RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2011.37.225

ScienceAsia 37 (2011): **225–233**

Oxidation of polycyclic aromatic hydrocarbons by a tropical isolate of *Pycnoporus coccineus* and its laccase

Paweena Thongkred[a], **Pongtharin Lotrakul**[a,*], **Sehanat Prasongsuk**[a], **Tsuyoshi Imai**[b], **Hunsa Punnapayak**[a]

[a] Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand

[b] Department of Civil and Environmental Engineering, Yamaguchi University, Ube, Japan

*Corresponding author, e-mail: Pongtharin.L@Chula.ac.th

Received 23 Feb 2011
Accepted 30 Aug 2011

ABSTRACT: Basidiocarps of *Pycnoporus coccineus* and *P. sanguineus* were collected from Kanchanaburi, Phetchaburi, and Mae Hong Son provinces in Thailand. Liquid culture of *P. coccineus* isolates provided higher laccase and manganese peroxidase activities than those of *P. sanguineus*. Lignin peroxidase activity was not detectable in any of the three *Pycnoporus* isolates. *P. coccineus* Thongkred 013 BCU, the best laccase producer among these isolates, was selected to optimize laccase production. The highest laccase yield (5.97 ± 0.40 U/ml) was obtained when grown at 28 °C for 8 days in basal medium containing 2% (w/v) glucose and 0.25% (w/v) peptone at an initial pH of 5.0. The optimum conditions for stability and activity of the crude laccase enzyme were at pH 3.5 and room temperature (25 ± 2 °C). $FeSO_4$ (10 mM) and SDS (1% w/v) were both inhibitory to the laccase activity. When five different types of polycyclic aromatic hydrocarbons (PAHs) were individually added to liquid culture of *P. coccineus* at 100 ppm, the oxidation of anthracene, pyrene, and fluoranthene detected within 24 h were 59.7, 50.7, and 49.8%, respectively, whereas only 25.3% of benzo[a]pyrene and 32.4% of phenanthrene were oxidized. When *P. coccineus* crude laccase (1 U/ml) was used, these PAHs were more readily oxidized, with 76.4 and 74.3% of pyrene and anthracene, respectively, being oxidized within 2 h and 84.2% of fluoranthene oxidized within 24 h. Benzo[a]pyrene and phenanthrene oxidation by the enzyme were enhanced when 1 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was added as a mediator to the reaction mixture, resulting in 66.3 and 50.5% of the initial 100 ppm being oxidized after 24 h.

KEYWORDS: PAHs, anthracene, ABTS, bioremediation, white-rot fungi

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are toxic organic pollutants that occur in the environment mainly from the incomplete combustion of petroleum fuels[1]. Their common structure consists of two or more benzene rings fused together, which makes them hydrophobic. Because of their low water solubility, PAHs mainly accumulate in the soil and sediments. Some PAHs are mutagenic and carcinogenic and are, therefore, classified by the US Environmental Protection Agency as priority pollutants[2]. Due to their complex structures and hydrophobicity, PAHs are difficult to degrade. PAHs can be removed from the environment by many processes, including volatilization, photo-oxidation, and chemical oxidation[3]. However, the cost and adverse environmental effects caused by these methods discourage their widespread practice. The alternative methods for removing toxic PAHs are biological approaches, such as bioremediation that is of increasing interest due to its low cost and environmental friendliness. Bioremediation is a practical alternative to the traditional physical and chemical processing. Both bacteria and fungi, or mixtures thereof, may be used in bioremediation[4]. White-rot fungi capable of producing lignin-modifying enzymes (LMEs) are able to degrade various recalcitrant aromatic compounds including PAHs[5]. Compared to most PAH-degrading enzymes produced by bacteria that have a narrow substrate specificity, the extracellular LMEs of these fungi are non-specific and so can degrade a wider range of PAHs[3]. Many species of white-rot fungi, such as *Ganoderma lucidum*, *Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus*, and *Trametes versicolor*, produce the secreted (extracellular) enzyme laccase and have been reported as efficient degraders of PAHs[6–9].

A great diversity of white-rot fungi has been reported in Thailand, with representatives of more than 28 orders, 63 families, and 141 genera of fungi in this

group, including *Pycnoporus* spp[10]. However, most of these fungi have not been characterized and their ability to produced laccase is unknown. Recently, a number of laccase producing *Pycnoporus sanguineus* isolated in Thailand has been studied for their ability to degrade a mixture of 3 PAHs, anthracene, phenanthrene, and pyrene[11]. Phenanthrene and pyrene were rapidly degraded by laccases from these *P. sanguineus* isolates. Degradation of anthracene was less efficient as more than half of the anthracene remained after 24-h incubation[11]. It is interesting to explore whether different species or isolates of *Pycnoporus* produce laccases that can degrade anthracene and other PAHs with more complicated structures such as fluoranthene and benzo[a]pyrene. Therefore, the aims of this study are (1) to isolate and screen for laccase producing *Pycnoporus* spp., (2) to preliminarily optimize the culture conditions for laccase production, (3) to characterize the crude enzyme and explore the potential of using live cultures and crude laccase enzyme preparations for PAH oxidation, and (4) to investigate the effect of a mediator on PAH oxidation by the selected laccase.

MATERIALS AND METHODS

Fungal collection and screening for laccase producers

Basidiocarps of *Pycnoporus* spp. were collected from the Kanchanaburi, Phetchaburi, and Mae Hong Son provinces in Thailand. All fungal isolates were identified based on macro- and microscopic morphological characteristics, as described by Ryvarden and Johansen[12] and Gilbertson and Ryvarden[13]. Pure fungal cultures were obtained by aseptically placing small pieces of fruiting body tissue on potato-dextrose agar (PDA) containing chloramphenicol (50 mg/l). Stock cultures of fungi were maintained on PDA slants at 4 °C.

All *Pycnoporus* isolates were screened for the ability to produce laccase using a plate assay with PDA containing 250 ppm of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). One agar plug (1 mm in diameter) of a 7-day old culture, pregrown on PDA, was placed onto each plate. The cultures were then incubated at 28 °C for 7 days. Any colony that formed a green halo on this medium was determined as potentially laccase positive and was selected for further screening.

Culture conditions and LME assay

Five agar plugs, cut from each actively growing fungal culture on a PDA plate, were used as an inoculum. Each isolate was grown for 10 days in a 250-ml Erlenmeyer flask containing 50 ml basal medium (all (w/v): glucose 2.0%, L-asparagine 0.25%, D,L-phenylalanine 0.015%, adenine 0.00275%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2$ 0.001%, $FeSO_4 \cdot 7H_2O$ 0.001%, $MnSO_4 \cdot 4H_2O$ 0.0001%, $ZnSO_4 \cdot 7H_2O$ 0.0001%, and $CuSO_4 \cdot 5H_2O$ 0.0002%), pH 5.0, at room temperature (25 ± 2 °C) with continuous shaking at 150 rpm[14]. Fungal mycelia were removed from the cultures by filtration through Whatman filter paper No. 1, and the filtrates were then used for quantitative determination of the LME activity.

Laccase activity was determined as described by Revakar and Lele[14], with 1 U of laccase activity being defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute. Manganese peroxidase activity was assayed following the method of Watanabe et al[15], with 1 U of manganese peroxidase activity defined as to the amount of enzyme that oxidized 1 μmol 2,6-dimethoxyphenol per minute. Lignin peroxidase activity was measured as described by Tuisel et al[16], where 1 U of peroxidase is defined as the amount of enzyme that oxidized 1 μmol of veratryl alcohol per minute.

Optimization of laccase production

Three types of carbon source (glucose, fructose, and sucrose) were used for the optimization of laccase production. Each sugar was added to 50 ml basal medium at 2% (w/v) as the sole carbon source. The cultures were grown in a 250-ml Erlenmeyer flask and incubated at room temperature with continuous shaking at 150 rpm for 14 days. Cultures were harvested every 2 days and the fungal growth (dry weight) and extracellular laccase enzyme activity in the culture media were determined. The carbon source that yielded the highest extracellular laccase activity was then selected for further nitrogen source optimization. Three types of nitrogen source (L-asparagine, peptone, and ammonium sulphate) were separately added to the basal medium at 0.25% (w/v), to evaluate different nitrogen sources. The cultures were incubated under the same conditions for the carbon source optimization.

Enzyme preparation and characterization

The culture filtrate of each eight-day old *Pycnoporus* culture was concentrated by ultrafiltration (VivaFlow 250 with a 10 kDa molecular weight cut-off, Sartorius, Germany) prior to ammonium sulphate precipitation. The proteins that precipitated in 60–80% (saturation) ammonium sulphate were resolved in 0.1 M glycine HCl buffer (pH 3.5) and dialysed (CelloSep with a 12–

14 kDa molecular weight cut-off, Membrane Filtration Products, Inc., USA) in the same buffer at 4 °C for 24 h. The amount of protein was then quantitatively determined as described by Lowry et al[17].

The optimal pH of the crude laccase was detected by measuring the enzyme activity in the reaction mixtures adjusted to different pH values using 0.1 M glycine HCl buffer (pH 3.0 and 4.0), 0.1 M tartrate buffer (pH 4.0, 5.0, and 6.0), 0.1 M sodium phosphate buffer (pH 6.0, 7.0, and 8.0), and 0.1 M glycine NaOH buffer (pH 8.0 and 9.0). The stability of laccase at each pH was investigated by incubating the crude enzyme in each different pH buffer for 1 h prior to assaying the enzyme activity at pH 3.5.

The optimal temperature of the crude laccase was determined by incubating the reaction mixtures at various temperatures ranging from 15 °C to 60 °C during the kinetic assay. The temperature stability was studied by incubating the crude enzyme at different temperatures for 1 h before measuring the enzyme activity at room temperature.

The effect of ions on activity of the crude laccase was determined by individually adding $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, NaCl, $ZnCl_2$, or EDTA (all at 10 mM final concentration) or the effect of surfactant by adding SDS (1% w/v final concentration) to the reaction mixtures. Controls were carried out in the absence of ion, EDTA and SDS.

The relative enzyme activity was determined by comparing the laccase activity in reaction mixtures incubated at various pH and temperatures to those incubated under the standard conditions (pH 3.5 at room temperature). The residual activity was calculated in comparison to activity of the crude enzyme stored at 4 °C in 0.1 M glycine HCl (pH 3.5) prior to evaluating the residual enzyme activity level by the standard enzyme assay.

PAH OXIDATION AND ANALYSIS

PAH tolerance test

The ability of each fungal isolate to tolerate PAHs was tested by placing one agar plug on basal medium agar containing 100 ppm of one of the five tested PAHs (anthracene, phenanthrene, fluoranthene, pyrene, or benzo[a]pyrene). Mycelial growth was measured as the colony diameter after a 7-day incubation in the dark at room temperature. Growth inhibition (%) was calculated based on the comparison with fungi grown under the same condition but without PAH. Acetonitrile (PAH solvent) was used as a control at the same final concentration.

Oxidation in liquid culture

Each PAH (1000 ppm in acetonitrile) was individually added to an eight-day old *Pycnoporus* culture, grown in 50 ml of the optimized basal medium in a 250-ml flask, to a final PAH concentration of 100 ppm. The mixture was then further incubated in the dark at room temperature with continuous shaking (200 rpm) for 24 h. Fungal mycelia were removed from the cultures by filtration through Whatman filter paper No. 1 and the residual PAHs in the filtrate were extracted and then quantitatively determined by HPLC, as described by Cho et al[18], using a 4.6×150 mm C18 reverse-phase column and eluted with a 7:3 (v/v) acetonitrile:water mobile phase at a flow rate of 1 ml/min. PAHs were measured by UV detection at 295, 250, 236, 235, and 225 nm for benzo[a]pyrene, phenanthrene, pyrene, fluoranthene, and anthracene, respectively. In control experiments, each PAH was added to the optimized basal medium and incubated under the same conditions but with no fungus added.

Oxidation by laccase

Each PAH was added separately to 0.1 M sodium acetate buffer (pH 5.0) to a final concentration of 100 ppm. One unit of crude laccase was then added to each reaction mixture before adjusting the total volume to 1 ml with the same buffer and incubating the reaction mixture in the dark at room temperature with continuous shaking at 200 rpm. No enzyme was added in the control experiments. The residual PAHs were extracted and quantitatively determined after 2 and 24 h of incubation.

RESULTS AND DISCUSSION

Fungal collection and screening for laccase producer

Basidiocarps collected were identified as two isolates of *Pycnoporus coccineus* and one of *P. sanguineus*, based on the morphological characteristics of their spores, basidiocarp and mycelium. ITS sequence comparison supported the morphological identification (GenBank accession number JF792517-18 and JF792520). Both species have been previously reported in Thailand[19,20]. Each fungus was determined to be a potential laccase producer if a dark green coloration appeared in the first week of incubation and the ratio of the green halo diameter to that of colony was greater than 1[21]. All three isolates were found to be positive for laccase by this assay, which agrees with a previous report that *P. coccineus* has the ability to oxidize ABTS and $MnCl_2$[20].

Table 1 Maximal extracellular laccase and manganese peroxidase activity in the culture media filtrate of the three Thai isolates of white-rot fungi, determined daily during fungal growth in basal medium at room temperature over 10 days.

Fungal isolate[a]	Maximal laccase activity		Maximal manganese peroxidase activity	
	(U/ml)[b]	Day[c]	(U/ml)[b]	Day[c]
PcT013	3.61 ± 1.04	8	0.04 ± 0.01	10
PcT014	3.31 ± 1.04	8	0.13 ± 0.04	10
PsT015	2.65 ± 0.13	6	0.03 ± 0.02	10

[a] PcT013: *Pycnoporus coccineus* Thongkred 013 BCU, PcT014: *Pycnoporus coccineus* Thongkred 014 BCU, PsT015: *Pycnoporus sanguineus* Thongkred 015 BCU.
[b] Data are shown as the average $\pm$ standard deviation, derived from three replications.
[c] Laccase activity measured on the culture day of maximum production.

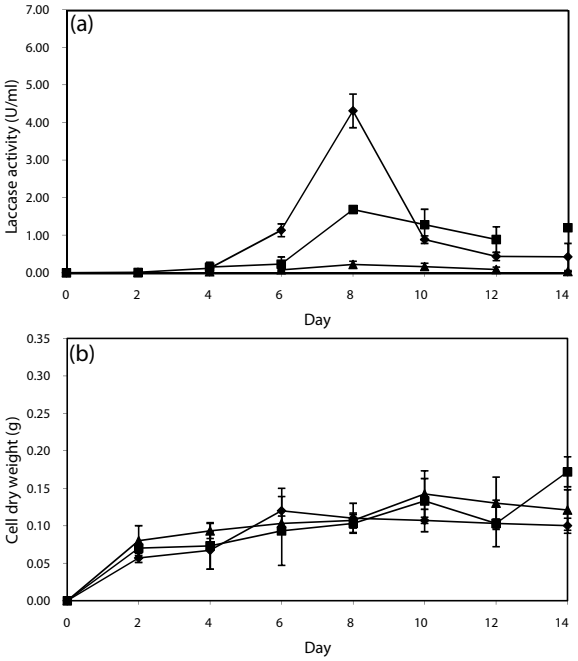

Fig. 1 Effect of the carbon source on the (a) extracellular laccase production and (b) fungal growth as cell dry weight for *Pycnoporus coccineus* Thongkred 013 BCU grown on basal medium with 0.25% (w/v) L-asparagine as the nitrogen source and 2% (w/v) glucose (diamonds), 2% (w/v) fructose (squares), and 2% (w/v) sucrose (triangles) as the sole carbon source. Cultures were incubated at room temperature for 14 days with shaking at 150 rpm. Data are shown as the average $\pm$ standard deviation, derived from three replications.

LME production and characterization

P. coccineus Thongkred 013 BCU, *P. coccineus* Thongkred 014 BCU, and *P. sanguineus* Thongkred 015 BCU were found to be comparatively good laccase producers, with the highest laccase activity found in *P. coccineus* Thongkred 013 BCU (Table 1). Both *P. coccineus* isolates produced higher laccase activity (3.31–3.61 U/ml) than that of *P. sanguineus* Thongkred 015 BCU (2.65 U/ml) and the other previously reported Thai isolates (0.46–2.53 U/ml)[11]. A broadly similar level of extracellular laccase activity has also been reported in some *P. coccineus* strains[22,23]. Manganese peroxidase activity was also detected in the culture filtrate of all three isolates, ranging from 0.03 U/ml in *P. sanguineus* Thongkred 015 to 0.13 U/ml in *P. coccineus* Thongkred 014 BCU (Table 1). No lignin peroxidase was detected in any of the isolates (data not shown). It was also reported that *P. coccineus* did not produce any detectable lignin peroxidase even though a lignin peroxidase-like DNA sequence has been annotated in the genome[22,24].

As the best laccase producer among the three *Pycnoporus* isolates, *P. coccineus* Thongkred 013 BCU was selected for the optimization of extracellular laccase production. Of the three sugars tested, glucose resulted in the highest culture filtrate laccase activity (4.31 ± 0.46 U/ml) (Fig. 1). However, this was dependent upon the age of the culture with the enzyme activity rising sharply from day 4 to peak at day 8. In contrast, when grown on sucrose a very low laccase activity was observed (0.22 ± 0.09 U/ml). When grown with fructose, the peak culture filtrate activity was also seen at 8 days of cultivation (1.68 ± 0.08 U/ml). In terms of fungal growth (as dry weight), all three sugars supported fungal growth well, but the fungal mass showed no correlation with the production of extracellular laccase activity seen in the culture filtrates in terms of the kinetics or maximal yields.

With respect to the nitrogen source, the production of extracellular laccase peaked at 8 days of cultivation with peptone (5.97 ± 0.40 U/ml) and L-asparagine (4.53 ± 0.34 U/ml) (Fig. 2). In contrast, when grown on ammonium sulphate, a very low level of laccase activity was detected in the culture filtrate (0.46 ± 0.24 U/ml). In terms of fungal growth, the highest cell dry weight was obtained in the culture supplemented with peptone following by L-asparagine. The lowest growth rate was observed in culture supplemented with ammonium sulphate. Therefore, laccase production was likely to be growth-associated phenomenon.

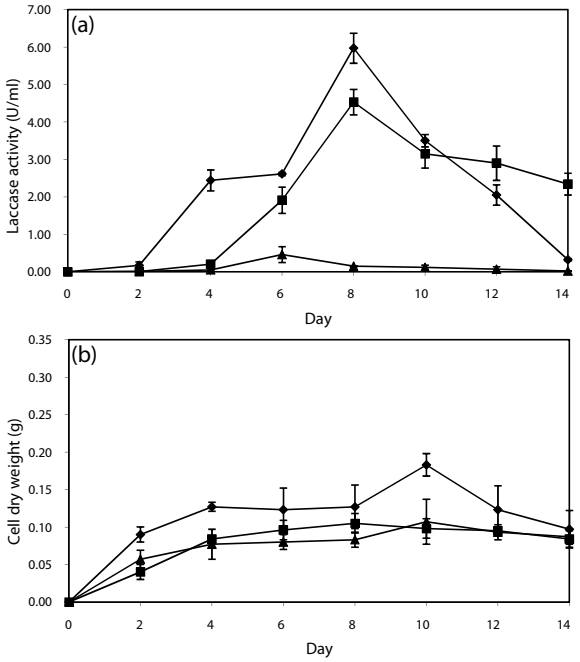

Fig. 2 Effect of the nitrogen source on the (a) extracellular laccase production and (b) fungal growth as cell dry weight for *Pycnoporus coccineus* Thongkred 013 BCU grown on basal medium with 2% (w/v) glucose as the carbon source and 0.25% (w/v) peptone (diamonds), 0.25% (w/v) L-asparagine (squares), and 0.25% (w/v) ammonium sulphate (triangles) as the nitrogen source. Cultures were incubated at room temperature for 14 days with shaking at 150 rpm. Data are shown as the average $\pm$ 1 standard deviation, derived from three replications.

A number of studies reported that addition of copper to the production medium enhanced fungal laccase production [14,25]. However, when $CuSO_4 \cdot 5\,H_2O$ was added to the production medium to the final concentration of 0.005 and 0.01% (w/v), *P. coccineus* Thongkred 013 BCU laccase activity was reduced to 92.94% and 39.80% of that grown in the production medium with lesser amount of $CuSO_4 \cdot 5\,H_2O$ (0.0002% w/v), respectively, (data not shown). The reduction in laccase activity may be caused by the copper toxicity since copper has been used as a key ingredient in many fungicides [25–27].

The pH optimum for enzyme activity and for the stability of the crude laccase was both found to be at pH 3.5. However, increasing pH above 3.5 reduced the activity of the laccase and no activity was detected at pH above 7. Relative activities at pH 4, 5, 6, and 7 were 71.8 ± 1.7, 62.4 ± 1.5, 54.8 ± 1.8, and 0.43 ± 0.04%, respectively. A similar pH optimum was previously reported for the purified laccase from

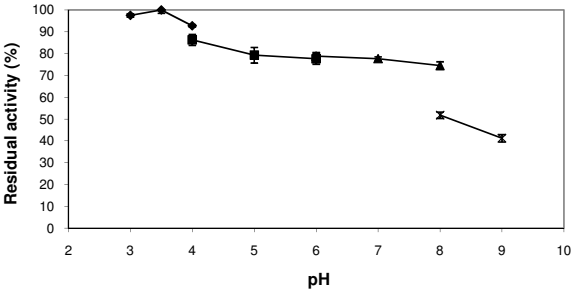

Fig. 3 pH stability of the crude laccase from *Pycnoporus coccineus* Thongkred 013 BCU incubated in 0.1 M glycine HCl buffer (diamonds, pH 3.0 and 4.0), 0.1 M tartrate buffer (squares, pH 4.0, 5.0, and 6.0), 0.1 M sodium phosphate buffer (triangles, pH 6.0, 7.0, and 8.0), and 0.1 M glycine NaOH buffer (crosses, pH 8.0 and 9.0). Data are shown as the average $\pm$ standard deviation, derived from three replications.

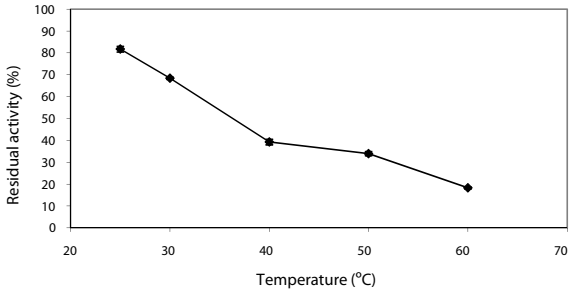

Fig. 4 Thermostability of the crude laccase from *Pycnoporus coccineus* Thongkred 013 BCU incubated at different temperatures for 1 h. Data are shown as the average $\pm$ standard deviation, derived from three replications.

P. coccineus MUCL38527 [22]. The enzyme seemed to be relatively stable up to pH 8, with around 75% and 40% activity remaining after 1-h incubation at pH 8 and 9, respectively, (Fig. 3).

The temperature optimum for enzyme activity was at ambient temperature (25 ± 2 °C). Relative activities at 15, 20, 30, 40, 50, and 60 °C were 67.0 ± 1.0, 72.8 ± 0.3, 84.2 ± 1.9, 63.0 ± 2.7, 50.5 ± 0.8, and 47.6 ± 0.7%, respectively, compared to that at ambient temperature. The crude laccase was found to be relatively stable at room temperature (25 ± 2 °C), but its activity declined quickly as the storage temperatures rose above 30 °C (Fig. 4). The calculated half life of the enzyme was approximately 1 h at 34 °C.

Finally, the addition of 1% (w/v) SDS, or 10 mM of EDTA, $MgSO_4$, $CaCl_2$, NaCl, $ZnCl_2$, or $FeSO_4$

Table 2 The effect of ionic salts and the divalent chelator EDTA at 10 mM, or the surfactant SDS at 1% (w/v), upon the activity of the crude laccase from *Pycnoporus coccineus* Thongkred 013 BCU.

Component	Relative activity[a]
$CuSO_4$	99.07 ± 2.09
$MnSO_4$	95.09 ± 1.92
$MgSO_4$	41.87 ± 2.26
$ZnCl_2$	25.15 ± 0.96
$CaCl_2$	34.35 ± 0.96
$FeSO_4$	2.15 ± 0.96
NaCl	36.81 ± 3.15
EDTA	69.32 ± 0.83
SDS	1.84 ± 0.96

[a] Data are shown as the average $\pm$ 1 standard deviation, derived from three replications.

were all found to be inhibitory to the crude laccase activity, with $FeSO_4$ and SDS almost completely inhibiting the enzyme (Table 2). EDTA and the other salts caused a 30.1% (EDTA) to 74.8% ($ZnCl_2$) reduction in the residual activity. In contrast, $CuSO_4$ or $MnSO_4$ (10 mM) showed no inhibition to the enzyme activity. In comparison to the laccases from other white-rot fungi[28–30] including an isolate of *Pycnoporus*[31], it was found that *P. coccineus* Thongkred 013 BCU laccase was more sensitive to SDS, EDTA, and almost all ions tested, except for Mn^{2+}.

PAH OXIDATION AND ANALYSIS

PAH tolerance test

The two *P. coccineus* isolates (Thongkred 013 BCU and Thongkred 014 BCU) and *P. sanguineus* Thongkred 015 BCU grew in the presence of all five tested PAHs (anthracene, phenanthrene, fluoranthene, pyrene, and benzo[a]pyrene) at an initial concentration of 100 ppm, but their growth was significantly inhibited in the presence of four of these PAHs, ranging from 75–85.6% inhibition. Anthracene was less inhibitory for the two *P. coccineus* isolates, compared to *P. sanguineus* Thongkred 015 BCU (Table 3). Indeed, the two *P. coccineus* isolates showed a lower percent growth inhibition on four of the five PAH species tested, compared to *P. sanguineus* Thongkred 015 BCU. However, the growth with pyrene was not significantly different among all three isolates.

Based on the yield of laccase production and the PAH tolerance, *P. coccineus* Thongkred 013 BCU was selected for further PAH degradation tests.

Table 3 Effect of PAHs on the growth of the two *P. coccineus* isolates (Thongkred 013 BCU *and* Thongkred 014 BCU) and *P. sanguineus* Thongkred 015 BCU when grown on basal medium agar containing the indicated PAH (100 ppm) at room temperature for 7 days.

PAH species	Growth inhibition[a] (%)		
	PcT013[b]	PcT014[b]	PsT015[b]
Anthracene	5.4 ± 5.0	11.0 ± 0.9	60.0 ± 1.4
Phenanthrene	77.9 ± 5.9	77.9 ± 2.2	80.4 ± 1.8
Fluoranthene	79.1 ± 1.9	78.8 ± 1.7	84.4 ± 1.2
Pyrene	75.4 ± 0.8	79.4 ± 2.2	76.8 ± 5.6
Benzo[a]pyrene	80.9 ± 2.6	83.7 ± 0.9	85.6 ± 1.2

[a] Growth was measured as colony diameter. The percent inhibition was calculated in comparison to those of fungi grown on media without PAH (but with the solvent), and are shown as the average $\pm$ standard deviation, derived from three replications.

[b] PcT013: *Pycnoporus coccineus* Thongkred 013 BCU, PcT014: *Pycnoporus coccineus* Thongkred 014 BCU, PsT015: *Pycnoporus sanguineus* Thongkred 015 BCU.

PAH oxidation in liquid culture

More than half (59.7%) of anthracene was degraded by a liquid culture of *P. coccineus* Thongkred 013 BCU within 24 h (Table 4), whereas about half of the pyrene and fluoranthene (50.7 and 49.8%, respectively), a third (32.4%) of the phenanthrene and a quarter (25.3%) of the benzo[a]pyrene were degraded by the fungus during the same period. Note that the total laccase activity in each culture was not significantly different, ranging from 6.04–6.23 U/ml (data not shown), and so this is likely to reflect the enzymes ability to degrade the different PAHs rather than the effect of each PAH on enzyme production by the fungi. These results suggest the possibility of using *P. coccineus* Thongkred 013 BCU for the bioremedial treatment of PAH-contaminated wastewater. *P. coccineus* grown in a sand culture was shown to degrade more than 50% of the initial phenanthrene level within a week of incubation[32]. However, less than 25% of the pyrene was degraded by this *P. coccineus* strain[32]. More than 80% of anthracene and pyrene were degraded by *Pleurotus ostreatus* growing in a soil culture after an eight-week incubation[1], whilst a complete removal of phenanthrene and benzo[a]pyrene was reported in a soil culture inoculated with spent compost of *P. pulmonarius* after a 2-day incubation. Note that in this case the degradation was likely to have been performed by a consortium of microorganisms in addition to *P. pulmonarius*, including other fungi, bacteria, and even enzymes immobilized in the compost[33]. Finally, PAH degradation in a saline marsh soil

Table 4 Degradation of 100 ppm PAHs by *Pycnoporus coccineus* Thongkred 013 BCU grown in liquid basal medium. Each PAH was added to an eight-day old fungal culture to a final concentration of 100 ppm, and then incubated in the dark at room temperature with continuous shaking at 200 rpm for 24 h.

PAHs	Residual PAH (%)[a]
Anthracene	40.3 ± 8.9
Phenanthrene	67.7 ± 1.9
Fluoranthene	50.2 ± 7.3
Pyrene	49.3 ± 2.4
Benzo[a]pyrene	74.7 ± 3.0

[a] Data are shown as the % residual level of PAH relative to the control (PAH added to the autoclaved basal medium), and are shown as mean $\pm$ 1 standard deviation.

by nine strains of white rot fungi revealed more than 50% of phenanthrene, fluoranthene, and pyrene were degraded in 30 days by *Bjerkandera adusta*, *Irpex lacteus*, and *L. tigrinus*[34]. Thus with its shorter incubation time, it is likely that *P. coccineus* Thongkred 013 BCU could potentially degrade PAHs relatively efficiently compared to these other fungi.

PAH oxidation by crude laccase

Compared to the degradation of PAHs using the live mycelia, it was found that PAHs were more efficiently oxidized when the crude laccase from *P. coccineus* Thongkred 013 BCU was used (Table 5). Under these conditions 76.4 and 74.3% of pyrene and anthracene, respectively, were oxidized within 2 h of incubation, compared to 50.7 and 59.7% after 24 h with the fungal culture. Likewise an almost 1.7-fold higher level of oxidation of benzo[a]pyrene was observed with the liquid enzyme solution in 2 h than the fungal culture in 24 h (42.7 versus 25.3%). However, no improvement was seen for phenanthrene and fluoranthene oxidation (35.9 and 31.9% versus 32.4 and 49.8%, respectively). When the incubation time was prolonged to 24 h, more than 84% of the anthracene, pyrene, and fluoranthene were oxidized, but only an extra 6–11% of the benzo[a]pyrene and phenanthrene were oxidized compared to that observed at 2 h. Laccase from *P. coccineus* Thongkred 013 BCU oxidized anthracene more rapidly (more than 85% oxidized after 24 h) than laccases from the previously reported *P. sanguineus* isolates from Thailand (37–43% oxidized after 24 h)[11]. *P. coccineus* Thongkred 013 BCU laccase oxidized pyrene and phenanthrene slower (85 and 42%, respectively, after 24 h) than laccases from those *P. sanguineus* isolates (95 and 89%, respectively, after 24 h)[11]. In addition, the amount of *P. coccineus* Thongkred 013 BCU laccase used in this study (1 U/ml) was much less than those of *P. sanguineus* isolates (30 U/ml)[11].

When 1 mM ABTS (mediator) was added to the reaction mixtures, the oxidation of recalcitrant phenanthrene and benzo[a]pyrene were slightly enhanced (1.25- and 1.15-fold, respectively) after 2 h of incubation (Table 5). Oxidation of benzo[a]pyrene was clearly enhanced (1.34-fold) by ABTS when the incubation time was extended to 24 h, but this effect was not as pronounced in phenanthrene (1.18-fold). In contrast, the addition of ABTS adversely affected the oxidation of pyrene, anthracene, and fluoranthene with an approximately 0.07- to 0.42-fold reduction in the level of oxidation at 2 h and a 0.09- to 0.34-fold reduction at 24 h. Perhaps, ABTS mediator was not necessary for these PAHs since it did not enhance the oxidation reaction.

The ability to oxidize different species of PAHs by the crude laccase from *P. coccineus* Thongkred 013 BCU varied. No apparent correlation between the oxidation by laccase and the molecular weight of the PAH, structures, or ionization potential (IP) was found, as has been previously noted[35,36]. Anthracene, with its low molecular weight and linear structure, was quickly oxidized compared to those with a higher molecular weight and/or benzene rings arranged in an angular or clustered manner, such as benzo[a]pyrene and phenanthrene. However, fluoranthene and pyrene, which have clustered structures, were oxidized relatively quickly. The IP values of these PAHs are 7.12, 7.40–7.55, 7.43–7.72, 7.76–7.89, and 7.91–8.03 eV for benzo[a]pyrene, pyrene, anthracene, fluoranthene, and phenanthrene, respectively[35–38], which mostly fall within the range of the reported threshold IP value for laccase (7.45–7.80 eV)[39]. Similar effects of the addition of ABTS to the reaction on the degradation of PAHs were also reported for the *Trametes* laccase, where the addition of ABTS only slightly enhanced the degradation of some PAHs while it reduced the degradation of other[1]. However, the different incubation conditions and compositions of the reaction mixtures make it difficult to validly compare the ability of laccases from different fungal species in PAH degradation. Regardless, the crude laccase from *P. coccineus* Thongkred 013 BCU appeared to be more efficient in the oxidation of all five PAHs tested here compared to the laccase from *Trametes* after incubation for 24 h or longer[1,31]. In the presence of ABTS, blue and yellow laccase from *Pleurotus ostreatus* can degrade anthracene, fluoranthene, pyrene, and

Table 5 Degradation of PAHs by the crude laccase from *Pycnoporus coccineus* Thongkred 013 BCU with and without 1 mM ABTS as the mediator.

PAHs	Residual PAH[a] (%)			
	2-h incubation period		24-h incubation period	
	Without ABTS	With 1 mM ABTS	Without ABTS	With 1 mM ABTS
Anthracene	25.7 ± 3.4	30.7 ± 1.9	12.2 ± 3.4	29.5 ± 0.7
Phenanthrene	68.1 ± 2.2	60.1 ± 2.4	57.4 ± 3.2	49.5 ± 1.8
Fluoranthene	64.1 ± 4.8	74.9 ± 2.3	15.8 ± 3.0	38.0 ± 4.6
Pyrene	23.7 ± 0.9	30.7 ± 9.3	15.7 ± 6.9	23.2 ± 13.1
Benzo[a]pyrene	57.3 ± 0.5	50.9 ± 1.1	50.6 ± 4.2	33.8 ± 2.7

[a] The reaction mixtures were incubated in the dark at room temperature with continuous shaking at 200 rpm for the indicated times. No enzyme was added in the control experiments. Data are shown as mean $\pm$ 1 standard deviation.

phenanthrene at a comparable or higher rate compared to the *P. coccineus* Thongkred 013 BCU crude laccase, but the *P. ostreatus* blue laccase is unable to degrade these PAHs without adding the mediator[36,40].

In conclusion, laccase, produced as a culture medium filtrate by a Thai isolate of *P. coccineus* (Thongkred 013 BCU), could oxidize all five tested PAHs, from an initial level of 100 ppm to nearly 90% of anthracene within 24 h without a mediator. The addition of 1 mM ABTS, as the mediator, enhanced the oxidation of the more recalcitrant benzo[a]pyrene and phenanthrene up to 50% or above within 24 h. In addition, the live mycelia of *P. coccineus* Thongkred 013 BCU could also degrade all five tested PAHs by 25–60% of the initial applied dose within 24 h. Thus the fungus and its crude laccase have a potential to be used in the microbial consortium for decontamination of PAH in environment.

Acknowledgements: The authors are thankful to The Biotechnology Program, Faculty of Science, Chulalongkorn University, and Ratchadaphiseksomphot Endowment Fund for the Plant Biomass Utilization Research Unit, Department of Botany, Chulalongkorn University, for financial support. This study was also financially supported by The 90th Anniversary of Chulalongkorn University Fund, the Higher Education Research Promotion and National Research University Project of Thailand (EN281B), Office of the Higher Education Commission and Asian Core Program, the Japan Society for the Promotion of Science – National Research Council of Thailand (JSPS – NRCT). Guidance for fungal identification from Aniwat Chalermpongse and valuable comments on the manuscript from Robert Butcher were appreciated.

REFERENCES

1. Wu Y, Teng Y, Li Z, Liao X, Luo Y (2008) Potential role of polycyclic aromatic hydrocarbons (PAHs) oxidation by fungal laccase in the remediation of an aged contaminated soil. *Soil Biol Biochem* **40**, 789–96.
2. Office of the Federal Register (ORF) (1982) Appendix A: priority pollutants. *Fed Regul* **47**, 52309.
3. Zhao HP, Wang L, Ren JR, Li Z, Li M, Gao HW (2008) Isolation and characterization of phenanthrene-degrading strains *Sphingomonas* sp. ZP1 and *Tistrella* sp. ZP5. *J Hazard Mater* **152**, 1293–300.
4. Juan D, Jun C, Juan Z, Shixiang G (2008) Polycyclic aromatic hydrocarbon biodegradation and extracellular enzyme secretion in agitated and stationary cultures of *Phanerochaete chrysosporium*. *J Environ Sci* **20**, 88–93.
5. Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. *Appl Microbiol Biotechnol* **57**, 20–33.
6. Han MJ, Choi HT, Song HG (2004) Degradation of phenanthrene by *Trametes versicolor* and its laccase. *J Microbiol* **42**, 94–8.
7. Punnapayak H, Prasongsuk S, Danmek K, Messner K, Lotrakul P (2009) Polycyclic aromatic hydrocarbons (PAHs) degradation by laccase from a tropical white rot fungus *Ganoderma lucidum*. *Afr J Biotechnol* **8**, 5897–900.
8. Songulashvili G, Elisashvili V, Wasser SP, Nevo E, Hadar Y (2007) Basidiomycetes laccase and manganese peroxidase activity in submerged fermentation of food industry wastes. *Enzym Microb Tech* **47**, 57–61.
9. Rama R, Mougin C, Boyer FD, Kollmann A, Malosse C, Sigoillot JC (1998) Biotransformation of benzo[a]pyrene in bench scale reactor using laccase of *Pycnoporus cinnabarinus*. *Biotechnol Lett* **20**, 1101–4.
10. Chandrasrikul A, Suwanarit P, Sangwanit U, Morinaga T, Nishizawa Y, Muragami Y (2008) *Diversity of Mushrooms and Macroscopic Fungi in Thailand*. Kasetsart Univ, Bangkok.
11. Munusamy U, Sabaratnam V, Muniandy S, Abdullah N, Pandey A, Jones EGB (2008) Biodegradation of polycyclic aromatic hydrocarbons by laccase from *Pycnoporus sanguineus* and toxicity evaluation of treated PAH. *Biotechnology* **7**, 669–77.
12. Ryvarden L, Johansen I (1980) *A Preliminary Polypore*

Flora of East Africa. Fungiflora, Oslo.
13. Gilbertson RL, Ryvarden L (1987) *North American Polypores. Volume 2*. Fungiflora, Oslo.
14. Revankar MS, Lele SS (2006) Enhanced production of laccase using a new isolate of white rot fungus WR-1. *Process Biochem* **41**, 581–8.
15. Watanabe T, Shirai N, Okada H, Honda Y, Kuwahara M (2001) Production and chemiluminescent free radical reactions of glyoxal in lipid peroxidation of linoleic acid by the ligninolytic enzyme, manganese peroxidase. *Eur J Biochem* **268**, 6114–22.
16. Tuisel H, Sinclair R, Bumpus JA, Ashbaugh W, Brock BJ, Aust SD (1990) Lignin peroxidase H2 from *Phanerochaete chrysosporium*: purification characterization, and stability to temperature and pH. *Arch Biochem Biophys* **279**, 158–66.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193**, 265–75.
18. Cho SJ, Park SJ, Lim JS, Rhee YH, Shin KS (2002) Oxidation of polycyclic aromatic hydrocarbons by laccase of *Coriolus hirsutus*. *Biotechnol Lett* **24**, 1337–40.
19. Biodiversity-Based Economy Development Office (2010) Biodiversity Database. http://app1.bedo.or.th/biolistdb/public/viewmushroom_list.asp?cmd=resetall.
20. Chairattanamanokorn P, Imai T, Kondo R, Sekine M, Higuchi T, Ukita M (2004) Decolorization of alcohol distillery wastewater by thermotolerant white rot fungi. *Appl Biochem Microbiol* **41**, 583–8.
21. Dhouib A, Hamza M, Zouari H, Mechichi T, Hmidi R, Labat M, Jesus MM, Sayadi S (2005) Screening for ligninolytic enzyme production by diverse fungi from Tunisia. *World J Microbiol Biotechnol* **21**, 1415–23.
22. Jaouani A, Guillen F, Penninckx MJ, Martinez AT, Martinez AJ (2005) Roles of *Pycnoporus coccineus* in the degradation of aromatic compounds in olive oil mill wastewater. *Enzym Microb Tech* **36**, 478–86.
23. Lomascolo A, Cayol J-L, Roche M, Guo L, Robert J-L, Record E, Lesage-Meessen L, Ollivier B, Siggoillot J-C, Asther M (2002) Molecular clustering of *Pycnoporus* strains from various geographic origins and isolation of monokaryotic strains for laccase hyperproduction. *Mycol Res* **106**, 1193–203.
24. Pointing SB, Pelling AL, Smith GJD, Hyde KD (2005) Screening of basidiomycetes and xylariaceous fungi for lignin peroxidase and laccase gene-specific sequences. *Mycol Res* **109**, 115–24.
25. Fonseca MI, Shimizu E, Zapata PD, Villalba LL (2010) Copper inducing effect on laccase production of white rot fungi native from Misiones (Argentina). *Enzym Microb Tech* **46**, 534–9.
26. Baldrian P (2003) Interactions of heavy metals with white-rot fungi. *Enzym Microb Tech* **32**, 78–91.
27. Fernández-Calviño D, Soler-Rovira P, Polo A, Díaz-Ravina M, Arias-Estévez M, Plaza C (2010) Enzyme activities in vineyard soils long-term treated with copper-based fungicides. *Soil Biol Biochem* **42**, 2119–27.
28. Farnet AM, Gil G, Ferre E (2008) Effects of pollutants on laccase activities of *Marasmius quercophilus*, a white-rot fungus isolated from a Mediterranean schlerophyllous litter. *Chemosphere* **70**, 895–900.
29. Min KL, Kim YH, Kim YM, Jung HS, Hah YC (2001) Characterization of a novel laccase produced by the wood rotting fungus *Phellinus ribis*. *Arch Biochem Biophys* **392**, 279–86.
30. Zhang GQ, Wang YF, Zhang XQ, Ng TB, Wang HX (2010) Purification and characterization of a novel laccase from the edible mushroom *Clitocybe maxima*. *Process Biochem* **45**, 627–33.
31. Wang ZX, Cai YJ, Liao XR, Tao GJ, Li YY, Zhang F, Zhang DB (2010) Purification and characterization of two thermostable laccases with high cold adapted characteristics from Pycnoporus sp. SYBC-L1. *Process Biochem* **45**, 1720–9.
32. Matsubara M, Lynch JM, De Leij FAAM (2006) A simple screening procedure for selecting fungi with potential for use in the bioremediation of contaminated land. *Enzym Microb Tech* **39**, 1365–72.
33. Lau KL, Tsang YY, Chiu SW (2003) Use of spent mushroom compost to bioremediate PAH-contaminated sample. *Chemosphere* **52**, 1539–46.
34. Valentin L, Feijoo G, Moreir MT, Lema JM (2006) Biodegradation of polycyclic aromatic hydrocarbons in forest and salt marsh soils by white-rot fungi. *Int Biodeter Biodegr* **58**, 15–21.
35. Majcherczyk A, Johannes C, Hüttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzym Microb Tech* **22**, 335–41.
36. Pozdnyakova NN, Rodakiewicz-Nowak J, Turkovskaya OV, Haber J (2006) Oxidative degradation of polyaromatic hydrocarbons catalyzed by blue laccase from *Pleurotus ostreatus* D1 in the presence of synthetic mediators. *Enzym Microb Tech* **39**, 1242–9.
37. Pickard MA, Roman R, Tinoco R, Vazquez-Duhalt R (1999) Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Appl Environ Microbiol* **65**, 3805–9.
38. Valentín L, Lu-Chau TA, López C, Feijoo G, Moreira MT, Lema JM (2007) Biodegradation of dibenzothiophene, fluoranthene, pyrene and chrysene in a soil slurry reactor by the white-rot fungus *Bjerkandera* sp. BOS55. *Process Biochem* **42**, 641–8.
39. Collins PJ, Kotterman MJJ, Field JA, Dobson ADW (1996) Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versicolor*. *Appl Environ Microbiol* **62**, 4563–7.
40. Pozdnyakova NN, Rodakiewicz-Nowak J, Turkovskaya OV, Haber J (2006) Oxidative degradation of polyaromatic hydrocarbons and their derivatives catalyzed directly by the yellow laccase from *Pleurotus ostreatus* D1. *J Mol Catal B* **41**, 8–15.