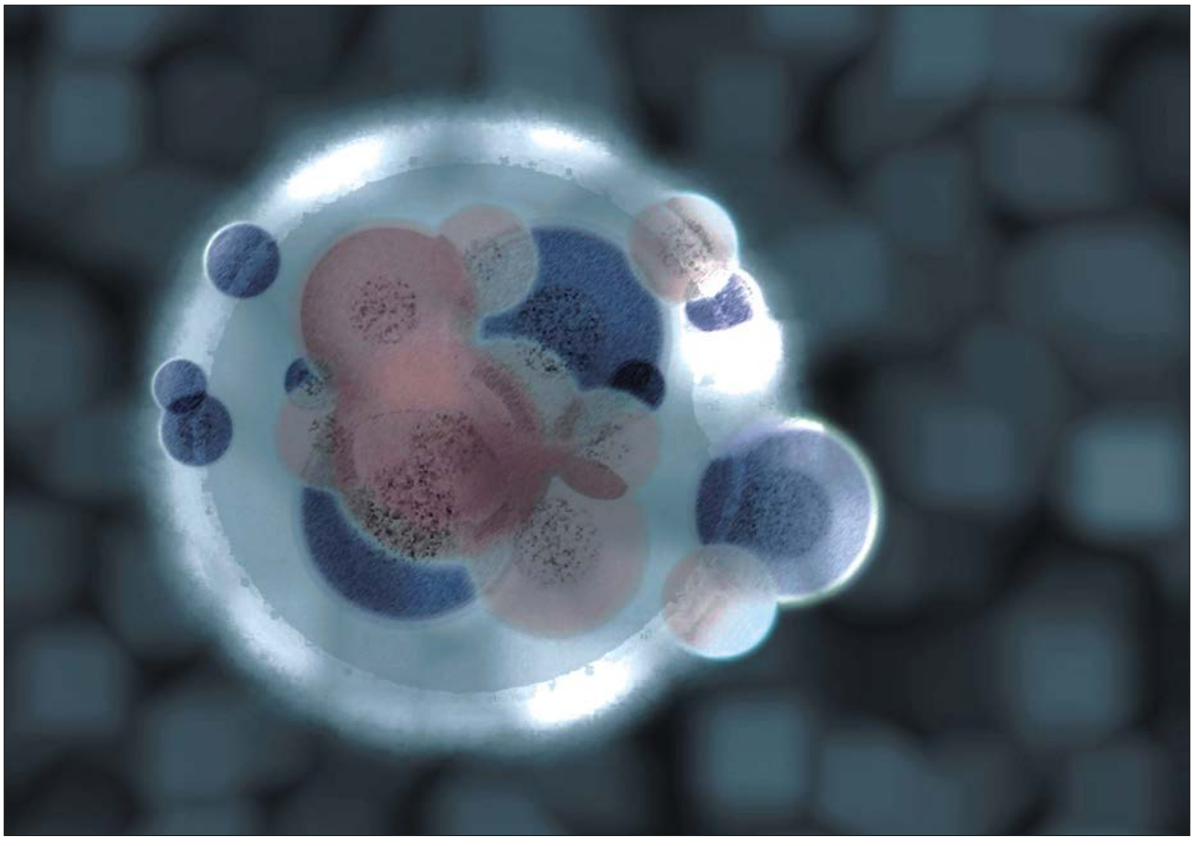

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Characterisation of Laccase from *Pycnoporus sanguineus* KUM 60953 and KUM 60954

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Abstract: Four strains of *Pycnoporus sanguineus* isolated from Shah Alam (KUM 60954), Endau Rompin (KUM 60955), Cherating (KUM 60956), Gombak (KUM 60957) and the standard strain from Thailand (KUM 60953) were evaluated for their ability to grow on Polycyclic Aromatic Hydrocarbon (PAH)- and acetonitrile-incorporated Potato Dextrose Agar (PDA). The mycelial growth was then compared to the growth of *P. sanguineus* on PAH and acetonitrile free PDA. All strains tested showed a high tolerance to PAH up to 25 ppm and 2.5% of acetonitrile. Laccase derived from the best strains KUM 60954 and KUM 60953 which exhibited higher tolerance to both PAH and acetonitrile was further characterised. The activity was maximum for both strains at pH 5.0 and 40°C. Laccase from both strains was stable over a wide range of pH (3-5) and at temperatures below 60°C. Laccase was stored as a concentrate or as a lyophilised powder to minimise the degradation of enzyme activity during storage. The laccase activity in the extract remained a high activity up to four months at optimum pH 5.0 and at both 0 and 4°C. Further laccase was also stable up to 24 h at 40°C when mixed with 1% of acetonitrile.

Key words: Tolerance, PAH, acetonitrile, laccase activity, stability, bioremediation

INTRODUCTION

Laccases are a family of blue-copper oxidase proteins containing four copper ions. It has a low redox potential ca. 0.708 V/NHE, therefore it is able to catalyse single electron oxidation steps with concomitant reduction of O_2 to water (Baiocco *et al*., 2003). Laccase is able to catalyse one electron abstraction from phenolic hydroxyl group compounds, ortho-and para-diphenols, methoxy-substituted phenols, aromatic amines and phenolic acids to form phenoxy radicals coupled to the reduction of molecular oxygen (Robles *et al*., 2002).

Laccases have gained considerable interest in various industrial areas due to their interesting catalytic properties and broad variety of reaction that can be catalysed. The most intensively studied applications include pulp delignification, textile dye bleaching, detoxification of effluent, as well as in biopolymer modification (Palonen *et al*., 2003). In these applications, many of the processes occur at high pH and temperatures. The enzymes secreted by basidiomycetes depend on the species of fungus (Zouari-Mechichi *et al*., 2006). For example, laccase from *Trichophyton rubrum* LKY-7 was stable at temperatures below 50°C and pH 5-7 (Jung *et al.,* 2002), while laccase from *Pycnoporus coccineus* was stable at 65°C and pH 4-9 (Schneider *et al*., 1999). This characteristic of laccase is therefore deemed critical for commercial applications of laccases in bioremediation.

The aims of this study were (a) evaluate the laccase of several strains of *Pycnoporus sanguineus* for their tolerance towards different levels of acetonitrile and PAH-mixture concentration, (b) verify the effects of pH, temperature and acetonitrile on laccase activity and stability, (c) estimate the storage stability in pH 5.0 and pH 7.0 and (d) determine the molecular sizes of the laccases obtained via solid state fermentation.

MATERIALS AND METHODS

Fungal cultures: Fungal cultures designated KUM 60953, 60954, 60955, 60956 and 60957 were obtained from the fungal culture collection (Kulat Universiti Malaya) at Institute Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

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Growth: Stock cultures derived from tissue culture were revived by subculturing on Potato Dextrose Agar (PDA) media and incubated for three days at 27±2°C. Mycelial plugs with diameter of 0.5 cm cut from the actively growing region were subcultured on a fresh PDA media. The inoculated plates were incubated for seven days at 27±2°C.

Selection of indigenous strains of *P. sanguineus* tolerant to acetonitrile and PAH: Acetonitrile-incorporated PDA plates were prepared by adding sterilised acetonitrile to autoclaved PDA medium to provide final concentrations of 1, 2.5 and 5%. The plates were cooled overnight at room temperature. Stock solution of a mixture of PAHs (phenanthrene, anthracene and pyrene) at a concentration of 1000 ppm was prepared by dissolving 50 mg of each individual PAH in a total of 50 mL of acetonitrile and the solutions were thoroughly mixed. The filter-sterilised stock solutions of PAH-mixture solutions were incorporated into 100 mL of sterilised PDA medium providing final concentrations of 10, 25 and 50 ppm of PAH-mixture. Twenty milliliter of acetonitrile and PAH-mixture incorporated PDA was dispensed into several Petri dishes and allowed to solidify overnight at room temperature. The negative controls were plates of PDA medium. The prepared plates were then inoculated with a seven day old mycelium plug and were incubated at 27±2°C for seven days. Three replicates were set up for each strain tested. Radial growth of the mycelium on the plate was measured daily. Mycelial extension of *P. sanguineus* on control PDA plates were compared with growth on acetonitrile and PAH mixture-incorporated PDA.

Detection of laccase using zymogram assay: Forty milligram of lyophilised laccase was dissolved in loading buffer and aliquots were stored at -20°C for one month prior to use. A zymogram assay was carried out for detection of laccase activity by native-PAGE performed with 10% (w/w) gels according to the method of Laemmili (1970). The gel was stained in 50 mL of 0.1 mM syringaldazine in 50% ethanol for 15 min to detect laccase activity.

Determination of optimum pH and temperature for laccase activity: Lyophilised enzyme powder was dissolved in distilled water and 0.2 mL aliquot was added to 3 mL buffer solution from pH 3.0 - 9.0. Laccase activity was determined under the standard assay conditions. While, for determination of the optimum temperature for laccase activity, the prepared buffer and enzyme mixture was examined at pH 5.0 at temperatures ranging from 30 to 80°C. All assays were carried out in triplicates.

Effect of acetonitrile mixtures on laccase activity and stability: Laccase assay was carried out with slight modification of standard assay procedure. Various concentration (0-30%) of pure acetonitrile was added in the standard assay and was incubated at 40±2°C for 1 h. Acetonitrile was included in the control, enzyme blank and reaction mixture. The stability of laccase of KUM 60954 and KUM 60953 were evaluated in experimental mixtures. Experimental mixtures consisted of 1 mL of acetonitrile (equivalent to final concentration of 1%), 1.0 mL of laccase (equivalent to 30 U mL^{-1}) and 8 mL of pH 5.0 buffer was incubated at 40±2°C for 4, 8 and 24 h. Samples were removed at intervals of 4, 8 and 24 h. Control without acetonitrile was incubated parallely. All assays were carried out in triplicates.

Determination of stability as a function of pH and temperature: The stability of laccase at different pH values was determined by preincubating the laccase solution in the same buffer systems as described above. Two milliliter samples of laccase were incubated at the optimum temperature (40°C) at various pH, ranging from 3.0 to 9.0 for 1 h with moderate shaking (80 rpm). The measurement of the activity was carried out in triplicates under standard assay conditions. pH stability was expressed as percentage of relative activity. For measuring stability as a function of temperature, laccase in buffer at optimum pH (pH 5.0) was incubated at temperatures ranging from 30 to 80°C in the absence of substrate in a water bath for one hour. Samples were cooled at room temperature prior to determination of relative activity. Assays were carried out in triplicates.

Determination of storage stability in pH 5 and pH 7 at 4 and 0°C of laccase activity: Aliquots of laccase in pH 5 and pH 7 were stored at 4 and 0°C for up to 3 month. At the end of each month samples were assayed in triplicates.

Gel electrophoresis: Laccase (15 μL) and marker (10 μL) were electrophoresed by applied current (30 mA/ 200 V). Electrophoresis was terminated when the tracking dye reached about one centimeter from the bottom of the gel. Once electrophoresis was completed, the gel was stained in ABTS solution for 15 min. While for staining marker, the gel was stained overnight with 0.025% coomassie blue solution. The background stain was removed by washing with several changes of destaining solution contained 5% methanol and 7% glacial acetic acid in deionised water.

Molecular weight determination: Molecular weight was determined by the method of Fergusson (1964). The R_f

value (the electrophoretic mobility) of the protein in each gel relative to the tracking dye was determined. The value of 100 [log (R_f x 100)] against the percentage of gel concentration for each protein was plotted. The slope from each protein known as retardation coefficient (K_r) value was determined for each protein standard. The negative slope value was plotted against the logarithm of the known molecular weight of each protein standard to produce a linear plot. While, for unknown sample (laccase), K_r value was determined similarly. The K_r value from the unknown samples was used to determine molecular weight from the standard graph of the known molecular weight of protein standards (negative slope value against the logarithm of the known molecular weight of protein standards).

Statistical analysis: Triplicate data values obtained were 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 differences.

RESULTS

Tolerance of indigenous strains to acetonitrile and PAH-mixture among *P. sanguineus* strain: Table 1 gives the radial extension of mycelium growth of *P. sanguineus* on control (PDA agar) for each strain after seven days. KUM 60954 rapidly colonised the PDA plate and the diameter of mycelial growth was 8.5 cm after seven days of incubation at 27±2°C. This was followed by KUM 60957 and KUM 60956 strains. The strains KUM 60953 and KUM 60955 were slower growing compared to the strains KUM 60954, KUM 60957 and KUM 60956. The strain differences did have a significant ($p<0.01$) effect on the growth rate of the five strains tested. The radial growth of all strains of *P. sanguineus* on control plates was similar with an average growth rate of 1.16 cm day^{-1}.

Radial extension of all strains tested on PDA plate with 1% acetonitrile was inhibited to 0.97 cm day^{-1} compared to the control plates (Table 1). Significant inhibition ($p<0.0039$) in fungal growth of *P. sanguineus* among strains occurred at 1% acetonitrile. The average growth rate among all the strains at 2.5% of acetonitrile was about 0.68 cm day^{-1}, which was less than the average of growth rate observed at 1% acetonitrile. This shows that the concentration of acetonitrile affected the growth rate of mycelium. Mycelium growth among all the strains tested was slightly visible in 5% of acetonitrile with a growth rate of only 0.20 cm day^{-1}. This indicated that the decrease in growth of mycelium increased with increase in concentration of acetonitrile.

Table 1 shows that 10 ppm of PAH-mixture supported mycelial growth of *P. sanguineus* by tolerates its toxic effects. The different strains showed significant ($p<0.0085$) differences in the inhibition of the mycelium growth. The effect of toxicity of PAH-mixture started at 25 ppm, when growth of mycelium was inhibited. Therefore, the concentrations of PAH had a significant ($p<0.05$) effect on the mycelium growth of *P. sanguineus*. Meanwhile, mycelium was not visible in plates containing 50 ppm of PAH-mixture due to complete inhibition of mycelium growth.

Zymogram: Two isoforms of laccase of KUM 60953 and KUM 60954 designated as Lac 1 and Lac 2 were detected in native-PAGE (Fig. 1). Lac 1 (minor band) being the

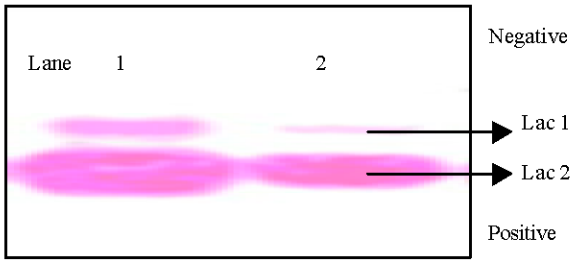

Fig. 1: Electrophoretic analysis of laccase of *P. sanguineus* isolates using native-PAGE. Samples loaded in lane 1: KUM 60954; lane 2: KUM 60953

Table 1: Radial extension (cm) of mycelium of *P. sanguineus* strains on control, 1, 2.5 and 5.0% of acetonitrile and 10, 25 and 50 ppm of PAH mixture incorporated into PDA after seven days of incubation at 27±2°C

	Condition						
		PDA+Acetonitrile (%)			PDA+PAH mixture (ppm)		
Strain	PDA-only (control)	1.0	2.5	5.0	10	25	50
KUM 60953	7.90±0.10^{a}	6.37±0.35^{a}	3.47±0.55^{a}	1.52±0.48^{ac}	2.70±0.10^{a}	1.10±0.00^{a}	0
KUM 60954	8.50±0.00^{b}	7.43±0.45^{b}	6.35±0.00^{b}	1.05±0.13^{b}	2.50±0.40^{a}	1.12±0.13^{a}	0
KUM 60955	7.97±0.06^{ac}	6.67±0.15^{ac}	3.85±0.22^{a}	1.35±0.15^{bc}	2.43±0.15^{a}	1.07±0.24^{a}	0
KUM 60956	8.00±0.20^{ac}	6.33±0.15^{a}	4.12±0.28^{a}	1.13±0.08^{d}	1.87±0.21^{b}	0.82±0.13^{b}	0
KUM 60957	8.13±0.12^{c}	7.17±0.29^{ab}	5.88±0.80^{b}	1.80±0.30^{c}	2.53±0.06^{a}	1.27±0.06^{a}	0

Same letter denotes not significant; ±refers to standard deviation; (0) mycelial growth was not observed

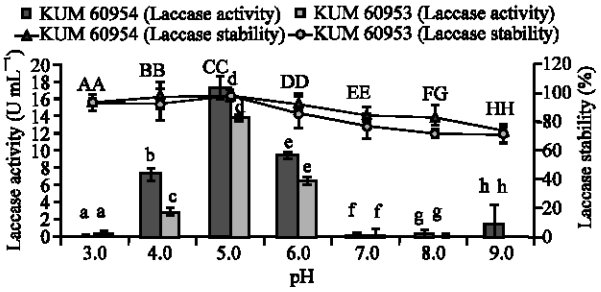

Fig. 2: Effect of pH on laccase activity and stability of KUM 60954 and KUM 60953, same letter denotes not significant; error bars refers to standard deviation

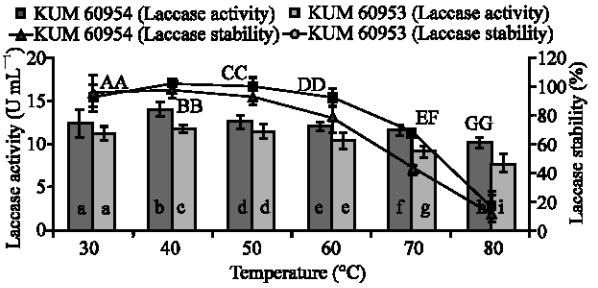

Fig. 3: Effect of temperature on laccase activity and stability of KUM 60954 and KUM 60953, same letter denotes not significant; error bars refers to standard deviation

slower migrating band was a charge isomer and the fastest migrating band Lac 2 was considered for molecular weight determination.

Effect of pH on activity and stability: Figure 2 shows the effect of pH on activity and stability of laccase. Laccase activity was low at pH below 4.5 and reached maximum activity at pH 5.0. A sharp drop in activity was seen from pH 6 to 7. The optimum pH for activity for both strains was not distinctly different and the overall pH-activity profiles were similar. Laccase from both strains showed broad pH stability. Laccase showed stability between pH 3.0 to 5.0 and held lower stability between pH 6 to 9. Eventhough, pHs had a significant ($p<0.01$) effect on laccase activity and stability but overall showed not significant between the strain tested.

Effect of temperature on activity and stability: Figure 3 shows temperature-activity and stability profiles of laccase of KUM 60954 and KUM 60953. A general trend was that the activity increased rapidly and peaked at 40°C and then decreased at 80°C. A similar trend was also observed for KUM 60953. The stability of laccase of KUM 60954 increased steadily with an increase in temperature up to 50°C and then it dropped sharply towards 80°C. Similar patterns were also observed in KUM 60953. Both laccases have the same stability pattern at 40°C. Hence, temperature did not have a significant effect on laccase activity and stability of the two strains tested.

Effect of acetonitrile on laccase activity and stability of KUM 60954 and KUM 60953: Figure 4 shown that addition of higher concentration than 1% acetonitrile will caused reduction in laccase activity of KUM 60954 but for KUM 60953 only more than 5% caused the reduction. Figure 5 shown that the stability of laccase of KUM 60953 was showed unaffected by acetonitrile. Laccase activity

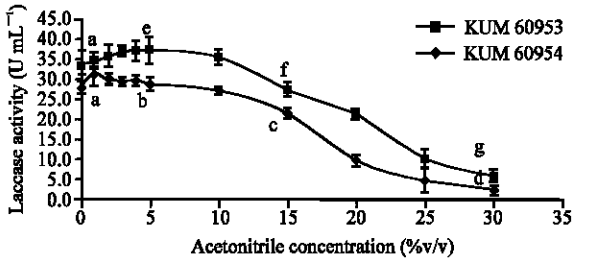

Fig. 4: Effect of acetonitrile at various concentrations (%) on laccase activity of KUM 60954 and KUM 60953, same letter denotes not significant; error bars refers to standard deviation

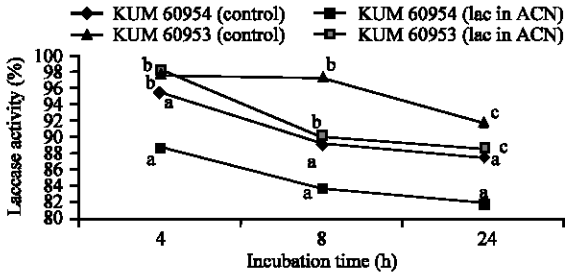

Fig. 5: Stability of laccase activity of KUM 60954 and KUM 60953 in acetonitrile (ACN) from 4 to 24 h of incubation time at 40±2°C, same letter denotes not significant; error bars refers to standard deviation

did not decrease sharply but steadily off instead in both control and experimental setup. This indicated that laccase of both strains were very stable in acetonitrile (final concentration ≤1%). Since, both strains did not show any significant effect in the activity and stability at 1% of acetonitrile concentrations, this concentration was choose to carry out PAH degradation in the latter part of the studies.

Table 2: Effect of storage time on laccase activity of KUM 60954 and KUM 60953 in pH 5.0 and pH 7.0 condition at 4±2°C stored for four months

	Strain			
	pH 5.0 condition		pH 7.0 condition	
Incubation month	KUM 60954	KUM 60953	KUM 60954	KUM 60953
1	92.05±3.58^{Aa}	93.27±2.81^{Aa}	57.97±0.66^{Ab}	60.12±4.82^{Ab}
2	84.00±4.11^{Ba}	88.65±4.22^{Aa}	43.46±0.52^{Bb}	36.37±5.23^{Bb}
3	75.39±3.54^{Ca}	80.67±6.75^{Ba}	42.30±0.21^{Bb}	32.55±5.73^{BCc}
4	55.85±1.34^{Da}	67.00±0.75^{Cb}	33.61±2.10^{Cc}	27.26±2.57^{Cd}

Table 3: Effect of storage time on laccase activity of KUM 60954 and KUM 60953 in pH 5.0 and pH 7.0 condition at 0±2°C stored for four months

	Strain			
	pH 5.0 condition		pH 7.0 condition	
Incubation month	KUM 60954	KUM 60953	KUM 60954	KUM 60953
1	92.49±2.53^{Aa}	88.28±3.24^{Aa}	52.18±1.88^{Ab}	8.53±0.55^{Ac}
2	81.02±7.87^{Ba}	81.92±0.75^{Ba}	30.03±2.93^{Bb}	8.47±0.46^{Ac}
3	76.38±3.27^{Ba}	75.69±1.77^{BCa}	18.32±0.13^{Cb}	1.07±0.42^{Bc}
4	67.77±0.38^{Ca}	74.10±1.20^{Cb}	16.44±0.60^{Cc}	0.97±0.21^{Bd}

Same letter denotes not significant; ± refers to standard deviation

Storage stability in pH 5.0 and pH 7.0 at 4 and 0°C: Table 2 shows the effects of storage time on laccase activity in pH 5.0 and pH 7.0 at 4°C. In pH 5.0 , the drop in activity was approximately linear and the activity gradually declined with time during four month storage period. It was observed that after four months of incubation, laccase stored in pH 5.0 retained 56% of activity. Meanwhile, in pH 7.0 condition, only 34% of activity was retained by KUM 60954. However, when laccase of KUM 60953 stored in pH 5.0 for four months, 67% of activity was retained but only 27% was detected in pH 7.0. Storage condition at pH 5.0 and pH 7.0 at 4°C had a significant ($p<0.01$) effect on laccase activity of KUM 60954 and KUM 60953. These results showed that laccase needs to be stored in pH 5.0 condition to maintain its activity during prolonged incubation.

The effect of storage time on laccase activity was also studied in pH 5.0 and pH 7.0 at 0°C (Table 3). Laccase activity of KUM 60954 was retained up to 68% of activity in pH 5.0 condition after four months of storage, meanwhile laccase of the similar strain in pH 7.0 only retained approximately 16% of laccase activity. While, laccase activity of KUM 60953 was detected up to 74% in pH 5.0 whereas in pH 7.0 only 1% of activity was observed after four months of storage period. From the results, it can be concluded that storage of laccase in pH 5.0 were necessary as it had a significant effect ($p\leq 0$) on the stability of the stored enzyme.

Molecular weight determination: These gels exhibited charge isomers, the migration distance of the darkest band

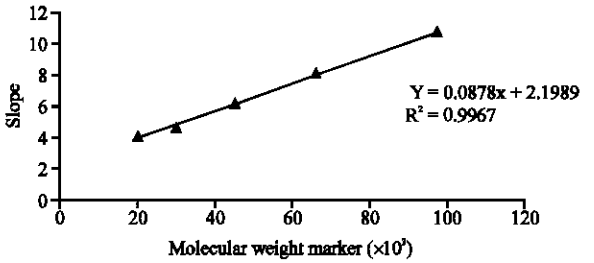

Fig. 6: Calibration curve from Ferguson plot analysis for the determination of molecular weight of unknown sample from standard markers. The proteins used were Phophorylase b (97 kDa), albumin (66 kDa), Ovalbumin (45 kDa), carbonic anhydrase (30 kDa), Trypsin inhibitor (20.1 kDa)

Table 4: Molecular parameters obtained from a series of Fergusson (1964) standard graph plot for raw data and unknown samples

Standard protein	Molecular weight (kDa)	Y = mX±C	K_r (slope)	R^2
Phosphorylase b	97	Y = -10.642X±217.46	-10.642	0.9730
Albumin	66	Y = -8.1456X±211.23	-8.1456	0.9606
Carbonic anhydrase	45	Y = -6.192X±230.00	-6.1920	0.9379
Trypsin inhibitor	30	Y = -4.6029X±223.23	-4.6029	0.9737
Ovalbumin	20.1	Y = -4.0752X±182.16	-4.0752	0.9935
KUM 60954	Unknown	Y= -8.4253X±252.71	-8.4253	0.9937
KUM 60953	Unknown	Y = -8.7327X±252.44	-8.7327	0.9938

was used to determine the molecular weight of laccase. A graph of 100 [log (R_f x 100)] versus the gel concentration showed parallel lines did not crossed each other, proving that separation between these two bands was caused by differences in charge isoenzymes and not due to the differences in molecular weight (Figure not shown). The band of the protein corresponding to the upper line was used to calculate the molecular weight (Gauldie *et al*., 1972) for laccase of KUM 60954 and KUM 60953. The slope known as retardation coefficient ($K_{r\ value}$) obtained from the standard graph was shown in Table 4. The determination of the $K_{r\ value}$ for standards with known molecular weight was determined from a calibration curve (Fig. 6). Molecular weight of laccases of KUM 60954 was 71±2 kDa while KUM 60953 was 74±2 kDa.

DISCUSSION

Fungi play an important role in the metabolism of PAH in aquatic and terrestrial environments (Bezalel *et al*., 1996). Fungi oxidise PAH via cytochrome P-450 monooxygenase and epoxide hydrolase-catalysed reactions (Bezalel *et al*., 1996). In this study, *P. sanguineus* was able to grow in a medium containing a mixture of PAHs up to 25 ppm. *Pycnoporus sanguineus* demonstrated a high tolerance towards these toxic

compounds compared to other white rot fungi that have been studied. For example, Baborova *et al.* (2006) had shown that *Irpex lacteus* was able to grow and degrade 15 ppm of PAH. Meanwhile, Bezalel *et al.* (1996) reported that *Pleurotus ostreatus* was tolerant to 25 ppm of PAH and a high degradation rate was detected at this concentration. Pickard *et al.* (1999) studied tolerance of PAH by *Criolopsis gallica* and showed that the fungal growth was not affected at 5 ppm.

According to cometabolism studies, fungal strains grow well in the presence of PAHs only when there was an easily available free carbon source in the solid media. At higher concentration (25 and 50 ppm) dilution of carbon source occurred compared to a lower concentration (10 ppm) of PAH (Giraud *et al.*, 2001). Therefore, mycelium growth was higher at lower concentration of PAH-mixture. One percent of acetonitrile was not toxic for all the tested strains as only 10 to 20% of reduction in mycelium growth had occurred compared to the mycelium growth on the control PDA plates. Further, at a concentration of 5% (v/v) of acetonitrile, an average of 0.20 cm of mycelial growth per day was recorded. This showed only a lower degree of mycelial inhibition compared to inhibition of mycelial growth at 50 ppm of PAH-mixture. Hence, growth inhibition was solely by the PAH-mixture itself and not from addition of acetonitrile.

For pH optimum, Ng and Wang (2004) reported that a dramatic reduction in laccase activity occurred with an increase in pH. In this study, laccase oxidised syringaldazine optimally at pH 5.0. Laccase from different strains exhibited optimal oxidative activity on similar substrates at a wide range of pH. For example, laccase of *Sinorhizobium meiloti* oxidised syringaldazine optimally at pH 7.2 (Rosconi *et al.*, 2005) and *Coprinus cinereus* at pH 6.5 (Scneider *et al.*, 1999). Furthermore laccase also oxidised different substrates at different optimal pHs. For example, UD4 strain oxidised ABTS at pH 4.0 (Jordaan *et al.*, 2004) and *Trichophyton rubrum* LKY-7 oxidised guaiacol at pH 3.5 (Jung *et al.*, 2002). However, it is generally accepted that most laccases of fungi exhibit pH optimum in an acidic range.

The optimum temperature for optimum activity of laccase of both strain that was determined in this study was 40°C, similar to that of laccase from fungal in *Mauginiella* sp. (Palonen *et al.*, 2003). However, the optimal temperature of laccases can also differ from strain to strain (Nilsson *et al.*, 2006). For example, laccase of *Panus rudius* was most active at 60°C (Zhang *et al.*, 2005) and laccase of *Cerena maxima* had a temperature optimum at 50°C (Koroleva *et al.*, 2002).

In this study, concentrated laccase produced by OPFPt was stable between pH 3.0 to 9.0. Other studies have shown that crude extract of laccase produced by liquid medium was stable at pH 5.0 (Record *et al.*, 2002), while the purified laccase was found stable over the pH range of 3.0 to 7.5 (Motoda, 1999). In the present study it had shown that laccase of *P. sanguineus* had a wider range of pH stability than that reported by other researchers. The only reasons for these characteristics were believed to be due to differences in strain and/or methods of production and the form of laccases (crude, concentrated or purified) that was studied (Dedeyan *et al.*, 2000). However, further studies need to be carried out to prove the above statement.

Most white rot fungi prefer to grow on lignocelluloses waste where during bioconversion processes many organic chemicals that created acidic condition will be produced. Since the enzyme is present in this acidic state, biochemically it become resistant to this condition. Therefore, laccase was not inactivated easily (Motoda, 1999). The enzyme was stable at 80°C for one hour although Motoda (1999) reported at 60°C. The main factor was thought to be the effect of acid residues content on laccases (Bonomo *et al.*, 1998). Hence, laccase of *P. sanguineus* may be a good target in development as biotechnological tools (Dedeyan *et al.*, 2000) as utilisation of enzymes in industrial applications usually requires high activity at high temperature where laccase able to retain high activity at this condition.

Storage in pH 5.0 is necessary for laccase. If these requirements are not satisfied, laccase can rapidly lose their ability to perform specific function. Laccase can lose activity through proteolysis and aggregation if stored in less than optimal storage conditions (Pierce, 2003). In this study, increase in stability of laccase was observed when it was stored in pH 5.0 compared to its stability in pH 7.0 at both tested temperatures (4 and 0°C). This indicated that laccases have increased its stability in acidic condition through a proper storage (Ryan *et al.*, 2003). Low temperature of storage gives prolonged enzyme activity. Storage at 4°C and 0°C for laccase of KUM 60954 and KUM 60953 had a significant ($p<0.01$) effect on the stability of laccase, respectively after four month of storage. From this study, it was shown that laccase of both strains can be stored either at 4 or 0°C for about three month but for longer storage it was advisable to store laccase at 0°C.

The effects of acetonitrile (the solvent used to dissolved PAH) on laccase stability was crucial to maintain native conformation of laccase throughout the PAH biodegradation studies. Solvents with high values of partition coefficient between water and *n*-octanol (K_{ow}) types of solvent will be more favourable for preserving enzymatic activity. Besides that, stability may not only effect through hydrophobicity but also by other

characteristics of the solvents, such as hydrogen bonding, anion stabilisation and free energy of solvation (Eibes *et al*., 2005). Present results confirm that usage of acetonitrile (final concentration of ≤ 1% v/v) did not affect the activity of laccase up to 24 h of incubation time. Laccase of *P. sanguineus* obtained in this study was more stable than that in the study conducted by Rodakiewicz-Nowak *et al.* (2000) which showed reduction in enzyme activity in solvent such as acetonitrile, acetone and DMSO.

In the present study, it was found that the molecular weight of laccases for KUM 60954 and KUM 60953 was approximately 70 and 74 kDa, respectively. This result was within the range that was reported by other researches. For example, the molecular weight of laccases of *Physisporinus rivulosus* was found between 66 kDa and 68 kDa (Hakala *et al*., 2005) and laccase from *Pycnoporus cinnabarinus* had a molecular weight of approximately 70 kDa (Record *et al*., 2002). The differences in molecular weight may also be attributed to the copy number. Laccase gene in which the genes encode two to five laccase located on the same chromosome was found in *T. villosa.* In *Rhizoctonia solani* three clustered laccase genes were found (Eggert *et al*., 1998) and that will give variation in the molecular weight.

CONCLUSION

Based on the series of tolerance tests done, *P. sanguineus* of Shah Alam (KUM 60954) and Thailand (KUM 60953) showed a tolerance to 1% (v/v) acetonitrile and 10 ppm of PAH-mixture concentration. This characteristic is crucial if laccase is utilised in the biodegradation of toxic compounds such as PAH. The laccase produced by the two strains exhibited high activity at pH 5.0 and at 40°C. It was also stable at a broad range of pH from 3.0 - 9.0, temperatures from 30 to 60°C for one hour and stable in the presence of not more than 1% of acetonitrile (final concentration) for 24 h. Laccase activity at pH 5 was maintained by storing at 0 and 4°C up to four and three months, respectively. These characteristics of laccase may ensure the continued activity of the enzyme when applied to PAH contaminated sites or wastewater treatment system where extreme temperatures and pH may prevail.

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REFERENCES

Baborova, P., M. Moder, P. Baldrian, K. Catjthamlova and T. Cajthaml, 2006. Purification of a new manganese peroxidase of the white rot fungus *Irpex lacteus* and degradation of polycyclic aromatic hydrocarbons by the enzyme. Res. Microbiol., 157: 248-253.

Baiocco, P., A.M. Barreca, M. Fabbrini, C. Galli and P. Gentili, 2003. Promoting laccase activity towards non-phenolic substrates: A mechanistic investigation with some laccase-mediator system. Org. Biomol. Chem., 1: 191-197.

Bezalel, L., Y. Hadar and C.E. Cerniglia, 1996. Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Applied Environ. Microbiol., 62: 292-295.

Bonomo, R.P., A.M. Boudet, R. Cozzolino, E. Rizzarelli, A.M. Santoro, R. Sterjiades and R. Zappala, 1998. A comparative study of two isoforms of laccase secreted by the white rot fungus *Rigidoporus lignosus*, exhibiting significant structural and functional differences. J. Inorg. Biochem., 71: 205-211.

Dedeyan, B., A. Klonowska, S. Tagger, T. Tron, G. Iacazio, G. Gil and J.L. Pett, 2000. Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. Applied Environ. Microbiol., 66: 925-929.

Eggert, C., P.R. Lafayette, U. Temp, K.E.L. Eriksson and J.F.D. Dean, 1998. Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*. Applied Environ. Microbiol., 64: 1176-1772.

Eibes, G., T. Lu-Chau, G. Feijoo, M.T. Moreira and J.M. Lema, 2005. Complete degradation of anthracene by manganese peroxidase in organic solvent mixtures. Enzyme Microbiol. Technol., 37: 365-372.

Gauldie, J., L. Marshall and B.L. Hillcoat, 1972. Purification and properties of dihydrofolate reductase from cultured mammalian cells. J. Biochem., 133: 349-356.

Giraud, F., P. Guiraud, M. Kadri, G. Blake and R. Steiman, 2001. Biodegradation of anthracene and fluoranthene by fungi isolated from an experimental constructed wetland for wastewater treatment. Water Res., 35: 4126-4136.

Hakala, T.K., T. Lundell, S. Galkin, P. Maijala, N. Kalkkinen and A. Hatakka, 2005. Manganese peroxidases, laccases and oxalic acid from the selective white rot fungus *Physisporinus rivulosus* grown on spruce wood chips. Enzyme Microbiol. Technol., 36: 461-468.

Jordaan, J., B.I. Pletschke and W.D. Leukes, 2004. Purification and partial characterization of a thermostable laccase from an unidentified basidiomycete. Enzyme Microbiol. Technol., 34: 635-641.

Jung, H., F. Xu and K. Li, 2002. Purification and characterization of laccase from wood-degrading fungus *Trichophyton rubrum* LKY-7. Enzyme Microbiol. Technol., 30: 161-168.

Koroleva, O.V., V.P. Gavrilova, E.V. Stepanova, V.I. Lebedeva, N.I. Sverdlova, E.O. Landesman, H.S. Yavmetdinov and A.I. Yaropolov, 2002. Production of lignin modifying enzymes by co-cultivated white rot fungi *Cerrena maxima* and *Coriolus hirsutus* and characterization of laccase from *Cerrena maxima*. Enzyme Microbiol. Technol., 30: 573-580.

Laemmili, U.K., 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T_4. Nature, 227: 680-685.

Motoda, S., 1999. Purification and characterization of polyphenol oxidase from *Trametes* sp. MS39401. J. Biosci. Bioeng., 87: 137-143.

Ng, T.B. and H.X. Wang, 2004. A homodimeric laccase with unique characteristics from the yellow mushroom *Cantharellus cibarius*. Biochem. Biophys. Res. Commun., 313: 37-41.

Nilsson, I., B. Mattiasson, M.S.T. Rubindamayugi and U. Welander, 2006. Decolorization of synthetic and real textile wastewater by the use of white rot-fungi. Enzyme Microbiol. Technol., 38: 94-100.

Palonen, H., M. Saloheimo, L. Viikari and K. Kruus, 2003. Purification, characterization and sequence analysis of a laccase from the Ascomycetes *Mauginiella*. Enzyme Microbiol. Technol., 33: 854-862.

Pickard, M.A., R. Roman, R. Tinoco and R. Vazquez-Duhalt, 1999. Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Applied Environ. Microbiol., 65: 3805-3809.

Pierce, 2003. Protein Stability and Storage. 1st Edn., Technical Resource Pierce Biotechnology INC., USA., pp: 6.

Record, E., P.J. Punt, M. Chamkha, M. Labat, C.A.M.J.J. Van Den Hondel and M. Asther, 2002. Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme. Eur. J. Biochem., 369: 602-609.

Robles, A., R. Lucas, M. Martinez-Canamero, N.B. Omar, R. Perez and A. Galvez, 2002. Characterization of laccase activity produced by the hyphomycete *Chalara* (syn. *Thielaviopsis*) *paradoxa* CH32. Enzyme Microbiol. Technol., 31: 516-522.

Rodakiewicz-Nowak, J., S.M. Kasture, B. Dudek and L. Aber, 2000. Effect of various water-miscible solvents on enzymatic activity of fungal laccases. J. Mol. Cataly. B. Enzymatic, 11: 1-11.

Ryan, S., W. Schnitzhofer, T. Tzanov, A. Cavaco-Paulo and G.M. Gubitz, 2003. An acid stable laccase from *Sclerotium rolfsii* with potential for wool dye decolourization. Enzyme Microbiol. Technol., 33: 766-774.

Schneider, P., M.B. Caspern, K. Mondorf, T. Halkier, L.K. Skov, P.R. Ostergaard, K.M. Brown, S.H. Brown and F. Xu, 1999. Characterization of a *Coprinus cinereus* laccase. Enzyme Microbiol. Technol., 25: 502-508.

Zhang, M., F. Wu, Z. Wei, Y. Xiao and W. Gong, 2005. Characterization and decolorization ability of a laccase from *Panus rudis*. Enzyme Microbiol. Technol., 39: 92-97.

Zouari-Mechichi, H., T. Mechichi, A. Dhouib, S. Sayadi, A.T. Martinez and M.J. Martinez, 2006. Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: Decolorization of textile dyes by the purified enzyme. Enzyme Microbiol. Technol., 39: 141-148.