MICROSCOPIC ANALYSIS OF SUGARCANE BAGASSE FOLLOWING CHEMICAL AND FUNGAL TREATMENT

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Sugarcane is widely used as a raw material for producing sugar and alcohol. Bagasse is generated as a by-product and it is suitable for further degradation and fermentation for fuel generation. In the present study, the efficiency of alkaline pretreatments in the degradation of bagasse is assessed through microscopic analysis. Results reveal that the alkaline treatments expose the cellulose and hemicellulose residues in the bagasse fiber, enabling further fungus establishment. *Penicillium* colonization in the open fibers enhanced the biodegradation of sugarcane bagasse. The proposed methodology allows a detailed investigation of the deterioration process in the bagasse fibers – a vital step for biofuel production – as well as a comprehensive assessment of combined treatments.

Keywords: alkaline pretreatment, lignin, *Penicillium*, sugarcane

INTRODUCTION

The economic importance of sugarcane is directly related to its primary products (sugar and alcohol) and secondary products (bagasse, vinasse, yeast *etc*.). Recently, sugarcane bagasse has been vastly used in wastewater treatment, energy co-generation, cellulose production and as fertilizer.[1] As a lignocellulosic material, it mainly consists of cellulose (41-44%), hemicellulose (25-27%) and lignin (20-22%), as well as small amounts of unidentified compounds.[2-4] The main feature of sugarcane bagasse is its high fiber content, which depends on the genotype of the cultivated plant and is directly correlated to the biomass produced by this culture.[5] Therefore, the great amount of fibers produced can be used as a substrate for the action of chemical and biological agents, to promote degradation of simple sugars for fermentation and alcohol production.

Fiber transformation can be achieved using biological agents that act on its structure enzymatically. However, the susceptibility of the lignocellulosic residues to enzymatic attacks depends on the accessibility of cellulose and hemicellulose.[6] In order to reach optimal conditions for enzymatic activity, lignocellulosic materials are exposed to pretreatment processes that seek to modify the fibers structurally, allowing a higher rate and extent of hydrolysis.

Sodium and calcium hydroxides are commonly used alkaline treatments. The application of these compounds results in the breakage of the lignin structure, promoting fiber reactivity, hydration, cellulose swelling and reduction in crystallinity.[7-9] These treatments are widely used in the delignification of a variety of materials to enhance the digestibility of the

compounds used in animal food.[10-13] An effective pretreatment process should disrupt the crystalline structure, promoting a shift in the superficial area of the cellulose fiber, reducing the protective lignin association and maintaining the fiber capillary structure.[7-14] There are several methods available for lignocellulosic pretreatment, such as physical, physico-chemical, chemical and biological, or even a combination of these methods.[7-8] Nevertheless, none of them was analyzed combined with further biological degradation of modified bagasse fiber. This technical note seeks to fulfill this gap and to analyze microscopically the differences found in the fiber structures of sugarcane bagasse pretreated with alkali chemical agents ($Ca(OH)_2$, NaOH and NaOH + $Ca(OH)_2$) and assess the efficiency of *Penicillium* sp. colonization of the fibers upon treatment.

EXPERIMENTAL

Chemical treatments of sugarcane bagasse

Sugarcane bagasse samples were obtained from Raizen Sugar Plant (Piracicaba – SP, Brazil) from the 2006/2007 harvest season. The bagasse was air-dried and separated into appropriate Erlenmeyer flasks. The proportion of bagasse to chemical solution was 1:5 w/v. Three chemical treatments were prepared as follows: 1) addition of 4% sodium hydroxide (NaOH); 2) addition of 4% calcium hydroxide ($Ca(OH)_2$), and 3) addition of 4% NaOH and 4% $Ca(OH)_2$. All chemical compounds were of analytical grade and purchased from Merck. A control sample was prepared with bagasse and distilled water. The flasks were all autoclaved for 20 minutes (1.0 atm, 121 °C) and cooled to room temperature before further handling.

Samples were rinsed with sterilized distilled water and neutralized (pH between 5.5 and 6.0) with a solution of sulfuric acid (1 M H_2SO_4) or hydrochloric acid (0.5 M HCl 0.5). Samples were submitted to drying at 60 °C for 48 hours, after which the samples were prepared for microscopy and fungus inoculation.

Biological treatment and lignocellulolytic enzyme assay

The biological treatment with *Penicillium* sp. was carried out on all the samples treated as described above. Culture medium disks of 5 cm diameter, containing mycelia of the isolated fungus, were used to inoculate the samples after their neutralization and drying. The samples were then incubated at 27 °C for 15 days, then prepared for analysis.

The activity of laccase was determined using a mixture containing 300 µL of a 0.05 M citrate-phosphate buffer with pH 5.0; 100 µL solution of 0.1% solution of syringaldazine in ethanol and 600 µL of the enzyme supernatant. The oxidation of syringaldazine was measured by monitoring the absorbance at 525 nm after 10 minutes of reaction. A unit of the enzymatic activity was defined as the quantity of enzyme necessary to oxidize 1.0 µmol of syringaldazine per minute, utilizing the molar extinction coefficient of 6.5 x 10^4/mol/cm for oxidized syringaldazine. The laccase activity was expressed in international enzyme unit per liter (U/L) of lignocellulolytic enzyme extract.[15]

The manganese peroxidase (MnP) activity was determined by evaluating the oxidation of phenol red (ϵ_{610} = 4460 mol cm^{-1})[16] in the presence of manganese and H_2O_2 at 30 °C in a solution of 100 µL of 0.25 M sodium-lactate buffer; 50 µL of 0.002 M $MnSO_4$; 50 µL of H_2O_2 in a 0.2 M sodium-succinate buffer with pH of 4.5; 200 µL 0.5% bovine serum albumin and 600 µL of the enzyme extract. The reaction was initiated with the addition of 100 µL of 0.1% phenol red and the absorbance was measured at 610 nm. 40 µL of 2.0 N NaOH was added to stop the reaction 10 minutes after the first reading. One unit of enzyme activity was defined as the quantity of enzyme necessary to form 1.0 µmol of Mn^{+3} per minute, using the molar extinction coefficient of 4.460 x 10^3/mol/cm.[16] The MnP activity was expressed in international enzyme unit per liter (U/L) of the lignocellulolytic crude extract.

All absorbance readings were performed by a FEMTO-432 spectrophotometer in triplicates.

Scanning and transmission electron microscopy

Sample preparation for scanning and transmission electron microscopy was carried out according to the methodology described by Dellamatrice.[14] For transmission electron microscopy (TEM), samples were incubated for 3 hours in a solution containing 2% glutaraldehyde, 2% paraformaldehyde, 0.001 M $CaCl_2$ and 0.05 M sodium cacodylate, of pH 7.2. Three transfers of 15 min in 0.1 M sodium cacodylate buffer, followed by fixation in 1% OsO_4 for 2 hours at 4 °C, were carried out. After fixation, the material was washed three times (5 minutes each) in a saline solution (0.9% NaCl) and stained using a 2.5% uranyl acetate solution for 16 hours at 4 °C. The samples were then dehydrated in increasing concentrations of acetone every 5 minutes (25, 50, 75, 90 and 100%). The material was then immersed in Spurr resin in increasing ratios to acetone at 1:1, 2:1 pure resin, and then stored for 48 hours at 60 °C. Sections were cut in an ultramicrotome and dyed with 2.5% uranyl acetate for 10 min and lead citrate for 6 minutes.

For scanning electron microscopy (SEM), samples were treated as described above. However, after the acetone dehydration process, samples were submitted to drying to a critical point, placed over stubs and covered with a 200 nm gold film.

Analysis of lignocellulosic material

Seeking to verify the efficiency of chemical treatments, lignin, cellulose and hemicellulose levels

were determined for all chemically treated samples, the control (bagasse + distilled water), as well as a negative control (pure non-treated bagasse). Analyses were carried out at the Zootechnical Institute of the University of São Paulo (Nova Odessa – SP, Brazil), following the methodology of Goering and Van Soest,[17] and Van Soest.[18]

RESULTS AND DISCUSSION

Disruption of bagasse fibers

An initial observation of the control treatment (bagasse + distilled water) shows the bagasse structural fibers intact and the absence of any fungal growth (Fig. 1). This confirms that only a thermal treatment is not enough to alter the material or to induce fiber breakage. After the treatment of the bagasse with NaOH, it is possible to clearly observe modifications in the superficial area of the bagasse fibers (Fig. 2A, B and C). Previous studies describe that NaOH pretreatments can not only break the lignin structure, but also hydrate and swell the cellulose fibers, reducing crystallinity.[9,19-23]

Slightly less disintegration is observed in the bagasse treated with calcium chloride. Small stratifications in the fibers treated with $Ca(OH)_2$ are visible (Fig. 2D, E and F).

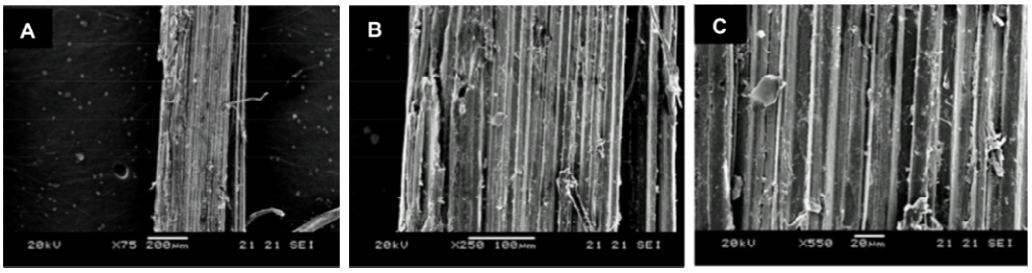

Figure 1: Cellular structures of sugarcane bagasse at (a) 75X, (b) 250X and (c) 550X – before chemical and biological treatments

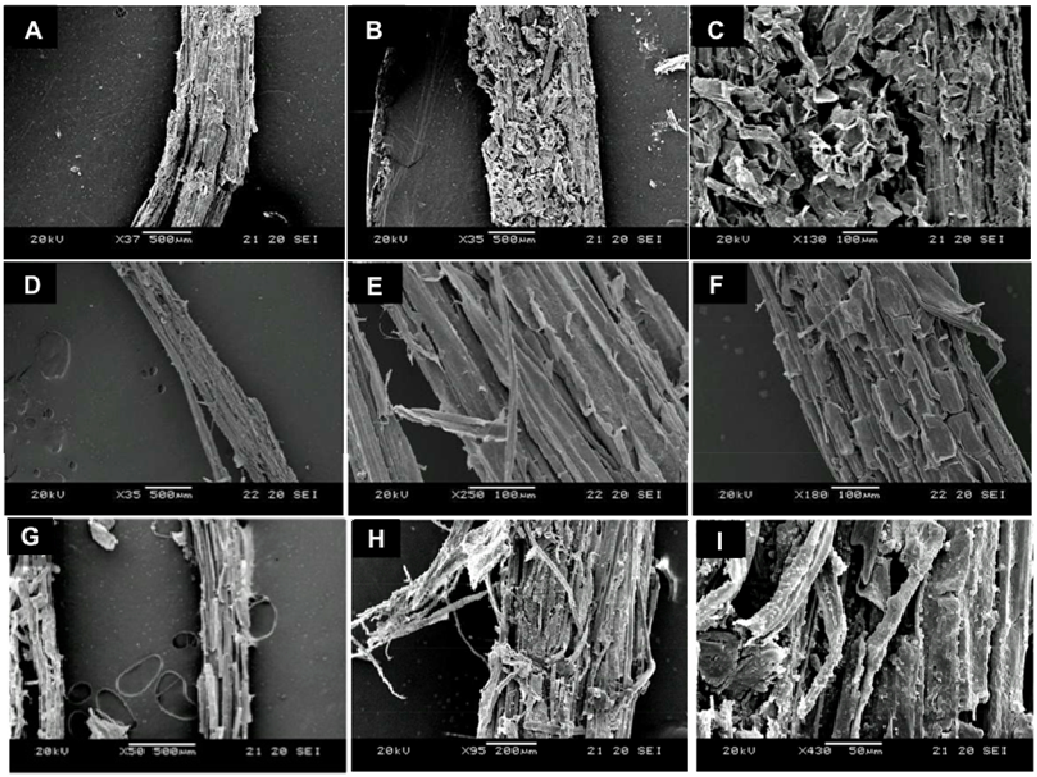

Figure 2: SEM images of chemically treated sugarcane bagasse: NaOH (A, B, C); $Ca(OH)_2$ (D, E, F) and NaOH + $Ca(OH)_2$ (G, H, I)

Table 1
Characterization of sugarcane bagasse with and without alkaline or acid treatment (as percentage of dry matter)

Samples	Cellulose, %	Lignin, %	Hemicellulose, %
Negative control (pure non-treated bagasse)	54.55	10.44	26.75
Positive control (bagasse + distilled water)	40.28	8.57	23.00
Bagasse + NaOH	48.57	8.26	12.32
Bagasse + $Ca(OH)_2$	42.06	10.68	13.00
Bagasse + NaOH + $Ca(OH)_2$	50.28	6.06	6.08

The extraction of hemicellulose and lignin by alkaline solutions promotes modifications in the fibers' chemical composition, as a consequence of cellular wall stratification – the fibers are seen in the image as composed of various layers. Nevertheless, the treatment carried out with the two bases combined (NaOH and $Ca(OH)_2$) showed an intense disruption in the capillary structure of the bagasse fibers. This combined treatment caused changes in the superficial area of the cellulose fibers, while reducing the protective association of lignin (Fig. 2G, H and I). Although it may be noted that the fibers seem to be aggregated, they are very brittle, easily collapsing, and show no symmetry. The outcome is associated with the synergetic effect of the alkali solutions used, which altered the lignin and cellulose structure simultaneously. Additionally, in these SEM images it is also possible to detect an increase in fiber porosity, promoted by the removal of waxy compounds.

Composition of treated bagasse fibers

There is a correlation between the chemical treatments used and a higher degradation of fibers as presented in Table 1. As regards the cellulose, lignin and hemicellulose content in the bagasse fibers, a reduction in the content of lignin and hemicellulose is obtained after all the chemical treatments. Nonetheless, the combined alkali treatment of NaOH + $Ca(OH)_2$ promoted the sharpest reduction in the amount of lignin (42%) and hemicellulose (77%), while keeping a high cellulose content. It has been shown that hemicellulose presents higher solubility in alkaline medium,[24-26] which recommends this alkali treatment as an alternative to promote the susceptibility of lignocellulosic residues to enzymatic attack.[27-29] It is worth highlighting that the combined action of alkaline compounds and the heat treatment has a fundamental role in the disruption of fibers, increasing considerably the superficial area and, therefore, its susceptibility to enzymatic hydrolysis.

Enzymatic activity of *Penicillium* sp.

Comparing the control fiber (bagasse + distilled water) shown in Figure 1 and the chemically treated samples (Fig. 2), it is evident that the material is free of fungal contamination. This is basically due to the autoclaving process, as well as to the highly basic environment after the treatment. Upon addition of *Penicillium* sp. to the samples, fungal development spread over the sugarcane bagasse can be clearly seen in microscopy images (Fig. 3A and B). The fungus can exploit the compounds released in the substrate for its own development. Furthermore, the reproductive structures of the fungus are seen in the interior of the cells (Fig. 3C-1, C-2, C-3), in addition to the presence of hyphae in the inter-cellular spacing (Fig. 3C-4). Biodegradation of lignocellulosic materials, especially by *Penicillium* sp., is highly associated with extracellular enzyme activities.[30] These lignocellulolytic enzymes initially depolymerize the lignocellulosic matrix into smaller fragments suitable for intracellular transportation and metabolizing by the fungus.[30-32] *Penicillium* secretes non-specific oxidoreductases, among which, laccase and manganese peroxidase (MnP) are thought to play a crucial role in the initial lignin depolymerization. These enzymes are capable of oxidizing and depolymerizing natural and synthetic lignins, as well as entire lignocelluloses (milled straw or wood, pulp) in cell-free systems.[4,7,21] Therefore, an increase in activity in the treated samples is expected. In fact, increases in the activities of MnP in the chemically treated samples inoculated with *Penicillium* are detected in Figure 4A. Remarkably, laccase activity (Fig. 4B) in the samples subjected to the combined treatment was similar to the control, while the sample treated

with $Ca(OH)_2$ alone yielded higher activities. The synthesis and secretion of laccases and peroxidases are strictly influenced by nutrient levels, culture conditions, developmental stage, as well as the addition of a wide range of inducers to the culture media.[24,30,31] Nonetheless, the occurrence of lignocellulolytic degradation is corroborated with the high enzymatic activity of the fungus.

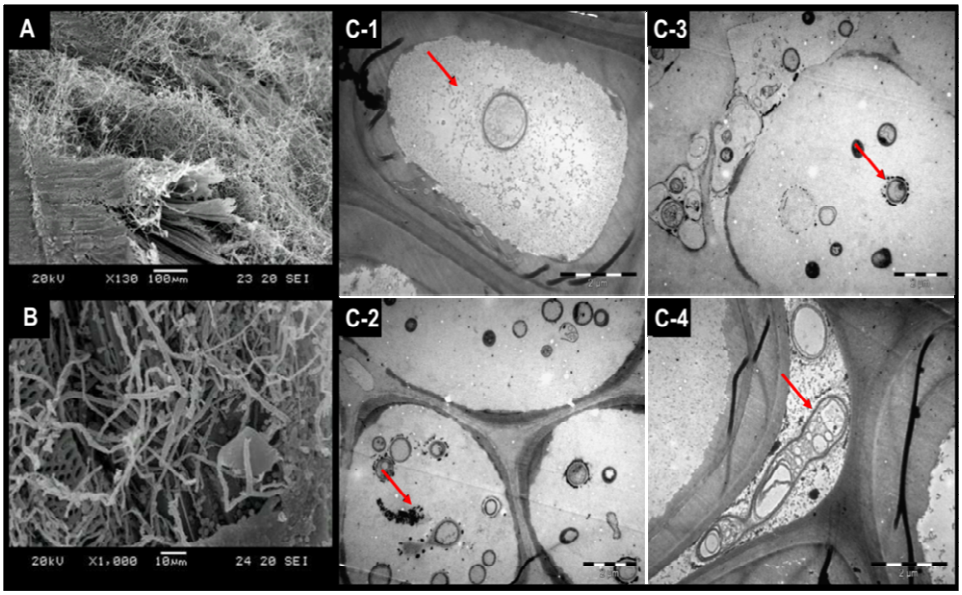

Figure 3: Bagasse chemically treated with NaOH + $Ca(OH)_2$ colonized with *Penicillium* sp. (A, B); TEM observation of *Penicillium* sp., on the same sample, its reproductive structures in the cells (C-1, C-2, C-3) and in the intercellular spacing (C-4)

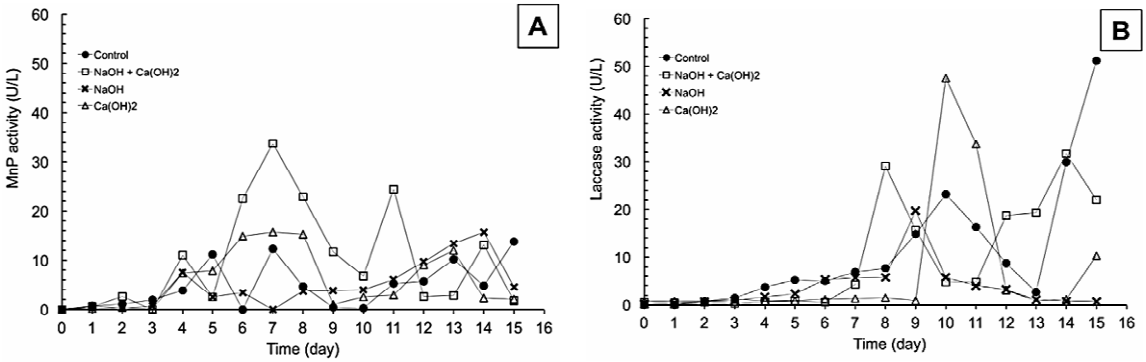

Figure 4: Enzyme activities of manganese peroxidase (A) and laccase (B) for *Penicillium* sp.

CONCLUSION

Although several treatment methods are applicable for preparing sugarcane bagasse for enzymatic hydrolysis, the importance of further knowledge concerning differences among the pretreatment options and the effect of each process on the remaining operations is crucial. The disruption in the bagasse fibers varies in accordance with the chemical treatment applied. Further, the use of *Penicillium* as a biological agent is effective in converting complex compounds as lignin and cellulose into simple sugars. These sugars possess great economic value in fermentation processes and ethanol production. The results obtained in this technical note support the hypothesis that an optimization of sugarcane bagasse treatments is possible through the combined action of chemical and

biological agents, considerably improving bioethanol productivity from bagasse fibers.

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