— **Regular Article** —

Optimization of Laccase-mediated Benzo[*a*]pyrene Oxidation and the Bioremedial Application in Aged Polycyclic Aromatic Hydrocarbons-contaminated Soil

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Laccase is a polyphenol oxidase with the ability to oxidize a broad range of persistent organic pollutants, including benzo[*a*]pyrene, the most carcinogenic, mutagenic and teratogenic polycyclic aromatic hydrocarbons (PAHs). In this study, the reaction conditions for benzo[*a*]pyrene oxidation by laccase from *Trametes versicolor* were optimized in a liquid medium by a series of single factor experiments. The maximal benzo[*a*]pyrene oxidation rate was observed at 40°C, pH 4, 10% of acetonitrile and an incubation time of more than 24 hr, and the benzo[*a*]pyrene oxidation was enhanced significantly by the addition of a mediator, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS). Laccase was also applied to aged PAHs polluted soil to examine the efficiency of enzymatic bioremediation. The results showed that the enzyme was still effective in the degradation of anthracene, benzo[*a*]pyrene and benzo[*a*]anthracene in soil. Moreover, the degradation rate of most PAHs increased by the addition of ABTS. Our results indicated that the bioremediation of PAHs contaminated soil using laccase is feasible but a suboptimal pH might be a limiting factor in the enzymatic treatment of soil.

Key words —— laccase, benzo[*a*]pyrene, oxidation, soil, bioremediation

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds consisting of two or more fused benzene rings arranged in various structures. For their high toxicity and recalcitrance, sixteen PAHs were recognized as a priority pollutant by the U.S. Environmental Protection Agency (EPA).[1)] When the PAHs' molecular weight increases, their water solubility decreases and their persistence in the environment increases. For example, benzo[*a*]pyrene, a typical high molecular weight (HMW) PAHs with five rings, is one of the most recalcitrant and toxic PAHs, and up to now, few microbes have been isolated to metabolize it as the sole carbon source.[1)] Though PAHs are a chief contaminant of air, soil acts as their ultimate depository.[2)] PAHs in soil can do harm to people's health via the food chain,[3)] therefore, soil pollution by PAHs is of great concern.[4)] Presently, bioremediation has been shown to be effective in the treatment of soils contaminated with low molecular weight (LMW) PAHs, while HMW PAHs are generally recalcitrant to microbial attack.[1)] In addition, some negative conditions (such as lack of nutrition, competition with native microbes and pollutant stress) could also cause the failure of bioremediation.[5, 6)] However, such conditions would only minimally obstruct the remediation using oxidative enzymes.[6)]

Laccase is one such oxidative enzyme that belongs to a copper-containing polyphenol oxidase. Many white rot fungi (WRF) can produce laccase extracellularly,[7)] and the enzyme has already been widely used in the food, pulp and textile industries.[8)] Recently, laccase has attracted new research interest due to its potential in detoxifying

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a wide range of recalcitrant organic contaminants including benzo[*a*]pyrene.[9, 10] The laccase catalysis reaction depends on monoelectronic oxidation, which transforms the substrates to corresponding reactive radicals. With the aid of special compounds, called "mediators," which act as a single electron donor and activator of the enzyme,[11] oxidation rates would be enhanced and the types of substrates would be extended.[12, 13] 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS) is one of the most popular artificial mediators.[9, 14]

The activity of laccase usually varies with environmental factors such as temperature, co-existing chemicals, and pH.[15] So far, the optimal oxidation conditions for the laccase-transformation of many contaminants such as hydroxy polychlorinated biphenyls,[13] chlorophenols,[16] bisphenol A[17] and the polymerization of catechol[18] have been well studied; however, little is known about benzo[*a*]pyrene oxidation, though it is a very hot contaminant.

In this study, benzo[*a*]pyrene was chosen as a model compound to investigate how organic solvents, reaction pH, temperature, incubation time and mediator ABTS affect the PAHs biodegradation by fungi laccase in a liquid system. Finally, a tentative experiment of enzymatic bioremediation of aged PAHs-contaminated soil was performed.

METHODS AND MATERIALS

Enzyme and Chemicals —— Pure laccase from *Trametes versicolor* was obtained from Sigma-Aldrich (Shanghai, China). benzo[*a*]pyrene was obtained from Supelco (Bellefonte, PA, U.S.A.). ABTS was purchased from Sigma-Aldrich (Shanghai).

Soils —— The PAHs-contaminated soil was collected from a petroleum gas station in Wuxi City, Jiangsu Province, China (30°36′14″N, 120°28′33″E). The soil has a total of fifteen PAHs in a concentration of 14555.7 μg/kg and benzo[*a*]pyrene of 616.1 μg/kg, pH 5.5, organic matter of 19.2 g/kg, total nitrogen of 1.0 g/kg, total phosphorus of 0.5 g/kg, total potassium of 14.2 g/kg, cation exchange capacity of 21.5 cmol/kg, and water-holding capacity 35.0%. The soil was air-dried, sieved (2 mm) and stored at 4°C in darkness prior to use.

Laccase Activity Assay —— Laccase activity was determined by the oxidation of ABTS at 30°C. The 2 ml reaction mixture included 1.8 ml Britton-Robinson (B&R) buffer (0.1 mol boracic acid, 0.1 mol phosphoric acid and 0.1 mol acetic acid, pH adjusted to 5.0 with NaOH), 0.1 ml ABTS (20 mmol) and 0.1 ml enzyme solution. The increase in absorbance at 420 nm was monitored with a spectrophotometer (model752, CANY, Shanghai, China) to determine laccase activity (ε_{420} = 36000/mol par cm). The laccase activity was calculated using the formula $\Delta A \times 20 \times 10^6/36000$, where ΔA is the incremental change in absorbance per min when it was stable. One unit of laccase activity was defined as the amount of enzyme able to oxidize 1 μmol ABTS per min.

Optimization of Laccase-mediated Benzo[*a*]pyrene Oxidation —— A series of single factor experiments was performed in 15 ml glass tubes with 5 ml of an acetonitrile-buffer solution mixture containing 30 U of laccase and 1 mg/l benzo[*a*]pyrene. Treatment with boiled laccase served as the control, and each treatment had three replicates. Reaction tubes were closed tightly with screw caps and shaken violently by hand, and then incubated in darkness. After another 5 ml of acetonitrile was added to terminate the reaction, the tubes were closed tightly and shaken again. After 1 hr incubation, the reaction mixture was centrifuged at 13000 g for 10 min, and 10 μl supernatant was analyzed by an Ultra Fast Liquid Chromatograph (UFLC) system.

Acetonitrile: Acetonitrile was used as an organic solvent to improve the bioavailability of benzo[*a*]pyrene. The effect of acetonitrile concentration on enzymatic catalysis was determined using 1%, 2%, 5%, 10% or 20% (v/v) of acetonitrile in phosphate buffer (pH 5.0, 50 mmol), respectively. The experiment was performed at 25°C, pH 5. The samples were analyzed after 48 hr of incubation.

pH: The effect of pH on enzymatic catalysis was also determined. The pH value of the medium varied from 3 to 7 by using citrate buffer (pH 3.0 and 4.0, 50 mmol) and phosphate buffer (pH 5.0, 6.0 and 7.0, 50 mmol). The experiment was performed at 25°C, 10% (v/v) of acetonitrile-buffer. The samples were analyzed after 48 hr of incubation.

Temperature: The effect of temperature on enzymatic catalysis was determined at 20, 30 and 40°C, respectively. The experiment was performed at pH 4, 10% (v/v) of acetonitrile-citrate buffer (pH 4.0, 50 mmol). The samples were analyzed after 48 hr of incubation.

Reaction Time: To determine the effect of reac-

tion time on enzymatic catalysis, the samples were collected and analyzed after 1, 2, 5, 10, 24 and 48 hr of incubation. The experiment was performed at 40°C, pH 4, 10% (v/v) of acetonitrile-citrate buffer medium.

Mediator: To determine the effect of a mediator on enzymatic catalysis, ABTS was added to one treatment at 1 mmol/l. The experiment was performed at 40°C, 10% (v/v) of acetonitrile-citrate buffer. The samples were analyzed after 48 hr of incubation.

Bioremediation of PAHs-contaminated Soil by Laccase —— The bench scale experiment was carried out to assess the potential of remediation of aged PAHs-contaminated soil by laccase form *Trametes versicolor*. 100 U laccase were added to 10 g air-dried soil and mixed thoroughly, and the soil water content was adjusted to 70% of water-holding capacity. ABTS were added to the soil at a concentration of 1 mmol/kg to test the effect of a mediator on enzymatic biodegradation. The soil with boiled laccase served as the control. All treatments were done in triplicate and incubated at 30°C in darkness. Pure water was added every day to maintain the water content. After 10 days of incubation, the soil was air-dried and screened by a 2 mm-sieve.

The Soxhlet extraction method was introduced to determine the PAHs concentration. Soil samples of 2.0 g were extracted by 60 ml dichloromethane in a Soxhlet apparatus for 24 hr. Extracts were concentrated using a rotary evaporator and purified with a chromatography column filled with activated silica gel before analysis by UFLC system.

UFLC Determinations —— PAHs samples were analyzed by a Shimadzu UFLC-20 system with a fluorescence detector. A reversed phase column C_{18}(3×150 mm, particle size 2.2 μm), using a mobile phase with acetonitrile/water (gradient elution 20 min, 0 min: 60/40; 10 min: 80 : 20; 14 min: 98 : 2; 17 min: 60/40; 20 min: 60/40, at a constant flow rate 0.8 ml/min, 50°C), was used to separate PAHs.

Statistics —— SPSS for Windows software was used for statistical analysis, and One-Way analysis of variance (ANOVA) to determine difference between treatments at a significant level of 0.05.

RESULTS AND DISCUSSION

Effect of Organic Solvent on Enzymatic Catalysis

Low-bioavailability was always considered to be the main obstacle in organic pollutant biodegradation,[1] and organic solvents were usually employed to increase the solubility of those hydrophobic substrates.[13, 19] In our work, acetonitrile was used as a solubilizer to investigate the effect on the benzo[*a*]pyrene oxidation. The results showed that the degradation rate of benzo[*a*]pyrene increased first, and then decreased, with an increasing concentration of acetonitrile, and the maximal oxidation rate was observed at 10% of acetonitrile (Fig. 1). At 1% of acetonitrile, the reaction rate was only approximately half of the maximum, suggesting that solubility affected benzo[*a*]pyrene oxidation enormously, and the addition of organic solvents substantially improved the benzo[*a*]pyrene oxidation through increasing in the bioavailability of the substrate.

Effect of pH on Enzymatic Catalysis

pH could affect laccase activity by changing the electrostatic properties of the protein surface and reaction center, or by influencing the stability of the enzyme.[20] In our work, the effect of pH on benzo[*a*]pyrene oxidation was determined by providing a medium pH value, from 3 to 7. The benzo[*a*]pyrene degradation rate was highest at up to pH 4 but decreased rapidly with either decreasing or increasing pH, and the activity of laccase was almost lost at pH 7 (Fig. 2). This suggests that laccase

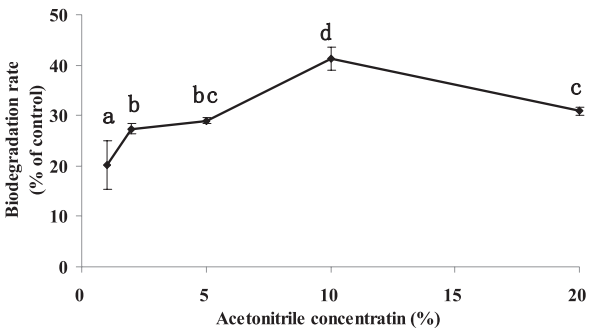

Fig. 1. Effect of Organic Solvent Concentration on Benzo[*a*]pyrene Oxidation

The experiment was carried out at pH 5, 25°C, 1 mg/l benzo[*a*]pyrene, 30 U total laccase activity and an incubation term of 48 hr. Values were means of triplicates, and error bars stood for standard deviations. Means with the same letter are not significantly different ($p > 0.05$).

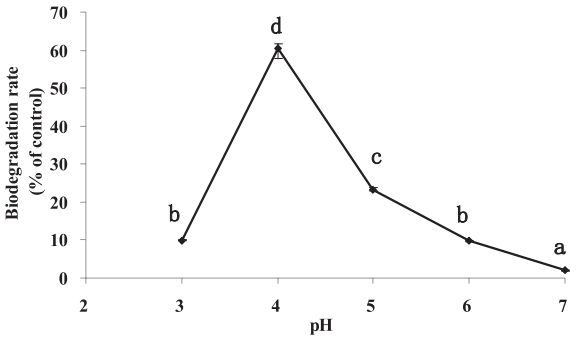

Fig. 2. Effect of pH on Benzo[*a*]pyrene Oxidation
The experiment was carried out at 25°C, 10% acetonitrile, 1 mg/l benzo[*a*]pyrene, 30 U total laccase activity and an incubation term of 48 hr. Values were means of triplicates, and error bars stood for standard deviations. Means with the same letter are not significantly different ($p > 0.05$).

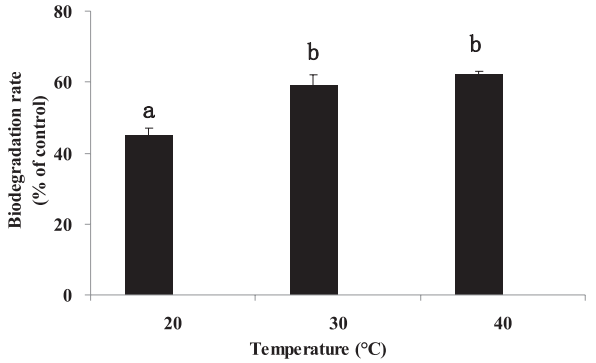

Fig. 3. Effect of Temperature on Benzo[*a*]pyrene Oxidation
The experiment was carried out at pH 4, 10% acetonitrile, 1 mg/l benzo[*a*]pyrene, 30 U total laccase activity and an incubation term of 48 hr. Values were means of triplicates, and error bars stood for standard deviations. Means with the same letter are not significantly different ($p > 0.05$).

is quite sensitive to medium pH, and the enzyme can only transform benzo[*a*]pyrene effectively around pH 4. The results were undesired because the optimal reaction pH of the enzyme was so narrow that it might be impotent in bioremediation of the environment with suboptimal pH.

Effect of Temperature on Enzymatic Catalysis

A relative high temperature is favorable to laccase catalysis. However, high temperatures would lead to the loss of enzyme activity faster, and the reduction of dissolved oxygen in the reaction system which is adverse for enzyme catalysis.[16, 18] In our work, the effect of temperature on benzo[*a*]pyrene oxidation was determined at 20, 30 and 40°C. The results showed that the maximal benzo[*a*]pyrene oxidation rate was observed at 40°C, which was slightly higher than that at 30°C (Fig. 3, $p > 0.05$). At a relatively low temperature of 20°C, the degradation rate of benzo[*a*]pyrene oxidation was still 72.5% of that at 40°C. These results indicated that laccase is more active at a relatively higher temperature; however, temperature usually would be not the limiting factor in enzymatic remediation.

Effect of Incubation Time on Enzymatic Catalysis

The benzo[*a*]pyrene oxidation rate was determined at intervals in an incubation term of 48 hr. The results showed that with prolonged time, the benzo[*a*]pyrene oxidation rate increased, but with a reduced rate of increase (Fig. 4). Reaction systems reached the maximal oxidation rate at 48 hr; however, there was no significant difference between 48 and 24 hr, suggesting that an incubation term of

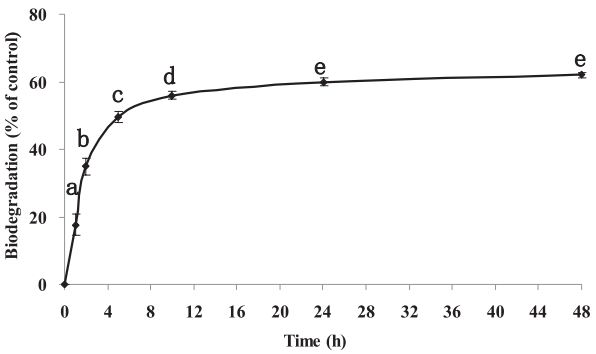

Fig. 4. Effect of Incubation Time on Benzo[*a*]pyrene Oxidation
The experiment was carried out at pH 4, 40°C, 10% acetonitrile, 1 mg/l benzo[*a*]pyrene and 30 U total laccase activity. Values were means of triplicates, and error bars stood for standard deviations. Means with the same letter are not significantly different ($p > 0.05$).

24 hr was adequate for benzo[*a*]pyrene oxidation. To our knowledge, this is much faster than conventional biodegradation using bacteria or fungi, which usually required several weeks,[21, 22] suggesting the rapidity advantage of enzymatic remediation.

Effect of Mediator on Enzymatic Catalysis

As in many other enzymatic reactions, coexisting chemicals can affect the reactivity of laccase.[23] In our work, the effect of a familiar mediator, ABTS, on benzo[*a*]pyrene oxidation was tested. The results showed that a laccase-mediator system could completely transform 1 mg/l benzo[*a*]pyrene in 24 hr, which was much more efficient than that by laccase alone (Fig. 5). The enhancement roles of mediators were also observed in previous research[5, 23, 24] and its mechanism was also partly elu-

Table 1. Degradation of PAHs in Soil by Laccase

PAHs	IP value (eV)	Control	Laccase		Laccase + ABTS	
		Concentration (μg/kg)	Concentration (μg/kg)	Degradation (%)	Concentration (μg/kg)	Degradation (%)
Naphthalene	8.13	ND	ND	0	ND	0
Acenaphthylene	7.86	488.2 ± 91.9[a)]	266.8 ± 144.3[a)]	45.4 ± 29.6	143.8 ± 125.6[b)]	70.5 ± 25.7
Fluorene	7.89	ND	ND	0	ND	0
Phenanthrene	7.91	660.1 ± 54.8[a)]	557.2 ± 102.2[a)]	15.6 ± 15.5	446.2 ± 142.1[a)]	32.4 ± 21.5[a)]
Anthracene	7.43	35.8 ± 4.6[a)]	17.0 ± 5.5[b)]	52.7 ± 15.3	9.1 ± 4.9[b)]	74.6 ± 13.8
Fluoranthrene	7.95	2661.8 ± 48.7[a)]	2622.1 ± 60.0[a)]	1.5 ± 2.3	2497.3 ± 142.1[a)]	6.2 ± 8.4
Pyrene	7.43	2033.1 ± 31.8[a)]	1976.4 ± 32.1[a)]	2.8 ± 1.6	1880.4 ± 129.5[a)]	7.5 ± 6.4
Benzo[*a*]anthracene	7.44	1172.2 ± 34.1[a)]	1032.3 ± 30.7[b)]	11.9 ± 1.6	934.7 ± 91.9[c)]	20.3 ± 3.4
Chrysene	7.59	1384.9 ± 44.3[a)]	1458.5 ± 22.7[a)]	−5.3 ± 2.6	1430.7 ± 47.5[a)]	−3.3 ± 7.8
Benzo[*a*]fluoranthene	7.70	1945.1 ± 47.3[a)]	1941.9 ± 8.0[a)]	0.2 ± 0.4	1917.0 ± 91.0[a)]	1.4 ± 4.7
Benzo[*k*]fluoranthene	7.48	725.2 ± 4.0[a)]	721.6 ± 8.5[a)]	0.5 ± 1.2	701.6 ± 30.0[a)]	3.3 ± 4.1
benzo[*a*]pyrene	7.12	616.1 ± 87.8[a)]	364.5 ± 87.8[b)]	40.8 ± 14.3	266.7 ± 52.0[b)]	56.7 ± 7.3
Dibenzo[*a*,*h*]anthrecene	—	319.6 ± 6.5[a)]	327.8 ± 6.2[a)]	−2.6 ± 1.9	313.2 ± 14.7[a)]	2.0 ± 4.6
Benzo[*g*,*h*,*i*]perylene	—	1262.3 ± 11.4[a)]	1251.0 ± 44.3[a)]	0.9 ± 3.5	1180.1 ± 56.2[a)]	6.5 ± 4.4
Indeno[1,2,3-*cd*]pyrene	—	1155.2 ± 22.7[a)]	1103.3 ± 21.8[a)]	4.5 ± 1.9	1091.7 ± 46.6[a)]	5.5 ± 4.0
Total		14555.7 ± 175.4[a)]	13662.5 ± 370.0[a)]	6.1 ± 2.5	12912.4 ± 903.6[a)]	12.0 ± 6.1

The experiment was carried out at 30°C and a 10-day incubation term. Laccase was added to 10 U/g with or without 1 mmol/kg ABTS, and treatment with boiled laccase served as the control. Data are presented as mean ± S.D. Means in the same PAH row with the same letter are not significantly different ($p > 0.05$). ND: not detected.

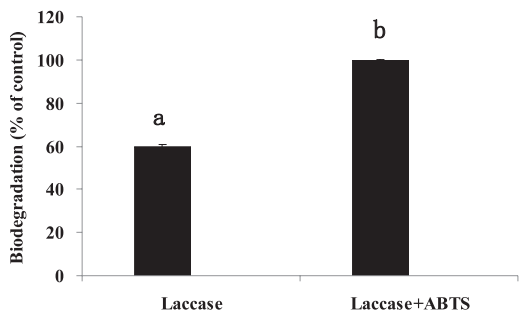

Fig. 5. Effect of ABTS on Benzo[*a*]pyrene Oxidation

The experiment was carried out at pH 4, 40°C, 10% acetonitrile, 1 mg/l benzo[*a*]pyrene, 30 U total laccase activity and an incubation term of 48 hr. Values were means of triplicates, and error bars stood for standard deviations. Means with the same letter are not significantly different ($p > 0.05$).

cidated by in which laccase could oxidize the ABTS to $ABTS^{+\cdot}$ or $ABTS^{2+}$ cation radicals first, and then these cation compounds oxidized the substrates with a higher efficiency. Simultaneously, radicals were reduced to their initial forms and the reaction cycle continued.[24)]

Potential of Bioremediation of PAHs-contaminated Soil by Laccase

Biodegradation of PAHs in soil is much more complicated than in liquid, either due to the low bioavailability of substrates[25)] or to suboptimal environmental conditions. Aged polluted soil, which has been contaminated by a gas plant for more than ten years, was used to assess the potential of enzymatic bioremediation. Considering the rapidity of enzymatic catalysis in liquid medium, the soil remediation term was set at only 10 days, which was much shorter than existing bioremediation practices.[26,27)] A mild temperature of 30°C was set as the incubation temperature. The results showed that laccase was effective in removing anthracene, benzo[*a*]pyrene and benzo[*a*]anthracene, but failed to degrade other PAHs significantly compared to the control (Table 1). Traditionally, the specificity of laccase catalysis was believed to relate to the ionization potentials (IPs) of the substrates, in which laccase could only oxidize compounds with an IP value less than 7.45 eV.[10)] In our work, the IP of each of the three top transformable PAHs was below 7.45 eV, which partly justified the IP theory. However, we also observed that the IP of pyrene (7.43 eV) was also below 7.45 eV, but it failed to be degraded, suggesting that further studies are required for full elucidation of the catalytic mechanism of laccase. However, the total 15 PAHs degradation rate was only 6.1%, which was not very high. Yet, benzo[*a*]pyrene, which is one of the most carcinogenic, mutagenic, teratogenic and persistent PAHs, was considerably removed, by 40.8%; moreover, the results were without using a employing

of solubilizer, suggesting the promising applicable potential of laccase in the future bioremediation of PAHs contaminated soil.

When a laccase-mediator system was added to the soil, almost all the degradation rates of the fifteen PAHs were enhanced, but only those of acenaphthylene and benzo[*a*]anthracene increased significantly ($p < 0.05$), compared to the addition of laccase alone. Unlike in a liquid system, the degradation rate of benzo[*a*]pyrene didn't increase significantly ($p > 0.05$), probably because a laccase-meditor system couldn't work actively in an environment lacking water. Anthracene was still the most degradable PAHs, followed by acenaphthylene, benzo[*a*]pyrene and benzo[*a*]anthracene. To our knowledge, this is the first time that a mediator has been used in the enzymatic remediation of soil. Our results suggest that addition of a mediator could improve the efficiency of enzymatic remediation to some extent. But, the high cost of an artificial mediator has always been limitation to its application in bioremediation. However, the discovery of natural mediators existing in crude enzymes from many laccase-produced WRF[9, 24] implies that the direct use of crude enzymes in environmental bioremediation might be a better alternative to pure laccase.

Though the PAH degradation rate was enhanced by optimizing the reaction conditions, it was inadvisable to apply these conditions to soil remediation because it might result in negative effects on the soil ecology system. Considering the sensitivity to pH, it was proposed that laccase, whose optimal reaction pH was approximate to soil pH, be used to remediate polluted soil in future work. A number of reports have indicated that the optimal pH for fungal laccase varies from 3 to 7, depending on the fungal species,[28] implying that alkali soil was never suitable for remediation by laccase.

In conclusions, in a liquid system, 10% of acetonitrile concentration, pH of 4, temperature of 40°C and incubation term of more than 24 hr were most conducive to benzo[*a*]pyrene oxidation by laccase from *Trametes versicolor* and the addition of ABTS could significantly enhance the oxidation rate. In a soil system, laccase was still effective in the degradation of anthracene, benzo[*a*]pyrene and benzo[*a*]anthracene, though a solibilizer was absent, suggesting the potential for enzyme treatment of aged PAHs-contaminated soil. The addition of ABTS increased the removal rate of several PAHs by laccase. In view of the high cost of laccase and artificial mediators, the direct use of crude enzymes form laccase-producing fungi to soil remediation was proposed. In order to enhance the remedial efficiency, the application of laccase with optimal reaction pH approximate to that of soil, was also proposed.

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