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Enzymatic and Fungal Treatments on Sugarcane Bagasse for the Production of Mechanical Pulps

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Crude ligninolytic enzyme extracts from *Phanerochaete chrysosporium* fungi were applied to sugarcane bagasse, prior to thermomechanical (TMP) and chemithermomechanical pulping (CTMP), and their properties were compared with the normal TMP and CTMP and also with TMP and CTMP pretreated with *Ceriporiopsis subvermispora* and *P. chrysosporium* fungi. The sugarcane bagasse was impregnated with the crude enzyme extract containing lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac). The results show that pretreatment with enzyme crude extract is an advantageous way to produce TMP and CTMP from sugarcane bagasse, as compared with only fungal pretreatment. Enzymatic pretreatments need only hours to enhance pulping and paper properties, compared with the weeks necessary for fungal treatments. Higher pulp yields were obtained compared with the fungal pretreatments. Enzymatic pretreatment reduced the energy consumption in a proportion similar to that of *C. subvermispora* fungal pretreatment and increased the pulp tensile index compared with the normal TMP and CTMP pulps, although the tensile strength was somewhat lower than that for pulps from *C. subvermispora* fungal pretreatment before CTMP processing. An advantage of enzymatic pretreatment is that brightness is increased compared with normal TMP and CTMP processes, whereas fungal pretreatments reduce the brightness.

KEYWORDS: Biotechnology; biomechanical pulping; enzyme treatment; white-rot fungi; mechanical pulps; pulping energy consumption; sugarcane bagasse

INTRODUCTION

The preservation of forests and increasing environmental awareness have focused research on the exploration of new, renewable fibrous resources and less toxic pulp and bleaching processes. Current mechanical pulping methods are popular because they produce high-yield pulps, which provide good optical properties for printing-grade papers. Mechanical pulp mills also require a much lower capital investment than chemical pulp mills. The major disadvantage of mechanical pulping processes is that they require large quantities of expensive electrical energy. The disadvantages of chemical pretreatments and high energy requirements have prompted interest in biological treatments prior to mechanical or chemical pulping (*1*). Studies so far have been generally based on the concept of application of a lignin-degrading fungi or an isolated enzyme to selectively remove or modify lignin. By requiring fewer pulping chemicals, biomechanical pulping has the potential to be less polluting than chemical and chemimechanical pulping processes.

Ligninolytic fungi secrete various oxidative extracellular enzymes—lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), laccase, and hydrogen peroxidase, which together with secondary metabolites and manganese form a complex extracellular system responsible for the ligninolysis (*2*). Numerous investigations on the use of fungi for the pretreatment of wood prior to mechanical refining have been reported, and considerable energy savings and enhancement in strength properties of aspen, pine, and poplar pulps have been realized by the use of white-rot fungi (*3*).

The use of fungi for nonwoody plants prior to chemical treatment or mechanical refining has also received attention in recent years. There have been investigations on the biopulping of wheat straw (*4*), kenaf, jute, and other nonwoody plants (*5*−*7*). In an earlier study we evaluated the biomechanical pulping of sugarcane bagasse with the white-rot fungi *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* (*8*). Also in later work, we have further evaluated the effects of *C. subvermispora* and

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a xylanase enzyme pretreatment for the production of biomechanical and biochemical pulps from sugarcane bagasse (*9*).

The main disadvantages of these treatments with microorganisms are the slow fungal growth and the yield loss due to the degradation of cellulose by the fungi. To overcome these problems, it is of interest to use isolated ligninolytic enzymes. One of the advantages of enzymatic treatment is that it takes only a few hours, which is a relatively short time, in comparison with the period of some weeks required by fungal treatment; this makes enzymatic delignification or modification industrially more feasible. In this investigation we have used a well-defibrated sugarcane bagasse after the sugar mill extraction and the mechanical depithing treatment, which allowed good accessibility to enzyme action (*10*).

In this study we evaluated the effects of crude ligninolytic enzymes extracts from the *Phanerochaete chrysosporium* fungi, prior to thermomechanical (TMP) and chemithermomechanical pulping (CTMP) of sugarcane bagasse, and compared the results with TMP and CTMP pretreated with *C. subvermispora* and *P. chrysosporium* fungi.

MATERIALS AND METHODS

Raw Material. Sugarcane bagasse, obtained from a sugar mill in Jalisco, Mexico, was depithed from 27.3% to a final pith content of 9.4%. Depithed bagasse was placed in plastic bags and frozen until use to prevent the growth of contaminating microorganisms.

Crude Enzyme Extract Preparation. The crude enzyme extract was produced from the white-rot fungi *P. chrysosporium*. The fungi were cultured in a basal liquid medium containing a mineral elixir with nitriloacetic acid and several minerals, ammonium tartrate as a nitrogen source (2 g/100 mL), KH_2PO_4 (20 g/100 mL), $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$ (1 g/100 mL), thiamin (5 mg/100 mL), and glucose (25 g/100 mL). The filtered pH of the medium was 6.5. Stationary cultures were carried out at 27 °C for 15 days to find the maximum enzyme activity, which was for 9 days, and this time was used to scale up the production of the crude extract. After the 9 days of culture, the extract was filtered, centrifuged, and refrigerated for 24 h.

Enzyme Activities. Enzyme activities were assayed spectrophotometrically in the UV−visible range using specific substrates: Lignin peroxidase (LiP) activity was measured by monitoring the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2 and sodium tartrate (pH 3) at 310 nm (*11*). Manganese peroxidase (MnP) activity was assayed by the oxidation of 2,6-dimethoxyphenol to its dimeric product, also in the presence of hydrogen peroxide, sodium tartrate (pH 4), and $MnSO_4$ at 469 nm (*12*). Laccase activity was measured in the presence of sodium tartrate (pH 3) and veratryl alcohol, but no hydrogen peroxide, with the activity measured at 310 nm.

Inoculum Preparation. The white-rot fungi *P. chrysosporium* BKM-F-1767 and *C. subvermispora* L-14807 SS-3 were obtained from potato dextrose agar (PDA) from the Center for Mycology Research at the UDSA Forest Products Laboratory, Madison, WI. The inoculum was prepared according to the Fischer protocol (*13*). PDA (39 g/L concentration) plate cultures were inoculated from these slants and then incubated at 27 °C and 70% relative humidity during 5 days for *P. chrysosporium* and during 10 days for *C. subvermispora*. The liquid culture medium was prepared in 2800 mL of water containing potato dextrose broth (PDB) and yeast extract as described previously (*8*).

Nutrient Medium. To increase the fungal biomass and suppress cellulose degradation, corn steep liquor from a starch-producing factory (Arancia S.A.) was used, as it contains proteins, lactic acid, and reducing sugars, described in a previous work (*9*). This nutrient medium was shown to be very effective during biomechanical pulping (*14*). It was added to the bagasse at a 1% oven-dry weight basis. A part of the mycelium, equivalent to 5 g of fungus per ton of dry weight of raw material, was added to the nutrient solution and then blended aseptically.

Raw Material Inoculation and Enzyme Treatment. The nutrient solution, with some water, was added to 115 g of bagasse with a known initial moisture content to increase the moisture to 70%. The mixture was autoclaved for 15 min at 121 °C. The bagasse was inoculated with *P. chrysosporium* or with *C. subvermispora* (in a laminar flow hood previously sterilized with ethanol at 70%) prior to incubation during 2 weeks at 27 °C. Other samples of bagasse were washed with 0.5% diethylenetriaminepentaacetic acid (DTPA, Sigma Aldrich Co.) on o.d. pulp and diluted to 5% consistency with distilled water and stirred for 30 min at room temperature. Samples of this bagasse (100 g) were autoclaved under the same conditions; 5 mL of hydrogen peroxide (30%) was added to activate peroxidase enzyme, which was then impregnated with the crude enzyme extract using a water-to-bagasse ratio of 8:1. The suspension was stirred for a few minutes to obtain a homogeneous pulp suspension. Residence times for the enzyme treatments were 12 h ($ETMP_{12}$ and $ECTMP_{12}$), 24 h ($ETMP_{24}$ and $ECTMP_{24}$), and 36 h ($ETMP_{36}$ and $ECTMP_{36}$) at 25−29 °C. All of the tests were made in triplicate.

Bioreactor Incubation. Several aerated static-bed bioreactors as described by Akhtar et al. (*15*) were used in this work. The bioreactors were sterilized with high-pressure steam prior to incubation. The bagasse (115 g) was placed in each bioreactor, and the inoculated material was incubated for 2 weeks at 27 °C and 70% relative humidity.

Pulping and Refining. The bagasse for the control TMP and CTMP, or after the fungal (BTMP and BCTMP) or enzyme treatment (ETMP and ECTMP), was cooked using a liquor-to-bagasse ratio of 4:1 at 130 °C for 20 min. The bagasse was cooked and defibrillated under pressure in the same equipment as described by Ramos (*16*). For CTMP, BCTMP, and ECTMP, 3% sodium hydroxide (98.2% Baker) and 4% sodium sulfite (98.0% Baker) were used. The pulp was refined in a 30 cm disk refiner, using disk model D2A 509NH at 5% consistency. Four different levels of refining were performed to around 25, 40, 55, and 70 °SR freeness. The energy consumption during refining was measured with a kilowatt/kilovar Amprobe ac meter, connected to the power supply of the electric motor. Energy consumption during the defiberizing step was not measured. Latency was removed before the pulp was screened in a diaphragm cleaner with a 0.15 mm wide slotted screen.

Hand Sheet Preparation and Evaluation. Hand sheets of 100 g/m^2 were made according to Tappi test method T-205. The sheets were evaluated for density, burst, tear, tensile, brightness, and opacity according to the corresponding Tappi test methods. Fiber classification was made in a Bauer McNett classifier according to Tappi test method T-233 cm-82.

RESULTS AND DISCUSSION

Although it has been stated that *P. crysosporium* fungus secretes other enzymes such as cellobiose dehyrogenase, endoglucanase, and β-glucosidase, the three main phenoloxidases produced are lignin peroxidase, manganese peroxidase, and laccasse (*17*). Therefore, we analyzed the activity of only these latter three enzymes. Crude enzyme extracts produced from the white-rot fungus *P. chrysosporium* showed a lignin peroxidase (LiP) activity of 300 units/L, a manganese peroxidase (MnP) of 250 units/L, and a laccase activity of 300 units/L. TMP obtained from bagasse impregnated with the crude enzyme extract during 12, 24, and 36 h at 25−29 °C showed an average yield of 89.5% compared with a yield of 94% when no enzyme pretreatment was made on the bagasse. There was no difference in yield for the three different treatment times.

When bagasse is pretreated with the white-rot fungi *P. chrysosporium* BKM-F-1767 and *C. subvermispora* L-14807 SS-3 during 2 weeks at 27 °C, the pulp yield is reduced more drastically. **Figure 1** shows the different yields for mechanical pulps with and without enzymatic and fungal treatment. TMP gave the highest yield of 94%, whereas enzymatically pretreated bagasse (ETMP) gave a 89.5% yield. Enzymatic pretreatment does not reduce the yield much when used in a mixture as a crude enzyme extract. It has been proposed that MnP oxidizes Mn^{2+} to Mn^{3+}, which in turn oxidizes lignin, and also it has been demonstrated that MnP is able to oxidize phenolic lignin

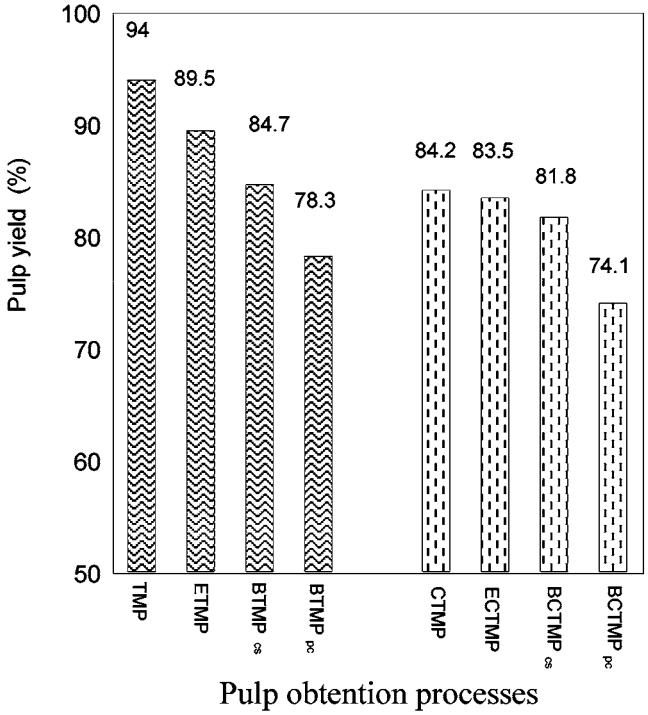

Figure 1. Pulp yield for mechanical pulps with biological treatment.

Table 1. Klason Lignin Content for TMP and CTMP, with and without Enzymatic and Fungal Pretreatments

pretreatment	Klason lignin (%)	pretreatment	Klason lignin (%)
TMP	19.15	CTMP	18.75
ETMP	19.02	ECTMP	17.99
$BTMP_{Cs}$	18.98	$BCTMP_{Cs}$	18.42
$BTMP_{Pc}$	19.05	$BCTMP_{Pc}$	18.50

subunits (*18*). It has been suggested that both laccase and MnP are able to oxidize only phenolic lignin structures (*18*, *19*).

C. subvermispora fungus pretreated pulp ($BTMP_{CS}$) gave an 84.7% yield, and the lowest yield value was for *P. chrysosporium* pretreated bagasse ($BTMP_{PC}$), at 78.3%. Most of the white-rot fungi produce both laccase and MnP for lignin degradation (*20*); it is also well documented that white-rot fungi produce a wide array of carboxylic acids that have various functions in the fungal degradation of lignin (*21*). The behavior for the CTMP was similar to that of TMP but with lower yield values; the highest yield for CTMP was 84.2%, followed by the enzymatically pretreated bagasse (ECTMP) at 83.5% and then *C. subvermispora* fungally pretreated pulp ($BCTMP_{CS}$) at 81.8% yield. The lowest value was for *P. chrysosporium* pretreated bagassse ($BCTMP_{PC}$) at 74.1%.

There is not much difference in Klason lignin content for the TMP pulps, with all values being ~19% (**Table 1**). The Klason lignin content for CTMP pulps was somewhat lower, 18.8% for CTMP and 18.0% for the enzymatically pretreated CTMP. It has been suggested that degradation by lignin enzymes is a nonspecific link reaction on the basis of free radicals formed in the lignin polymer, which results in destabilization of bonds and finally in the breakdown of the macromolecule (*22*). Kim et al. (*23*) found that laccase treatment alone for kraft pulp removed ~7% lignin; also, Galliano et al. (*24*) reported the solubilization of some *Hevea* lignin when MnP and laccase were present at the same time in the reaction mixture.

Figure 2 shows the energy consumption for TMP and CTMP treated with crude enzyme extracts during 12, 24, and 36 h.

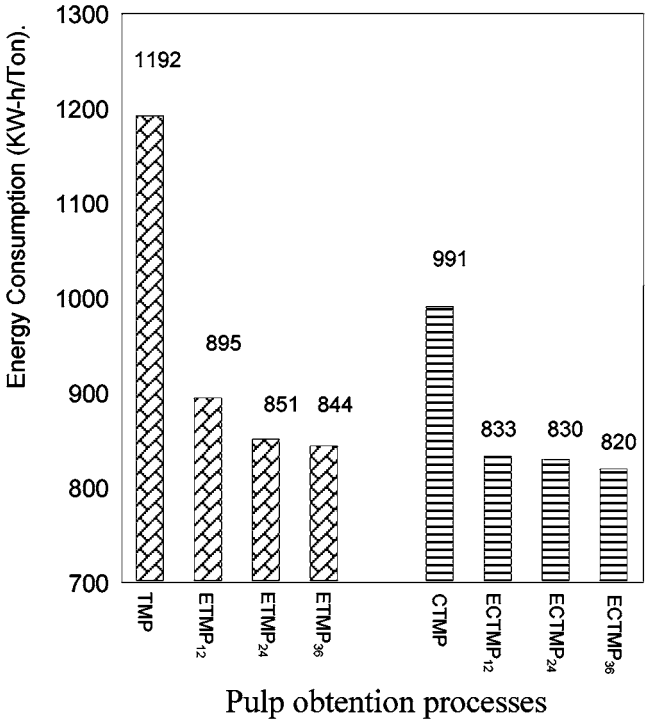

Figure 2. Energy consumption for TMP and CTMP pretreated with enzymes (refining degree for all of the pulps was 70 °SR).

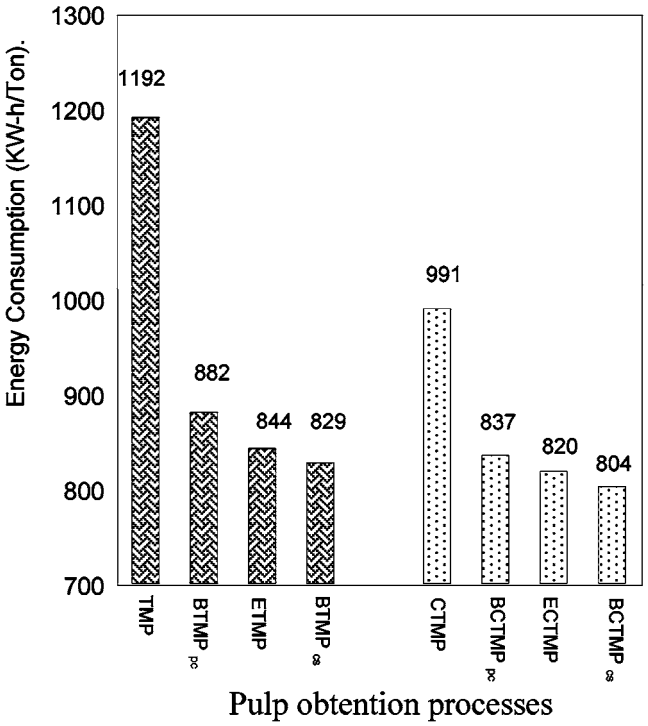

Figure 3. Energy consumption for TMP and CTMP, with and without bioligical pretreatments (refining degree for all of the pulps was 70 °SR).

Normal TMP processing consumes the highest energy, 1192 kW-h/ton, whereas $ETMP_{12}$ requires 895 kW-h/ton, $ETMP_{24}$ 851 kW-h/ton, and $ETMP_{36}$ only 844 kW-h/ton, a reduction in energy consumption of 29.2% for 36 h of pretreatment time. Crude enzyme extract pretreatment before CTMP processing also reduces the energy consumption as shown in **Figure 2**; in this case the reduction in energy consumption was 17.3% for 36 h of treatment time. It has been suggested that laccase treatment alone reduces energy consumption by 5% during

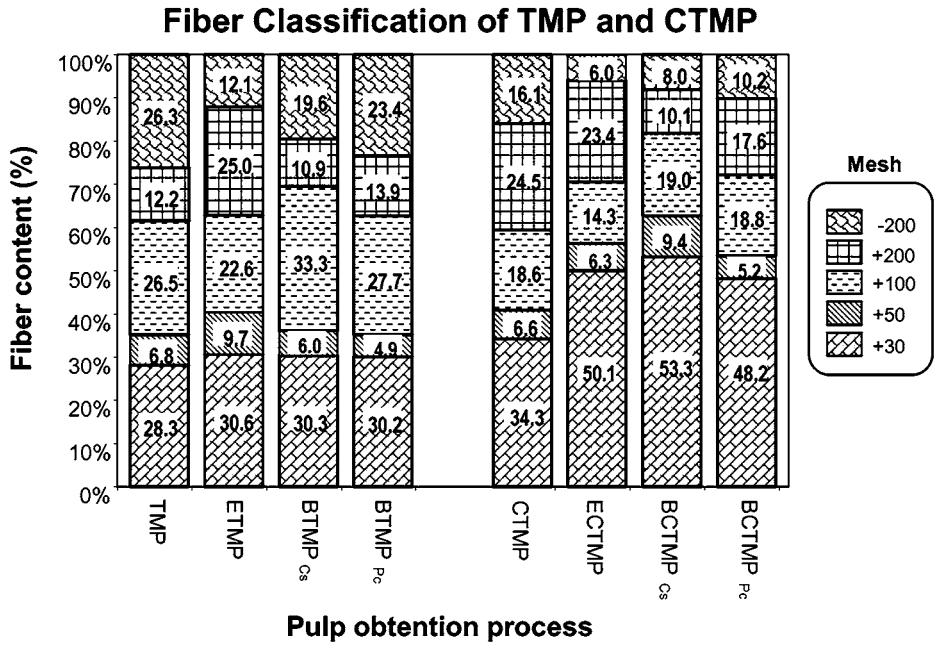

Figure 4. Fiber classification of TMP and CTMP, with and without enzymatic and fungal pretreatments (%).

mechanical pulping (*25*); also, Giovannozzi-Sermanni et al. (*26*) showed that biological treatment of nonwoody plants with enzymatic cocktails from *Lentinus edodes* resulted in significant energy savings during grinding and refining.

P. chrysosporium fungal pretreatment before TMP processing reduced the energy consumption by 26%, compared with the enzymatic pretreatment during 36 h, which reduced the energy consumption by 29.2%. The biological pretreatment with the *C. subvermispora* fungus gave the greatest reduction in energy consumption during refining, 30.5%, as shown in **Figure 3**. Energy consumption for all CTMP processes was lower than that for the TMP processes; for example, 991 kW-h/ton was needed for normal CTMP processing. Pretreatment with *P. chrysosporium* fungus before CTMP processing reduced the energy consumption by 15.5% compared with enzymatic pretreatment during 36 h, which reduced the energy consumption by 17.3%. Biological pretreatment with the *C. subvermispora* fungus gave the greatest reduction in energy consumption, 18.9%. The reduction in energy consumption with biological pretreatment with *C. subvermispora* is in accordance with previous investigations (*8, 27*).

Figure 4 shows the fiber classification for TMP and CTMP, with and without enzymatic and fungal pretreatments. As we see in the figure, long fibers comprise 28.3% of total TMP pulp; this long fiber content is increased 2% with biological pretreatments. On the other hand, fines for normal TMP are 26.3% of total pulp; this fine material is reduced to 23.4% for *P. chrysosporium* fungal pretreatment, to 19.6% with *C. subvermispora* fungal pretreatment, and to 12.1% for enzymatic pretreatment during 36 h.

CTMP normally has higher quantities of long fiber than TMP, 34.3% for CTMP, and these values are increased to 48.2% for *P. chrysosporium* fungal pretreatment, to 50.1% for enzymatic pretreatment during 36 h, and to 53.3% for *C. subvermispora* fungal pretreatment. Fine material is reduced with all of the pretreatments from 16.1% for the normal CTMP, to 10.2% for *P. chrysosporium* fungal pretreatment, to 8.0% for *C. subvermispora* fungal pretreatment, and to only 6.0% for enzymatic pretreatment during 36 h.

Figure 5 shows the tensile index behavior for different degrees of refining for TMP and CTMP, with and without

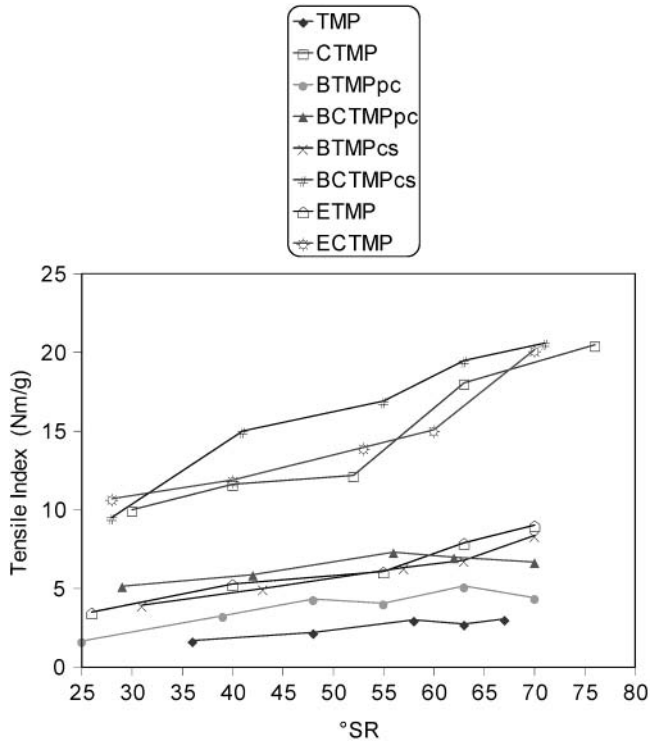

Figure 5. Tensile index for TMP and CTMP, with and without biological pretreatments.

biological pretreatments. TMP from bagasse has a very low tensile index of only 3.1 Nm/g at 60 °SR. Crude enzyme extract pretreatments before TMP processing continuously increased this property with treatment time from 12 to 36 h, but the strength values were still low. CTMP processes produced higher values for tensile index compared with TMP. Enzymatic pretreatment during 36 h before CTMP pulping resulted in a small increase in tensile index. Wong et al. (*28*) found that treating a long-fiber-rich fraction of mechanical pulp with laccase consistently increased tensile index at a given sheet density, and there were corresponding increases in burst index. Sigoillot et al. (*29*) studied enzymatic treatment of wheat straw

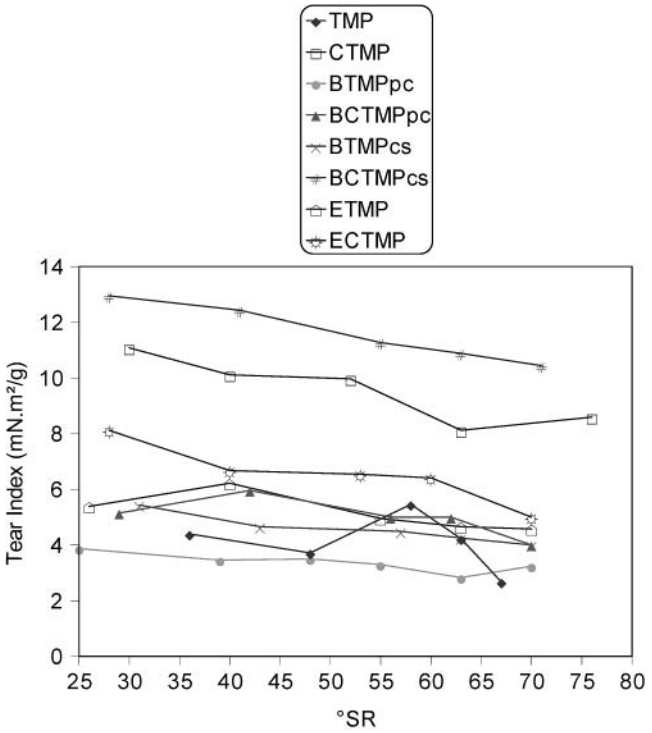

Figure 6. Tear index for TMP and CTMP, with and without biological pretreatements.

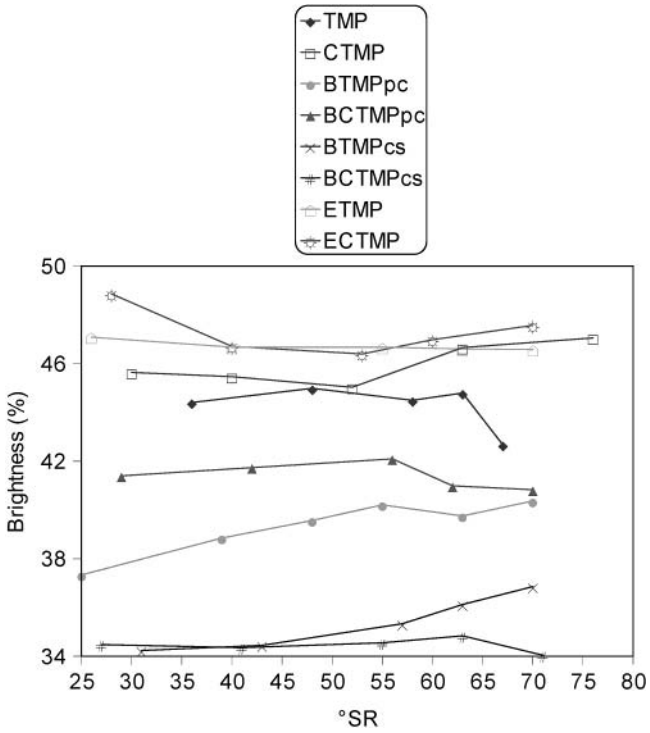

Figure 7. Pulp brightness for TMP and CTMP, with and without biological pretreatments.

with manganese peroxidase and found an improvement of physical properties of the pulp, especially the breaking length. Giovannozzi-Sermanni et al. (*26*) treated nonwoody plants with enzymatic cocktails from *L. edodes* followed by CTMP pulping and found that treated materials showed comparable and, in some cases, higher physical−mechanical properties than those obtained from untreated controls. *P. chrysosporium* fungal pretreatment gives pulps with lower tensile than normal CTMP, quite similar to the effect of enzymatic TMP pretreatment; the best values were obtained with *C. subvermispora* fungal pretreatment before CTMP processing, where the tensile strength was close to 16 Nm/g around 50 °SR degree of refining. All of the pulp tensile index properties increased as the degree of refining increased.

Figure 6 shows the tear index for TMP and CTMP, with and without biological pretreatments. The values of this property for TMP are quite low, even with biological pretreatments. The tear values were in a similar range to where pulps are normally used. CTMP processes have higher values in tear index than TMP, and, from the different biological pretreatments, only *C. subvermispora* fungal pretreatment gave higher values than normal CTMP, close to 12 mNm^2/g around 50 °SR refining degree, compared to around 10 mNm^2/g for CTMP without biological pretreatment.

Figure 7 shows brightness behavior at different degrees of refining for TMP and CTMP. As shown in this figure, the fungal pretreatment substantially reduced the brightness for TMP and CTMP, especially with *C. subvermispora* fungal pretreatment. The loss in brightness was close to 5 points for TMP at 60 °SR and >10 points for CTMP at 50 °SR. In contrast, pretreatment with enzymes for 36 h before TMP pulping increased the brightness by ∼2 points at 60 °SR, compared to normal TMP, and also by 2 points for CTMP pulping at 50 °SR. It has been shown that manganese peroxidase is the enzyme most consistently associated with pulp bleaching activity (*30*). Wong et al. (*28*), after treating a long-fiber-rich fraction of mechanical pulp with laccase, did not find any substantial change in pulp brightness. Giovannozzi-Sermanni et al. (*26*) showed that pretreatment of several nonwoody plants with enzymatic cocktails, followed by CTMP pulping, had little or no effect on opacity and brightness, except for wheat and rice straws, for which brightness decreased by 12 and 15%, respectively.

In summary, enzymatic pretreatment with crude enzyme extracts is an additional way to produce TMP and CTMP from sugarcane bagasse, as compared with fungal pretreatment. From this investigation we can conclude the following:

- Pretreatment of sugarcane bagasse with crude enzyme extract is a viable alternative for the production of TMP and CTMP.
- Enzymatic pretreatments do not require the prolonged treatments necessary with fungi (2 weeks); only 36 h was necessary with the enzymes.
- Enzymatic pretreatments of bagasse produce pulps with higher yield than those pretreated with fungi.
- When bagasse is pretreated with crude enzyme extract, a 29% reduction in energy consumption is realized in refining to produce ETMP, and a 17.3% energy reduction is found for ECTMP. Both results are close to the energy savings from pretreatment with *C. subvermispora* fungi.
- Pretreatment of bagasse with crude enzyme extract increases the tensile strength compared with normal TMP and CTMP, although the increase is somewhat less than that realized from *C. subvermispora* fungal pretreatment for CTMP.
- The brightness of the pulps obtained with enzymatic pretreatments is 2% higher compared with normal TMP and CTMP, whereas fungal pretreatment reduced the brightness 5% for TMP and 10% for CTMP.

Enzymatic pretreatments require much less time, only 36 h or less, compared with the 2 weeks needed for fungal treatments. We obtained a ∼5% higher yield for TMP (89.5%) compared with *C. subvermispora* pretreatment and a >11% higher yield compared with *P. chrysosporium* pretreatment at only 78.3%.

With enzymatic pretreatment the energy consumption was reduced 29% for the TMP process, similar to the 30.5%

reduction with *C. subvermispora* fungal pretreatment. This is higher than the 26% reduction in energy for *P. chrysosporium* pretreatment. Also, for CTMP enzyme pretreatment, we realized a 17.3% energy reduction, similar to the 18.9% for *C. subvermispora fungi* pretreatment and also higher than the 15.5% reduction for *P. chrysosporium* pretreatment.

Enzymatic pretreatment with crude enzyme extracts also increases the pulp tensile index compared with the normal TMP and CTMP pulps, although it is somewhat lower than from *C. subvermispora* fungal pretreatment before CTMP processing. An important advantage from enzymatic pretreatment is that brightness is somewhat increased compared with normal TMP and CTMP processes, whereas the fungal pretreatments reduce the brightness, especially with *C. subvermispora*, with losses close to 5% for TMP at 60 °SR and >10% for CTMP at 50 °SR.

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