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PRETREATMENTS EFFECTS ON SUGARCANE BAGASSE FOR HIGH YIELD OF CELLULLASE PRODUCTION

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RESUME - Sugarcane bagasse is a low-cost, abundant Brazilian feedstock and a potential substrate for producing high specificity cellulases tailored to allow the viability of 2nd generation ethanol production. Due to its poor digestibility, it needs suitable pretreatments to obtain high yields for biological conversion. The present work aims a comparison of physicochemical pretreatments (sulfuric acid, sodium hydroxide and its combination) and its effects on cellulases production using solid state fermentation (SSF) of *Aspergillus niger*. The acidic/alkaline pretreatment resulted in maximum concentration of cellulose (46.6% to 85.8%). Micrographs showed structural cells loosening with partial exposition of cellulose network and lignin removal. Crystalline degree increased with alkaline pretreatment indicating the loosing of amorphous components or formation of microcrystalline cellulose. Natural bagasse showed maximum FPase and xylanase production (0.3 and 23.20UI/g respectively) Lower enzymatic production of acidic, alkaline and acidic/alkaline substrates (in decreasing order) suggest formation and recalcitrance of toxins/inhibitors hindering fungal uptake.

Keywords: Agroenergy, cellulases, solid state fermentation, *Aspergillus niger*.

1. INTRODUCTION

In last 15 years, an increasing effort has made towards developing efficient and sustainable technologies to attend the growing world energy demands.

Lignocellulose shows enormous potential for conversion into bioethanol, this feedstock is made up predominantly of cellulose, hemicellulose, and lignin. These fractions must be hydrolyzed in order to produce fermentable sugars. There are numerous structural factors (e.g., crystallinity, available fiber surface area, pore structure and distribution) and enzymatic mechanistic factors that lead enzymatic hydrolysis

(Weng *et. al.*, 2008; Hendriks and Zeeman, 2009). The factors mentioned above all need to be addressed for cellulosic ethanol to emerge on an industrial scale. Constraints in the large scale process summarize in three critical steps: pretreatment, enzymatic hydrolysis, and fermentation. An additional associated factor has been the high price of cellulase enzymes capable of hydrolyzing lignocellulose.

Efforts on cost reduction have been directed towards increasing enzyme production by developing better microbial strains, efficient fermentation technologies and recovery systems (Xu *et. al.*, 2005). Solid state fermentation (SSF) is one such technique; it enables fungi growth in the absence of free water and in a relative short time.

The use of SSF for production of enzymes has many advantages like no need for complex and sophisticated machinery, easy product recovery, simple and inexpensive substrates for the fermentation, low energy demand, high volumetric productivity and often a high yield of products (Shah and Madamwar, 2005; Gessesse and Mamo, 1998).

However all these opportunities should be supported by an essential operation of pretreatment if we are to achieve high yields from biological operations.

In this context, the present work aims a comparison of physicochemical pretreatments (sulfuric acid, sodium hydroxide and its combination) and its effects on cellulases production. The substrate chosen, sugarcane bagasse (SCB) is an abundant and available byproduct from Brazilian industry and suitable for the solid state culture of *Aspergillus niger*. Resultant low-cost and highly specific celluloytic enzyme complex will expect to efficiently hydrolyze abundant SCB in sugars for the viable bioethanol production.

2. MATERIALS AND METHODS

2.1 Microorganism

The microorganism used in this study was a wild-type strain of *A. Niger* from the Embrapa Food Technology collection. The culture was kept on dry sand and activated in basic agar slants, formulated by Couri and Farias, 1995. Conidia were suspended in sterilized Tween 80 solution 0.3% (v:v).

2.2 Bagasse Pretreatments

A mass of 50 g of dried sugar cane bagasse was grinded to attain 0.5 mm size of mesh powder and then treated with 2% (w/v) concentrations of NaOH, H_2SO_4 and NaOH + H_2SO_4, with 1:5 (v/w) ratio in an autoclave at 121°C for 30 min. After the treatment, all the samples were washed three times with 3L (per wash) of distilled water acidified to pH 2 with H_3PO_4. The final pH of the wet solids was approximately 5. Then the substrates were dried in an oven at 60 °C for 5 h.

2.3 Solid State Fermentations

Solid state fermentations (SSF) were carried out in 500 mL flask with 5 g of the sterile pretreated substrate supplemented with Mandels & Weber medium (Mandels and Weber, 1969). The medium was inoculated with 1 x 10^7 conidia/g and incubated at 32°C for 72 h in a temperature-controlled chamber.

At the end of the process each flask was supplemented with 100 ml of an acetate buffer 200 mM (pH 4.5). The mixture was stirred for 1 h at 120 rpm and 32°C. The solid residue was separated by filtration through Whatman 40 filter paper.

2.4 Analytical Methods

2.4.1 Chemical composition

Van Soest's methodology (Van Soest and Wine, 1967) was used to determine cellulose, hemicellulose and lignin as they correlate with neutral-detergent fiber (NDF), acid-detergent fiber (ADF) and lignin as follows:

Cellulose = ADF-Lignin

Hemicellulose = NDF - Lignin-Cellulose

Dry matter and ash were determined by AOAC methodology (AOAC, 1980).

2.4.2 Fourier transform infrared (FT-IR)

Samples spectra were obtained on an FT-IR spectrophotometer (Spectrum One Perkin Elmer) in the range 4000 e 400 cm^{-1} using a KBr disc containing 1% of finely ground pretreated sample.

2.4.3 X-ray diffraction analysis

Crystallinity of the untreated and pretreated SCB samples were measured using a Shimadzu, 6000 X-ray diffractometer with Cu Tg (λ = 1,5418 Å). The crystallinity index (IC%) was calculated based on the method of Segal et al. (1959).

2.4.4 Scanning electron microscopy (SEM)

Samples were prepared for SEM inspection by sticking them on carbon glue and allowed to Au-coated (Coating System BAL-TEC MED 020).

The coated samples were examined by scanning electron microscopy (LEO 440 model) with OXFORD detector at 20 kV).

2.4.5 Enzyme assays

Each filtrate of the enzymes produced was monitored for filter paperase (FPase), carboxymethyl cellulase (CMCase) and xylanase activity.

FPase and CMC activities were determined according Mandels et al. (1974). For FPase, assay mixture (3 ml) consisted of 2 ml citrate buffer (50 mM, pH 4.5), filter paper Whatman No.1 (100 mg, 1 x 6 cm^2) and 1 ml of enzyme. The reaction mixture was incubated at 50 °C for 60 min.

For CMCase, the total reaction volume of 1 ml (0.5 ml sample of suitably diluted enzyme and 0.5 ml of 4% CMC solution in citrate buffer) was incubated at 50 °C for 10 min.

Xylanase activity was assayed in 3 ml of a reaction mixture containing 1 ml of diluted enzyme solution and 2 ml of 1.0% (w/v) xylan (Sigma) in 0.05 M citrate buffer (pH 4.8). The mixture was incubated at 50°C for 30 min according to Bailey (1992).

After all the incubation periods, the reducing sugars were determined by the dinitrosalicylic acid method (Miller, 1959) with glucose as standard.

One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmol of glucose in 1 min under the assay conditions.

3. RESULTS

3.1 Chemical composition

The Table 1 presents the chemical characterization of untreated and

pretreated SCB samples and the mass yield associated with each pretreatment.

Table 1. Relative chemical composition of SCB before and after pretreatments.

Component (%)	SCB	NaOH	H_2SO_4	NaOH + H_2SO_4
Cellulose	46,62	76,44	72,72	85,78
Hemicellulos	26,51	15,48	2,89	4,69
Lignin	21,7	2,42	19,98	2,57
Ash	2,51	1,87	2,22	1,12
Extractives	2,44	-	-	
Mass Yield	-	60	52	41

It can be observed that cellulose values increased in comparison to the untreated material indicating a relative concentration of this fraction due to the hemicellulose and lignin elimination.

Results in Table 2 show the total composition of the pretreated samples in relation of the final mass yield obtained by each pretreatment (Table 1). These information permit to calculate efficiencies in relation to each biomass component.

Table 2. Quantification of chemical components in the pretreated samples.

Biomass component (%)	NaOH	H_2SO_4	NaOH + H_2SO_4
Cellulose	45,87	37,82	35,17
Hemicellulose	9,29	1,50	1,92
Lignin	1,45	10,39	1,05
Ash	1,12	1,15	0,46

As suggested by the values, the pretreatments used were not selective for the lignin and hemicellulose, since they promoted the cellulose and carbohydrates solubilization (Fengel e Wegener, 1989).

The final concentration of lignin indicates an efficiency of 93% in consideration of this component. As reported by Silvertein *et al.*, 2007 and Cardona et al., 2010; the main effect of NaOH is delignification by breaking the ester cross-linking lignin and xylan, thus increasing the porosity of biomass. It resulted in lesser cellulose and hemicellulose solubilization than acid and combined pretreatments.

On the other hand, the main objective of the acid pretreatments is to solubilize the hemicellulosic fraction in sugars (xylose, arabinose, etc.) to make the cellulose more accessible (Alvira *et al.*, 2010). A decrease of 94% in hemicellulose content after acid treatment confirms this statement. However it resulted too in 18,8% of cellulose remotion.

After the combination of the acid and alkaline pretreatments both reduction in lignin and hemicellulose was 95% and 93%, respectively. This reduction influenced in the decreasing of cellulose concentration of approximately 25%.

In general, it is expected a better efficiency in fungal growth and uptake with the resultant major concentrations of cellulose.

3.2 Fourier transform infrared (FT-IR)

FT-IR technique was performed to infer structural changes in chemical groups derived by the pretreatments application.

Figure 1 shows the resultant spectra of the analysis of untreated and pretreated SCB. It can be seen that the carbonyl band at 1735 cm^{-1}, which has been

ascribed to hemicelluloses (Zhao *et al.*, 2008) is reduced in all pretreated SCB. As well as, lignin bands at approximately 1595 and 1510 cm^{-1} (aromatic ring stretch) are strongly reduced in the alkaline and acid/alkaline pretreated samples compared with both acid treated and untreated SCB (Kristensen *et al.*, 2008).

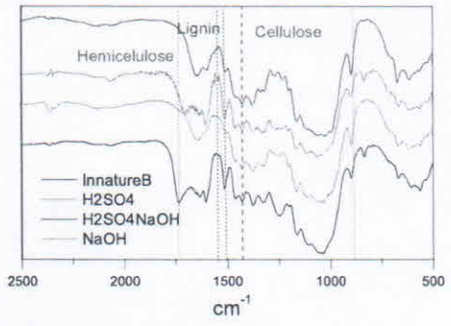

Figure 1. FTIR spectra of untreated, acid, alkaline and its combination pretreated SCB. Excerpt of spectra. All spectra are separated to ease comparison. The vertical lines in red, blue and green mark the positions of the bands ascribed to hemicellulose, lignin and cellulose respectively.

Differences between samples with regard to the relative amounts of amorphous and crystalline cellulose have earlier been described through infrared peak ratios at 1429 cm^{-1} (crystalline) and 893 cm^{-1} (amorphous) (Hulleman *et al.*, 1994; Wistara *et al.*, 1999).

Tough the FTIR spectra in this study were not quantitative; comparison between cited bands will allow inferring qualitative alterations. As observed the intensification in 1429 cm^{-1} should correspond to the concentration of crystalline cellulose. To complement this information, X-ray spectroscopy was performed in order to quantify the cellulose crystallinity.

3.3. X-ray diffraction (DRX) analysis

Crystallinity indexes (%IC) of the samples in Table 3 were calculated from DRX spectra (data not shown) as cited in methodology. %IC of a biomass measures the relative quantity of crystalline cellulose in the whole solid; therefore it should be corrected by the mass yield of each pretreatment to have a net result of each pretreatment.

Table 3. Comparison of crystallinity indexes, between untreated and pretreated SCB.

Pre-treatment	IC (%)	Mass yield (%)	Corrected IC (%)
SCB	52,83		52,83
NaOH	88,40	60	53,04
H_2SO_4	60,17	52	31,29
NaOH + H_2SO_4	73,53	41	30,15

As can be observed alkaline pretreatment caused a slight increase in %IC. This can be explained as the relative concentration of crystalline cellulose after the massive removal of amorphous components (Zhao *et al.*, 2008).

But then, acid and combined pretreatments caused a reduction in %IC of about 40.8% and 42.9% respectively. This fact suggests a significant recalcitrance reduction in the remaining cellulose and a rupture of the natural physical barrier Lin biomass (Himmel *et al.*, 2007).

3.4 Scanning electron microscopy observation (SEM)

Morphological modifications caused by pretreatments were determined from SEM (Fig. 2).

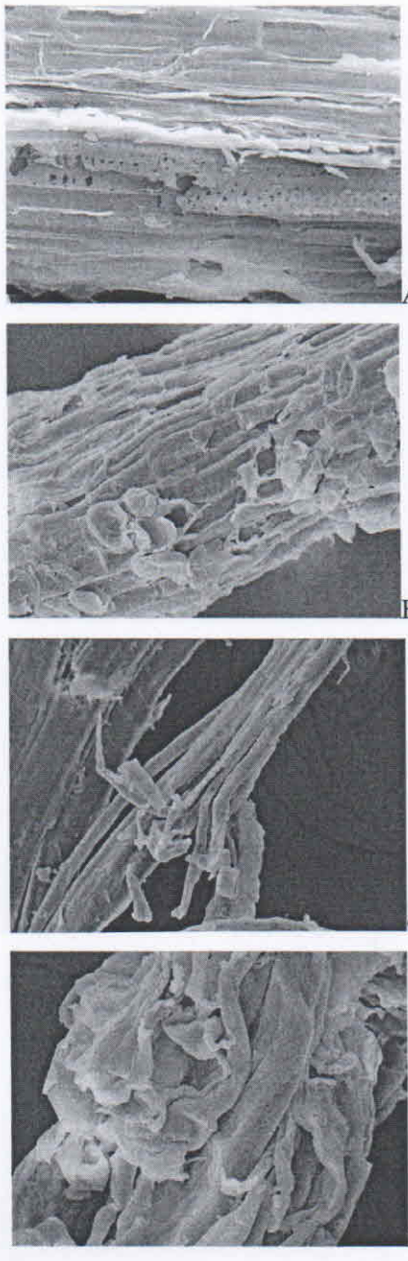

Figure 2. SEM micrographs of untreated and pretreated SCB. A) untreated SCB 500x. B) acid treated SCB 1000x. C)alkaline treated SCB 1000x and D)acid/alkaline treated SCB 2000x.

An entire comparison between micrographs of untreated SCB 2A and 2B, 2C and 2D reveals in general structural loosening of cells including epidermis and parenchyma tissue. Untreated SCB micrograph (Fig 2A) shows a disrupted structure derived from previous operations of milling and washing, original from sugarcane processing.

Although the whole pretreated material is heterogeneous and contains larger pieces easily recognized as SCB, Fig 2B displays a partial defibration and the exposition of vascular bundles (phloem and xylem) after acid pretreatment.

Moreover, in relation to alkaline pretreatment (Fig 2C), it can be observed a highlighted exposure of mycrofibrilar cellulose structure derived from the solubilization of lignin and hemicellulose.

Finally the acid/alkaline pretreatment (Fig 2D) promoted a partial removal of mycrofibrils and the appearance of amorphous cellulose aggregates.

3.5 Enzyme production

Table 4 summarizes the enzyme activity obtained at the end of the solid state fermentations (SSF).

Table 4. Mean final values of enzymatic activities of *A. Niger* SSF.

Pre-treatment	CMCase	Xilanase	Fpase
	(UI/g)		
SCB	9,32	23,20	0,30
NaOH	2,34	12,30	0,07
H_2SO_4	13,70	14,86	0,16
NaOH+ H_2SO_4	1,72	9,87	0,03

Highest FPase and xylanase activities indicate the more efficient fungal

utilization of natural SCB, yielding 2-fold increase of these enzyme productions when compared to the second better substrate (acid pretreated SCB).

On the other hand, CMCase activities were the highest in SSF of acid pretreated SCB. As established by chemical compositions, the acid pretreatment solubilized hemicellulose favoring its conversion to easily useful sugars for the fungi growth and endoglucanase production. Nevertheless, the lower FPase and xylanase fungal expression in comparison with natural SCB could be attributed to the specific inhibition by some other sugars formed too as a consequence of the pretreatment (Pathak and Ghose, 1973; Holtzapple *et al.*, 1990).

The lowest activities in alkali and combined pretreated SCB could be due to absorption of cellulase on cellulose with high porosity (Sutcliffe and Saddler, 1986; Chernoglazov *et al.*, 2008) and/or to inhibition by the presence of sugars degradation compounds. It has been documented that furfural, hydroxymethylfurfural and aromatic lignin degradation compounds inhibit fungi growing (Adsul *et al.*, 2005).

However combined treatment leads to significant concentration of cellulose. The difference in cellulase activities may be due to variation in the amounts of utilizable amorphous components present in treated samples and expressed as an increasing in the % IC of the remaining cellulose. These observations led to the conclusion that physical structure of bagasse cellulose is an important factor in the SSF for the production of cellulases.

4. CONCLUSSIONS

Between the substrates evaluated; untreated SCB represent the most efficient and economical substrate for FSS and a feasible alternative to save costs on the enzyme production process.

Although, partial hemicellulose and lignin removal is an important factor in increasing the digestibility of FSS substrates, it is shown that the more cellulose remains undegraded the more suitability for fungal uptake.

Finally, the study shows the relevance of the washing stage in the pretreatment process in order to extract fungal and yeast toxic substances that may have been formed.

5. REFERENCES

ADSUL, M.G.; GHULEB, J.E.; SINGHB, R.; SHAIKHB, H. Enzymatic hydrolysis of delignified bagasse polysaccharides. *Carbohyd Polym.*, v. 62, p.6-10, 2005.

AOAC (1980). *Official Methods of Analysis*, 13th edn. Association of Official Analytical Chemists, Washington, D.C.

ALVIRA, P.; TOMAS-PEJO, E.; BALLESTEROS, M.; NEGRO, M.J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technol.*, v. 101, p. 4851-4861, 2010.

BAILEY, M.J.; BIELY, P.; POUTANEN, K., Interlaboratory testing of methods for assay of xylanase

activity. *J Biotech*, v. 23, p. 257-270, 1992.

CARDONA, C.A.; QUINTERO, J.A.; PAZ, I.C. Production of bioethanol from sugarcane bagasse: Status and perspectives, *Bioresource Technol.*, v. 101, p. 4754-4766, 2010.

COURI S., FARIAS A.X. Genetic manipulation of *Aspergillus niger* for increased synthesis of pectinolytic enzymes. *Rev Microbiol* 1995; 26:314–7.

CHERNOGLAZOV, V. M., ERNOLOVA, O. V. & KLYOSOV, A. A. Adsorption of high purity endo-l,4-β-glucanases from Trichoderma reesei on components of lignocellulosic materials: cellulose, lignin and xylan. *Enzyme Microbial Technol.*, v. 10, p. 503-507, 2008.

CURTY, Marlene Gonçalves; CRUZ, Anamaria da Costa; MENDES, Maria Tereza Reis. Apresentação de trabalhos acadêmicos, dissertações e teses: (NBR 14724/2002). Maringá: Dental Press, 2002.

Fengel, D.; WEGENER, G. *Wood Chemistry, Ultrastructure, reactions*, Berlin: Walter de Gruyter, 1989.

FISCHER, E. H.; STEIN, E. A. *Biochem Preparat.*, v. 8, p. 27–33, 1961.

GESSESSE, A.; MAMO, G. Purification and characterization of an alkaline xylanase from alkalophilic Micrococcus sp. AR-135, *J. Ind. Microbiol. Biotechnol.* v. 20, p. 210–214, 1998.

GHOSH, T. K. Measurement of cellulose activities. *Pure Appl Chem.*, v. 59, p. 257–268, 1987.

HENDRIKS, A. T. W. M.; ZEEMAN, G. Pretreatments to enhance the digestibility of lignocellulosic biomass-Review. *Bioresource Technol.*, v. 100 p. 10–18, 2009.

HIMMEL, M. E.; DING, S. Y.; JOHNSON, D. K.; ADNEY, W. S.; NIMLOS, M. R.; BRADY, J. W.; FOUST, T. D. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. *Science*, v. 315, p 804-807, 2007.

HOLTZAPPLE, M.; COGNATA, M.; SHU, Y.; HENDRICSON, C. Inhibition of T. reesei cellulase by sugars and solvents. *Biotechnol. Bioengng*, v. 36, p. 275-287, 1990.

HULLEMAN, S. H. D.; VANHAZENDONK, J.M.; VANDAM, J. E. G. Determination of crystallinity in native cellulose from higher-plants with diffuse-reflectance Fourier-transform infrared-spectroscopy. *Carbohydr Res.*, v. 261, p. 163-172, 1994.

KRISTENSEN, J.; THYGESEN, L.; FELBY, C.; JORGENSEN, H.; ELDER, T. Cell-wall structural changes in wheat straw pretreated for bioethanol production. *Biotechnol Biofuels.*, v. 1, p. 1754-6834, 2008.

LYND, L. R. Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy. *Annu Rev Energy Environ.*, v. 21, p. 403–465, 1996.

LYND, L. R.; ELANDER, R. T., & WYMAN, C. E.. *Appl Biochem Biotech.*, v. 57, p. 741–761, 1996.

MANDELS, M.; WEBER, J. The production of cellulases. *Adv. Chem.*, v. 95, p. 391–414, 1969.

MANDELS, M.; HONTZ, L.; NYSTROM, J. Enzymatic hydrolysis of waste cellulose. *Biotech Bioengng*, v. 16, p. 1471–1493, 1974.

MADAMWAR, D.; PATEL, S.; PARIKH, H. Solid-state fermentation for cellulase and b-glucosidase production by A. niger, *J. Ferment. Bioengng.*, v. 67, p. 424-426, 1989.

MILLER, G. L. Use of dinitrisalicylic acid reagent for determination of reducing sugar, *Anal. Chem.* v. 31, p. 426–428, 1959.

PATHAK, A. N.; GHOSE, T. K. Cellulases-1 Sources technology, *Process Biochem.*, v. 8, p. 35-38, 1973.

SHAH, A. R.; MADAMWAR, D. Xylanase production under solid state fermentation and its characterization by an isolated strain of Aspergillus foetidus in India, *World J. Microbiol. Biotechnol.*, v. 21, p. 233–243, 2005.

SILVERSTEIN, R. A.; CHEN, Y.; SHARMA-SHIVAPPA, R. R.; BOYETTE, M. D.; OSBORNE, J. A. Comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technol.* v. 98, p. 3000–3011, 2007.

SEGAL, L.; CREELY, J. J.; MARTIN, A. E.; CONRAD, C. M. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Text. Res. J.* v. 29, p. 786–794, 1959.

SUTCLIFFE, R.; SADDLER, J. N. The role of lignin in the adsorption of cellulases during enzymatic treatment of lignocellulosic material. *Biotechnol. Bioengng Syrup.* v. 17, p. 749-762, 1986.

TILMAN, D; SOCOLOW, R.; FOLEY, J. A.; HILL, J.; LARSON, E.; LYND, L.; PACALA, S.; REILLY, J.; SEARCHINGER, T.; SOMERVILLE, C.; WILLIAMS, R. Beneficial biofuels-the food, energy, and environment trilemma, *Science*, v. 325, p. 270–271, 2009.

VAN SOEST, P. J.; WINE, R. H.. Use of detergents in the analysis of fibrous feeds: IV Determination of plant cell wall constituents. *J. AOAC*, v. 50, p. 50-55, 1967.

XU, Z. H.; BAIL, Y. L.; XU, X; SHI, J. S.; TAO, W.I. Production of alkali-tolerant cellulose free xylanase by Pseudomonas sp.UN024 with wheat bran as the main substrate, *World J. Microbiol. Biotechnol.* v. 21, p. 575–581, 2005.

WENG, J.; LI, X.; BONAWITZ, N. D.; CHAPPLE, C. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. *Curr Opin Biotech.*, v. 19, p. 166–172, 2008.

WISTARA, N.; ZHANG, X.; YOUNG, R. A. Properties and treatments of pulps from recycled paper. Part II. Surface properties and crystallinity of fibers and fines. *Cellulose*, v. 6, p. 325-348, 1999.

ZHAO, X. B.; WANG, L.; LIU, D. H. Peracetic acid pretreatment of sugarcane bagasse for enzymatic hydrolysis: a continued work. *J Chem Tech Biotech*, v. 83, p. 950-956, 2008.