII).

Another indicator of conversion is the degree of mercerization of cellulose (IIC-%), (Mansikkamaki

et al., 2007). The results of this indicator are only comparable for materials that are within the same series of experiments; a value of IIC-% of zero indicates large amounts of amorphous material (Equations 6 and 7).

$$\text{CII} - \% = \frac{\text{CII}_r - \text{CII}_{\text{t,min}}}{\text{CII}_{\text{t,max}} - \text{CII}_{\text{t,min}}} \times 100 \quad (6)$$

where CII-% = Degree of cellulose mercerization

$$\text{CII}_r = \frac{I_{r,12.1}}{I_{r,12.1} + 0.5(I_{r,14.6} + I_{r,16.1})} \quad (7)$$

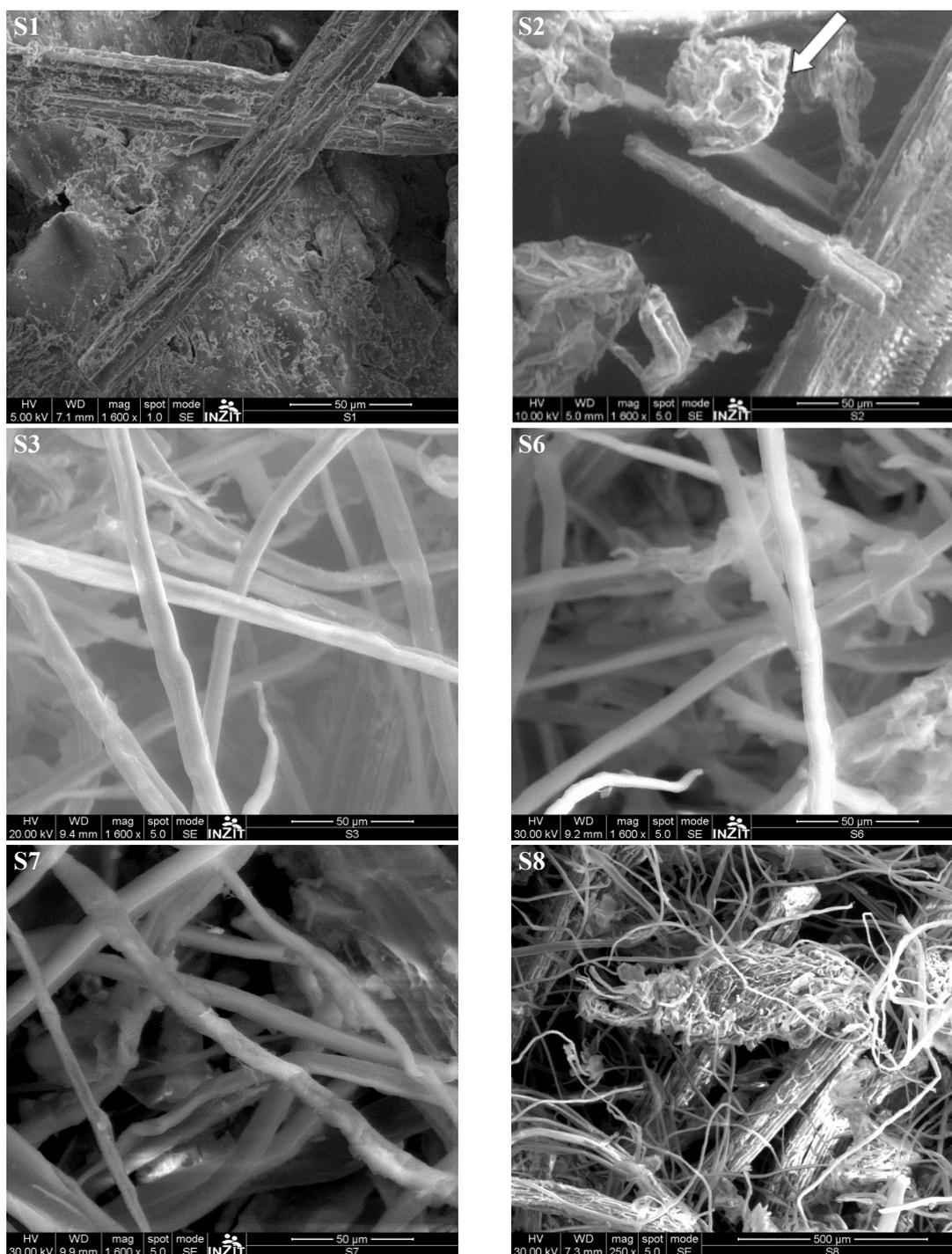
where  $\text{CII}_r$  = Degree of relative mercerization;  $\text{CII}_{\text{t,min}}$  = corresponds to the lowest value of the samples tested in CIIr;  $\text{CII}_{\text{t,max}}$  = the highest value of the samples tested in CIIr

Table 4 shows that the highest rate of crystallinity (CrI) corresponds to substrates that, in addition to the organosolv pretreatment, continued with additional treatments, as is the case of the substrate S6, with a CrI of 64.24, and substrate S3 with CrI of 62.17. In substrates pretreated only with organosolv treatment, an influence on CrI of sodium hydroxide added in treatment is shown.

The lowest CrI value is for the substrate S1 (41.41). No pretreatment was applied to this material. Also, it is observed that the value of the Number of Relative Intensity ( $I_r$ ) nearly approaching zero corresponds to the substrate S6 (0,313), and the highest value is

**Table 4.** Influence of Lignin Content, kappa number, CrI, IIC-% and Ir on the enzymatic hydrolysis expressed as GIS.

Substrates	S1	S2	S3	S5	S6	S7	S8
Residual lignin	7.12	7.8	0.92	1.93	1.8	2.8	2.2
Kappa number	47	38.56	5.85	27.39	6.21	16.87	13.24
CrI	41.41	46.76	62.17	56.54	64.24	57.85	58.61
CII-%	100.00	77.39	13.29	35.89	0.00	33.77	53.74
Ir	0.40	46.76	0.32	0.34	0.31	0.34	0.36
GIS	1.013	0.655	77.78	56.34	82.09	67.14	56.24

**Figure 3.** Scanning electronic microscopy (SEM) images of sugarcane (leaves and tops) harvest residues: (S1) material without pretreating; (S2) after enzyme pretreatment; (S3), (S6) (S7) and (S8) after the organosolv pretreatment.

the substrate S1 (0.395). This indicator reveals that pre-treatments remove lignin, hemicellulose and amorphous cellulose, CII. The same behavior is observed in the mercerizing degree (IIC-%): substrate S6 has the lowest degree of mercerization and the S1 substrate the highest. It is important to conclude that Ir and IIC-% indicators can also be used to interpret different cellulose types in pretreatments different from mercerization. In all cases mentioned above, the values of the S2 substrate, which was subjected to enzymatic delignification, are likely to approach the S1 substrate.

S1 and S2 substrates have the highest content of residual lignin (7.8 and 7.12) and kappa number (47 and 38.56) respectively, while substrates S3, S5 and S6, have the lowest values. However, there is no correlation between residual lignin and GIS, indicating that there are probably changes that occur during the lignin removal which affect the enzymatic hydrolysis, because changes in crystallinity, pore volume and surface area that facilitate the adsorption of the enzyme on the pre-treated material, as stated by Koo et al. (2012). It is also important to observe how the pretreated material releases many more sugars compared to the untreated (especially S1 and S2). The highest release of sugars was observed in waste pretreated at 160° C, with 3% NaOH and ethanol 45%, followed by bleaching with 1N sulphuric acid in EDTA (S6). Also, substrates that have a lower value of CrI, higher Relative Intensity (Ir) and high degree of cellulose mercerization have high resistance to enzymatic hydrolysis, expressed in low Global Index of Saccharification (GIS). S6 and S3 substrates contain less residual lignin content and lower kappa numbers, presented the highest CrI values, GIS and lower Ir, IIC-%, which corresponds to a production of crystalline cellulose. This presumably means that during the pretreatment, more amorphous materials or non-crystalline fractions (Hsu et al., 2010) were removed, which included cellulose II. The substrates to which only organosolv pretreatment was applied (S5, S7 y S8), without any additional treatment, have good behavior in enzymatic hydrolysis, a high CrI, and tends to form cellulose I, which is represented by the average values of Ir and IIC-%. It can be seen that the changes in the crystalline structure of cellulose, such as the delignification, favored the enzymatic hydrolysis. Based on these results, there is no tendency to increase the enzymatic hydrolysis with the decline of crystallinity, as some research says (Laureano Pérez et al., 2005; Hall et al., 2010). The opposite phenomenon is present, as has been observed in other studies (Chan and Holtzapfle, 2000; Koo et al., 2012), probably due to the correlation between crystallinity and enzymatic hydrolysis that cannot be expressed by only the relative Crystallinity Index (Koo et al., 2012). Equally there is no relationship between the residual

lignin content and changes in the crystalline structure after the pretreatment. SEM images of substrates that were used in this research show large differences between the substrate S1 and pretreated substrates. S1 and S2 substrate images were presented at the surface level without any defibrillation. Otherwise, in images of pretreated substrates, a destruction of the cell wall and a large defibrillation can be seen (see Figure 3), without deposited droplets of lignin after washing, as was observed in *Liriodendron tulipifera* after organosolv pretreatment, without washing (Koo et al., 2012), indicating the effectiveness of the conditions applied in this research for the removal of lignin.

## CONCLUSIONS

Pretreatment is an important factor to consider in the process of hydrolysis of lignocellulosic materials (sugarcane residues). Depending on the pretreatment, it can facilitate or hinder the lignocellulosic enzyme attack. The interaction of the chemical and physical effects produced during the pretreatment on the structure of lignocellulosic material facilitates the hydrolysis. This research showed the influence of the lignin content on the enzymatic hydrolysis, concluding that lignin can act as an inhibitor in enzymatic hydrolysis of sugarcane residues. However, the lignin content is not the only parameter to take into account for a successful development of the hydrolysis of lignocellulosic materials. The type of pretreatment applied to the original material is also important. Organosolv pretreatment attacks mainly components of hemicellulose and lignin, and contributes to changes in the crystalline structure of cellulose, generating a decrease in the amorphous material, which includes cellulose II, that facilitates enzymatic hydrolysis

## NOMENCLATURE

CrI	The Crystallinity Index
IIC%	Degree of cellulose mercerization
CII <sub>r</sub>	Degree of relative mercerization
X-ray	Diffraction of X-rays
E/S	Enzyme/substrate ratio
FPU	Filter paper unit
IGH	Global Index of Hydrolysis
GIS	Global Index of Saccharification
CII	Indicator of conversion of cellulose
FTIR	Fourier Transform Infrared Spectroscopy
Ir	Number of Intensity
%S	Percentage of saccharification
% w.b	Percentage wet base
KBr	Potassium bromide
pNPG	p-nitrophenol-glucoside
IS	Saccharification Index
SEM	Scanning Electron microscopy

s/v	Solid/liquid ratio
S1, S2, S3, S5, S6	Substrates
CII <sub>t, min</sub>	The lowest value of the samples tested in CIIR
CII <sub>t, max</sub>	The highest value of the samples tested in CIIR
UI	International units

### ACKNOWLEDGEMENTS

Authors thank the financial support given to the grant 2007-3719-346-07 by the Ministry of Agriculture and Rural Development, Universidad Autónoma de Occidente (Laboratory of BioProcess), Universidad del Valle and the Zuliano Institute of Technology Research "INZIT", as well as the Swiss Cooperation Agencies through the project: EPFL DDC 2009-2012 SCIENTIFIC COOPERATION FUND.

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