

Interference of a Commercial Catalase preparation in Laccase and Peroxidase Activities

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ABSTRACT

The influence of commercial catalase preparations (fungal and bovine origin) on laccase and peroxidase activity assays was evaluated using enzymatic extracts obtained from several basidiomycetes grown under different culture conditions. No hydrogen peroxide was detected in the extracts. Inhibition of laccase activity by 40 to 80% was related to the catalase source. In addition, oxidation of the substrate (ABTS) by fungal catalase in the absence of the enzymatic extract from basidiomycetes was observed. The results demonstrated the need for the evaluation of interference of the commercial catalase preparation when its use was required in the reaction mixture.

Key words: Peroxidase, fungi, basidiomycetes, hydrogen peroxide, enzymatic inhibition

INTRODUCTION

The study of the extracellular ligninolytic activity of basidiomycetes started in the 1930s, but lignin peroxidase (E.C. 1.11.1.14), an enzyme which is able to degrade phenolic and non-phenolic compounds in the presence of hydrogen peroxide (H₂O₂), was only discovered in 1983 (Tien and Kirk, 1983; Glenn et al., 1983). A new manganese-dependent peroxidase (E.C. 1.11.1.13) was described in 1984 (Kuwarara et al., 1984). Laccase (E.C. 1.10.3.2), a copper-containing polyphenoloxidase, has been considered to be an integrating part of the ligninolytic enzymatic system of basidiomycetes (Claus 2004; Baldrian 2005). Known for its capacity to catalyze the reduction of molecular oxygen to water, with the concomitant oxidation of phenolic and polyphenolic substrates in the absence of the

formation of H₂O₂, this enzyme became more important when its ability to degrade non-phenolic substructures of lignin in the presence of mediating substances was demonstrated (Bourbonnais and Paice, 1988).

The determination of laccase activity in crude extracts from basidiomycete fungi requires a series of precautions because the capacity of H₂O₂ production is common in this group of fungi (Moreira et al. 1998; Böckle et al., 1999; Guillén et al. 2002; Srebotnik and Boisson 2005). Thus, catalase is usually added to guarantee the complete absence of H₂O₂ in the reaction medium (Srinivasan et al., 1995; Lonergan and Baker 1995; Mayer and Staples, 2002; Gonzales et al. 2002).

Previous studies conducted have indicated the influence of fungal catalase on the laccase assay. In an attempt to standardize the method for the determination of laccase activity, the present study evaluated i) the influence of commercial catalase

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preparations of fungal and bovine origins on laccase and peroxidase activities assays, and ii) the influence of pH on the oxidation of ABTS by the fungal catalase preparation. For this, enzymatic extracts obtained from several basidiomycetes grown under different culture conditions (basal liquid medium, solid substrate consisting of sawdust or sugarcane bagasse) were used.

MATERIAL AND METHODS

Fungi/enzymes

Psilocybe castanella CCB444, *Lentinus crinitus* CCB274 and *Trametes villosa* CCB176 obtained from the Basidiomycete Culture Collection (CCB), Instituto de Botânica, São Paulo, were maintained on 2% malt extract agar (MEA) at 4°C. Horseradish peroxidase and sawdust colonized with *Pleurotus ostreatus* were provided by Toyobo do Brasil S.A. Commercial catalase preparations of bovine (Sigma, C-1345) and *Aspergillus niger* origins (ICN Biomedicals Inc, 9001-05-2) were used. Laccase from *Rhus vernicifera* (Sigma) was used as reference.

Growth on solid substrate

Five MEA discs of *P. castanella* and *L. crinitus* measuring 5 mm in diameter were used to inoculate 100 g of solid sterilized substrate (121°C). The substrate was sugarcane bagasse and soy flour (C:N ratio = 90). The substrate humidity was kept close to 70%. The fermentation was carried out in triplicate and the incubation was maintained at 28°C.

Growth in liquid medium

T. villosa was previously grown on 2% MEA at 28°C until the mycelium occupied 3/4 of the medium surface. Two fungal discs measuring 5 mm in diameter were removed to inoculate 250 mL flasks containing 50 mL basal medium (Kirk et al., 1976, modified). The final composition of the medium (in 1 L) was as follows: 5 g glucose, 1 mL thiamine HCl, 0.2298 g ammonium tartrate, 0.2 g KH_2PO_4 , 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.013 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.016 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1 mL mineral solution (3 g MgSO_4 , 1 g NaCl, 100 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg $\text{CoSO}_4 \cdot \text{H}_2\text{O}$, 82 mg CaC_2 , 100 mg ZnSO_4 , 10 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 mg $\text{AlK}(\text{SO}_4)_2$, 10 mg H_3BO_3 , 10 mg NaMoO_4), 0.5 mL vitamin supplement (2 mg biotin, 2 mg folic

acid, 5 mg thiamine HCl, 10 mg pyridoxine, 5 mg nicotinic acid), and 10 mL 50 mM sodium acetate buffer, pH 4.7. The content of the flasks was filtered at different time intervals and the extract was used for the determination of enzymatic activity. The tests were carried out in triplicate.

Enzyme extraction

Solid cultures

At given time intervals, 100 g of the flask content was extracted with 50 mM sodium acetate buffer, pH 4.5, at a proportion of 1:3 (w/v). The mixture was homogenized manually (3 min), followed by shaking at 120 rpm for one hour and manual stirring for 3 min. The filtrate was centrifuged at 10,000 rpm for 10 min and supernatant was stored to more enzymatic assays.

Liquid cultures

At given time intervals, the content of the flasks was filtered through filter paper and the biomass was determined as dry weight (60°C for 24h). The filtrate was used for the determination of enzymatic activities.

Determination of H_2O_2

The presence of H_2O_2 in the enzymatic extract was determined using horseradish peroxidase (Moreira et al., 1998).

Enzymatic activities

Total oxidation of ABTS

The reaction mixture contained (in 1 mL) 0.25 mL 50 mM citrate-phosphate buffer, pH 4.0, 0.1 mL 5 mM ABTS, 0.6 mL of the enzymatic extract, and 0.05 mL 2 mM H_2O_2 . Enzymatic kinetics was assayed by absorbance at 420 nm for 10 min. One unit of enzymatic activity was defined as the amount of enzyme necessary to oxidize 1 μmol of substrate per liter per minute (Machado and Matheus 2006).

Laccase activity

Laccase activity was determined as described for total oxidation of ABTS in the absence of H_2O_2 (Machado and Matheus 2006) or using the enzymatic extract preincubated for 10 min with 0.05 mL of fungal or bovine commercial catalase.

Peroxidase activity

Peroxidase activity was calculated as the difference between the values obtained for total ABTS oxidation and laccase activity (Eggert et al., 1996).

Effect of reaction pH on ligninolytic activities

The effect of pH on ABTS oxidation by the commercial catalase preparation was therefore evaluated using 50 mM citrate-phosphate buffers (pH 2.78, 3.80, 4.78 and 6.90) and 50 mM sodium acetate buffer (pH 4.50).

RESULTS AND DISCUSSION

Laccase activity of the enzymatic extracts obtained from *P. castanella* and *P. ostreatus* grown on solid substrate was inhibited by 80% in the presence of the fungal catalase preparation (Fig. 1). The laccase activity of *Rhus vernicifera*, used as a reference, was also inhibited (37%). In addition, the evaluation of *P. castanella*'s peroxidase activity yielded overestimated results in the presence of fungal catalase (113 U/L). No H₂O₂ was detected in the enzymatic extracts from *P. castanella* and *P. ostreatus*, confirming that the oxidation of ABTS, in the absence of catalase, was only due to the action of laccase. Also, no H₂O₂ was detected in the enzymatic extracts of *T. villosa* grown in liquid synthetic medium; however, the presence of fungal catalase inhibited laccase activity (Fig. 2).

Recently, Rescigno et al. (2007) evaluating several commercial preparations of tyrosinase as regards to their composition and purity, detected enzyme activities different from tyrosinase. The authors showed that the presence of enzymes other than tyrosinase could lead to misinterpretation of results ascribed to tyrosinase activity under certain experimental conditions. Probably, the commercial catalase employed in the present study contained other enzymes, *Aspergillus* produced oxiredutases (laccase alike) and many other forms of catalase, including catalase with similar behavior to laccase (Navarro and Aguirre 1998).

The inhibition caused by the catalase preparation varied according to the time of the fungus cultures (Fig. 2) and was more pronounced at 17, 21 and 26 days (inhibition of laccase activity of 43, 75 and 84%, respectively). No inhibition was observed at 31 days. This could indicate the production of laccase isoenzymes by *T. villosa* as reported about other basidiomycetes (Bonnarmet and Jeffries, 1990; Hakala et al., 2005). Probably, at 31 days the produced isoenzymes pattern suffered fewer influence by contaminants of fungal catalase preparation.

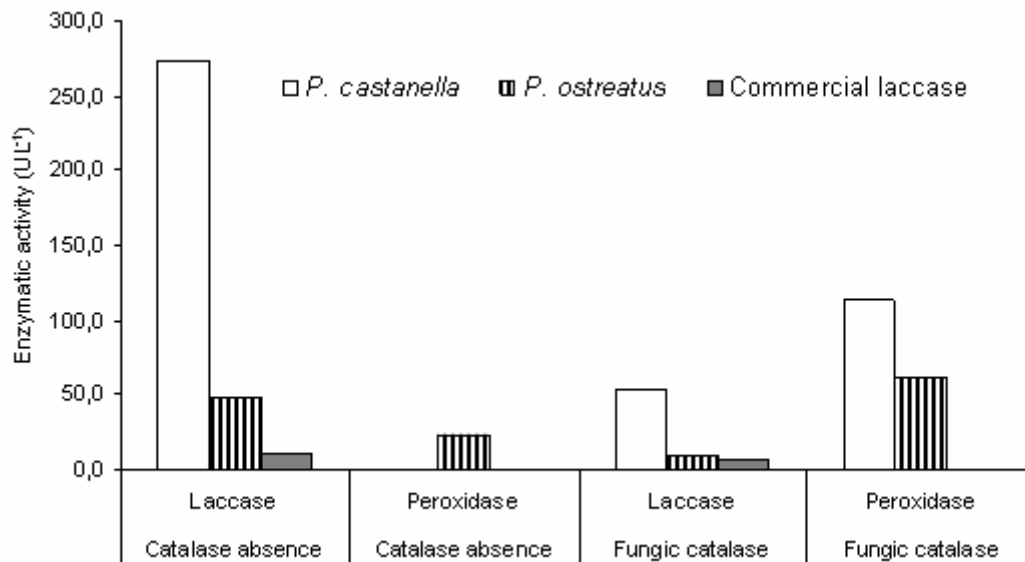


Figure 1 - Activities of laccase and peroxidases produced by *Psilocybe castanella* and *Pleurotus ostreatus* during growth on solid substrate determined in the absence and presence of a fungal commercial catalase preparation. Commercial laccase from *Rhus vernicifera* was used as reference.

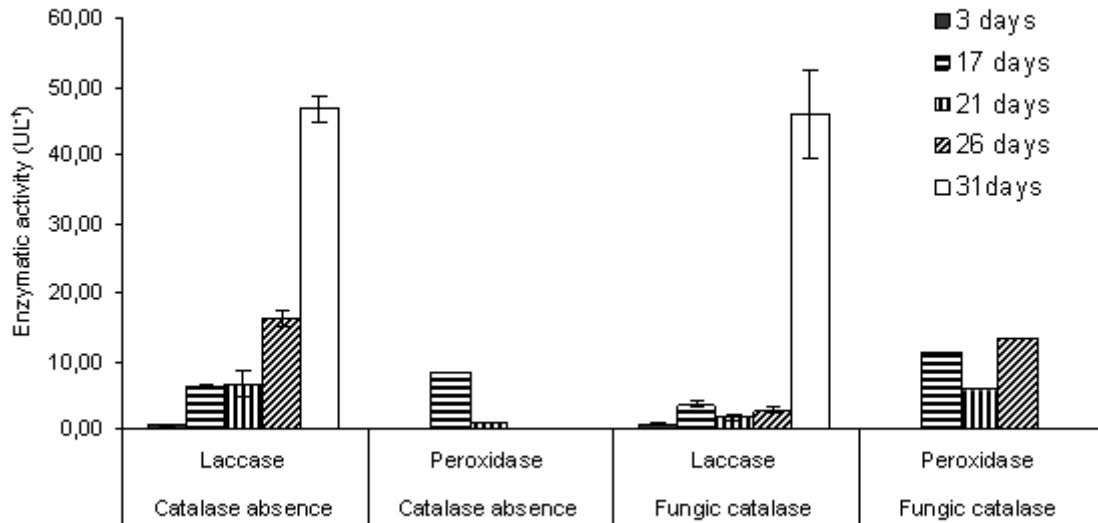


Figure 2 - Activities of laccase and peroxidases produced by *Trametes villosa* during growth in liquid synthetic medium determined in the absence and presence of a fungal commercial catalase preparation.

The effect of the origin of catalase preparation (fungal and bovine) was evaluated applying the enzyme on the extracts obtained from *L. crinitus* grown on solid substrate. The fungal catalase inhibited 76 and 47% of laccase activity in extracts obtained after 15 and 20 days of incubation, respectively (Fig. 3). No inhibition of laccase activity was observed in the presence of bovine catalase, making clear that laccase activity produced by basidiomycetes fungi suffered inhibition when *A. niger* catalase was added to the reaction. No similar studies were found in the literature about catalase preparation influence on activities assays of lignolytic enzymes produced by basidiomycetes.

Another problem observed previously with the fungal commercial catalase preparation was the oxidation of ABTS in the absence of enzymatic basidiomycete extract at acid pH. Oxidation of ABTS (activity of 879.7 U/L) in the absence of basidiomycete extract was observed only when the commercial catalase preparation was incubated in citrate-phosphate buffer at pH 2.78. When this enzymatic assay was performed with catalase preincubated with 50 μ L 1 mM sodium azide, 98% inhibition of activity was observed. These results suggested the presence of others enzymes as contaminants in the fungal commercial catalase preparation as described by Rescigno et al. (2007) for commercial sources of mushroom tyrosinase.

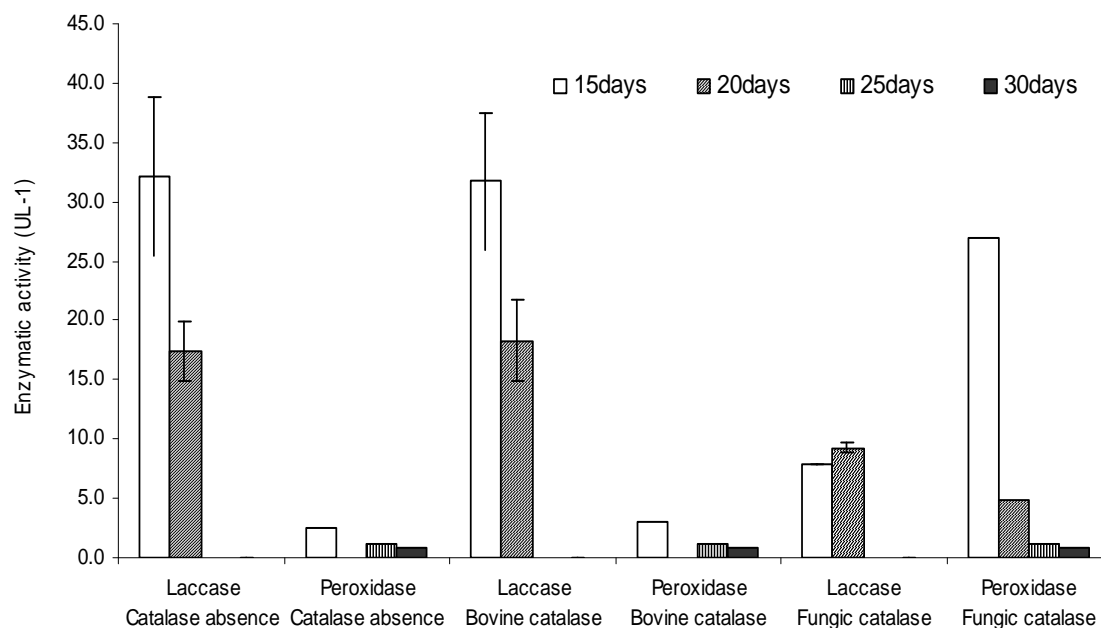


Figure 3 - Activities of laccase and peroxidases produced by *Lentinus crinitus* during growth on solid substrate determined in the absence and presence of a fungal or bovine commercial catalase preparation.

CONCLUSIONS

The enzymatic activity assay of the ligninolytic system of basidiomycetes was interfered by the commercial catalase preparation, underestimating laccase activity and overestimating peroxidase activity. The determination of H_2O_2 in enzymatic extracts obtained from basidiomycetes grown in liquid or solid media is necessary to indicate the need for the use of catalase in the reaction mixture.

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RESUMO

A influência da preparação comercial de catalase (origem fúngica e bovina) nos ensaios de atividade

de lacase e de peroxidases foi avaliada empregando-se extratos enzimáticos obtidos do crescimento de diversos basidiomicetos em diferentes condições de cultivo. Não foi detectado H_2O_2 nos extratos. Inibição de 40 a 80% da atividade de lacase foi relacionada à fonte de catalase. Além disso, foi observada oxidação do substrato (ABTS) pela catalase fúngica, na ausência de extrato enzimático do basidiomiceto. Os resultados evidenciaram a necessidade de se proceder a uma avaliação da interferência da preparação comercial de catalase, quando o seu uso se fizer necessário na mistura reacional.

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