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Ceriporiopsis subvermispora Used in Delignification of Sugarcane Bagasse Prior to Soda/Anthraquinone Pulping

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Abstract

Sugarcane bagasse was pretreated with the white-rot fungus *Ceriporiopsis subvermispora* for 30 d of incubation. The solid-state fermentation of 800 g of bagasse was carried out in 20-L bioreactors with an inoculum charge of 250 mg of fungal mycelium/kg of bagasse. The oxidative enzymes manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac) and the hydrolytic enzyme xylanase (Xyl) were measured by standard methods and related to the fungus's potential for delignification. Among the lignocellulolytic assayed enzymes, Xyl was detected in larger quantity (4478 IU/kg), followed by MnP (236 IU/kg). LiP and Lac were not detected. The results of chemical analysis and mass component loss showed that *C. subvermispora* was selective to lignin degradation. Pretreated sugarcane bagasse and control pulps were obtained by soda/anthraquinone (AQ) pulping. Pulp yields, kappa number, and viscosity of all pulps were determined by chemical analysis of the samples. Yields of soda/AQ ranged from 46 to 54%, kappa numbers were 15–25, and the viscosity ranged from 3.6 to 7 cP for pulps obtained from pretreated sugarcane bagasse.

Index Entries: *Ceriporiopsis subvermispora*; sugarcane bagasse; selective biodegradation; hydrolytic enzymes; oxidative enzymes; soda/anthraquinone pulping; lignin.

Introduction

Brazil is now the world's largest producer of sugarcane, harvesting approx 300 millions t/yr *(1–3)*. Sugarcane is mainly cultivated on flat terrain, whereas other crops are planted on hills, where sugarcane has been proven not to be competitive *(4)*. Mechanized harvesting of sugarcane

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reduces production costs by up to 50% when compared with the traditional manual technique *(4)*.

Sugarcane can be used for the production of sugar and ethanol. Both products generate bagasse as residue after sugarcane has been crushed for juice extraction *(4)*. Bagasse is a complex material that consists of approx 50% cellulose and 25% each of hemicellulose and lignin *(5,6)*.

In recent years, there has been an increasing trend toward more efficient utilization of agroindustrial residues, including sugarcane bagasse. Several processes and products have been utilizing sugarcane bagasse as a raw material. Among them are electricity generation, pulp and paper production, and products based on fermentation *(5)*.

The purpose of the present study was to determine the ability of the white-rot fungus *Ceriporiopsis subvermispora* to delignify sugarcane bagasse prior to soda/anthraquinone (AQ) pulping. The use of fungi for nonwoody plants prior to chemical treatment or mechanical refining has also received attention in recent years *(7,8)*.

White-rot fungi are able to fragment the major structural polymers of lignocellulosics: lignin, cellulose, and hemicelluloses *(9,10)*. Ligninolytic systems have an extracellular degradative mechanism that is oxidative rather than hydrolytic. The main enzymes acting directly or indirectly on lignin are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac).

C. subvermispora is a white-rot basidiomycete that selectively degrades lignin when growing on wood *(11–13)*. The ligninolytic system of this fungus is composed of MnP and a copper-containing phenol oxidase called Lac *(14)*.

Materials and Methods

Sugarcane Bagasse

Sugarcane bagasse, approx 2.1 cm long and 0.12 cm wide, was kindly furnished by the Usina Ester of Cosmópolis, SP, Brazil. It was air-dried to a final humidity of 10% and also passed through a 10-mesh sieve and stored at 4°C in a refrigerated chamber. The chemical composition of the bagasse was (43.8%) glucan, (28.6%) pentoses, (2%) acetyl groups, (23.4%) Klason, (0.14%) H_2SO_4-soluble lignin, and (1.3%) ash.

Fungus

C. subvermispora, kindly provided by Prof. M. Speranza from the Universidad de la Republica de Montevideo (Uruguay), was maintained in medium containing 2% malt extract, 0.2% yeast extract, and 2% agar.

Preparation of Inoculum

Fifteen milliliters of culture medium containing agar malt extract (2% malt extract, 0.2% yeast extract, and 2% agar) was added to 10-cm-diameter

plates, previously sterilized. The plates were inoculated and incubated at 27 ± 2°C. The growth of fungal mycelium was monitored until it reached the borders of the plate (7–10 d). The mycelium was removed from those outlying areas of the plates by cutting out 8-mm circles of mycelium. In an Erlenmeyer flask containing 200 mL of liquid culture medium (2.4% potato extract and 0.7% yeast extract sterilized in an autoclave at 121°C for 20 min and then coolled), 20 circles of the mycelium cultivated in solid medium were introduced. This Erlenmeyer flask was incubated for 10 d at 27 ± 2°C. Then, the mycelium was filtered using a sterile Buchner funnel and washed with 300 mL of sterile water. The washed mycelium obtained from the fungus *C. subvermispora* was blended with 100 mL of sterile water in three 15-s cycles. A 25-mL aliquot of extract was used for determination of fungal mass (grams/liter) by filtration through quantitative filter paper. The retained material was dried at 60°C for 2 h and afterward at 105°C until constant weight, reaching 4 g/L of fungus. The aliquot of inoculum was calculated using as standard 100 g of fungal mass for 1000 kg of bagasse *(12,13,15,16)*. The constant mass of the obtained inoculum fungi was considered to be 0.4 g for 100 mL of suspension.

Bioreactor

Each of nine bioreactors was fabricated from a polypropylene vessel. The top of the vessel was sealed with a lid, which was vented to the atmosphere through an exit tube. The perforated polypropylene floor, suspended above the bottom of the reactor, was supported by a stand. The air for the bioreactor came from a regulated supply, passed through tubing, and was sterilized with potassium permanganate and humidified with sterile water. Humidified air, passing through a 0.2-µm membrane, was injected at the bottom of the reactor by low-pressure pumps at 23 L/h. The 20-L bioreactors had a 27-cm inferior diameter, 33-cm superior diameter, and 32-cm height.

Inoculation of Bioreactor

Each 20-L bioreactor was loaded with 800 g of sugarcane bagasse immersed in water for a 12-h period after the water was drained. The bagasse was sterilized (121°C, 15 min) for two consecutive periods intermediated by of 24 h of cooling. After sterilization, the bagasse was inoculated with an inoculum charge of 250 mg/kg of bagasse and stored at 27°C for 30 d. Humidified air, passing through a 0.2-µm membrane, was provided for the 20-L bioreactor throughout the biodegradation. One cylindrical container (9-cm height and 5-cm diameter) made of a 0.84-mm steel screen was filled with 7.5 g of bagasse and inserted into each bioreactor just before inoculation. After the biotreatment, the bioreactors were opened, and the bagasse was washed with water in order to remove the

superficial mycelium. The decayed bagasse was air-dried. Nine bioreactors were inoculated with 800 g of sugarcane bagasse.

Extraction of Enzymes

The bagasses fermented in the cylindrical containers inserted into the center of each bioreactor were transferred to 0.5-L Erlenmeyer flasks with 100 mL of 50 m*M* sodium acetate buffer, pH 5.5, with 0.01% Tween-60 according to refs. *15* and *17.* The extraction solution was mixed into flasks with fungal mycelium and bagasse with the aid of a glass rod. Two sequential 6-h extractions were made under agitation at 120 rpm and 10 ± 1°C. Enzymatic extracts were recovered by filtration through qualitative filter paper through a Millipore membrane (0.45 μm). Activities of LiP *(18)*, MnP *(19)*, Lac *(20,21)*, cellulase *(22)*, and protease *(23)* cited in Germano et al. *(24)* were determined by published methods and are expressed in international units per kilogram of initial bagasse.

Chemical Analysis of Decayed Sugarcane Bagasse

The modified Klason method was utilized *(25)*. Samples of 1.0 g of decayed and nondecayed sugarcane bagasse were treated with 5 mL of 72% H_2SO_4. After 7 min of stirring at 45°C, 25 mL of water was added to the mixture, which was posthydrolyzed under 1.05 bar for 30 min. The product was filtered and the insoluble portion (Klason lignin) was quantified. The hydrolyzed was acidified to pH 1.0 to 3.0, filtered in a Sep-Pak C_{18} cartridge, and analyzed by high-performance liquid chromatography in a Shimadzu LC10 chromatograph using an Aminex HPX-87H column at 45°C. The mobile phase was 0.005 mol/L of H_2SO_4 at 0.6 mL/min. The products were determined by refraction index and quantified by using calibration curves *(26)*. Soluble lignin was determined as described by Rocha et al. *(27)* using the absorption at 280 nm of alkaline solutions obtained from the hydrolyzed. The mass loss and selectivity of the components were determined.

The mass loss of sugarcane bagasse and the loss of each sugarcane bagasse component were calculated using Eqs. 1 and 2, respectively. For Eq. 1,

$$L_m = \left(\frac{m_i - m_f}{m_i} \right) \times 100\% \quad (1)$$

in which L_m is the mass loss; m_i is the initial mass of bagasse (dry basis); and m_f is the final mass of bagasse, after treatment (dry basis). For Eq. 2,

$$L_c = \left(\frac{m_{ci} - m_{cf}}{m_{ci}} \right) \times 100\% \quad (2)$$

in which L_c is the component loss, m_{ci} is the amount of component in the original bagasse (%) × initial mass of bagasse, and m_{cf} is the amount of component in bagasse after treatment (%) × final mass of bagasse.

Soda/AQ Pulping of Sugarcane Bagasse

Pulping conditions were 9.7% Na_2O, 0.15% AQ, and a liquor:bagasse ratio of 12:1 (v/w). Soda cooking was performed in an 80-mL stainless steel autoclave at 160°C, for 15, 30, 45, 60, 90, and 120 min. The obtained pulps were washed and filtered until all of the liquor was removed. Pulps were air-dried and the yield was calculated. The kappa number and viscosity of the bagasse pulps were determined by TAPPI methods.

Calculation of Kinetic Constants of Delignification

The kinetic constants of delignification were calculated considering a pseudo-first-order process as shown in Eq. 3 (*28–30*) cited by Mendonça *(31)*:

$$\frac{dL}{dt} = -k.L \tag{3}$$

in which: dL/dt: is the delignification rate, L is the lignin content (in bagasse basis), and k is the delignification constant.

Equation 4 is obtained by integrating Eq. 3:

$$\text{Ln } L - \text{Ln } L_0 = -k \cdot t, \tag{4}$$

in which L is the residual lignin content in the pulp (in bagasse basis), L_0 is the initial lignin content in the bagasse, and t is the reaction time.

Equation 3 is analogous to that of a straight line $Y = a + bX$, in which $Y = \text{Ln } L$, $a = \text{Ln } L_0$, $b = -k$ and $X = t$.

The rate of lignin solubility is governed by the faster phase (bulk phase), whose rate constant can be obtained from the straight line fitted to the points that compose this phase, in a graph of natural logarithm of residual lignin content (in initial bagasse basis) in function of the reaction time (Eq. 4). The inclination of the straight line (*b*) corresponds to the value of delignification constant (*k*) for the studied sample. The Ln graphics of residual lignin were made in function of time.

Residual lignin content (in bagasse basis) was calculated as follows:

$$L_{bm}(\%) = \left(\frac{L_p \times RP}{100} \right) \tag{5}$$

in which: L_{bm} is the residual lignin in bagasse basis (%), L_p is the residual lignin in the pulp (%), and RP is the pulp yield (%).

In the case of the biologically pretreated bagasse samples, the process yield and the residual lignin content were expressed based on the biodegraded bagasse.

Results and Discussion

Enzymatic Activity

The results of enzymatic activity refer to solid fermentation of nine bioreactors that were inoculated and incubated under the same conditions. The fermentation of sugarcane bagasse was carried out with an inoculum charge of 250 mg/kg for 30 d of incubation.

The mean value of Xyl activity found for nine solid fermentations of sugarcane bagasse was in the range of 4478 ± 491 IU/kg, followed by MnP 236 ± 53 IU/kg and cellulases 17.4 ± 4.4 IU/kg. In a study by Guerra et al. *(11)*, the solid fermentation of *Pinus taeda* with the same fungus and the same incubation period, resulted in an Xyl activity of 4257 IU/kg *(11)*, smaller than that found by Ferraz et al. *(32)* for fermentation of 50 g of *Eucalyptus grandis* with the same fungus (942 IU/culture). For comparison, the Xyl activity of *Panus tigrinus* in sugarcane bagasse with 30 d of incubation was found to be from 500 to 700 IU/kg *(33,34)*.

The mean activity values of MnP found for the solid fermentation of sugarcane bagasse with *C. subvermispora* for 30 d (238 IU/kg) were higher than peroxidase values found for the solid fermentation of *P. taeda* with the same fungus and the same incubation period (125 IU/kg) *(11)*. Cellulase activity was low, which favored carbohydrate preservation, and Lac and LiP were not detected. The effect of veratryl alcohol on Lac production varied according to the fungus used and also to the different nutritional conditions media used *(35)*.

To confirm the absence of interference in the determination of lignocellulolytic activities, protease was measured and showed very low activity even before (0.082 ± 0.026 IU/mL) or after (0.0088 ± 0.0063 IU/mL) dialysis. The presence of protease could explain the decrease in MnP activity.

Chemical Analysis and Mass and Components Losses

Table 1 shows the average values of the chemical composition determined after growth of *C. subvermispora* for 30 d in sugarcane bagasse and undecayed bagasse (control).

Mass and components (%) losses were calculated for a better evaluation of the action of *C. subvermispora* on bagasse, because chemical analysis is restricted to verification of changes in the samples' composition. Table 2 presents these values together with selectivity values (lignin loss/glucan loss ratio).

The biotreatment of sugarcane bagasse with *C. subvermispora* for 30 d resulted in a bagasse weight loss of 8.4 ± 2%. Lignin, polyoses, and glucan losses were 19 ± 5, 19.4 ± 4.5, and 10.6 ± 1.7%, respectively.

In the nine bioreactors, chemical analysis confirmed that *C. subvermispora* was selective to lignin degradation, reaching selectivity higher than 1.4 in most of the experiments.

Table 1
Chemical Composition of Decayed and Undecayed (Control) Sugarcane Bagasses After 30 d of Incubation with *C. subvermispora*

	Control	Decayed
Glucan (%)	34.6 ± 1.5	37.4 ± 2.0
Acetyl (%)	3 ± 0.1	2.3 ± 0.4
Xylan (%)	24.7 ± 0.3	23.2 ± 0.9
Arabinan (%)	1.6 ± 0.1	1.5 ± 0.1
Total carbohydrates (%)	63.9 ± 1.9	64.4 ± 3.4
Klason lignin (%)	21.7 ± 0.3	19.5 ± 1.3
Soluble lignin (%)	2.1 ± 0.1	3.2 ± 0.5
Total lignin (%)	23.8 ± 0.4	22.8 ± 1.8
Ash (%)	1.2 ± 0.1	1.2 ± 0.2
Total ash (%)	2.1 ± 0.2	2.4 ± 0.4
Mass balance (%)	89.8 ± 2.5	87.2 ± 5.6
Xylan/glucan ratio	0.7	0.6
Polyoses/glucan ratio	0.7	0.7

Table 2
Mass and Components (%) Losses and Selectivity

	% Losses
Mass	8.4 ± 2
Glucan	10.6 ± 1.7
Polyoses	19.4 ± 4.5
Lignin	19 ± 5
Selectivity (lignin/glucan)	1.8 ± 0.4

For comparison, Dorado et al. *(36)* found that the mass loss of wheat straw after 60 d of fermentation with *Phanerochaete chrysosporium* was up to 45% and from 20 to 30% at the end of the incubation period with *Pleurotus eryngii*, *Phlebia radiata,* and *C. subvermispora.*

Soda/AQ Pulping

Tables 3 and 4 present the values of yield, viscosity, and kappa number for the pulps obtained from decayed sample and control medium. A reduction in kappa number was higher at the beginning of pulping. By increasing cooking time, however, the difference in kappa number became smaller. Viscosity was preserved, ranging from 4 to 10 cP.

Chemical Analysis of Soda/AQ Pulps

Chemical analysis of the soda/AQ pulps obtained with a concentration of 12.5% NaOH of decayed and undecayed (control) medium sugarcane bagasses with a cooking time from 15 to 120 min was performed.

Table 3
Results of Soda/AQ Pulping of Undecayed (Control) Sugarcane Bagasse with Cooking Time from 15 to 120 min

	Pulp characteristic			
Cooking time (min)	Yield (%)	kappa number	Viscosity (cP)	Viscosity/kappa number
15	54 ± 0.4	26.1 ± 7.0	4.6 ± 0.6	0.18
30	52 ± 0.9	24.0 ± 0.6	5.8 ± 0.6	0.24
45	51 ± 0.4	22.2 ± 1.6	8.7 ± 1.4	0.39
60	48 ± 2.0	22.9 ± 1.0	9.8 ± 1.6	0.43
90	48 ± 0.2	21.6 ± 3.0	8.2 ± 3.0	0.38
120	46 ± 0.4	19.2 ± 1.1	5.8 ± 0.1	0.30

Table 4
Results of Soda/AQ Pulping of Decayed Sugarcane Bagasse with Cooking Time from 15 to 120 min

	Pulp characteristic			
Cooking time (min)	Yield (%)	kappa number	Viscosity (cP)	Viscosity/kappa number
15	54 ± 0.9	25.2 ± 2.0	3.6 ± 0.1	0.14
30	49 ± 0.7	20.9 ± 1.3	5.4 ± 2	0.26
45	47 ± 0.8	21.8 ± 0.3	8 ± 2	0.37
60	46 ± 1.2	19.7 ± 0.3	6.6 ± 2	0.34
90	44 ± 1.0	18.6 ± 2.0	8.2 ± 2	0.44
120	44 ± 1.0	17.4 ± 1.0	6.1 ± 0.4	0.35

In all the cases, the residual lignin contents of the pulps prepared from pretreated sugarcane bagasse were lower than those of the control pulps.

Delignification Kinetics of Sugarcane Bagasse Under Soda/AQ Cooking

The purpose of studying delignification kinetics was to investigate the lignin removal during the soda/AQ pulping process obtained from decayed and undecayed (control) medium sugarcane bagasses. In other words, the aim was to determine whether the lignin removal rate by the soda/AQ process was affected by pretreatment and whether fungi caused selective delignification.

Biotreated bagasse and undecayed controls were isothermally cooked at 160°C for 15–120 min in 9.7% Na_2O and 0.15% AQ. The residual lignin contents in the pulps prepared from the biotreated sugarcane bagasse were lower than those observed in pulps from the undecayed control; this is

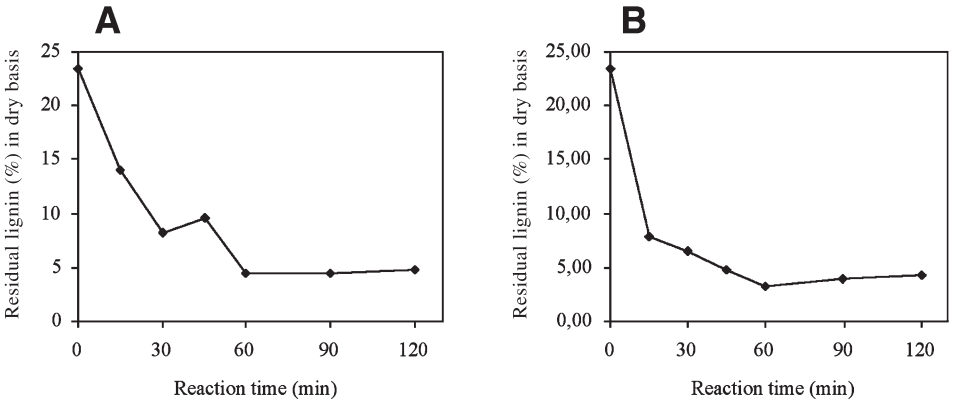

Fig. 1. Residual lignin in initial sugarcane bagasse basis in soda/AQ pulping with concentratation of 12.5% NaOH: **(A)** undecayed (control); **(B)** decayed sugarcane bagasse.

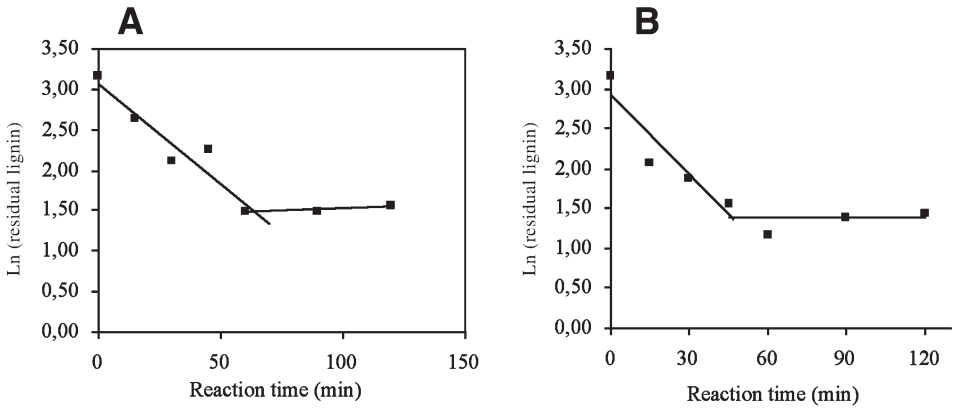

Fig. 2. Delignification kinetics of sugarcane bagasse during soda/AQ pulping with concentration of 12.5% NaOH: **(A)** undecayed (control); **(B)** decayed sugarcane bagasse.

illustrated in Figs. 1 and 2. The delignification kinetics of sugarcane bagasse under soda/AQ cooking were obtained from the data in Tables 4–7. The delignification processes showed two pseudo-first-order delignification phases (bulk and residual) (Fig. 2). Table 7 summarizes these results, showing the values of delignification constants for each observed phase, as well as the time required for the phase changes. These data confirm that maximal benefits are obtained from the fungal pretreatment when the subsequent chemical pulping is performed at low severity *(37–40)*.

Conclusion

The action of the white-rot decomposition fungus *C. subvermispora* in the bagasse fibers was evaluated by the enzymatic activity and compared with the chemical composition, which showed that the fungus was selective for lignin degradation.

Table 5
Chemical Analysis of Soda/AQ Pulps Obtained from Undecayed (Control) Sugarcane Bagasse with Cooking Time from 15 to 120 min

	Cooking time					
	15 min	30 min	45 min	60 min	90 min	120 min
Glucan (%)	43.1 ± 0.1	52.3 ± 0.3	48.4 ± 0.4	53.5 ± 0.5	56.3 ± 0.5	54.3 ± 0.3
Xylan (%)	28.4 ± 0.4	28.5 ± 0.2	26.7 ± 0.6	27 ± 0.1	27.9 ± 0.1	28.4 ±0.1
Arabinan (%)	2.4 ± 0.1	2.3 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.6 ± 0.1
Total carbohydrates (%)	73.9 ± 0.6	83 ± 0.6	77.6 ± 1.1	83 ± 0.7	86.7 ± 0.7	85.3 ± 0.5
Klason lignin (%)	25.1 ± 0.1	15.3 ± 0.1	17 ± 0.1	8.3 ± 0.2	8.1 ± 0.2	9.1 ± 0.2
Soluble lignin (%)	0.9 ± 0.1	0.5 ± 0.1	1.7 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Total lignin (%)	26 ± 0.2	15.8 ± 0.2	18.7 ± 0.2	9.2± 0.3	9.3 ± 0.3	10.3 ± 0.3
Mass balance (%)	99.9	98.8	96.3	92.2	96	95.6
Xylan/glucan ratio	0.7	0.5	0.6	0.5	0.5	0.5
Polyoses/glucan ratio	0.6	0.3	0.4	0.2	0.2	0.4

Table 6
Chemical Analysis of Soda/AQ Pulps Obtained from Decayed Sugarcane Bagasse with Cooking Time from 15 to 120 min

	Cooking time					
	15 min	30 min	45 min	60 min	90 min	120 min
Glucan (%)	56.3 ± 0.3	56.7 ± 0.3	60 ± 0.6	61 ± 0.4	62 ± 0.5	60.7 ± 0.3
Xylan (%)	26.3 ± 0.3	22.8 ± 0.2	23.8 ± 0.2	24 ± 0.2	24.7 ± 0.2	24 ± 0.2
Arabinan (%)	2.2 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Total carbohydrates (%)	84.8 ± 0.7	81.4 ± 0.6	85.7 ± 0.9	86.9 ± 0.7	88.6 ± 0.8	86.6 ± 0.6
Klason lignin (%)	12.2 ± 0.1	10.6 ± 0.1	7.4 ± 0.1	5.5 ± 0.6	7.0 ± 0.5	7.3 ± 0.4
Soluble lignin (%)	2.5 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	1.5 ± 0.2	2.0 ± 0.3	2.3 ± 0.1
Total lignin (%)	14.7 ± 0.2	13.2 ± 0.2	10.1 ± 0.2	7.0 ± 0.8	9.0 ± 0.8	9.6 ± 0.5
Mass balance (%)	99.5	94.6	95.8	97.6	97.6	96.2
Xylan/glucan ratio	0.5	0.4	0.4	0.4	0.4	0.4
Polyoses/glucan ratio	0.3	0.2	0.2	0.2	0.2	0.2

Table 7
Results of Delignification Kinetics for Soda/AQ Pulping with Concentration of 12.5% NaOH of Decayed and Undecayed (Control) Sugarcane Bagasse Samples

	Delignification rate constant for bulk phase (10^{-2} min^{-1})	Time of change from bulk phase to residual phase (min)	Delignification rate constant for residual phase (10^{-2} min^{-1})
Undecayed (control)	2.49	64	0.12
Decayed	3.34	46	0.01

The effects of biologic treatment were also evaluated in a soda/AQ pulping process with a concentration of 12.5% NaOH. Through delignification kinetics of the pulps, a 28% decrease in the time of change from the bulk phase to the residual phase of the biodegraded bagasse in relation to the control medium was verified. This can represent an energy economy in the cooking of the pulps.

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