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Purification, partial characterization, and reactivity with aromatic compounds of two laccases from *Marasmius quercophilus* strain 17

A.M. Farnet, S. Criquet, S. Tagger, G. Gil, and J. Le Petit

Abstract: Two isozymes of laccase were obtained from an induced liquid culture of *Marasmius quercophilus* with *p*-hydroxybenzoic acid as the inducer. Both the constitutive and the induced isozyme have a molecular mass of 60 kDa as determined by polyacrylamide gel electrophoresis. Using isoelectric focusing, we found three isozymes with the constitutive enzyme (pI 4, 4.2, 4.4) and four of the induced form (pI 4.75, 4.85, 4.95, 5.1). We observed certain differences between these two isozymes; the specific activity of the induced isozyme was twice as high, and two optimum pH levels (5 and 6) were observed with the induced isozyme (only one, pH 5, for the constitutive isozyme). However, both of these enzymes have the same thermal stability and the same temperature for their highest activity (80°C). Furthermore, the reactivity of both these enzymes with aromatic compounds was similar. The use of mediators extended the oxidized substrate range of the laccases studied. Various products of degradation were observed, depending on the mediator used. When laccase was used alone, the decrease of the signal corresponding to the aromatic cycle, without any formations of other peaks at different wavelengths, suggested polymerisation of aromatic compounds.

Key words: laccase, *Marasmius quercophilus*, mediator, phenol degradation.

Résumé : Deux isozymes de laccases ont été obtenues à partir d'une culture liquide induite de *Marasmius quercophilus* en utilisant l'acide *p*-hydroxybenzoïque comme inducteur. Les isozymes constitutives et induites ont toutes deux un poids moléculaire de 60 kDa mesuré en électrophorèse sur gel de polyacrylamide. En électrofocalisation, nous avons observé trois isozymes pour l'enzyme constitutive (pI 4, 4.2, 4.4) et quatre pour l'isozyme induite (pI 4.75, 4.85, 4.95, 5.1). Nous avons observé certaines différences entre ces deux isozymes; l'activité spécifique de l'isozyme induite était deux fois plus importante que celle de l'isozyme constitutive et deux pH optimum (5 et 6) ont été notés pour l'isozyme induite (seulement un, pH 5, pour l'isozyme constitutive). Les deux enzymes avaient la même stabilité thermique et la même température d'activité maximale (80°C). De plus, la réactivité de ces deux laccases vis-à-vis des composés aromatiques était semblable. L'utilisation de médiateurs a augmenté la gamme des substrats oxydés par les laccases étudiées. Différents produits de dégradation ont été observés selon le médiateur utilisé. Quand seule la laccase est utilisée, la diminution du signal correspondant au composé aromatique et l'absence d'autres pics à différentes longueurs d'ondes, suggèrent une polymérisation des composés aromatiques.

Mots clés : laccase, *Marasmius quercophilus*, médiateurs, dégradation des phénols.

Introduction

Marasmius quercophilus is a white-rot fungus which colonizes evergreen oak litter, a typical Mediterranean ecosystem. The potential role of this basidiomycete in litter biodegradation has been described in a previous study (Tagger et al. 1998). This fungus is particularly involved in ligninolysis

because of its production of laccases (EC 1.10.3.2.). The role of these phenoloxidases in ligninolysis has already been described by several authors, such as Bourbonnais et al. (1997), using kraft lignin as a lignin model. Ander and Eriksson (1976) have also demonstrated that laccases were essential in lignin degradation by *Sporotrichum pulverulentum*, as did Eggert et al. (1997) with *Pycnoporus cinnabarinus*. This enzyme belongs to the family of blue copper oxidases, which includes ceruloplasmine and ascorbate oxidase. Laccases have provoked considerable interest in biotechnologies such as kraft pulp bleaching or degradation of aromatic pollutants because of the reactions they catalyze. They oxidize aromatic compounds such as *ortho*-, *para*-diphenols, and aminophenols, and simultaneously reduce oxygen to water. Recently, authors have reported that the use of mediators such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), promazine, Remazol brilliant blue, or 1-hydroxybenzotriazole (HBT), extended the substrate range of laccases (Xu et al. 1997; Bourbonnais et al. 1998). These

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mediators seem to act as diffusible lignin-oxidizing agents, since laccase is a large molecule which can not easily enter the plant cell wall structure because of steric hindrance. The purpose of our study was to describe the biochemical characteristics of two laccases from *M. quercophilus*. Furthermore, we investigated the enzymatic potential of both these enzymes by testing various aromatic compounds (monolignols, hydrolysable tannins). We also studied the effect of two mediators (ABTS and HBT) on the laccase degradation potential. The two isozymes studied included a constitutive and an induced enzyme. In a previous study (Farnet et al. 1999), the induction was performed using *p*-hydroxybenzoic acid as described. Numerous aromatic compounds have already been reported as inducers of laccases (Rogalski and Leonowicz 1992; Vasdev and Kuhad 1994). Moreover, we wanted to compare these two isozymes in their reactivity on aromatic compounds or in their biochemical characteristics. This may explain their respective roles in the laccase enzymatic system of *M. quercophilus*.

Material and methods

Isolation of the strain

Marasmius quercophilus strain 17 was isolated from La Gardiole de Rians, Var, France, using the rhizomorphic form of the fungus. A fungal cap culture was first made on a 20 g·L⁻¹ malt-agar medium (Bio Mérieux, Marcy l'Etoile, France), and 50 mg·L⁻¹ chloramphenicol (Sigma). Next, the pure mycelial culture obtained was used to inoculate an agar medium (whole wheat flour, 20 g·L⁻¹) favouring rhizomorph production. Finally, one rhizomorph was used to inoculate a malt-agar plate to obtain a pure dicaryotic culture.

Culture conditions

Precultures were performed in 200-mL Erlenmeyer flasks containing 50 mL of malt extract medium (20 g·L⁻¹) in static conditions at 28°C. They were inoculated with a plug of agar culture (15 g·L⁻¹) with malt extract (20 g·L⁻¹). These liquid precultures were used to inoculate two 2000-mL Erlenmeyer flasks with 500 mL of malt extract medium (20 g·L⁻¹), 0.05% Tween 80, 5 mg·L⁻¹ CuSO₄, and 500 mg·L⁻¹ *p*-hydroxybenzoic acid. They were incubated at 28°C for five days under axial shaking. Enzyme activity was measured by following the oxidation of syringaldazine (N,N'-bis-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine), which leads to its quinone (ϵ^M : 6.5 × 10⁴·M⁻¹·cm⁻¹) at 525 nm (Harkin and Obst 1973) on a Kontron Uvikon 860 spectrophotometer. The assay contained 500 µL of the filtered culture medium, 2.5 mL of 0.1 M phosphate buffer pH 5.7, and 10 µL of syringaldazine 0.6% (w/v), diluted in methanol. The blank consisted of 500 µL of the filtered culture medium, and 2.5 mL of the same phosphate buffer.

Purification of the two laccase isozymes

Cultures were filtered on a 2.7-µm glass microfibre filter GF/D (Whatman, U.K.). These filtered media were concentrated on an ultrafiltration cell, model 8200 (Amicon, Beverly, Mass.) until the volume reached 15 mL. The membrane used (Amicon), was rated at 10 MW (10 kDa) cut-off. This membrane was rinsed with an additional volume of 1 mL of 0.1 M phosphate buffer, pH 5.7, to remove any bound laccases from its surface. This sample was then loaded on a Mono Q column. A step gradient system of NaCl in Tris-HCl buffer 10 mM pH 6 (0, 0.2, 0.4, 1) was used to elute active fractions. Laccase activity was detected using syringaldazine. The active fractions that eluted as one peak were pooled and ultrafiltered as described before.

Effects of pH and temperature on laccase activity

Laccase activity was detected as previously described using 15 µg of each purified fraction. Acetate buffer at 0.1 M was used for pH range from 4 to 5, and 0.1 M phosphate buffer was used for pH range from 5.7 to 8. The optimum temperature for laccase activity was determined using 15 µg of each purified fraction in 0.1 M acetate buffer at pH 5. Six temperatures were tested: 30, 40, 50, 60, 70, and 80°C. The thermal stability of laccase activity was studied using the temperature mentioned above after several incubation times (1, 2, 3, 5, 7, 9, 24, and 36 h). Laccase activity was also measured using 15 µg of each purified fraction in 0.1 M acetate buffer at pH 5.

Electrophoresis analysis

Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli (1970) using a 4% stacking gel and 7.5% separating gel at 220 V with the Mini-Protean II electrophoresis cell (Bio-Rad). Molecular masses of the two laccase isozymes were determined using a high-range molecular mass prestained standard (Bio-Rad). Protein bands were revealed using the Coomassie blue standard method. IEF analysis were performed on Servalyt Precotes polyacrylamide gels (Serva, 3–6 pH gradient, 150 µm, 125 × 125 cm, pI markers) according to the manufacturer. Gel staining was performed using *p*-phenylenediamine at a final concentration of 0.1% (v/v) in 0.1 M acetate buffer, pH 5, during 30 min.

HPLC analysis of aromatic compound degradation

HPLC system was equipped with a C18 column (Merck, 4.6 × 250 mm) in the following gradient system: solvent A, water/trifluoro acetic acid 0.1%/methanol 90/10 v/v; solvent B water/trifluoro acetic acid 0.1%/methanol 5/95 v/v; gradient = 0 – 25 min A 100% to B 100%; 25 to 40 min B 100% (flow rate 1 mL·min⁻¹; detection at 270 nm). The aromatic compound degradation was assayed using a reaction mixture (10 mL) containing 1 mM ABTS or HBT, 1000 mg·L⁻¹ aromatic compound, and 5 U of the laccase isozyme studied (constitutive or induced) in 0.1 M acetate buffer pH 5. The same reaction was performed without mediators. Controls with only ABTS or HBT with laccase, and using ABTS or HBT with the aromatic compound but without laccase, were also used. Samples were collected at 0, 2, and 4 h, and 20 µL of each sample were injected in the HPLC system.

Results and discussion

A one-step purification using ion exchange chromatography allowed us to separate the two laccase isozymes (Fig. 1). The purity of these enzymes was shown on SDS-PAGE, where each one of them was resolved as a single band (Fig. 2). We found a molecular mass of 60 kDa for both the induced and the constitutive forms. In a previous study, we observed a difference of migration in non-denaturing conditions (Farnet et al. 1999); the constitutive isozyme had the highest molecular weight. This may be due to various degrees of glycosylation of the protein. Most laccases have molecular masses ranging from 60 to 140 kDa (Thurston 1994; Binz and Canevascini 1997). However, a previous study in our laboratory has shown that the laccase from another *M. quercophilus* strain (strain C30) had a different molecular mass, measuring 63 kDa (data unpublished). This may be explained by the fact that strain C30 and strain 17 under study were collected in two different areas at the site of La Gardiole de Rians. Other strains isolated from various distant sites may exhibit new isoenzyme profiles

Fig. 1. The chromatogram of the two laccase isozymes from *Marasmius quercophilus* with Mono Q ion exchange chromatography. The NaCl step gradient system used is shown (---) as well as laccase activity (\square) and protein concentration (\blacksquare). The peaks of the two laccases are labelled C and I for the constitutive and the inducible isozymes, respectively.

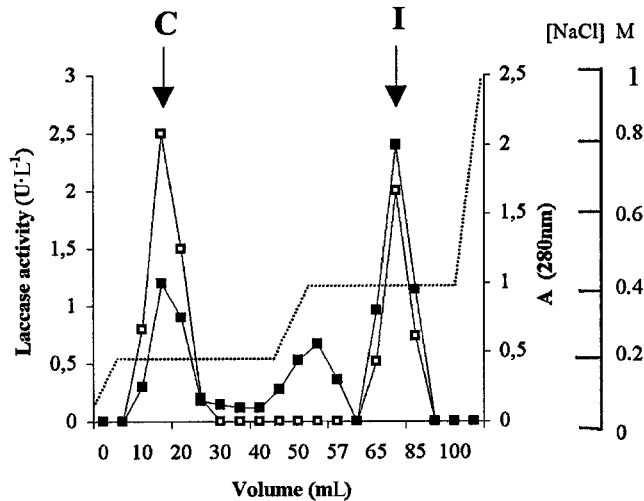
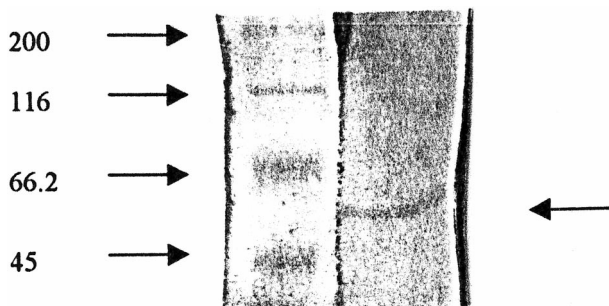


Fig. 2. SDS-PAGE of the purified extracellular laccases. The same band was observed for the constitutive and the induced isozyme. Molecular mass markers are shown in the left lane.



depending on the geographical areas where they are collected. Many studies have already described this phenomenon (Kerrigan and Ross 1988; Dupré et al. 1993; Sagawa et al. 1993). Authors have successfully distinguished the strains studied depending on the crop localization using their laccase zymograms. The two isozymes under study were monomeric enzymes. These are common properties for laccases. These enzymes are indeed composed of only one subunit, except in the case of the laccase from *Podospora*, where four subunits were found (Reinhammar and Malmström 1981). Laccase molecular properties are indeed quite homogenous; the typical laccase is a monomeric enzyme with a molecular mass ranging from 60 to 80 kDa with 10%–15% of carbohydrates.

In our study, the presence of other laccase isozymes was demonstrated using the isoelectric focusing experiment (Fig. 3). We also used syringaldazine to check for the staining of the gel, since this substrate is specific to laccase under the conditions of our experiment (Harkin and Obst 1973; Leonowicz and Grzywnowicz 1981). However, syringaldazine did not lead to stable staining like *p*-phenylenediamine (data not

Fig. 3. Isoelectric focusing of the two isozymes of laccase from *Marasmius quercophilus* revealed by *p*-phenylenediamine. Lane 1: pI standards; lane 2: zymogram of the constitutive isozyme; lane 3: zymogram of the induced isozyme.

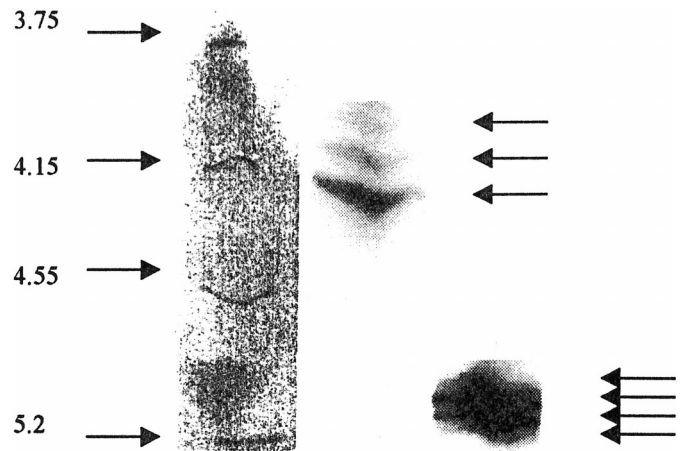
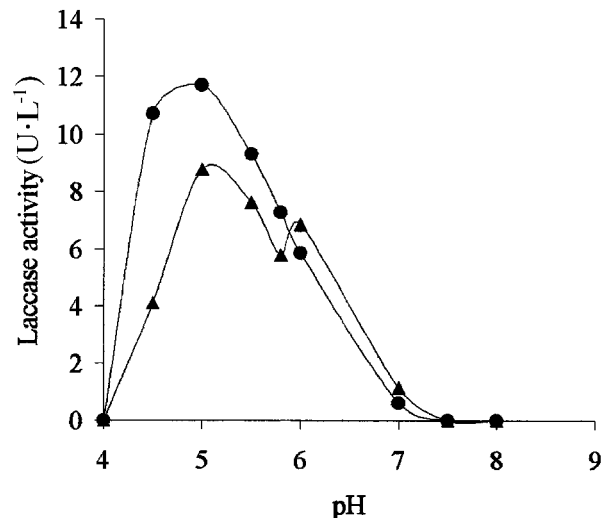


Fig. 4. The effect of pH on the laccase activity (U·L⁻¹) of *Marasmius quercophilus* was estimated with the constitutive (\bullet) and the induced (\blacktriangle) isozyme. All the standard deviations were below 3%.



shown), and thus this last substrate was chosen for the staining of the gel. Three other isozymes (pI 4, 4.2, and 4.4) were found on the electrophoretic pattern of the pure constitutive laccase, and four other isozymes (pI 4.75, 4.85, 4.95, 5.1) were detected with the induced laccase. Several isozymes detected with isoelectric focusing have already been reported in the case of fungal laccases. For example, three isozymes were revealed by IEF from a 32-kDa laccase fraction purified from a *Botrytis cinerea* strain (Pezet 1998). Different biochemical characteristics have been pointed out in this study, which may be explained by the various isozymes observed in the isoelectric focusing experiment. The specific activity of the induced isozyme was twice as high as that of the constitutive isozyme, respectively 12.35 and 6.71 U·mg⁻¹ protein. The optimum pH was 5 for both

Fig. 5. The effect of temperature on the laccase activity ($\text{U}\cdot\text{L}^{-1}$) of *Marasmius quercophilus* was estimated with the constitutive (\blacksquare) and the induced (\square) isozyme. All the standard deviations were below 3%.

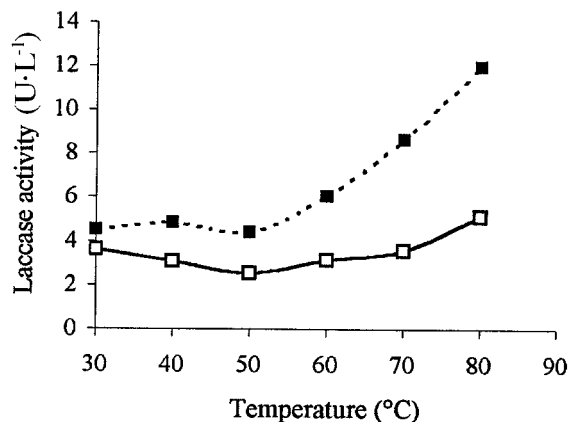


Fig. 6. The thermal stability of the two laccase isozymes of *Marasmius quercophilus* after various incubation times. The constitutive (A) and the induced (B) isozyme was tested using various temperatures: 40°C (—); 50°C (— — —); 60°C (- - - -), 70°C (- - - -); 80°C (- - -). All the standard deviations were below 3%.

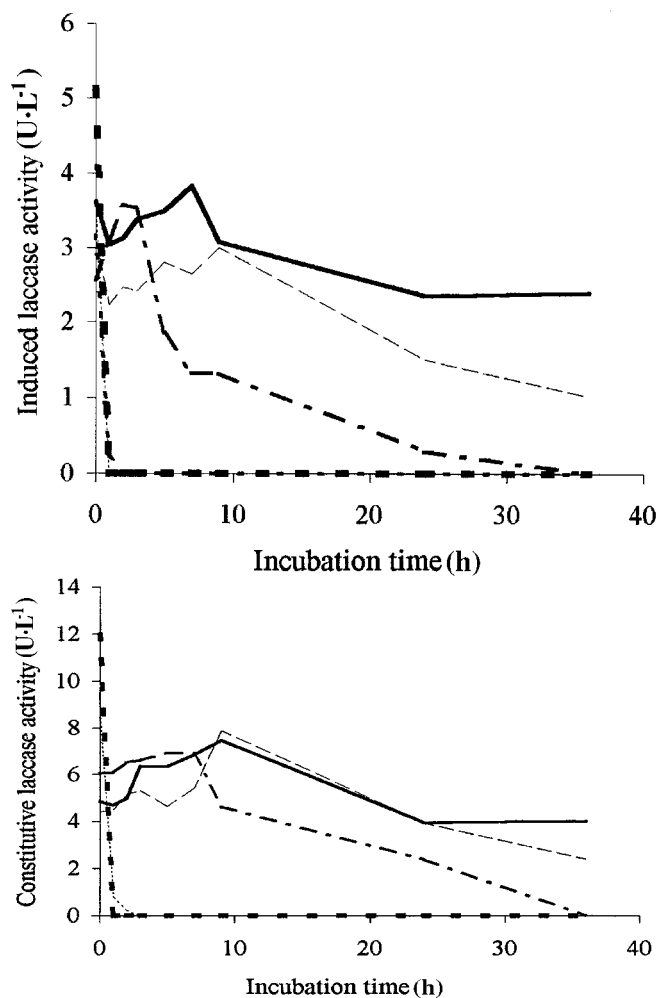
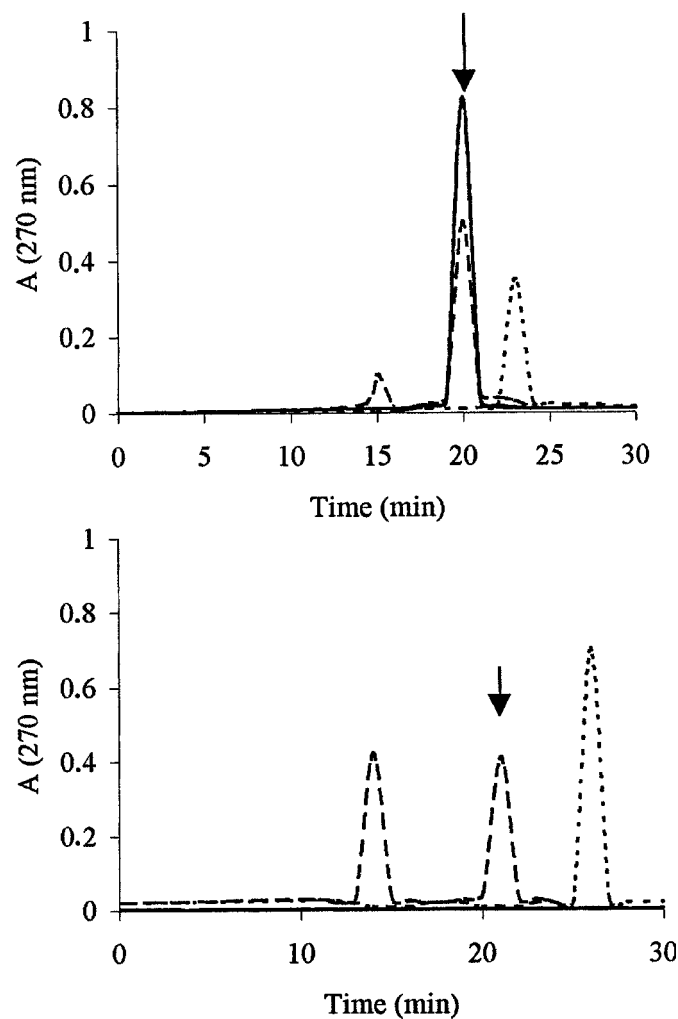


Fig. 7. The degradation of aromatic compounds with the two isozymes of *Marasmius quercophilus* after 4 h of incubation with or without mediators using RP C18 HPLC column. Results were identical regardless of isozyme used. The degradation of veratryl alcohol (A) and coniferyl alcohol (B) with ABTS (- - -), with HBT (- - -), and without mediators (—) is shown. The arrow points out the elution time of the peak of the tested aromatic compound alone.



these enzymes as determined with acetate buffer, but high activity was also observed at pH 6 with the induced isozyme (Fig. 4). However, the activity of this induced isozyme was lower at pH 6 than at pH 5. Fungal laccases are generally active at low pH values as already reported by many authors (Bollag and Leonowicz 1984; Eggert et al. 1996; Palmieri et al. 1997). We observed that the constitutive and the induced isozymes of laccase had certain similar properties, such as thermal stability and optimum temperature. The temperature tested at the highest laccase activity was 80°C for both isozymes (Fig. 5), and the activity was stable after 1 h of preincubation at 40, 50, and 60°C (Figs. 6A and B). Preincubation at 40°C and 50°C strongly increased their activity, mostly in the case of the constitutive isozyme. After 1 h of preincubation at 70°C, the enzymes retained only 9% and 6% of their initial activity for the constitutive and the

induced isozyme, respectively. After 2 h, only the constitutive form was still active (2.7% of residual activity). No activity was detected after 1 h of preincubation at 80°C in both cases. Most fungal laccase activities have an optimum temperature ranging from 30 to 60°C. Moreover, these enzymatic activities are not often stable between 50 to 70°C (Périer et al. 1998; Scherer and Fischer 1998). Thus, we found very particular properties for the laccases of *M. quercophilus* in terms of optimum temperature and thermal stability. These particular properties have been found only in the case of laccases isolated from a composted municipal solid waste (Chefetz et al. 1998a, 1998b), and from paper factory wastewater (Coll et al. 1993). In this last study, the laccase of basidiomycete PM1 exhibited very similar characteristics to *M. quercophilus* laccases; the optimum temperature was 80°C, and thermal stability for a range of temperatures between 50°C–70°C was also observed. These biochemical properties are particularly interesting for biotechnological applications which may involve high temperatures.

All the aromatic compounds tested were degraded by the constitutive and induced isozyme of laccase, except veratryl alcohol and *p*-hydroxybenzoic acid. These aromatic compounds were not degraded without the addition of mediators, nor with constitutive isozymes, or the induced form (Fig. 7A). This result with veratryl alcohol is consistent with that observed by Bourbonnais et al. (1998). The oxidation of this non-phenolic compound was explained by the role of the dication ABTS²⁺, the oxidation product of laccase, as the oxidative intermediate agent. Thus the oxidation of laccase was indirect; the aromatic compound degradation is probably caused by the oxidizing intermediates produced by the laccase.

When ABTS was used as the mediator, we always observed new peaks at 270 nm. With HBT, we did not find new peaks with gallic acid and tannic acid; we only observed the decrease of the peak of the aromatic compound. We also did not observe degradation products when only the laccase was added to the reaction mixture in the case of these two aromatic compounds. *p*-Hydroxybenzoic acid was transformed only when laccase was associated with ABTS.

In each case, the new peaks observed at 270 nm with ABTS and HBT differed depending on the mediator used (Fig. 7B). They were also different to those observed in the experiments involving only laccase. This would suggest that the mechanism of aromatic compound oxidation is different, depending on the oxidized mediator. These results can be explained using the experiment involving only the enzyme and the mediator; we observed new peaks which may be the oxidized form of the mediator. However, further studies are needed to clearly identify these components. These results showed that the mediators used had an effect on oxidation, and their respective action on aromatic compounds appears to be different.

The two laccase isozymes did not exhibit different properties in the degradation of aromatic compounds. The responses were identical for both the enzymes; the new peaks observed and the decrease of the peaks were the same. In this part of our study we observed only the conversion of aromatic compounds into other organic compounds. The aim of our further studies will be the identification of these products, which

would allow us to investigate the mechanism of laccase oxidation at the same time. When the chromatograms showed only the decrease of the peak of the aromatic compound, this may have suggested a polymerization of aromatic compounds, which have already been described in several studies (Lundquist and Kristersson 1985; Dec and Bollag 1990). On the other hand, the cleavage of aromatic compounds by laccase has not been reported in literature before; most laccases are involved in demethylation, decarboxylation, or C_α–C_β cleavage in lignin model compounds (Higuchi 1993). Thus the cleavage of aromatic compounds is not likely to occur, and does not explain the decrease of the peak. However, the oligomers that may be produced in this reaction can not be detected under our experimental conditions.

It seems that, in this study, the main differences observed between the two laccase isozymes are their specific activity and the pH where the maximum activity is observed; the induced isozyme has a higher specific activity and exhibits a high activity for two pH values.

This study has shown the involvement of the laccase enzymatic system in the degradation of phenolic compounds. Furthermore, this work has also pointed out that when laccase is associated with certain mediators, its potential to degrade aromatic compounds is extended to a wider range. In a further study, we will also focus on the mechanism of the reaction by evaluating, for example, the redox potentials of the enzyme (Bonomo et al. 1998).

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