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# Immobilized Laccase Mediator-Catalyzed Oxidation of Aqueous Mixtures of Polycyclic Aromatic Hydrocarbons

Daniel E. Dodor [a], Michael Miyittah[b], and Benjamin D. K. Ahiabor[c]

[a]Department of Soil Science, School of Agriculture, University of Ghana, Legon, Ghana; [b]Department of Environmental Sciences, University of Cape Coast, Cape Coast, Ghana; [c]Department of microbiology, CSIR-Savanna Agricultural Research Institute, Tamale, Ghana

## ABSTRACT

Although terrestrial and aquatic environments are polluted with mixtures of polycyclic aromatic hydrocarbons (PAHs), much of the research efforts toward bioremediation of these toxic, mutagenic and/or carcinogenic pollutants have focused on individual compounds. The potential of laccase (E.C. 1.10.3.2) from *Trametes versicolor* immobilized on soil and kaolinite for *in vitro* oxidation of equimolar concentration of anthracene (AnT), benzo[a]pyrene (BaP), benz[a]anthracene (BaA) and pyrene (PyR) in sole, binary, ternary and quaternary substrate systems, in the presence of 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) as mediator compounds was investigated. Results indicated that HBT was a better mediator compared to ABTS due to favorable redox potential difference ($\Delta E_o$) between laccase-HBT and PAH couples. The amount of each PAH oxidized depends on the type and number of PAHs present in the mixture. Due to its low molecular weight and high $E_o$, AnT inhibited the oxidative transformation of the other PAHs, and appeared to be competing for the same active site of the enzyme with BaP. Except for AnT, the oxidation of all the PAHs decreased with complexity of the mixture, with PyR oxidation inhibited by the presence of BaA. The oxidation of the PAHs was significantly low in the quaternary substrate system, probably due to enzyme inhibition from substrate overload. The results indicated that competitive effect may arise when more than one PAH is present in the reaction solution to interact with the enzyme and offer the possibility of the use of immobilized LMS for remediating complex PAH mixtures in the environment.

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute an important class of toxic, mutagenic and/or carcinogenic organic pollutants that are widely distributed in terrestrial and aquatic environments. These ubiquitous environmental contaminants are formed and released in significant levels into our drinking water, food and air through natural and man-made sources. The US Environmental Protection Agency (EPA) classifies 16 PAHs as priority pollutants whose remediation are considered indispensable for environmental cleanup and human health.[1] Furthermore, 15 of the PAHs are designated as known or reasonably anticipated human carcinogens.[2] Therefore, lots of research efforts have been expended in designing environmentally friendly and feasible remediation techniques to minimize human and environmental health risk associated with exposure to these contaminants.

Various extracellular peroxidases capable of oxidizing phenolic pollutants and transforming them into innocuous products have been used for biodegradation of PAHs.[3] Laccases (benzenediol: oxygen

oxidoreductases; E.C. 1.10.3.2), copper-containing oxidoreductive enzymes known to catalyze a one-electron oxidation of substrates, are considered the most promising among these groups of enzymes.[4] In addition, the relaxed substrate specificity of laccases has enable them to oxidize broad range of polyphenols and aromatic substrates.

Laccases are capable of oxidizing substrates with lower or comparable redox potential ($E_o$) values than their own,[5] with the oxidative potency dependent on the redox potential difference ($\Delta E_o$) between the enzyme and the substrate.[6] In addition, as molecular size and complexity of substrates increase, accessibility to the active site of the enzyme decreases due to steric hinderances,[7,8] thereby decreasing the reactivity and oxidation of the substrates. However, inclusion of certain exogenous compounds in the reaction mixture to form laccase-mediator systems (LMSs) can boost the oxidative potential of the enzyme, thereby enabling it to oxidize substrates it could not hitherto oxidize directly due to their high $E_o$ values or molecular size.[9]

Other important parameters that need to be optimized for practical application of an enzyme on a large-scale basis include the capability of use under varied environmental conditions, prolong storage and reusability. To these ends, immobilization of laccase on different carriers have been shown to improve the thermal stability and pH range of the enzyme, enabling it to withstand wider external denaturing conditions compared to its soluble or free counterpart.[10,11] The ability of immobilized laccase from *Trametes versicolor* to oxidize AnT and BaP on repeated basis without loss of activity has been demonstrated.[12]

Although terrestrial and aquatic environments are often polluted with complex mixtures of PAHs, much of the research efforts toward bioremediation have focused on individual compounds. The heterogeneity of occurrence of PAHs in the environment could trigger substrate interactions that can manifest itself in the form of competition for the enzyme active site, leading to varied degree of oxidative transformation of xenobiotic compounds. This can substantially complicate the fate and effect, remediation as well as risk assessment of these compounds.

The potential of immobilized LMS for *in vitro* oxidation of individual PAHs have been reported.[12] However, to the best of our knowledge, the application of immobilized LMS for biotransformation of mixtures of PAHs in aqueous systems has not been studied. Therefore, in the present study, laccase from *T. versicolor* was immobilized on soil and kaolinite, and the potential of the free and immobilized laccases to oxidize anthracene (AnT), benzo[a]pyrene (BaP), benzo[a]anthracene (BaA) and pyrene (PyR) in sole, binary, ternary and quaternary substrate systems, in the presence of 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and N-hydroxybenzotriazole (HBT) was investigated.

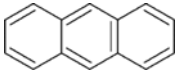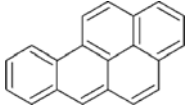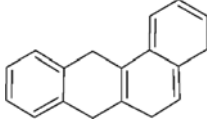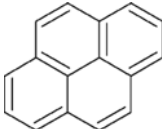## Materials and methods

### Chemicals

AnT, BaP, BaA and PyR, ABTS, HBT, acetone and acetonitrile were obtained from Sigma (St. Louis). The four PAHs are on US EPA's priority pollutants list,[1] and are designated as known or reasonably anticipated human carcinogens;[2] AnT and BaP are considered markers of toxicity and biotransformation. Selected physical and chemical properties of the PAHs are shown in Table 1. All other reagents used in the present study were of analytical grade.

### Enzyme immobilization, kinetics and stability studies

A commercial laccase (E.C. 1.10.3.2) from *T. versicolor* with 34 U/mg activity was purchased from Sigma and used without further purification. Laccase was immobilized on soil and kaolinite as described previously.[12] The activity of the free and immobilized laccase was assayed at 25 °C using ABTS as substrate.[12] The standard condition for the assay contains 1.5 mM of ABTS in 0.1 M Na-acetate buffer (pH 4.5) and a suitable amount of the free or immobilized enzyme. The oxidation of ABTS was monitored by measuring the increase in absorbance at 420 nm ($\varepsilon_{420} = 3.6 \times 10^{-4}$ M$^{-1}$ cm$^{-1}$). Laccase activity is expressed in enzyme units (U), with 1 U of enzymatic activity defined as the amount of the enzyme required to oxidize 1 µmol of ABTS per minute. The kinetic parameters of the free and immobilized laccases were determined using ABTS in the concentration ranges 0.05–2 mM. A non-linear regression

**Table 1.** Selected physical–chemical properties of the PAHs studied.

| Compound | CAS no. | Molecular weight (g/Mol) | Solubility at 25 °C (mg/l)[a] | Half-wave oxidation potential, $E_{1/2\text{-ox}}$ (V, vs. SCE)[b] | Structure |
|---|---|---|---|---|---|
| Anthracene | 120–12-7 | 178 | 4.3E—02 | 1.09 | |
| Benzo[a]pyrene | 50.32–8 | 252 | 1.6E—03 | 1.27 | |
| Benz[a]anthracene | 56–55–3 | 228 | 9.4E—03 | 1.18 | |
| Pyrene | 129–00-0 | 202 | 7.7E—02 | 1.16 | |

[a]ChemIDplus 2017.
[b]Values are from Pysh and Yang.[28]

model was used to estimate the Michaelis–Menten constants, $K_m$ and $V_{max}$ of the enzyme preparations. Except for the soil immobilized laccase, other kinetic parameters of the enzyme preparations, including temperature-activity profile, stability to elevated temperatures, inhibitors, organic solvents and effect of storage condition have been reported previously.[12]

### *Enzymatic treatment of PAHs with laccase and laccase-mediator systems*

For the single substrate systems, a 25-µL aliquot of 5 mM of AnT, BaP, BaA or PyR in acetone was added to a 50-ml amber bottles containing free or immobilized laccase adjusted to 4 U/ml with 0.1M Na-acetate buffer (pH 4.5). Tween 80 was added to all treatments to a final concentration of 1% to increase PAH bioavailability, and the mediator compounds, HBT and ABTS were added to a final concentration of 0.1 and 1.0 mM, respectively. The reaction mixture was brought to a final volume of 5 ml to provide a final PAH concentration of 25 µM. The bottles were closed tightly with Teflon-lined screw cap and incubated on a rotating shaker at room temperature for 24 h. The dependence of substrate transformation on the presence of other PAHs was evaluated by using laccase immobilized on kaolinite. The competitive experiment was carried out by incubating immobilized LMSs containing AnT, BaA, BaP and PyR in binary, ternary or quaternary mixtures (25 µM final concentration of each PAH) under conditions described above. The relative reactivity or intermolecular selectivity of the LMSs toward equimolar amount of the substrates was determined by measuring the amount of each PAH oxidized in the reaction system after 24-h incubation.

At the end of the incubation period, the complete reaction mixture was centrifuged at 13,000*g* for 10 min, and the supernatant decanted and shaken with 5 ml of acetonitrile in complete darkness for 1 h. A 1-ml aliquot of the extract was centrifuged at 5,000 *g* for 10 min, filtered through 0.45 mm nylon membrane, and the concentration of the parent PAH compounds remaining in the reaction mixture determined by High Performance Liquid Chromatography (HPLC) as described previously.[12] In all cases, control samples were treated in the same manner, but the free and immobilized enzyme preparations were boiled for 30 min before the mediator compounds and PAHs were added. This was necessary to deactivate the enzyme and to evaluate sorption of the PAHs by the solid support. The percentage of

PAH oxidized by the free and immobilized LMSs were calculated from the difference between the concentration of PAH in the reaction mixtures and that in the corresponding controls without enzyme. All treatments, including controls were replicated three times.

### Reusability of immobilized laccase

Laccase immobilized on kaolinite was used to investigate the reusability of immobilized LMS in a batch experiment under similar conditions described for the single substrate systems above. After incubation of PAHs with the immobilized enzyme for 24 h, the reaction mixture was centrifuged at 13,000 *g* for 10 min and the supernatant filtered and extracted for PAH analysis. The enzyme preparation was washed several times with phosphate buffer, after which the oxidative potential of the immobilized laccase was measured again by adding fresh PAHs. The oxidative transformation was repeated four times.

### Statistical analysis

Separation of means was performed by ANOVA and Duncan's multiple range test was used to evaluate the significant differences between means at the 95% level of probability. All statistical analysis and graphs were generated using Graph Pad Prism 7 for windows.

## Results and discussion

### Kinetic parameters of free and immobilized laccases

Based on the $K_m$ values of $\leq 261$ µM for the enzyme preparations (Table 2), the 1.5 mM ABTS concentration chosen for the standard assay condition was sufficient to achieve substrate saturation conditions. At this concentration, the reaction followed a first-order kinetics, where the rate of the reaction was independent of the substrate concentration but dependent on the amount of the enzyme. The relatively lower $K_m$ values of the immobilized laccases compared to the free enzyme indicates potential conformational change of the enzyme due to immobilization, leading to a higher affinity for ABTS used as a substrate for the enzyme activity assay.

Immobilization on the soil shifted the optimum pH of laccase slightly to acidic region (pH optimum of 4.0) compared to the free enzyme (pH optimum of 4.5); however, the pH optimum was unchanged when kaolinite was used as the support (Table 2). The optimum pH for laccase activity has been reported to be in the acidic region, with typical pH values ranging from 3.5 to 7.0.[3,13] The shift in the optimum pH of laccase after immobilization on the soil agrees with the results of other researchers.[14,15] Improved stability of immobilized laccase at wider pH ranges compared to the free counterpart, as well as kinetic parameters of the enzyme preparations, such as temperature-activity profile, stability to elevated temperatures, inhibitors, organic solvents and effect of storage conditions have been reported previously.[12]

### Oxidation of PAHs in sole substrate systems

Oxidation of the PAHs in the sole substrate systems by free and immobilized laccases are shown in Figure 1. The control experiment using boiled immobilized laccases showed that adsorption of the PAHs onto the solid supports was not significant, indicating that biological processes are responsible for the

**Table 2.** Kinetic parameter of free and immobilized laccases.

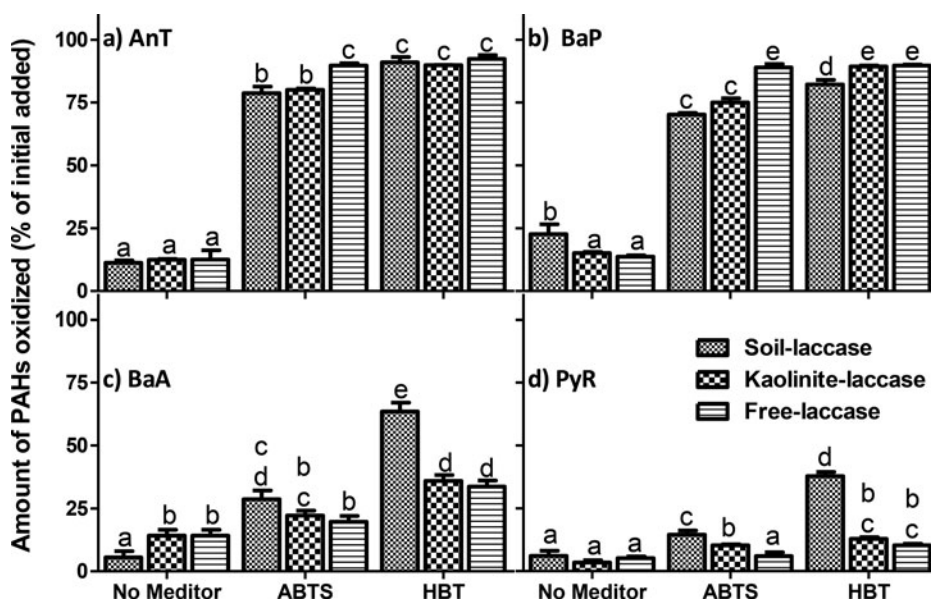| Enzyme | Michaelis–Menten constants | | Optimum pH |
|---|---|---|---|
| | $K_m$ (µM) | $V_{max}$ (µM min$^{-1}$) | |
| Free-laccase | 261.4 | 531.2 | 4.5 |
| Kaolinite-laccase | 119.0 | 720.1 | 4.5 |
| Soil-laccase | 165.0 | 1381.0 | 4.0 |

**Figure 1.** Oxidation (%) of 25 mM of a) AnT, b) BaP, c) BaA and d) PyR by free and immobilized laccase in the presence of different mediator compounds during 24-h incubation. Error bars represent standard error of the means ($n = 3$). Within each PAH, bars with the same letter(s) are not significantly different at $p < 0.05$.

observed transformations. There was no significant oxidation of the PAHs by the free and immobilized laccases alone (Figure 1). However, the addition of 1 mM ABTS or 0.1 mM HBT to the reaction mixtures resulted in significant ($p < 0.001$) increase in the rate of oxidation of the PAHs during the 24-h incubation. The largest increase in oxidation was observed with AnT and BaP; oxidation of the two PAHs increased from ≤22.7% without mediators to almost total disappearance after 24-h incubation (Figure 1a and b). Oxidation of BaA increased from 2% without mediators to 55% in the presence of ABTS and HBT (Figure 1c). Although the overall oxidation of PyR was negligible, there was significant increase in its oxidation in the presence of the mediator compounds compared to that in their absence (Figure 1d).

Overall, HBT showed superior catalytic efficiency in influencing the extent of the oxidation of the PAHs studied compared to ABTS. The results of the present study agree with previous reports showing significant increase, up to 75% in the transformation of AnT and BaP in mixtures of synthetic PAHs in the presence of laccase-ABTS system, and higher oxidation rate of PAHs by immobilized laccase-HBT compared to laccase-ABTS systems.[16] The results, however, disagree with other researchers who reported no transformation of PAHs when HBT was used as the redox mediator, and attributed it to instability and decay of the HBT radicals formed.[17]

With regards to the state of laccase (whether free or immobilized), generally, the oxidation of AnT and BaP were similar without mediator compounds (Figure 1a and b). There was statistically higher oxidation of BaA and PyR with the immobilized compared to the free LMSs. However, when all the oxidation data were pooled across the four PAHs studied, analysis of variance indicated that the state or form of the enzyme preparations did not influence the rate of oxidation of the PAHs. This indicates that immobilization did not affect the catalytic efficiency of the PAH oxidation by the enzymes. These findings agree with previous studies showing that oxidation of AnT and BaP was not significantly different between free and immobilized LMSs.[12] However, there was a significant ($p < 0.05$) enzyme-state × mediator interactive effects, suggesting a complex interplay of the two factors in influencing oxidation of the PAHs studied.

### Role and mechanism of the mediator compounds

The role of mediator compounds in the oxidation of non-phenolic compounds, such as PAHs is by "shuttling" electrons between the enzyme and the substrate, with the catalytic efficiency of the oxidation

process being directly proportional to the redox potential difference ($\Delta E_o$) between the enzyme and the substrate.[9,18] The mechanism of the mediator-facilitated laccase-catalyzed reaction involves oxidation of the mediator by the enzyme to form stable intermediate compounds with higher $E_o$ values.[9,19] The $E_o$ value of a fully oxidized ABTS and HBT were reported to be 1.09 V and 1.2 V, respectively.[6,20] The oxidized cation radicals with higher $E_o$ values then diffuse away from the enzyme to oxidize substrates with higher $E_o$ values than those of the enzyme.[16,21]

The oxidation of substrates by laccase has been reported to be limited to those with $E_o$ values ≤ 0.94 V.[22] Due to the low redox potential of laccase from *T. versicolor* (0.78–0.8 V),[23] the enzyme could not oxidize directly the PAHs studied that have higher $E_o$ values (≥0.94 V; Table 1). Inclusion of the mediator compounds with $E_o$ values higher than 0.94 V in the reaction mixtures boosted the oxidative capability of the enzyme, enabling it to oxidize the PAHs. The relatively low percent transformation of BaA and PyR can be partially attributed to the unfavorable $\Delta E_o$ value between the LMSs and the two PAHs (Table 1). In addition, as molecular size and complexity of substrates increases, water solubility decreases, resulting in decreased accessibility to the active site of the enzyme due to steric hinderances,[6,7] thereby decreasing the reactivity of laccase. Except for BaP, the molecular weight of BaA and PyR are relative large, and with low aqueous solubility.

Between the two mediator compounds used in the present study, HBT showed superior catalytic potency in oxidizing the PAHs compared to ABTS. The oxidization of ABTS involves an initial partially oxidized cation radical (ABTS$^+$) with $E_o$ value of 0.68 V, which is further oxidized to the dication radical (ABTS$^{++}$) with $E_o$ value of 1.09 V.[20] The two oxidized forms of ABTS are electrochemically reversible.[21] The nature of the oxidized state(s) of ABTS responsible for oxidation of the PAHs depends on pH of the medium, with the dication radical (ABTS$^{++}$) being the dominant and stable form at acidic pH of 3.5.[21,24] It has also been shown that ABTS$^{++}$, which is the most oxidized form of ABTS is responsible for PAH oxidation.[19,21] Because the PAHs oxidation experiment was conducted at pH 4.5, in theory, ABTS$^+$ will be present to some extent in the reaction mixture. Since the $E_o$ value of a fully oxidized dicationic form of ABTS (ABTS$^{++}$) is 1.09 V,[20] the presence of ABTS$^+$ with $E_o$ value of 0.68 V in the reaction mixture will decrease the equilibrium $E_o$ to a value lower than 1.09 V. This decrease in the $E_o$ value of ABTS resulted in the slower oxidation rate of the PAHs when it was used as the mediator compound. There was, however, no correlation between $E_o$ values and oxidation of the PAHs studied, probably due to the small number of substrates used. Another mechanism by which HBT could be a better mediator compared to ABTS is through the radical H-atom transfer pathway that neglects the $E_o$ features of the substrate.[24]

### *Repeated oxidation of PAHs by immobilized laccase*

Laccase immobilized on kaolinite was used to study the ability of immobilized LMS to oxidize AnT, BaP and BaA on repeated basis. As shown in Figure 2, the ability of immobilized laccase to oxidize the PAHs on continuous basis decreased sharply without mediator compounds. In the presence of ABTS and HBT, however, immobilized laccase oxidized AnT and BaP without appreciable loss of catalytic activity after four cycles of use (Figure 2a and b). The maintenance of catalytic activity of the immobilized LMS can be attributed to the prevention of cross-linking of the enzyme with quinine reaction products by the mediator compounds.[25]

Given the low percent BaA oxidation in the single batch experiment, the loss of catalytic activity by the immobilized LMS after three cycles of its oxidation was not surprising. The observed decrease in the oxidation of BaA in the absence of mediator compounds agrees with the result of other workers who reported over 36% decrease in laccase activity following incubation with mixtures of PAHs.[17] A complementary decrease in laccase activity following its use to remove phenolic compounds from a synthetic waste water, albeit without quantitative relationship between percent phenol removed and enzymatic activity, has also been reported.[26,27] The authors attributed this to adsorption and incorporation of the enzyme protein into organo-mineral complexes formed in the reaction mixture. As observed in the sole PAH oxidation experiment and discussed in section *Role and mechanism of the mediator compounds*, the superiority of HBT in influencing oxidation of the PAHs was once again apparent in the repeated oxidation experiments.
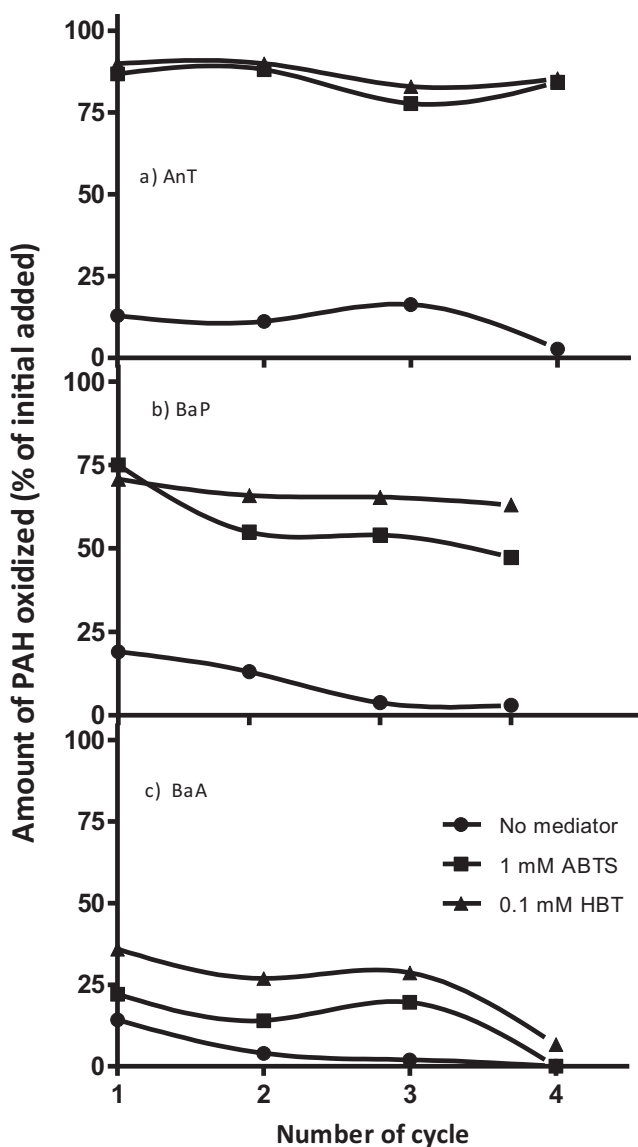
**Figure 2.** Reusability of immobilized laccase for biotransformation of PAHs over four cycles in the presence of mediator compounds.

### *Oxidation of PAHs in binary substrate systems*

Due to the low oxidation of the PAHs in the absence of mediator compounds, results presented in Figure 3 are those in binary mixtures with immobilized LMSs only. As observed in the sole substrate system, significantly higher PAHs were oxidized with HBT as the mediator compound compared to ABTS. The oxidation of AnT, a low molecular weight PAH, was significantly ($p < 0.05$) higher in the binary system with BaA and PyR compared with the sole substrate systems (Figure 3a). This indicates that AnT oxidation was stimulated by the presence of BaA and PyR in the binary mixtures and suggests the presence of a distinct catalytic site responsible for its oxidation for which the two PAHs cannot compete. The oxidation of AnT in the binary system with BaP was similar to that observed in the sole substrate system.

Biotransformation of BaP was significantly ($p < 0.05$) lower in the binary compared to the sole substrate systems when ABTS was used as the mediator compound, with the greatest inhibition occurring in the presence of AnT (Figure 3b). In the presence of HBT, however, the inhibitory effect due to the presence of BaA and PyR was suppressed, resulting in similar amount of BaP oxidation in the binary
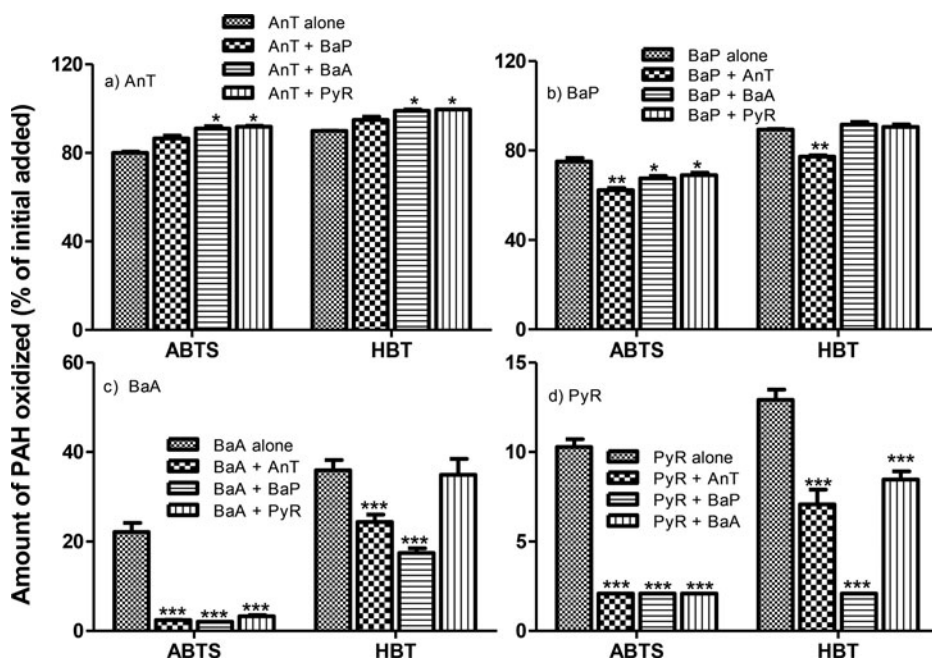
**Figure 3.** Oxidation (%) of 25 mM of a) AnT, b) BaP, c) BaA and d) PyR in binary mixtures by immobilized laccase in the presence of different mediator compounds after 24-h incubation. Error bars represent standard error of the means ($n = 3$). Within each PAH system, bars with *$p < 0.05$, **$p < 0.01$, ***$p > 0.001$ compared to the respective PAH alone.

compared to that observed in the sole substrate systems. It is interesting to note that HBT was not able to relieve the inhibitory effect of the presence of AnT on oxidation of BaP. The higher but statistically insignificant oxidation of AnT in the presence of BaP (Figure 3a), coupled with the significantly lower oxidation of BaP in the presence of AnT with both mediator compounds (Figure 3b) suggest that the two PAHs are probably competing for the same active site of the enzyme. The observed efficient and higher oxidation rate of AnT in the binary system containing BaP can be attributed to the smaller size, relatively higher aqueous solubility and lower $E_o$ value of AnT compared to BaP (Table 1).

The oxidation of BaA was significantly ($p < 0.05$) inhibited by the presence of the other three PAHs when ABTS was acting as the redox mediators (Figure 3c); BaA oxidation decreased significantly ($p < 0.05$) from 22.1% in the sole substrate system to $\leq 1.3\%$ in the binary systems. However, HBT suppressed the inhibitory effect of the presence of PyR on BaA oxidation, resulting in similar amounts of the two PAHs being oxidized in the binary compared to the sole substrate systems. The results suggest that the same active site might be responsible for oxidation of BaA and PyR. Because there was no correlation between $E_o$ values and the percentage of PAHs oxidized, the $\Delta E_o$ differences between the two PAHs and the LMS cannot be used to explain the observed phenomena. We speculated that HBT suppressed the inhibitory effect of PyR on BaA oxidation through the radical H-atom transfer pathway that neglects the $E_o$ features of the substrates.[24]

Although the oxidation of PyR was low in the sole substrate system, its bio-oxidation was further reduced to almost zero in the binary substrate systems when ABTS was used as the mediator compound (Figure 3d). Although statistically insignificant, HBT relieved the inhibitory effect of the other three PAHs on oxidation of PyR in the binary compared to the sole substrate systems, probably through the radical H-atom transfer pathway.

### *Oxidation of PAHs in ternary and quaternary substrate systems*

The catalytic superiority of HBT compared to ABTS in influencing the oxidation of the four PAHs studied was once again apparent in the ternary and quaternary substrate systems (Table 3). As discussed

**Table 3.** Oxidation of PAHs in ternary and quaternary mixtures by immobilized laccase in the presence of different mediator compounds.

| | Oxidation (%)[#] | | | | | | | |
| | Anthracene | | Benzo[a]pyrene | | Benzo[a]anthracene | | Pyrene | |
| PAH system | ABTS | HBT | ABTS | HBT | ABTS | HBT | ABTS | HBT |
|---|---|---|---|---|---|---|---|---|
| Sole systems | | | | | | | | |
| Anthracene (AnT) | 80 b | 90 b | — | — | — | — | — | — |
| Benzo[a]pyrene (BaP) | — | — | 75 d | 89 b | — | — | — | — |
| Benzo[a]anthracene (BaA) | — | — | — | — | 22 b | 36 b | — | — |
| Pyrene (PyR) | — | — | — | — | — | — | 11 b | 13 a |
| Ternary systems | | | | | | | | |
| AnT + BaP + BaA | 91 c | 93 c | 61 b | 94 c | 5 a | 38 b | — | — |
| AnT + BaP + PyR | 81 b | 99 d | 68 c | 91 b | — | — | 43 c | 55 c |
| BaP + BaA + PyR | — | — | 71 c | 99 d | 32 c | 39 b | 9 b | 25 b |
| Quaternary systems | | | | | | | | |
| AnT + BaP + BAA + PyR | 55 a | 66 a | 42 a | 81 a | 5 a | 11 a | 5a | 11a |

[#]Symbol "–" indicates not applicable.
Within each column, means followed by the same letter are not statistically different ($p < 0.05$) by Duncan's multiple range test.

in section *Role and mechanism of the mediator compounds*, this can be attributed to the favorable $\Delta E_o$ between HBT and the PAHs compared with ABTS, or the use of the radical H-atom transfer pathway of oxidation by HBT. Statistically similar or higher amount of AnT was oxidized in the ternary compared to the sole or binary substrate systems with either ABTS or HBT as the redox mediator. This affirms our assertions earlier that a distinct active site of the enzyme was responsible for oxidation of AnT, which was not assessible to BaA and PyR, and that the comparatively smaller size, relatively high aqueous solubility and lower $E_o$ value of AnT favored its oxidation in the ternary system containing BaP.

The oxidation of BaP in the ternary substrate systems was significantly ($p < 0.05$) lower than that observed in the sole substrate system when ABTS was used as the redox mediator. However, as was observed in the sole and binary substrate systems, HBT was effective in relieving the inhibitory effect of the presence of the other PAHs, resulting in significantly ($p < 0.05$) higher or similar BaP oxidation in the ternary compared to the sole and binary substrate systems (Table 3). The oxidation of BaA was low in the ternary system containing AnT, but was stimulated in its absence when ABTS was used as the redox mediator, resulting in significantly ($p < 0.05$) higher oxidation than that observed in the sole substrate systems (Table 3). It is interesting to note that oxidation of PyR, which was negligible in the sole and binary substrate systems increased significantly ($p < 0.05$) in the ternary mixture without BaA with both ABTS and HBT as mediators, and in the absence of AnT when HBT was the redox mediator (Table 3). The results affirm our assertion that the same active site might be responsible for oxidation of BaA and PyR.

Except for the oxidation of BaP with HBT as redox mediator, the oxidation of all four PAHs in the quaternary substrate systems was significantly ($p < 0.05$) lower than that observed in the sole, binary or ternary substrate systems (Table 3). The significant lower oxidation of the PAHs in the quaternary systems could be attributed to combinations of unfavorable effects. First, because the volume of the reaction mixture and the amount of Tween 80 added was the same in all treatments, the overall solubility of the PAHs might have decreased with increasing numbers in the reaction mixtures. This probably resulted in decreased availability and consequently, decreased rate and extent of oxidation of the PAHs by the LMSs. Second, the amount of the mediator compounds added to the quaternary system was the same as in the other systems. The reduced amount of mediator compound per mole of PAHs will affect availability of the cationic radicals required to "shuttle" electrons between the enzyme and the substrate, thereby reducing the overall oxidative capacity of the system. Third, there could be accumulation of oxidation products generated in the reaction mixture when the system was overloaded with the four PAHs, leading to inhibition and partial loss of enzymatic activity.

## Conclusions

In this study, the ability of immobilized LMS to oxidatively transform PAHs in aqueous mixtures was demonstrated. Variable amounts of PAHs were oxidized by the LMS, with the extent of oxidation dependent on the type of PAH and the complexity of the mixture. Therefore, competitive effect may arise when more than one PAH is present in the reaction solution and interact with the enzyme. These results are encouraging for the possible use of immobilized LMS as a remediating agent in the treatment of complex mixtures of PAHs.

## Acknowledgments

## ORCID

Daniel E. Dodor  http://orcid.org/0000-0002-0640-2815

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