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RESEARCH ARTICLE

Production of laccase and manganese peroxidase by white-rot fungi from sugarcane bagasse in solid bed: Use for dyes decolourisation

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Abstract Three strains of white-rot fungi were tested in solid state fermentation and submerged culture to obtain enzymes for dyes biotransformation. Both sugarcane bagasse and dyes induced laccase and manganese peroxidase biosynthesis but laccase seems to be the main enzyme related to the decolourisation profiles. A variable behavior of strains was observed depending on inducers, fermentation system and characteristics of the strains. Crude enzyme of *Earliella scabrosa* obtained in solid state fermentation showed higher decolourisation percentage of Navy FNB and Red FN-3G dyes than *Trametes maxima* (13) and *Ganoderma zonatum* (B-18). *T. maxima* exhibited the best decolourisation percentage in submerged cultures supplemented with Navy FNB, Red FN-3G and yellow P-6GS dyes. Growing biomass of *T. maxima* could supply other enzymes and mediators for dyes transformation. Peculiar behaviour was observed with *Ganoderma zonatum* (B-18), it had a similar dyes biodegradation in both liquid and solid bed fermentation and there was not positive correlation between ligninolytic enzymes production and decolourisation pattern. The employment of crude enzymes produced in solid bed of bagasse could be an attractive option for biological removal of textile dyes.

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Introduction

Lignin, a complex and heterogeneous aromatic biopolymers in woody and herbaceous plants, is one of the most abundant natural polymers on earth (Sasaki *et al.,* 2001). Lignin degradation by basiodiomycetes is the key step in lignocelluloses decay which is considered a central step for carbon recycling in terrestrial ecosystems. White-rot fungi are known for their ability to degrade or modify lignin by an enzymatic process (Martínez *et al.,* 2002). The extracellular ligninolytic enzyme system of these fungi has been studied extensively in recent years and the most information comes from studies with *Phanerochaete chrysosporium* and a few others species (Novotny *et al.,* 2000). Lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.2) so called "lignin- modifying enzymes", are involved in degradation of lignin and other recalcitrant molecules due to the non-specificity of this enzymatic machinery. Many of these compounds are major environmental pollutants such as pesticides, synthetic dyes, polycyclic aromatic hydrocarbons and synthetic polymers. Textiles dyes are decolorized by a specific mixture of enzymes which are induced during the fungal growth on media supplemented with different dyes. Much information on the degradation and decolourisation of synthetic dyes by ligninolytic fungi has been obtained in submerged culture with some species of *Phanerochaete, Trametes* and *Pleurotus* (Eichlenová *et al.,* 2005). There have been only a few studies in which the production and action of these enzymes have been investigated

in solid state fermentation (SSF). Mn peroxidase and Laccase activities was found during SSF of some insoluble substrate, such as sawdust, wheat straw and bagasse (Hofrichter *et al.,* 1999). Employ SSF of lignocelluloses materials for enzyme production provide important advantages, such as: it mimics the natural environment of the white- rot fungi, simplifying the culture media and permits low content of water to obtain concentrated crude enzymes (Guerra *et al.,* 2006). This investigation was carried out to obtain fungal ligninolytic enzymes in solid bed supplemented with sugarcane bagasse for their use in the biotransformation of textile dyes.

Materials and Methods

Microorganisms

Earliella scabrosa and *Trametes maxima* belonging to CUBACHAM project collection and *Ganoderma zonatum* (B-18) isolated by Manzano (2004) were used throughout this study. Stock cultures were growth on malt agar slants at 30 °C during 7 days and latter stored at 4°C.

Culture conditions

Solid state fermentation was carried out in 100mL flasks containing 4g of sifted bagasse (1.6 mm particle diameter), 0.25 % (w /v) $(NH_4)_2SO_4$ and 70 % humidity. Media were sterilized by heating at 121 °C for 30 min. All flasks were inoculated with two agar plugs (5x5mm) from the margin of the actively growing colony in Petri dish with malt agar media. After harvesting bagasse from a culture flasks were suspended in 32 mL of tris sodium tartrate buffer (10m mol, pH 5) and incubated on a rotary shaker for 1 hour and subsequently it was filtered and centrifuged at 4000 $r.min^{-1}$ to separate the extracellular fungal enzymes. Submerged culture was developed in 100 mL Erlenmeyer's containing Kimura media (Manzano, 2004) with 0.01 % of dyes. Cultures were incubated at 30 °C in agitation during 21 days.

Enzymes assays

Laccase activity was determined by Muñoz (1997) method employing sodium acetate buffer (0.1 M, pH 5) and ABTS 10mM. Complex formation was monitored by measuring the increase in absorbance at 436 nm (ξ_{436}= 29,300 $M^{-1} cm^{-1}$). Mn peroxidase activity was measured by direct oxidation of $MnSO_4$ (1mM) in the presence of 0.1mM H_2O_2 and sodium tartrate buffer (0.1 M, pH 5). The increase in absorbance at 238 nm was determined at 30 °C and the activity calculated using ξ_{238}= 6,500 $M^{-1} cm^{-1}$.

Dyes

The dyes used in the decolourisation studies were courtesy from CIBA Company in Havana, Cuba. The bi-functional reactive dyes studied were yellow P-6GS Cibacron, Red FN-3G Cibacron and Navy FNB Cibacron.

Decolourisation assays

For decolourising assay was employed 1 mL of dye solution (0.01 % w/v) and 1 mL of crude enzyme obtained by solid state fermentation. Mix was incubated at 30 °C during 24 hours. Colour reduction was calculated from the difference in absorbance between samples and the respective abiotic controls at the maximum visible absorbance peak for each dye using a Genesys 10 UV-vis spectrophotometer (yellow P-6GS: λ= 420nm., Red FN-3G: λ=500nm and Navy FNB: λ=600nm).

Statistical analysis

Normality and variance homogeneity were investigated prior to carrying out the statistical analysis of the data, by means of the Kolmogorov-Smirnov test and Bartlett test, respectively. In case of the data meeting the two previous criteria, an analysis of simple classification variance was used and media were compared by means of Student- Newman-Keuls test (SNK parametric). The data were processed with package Statistic 6.0.

Results

Production of ligninolytic enzymes from sugarcane bagasse in solid bed

The bagasse employment in culture media showed the capacity of this substrate to induce the synthesis of laccase and manganese peroxidase (MnP) enzymes. *Earliella scabrosa* (7), *Trametes maxima* (13) and *Ganoderma zonatum* (B-18) were able to grow and produce ligninolytic enzymes in solid state fermentation using sugarcane bagasse as sole carbon and energy sources.

The kinetic studies with *E. scabrosa* in solid bed with sifted bagasse and ammonium sulfate as nitrogen source are shown in Fig. 1a. The production of MnP was detected from the beginning of the fermentation with to gradual increase of the activity reaching its maximum values at the day 11 (3.692 $U.g^{-1}$) and 21 (3.655 $U.g^{-1}$). Laccase showed its maximum activity to the 11 days (44.3 $U.g^{-1}$) and fall down ostensibly until the 18 days of incubation, in which it evidenced a slight increase of enzyme activity at the end of the fermentation. Only few fluctuations of pH were appreciated during the incubation period of culture. It showed a slight increase to pH 6 during the first 4 days, with a light oscillation of pH among 5.1 and 5.4 at the end of the fermentation process.

The fermentation profile with *Trametes maxima* (Fig. 1b) indicated different behavior to those reached by *E. scabrosa*. Manganese-peroxidase production began to the seventh day with a maximum of activity the day 14 (2.166 $U.g^{-1}$). Laccase

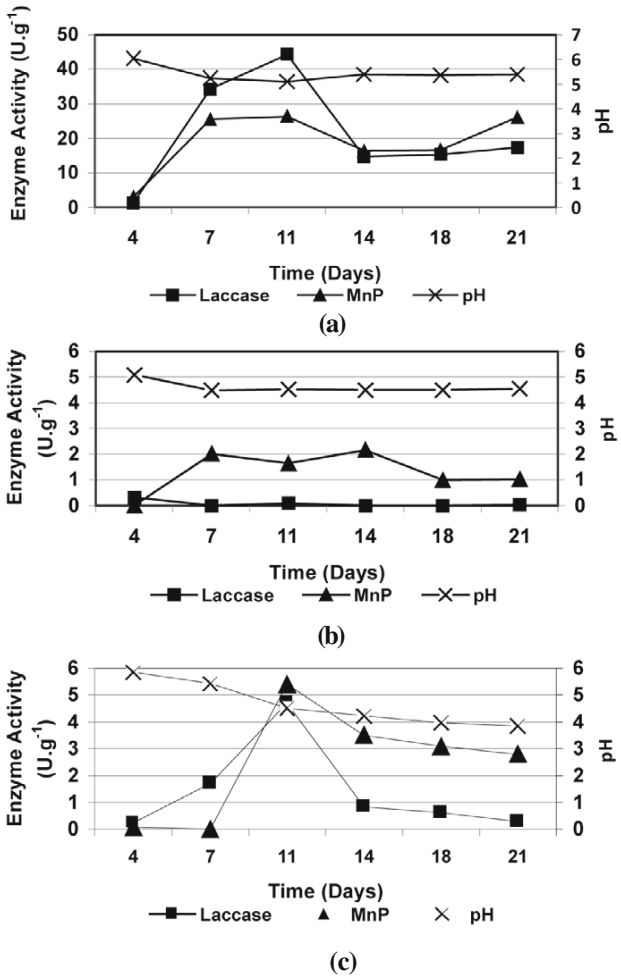

Fig. 1. Time course of ligninolytic enzymes production of a) *Earliella scabrosa*, b) *Trametes maxima* and c) *Ganoderma zonatum* growing in solid bed during 21 days.

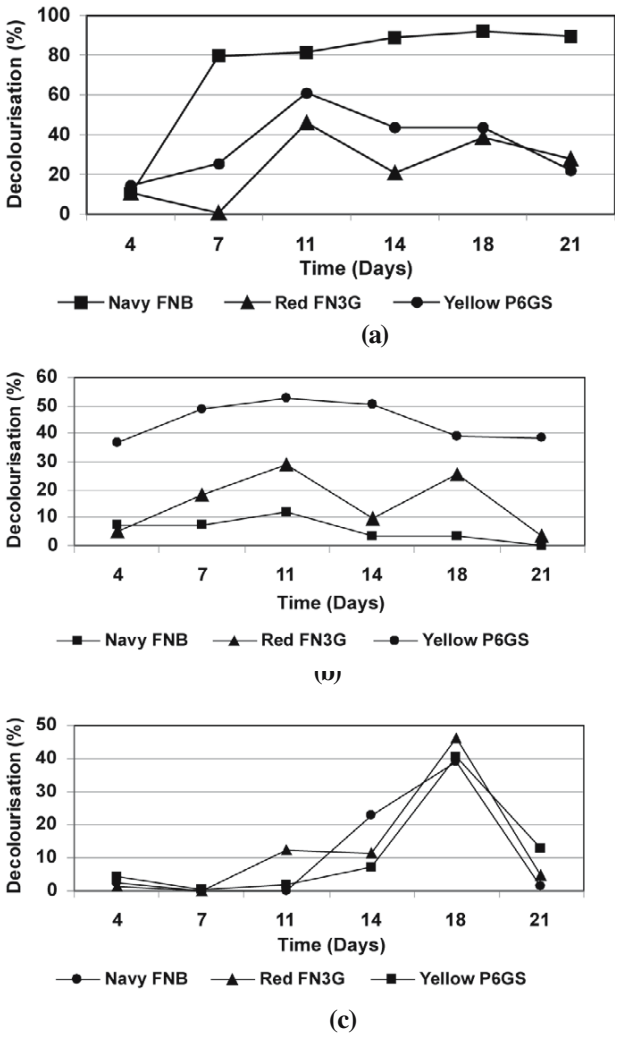

Fig. 2. Time course of decolourisation of the strains a) *Earliella scabrosa*, b) *Trametes maxima* and c) *Ganoderma zonatum* growing in solid bed during 21 days.

activity was inferior to 1 $U.g^{-1}$, with a maximum value at the fourth day of fermentation (0.313 $U.g^{-1}$). During the period of culture incubation the pH showed only a few fluctuations. A slight decrease was observed during the first seven days reaching a pH value of 4.48, staying stable until the end of the fermentation.

The kinetic studies with *G zonatum* (B-18) which appear in Fig. 1c showed different results to those obtained for *E. scabrosa* (7) and *T. maxima* (13). The production of the enzyme laccase had an increase until the 9 day, when a maximum pick of activity (4.7 $U.g^{-1}$) was reached. Starting from this day, the value of activity laccase diminished until succeeding in 0.3 $U.g^{-1}$ concluding the fermentation. Mn Peroxidase enzyme had fluctuations during the incubation period of cultures, it was 0.08 $U.g^{-1}$ at the 3 day of fermentation and later fallen up to 0. Coinciding with the pick of maximum laccase secretion at the 9th day, the highest value of MnP activity took place (5.4 $U.g^{-1}$) and later diminish up to 2.8 $U.g^{-1}$ at the end of the fermentation. During the 21 days of fermentation the pH of crude enzyme extracts suffered a descent in 2 units from 5.85 up to 3.85.

Dyes biotransformation studies with crude Ligninolytic enzymes produced in SSF.

As we can see in Fig. 2a the kinetic study of dyes decolourisation using crude enzymes of *E. scabrosa* obtained from SSF exhibited high biotransformation of Navy FNB (92.05% at the 18 days of incubation). Biotransformation of textile dyes Red FN-3G and Yellow P6GS showed a decolourisation percentage lower than Navy FNB, the highest values were obtained using crude enzyme at the 11th day of fermentation (45.91% and 60.91% respectively).

Decolourisation experiments with *T. maxima* (Fig. 2b) indicated that higher decolorizing activities were raised from crude enzymes to 11th day of fermentation. Decolourisation profile showed a poor biotransformation of Navy FNB whose maximum value was 11.86%. The degradation of red FN-3G increased also reaching its maximum value (29.06%). The Yellow P6GS was transformed easier and showed an increase

in decolourisation percentage since the initial phase of culture. The maximum value of 52.40 % was obtained at the 11 days, and then diminished gradually until the end of the incubation period.

Experimental results with *G. zonatum* indicated a very similar biotransformation for the three dyes tested. The maximum values were reached to the 16 day, with 38.89% for Navy FNB, 46.01% for red FN-3G and 40.59% for yellow P6GS followed by an abrupt decrease until the end of the fermentation (Fig. 2c).

Studies in submerged culture. Dyes decolourisation.

Studies in submerged culture using dyes as inducers of ligninolytic enzymes was carried out to evaluate the biotransformation capacity of crude enzyme obtained in SSF for decolorizing purpose and to determine the strains behavior in both fermentation system.

As it can be observed in Fig. 3b and Fig. 2b, biotransformation of Navy FNB and Red FN-3G dyes in cultures of *Earliella scabrosa* (7) at 11 days of incubation was very similar in both fermentation process liquid and solid state fermentation. The biggest difference in decolourisation values was obtained with yellow P6GS with an acceptable removal of color using crude enzymes obtained in solid bed with bagasse (61%) and very poor biotransformation with growing biomass in liquid culture supplemented with dye (3%).

In contrast with the previous results *Trametes maxima* (13) showed a non homogeneous behavior. The decolourisation percent obtained with crude enzyme (12 blue%, 29 red% and 52 yellow %) were lower than those reached by

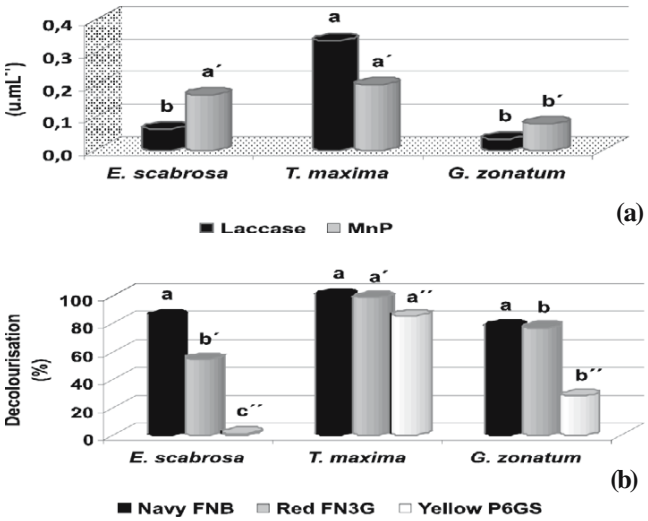

Fig. 3. Cultures behaviour in liquid Kimura media with dyes (0.01 %) to the 11th days of fermentation. a) Ligninolytic enzymes production, b) Biotransformation of dyes. Different letters means significant differences (p=0.05) between media values.

Earliella scabrosa (7) (Fig. 2b), however, as we can see in Fig. 2b and Fig. 3b the significant highest values in removal of the color were obtained in submerged culture (99 blue%, 98 red% and 85 yellow %).

Ganoderma zonatum (B-18) had a lower decolourisation percent (39% Navy FNB, 46% Red FN-3G and 41% Yellow P-6GS) than those obtained for crude enzyme of *E. scabrosa* (Fig. 2c) and lightly higher than obtained by *T. maxima* (13). However this strain showed a good behavior in submerged cultures (79% Navy FNB, 76% Red FN-3G y 28% Yellow P6GS) (fig.3c).

Discussion

Crude enzymes with laccase and Mn-peroxidase activities produced in SSF were able to decolorized the three azodyes tested witch have different chemicals structure. The kinetic studies indicated that between 4-18 days take place the biggest production of both enzymes. Laccase activity was observed in all cases and was the main component in the crude enzymes extracts, similar results were informed by Muñoz *et al.* (1997). Kirst *et al.* (2004) found laccase as majority activities in cultures using corn straw as sole carbon and energy source, but Gandolfi *et al.* (2004) informed the detection of manganese peroxidase, as a main component in culture of *Lentinus edodes* produced on SSF for dying decolourisation purpose. These researchers reported variable responses of cultures depending on the strains and the substrate (inducer) used for ligninolytic enzyme production.

It has been confirmed by several authors that the enzymes laccase and MnP are involved in transformation of industrial dyes (Swamy and Ramsay, 1999; Kirby *et al.,* 2000; Conneely *et al.,* 2002., Novotny *et al*., 2000). Decolourisation profiles of *E. scabrosa* indicated that biotransformation of Navy FNB is close associated to the pattern of Manganese- peroxidase activity while laccase activity seem to be related to transformation of both Red and Yellow dyes. The relationship between enzymatic and decolourisation profiles was different in *Trametes maxima* cultures. The biotransformation of yellow Cibacron seems to be closely related to the production of manganese peroxidase while the decolourisation of the Red and Navy dyes can not be directly related to the presence of these laccase and Mn peroxidase enzymes. Low level of laccase activities detected in enzyme crude extract of *T. maxima* could explain the low percentage of decolourisation and the essential role of laccase in dyes degradation. Decolourisation and enzyme profiles of *G. zonatum* showed not direct relationship between maximal enzyme activities and dyes transformation.

It has been described the increment of the laccase by white-rot fungi in some fermentation with sugarcane bagasse in SSF. Laccase is considered the most active enzyme in decolourisation process (Kahraman and Gurdal, 2002; Levin *et al.,* 2004; Abadulla *et al.,* 2000; Kirst *et al.,* 2004). Our results reaffirm the participation of the enzyme laccase in the transformation of tints, fundamentally in cultures of *E. scabrosa* which reached the highest enzyme productions and high percent of dyes decolourisation.

Several authors have described that MnP activity is

stimulated by the presence of Mn^{+2} ions in culture media being detected high enzyme levels during the fermentation process (Kuan and Tien, 1993; Hofrichter *et al.,* 1999). Otherwise there is not Manganese salt in culture media but Mn^{+2} levels in sugarcane bagasse could be enough to stimulate the enzyme production.

The results obtained with all strains suggest that other enzymes and/ or low molecular weight mediators, none monitored in this work, may be involved. These molecules could play an important role in dyes biotransformation together with ligninolytic enzymes.

The pH profile of all crude enzymes was closing similar, with a slight decrease at the end of fermentation. The same behavior was informed by Kirst *et al.* (2004) in cultures of *Pleurotus pulmonaris*.

Studies carried out in submerged culture and in solid bed showed that both dyes and sugarcane bagasse are able to induce the biosynthesis of the ligninolytic enzymes, which are involved in dyes transformation but the enzymes level and the decolourisation percentage were so different in studied strains. *T. maxima* had to good acting in submerged cultures while *E. scabrosa* obtained better result in solid bed. Levin *et al*. (2004) and Rodríguez *et al*. (1999) reported bigger decolorizing activity in submerged culture that in cell- free crude enzyme from SSF of *Coriolus versicolor*, *Trametes versicolor* and *Pleurotus ostreatus*. These variable responses found in some strains of white- rot fungi suggest that the growing biomass could supply other metabolites and other enzymes needed for dyes biodegradation (Rodríguez *et al*., 2004; Cha *et al*., 2001).

On the other hand many strains of white-rot fungi like *E. scabrosa* have a good acting in solid state fermentation; the use of crude enzyme offers an alternative for the biological degradation of textile dyes without necessity of applying the fungal biomasses. The rational use of fungal crude enzyme for dyes transformation could be considered a friendly proposal with the environment for biological treatment of polluted effluents.

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