

## Use of Vinasse and Sugarcane Bagasse for the Production of Enzymes by Lignocellulolytic Fungi

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### ABSTRACT

*In this present work, three strains of Pleurotus and Trichoderma reesei were cultivated in media with pre-treated bagasse and vinasse. Cellulolytic and lignolytic activities and biomass production were analyzed. The treatment of the bagasse with 2% H<sub>2</sub>O<sub>2</sub> + 1.5% NaOH + autoclave resulted in a greater fiber breakage increasing the cellulose level up to 1.2 times and decreasing 8.5 times the hemicellulose content. This treatment also resulted in a high lignolytic activity for all cultures utilized. T. reesei produced laccase, peroxidase and manganese-peroxidase in all the treatments, having its manganese-peroxidase activity raging from 1.9 to 4.8 times higher than the basidiomycetes.*

**Key words** Bagasse, Enzyme, Fungi, Lignocellulolytic, *Pleurotus*, Sugarcane

### INTRODUCTION

The sugar-alcohol industry is an excellent representation of the developing process of Brazil, having sugarcane as one of the largest monocultures according to the Brazilian Service of Support for Micro and Small Companies (SEBRAE, 2008). The prediction for the sugarcane production for the 2008/2009 growing season is that the total amount will reach approximately 558.72 million tons, which will be processed in the sugar industry. This amount corresponds to an increase of 11.4% relating to the 2007/2008 growing season (CONAB, 2008). With this, there will also be an increase in the residues, among them, bagasse and vinasse. For each ton of sugarcane processed, approximately 250 to 280 kg of bagasse is produced; and between 700 and 900

liters of vinasse is obtained (CONAB, 2008). These residual products have the capacity to aggregate value to the final production depending in the sector in which are applied.

The biological degradation of the sugarcane bagasse involves the enzymatic hydrolysis of the glycosidic linkages from the cellulose chains and an attack on the lignin polymer. These cellulose fibers, in nature, are generally fixed with other polymers (Leshine, 1995; Melo and Azevedo, 1997), especially hemicellulose and pectin (Enari, 1983). The microorganisms capable of hydrolyzing these materials contain a complex system of a variety of enzymes. Such systems adhere to the cellular envelope and to the substrate, mediating a greater proximity necessary for the enzyme actions (Fengel and Wegener, 1989). Different microorganisms act in

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cooperation at several stages in the degradation of cellulose, releasing multiple compounds which are utilized in the metabolism (Melo and Azevedo, 1997; Esposito and Azevedo, 2004).

Lignin is a constituent of the cell wall of all the vascular plants, is a macromolecule consisting of units of phenylpropane, with a tridimensional and amorphous conformation, representing from 20 to 30% of the lignocellulosic material (Fagerstam et al., 1980). Due to the strict association between cellulose, hemicellulose and lignin, these compounds are not homogeneously distributed within the cell wall of the plant. The secondary wall contains high quality cellulose, while in the middle lamella, a higher quantity of lignin is found. However, all the three compounds can be found in all the layers of the cell wall.

The enzymes that constitute the multi-enzymatic lignocellulolytic include laccase (E.C. 1.10.3.2) (Kuhad et al. 1990; Kuwahara et al. 1984), lignin peroxidase (E.C. 1.11.1.14) (Kuhad et al. 1990) and manganese-peroxidase (E.C. 1.11.1.13) (Bon et al., 2008). These enzymes participate in the degradation of lignin. The enzymes responsible for the degradation of cellulose are exoglucanases (E.C. 3.2.1.91) (Kuwahara et al. 1984; Bon et al., 2008) and the endoglucanases (E.C. 3.2.1.4) (Bon et al., 2008).

The conversion of plant macromolecules into low molecular weight sugars that can be utilized directly or fermented may involve the following steps: reduction in the size of the material, pre-treatment, enzymatic hydrolysis and simultaneous fermentation, and isolation of the reaction compounds. The pre-treatment allows a contact surface and a rupture in the structure of the lignin and cellulose fibers, consequently facilitating the hydrolysis to the simple sugar (Kuhad et al., 1990; Kuwahara *et al.*, 1984; Bon et al., 2008). The pre-treatments can be physical (as defibrillation by pressure and explosion) or chemical (acid and alkaline hydrolysis).

The alkaline pre-treatment solubilizes the hemicellulose content. The physical-chemical pre-treatments utilizing diluted acid and vapor pressure, allow the selective removal of the hemicellulose, producing sugar solutions with elevated pentose content and therefore reducing the lignin content. The alkali processes tend to promote a high dissolution of the lignin and lower the solubilization/fragmentation of the hemicelluloses (Melo and Azevedo, 1997; Enari,

1983; Esposito and Azevedo, 2004; Kuwahara et al. 1984; Bon et al., 2008).

The objective of this work was the selection of lignocellulolytic fungi and the evaluation of the enzyme production of these microorganisms, cultivated in chemically treated sugarcane bagasse with vinasse, under a range of temperatures, aiming towards the recovery of these residues for an eventual enzyme production.

## MATERIALS AND METHODS

### Microorganisms

The cultures of lignocellulolytic fungi were obtained from the Applied Ecology Laboratory, CENA-USP, Piracicaba, SP. The cultures were: *Pleurotus sajor-caju* CCB020, *Pleurotus ostreatus*, *Pleurotus ostreatoroseus* CCB440 and *Trichoderma reesei*.

### Bagasse substrate

Sun-dried sugarcane bagasse donated by a local sugar-alcohol industry was collected. The shredded pieces were sieved at 1.19 mm (tyler14) to obtain smaller particles.

### Determination of cellulase and ligninase production

The fungi were tested according to the capacity of producing cellulases and ligninases utilizing the methodology of Hankin and Anagnostakis (1975), with modifications in the media tested. For the production of ligninases, the medium contained 2.0 g sugarcane, 100  $\mu$ L guaiacol, 16 g agar and 1000 mL of distilled water. For cellulase production, the medium contained 10 g carboxymethyl cellulose (CMC), 10 g agar and 1000 mL of distilled water. Both, cellulase and ligninases media were autoclaved at 121°C for 15 minutes. In the medium containing cellulose (CMC), 10 mL of a Lugol-iodine solution was added to the Petri dish after growing the fungus for cellulase production.

The fungi were inoculated using 5.0 mm diameter disks from seven-days old cultures cultivated on malt-extract media. The Petri dishes were sealed and placed in an incubator at 28°C for seven days. The production of ligninases and cellulases were verified by the formation of a reddish growth halo in the guaiacol medium and a blue coloration in the medium with CMC.

### Bagasse pre-treatment

The bagasse was treated with 2% H<sub>2</sub>SO<sub>4</sub>, 1.5% NaOH, 2% H<sub>2</sub>O<sub>2</sub> and 2% H<sub>2</sub>O<sub>2</sub> + 1.5% NaOH in the proportion of 1:10 (w/v) and autoclaved at 121°C for 15 minutes. The control consisted in non-treated bagasse added with distilled water + autoclave. After the treatment, all the samples were washed with distilled water in order to neutralize the effects of the reagents and dried at 60°C for 18 h and then analyzed for cellulose, hemicellulose and lignin as described by Goering and Van Soest, 1970.

### Vinasse characterization

The vinasse was collected from the same local sugar-alcohol industry and was kept in sealed 5 L gallons and stored in cold room. Samples of this vinasse were characterized for its contents.

### Incubation and sampling

The cellulase and ligninase producing cultures were transferred aseptically to 250 mL Erlenmeyer flasks containing 20 g bagasse with 60 mL of sterilized vinasse. The pH was adjusted to 6.0. The incubation was carried out in a controlled temperature incubator at 180 rpm and 25, 30, 35 and 40° C. The control consisted of 20 g bagasse and 60 mL minimal mineral medium (g L<sup>-1</sup>: 1.4 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 2.0 KH<sub>2</sub>PO<sub>4</sub>; 0.3 urea, 0.3 MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.0014 ZnSO<sub>4</sub>·7H<sub>2</sub>O; 0.005 FeSO<sub>4</sub>·7H<sub>2</sub>O; 0.0016 MnSO<sub>4</sub>; 0.002 CoCl<sub>2</sub>; 0.002 CaCl<sub>2</sub>; 2.0 mL Tween-80 and 1.0 mL peptone). The experiment was carried out with three replicates during a period of 30 days. Every three days of incubation, a flask from each culture was vacuum filtered, using a Whatman N° 1 filter-paper (85 mm Ø). The supernatant was then transferred to a 50 mL Falcon tube for enzymes analysis.

The crude extract was frozen and lyophilized (Thermo Savant, model modulyod 115) for 72 h at -48° C and 1.2 mbar, and the biomass was evaluated by gravity until a constant weight.

### Enzymatic analysis

The activity of endoglucanase, exoglucanase, laccase, manganese-peroxidase and peroxidase were determined in triplicate by reading the absorbance by a FEMTO-432 spectrophotometer.

### Endoglucanase and exoglucanase activities

The activity of endoglucanase (endo-1,4-β-D-glucanase) was determined by using

carboxymethyl cellulose (CMC) as the substrate and the exoglucanase (exo-1,4-β-D-glucanase) by the dinitrosalicylic acid (DNS) technique, according to the methodology of Miller (1959). One unit (IU) of enzyme activity was defined as the amount required to release 1.0 μmol of glucose from the appropriate substrates per minute per milliliter of crude filtrate under the assay conditions.

### Determination of laccase activity

The activity of laccase was determined using a mixture containing 0.3 mL of a 0.05M citrate-phosphate buffer with pH 5.0; 0.1 mL solution of 0.05 g syringaldazine in 50 mL ethanol and 0.6 mL of the enzyme supernatant. The oxidation of syringaldazine was measured by monitoring the absorbance at 525 nm after 10 minutes of reaction. A unit of the enzymatic activity was defined as the quantity of the enzyme necessary to oxidize 1.0 μmol of syringaldazine per minute, utilizing the molar extinction coefficient of 6.5 x 10<sup>4</sup>/mol/cm for oxidized syringaldazine. The laccase activity was expressed as international unit per liter (UI L<sup>-1</sup>) of the extract (Szklar et al., 1989).

### Determination of manganese-peroxidase (MnP) activity

The MnP activity from the crude enzyme extract was determined evaluating the oxidation of phenol red (ε<sub>610</sub> = 4460 mol cm<sup>-1</sup>) (Hankin and Anagnostakis, 1975) in the presence of manganese and H<sub>2</sub>O<sub>2</sub> at 30° C in a mixture composed of 100 μL of sodium-lactate buffer (0.25 mol L<sup>-1</sup>); 50 μL of a MnSO<sub>4</sub> (2.0 mmol L<sup>-1</sup>) solution; 50 μL of H<sub>2</sub>O<sub>2</sub> in a sodium-succinate buffer (0.2 mol L<sup>-1</sup>) with pH of 4.5; 200 μL bovine serum albumin (0.5%) and 600 μL of the crude enzyme extract. The reaction was initiated with the addition of 100 μL phenol red (0.1%) and the absorbance was measured at 610 nm. To stop the reaction, 40 μL of 2.0 N NaOH was added after 10 minutes of the first reading. One unit of the enzyme activity was defined as the quantity of the enzyme necessary to form 1.0 μmol de Mn<sup>+3</sup> per minute, using the molar extinction coefficient of 4.460 x 10<sup>3</sup>/mol/cm (Hankin and Anagnostakis, 1975). The MnP activity was expressed as international unit per liter (UI L<sup>-1</sup>) of the extract.

### Determination of peroxidase activity

The activity of peroxidase was performed according to the protocol established by Archibald

(1992). Two 10 x 100 mm tubes were used, one for the non-boiled sample and the other for the boiled sample. In each tube, 0.6 mL of the sample to be analyzed was added with 0.2 mL of citrate-phosphate buffer and 0.1 mL of H<sub>2</sub>O<sub>2</sub>. One tube was put into boiling water for 10 minutes (control sample) and cooled for the analysis. The initial time was determined by the measure of the absorbance at the time at which 0.1 mL of syringaldazine was added. The final time was the measurement at 10 minutes after the reaction started. From each tube, 1.0 mL aliquots were taken from the boiled and non-boiled samples to measure against a blank at 460 nanometers. The peroxidase activity was expressed in international units per liter (UI L<sup>-1</sup>) of the extract.

### Statistical analysis

The statistical tests were performed utilizing the statistical analysis software Assistat 7.5 by the Tukey's test with a significance level,  $p < 0.05$  (5%).

## RESULTS AND DISCUSSION

### Cellulase and ligninase production

A blue color halo was observed in the CMC-Lugol medium. The reddish color was due to the

oxidation of guaiacol to tetraguaiacol (Hankin and Anagnostakis, 1975), which confirmed the lignolytic activity of the fungus, because the oxidation of guaiacol would occur only in the presence of peroxidase. The lugol reacts with some polysaccharides (Hankin and Anagnostakis, 1975). In the presence of starch, a blue color is formed due to the potassium iodide, rendering the elementary iodine soluble in water through the formation of the I<sub>3</sub><sup>-</sup> ion in the helix formed by the amylase (Pereira, et al., 2003). The starch was produced by the degradation of the carboxymethyl cellulose in the medium into lower molecular weight saccharides, consequently confirming the presence of cellulases.

This experiment allowed the comparison of the capability of the fungi in producing cellulolytic and lignolytic enzymes and also demonstrated a promising method for selection of cultures for these enzymatic studies.

### Analysis of collected vinasse

The temperatures at the time of the collection of vinasse varied from 95.5°C to 118°C and after cooling, the pH was between 3.95 and 4.5. The sample had high concentration of potassium and phosphorus and high values of COD and BOD as shown on Table 1.

**Table 1** – Characterization of the vinasse *in natura*.

Parameters of vinasse <i>in natura</i>	
pH (at 20°C)	3,95
COD (mg L <sup>-1</sup> )	42000
BOD (mg L <sup>-1</sup> )	11310
Total suspended solids (mg L <sup>-1</sup> )	5969
Total dissolved solids (ppm)	152126
Reducing sugars (mg L <sup>-1</sup> )	962
Total Kjeldahl Nitrogen (mg L <sup>-1</sup> )	70
Potassium (mg L <sup>-1</sup> )	2272
Phosphate (as P) (mg L <sup>-1</sup> )	200

### Analysis of the cellulose, hemicellulose and lignin content

The treatments with the alkaline and acid solutions, with the exception of the 1.5% NaOH resulted in increase in the level of cellulose in relation to the control, in the treatments with 2% H<sub>2</sub>SO<sub>4</sub> and with 2% H<sub>2</sub>O<sub>2</sub> + 1.5% NaOH, the cellulose levels were increased by 1.2 times (Table 2). These treatments promoted the rupture of the

lignin and cellulose fibers found in the internal part of the bagasse fiber, being released or solubilized. The alteration in the level of hemicellulose occurs by the fact that the acidic treatments break the hemicellulose fibers, while the alkaline treatment solubilizes the hemicellulose, generating pentoses and hexoses such as xylose and arabinose in variable quantities (Mane et al., 2006; Fasanella, 2008; Doerge et al.,

1997). The bagasse treated with 2% H<sub>2</sub>O<sub>2</sub> + 1.5% NaOH + autoclave at 121° C for 15 minutes was selected as it resulted high level of cellulose and lignin and decreased the level of hemicellulose. However the high content of cellulose was obtained by the 2% H<sub>2</sub>SO<sub>4</sub> treatment, but this treatment was not chosen because the hemicellulose content was of 5.87%, not facilitating the attack of the microbial enzymes in the free cellulose and lignin (Alborés et al., 2007). The remaining percentage as observed in Table 2 was due to other components such as proteins and lipids as stated by Goering and Van Soest, 1970.

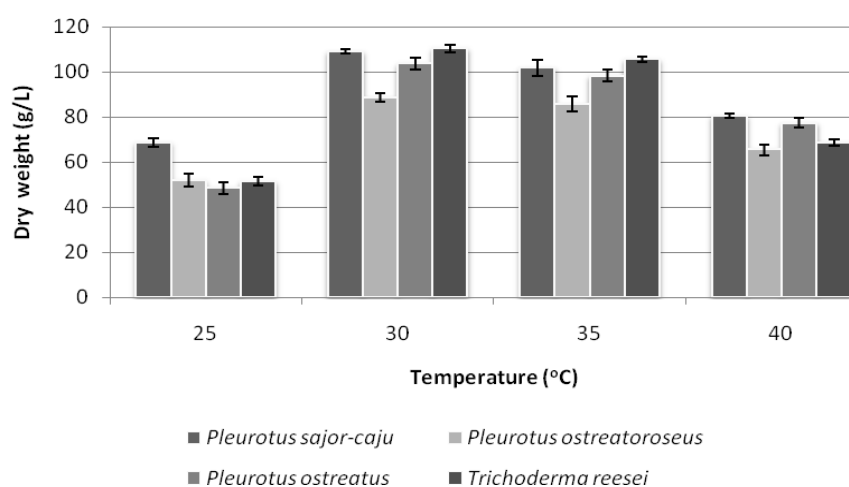
### Mycelium quantification

The quantity of the biomass produced after 30 days of cultivation in the mineral medium (Fig. 1) showed a significant variation among the cultures at 25° C in comparison to 30° C, where the quantity of biomass produced by the fungi was much higher in *T. reesei* (110.43 g L<sup>-1</sup>); *P. sajor-caju* (109.10 g L<sup>-1</sup>); *P. ostreatus* (103.62 g L<sup>-1</sup>) and *P. ostreatotroseeus* (88.67 g L<sup>-1</sup>). At 35° C also a high biomass production was observed by *T.*

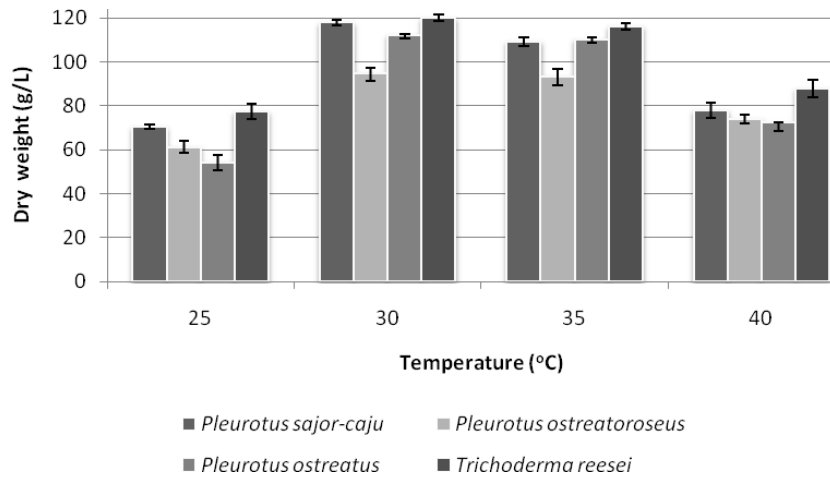
*reesei* (105.49 g L<sup>-1</sup>); *P. sajor-caju* (101.78 g L<sup>-1</sup>); *P. ostreatotroseeus* (85.80 g L<sup>-1</sup>) and *P. ostreatus* (98.27 g L<sup>-1</sup>). Figure 2 showed that the mycelial biomass had an increment in the medium containing sieved bagasse + vinasse, as compared to the mineral medium (Fig. 1). The most adequate temperature for a higher mycelial growth was between 30 and 35° C. This range of temperature was expected since the microorganisms studied were mesophiles (Madan et al., 1983; Zhang, et al 2009). The fungal biomass at 30° C was 117.87 g L<sup>-1</sup> for *P. sajor-caju*, 94.52 g L<sup>-1</sup> for *P. ostreatotroseeus*, 112.31 g L<sup>-1</sup> for *P. ostreatus* and 120.04 g L<sup>-1</sup> for *T. reesei*. The relatively high production of biomass could be attributed to highly nutritious nature of the media utilized. The mineral medium contains the basic minerals while in the vinasse contains high values of nitrogen, potassium, phosphorus and organic matter; necessary elements in the composition of the cellular material (Esposito and Azevedo, 2004). The temperature of 30° C showed to be the most appropriate temperature for the production of biomass for the selected fungi.

**Table 2** – Cellulose, lignin and hemicellulose levels of treated and un-treated sugarcane bagasse

Treatment	Cellulose (%)	Lignin (%)	Hemicellulose (%)
Bagasse <i>in natura</i> (Control)	55.34	11.21	25.87
2% H <sub>2</sub> SO <sub>4</sub>	70.08	12.22	5.87
1.5% NaOH	53.44	9.75	11.98
2% H <sub>2</sub> O <sub>2</sub>	59.50	13.37	8.46
2% H <sub>2</sub> O <sub>2</sub> + 1.5% NaOH	67.31	15.87	2.98



**Figure 1** – Dry weight (g L<sup>-1</sup>) of selected fungi in relation to the temperature (°C) cultivated in mineral (control) medium.



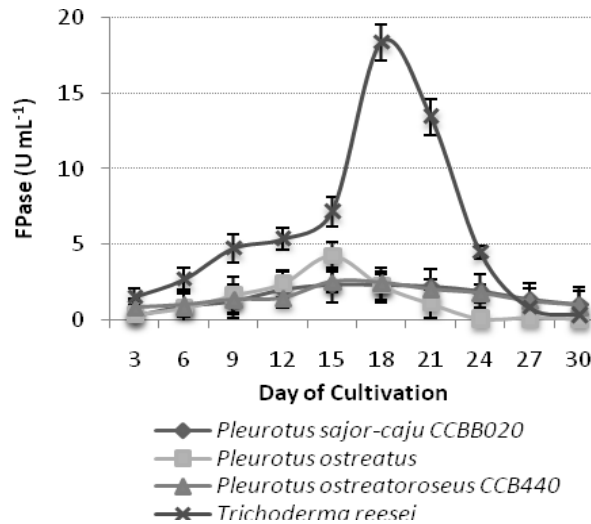
**Figure 2** – Dry weight ( $\text{g L}^{-1}$ ) of selected fungi in relation to the temperature ( $^{\circ}\text{C}$ ) cultivated in vinasse medium.

### Enzymatic activity analysis

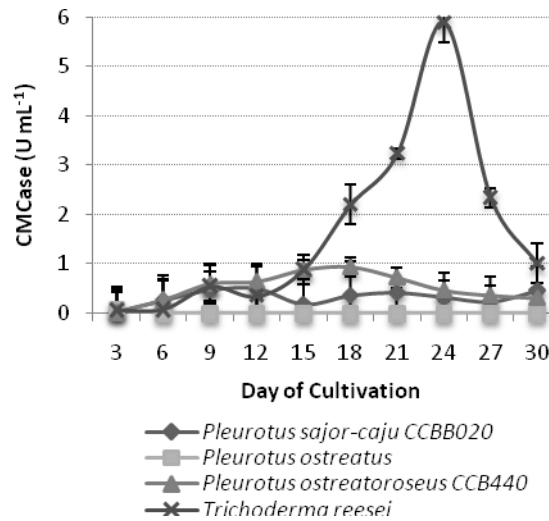
The *Pleurotus* showed a low enzymatic activity of exo- and endoglucanase (Fig. 3 and 4), while the *T. reesei* demonstrated endoglucanase activity (Fig. 4) with 3.23 and 5.88  $\text{U mL}^{-1}$  at the 21<sup>st</sup> and 24<sup>th</sup> day of cultivation, respectively. *T. reesei* also

showed a high exoglucanase activity (Fig. 3) with 18.35  $\text{U mL}^{-1}$  at the 18<sup>th</sup> day of growth.

The presence of endoglucanase may affect the activity of exoglucanase, since these microorganisms possess multiple distinct varieties of endo- and exoglucanases, acting together in a synergic manner.



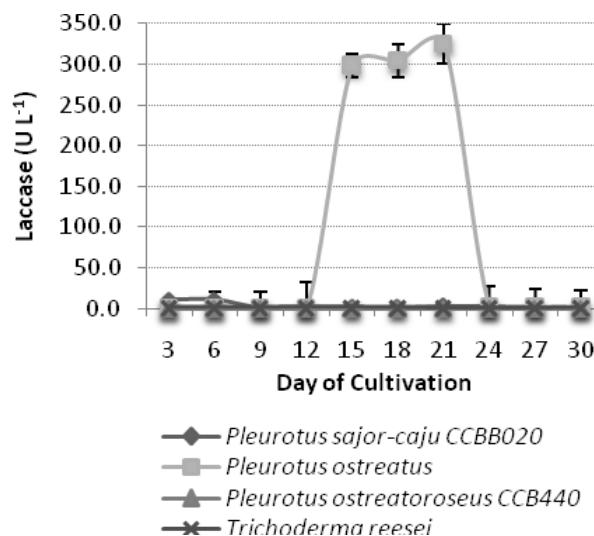
**Figure 3** - Exoglucanase activity (FPase,  $\text{U mL}^{-1}$ ) during 30 days of cultivation at 30 $^{\circ}\text{C}$  cultivated in vinasse medium.



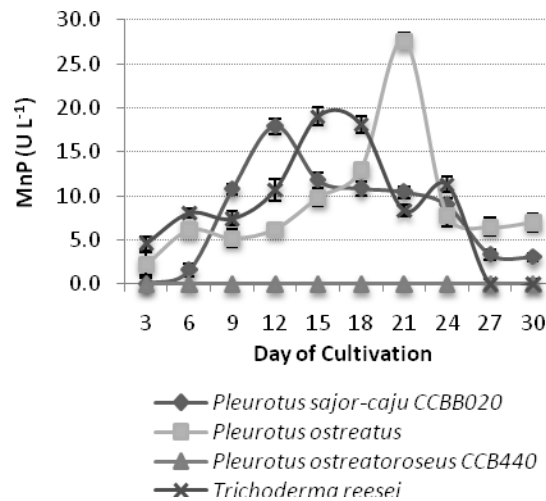
**Figure 4** – Endoglucanase activity (CMCase, U mL<sup>-1</sup>) during 30 days of cultivation at 30°C cultivated in vinasse medium.

Figure 5 shows the activity of laccase. *P. sajor-caju* at the first six days of cultivation showed 10.53 UI L<sup>-1</sup>, then decreased to 1.09 UI L<sup>-1</sup>. *P. ostreatoroseus* and *T. reesei* did not produce laccase. *P. ostreatus* produced 298.46 and 325.23 UI L<sup>-1</sup>. The *P. sajor-caju* showed high MnP activity starting at the sixth day of incubation, reaching a peak of 17.93 UI L<sup>-1</sup> at the 12<sup>th</sup> day of

cultivation (Fig. 6). The activity for *P. ostreatoroseus* was undetectable for this enzyme. The activity of MnP for the *P. ostreatus* was high (27.69 UI L<sup>-1</sup>) at the 21<sup>st</sup> day of cultivation, while the *T. reesei* obtained a high manganese-peroxidase activity between the 15<sup>th</sup> and 18<sup>th</sup> day (19.02 and 18.08 UI L<sup>-1</sup>, respectively).



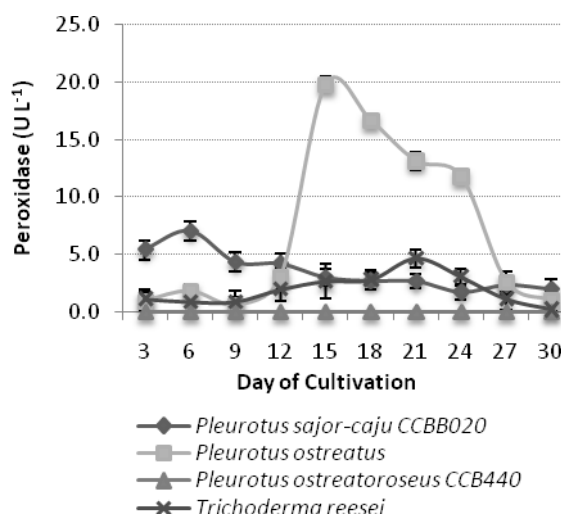
**Figure 5** - Laccase activity (UI L<sup>-1</sup>) during 30 days of cultivation at 30°C cultivated in vinasse medium.



**Figure 6** – Manganese-oxidase activity (UI L<sup>-1</sup>) during 30 days of cultivation at 30°C cultivated in vinasse medium.

According to Hatakka (1994), since the decade of 1980s, manganese-oxidases together with other peroxidases were discovered and certain groups of fungi were classified according to their enzymatic production capacity: those capable of producing MnP + peroxidases, MnP + laccases and those that produced peroxidases + laccases, however some exceptions were applied (Mane et al., 2006; Fasanella, 2008; Doerge et al., 1997; Flachner and Réczey, 2004). According to Figure 7, the

peroxidase activity demonstrated by the *P. sajor-caju* was high at the first six days of incubation, and then decreased gradually. For *P. ostreatus* an activity of 19.84 UI L<sup>-1</sup> was observed at the 15<sup>th</sup> day, being the highest among the studied cultures. The *P. ostreatoroseus* did not show any activity of peroxidase, while *T. reesei* had a low activity of peroxidase, reaching 4.64 UI L<sup>-1</sup> at the 21<sup>st</sup> day of growth.



**Figure 7** – Peroxidase activity (UI L<sup>-1</sup>) during 30 days of cultivation at 30°C cultivated in vinasse medium



## CONCLUSION

The *P. sajor-caju* CCB020, *P. ostreatus* and the *T. reesei* showed a higher mycelial biomass when cultivated in the medium containing vinasse, between 30 and 35° C. The chemical pre-treatment of the bagasse which resulted in a better breakage of the fibers and the production of exoglucanases and endoglucanases, was the treatment with 2% H<sub>2</sub>O<sub>2</sub> + 1.5% NaOH + autoclave at 121° C for 15 minutes, making a better accessibility of the cultures to the fibers. The *T. reesei* had an oxidative activity of manganese-peroxidase when cultivated in medium containing bagasse and vinasse. It was concluded that the pre-treatment of the bagasse was an important parameter for increasing the cellulolytic and lignolytic enzyme production. The vinasse, in combination with bagasse, could be reused as a supplement to the production for these enzymes as well as the production of fungal biomass.

## RESUMO

Recentemente o uso de material lignocelulolítico tem mostrado um importante avanço na produção de biocombustíveis. O bagaço e a vinhaça são resíduos oriundos do processamento da cana de açúcar e contem um alto teor de carbono, que geralmente é usado na co-geração de energia e ração animal. Três linhagens de *Pleurotus* e um ascomiceto, *Trichoderma reesei*, foram cultivados em bagaço pré-tratado e vinhaça. As atividades lignolíticas e celulolíticas foram analisadas, tanto quanto a produção de biomassa. Foi observado que o tratamento no bagaço com 2% H<sub>2</sub>O<sub>2</sub> + 1.5% NaOH + autoclave resultou numa maior quebra da fibra, aumentando o teor de celulose em 1.2 vezes mais e diminuiu em 8.5 vezes o conteúdo de hemicelulose. Este tratamento também resultou numa alta atividade lignolítica pelos fungos utilizados. O ascomiceto *T. reesei* produziu lacase, peroxidase e manganês-peroxidase em todos os tratamentos, tendo uma atividade de manganês-peroxidase variando entre 1.9 a 4.8 vezes mais que nos basidiomicetos.

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## REFERENCES

- Alborés, S.; Pianzolla, M.J.; Soubes, M.; Cerdeiras, M.P. (2006), Biodegradation of agroindustrial wastes by *Pleurotus* spp for its use as ruminant feed. *Electronic Journal of Biotech.*, **9**, 3.
- Archibald, F.S. (1992), A new assay for lignin-type peroxidases employing the dye azure B. *Appl. Env. Microbiol.*, **58**, 3110.
- Bon, E.; Ferrara, M.; Corvo, M. (2008), Eds. In *Enzimas em Biotecnologia – Produção, Aplicações e Mercado*. Interciência: Rio de Janeiro.
- Companhia Nacional De Abastecimento – CONAB (2008), [homepage on the Internet]. Brasília, DF. Available from: <http://www.conab.gov.br>.
- Doerge, D.R.; Divi, R.L.; Churchwell, M.I. (1997), Identification of the colored guaiacol oxidation product produced by peroxidases. *Anal. Biochem.* **250**, 10.
- Enari, T. M. (1983), *Microbial lipases*. London: Applied Science Publishers.
- Esposito, E.; Azevedo, J. L. Eds. (2004), *Fungos decompositores de materiais lignocelulolíticos*. Caxias do Sul, RS.
- Fagerstam, L.G.; Pettesson L.G. (1980), The 1,4-h-glucan cellobiohydrolases of *Trichoderma reesei* QM9414. *FEMS Microbiol. Letters*, 119.
- Fasanella, C.C. (2008). *Master of Science Dissertation*, [homepage on the Internet]. Piracicaba, SP. Available from: <http://www.teses.usp.br>
- Fengel, D.; Wegener, G. (1989), *Wood chemistry, ultrastructure and reactions*. New York: Walter de Gruyter.
- Flachner, B.; Réczey, K. (2004),  $\beta$ -glucosidase production and characterization of some *Aspergillus* strains. *Chem. Biochem. Engin. Quart.*, **18**, 30.
- Goering, H.K; Van Soest, P.J (1970), *Forage fiber analysis (Apparatus, reagents, procedures and some applications)*. Washington: United States Department of Agriculture.

- Hankin, L.; Anagnostakis, S.L. (1975), The use of solid media for detection of enzyme production by (pathogenic and saprophytic) fungi. *Mycol*, 67.
- Hatakka A. (1994), Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol. Lett.* **13**, 125.
- Kuhad, R.C. (1990). Singh, A.; Eriksson, K.E.L. Microorganisms and enzymes involved in the degradation of plant fiber cell walls. *Adv. Bioch. Biotech.*, **57**, 45.
- Kuwahara, M.; Glenn, J. K.; Morgan, M. A.; Gold, M. H. (1984), Separation and characterization of two extracellular H<sub>2</sub>O<sub>2</sub> dependent oxidases from lignolytic cultures of *Phanerochaete chrysosporium*. *FEMS Microbiol. Lett.*, 169.
- Leschine, S. (1995), Cellulose degradation in anaerobic environments. *Annu. Rev. Microbiol.*, 49: 399.
- Madan, M.; Bisaria, R. C. (1983), cellulolytic enzymes from an edible mushroom *Pleurotus sajor-caju*. *Biotech Letters.*, **5**, 9
- Mane, J.D.; Modi, S.; Nagawade, S.; Phadnis, S.P.; Bhandari, V.M. (2006), Treatment of spentwash using chemically modified bagasse and colour removal studies. *Bioresour. Technol.*, **97**, 1752.
- Melo, I.S.; Azevedo, J.L. Eds. (1997) *Biodegradação de lignina e tratamento de efluentes por fungos lignolíticos*. Jaguariúna, SP: CNPM/EMBRAPA.
- Miller, G.L. (1959), Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem.*, **31**, 426.
- Pereira Júnior, J.; Correia, M. J.; Oliveira, N. T. (2003), Cellulase activity of a *Lentinula edodes* (Berk.) Pegl. strain grown in media containing carboximetilcellulose or microcrystalline cellulose. *Braz. arch. biol. technol.*, **46**, 3.
- Serviço Brasileiro De Apoio A Micro E Pequenas Empresas – SEBRAE. (2008), [homepage on the Internet]. Brasilia, DF. Available from: <http://www.sebrae.com.br/paginaInicial>.
- Szklar, G.; Antibus, R. K.; Sinsabaugh, R. L.; Linkins, A. (1989), Production of phenoloxidases and peroxidases by wood-rotting fungi. *Mycol.*, **81**, 234.
- Zhang, Y.; Lu, X.; Dan, H.; Sun, Y. (2009). Screening and enzymatic study of a composite microbial system FH3. *Braz. arch. biol. technol.* **52**, 1.

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