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Enzymatic Bleaching of Organosolv Sugarcane Bagasse Pulps with Recombinant Xylanase of the Fungus *Humicola grisea* and with Commercial Cartazyme HS Xylanase

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Abstract

Organosolv (ethanol/water and acetosolv) pulps were treated with *Humicola grisea* var. *thermoidea* and compared with Cartazyme HS xylanase–treated pulp. The ethanol/water pulps treated with *H. grisea* had the same viscosity as unbleached pulps (8 cP). Ethanol/water pulps treated with Cartazyme had higher viscosity than *H. grisea*–treated pulps (12 cP). Acetosolv pulps treated with *H. grisea* and Cartazyme presented a reduction in viscosity; however, the pulps treated with *H. grisea* had a lower reduction in viscosity than Cartazyme-treated pulps. Ethanol/water pulps treated with *H. grisea* had a 23% reduction in kappa number in 4 and 8 h of treatment, compared with the unbleached pulps. Cartazyme-treated pulps had a kappa number similar to that of the control pulps for 4 h of treatment. Extending the treatment time to 12 h resulted in a reduction of 33%. The acetosolv pulp treated with *H. grisea* had a kappa number reduced to 23% in 4 h. Cartazyme treatment resulted in a reduction of 55 and 44% in kappa number for 4 and 8 h of treatment, respectively, when compared with control pulp. Extending the treatment time to 12 h decreased the kappa number 72%. Fourier transform infrared spectra and principal component analysis showed differences among unbleached, *H. grisea*–treated, and Cartazyme-treated pulps.

Index Entries: *Humicola grisea*; sugarcane bagasse pulps; cartazyme; viscosity; xylanase.

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Introduction

Environmental and legislative pressures have forced the pulp and paper industry to modify pulping, bleaching, and effluent treatment technologies in order to reduce the environmental impact of mill effluents *(1)*.

Worldwide the most utilized process of wood transformation into paper is the kraft process *(2)*. After cooking, the yellow/brownish kraft pulp must be bleached before paper production. This is accomplished by removing the residual lignin in a multistage bleaching process using chlorine, chlorine dioxide, and alkaline extraction *(3)*. Organic chlorine byproducts formed during these processes are toxic and, consequently, alternative processes have been studied to reduce and/or eliminate the use of chlorine and chlorine dioxide *(4)*.

Biobleaching and bioprocessing of pulps using xylanases is one of the most suitable applications to be used in the pulp and paper industry *(5)*. In biobleaching, it is essential that enzymic preparations not show cellulase activity; otherwise, it would cause morphologic changes in the cellulose fibers, which would reduce the ultimate quality of paper *(6)*.

Microorganisms with the greatest amount of xylanase need to be identified in order to improve the efficiency of pulp bioprocessing. The thermophilic fungus *Humicola grisea* var. *thermoidea* isolated from Brazilian soil *(7)* produces several hydrolytic enzymes including cellulases and xylanases. Molecular cloning of two *H. grisea* xylanase genes has been reported. Faria et al. *(8)* isolated and characterized a gene (*xyn2*) encoding a putative 23-kDa xylanase protein that belongs to the glycosyl hydrolase family G/11.

The aim of the present work was to study the bleaching of organosolv sugarcane bagasse pulps with *H. grisea* xylanase and to compare this method to commercial enzymatic treatment.

Materials and Methods

Acetosolv Pulping

Acetosolv sugarcane bagasse pulping was carried out with 93% acetic acid (v/v), with HCl as catalyst according to Benar *(9)*. The bagasse/solvent ratio was 1:14 (w/v). Pulping was done at 110°C (temperature of solvent mixture) for 2 h. The pulp was washed with 93% acetic acid (v/v) and then washed thoroughly with water until the wash water attained neutral pH.

Ethanol/Water Pulping

Ethanol/water pulping of sugarcane bagasse was performed in a 200-mL closed vessel using a 1:1 (v/v) ethanol/water mixture, pH 7.0, with a bagasse-to-solvent ratio of 1:10 (m/v). The cooking time was 2.5 h, and the temperature was 185°C. The pulp was filtered and washed with 2500 mL of ethanol/water according to Gonçalves and Ruzene *(10)*.

Xylanase Assay

Xylanase from *H. grisea* var. *thermoidea* was supplied by Faria et al. *(8)*. Xylanase was assayed as described by Bailey et al. *(11)* by incubating the diluted enzyme solution (citrate buffer, pH 4.8) at 50°C for 5 min using a substrate solution of 1% (w/v) birchwood xylan (Roth, Karlsruhle, Germany). One unit of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of xylose equivalents/min of reaction.

Xylanase Pretreatment of Pulp

Samples of acetosolv or ethanol/water pulp with 3% consistency (w/w) were incubated in Erlenmeyer flasks in a shaker at 50°C for 4, 8, and 12 h. One set of samples was incubated with 18 IU of xylanase obtained from the fungus *H. grisea* var. *thermoidea*, and another set was incubated with 18 IU of Cartazyme HS (Sandoz, Leeds, UK). After incubation, the pulp was filtered in a Büchner funnel, and the pulp was washed thoroughly with distilled water. One set of samples with 3 g (dry wt) of enzymatically pretreated bagasse pulp was placed in Erlenmeyer flasks and treated with 2% NaOH at 60°C for 1 h. The pulp was filtered and washed with distilled water. Standard samples were incubated without enzyme. Samples were also treated with NaOH under the same conditions as just described; these were control pulps. All the experiments were carried out independently in triplicates, and the results presented are the mean of the three. The standard deviations (SDs) were within 5%.

Estimation of Kappa Number

Samples were exposed to 0.1 *N* $KMnO_4$ at 25°C for 10 min. The reaction was stopped by adding excess KI solution. The consumed $KMnO_4$ was determined from the results of back-titrating the liberated iodine with standard sodium thiosulfate. The kappa number was obtained from the volume in milliliters of 0.1 *N* $KMnO_4$ consumed per gram of pulp *(12)*.

Determination of Viscosity

Viscosity was determined by dissolving bagasse pulp in cupriethylenediamine and measuring the viscosity of a 0.5% solution with an Ostwald viscometer *(13)*.

Determination of Chemical Compounds of Pulp

One gram of dry pulp was treated with 10 mL of 72% H_2SO_4 and stirred at 45°C for 7 min. The reaction was interrupted by adding 25 mL of distilled water, the mixture was transferred to a 250-mL Erlenmeyer flask, and distilled water was added to 140 mL. The flask was autoclaved for 30 min at 1.05 bar pressure for complete oligomer hydrolysis. The mixture was filtered and the filtrate (hydrolysate) completed to 250 mL. A 20-mL

sample of the hydrolysate was diluted to 25 mL, and the pH was adjusted to 2.0 with 2 mol/L of NaOH. After filtration in a Sep-Pak C_{18} cartridge to remove aromatic compounds, the hydrolysate was analyzed in an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, Hercules, CA) at 45°C using a Shimadzu chromatograph and refraction index detector. The mobile phase was 0.005 mol/L of H_2SO_4 at 0.6 mL/min. Sugar concentrations reported as xylan and glucan were determined using calibration curves of pure compounds. Lignin was determined by gravimetric analysis *(14)*.

Fourier Transform Infrared and Principal Component Analysis of Bleached and Unbleached Pulps

Fourier transform infrared (FTIR) spectra were directly obtained from the bleached and unbleached refined pulps utilizing attenuated reflexion technique under the conditions described by Faix et al. *(15)*. Spectra were recorded (16 scans) in a Perkin-Elmer spectrometer. After polygonal baseline correction *(15)*, the spectra were normalized by the absorption at 900 cm^{-1}, which corresponds to the anomeric carbon atom of the O-C-O group in polysaccharides and is not influenced by other groups *(15)*. The spectra were converted to text files using OMNIC software (Nicolet). The normalized absorbances in the range of 650–4000 cm^{-1} were submitted to principal component analysis (PCA) calculations using BIOTEC and FAEN programs compiled in FORTRAN, which were written in our laboratory based on the work of Scarminio and Bruns *(16)*. Graphic presentations were easily made using Microsoft EXCEL 5.0.

Results and Discussion

Viscosity and Kappa Number of Pulps

Varying the time of ethanol/water treatment did not affect the viscosity (8 cP) of the pulps treated with *H. grisea* (Fig. 1A). The enzymatically treated pulps had the same viscosity as unbleached pulps. Alkaline extraction after treatment of pulp with *H. grisea* increased viscosity by 12% compared with alkaline extracted pulp. Ethanol/water pulp treated with Cartazyme had higher viscosity than *H. grisea*–treated pulps in 8 and 12 h of treatment (12 cP) (Fig. 1A).

Acetosolv pulps treated with Cartazyme and *H. grisea* had the same viscosity at the three times studied, considering the SD. Acetosolv pulps treated with Cartazyme followed by alkaline extraction showed the higher viscosity (13 cP) in 8 h of treatment (Fig. 1B).

Ethanol/water pulps treated with *H. grisea* xylanase presented a 23% reduction in kappa number in 4 and 8 h of treatment, compared with the unbleached pulps (Fig. 2A). Ethanol/water pulp treated with Cartazyme presented the same kappa number (40) as *H. grisea*-treated pulp. Ethanol/water pulps enzymatically treated with *H. grisea* or Cartazyme

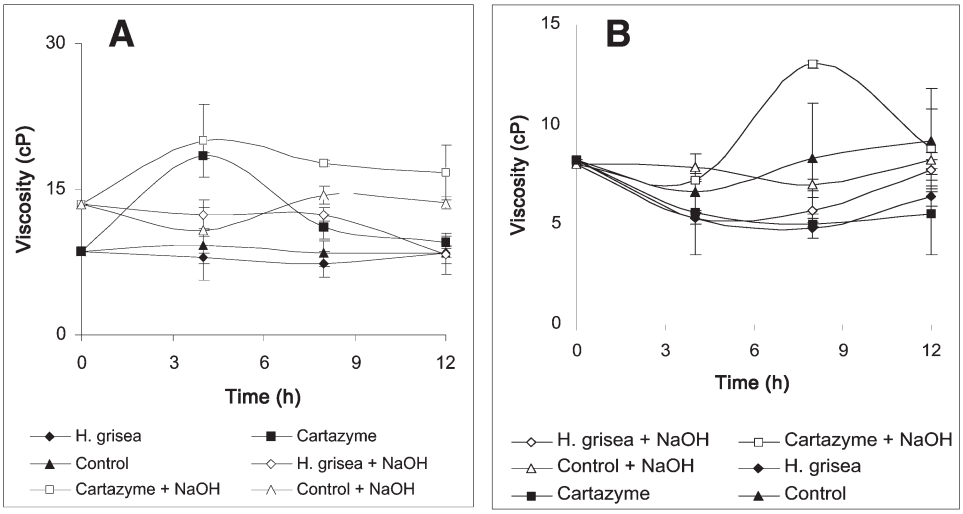

Fig. 1. Viscosity of unbleached Cartazyme and *H. grisea* sugarcane bagasse pulps: **(A)** ethanol/water pulps; and **(B)** acetosolv pulps.

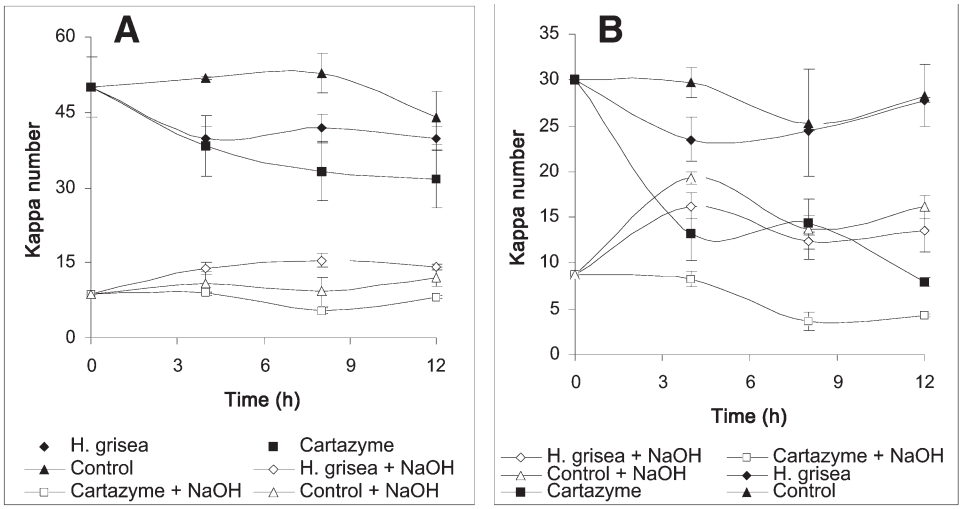

Fig. 2. The kappa number of unbleached, Cartazyme and *H. grisea* sugarcane bagasse pulps: **(A)** ethanol/water pulps; and **(B)** acetosolv pulps.

followed by alkaline extraction showed a reduction in kappa number of 80% compared with control pulps (Fig. 2A).

The acetosolv pulps treated with *H. grisea* showed a kappa number (30) similar to that of the control pulps (unbleached pulps). Cartazyme-treated pulps had a 57% reduction in kappa number when compared with unbleached pulps. Pulps treated with Cartazyme followed by alkaline extraction had the lowest kappa number (3.7) in 8 h of treatment (Fig. 1B).

Table 1
Chemical Composition of Ethanol/Water Pulps Treated with *H. grisea* and Commercial Xylanase

	H. grisea			Cartazyme		
Treatment[a]	Glucan (%)	Xylan (%)	Total lignin (%)	Glucan (%)	Xylan (%)	Total lignin (%)
X (4 h)	66.8 ± 4.8	9.6 ± 0.5	20.1 ± 0.7	72.2 ± 0.7	6.8 ± 0.1	18.5 ± 1.5
XE (4 h)	86.5 ± 2.1	5.4 ± 0.1	7.1 ± 0.6	87.8 ± 0.5	5.0 ± 0.1	6.5 ± 0.2
X (8 h)	62.4 ± 4.8	8.6 ± 0.5	25.0 ± 1.2	73.1 ± 0.1	7.5 ± 2.3	17.2 ± 1.4
XE (8 h)	88.1 ± 4.5	5.4 ± 1.0	5.6 ± 0.4	86.7 ± 3.0	5.8 ± 0.3	6.9 ± 1.0
XE (12 h)	68.0 ± 3.8	9.2 ± 0.1	20.5 ± 1.0	70.2 ± 3.2	9.4 ± 1.6	18.2 ± 0.3
XE (12 h)	83.8 ± 0.2	5.8 ± 0.1	9.3 ± 1.8	86.4 ± 0.7	5.7 ± 0.2	7.2 ± 0.8

[a]X, xylanase; E, alkaline extraction.

Salles et al. *(1)* treated eucalyptus kraft pulp with xylanase preparations of two different fungi: *H. grisea* and *Acrophialophora nainiana*. They applied 1.5, 2.5, and 5 IU/g of moisture-free pulp and observed that all the effects of enzyme application on cellulose fibers revealed by scanning electron microscopy were similar. Garg et al. *(17)* obtained comparable results. They observed an increase in brightness and a reduction in kappa number by increasing enzyme charges without significant morphologic changes among the pulp samples.

Analysis of Chemical Composition of Pulps

The ethanol/water pulps treated with *H. grisea* xylanase presented a chemical composition similar to that of the Cartazyme-treated pulps. The pulps treated during different times did not present differences in composition (Table 1).

Varying the treatment time in acetosolv pulps did not affect the composition of the pulps (Table 2). The similar chemical compositions in the acetosolv pulps treated with *H. grisea* and Cartazyme corresponded with the similar viscosity of the enzyme-treated pulps presented in Fig. 1.

FTIR and PCA of Pulps

FTIR spectra and multivariate analysis, namely PCA, were used to discriminate the different enzymes and treatment times of the sugarcane bagasse pulps. The spectra of unbleached and *H. grisea*–treated pulps are similar to those of Cartazyme-treated pulp followed by alkaline extraction (Fig. 3A). The ethanol/water and acetosolv are different pulps, presenting different composition and physical properties owing to the different processes of pulping. However, the spectra for ethanol/water and acetosolv are similar. In the spectra of ethanol/water and acetosolv pulps, there

Table 2
Chemical Composition of Acetosolv Pulps Treated with *H. grisea* and Commercial Xylanase

	H. grisea			Cartazyme		
Treatment[a]	Glucan (%)	Xylan (%)	Total lignin (%)	Glucan (%)	Xylan (%)	Total lignin (%)
X (4 h)	68.7 ± 1.2	6.6 ± 0.1	19.0 ± 2.4	61.3 ± 0.3	10.9 ± 0.1	20.7 ± 0.4
XE (4 h)	88.9 ± 0.6	5.3 ± 0.4	5.4 ± 1.3	79.3 ± 3.5	9.4 ± 1.4	9.1 ± 3.3
X (8 h)	64.3 ± 0.3	6.0 ± 0.1	23.7 ± 2.5	70.4 ± 2.7	7.9 ± 1.5	16.3 ± 1.7
XE (8 h)	88.2 ± 0.3	4.7 ± 0.5	5.8 ± 1.2	84.9 ± 4.3	7.8 ± 2.9	5.5 ± 1.0
X (12 h)	66.5 ± 3.1	6.8 ± 1.1	20.7 ± 1.2	68.3 ± 2.7	9.0 ± 0.1	16.7 ± 1.0
XE (12 h)	83.3 ± 4.6	5.6 ± 0.1	9.8 ± 1.5	87.9 ± 4.2	6.5 ± 0.3	4.0 ± 1.2

[a]X, xylanase; E, alkaline extraction.

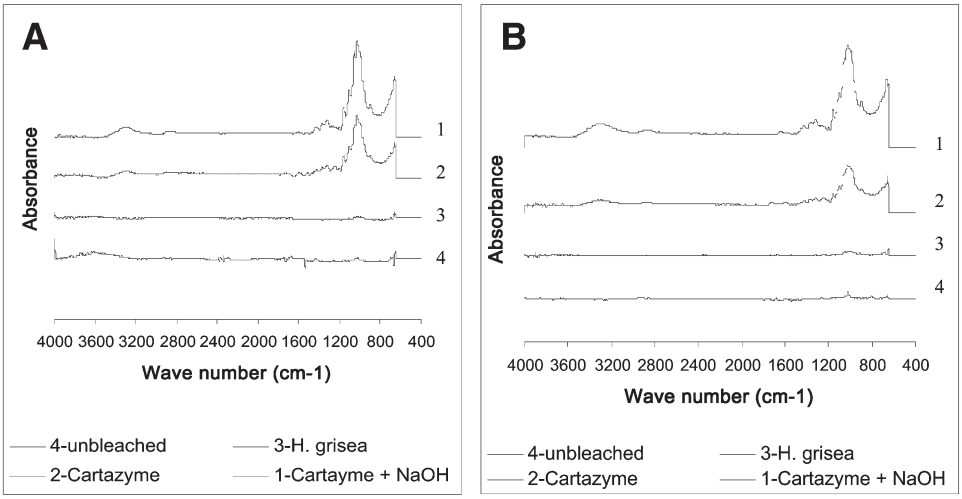

Fig. 3. FTIR spectra of sugarcane bagasse pulps: **(A)** ethanol/water pulps; **(B)** acetosolv pulps.

is a band at 1000 cm^{-1}, which is characteristic of the C-O bond in ester (Fig. 3).

The first three principal components (PCs) explain more than 94% of the total variance of the system. From the PC (biplot analysis), it is possible to differentiate the Cartazyme-treated pulps (enclosed in the oval in Fig. 4A) and unbleached pulps (enclosed in the circle in Fig. 4A). In Fig. 4B (PC2 × PC1 biplot), the Cartazyme-treated pulps are enclosed in the oval and the unbleached pulps are enclosed in the circle. The unbleached pulps and *H. grisea*–treated pulps did not present greater differences because the points were close in the PC2 × PC1 graph (Fig. 4B).

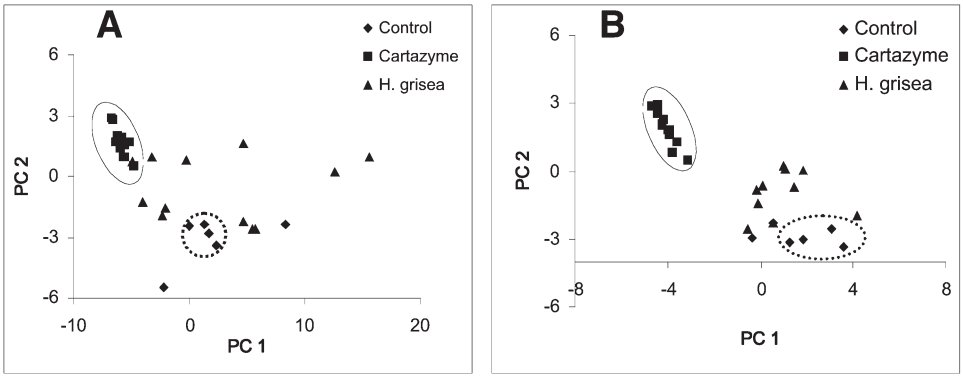

Fig. 4. Score values (PC 2 × PC 1) from FTIR spectra of *H. grisea*–bleached and Cartazyme xylanase–bleached and unbleached **(A)** ethanol/water and **(B)** acetosolv sugarcane bagasse pulps.

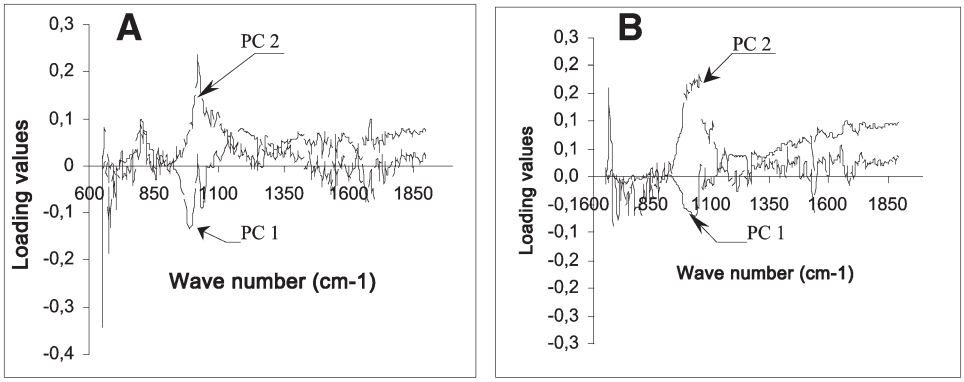

Fig. 5. (A) Ethanol/water and **(B)** acetosolv loading values of PCs 1 and 2 of FTIR spectra of bleached *H. grisea* and Cartazyme xylanase and unbleached bagasse pulps.

The loading values provide information about the bands' weight, and by the wave number, it is feasible to infer the modification in the pulps. From Fig. 5A, in the ethanol/water pulps, the influence of IR bands on PC scores can be evaluated. The plot of PC2 is infuenced by C-O (1022 cm^{-1}) bonds, characteristic of esters. From Fig. 5B, in the acetosolv pulps, PC2 is influenced by C-O (1026 cm^{-1}) bonds, also characteristic of esters. Ethanol/water and acetosolv pulps are different; however the loading values × absorbance plots are very similar.

Conclusion

H. grisea xylanase did not reduce kappa number as Cartazyme did. On the other hand, the viscosity of the pulps was not affected by treatment with *H. grisea* xylanase. *H. grisea* xylanase can be used to bleach pulps as an alternative commercial source of enzyme.

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