



EXTRACTION OF MANGANESE PEROXIDASE PRODUCED BY *LENTINUS TUBERREGIUM*

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ABSTRACT

Lentinus tuberregium, is considered a choice edible mushroom with exotic taste and medicinal quality. *L. tuberregium* grows very well and produces a range of enzymes when cultivated on eucalyptus residues. Development of appropriate experimental procedures for recovery and determination of enzymes became a widely important cash crop. In this work, enzymes produced by *L. tuberregium* were extracted using different pH buffer and determined regarding peroxidases and proteases. Lignin peroxidase (LiP) was not detected in the extracts based on veratryl alcohol or azure B oxidation. Proteases were very low while Mn-peroxidases (MnP) predominated. The optimal pH for MnP recovery was 5., under agitation at 25 °C. The oxidation of phenol red decreased after dark-colored small compounds or ions were eliminated by dialysis. The extract of *L. edodes* contained components of high molecular weight, such as proteases or high polyphenol, that could be involved in the LiP inactivation.

INTRODUCTION

The white rot basidiomycete *L. tuberregium* Pegler, also known as the edible mushroom, is the most widely cultivated mushroom because of the exotic taste and medicinal quality. *L. edodes* and *L. tuberregium* degrade individual components of lignocellulose, i.e. lignin, cellulose and hemicellulose, by secreting an array of oxidative and hydrolytic enzymes (Leatham, 1986). The major enzymes associated with the lignin-degrading ability of white-rot fungi are lignin peroxidase (LiP) (EC 1.11.1.14), manganese peroxidase (MnP) (EC 1.11.1.13) and laccase (EC 1.10.3.2) (Hatakka, 1994), which have been attracting considerable attention because their ability to degrade lignin, environmentally persistent xenobiotics and dyes (Boer et al., 24;

Wesenberg et al., 23; Baldrian et al., 2). Some white-rot fungi produce all these enzymes while others produce only one or two of them. The ligninolytic enzymes have been well studied for *L. edodes* which has been described as a good producer of laccase and MnP (Boer et al., Hatvani and Mecs, 22). *L. tuberregium* apparently lacks LiP capable of oxidizing veratryl alcohol (D'Annibale et al., 1996), however, it has been reported that incubations of veratryl alcohol with crude extracellular preparations from *L. tuberregium* yielded several products, quite different from those identified from other white-rot fungi (Crestini and Sermanni, 1995). Thus, from the result of absence of LiP in the extracts of *L. tuberregium* arise the question if this fungus

really produces LiP, or if it cannot be detected by conventional analytical methods. The cultivation of *L. tuberregium* in solid medium based on lignocellulose is an attractive means of enzyme production, however it has to be considered that LiP and MnP production are regulated by carbon and nitrogen (Nakamura et al., 1999). Moreover, Mn^{2+} is a specific effector that induces MnP and represses LiP (Zhao et al., 1996). Nevertheless, the usefulness of lignocellulose materials for enzyme production is limited by the lack of a simple and reproducible technique for preparing active extracts, since extraction conditions influence greatly the values obtained. Temperature and type of solvent are known as important parameters in the extraction of solutes from solids. Additionally, when dealing with enzymes, it is necessary to take into account the enzyme thermal stability, which in turn is a function of the exposure time. The activity and stability of MnP is strongly influenced by the pH, as well as temperature and time of incubation (Sutherland and Aust, 1996). However, the optimum pH for recovering enzymes should take into consideration the condition, which reduces at maximum the protease activity, normally present in the extracts. In this study, we report the investigation of a pH buffer used for maximizing the extraction of MnP from *L. tuberregium* grown on eucalyptus residues. Due to the importance of this enzyme in industrial applications, an attempt has been made to improve the amount of enzymes extracted from biodegraded eucalyptus residues, with low protease activity. In addition, several conditions and substrates were tested to identify LiP activity in extracts.

2. METHODS

2.1. Fungal strain and culture conditions:

L. tuberregium was maintained on potato-dextrose-agar (PDA 2%) medium at 4 °C. Inoculum of *L. tuberregium* was prepared from mycelia grown on the same media incubated at 25 °C during 21 days and for *L. tuberregium* the incubation conditions were 37 °C for 7 days. *L. tuberregium* was

cultivated on eucalyptus waste (leaves, branches and bark) milled using a hammer-type mill (0.5 mm) and immersed in tap water for 24 h. The excess of water was drained and the moisture adjusted (65–70%). Polypropylene bags (25 × 3 cm) were packed with 75 g of substrate. The bags were sealed with water proof paper and autoclaved at 115 °C for 1 h. This procedure was repeated twice obeying interval of 24 h. After cooled, the bags were inoculated in sterile conditions by introducing seven discs cut from the plate (B = 8 mm), sealed again and incubated at 27 ± 2 °C for 3 days in the dark until the substrate was fully colonized with mycelium. *P. chrysosporium* was cultivated in liquid media, low nitrogen conditions (C/N = 9.3) containing the following (per liter): 2.0 g of glucose, 0.66 g di-ammonium tartrate, 2.5 mM veratryl alcohol, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.184 g $FeSO_4 \cdot 7H_2O$, 0.7 g H_3PO_4 , 1 mg of thiamine, 4 mg of biotin, 4 mg of folic acid, 1 mg of riboflavin, 1 mg of pantoic acid, 1 mg of p-aminobenzoic acid, 0.2 mg of cyanocobalamin, 2 mg of pyridoxine hydrochloride, 1 mg of nicotinic acid, 1 mg calcium pantothenate. The following trace elements were also added (per liter): 1.5 mg sodium nitrolotriacetate, 1 mg $MgSO_4 \cdot H_2O$, 1 mg $CoCl_2 \cdot 6H_2O$, 3 mg $ZnSO_4 \cdot 7H_2O$, 1 mg H_3BO_3 , 0.1 mg Na_2MoO_4 . The pH was adjusted to 4.5 with tartaric acid buffer (3 g/L). Media (5 ml) were dispensed in 25 ml Erlenmeyer flasks. After sterilization for 2 min at 115 °C, five discs (B = 8 mm) of agar carrying mycelium of *P. chrysosporium* were introduced into media in sterile conditions. The Erlenmeyer flasks were incubated at 37 ± 2 °C in stationary condition for 7, 14 and 21 days.

2.2. Enzyme extraction: The enzymes were extracted with 5 mM citrate-phosphate buffer at pH 4., 5. and 6.. Twenty grams of fermented substrate was extracted with 1 ml of buffer, at 1 and 25 °C. Extractions were performed at 1 rpm for 3 h, and in stationary condition for 1 h. The enzymatic extracts were centrifuged at 7 rpm for 2 min and the supernatants were recovered by filtration through

porous ceramic filter number 4 under vacuum.

2.3. Enzymatic activities assays: MnP was determined using phenol red .1% ($\epsilon_{61} = 22, M_{-1} \text{ cm}_{-1}$) (Kuwahara et al., 1984) and LiP activity was determined using azure B ($\epsilon_{651} = 48,8 M_{-1} \text{ cm}_{-1}$) (Archibald, 1992) and veratryl alcohol ($\epsilon_{31} = 39 M_{-1} \text{ cm}_{-1}$) (Tien and Kirk, 1988). Protease activity was determined according to the method of Anson (1938) using tyrosine as standard. All enzymatic activities were expressed as IU g_{-1} defined as the amount of enzyme producing 1 μmol of product per min per g of fungal biomass.

2.4. Determination of fungal biomass (FB): The FB was determined by extraction and quantification of ergosterol contained in the phospholipid bilayer of cell membranes. The extraction of ergosterol was carried out by saponification of the phospholipid bilayer with NaOH micro-wave-assisted and subsequent extraction with pentane (Montgomery et al., 2). Ergosterol was detected at 282 nm. FB was determined using an ergosterol-to-fungal biomass conversion factor from cells growing in liquid culture ($y = 4.96x - 3.349/r^2 = .87$) determined in previous work (Silva et al.,).

2.5. Determination of metal contents in wood chips: Eucalyptus residue was milled to pass through a .5 mm screen. Milled samples (5 mg) were hydrolyzed with 6 μmol sulfuric acid (72% w/w) in a Digesdahl Hach digester heated for 5 min at 44 $^{\circ}\text{C}$. Afterward, 3 ml of 3% (w/w) hydrogen peroxide was added and the mixture heated at 44 $^{\circ}\text{C}$ for additional 3 min. After cooling, the solution was raised to 5 ml with deionized water. This solution was analyzed in ICP-GBC Integra XM equipment to determine the manganese, iron, calcium, potassium and copper contents.

3. RESULTS AND DISCUSSION: Biodegraded eucalyptus residue was extracted with buffer solutions to recover the extracellular enzymes produced during fungal growth. Considering that the stability of enzymes produced by *L. tuberregium*

during lignocellulose degradation are pH and temperature dependent, different extraction conditions were evaluated.

3.1. Effect of extraction parameters on MnP and LiP activity: MnP was detected in the range of 38–7 UI/g. The lowest activities were recovered when the extraction was performed at pH 6. In addition, MnP recovered at pH 4. or 5. at 1 $^{\circ}\text{C}$ were not statistically different, however higher activity was observed at pH 5. when the enzyme was recovered at 25 $^{\circ}\text{C}$ for 3 h ($P < .1$). LiP was not detected in *L. tuberregium* extracts using veratryl alcohol assay, however it cannot be assumed that the enzyme is absent in the extracts, since the presence of compounds such as phenolics and other aromatics typical from lignocellulose compounds interferes with the assay employed. The interference due to aromatic compounds was completely eliminated by using azure B as substrate to evaluate LiP, which was monitored in visible region (651 nm) (Archibald, 1992). Therefore, the non-oxidation of azure B by the extracts of *L. tuberregium* was an evidence that LiP was not produced. This result is consistent with several reports in oxidative systems of *L. tuberregium*, which indicate that this fungus lacks LiP activity (Leatham, 1986; Crestini and Sermanni, 1995). However, the high content of manganese (183 ppm) in the eucalyptus residue could be regulating the production of LiP. This enzyme is formed almost exclusively when Mn^{2+} is low (1.6–.3 ppm) (Bonnarme and Jeffries, 199). Otherwise, it was reported that veratryl alcohol could be oxidized by incubation with purified MnP of *L. tuberregium* yielding different metabolic products in the absence of veratryl alcohol oxidase activity (D'Annibale et al., 1996). This result indicates that there are non-enzymatic mechanisms displayed by *L. tuberregium* to oxidize nonphenolic structures, such as peroxyl or thiol radicals formed through lipid peroxidation (Kapich et al., 1999) and amino acid oxidation (D'Annibale et al., 1996). Enzymatic extracts with high MnP activity were submitted to dialysis to remove small molecular weight compounds.

After this step, the recovery of MnP activity, for 1 or 3 h at pH 4, strongly decreased. The lowering of MnP should be connected with the action of proteases normally present in the extracts submitted to dialysis for a long time. The absorbance at 28 nm was completely reduced in these extracts due to elimination of low molecular weight compounds that could be causing interference in the LiP activity (Harvey and Palmer, 199). However, LiP activity was not detected in the extracts after dialysis, assayed with azure B or veratryl alcohol oxidation.

3.2. Inhibition of LiP by *Lentinus tuberregium* extracts: *L. tuberregium* is probably the best studied microorganism with LiP activity and it is often used as a reference. In order to better clarify a possible LiP inhibition, *L. tuberregium* extract was added to the supernatant of *P. chrysosporium* in the proportions of 1:1, 1:2, 1:5 and 1:1 and the LiP activity was newly determined. *P. chrysosporium* cultivated in liquid media presented LiP activity during azure B and veratryl alcohol oxidation at pH 3.5 (13.8 and 4.6 IU/L, respectively) and 4.5 (9.1 and 22.4 IU/L, respectively), after 14 days of cultivation. The high LiP activity found by veratryl alcohol oxidation could be connected with low molar extinction coefficient of veratryl alcohol or often by probable presence of veratryl alcohol oxidase, since aryl alcohol oxidase eventually present in the extracts does not oxidize azure B, but oxidize veratryl alcohol by a H₂O₂-independent reaction (Archibald, 1992; Arora and Gill, 21). The sample previously submitted to dialysis was also joined to LiP of *L. tuberregium* at the same conditions described above, however total inhibition of LiP was observed which was probably caused by compounds with high molecular weight released from eucalyptus residue.

3.3. Effect of extraction parameters on protease activity: Protease was analyzed in the extracts and high activities were detected at pH 4. and 6., while at pH 5. low protease activity was determined. These

results indicate that proteases secreted by *L. edodes* are less active at pH 5. and provided greater recovery of MnP. Several hypotheses describe the function of proteases in wood degradation by white-rot fungi. Eriksson and Pettersson (1988) suggested a possible action of proteases in the releasing of ligninolytic enzymes contained in the cellular wall of fungi. Otherwise, the function of proteases produced by white-rot fungi is to release nitrogen from the substrates or to recycle extracellular proteins by autolysis mechanism (Dosoretz et al., 199a,b; Cabaleiro et al., 22). The results obtained by us seem to sustain the second hypothesis since high levels of proteases coincide with low levels of ligninolytic enzymes. In fact, the action of proteases for nitrogen disposal is more important in solid-state culture than in submerged, because of the low nitrogen content in wood materials. In fact, nitrogen present in culture extracts of *L. tuberregium* is provided mainly by the hydrolysis of mycelia and extracellular proteins (Matsumoto, 1988). In conclusion, the pH is an important factor to control the activity of MnP of *L. tuberregium*. MnP functions over a range of pH (4.-6.), while the maximum MnP was recovered at pH 5., the same pH at which was recovered the lowest levels of protease. Furthermore, other factors are important to obtain higher MnP recovery, thus the optimization of fermentation conditions, such as the fungal strain, growth medium, moisture content, aeration level and culture age are crucial to improve the MnP titers (Leatham et al., 1991). In fact, in previous work we observed a markedly effect on MnP activity when nine strains of *L. tuberregium* were grown on eucalyptus waste supplemented with 2% of rice bran (Silva et al., 25). The lack of LiP activity in the extracts of *L. tuberregium* is a common characteristic for this species, but it could also be related with the concomitant presence of protease activity.

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