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Journal of Molecular Catalysis B: Enzymatic 30 (2004) 19–24
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Catalytic properties of yellow laccase from *Pleurotus ostreatus* D1

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Received 4 August 2003; received in revised form 27 February 2004; accepted 4 March 2004

Abstract

The catalytic activity of yellow laccase from *Pleurotus ostreatus* D1 (YLPO) in the oxidation of 2,6-dimethoxyphenol (DMOP), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and syringaldazine in aqueous buffers of different pH was studied. YLPO showed lower values of V_{max}, and higher K_m values, in the oxidation of DMOP and syringaldazine than yellow laccase from *Panus tigrinus* 8/18, and comparable values with blue laccase from other *P. ostreatus* strain. In order to find the optimum conditions for the oxidation of polyaromatic hydrocarbons (PAHs), the effect of acetonitrile (0–5% (v/v)) on the oxidation of DMOP in acetate buffer, pH 4.0 was investigated. Then, the effect of two surfactants, AOT and Tween 80, on DMOP oxidation in 1% (v/v) acetonitrile solutions was measured. Finally, the oxidation of anthracene and fluoranthene in 2 mM AOT solutions of 1% (v/v) acetonitrile solutions, in the absence of ABTS and presence of ABTS as a mediator of oxidation was examined. YLPO showed a relatively high activity in the degradation of the studied PAHs in the presence of ABTS. For the first time it was shown that the yellow laccase is able to convert PAHs in the absence of exogenous mediators and the observed activity was comparable to that observed in the presence of the mediator (ABTS).

Keywords: Yellow laccase; Anthracene; Fluoranthene; Polyaromatic hydrocarbons; Oxidation

1. Introduction

Laccases (EC. 1.10.3.2) belong to the group of polyphenol oxidases and are produced by fungi, plants and bacteria. The most extensively studied are the extracellular laccases from lignin degrading basidiomycetes [1].

It is commonly accepted that laccases are enzymes able to oxidise aromatic compounds with a phenolic functional group. Typical substrates of laccases are substituted monophenols, polyphenolic compounds and phenolic groups of lignin polymer. However, it was shown recently, that laccases could oxidise also non-phenolic aromatic compounds in the presence of aromatic electron-transfer or radical-forming mediators. In the presence of mediators such as ABTS, 1-hydroxybenzotriazole (HBT), and hydroxyanthranilic acid, laccases are capable to oxidise such compounds as for instance veratryl alcohol, benzyl alcohols, non-phenolic groups of lignin polymer, and lignin model substances [2–4]. Besides, it was reported that the *Trametes versicolor* laccase catalysed the oxidation of the three-ring polycyclic aromatic hydrocarbon (PAH), anthracene in the presence of HBT or ABTS [5,6] and the oxidation of benzo[a]pyrene in the presence of ABTS [7].

Recently it was shown that white-rot fungi cultivated on natural solid lignin-containing substrates produce another form of laccases, so called “yellow laccases” [8]. It was shown that yellow laccases isolated from a few white-rot fungi cultures do not show the characteristic absorption spectrum at about 600 nm (blue colour of blue laccases) and the EPR spectrum [9], and differ in CD spectrum from the respective blue laccases [10]. They show typical activity towards the typical substrates of the more commonly-studied blue laccases, obtained during submerged cultivation, but in contrast to blue laccases, the yellow laccases are capable of oxidising non-phenolic compounds in the absence of mediators [11,12]. Therefore, it was assumed that yellow laccase forms as a result of binding of aromatic products of lignin degradation with the blue laccase. It was postulated that yellow laccases might contain endogenous mediators derived from lignin, which carry out a role of exogenous

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doi:10.1016/j.molcatb.2004.03.005

mediators in the reaction of oxidation of non-phenolic compounds [10,11].

However, at the present time information about the purification and characteristics of the yellow laccases are extremely limited and their catalytic properties are still seldom investigated. There is no information about the ability of yellow laccases to oxidise PAHs. The study of properties of yellow laccases, their ability to oxidise non-phenolic substrates directly, including PAH represents not only theoretical, but also of practical interest. The information obtained about the catalytic properties of these enzymes could serve as a basis for the development of bio-preparations and creation of an effective technology for the bioremediation of PAH-contaminated soils and waters.

The ligninolytic fungus *Pleurotus ostreatus*, which is known as a powerful producer of laccase and an active degrader of polyaromatic hydrocarbons [13], was selected as the object for our study [14].

The aim of this work was to study the catalytic properties of the yellow laccase from *P. ostreatus* D1, in the oxidation of the selected low weight phenolic compounds and polyaromatic hydrocarbons. In particular we wanted to check whether this laccase is capable of catalysing the oxidation of PAHs in the absence of any exogenous mediator, and to compare its activity in the absence and presence of ABTS, the mediator studied earlier in PAH oxidation.

2. Experimental

2.1. Materials

2.1.1. Yellow laccase from P. ostreatus

Laccase was purified from a solid-state culture of the white-rot fungus [12]. The purification included three steps: the ion exchange chromatography on TEAE-Servacel 23 (Chemapol, Czech Republic), ammonium sulphate precipitation (80%), and gel filtration on the Sephadex G-100 (Aldrich, USA). An electrophoretically homogeneous protein was obtained [14].

Protein concentration was determined using the Bradford method [15].

2.1.2. Chemicals

4-Hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,6-dimethoxyphenol (DMOP), polyoxyethylene sorbitan monooleate (Tween 80) and acetonitrile were products of Sigma–Aldrich (Germany), acetic acid and fluoranthene were products of Fluka (Switzerland), sodium acetate and anthracene were from POCh (Poland), disodium hydrogen phosphate and potassium dihydrogen phosphate were the products of Merck (Germany).

Bis-2-(ethylhexyl) sulfosuccinate sodium salt (AOT) was a product of Fluka (Switzerland). It was purified acc. to Menger and Yamada [16].

2.2. Measurements

Oxidation of syringaldazine, ABTS and DMOP was studied spectrophotometrically, at 25 °C, in quadruplicate. 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], and $\varepsilon_{525} = 65{,}000\,M^{-1}\,cm^{-1}$ for syringaldazine [19]. The Shimadzu PC2101 spectrophotometer was used. The concentrated solution of the enzyme was added directly to the cuvettes containing all other components of the system, thermostatically controlled.

The enzyme activity was expressed in units defined as 1 U = 1 μmole of substrate oxidised (or 1 μmole product formed) in one minute by 1 mg yellow laccase from *Pleurotus ostreatus* D1 (YLPO).

PAH oxidation was studied with HPLC. SpectraSeries P200 manufactured by Spectra-Physics Analytical Inc., using a SpectraSeries UV 100 detector, produced by Thermo Separation Products, USA were used. The separation was carried out in the Spherisorb S5 PAH column. Five microlitres of sample was injected. After 2 min of isocratic flow of 40% acetonitrile:water, the analyses were performed with a gradient varying from 40 to 100% acetonitrile, at a rate of 1.6 ml/min, for 35 min. The plates with Silufol UV-254 (“Kavalier”, Czechia) was used for TLC. TLC carried out in system benzol:ethylacetate (2:1).

2.3. Enzymatic treatment of PAH

All experiments were carried out in reaction tubes containing 1 ml of 50 mM acetate, pH 4.0 or Na-phosphate pH 6.0 buffer, 1% (v/v) acetonitrile, with or without 2 mM AOT, and with or without 0.5 mM ABTS. Samples of 100 μl laccase were added to each tube to final concentration of 0.176 U/ml (0.156 mM). PAHs were dissolved in acetonitrile and added to produce a final concentration of 10 or 20 μM. Closed reaction tubes were incubated at 30 °C for the desired period of time. PAHs and the products of their oxidation were extracted with 1 ml chloroform, evaporated and re-dissolved in 100 μl acetonitrile. All experiments were performed in duplicate.

Control samples were prepared in the same manner, but the enzyme was deactivated by boiling for 10 min before its addition.

3. Results and discussion

3.1. Specific activity of YLPO in aqueous solutions

In Fig. 1 the dependence of the catalytic activity of YLPO towards DMPO, syringaldazine and ABTS, upon pH of the

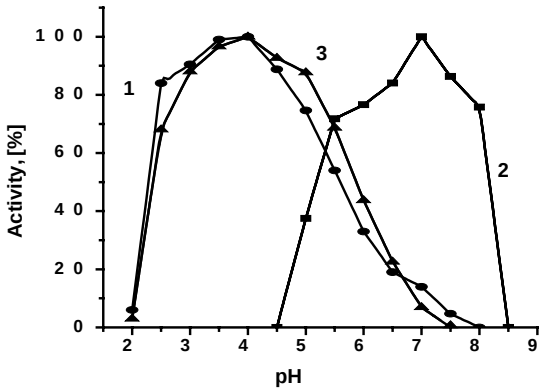

Fig. 1. Dependence of YLPO activity on pH; YLPO concentration 0.01 U ml^{-1}; DMOP concentration 0.5 mM; syringaldazine concentration = 0.04 mM, ABTS concentration = 0.2 mM; 1-ABTS (●), 2-syringaldazine (■), and 3-DMOP (▲).

solutions is presented. It may be seen that the optimum pH values are: 4.0 for ABTS, 4.0 for DMOP, and 7.0 for syringaldazine [14]. The results obtained agree closely with the data in the literature. The optimum pH of other species of *Pleurotus* in ABTS oxidation varies between 3.0 and 4.0, and for syringaldazine oxidation it varies between 6.0 and 7.0 [20].

Table 1 shows the Michaelis–Menten constants for YLPO. These values are much greater than the ones observed in syringaldazine and DMOP oxidation catalysed by the yellow laccase from *Panus tigrinus* 8/18 (K_m = 0.011 mM for DMOP, K_m = 0.001 mM for syringaldazine and K_m = 0.033 for ABTS) [9,11]. The values are however comparable with those obtained for blue laccase from the other strain of *P. ostreatus* (K_m = 0.015 mM for syringaldazine and 0.28 mM for ABTS) [20]. The K_m values for DMOP are relatively high and are comparable in their order of magnitude only with that of blue laccase from *Phlebia radiata* (0.25 mM) [21].

3.2. Optimum conditions for PAH oxidation

The solubility of PAHs in aqueous solutions is very low (0.003–1.3 mg/l) [22]. Their precipitation in aqueous solutions with a low content of organic solvents causes a very high variation of reaction yields. The addition of the detergent increases the solubility of PAHs and allows a repeatable determination of the substrate and products.

Therefore the published attempts of oxidation of PAHs with laccases are based on such systems [5,7]. To increase

Table 1
Michaelis–Menten constants for YLPO

Substrate	K_m (mM)	V_{max} (μmol/(min mg))	k_{cat} (min^{-1})
Syringaldazine	0.0087 ± 0.00088	5.71 ± 0.17	365.44
ABTS	0.11 ± 0.0075	11.0 ± 0.29	704.00
DMOP	0.43 ± 0.02	8.37 ± 0.16	536.50

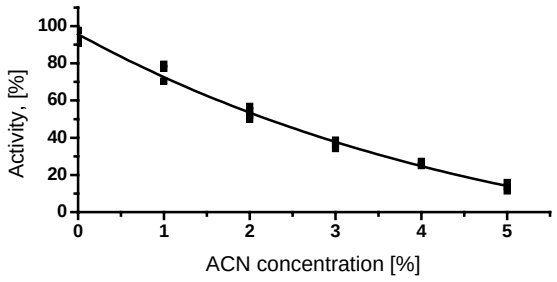

Fig. 2. Dependence of YLPO activity upon acetonitrile concentration; YLPO concentration 0.01 U ml^{-1}; DMOP concentration 0.5 mM.

the substrate availability to enzymes and cells non-ionic surfactants such as Tween 20 and Tween 80 are usually used [23]. On the other hand, AOT is one of the most biocompatible ionic surfactants, widely used for bioconversions in apolar organic systems [24]. In this work Tween 80 and AOT have been tested.

In attempt to find an optimal solution, the effect of acetonitrile, and Tween 80, and AOT on the rate of oxidation of DMOP by the YLPO, and on stability of the enzyme were studied.

Fig. 2 shows the effect of acetonitrile concentration on the initial rate of oxidation of 0.5 mM DMOP by 0.01 U YLPO. It may be seen that even 1% acetonitrile reduces laccase activity by 25% of that observed in the absence of a surfactant. At the same time Pickard et al. have shown, that laccases of fungi *Coriolopsis gallica* and another strain *P. ostreatus* keep up to 91% of activity at the incubation in 15% ACN within a day [25]. The result obtained for YLPO is however consistent with our earlier work on the effect of ethanol on blue and yellow laccases from *P. tigrinus* 8/18, where the yellow laccase was also relatively very sensitive (more than the blue one) to the presence of the organic solvent. It may be assumed, that such a high sensitivity of yellow laccase to the solvents can be a consequence of the presence of the endogenous mediators at the active centre of enzyme, which, as it is visible from CD-spectrum, destabilized the laccase structure [10]. This property of the yellow laccases will be investigated further.

Figs. 3 and 4 show the effect of AOT and Tween 80 on the initial rate of DMPO oxidation by YLPO in 1% acetonitrile

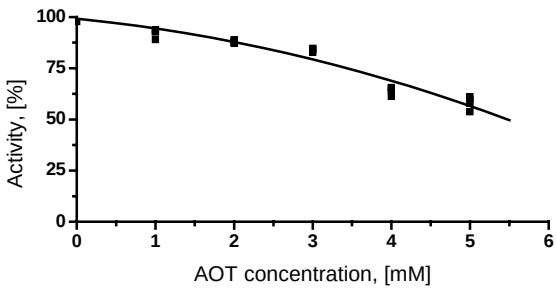

Fig. 3. Dependence of YLPO activity upon AOT concentration in 1% (v/v) acetonitrile solutions; YLPO concentration 0.01 U ml^{-1}; DMOP concentration 0.5 mM.

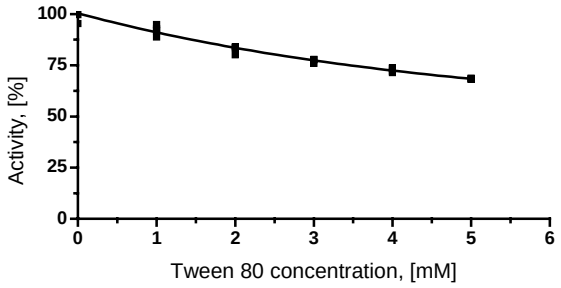

Fig. 4. Dependence of YLPO activity upon Tween 80 concentration in 1% (v/v) acetonitrile solutions; YLPO concentration 0.01 U ml^{-1}; DMOP concentration 0.5 mM.

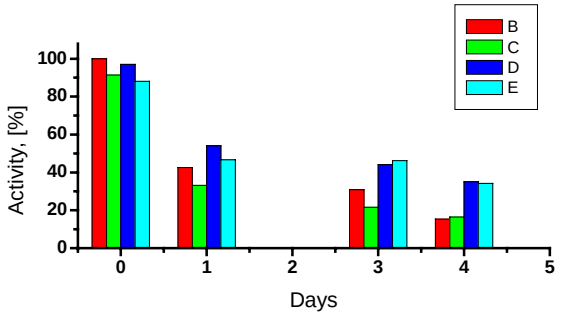

Fig. 5. YLPO stability, at pH 4.0; (B) 50 mM acetate buffer; (C) 1% (v/v) acetonitrile; (D) 1% (v/v) acetonitrile, 2 mM AOT; (E) 1% (v/v) acetonitrile, 2 mM Tween 80.

solutions. Both surfactants reduce activity in a similar way, 2 mM AOT reduces it by12% and 2 mM Tween 80 by 17%. The stability of YLPO in 2 mM AOT and Tween 80 solutions was then checked (Figs. 5 and 6).

Figs. 5 and 6 show the results of the measurements of the initial rate of DMOP oxidation by YLPO incubated for 0, 24, 72 and 96 h in the following solutions: B = control, C = 1% (v/v) acetonitrile solutions, D = 1% (v/v) acetonitrile, 2 mM AOT, and E = 1% (v/v) acetonitrile, 2 mM Tween 80, of pH 4.0 (Fig. 5) or pH 5.0 (Fig. 6). It may be seen that YLPO is more stable in solutions of pH 5.0. Moreover, the presence of surfactants stabilizes YLPO, even in comparison with the control aqueous buffer solutions. The effect is more

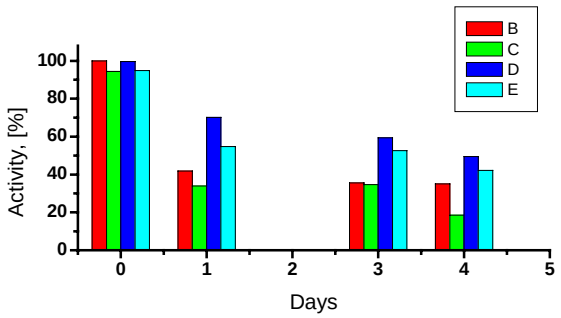

Fig. 6. YLPO stability, at pH 5.0; (B) 50 mM acetate buffer; (C) 1% (v/v) acetonitrile; (D) 1% (v/v) acetonitrile, 2 mM AOT; (E) 1% (v/v) acetonitrile, 2 mM Tween 80.

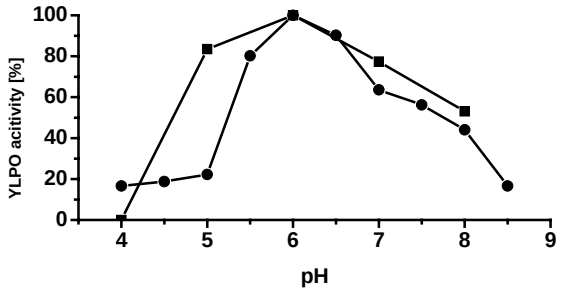

Fig. 7. Optimum pH levels for anthracene and fluoranthene conversion in 1% (v/v) acetonitrile, 2 mM AOT. (■) Anthracene, (●)fluoranthene. The value observed at pH 6.0 was taken as 100% for both PAH, so as to show both curves in one figure.

pronounced for 1% (v/v) acetonitrile solutions. AOT seems to be a more effective YLPO stabilizer than Tween 80, at least under the experimental conditions.

To check, whether the selected system would not inhibit the reaction in the presence of PAHs of low water solubility the activity of YLPO in oxidation of 40 mM syringaldazine in 1% acetonitrile, pH 6.0, in the absence and presence of 2 mM AOT was checked. No effect by AOT on the initial reaction rate was found.

On the basis of these results the experiments to determine anthracene and fluoranthene oxidations were carried out in solutions containing 1% (v/v) acetonitrile and 2 mM AOT.

3.3. Oxidation of anthracene and fluoranthene

Laccase alone oxidised the studied PAHs, which might be considered to be the result of the action of an endogenous mediator postulated earlier [8,9,11]. In such a case the presence of exogenous mediators would not be necessary.

The use TLC showed the presence of products of reactions with Rf = 0.64 and 0.6, for anthracene and fluoranthene, accordingly.

The formation of 9,10-anthraquinone from anthracene was observed in HPLC analysis and by UV-Vis spectroscopy. At pH 6.0, in the absence of ABTS, TLC also showed the formation of one product only. In the presence of ABTS, at low pH optimal for its oxidation some coupling products were also observed in HPLC analyses. This is similar to Hüttermann's findings [5]. Both by TLC and HPLC a single fluoranthene oxidation product was observed. We have not identified this product. Whereas the literature data show that laccases oxidise anthracene to 9,10-antraquinone, data is not available on the product of fluoranthene oxidation by laccase [5,6]. Identification of these products is not trivial [26] and requires separate study.

The quantitative analysis of the conversion was carried out by comparison of the concentration of substrate in the experimental tube and in the control, after the reaction had taken place for the desired time.

First, the pH optimum of PAH oxidation was tested. Fig. 7 shows the similar pH maximum of pH 6.0 both for

Table 2
Anthracene conversion (2 days)

System	Substrate conversion (%)
YLPO, pH 6.0	95 ± 1
YLPO + AOT, pH 4.0	0
YLPO + AOT, pH 6.0	89 ± 3
YLPO + ABTS, pH 6.0	76 ± 4
YLPO + ABTS, pH 4.0	96 ± 0.5
YLPO + ABTS + AOT, pH 4.0	98 ± 1

anthracene and fluoranthene. Therefore, subsequent reactions in the absence of ABTS were carried at pH 6.0.

To compare the effectiveness of YLPO with that of other laccases, the experiments in the presence of an exogenous synthetic mediator were also carried out. To discriminate between the reaction catalysed by YLPO with and without such mediator, ABTS was chosen. The pH optimum for ABTS oxidation catalysed by YLPO is equal to 4.0. At pH 6.0 its oxidation is relatively low. Conversely, the activity of YLPO alone in PAH oxidation is low at pH 4.0 and high at pH 6.0. The comparison of the PAH conversion at both pH allows the comparison of the effects of the laccase action with and without exogenous mediation.

Table 2 shows the results of anthracene (10 μM) conversion during the first 2 days of incubation at 30 °C. The following can be seen:

(i) laccase alone does not oxidise anthracene at pH 4.0 during the first 2 days of incubation;
(ii) at pH 6.0 the YLPO alone oxidises the substrate to a greater extent than in the presence of 0.5 mM ABTS. The effect of ABTS is however most significant at pH = 4.0, which is the pH optimum for ABTS oxidation by YLPO and corresponds to very low activity level of YLPO alone;
(iii) laccase with ABTS is only slightly better than laccase alone, when ABTS is added at the pH optimum for its oxidation and laccase alone works at the optimum pH for anthracene oxidation;
(iv) the presence of AOT does not practically improve anthracene oxidation (during the first 48 h).

It may be seen that anthracene oxidation in the absence and presence of ABTS reaches comparable degree. It is not surprising if we assume that all molecules of yellow laccase catalyse the reaction. In both cases there is an excess of ABTS and YLPO (and possibly its endogenous mediator) in relation to the substrate molecules (although the excess is greater in the case of ABTS).

The above results may be compared with the results of action of laccases from *Trametes versicolor*, *P. ostreatus* and *Coriolopsis gallica* on anthracene [5,23,25].

First, YLPO is much more efficient catalyst of anthracene oxidation than the laccases from the above mentioned fungi. Moreover, under the experimental conditions applied in the work of Hüttermann, ABTS considerably increased the anthracene oxidation: 1 mM ABTS, at pH 4.5 increased the anthracene degradation from 35 to 64%, and 2 mM ABTS increased this value to 89% [5]. In the presence of 2 mM ABTS besides 9,10-anthraquinone also the coupling products were formed [5]. The stoichiometric amount of 9,10-anthraquinone was observed only at low mediator mediator and product concentration, where the quinone was the final product. At the high concentration of the mediator the observed 9,10-anthraquinone accumulation was lower than the substrate degradation [5]. In the studied here case; of YLPO, for relatively high substrate degradation, also no clear correlation between anthraquinone formation and anthracene degradation was found either.

Table 3
Fluoranthene degradation (10 days)

System	Substrate conversion (%)
YLPO, pH 6.0	14 ± 4
YLPO + AOT, pH 6.0	49 ± 5
YLPO + ABTS, pH 6.0	24 ± 0.5
YLPO + AOT + ABTS, pH 6.0	26 ± 0.5

Table 3 presents the results of fluoranthene (20 μM) conversion during the first 10 days of incubation (no substrate concentration reduction was observed during the first 2 days of incubation). It may be seen that:

(i) YLPO alone oxidises fluoranthene;
(ii) AOT improves the results; to a significant extent in the absence of ABTS, to a slight extent in the presence of ABTS;
(iii) the effect of ABTS at pH 6.0 is not clear.

Similarly, the fluoranthene degradation or oxidation catalysed by *Trametes versicolor* laccase was not sensitive to the addition of ABTS, even at pH 4.5 [27]. On the other hand, Pickard et al. have shown, that laccases of fungi *Coriolopsis gallica* and *P. ostreatus* do not oxidise or degrade fluoranthene, either in presence mediator, or directly (reaction time not given) [24].

4. Conclusions

Yellow laccase from *P. ostreatus* shows pH optima in oxidation of typical phenolic substrates (ABTS, DMOP and syringaldazine), comparable with laccases from other *Pleurotus* species. V_{max} values observed at optimal pH levels for these substrates are generally lower and K_m values are higher than those observed for blue laccases.

However, the enzyme seems to be a very good catalyst of anthracene and fluoranthene oxidative degradation by comparison, both in the absence and presence of ABTS (Fig. 2), to other studied laccases. Anthracene oxidation in the absence and presence of ABTS reaches comparable state. This outcome is not surprising if we assume that all molecules of yellow laccase catalyse the reaction. Then, in both cases

there is an excess of the mediator in relation to the substrate molecules (greater in the case of ABTS).

Johannes and Majcherczyk [6] have shown recently, that some aromatic substances produced by white-rot fungi at the process of lignin degradation, can act as exogenous mediators of laccases. For example, 4-hydroxybenzoic the acid served as a similarly effective mediator as HBT in the oxidation of benzo[a]pyrene catalysed by laccase [28]. Our early studies have shown, that such natural mediators, apparently, can be bound by the enzyme molecule. It results in change as molecular (yellow colour, UV-Vis-, EPR- and CD-spectra) and catalytic (oxidation of non-phenolic substrates) properties of laccase [8,10].

The research into the catalytic mechanism of yellow laccase will be continued.

Acknowledgements

The authors thank V.E. Nikitina, DSc, for the strain of the fungus *P. ostreatus* D1. The authors thank for the financial support of Dr. N.N. Pozdnyakova stay in Krakow (KBN Grant SPB–CATCOLL).

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