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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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A B S T R A C T

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-ethylbenzothiazoline-6-sulfonic acid (ABTS) as a mediator. The percentage of *p*-*t*-octylphenol disappearance in liquid cultures was $69.0 \pm 1.5\%$ and $81 \pm 5\%$ after a 8-d or 15-d incubation, respectively, with *p*-nonylphenol, these percentages were $62 \pm 4\%$ and $91 \pm 6\%$ and with *p*-octylphenol $37 \pm 3\%$ and $65 \pm 1\%$ 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 $91.0 \pm 0.5\%$ and $97 \pm 1\%$ 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.

Keywords:

Endocrine disruptive chemicals

Laccase

Mediators

Polymerisation

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 anthraquinone 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 cofactors 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 m²) 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%, CuSO₄, 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 × 10⁴ 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 H₂O₂ and MnSO₄. 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-Protein 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-Protein 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

ABTS with the alkylphenol but without laccase. All these controls were incubated at 30 °C for 6 h. *p*-Hydroxybenzoic acid was used as reference in each injection. A calibration curve was also performed for alkylphenol concentrations ranging from 50 to 400 µM. Results were expressed as percentages of alkylphenol disappearance.

2.7. MS analysis

Mass spectrometry was performed with a Spectrophotometer QStar Elite (Applied Biosystems SCIEX) equipped with an electrospray atmospheric pressure ionisation (ESI) source in the positive ion monitoring mode. The ESI conditions were set as follows: electrospray voltage (ESV), 5500 V, orifice voltage (OR), 70 V, gas nebulisation pressure, 20 psi (138 kPa), collision energy (CE), 15 eV.

3. Results and discussion

M. quercophilus is a white-rot basidiomycete whose laccases have been involved in the transformation of various organic pollutants, mainly chlorophenols and PAH such as anthracene (Farnet et al., 2004, 2008). Here, we investigate the potential of these enzymes to transform different alkylphenols using both liquid cultures and a partially purified isoform of laccase.

Different culture conditions were used to test alkylphenol transformation in liquid cultures with *M. quercophilus*. Both liquid medium and mycelium were extracted to check whether any adsorption of alkylphenols occurred on the hyphae. Liquid cultures of 200 mL containing alkylphenols at 200 mg L⁻¹ were performed. Under these conditions, the percentages of transformation were particularly high after 15-d incubation with *p*-*t*-octylphenol and *p*-nonylphenol (81 ± 5% and 91 ± 6% respectively), while the weaker percentage was obtained with *p*-octylphenol (37.0 ± 3.5%). The transformation of these molecules was also tested in cultures of 800 mL with 200 mg L⁻¹ of alkylphenols. After a 21-d incubation, the percentages of disappearance of *p*-octylphenol, *p*-nonylphenol and *p*-*t*-octylphenol were 100%, 92% and 90%, respectively (data not shown). Thus, the partial solubility of alkylphenols in water did not affect their transformation by the *M. quercophilus* laccase. No solvents were added in the culture medium since their toxicity towards the fungus may have altered its growth. It is also noteworthy that the only phenoloxidase activity detectable in the culture medium was that of laccase, which shows that neither Mn-peroxidase nor lignin-peroxidase were responsible for this transformation.

In order to enhance *p*-octylphenol transformation, laccase induction *via* pre-cultures with *p*-octylphenol (50 mg L⁻¹) were performed. These pre-cultures were then used to inoculate the cultures to test *p*-octylphenol transformation (250 mg L⁻¹). This time, the percentages of disappearance were 91.0 ± 0.5% and 97 ± 1% after a 8-d and a 15-d incubation, respectively (Table 1). Thus inducing pre-cultures significantly enhances the percentage of *p*-

Table 1
Percentages of transformation of alkylphenols in cultures of *Marasmius quercophilus* after different incubation times.

Incubation time (d)	% of Transformation		
	<i>p</i> -Octylphenol	<i>p</i> - <i>t</i> -Octylphenol	<i>p</i> -Nonylphenol
8	15.0 ± 1.5 (57 ± 1 ^a)	69.0 ± 1.5	62 ± 4
15	37.0 ± 3.5 (97 ± 1 ^a)	81 ± 5	91 ± 6
21	65 ± 0.8	90 ± 2	92 ± 1

^a % of transformation of *p*-octylphenol obtained with cultures inoculated with induced pre-cultures.

octylphenol transformation. Laccase induction has been reported under different conditions such as antagonisms between microorganisms (Velazquez-Cedeno et al., 2007) or when cultures are complemented with aromatic compounds (Farnet et al., 1999). Induction can either lead to the production of new isoforms of enzymes or enhance the production of the constitutive isoform with an increase in laccase activity as a result. Here, laccase was produced for a longer time in cultures inoculated with pre-cultures induced with *p*-octylphenol (Fig. 1a). Laccase activity indeed increased after 11- and 14-d incubations in induced cultures while very low activity was observed after an 11-d incubation. SDS-PAGE did not reveal new laccase isoforms when *p*-octylphenol was added to the culture medium: only one isoform was produced ($R_f = 0.47$) whatever the experimental conditions (Fig. 1b). However, on lane b, with induced pre-cultures, the laccase band is strongly stained, showing higher activity under these culture conditions and for the same quantity of proteins in the samples loaded into the gel. Thus, this result suggests that, in our study, the production of the constitutive laccase isoform was enhanced, leading to higher *p*-octylphenol transformation yields. Tanaka et al. (2003) have found that laccase from *Trametes sp.* and *Pycnoporus coccineus* decrease the amount of *p*-nonylphenol, *p*-octylphenol and bisphenol A adsorbed on sea sand and the products of oxidation of *p*-nonylphenol are polymers. Sakuyama et al. (2003) have also observed polymers from the oxidation of alkylphenols with Horseradish peroxidase.

Here, whatever the experimental conditions used, no products of transformation were observed in the extract from cultures. In order to identify the oxidation products, we also tested the transformation of these compounds using the purified isoform of laccase with and without mediators. In these experiments, acetone was used as a solvent for alkylphenols since a previous study has shown that a concentration of 10% of acetone alters weakly *M. quercophilus* laccase activity (Farnet et al., 2008). After laccase purification from *M. quercophilus* cultures on MEL one band on SDS-PAGE (data not shown) was obtained with a molecular weight of 60 KDa as described previously (Farnet et al., 2008). Without ABTS, alkylphenol oxidation by laccase was weak: the percentages of transformation were 37 ± 2% and 20.0 ± 1.5% for both *p*-*t*-octylphenol and *p*-nonylphenol and for *p*-octylphenol respectively. When ABTS was added to the reaction mixture, oxidation yields were 92 ± 1%, 67 ± 2% and 47.0 ± 1.5% for *p*-*t*-octylphenol, *p*-nonylphenol and *p*-octylphenol respectively. Thus, the use of mediators leading to chemical oxidation strongly increases the yields of the reaction. Cabana et al. (2007) tested the oxidation of *p*-nonylphenol and bisphenol A with laccases from *Coriolopsis polyzona*. They identified oligomers as products of oxidation with both the endocrine disrupting chemicals tested. Here, MS analysis of *p*-octylphenol products of oxidation (Fig. 3a) indeed show a compound of *m/z* 411 which can be assigned to dimers of *p*-octylphenol (C₂₈H₄₃O₂). This compound is indeed ionised in two fragments with *m/z* 299 and 187: the loss of two fragments of *m/z* 112 (411 – 112 = 299 and 299 – 112 = 187) may be explained by the loss of ethylene and 1-hexene from the octyl chain from *p*-octylphenol. This mechanism can be extrapolated for both dimers of *p*-octylphenol (C–C and C–O dimers). This result can be of major importance since steric hindrance of the dimers obtained may limit the biological effects of these molecules. Cabana et al. (2007) have demonstrated that the estrogenic effect of nonylphenol and bisphenol A dramatically decreases after oxidation with laccases. Tamagawa et al. (2007) have also observed this result with the oxidised products of *p*-*t*-octylphenol after laccase and Mn-peroxydase oxidation. Thus, though laccase oxidation does not lead to the degradation of such compounds, estrogenic effects of alkylphenols on organisms may decrease because of their structure modification *via* polymerisation.

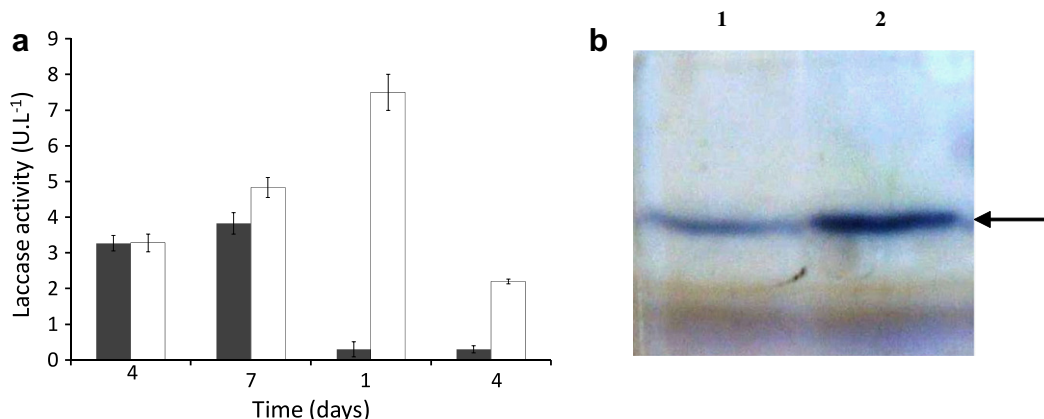


Fig. 1. (a) Laccase activity in cultures of *Marasmius quercophilus* with *p*-octylphenol (200 mg L⁻¹). Cultures were inoculated: (■) with standard pre-cultures or (□) with pre-cultures induced with *p*-octylphenol (50 mg L⁻¹). (b) SDS-PAGE patterns of laccase isoform from cultures of *Marasmius quercophilus* on malt extract without (1) and with (2) *p*-octylphenol. The arrow indicates the laccase isoform stained with *p*-phenylenediamine.

We also tested alkylphenol oxidation using ABTS as a mediator of laccase activity. This mediator was chosen since previous studies (Pickard et al., 1999; Baiocco et al., 2003; Saito et al., 2004) have shown that ABTS is more efficient than HBT (hydroxybenzotriazole) most probably because of the high redox potential of this molecule. Whatever the alkylphenol tested, the reaction mixture became purple and the chromatogram from HPLC revealed a compound absorbing at 550 nm (Fig. 2). HPLC-MS-MS was used to identify this compound which has a mass of 476 *m/z* (C₂₃H₃₀O₄N₃S₂). The mass spectrum of this fragment contained only one fragment of 244 *m/z*. Åkerström et al. (2007) have studied the oxidation of tyrosin with ABTS⁺ and found a similar purple compound (451 *m/z*). They show that the mass spectrum of ABTS⁺ contains three fragments of 244, 259 and 272 *m/z* and suggest that the purple compound combines both the fragment from ABTS⁺ (244 *m/z*) and tyrosine. This result suggests that, in our study, ABTS⁺ reacts with alkylphenols and that this reaction leads to a

purple product. The structure of the fragment of 244 *m/z* described by Åkerström et al. (2007) is presented in Fig. 3b and indicates the presence of a π -conjugated system which explains the purple colour of the compound. Fig. 3b also suggests the probable structure of the purple compound (476 *m/z*) produced from the reaction between ABTS⁺ and *p*-octylphenol. Thus, this study shows that ABTS⁺ interacts with the substrate which has to be oxidised and this reaction produce another molecule. This result should be taken into account when ABTS is used as a mediator since it leads to by-products and affects oxidation yields.

Previous studies have demonstrated that ABTS cannot really be considered as a relevant substrate for laccase activities since the oxidised form, ABTS⁺, is highly reactive and used as a mediator of laccase oxidation (Terrón et al., 2004; Farnet et al., 2009). Thus, abiotic reactions may occur during enzyme measurement, leading to a misinterpretation of the results. The present study confirms that, in complex environments such as soils or litters where many

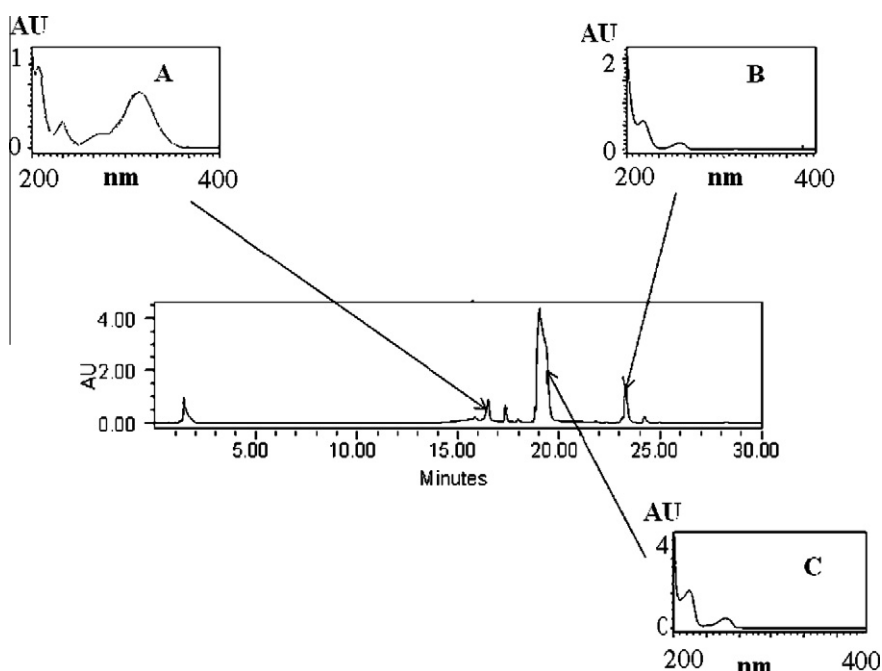


Fig. 2. Chromatogram of *p*-octylphenol (C) oxidised by the laccase-ABTS system with different products of oxidation: (A) a purple compound derived from a reaction between ABTS⁺ and *p*-octylphenol and (B) dimers.

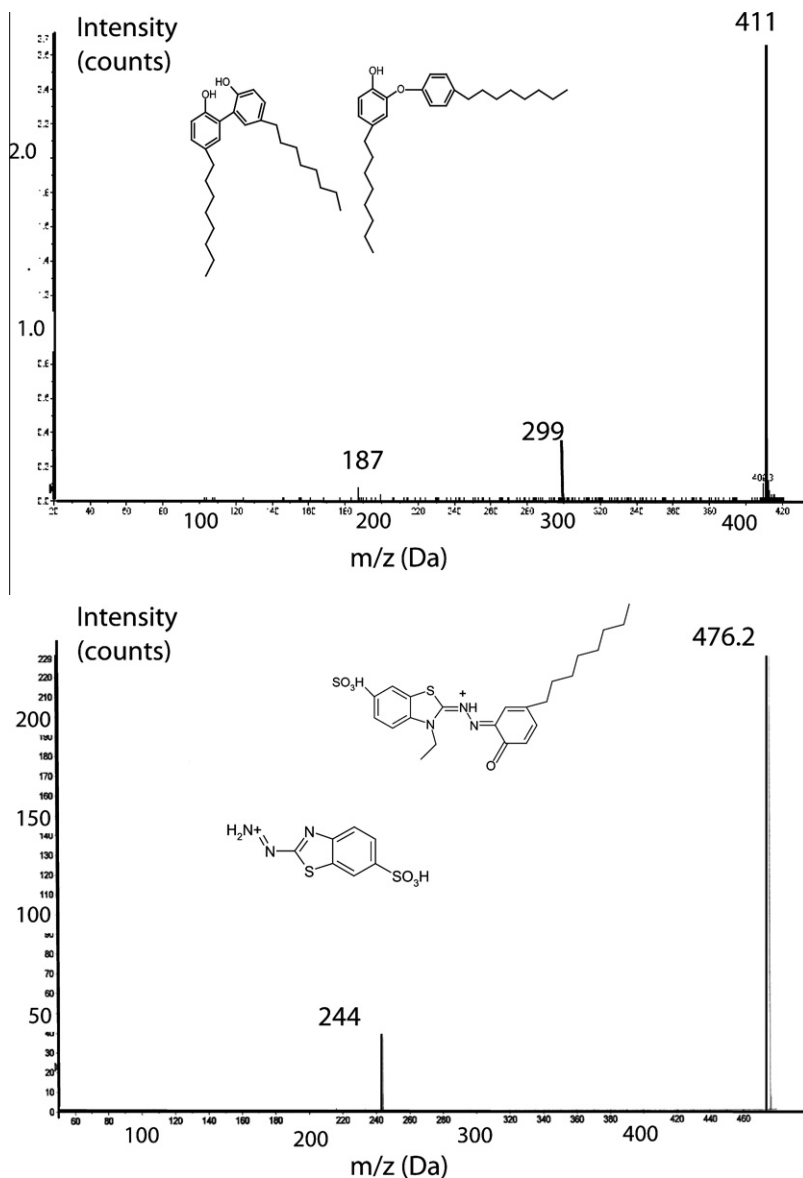


Fig. 3. MS spectrum of: (a) dimers (m/z 411) from *p*-octylphenol and of (b) a purple compound obtained from the reaction between ABTS⁺ and *p*-octylphenol.

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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