0273-2289/02/98-100/0373/$12.50

Panus tigrinus Strains Used in Delignification of Sugarcane Bagasse Prior to Kraft Pulping

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Abstract

Three strains of the white-rot fungus *Panus tigrinus* (FTPT-4741, FTPT-4742, and FTPT-4745) were cultivated on sugarcane bagasse prior to kraft pulping. Pulp yields, kappa number, and viscosity of all pulps were determined and Fourier transform infrared (FTIR) spectra from the samples were recorded. The growth of *P. tigrinus* strains in plastic bags increased the manganese peroxide and xylanase activities. Lignin peroxidase was not detected in the three systems (shaken and nonshaken flasks and plastic bags). FTIR spectra were reduced to their principal components, and a clear separation between FTPT-4742 and the control was observed. Strain FTPT-4745 decayed lignin more selectively in the three systems utilized. Yields of kraft pulping were low, ranging from 20 to 45% for the plastic bag samples and from 12 to 38% for the flask samples. Kappa numbers were 1–18 and viscosity ranged from 2.3 to 6.8 cP.

Index Entries: *Panus tigrinus* strains; sugarcane bagasse; kraft pulping; biological pretreatment

Introduction

Sugarcane bagasse is a lignocellulosic residue with a complex structure. It can be efficiently decayed especially by white-rot fungi *(1–5)*. Production of sugarcane bagasse in Brazil has increased during recent decades, and the integral use of its components would be economically and environmentally desirable.

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The purpose of the present study was to determine the ability of three strains of white-rot fungus *Panus tigrinus* (FTPT-4741, FTPT-4742, and FTPT-4745) to delignify sugarcane bagasse prior to kraft pulping.

In previous work, the ligninolytic fungus *P. tigrinus* was utilized in the pretreatment of surgarcane bagasse, and its action was evaluated by enzymatic activities *(6)*, chemical analysis and component losses *(6–7)*, scanning electronic microscopy (SEM), and infrared spectroscopy (Fourier transform infrared [FTIR]).

White-rot fungi are able to fragment the major structural polymers of lignocellulosics: lignin, cellulose, and hemicelluloses *(8)*. Ligninolytic systems have extracellular degradative mechanisms that are oxidative rather than hydrolytic. The main enzymes acting directly or indirectly on lignin are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac). The laccases from *P. tigrinus* were isolated, purified, and studied in acetate buffer solutions, with and without the addition of various amounts of ethanol, using syringaldazine and 2,6-dimethoxyphenol as substrates. The effect of ethanol on blue laccases could be successfully described using the mixed inhibition model, over the range of 0–2.5 *M* ethanol *(9)*. Characterization of oxidase-1 from a strain of *P. tigrinus* was also carried out *(10)*.

The kraft process is currently the dominating chemical pulping procedure, despite some inconveniences such as odor release and the excessive capital demand. The biologic pretreatment of sugarcane bagasse can be used together with kraft pulping and has a high economic and environmental potential.

Materials and Methods

Fungi

Three strains of *P. tigrinus* (FTPT-4741, FTPT-4742, FTPT-4745) from a collection belonging to Fundação Tropical de Pesquisa e Tecnologia André Tosello (Campinas, SP, Brazil) were used. These strains, grown from a vegetative inoculum on malt extract agar (4%) at 28°C for 5 d, had been previously screened by Esposito et al. *(3)* and Gonçalves et al. *(11)*.

Sugarcane Bagasse

The depithed sugarcane bagasse contained 51% glucane, 23% polyoses, 2% acetyl groups, 21% Klason lignin, and 3% ash. The mean particle size was 2.1 cm long and 0.12 cm wide.

Culture Conditions

Experiments were realized in triplicate using three different systems.

Series 1

Semi-solid-state fermentation cultures were grown at 28°C using 250-mL Erlenmeyer flasks for 10 d with agitation at 150 rpm. Six-gram samples of depithed and sterilized sugarcane bagasse were supplemented

only with 1 mL of salts solution (2 g/L of $NaNO_3$, 1 g/L of K_2HPO_4, 0.5 g/L of $MgSO_4$, 0.5 g/L of KCl, 0.3 g/L of $CaCl_2$, 0.10 mg/L of $CuSO_4 \cdot 5H_2O$, 0.20 mg/L of $FeSO_4 \cdot 7H_2O$, 0.02 mg/L of $MnSO_4$, 0.15 mg/L of $ZnCl_2$) and 49 mL of distilled water. No additional carbon source was included.

Series 2

The same conditions as for series 1 were used but with 1 mL of salts solution ($CaCl_2$, $[NH_4]_2HPO_4$, $FeCl_2$, KH_2PO_4, $MgSO_4$) with and without agitation at 150 rpm. This series was carried out to make possible a comparison between series 1 (6-g scale) and 3 (100-g scale).

Series 3

Semi-solid-state fermentation cultures were grown in sterilized polyethylene bags at 28°C for 30 d. Samples of 100 g of depithed and sterilized sugarcane bagasse were supplemented only with 17 mL of salts solution ($CaCl_2$, $[NH_4]_2HPO_4$, $FeCl_2$, KH_2PO_4, $MgSO_4$) and 833 mL of distilled water. No additional carbon source was included.

Ligninolytic and Hydrolytic Enzymes

After fermentation, the extracellular enzymes present in the liquid extract were filtered through Millipore membranes (0.47 µm), and the activities of LiP, MnP, Lac and xylanase (Xyl) were measured by standard methods *(12–15)*.

Dialysis

Dialysis of 20 mL of the liquid extract with enzymes obtained in series 3 was carried out using a cellulosic membrane with a 12-kDa cut-off and 50 m*M* sodium acetate buffer, pH 5.5.

FTIR Spectroscopy of Bagasse Samples

FTIR spectra were obtained directly from decayed and controlled bagasse samples utilizing the diffuse reflectance infrared with Fourier transform (DRIFT) technique. Spectra were recorded in a Nicolet 520 spectrometer. After polygonal baseline correction *(16)*, the spectra were normalized by the absorption at 1512 cm^{-1}, which corresponds to the aromatic ring vibrations typical for lignins and suffers no influence by other groups.

Principal Component Analysis of the FTIR Spectra

Spectra of sugarcane bagasse were converted to text files using OMNIC software (Nicolet). The normalized absorbances in the range of 792–1865 cm^{-1} (557 data points per each bagasse spectrum) were submitted to principal component analysis (PCA) calculations using the BIOTEC and FAEN programs compiled in FORTRAN, which were written in our laboratory and adapted from the literature *(17)*. Graphic presentations were easily made with Microsoft EXCEL 5.0.

Chemical Analysis of Decayed Sugarcane Bagasse

The modified Klason method was utilized (ASTM 1956). Samples of 0.5 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) quantified by weighing. The hydrolysate was acidified to pH 1.0–3.0, filtered in a Sep-Pak C_{18} cartridge, and analyzed by high-performance liquid chromatography in a Shimadzu LC10 chromatograph by using an Aminex HPX-87H column at 45°C. Mobile phase was 0.005 mol/L of H_2SO_4 at 0.6 mL/min. The products were determined by refractive index and quantified by using calibration curves *(18)*. Soluble lignin was determined using the absorption at 280 nm of alkaline solutions obtained from the hydrolysate *(19)*, and the mass losses of components and selectivity were calculated.

Kraft Pulping

Kraft pulping of control sample and pretreated samples of sugarcane bagasse was performed in an 80-mL autoclave under the following conditions: 15% active alkali; 20% sulfidity; liquor ratio of 4:1 (v/w); cooking temperature of 170°C; cooking time of 20, 40, and 60 min. The kappa number and viscosity of bagasse pulps were determined by TAPPI methods.

Results and Discussion

Enzymatic Activity

Table 1 shows the enzymatic activities determined for the three strains after growth in sugarcane bagasse. Three strains of *P. tigrinus* exhibited higher Lac activities in agitated systems. The MnP is an enzyme depending on H_2O_2 and Mn^{+2}. Maltseva et al. *(20)* proved that MnP produced by *P. tigrinus* is able to degrade lignin models, related to phenolic and nonphenolic structures. Lac is also involved in the degradation of lignin, since it reduces O_2 totally to H_2O with low specificity for electron-donor substrates. These two oxidative enzymes should be directly involved in the removal of lignin, and LiP has no effect, since it was not detected in the strains. Xyl seems to have an important role in the degradation of xylan present in the bagasse fibers. Release of xylans gives rise to the most effective action of oxidative enzymes (MnP and Lac) on the lignin as reported previously *(11)*. The salts solutions practically did not affect Lac activity. MnP activity could be influenced by the absence of Mn^{+2} ions in the salts solution of series 2, but as can be seen from Table 1, this fact was not observed. Probably, the ions present in the original sugarcane bagasse were enough to supply Mn^{+2} for the enzyme expression. Only Xyl activities for FTPT-4741 and FTPT-4745 decreased by using series 2.

Dialysis was carried out on the liquid extract resulting from 10- and 30-d fermentations, and the results are shown in Table 2. After dialysis, the

Table 1
Enzymatic Activities Produced by *P. tigrinus* in Sugarcane Bagasse Determined After 10 d of Incubation

	Lac (U/g)			
Strains/ Enzyme	Agitation (series 1)	Agitation (series 2)	Static (series 2)	Plastic bags (series 3)
FTPT-4741	0.01 ± 0.02	0.013 ± 0.003	0.013 ± 0.002	0.004 ± 0.001
FTPT-4742	0.017 ± 0.009	0.010 ± 0.002	0.008 ± 0.003	0.0010 ± 0.005
FTPT-4745	0.02 ± 0.02	0.02 ± 0.01	0.007 ± 0.001	0.004 ± 0.003
	MnP (U/g)			
Strains/ Enzyme	Agitation (series 1)	Agitation (series 2)	Static (series 2)	Plastic bags (series 3)
FTPT-4741	0.13 ± 0.05	0.09 ± 0.02	0.13 ± 0.03	0.14 ± 0.02
FTPT-4742	0.16 ± 0.03	0.10 ± 0.01	0.13 ± 0.03	0.09 ± 0.02
FTPT-4745	0.17 ± 0.01	0.22 ± 0.01	0.17 ± 0.04	0.07 ± 0.02
	Xyl (U/g)			
Strains/ Enzyme	Agitation (series 1)	Agitation (series 2)	Static (series 2)	Plastic bags (series 3)
FTPT-4741	7 ± 2	2.5 ± 0.8	5.8 ± 0.8	0.4 ± 0.3
FTPT-4742	8 ± 2	5.8 ± 0.8	5.8 ± 0.8	1.1 ± 0.1
FTPT-4745	6 ± 1	<0.1	4.2 ± 0.1	1.5 ± 0.1

enzymatic extract was concentrated and MnP and Lac activities increased. For Lac, the activity was two to eight times higher for FTPT-4741, FTPT-4742, and FTPT-4745. For MnP, the increase in activity was smaller, 59% for FTPT-4741. Xylanase activity for the three strains decreased after dialysis, indicating that the membrane of 12-kDa cutoff could not adequately concentrate xylanase in the extract. There is no information about the molecular weight of Xyl from *P. tigrinus*, but Kirk and Cullen *(8)* reported that the molecular weight of endoxylanases from *Trametes reesei* are in the range of 16–75 kDa. Another possibility is the contamination of the enzymatic extract with xylan obtained from the polysaccharides, mainly at the beginning of the fermentation. This can be evaluated by comparing the Xyl activities at 10 and 30 d of incubation.

Chemical Composition

Table 3 shows that the loss of mass was higher in the flasks, principally in the flask with agitation. The higher lignin loss and better selectivity were verified in the plastic bags. Strain FTPT-4745 was the most stable in the three studied systems.

Table 2
Enzymatic Activities Produced by *P. tigrinus* in Sugarcane Bagasse Determined After 10 and 30 d of Incubation Before and After Dialysis

	Lac (U/g)		
Strains/Enzyme	10 d	30 d before dialysis	30 d after dialysis
FTPT-4741	0.004 ± 0.001	0.00010 ± 0.00005	0.0009 ± 0.0002
FTPT-4742	0.0010 ± 0.0005	0.0003 ± 0.0002	0.0008 ± 0.0004
FTPT-4745	0.004 ± 0.003	0.0003 ± 0.0002	0.0025 ± 0.0002
	MnP (U/g)		
Strains/Enzyme	10 d	30 d before dialysis	30 d after dialysis
FTPT-4741	0.14 ± 0.02	0.14 ± 0.03	0.22 ± 0.01
FTPT-4742	0.09 ± 0.02	0.17 ± 0.04	0.19 ± 0.02
FTPT-4745	0.07 ± 0.02	0.13 ± 0.01	0.14 ± 0.02
	Xyl (U/g)		
Strains/Enzyme	10 d	30 d before dialysis	30 d after dialysis
FTPT-4741	0.4 ± 0.3	0.7 ± 0.1	0.22 ± 0.01
FTPT-4742	1.1 ± 0.1	0.5 ± 0.2	0.27 ± 0.01
FTPT-4745	1.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1

Table 3
Loss of Mass and Components (%)

	FTPT-4741	FTPT-4742	FTPT-4745
Agitation for 10 d			
Mass	13.32	24.1	7.4
Glucan	16.58	30.0	8.50
Polyoses	17.10	30.84	8.32
Lignin	9.42	13.61	9.46
Selectivity (lignin/glucan)	0.57	0.45	1.28
Static for 10 d			
Mass	21.1	16.65	7.76
Glucan	20.97	16.02	2.03
Polyoses	18.42	10.13	3.48
Lignin	15.79	9.82	4.98
Selectivity (lignin/glucan)	0.75	0.61	2.45
Plastic bags for 30 d			
Mass	5.10	5.15	9.31
Glucan	1.34	8.48	15.50
Polyoses	3.92	7.25	10.93
Lignin	25.4	12.26	16.04
Selectivity (lignin/glucan)	18.96	1.45	1.03

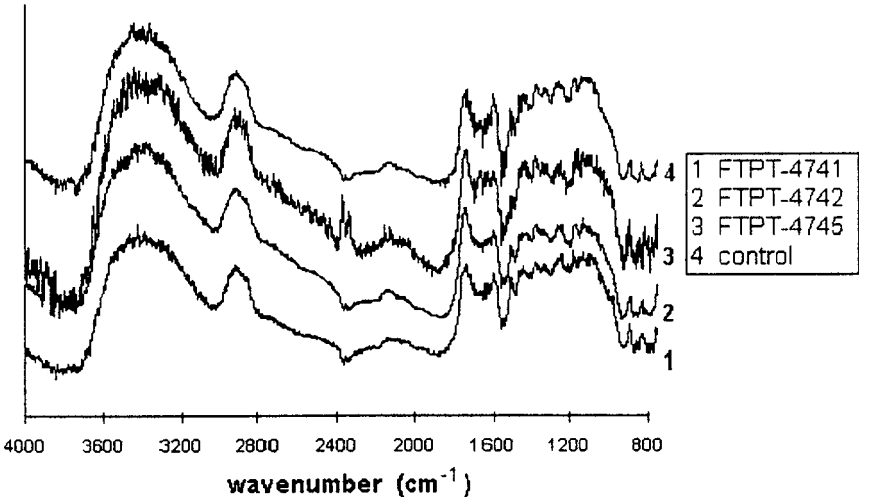

Fig. 1. FTIR spectra for decayed and nondecayed samples obtained in agitation experiments, series 1.

FTIR Principal Components Analysis

The FTIR spectra of the decayed samples were very similar (Fig. 1), and with a PCA treatment the differences between them can be easily observed and evaluated. After PCA treatment of all spectra, the seven first principal components describe more than 95% of the variance of the spectra. Only the first (PC1) and the second (PC2) principal components describe 47 and 27% of this variance, respectively, and the correlation of scores PC2 × PC1 (Fig. 2) explains 74% of the modifications observed in the FTIR spectra. For agitation flasks, series 1, a clear separation between strain FTPT-4741 and the control was observed and is highlighted by the ellipses (Fig. 2). FTPT-4741 had the highest carbohydrate preservation value, the second highest hemicellulose/glucan ratio, and the lowest lignin content. Strain FTPT-4745 showed the most efficient lignin modification (Table 1), but in the PC2 × PC1 correlation of FTIR spectra, the points of FTPT-4745 are very disperse and close to the control. For the other systems, the separation is not clear, as can be seen by Fig. 2 A–C.

Kraft Pulping

Table 4 shows the values of yield, viscosity, and kappa number for the pulps obtained from the samples of decayed bagasse and control. In the experiments carried out for 40 and 60 min, the yields decreased owing to the drastic pulping conditions utilized; kappa number and viscosity values for bagasse kraft pulp were very low. The pulps obtained from decayed sugarcane bagasse with 20 min of reaction presented a viscosity/kappa ratio higher than the control and lower kappa number, showing that the biodegradation is favorable as a pretreatment for the bagasse fibers.

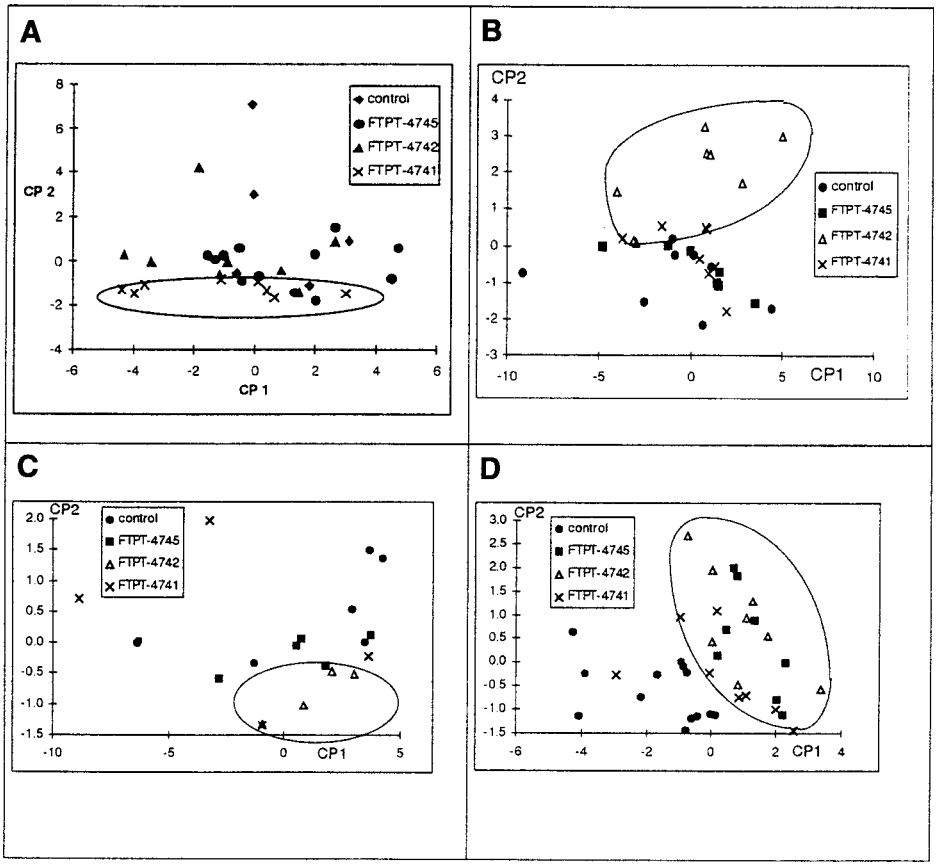

Fig. 2. Scores of PC1 and PC2 of the FTIR of decayed and nondecayed sugarcane bagasse samples: **(A)** agitation series 1; **(B)** agitation series 2; **(C)** static; and **(D)** plastic bag.

Conclusion

The action of *P. tigrinus* on the sugarcane bagasse fibers was evaluated by the enzymatic activity and compared with chemical composition and spectroscopic properties. The results showed that this fungus is adequate for the pretreatment of sugarcane bagasse, causing delignification and preserving the fibers' properties. This process can be utilized in pulp production, but the pulping conditions should be optimized. After biologic pretreatment, the use of chemicals can be reduced significantly. Strain FTPT-4745 was the more stable and appropriate to decay sugarcane bagasse.

Acknowledgments

This work was supported by Conselho Nacional do Desenvolvimento Científico e Tecnológico and Fundação de Amparo à Pesquisa do Estado de São Paulo.

Table 4
Results of Kraft Pulping of Pretreated Bagasse and Control in Plastic Bags with Reaction Times of 20, 40, and 60 min

	Yield (%)	kappa number	Residual lignin (%)[a]	Viscosity (cP)	Viscosity per kappa
Time (20 min)					
Control	46.9	10.2	1.5	5.9 ± 0.5	0.578
FTPT-4741	32.2	7.1	1.1	4.7 ± 0.4	0.662
FTPT-4742	33.0	7.9	1.2	5.5 ± 0.1	0.696
FTPT-4745	32.5	7.0	1.1	5.5 ± 0.1	0.786
Time (40 min)					
Control	33.6	1.9	0.3	4.6 ± 0.2	2.421
FTPT-4741	30.5	5.0	0.8	3.5 ± 0.2	0.700
FTPT-4742	31.9	5.2	0.8	4.1 ± 0.2	0.788
FTPT-4745	30.0	4.0	0.6	4.3 ± 0.1	1.075
Time (60 min)					
Control	30.3	0.9	0.1	3.5 ± 0.2	3.889
FTPT-4741	30.3	1.5	0.2	3.1 ± 0.4	2.067
FTPT-4742	30.6	2.0	0.3	3.2 ± 0.1	1.600
FTPT-4745	27.8	3.3	0.5	3.7 ± 0.2	1.121

[a] Calculated from the ratio of residual lignin = kappa × 0.15.

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