



HAL
open science

Purification of a laccase from *Marasmius quercophilus* induced with ferulic acid: reactivity towards natural and xenobiotic aromatic compounds

Anne Marie Farnet da Silva, S. Criquet, Mireille Cigna, Gérard Gil, Elisée Ferré

► To cite this version:

Anne Marie Farnet da Silva, S. Criquet, Mireille Cigna, Gérard Gil, Elisée Ferré. Purification of a laccase from *Marasmius quercophilus* induced with ferulic acid: reactivity towards natural and xenobiotic aromatic compounds. *Enzyme and Microbial Technology*, 2004, 34 (6), pp.549-554. 10.1016/j.enzmictec.2003.11.021 . hal-02071810

HAL Id: hal-02071810

<https://hal.science/hal-02071810>

Submitted on 18 Mar 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Purification of a laccase from *Marasmius quercophilus* induced with ferulic acid: reactivity towards natural and xenobiotic aromatic compounds

Anne-Marie Farnet^{a,*}, Stéven Criquet^a, Mireille Cigna^a, Gérard Gil^b, Elisée Ferré^b

^a Laboratoire de Microbiologie, Service 452, UMR CNRS 6116, Institut Méditerranéen d'Ecologie et de Paléoécologie, Université d'Aix-Marseille III, Marseille, France

^b Laboratoire de Chimie, Biologie et Radicaux Libres, Service 532, UMR CNRS 6517, Université d'Aix-Marseille III, Marseille, France

Abstract

In this study, we describe the biochemical features and the ability of a new laccase isoform from a *Marasmius quercophilus* strain collected on evergreen oak litter to transform various aromatic compounds. This laccase, induced with ferulic acid, exhibits interesting enzymatic properties, such as great thermal stability at 30 and 40 °C (24 h), no inhibition with EDTA, Cystein or SDS. These biochemical features are quite different from those observed with the laccase of another *M. quercophilus* strain collected in a different area of the South of France. Thus, this study shows the degree of variation in the properties of this enzymatic system within the same species. Furthermore we demonstrate that several natural aromatic compounds were transform leading to polymerization. The transformation of chlorophenols such as 2-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol is also observed without adding mediators (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) or 1-hydroxybenzotriazole) to the reaction mixture. Thus, the mediators used do not seem to extend the substrate range for this laccase under these experimental conditions.

Keywords: Aromatic compounds; Laccase; Mediators; White-rot fungi

1. Introduction

Marasmius quercophilus is a predominant basidiomycete in typical Mediterranean ecosystems such as evergreen (*Quercus ilex* L.) or cork oak (*Quercus suber* L.) litters. This white-rot fungus has been implicated in lignin biodegradation and thus plays an important ecological role since it contributes to the carbon recycling. Phenoloxidases, i.e. laccases, peroxidases or tyrosinases are the enzymatic systems which degrade lignin [1,2]. We have studied different laccases (EC 1.10.3.2.) produced by certain *M. quercophilus* strains collected in various areas in the south of France [3,4]. These studies attempt to determine whether the biochemical characteristics and the aromatic compound degradation potential of such a polymorphic enzymatic system may vary depending on different parameters: ecosystem, fungal strain or culture conditions. Finding a laccase which has a strong abil-

ity to transform aromatic compounds is an important aim for biotechnological applications. Laccases have indeed aroused considerable interest in the last few years because of the reaction they catalyze. They reduce oxygen to water while oxidizing various phenolic compounds in a non-specific radical-based reaction. One of the applications is the degradation of chlorophenols which have been used extensively as pesticides and thus are now widely spread pollutants.

In this study, we described a new isoform of laccase produced by a *M. quercophilus* strain isolated from evergreen oak litter and which has been induced with ferulic acid. We also compared its biochemical characteristics with those of another isoform also induced with ferulic acid but produced by a *M. quercophilus* strain isolated from a cork oak litter. Furthermore, this new isoform has been tested on different phenolic compounds both with and without two mediators: 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT), which have been extensively used to extend the range of substrates that can be oxidized and also to enhance the transformation of aromatic compounds [5].

* Corresponding author. Tel.: +33-4-91-28-81-90; fax: +33-4-91-28-80-30.

E-mail address: amfarnet@netscape.net (A.-M. Farnet).

2. Materials and methods

2.1. Isolation of the strain

M. quercophilus strain 19 was isolated from a part of the site of La Gardiole de Rians, Var, France (1 ha) using the rhizomorphic form of the fungus. A fungal cap culture was firstly made on a malt–agar medium, 20 g l⁻¹ (Bio Mérieux, Marcy l’Etoile, France) and chloramphenicol, 50 mg l⁻¹ (Sigma). Secondly, the pure mycelial culture obtained was used to inoculate an agar medium (whole wheat flour, 20 g l⁻¹) which favours rhizomorph production. Then one rhizomorph was used to inoculate a malt–agar plate to obtain a pure dicaryotic culture.

2.2. 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 cultures (15 g l⁻¹) with malt extract (20 g l⁻¹). These liquid precultures were used to inoculate two 3000-ml Erlenmeyer flasks with 800 ml of malt extract medium (20 g l⁻¹); Tween 80, 0.05%; CuSO₄, 5 mg l⁻¹; ferulic acid, 500 mg l⁻¹. They were incubated at 28 °C for 12 days under an axial shaking. Enzyme activity was measured by following the oxidation of syringaldazine [*N,N'*-bis-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine] that leads to its quinone (ϵ^M : 6.5 10⁴ M⁻¹ cm⁻¹) at 525 nm [6] on a spectrophotometer Kontron Uvikon 860. The assay contained 100 μ l of the culture medium, 2.5 ml of phosphate buffer 0.1 M, pH 5.7 and 10 μ l of syringaldazine 0.6% (w/v), diluted in methanol. The blank consisted of 100 μ l of the culture medium and 2.5 ml of the same phosphate buffer.

2.3. Purification of the two laccase isozymes

Cultures were filtered on a glass microfiber filter GF/D, 2.7 μ m (Whatman, England). Then, the filtered culture medium was concentrated in dialysis tubes rated at 12 kDa (32 mm diameter, Poly Labo) using polyethyleneglycol. The samples from the different tubes were pooled using a phosphate buffer 20 mM, pH 5.7 in a final volume of 15 ml. 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 M) was used to elute active fractions. Laccase activity was detected using syringaldazine. The active fractions eluted as one peak were pooled and concentrated on an ultrafiltration cell, Model 8200 (Amicon, Beverly, USA) till the volume reached 5 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 acetate buffer, 0.1 M, pH 4 to remove any bound laccases from its surface.

2.4. Effects of pH and temperature on laccase activity

Laccase activity was detected as previously described using 15 μ l of the purified fraction. Acetate buffer 0.1 M was used for pH range from 4 to 5 and phosphate buffer 0.1 M for pH range from 5.7 to 8. The optimum temperature for laccase activity was determined using 15 μ l of the purified fraction in acetate buffer 0.1 M pH 4.5. Seven temperatures were tested: 30, 40, 50, 60, 70, 80, 90 °C. The thermal stability of laccase activity was studied using the temperatures mentioned above and after several incubation times (1, 2, 3, 5, 7, 9, 24 and 36 h). Laccase activity was also measured using 15 μ l of the purified fraction.

2.5. Effects of inhibitors on laccase activity

Laccase activity was measured using 15 μ l of the purified enzyme in acetate buffer 0.1 M, pH 4.5 with ABTS 100 μ M as the substrate. The inhibitors used at a final concentration of 1 mM were: sodium azide, EDTA, cystein, and sodium diethyldithiocarbamate (DDC). All the chemical products were purchased from Sigma.

2.6. Determination of kinetic parameters

Syringaldazine and ABTS were used as substrates for the determination of the V_m and K_m which were calculated using the Lineweaver–Burk transformation of Michaelis–Menten equation. The concentrations ranged from 0.5 to 50 μ M and from 1 to 10 μ M for ABTS and syringaldazine respectively in acetate buffer 0.1 M pH 4.5.

2.7. Electrophoresis analysis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) were carried out according to Laemmli [7] using 4% stacking gel and 12% separating gel at 220 V with the Mini-Protean II electrophoresis cell (Bio-Rad). Molecular weight of the laccase isozyme was determined using high range molecular weight prestained standard (Bio-Rad). Protein band was revealed using the Coomassie blue standard method. For laccase activity staining, acetate buffer, 0.1 M, pH 4.5, with syringaldazine 0.1% or *p*-phenylenediamine 0.1% was used as the staining solution.

2.8. HPLC analysis of aromatic compound degradation

HPLC system was equipped with a C18 column (Merck, 4.6 mm \times 250 mm) in the following gradient system: solvent A, water/0.1% trifluoro acetic acid/methanol 90/10 (v/v), solvent B water/0.1% trifluoro acetic acid/methanol 95/5 (v/v), gradient = 0–30 min A 100% to B 100%, 30–40 min B 100% (flow rate 1 ml min⁻¹). The aromatic compound degradation was assayed using a reaction mixture (10 ml) containing ABTS or HBT 1 mM, aromatic

compound 1000 mg l^{-1} and 5 U of laccase in acetate buffer 0.1 M pH 4.5. The aromatic compounds used were: catechin, ellagic acid, ferulic acid, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, veratryl alcohol, 2-chlorophenol (2-CP), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP). All the aromatic compounds were purchased from Sigma. The same reaction was performed without mediators. Controls were also realized using only ABTS or HBT with laccase and using ABTS or HBT with the aromatic compound but without laccase. Samples were collected at 0, 2, 4 and 48 h and $30 \mu\text{l}$ of each sample was injected in the HPLC system. *P*-hydroxybenzoic acid was used as reference in each injection.

3. Results

The production of the induced isoform was observed on SDS-PAGE after a 5-day incubation and the maximum global laccase activity was obtained after a 12-day incubation (Fig. 1). The laccase isoforms in the culture supernatant were separated by SDS-PAGE: three isoforms were stained with *p*-phenylenediamine as previously described [2]. The isoform with the lowest molecular weight was then purified using a Mono Q chromatography column (Fig. 2). This purification step allowed us to obtain a single band on the gel using syringaldazine as the substrate. Under denaturing conditions we also observed a major protein band with a molecular weight of about 60 kDa (Scheme 1).

The effect of pH (values ranging from 3 to 8) was examined on laccase activity. The pH optimum of the induced laccase was 4 using ABTS and 4.5 using syringaldazine (Fig. 3). The maximum activity was obtained at 80°C with these two substrates. The thermal stability of this enzyme was greatest at 30 and 40°C (100% of the maximal activity remained after a 24 h incubation) while the enzymatic activity quickly decreased (after a 1-h incubation) however high temperatures tested (50, 60, 70, 80, 90°C). Furthermore, we observed an increase in enzymatic activity from 1250 to 2330 U l^{-1} for 30°C and from 1300 to 2700 U l^{-1} for 40°C .

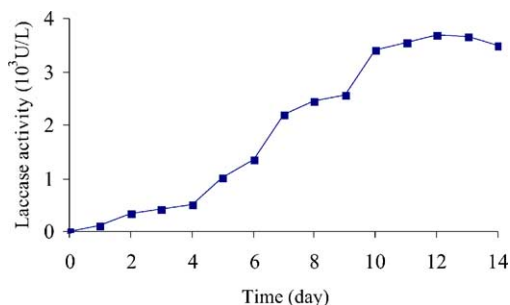


Fig. 1. Time course of total laccase activity (U l^{-1}) in liquid cultures with malt extract medium (20 g l^{-1}), Tween 80 (0.05%), CuSO_4 (5 mg l^{-1}) and ferulic acid (500 mg l^{-1}) at 28°C . All the standard deviations were below 3%.

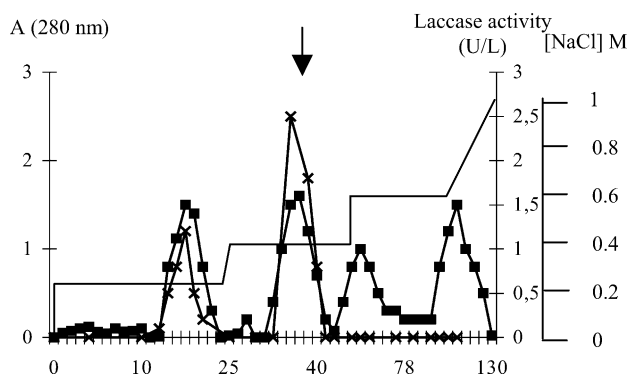
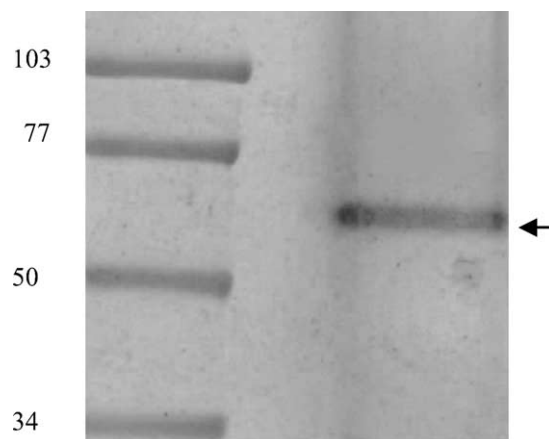


Fig. 2. Purification profile of the laccase induced with ferulic acid from strain 19 using a step gradient of NaCl: (x) laccase activity (U l^{-1}), (■) absorbance at 280 nm. The arrow indicates the peak of the induced isoform.

The first values (1250 and 1300 U l^{-1}) are the basal laccase activities at room temperature. The kinetic parameters V_m and K_m were 2 mM min^{-1} , $4.2 \mu\text{M}$ for syringaldazine and 3.3 mM min^{-1} , $11.3 \mu\text{M}$ for ABTS. Laccase activity was not affected by EDTA or cysteine as inhibitors while strong inhibition was observed with azide. With DDC, first a decrease in absorbance was observed for 11 min, followed by an increase for four more minutes and the absorbance finally stabilized for the last 15 min of the experiment (Fig. 4).

All the natural aromatic compounds, gallic acid, tannic acid, protocatechuic acid and vanillic acid were transformed with or without the addition of mediators. For gallic acid, ferulic acid, tannic acid or vanillic acid, several new peaks were obtained (Fig. 5) while the transformation of protocatechuic acid led to a single major peak. All these newly transformed compounds eluted after a longer elution time than the initial peaks.

The transformation of chlorophenols was efficient for 2-CP, 2,4-DP and 2,4,6-TCP. A decrease of about 50% of the initial absorbance was observed for 2-CP with the enzyme alone and with 2,4-DP with the enzyme added



Scheme 1. The profile of the purified laccase induced with ferulic acid from strain 19 on a SDS-PAGE (low molecular weight markers (kDa) on the left line).

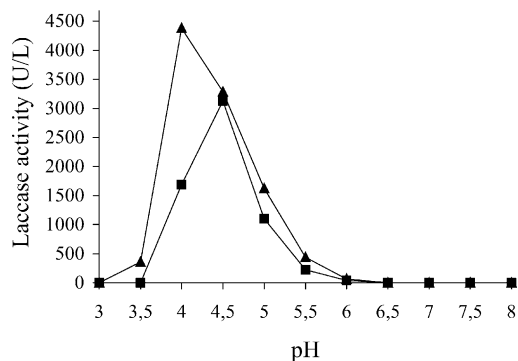


Fig. 3. The effect of pH on the induced laccase activity ($U l^{-1}$) using ABTS (■) or syringaldazine (▲). All the standard deviations were below 3%.

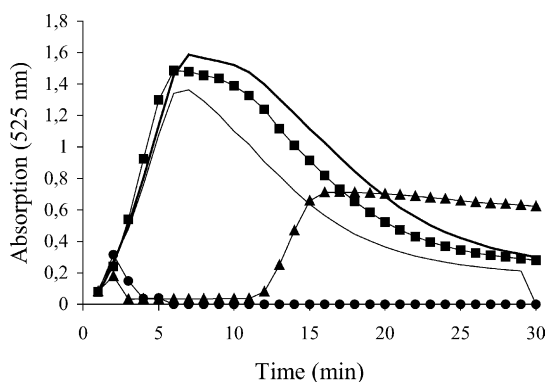


Fig. 4. The effect of inhibitors on the production of the quinone of syringaldazine at 525 nm: (■) without inhibitor, (■) with EDTA, (—) with Cystein, (●) with sodium azide, (▲) with DDC. All the standard deviations were below 3%.

with ABTS. Furthermore, with 2,4,6-TCP the decrease in initial absorption was stronger (about 23%) with or without mediators. Moreover, for these three chlorophenols, we detected several peaks which eluted after longer retention times. We did not observe the transformation of pen-

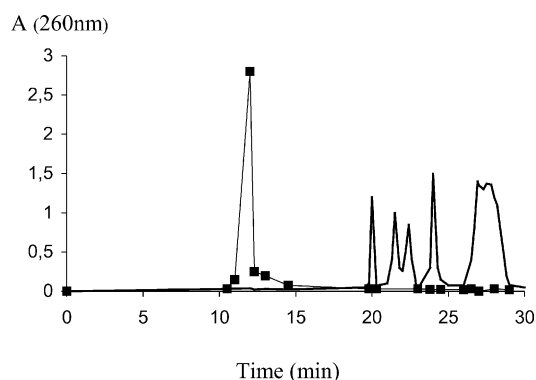


Fig. 5. The analysis of the transformation of vanillic acid with the induced laccase using a RP C18 column: (■) vanillic acid alone, (■) vanillic acid added with the induced laccase.

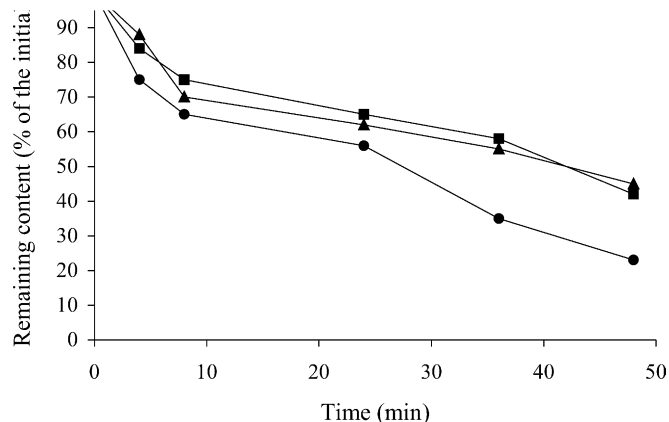


Fig. 6. Transformation of 2-CP, 2,4-DCP and 2,4,6-TCP through time. The results are expressed as the percentage of remaining absorption of the initial peak at 280 nm: (■) 2-CP; (▲) 2,4-DCP; (●) 2,4,6-TCP.

tachlorophenol whatever the experimental conditions used (Fig. 6).

4. Discussion

Previous studies have allowed us to purify various isoforms of laccases produced by strains isolated from evergreen oak [3] or cork oak litter [4]. We have shown that the strains isolated from evergreen oak litter exhibited similar laccase patterns under ferulic acid induction: three isoforms were observed [2]. We have already purified and described the two laccase isoforms with higher molecular weights [3]. In this study we wanted to characterize the third laccase isoform induced with ferulic acid from a *M. quercophilus* strain isolated from a *Q. ilex* L. litter. A similar experiment of induction with ferulic acid [4] has been realized with a *M. quercophilus* strain collected from a cork oak litter (*Q. suber* L.). The biochemical characteristics of this two laccase isoenzymes induced with ferulic acid differ in various ways: optimum pH, thermal stability and V_m and K_m values (Table 1). The optimum pH for laccase activities is usually low, ranging from 3.0 to 4.5 [8] but great differences have been observed between the two isoforms using ABTS and syringaldazine as the substrates. Furthermore, stability at 40 °C was significantly greater for the laccase from strain 19 (48 h instead of 7 h). Decreases in thermal stability of laccases at higher temperatures have already been described in several studies [9]. The optimum temperature found (80 °C) was similar for both laccases from strains 19 and C7. Laccases can exhibit very different optimum temperatures from 30 °C for example with the hyphomycete *Chalara paradoxa* [10] to 80 °C with basidiomycete PM1 [11]. The V_m and K_m values differed considerably from one isoform to another. The values found for strain 19 were close to those observed for other laccases from white-rot fungi such as *Trametes*

Table 1

The biochemical features of the laccases induced with ferulic acid from strains C7 and 19 of the white-rot fungus *Marasmius quercophilus*

	Strain C7	Strain 19
Molecular weight (kDa)	65	60
pH ABTS	2.6	4
pH syringaldazine	6.2	4.5
V_m ABTS ($\mu\text{M min}^{-1}$)	1.1 ^a	3.3
K_m ABTS (mM)	7.7	113×10^{-3}
V_m syringaldazine ($\mu\text{M min}^{-1}$)	7.8×10^{-2}	2
K_m syringaldazine (mM)	50 ^a	4.2×10^{-3}
Optimum temperature ($^{\circ}\text{C}$)	80 ^a	80
Thermal stability at 40 $^{\circ}\text{C}$ (50% activity remaining)	After 7 h ^a	After 48 h
No inhibition	EDTA ^a SDS ^a	EDTA, Cystein SDS

^a Data from Farnet et al. [4].

pubescens [12] or *Ganoderma lucidum* [13]. We also observed that EDTA and cystein did not inhibit laccase from strain 19 although the absence of inhibition was shown only when EDTA was added to the laccase from strain C7. Furthermore, the staining of SDS-PAGE with syringaldazine was possible, which may suggest the absence of inhibition of laccase with SDS for the concentration used. These properties are particularly interesting since other studies have demonstrated a loss of activity of this enzyme with similar inhibitor concentrations when EDTA, cystein or SDS were used as the inhibiting agents. When compared to the other isoforms of laccases previously purified [3], biochemical differences were also pointed out: those isoforms exhibited different optimum pHs for laccase activity with syringaldazine as the substrate (pH 5; pH 5 and 6) and also a greater thermal stability of laccase activities. Thus, this study shows the great variations of the biochemical properties of laccases produced by a single fungal species.

The transformation with laccase of most of the natural aromatic compounds tested showed several new peaks with hydrophobic properties (longer elution times). This suggests a polymerization of these compounds which was probably due to the formation of radicals by the laccase. Few studies have sought to identify the by-products of laccase transformation. Recently, Carunchio et al. [14] have described dimerization of ferulic acid with a laccase. In this study, the authors have identified three intermediates which react to form six different dimers. They separated two major by-products of oligomerization, which allowed them to identify these two compounds using mass spectrometry. It seems that the laccase from *M. quercophilus* forms many different compounds from gallic acid, ferulic acid and tannic acid. This result is observed with or without mediators thus these non-specific reactions are not due to the action of oxidized mediators alone. The radicals formed with laccase seem to interact in different ways, leading to various polymerized compounds.

The transformed chlorophenols tested have chlorine atoms in ortho or para position. The meta-substituted com-

pounds have been found not to be easily transformed [15]. Indeed, dechlorination of halogenated compounds has been reported with laccase in ortho or para positions with respect to the hydroxyl group of the same ring [16]. In this study, the transformation of chlorophenols has shown that the order of reactivity of the induced laccase was 2,4,6-TCP > 2,4-DCP > 2-CP. This is in correlation with the study of Itoh et al. [17] which have described the action of a laccase towards several different chlorophenols. Thus, the reactivity of laccase increases with the number of chlorine on the cycle. This may be better explained by the increase in proton acidity of the hydroxyl group because of the chlorine atoms than by a higher specificity of laccase for this chemical compound. The formation of the radical may be favored by the electroattractive property of the chlorine atoms. With chloroaromatic, we have also observed new several peaks at the end of the elution gradient that suggests an oligomerization as described by Leontievsky et al. [18]. For PCP, Aiken and Logan [19] have reported its biodegradation with the white-rot fungus *Phanerochaete chrysosporium*. Nevertheless, few studies have described its transformation by white-rot fungus polyphenoloxidases.

In further studies, we will attempt to assess the transformation of aromatic compounds using the supernatant of a culture from *M. quercophilus*. This would allowed us to evaluate the potential effect of other enzymes such as peroxidases which are also produced by this fungus. The synergistic action of both phenoloxidases may improve the biotransformation of such recalcitrant compounds.

References

- [1] Tagger S, Périssol C, Gil G, Vogt G, Le Petit J. Phenoloxidases of the white-rot fungus *Marasmius quercophilus* isolated from an evergreen oak litter (*Quercus ilex* L.). *Enzyme Microbial Technol* 1998;23:372–9.
- [2] Farnet AM, Tagger S, Le Petit J. Effects of copper and aromatic inducers on the laccases of the white rot fungus *Marasmius quercophilus*. In: *Comptes Rendus de l'Académie des Sciences, Paris. Sciences de la Vie/Life Sciences* 1999;322:499–503.
- [3] Farnet AM, Criquet S, Tagger S, Gil G, Le Petit J. Purification, partial characterization and reactivity with aromatic compounds of two laccases from *Marasmius quercophilus* strain 17. *Can J Microbiol* 2000;46:1–6.
- [4] Farnet AM, Criquet S, Pocachard E, Gil G, Ferré E. Purification of a new isoform of laccase from a *Marasmius quercophilus* strain isolated from a cork oak litter (*Quercus ilex* L.). *Mycologia* 2002;94:735–40.
- [5] Bourbonnais R, Leech D, Paice MG. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochem Biophys Acta* 1998;1379:381–90.
- [6] Harkin JM, Larsen MJ, Obst JR. Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. *Mycologia* 1974;66:469–76.
- [7] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.
- [8] Heinzkill M, Bech L, Halkier T, Schneider P, Anke T. Characterization of laccases from a wood-rotting fungi (family *Coprinaceae*). *Appl Environmental Microbiol* 1998;64:1601–6.
- [9] Nyanhongo GS, Gomes J, Gübitz G, Zvauya R, Read JS, Steiner W. Production of laccase by a newly isolated strain of *Trametes modesta*. *Bioresource Technol* 2002;84:259–63.

- [10] Robles A, Lucas R, Martinez-Canamero M, Ben Omar N, Perez R, Galvez A. Characterisation of laccase activity produced by the hyphomycete *Chalara* (syn. *Thielaviopsis*) *paradoxa* CH 32. *Enzyme Microbial Technol* 2002;31:516–22.
- [11] Coll PM, Tabernero C, Santamaria R, Perez P. Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). *Appl Environ Microbiol* 1993;59:4129–35.
- [12] Galhaup C, Goller S, Peterbauer CK, Strauss J, Haltrich D. Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiology* 2002;148:2159–69.
- [13] Ko E-M, Leem Y-E, Choi HT. Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. *Appl Microbiol Biotechnol* 2001;57:98–102.
- [14] Carunchio F, Crescenzi C, Girelli A-M, Messina A, Tarola A-M. Oxidation of ferulic acid by laccase: identification of the products and inhibitory effects of some peptides. *Talanta* 2001;55:189–200.
- [15] Filazzola MT, Sannino F, Rao MA, Gianfreda L. Effect of various pollutants and soil-like constituents on laccase from *Cerrena unicolor*. *J Environ Quality* 1999;28:1929–38.
- [16] Schultz A, Jonas U, Hammer E, Schauer F. Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. *Appl Environ Microbiol* 2001;67:4377–81.
- [17] Itoh K, Fujita K, Suyama K, Yamamoto H. Phenolic acids affect transformations of chlorophenols by a *Coriolus versicolor* laccase. *Soil Biol Biochem* 2000;32:85–91.
- [18] Leontievsky AA, Myasoedova NM, Baskunov BP, Golovleva LA, Bucke C, Evans CS. Transformation of 2,4,6-trichlorophenol by free and immobilized fungal laccase. *Appl Microbiol Biotechnol* 2001;57:85–91.
- [19] Aiken BS, Logan B. Degradation of pentachlorophenol by the white-rot fungus *Phanerochaete chrysosporium* grown in ammonium lignosulfonate media. *Biodegradation* 1996;7:175–82.