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Oxidative degradation of polyaromatic hydrocarbons and their derivatives catalyzed directly by the yellow laccase from *Pleurotus ostreatus* D1

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Abstract

The catalytic activity of the yellow laccase from *Pleurotus ostreatus* D1 (YLPO) towards a range of nonphenolic aromatic compounds, including polyaromatic hydrocarbons (PAHs), their derivatives, and anthracene-like synthetic dyes, was investigated. YLPO did not catalyze the oxidation of the two-ring PAH naphthalene, but the naphthalene derivatives α- and β-naphthols, α-nitroso-β-naphthol, α-hydroxy-β-naphthoic acid, and α-naphthylamine were all good laccase substrates. YLPO degraded all the PAHs containing from three to five rings, with the following efficiencies: 91% for anthracene, 40% for pyrene, 95% for fluorene, 47% for fluoranthene, 82% for phenanthrene, and 100% for perylene. These efficiencies were higher than that observed for a blue laccase from the same fungus in both absence and presence of the typical synthetic mediators 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1-hydroxybenzotriazole (HBT) under the same experimental conditions. YLPO oxidized a model mixture of PAHs and all the synthetic dyes except anthrone. The same product of anthracene oxidation and various unidentified products of fluorene oxidation were observed in solutions of various solvents. Although the blue and yellow laccases of *P. ostreatus* D1 oxidized the typical phenolic substrates in a similar way, all the collected data show that the catalytic properties of the blue and yellow laccases towards nonphenolic substrates differed significantly. Further catalytic and structural studies are needed to explain the catalytic mechanism of action of yellow laccases.

Keywords: Yellow laccase; Polyaromatic hydrocarbons; Synthetic dyes

1. Introduction

Contamination of the environment by aromatic xenobiotics has become a problem of global dimensions. Many xenobiotics, such as PAHs and synthetic dyes, constitute a serious danger to humans and animals [1,2]. Recently, the possibility of using a special group of organisms, white-rot fungi, in bioremediation has been discussed widely. These fungi are the most active degraders of lignin in nature, and they can degrade a wide range of aromatic xenobiotics [3]. Current research includes investigating the biochemical mechanisms of xenobiotic degradation and the enzymatic systems involved in this process. It was assumed that the degradation of aromatic compounds is catalyzed by the extracellular enzyme system of these fungi, in which laccase plays a key role [4].

Laccases (*p*-diphenol oxidase, EC 1.10.3.2) belong to the family of multicopper oxidases that catalyze the oxidation of various aromatic substances, such as diphenols, arylamines, and aminophenols, with the concomitant reduction of O_2 to H_2O [5]. It is commonly accepted (see, e.g., [5]) that laccases oxidize aromatic compounds with a phenolic functional group. However, the overlapping substrate specificity of these enzymes can be extended to nonphenolic aromatic compounds with the use of aromatics called redox mediators. In the presence of some synthetic and natural mediators, laccases can oxidize veratryl and benzyl alcohols, nonphenolic groups of the lignin polymer, and lignin model substances [6–8].

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ACN, acetonitrile; ANT, anthracene; AOT, bis-2-(ethylhexyl) sulfosuccinate sodium salt; DMOP, 2,6-dimethoxyphenol; DMSO, dimethylsulfoxide; FLA, fluoranthene; FLU, fluorene; HBT, 1-hydroxybenzotriazole; K_m, the Michaelis constant; PAH, polyaromatic hydrocarbon; PER, perylene; PHE, phenanthrene; PYR, pyrene; syringaldazine, 4-hydroxy-3,5-dimethoxybenzaldehyde azine; Tween-80, polyoxyethylene sorbitol monooleate; YLPO, yellow laccase from *Pleurotus ostreatus* D1

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During solid-state fermentation of a natural lignin-containing substrate, the white-rot fungi produce what is known as a yellow form of laccase. The active center of this enzyme is modified by lignin-degradation products. As a result of this modification, laccase gains the ability to catalyze the oxidation of nonphenolic compounds without addition of mediators. Yellow laccases were first discovered and investigated by Leontievsky et al. [9], who showed that the yellow laccase from the fungus *Panus tigrinus* 8/18 can oxidize veratryl alcohol and nonphenolic lignin model compounds without any synthetic mediator in the reaction mixture. Subsequently, yellow laccases were found in other white-rot fungi, such as *Phlebia radiata* and *Phlebia tremellosa* [10], and were shown to be able to oxidize nonphenolic lignin model compounds as well [11]. Previously [12], we obtained yellow laccase from the fungus *Pleurotus ostreatus* D1 (YLPO). The enzyme can oxidize the polyaromatic hydrocarbons (PAHs) anthracene and fluoranthene in the absence of any synthetic mediator in the reaction mixture [12]. The full substrate range for the yellow laccases has not yet been determined.

In this study, we examine the catalytic activity of YLPO towards a range of PAHs containing from two to five aromatic rings and also towards several anthracene-like synthetic dyes.

2. Experimental

2.1. Materials

2.1.1. Organism

The fungus *P. ostreatus* D1 was isolated and identified by Dr. V.E. Nikitina, Laboratory of Microbiology and Mycology, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov [13].

2.1.2. YLPO purification

YLPO was purified from a solid-state culture of the fungus. The purification included ion exchange chromatography on TEAE-Servacel 23 (Chemapol, Czech Republic), ammonium sulfate precipitation (80%), and gel filtration on Sephacryl HR 200 by using an FPLC system (Pharmacia, Sweden) [12]. SDS-PAGE was performed by the method of Laemmli on 12% polyacrylamide gels [14]. The resulting protein was electrophoretically homogeneous. Protein concentration was determined according to Bradford [15].

2.1.3. Chemicals

4-Hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,6-dimethoxyphenol (DMOP), polyoxyethylene sorbitol monooleate (Tween-80), and acetonitrile were from Sigma–Aldrich (Germany); acetic acid, fluoranthene, fluorene, phenanthrene, naphthalene, α-hydroxy-β-naphthoic acid, and pyrene were from Fluka (Switzerland); sodium acetate, anthracene, and perylene were from POCh (Poland); disodium hydrogen phosphate and potassium dihydrogen phosphate were from Merck (Germany); bis-2-(ethylhexyl) sulfosuccinate sodium salt (AOT), purified according to Menger and Yamada [16], was from Fluka; 1-hydroxybenzotriazole (HBT) was from ICN Biomedicals (Germany); dyes, α- and β-naphthols, α-nitroso-β-naphthol, and α-naphthylamine were from Reachim Co. (Russia).

2.2. Assay for laccase activity

The oxidation of syringaldazine, ABTS, DMOP, and catechol was studied spectrophotometrically at 25 °C in quadruplicate, by using a Shimadzu PC2101 spectrophotometer. The following absorption coefficients were used: $\varepsilon_{468} = 14{,}800\ M^{-1}\ cm^{-1}$ for DMOP [17], $\varepsilon_{436} = 29{,}300\ M^{-1}\ cm^{-1}$ for ABTS [18], $\varepsilon_{525} = 65{,}000\ M^{-1}\ cm^{-1}$ for syringaldazine [19], and $\varepsilon_{410} = 2211\ M^{-1}\ cm^{-1}$ for catechol [20]. One unit of enzyme activity is 1 μmol of substrate oxidized (or 1 μmol of product formed)/min.

The oxidation of *p*-phenylenediamine and *N*-acetyl-*p*-phenylenediamine was studied spectrophotometrically at 530 and 355 nm, respectively. The oxidation of substituted naphthalenes was studied at 530 nm for α-naphthol, 360 nm for β-naphthol [21], 480 nm for α-naphthylamine, and 405 nm for α-nitroso-β-naphthol. HBT oxidation was studied at 408 nm ($\varepsilon = 280\ M^{-1}\ cm^{-1}$) [22].

2.3. Enzymatic treatment of synthetic dyes

All experiments were carried out in reaction tubes containing 3 ml of 50 mM NaK-phosphate buffer (pH 6.0) and 0.176 U/ml laccase (0.156 mM). The dyes were added to a final concentration of 50 μM. The reaction tubes were sealed and were incubated at 30 °C for the desired period of time. Decolorization of the dyes was studied spectrophotometrically at the following wavelengths: anthrone, 255 nm; alizarin red, 505 nm; acridine orange, 470 nm; neutral red, 525 nm; rhodamine C (butyl ester), 560 nm; rhodamine 6G, 500 nm; safranin T, 515 nm; and eosin Y, 520 nm.

2.4. Enzymatic treatment of PAHs

All experiments were carried out in reaction tubes containing 2 ml of 50 mM NaK-phosphate buffer (pH 6.0), 1% (v/v) acetonitrile, and 2 mM AOT. Samples of 100 μl laccase were added to each tube to a final concentration of 0.176 or 1.76 U/ml. PAHs (naphthalene, α-hydroxy-β-naphthoic acid, anthracene, fluorene, phenanthrene, pyrene, fluoranthene, and perylene) were dissolved in acetonitrile and were added to a final concentration of 10 μM. The reaction tubes were sealed and were incubated at 30 °C for the desired period of time. PAHs and their oxidation products were extracted with 2 ml of chloroform and were studied by HPLC and spectrophotometry. Control samples were prepared identically, except that the enzyme was deactivated by boiling for 10 min before being added to the tubes.

A SpectraSeries P200 device (Spectra-Physics Analytical, Inc.) fitted with a SpectraSeries UV 100 detector (Thermo Separation Products, USA) was used. Separation was carried out in a Spherisorb S5 PAH column. The sample volume was 5 μl. After 2 min of isocratic flow of 40% acetonitrile:water, analyses were performed with a gradient varying from 40 to 100% acetonitrile, at a rate of 1.6 ml/min, for 35 min.

Table 1
Michaelis–Menten constants for YLPO

Substrate	K_m (mM)	References
Syringaldazine	0.0087 ± 0.0009	[12]
ABTS	0.11 ± 0.0075	[12]
Veratryl alcohol	0.29 ± 0.0064	[49]
2,6-Dimethoxyphenol	0.43 ± 0.02	[12]
p-Phenylenediamine	0.66 ± 0.008	This work
Catechol	3.65 ± 0.53	[12]
N-Acetyl-*p*-phenylenediamine	6.70 ± 0.085	This work

3. Results and discussion

3.1. Specific activity of YLPO in aqueous solutions

The K_m values varied from the lowest value of 0.0087 mM with syringaldazine to the highest value of 6.7 mM with *N*-acetyl-*p*-phenylenediamine (Table 1). Analysis of the literature data shows that with the substrates used in this study, the K_m values for the laccases from *Rigidoporus lignosus*, *Lentinus edodes*, *Polyporus pinsitus*, *Ceriporiopsis subvermispora*, *P. ostreatus*, *Trametes villosa*, and *Trametes pubescens* follow the sequence syringaldazine < ABTS < 2,6-dimethoxyphenol < catechol [23–31]. It should be noted that syringaldazine is the preferred substrate and catechol is the poorest substrate for laccase, no matter which fungus is producing the enzyme.

The K_m values obtained with ABTS, veratryl alcohol, 2,6-dimethoxyphenol, and *p*-phenylenediamine were within one order of magnitude. Most probably, the active center of YLPO is equally accessible to substrates containing a phenolic hydroxyl group or an amino group and to nonphenolic substrates. The K_m value obtained with *p*-phenylenediamine was higher than that observed for *p*-phenylenediamine oxidation catalyzed by *L. edodes* and *Agaricus bisporus* laccases [25,32]. This is the first time that the oxidation of *N*-acetyl-*p*-phenylenediamine by a fungal laccase has been shown. The K_m value found with *N*-acetyl-*p*-phenylenediamine was higher than that found with catechol by only two times.

The rate of HBT oxidation by YLPO was very slow (only 1.5 U/mg YLPO). This result is similar to the data obtained by other authors, who showed that HBT is a very poor substrate for *Trametes hirsuta* and *Trichophyton rubrum* laccases. With HBT, the K_m values were 8 mM for *T. hirsuta* laccase [33] and 24 mM for *T. rubrum* laccase [22].

3.2. Oxidation of naphthalene and its derivatives

Naphthalene is the simplest PAH—it contains only two condensed aromatic rings. No activity of fungal laccases towards naphthalene has been found yet, probably because of the high ionization potential of this substrate, which reaches 8.13 eV [34]. In this study we investigated the catalytic activity of YLPO towards naphthalene. YLPO did not oxidize 10 μM naphthalene at pH 6.0 within 10 days, when 0.176 or 1.76 U/ml enzyme was added to the reaction mixture.

α- and β-naphthols were identified as fungal metabolites of naphthalene degradation [35]. To find a way to make naphthalene more accessible to laccase, we investigated YLPO degradation of several substituted naphthalenes, including α- and β-naphthols, α-naphthylamine, α-nitroso-β-naphthol, and α-hydroxy-β-naphthoic acid. The formation of the α-naphthol, α-nitroso-β-naphthol, and α-naphthylamine products and the oxidation of β-naphthol are shown in Fig. 1A; and the formation of the α-naphthol product is depicted in Fig. 1B.

We found that inserting one hydroxyl group into the aromatic ring improved the availability of the aromatic structure of naphthalene to laccase. The oxidation of α-naphthol gave rise to a violet product (λ_{max} = 530 nm), whereas the product of β-naphthol oxidation was a white insoluble precipitate. Therefore, we studied the oxidation of β-naphthol by measuring the decrease in substrate concentration. The position of the hydroxyl group was important for substrate availability to the enzyme. For example, the naphthol containing the hydroxyl group at the α-position was oxidized four times more rapidly than was the β-substituted derivative (Fig. 1). These results are consistent with those obtained by Kulys et al. [21], who showed that α-naphthol is the better substrate for *P. pinsitus* laccase than is β-naphthol. With α-naphthol the K_m value was 0.0095 mM, whereas with β-naphthol it was 0.374 mM [21]. However, we did not find any YLPO inactivation, either by the naphthols or by their oxidation products, for 1 mM substrate concentration. Moreover,

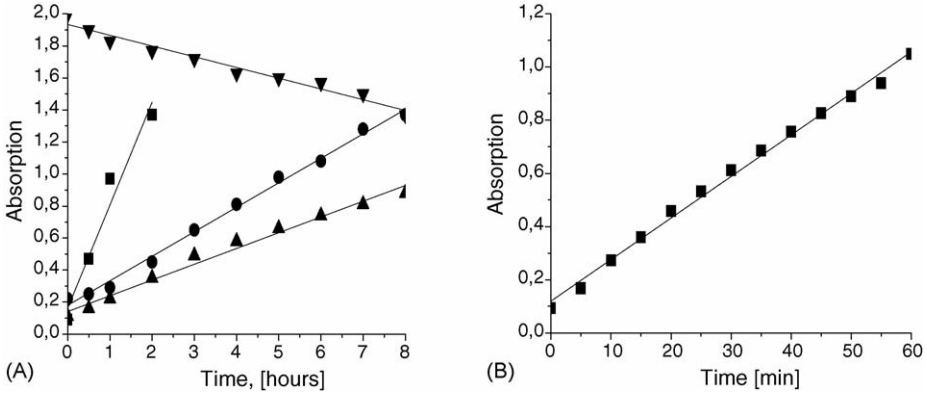


Fig. 1. Oxidation of the substituted naphthalenes by YLPO in 1% (v/v) acetonitrile solutions: YLPO concentration 0.0176 U/ml; 50 mM Tris–HCl, pH 6.0; substrate concentrations 1 mM; (A) α-naphthol (■), β-naphthol (▼), α-naphthylamine (●), and α-nitroso-β-naphthol (▲); (B) α-naphthol (■). For details, see Section 2.2.

we found that replacing the hydroxyl group (α-naphthol) by an amino group (α-naphthylamine) decreased YLPO activity by almost six times (Fig. 1). α-Nitroso-β-naphthol was even less available to YLPO than were the simple naphthols.

Finally, we studied YLPO activity towards α-hydroxy-β-naphthoic acid, which contains the hydroxyl group at the α-position and the carboxyl group at the β-position. YLPO degraded only 25.9 ± 2.2% of the substrate within 10 days, when 0.176 U enzyme was added to the reaction mixture containing 10 μM substrate, 1% (v/v) acetonitrile, and 50 mM NaK-phosphate buffer (pH 6.0). Of the five naphthalene derivatives, α-hydroxy-β-naphthoic acid was the worst laccase substrate.

3.3. Oxidation of synthetic dyes of the anthraquinone type

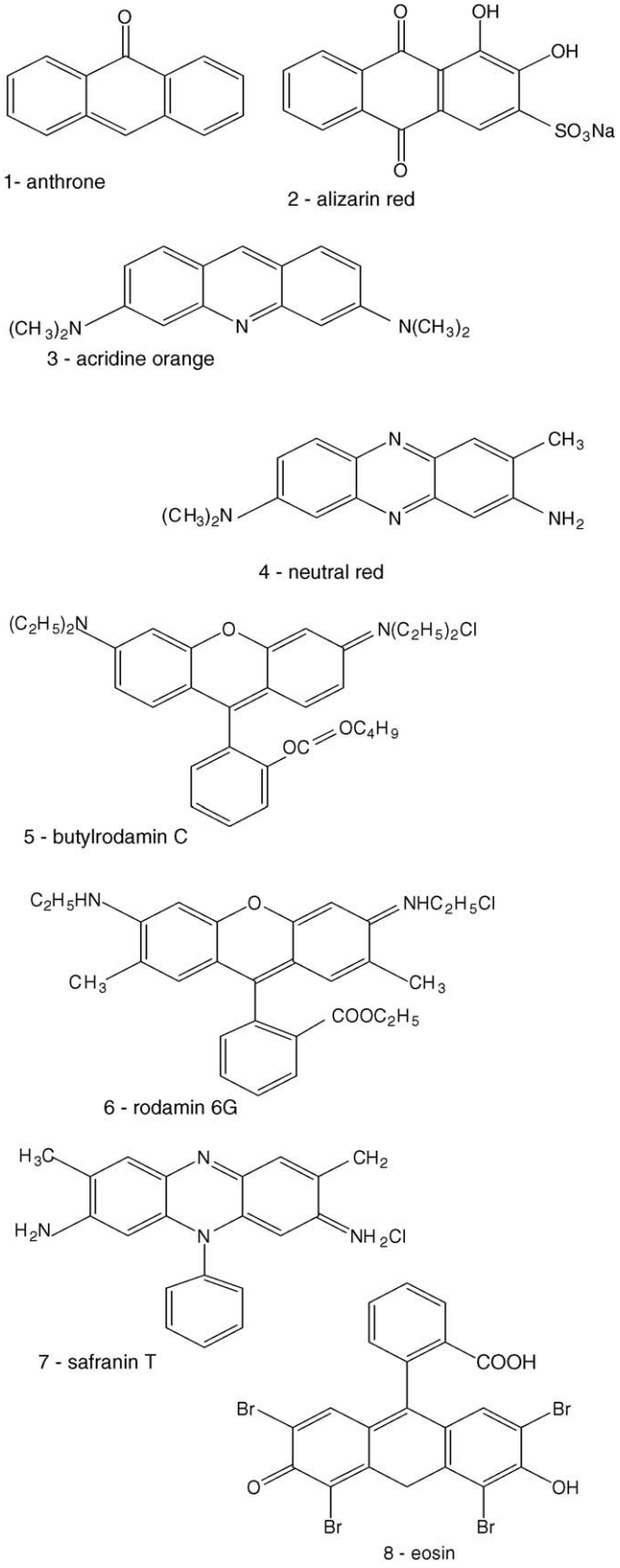
Recently [12] we showed that YLPO oxidizes anthracene with the formation of anthraquinone, as do other fungal laccases. Anthraquinone is the central part of the molecules of synthetic dyes called the anthraquinone-type dyes. Many authors believe that only anthraquinone-type synthetic dyes can be directly oxidized by laccase [36,37]. We investigated the catalytic activity of YLPO towards several anthraquinone-type and anthracene-derived dyes, including anthrone, alizarin red, acridine orange, neutral red, rhodamine C (butyl ester), rhodamine 6G, safranin T, and eosin Y. All these dyes have an anthracene-like three-ring aromatic structure in the central part of their molecule plus various substituents (Scheme 1).

The examined substrates were divided into three categories: those that contained quinone groups (anthrone and alizarin red), those that did not contain aromatic substituents (acridine orange and neutral red), and those that contained an aromatic ring as the substituent (rhodamine C (butyl ester), rhodamine 6G, safranin T, and eosin Y).

Of the category 1 dyes, only anthrone was not oxidized by YLPO with either 0.176 or 1.76 U/ml enzyme added to the reaction mixture containing 50 μM substrate and 50 mM NaK-phosphate buffer (pH 6.0). Alizarin red, containing two quinone groups and two hydroxy groups, was the best substrate for YLPO. Fig. 2 shows that this dye was decolorized most rapidly—within the first hour after the addition of 0.176 U/ml YLPO to the reaction mixture containing 50 μM dye and 50 mM NaK-phosphate buffer (pH 6.0). The reaction then slowed down. Decolorization reached 91.2% within 3 h (Fig. 3) and was accompanied by absorption decreases at 332 and 505 nm and by simultaneous absorption increases at 230 and 290 nm.

The decolorization of the substrates that did not contain quinone groups was very slow. Within 10 days it reached only 8.5 and 14.7% for acridine orange and neutral red, respectively (Fig. 3).

The dyes containing an aromatic ring as the substituent were good substrates for YLPO. The decolorization of rhodamine C (butyl ester), rhodamine 6G, safranin T, and eosin Y reached 70.8, 47.8, 43.2, and 64.8%, respectively, at 10 days after the addition of 0.176 U/ml YLPO to the reaction mixture containing 50 mM NaK-phosphate buffer (pH 6.0) and 50 μM substrate (Fig. 3).



Scheme 1. Structures of the anthracene-like synthetic dyes.

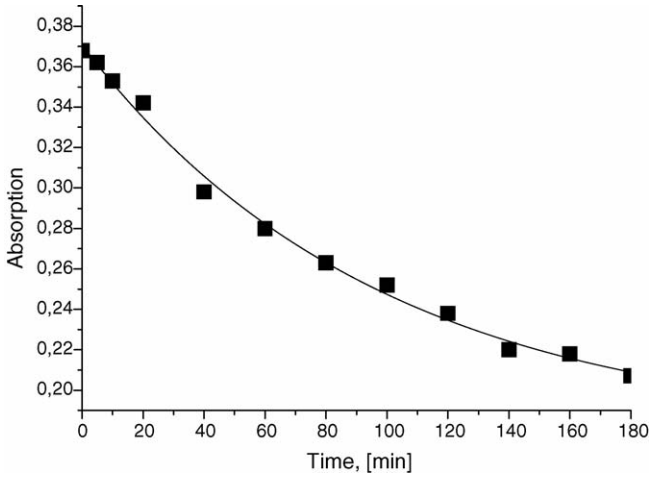


Fig. 2. Decolorization of alizarin red by YLPO: 0.176 U/ml YLPO; 50 mM Tris–HCl, pH 6.0; substrate concentration 50 μM.

3.4. PAH oxidation by YLPO in the absence of any mediator

Generally, an increase in the size and angularity of a PAH molecule results in a concomitant increase in hydrophobicity and electrochemical stability [38]. The stability and hydrophobicity of PAH molecules are the two primary factors contributing to the persistence of high-molecular-weight PAHs in the environment [35]. PAH recalcitrance for microbial degradation increases directly with increasing molecular weight and octanol:water partition coefficient (log K_{ow}) and depends inversely on aqueous water solubility, since high-molecular-weight PAHs are more slowly desorbed and, therefore, are less available for biological uptake than low-molecular-weight PAHs [39]. The recalcitrance of the PAHs used in this study increases in the order naphthalene → anthracene → phenanthrene → fluoranthene → pyrene,

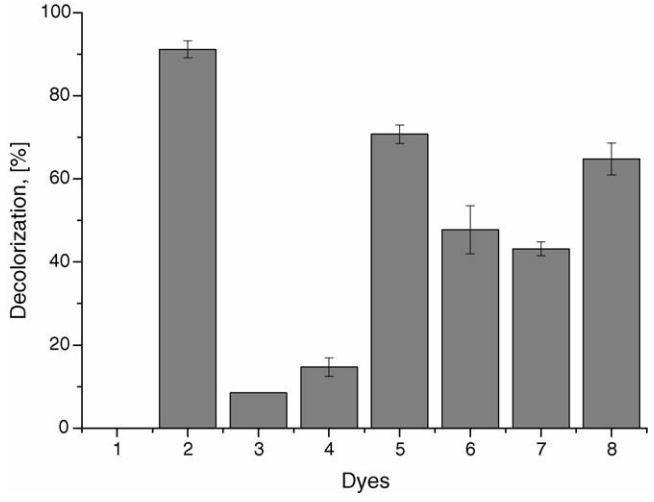


Fig. 3. Oxidation (decolorization) of the anthraquinone-type and anthracene-derived synthetic dyes: (1) anthrone (λ = 255 nm), (2) alizarin red (λ = 505 nm), (3) acridine orange (λ = 470 nm), (4) neutral red (λ = 525 nm), (5) rhodamine C (butyl ester) (λ = 560 nm), (6) rhodamine 6G (λ = 500 nm), (7) safranin T (λ = 515 nm), and (8) eosin Y (λ = 520 nm); 0.176 U/ml YLPO, 50 mM Tris–HCl, pH 6.0; substrate concentrations 50 μM.

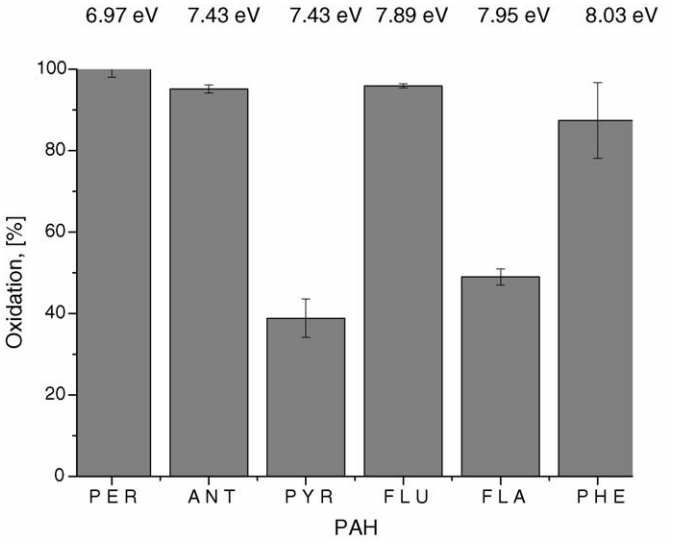


Fig. 4. PAH oxidation by YLPO: PER, perylene; ANT, anthracene; PYR, pyrene; FLU, fluorene; FLA, fluoranthene; PHE, phenanthrene; 50 mM Tris–HCl, pH 6.0, 2 mM AOT, 10 μM PAH, 0.176 U YLPO; duration: 2 days for ANT, FLU, PYR, and PER and 10 days for PHE and FLA. The data in the figure are arranged in line with the IP values.

and their solubilities are as follows: naphthalene, 31.7 mg/l; anthracene, 0.07 mg/l; phenanthrene, 1.3 mg/l; fluoranthene, 0.26 mg/l; and pyrene, 0.14 mg/l [35].

We investigated the catalytic activity of YLPO towards six PAHs containing from three to five aromatic rings. We found that all the PAHs were oxidized by laccase without addition of any mediator to the reaction mixture. After a 2-day incubation, 100 ± 2.0% of perylene, 95.1 ± 1.0% of anthracene, 95.9 ± 0.5% of fluorene, and 38.9 ± 4.7% of pyrene were oxidized by 0.176 U/ml YLPO (Fig. 4). Extending the reaction time to 10 days led to 87.4 ± 9.3% of phenanthrene degraded and to 49.0 ± 5.0% of fluoranthene degraded.

Likewise, in a previous study with blue laccase [40] we did not find any dependence of the PAH ionization potential and the number of aromatic rings in the PAH structure on the output of the oxidation reaction.

HPLC analysis showed the presence of peaks corresponding to the reaction products with the following retention times: anthracene, 13.8 min; phenanthrene, 13.5 min; fluorene, 12.8 min; pyrene, 16.0 min; and perylene, 7.3 and 8.6 min. The most probable initial step of the reaction was the formation of the corresponding quinones. As in a previous study, with blue laccase [40], the product of anthracene oxidation was anthraquinone (Fig. 5A). The formation of 9,10-anthraquinone from anthracene was confirmed by HPLC and by UV–vis spectroscopy. The HPLC retention time (13.8 min) and the UV–vis spectrum, with a maximum at 326 nm, corresponded to anthraquinone. The absorption spectrum of an extract of the reaction mixture after fluorene oxidation is shown in Fig. 5B, in which the appearance of a new absorbance peak at 330 nm, probably corresponding to a fluorenone peak, can be seen. 9-Fluorenone formation from fluorene was shown previously for *Trametes versicolor* laccase [34]. Identifying the oxidation prod-

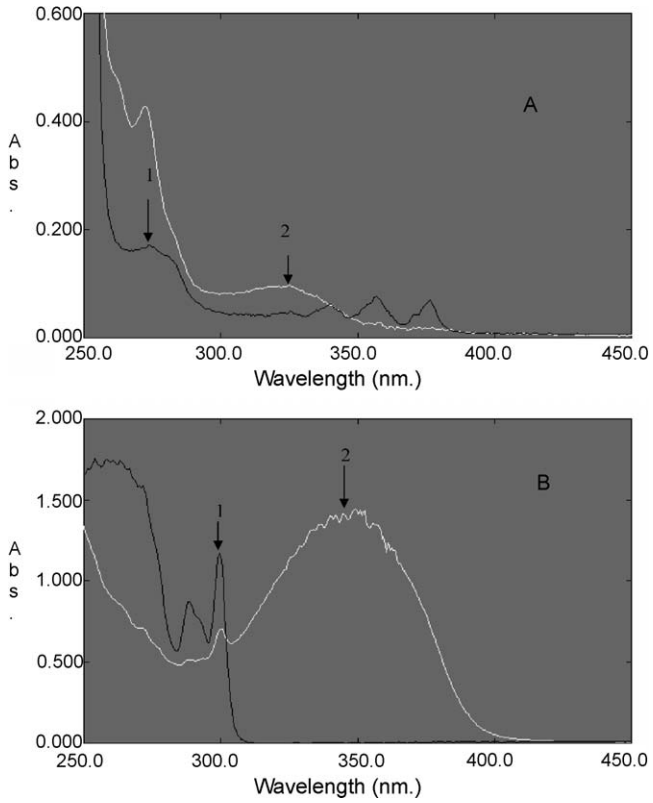


Fig. 5. UV–vis spectra of the chloroform extracts of the reaction mixtures: (A) anthracene: control (1) and the reaction product anthraquinone (2) (50 mM Tris–HCl, pH 6.0, 2 mM AOT, 1% ACN, 10 μM anthracene, 0.176 U YLPO; duration, 2 days); (B) fluorene: control (1) and the reaction product (2) (50 mM Tris–HCl, pH 6.0, 2 mM AOT, 1% ACN, 10 μM fluorene, 0.176 U YLPO; duration, 2 days).

ucts of fluorene, fluoranthene, phenanthrene, pyrene, and perylene will be the subject of separate study.

3.5. Mediator effects on PAH oxidation by YLPO

The presence of certain synthetic mediators greatly increases laccase activity towards PAHs [34]. However, yellow laccases do not need an exogenous mediator for the oxidation of nonphenolic compounds [9,11]. We compared YLPO activities towards the three-ring PAH anthracene and the four-ring PAHs pyrene and fluoranthene, with and without ABTS and HBT. The presence of ABTS almost did not influence anthracene or pyrene oxidation; however, it reduced fluoranthene oxidation almost two-fold (Fig. 6). Addition of 1 mM HBT, instead of ABTS, to the reaction mixture decreased fluoranthene and anthracene oxidation almost four-fold and insignificantly increased pyrene oxidation (Fig. 6).

It may be assumed that the decrease in YLPO catalytic activity in the presence of the synthetic mediators was a consequence of competition between the mediators and the PAH molecules for the active center of the enzyme, as PAH oxidation in the active center proceeds faster than that by the mediator outside the enzyme molecule. This hypothesis needs independent confirmation.

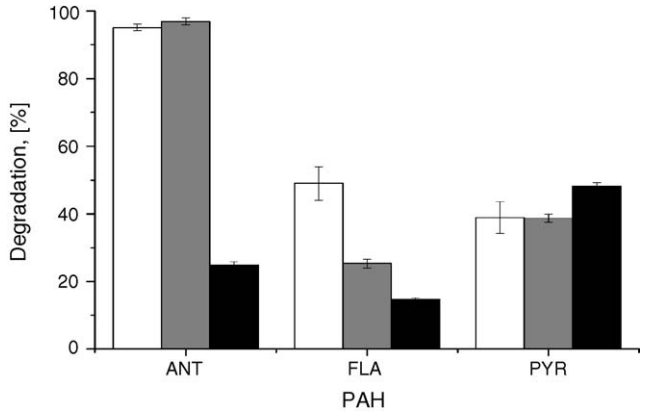


Fig. 6. Effect of ABTS on PAH oxidation by YLPO: (□) without mediator, (▨) with ABTS, and (■) with HBT.

3.6. Phenanthrene oxidation by YLPO under various conditions

For a very long time, it has been assumed that the catalytic activity of a laccase/mediator pair towards PAHs is limited by the ionization potential of a substrate, which should not exceed 8.0 eV. Collins et al. [4] did not observe phenanthrene oxidation by *T. versicolor* laccase, attributing this result to the relatively high ionization potential of phenanthrene. The *Coriolopsis gallica* laccase catalyzed low-level phenanthrene degradation in the presence of ABTS [41]. However, Böhmer et al. [42] showed that phenanthrene is oxidized (IP = 8.03 eV) by the laccase/HBT pair in the presence of unsaturated lipids. They assumed that two reactions took place: lipid peroxidation and phenanthrene oxidation. They showed that the process can be mediated only by HBT, but not by ABTS or 3-hydroxyanthranilic acid. This phenomenon has not been further investigated, but it is likely that HBT forms more reactive intermediates than does ABTS or 3-hydroxyanthranilic acid [42].

We investigated the influence of two different detergents on YLPO activity towards phenanthrene. We found that YLPO oxidized phenanthrene without any synthetic mediator in the reaction mixture. Moreover, YLPO did not need the presence of any detergent in the reaction mixture, which is contrary to Böhmer et al.'s [42] data and to our earlier results for the blue laccase of *P. ostreatus* D1 [40]. The degradation of phenanthrene was 73.6 ± 2.0% in the absence of any detergent, 68.8 ± 1.0% in the presence of Tween-80, and 87.4 ± 9.3% in the presence of AOT, within 10 days (Fig. 7). The greatest oxidation effectiveness obtained in the presence of AOT may mean that this detergent protects the enzyme better than does Tween-80. Apparently, the mechanisms of phenanthrene oxidation by the blue and yellow laccases from the same source can differ.

3.7. Oxidation of anthracene and fluorene in solutions of various water-miscible organic solvents

The bioavailability of contaminants decreases with an increase in the hydrophobicity of chemicals [35]. Surfactants and

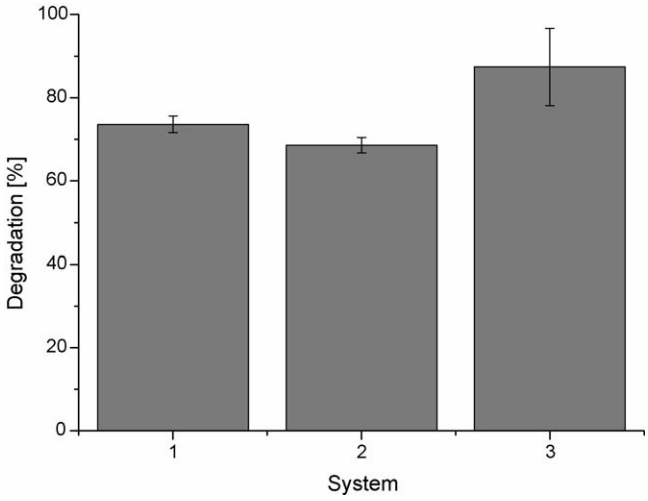


Fig. 7. Phenanthrene oxidation: (1) without detergent, (2) with Tween-80, and (3) with AOT.

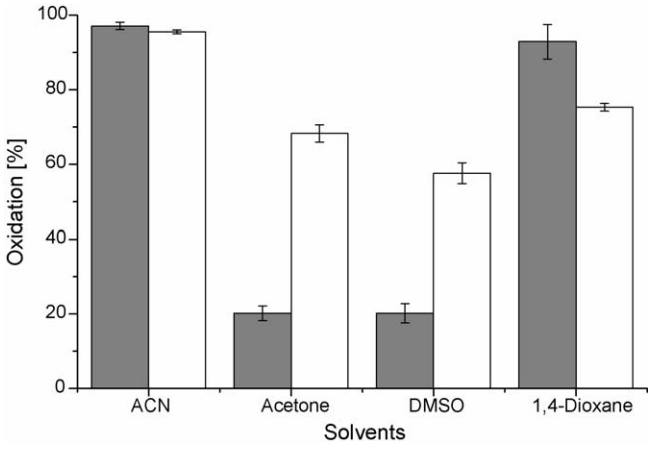


Fig. 8. Degradation of anthracene (■) and fluorene (□) in solutions of various water-miscible solvents (50 mM Tris–HCl, pH 6.0, 2 mM AOT, 1% ACN, 10 μM anthracene or fluorene, 0.176 U YLPO; duration, 2 days).

water-miscible organic solvents are frequently used to increase the bioavailability of environmental contaminants for bioremediation [43,44]. However, the addition of organic solvents often causes problems, e.g., enzyme inactivation. Keum and Li [45] reported that the activities of *T. versicolor* and *P. ostreatus* laccases changed little in up to 10% of acetone, acetonitrile, and DMSO; however, the laccases were rapidly inactivated in over 10% of the organic solvents and were completely inhibited at 80 and 60% of the organic solvents, respectively [45]. Organic solvents can inactivate the enzymes by several mechanisms, e.g., by substituting for the water molecules at the active sites or by altering the effective pH [46,47]. Hydrophobic effects, followed by deformation of the three-dimensional structure, can also inactivate the enzyme [48].

We investigated the oxidation of anthracene and fluorene in solutions of various water-miscible organic solvents. All solvents were used at a 1% concentration because in this case, YLPO activity was unaffected. Anthracene oxidation reached 95% in acetonitrile and 92.9% in 1,4-dioxane solutions. Replacing these solvents by dimethylsulfoxide or acetone resulted in a four-fold decrease in degradation (Fig. 8). Fluorene oxidation reached 95.5% in acetonitrile and 75.3% in 1,4-dioxane solutions. Replacing these solvents by dimethylsulfoxide or acetone resulted in a 1.5-fold decrease in degradation (Fig. 8). Possibly, fluorene protects the enzyme from solvent inactivation more effectively than does anthracene.

Depending on the type of solvent used, HPLC analysis of the reaction-mixture extracts revealed the presence of the same product of anthracene oxidation and various products of fluorene oxidation. In acetonitrile solutions, a product with a retention time of 14.8 min was found, and in DMSO solutions, a product with a retention time of 12.9 min was found. The appearance of both these products in acetone solutions was a surprise. More work is needed to identify these products and to explain the mechanisms of their formation.

3.8. Oxidation of a model PAH mixture

PAH contamination of soil and groundwater usually includes a mixture of several PAHs. Little is known about the biodegradation of complex mixtures of PAHs, especially about the effect of one PAH component on the biodegradability of another [35]. In this study, we chose anthracene and pyrene as representatives of three- and four-ring PAH contaminants.

We investigated the oxidation of a model PAH mixture (10 μM anthracene and 10 μM pyrene) by YLPO in the absence of any mediator. We found an 88% anthracene loss and a 28% pyrene loss within 2 days of incubation.

4. Conclusions

The catalytic activity of YLPO towards a range of phenolic and nonphenolic compounds, including PAHs, their derivatives, and anthracene-like synthetic dyes, was investigated. It was found that the two-ring PAH naphthalene was not oxidized by the enzyme, but naphthalene derivatives (α- and β-naphthols, α-nitroso-β-naphthol, α-hydroxy-β-naphthoic acid, and α-naphthylamine) were. The YLPO-catalyzed oxidation of α-nitroso-β-naphthol and α-hydroxy-β-naphthoic acid was shown for the first time. The enzyme decolorized a wide variety of anthracene-like synthetic dyes, including alizarin red, acridine orange, rhodamine C (butyl ester), eosin, rhodamine 6G, neutral red, and safranin T. Of the dyes tested, only anthrone was not oxidized.

All the PAHs tested, containing from three to five rings, were degraded by YLPO. The degradation effectiveness was higher than that observed for the blue laccase from the same fungus [40] in both absence and presence of standard amounts of typical synthetic mediators (ABTS and HBT). The addition of the mediators reduced YLPO-catalyzed PAH oxidation but enhanced oxidation when the blue laccase was used. However, phenanthrene oxidation by YLPO, unlike that by the blue laccase, proceeded in the absence of any mediator or surfactant in the reaction mix-

ture. The presence of various products of fluorene oxidation, depending on the solvent used, was found.

Although the blue and yellow laccases of *P. ostreatus* D1 oxidized the typical phenolic substrates in a similar way, all the collected data show that the catalytic properties of the blue and yellow laccases towards nonphenolic substrates differ significantly. The mechanism of the laccase-catalyzed degradation of nonphenolic substrates is intriguing and needs further study.

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