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# Purification and partial characterisation of a 60 KDa laccase from *Fomitiporia mediterranea*

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**Summary.** A 60-kDa extra-cellular laccase enzyme was purified to homogeneity from a liquid culture of *Fomitiporia mediterranea,* the main wood rot agent of the grapevine disease esca. The enzyme exhibited thermal stability. Substrate specificity and inhibitor studies indicated that the enzyme was a typical fungal laccase. Several natural phenolic and polyphenolic compounds were oxidised by the isolated laccase.

**Key words**: *Vitis vinifera*, esca, wood-rot fungi.

## Introduction

Esca is a trunk disease of grapevine frequent in vineyards all over the world, that leads to a slow decline and the death of the plant. The disease is a complex disease including a vascular disease and an internal white rot of the trunk, which gradually changes the hard wood to a soft, friable, spongy mass (Graniti *et al*., 1994; Mugnai *et al*., 1999). *Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and the basidiomycete *Fomitiporia mediterranea* (*Fmed*) (Fischer, 2002) constitute the complex of pathogens associated with the diseases forming the esca complex. Some of these xylem-inhabiting fungal pathogens are known to produce several phythotoxins (Evidente *et al*., 2000; Tabacchi *et al*., 2000; Abou-Mansour *et al*., 2004). *F. mediterranea* produces various extra-cellular lignin-degrading enzymes, including peroxidases and laccases (Mugnai *et al*., 1999). Most known laccases are of fungal origin, especially from fungi causing white rot (Mayer *et al*., 2002). The laccase family is involved in several physiological functions, the plant laccases in lignin biosynthesis, and the fungal laccases in plant pathogenesis, insect sclerotisation, and depolymerisation of lignin (Thurston, 1994).

*Phellinus igniarius* (L.) Quél. and *Fomitiporia punctata* (P. Kast.) Murrill (formerly called *Phellinus punctatus* [P. Kast.] Pilat) are the basidiomycetes most frequently implicated in the white rot of grapevine trunks. *Fomitiporia mediterranea* has been distinguished as a new species distinct from *Fomitiporia punctata* (Fisher, 2002). The genus *Phellinus* is known to produce laccases as described by Min *et al*., (2001), therefore it is not surprising that *Fomitiporia punctata* and also *Fomitiporia mediterranea* produce wood-degrading enzymes (Bruno and Sparapano, 2007).

In this study, we report for the first time the purification and characterisation of an extra-cellular laccase enzyme from *F. mediterranea*. This experiment constitutes a first step in the analysis of the degradation of vine wood by enzymes of *F. mediterranea*.

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## Materials and methods

### Fungal strain

*Fomitiporia mediterranea* strain 1413 was kindly supplied by Paolo Cortesi (University of Milan, Milan, Italy) and cultivated on potato dextrose agar (Fluka Chemie GmbH, Buchs, Switzerland) in our culture collection.

### Medium and culture conditions

A twenty-day-old culture of *F. mediterranea* was used for laccase preparation. Prior to large-scale cultivation, the best culture conditions for laccase production were determined by culturing *Fmed* in four different conditions: with a 12 h day, in the dark, in shake culture, and in stationary culture. The inoculum was prepared by taking eight disks (0.5 cm diameter) from the edge of mycelium growing in Petri dishes containing potato dextrose agar, and transferring them to four 500 mL Erlenmeyer flasks each containing 250 mL of potato dextrose broth (Fluka). After ten days of shake culture in the dark, the inoculum was homogenised by magnetic stirring. Ten litres of culture medium was prepared as above by adding 20 mL of inoculum to each Erlenmeyer flasks, and shake-culturing at 105 rpm, 25°C, in the dark. Laccase activity in the culture filtrates was measured daily and the enzyme was collected when laccase activity peaked, after which the mycelium was removed by filtration through a cheesecloth.

### Protein and enzyme activity determination

Laccase activity in the culture medium was determined by oxidation of ABTS (Fluka) used as a substrate according to Niku-Paavola (1988). The reaction mixture consisted of 1 mL of fungal culture medium and 1 mL of a solution of 1 mM ABTS dissolved in 100 mM acetate buffer pH 5. After incubation for 10 min at 25°C, the reaction was stopped by adding 1 mL of 5% (w:v) trichloroacetic acid. Formation of the cation radical was detected by measuring the absorbance increase at 420 nm $\varepsilon_{420}$ 36000 $M^{-1}$ $cm^{-1}$ on a UV-visible recording spectrophotometer (Shimadzu UV-160, Kyoto, Japan). During purification, laccase activity was measured with 2,6-dimethoxyphenol (DMP, Sigma, St Louis, MO, USA) according to Wariishi (1993) at 469 nm by monitoring dopachrome formation and using the molar extinction coefficient $\varepsilon_{469}$ 36400 $M^{-1}$ $cm^{-1}$. Activities were expressed as µM $min^{-1}$. One unit of laccase activity was defined as the amount of enzyme that catalysed the oxidation of 1 µM of DMP at 25°C. Protein concentration was determined by the method of Bradford (1976) using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

### Purification of the enzyme

Laccase was purified according to the method of Pezet (1998) partially modified as follows. Clear filtrate (10 L) was concentrated by tangential ultra-filtration (Filtron, 30-kDa cut off, Northborough, MA, USA) to a final volume of 200 mL. Concentrated filtrate was brought to 40% saturation $(NH_4)_2SO_4$, overnight at 4°C and centrifuged at 12,000 rpm for 10 min. The resulting supernatant was brought to 80% saturation $(NH_4)_2SO_4$, overnight at 4°C and centrifuged. The resulting pellets were dissolved in distilled water and dialysed against distilled water overnight at 4°C. The sample (35 mL) was centrifuged and concentrated over polyethylene glycol (PEG 20000, Fluka) to 13 mL (crude extract). The crude extract was loaded on a DEAE column (2.5×16 cm) and suspended in sodium phosphate buffer (0.05 M, pH 5). A step gradient system of NaCl in the buffer (0, 0.1, 0.2, 0.5 M) was used to elute four fractions monitored by a UV detector (Variable Wavelength Monitor, Bruker, Fallenden, Switzerland) at 280 nm. DMP oxidation activity was measured as above. The active fraction was dialysed overnight and the membrane concentrated to 4 mL. Samples of 0.2 mL were loaded on a Superdex 200 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.05 M phosphate buffer (pH 7) containing 0.15 M NaCl at a flow rate of 0.5 ml $min^{-1}$ on a BioLogic DuoFlow System (BioRad Laboratories, Hercules, CA, USA). Active fractions were pooled, dialysed overnight, concentrated and purified a second time on the same column under the same conditions.

### $K_m$ determination

The $K_m$ constant was determined using various concentrations of DMP (2.5–250 mM) as substrates in the normal assay procedures. The experimental data were analysed according to the Lineweaver-Burk plot (Sigma Graphit computer software).

### Electrophoretic analysis

SDS-PAGE was performed with precoated gels (Bio-Rad) according to manufacturer's instructions. The molecular mass of laccase was determined using low range and broad range molecular mass pre-stained standards (103 to 20.7 kDa and 209 to 7.1; Bio-Rad).

### Determination of isoelectric point

The isoelectric point of *F. mediterranea* laccase was determined by isoelectric focusing on precoated gels (Serva, 3–10, pH gradient, 150 mM 125×125 cm, p*I* marker protein mixture) on an LKB 2117 Multiphor II Electrophoresis System (LKB Pharmacia, Bromma, Sweden) according to

manufacturer's instructions. Bands containing laccase activity were visualised by staining the gel with *p*-phenylenediamine (35 mg 100 $mL^{-1}$, in citrate buffer, 0.1 M, pH 5.2) as described by Pezet *et al*. (1991) and proteins were visualised by the Coomassie Blue staining method (Bio-Rad).

**Spectroscopic studies**

The absorption spectrum was determined at room temperature between 600 and 220 nm on a UV detector (Bruker variable wavelength monitor).

**Temperature stability and pH optimum**

The optimum pH for purified *F. mediterranea* laccase was estimated by monitoring laccase activity at pH 2–4 in 50 mM citrate buffer and at pHs 4.1–8.9 in 50 mM sodium phosphate buffer, using DMP and ABTS as substrates. To determine the optimum temperature at which the laccase reacts, the activity was measured at temperatures ranging from 10 to 70°C. Temperature was measured inside the spectrophotometer cell. Temperature stability was determined by incubating the enzyme solution in 50 mM citrate buffer (pH 3) at 50°C for 16 h, and determining the residual enzyme activity with DMP as a substrate.

**Inhibition and substrate specificity studies**

Effect of the potential inhibitor dithiothreitol on laccase activity was determined with 25 mM DMP as the substrate, in 50 mM sodium phosphate buffer (pH 3) at 50°C and the presence of the inhibitor at a concentration of 0.1 and 0.05 mM.

**HPLC analysis of oxidation of phenolic and aromatic compounds**

The oxidation of fifteen phenolic and aromatic compounds was assayed. Ten µL of the enzyme (86 ng proteins $\mu L^{-1}$) was added to 100 µL of the substrate at 5 mM (dissolved in 20 µL of ethanol) and adjusted to 1 mL with 0.05 M citrate buffer pH 3 at 40°C. All compounds were purchased from Fluka. Controls were done using the enzyme and the solvent without the compounds, and the substrates without the enzyme. The reactions were stopped after 30 min by freezing at –20°C. Before analysis, each sample was thawed and 50 µL of the mixture was analysed by an HPLC system (Agilent 1100 series) coupled with a diode array detector and an LC/MSD trap. Analysis was carried out on a Nuclesosil column (MN, 100-5 C18, 250×4 mm i.d.), with a gradient of eluent as follows: A, water/acetonitrile/acetic acid (93/3/2, v:v:v) and B, acetonitrile/water/acetic acid (95/3/2, v:v:v), starting from A 0% and reaching 40% of B in 20 min, and 100% in 26 min at a flow rate of 0.5 mL $min^{-1}$. The decrease of the compounds in the reaction mixture was estimated by measuring the peak areas of UV absorbance at 280 nm except for 2,5-dihydroxybenzaldehyde and 2,3-dihydroxybenzoic acid, which were measured at 260 nm. All experiments were duplicated and the results are shown as means of the average values.

## Results and discussion

**Laccase production in batch culture**

In a preliminary study, oxygen and light were investigated as physiological factors in relation to the expression of laccase activity. Four different conditions were chosen: with a 12 h day, in darkness, with shaking, and without shaking. Maximum laccase expression was reached after 9 days of growth in the shake culture in the dark. With the 12h day, laccase activity peaked after 8 days but was two-fold less active than in darkness (Figure 1). In stationary culture, laccase activity did not increase after 14 days of growth. Laccase activity is apparently constitutive, but oxygenation is crucial to induce laccase production and enhance its activity. Enzyme production increased steadily in the extra-cellular fluid, and peaked at day 9. In the following day activity decreased however, as previously noted with other fungi, including *Rigidoporus lignosus* (Bonomo *et al*., 1998), *Trametes pubescens* (Galhaup *et al*., 2002) and *Phanerochaete chrysosporum* (Dittmer *et al*., 1997).

**Enzyme purification**

Laccase activity in a culture medium was determined by the oxidation of ABTS, a known peroxidase substrate, but for enzyme purification, DMP was used, since this is more suitable for detecting phenoloxidases and laccases (Solano *et al*., 2001).

Enzyme was purified to near homogeneity by $(NH_4)_2SO_4$ (Figure 2) selective precipitation, ion exchange and gel filtration chromatography. Overall, an approximately about 10-fold purification and a yield of 0.5% with a specific activity of 3658 µmol • $min^{-1}$ $mg^{-1}$ of protein was achieved (Table 1).

The molecular mass of the laccase was estimated at 60.8 kDa by Superdex chromatography (data not shown) and by SDS-Page (Figure 2). The molecular mass of the purified laccase was consistent with the molecular masses of most other fungal laccases, which are reported to be between 60 and 80 kDa (Thurston, 1994). Isoelectric focusing analysis of purified laccase showed three major isoform bands at pHs 4.0, 3.9 and 3.2, indicating different glysolated isoforms when stained with *p*-phenylenediamine (Figure 3). The p*I*s of *F. mediterranea* laccase were in the acidic range, which was in accordance with p*I*s reported for the laccases of other white rot fungi, including *Pleurotus eryngii* and *P. ostreatus* (p*I* 3.9 to 4.7) (Palmieri *et al*., 1993; Munoz *et al*., 1997; Giardina *et al*., 1999) and *Pycnoporus cinnabarinus* (p*I* 3.7) (Eggert *et al*., 1996).

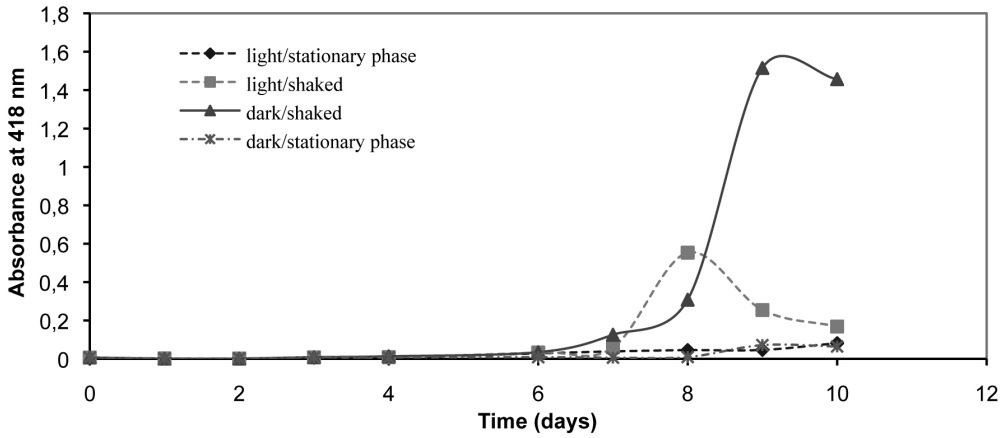


Figure 1. Time course of laccase production by *Fomitiporia mediterranea*.

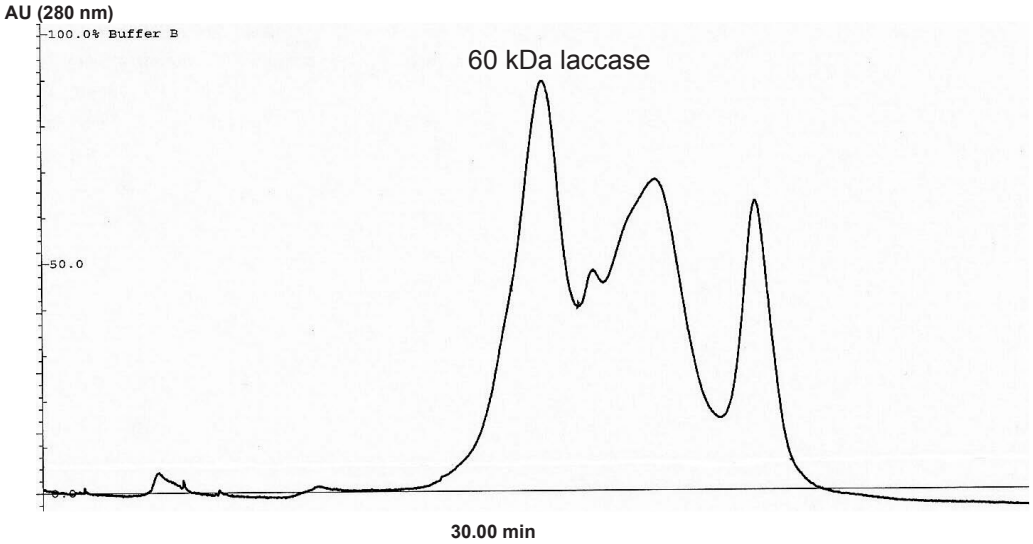


Figure 2. Purification profile of the laccase isolated from *Fomitiporia mediterranea* with sodium phosphate buffer 50 mM pH7 and 15 M NaCl.

Table 1. Purification steps for the laccase produced by *Fomitiporia mediterranea*.

| Purification step | Volume (ml) | Total protein (mg) | Total enzyme ($\mu$moles $min^{-1}$) | Specific activity units ($\mu$moles $min^{-1}$ $mg^{-1}$) |
|---|---|---|---|---|
| Culture filtrate | 10 000 | 329 | 124 740 | 379 |
| Concentrated ultrafiltrate | 200 | 118.51 | 75 449 | 636,7 |
| Crude extracts $(NH_4)_2SO_4$ | 35 | 47.85 | 27 804 | 581,1 |
| DEAE (0,1 M NaCl) | 8 | 4.53 | 45 491 | 10 055 |
| Superdex | 1 | 0.16 | 600 | 3658,5 |

**$K_m$ determination**

The relationship between laccase activity and substrate concentration produced a typical Michaelis-Menten curve. The Km was 25 mM for DMP, which is considered as specific substrate for laccase.

**Spectrum**

The UV-visible absorption spectrum of the purified enzyme showed three peaks at 280, 340 and 620 nm. The peak at 620 nm is typical for type I Cu (II), and the peak at 340 nm suggests the presence of the type III binuclear Cu (II) pair. These characteristics indicated that all three types of copper atoms were present (Eggert *et al*., 1996).

**Laccase activity in relation to pH and temperature**

The pH optima for the purified laccase were determined with ABTS and DMP as substrates (Figure 3A). The optimum pH was 3 for both substrates. However, oxidation with DPM showed a broad optimum pH from 2 to 6, like that of many other fungal laccases, whereas for ABTS it was narrower (Xu, 1997; Robles *et al*., 2000). The purified enzyme retained high activity at pH 2 with both ABTS and DMP (68 and 86% respectively). However, at pH 8 the enzyme lost all activity.

The activity of purified laccase from *F. mediterranea* was determined at various temperatures (10–70°C). The enzyme showed its highest activity at 40°C using ABTS and at 50°C using DMP. Activity with ABTS dropped sharply above 40°C. Below 10°C, laccase activity dropped with both substrates (Figure 3B). After 16 h incubation of the enzyme at 50°C and at pH 3, 13% of activity against DMP remained, indicating relatively good thermostability.

**Substrates and inhibitors**

The purified enzyme oxidised many phenolics and polyphenolic compounds (Table 2). Several classes of chemical compounds were investigated: (i) *p*- and *m*- substituted phenols, such as L-tyrosine and 2,6-dimethoxyphenol; (ii) mono and disubstituted phenolic acids (4-hydroxybenzoic acid, caffeic acid, vanillic acid, ferulic acid, 2,3 dihydrobenzoic acid, veratric acid); (iii) mono, di and trisubstituted phenolic aldehydes (2,5-dihydroxybenzaldehyde, vanillin, syringaldehyde); (iv) other substrates, such as catechin resveratrol and lignin. Laccase oxidised phenolic acids such as ferulic, vanillic and caffeic acids (lignin depolymerisation products) and, to a lesser extent veratric acid, but it did not oxidise *p*-hydroxybenzoic acid. Phenolic aldehydes, syringaldehyde, vanillin (4-hydroxy-3-methoxybenzaldehyde) and 2,5 dihydroxybenzaldehyde were likewise not oxidised by laccase. Maximum laccase activity was associated with the substitution of a hydroxyl in *para* associated with a methoxyl in position *meta* in the phenolic acids. Resveratrol, catechin and lignin were oxidised at 26, 34 and 39% respectively. The oxidation products of resveratrol were analysed by HPLC-MS and four compounds were detected. Three of these compounds exhibited a molecular ion as base peak at m/z 471 $[M-H]^-$ and one

Table 2. Substrate specificity of purified laccase from *Fomitiporia mediterranea*.

| Chemical Family | Substrate | Oxidation (%) [a] |
|---|---|---|
| Phenolic acids | Phenolic acids 4-hydroxybenzoic acid | No |
| | 2,3-dihydroxybenzoic acid | 14 |
| | ferulic acid | 92 |
| | vanillic acid | 91 |
| | caffeic acid | 68 |
| | veratric acid | 24 |
| | 4-hydroxybenzaldehyde | No |
| | 2,5-dihydroxybenzaldehyde | 3 |
| Phenolic aldehydes | syringaldehyde | 7 |
| | vanillin | No |
| | tyrosine | 7 |
| Phenols | 2,6-dimethoxyphenol | 58 |
| | resveratrol | 26 |
| Polyphenolic compounds | catechine | 34 |
| | lignin | 39 |

[a] No, no oxidation detected.

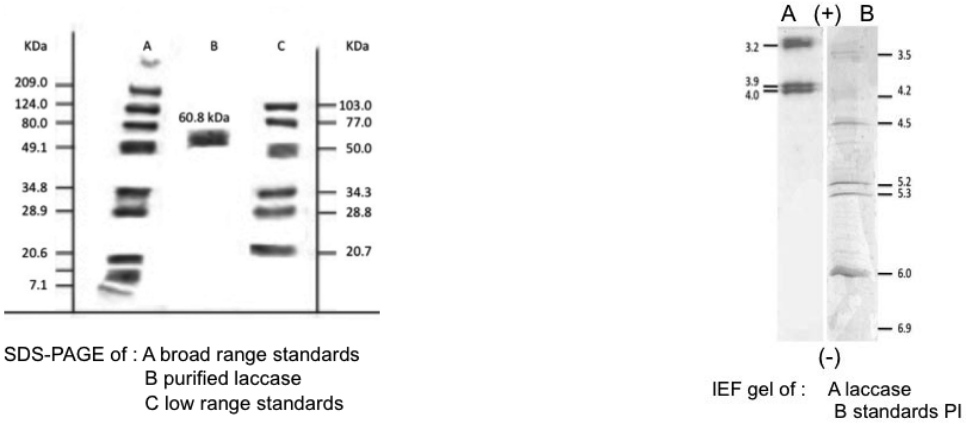


Figure 3. SDS-PAGE and IEF of purified laccase from Fomitiporia *mediterranea*.

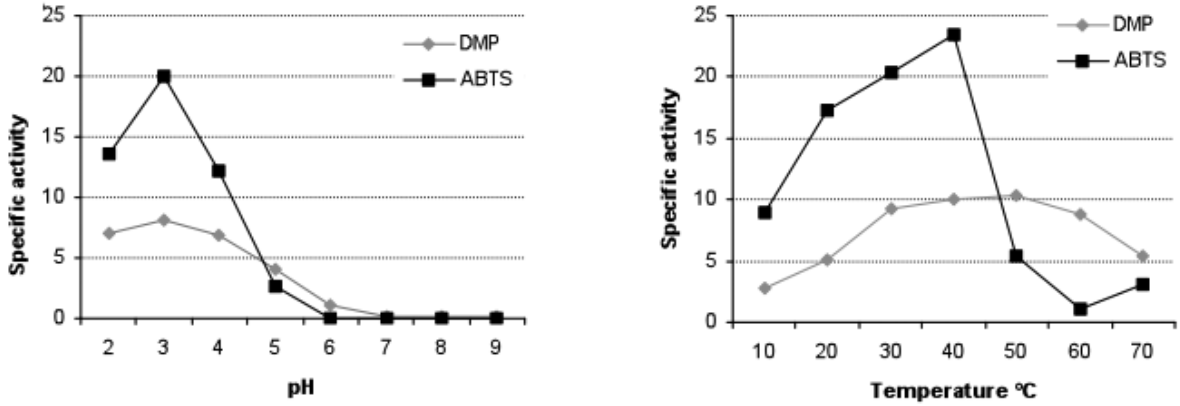


Figure 4. pH A, and B, temperature activity profiles of purified laccase from *Fomitiporia mediterranea*.

at 499. The UV spectra of these compounds exhibited a maximum at 280 nm and a loss of the extended conjugation of the resveratrol at 307 nm. Tyrosine was not a substrate, so that laccase does not belong to the tyrosinase-type polyphenol oxidases. However, dithiothreitol, a classical inhibitor of copper-containing oxidases, at a concentration of 0.1 mM inhibited 85% of laccase activity. Many sulfhydryl-containing compounds are laccase-inhibitors (e.g. L-cysteine) (Maicherczyk *et al*., 1999). Based on the substrate specificity and the spectral data we conclude that the enzyme purified from *F. mediterranea* is a true laccase.

## Conclusion

For the first time a laccase that oxidised lignin substrates was purified from *Fomitiporia mediterranea*. We describe the purification and the characterisation of some of the properties of laccase after its large-scale production. *F. mediterranea* can be associated with approximately 50% of grapevines with esca symptoms (Cortesi *et al*., 2000). White-rot fungi produce various isoforms of extra-cellular lignin-degrading enzymes, including lignin peroxidase, manganese peroxidase and laccase. Laccases protect the fungal pathogen from the toxic phytoalexins and tannins in the host environment (Pezet *et al*., 1991). However, such a role has not been investigated for *F. mediterranea* laccases or enzymes. The purification of laccase from *F. mediterranea* is here reported for the first time.

Purification of laccase is an important step to study the expression of this enzyme in gene disruption and to study *in vivo* the biochemical role of laccase enzymes in the infection of grape trunk by *F. mediterranea*.

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