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Expression of manganese peroxidase by *Lentinula edodes* and *Lentinula boryana* in solid state and submerged system fermentation

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ABSTRACT

The production of ethanol from lignocellulosic biomass is referred as a second generation biofuel, whose processing is one of the most promising technologies under development. There are few available studies on the use of enzymes produced by fungi as active for the biodegradation of lignocellulosic biomass. However, the manganese peroxidase (MnP) enzyme presents high potential to degrade lignin and the basidiomycetes are the major producers of this oxidase. Thus, this study aimed at evaluating the ability of fungi *Lentinula edodes* and *Lentinula boryana* to produce this enzyme when cultivated in submerged fermentation system (SS) and also in solid-state fermentation system (SSF) containing *Eucalyptus benthamii* sawdust with or without corn cob meal. In the SS the greatest MnP expression occurred on the 25th day, being of 70 UI.L^{-1} for *L. boryana* and of 20 UI.L^{-1} for L. edodes. In the SSF, the best results were obtained on the 10th day for *L. edodes*, while for L. boryana it happened between the 20th and the 25th days, despite both species presented values close to 110 UI.L^{-1}. Therefore, the results indicated that the studied fungi express the enzyme of interest and that its production is enhanced when cultivated in solid system.

Key words: basidiomycetes, *Eucalyptus benthamii*, enzyme, ethanol.

INTRODUCTION

The increase in ecological awareness starting in the late 20th century made it clear that the great challenge facing humanity for the coming decades is balancing the production of goods and services, also economic growth, social equity and environmental sustainability. Among the issues that have been studied lately, lignocellulosic biomass has received increasing attention from the Brazilian government and researchers who have dedicated time and thought to study the transformation of this material, often an environmental problem, into a value-added product, such as the second generation ethanol, also known as cellulosic biomass ethanol or bioethanol.

The lignocellulosic material from wood biomass has been viewed as a promising energy source due to its renewable capacity and the

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amout of carbohydrates (Sun and Cheng 2002). However, the use of lignocellulosic biomass to produce ethanol from the enzymatic hydrolysis of pretreated biomass has as its main technical and economic barriers the difficulty to establish an efficient and appropriate pretreatment of raw material (McMillan 1994).

The basidiomycetes are highlighted in the degradation of lignocellulosic materials due to the fact that they produce enzymes that hydrolyze cellulose, such as: avicelase, carboxymethyl-cellulase and glucosidase (cellulase), which act on the cellulosic portion; xylanases, mannanases, glucanases and galactanases (hemicellulases), acting on the hemicellulosic portion and oxidative enzymes, as lignin peroxidase, manganese peroxidase and laccase, defined as phenoloxidases. They have the ability of acting on phenolic compounds as lignin (Coelho 2007). These enzymes degrade lignocellulosic materials to produce fermentable sugars for biofuels as an example, the second generation ethanol, making it possible through the bioconversion carried out by enzymes like manganese peroxidase (MnP).

MnP is one of the lignolytic enzymes expressed by white rot basidiomycetes and has been widely studied as active in the decolorization of synthetic dyes used in textile industries (Boer et al. 2004, Mohorcic et al. 2006, Park et al. 2007). Furthermore, it has been used in studies of biodegradation of lignin, polycyclic aromatic hydrocarbons, humic acids and chlorinated pollutants (Coelho 2007, Hamid and Rehmana 2009). Therefore, the remarkable degradation potential of MnP makes it an attractive enzyme for biotechnological applications. The production of commercial enzymes is usually performed by biological processes in liquid medium - submerged fermentation system (SS) - in mixed reactors (Dalsenter and Tavares 1999). However, several studies have shown that the biological processes carried out in solid state fermentation (SSF) promote higher enzymatic activity, despite the difficult control performance (Palma 2003).

Throughout the production of enzymes by basidiomycetes, the SSF system seems quite appropriate given the characteristics of the substrate involved in the process, which consists essentially of lignocellulosic material (Wisniewski et al. 2010). Thus, as the SS presents better process conditions, the addition of lignocellulosic supplements such as cob corn meal has been proposed to stimulate the expression of lignolytic enzymes, in particular MnP (Boer et al. 2004). Therefore, the corn cob and the presence of lignocellulosic material suitable for the development of basidiomycetes are nutritionally adequate for mycelial growth (Valadares Filho et al. 2002).

Accordingly, this study aims to evaluate the expression of MnP by the basidiomycetes *Lentinula edodes* and *Lentinula boryana* in SSF, cultivated in lignocellulosic biomass of *Eucalyptus benthamii*, and in SS, seeking knowledge on the differences between the two systems in order to define the best conditions for enzyme express.

MATERIALS AND METHODS

Fungi and Cultivation Conditions

L. edodes (CNPF 21) and *L. boryana* (CNPF 24) were both maintained on Potato Dextrose Agar medium (PDA - prepared according to manufacturer's instructions) at 4 °C (called as primary matrix). For the production of secondary matrix, 7 mm plugs of the primary matrix were placed separately in Petri dishes containing PDA medium. These plates were incubated in a B.O.D chamber, at 20 °C for *L. boryana* (Faria et al. 2007) and at 25 °C for *L. edodes* (Regina and Broetto 2005), in the absence of light, until the complete outspreading of the fungus on the plate. After that, the matrix was used as inoculum for SS and SSF.

For SS, a modified SOCAREAN medium was prepared (Couri and Farias 1995), containing

3.0 $g.L^{-1}$ of sodium nitrate p.a. ($NaNO_3$), 0.5 $g.L^{-1}$ of magnesium sulphate p.a. ($MgSO_4$), 0.5 $g.L^{-1}$ of potassium chloride p.a. (KCl), 0.01 $g.L^{-1}$ of ferrous sulfate heptahydrate p.a. ($FeSO_4.7H_2O$), 1.0 $g.L^{-1}$ of dibasic potassium phosphate p.a. (K_2HPO_4) and 12.0 $g.L^{-1}$ of corn cob meal (obtained from Embrapa Florestas). The medium (50 mL) was placed into 125 mL Erlenmeyer flasks and sterilized in an autoclave at 121 °C for 15 min. The flasks were inoculated with 6 plugs of the secondary matrix and incubated for 30 days in a B.O.D chamber at 20 °C for *L. boryana* and at 25 °C for *L. edodes*, in the absence of light. Four flasks were randomly taken every five days for kinetic analysis of cellular growth, glucose residual value, pH and MnP activity.

For SSF, two treatments (culture medium) were used and both were prepared with sawdust (≤ 3 mm) of *Eucalyptus benthamii* (obtained from Embrapa Florestas). T1 treatment consisted of 95% sawdust and 5% soybean meal. T2 treatment consisted of 75% sawdust, 5% soybean meal, 10% cassava bagasse and 10% corn cob meal. Forty grams (40 g) of the treatments were transferred to 500 mL flasks and 50 mL of distilled water were added to each of them. The flasks were sterilized in an autoclave at 121 °C for 1 hour. The two media were inoculated with 5 plugs of the secondary matrix and incubated for 30 days in a B.O.D chamber at 20 °C for *L. boryana* and at 25 °C for *L. edodes*, in the absence of light. Three flasks were randomly taken every five days for kinetic analysis of the moisture content, a_w, pH and MnP activity.

Kinetic Analysis of Submerged System

The fungi mycelium was separated from the culture medium through vacuum filtration and, in order to obtain the enzymatic extract, the medium was centrifuged (Hermle refrigerated centrifuge) at 2,990.65 × g and 4 °C during 15 min. The supernatant (extract) was collected for MnP quantification.

To estimate the biomass, the gravimetric method of dry weight was used. The mycelium and corn cob meal retained on the filter paper, after vacuum filtration, were placed in a laboratory oven at 70 °C until constant weight was achieved. Mycelium mass was calculated by the difference between total mass and corn cob meal mass added to the flask. Therefore, cell concentration (*Cc*) was calculated as the ratio between mycelium mass and the medium's volume.

The average growth rate (*Gr*) was calculated as the ratio between the variation of cell concentration and cultivation time. Maximum specific growth rate (μ_{max}) was determined using a linear regression of natural logarithm of cell concentration against cultivation time. The slope obtained during logarithm phase represented μ_{max} (Pirt 1967).

The residual glucose concentration in the culture medium was determined by spectrophotometry (in Shimadzu UV-1650 spectrophotometer) using the enzymatic method of glucose oxidase (GOD), performed as described by the manufacturer of the enzymatic glucose test kit (Doles). The pH was directly measured in all samples using the potentiometric method (Tecnal pH meter).

Kinetic Analysis of Solid State Fermentation

Humidity (H) was determined by drying the substrate samples in a laboratory oven at 70 °C until reaching constant weight. The difference between wet mass (m_W) and dry mass (m_D) was calculated as shown in Equation 1.

$$H- \left(\frac{m_W - m_D}{m_W}\right)100 \quad (1)$$

Water activity ($a_w = Pm / Ps$) of the medium depends on its moisture content and composition as it concerns the ratio between the vapor pressure of the culture medium (*Pm*) and the vapor pressure of pure water (*Ps*) at the same temperature (Wenqing et al. 2003). In each sample of the SSF, a_w was determined by direct measurement of substrate samples in an Aqualab® device (Decagon).

To obtain the enzymatic extract from the solid medium, it was placed on Erlenmeyer flasks, in a ratio of 6 mL of distilled water/gram of substrate, and shaken for 3 hours at 20 °C. After that, the mixture was vacuum filtered and the liquid portion was centrifuged at 2,990.65 × g at 4 °C during 15 min. The supernatant (extract) was collected for MnP quantification and pH direct measurement.

Quantification of MNP in the Extracts

The MnP activity in the extracts obtained from SS and SSF was determined through spectrophotometry, using the modified method of Wariishi et. al (1992). A reaction mixture containing 0.4 mL of extract, 0.4 mL of maganese sulfate p.a. ($MnSO_4$) 10 mM, 2.8 mL of sodium malonate buffer 50 mM, pH 4.5, and 0.4 mL of hydrogen peroxide p.a. (H_2O_2) 0.5 mM was prepared. The oxidation of MnSO4 by H2O2 results in the formation of the complex Mn(III)-malonate which was monitored at 30 °C, during 5 min, at 270 nm (ε270=11,590 M-1.cm-1).

The enzymatic activity (*EA*) expressed in International Unit per liter ($UI.L^{-1}$), defined as the amount of enzyme required to oxidize 1 μM of substrate per minute per liter of extract was calculated by:

$$EA = \frac{\Delta A}{t}\,\frac{1}{d}\,\frac{1}{\varepsilon}\,\frac{V_R}{V_E} \qquad (2)$$

where $\Delta A/t$ is the graph slope of absorbance variation (ΔA) over reaction time (t), d is the path length, ε is the molar extinction coefficient, V_R is the total reaction volume and V_E is the extract volume.

Statistical Analysis

All analyses were performed in triplicate. The results of biomass production, MnP activity, pH, moisture and a_w were examined by analysis of variance (ANOVA). Means with p-values under 0.05 were considered statistically different, and, in these cases the Tukey´s test was also applied.

RESULTS AND DISCUSSION

Fermentation in Submerged System

Biomass production (expressed by cell concentration) and MnP activity analyzed during the SS of *L. edodes* and *L. boryana* are shown in Figure 1. It is possible to notice that there were significant differences between the cell concentration means of the two species only on the 5th and 25th day of cultivation, indicating that they grow in a similar way, although the Gr of *L. edodes* (0.14 $g.L^{-1}.day$-1) was greater than that of *L. boryana* (0.08 $g.L^{-1}.day^{-1}$).

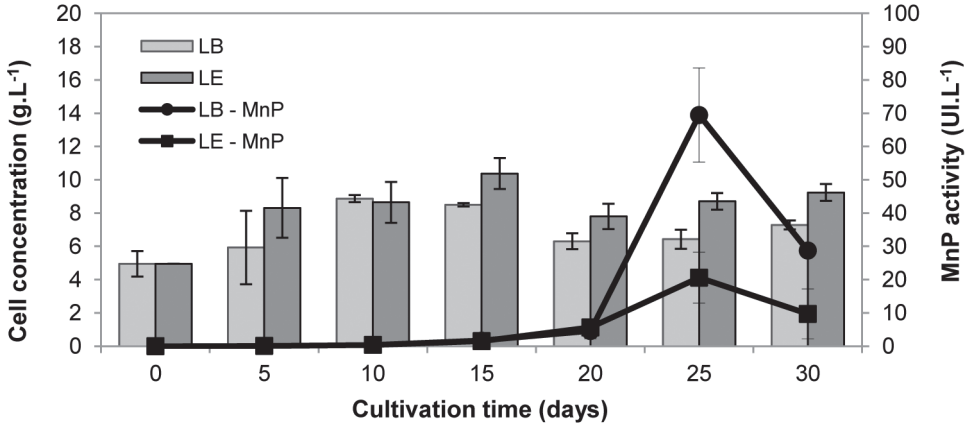

Fig. 1 - Cell concentration and MnP activity during the SS of *L. boryana* (LB) and *L. edodes* (LE). (The vertical bars represent standard deviation).

Due to the fact that the cultivation was performed in multiple flasks for sample collection, the occurrence of oscillations during growth is acceptable, such as the peak cell concentration of *L. edodes* on the 15th day followed by a decay of biomass, which is not usual in reality. For this reason it was not possible to precisely define the stages of fungal growth. The use of natural logarithm made possible to verify the absence of a lag phase. To calculate the μmax, the *L. boryana* logarithmic's phase was defined between 0 and 10 days (reaching $_{\mu max}$ of 0.05.day^{-1}) and also *L. edodes*' between 0 and 5 days ($_{\mu max}$ of 0.10.day^{-1}) (Figure 2). These values confirm what was observed visually during the experiment, that *L. edodes* presented higher mycelial density in a shorter cultivation time.

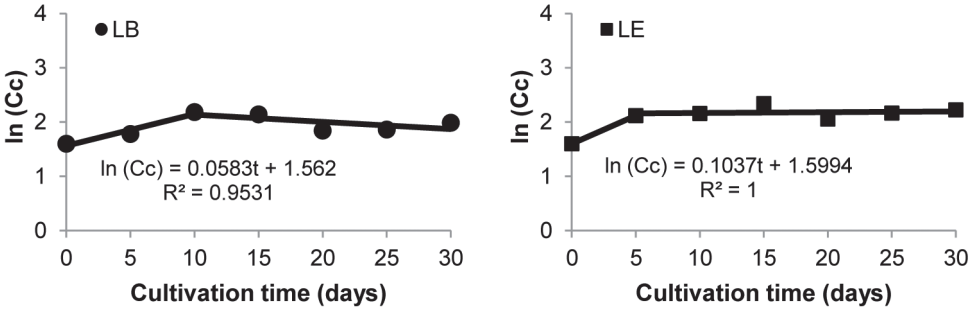

Fig. 2 - Natural logarithm of cell concentration against cultivation time to obtain the maximum.

After the period of maximum growth the system got into a phase of reduction in the rate of biomass production (characterized as stationary phase), in which secondary metabolism is activated, resulting in the production of bioactive compounds such as phenol oxidative enzymes. Ferreira et al. (2010) states that nutritional stress may be one of the responsible factors for MnP synthesis, explaining why this enzyme expression was observed only after the 15th day (Figure 1). Maximum levels of enzyme activity were obtained at 25 days of cultivation for both species. For *L. boryana* this value was much higher (70 UI.L^{-1}). The values obtained at 30 days for both species. The one obtained at 25 days for *L. edodes* showed no statistical difference.

MnP activity expressed by *L. edodes* in this study was lower than the one found in the literature for the same fungus. Regina and Broetto (2005) obtained maximum values of MnP activity around 1,400 UI.L^{-1} for *L. edodes* grown in liquid media composed by an infusion of cassava bagasse and dextrose. However, these authors also observed the influence of substrate on enzyme expression, as in an infusion of sugar cane bagasse and dextrose, the maximum value obtained was of 400 UI.L^{-1}.

The SS medium used in this study contained corn cob meal which has more than 60% of polysaccharides with high concentration of xylan, followed by the monosaccharides xylose and glucose (R.F.M. Silveira, unpublished data). However, the presence of residual glucose during the cultivations was not detected, showing that this lignocellulosic substrate may not have been used by the fungi during its development. Another hypothesis is that the low enzymatic activity promoted a reduced biodegradation, influencing the low production of sugars for cellular metabolism and thus influencing global cellular metabolism.

The medium presented an initial pH near 5.0 (Table I), but a statistically significant decrease in this value after the beginning of both species of *Lentinula*'s growth was observed.

The assays for quantification of MnP use buffer with pH 4.5, and then it seems that values of pH near this one help in the enzyme stability.

Although pH has fluctuated throughout the growth of fungi, it has remained between 3.6 and 4.6. It was noted that for both species, the remarkable lower values occurred at 20, and especially at 25 days; periods at which a greater expression of the enzyme was observed. It indicates that the biosynthesis of metabolites of this species could significantly change the pH in the case of SS, a fact that should be better studied.

Analyzing the obtained data, it seems that SS presents suitable characteristics for MnP production and it is feasible to study its application in the development of second generation ethanol. However, considering what was observed in this study, the time required for the occurrence of enzyme expression in this system becomes a barrier for its application in an industrial process.

Solid State Fermentation

Values of a_w, pH and moisture concerning SSF are presented in Table II. Despite the samplings being performed in periods of 5 days, the values mentioned in Table II only correspond to the beginning, middle and end of cultivation time. Unlike submerged culture, in SSF there was no statistical difference in the values of pH throughout the cultivation of both species neither between the species for the same day.

TABLE I
Values of pH obtained during SS for *L. edodes* and *L. boryana* during cultivation time.

Fungi	Cultivation time (days)						
	0	5	10	15	20	25	30
L. boryana	5.06±0.13Aa	4.57±0.13Ab	4.65±0.08Ab	4.56±0.07Ab	4.26±0.05Ac	4.10±0.07Ac	4.50±0.01Ab
L. edodes	5.06±0.013Aa	4.39±0.20Ab	4.56±0.11Ab	4.23±0.16Ab	3.77±0.04Bc	3.59±0.16 Bc	4.16±0.05Bb

Means ± standard deviation followed by the same lowercase letters in the line and by the same uppercase letters in the row do not differ by Tukey's test ($p \leq 0.05$).

TABLE II
Values of pH, moisture content (%) and a_w obtained during SSF.

Treatment	T1			T2		
Fungi	pH					
	0 day	15 days	30 days	0 day	15 days	30 days
L. boryana	4.64 ± 0.05Aa	4.54 ± 0.04Aa	4.99 ± 0.13Aa	4.60 ± 0.02Aa	4.46 ± 0.30Aa	5.12 ± 0.04Aa
L. edodes	4.64 ± 0.05Aa	4.57 ± 0.09Aa	4.87 ± 0.10Aa	4.60 ± 0.02Aa	4.44 ± 0.03Aa	4.77 ± 0.09Aa
	Moisture content (%)					
L. boryana	53.01 ± 0.67Aa	52.95 ± 3.10Aa	39.65 ± 2.65Aa	53.37 ± 0.58Aa	52.05 ± 3.9Aa	44.29 ± 3.97Aa
L. edodes	53.01 ± 0.67Aa	55.52 ± 2.44Aa	54.73 ± 5.91Aa	53.37 ± 0.58Aa	56.13 ± 2.55Aa	47.03 ± 3.10Aa
	a_w					
L. boryana	0.999±0.001Aa	0.997±0.003Aa	0.988±0.006Aa	1.000±0.003Aa	0.997±0.002Aa	0.991±0.007Aa
L. edodes	0.999±0.001Aa	0.997±0.002Aa	1.002±0.003Aa	1.000±0.003Aa	0.999±0.002Aa	0.997±0.003Aa

Means ± standard deviation followed by the same lowercase letters in the line and by the same uppercase letters in the row do not differ by Tukey's test ($p \leq 0.05$).

V.M.C.S Santos (unpublished data) reports that some species of basidiomycetes have the characteristic of self-regulating pH, tending to stabilize its value in the optimum pH for growth, regardless the initial pH value. This fact was also observed by Vieira et al. (2008) in a study with

Polyporus tricoloma for antibiotic production. The initial pH value does not seem to influence the lengthening of hyphae in *Macrocybe* titans as well. Wisniewski et al. (2010) evaluated the effect of initial pH values (5, 6, 7 and 8) in radial mycelial growth of *M. titans* in PDA medium. According to the author, there was no influence on mycelial growth, which can be explained by self-regulation.

Regarding humidity, there were no statistical differences between fungi neither throughout cultivation time (Table II). The values remained constant until the 15th day for the *L. boryana*'s culture, being reduced after that by over 15%. For the cultivation of *L. edodes*, in T1 treatment values remained constant throughout the whole cultivation, unlike the values of T2 treatment, which have decreased. Concerning the humidity achieved during SSF, the aw values showed no statistically significant differences (Table II).

However, analyzing the data from a physiological point of view, it is known that an aw of 0.988 may be different from 1.0, since it is possible to have aw values close to 1.0 with moisture contents between 20 and 100%, which can lead to the verification of different consequences for cell growth and enzymes expression.

MnP activity values obtained throughout the SSF of fungi is shown in Figure 3. It was realized that although the maximum value of fungi's enzymatic activity was very close, around 110 $UI.L^{-1}$ (equivalent to 0.66 $UI.g^{-1}$), the enzyme expression occurred at different times. For *L. edodes* the maximum value was obtained at 10 days of cultivation, while for *L. boryana* the maximum value was observed between 20 and 25 days. It was also noted that the supplementation of culture medium with cassava bagasse or corn cob (which made T1 different from T2) showed no significant difference for the species of *Lentinula*. Consequently, the combined addition of these carbon sources, in the studied concentrations, did not contribute to increase the production of the enzyme MnP.

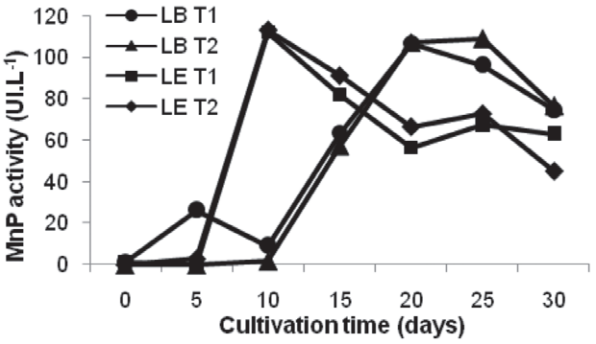

Fig. 3 - MnP activity during the SSF of *L. edodes* (LE) and *L. boryana* (LB) in the different treatments (T1 and T2).

MnP activity showed a decrease in both treatments in SSF after reaching the highest activity level. This was also reported by Regina (2004), who found a significant decreasing in MnP activity after the 8th day of incubation of different *L. edodes*' strains. According to Kadimaliev (2003), changes in lignolytic activity and in peroxidase biosynthesis activity by fungi during solid state cultivation, depend on the type of substrate used. The author states that the amount of substances that restrict the mycelium growth (such as resins) and the existence of a more intricate supramolecular structure may be linked to this outcome.

According to Silva (2004), many basidiomycetes are developed in a simple media which presents availability of assimilable carbon, nitrogen and phosphorus sources and essential mineral salts. The ability of the fungus to grow in lignocellulosic substrates is related to the vigor of the inoculated mycelium and to the capacity to activate physiological mechanisms necessary to use the nutrients from the culture medium (Mata et al. 2001). Thus, the nutritional needs for a satisfactory mycelium growth, especially at the initial stage of cultivation, can be achieved depending on the type of material used in culture medium supplementation. The addition of certain meals, for example, provides nitrogen sources and can stimulate the enzymatic action of mycelium (Donini et al. 2006).

However, J.H.A. Betini (unpublished data) proved that the fungus *Aspergillus niveus* produced a 30% higher concentration of xylanase when grown in a medium containing only wheat bran as carbon source, when compared to the medium which also contained corncob. This demonstrates that supplementation with cob meal does not seem to respond satisfactorily to the fungal enzyme production, although Kadowaki et al. (1997), in their studies, have obtained maximum production of xylanase by *Aspergillus tamarii* when it was cultivated in medium supplemented with high concentration of corncob.

CONCLUSION

When considering the two cultivation systems, it seems clear that the process developed in liquid medium, apart from presenting a lower enzymatic activity, reduced the kinetics of MnP's production. On the other hand, lignocellulosic materials, such as eucalyptus wood, proved to be promising for enzymes production by basidiomycetes, for their stimulation capacity of MnP expression. Thus, considering the importance of eucalyptus in the energy matrix to obtain bioethanol, studies on how to optimize the production processes of enzymes able to degrade lignocellulolytic compounds should be encouraged.

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RESUMO

A produção de etanol a partir de biomassa lignocelulósica é referida como uma segunda geração de biocombustíveis, sendo que este processo é uma das mais promissoras tecnologias em fase de desenvolvimento. Poucas são as pesquisas disponíveis sobre o uso de enzimas produzidas por fungos como ativos para biodegradação de biomassa lignocelulósica. No entanto, a enzima manganês peroxidase (MnP) apresenta alto potencial para degradar a lignina e os fungos basidiomicetos são os principais produtores desta oxidase. Portanto, esse estudo teve por objetivo avaliar a capacidade dos fungos *Lentinula edodes* e *Lentinula boryana* em produzir essa enzima quando cultivados em fermentação em sistema submerso (FSS) e também em fermentação em sistema sólido (FES) contendo serragem de *Eucalyptus benthamii* suplementada ou não com farelo de sabugo de milho. Na FSS, a maior expressão de MnP ocorreu no 25° dia de cultivo, sendo 70 $UI.L^{-1}$ para *L. boryana* e 20 $UI.L^{-1}$ para *L. edodes*. Na FES, os melhores resultados foram obtidos no 10º dia para *L. edodes*, enquanto que para *L. boryana* foram entre o 20° e o 25° dia, apesar de ambas as espécies terem apresentado valores próximos a 110 $UI.L^{-1}$. Portanto, os resultados indicaram que os fungos estudados expressam a enzima de interesse e que a sua produção é otimizada quando cultivados em sistema sólido.

Palavras-chave: basidiomicetos, *Eucalyptus benthamii*, enzima, etanol.

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