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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 inthe extracts based on veratryl alcohol or azure B oxidation. Proteases were very low while Mn-peroxidases (MnP) predominated. Theoptimal pH for MnP recovery was 5., under agitation at 25 _C. The oxidation of phenol red decreased after dark-colored small compoundsor ions were eliminated by dialysis. The extract of L. edodes contained components of high molecular weight, such as proteases orhigh polyphenol, that could be involved in the LiP inactivation.

INTRODUCTION

The white rot basidiomycete*L. tuberregium*Pegler, also known as the edible mushroom, isthe most widely cultivated mushroom because of the exotictaste and medicinal quality. L. edodesand *L. tuberregium*degrades individual components of lignocellulose, i.e. lignin, cellulose andhemicellulose, by secreting an array of oxidative andhydrolytic enzymes (Leatham, 1986). The major enzymesassociated with the lignin-degrading ability of white-rotfungi are lignin peroxidase (LiP) (EC 1.11.1.14), manganeseperoxidase (MnP) (EC 1.11.1.13) and laccase (EC1.1.3.2) (Hatakka, 1994), which have been attracting considerableattention because their ability to degrade lignin,environmentally persistent xenobiotics and dyes (Boeret al., 24; Wesemberg et al., 23; Baldrian et al., 2).Some white-rot fungi produce all these enzymes whileothers produce only one or two of them. The ligninolyticenzymes have been well studied for *L. edodes* which hasbeen 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 reportedthat incubations of veratryl alcohol with crude extracellularpreparations from *L. tuberregium*yielded several products,quite different from those identified from other white-rotfungi (Crestini and Sermanni, 1995). Thus, from the resultsof

absence of LiP in the extracts of *L. tuberregium*arise the questionif this fungus really produces LiP, or if it cannot bedetected by conventional analytical methods.The cultivation of *L. tuberregium*in solid medium based onlignocellulose is an attractive mean of enzymes production,however it has to be considered that LiP and MnP productionare regulated by carbon and nitrogen (Nakamuraet al., 1999). Moreover, Mn2+ is a specific effector thatinduces MnP and represses LiP (Zhao et al., 1996).Nevertheless, the usefulness of lignocellulose materialsfor enzyme production is limited by the lack of a simpleand reproducible technique for preparing active extracts,since extraction conditions influence greatly the valuesobtained. Temperature and type of solvent are known asimportant parameters in the extraction of solutes from solids.Additionally, when dealing with enzymes, it is necessaryto take into account the enzyme thermal stability,which in turn is a function of the exposure time. The activityand stability ofMnP is strongly influenced by the pH, aswell as temperature and time of incubation (Sutherlandand Aust, 1996). However, the optimum pH for recoveringenzymes should take in consideration the condition, whichreduces at maximum the protease activity, normally present in the extracts. In this study, we report the investigation of a pH bufferused for maximizing the extraction of MnP from *L. tuberregium*grown on eucalyptus residues . Due to the importance ofthis enzyme in industrial applications, an attempt has beenmade to improve the amount of enzymes extracted frombiodegraded eucalyptus residues, with low protease activity.In addition, several conditions and substrates weretested 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 samemedia 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(.5 mm) and immersed in tap water for 24 h. The excessof water was drained and the moisture adjusted (65–7%).Polypropylene bags (25 · 3 cm) were packed with 75 g ofsubstrate. The bags were sealed with water proof paper andautoclaved at 115 _C for 1 h. This procedure was repeatedtwice obeying interval of 24 h. After cooled, the bags wereinoculated in sterile conditions by introducing seven discscut from the plate (B = 8 mm), sealed again and incubatedat 27 ± 2 _C for 3 days in the dark until the substrate wasfully colonized with mycelium.P. chrysosporium was cultivated in liquid media, lownitrogenconditions (C/N = 9.3) containing the following(per liter): 2. g of glucose, .66 g di-ammonium tartrate,2.5 mMveratryl alcohol, .5 g MgSO4 Æ 7H2O, .1 gCaCl2 Æ 2H2O, .184 g FeSO4 Æ 7H2O, .7 g H3PO4,1 lg of thiamine, 4. lg of biotin, 4 lg of folic acid,1 lg of riboflavin, 1 lg of tioic acid, 1 lg of p-aminobenzoicacid, .2 lg of cyanocobalamin, 2 lg of pyridoxinehydrochloride, 1 lg of nicotinic acid, 1 lg calciumpantothenate. The following trace elements were alsoadded (per liter): 1.5 lg sodium nitrolotriacetate, 1. lgMgSO4 Æ H2O, 1. lg CoCl2 Æ 6H2O, 3. lg ZnSO4 Æ 7H2O,.1 lg H3BO3, .1 lg Na2MO4. The pH was adjustedto 4.5 with tartaric acid buffer (3. g/L). Media (5 ml)were dispensed in 25 ml Erlenmeyer flasks. After sterilizationfor 2 min at 115 _C, five discs (B = 8 mm) of agarcarrying mycelium of P. chrysosporium were introducedinto media in sterile conditions. The Erlenmeyer flasks wereincubated at 37 ± 2 _C in stationary condition for 7, 14 and21 days.

2.2. Enzyme extraction: The enzymes were extracted with 5 mM citrate–phosphatebuffer at pH 4., 5. and 6.. Twenty grams offermented substrate was extracted with 1 ml of buffer,at 1 and 25 _C. Extractions were performed at 1 rpmfor 3 h, and in stationary condition for 1 h. The enzymaticextracts were centrifuged at 7 rpm for 2 min and thesupernatants were

recovered by filtration through porousceramic filter number 4 under vacuum.

2.3. Enzymatic activities assays: MnP was determined using phenol red .1%(e61 = 22, M_1 cm_1) (Kuwahara et al., 1984) andLiP activity was determined using azure B (e651 =48,8 M_1 cm_1) (Archibald, 1992) and veratryl alcohol(e31 = 39 M_1 cm_1) (Tien and Kirk, 1988). Proteaseactivity was determined according to the method of Anson(1938) using tyrosine as standard. All enzymatic activitieswere expressed as IU g_1 defined as the amount of enzymeproducing 1 lmol of product per min per g of fungalbiomass.

2.4. Determination of fungal biomass (FB): The FB was determined by extraction and quantificationof ergosterol contained in the phospholipidbilayer of cellmembranes. The extraction of ergosterol was carried outby saponification of the phospholipidbilayer with NaOHmicro-wave-assisted and subsequent extraction with pentane(Montgomery et al., 2). Ergosterol was detectedat 282 nm. FB wasdetermined using an ergosterol-to-fungal biomass conversionfactor from cells growing in liquid culture (y =4.96x _ 3.349/r2 = .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 mmscreen. Milled samples (5 mg) were hydrolyzed with 6 mlof sulfuric acid (72% w/w) in a DigesdahlHach digesterheated for 5 min at 44 _C. Afterward, 3 ml of 3% (w/w)hydrogen peroxide was added and the mixture heated at44 _C for additional 3 min. After cooling, the solutionwas raised to 5 ml with deionized water. This solutionwas analyzed in ICP-GBC Integra XM equipment to determinethe manganese, iron, calcium, potassium and coppercontents.

3. RESULTS AND DISCUSSION:

Biodegraded eucalyptus residue was extracted with buffersolutions to recover the extracellular enzymes producedduring fungal growth. Considering that the stability ofenzymes produced by *L. tuberregium* during lignocellulose degradationare pH and temperature dependent, differentextraction 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 extractionwas performed at pH 6.. In addition, MnP recovered atpH 4. or 5. at 1 _C were not statistically different, howeverhigher activity was observed at pH 5. when theenzyme was recovered at 25 _C for 3 h (P < .1).LiP was not detected in *L. tuberregium*extracts using veratrylalcohol assay, however it cannot be assumed that theenzyme is absence in the extracts, since the presence ofcompounds such as phenolics and other aromatics typicalfrom lignocellulose compounds interferes with the assayemployed. The interference due to aromatic compoundswas completely eliminated by using azure B as substrateto evaluate LiP, which was monitored in visible region(651 nm) (Archibald, 1992). Therefore, the non-oxidationof azure B by the extracts of *L. tuberregium*was an evidence thatLiP was not produced. This result is consistent with severalreports in oxidative systems of *L. tuberregium*, which indicatethat this fungus lacks LiP activity (Leatham, 1986; Crestiniand Sermanni, 1995).However, the high content of manganese (183 ppm) inthe eucalyptus residue could be regulating the productionof LiP. This enzyme is formed almost exclusively whenMn2+ is low (1.6–.3 ppm) (Bonnarme and Jeffries, 199).Otherwise, it was reported that veratryl alcohol could beoxidized by incubation with purified MnP of *L. tuberregium*yielding different metabolic products in the absence ofveratryl alcohol oxidase activity (D'Annibale et al., 1996). This result indicates that there are non-enzymatic mechanismsdisplayed by *L. tuberregium*to oxidize nonphenolic structures,such as peroxil or thiol radicals formed through lipidperoxidation (Kapich et al., 1999) and aminoacid oxidation(D'Annibale et al., 1996).Enzymatic extracts with high MnP

activity were submittedto dialysis to remove small molecular weight compounds.

After this step, the recovery of MnP activity, for1 or 3 h at pH 4, strongly decreased. The loweringof MnP should be connected with the action of proteasesnormally present in the extracts submitted to dialysis fora long time. The absorbance at 28 nm was completely reduced inthese extracts due to elimination of low molecular weightcompounds that could be causing interference in the LiPactivity (Harvey and Palmer, 199). However, LiP activitywas not detected in the extracts after dialysis, assayed withazure B or veratryl alcohol oxidation.

3.2. Inhibition of LiP by *Lentinus tuberregium* extracts: *L. tuberregium* is probably the best studied microorganismwith 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. chrysosporiumin the proportions of 1:1, 1:2, 1:5 and 1:1 andthe LiP activity was newly determined.P. chrysosporium cultivated in liquid media presentedLiP activity during azure B and veratryl alcohol oxidationat pH 3.5 (13.8 and 4.6 IU/L, respectively) and 4.5 (9.1and 22.4 IU/L, respectively), after 14 days of cultivation.The high LiP activity found by veratryl alcohol oxidationcould be connected with low molar extinction coefficient of veratryl alcohol or often by probable presence of veratrylalcohol oxidase, since aryl alcohol oxidase eventuallypresent in the extracts does not oxidize azure B, but oxidizeveratryl alcohol by a H2O2-independent reaction (Archibald,1992; Arora and Gill, 21).The sample previously submitted to dialysis was alsojoined to LiP of *L. tuberregium*at the same conditionsdescribed above, however total inhibition of LiP wasobserved which was probably caused by compounds withhigh molecular weight released from eucalyptus residue.

3.3. Effect of extraction parameters on protease activity: Protease was analyzed in the extracts and high activitieswere detected at pH 4. and 6., while at pH 5. low proteaseactivity was determined . These results indicatethat proteases secreted by L. edodes are less active atpH 5. and provided greater recovery of MnP. Severalhypothesis describe the function of proteases in wood degradationby white-rot fungi. Eriksson and Pettersson (1988)suggested a possible action of proteases in the releasing ofligninolytic enzymes contained in the cellular wall of fungi.Otherwise, the function of proteases produced by white-rotfungi is to release nitrogen from the substrates or to recycleextracellular proteins by autolysis mechanism (Dosoretzet al., 199a,b; Cabaleiro et al., 22). The results obtainedby us seem to sustain the second hypothesis since high levelsof proteases coincide with low levels of ligninolyticenzymes. In fact, the action of proteases for nitrogen disposalis more important in solid-state culture than in submerged,because of the low nitrogen content in woodmaterials. 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 controlthe activity of MnP of *L. tuberregium*. MnP functions over arange of pH (4.–6.), while the maximum MnP was recoveredat pH 5., the same pH at which was recovered thelowest levels of protease. Furthermore, other factors areimportant to obtain higher MnP recovery, thus the optimizationof fermentation conditions, such as the fungalstrain, growth medium, moisture content, aeration leveland culture age are crucial to improve the MnP titers(Leatham et al., 1991). In fact, in previous work weobserved a markedly effect on MnP activity when ninestrains of *L. tuberregium*were grown on eucalyptus waste supplementedwith 2% of rice bran (Silva et al., 25).The lack of LiP activity in the extracts of *L. tuberregium*is acommon characteristic for this species, but it could also berelated with the concomitant presence of protease activity.

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