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Short Communication

Alkylphenol oxidation with a laccase from a white-rot fungus: Effects of culture induction and of ABTS used as a mediator

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ABSTRACT

We investigated the potential of the laccase from the white-rot fungus *Marasmius quercophilus* to transform certain alkylphenols (*p*-nonylphenol, *p*-octylphenol and *p*-*t*-octylphenol). We tested the reactivity of this enzyme under different conditions: in liquid cultures and using the partially purified laccase with and without 2,2′-azino-bis-3-ehtylbenzothiazoline-6-sulfonicacid (ABTS) as a mediator. The percentage of *p*-*t*-octylphenol disappearance in liquid cultures was 69.0 ± 1.5% and 81 ± 5% after a 8-d or 15-d incubation, respectively, with *p*-nonylphenol, these percentages were 62 ± 4% and 91 ± 6% and with *p*-octylphenol 37 ± 3% and 65 ± 1% after a 15-d and a 21-d incubations, respectively. Induced pre-cultures were also used to inoculate the liquid cultures to enhance *p*-octylphenol transformation: the percentages of disappearance were 91.0 ± 0.5% and 97 ± 1% after a 8-d and a 15-d incubation, respectively. Mass spectrometry analysis showed that the products of oxidation of *p*-octylphenol were dimers with a mass of 411 *m/z*. Furthermore, we identified a purple compound (*m/z* 476) formed when ABTS was added to the reaction medium with the purified laccase. This result confirms that, in complex environments such as soils or litters where many molecules can interact with the enzyme substrate or the product of oxidation, laccase activities and those of other phenoloxidases should not be measured with ABTS.

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1. Introduction

Many natural and synthetic chemical compounds exhibit estrogenic activities. These are known as endocrine disrupting compounds, EDCs (Mc Lachland, 1985) and include bisphenol A (2,2-bis(4-hydroxy-phenol)propane), various alkylphenols or triclosan (5-chloro-2(2,4-dichloro-phenoxy)phenol). These compounds have significant ecotoxicological effects in aquatic environments contaminated with wastewaters in intensively urbanised areas (Svenson et al., 2003; Peck et al., 2004; Schiliro et al., 2003; Nagarnaik et al., 2010). For instance, these molecules may affect sex ratio changes or reduce fecundity of fish (Nagler et al., 2001; Chowen and Nagler, 2004). Alkylphenols, such as *p*-nonylphenol and *p*-octylphenol, are environmentally persistent molecules with estrogenic activities: they are produced *via* the microbial transformation of alkylphenol polyethoxylates in sewage treatment plants (Tollefsen et al., 2008). Lignin-degrading enzymes i.e. phenoloxidases (laccases, Mn-peroxidases or lignin-peroxidases) have been extensively used to transform many aromatic pollutants such as PAHs (polycyclic aromatic hydrocarbons), chlorophenols or EDCs (Tanaka et al., 2003; Saito et al., 2004; Torres-Duarte et al., 2009; Uhnakova et al., 2009). These enzymes, mainly produced by white-rot fungi, are involved in the transformation of natural aromatic compounds. Moreover, their non-specific oxidation makes the transformation of pollutants with similar aromatic structure possible. Thus these enzymes, which have a broad substrate specificity, have been extensively tested for biotechnological applications (Ghosh et al., 2008).

Marasmius quercophilus, a white-rot fungus which was isolated from Mediterranean ecosystems such as *Quercus ilex* or *Quercus suber* litters, mainly produces laccases. Recent studies have demonstrated their potential in transforming aromatic pollutants such as PAHs or chlorophenols (Farnet et al., 2004, 2008). For instance, these laccases were able to oxidise anthracene to anthaquinone and to remove chloride from chlorophenols. Thus the laccases produced by this fungus seem to exhibit an oxidative potential which can be used towards various aromatic pollutants. This potential can be of major importance in litters colonised by this fungus since it can be involved in detoxification of natural environments. Furthermore these enzymes can easily be used in biotechnological

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applications since they can be produced in high amounts, no co-factors are needed for the reaction they catalyse and they are very stable at quite high temperatures (80% of remaining activity after 48 h at 40 °C, Farnet et al., 2004).

Here, we examine the potential modification of various alkylphenols using the laccase of *M. quercophilus* in liquid cultures or using the purified laccase with or without a common laccase mediator, ABTS. We partially describe the mechanisms of oxidation of laccase with or without ABTS *via* the products of *p*-*t*-octylphenol oxidation which were determined using mass spectrometry.

2. Materials and methods

2.1. Isolation of the strain

M. quercophilus strain 19 was isolated from an area of the site of La Gardiole de Rians, Var, France (10 000 m^2) using the rhizomorphic form of the fungus. First, a fungal cap culture was made on a malt-agar medium, 20 g L^{-1} (Bio Mérieux, Marcy l'Etoile, France) and chloramphenicol, 50 mg L^{-1} (Sigma). Second, 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. Partial purification of the laccase of M. quercophilus

The partial purification was performed as described by Farnet et al. (2004). Pre-cultures were realised in 200 mL Erlenmeyer flasks containing 50 mL of malt extract liquid medium, 20 g L^{-1} (MEL) 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 pre-cultures were used to inoculate two 3000 mL Erlenmeyer flasks with 800 mL of malt extract medium (20 g L^{-1}), Tween 80, 0.05%, $CuSO_4$, 5 mg L^{-1}. They were incubated at 25 °C for 12 d under axial shaking. Enzyme activity was measured by monitoring the oxidation of syringaldazine [N,N′-bis-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine] to its quinone (ε^M: 6.5×10^4 M^{-1} cm^{-1}) at 525 nm (Harkin et al., 1974) on a spectrophotometer Kontron Uvikon 860 (Serlabo Technologies, Entraigues, France). The assay contained 100 µL of the culture medium, 2.5 mL of acetate buffer 0.1 M, pH 4.5 and 10 µL of syringaldazine 0.6% (w/v), diluted in methanol. The blank consisted of 100 µL of the culture medium and 2.5 mL of the same acetate buffer. Peroxydase activities were also measured using H_2O_2 and $MnSO_4$. When laccase activity was detected, cultures were filtered on a glass microfibre filter GF/D, 2.7 µm (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 acetate buffer 10 mM, pH 4.5 in a final volume of 15 mL. The concentrated filtered medium was loaded on an ion-exchange Mono Q column in fast performance liquid chromatography (FPLC) equilibrated with phosphate buffer 0.1 M pH 6.0. Laccase was eluted with a step gradient (0.2 M, 0.4 M, 2 M NaCl) for 30 min at a flow rate of 1 mL min^{-1} (Farnet et al., 2004). Laccase activity was measured as described above to detect fractions containing laccase. Laccase purity was checked on a polyacrylamide gel electrophoresis (PAGE) carried out according to Laemmli (1970) using 4% stacking gel and 7.5% separating gel at 220 V with the Mini-Protean II electrophoresis cell (Bio-rad) and protein was stained using the Coomassie Blue standard method.

2.3. Cultures of M. quercophilus with alkylphenols

Cultures, previously inoculated with 10 mL of preculture of strain 19, were performed for 8 or 15 d on a rotary shaker (200 rpm) at 25 °C in 1 L-Erlenmeyer flasks with 200 mL of MEL including 50 mg of *p*-octylphenol, 4-(1,1,3,3-tetramethylbutyl)-phenol, (*p*-*t*-octylphenol) or *p*-nonylphenol. From day four, laccase activities were measured every day with syringaldazine as the substrate as described above. Cultures with 800 mL of MEL and 200 mg of alkylphenols were also performed. Different control cultures without mycelium were performed: control cultures after autoclaving and control cultures incubated in the same conditions and for both incubation periods assess alkylphenol evaporation during sterilisation or incubation time, respectively. Each experiment was performed in three replicates. Induction experiments were also performed using pre-cultures with 200 mL of MEL with *p*-octylphenol (50 mg L^{-1}). These pre-cultures were incubated for 8 d at 25 °C and then were used to inoculate cultures with 200 mL of MEL with *p*-octylphenol (250 mg L^{-1}).

For all these experiments, cultures were filtered and both the mycelium and the supernatant were extracted three times with 200 mL of dichloromethane. The solvent was then evaporated and the solid extract solubilised in 1 mL of ethanol. Thirty microliter of dilution 10^{-3} were injected in the HPLC system. Alkylphenol transformation was calculated as percentages using calibration curve described previously.

2.4. Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE)

For all the cultures with alkylphenols, SDS-PAGE were performed in order to check whether any laccase isoforms were induced. Electrophoresis was carried out according to Laemmli (1970) using 4% stacking gel and 12% separating gel at 220 V with the Mini-Protean II electrophoresis cell (Bio-Rad). For laccase activity staining, *p*-phenylenediamine (0.1%) was used as the substrate in acetate buffer 0.1 M, pH 4.

2.5. Statistical analysis

The non-parametric U-test of Mann-Whitney was used to separate significantly different means ($P < 0.05$) of remaining alkylphenols under the different experimental conditions. These analyses were performed using SPSS 11.0 statistical software (SPSS Inc., Chicago, IL).

2.6. Analysis of alkylphenol transformation with high performance liquid chromatography (HPLC)

HPLC system was equipped with a C18 Reverse Phase column (Merck, 4.6 × 250 mm) in the following gradient system: solvent A, (water/trifluoro acetic acid 0.1%)/acetonitrile 90/10 v/v, solvent B (water/trifluoro acetic acid 0.1%)/acetonitrile 95/5 v/v, gradient = 5–15 min, A 100% to B 100%; 15–25 min B 100% (flow rate 1 mL min^{-1}). The alkylphenols tested were transformed realised using the laccase extract. The reaction mixture (5 mL) contained ABTS 1 mM, alkylphenol 250 mg L^{-1} and 5 U of laccase in acetate buffer 0.1 M pH 4.5 and 10% ethanol. The reaction mixtures were incubated at 30 °C for 6 h in the dark. An extraction with 5 mL of dichloromethane was performed for each reaction mixture prior to injection. The organic phase was evaporated and the products of extraction were dissolved in 1 mL of acetonitrile (30 µL injected). The alkylphenols used were: *p*-octylphenol, *p*-*t*-octylphenol and *p*-nonylphenol. All alkylphenols were purchased from Sigma. The same reactions were performed without mediators. Controls were also realised using only ABTS with laccase and using

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