Appl Biochem Biotechnol (2008) 149:1–8
DOI 10.1007/s12010-007-8100-4

Degradation of Polycyclic Aromatic Hydrocarbons by *Rigidoporus lignosus* and its Laccase in the Presence of Redox Mediators

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Received: 26 May 2007 / Accepted: 24 November 2007 /
Published online: 14 February 2008

Abstract The metabolism of polycyclic aromatic hydrocarbons (PAHs) was studied *in vivo* and *in vitro* in systems consisting of *Rigidoporus lignosus* and its laccase, in the presence of so-called "mediator" compounds. The static culture of the native fungal strain was able to metabolize anthracene and 2-methylanthracene, but not 9-nitroanthracene. The addition of redox mediators 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT) or violuric acid (VA) led to a significant increase in the degradation of substrates. The oxidation of PAHs was not significant when purified laccase was used without the addition of mediators. The addition of these compounds increased the oxidation of all substrates by approximately 70–80% after 72 h of incubation. The degradation rate was highest for 2-methylanthracene in the presence of VA.

Keywords *Rigidoporus lignosus* · Laccase · Polycyclic aromatic hydrocarbons · Degradation · Redox potential · Mediators

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are classified as hazardous organic compounds containing several fused benzene rings in different stereochemical arrangements. PAHs are commonly found as ubiquitous air, soil, and water pollutants. They can be produced by natural events, such as the incomplete combustion of organic forest matter, but a much greater amount is released into the environment as a result of human activity [1, 2]. Many PAHs are toxic to living organisms and some are potentially mutagenic and carcinogenic in man [3]. High molecular weight PAHs are resistant to bacterial degradation in soil and

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sediments [4]. This has been attributed to their limited bioavailability because of low PHA water dissolution rates, sorption by solid matrices, and incorporation into solid particle micropores [5–7]. An investigation into the activity of microorganism with high PAH degrading capacity is, therefore, essential for an efficient bioremediation of these compounds. Some fungal species, such as Basidiomycetes, produce highly nonspecific ligninolytic enzymes and secrete extracellular oxidative enzymes with greater access to bioavailable substrates. The activity of these enzymes is more efficient than most degrading enzymes produced by bacteria, which have narrow substrate specificity [8–10]. Therefore, the potential usefulness of white-rot fungi and its laccase in the bioremediation of PAHs has been studied [11–14].

Laccases are a family of multicopper oxidases that catalyze the one-electron oxidation of phenol or aromatic amine substrates with concomitant reduction of O_2 to water [15]. However, it has been demonstrated that in the presence of oxidizable low molecular weight compounds, so-called mediators, laccase is able to oxidize a wide range of other aromatic compounds. Among these, PAHs show redox potential values ($E°$) higher than that of laccase (500–800 mV) [16–17]. The most common mediators used are 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [18] and, more recently, -N-OH type mediators such as 1-hydroxybenzotriazole (HBT) and violuric acid (VA) [19, 20].

Over the past few years, we have extracted and purified, apparently to isoelectrophoretic homogeneity, laccase isoenzymes obtained from the culture medium of white-rot fungus *Rigidoporus lignosus* (RIL) [21]. We recently reported the three-dimensional structure of one purified RIL isoenzyme, as determined by x-ray crystallography at a resolution of 1.7 Å [22].

The aims of this study were (1) to describe the biodegradation of selected PAHs by a previously uncharacterized fungal strain of *Rigidoporus lignosus* in a medium supplemented by mediators and (2) to measure the oxidation of these PAHs by purified laccase *in vitro*, in the presence of different mediator compounds, to determine the potential usefulness of RIL and its laccase in the bioremediation of PAH pollutants in soil and aquatic environments.

Materials and Methods

Chemicals

Anthracene, 2-methylanthracene, 9-nitroanthracene, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), violuric acid (VA), and all organic solvents of high-performance liquid chromatography (HPLC) grade were obtained from Sigma.

Biodegradation of PAHs by the RIL K1 Strain

RIL K1 native strain was provided by Dr. A.M. Boudet, Paul Sabatier University, Toulouse, France. The fungus was maintained in solid medium containing agar 2%, yeast extract 4 g, malt extract 10 g, glucose 4 g, per liter H_2O. For laccase production, a small portion of mycelial implant taken from a solid disk preculture was distributed in 250-ml Erlenmeyer flasks containing basic synthetic liquid medium (25 mM nitrogen, provided by ammonium tartrate, and 0.1% glucose as the carbon source) supplemented with 1 mM *p*-hydroxybenzoic acid, 500 μg per liter $CuSO_4$, and buffered at pH 6.5 with 10 mM Tris–HCl. The flasks were

inoculated with a mycelial implant taken from a solid disk preculture. Cultures were incubated at 28°C, under static conditions, for 11 days. One hundred forty milllimolar PAHs dissolved in Tween 20 1% and 0.9 mM redox mediators (ABTS, HBT, VA) dissolved in 20% ethanol were then added to the pregrown fungal cultures, which were incubated at 28°C for up to 28 days. Samples were taken periodically from three replicates. The residual PAHs were determined using a Perkin-Elmer LC 200 HPLC system equipped with a C18 column (VYDAC), 5 μm reverse-phase 150×4.6 mm and UV/Visible detector, at λ =254 nm. The analyses were carried out by injection of a 20-μL sample into the column and using isocratic elution with acetonitrile–water (30:70, *v*/*v*), at 1 ml min^{-1} flow rate. The amount of unreacted substrate was quantified using para-terphenil (200 mg/l) as internal standard. The standard was added to the reaction mixture, which was then filtered through SPE LC-18 (Agilent) and eluted with 2 ml of acetonitrile.

Mediated Oxidation of PAHs by Purified Laccase

Laccase was extracted and purified from extracellular fluid of RIL cultures as previously described [21]. Laccase mediated oxidation of PAHs was performed in the reaction mixture (5 ml) containing 100 mM acetate buffer at pH 4.5, 5 U/ml of purified laccase, 100 μM of PAH substrates (anthracene, 2-methyl-antracene, 9-nitro-anthracene) in the presence or absence of different redox mediators at 0.9 mM (ABTS, HBT, VA). Samples were incubated at 30°C in an agitated water bath. At set time intervals, aliquots of 200 μl were removed from the reaction mixture and added to 200 μl of 3.7 mM $NaBH_4$. After 15 min of incubation at room temperature, the conversion of 9,10-anthraquinone (reaction product) into 9,10-anthrahydroquinone was followed spectrophotometrically, measuring the absorbance at λ = 419 nm [23]. The enzymatic oxidation is expressed as % of unreacted (residual) substrate at the end of reaction.

Results and Discussion

Metabolism of PAHs by RIL Fungal Strain

The time course and degree of anthracene and 2-methylanthracene degradation by RIL cultures in the absence and presence of redox mediators are shown in Figs. 1 and 2. Both compounds were also degraded in the absence of mediating substrates. The methyl derivative was almost completely transformed after 14 days incubation (0.17% residual),

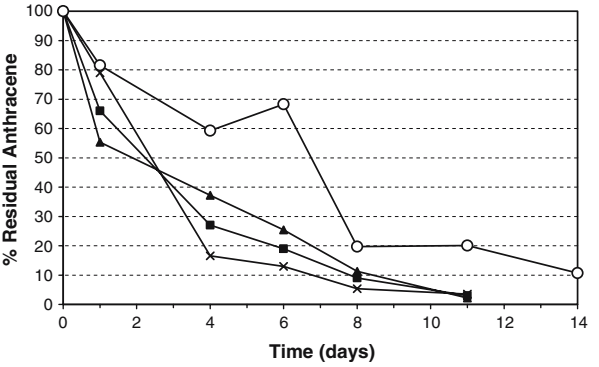

Fig. 1 Time course of 140 mM anthracene biodegradation in RIL cultures. Mediators 0.9 mM. Anthracene (o), anthracene + HBT (▪), anthracene + ABTS (▲), anthracene + VA (x)

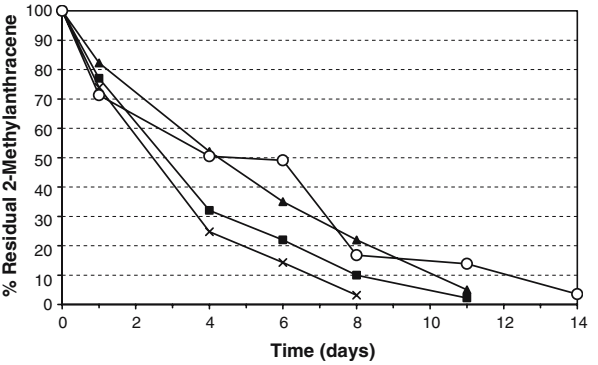

Fig. 2 Time course of 140 mM 2-methylanthracene biodegradation in RIL cultures. Mediators 0.9 mM. 2-Methylanthracene (o), 2-methylanthracene + HBT (■), 2-methylanthracene + ABTS (▲), 2-methylanthracene + VA (x)

while anthracene was not fully metabolized at that time (10.7% residual). The addition of mediating compounds on the first day of cultivation considerably enhanced the oxidation of both PAHs. The greatest effect was produced by VA on the eighth day of cultivation with 5.32% residual anthracene and 3.12% residual 2-methylanthracene. ABTS and HBT were less effective. The highest degradation rate was, in fact, obtained by 2-methylanthracene in the presence of VA. 9-nitroanthracene was not metabolized (data not shown).

Our results on the biodegradation of PAHs without the addition of synthetic mediators are in accord with those obtained previously on whole fungal cultures [24–26]. The results may be explained by assuming that some aromatic compounds produced by the RIL fungal strain during growth can act as natural mediators in the oxidation of PAHs. The secretion by white-rot fungi of low molecular weight aromatic compounds, some of which were potential laccase mediating substrates, has been reported [27]. The oxidation efficiency of the RIL fungal strain was higher for 2-methylanthracene, which showed a lower ionization potential (E=7.42) than anthracene (E=7.55) [28].

In Vitro Oxidation of Pahs by Purified Ril Laccase

When purified RIL laccase (5 U/ml) was used, only 8.7% of anthracene was oxidized over 72 h incubation in the absence of a mediator. The addition of 0.9 mM mediators considerably increased substrate oxidation: after 8 h incubation the greatest effect was obtained by VA (25.5% residual) followed by HBT (44.4% residual) and ABTS (69.5% residual). Complete degradation was observed after 48 h for all three mediators (Fig. 3). In

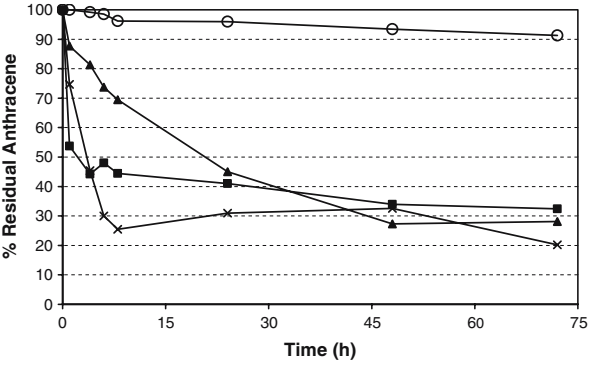

Fig. 3 Effect of mediating substrates HBT, ABTS, VA (0.9 mM) on 100 mM anthracene oxidation by RIL laccase. Anthracene (o), anthracene + HBT (■), anthracene + ABTS (▲), anthracene + VA (x)

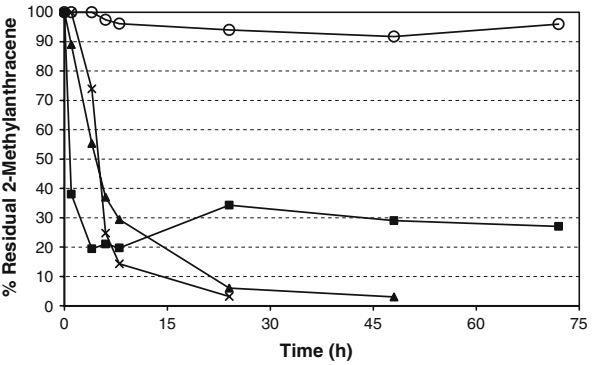

Fig. 4 Effect of mediating substrates HBT, ABTS, VA (0.9 mM) on 100 mM 2-methylanthracene oxidation by RIL laccase. 2-methylanthracene (o), 2-methylanthracene + HBT (■), 2-methylanthracene + ABTS (▲), 2-methylanthracene + VA (x)

the absence of mediators, only 4% of 2-methylanthracene was oxidized at the end of incubation (28 days). The addition of mediators increased oxidation, giving % residual values of 14.5% for VA, 20.0% for HBT, and 29.5% for ABTS after 8 h of incubation. The full oxidation of 2-methylanthracene was obtained after 24 h for VA and after 48 h and 72 h for ABTS and HBT, respectively. The highest transformation rate was obtained by VA (Fig. 4). 9-nitroanthracene was not oxidized in the absence of mediators: the addition of these compounds activated the oxidation, which increased progressively and reached a maximum after 72 h incubation (Fig. 5).

A comparison of the results obtained by 2-methyl- and 9-nitro-substituted on anthracene indicates that, similar to phenols [29], the presence of an electron-donating ($-CH_3$) or electron-withdrawing group ($-NO_3$), modified the electron density on the anthracene ring, thus increasing or decreasing the reactivity of the substrate [30]. It has been observed that the different activity of various laccases toward substrates can mainly be attributed to differences in their redox potential [31]. In recent publications, a new classification scheme for laccases has been developed [32, 33]. Indeed, all laccases can be divided into three groups according to their redox potential ($E°$) at the T1 Cu site: (a) low $E°$ enzymes with $E°\approx 430$ mV vs. NEH; (b) a middle $E°$ group including enzymes with potentials 470÷710 mV; (c) high $E°$ enzymes with redox potentials of 730÷800 mV. On the basis of this classification, RIL has a high $E°$ laccase with a redox potential of 730 mV [34].

Laccase-based biocatalysis has recently been extended, with the aid of "small mediators," to PAH degradation. A conceivable role of the mediators would be that of a kind of "electron shuttle" between the enzyme and the substrate. Once the mediator is

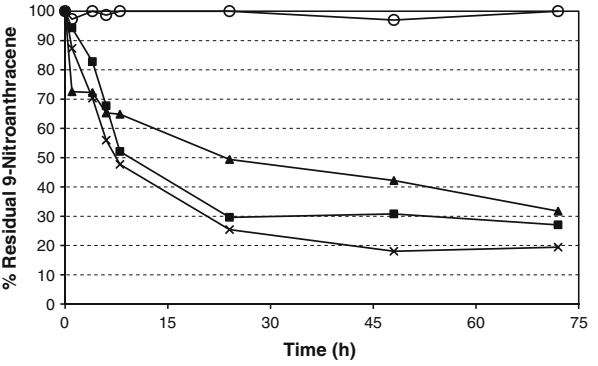

Fig. 5 Effect of mediating substrates HBT, ABTS, VA (0.9 mM) on 100 mM 9-nitroanthracene oxidation by RIL laccase. 9-nitroanthracene (o), 9-nitroanthracene + HBT (■), 9-nitroanthracene + ABTS (▲), 9-nitroanthracene + VA (x)

oxidized by the enzyme, it diffuses away from the active enzymatic site and then oxidizes substrates that, because of steric hindrance, could severely interfere with their proper docking in the substrate binding pocket of laccase [35]. Currently, the most studied mediators include ABTS and -N(OH)- compounds: HBT and VA. Previous studies have indicated that the oxidation of HBT and VA by laccase is controlled by the first single-electron transfer from the -N(OH)- site to T1 Cu in laccase, similar to the oxidation of phenols and other laccase substrates [19, 20]. The thermodynamic driving force $\Delta E = E°$ (laccase) − E° (N-OH) controls the oxidation rate. A laccase with a higher $E°$ or an -N(OH)-compound with a lower E° tends to have a higher oxidation rate. The redox potential values of ABTS, HBT and VA are 1.09 V [36], 1.12 V and 0.91 V [19], respectively. A greater $\Delta E°$ could create a more favorable transitional energy state for the interaction between molecular orbitals of mediator and enzyme, resulting in faster electron transfer [19, 20].

An important problem is to determine whether the differences in the $E°$ of laccases can be explained at the structural level. Detailed structural comparison of selected laccases of known three-dimensional structure have shown that the most significant structural differences, possibly accounting for the different $E°$ values, are found in the Cu1….NE coordination distance [37]. When comparing this coordination distance between different high $E°$ laccases, it is seen that RIL laccase shows the greatest values (2.20 Å). The stretching of the bond between Cu1 and the coordinated His-457 in RIL laccase decreases the electron density contribution at the metal cation, thereby increasing the redox potential of the copper site [22].

In conclusion, it must be pointed out that these results represent a useful basis for the development of a future research project aimed at directly testing these systems *in situ*, i.e., a polluted area. Such an investigation would certainly help verify whether the degradation of polycyclic aromatic hydrocarbons, in the presence of redox mediators, could be equally (or more) efficient in an environment representative of an "*in vivo* degradation target."

Acknowledgments This work was supported in part by a grant from the Regione Siciliana, in the framework of Project 1999/IT.16.1.PO.011/3.13/7.2.4/339 Prot.238 "Formazione per la ricerca nel campo della bonifica dei siti contaminati" and from the Istituto Nazionale Biostrutture e Biosistemi.

We would like to thank M. D. Wilkinson for his revision of the English version of the paper.

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