International Biodeterioration & Biodegradation 65 (2011) 340–344

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Influence of NaCl and Na_2SO_4 on the kinetics and dye decolorization ability of crude laccase from *Ganoderma lucidum*

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ARTICLE INFO

Article history:
Received 12 November 2010
Received in revised form
13 December 2010
Accepted 14 December 2010
Available online 7 January 2011

Keywords:
Dye decolorization
Ganoderma lucidum
Laccase
Kinetics
Salt concentration
Solid state fermentation

ABSTRACT

In a solid state medium using yellow passion fruit waste as substrate, the basidiomycete *Ganoderma lucidum* produced a laccase as the main ligninolytic enzyme. This crude enzyme presented Michaelian behavior with both substrates tested, namely 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and the anthraquinone dye remazol brilliant blue R (RBBR). The K_M's for these substrates were, respectively, 0.232×10^{-3} and 0.602×10^{-3} M. The actions of NaCl and Na_2SO_4, two important salts usually found in textile wastewaters, were investigated. 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 decolorize RBBR in the presence of 1.0 M Na_2SO_4 and 50% decolorization was found in the presence of 0.1 M NaCl. Such properties certainly make the enzyme a good agent for textile dye effluent treatment considering the fact that wastewaters of this industry usually contain high concentrations of NaCl and Na_2SO_4.

1. Introduction

Synthetic dyes are extensively used for various industrial applications including textile dyeing. It is estimated that between 10% and 15% of the total dyes used in the dyeing processes can be found in wastewaters (Arantes et al. 2006). As they are relatively recalcitrant to biodegradation, the elimination of colored effluents in wastewater treatment systems is mainly based on physical or chemical procedures such as adsorption, concentration, chemical transformation and incineration; these clean-up procedures are expensive, and sometimes produce hazardous by-products which limit their application (Moreira et al. 2000). Biological processes have received increasing interest owing to their lower cost, higher efficiency and, in particular, environmental friendliness (Li et al. 2009).

White rot fungi (WRF) have been considered as a biological alternative for decolorization of synthetic dyes due to their capability to produce non-specific oxidative enzymes involved in the degradation of lignin, mainly, lignin peroxidase, manganese peroxidase and laccase (Eichlerová et al. 2005; Chander and Arora 2007; Asgher et al. 2008). Laccases are multi-copper oxidases widely found in WRF (Arora and Sharma 2010). The enzyme can catalyze the one-electron oxidation of a wide variety of organic and inorganic substrates, including mono-, di- and polyphenols, methoxyphenols, aromatic amines, and ascorbate, with concomitant four-electron reduction of oxygen to water (Riva 2006). Since laccases have a wide substrate specificity, are secreted extracellulary and are tolerant to pollutants at high concentrations, they have attracted growing attention in many industrial and environmental fields, including textile dye decolorization (Michniewicz et al. 2008; Majeau et al. 2010).

Ganoderma lucidum is one of the most important and largely distributed WRF in the world and is associated with the degradation of a wide variety of woods (D'Souza et al. 1999). Most studies with *G. lucidum* are related to its medicinal and pharmacological properties (Boh et al. 2007). However, some studies have explored the use of *G. lucidum* and its enzymes in the degradation of xenobiotics, including dyes (Murugesan et al. 2007, 2009), and organic compounds (Jeon et al. 2008; Coelho et al. 2010a). Among the major extracellular enzymes involved in the lignin degradation, laccase appears to be the main enzyme produced by *G. lucidum* in the majority of culture conditions, although Mn peroxidase, even at low activity, has also been found (Songulashvili et al. 2007; Coelho et al. 2010a). The capability of *G. lucidum* laccase to decolorize azo and

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doi:10.1016/j.ibiod.2010.12.007

anthraquinone dyes has already been described as well as the effects of pH and temperature on the decolorization efficiency (Murugesan et al. 2007). Laccases can be sensitive to denaturing conditions typically found in dye containing effluents, such as high Na_2SO_4 or NaCl concentrations, usually found in wastewaters from the textile industry. Taking these considerations into account, the objectives of this work were to produce *G. lucidum* laccase under solid state conditions using a new and inexpensive substrate, namely yellow passion fruit waste, and to investigate the effect of ionic strength on both enzyme activity and kinetics. Furthermore, in order to evaluate the potential of laccase as a decolorizing agent its efficiency in the decolorization of one of the most important dyes in the textile industry, namely remazol brilliant blue (RBBR), was investigated at various ionic strengths.

2. Material and methods

2.1. Waste material

Yellow passion fruit (*Passiflora edulis*) wastes (YPFW) were obtained from local producers represented by the Cooperativa Agroindustrial de Corumbataí do Sul e Região (COAPROCOR, Corumbataí, Paraná, Brazil). The YPFW was washed and dried in an air-circulating oven at 50 °C until their weight became constant. The dried material was then milled and the resulting product was used as substrate. The YPFW contained (in %): moisture, 9.40; ash, 7.00; lipids, less than 0.10; proteins, 7.75; soluble and insoluble fibers, 57.10; carbohydrates, 18.65.

2.2. Microorganism and culture conditions

G. lucidum was obtained from the Culture Collection of the Botany Institute of São Paulo, Brazil. It was cultured on potato dextrose agar medium (PDA) for 2 weeks at 28 °C. When the plates were fully covered with the mycelia, mycelial plugs (diameter 10 mm) were used as inoculum. The cultures were performed in cotton-plugged Erlenmeyer flasks (0.25 L) containing 0.01 kg of YPFW and 0.07 L of Vogel salt solution (Vogel 1956) to obtain an initial moisture content of 88%. Prior to use, the mixtures were sterilized by autoclaving at 121 °C for 15 min. Inoculation was done directly in the Erlenmeyer flasks. Each flask received three mycelial plugs and was incubated statically under an air atmosphere at 28 °C and in complete darkness.

2.3. Enzyme extraction

Crude extracts were obtained by adding 0.04 L of water to the contents of each flask. This was followed by stirring for 30 min at 8 °C and afterwards by filtration and centrifugation (10 min at 3000 rpm). The residual total and reducing carbohydrates in the culture filtrates were determined by the phenol sulfuric (Dubois et al. 1956) and dinitrosalicylic acid (Miller 1959) methods, respectively, and expressed as glucose equivalents. For experiments of kinetics, dye decolorization and electrophoretic analysis, the crude extracts were exhaustively dialysed against deionized water and ultra-filtered via a Biomax (Mr 10,000 Da cut-off) ultra-filtration membrane (Millipore Corp. Bedford, Massachusetts, USA). The material was stored at −20 °C until use.

2.4. Enzyme assays

Laccase activity (EC 1.10.3.2) was measured with 2,2′-azino bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in 0.05 M sodium acetate buffer (pH 4.0) at 40 °C (Murugesan et al. 2007). Oxidation of ABTS was determined by the increase in A_{420nm} ($\varepsilon = 36{,}000\ M^{-1}cm^{-1}$). Mn peroxidase activity (MnP) (EC 1.11.1.13) was assayed spectrophotometrically by following the oxidation of 1 mM $MnSO_4$ in 0.05 M sodium malonate, pH 4.5, in the presence of 10^{-4} M H_2O_2. Manganese íons, Mn^{3+}, form a complex with malonate, which absorbs at 270 nm ($\varepsilon = 11{,}590\ M^{-1}\,cm^{-1}$) (Wariishi et al. 1992). The lignin peroxidase activity was determined by spectrophotometric measurement at 310 nm of the H_2O_2-dependent veratraldehyde formation ($\varepsilon = 9300\ M^{-1}\,cm^{-1}$) from veratryl alcohol (Tien and Kirk 1984). The enzyme activities were determined at 40 °C and expressed in (U) international enzymatic units ($mol \times 10^{-6}\,min^{-1}$).

2.5. Polyacrylamide gel electrophoresis of crude laccase

Native SDS-PAGE was carried out on 12% polyacrylamide gel (Laemmli 1970). The laccase activity was visualized in the gel with ABTS as the substrate (Souza et al. 2004). The native gel was also stained with RBBR in 0.05 M sodium acetate buffer. After 2 h incubation at 30 °C, the dye solution was discarded and the gel was visualized for RBBR decolorization (Murugesan et al. 2007). The molecular weight of laccase was evaluated by comparison of the electrophoretic mobility with standard protein markers (MW-70 kit-Sigma). The standard protein bands were visualized by silver staining.

2.6. Kinetic analyses

Initial reaction rates were measured at various substrate (ABTS or RBBR) concentrations in the presence and absence of salts (NaCl or Na_2SO_4). The saturation curves obtained in the absence or presence of salts were analyzed by means of the following equation, which describes mixed inhibition (Schulz 1994):

$$v = \frac{V_{max}[S]}{[S](1+[I]/K_{i1}) + K_M(1+[I]/K_{i2})} = \frac{\frac{V_{max}}{(1+[I]/K_{i1})}[S]}{[S] + K_M\frac{(1+[I]/K_{i2})}{(1+[I]/K_{i1})}} \quad (1)$$

In equation (1) v represents the reaction rate, [*S*] the substrate concentration, [*I*] the inhibitor (NaCl) concentration, K_M the Michaelis constant, V_{max} the maximal reaction rate and K_{i1} and K_{i2} the inhibition constants. Equation (1) predicts that an inhibitor will produce apparent changes in both K_M and the V_{max} according to the following relations:

$$V_{max,apparent} = \frac{V_{max}}{1+[I]/K_{i1}} \quad (2)$$

$$K_{M,apparent} = K_M\left(\frac{1+[I]/K_{i2}}{1+[I]/K_{i1}}\right) \quad (3)$$

Fitting of equation (1) to the experimental data was performed by means of an iterative non-linear least-squares procedure, using the Scientist® software from MicroMath Scientific Software (Salt Lake City, USA).

2.7. Enzymatic RBBR decolorization

To study the capability of the crude laccase to decolorize RBBR, a reaction mixture containing 2×10^{-7} kg RBBR, 1.0 U crude enzyme in 0.05 M sodium acetate buffer (pH 4.0) in a total volume of 0.002 L was incubated at 40 °C in the dark. Periodically, the decrease in absorbance was measured at 595 nm in a UV–VIS Shimadzu Spectrophotometer and expressed in terms of percentage. In parallel, control samples were maintained with heat inactivated enzyme. The effect of ionic strength (NaCl and Na_2SO_4)

on the capability of laccase to decolorize RBBR was investigated by adding in the same reaction mixture several amounts of NaCl (up to 1.0 M) or Na_2SO_4 (up to 1.0 M).

2.8. Statistical analysis

The data were analyzed by ANOVA and compared by the Tukey test ($p < 0,05$) using the GraphPad Prism® program (Graph Pad Software, San Diego, USA). All measurements, including the kinetic assays, were repeated at least three times.

3. Results and discussion

3.1. Production of ligninolytic enzymes by G. lucidum in YPFW medium

The solid state system consisting of YPFW plus mineral solution revealed to be an optimal medium for growing *G. lucidum*. At 28 °C, the first signs of fungal mycelial growth were seen 24 h after inoculation and the medium was completely colonized by fungal mycelia within 6 days. Among the main known ligninolytic enzymes, namely laccase, Mn peroxidase and lignin peroxidase, only the first of them was produced by *G. lucidum* in this medium (Fig. 1). Laccase production reached its maximum within 16–30 days of cultivation (9600 U/L). It is possible to note that maximal laccase production was associated with depletion of the carbohydrate content of the medium. Non-denaturing SDS-PAGE followed by incubation of the gel with the substrate ABTS and the dye RBBR revealed that apparently a single laccase, with molecular weight of 43 kDa, was produced in YPFW solid state cultures (data not shown). These results are in agreement with most studies with *G. lucidum*, suggesting that laccase is the main ligninolytic enzyme of this fungus. However, two, three and even five isoforms of laccases have been visualized in previous electrophoretic studies of *G. lucidum* culture filtrates (D'Souza et al. 1999; Ko et al. 2001; Coelho et al. 2010b), whereas only a single laccase isoform was detected by SDS-PAGE in the present study. However, this is not the first report about the production of a single laccase by *G. lucidum* in solid state conditions. A single laccase was also found in wheat bran solid state cultures (Murugesan et al. 2007). These differences in enzyme expression may be the result not only of the different culture conditions but also of the different extraction and manipulation procedures. Furthermore, it should also be considered that different studies used different *G. lucidum* isolates. It is interesting to note that an isolate identified as *G. lucidum* IBL-05 produced Mn peroxidase activity without significant laccase activity and the former enzyme was associated with the capability of submerged cultures to decolorize several dyes (Bibi et al. 2009).

Solid state cultures have been considered as an efficient method for enzyme production due its potential advantages, high productivity and low cost (Pandey et al. 1999). Inexpensive agriculture and agro-industrial residues represent one of the most energy-rich sources of the planet and can be used as substrates in this type of cultivation. Wheat bran is the most commonly substrate used for enzyme production. However, the list of possibilities is very large and includes several lignocellulolytic wastes and food processing wastes (Pandey et al. 1999; Rodriguez-Couto and Sanromán 2005; Osma et al. 2007; Arora and Sharma 2010). The choice of YPFW as substrate for cultivation of *G. lucidum* and production of laccase presents several advantages. First, YPFW is a very abundant and inexpensive residue in Brazil. In 2004, the annual production of yellow passion fruit (*Passiflora edulis*) was around 492,000 ton (Agrianual 2007) and the YPFW represents around 52% of the fruit weight. In spite of the use of YPFW as a supplement to animal rations, its general use is still at an incipient phase. Second, the YPFW has a high capability to induce laccase production, probably due to its phenolic compound content. Phenolic compounds are usually described as very efficient laccase inducers (Souza et al. 2004). Third, the YPFW contains only small amounts of pigments. The use of substrates rich in pigments, such as wheat bran and wheat straw can be disadvantageous. These substrate pigments remain associated with the crude fungal enzymes, are difficult to eliminate through simple methods of fractionation and can interfere with the determination of dye decolorization. And finally, laccase was the main protein secreted by *G. lucidum* in YPFW cultures and our results have implicated that this laccase is solely responsible for the RBBR decolorization capability of the *G. lucidum* culture filtrates. This condition allows the conduction of experiments with crude enzymatic extracts, without the necessity of expensive and time-consuming enzyme purification procedures.

3.2. Effects of NaCl and Na_2SO_4 on the laccase activity and kinetics

The effects of the salts Na_2SO_4 and NaCl on the *G. lucidum* laccase activity determined as ABTS oxidation and RBBR decolorization were studied. The enzyme used in these assays was obtained from the solid state cultures at day 24 (see Fig. 1). Fig. 2 shows the oxidation of ABTS by laccase at different ionic strengths. The influence of the ionic strength on the laccase activity is dependent on the nature of the salt (chloride or sulfate). Na_2SO_4 slightly stimulated the laccase activity, even at the high concentration of

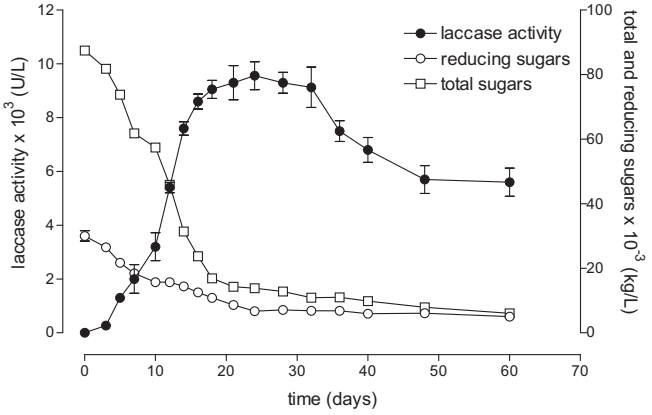

Fig. 1. Time course of laccase production by *G. lucidum* in YPFW solid state cultures.

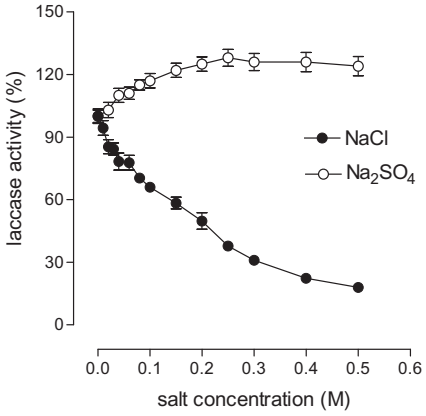

Fig. 2. Effects of the salts Na_2SO_4 and NaCl on the activity of *G. lucidum* crude laccase. The enzyme used in the assays was obtained from solid state cultures at day 24 (see Fig. 1).

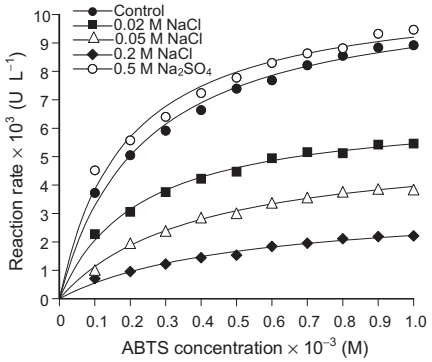

Fig. 3. Saturation curves of laccase with respect to ABTS in the absence and presence of Na_2SO_4 and NaCl. The lines joining the experimental points represent the theoretical curves calculated according to the equation (1) using the optimized kinetic constants obtained in the least-squares fitting procedure. The enzyme used in the assays was obtained from solid state cultures at day 24 (see Fig. 1).

1.0 M. Opposite to this, NaCl inhibited the enzyme even at low concentrations. In order to characterize further the effects of salts on the laccase activity, saturation curves using ABTS as substrate were measured at various NaCl concentrations (0.02, 0.05 and 0.2 M) as well as in the presence of 0.5 M Na_2SO_4. The results are shown in Fig. 3. It is apparent that NaCl inhibited the enzyme over the whole substrate concentration range. Sulfate, on the other hand, caused a slight stimulation. The continuous lines in Fig. 3 were calculated with the optimized parameters obtained by fitting equation (1) to the experimental reaction rates. It is apparent that equation (1) describes reasonably well the experimental data. The true K_M and V_{max} values (absence of salts i.e., $[I] = 0$), derived from the fitting procedures, were equal to 0.232 ± 0.015 mM and $10.897 \pm 0.224 \times 10^{-3}$ moles L^{-1} min^{-1}. As predicted by equations (2) and (3), NaCl produced apparent changes in both kinetic parameters, as revealed by Fig. 4. In this graph, $K_{M,apparent}$ and $V_{max,apparent}$ were represented as a function of the NaCl concentration. $V_{max,apparent}$ decreased and $K_{M,apparent}$ increased as the NaCl concentration was raised. These changes were not only function of the NaCl concentration, but also of K_{i1}, as can be inferred from the data in Table 1. The latter lists the K_{i1} and K_{i2} values obtained from the fitting procedures. Table 1 reveals that K_{i2} was essentially the same for all NaCl concentrations, but K_{i1} increased in a systematic way when the salt concentration was raised. Fitting of the saturation curve obtained in the presence of 0.5 M sulfate resulted in a $V_{max,apparent}$ value of $10.88 \pm 0.36 \times 10^{-3}$ moles L^{-1} min^{-1} and a $K_{M,apparent}$ of 0.182 ± 0.023 mM. The stimulatory action of sulfate

Table 1
Values of the NaCl inhibition constants K_{i1} and K_{i2}, obtained by fitting equation (1) to the experimental data.

NaCl concentration (M)	K_{i1} (M)	K_{i2} (M)
0.02	0.0315 ± 0.0033	0.0347 ± 0.0094
0.05	0.0502 ± 0.0066	0.0219 ± 0.0034
0.2	0.088 ± 0.017	0.0328 ± 0.0067

is, thus, the consequence of a small decrease in the apparent K_M value.

In kinetic terms, thus, the inhibition caused by NaCl presented the general characteristics of the mixed type in that both K_M and V_{max} were affected. In other words, NaCl changed both the affinity of the enzyme for ABTS as well as the catalytic step. The different effects of NaCl on K_{i1} and K_{i2} probably reflect two different levels of interaction of NaCl with the enzyme. That one expressed by K_{i1} became less potent as the NaCl concentration was raised. This kind of behavior is usually classified as incomplete inhibition (Plowman 1972). The second level of interaction, however, retained its potency, at least in the range between 0.02 and 0.2 M NaCl. With respect to the action of Na_2SO_4 it is worth noting that the small stimulation resulted from an increased affinity of the enzyme for the substrate rather than from an acceleration of the catalytic act. This is at least the most simple conclusion that can be drawn from the finding that Na_2SO_4 diminished K_M without affecting V_{max}.

The RBBR decolorization ability of crude laccase was also evaluated. With RBBR as substrate, the enzyme was considerably less effective when compared to ABTS: V_{max} was equal to $0.089 \pm 0.011 \times 10^{-3}$ moles L^{-1} min^{-1}, with a K_M of $0.602 \pm 0.085 \times 10^{-3}$ M. The reaction was also affected by salts (Fig. 5). Addition of Na_2SO_4 (0.5 and 1.0 M) caused a slight inhibition, but with NaCl (0.05 at 0.2 M) inhibition was significant (Fig. 6). In spite of these effects on the reaction rate, the degree of decolorization after 12 h was only barely affected by Na_2SO_4 (Fig. 6). NaCl, however, inhibited RBBR decolorization to some extent. Even so, after 12 h more than 50% of RBBR decolorization was obtained in the presence of low NaCl concentrations (up to 0.1 M). These results show clearly that NaCl diminished the capability of the enzyme to decolorize RBBR while Na_2SO_4 acted by slightly activating the enzyme. It should be noted that the addition of chloride ions usually inhibits the activity of laccases (Trovaslet et al. 2007; Li et al. 2009). Recently, however, it was described that NaCl acts as an activator of the *Bacillus halodurans* laccase (Ruijssenaars and Hartmans 2004).

One form of evaluating the catalytic efficiency of an enzyme with different substrates is to compare the corresponding V_{max}/K_M ratios. In the case of *G. lucidum* crude laccase, these ratios are 46.970 and 0.148, respectively, for ABTS and RBBR. This analysis

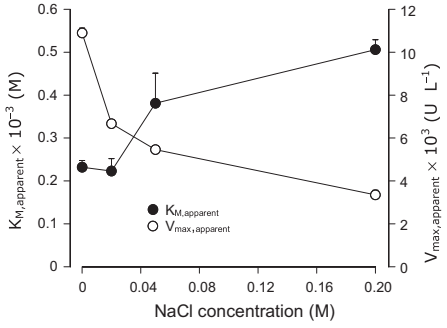

Fig. 4. The kinetic constants of laccase with respect to ABTS at various NaCl concentrations. The enzyme used in the assays was obtained from solid state cultures at day 24 (see Fig. 1).

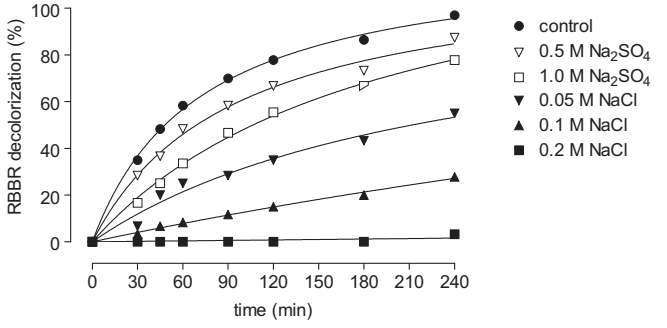

Fig. 5. Time course of RBBR decolorization by crude laccase from *G. lucidum* in the presence of different concentrations of NaCl and Na_2SO_4. The enzyme used in the assays was obtained from solid state cultures at day 24 (see Fig. 1).

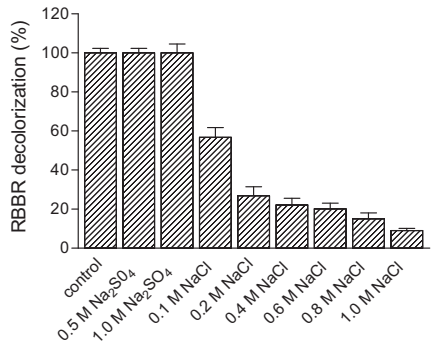

Fig. 6. Effect of various NaCl and Na_2SO_4 concentrations on RBBR decolorization by crude *G. lucidum* laccase after 12 h of treatment. The enzyme used in the assays was obtained from solid state cultures at day 24 (see Fig. 1).

reveals a much lesser catalytic efficiency with RBBR when compared to ABTS. Even so, compared with other laccases (Palmieri et al. 2005; Chander and Arora 2007; Michniewicz et al. 2008; Li et al. 2009), the crude laccase from *G. lucidum* presented an excellent capability to decolorize the anthraquinone dye RBBR (1×10^{-4} kg L^{-1} RBBR were removed in 1 h).

Acknowledgments

The authors thank the Foundation for Research Support of Pa-raná (Fundação Araucária), Fundo Paraná and CNPq (Conselho Nacional de Pesquisa e Desenvolvimento) for the financial support for this research. R.M. Peralta and A. Bracht are research fellows of CNPq.

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