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AGREGAÇÃO DE VALOR A RESÍDUOS AGROINDUSTRIAIS: PRODUÇÃO DE ENZIMAS OXIDATIVAS POR BASIDIOMICETOS E APLICAÇÃO NA DESCOLORAÇÃO DE CORANTES INDUSTRIAIS

GISELE CRISTINA DOS SANTOS BAZANELLA

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Tese apresentada ao programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de doutor em Ciência de Alimentos.

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Maringá - 2013

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Dedico

AO ALEXANDRE, MEU QUERIDO ESPOSO, PELO AMOR, DEDICAÇÃO, FORÇA NOS MOMENTOS DIFÍCEIS, PACIÊNCIA E COMPREENSÃO, ENFIM, POR FAZER PARTE DE MINHA VIDA. E AOS MEUS PAIS, NIRA E JOÃO, POR INVESTIREM EM MINHA EDUCAÇÃO E ACREDITAREM EM MIM, POR ME ENSINAREM O SIGNIFICADO DO AMOR.

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APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de três artigos científicos.

1. **ARTIGO DE REVISÃO.** Gisele Cristina dos Santos Bazanella, Caroline Aparecida Vaz Araújo, Rafael Castoldi, Giselle Maria Maciel, Fabíola Dorneles Inácio, Cristina Giatti Marques de Souza, Adelar Bracht and Rosane Marina Peralta. Ligninolytic enzymes from white-rot fungi and application in the removal of synthetic dyes. In: Maria de Lourdes T.M. Polizeli & Mahendra Rai (Eds). Fungal Enzymes. CRC Press,

2. **ARTIGO EXPERIMENTAL 1**. Gisele Cristina dos Santos Bazanella, Daniela Farani de Souza, Rafael Castoldi, Roselene Ferreira Oliveira, Adelar Bracht and Rosane Marina Peralta. Production of laccase and manganese peroxidase by *Pleurotus pulmonarius* in solid state cultures and application in dye decolorization. Folia Microbiologica, prelo.

3. **ARTIGO EXPERIMENTAL 2.** Gisele Cristina dos Santos Bazanella, Sílvio Cláudio da Costa, Thatiane Mota, Rosane Marina Peralta. Uso de *Moringa oleifera* e carvão ativado para produção de uma lacase de *Pleurotus ostreatus* hábil na descoloração do corante remazol brilliant blue R. Provável submissão ao periódico: Enzyme and Microbial Technology.

RESUMO GERAL

Corantes sintéticos são liberados no ambiente, através de efluentes decorrentes de indústrias têxteis, de impressão, de artefatos de couro, papel, alimentos, cosméticos, tintas e plásticos. Persistem na natureza, pois são estáveis à luz, temperatura e ataque microbiano. Podem obstruir a passagem de luz solar através dos recursos hídricos, levando a uma diminuição da fotossíntese e consequente diminuição da concentração de oxigênio dissolvido, essencial para sobrevivência de organismos aeróbicos e a uma diminuição da biodegradação de matérias orgânicas. Diante deste contexto, diferentes tecnologias para a descoloração de corantes têm sido estudadas. Atualmente, a remoção de corantes a partir de efluentes é realizada por meios físico-químicos, incluindo adsorção, precipitação, coagulação-floculação, oxidação, filtração e fotodegradação. Abordagens biotecnológicas são também comprovadamente eficazes e potencialmente eco-eficientes no tratamento destas fontes de poluição. Vários microrganismos, incluindo fungos ligninolíticos têm a capacidade de descolorir e degradar uma vasta gama de corantes. A baixa especificidade das enzimas ligninolíticas permite que elas sejam capazes de atacar uma série de moléculas quimicamente semelhantes à lignina, incluindo os corantes sintéticos.

No primeiro artigo, uma extensa revisão da capacidade de fungos da podridão branca da madeira e suas enzimas descolorir corantes sintéticos foi realizada. No segundo artigo, a produção de enzimas ligninolíticas lacase e Mn peroxidase pelo fungo da podridão branca *Pleurotus pulmonarius* (Fr.) Quélet foi estudada em culturas em estado sólido utilizando resíduos agrícolas e de alimentos como substrato. As maiores atividades de lacase foram encontradas nos cultivos com farelo de trigo (2.860 ± 250 U/L), casca de abacaxi (2.450 ± 230 U/L) e bagaço de laranja (2.100 ± 270 U/L) todas elas com uma umidade inicial de 85%. As maiores atividades de Mn peroxidase foram obtidas em culturas com casca de abacaxi (2.200 ± 205 U/L) a uma umidade inicial de 75%. Em geral, a condição de elevada umidade inicial (80-90%) foi a melhor condição para a produção de lacase, enquanto a melhor condição para a produção de Mn peroxidase foi cultivo a baixas umidades iniciais (50-70%). Culturas contendo as atividades da Mn peroxidase elevadas foram mais eficientes na descoloração dos corantes industriais vermelho do congo, azul de metileno e violeta de etila. O corante remazol brilliant blue R (RBBR) foi eficientemente descolorido em culturas com alta atividade de Mn peroxidase e lacase.

Finalmente, no terceiro artigo, a produção de lacase por *Pleurotus ostreatus* foi avaliada em cultivos em estado sólido utilizando-se semente de *Moringa oleifera* e farelo de trigo como substrato na presença e ausência de carvão ativado como agente clarificador. Os resultados obtidos demonstram que elevadas atividades de lacase foram obtidas nas culturas com *M. oleifera* (2338^{a} ± 220 U/L) quando comparada às obtidas com o tradicional substrato farelo de trigo (1520^{c} ± 285 U/L). A presença do agente clarificador carvão ativado teve um efeito significativo na produção de lacase nos cultivos em farelo de trigo ($2081^{a,b}$ ± 202 U/L), mas não nos cultivos em *M. oleifera* ($2126^{a,b}$ ± 48 U/L). O uso de uma mistura de farelo de trigo-semente de Moringa na proporção de 4:1 também resultou em um pequeno incremento na produção de lacase ($2252^{a,b}$ ± 475 U/L). O carvão ativado apresentou um efeito clarificador nos extratos enzimáticos brutos obtidos dos cultivos em *M. oleifera.* A lacase obtida em cultivos com semente de *M. oleifera* mais carvão ativado a 1% foi concentrada através de filtração por membrana com *cut off* de 5 kDa seguido de liofilização e utilizada com sucesso na descoloração do corante antraquinoníco RBBR.

Palavras chaves: aproveitamento de resíduos, descoloração de corantes, enzimas ligninolíticas, *Pleurotus pulmonarius*, *Pleurotus ostreatus*, *Moringa oleifera*.

GENERAL ABSTRACT

Large amounts of chemical dyes are used for various industrial applications such as textile and printing industries. It is estimated that between 10 and 20% of about 0.7 million tons of dyestuff that are manufactured each year and used in dyeing processes may be found in wasterwater. Several of these dyes are very stable to light, temperature and microbial attack, making them recalcitrant compounds. Dyes can obstruct the passage of the sunlight through the water resources, leading to decreased photosynthesis by aquatic plants coupled to a decreased concentration of dissolved oxygen, and to a diminished biodegradation of organic matters. Currently the removal of dyes from the effluents is brought about by physicochemical means, including adsorption, precipitation, coagulation-flocculation, oxidation, filtration and photo-degradation. At the present, the biotechnological approaches are proven to be potentially effective and ecoefficient in the treatment of these pollution sources. Several microorganisms including the ligninolytic fungi have the ability to decolorize and degrade a wide range of dyes. The possibility of using the filamentous fungi associated with the decay of wood, usually called white-rot fungi, has attracted considerable attention. White-rot fungi possess a group of oxidative enzymes named lignin-modifying enzymes or ligninolytic enzymes involved in lignin degradation, mainly laccases and peroxidases. Due to the fact that ligninolytic enzymes are non-specific, they are able to attack a series of molecules chemically similar to lignin including the synthetic dyes.

In the first paper, a review of implication of white-rot fungi and their ligninolytic enzymes in dye decolorization processes was carried out. In the second article, the production of ligninolytic enzymes (laccase and Mn dependent peroxidase) by the white-rot fungus *Pleurotus pulmonarius* (FR.) Quélet was studied in solid state cultures using agricultural and food wastes as substrate. The highest activities of laccase were found wheat bran (2,860±250 U/L), pineapple peel (2,450±230 U/L) and orange bagasse (2,100±270 U/L) cultures, all of them at an initial moisture level of 85%. The highest activities of Mn peroxidase were obtained in pineapple peel cultures (2,200±205 U/L) at an initial moisture level of 75%. In general, the condition of high initial moisture level (80-90%) were the best condition to laccase activity, while the best condition to Mn peroxidase activity was cultivation at low initial moisture (50-70%). Cultures containing high Mn peroxidase activities were more efficient in the decolorization of the industrial dyes congo red, methylene blue and ethyl violet than those containing high laccase activity. Also, crude enzymatic extracts with high Mn peroxidase activity were more efficient in the *in vitro* decolorization of methylene blue, ethyl violet and congo red. The dye remazol brilliant blue R (RBBR) was efficiently decolorized by both crude extracts, rich in Mn peroxidase activity or rich in laccase activity. Finally, in the third article, the production of laccase by *Pleurotus ostreatus* was evaluated in solid-state cultivation using *Moringa oleifera* seed and wheat bran as substrate in the presence and absence of activated charcoal as a clarifying agent. The results show that high laccase activity was obtained in cultures with *M. oleifera* (2338 ± 220 U/L) compared to those obtained with traditional wheat bran substrate (1520 ± 285 U/L). The presence of the clarifying agent activated carbon had a significant effect on the laccase production cultures in wheat bran ($2081^{a,b}$ ± 202 U/L) but not in the cultures with *M. oleifera* ($2126^{a,b}$ ± 48 U/L). The use of a mixture of bran seed of Moringa in 4:1 ratio also resulted in a small increase in the production of lacase ($2252^{a,b}$ ± 475 U/L). The activated charcoal showed a clarifying effect on crude enzyme extract obtained from cultures in *M. oleifera.* Laccase obtained in seed crops with *M. oleifera* plus 1% activated carbon was concentrated by membrane

filtration with 5 kDa cut off followed by lyophilization and used successfully in decolorization of the anthraquinone dye RBBR.

Key words: waste recovery, dye decolorization, ligninolytic enzymes, *Pleurotus pulmonarius*, *Pleurotus ostreatus, Moringa oleifera.*

ARTIGO 1

Ligninolytic enzymes from white-rot fungi and application in the removal of synthetic dyes

Gisele Cristina dos Santos Bazanella, Caroline Aparecida Vaz Araújo, Rafael Castoldi, Giselle Maria Maciel, Fabíola Dorneles Inácio, Cristina Giatti Marques de Souza, Adelar Bracht and Rosane Marina Peralta*.

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Introduction

Large amounts of chemical dyes are used for various industrial applications such as textile and printing industries. It is estimated that between 10 and 20% of about 0.7 million tons of dyestuff that are manufactured each year and used in dyeing processes may be found in wasterwater (Soares et al., 2001, Arantes et al., 2006). Several of these dyes are very stable to light, temperature and microbial attack, making them recalcitrant compounds (Nyanhongo et al., 2002). Dyes can obstruct the passage of the sunlight through the water resources, leading to decreased photosynthesis by aquatic plants coupled to a decreased concentration of dissolved oxygen, and to a diminished biodegradation of organic matters. Currently the removal of dyes from the effluents is brought about by physicochemical means, including adsorption, precipitation, coagulation-flocculation, oxidation, filtration and photo-degradation (Vandevivere et al., 1998, Ahn et al., 1999, Robinson et al., 2001). At the present, the biotechnological approaches are proven to be potentially effective and ecoefficient in the treatment of these pollution sources. Several microorganisms including bacteria, algae, yeast and filamentous fungi have the ability to decolorize and degrade a wide range of dyes (Forgacs et al., 2004, Pandey et al., 2007, El-Sheekh et al., 2009, Kaushik and Malik, 2009). The possibility of using the filamentous fungi associated with the decay of wood, usually called white-rot fungi, has attracted considerable attention. White-rot fungi possess a group of oxidative enzymes named lignin-modifying enzymes or ligninolytic enzymes involved in lignin degradation, mainly laccases and peroxidases. Due to the fact that ligninolytic enzymes are non-specific, they are able to attack a series of molecules chemically similar to lignin including pesticides, polyclyclic aromatic hydrocabons, synthetic polymers and synthetic dyes (Asgher et al., 2008).

White-rot fungi

White-rot fungi are Basidiomycetes that participate in the biodegradation of lignin in nature, which is essential for global carbon recycling. Lignin is a polymer found in wood and vascular tissues (ten Have and Teunissen, 2001). In the cell wall, lignin is covalently associated to carbohydrate components (cellulose and hemicelluloses) forming a matrix that protects the cell wall against microbial attack (Leonowicz at al., 2001). Unlike most natural polymers, lignin is irregular and non-repeating (Fig. 1). Its biosynthesis is the result of oxidative polymerization of several phenyl-propanoid precursors, such as coniferyl alcohol,

sinapyl alcohol and *p*-coumaryl alcohol. The polymerization occurs at random by various carbon-carbon and ether bonds resulting in an irregular structure (Ralph et al., 2004).

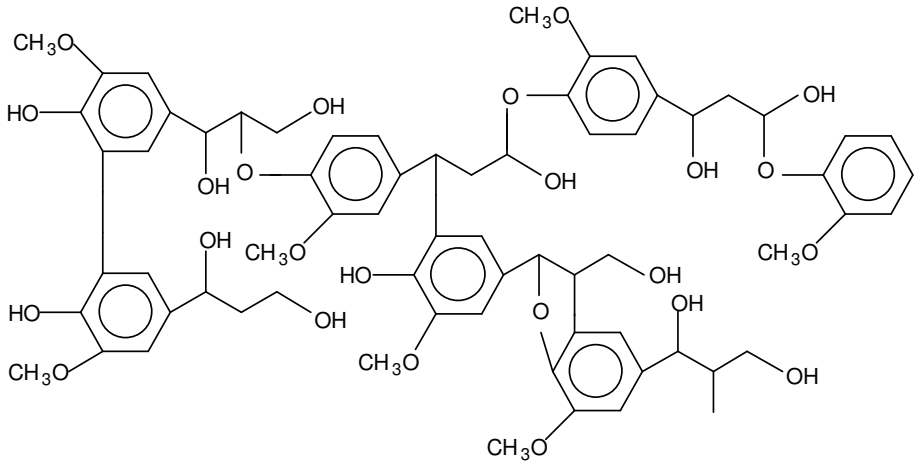


Figure 1. Lignin: some possible structures

Lignin resists to the attack by most microorganisms; anaerobic processes tend not to attack the aromatic rings at all and the aerobic breakdown of lignin is slow. In nature, only basidiomyceteous white-rot fungi are able to degrade lignin efficiently. Wood decayed by white-rot fungi is pale in color because of oxidative bleaching and loss of lignin and often retains a fibrous texture. There are about 10,000 species of white-rot fungi, with varying capacities to degrade lignin, cellulose, and hemicelluloses. However, not more than 3 dozen have been more properly studied. Among these, the majority of studies have focused on *Phanerochaete chrysosporium*, followed by *Trametes versicolor, Bjerkandera adusta and Pleurotus sp* (Dashtban et al., 2010).

Isolation and laboratory maintenance of white-rot fungi

White-rot fungi have great potential for biotechnological applications. For this reason, there has been a growing interest in screening for new white rot fungi species and strains. A classical strategy is to collect basidioma or mycelia of white rot fungi in forests, dead trees, and lignocellulosic crop residues showing signs of attack by fungi. Samples of basidioma or mycelia are aseptically transferred onto potato dextrose agar or malt extract agar and sub-cultured until the obtainment of pure mycelia. Identification is based on morphological, physiological, biochemical and genetic characteristics of the basidioma, hyphae and spores. More recently, molecular biology techniques are being used in the identification of new isolates. Advancements in molecular methods have permitted a more rational study of the phylogenetic relationships within the various organisms. Non-coding internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA seem to be one of the most frequently employed analytical tools (Prewitt et al., 2008).

There are several options for maintenance of white rot fungi in the laboratory. Continuous growth methods of preservation in which white rot fungi are grown on agar (e.g., malt extract agar, potato dextrose agar and yeast extract agar) are typically used for short-term storage. For long-term storage, preservation in distilled water called method of Castellani (Castellani, 1939), mineral oil and anhydrous silica gel are some of the indicated methods. These are low cost methods but none is considered permanent. Lyophilization and liquid nitrogen refrigeration (cryo-preservation) are expensive methods but are considered permanent.

Ligninolytic enzymes

Lignin is not used as an energy source by white-rot fungi, thus it is not a substrate of primary metabolism. The biodegradation of lignin is an oxidative process that occurs during secondary metabolism in order to gain access to the energy-rich cellulose and hemicelluloses, which are chemically bound to lignin in the plant cell wall. For becoming the most effective wood degraders in nature, the white rot fungi developed non-specific and radical-based mechanisms for degrading lignin. White-rot fungi secrete one or more of four extracellular enzymes that are involved not only in lignin degradation but also in the degradation of several pollutants. The four major ligninolytic enzymes are: lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16) and laccase (Lac, EC 1.10.3.2). All these enzymes act via generation of free radicals which represent an efficient way to reach their substrates. Laccase was firstly described over 128-years ago, becoming one of the oldest known enzymes (Mayer and Staples, 2002). LiP and MnP were firstly discovered in *Phanerochaete chrysosporium* (Tien and Kirk, 1984, Glenn and Gold, 1985). Versatile peroxidase (VP) is a new addition to the group of ligninolytic enzymes and was discovered in a strain of *Pleurotus eryngii* (Martinez et al., 1996, Martinez, 2002). The genomes of some species of white-rot fungi such as *Phanerochaete chrysosporium* (Martinez et al., 2004) and *Schizophyllum commune* (Ohm et al., 2010) have already been sequenced and are useful tools for understanding the enzymatic systems used by different fungal species in lignocelluloses degradation. *P. chysosporium* has genes encoding peroxidases but not laccases, whereas *S. commune* has genes encoding laccases but not peroxidases. Therefore, these fungi probably have different mechanisms to degrade lignin. Due to the redox potential, laccase alone can only oxidize phenolic lignin structures. The addition of a mediator, a small chemical compound, extends the substrate range to non-phenolic lignin structures. It is assumed that the mediator is needed because the large laccase molecule can not enter the secondary cell wall and oxidize lignin directly. Most of the mediators found contain N-hydroxy-groups, such as 1-hydroxybenzotriazole (HBT), violuric acid (VA) and N-hydroxy-acetanilide (NHA). For an extensive list of native and synthetic mediators in ligninolytic systems see the review articles of Wesenberg et al. (2003) and Morozova et al., (2007).

White-rot fungi usually secrete one or more of ligninolytic enzymes in different combinations. Distribution of white-rot fungi into groups according to their enzymatic systems have been undertaken (Hatakka, 1994, Tuor et al., 1995, Nerud and Misurcova, 1996). Generically, white-rot fungi can be distributed into four groups, according to their ability to produce laccases and peroxidases (LiP, MnP and VP):

1). laccase and the peroxidase MnP and LiP such as in *Trametes versicolor* and *Bjerkandera adusta*;

2). laccase and at least one of the peroxidases such as in *Lentinula edodes*, *Pleurotus eryngii* and *Ceriporiopsis subvermispora*;

3). only laccase such as in *Schizophyllum commune*;

4). only peroxidases such as in *Phanerochaete chrysosporium*.

The most frequently observed ligninolytic enzymes among the white-rot fungi species are laccases and MnP and the least are LiP and VP.

The ligninolytic enzymes may function cooperatively or separately from each other, however auxiliary enzymes (unable to degrade lignin on their own) are necessary to complete the process of lignin and/or xenobiotics degradation: aryl alcohol oxidase (AAO, EC 1.1.3.7), aryl alcohol dehydrogenase (AAD, EC 1.1.1.90), glyoxal oxidase (GLOX, EC 1.2.3.5), quinone reductase (QR, EC 1.1.5.1), cellobiose dehydrogenase (CDH, EC 1.1.99.18),

superoxide dismutase (SOD, EC 1.15.1.1), glucose 1-oxidase (GOX, EC 1.1.3.4), pyranose 2-oxidase (P2Ox, EC 1.1.3.4) and methanol oxidase (EC 1.1.3.13). These are mostly oxidases generating H_2O_2 and dehydrogenases. Cytochrome P450 monooxygenases are also significant components involved in the degradation of lignin and many xenobiotics. Other important components of the lignin degradative system of white-rot fungi are low molecular mass oxidants such as hydroxyl radicals (•OH) and chelated Mn^{3+} (Mn^{3+} mainly chelated by oxalic acid), produced through the action of the ligninolytic enzymes. These are particularly important during the early stages of wood decay and can also act on the degradation of xenobiotics (ten Have and Teunissen, 2001, Hammel et al., 2002, Hofrichter, 2002, Watanabe et al., 2002). Recent additions to the enzymatic systems of white-rot fungi include dye-decolorizing peroxidases or DyP involved in the oxidation of synthetic high redox-potential dyes and nonphenolic lignin model compounds (Liers et al., 2010) and aromatic peroxygenases or APOs that catalyze diverse oxygen transfer reactions which can result in the cleavage of ethers (Hofrichter et al., 2010, Liers et al., 2011).

Laboratory cultivation for production of ligninolytic enzymes

For screening the ligninolytic capability of white-rot fungi, in recent years the traditional screening reagents tannic and gallic acid (Bavendam reaction) have been replaced by synthetic phenolic reagents such as guaiacol and syringaldazine. Therefore, guaiacol and syringaldazine are added to the malt extract agar or potato dextrose agar and the ligninolytic activity is observed as a brown halo around the microbial growth (Fig. 2A; Erden et al., 2009). Another very common technique for screening the ligninolytic capability of white rot fungi is to evaluate the decolorization halo of synthetic dyes on agar plates. The anthraquinone derivative dye remazol brilliant blue R (Machado et al., 2005) and the polymeric dye Poly R478 (Kiiskinen et al., 2004) are frequently used for this purpose (Fig. 2B).

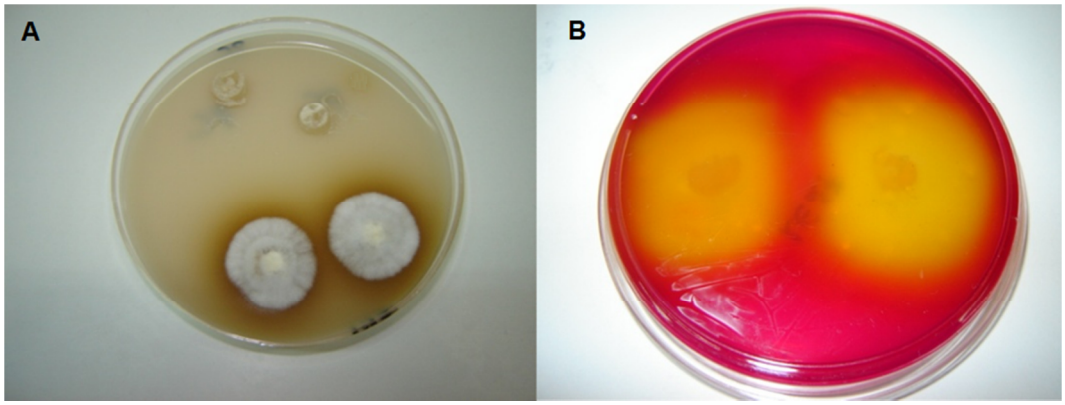
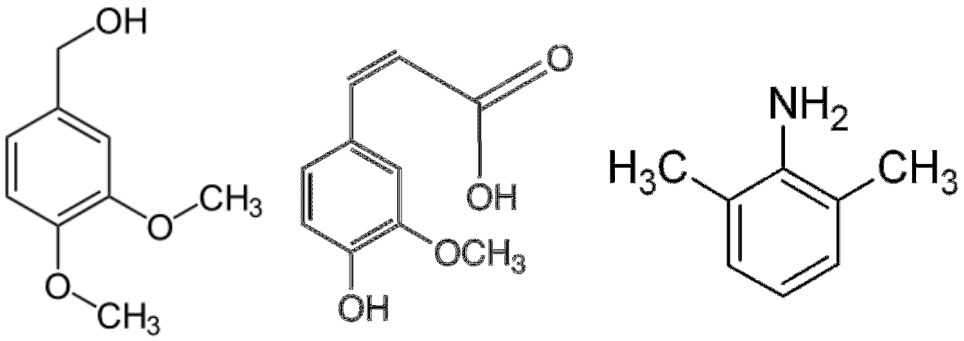


Figure 2. Evaluation of the ligninolytic activity on agar plates. In A: Test of oxidation of guaiacol. In B: Test of decolorization of the polymeric dye Poly R478

Industrially important enzymes have traditionally been obtained from submerged cultures because of the ease of handling and greater control of environmental factors such as temperature, pH and aeration. Additionally, specific factors of the media composition can be relevant in the production of ligninolytic enzymes. For example, the production of ligninolytic enzymes is considerably enhanced by aromatic and phenolic compounds related to lignin or lignin derivatives, such as ferulic acid, 2,5 xylidine and veratryl alcohol (Fig. 3), and cations such as manganese and copper (Leonowicz et al., 2001, Lo et al., 2001, Nyanhongo et al., 2002, Gill and Arora, 2003, Souza et al., 2004, Lorenzo et al., 2006,

Twchanowicz et al., 2006, Saeki et al., 2011). Factors like these are more easily controlled in submerged cultures. Several recent contributions have described the production of laccase and peroxidases, MnP, LiP and VP, in submerged cultures (Linke et al., 2005, Lorenzo et al., 2006, Rogalski et al., 2006, Janusz et al., 2007, Tellez-Tellez et al., 2008, Johnsy and Kaviyarasan, 2011, Ding et al., 2012)



Veratryl alcohol Ferulic acid 2,5 Xylidine

Figure 3. Chemical structures of some important inducers of ligninolytic enzymes.

Solid state culture (SSC) is also an attractive option for ligninolytic enzyme production because it mimics the natural environment of the white rot fungi and presents higher productivity, simpler operation, and lower cost when compared with the submerged culture. SSC reproduces the conditions under which the white rot fungi grow in nature and allows the use of a series of food, agricultural, and forestry wastes as substrates. These wastes are rich in carbohydrates, and most of them are easily assimilated by microorganisms and for this reason can be used for their cultivation in SSC without necessity of supplementation. Wheat bran is the most commonly used substrate for the cultivation of white-rot fungi. However, the list of possibilities is very large and includes several lignocellulolytic wastes such as cane bagasse, corn cob, wheat straw, oat straw, rice straw, and food processing wastes such as banana, kiwi fruit, and orange wastes (Holker et al. 2004, Tychanowicz et al., 2004, Alexandrino et al., 2007, Osma et al., 2007, Couto, 2008). Even so, it is worth to search for new substrates, especially if they are available in large amounts, allow the growth of white-rot fungi without further supplementations, and facilitate obtainment of valuable products (Zilly et al., 2012).

Environmental applications of ligninolytic enzymes

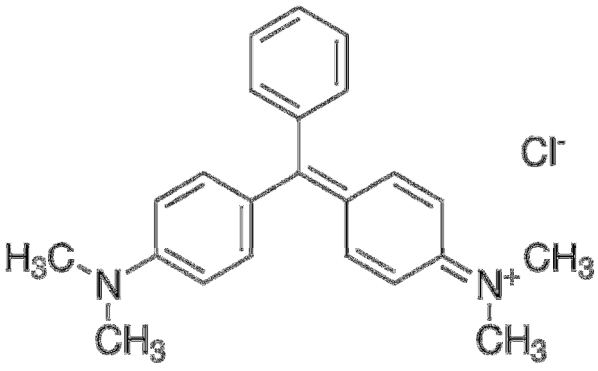
The ligninolytic enzymes are highly non-specific and, thus, capable of degrading a wide range of recalcitrant compounds structurally similar to lignin. In recent years, the capability of white rot fungi and their enzymes to biodegrade several xenobiotics and recalcitrant pollutants has generated considerable research interest in the area of industrial/enviromental microbiology. As a consequence, a considerable number of reviews detailing the numerous characteristics and applications of ligninolytic enzymes have been published (Conesa et al., 2002, Hofrichler, 2002, Mayer and Staples, 2002, Wesenberg et al., 2003, Baldrian, 2006, Kersten and Cullen, 2007, Ruiz-Duenas and Martinez, 2009). Among the industrial applications of the ligninolytic enzymes found in WRF are a). paper whitening, b). degradation of industrial dyes from the textile industry and c). degradation of xenobiotic compounds, including polycyclic aromatic hydrocarbons, phenolics, herbicides and other pesticides (Asgher et al., 2008).

Synthetic dyes

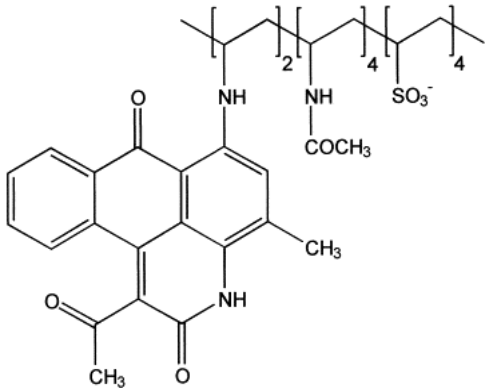
Synthetic dyes are aromatic compounds. According to their dissociation in an aqueous solution, dyes can be classified as acid, direct reactive dyes (anionic), basic dyes (cationic) and disperse dyes (nonionic). They are used in textiles, paper printing, color photography, cosmetics, plastics, pharmaceutical and leather industries. Synthetic dyes have been used increasingly in textile and dyeing industries because of their ease and cost effectiveness in synthesis, firmness and variety in color when compared to that of natural dyes.

About 100,000 commercial dyes are manufactured including several varieties of dyes such as acidic, basic, reactive, azo, diazo and anthraquinone based meta complex dyes. Over 10,000 dyes with an annual production of over 7×10^5 metric tons are commercially available (Campos et al., 2001). Depending on the class of the dye, up to 50% of them are released into the industrial effluents. Colored industrial effluents from the dyeing industries represent major environmental problems. Unbound reactive dyes undergo hydrolysis favored by the temperatures and pH values during the dyeing processes. Many dyes are visible in water at concentrations as low as 1 mg/L and have a huge impact on the aquatic environment caused by its turbidity and high pollution strength, in addition to the toxic degradation products that can be formed. Dyes that absorb light with wavelength in the visible range (350-700 nm) are colored. Dyes contain chromophoric electron systems with conjugated double bonds and auxochromes. The latter are electron withdrawing or electron donating substituents that cause the color of the chromophore by altering the overall energy of the electron system. Usual chromophores are –C=C–, –C=N–, –C=O–, –N=N–, $–NO_2$ and quinoid rings, and usual auxochromes are $–NH_3$, –COOH, $–SO_3H$ and –OH.

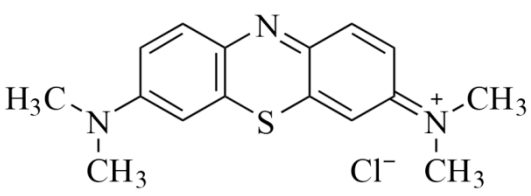
Dyes can also be classified according to the chemical structure of the chromophoric group. According to this criterion they can be classified, for example, as azo, anthraquinone, triphenylmethane, heterocyclic, and polymeric dyes (Fig. 4). Among these, the versatile azo and triphenylmethane dyes account for most textile dyestuffs produced.



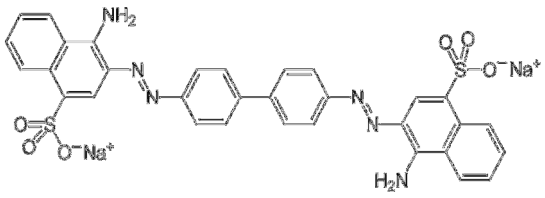
Triphenylmethane dye: malachita green



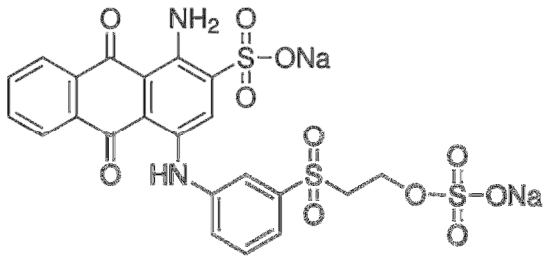
Polymeric dye: poly R478



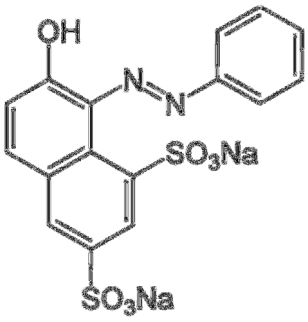
Heterocyclic dye: methylene blue



Azo dye: Congo red



Anthraquinone dye: remazol brilliant blue R



Azo dye: orange G

Figure 4. Some examples of azo, anthraquinone, triphenylmethane, heterocyclic and polymeric dyes

Methods for evaluting the removal of synthetic dyes

The most simple and most used methods to evaluate dye decolorization are the spectrophotometric assays. To test the ability of white rot fungi to decolorize synthetic dyes in vivo, a dye is added to submerged, liquid or solid state cultures and at different times of cultivation the decrease of visible light absorbance at the wavelength of maximum absorbance of the dye is measured in a spectrophotometer. In the same way, it is possible to evaluate the capability of a crude or purified ligninolytic enzyme to decolorize a specific dye. As examples, the decolorization of RBBR, congo red and poly R478 can be estimated at 595, 497 and 520 nm, respectively (Boer et al., 2004). A more accurate method is to obtain UV-VIS or VIS spectra of the material before and after treatment (Fig. 5).

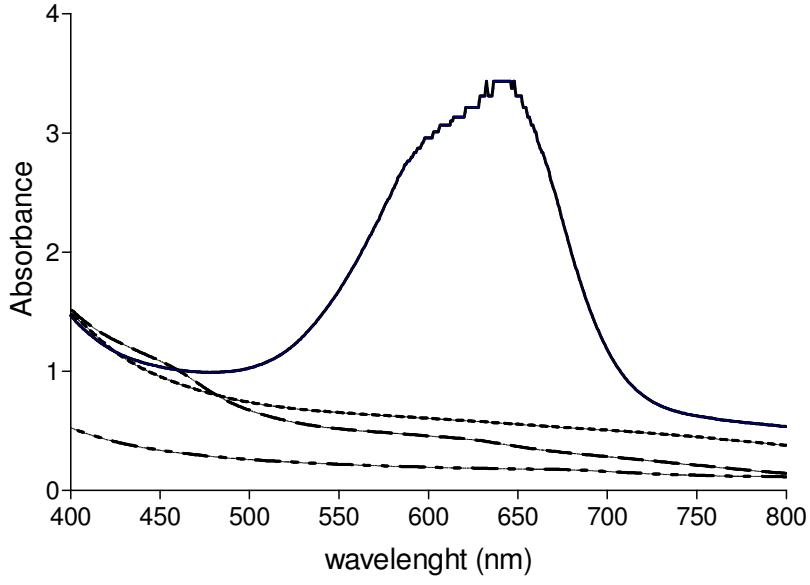


Figure 5. Visible spectra of laundry effluent before and after treatment with *Ganoderma lucidum, Pycnoporus* sp and *Trametes* sp crude laccases. Crude laccases from white rot fungi were added to an effluent containing the textile effluent and the mixtures maintained under agitation of 120 rpm for 2 h at 28^{o} C Before treatment (continues line); after

treatment with laccase from *G. lucidum*, *Pycnoporus* sp *Trametes* sp (dashed lines) (unpublished data).

Although the majority of the studies of dye decolorization are carried out using spectrophotometric analysis, this technique is limited as an analytical tool considering that, in essence, it allows only the evaluation of the chemical modifications that occur in the chromophore. New methodologies have been recently introduced with the objective of identifying soluble and insoluble compounds produced by the action of ligninolytic enzymes when acting on the dyes. Some examples are:

1. Zille et al. (2005) used liquid chromatography-mass spectrometry (LC-MS) and ^{13}C-nuclear magnetic resonance (^{13}C-NMR) to identify, respectively soluble and insoluble compounds produced from the decomposition of the azo dye 3-(2-hydroxy-1-naphthylazo) by *Trametes villosa* laccase. By using LC-MS it was possible to identify at least two metabolites: hydroxybenzenesulfonic acid and benzenesulfonic acid. ^{13}C-NMR allowed the identification of compounds produced by polymerization reaction during the long-term batch decolorization processes of the azo dye.

2. Michniewicz et al. (2008) used a high performance liquid chromatography system equipped with a diode array detector (HPLC-DAD) to evaluate the modifications caused by a purified *Cerrena unicolor* laccase in the anthraquinone dye acid blue 62. The analysis did not allow the chemical identification of the oxidation products, but revealed the formation of at least two new more polar oxidation products.

3. Murugesan et al. (2009) used HPLC and liquid chromatography-mass spectrometry-electrospray ionization (LC-ESI-MS) analysis and concluded that *N*-demethylation was the key mechanism of decolorization of the tryphenylmethane dye malachita green by *Ganoderma lucidum* laccase.

4. Zhao et al. (2006) and Zhao and Hardin (2007) used HPLC-DAD to analyze the metabolites produced from the degradation of the azo dyes (in special disperse orange 3) by *Pleurotus ostreatus*. In these studies, no attempt was made to correlate the dye degradation with ligninolytic enzymes, but nitrobenzene, 4-nitroanisole, 4-nitrophenol and 4-nitroaniline were identified as metabolite products of dye degradation.

An aspect normally neglected in dye decolorization studies is the toxicity changes occurring during fungal biodegradation. It is well known that synthetic dyes are toxic, most of them carcinogenic (Wesenberg et al., 2003), but the question is: how much safer are the products formed through chemical transformation of synthetic dyes by white rot fungi and their ligninolytic enzymes? The knowledge of this point has a tremendous importance, but only a few works have attempted to present answers. Some examples are:

1. Ramsay and Nguyen (2002) used the white rot fungus *Trametes versicolor* to decolorize eight textile dyes and also evaluated the toxicity of the material before and after the treatment using a Microtox 500 Analyzer (Azur Environmental, Newark, Del.). After decolorization the toxicity of the solutions containing Amaranth, Tropaeolin O and Reactive Black 5 was unchanged; Reactive Blue 15, Remazol

Brilliant Blue R and Cibacron Brilliant Red 3G-P decreased to non-toxic levels; and Cibacron Brilliant Yellow 3B-A and Congo Red became more toxic.

2. Abadulla et al. (2000) observed a reduction of toxicity (based on the oxygen consumption rate of *Pseudomonas putida*) of triarylmethane, indigoid, azo, and anthraquinonic dyes after treatment with an immobilized laccase from *Trametes hirsuta*.

3. Apohan and Yesilada (2005) observed a reduction in the toxicity of azo dyes, astrazon blue and astrazon red, after treatment with pellets of the white rot fungus *Funalia trogii.*

4. Anastasi et al. (2010) used Mn-peroxidase and Mn-independent peroxidase from *Bjerkandera adusta* to decolourize 13 industrial dyes. Toxicity of the simulated wastewaters was also evaluated, before and after the fungal treatment, by means of a phytotoxicity test on *Cucumis sativus* L. seeds.

5. Casieri et al. (2008) used *Trametes pubescens* and *Pleurotus ostrealus,* immobilized on polyurethane foam cubes in bioreactors to decolorize two anthraquinone dyes (B49 and RBBR) and one azo dye (R243). To evaluate the toxicity before and after treatments, the authors used the *Lemna minor* (duckweed) ecotoxicity test.

6. Zhuo et al. (2011) used laccase-producing white-rot fungi strain *Ganoderma* sp.En3 to decolorize synthetic dyes and real textile dye effluent. Phytotoxicity study with respect to *Triticum aestivum* and *Oryza sativa* demonstrated that *Ganoderma* sp.En3 was able to detoxify four synthetic dyes, two simulated dye effluents and the real textile dye effluent.

7. Eichlerová et al. (2007) used a bioassay based on the growth inhibition of duckweed *Lemna minor* to evaluate the toxicity of synthetic dyes orange G ans remazol brilliant blue before and after treatment with *Dichomitus squalens* (static and skaken cultures). The toxicity of both dyes decreased after the decolorization process.

Ligninolytic enzymes and dye decolorization

Representative studies published after year 2000 where ligninolytic enzymes were used for removing synthetic dyes are presented below. Only those studies are listed in which direct or strong indirect evidence is presented about the participation of enzymes in the dye decolorization processes. For analyses of the articles published before 2000, see previous reviews such as Wesenberg et al., 2003, Forgacs et al., 2004, Husain, 2006.

1. Several structurally different synthetic dyes (including azo, triphenylmethane, heterocyclic and polymeric dyes) were decolorized in submerged and solid cultures of *Pleurotus pulmonarius* producing laccase as the main ligninolytic enzyme. Both laccase activity and dye decolorization were related to glucose and ammonium starvation or to induction by ferulic acid. Additionally, decolorization and laccase activity were equally affected by pH and temperature. Laccase can thus be

considered to be the major enzyme involved in the ability of *P. pulmonarius* (Zilly et al., 2002).

2. Crude laccase enzyme from *Ganoderma lucidum* obtained in wheat bran solid state cultures demonstrated excellent decolorization activity to anthraquinone dye remazol brilliant blue R (RBBR) without redox mediator, whereas the diazo dye remazol black-5 (RB-5) requires a redox mediator. N-hydroxybenzotriazole at a concentration of 1 mM was the best redox mediator (Murugesan et al., 2007).

3. *Ganoderma lucidum* laccase obtained from wheat bran solid state cultures was able to tolerate mixtures of several metal ions. Treatment of simulates reactive dye effluent (RBBR and RB-5) by laccase showed that the redox mediator system is necessary for effluent decolorization. Syringaldehyde, a natural redox mediator, was more effective than the synthetic mediator 1-hydroxybenzotriazole (HBT) (Murugesan et al., 2009a)

4. *G. lucidum* laccase obtained from wheat bran solid state cultures was able to decolourize the dye malachite green. The dye decolourisation was improved when natural phenolic compounds such as acetovanillone, *p*-coumaric acid, ferulic acid, syringaldehyde and vanillin act as redox mediators. Characterization of MG transformation products by HPLC, UV-VIS and liquid chromatography-mass spectrometry-electrospray ionization analysis revealed that N-demethylation was the key mechanism of decolourization of MG by laccase (Murugesan et al., 2009b).

5. *G. lucidum* laccase obtained in passion fruit waste solid state cultures was used to decolourize RBBR. The enzyme was inhibited by NaCl but not by Na_2SO_4. Inhibition by NaCl was of the mixed type with two different inhibition constants. The enzyme was able to completely decolourise RBBR in the presence of 1.0 M Na_2SO_4 and 50% decolourisation was obtained in the presence of 0.1 M NaCl (Zilly et al., 2011).

6. Direct dye solar golden yellow R was decolourized by *G. lucidum* Mn peroxidase obtained from submerged culture. Various parameters such as optimum pH, incubation time, temperature and amounts of carbon and nitrogen additives were optimized to achieve maximal decolourisation of the dye. Addition of 1% starch accelerated the decolourization (96%), while the addition of nitrogen sources inhibited both enzyme induction and dye decolourisation (Bibi et al., 2009).

7. The dyes orange G and RBBR were treated using ligninolytic enzymes from *Dichomitus squalens*, *Ischnoderma resinosum* and *Pleurotus calyptratus* obtained in liquid cultures. All white rot fungi efficiently decolorized both Orange G and RBBR, but they differed in decolorization capacity depending on cultivation conditions and ligninolytic enzyme production. Two different decolorization patterns were found in these strains: Orange G decolorization in *I. resinosum* and *P. calyptratus* was caused mainly by laccase, while RBBR decolorization was effected by manganese peroxidase (MnP); in *D. squalens* laccase and MnP cooperated in the decolorization processes (Eichlerová et al., 2005, 2007).

8. Anthraquinone and azo dyes were decolourized by *Cerrena unicolor* laccase obtained from submerged cultures. The capability of crude and purified laccase from were evaluated for removing different classes of dyes using kinetic studies and HPLC analysis. The decolourization process can be satisfactorily described by the Michaelis–Menten kinetic model. The most suitable pH for decolorization was 3.5. Laccase was able to decolorize recalcitrant azo dyes all without any redox mediators (Michniewicz et al., 2008).

9. Two purified laccases (POXC and POXA3) from *Pleurotus ostreatus* obtained from liquid cultures were able to degrade RBBR *in vitro*, in the absence of any redox mediators. These laccases differ significantly in their efficiency of decolourisation of the tested dye, as suggested by comparison of their catalytic efficiency (k_{cat}/K_m values) towards RBBR. Furthermore, using a mixture of both POXC and POXA3 a remarkable improvement in the reaction rate and in the final level of dye decolorization was observed. The extent of RBBR decolourisation by laccase mixture also depends on incubation temperature and enzyme concentration. The dye is decolorized by laccase isoenzymes most efficiently under acidic conditions. Treatment of RBBR with the laccase mixture reduced its toxicity by 95% (Palmieri et al., 2005).

10. One laccase isoenzyme (LAC1) from *Pycnoporus sanguineus* obtained from malt extract cultures was able to decolourize several dyes. Regarding the activity profile of the biocatalyst against pH, salts, temperature and target substrates, LAC-1 appears to be a good candidate for application in acid dye bath treatments. Studying the model anthraquinonic dye ABu62 decolorization, it was proved that this dye was a good substrate for LAC-1. LAC-1 showed an unusual kinetic behaviour, suggesting that LAC-1 was activated in presence of ABu62 (Trovaslet et al., 2007).

11. *Trametes villosa* laccase (Novo Nordisk, Denmark) was used for direct azo dye degradation, and the reaction products that accumulated after 72 h of incubation were analyzed. Liquid chromatography-mass spectrometry (LC-MS) analysis showed the formation of phenolic compounds during the dye oxidation process as well as a large amount of polymerized products that retain azo group integrity. The amino-phenol reactions were also investigated by ^{13}C-nuclear magnetic resonance and LC-MS analysis, and the polymerization character of laccase was shown. This study highlights the fact that laccases polymerize the reaction products obtained during long-term batch decolorization processes with azo dyes. These polymerized products provide unacceptable color levels in effluents, limiting the application of laccases as bioremediation agents (Zille et al., 2005).

12. Various textile dyes were decolorized with the immobilized *Trametes hirsuta* laccase, and the toxicity of the degradation products was determined. The aquatic toxicities of samples were evaluated based on their inhibitory effects on the oxygen consumption rate of *Pseudomonas putida* (Abadulla et al., 2000).

13. The capability of white-rot fungi *Irpex lacteus* to decolourize reactive Orange 16 and Remazol Brilliant Blue R was mainly related to a laccase-like enzyme activity associated with fungal mycelium. The highest enzymatic activity was obtained with ABTS as substrate. Enzyme activity was fully inhibited with 50 mM NaN_3. Depending

on the chemical structure of dyes, redox mediators had a positive effect on the dye decolorization by fungal mycelium. Enzyme isolated from fungal mycelium was able to decolorize synthetic dyes *in vitro* (Svobodová et al., 2008).

14. Native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate PAGE confirmed the role of crude laccase from *Rigidoporus lignosus* in the decolorization of remazol brilliant blue and malachite green. Efficient enzymatic decolorisation without redox mediator was achieved. Unusual storage stability at alkaline pH was observed, with the laccase activity being enhanced 1.5–2-fold after 3 h of incubation (Li et al., 2009).

15. Original textile industry effluents were decolorized using *G. lucidum* Mn peroxidase obtained in submerged cultures. Process optimization could improve color removal efficiency of the fungus to 95% within only 2 days, catalyzed by Mn peroxidase (1295 U/L) as the main ligninolytic enzyme at pH 3 and 35^{o} C using 1% starch supplemented Kirk´s basal medium (Asgher et al., 2010).

16. Direct dye solar golden yellow R was decolorized by *Ganoderma lucidum* Mn peroxidase obtained from submerged culture. Various parameters such as optimum pH, incubation time, temperature and amounts of carbon and nitrogen additives were optimized to achieve maximal decolourisation of the dye. Addition of 1% starch accelerated the decolourization (96%). Addition of nitrogen sources inhibited both enzyme induction and dye decolourisation (Bibi et al., 2009).

17. The role of Mn peroxidase in dye decolorization of polymeric dye Poly R478 was assayed with isoenzyme PCH4 from *Phanerochaete chrysosporium* and isoenzyme BOS2 from *Bjerkandera* sp. BOS55. The assays were conducted with semi-continuous addition of H2O2. The rwo pure MnP have different catalytic properties because MnP from *Bjerkandera* produced a certain decolorization in the absence of Mn+2, whereas MnP from *P. chrysosporium* had a very little effect. The enzymatic treatment provoked not only the destruction of the chromophoric groups but also a noticeable breakdown of the chemical structure of the dye. As the experiments were conducted with pure enzymes, MnP proved to be the main factor responsible for the dye decolorization (Moreira et al., 2001a).

18. A novel strain of *Bjerkandera* sp. (B33/3), with particularly high decolourisation activities upon Poly R-478 and Remazol Brilliant Blue R (RBBR) dyes, was isolated. The role of the ligninolytic extracellular enzymes produced by this strain on decolorization of RBBR was studied in some depth. The basis of decolorization is an enzyme-mediated process, in which the main enzyme responsible is a recently described peroxidase with capacity for oxidation of manganese, as well as veratryl alcohol and 2,6-dimethoxyphenol in a manganese-independent reaction (Moreira et al., 2001b).

19. From the extracellular fluid of a novel strain of *Bjerkandera* sp., it was isolated, purified and identified the main enzyme responsible for Remazol Brilliant Blue R dye decolorization. Such an enzyme is able to oxidize manganese, as well as veratryl alcohol and 2,6-dimethoxyphenol in manganese-independent reactions; hence, it can

be included in the new group of versatile peroxidases. The enzyme substrate range for oxidation of several phenolic and non-phenolic aromatic compounds was determined and the corresponding Michaelis–Menten kinetic constants calculated (Moreira et al., 2006).

20. Manganese-peroxidase and manganese-independent peroxidase from *Bjerkandera adusta* were correlated with capability of fungus to decolourize 13 industrial dyes. In order to evaluate its true bioremediation potential, the fungus was packed in a fixed-bed bioreactor, for treatment of large volumes of a real wastewater. The fungus resulted effective during 10 cycles of decolourisation, remaining active for a very long period, in non-sterile conditions. Toxicity of the simulated wastewaters was also evaluated, before and after the fungal treatment, by means of a phytotoxicity test on *Cucumis sativus* L. seeds (Anastasi et al., 2010).

21. *Phanerochaete chrysosporium* was able to decolorize the azo dye orange in submerged cultures. The dye decolorization was dependent of pH and temperature. The main ligninolytic associated to dye descolorization was Mn peroxidase (Sharma et al., 2009).

22. Manganese peroxidase was the main enzyme involved in dye decolorization of decolorize azo dyes Reactive Orange 16 and Naphthol Blue Black by two white-rot fungi, *Irpex lacteus* and *Phanerochaete chrysosporium*, A significant increase in the Reactive Orange 16 decolorization by the agitated *I. lacteus* cultures was observed after adding 0.1% Tween 80, following a higher Mn-dependent peroxidase production. The in vitro dye decolorization using the purified enzyme proved its decolorization ability (Svodová et al., 2006).

23. Manganese peroxidase (MnP) from *Phanerochaete chrysosporium* was also responsible by the decolorization of a series of sulfonphthalein dyes, such as bromocresol green, bromophenol blue (Cristian et al., 2003).

24. Manganese peroxidase was the main enzyme responsible by decolorization of azo and anthraquinone dyes by *Phanerochaete sordida*. Decolorization of dye mixtures by *P. sordida* was partially inhibited by polyvinyl alcohol (PVA) that wastewaters from textile industries often contain (Harazono et al., 2005).

Concluding remarks

The majority of the synthetic dyes is chemically stable and resistant to microbiological attack. White rot fungi are excellent candidates for biodegradation of these recalcitrant compounds. The isolation of new strains or the adaptation of existing ones to the decomposition of dyes will probably increase the efficacy of dye bioremediation in the near future.

The employment of ligninolytic enzyme preparations can bring considerable benefits over the direct use of white rot fungi, considering that preparations can be more easily standardized, facilitating accurate dosage. Enzyme application is simpler than the use of microorganisms and can be rapidly modified according to the characteristics of the dye or dyes to be removed. In addition to this, analysis of metabolite compounds produced by

enzyme preparations is easier than analysis of metabolites produced by using the whole white rot fungus.

There is a need for including new methodologies in the elucidation of the chemical structures of metabolites produced by the action of ligninolytic enzymes. This should also facilitate the elucidation of the metabolic routes used by white rot fungi in dye degradation. Furthermore, the verification that the process of decolorization corresponds to detoxification is a crucial step in order to indicate a real application.

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ARTIGO 2

Production of laccase and manganese peroxidase by *Pleurotus pulmonarius* in solid state cultures and application in dye decolorization

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ABSTRACT

The production of ligninolytic enzymes (laccase and Mn dependent peroxidase) by the white-rot fungus *Pleurotus pulmonarius* (FR.) Quélet was studied in solid state cultures using agricultural and food wastes as substrate. The highest activities of laccase were found wheat bran (2,860±250 U/L), pineapple peel (2,450±230 U/L) and orange bagasse (2,100±270 U/L) cultures, all of them at an initial moisture level of 85%. The highest activities of Mn peroxidase were obtained in pineapple peel cultures (2,200±205 U/L) at an initial moisture level of 75%. In general, the condition of high initial moisture level (80-90%) were the best condition to laccase activity, while the best condition to Mn peroxidase activity was cultivation at low initial moisture (50-70%). Cultures containing high Mn peroxidase activities were more efficient in the decolorization of the industrial dyes congo red, methylene blue and ethyl violet than those containing high laccase activity. Also, crude enzymatic extracts with high Mn peroxidase activity were more efficient in the *in vitro* decolorization of methylene blue, ethyl violet and congo red. The dye RBBR was efficiently decolorized by both crude extracts, rich in Mn peroxidase activity or rich in laccase activity.

Key words: dye decolorization, ligninolytic enzymes, *Pleurotus pulmonarius*, white-rot fungi, synthetic dyes.

Introduction

White rot fungi are known for their ability to degrade or modify lignin by enzymatic processes. The major enzymes associated with the lignin-degrading ability of white-rot fungi are lignin peroxidase (LiP, EC 1.11.1.14), manganese dependent peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16) and laccase (EC 1.10.3.2) (Maciel et al. 2012). All these enzymes act via generation of free radicals which represent an efficient way to gain access to their substrates. White-rot fungi usually secrete one or more of the ligninolytic enzymes in different combinations. Generically, white-rot fungi can be distributed into four groups, according to their ability to produce laccases and peroxidases (LiP, MnP and VP) (Kuhar et al. 2007): 1). laccase and both peroxidases, MnP and LiP (*Trametes versicolor*, *Bjerkandera adusta*); 2). laccase and at least one of the peroxidases (*Lentinula edodes*, *Pleurotus eryngii*, *Ceriporiopsis subvermispora*); 3). only laccase (*Schizophyllum commune*); 4). only peroxidases (*Phanerochaete chrysosporium*). The most

frequently observed ligninolytic enzymes among the white-rot fungi species are laccases and MnP and the least are LiP and VP (Maciel et al. 2012).

The demand for application of ligninolytic enzymes in industry and biotechnology is ever increasing due to their use in a variety of processes. Ligninolytic enzymes have potential applications in a large number of fields, including the chemical, fuel, food, agricultural, paper, textile and cosmetic industrial sectors in addition to others (Karigar and Rao 2011). These enzymes are also directly involved in the degradation of various xenobiotic compounds. Due to the fact that ligninolytic enzymes are basically non-specific, they are able to attack a series of molecules chemically similar to lignin including pesticides, polycyclic aromatic hydrocarbons, synthetic polymers and synthetic dyes (Maciel et al. 2012).

Mushrooms of the genus *Pleurotus* are very easy to cultivate (Cohen et al. 2002). The two most important species cultivated in large scale are *Pleurotus ostreatus* and *Pleurotus pulmonarius* (formerly *P. sajor-caju*). In nature they grow on wood, usually on dead standing trees or on fallen logs. Various substrates that contain lignin and cellulose can be used for *Pleurotus* cultivation such as wood chips, corn cob, rice straw, cotton stalks, waste hulls, and other agricultural wastes, some of which can be recycled and upgraded for use as animal feed or for preparation of other products (Cohen et al. 2002). These substrates are also frequently used to study the production of MnP and laccase by *Pleurotus* spp in both submerged and solid state cultures. *P. pulmonarius,* when cultured under submerged and solid state conditions using wheat bran as a substrate, produces laccase as the main extracellular enzyme (Souza et al. 2002). The capability of *P. pulmonarius* to decolorize textile dyes in both types of cultures has already been described. This capability of the fungus was mainly due to its laccase activity if one considers that the Mn peroxidase production was very low in those cultures (Tychanowicz et al. 2004; Zilly et al. 2002). More recently however, it has been found that when *P. pulmonarius* was cultured in wheat bran solid state medium with a low initial moisture level, it produced elevated amounts of both enzymes Mn peroxidase and laccase (Souza et al. 2006). The objective of this study was to compare the production of laccase and Mn peroxidase by *P. pulmonarius* in solid state cultures using different agricultural residues at several initial moisture levels. An attempt was also done to evaluate the capability of *P. pulmonarius* and its ligninolytic enzymes to decolorize some synthetic dyes.

Material and methods

Waste material

Seven agricultural residues were used in this work and they were obtained from local producers: yellow passion fruit waste, orange bagasse, banana stalk, pineapple peel, corn cob, rice hull, sugar cane bagasse, and wheat straw. All residues were washed and dried in an air-circulating oven at 50º C until their weight became constant. The dried materials were then milled and the resulting products were used as substrates. Wheat bran was obtained from the local market and used without modification.

Microorganism

P. pulmonarius CCB-19 was obtained from the Culture Collection of the Botany Institute of São Paulo. It was cultured on potato dextrose agar (PDA) medium for 2 weeks at 28 °C. When the plates were fully covered with the mycelia, mycelial plugs measuring 10 mm in diameter were made and used as inocula.

Culture conditions

The cultures were performed in cotton-plugged Erlenmeyer flasks (250 mL) containing 5 g of one of the following nine substrates: yellow passion fruit waste, orange bagasse, banana stalk, pineapple peel, corn cob, rice hull, sugar cane bagasse, wheat bran or wheat straw. The following salts were added to give a final salt concentration of (mg/g): K_2HPO_4, 1; $MgSO_4 \cdot 7\ H_2O$, 0.2 and $CaCl_2 \cdot 2\ H_2O$, 0.1. Water was added to obtain initial moisture levels varying from 50% to 90%. A total of 9 different moistures were tested. The pH of the media was 6.0±0.1. Prior to use, the mixtures were sterilized by autoclaving at 121 °C for 15 min. Each flask received three mycelial plugs and was incubated statically under an air atmosphere at 28 °C and in complete darkness. After 10 days of cultivation, fungal growth was measured by glucosamine estimation of the fungal cell wall (Scotti et al. 2001).

Extraction of enzymes.

After 10 days of cultivation, a volume of 50 mL of cold water was added to the contents of each flask. The mixtures were stirred for 1 h at 4° C and centrifuged at 5,000 rpm for 10 min. The supernatants obtained were stored at 4 °C and used as crude enzyme extracts.

Enzyme assays

The laccase activity was determined by leasuring the oxidation of 1 mmol/L ABTS (2,2'-azino bis (3-ethylbenzthiazoline-6-sulphonic acid) in 50 mmol/L sodium acetate buffer (pH 4.5). Formation of the cation radical of ABTS was monitored at 420 nm (ε = 36 L $mmol^{-1}$ cm^{-1}) (Hou et al. 2004). The Mn peroxidase activity was assayed spectrophotometrically by following the oxidation of 1 mmol/L $MnSO_4$ in 50 mmol/L sodium malonate, pH 4.5, in the presence of 0.1 mmol/L H_2O_2. The reaction was initiated by adding H_2O_2, and the rate of Mn^{3+}-malonate complex formation was monitored by measuring the increase in absorbance at 270 nm (ε = 11.59 L $mmol^{-1}$ cm^{-1}) (Wariishi et al. 1992).

In vivo dye decolorization experiments

To test the ability of *P. pulmonarius* cultures to decolorize synthetic dyes, each dye was membrane-filtered through a 0.45 µm cellulose nitrate filter and mixed with the cultures previously autoclaved, to a final concentration of 200 ppm. After 10 days, the residual dyes in the cultures were extracted firstly with 50 mL of water followed by 50 mL of a mixture of methanol:acetone:water (1:1:1). Dye disappearance was determined spectrophotometrically by monitoring the absorbance at the wavelength of maximum absorbance for each dye: remazol brilliant blue (RBBR), 595 nm; ethyl violet, 596 nm; methylene blue, 665 nm; poly R-478, 530 nm and Congo red, 497 nm. In control cultures, either the dye or the fungus (abiotic control) was omitted. To calculate the residual dye in the cultures, the total dye extracted with water and organic mixture in the abiotic control was considered as 100%.

Dye decolorization by crude enzyme extracts

A volume of 0.5 mL of each dye to give a final concentration of 100 ppm and 0.5 mL of crude enzyme extract were added to 4.0 mL of 50 mmol/L malonate buffer, pH 4.5, containing 1 mmol/L $MnSO_4$ and 0.1 mmol/L H_2O_2. The mixtures were incubated in a rotary

shaker at 40^{o} C for 2 h. Dye disappearance was determined spectrophotometrically by monitoring the absorbance at the wavelength of maximum absorbance for each dye. Boiled crude enzyme extracts were used as negative controls. For determination of dye decolorization by crude enzyme extracts at pH 6.5, malonate buffer was replaced by phosphate buffer.

Statistical analysis

The data were analyzed by Student's t test, and one-way ANOVA with Tukey's multiple comparison test ($p<0.05$) using the statistical program pack GraphPad Prism® (Graph Pad Software, San Diego, USA). All data are presented as mean±SD of three independent experiments.

Chemicals

The enzymatic substrates were obtained from SIGMA Chemical Corp., St Louis, MO. PDA was obtained from DIFCO Laboratories, Detroit, MI. All other reagents were of analytical grade.

Results

Influence of the substrates and the initial moisture levels on the production of *P. pulmonarius* ligninolytic enzymes

The influence of the substrates on the growth of *P. pulmonarius* and on the activity of ligninolytic enzymes was evaluated using eight agricultural residues (banana stalk, corn cob, orange bagasse, rice hull, sugar cane bagasse, wheat straw, pineapple peel and yellow passion fruit waste) and wheat bran. The initial moisture level of these cultures was 75% (Table 1). After 10 days all media were completely colonized by the mycelial biomass. Fungal chitin hydrolysis into N-acetylglucosamine was used to determine the fungal biomass. This analysis showed that *P. pulmonarius* grew well in all solid systems, but that orange bagasse, yellow passion fruit waste, pineapple peel and wheat bran were the best substrates for growth. Under the conditions used in this experiment, laccase was the main ligninolytic enzyme produced by the fungus. The highest laccase activities were obtained by using the following substrates: wheat bran (830±90.0 U/L), pineapple peel (1,400±100 U/L), orange bagasse (1,800±160 U/L) and yellow passion fruit waste (1600±180 U/L). Mn peroxidase activities were low, with the exception of the pineapple peel cultures, where a high activity of Mn peroxidase (2,200±205 U/L) was present.

The effect of the initial moisture level on the production of ligninolytic enzymes was tested using four substrates: wheat bran, pineapple peel, corn cob and orange bagasse (Fig. 1). In pineapple peel cultures (Fig. 1A) both enzymes were produced at high amounts, being the initial moisture level of 80-90% the best condition for the production of laccase (around 2,400 U/L) and an initial moisture content of 70-75% the best condition for the production of Mn peroxidase (around 2,000 U/L). The substrate for which the initial moisture level had the strongest effect on the production of enzymes was corn cob (Fig 1B). The initial moisture level of 85-90% was the best condition for the production of laccase (900-1,000 U/L) whereas very low Mn peroxidase activities were detected in the filtrates. The best initial moisture level for the production of Mn peroxidase was 50-65% (500-600 U/L), a condition for which the production of laccase was very low. Laccase was the main ligninolytic enzyme

produced by the fungus in wheat bran cultures and its production was positively affected by increases in the initial moisture level. The highest laccase activities were obtained with an initial moisture level of 85% (2,860±250 U/L) (Fig. 1C). It is interesting to point out that in orange bagasse cultures, the production of laccase was enhanced (up to 2,000 U/L) by elevating the initial moisture level, and a low Mn peroxidase activity was found in all cultures, less than 100 U/L even in cultures developed with low initial moisture level (Fig. 1D).

Effect of the initial moisture level on the capability to decolorize industrial dyes by solid state cultures of *P. pulmonarius*

The synthetic dyes used in this work were selected on the basis of their stability over a wide range of pH (3-11), thermostability, stability under culture conditions in non-inoculated flasks and they were representative for each chemical category (anthracene derivative, azo, heterocyclic, polymeric and triphenylmethane dyes)

The fungal cultures developed with initial moisture levels of 60 and 85% were able to decolorize completely RBBR. The other dyes were partially decolorized in the following order: Congo red > ethyl violet > methylene blue > polyR 478 (Table 2). Alcoholic extracts from mycelia and residual substrates showed that less than 8% of the dyes were adsorbed by the mixture of fungi plus substrate.

Capability of crude extracts from *P. pulmonarius* corn cob cultures to decolorize synthetic dyes with high and low Mn peroxidase/laccase ratios

The selection of corn cob culture extracts for the *in vitro* decolorization experiments was based on two main points: first, in corn cob cultures it was possible to obtain the lowest and highest Mn peroxidase/laccase ratio, at 60 and 85% of initial moisture levels, repectively (Fig.1); second, the corn cob crude extracts were clear due its low amount of natural colored pigments, when compared to other crude extracts. Corn cob culture extracts were tested for their ability to decolorize the synthetic dyes under two different conditions: a). to obtain the best condition for Mn peroxidase activity (50 mmol/L malonate buffer pH 4.5 with 1 mmol/L $MnSO_4$ and 0.1 mmol/L H_2O_2); and 2). to obtain the best condition for laccase activity (50 mmol/L phosphate buffer, pH 6.5). The results are shown in Fig. 2. Both crude extracts (from 60% and 85% initial moisture content cultures) efficiently decolorized RBBR. The crude enzyme extracts obtained from cultures developed with an initial moisture level of 60% were more efficient to decolorize the dyes methylene blue, ethyl violet, Congo red and poly R-478.

Discussion

The potential application of ligninolytic enzymes in biotechnology has stimulated investigations for selecting promising enzyme producers and for finding convenient substrates to obtain large amounts of low-cost enzymes. Wheat bran is the most commonly used substrate for the cultivation of white-rot fungi in solid state cultures. However, the list of possibilities is very large and includes several lignocellulolytic wastes such as cane bagasse, corn cob, wheat straw, oat straw, rice straw and food processing wastes such as banana, kiwi fruit and orange wastes (Alexandrino et al. 2007; Couto 2008). Even so, it is worth to search for new substrates, especially if they are available in large amounts, allow the growth of white-rot fungi without further supplementations and facilitate the

obtainment of valuable products. Recently, yellow passion fruit waste was tested by our group as a substrate for growth and production of ligninolytic enzymes by several white rot fungi with results comparable to those obtained with wheat bran (Zilly et al. 2012). In the present work, the main oxidative enzyme produced by *P. pulmonarius* was laccase. This result is in agreement with the general observation that laccase is the main ligninolytic enzyme for the genus *Pleurotus* (Arora and Sharma 2010). However, corn cob cultures developed under low moisture levels produced elevated levels of Mn peroxidase. By varying only the initial moisture level, the use of corn cob as a substrate allowed the obtainment of crude extracts rich in either laccase or Mn peroxidase. Another point worth of being emphasized are the elevated activities of Mn peroxidase obtained in pineapple peel cultures. To our knowledge, this is the first report of the use of pineapple peel to produce ligninolytic enzymes.

Textile industries consume large volumes of water and chemicals for wet processing of textiles. The presence of very low concentrations of dyes in effluents is highly visible and undesirable (Nigam et al. 2000). Due to their chemical structure, dyes are resistant to fading on exposure to light, water and many chemicals (Poots and McKay 1976) and decolorization of textile dye effluents does not occur when they are treated aerobically by sewerage systems (Willmott et al. 1998). Many white rot fungi have been intensively studied in connection with their ligninolytic enzyme production and their decolorization ability (Boer et al. 2004; Chagas and Durrant 2001; Jarosz-Wilkolazka et al. 2002; Kasinath et al. 2003; Pointing and Vrjmoed 2000). However, most studies on dye decolorization have been carried out using liquid or solid cultures on agar plates, which do not reflect the natural living conditions (i.e. in wood and other lignocellulosic substrates) of the white-rot fungi. Our results show that solid state cultures of *P. pulmonarius* were able to decolorize, at least partially, all dyes tested. In addition to this, our results demonstrate the dye decolorization capability of corn cob culture extracts rich in laccase or Mn peroxidase activities. The employment of ligninolytic enzyme preparations can bring considerable benefits over the direct use of white rot fungi, considering that preparations can be more easily standardized, facilitating accurate dosage. Enzyme application is simpler than the use of microorganisms and it can be rapidly modified according to the characteristics of the dye or dyes to be removed. Furthermore, analysis of metabolite compounds produced by enzyme preparations is easier than analysis of metabolites produced when the whole white rot fungus is used.

An additional advantage for using the corn cob culture extracts instead of those obtained with wheat bran or pineapple peel, is the low amount of natural colored pigments in corn cob. This allows the use of corn cob crude extracts in experiments of decolorization without any additional treatments, because the color of the extracts does not interfere with the determination of residual dyes. From the results obtained in this work, it is possible to suggest that Mn peroxidase was the main enzyme responsible for the decolorization of polyR 478, Congo red, ethyl violet and methylene blue. Our data also suggest that laccase and Mn peroxidase cooperated in the RBBR decolorization process. To elucidate the mechanisms involved in the dye decolorization by the ligninolytic enzymes of *P. pulmonarius* as well as to evaluate more properly the toxicity of the decolorized products, it is necessary to conduct experiments using purified preparations.

Acknowledgements

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Figure legends

Figure 1. Effect of the initial moisture level on the laccase and Mn peroxidase activities by *P. pulmonarius* in solid state cultures. The cultures were developed for 10 days at 28^{o} C. Laccase: full columns; Mn peroxidase: dashed columns

Figure 2. *In vitro* decolorization of industrial dyes by *P. pulmonarius* corn cob crude extracts.

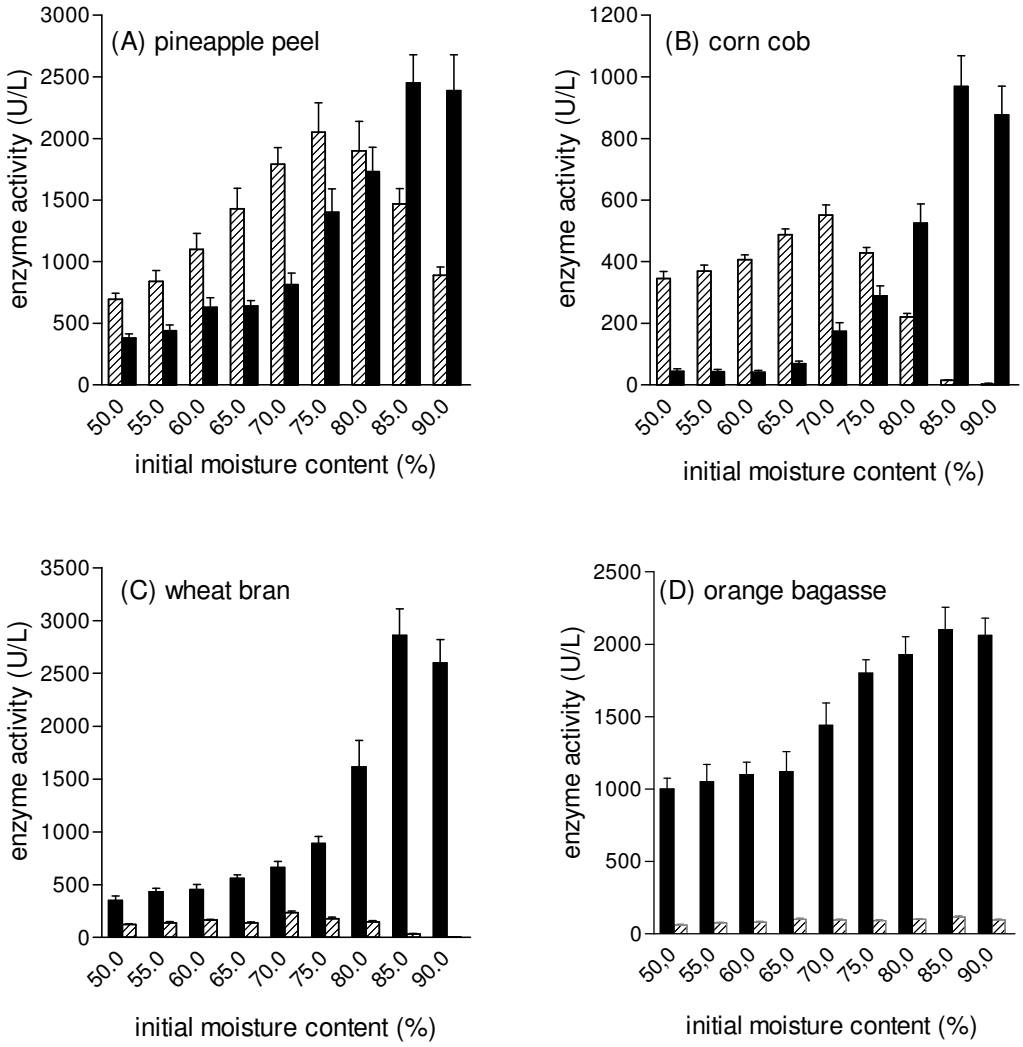
(A) pineapple peel
enzyme activity (U/L)
initial moisture content (%)
(B) corn cob
enzyme activity (U/L)
initial moisture content (%)
(C) wheat bran
enzyme activity (U/L)
initial moisture content (%)
(D) orange bagasse
enzyme activity (U/L)
initial moisture content (%)

Figure 1.

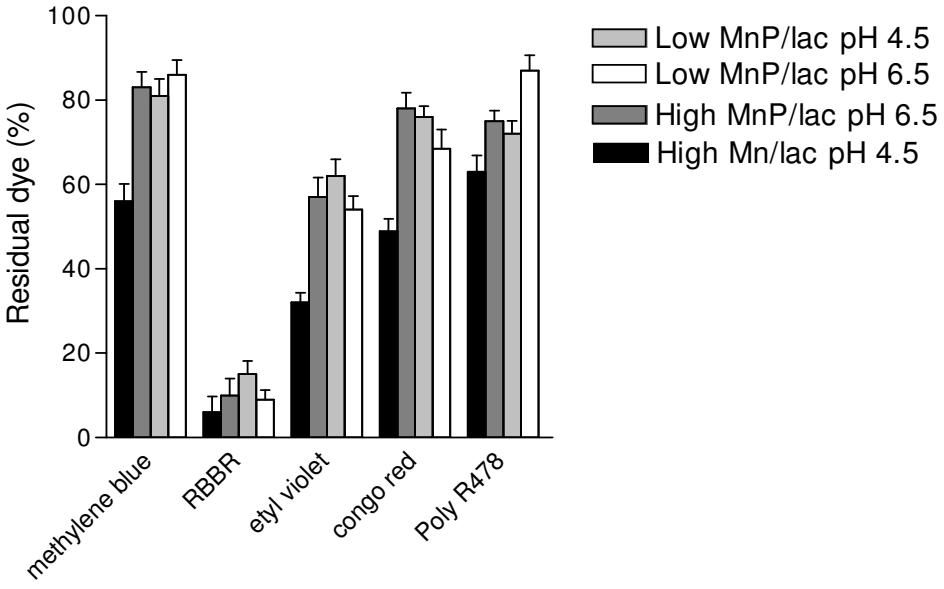
Residual dye (%)
100
80
60
40
20
0
methylene blue
RBBR
etyl violet
congo red
Poly R478
Low MnP/lac pH 4.5
Low MnP/lac pH 6.5
High MnP/lac pH 6.5
High Mn/lac pH 4.5

Figure 2

Table 1. Effect of substrate on the growth and laccase and Mn peroxidase activities by *P. pulmonarius* in solid state cultures. The cultures were developed for 10 days at 28^{o} C.

Substrate	Enzyme activity (U/L)		Fungal biomass (*)
	Laccase	Mn peroxidase	
Wheat bran	830.0± 90.0	110.0±14.0	24.0±6.0
Wheat straw	670.0± 54.0	315.0±30.0	20.0±7.0
Corn cob	450.0± 32.0	250.0±21.0	21.0±7.0
Sugar cane bagasse	640.0± 75.0	150.0±10.0	19.0±6.0
Rice hull	600.0±50.0	180.0±15.0	17.0±4.0
Pineapple peel	1400.0±100.0	2200.0±205.0	25.0±6.0
Banana stalk	510.0± 30.0	150.0±10.0	15.0±4.0
Orange bagasse	1800.0±160.0	90.0±15.0	26.0±8.0
Yellow passion fruit waste	1600.0±180.0	180.0±20.0	25.0±7.0

(*)as glucosamine content (mg/flask)

Table 2. Decolorization of synthetic dyes by solid state cultures of *P. pulmonarius* at two initial moisture levels.

Synthetic dye	Residual dye (%)	
	Initial moisture contente	
	60%	85%
Anthracene derivative dye: remazol brilliant blue R		
Corn cob cultures	3.7±2.8$^{(a)}$	4.4±2.2$^{(a)}$
Orange peel cultures	2.1±1.4$^{(a)}$	1.9±1.3$^{(a)}$
Wheat bran cultures	3.3±0.6$^{(a)}$	3.0±1.0$^{(a)}$
Pineapple peel cultures	1.4±1.0$^{(a)}$	2.0±0.7$^{(a)}$
Azo dye: Congo red		
Corn cob cultures	22.0±5.0$^{(a)}$	47.3±5.6$^{(b)}$
Orange peel cultures	50.4±7.6$^{(a)}$	45.6±5.6$^{(a)}$
Wheat bran cultures	25.4±3.7$^{(a)}$	49.1±4.8$^{(b)}$
Pineapple peel cultures	10.8±3.3$^{(a)}$	14.7±3.6$^{(a)}$
Heterocyclic dye: methylene blue		
Corn cob cultures	52.1±5.7$^{(a)}$	67.9±7.3$^{(b)}$
Orange peel cultures	60.0±4.7$^{(a)}$	63.0±4.2$^{(a)}$
Wheat bran cultures	51.0±4.9$^{(a)}$	68.0±8.1$^{(b)}$
Pineapple peel cultures	35.0±7.2$^{(a)}$	38.0±6.0$^{(a)}$
Triphenylmethane dye: ethyl violet		
Corn cob cultures	39.5±6.2$^{(a)}$	62.5±2.3$^{(b)}$
Orange peel cultures	58.4±7.1$^{(a)}$	61.3±5.8$^{(a)}$
Wheat bran cultures	43.1±4.9$^{(a)}$	56.1±5.4$^{(a)}$
Pineapple peel cultures	21.7±2.4$^{(a)}$	23.1±1.9$^{(a)}$
Polymeric dye: poly R478		
Corn cob cultures	67.8±6.8$^{(a)}$	81.8±7.2$^{(a)}$
Orange peel cultures	83.0±7.9$^{(a)}$	80.5±8.3$^{(a)}$
Wheat bran cultures	78.1±6.3$^{(a)}$	81.1±4.9$^{(a)}$
Pineapple peel cultures	66.7±6.5$^{(a)}$	63.0±9.1$^{(a)}$

Values labeled with different superscript letters in each line are significantly different ($p < 0.05$).

ARTIGO 3

Uso de *Moringa oleifera* e carvão ativado para produção de uma lacase de *Pleurotus ostreatus* hábil na descoloração do corante remazol brilliant blue R

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RESUMO

A produção de lacases por *Pleurotus ostreatus* foi avaliada em cultivos em estado sólido utilizando-se semente de *Moringa oleifera* e farelo de trigo como substrato na presença e ausência de carvão ativado como agente clarificador. Os resultados obtidos demonstram que elevadas atividades de lacase foram obtidas nas culturas com *M. oleifera* ($2338^{a} \pm 220$ U/L) quando comparada às obtidas com o tradicional substrato farelo de trigo ($1520^{c} \pm 285$ U/L). A presença do agente clarificador carvão ativado teve um efeito significativo na produção de lacase nos cultivos em farelo de trigo ($2081^{a,b} \pm 202$ U/L), mas não nos cultivos em *M. oleifera* ($2126^{a,b} \pm 48$ U/L). O uso de uma mistura de farelo de trigo-semente de Moringa na proporção de 4:1 também resultou em um pequeno incremento na produção de lacase ($2252^{a,b} \pm 475$ U/L). O carvão ativado apresentou um efeito clarificador nos extratos enzimáticos brutos obtidos dos cultivos com *M. oleifera.* A lacase obtida em cultivos com semente de *M. oleifera* mais carvão ativado a 1% foi concentrada através de filtração em membrana com cut off de 5 kDa seguido de concentração por liofilização e utilizada com sucesso na descoloração do corante antraquinoníco remazol brilhant blue R (RBBR).

Palavras-chave: lacase, *Pleurotus ostreatus*, *Moringa oleifera*, carvão ativado, descoloração.

1. Introdução

As lacases (benzenodiol: oxigênio oxidorredutases, EC 1.10.3.2) são oxido-redutases produzidas principalmente por basidiomicetos hábeis em degradar a lignina que são conhecidos como fungos da podridão branca ou fungos ligninolíticos [1-2]. São enzimas capazes de catalisar a oxidação de uma variedade de compostos fenólicos, assim como diaminas e aminas aromáticas com concomitante redução de oxigênio molecular à água. Esta oxidação resulta na geração de radicais livres de oxigênio [3]. A catálise pode ser estendida a substratos não fenólicos pela inclusão de mediadores, grupos de compostos orgânicos de baixo peso molecular que primeiro são oxidados pela lacase e depois oxidam os compostos não fenólicos que a lacase sozinha não é capaz de oxidar [1]. Na natureza, a lacase está envolvida na degradação do polímero recalcitrante lignina, de modo a permitir aos microrganismos acesso aos outros componentes da madeira, celulose e hemicelulose

[4]. Para esta tarefa, as lacases atuam em cooperação com outras enzimas ligninolíticas, como a lignina peroxidase, peroxidase dependente de manganês (Mn peroxidase) e peroxidase versátil que são capazes de oxidar seus substratos na presença e ausência do íon Mn [5].

A ampla gama de possíveis aplicações das enzimas ligninolítica sem diferentes processos na indústria de bebidas e alimentos, indústrias têxteis, papeleiras e indústrias farmacêuticas, assim como em processos de biorremediação de diferentes xenobióticos e o elevado custo de produção tem despertado o interesse de pesquisadores em alternativas convenientes para obter grandes quantidades de enzimas de baixo custo. A cultura em estado sólido, que é definida como o processo de fermentação que ocorre na ausência ou quase ausência de água livre, assemelha-se mais à condição de desenvolvimento dos fungos na natureza. O substrato sólido não só fornece os nutrientes à cultura, como também serve de ancoragem para as células microbianas [1, 3].

Tem a seu favor o fato de ser simples e proporcionar as condições necessárias de elevadas quantidades de enzima a custos reduzidos. O farelo de trigo é o substrato mais comumente utilizado para o cultivo de fungos da podridão branca em culturas de estado sólido. No entanto, a lista de possibilidades é muito grande e ao se escolher um determinado substrato, diversos fatores devem ser levados em conta, especialmente os relacionados a custo e facilidade de manuseio [4].

As sementes de *M. oleifera* tem sido objeto de diversos estudos. É uma espécie amplamente cultivada em várias partes do mundo da família *Moringaceae*, nativa da Índia, Paquistão, Bangladesh e Afeganistão rica em aminoácidos, ácidos graxos, vitaminas, glicosinolatos e fenólicos [15,16]. A semente, rica em proteínas e óleo, é utilizada para o tratamento de água e redução de turbidez, graças a uma proteína coagulante, além do tratamento de doenças tais como a artrite, reumatismo, doenças sexualmente transmissíveis, hipertensão e furúnculos [17].

A baixa especificidade da lacase ao substrato permite sua utilização para degradar compostos com uma estrutura semelhante à lignina, tais como hidrocarbonetos aromáticos policíclicos, corantes industriais e outros compostos xenobióticos [4]. Corantes industriais, a grande maioria deles sintéticos, são liberados no ambiente através de efluentes decorrentes de indústrias têxteis, impressão, farmacêuticas e de corantes [6, 7]. Persistem na natureza, causando graves problemas ambientais, pois podem obstruir a passagem de luz solar através dos recursos hídricos, levando a uma diminuição da fotossíntese e a uma diminuição da biodegradação de matérias orgânicas [8, 9]. Lacases de *P. ostreatus* têm sido objeto de pesquisas visando à aplicação de suas lacases na descoloração de diferentes corantes industriais [9-11].

Aplicações comerciais da lacase necessitam de obtenção de elevadas quantidades da enzima que possam ser facilmente concentradas e comercializadas na forma bruta, sem a necessidade de procedimentos de purificação que encarecem o produto final. Face ao exposto, o objetivo deste trabalho foi a obtenção de um preparado bruto de lacase de *P. ostreatus* capaz de ser utilizado em processos de descoloração do corante antraquinoníco RBBR.

2. Materiais e métodos

2.1. Materiais

Foram utilizados como substrato nos cultivos do *P. ostreatus* farelo de trigo, obtido em mercado local, e semente de *M. oleifera,* obtida da fazenda da Universidade Estadual de Maringá. O carvão ativado utilizado como agente clarificador foi fornecido pela Bonechar Carvão Ativado do Brasil LTDA. Este carvão é de osso bovino, sendo composto de esqueleto poroso de 80% de fosfato de cálcio, 10% de carbono e 10% de carbonato de cálcio. O corante utilizado nos experimentos de descoloração foi o RBBR (azul reativo 19, C.I. 61200) adquirido de Sigma Co (USA).

2.2. Microrganismo e condições de cultivo

P. ostreatus (CCB2) foi obtido junto a Coleção de Culturas do Instituto de Botânica de São Paulo, Brasil. Em laboratório ele foi mantido através de repiques sucessivos em ágar-batata-dextrose (BDA) a 28 ^{o}C. Para os cultivos, os inóculos foram obtidos de placas totalmente colonizadas com até 30 dias de idade e consistiram de discos miceliais com 1 cm de diâmetro. As culturas foram desenvolvidas em frascos Erlenmeyer de 125 mL contendo 5 g de substrato sólido ou combinações destes (farelo de trigo e/ou semente de *M. oleifera*). Solução mineral de Vogel foi adicionada para obtenção de umidade inicial de 75%. Quando indicado, quantidades variáveis de carvão ativado foram adicionadas com granulometrias variadas. Os meios foram esterilizadas em autoclave a 121 ^{o}C durante 15 minutos. Três discos miceliais de *P. ostreatus* foram adicionados a cada frasco. As culturas foram mantidas a 28 ^{o}C por 7, 14 e 21 dias. Um total de 24 misturas diferentes foram testadas e estão descritas na tabela 1.

2.3. Extração enzimática

Para extração das enzimas, um volume de 20 mL de água destilada foi adicionado às culturas e as misturas foram mantidas sob agitação em 120 rpm durante 10 min a 28 ^{0}C. Após este período, os materiais foram filtrados primeiramente em gaze e em seguida em papel de filtro e finalmente centrifugados a 5000 rpm durante 10 minutos. Os sobrenadantes foram considerados extratos enzimáticos brutos.

2.4. Avaliação da cor dos extratos brutos

Para avaliação da cor dos extratos enzimáticos brutos, espectros de varredura foram realizados entre 400 e 700 nm em um espectrofotômetro Shimadzu UV-1800.

2.5. Concentração dos extratos enzimáticos brutos

Os extratos enzimáticos brutos foram filtrados à vácuo em filtro zetaplus 90 s cunho e submetidos à concentração por membranas. Os ensaios de ultrafiltração (UF) por membranas foram efetuadas em uma unidade de UF, utilizando o princípio de filtração de fluxo cruzado. O módulo utilizado foi labscalemilipore TFF system do laboratório do departamento de bioquímica da Universidade Estadual de Maringá, Nepron. O sistema era equipado com manômetros na entrada e na saída para controlar a pressão transmembrana e ligado a um banho de água gelada, adaptada, para controle da temperatura do extrato enzimático contido no tanque de alimentação. Foi utilizada uma membrana de

polietersulfona de 5 KDa e 50 cm^2 de área. As condições de pressão e temperatura do processo foram de 10 psi e 14 ^oC, respectivamente. A concentração enzimática foi finalizada por liofilização após congelamento do extrato bruto resultante do processo de filtração por membranas a – 43 ^oC por 30 h. O liofilizador utilizado foi o modelo Alpha 1-4 Christ.

2.6. Determinação das atividades enzimáticas de lacase e Mn peroxidase e proteínas totais

A atividade da lacase foi realizada à 40 ^oC usando ABTS (2,2-azino-bis(3-etilbenzotiazolina-6-sulfonato) como substrato. A mistura de reação continha 200 µL de ABTS 10 mmol/L, 100 µL do extrato enzimático em 1,7 mL de tampão acetato de sódio 50 mmol/L (pH 4,0). A oxidação do substrato, após 5 minutos, foi monitorada a 420 nm [12]. Uma unidade de atividade enzimática foi definida como a quantidade de enzima capaz de oxidar 1 µmoL de ABTS por minuto.

A atividade da manganês peroxidase foi avaliada à 40 ^oC usando sulfato de manganês ($MnSO_4$) como substrato. A mistura de reação continha 1,4 mL de tampão malonato 50 mmol/L (pH 4,5); 0,2 ml de sulfato de manganês ($MnSO_4$) 10 mmol/L; 0,2 mL do extrato enzimático e 0,2 mL de água oxigenada (H_2O_2) 0,5 mmol/L. A reação foi iniciada pela adição do H_2O_2 e a oxidação do substrato foi monitorada a 270 nm durante 5 min [13].

Proteínas totais dos extratos de cultura concentrados por filtração em membranas foram quantificadas pelo método de Bradford [14] utilizando soro albumina bovina como padrão.

2.7. Ensaios de descoloração do corante RBBR

A descoloração do corante RBBR foi avaliada utilizando-se extrato bruto concentrado obtido dos cultivos em *M. oleifera*. O meio reacional foi constituído de 25 mL de tampão acetato de sódio 50 mmol/L (pH 5,0), corante na concentração final de 100 ppm e um volume de extrato bruto para obtenção de atividade final de lacase de 80 U/L. Os ensaios foram realizados em frascos Erlenmeyer de 125 ml a 28 ^oC sob agitação de 120 rpm. Alíquotas foram retiradas nos tempos zero, 5, 10, 15 e 39 h. O desaparecimento da cor foi determinado em espectrofotômetro Shimadzu UV-1800 por espectro de varredura entre 400 e 700 nm. Para avaliação do percentual de descoloração, utilizou-se a redução da cor na absorbância de 595 nm.

2.8. Análise estatística

A função ANOVA no programa R foi utilizada para comparar as diferenças entre os substratos utilizados nos cultivos com 5% de significância e comparados pelo teste de comparações múltiplas de Duncan usando o mesmo software.

3. Resultados

3.1. Produção de lacases por *P. ostreatus* em cultivos em estado sólido

A Figura 1 apresenta os melhores resultados para a produção de lacase para os cultivos em estado sólido utilizando *M. oleifera* triturada e farelo de trigo como substratos, com e sem adição de carvão ativado como agente clarificador, interrompidos em 7 dias de crescimento. Os cultivos foram mantidos por tempos maiores (até 21 dias), mas as atividades enzimáticas obtidas nestes cultivos mais longos foram semelhantes ou menores que as obtidas com os cultivos de 7 dias (dados não mostrados) Com o uso da semente de *M. oleifera* triturada como substrato a produção de lacase pelo fungo *P. ostreatus* foi superior às obtidas com o substrato farelo de trigo, (2338±220 U/L e 1520±220 U/L, respectivamente. O uso de combinações de semente de *M. oleifera* com o farelo de trigo em qualquer proporção, possibilitou a obtenção de lacase em valores maiores que os obtidos nos cultivos com farelo de trigo como único substrato. Na combinação de farelo de trigo + semente de *Moringa* na proporção de 4:1, a produção de lacase foi de 2027±147 U/L. Com relação ao agente clarificador carvão ativado, quando o substrato utilizado foi a semente de *Moringa* ou combinação de semente de *Moringa* mais farelo de trigo, nenhuma variação na produção da enzima ocorreu quando foi adicionado na concentração final de 1% (p/p) (Figura 1). Entretanto, a adição de carvão ativo aos cultivos com farelo de trigo como substrato, proporcionou um incremento significativo na produção de lacases. Os dados apresentados na Figura 1 foram obtidos com o carvão ativado com granulometria 20 x 50 mm. Outras granulometrias foram testadas (3 mm X 5 mm; 20 mm X 50 mm e 8 mm X 24 mm) e os resultados obtidos foram semelhantes aos apresentados. Atividade Mn peroxidase foi determinada e foram menores que 100 U/L em todos os extratos.

3.2. Avaliação da clarificação dos extratos enzimáticos pelo carvão ativo

A Figura 2 apresenta os espectros de varredura no visível dos extratos enzimáticos brutos de *P. ostreatus.* Observa-se que o uso de carvão ativado teve um efeito clarificador para os extratos dos cultivos desenvolvidos com a semente de Moringa, mas não para os extratos obtidos dos cultivos em farelo de trigo ou com a mistura 4:1 farelo de trigo: *Moringa*.

3.3. Concentração do extrato enzimático bruto por membranas

O extrato enzimático obtido dos cultivos com *M. oleifera* mais carvão ativado a 1% foi o que apresentou maior atividade lacase e menor interferentes coloridos. Este extrato que originalmente apresentava uma atividade específica de lacase de 3,39 U/mg de proteínas após concentração apresentou um atividade específica de 22,9 U/mg de proteínas.

3.4 Descoloração de RBBR pela lacase concentrada

A Figura 3 apresenta os espectros de absorção do corante RBBR na concentração final de 100 ppm antes e após a ação da lacase por até 39 h. Utilizando-se os valores de absorbância no comprimento de onda 595 nm, foi possível determinar a descoloração após 5, 10, 15 e 39 h e verificar que ocorreu uma descoloração de 70% no período de 39h.

4. Discussão

Neste trabalho foi avaliado pela primeira vez seu potencial como substrato para cultivo de *P. ostreatus* e produção da lacase. A capacidade de indução da lacase pela semente de Moringa deve-se, provavelmente, ao seu teor em fenólicos, considerando que a expressão de lacases é induzida por vários fenólicos tais como ácido ferúlico e vanilina [15,18].

Uma das características do uso de cultivos em estado sólido para a produção de enzimas é o fato dos extratos enzimáticos adquirirem um elevado teor de pigmentos, muitos associados fortemente às proteínas, que conferem cor aos extratos. Para várias aplicações esta cor pode atrapalhar e tornar necessária a aplicações de procedimentos de clarificação e/ou purificação que encarecem as enzimas. O carvão ativado pode ser utilizado em muitas aplicações industriais, incluindo processos que se deseja purificar, descolorir, recuperar e remover odores [19]. Os resultados mostram que o uso de carvão ativado como agente clarificador no cultivo que utilizou semente de *M. oleifera* como substrato proporcionou uma produção de extrato enzimático mais claro comparado ao sem adição de agente clarificador. A superfície de carvão ativado tem tanto cargas negativas (aniônicas) como cargas positivas (catiônicas) para atrair íons livres em solução ou suspensão. Por conta disso, o tratamento com carvão incrementaria a capacidade do carvão para a troca com ânions ou cátions de acordo com as características do meio [20].

Neste sentido, o resultado positivo pelo uso de carvão ativado como agente clarificador, apenas no cultivo que utilizou semente de *M. oleifera* como substrato, pode ser explicado pela presença de uma proteína catiônica em sua constituição. As sementes de *M. oleifera* contêm uma proteína coagulante que pode ser utilizada na clarificação da água potável ou tratamento de águas residuais. Pesquisadores identificaram o componente coagulante do extrato de semente de *M. oleifera* como uma proteína catiônica dimérica (MOCP), com um peso molecular na gama de 6,5-14 kDa [21,22]. A funcionalidade antimicrobiana da MOCP decorre da positividade, seções ricas em glutamina em torno de uma hélice-volta-hélice contendo uma prolina hidrofóbica. Esta porção da cadeia de proteína atua como uma "faca molecular", a seção de carga positiva é eletrostaticamente atraída para a parede da célula bacteriana durante o ciclo hidrofóbico penetra e perturba a parede [22].

A enzima lacase obtida dos cultivos com a semente de Moringa mostrou-se eficiente na descoloração do corante RBBR. Entretanto, um tempo longo de contato do corante com a enzima foi necessário para obter 70% de descoloração. É sabido que a adição de um mediador, tal como o ABTS, acelera o processo enzimático de descoloração de corantes pelas lacases [11] e tal procedimento pode ser tentado para otimizar o uso desta lacase na descoloração do RBBR.

5. Conclusões

Os resultados demonstram que valores significativamente maiores de atividades de lacase foram obtidos com o uso da semente de *M. oleifera* como substrato, comparados aos cultivos no tradicional farelo de trigo.

Com relação à incorporação de carvão ativado como agente clarificador, os resultados mostram que sua presença não alterou a produção de lacase. Entretanto foi obtida uma redução significativa da coloração do extrato enzimático apenas para o cultivo com a semente de Moringa. Portanto, novos estudos serão necessários para avaliar seu uso associado a diferentes substratos.

É sabido que os fungos de podridão branca são excelentes candidatos para a biodegradação de compostos recalcitrantes. Entretanto, selecionar substratos, fungos produtores e condições de operação para produção de enzimas ligninolíticas promissoras no processo de descoloração de corantes ou outros processos industriais é de grande relevância.

O emprego de preparações enzimáticas ligninolíticas no processo de descoloração de corantes é simples e pode ser rapidamente modificada de acordo com as características do corante a ser removido. Além disso, as enzimas podem ser facilmente padronizadas, facilitando a dosagem exata. Os resultados mostram que a lacase foi utilizada com sucesso na descoloração do corante RBBR. Com relação a otimização do uso da lacase na descoloração, novos experimentos associando o mediador ABTS ao processo de descoloração enzimática do RBBR estão sendo conduzidos no laboratório.

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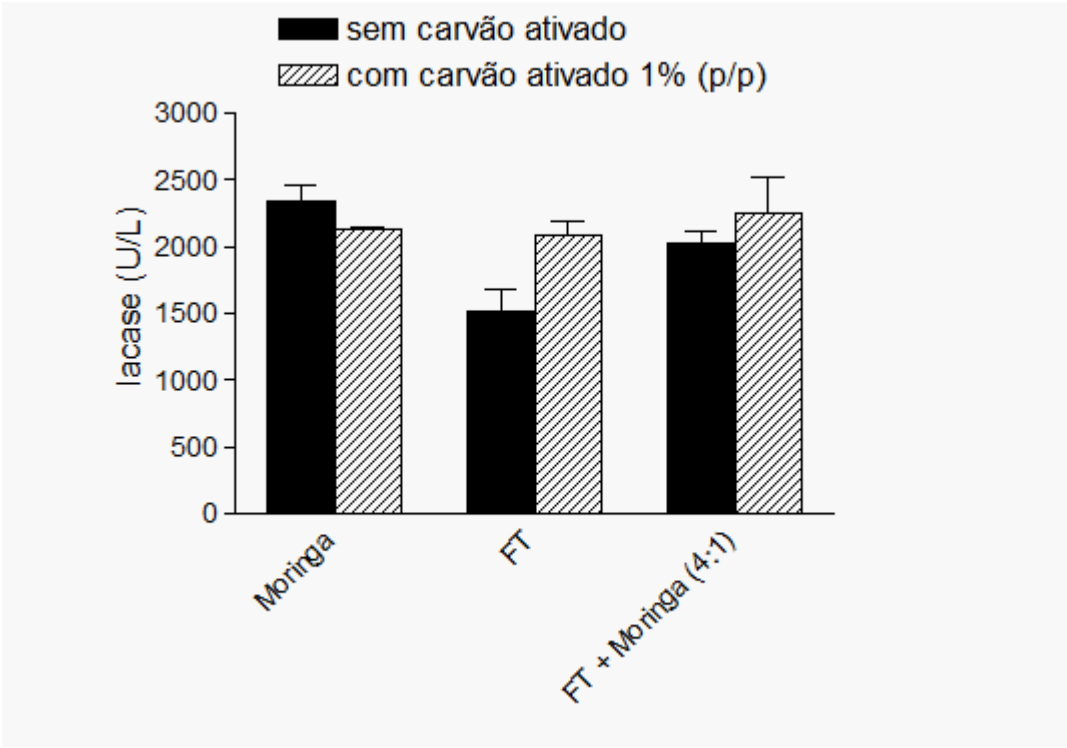

Fig. 1. Efeito do substrato e da presença do clarificador carvão ativado na produção de lacase por *P. ostreatus* em cultivos em estado sólido. FT= farelo de trigo; granulometria do carvão = 20 x 50 mm.