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Biodegradation of Polycyclic Aromatic Hydrocarbons by Laccase of *Pycnoporus sanguineus* and Toxicity Evaluation of Treated PAH

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Abstract: Four strains of *Pycnoporus sanguineus* isolated from Thailand (KUM 60953), Shah Alam (KUM 60954), Endau Rompin (KUM 60955), Cherating (KUM 60956) and Gombak (KUM 60957) were grown on PDA plates. Laccase of *P. sanguineus* was produced using oil palm frond parenchyma tissue (OPFPt) in solid substrate fermentation (SSF). Strain KUM 60954 produced significant (p = 0) levels of laccase at 2.53 U mL^{-1} (76 U g^{-1}) followed by KUM 60957 at 1.05 U mL^{-1} (32 U g^{-1}), KUM 60956 at 0.55 U mL^{-1} (17 U g^{-1}) and KUM 60955 at 0.46 U mL^{-1} (14 U g^{-1}). Meanwhile, KUM 60953 (reference strain) from Thailand produced 2.44 U mL^{-1} of laccase (73 U g^{-1} of OPFPt). Biodegradation of a mixture of 10 ppm each of phenanthrene, anthracene and pyrene by 30 U mL^{-1} of laccase in sodium citrate buffer pH 5 was studied. The reaction mixture was incubated at 40°C and was shaken at 80 rpm for 24 h. Laccase of KUM 60954 degrades 90% of phenanthrene, 37% of anthracene and 96% of pyrene. Meanwhile, laccase of KUM 60953 degrades 89% of phenanthrene, 43% of anthracene and 95% pyrene. Pyrene was rapidly biodegraded followed by phenanthrene and anthracene. However, a similar pattern of degradation was observed for both KUM 60953 and KUM 60954. Degraded PAH sample was further tested for toxicity using *Artemia*. The untreated PAH caused more than 50% of *Artemia* death while for the treated PAH no death was observed indicating that the toxicity level was reduced and possibly no any new toxic compound was produced during the degradation.

Key words: Fungus, biodegraded, phenanthrene, anthracene, pyrene, *Artemia*

INTRODUCTION

A wide variety of polycyclic aromatic hydrocarbons (PAHs) are found in the environment as a result of the incomplete combustion of organic matter, automobile exhausts and also by petroleum contamination on soils and waters (Dimashki *et al*., 2000). Polycyclic aromatic hydrocarbons are aromatic compounds made up of three or more fused benzene rings. Therefore, PAHs can persist in the environment for longer periods (Ang *et al*., 2005) and they are well known for their toxicity, carcinogenicity and mutagenicity (Wik and Dave, 2005). Polycyclic aromatic hydrocarbon exposure occurs by inhalation, ingestion and dermal contact which cause lung, intestine, liver, pancreases and skin cancer (Jacques *et al*., 2005).

Further, due to their low bioavailability they are not easily degraded by conventional remediation techniques.

The white rot fungus, which degrades lignin biopolymers by a range of non specific extracellular enzymes have been used to degrade and detoxify polycyclic aromatic hydrocarbon (Park *et al*., 2006). A major breakthrough in use of fungi for PAH bioremediation was in 1985 (Bumpus *et al*., 1985). However, approach of growing the white rot fungi directly or indirectly on the contaminated site can be a very slow process with incomplete removal of pollutants (Harayama, 1997). One of the strategies to overcome this limitation is through direct enzymatic treatment by using Lignin Modifying Enzymes (LME) (Valentin *et al*., 2006). The LMEs consist mainly of three types of extracellular phenoloxidase namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Ohkuma *et al*., 2001).

Laccases (ρ-diphenol: dioxygen oxidoreductase, E.C. 1.10.3.2) are multicopper oxidases having Type 1, Type 2 and 3 copper sites which belongs to a group of

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enzymes called blue copper oxidases. Laccases can catalyse oxidative reactions and detoxify phenolic contaminants (Ryan *et al*., 2003) by reducing molecular oxygen to water (Hou *et al*., 2004). The ability of laccases to utilise sufficient oxygen without addition of mediators makes these enzymes favorable for industrial and environmental application (Rosconi *et al*., 2005).

Production of laccases by solid substrate fermentation (SSF) of agro-industry waste need to gain much attention in biotechnology due to its higher productivities and low production cost. However, a majority of studies have been focused on liquid medium for laccase production which is more expensive than SSF (Kahraman and Gurdal, 2002). Besides that, in SSF, fungi are able to grow under natural conditions that contain soluble carbohydrates and inducers. The different available food industrial wastes such as wheat straw (Lechner and Papinutti, 2005) and oil palm frond parenchyma tissue (Vikineswary *et al*., 2006) can be used in SSF.

Pycnoporus sanguineus is a white rot fungus that produces laccase as the sole lignolytic enzyme (Vikineswary *et al*., 2006). However, there are limited studies was carried out on PAH degradation with laccase of *P. sanguineus* produced by SSF, as majority studies have been focused only on *Pleurotus ostreatus* (Pozdnyakova *et al*., 2006) and *Phanerochaete chrysosporium* (Liao *et al*., 1997). Polycyclic aromatic hydrocarbon degradation using laccase of *P. sanguineus* produced by SSF followed by the toxicity evaluation of the degraded PAH sample by the method of Togulga (1998) has not yet been reported. In this study, therefore we aimed to degrade PAHs by laccase produced by *P. sanguineus* during solid substrate fermentation of agro-residues and to evaluate the toxicity of treated PAH.

MATERIALS AND METHODS

Fungus: Indigenous strains of *Pycnoporus sanguineus* (Linn. ex FR.) isolated from the year 2000 to 2004 from different locations in Malaysia including Shah Alam (KUM 60954), Endau Rompin (KUM 60955), Cherating (KUM 60956) and Gombak (KUM 60957) which were selected for this research (Fig. 1) was obtained from the fungal culture collection in Institute Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia. KUM 60953 from Thailand was used as a reference strain was given by Prof E.B.G. Jones.

Chemicals, microbiological media, substrates and equipments: Analytical grade chemicals were used in the preparation of buffers and substrates. The chemicals were purchased from Sigma, Megalab, BDH and Difco. Binocular microscope was purchased from Nikon. ÄZOO *Artemia cysts* (brine shrimp eggs), Reef Salt (sea salt) and oxygen pump were purchased from a fish store. Table lamp (white lamp) and polypropylene tub was purchased from supermarket. Oil palm frond parenchyma tissue was provided by oil palm plantation at Teluk Intan.

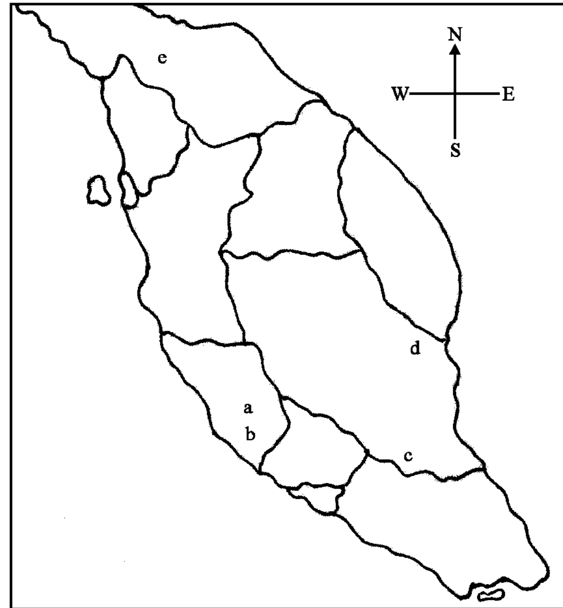

Fig. 1: Locations of the indigenous strains of *Pycnoporus sanguineus* were earlier collected in Peninsular Malaysia was obtained from the fungal collection in Institute Biological Science, Faculty of Science, University of Malaya.
a: University of Malaya Field Study Center Ulu Gombak (N 03° 19` E 101° 45`)
b: Shah Alam (N 03° 04` E 101° 30`)
c: Endau Rompin (N 02° 25` E 103° 30`)
d: Cherating (N 05° 04` E 103° 17`)
e: Thailand (N 15° 52` E 100° 59`)

Inoculum preparation: The stock cultures derived from the tissue culture were revived by subculturing on Potato Dextrose Agar (PDA) media and incubated for seven days at 27±2°C. Five plugs from this culture plate were used to inoculate sterilised wheat grains. The sterilised wheat grains was prepared by adding distilled water to cooked wheat grains (1:5) and was autoclaved at 121°C at 15 psi for 20 min. The flasks (koji) were then incubated in the dark at 28±2°C for 14 days.

Culture conditions: Solid Substrate Fermentation (SSF) was carried out according to Vikineswary *et al*. (2006).

Ten grams of OPFPt was autoclaved at 121°C at 15 psi for 15 min and allowed to cool overnight at 28±2°C. The sterilised OPFPt was then supplemented with 40 mL of nutrient solution containing filter sterilised urea with a final concentration of 0.21% nitrogen (N) and autoclaved calcium carbonate to give a final concentration of 1% (w/v). Each flask was then inoculated with 20% (w/w) of two week old *P. sanguineus* koji. Triplicate flasks were set up and incubated for six days in a dark chamber at 28±2°C in static condition.

Preparation of enzyme extract: Three hundred milliliter of distilled water at pH 4.0 was added to each of the 6 days old solid culture. This mixture was homogenised at 8000 rpm for 8 min at 28±2°C (Avneesh *et al*., 2003). The content was filtered and was centrifuged for 9000 rpm for 30 min at 4°C (Kumaran *et al*., 1997). Laccase activity of the crude extract was determined prior to concentration according to Szklarz *et al*. (1989). The supernatant was then concentrated and also lyophilized. All enzyme samples were stored at 4°C prior to use.

Laccase assay: The substrate blank consisted of 3.2 mL of 50 mM sodium citrate buffer and 0.2 mL syringaldazine. The enzyme blank consisted of all reagents except syringaldazine. The reaction mixture consisted of buffer and enzyme cocktail. The reaction mixture was allowed to equilibrate at 30±2°C and reaction was initiated by the addition of 0.2 mL of syringaldazine solution. The reaction was monitored at 525 nm by the production of tetramethoxy-azo-bis-methylenequinone resulting from the reaction of laccase with syringaldazine (Aldrich, USA). One unit (U) of laccase activity was defined as the amount of enzyme producing 1 OD unit/min/mL at λ = 525 nm.

Preparation of PAH standards: Polycyclic aromatic hydrocarbons standard was prepared based on a method of Liao *et al*. (1997). Fifty mg of individual PAH (anthracene, phenanthrene and pyrene) was dissolved in separate 50 mL of acetonitrile (HPLC grade) to make a stock concentration of 1000 ppm (mg L^{-1}). While, three individual PAH standards was mixed together and dissolved in 50 mL of acetonitrile to obtained a PAH mixture of a similar concentration as above. All volumetric flasks containing PAH working stock were tightly wrapped in aluminium foil and stored at 0°C. Standards were analysed using High Performance Liquid Chromatography (HPLC).

Effect of incubation time on PAHs stability at 40°C: In this study, the standard biodegradation assay was employed at 40±2°C at 80 rpm for 4, 8 and 24 h. The content was then extracted using DCM and left on the bench for 30 min. The lower layer of extract was then collected and 1 g of anhydrous sodium sulphate was added and incubated for another 10 min at 28±2°C to eliminate water from the extract. The lower layer of the extract was then collected slowly. The extract was then evaporated to dryness using a rotary evaporator. The dried extract of PAH was then re-dissolved in 1 mL of acetonitrile and the vials were covered with an aluminium foil.

Biodegradation of PAH mixture by laccase of *P. sanguineus* strains: The control (with autoclaved laccase) was prepared by spiking 10 ppm (final concentration) of PAHs mixture standard in a total volume of 10 mL of buffer. The reaction mixture consisted of 9.9 mL of 50 mM sodium citrate buffer, 1.0 mL of crude laccase extract (30 U mL^{-1}) and 0.1 mL of PAH mixture in acetonitrile (final concentration $\leq$1% (v/v). The control and reaction mixture was then incubated at 40±2°C and was shaked at 80 rpm for 4, 8 and 24 h. Laccase without PAHs mixture was also incubated parallel to test on laccase activity throughout the incubation periods.

At the end of each incubation period, the reaction in both control and reaction mixtures were stopped by adding pure dichloromethane. The content was extracted as mention earlier. The control was prepared in order to determine the degree of PAHs loss by volatilization, sorption to the flask during the whole incubation and losses during the extraction period. The amount of PAHs loss during extraction from the control at each incubation period was also compared to the total amount of recovered from the spiked sample at 0 h (Field *et al*., 1995).

Detection of PAH by HPLC: Polycyclic aromatic hydrocarbons were analysed using a Spectra-Physics HPLC system equipped with a pump, a detector (Spectra-Physics UV 2000) and an integrator integrated with JCL 6000-Chromatography Data System (v2.0) (Field *et al*., 1992). The column (250×4.6 mm ID) which was packed with Purospher® STAR RP-18 endcapped (5 µm particles) coupled with a guard column was used to determine the PAHs separation. Polycyclic aromatic hydrocarbons were analysed under isocratic condition with 90% of acetonitrile and 10% deionized water as elution solvents and were detected at 254 nm using an UV detector. Twenty microliter of working stock solutions, filtered extracts and spiked samples were injected separately into the injector and was allowed to run for 12 min.

Evaluation of toxicity using artemia cysts: The brine shrimp eggs were incubated in a polypropylene tub (21×14×7 cm) containing sterilised salt water (70 g L^{-1}) which was prepared a day before. Salinity of the salt water was measured by a hydrometer in the range of 1.020-1.023. Two scoops of cysts were suspended in the autoclaved salt water. After an average of 72 h old the nauplii shrimps were used for the experimental bioassay.

Determination of LD_{50} (lethal dose): The test was performed as described by Kanegusuku *et al.* (2002). A series of dilution, ranging from 30 to 400 μL of PAH sample was used. A suspension of 15 nauplii of 0-72 h old in 200 μL of PAH was placed in every 3 mL wells. The multiwell plates were incubated and the number of dead nauplii was counted after 24 h of incubation at room temperature (27+2°C). Finney (1971) statistical method was used to calculate the volume of PAH that would kill 50% of brine shrimps in 24 h exposure (LD_{50} with the 95% confidence intervals) by using Probit Analysis software.

Comparison of PAH toxicity: The untreated and treated PAH at 4, 8 and 24 h with laccase were tested in the total volume of 3 mL wells containing sterilised salt water. Fifteen nauplii suspension were incubated for 24 h with 37.16 μL of treated and untreated PAH. Salt water and acetonitrile as a control was also run parallel. After 24 h of incubation, by using binocular microscope Nikon SMZ-10 the number of dead and live nauplii in each well was counted. Replicates for each treatment were included to minimise the variability of the test organisms.

Statistical analysis: Mean values of triplicate data for all the parameters tested were obtained and subjected to one-way analysis of variance (ANOVA). The significance was tested by the multiple range tests at 95% Least Significant Difference (LSD) to detect any significant difference in the laccase activity by different strains of *P. sanguineus* and also in the biodegradation of PAH compounds by laccase of *P. sanguineus* KUM 60953 and KUM 60954.

RESULTS

Laccase activity of strains of *P. sanguineus* during their growth in optimised SSF:. A maximum of activities were produced by KUM 60954 followed by the KUM 60953. After concentrated, 45.63 and 35.35 units of activity were obtained from KUM 60954 and KUM 60953 respectively. KUM 60954 was able to produce more laccase by 4% increases in activity compared to other three strains (KUM 60955, KUM 60956 and KUM 60957). Less than 1 U mL^{-1} of activity was produced by KUM 60955 and KUM 60956 strains. KUM 60954 did not show significant differences in laccase activity compared to KUM 60953 during SSF of OPFPt but showed significant activity with other strains. Therefore, KUM 60954 was then selected for further studies with KUM 60953 as a reference strain (Table 1).

Table 1: Laccase activity during SSF of OPFPt by 20% (w/w) of two week old *P. sanguineus* strains after six days of incubation in a dark chamber at 28±2°C in static condition

Indigenous strain	Laccase activity (U mL^{-1})
KUM 60953 (reference)	2.44±0.16[a]
KUM 60954	2.53±0.10[a]
KUM 60955	0.46±0.01[b]
KUM 60956	0.55±0.02[b]
KUM 60957	1.05±0.07[c]

(p = 0.00); same letter denotes not significant; ± refers to standard deviation

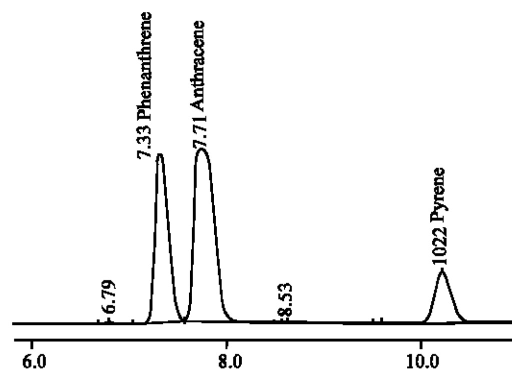

Fig. 2: Resolution of 10 ppm phenanthrene, 10 ppm anthracene and 10 ppm pyrene in PAH-mixture using 90% (v/v) acetonitrile and 10% (v/v) water and detected at 254 nm for 12 min using LiChroCart ® 250-4,6 HPLC-Cartridge Purospher ® STAR RP-18 endcapped (5 μm) column

Effect of incubation time on PAHs stability at 40°C during degradation: Resolution of 10 ppm phenanthrene, 10 ppm anthracene and 10 ppm pyrene in PAH-mixture using 90% (v/v) acetonitrile and 10% (v/v) water and detected at 254 nm for 12 min using LiChroCart ® 250-4,6 HPLC-Cartridge Purospher ® STAR RP-18 endcapped (5 μm) column produced three peaks (Fig. 2). Lowest detection was detected at 0.001, 0.0008 and 1.0 ppm, for phenanthrene, anthracene and pyrene, respectively. Coefficient of variation (%) determined from previous study for recoveries was 0.34 (phenanthrene), 0.77 (anthracene) and 19 (pyrene). Reproducibility of the peak height measurements from 0 to 24 h was consistent for phenanthrene, anthracene and pyrene with coefficient

Table 2: Percentage degradation of PAH-mixture incubated with 30 U mL^{-1} of laccase of *P. sanguineus* KUM 60953 and KUM 60954 at 40°C and was shaked at 80 rpm from 4 to 24 h

	Incubation time (h)					
	4		8		24	
	KUM 60953	KUM 60954	KUM 60953	KUM 60954	KUM 60953	KUM 60954
PAHs sample	Strain					
Phenanthrene	74.1 ± 0.8^{a}	34.7 ± 1.5^{b}	83.7 ± 0.4^{c}	60.6 ± 12.2^{d}	89.3 ± 0.5^{e}	89.5 ± 0.1^{e}
Anthracene	10.3 ± 1.0^{f}	8.1 ± 0.6^{g}	27.9 ± 1.6^{h}	10.3 ± 4.5^{i}	42.8 ± 10.1^{j}	36.8 ± 0.5^{k}
Pyrene	87.7 ± 0.8^{l}	44.4 ± 7.7^{m}	95.5 ± 0.1^{n}	84.1 ± 6.8^{o}	95.9 ± 0.6^{p}	95.7 ± 0.1^{p}

Same letter denotes not significant; ± refers to standard deviation

variation of 1.34, 2.42 and 2.72%, respectively. Thus, it was found that the combined errors of thermal degradation and recovery were minimal.

PAHs degradation by laccase of KUM 60953 and KUM 60954 at 4, 8 and 24 h: From preliminary studies, the level of laccase activity throughout 24 h of incubation at 40°C was studied and was detected that there was no significant reduction in the activity (data not shown). The percentage degradation of each PAH was calculated based on the peak height of the untreated PAH subtracted with the spiked treated PAH recovered at each sampling hours (4, 8, 24).

Laccase of KUM 60953 degraded 74.1% of phenanthrene, 10.3% of anthracene and 87.7% of pyrene in 4 h of incubation to give final concentration of 2.51, 8.84 and 0.85 ppm, respectively. The degradation percentage increased to 83.7, 27.9 and 95.5% at 8 and 24 h of incubation. Laccase of KUM 60954 degraded 34.7% of phenanthrene, 8.1% of anthracene and 44.4% of pyrene in 4 h of incubation (Table 2), with corresponding concentration of 6.53, 9.09 and 5.10 ppm, respectively. At 8 h of incubation phenanthrene, anthracene and pyrene was degraded up to 60.6, 10.3 and 84.1%, respectively. At the end of the incubation period 89.5% of phenanthrene, 36.8% of anthracene and 95.7% of pyrene was degraded and their corresponding final concentrations were 0.96, 5.92 and 0.33 ppm, respectively. Incubation time had a significant ($p<0.01$) effect on the percentage of PAHs degradation by laccase of KUM 60953 and KUM 60954. However as shown in Table 2, both strains had no significant difference in the percentage of degradation for phenanthrene and pyrene at 24 h of incubation.

Toxicity evaluation using brine shrimp: Results of brine shrimp lethality bioassay were estimated by probit software using mortality data that was obtained for pure PAH sample. PAH samples gave cytotoxicity effect at 37.16 μL of lethal dose (LD_{50}) equivalent to 0.12 ppm. The minimum toxicity was detected at 34.8 μL dose level and the maximum toxicity will be at 39 μL.

The final concentration of control (untreated PAH) was calculated based on standard graph was detected at 0.12 ppm of phenanthrene, anthracene and pyrene (data not shown). Final concentration of degraded PAHs which was incubated with laccase of KUM 60953 for 4 h of incubation was 0.03 ppm of phenanthrene, 0.1 ppm of anthracene and 0.01 ppm of pyrene. While, for PAHs incubated with laccase of KUM 60954 the concentration of the degraded PAHs was 0.08, 0.1, 0.06 ppm for phenanthrene, anthracene and pyrene, respectively. However the concentration of each PAH was reduced more by prolonged incubation time from 8 to 24 h with laccases.

Lethal dose (LD_{50}) value obtained from Probit Analysis was used to detect the level of toxicity in the untreated and treated PAH with laccase of KUM 60953 and KUM 60954. In this study, 36.17 μL lethal dose volume of PAH sample was used to compare the reduction in the toxicity between untreated and treated PAH and also with the positive controls (acetonitrile and salt water). Since, there was no death of brine shrimp in the positive control (acetonitrile and salt water) death of brine shrimp was occurred due to the toxicity of PAHs alone.

PAHs degradation was considered had occurred when *Artemia* in treated PAH showed less than 50% of mortality. The PAH sample treated with laccase of KUM 60954 for 4 h was considered toxic to brine shrimp as 9.1% of mortality was detected even though the corresponding concentration was only about 0.08 ppm of phenanthrene, 0.1 ppm of anthracene and 0.06 ppm of pyrene. PAH sample that was treated for a longer period however showed 0% mortality. PAH sample treated with laccase of KUM 60953 from 4 to 24 h showed a reduction in the toxicity as 0% of mortality was obtained. Hence, incubation time had a significant ($p<0.01$) effect on degradation of PAH sample which was treated with laccase of KUM 60954 but not for KUM 60953. The untreated and treated PAH sample for both strains had significant ($p<0.01$) differences in the percentage of mortality.

DISCUSSION

Pycnoporus sanguineus is a white rot fungus that offers a number of advantages in bioremediation. The

oxidising enzymes of lignin degradation which were located extracellularly allow substrates of low solubility to be oxidised by increasing the bioavailability and water solubility of the substrates (Park *et al*., 2006). Hence it also enabled these organisms to tolerate a relatively higher concentration of toxic pollutants (Reddy and Mathew, 2001). White rot fungi catalyse degradation of lignin and a wide variety of other pollutants by using non-stereoselective and non-specific substrates (Hammel, 1992).

In this study the types of lignocellulosic substrate used was oil palm frond parenchyma tissue (OPFPt). In Malaysia, this agroindustrial residue was produced in large amounts. About 11 tonnes of pruned fronds contain 35% of OPFPt have been reported to be suitable for the production of laccase via SSF (Vikineswary *et al*., 2006). Researches reported that *P. sanguineus* have been reported to produce laccase as the sole lignolytic enzyme by SSF (Vikineswary *et al*., 2006; Lomascolo *et al*., 2002). Therefore, in this study utilisation of agroindustries waste was used in the production of laccase from *Pycnoporus sanguineus*.

Various methods were applied by many researchers in order to produce high levels of laccase. According to Baldrian (2004) laccase was produced maximum during interspecific interaction between *Trametes versicolor* and *Pleurotus ostreatus*. Many studies have been carried out to enhance laccase productivity by addition of cooper as a micronutrient (Revankar and Lele, 2005) and by supplementation of inducers such as veratryl alcohol (Lin *et al*., 2003) and 25-xylidine (Alve-García, 2006). However, the preferred substrates for growth of white rot fungi in nature are lignocellulosic substrates. Therefore, inexpensive source of lignocellulosics such as OPFPt was chosen (Vikineswary *et al*., 2006).

In this study, laccase in the cocktail were obtained by SSF without addition of any mediators or mineral solutions. For example, Tong *et al*. (2007) in his studies produced 0.0068 U mL^{-1} of laccase and Pointing and Vrijmoed (2000) produced 1.4 U mL^{-1} of laccase by liquid culture by addition of mineral solution. Hence, by SSF alone, Vimala *et al*. (2001) was able to obtained 2.2 U mL^{-1} of laccase from KUM 60953 higher than that reported by the two researchers. According to Reddy and Mathew (2001), laccase levels can be increased by SSF rather than usage of expensive liquid media. It is because in SSF the substrates not only contain lignin but also cellulose and other carbon sources exist naturally which were preferred for the growth of white rot fungi.

The use of whole fungal cultures for degradation at contamination sites has become increasingly popular. However, according to the studies of Bumpus *et al*. (1985), though considerable success has been achieved in the laboratory in demonstrating extensive degradation by *P. chrysosporium*, field scale studies have not achieved a similar degree of success. In laboratory scale, the study of fungal bioremediation is dominated by studies using pure cultures, controlled environments, single chemical compounds, balanced culture media and other conditions that are amendable to experimental optimisation and replication. It is unrealistic to expect such conditions to exist in the field (Harvey and Thurston, 2001).

In general, laccase possesses a highly specific binding pocket that appears to be shallow and relatively non-stereospecific for oxygen and for reducing substrates. In fact, whether a compound will or will not oxidise depends on the differences in the redox potential between the reducing substrate and Type 1 copper in the active site (Harvey and Thurston, 2001). However, this property provides laccase with the ability to oxidise a broad range of substrates with high redox potentials of more than 1V/NHE (Harvey and Thurston, 2001). Therefore, the use of crude extracts of enzymes through direct application may be feasible and cost effective. However, the successful of such processes are not only dependent on initial enzyme activities, but also on their stabilities during the degradation processes.

Laccase of KUM 60953 was able to degrade PAH sample in 4 h of incubation periods. However, laccase of KUM 60954 degraded PAH sample in 8 h of incubation time. Both strains degraded PAH at different incubation times even though the laccase activity was controlled in the laboratory. The difference in the time taken by both laccases to degrade PAH sample may be due to that both strains were isolated from two different sites (Coates *et al*., 1997), KUM 60953 was isolated from Thailand which may isolated from a contaminated sites and on the other hand, KUM 60954 from Shah Alam may not be isolated from a contaminated sites. Therefore, both strains differ in the degradation pattern.

Both laccases were able to degrade PAHs mixture content with slight differences in the percentage of degradation. According to Bezalel *et al*. (1996) study, laccase activity will increase simultaneously with increase of the CO_2 levels. Accumulation of CO_2 by degradation in the early incubation hours contribute to the increases of laccase activity of KUM 60954 at latter part of incubation hours. This may be the reason why laccase of KUM 60954 was able to reach the same level of degradation with KUM 60953 at the end of incubation periods.

The oxidation of PAH by laccase was based on an ionization potential (IP) and solubility of PAH. In this study, percentage degradation of PAH was noted in the

following order: pyrene > phenanthrene > anthracene, similar order was reported by Zeng *et al.* (2000). But a different order was noted by Chang *et al.* (2003) such as in the degradation of PAH in petrochemical sludge the pattern was follows as: phenanthrene > anthracene > pyrene, while in PAH municipal sludge the trend was as follows phenanthrene > pyrene > anthracene.

Pyrene with the ionization potential (IP) value of 7.41 eV was rapidly biodegraded compared to phenanthrene and anthracene. It was because phenanthrene and anthracene have higher IP value of 7.90 and 7.44 eV respectively compared to pyrene. A similar finding was represented by Valentin *et al.* (2006), who stated that PAHs with lower IP value will be degraded faster than PAH with higher IP value. In pyrene degradation, IP value was correlated with the percentage of degradation (Pozdnyakova *et al.*, 2006).

Meanwhile, the percentage degradation between phenanthrene and anthracene showed no correlation on IP values, because anthracene should be an easily degradable substrate by both laccases than phenanthrene. However in this study, phenanthrene was degraded rapidly compared to anthracene. Therefore it was assumed that there was correlation between the solubility of PAHs between phenanthrene and anthracene instead of IP values (Pozdnyakova *et al.*, 2006). The solubility of PAHs of phenanthrene and anthracene increased in the order of phenanthrene > anthracene. Therefore, in this study, it was shown that phenanthrene which was more soluble (1.3 mg L^{-1}) than anthracene (0.07 mg L^{-1}) was degraded faster.

In a study of phenanthrene degradation by Bohmer *et al.* (1998), it was shown that phenanthrene degradation was achieved in 182 h by using 1 U mL^{-1} purified laccase of *Trametes hirsute* in the presence of mediator (HBT). In this study, PAH degradation was achieved in less than 24 h using 30 U mL^{-1} crude laccase from *P. sanguineus* without usage of any mediators. The form of laccase (crude or purified) used could explain the differences in the percentage degradation (Bonomo *et al.*, 1998). So far, percentage of degradation by using laccase cocktail directly to the PAH sample has been only reported by Pozdnyakova *et al.* (2004).

Toxicity evaluation is an important test to determine the toxicity level of the degraded product. The brine shrimp lethality assay represents a rapid, inexpensive, simple bioassay for testing the toxicity level of PAHs before and after the degradation process take place (Kanegusuku *et al.*, 2002). In this study, the final concentration of the untreated PAH in the volume of 3 mL of salt water was at 0.12 ppm showed > 50-56% < of

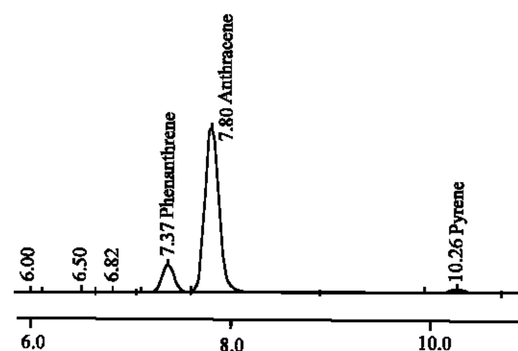

Fig. 3: Resolution of degradation at 24 h of incubation period on phenanthrene, anthracene and pyrene in PAH-mixture using 90% (v/v) acetonitrile and 10% (v/v) water and detected at 254 nm for 12 min using LiChroCart ® 250-4,6 HPLC-Cartridge Purospher ® STAR RP-18 endcapped (5 μm) column

mortality. Both salt water and acetonitrile were non-toxic to brine shrimp as zero mortality was recorded. The percentage of mortality was reduced from 9.1 to 0% in the PAH sample treated from 4 to 24 h with laccase of KUM 60954. Meanwhile, the mortality of the brine shrimp in the PAH sample treated by laccase of KUM 60953 for a similar length of incubation hours showed zero percentage of mortality. This showed that the degradation process with laccase had reduced the toxicity levels of PAH and did not produce any new toxicants as by products of PAH degradation. Further, there were no new peaks observed in the chromatogram obtained after degradation being carried out (Fig. 3). This study showed that laccase may have significant roles in bioremediation. However, further in- situ studies at the contaminated sites need to be under taken.

CONCLUSION

Solid substrate fermentation of OPFPt by *P. sanguineus* of KUM 60954 and KUM 60953 gave the highest amount of laccase of 2.53 and 2.44 U mL^{-1}, respectively, when compared to the other selected strains. Laccase produced by SSF of *P. sanguineus* was found to be dominant oxidative enzymes as reported in Vikineswary *et al.* (2006) was able to degrade PAHs. The degradation levels of the two tested strains were slightly different but longer incubation time produced similar level of degradation. Laccase (30 U mL^{-1}) of both strains were able to degrade PAH mixture at pH 5.0 and at 40°C within 24 h of incubation. Laccase of KUM 60953 showed rapid degradation at 4 h of incubation compared to laccase of KUM 60954. However both laccase showed similar

percentage of degradation at 24 h of incubation. Based on the toxicity studies done, degradation of PAHs by laccase did not lead to formation of new toxicants as there was zero mortality of *Artemia* exposed to the PAH sample that was treated for 24 h. Overall, PAH degradation was faster with laccase of KUM 60953 compared to laccase of KUM 60954.

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