# New Process for Fungal Delignification of Sugar-Cane Bagasse and Simultaneous Production of Laccase in a Vapor Phase Bioreactor

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We propose a new process using a vapor phase bioreactor (VPB) to simultaneously (i) delignify sugar-cane bagasse, a residue of sugar production that can be recycled in paper industry, and (ii) produce laccase, an enzyme usable to bleach paper pulp. Ethanol vapor, used as laccase inducer, was blown up through a VPB packed with bagasse and inoculated with *Pycnoporus cinnabarinus* ss3, a laccase-hyperproducing fungal strain. After 28 days, the laccase activity in the ethanol-treated bagasse was 80-fold higher (80 U $g_{ds}^{-1}$) and the bagasse delignification percentage was 12-fold (12%) higher than in the reference samples produced in the absence of ethanol, corresponding to a high overall pulp yield of 96.1%. In the presence of ethanol, the total soluble phenols amount was 2.5-fold (3 mg FA $g_{ds}^{-1}$) higher than that without ethanol. Six monomeric phenols were detected: *p*-coumaric (4-hydroxyphenyl-2-propenoic), ferulic (4-hydroxy-3-methoxyphenyl-2-propenoic), syringic (4-hydroxy-3,5-dimethoxybenzoic), vanillic (4-hydroxy-3-methoxybenzoic) and 4-hydroxybenzoic acids, and 2-methoxyhydroquinone. Higher concentrations of phenolic compounds were observed when ethanol vapor was added, confirming a more efficient bagasse delignification. After 28 days, the fungal-treated bagasse (with ethanol addition) was pulped and refined. For a freeness of 81 mL CSF, this processing required 50% less energy than with untreated bagasse (without inoculation and ethanol addition), which indicated a significant potential economy for the pulp and paper industry. Handsheets were made from pulp obtained after fungal-treated and untreated bagasse. Comparison of bagasse-pulp characteristics for freeness of 35 and 181 mL CSF showed an average increment by 35% for tensile index and breaking strength and length. VPB allowed a simultaneous production of laccase (90 U $g_{ds}^{-1}$, after pressing of the bagasse) that improved the overall profitability of the process.

**KEYWORDS: Ethanol vapor; bagasse; *Pycnoporus cinnabarinus*; laccase; delignification; mechanical pulp**

## INTRODUCTION

In many countries, the demand for pulp and paper is rising. The United States, Japan, and Europe represent 22% of the world population but consume 70% of the world paper, more than 30% of which is consumed by the United States.

Wood is the most important source of papermaking fiber. However, because wood is not sufficiently available in many countries (*1*) and because its consumption for pulp and paper is rapidly growing, alternative sources of raw material are now investigated. Moreover, reduction of the forest resources requires the use of new raw materials, such as cereal straws, bamboo, asparto, reeds and grasses, kenaf, and sugar-cane bagasse, as wood alternatives. Among nonwood plants commonly used, annual plant fibers provide cheaper sources than wood chips.

Sugar-cane bagasse is a ligno-cellulosic residue of the sugar industry obtained after crushing and juice extraction of cane stalks. Its composition is 50% cellulose, 25% hemicellulose, and 25% lignin (*2*) but varies with sugar-cane varieties, the plant maturity, and soil properties. It is generally burned to generate energy for ethanol and sugar factories. The remaining bagasse has mainly been used for the bioconversion process and pulp and paper productions. In the Middle East and several Latin American countries, the use of bagasse is important. For

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example, sugar-cane bagasse represents almost 20% of India's paper production, where bagasse supply is very abundant (*3*). In Mexico, bagasse is one of the most important raw materials for paper and pulp productions (*4*). Brazil is the first world producer of sugar-cane bagasse that is dedicated to ethanol and sugar production. Ninety percent of the bagasse is burned to produce steam, which in turn is used to generate energy; the remaining 10% is used for pulp and paper productions (*5*). Among the common nonwood fibers, sugar-cane bagasse offers the following advantages: a short growing time, large availability, lower cooking and bleaching chemical requirements, and less refining energy to attain the same freeness drops as in wood pulps.

To produce pulps, mechanical and chemical methods are traditionally used by the industry to remove lignin. However, these treatments have several disadvantages such as energy cost and the production of waste effluents. In the past two decades, interest has been paid to biological treatment based on lignin-degrading fungi and/or enzymes. A fungal pretreatment of wood (pine, aspen), prior to mechanical refining, enhanced strength properties and saved considerable energy (*6*). For different wood species and fungal treatments, the energy savings were from 20 to 37% (*7*, *9*). Pretreatment of bagasse with the white-rot fungus *Ceriporiopsis subvermispora* decreased lignin content and refining energy consumption and increased physical properties (burst, tear, and tensile indexes) (*6*, *10*).

Many fungal strains produce cellulases that degrade cellulose and consequently reduce the mechanical properties of the paper pulp. However, the white-rot fungus *Pycnoporus cinnabarinus*, known as an hyperproducing-laccase strain (*11*, *12*), does not degrade cellulose of natural substrates such as maize and wheat bran and sugar-beet pulp (*13*) because of its very low cellobiose dehydrogenase activity. Laccase used in pulping has been already reported to reduce refining energy cost and to improve the strength of the paper by increasing fiber bonding (*14*).

The addition of laccase inducers, such as ethanol in *P. cinnabarinus* submerged and solid-state cultures, stimulated laccase production (*12*, *15*). In the presence of 35 $g_{ethanol}$ $L^{-1}$, a laccase activity of 266 600 $UL^{-1}$ was found in *P. cinnabarinus* submerged cultures (*12*), which was 155-fold higher than in noninduced cultures. A maximum laccase production of 90 U $g^{-1}$ dry support (ds) was achieved in a vapor phase bioreactor (VPB) packed with bagasse and inoculated with *P. cinnabarinus*, in the presence of 7 g $m^{-3}$ ethanol in the gas phase (corresponding to 31 g $L^{-1}$ ethanol in the liquid phase). This laccase activity was 45-fold higher than in the absence of ethanol (*15*).

In this study, we investigated a new delignification process of sugar-cane bagasse in the presence of *P. cinnabarinus* and ethanol vapors as laccase inducer. The effects of this treatment on the saving of refining energy and the main mechanical characteristics of the bagasse pulp paper were studied.

## MATERIALS AND METHODS

**Fungal Strain.** The laccase-hyperproducing strain *Pycnoporus cinnabarinus* ss3 was obtained from the BRFM collection (Banque de Ressources Fongiques de Marseille, France). It was isolated from the fruit-like structure of the wild dikaryotic strain BRFM 145 by Herpoël et al. (*16*). The strain was kept on malt agar slants at 4 °C.

**Media and Culture Conditions.** Inoculum was obtained from 10-day-old nonagitated precultures grown in Roux flasks containing 200 mL of medium (*16*) and incubated at 30 °C. The flasks were inoculated with 5 mm agar disks of mycelium (five per flask) from a Petri dish. After 10 days, mats were recovered and ground (Ultraturax, 10 000 rpm, 60 s) in liquid culture medium. An inoculation rate of 18 mg dry
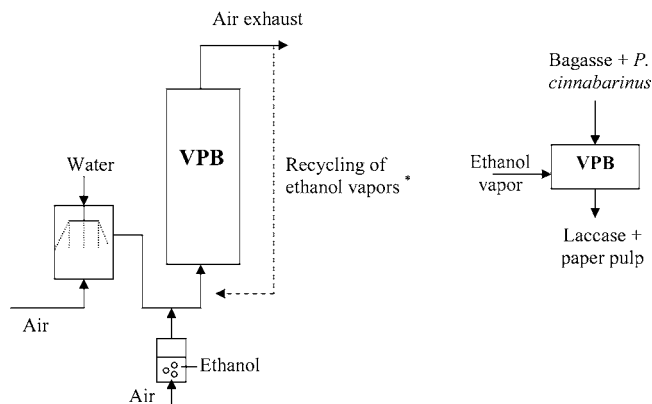


**Figure 1.** Schematic of the experimental setup and diagram of the laccase and paper pulp productions in a VPB. *Recycling of ethanol vapors could be considered because no ethanol degradation was observed during the process (*15*).

biomass/g dry support was used for solid-state culture. The final composition of the culture medium was as follows: maltose (2.5 g $L^{-1}$), yeast extract (1 g $L^{-1}$), sodium tartrate (2.3 g $L^{-1}$), ammonium tartrate (1.84 g $L^{-1}$), and $CuSO_4 \cdot 5H_2O$ (0.1 g $L^{-1}$).

**Packing Material.** Sugar-cane bagasse from Orizaba state (Mexico) was previously sterilized at 121 °C for 15 min and used as packing material. Water content was 70% (w/w). Bagasse contains particles with sizes between 0.4 and 0.8 mm and sticks of approximately 1 cm length.

**Culture Conditions.** *Columns.* Experiments were performed using glass columns (250 mL) placed in a controlled-temperature box at 24 ± 1.5 °C. They were packed with bagasse impregnated with the culture medium and inoculated with *P. cinnabarinus*. The humidified ethanol–air stream was distributed to the columns as previously described (*15*). Inlet ethanol concentration was 7 g $m^{-3}$ ± 0.2 g $m^{-3}$. The airflow rate was 40 mL $min^{-1}$, and the empty bed residence time was 6.3 min. Columns were analyzed at different incubation times to determine laccase activity, total and specific phenolic compounds, and percentage of delignification. Samples from the columns were taken in triplicate.

*Biofilter.* Experiments were conducted in a laboratory scale VPB (**Figure 1**). It consisted of a 150 cm long cylindrical acrylic column with an inner diameter of 18 cm, equipped with four axial sampling ports. The height of the VPB bed was 0.71 m, equivalent to a volume of 18 L. Air was provided by a compressor and divided into two flows. The main stream was passed through a humidifying column containing lava rock. The main airflow was controlled with an electronic mass-flow controller sensor (GFC371, Aalborg, USA). The second was sparged through a 0.5 L bottle containing liquid ethanol and controlled with a mass-flow controller (GFC371, Aalborg, USA). The ethanol–air stream was obtained by mixing the humidified air with the ethanol-laden air and flowed downward through the biofilter.

During the experiment, the inlet ethanol concentration was maintained at 7 g $m^{-3}$ ± 0.2 g $m^{-3}$. Empty bed residence time was 6 min. Experiments were performed in a temperature-controlled room (24 °C ± 2 °C). Samples of packing material were withdrawn at different times of incubation to measure laccase activity and percentage of delignification. Samples were taken in triplicate.

**Ethanol Measurement by Gas Chromatography Analysis.** Flame ionization detector (FID) gas chromatograph (Hewlett-Packard 6890, France) equipped with a HP-5 column (ME Siloxane, Hewlett-Packard, USA) and a 250 $\mu$L loop was used to measure ethanol concentration. The operating conditions were as follows: injector, 180 °C; oven, 250 °C; and detector, 180 °C with a carrier-gas ($N_2$) flow of 25 mL $min^{-1}$. To determine ethanol in the gas phase by gas chromatography, a 5 mL airtight syringe was used.

**Assay for Laccase Activity.** One gram of wet bagasse was diluted with 2 mL of distilled water, homogenized, and centrifuged. Laccase activity on the supernatant was determined as described by Herpoël et al. (*16*). Enzyme activity was expressed in international units (U). One

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unit of activity is defined as the amount of enzyme which leads to the transformation of 1 $\mu$mol substrate per min. Experiments were performed in triplicate, and the standard deviation was lower than 8% of the mean.

**Total and Specific Phenolic Determination.** *Phenolic Compound Extraction.* The soluble phenolic compounds were extracted from sugarcane bagasse by addition of 4 mL of water to 2 g of wet bagasse. The mixture was shaken for 2 h at room temperature and filtered, and the filtrate was used for analysis.

*Total Phenolic Content Determination.* Total soluble phenol (TSP) content was determined colorimetrically at 750 nm, using the Folin−Ciocalteau reagent (*17*), and expressed as milligrams of ferulic acid equivalents per gram of dry bagasse.

*Extraction and HPLC Quantitative Analysis of Monomeric Phenols.* The filtrate used as source of phenolic compounds was acidified with HCl until pH < 3 and then extracted three times with 4 mL of diethyl ether. The organic fractions were combined and evaporated to dryness under nitrogen gas. The residue was dissolved in 1 mL of methanol (50% (v/v)) and filtered through a 0.2 $\mu$m nylon filter (Gelman Sciences, Acrodisc 13, Ann Arbor, MI).

The filtrate was analyzed by HPLC (25 $\mu$L injected). HPLC analyses were performed at 280 nm and 30 °C on a model HP1100 (Hewlett-Packard, Rockville, MD) equipped with a variable UV/Vis detector and a 100-position autosampler autoinjector. Separations were achieved on a Merck RP-18 reversed-phase column (Chromolith 3.5 $\mu$m, 4.6 × 100 mm, Merck, Nogent-sur-Marne, France). The flow rate was 1.4 mL min$^{-1}$. The used mobile phases were 1% acetic acid and 10% acetonitrile in water (solvent A) and acetonitrile 100% (solvent B). The gradient changed as follows: solvent B started at 0% for 2 min, then increased to 50% in 10 min, to 100% in 3 min until the end of running (20 min). A HP 3365 ChemStation processed the data, and the quantification was performed by external standard calibration.

Specific phenolic compounds were identified by LC−mass spectrometry using a Perkin-Elmer ApI150EX apparatus (Perkin-Elmer Applied Biosystems, Courtaboeuf, France).

**Delignification.** The lignin content in the sample was measured gravimetrically as Klason lignin using TAPPI standard method (T-222). The lignin fraction is accounted as the acid insoluble fraction depleted from its ashes. Increasing the amount of fungal biomass has no influence on the result of the assay since the fungus was hydrolyzed in strong acidic conditions and the amount of its ashes withdrawn. All assays were duplicated. Bagasse delignification percentage (BDP) was reported as percentage loss referred to Klason lignin.

**Refining and Pulping.** *Refining.* The untreated bagasse (control) and the fungal-treated bagasse were pulped and refined in a Sprout−Waldron single-disk refiner (model R-12). The energy consumed during fibrillation and refining was measured on a wattmeter connected to the power supply of the electric motor. The primary-stage refining was carried out at 0.02 in. plate clearance, and for the subsequent stages of refining the plate clearance was adjusted to obtain pulps at 80−360 mL CSF (Canadian standard freeness). Refining was performed at 3% consistency.

*Pulping Quality Evaluation.* The physical properties of pulps were evaluated on paper handsheets. The sheets of paper (80 g m$^{-2}$; 20 cm of diameter) were made according to ISO 5270. The freeness (ISO 5267-2) and the strength properties such as burst, tensile (NF EN ISO 1924-2), and tear (NF EN 21974) indexes of the pulps were determined in accordance with ISO standards.

## RESULTS

The diagram, presented in **Figure 1**, describes a new process based on a VPB. VPB was packed with sugar-cane bagasse inoculated with *P. cinnabarinus* and fed with ethanol vapors. The main objective was the simultaneous production of a lignolytic enzyme (laccase) and paper pulp from bagasse.

The following abbreviations were used: i (*P. cinnabarinus*) and e (ethanol in vapor phase). For each experiment, three bagasse samples were obtained: i+/e+ (inoculated with *P.*
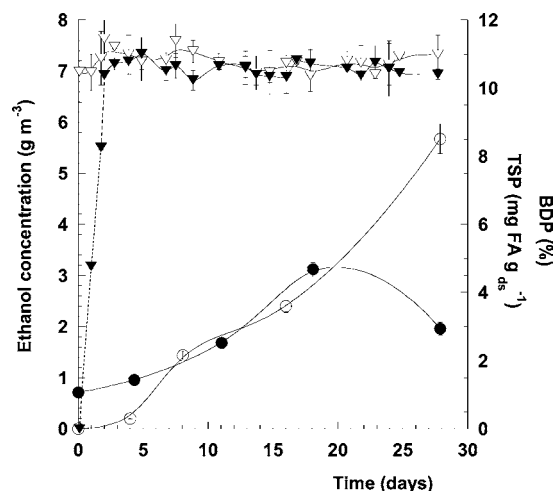


**Figure 2.** Dynamics of inlet ($\triangledown$) and outlet ($\blacktriangledown$) ethanol concentrations, total soluble phenols (TSP) ($\bullet$), and bagasse delignification percentage (BDP) ($\bigcirc$) over time in VPB of 250 mL. FA: ferulic acid; ds: dry support.

*cinnabarinus* and fed with ethanol vapor), i+/e− (inoculated with *P. cinnabarinus* without ethanol addition), and i−/e− (sterile sample without ethanol addition).

**Sugar-Cane Bagasse Delignification in Column Experiments.** *Influence of Ethanol on Delignification.* The laccase activity in the ethanol-treated bagasse (i+/e+) was 80 U $g_{ds}^{-1}$ at the end of experiment (28 days) as compared to 1 U $g_{ds}^{-1}$ in the reference sample (i+/e−). Concomitantly, BDP was 8-fold higher in the presence of ethanol (BDP = 8.5%) as compared to the reference sample (BDP = 1.1%). The TSP amount was 2.5-fold (3 mg FA $g_{ds}^{-1}$) higher with ethanol than without ethanol (1.2 mg FA $g_{ds}^{-1}$). In sterile control (i−/e−), laccase activity was not detected and TSP was 1.1 mg FA $g_{ds}^{-1}$.

*Delignification Time Course in the Presence of Ethanol (i+/e+).* The ethanol outlet concentration reached the inlet value only after 3 or 4 days of incubation. During these first days, laccase activity (data not shown) and BDP were very low while the TSP amount remained almost constant (Figure 2). Then, PDB continuously increased from day 4 to day 28 up to 8.5%. The TSP amount released during sugar-cane bagasse delignification increased until day 18 to a maximum of 5 mg FA $g_{ds}^{-1}$ and then decreased from 5 to 3 mg FA $g_{ds}^{-1}$ (**Figure 2**).

Among the TSP extracted from sugar-cane bagasse, six phenolic monomers were detected: *p*-coumaric (4-hydroxyphenyl-2-propenoic), ferulic (4-hydroxy-3-methoxyphenyl-2-propenoic), syringic (4-hydroxy-3,5-dimethoxybenzoic), vanillic (4-hydroxy-3-methoxybenzoic) and 4-hydroxybenzoic acids, and 2-methoxyhydroquinone. At day 0, none of those phenolic compounds were detected by HPLC, whereas TSP, determined by colorimetry, was quantifiable (**Figure 2**). That can be explained by the presence of other phenolic compounds in the bagasse. Release of phenolic compounds began after 4 days of incubation (**Figure 3**). Higher concentrations of phenolic compounds were observed when ethanol vapors were added. Maximum concentrations of *p*-coumaric and ferulic acids of 1.25 mg $g_{ds}^{-1}$ were attained after 16 days while syringic acid concentration (0.5 mg $g_{ds}^{-1}$) increased slightly until day 28 (**Figure 3**). The same pattern for the reference sample (i+/e−) was observed with values more than 2.5 times lower (**Figure 3**). With or without ethanol addition, vanillic, 4-hydroxybenzoic acids, and methoxyhydroquinone productions were very low (**Figure 4**). The maximum value, lower than 0.3 mg $g_{ds}^{-1}$, was reached on day 20 for vanillic acid, in the presence of ethanol.

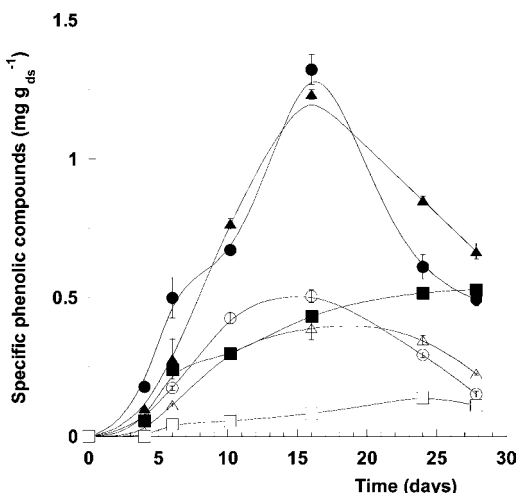Using VPB To Delignify Sugar-Cane Bagasse and Produce Laccase

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**Figure 3.** Dynamics of specific phenolic compounds (*p*-coumaric acid (▲; △), ferulic acid (●; ○), syringic acid (■; □)) over time in 250 mL VPB. Closed symbols: with addition of ethanol vapors, i+/e+. Open symbols: without addition of ethanol vapors: i+/e−. ds: dry support.
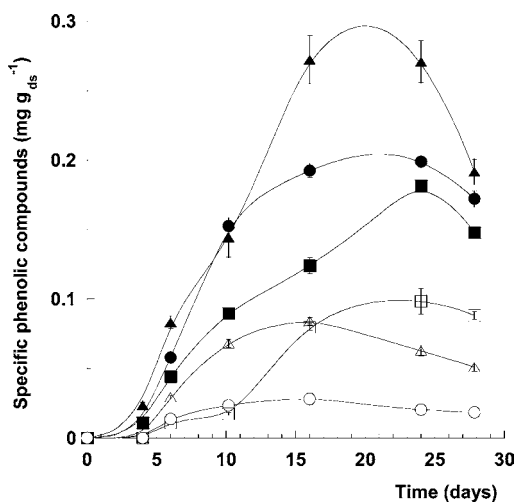


**Figure 4.** Dynamics of specific phenolic compounds (vanillicacid (▲; △), 4-hydroxybenzoic acid (●; ○), and methoxyhydroquinone (■; □)) over time in a 250 mL VPB. Closed symbols: with addition of ethanol vapors, i+/e+. Open symbols: without addition of ethanol vapors, i+/e−. ds: dry support.

However, an increment of 2 or 3 times of the vanillic, 4-hydroxybenzoic acids, and methoxyhydroquinone productions was observed in comparison with the reference sample (i+/e−).

**Characteristics of the Bagasse Pulps Obtained after *P. cinnabarinus* Treatment at Pilot Scale.** During the experiment, laccase activity increased until day 21 to reach a steady-state value averaging 80 U $g_{ds}^{-1}$, which was maintained for 9 days. Bagasse delignification was observed on the first 10 days (BDP = 2.5%). After 30 days, BDP attained 12% (**Figure 5**).

At the end of the experiment, bagasse was refined to obtain mechanical bagasse pulp. Handsheets were made to evaluate breaking length, tensile index, apparent mass volume, and density of untreated (i−/e−) and treated (i+/e+) pulps against the freeness (**Figures 6**, **7**, and **8**). In both cases, specific energy (**Figure 6**) and breaking length (**Figure 7**) decreased with the freeness. For a freeness of 81 mL CSF, specific energy was about twice less after laccase treatment and equal to 1839 kWh t$^{-1}$. For a freeness of 250 mL CSF, specific energy was similar. For freeness ranging from 181 to 257 mL CSF, an increase of
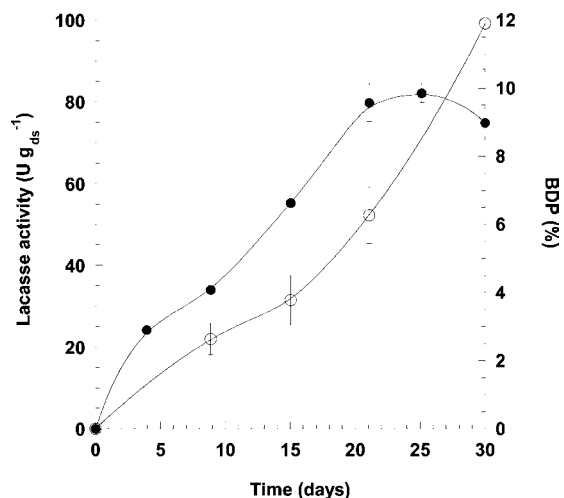


**Figure 5.** Dynamics of laccase activity (●) and BDP (○) over time in a 18 L VPB, in the presence of ethanol vapors as laccase inducer (i+/e+).
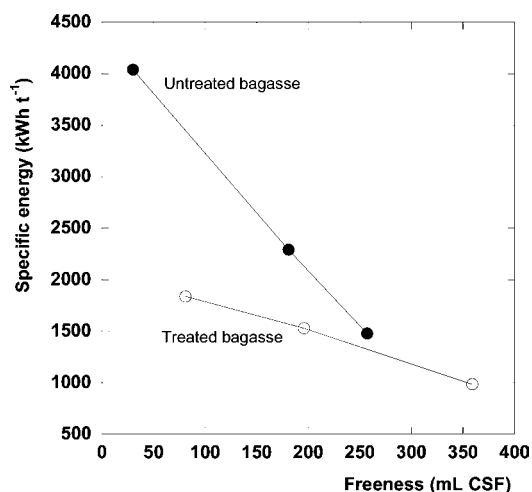


**Figure 6.** Freeness effect on specific energy of refining. (○) Treated bagasse (i+/e+) corresponds to bagasse inoculated with *P. cinnabarinus* with addition of ethanol vapors as laccase inducer. (●) Untreated bagasse (i–/e–) corresponds to bagasse without inoculation and addition of ethanol vapors.

the breaking length of about 30% was observed after enzyme treatment (**Figure 7**). For both untreated and treated bagasse, the apparent mass volume of sheets decreased when the refining increased. However, as compared to untreated pulp, laccase treatment allowed a decrease of the apparent mass volume from 2.96 to 2.64 cm$^3$ g$^{-1}$ for a freeness of 181 mL CSF (**Figure 8**).

Bagasse-pulp characteristics and refining energy for untreated (i−/e−) and treated (i+/e+) bagasse were compared at a freeness of 181 mL CSF (**Table 1**). Fibrillation and refining of treated pulp required 35% less energy than untreated pulp processing. Moreover, in **Table 1**, the characteristics of treated pulp were enhanced as compared to the control: tensile index and breaking strength and length were 14.7 N m g$^{-1}$, 1.23 kN m$^{-1}$, and 1.47 km, corresponding to an average increment of 30%. Thickness (230 $\mu$m) and apparent mass volume (2.72 cm$^3$ g$^{-1}$) of the sheets were 7% lower than control. Sheet density was 0.36 g cm$^{-3}$, which was an increase of around 6%. A repetition of the experiment confirmed the enhancement of pulp characteristics. Physical properties at freeness of 35 mL CSF (**Table 2**) were close to those obtained with the first experiment. An increment about 38% of tensile index (16.4 N m g$^{-1}$) and breaking strength (1.27 kN m$^{-1}$) and length (1.67 km) were

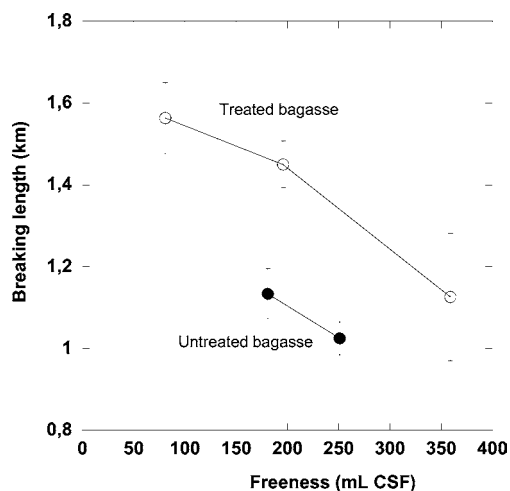**3856**   *J. Agric. Food Chem.,* Vol. 54, No. 11, 2006

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**Figure 7.** Freeness effect on specific breaking length. (○) Treated bagasse (i+/e+) corresponds to bagasse inoculated with *P. cinnabarinus* with addition of ethanol vapors as laccase inducer. (●) Untreated bagasse (i−/e−) corresponds to bagasse without inoculation and addition of ethanol vapors.



**Figure 8.** Freeness effect on apparent mass volume. (○) Treated bagasse (i+/e+) corresponds to bagasse inoculated with *P. cinnabarinus* with addition of ethanol vapors as laccase inducer. (●) Untreated bagasse (i−/e−) corresponds to bagasse without inoculation and addition of ethanol vapors.
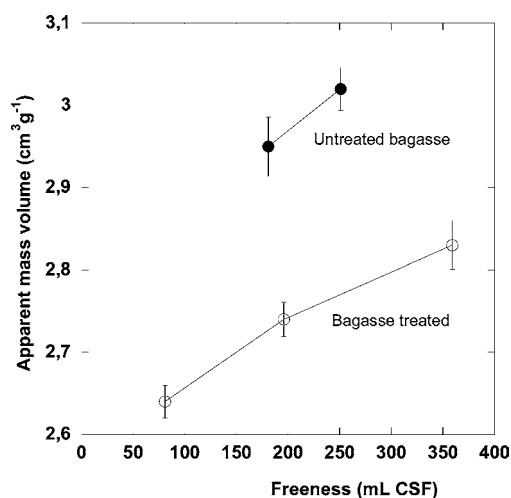
measured. The variation of thickness, apparent mass volume, and density were also similar. However, in this second experiment, tear index and tensile strength were improved by about 20%.

## DISCUSSION

**Laccase Production in VPB.** A previous study (*15*) showed that optimum laccase production on bagasse in a VPB was obtained at 7 g ethanol m$^{-3}$. In our study, the equilibrium between ethanol vapors and dissolved ethanol in the wet bagasse was attained within 3 days. Then, outlet ethanol concentration remained constant and equal to inlet concentration (**Figure 2**). As previously observed (*15*), the fungus, in these operating conditions, did not markedly metabolize ethanol. Therefore, to minimize atmospheric pollution, the recirculation of ethanol vapors should be considered. Inlet ethanol concentration in the vapor phase corresponded to 31 g ethanol L$^{-1}$ in the liquid

**Table 1.** Refining Energy and Mechanical and Physical Characteristics of Untreated and Treated Bagasse at Day 28 for a Freeness of 181 mL CSF[a]

| | untreated bagasse | treated bagasse | percentage |
|---|---|---|---|
| specific energy (kWh t$^{-1}$)[b] | 2290 | 1560 | −32[c] |
| energy of refining (kWh t$^{-1}$) | 1582 | 1035 | −35[c] |
| tensile index (N m g$^{-1}$) | 11.1 | 14.7 | +32 |
| breaking strength (kN m$^{-1}$) | 0.93 ± 0.05 | 1.23 ± 0.05 | +32 |
| breaking length (km) | 1.134 ± 0.06 | 1.470 ± 0.06 | +30 |
| tear index (mN m$^2$ g$^{-1}$) | 1.93 | 1.86 | −3.6 |
| tensile strength (%) | 0.9 ± 0.1 | 0.91 ± 0.1 | +1 |
| thickness ($\mu$m) | 247 ± 2.9 | 230 ± 2.1 | −7 |
| grammage (cm$^2$ g$^{-1}$) | 83.6 ± 1.7 | 84.8 ± 0.9 | +1.4 |
| apparent mass volume (cm$^3$ g$^{-1}$) | 2.95 | 2.72 | −7.8 |
| density (g cm$^{-3}$) | 0.34 | 0.36 | +5.9 |

[a] Treated bagasse (i+/e+) was previously inoculated with *P. cinnabarinus* and incubated with ethanol vapors as laccase inducer. Untreated bagasse (i−/e−) was not inoculated and incubated without ethanol. [b] Specific energy is defined as the sum of refining and defibering energy. [c] For a freeness of 81 mL CSF, reduction of specific and refining energy was 47% and 54%, respectively.

**Table 2.** Mechanical and Physical Characteristics of Untreated[a] and Treated[a] Bagasse at Day 28 for a Freeness of 35 mL CSF

| | untreated bagasse | treated bagasse | percentage |
|---|---|---|---|
| tensile index (N m g$^{-1}$) | 11.9 | 16.4 | +37.8 |
| breaking strength (kN m$^{-1}$) | 0.92 ± 0.01 | 1.27 ± 0.091 | +38 |
| breaking length (km) | 1.21 ± 0.13 | 1.670 ± 0.12 | +38 |
| tear index (mN m$^2$ g$^{-1}$) | 1.44 | 1.66 | +15.3 |
| tensile strength (%) | 0.99 ± 0.18 | 1.24 ± 0.13 | +25.4 |
| thickness ($\mu$m) | 196 ± 2.2 | 186 ± 2 | −5.1 |
| grammage (cm$^2$ g$^{-1}$) | 77.3 ± 1.53 | 77.3 ± 0.81 | 0 |
| apparent mass volume (cm$^3$ g$^{-1}$) | 2.54 | 2.41 | −5.1 |
| density (g cm$^{-3}$) | 0.39 | 0.42 | +7.7 |

[a] As defined in **Table 1**.

phase, which was optimal to produce high laccase activity in liquid culture (*12*). From days 0 to 20, laccase production increased up to 80 U g$^{-1}$ dry support, then it remained constant until day 30 (**Figure 5**). Bagasse delignification started after 5 days (**Figure 2**), corresponding to the end of biomass growth and the beginning of laccase production as a response to ethanol induction (data not shown). The startup of bagasse delignification was correlated with the total soluble phenol (**Figure 2**) and specific soluble phenol productions (**Figures 3** and **4**). It is known that phenolic acids are relatively abundant constituents in plant cell walls (*18*). Among these acids, *p*-coumaric, ferulic, and syringic acids were reported to be linked to components of lignin by ester or ether bonds. Release of these compounds is generally related to fungal or bacterial activity. The lignin degradation pathway involves *p*-coumaric, ferulic, and syringic acids production as well as intermediates of *p*-coumaric acid (*p*-hydroxybenzoic acid) and of ferulic acid (vanillic acid and methoxyhydroquinone) (*19*). The comparison of **Figures 2** and **3** shows that the maximum production of ferulic and *p*-coumaric acids occurred about day 15 while the maximum production of vanillic acid, methoxyhydroquinone, and 4-hydroxybenzoic acid occurred only after 20 and 25 days, respectively. This delay was consistent with the hypothesis of lignin degradation by the fungus following the classical metabolic pathway in which laccase is the first enzyme involved (*20*).

Similar results for laccase production and BDP were obtained by scaling up the process from 300 mL to 18 L. At the end of

Using VPB To Delignify Sugar-Cane Bagasse and Produce Laccase

*J. Agric. Food Chem.,* Vol. 54, No. 11, 2006 **3857**

the experiment, laccase was recovered by pressing and washing the bagasse. Overall yield was 90 U $g_{ds}^{-1}$ for about 2kg of dry bagasse, confirming values obtained in local sampling in the bioreactor during the experiment (**Figure 5**). Total recovery of laccase was easily obtained showing that VPB packed with sugar-cane bagasse was a good system to produce laccase at low cost.

**Paper Pulp Production in VPB.** Large quantities of energy are consumed for mechanical refining applied during paper production to enhance fiber characteristics by fibrillation. The level of refining is monitored by the measurement of freeness (water drainage from pulp), which decreases (swelling of fibers) when the refining increases.

In this study, a reduction up to 50% of energy consumption (freeness of 81 mL CSF), as compared to the untreated control bagasse (i−/e−) was obtained which represents a very significant economy for the pulp and paper industry (**Figure 5**). Different authors have reported a reduction in energy consumption during mechanical refining of wood chips inoculated with *Phanerochaete chrysosporium* and *C. subvermispora* (*7, 9, 21*). Inoculating red pine logs with *Phelbiopsis gigantea*, a white-rot fungus, reduced energy consumption of mechanical refining by 9 to 27% after 17 weeks of treatment, as compared with untreated logs (*22*).

Bustamente et al. (*10*) studied the effect of the biomechanical pulping of depithed bagasse with the white-rot fungi *C. subvermispora* and *Pleurotus ostreatus* on the refining energy, lignin losses, and physical properties of bagasse pulps. Despite that the bagasse used in our study was not depithed, energy saving was about twice more with *P. cinnabarinus* (54% instead of 22% and 34%). Regarding delignification and physical properties, results obtained with *P. cinnabarinus* were close to the best results obtained with *Pl. ostreatus* and *C. subvermispora*. For example, BDP was 12% as compared to 9% and 17% with *Pl. ostreatus* and *C. subvermispora*. Tensile index increased by about 33% for both *P. cinnabarinus* and *C. subvermispora* while *Pl. ostreatus* was less than 3%. The improvement of the characteristics was most probably due to an extensive delignification of the surface of the fiber, corresponding to the 12% delignification observed. Indeed, the main characteristics of the fibers, such as breaking length, are related to interfiber bonding. The removal of surface lignin by laccase favored external defibrillation and increased hydrophilic surface areas that are involved in fiber bonding.

The weight losses of bagasse measured after 30 days of fermentation correspond to 3.9% and 1.1% with (i+/e+) and without (i+/e−) ethanol, respectively. Weight-loss percentage difference (2.8%) can be attributed to the bagasse delignification under ethanol treatment. The initial percentage of lignin in bagasse being approximately 25%, these 2.8% correspond to a delignification of 11.2%, a value very close to that measured in the pilot VPB in the presence of ethanol (10.9%). Pulp yield, obtained in the case of ethanol addition in the VPB, was 96.1%.

This study shows that a clean original process can be applied to sugar-cane bagasse in order to (i) produce laccase that can be used to bleach paper pulp or sold to enhance the overall profitability of the process, (ii) enhance paper characteristics by superficial delignification of fibers, without the use of chemicals, and (iii) save refining energy. However, a techno-economic study should be necessary to verify the cost effectiveness of the whole process. Mechanical bagasse pulps generally exhibit poor strength properties and high-cost energy that hampered its industrial use despite high-yield pulping. However, biomechanical pulping with a vapor phase bioreactor, using ethanol as a laccase inducer, opens promising prospects for industrial utilization of agricultural wastes, as this study could also concern other annual plant fibers.

## LITERATURE CITED

(1) Saijonkari-Pahkala, K. Non-wood plants as raw material for pulp and paper. Academic dissertation. University of Helsinki, 2001.

(2) Pandey, A.; Soccol, C. R.; Nigam, P.; Soccol, V. T. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresour. Technol.* **2000**, *74*, 69−807.

(3) Viswanathan, K. S. K.; Gopalaratnam, N. Effect of bagasse pulp on quality. A study of various Indian paper grades. North American nonwood fiber symposium, Atlanta, GA; Tappi Press, 1998; pp 17−18, 277−290.

(4) Sanjuan, R.; Anzaldo, J.; Vargas, J.; Turrado, J.; Patt, R. Morphological and chemical composition of pith and fibers from mexican sugarcane bagasse. *Holz. Roh. Werkst.* **2001**, *59*, 447−450.

(5) Gonçalves, A. R.; Esposito, E.; Benar, P. Evaluation of *Panus tigrinus* in the delignification of sugarcane bagasse by FTIR−PCA and pulp properties. *J. Biotechnol.* **1998**, *66*, 177−185.

(6) Ramos, J.; Gonzalez, M.; Ramirez, F.; Young, R.; Zuñiga, V. Biochechanical and biochemical pulping of sugarcane bagasse with *Ceriporiopsis subvermispora* fungal and xylanase pretreatments. *J. Agric. Food Chem.* **2001**, *49*, 1180−1186.

(7) Akhtar, M.; Attridge, M. C.; Myers, G. C.; Kirk, T. K.; Blanchette, R. A. Biochemical pulping of loblolly pine with different strains of white-rot fungus *Ceriporiopsis subvermispora*. *Tappi J.* **1992**, *75*, 105−108.

(8) Bar-Lev, S. S.; Kirk, T. K.; Chang, H. M. Fungal treatment can reduce energy requirements for secundary refining of TMP. *Tappi J.* **1982**, *65*, 111−113.

(9) Leatham, G. F.; Myers, G. C.; Wegner, T. H. Biochemical pulping of aspen chips: Energy savings resulting from different fungal treatments. *Tappi J.* **1990**, *73*, 197−200.

(10) Bustamente, P.; Ramos, J.; Zuñiga, V.; Sabharwal, H. S.; Young, R. A. Biomechanical pulping of bagasse with the white rot fungi *Ceriporiopsis subvermispora* and *Pleurotus ostreatus*. *Tappi J.* **1999**, *82*, 123−128.

(11) Eggert, C.; Temp, U.; Eriksson, K. E. L. The lignolytic system of the white rot fungus *Pycnoporus* cinnabarinus: purification and characterization of the laccase. *Microbiology* **1996**, *62*, 1151−1158.

(12) Lomascolo, A.; Record, E.; Herpoel-Gimbert, I.; Delattre, M.; Robert, J. L.; Georis, J.; Dauvrin, T.; Sigoillot, J. C.; Asther, M. Overproduction of laccase by a monokarryotic strain of *Pycnoporus* cinnabarinus using ethanol as inducer. *J. Appl. Microbiol.* **2003**, *94*, 1−7.

(13) Sigoillot, C.; Lomascolo, A.; Record, E.; Robert, J. L.; Asther, M.; Sigoillot, J. L. lignocellulolytic system of *Pycnoporus cinnabarinus*: isolation and characterization of a cellobiose dehydrogenase and a new xylanase. *Enzyme Microb. Technol.* **2002**, *31*, 876−883.

(14) Kenealy, W. R.; Jeffries, T. W. Enzyme processes for pulp and paper: A review of recent developments. In *Wood Deterioration and Preservation: Advances In Our Changing World*; Goodell, B., Nicholas, D. D., Schultz, T. P., Eds.; American Chemical Society: Washington, DC, 2003; pp 210−239.

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(15) Meza, J. C.; Lomascolo, A.; Casalot, L.; Sigoillot, J. C.; Auria, R. Laccase production by *Pycnoporus* cinnabarinus grown on sugar-cane bagasse: Influence of ethanol vapors as inducer. *Process. Biochem.* **2005**, *40*, 3365−3371.

(16) Herpoel, I.; Moukha, S.; Lesage-Meessen, L.; Sigoillot, J. C.; Asther, M. Selection of *Pycnoporus cinnabarinus* strains for laccase production. *FEMS Microbiol. Lett.* **2000**, *183*, 301−306.

(17) Folin, O.; Ciocalteau, V. On tyrosine and tryptophan determination in protein. *J. Biol. Chem.* **1927**, *73*, 627−650.

(18) Topakas, E.; Kalogeris, E.; Kekos, D.; Macris, B. J.; Christa-kopoulos, P. Production of phenolic from corn cobs by coupling enzymic treatment and solid-state fermentation. *Eng. Life. Sci.* **2004**, *4*, 283−286.

(19) Kirk, T. K. Effects of microorganisms on lignin. *Annu. Rev. Phytopathol*. **1971**, *9*, 185−210.

(20) Kluczek-Turpeinen, B.; Tuomela, M.; Hatakka, A.; Hofrichter, M. Lignin degradation in a compost environment by the deuteromycete *Paecilomyces inflatus*. *Appl. Microbiol. Biotechnol.* **2003**, *4*, 374−379.

(21) Leatham, G. F.; Myers, G. C.; Wegner, T. H, Blanchette, R. A. Biomechanical pulping of aspen chips paper strength and optical properties resulting from different fungal treatments. *Tappi J.* **1990**, *73*, 249−254.

(22) Blanchette, R.; Behrendt, C. D.; Williams, D.; Iverson, S.; Akhtar, M.; Enebak, S. A. A new approach to effective biopulping: treating logs with *Phelbiopsis gigantea*. Proceedings of 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, 1998; pp A 51−A 54.