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Biodegradation of Polycyclic Aromatic Hydrocarbons by Immobilized *Pycnoporus sanguineus* on Ecomat

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Abstract: This study was aimed to evaluate the advantages of immobilization of *Pycnoporus sanguineus* on Ecomat for mycelial biomass yield, laccase production and hence, polycyclic aromatic hydrocarbons (PAHs) degradation. The addition of polycyclic aromatic hydrocarbons standard mixture increased the mycelial biomass yield of both immobilized and free mycelia of *P. sanguineus* with the highest dry weight obtained at 10 days of incubation of 0.46 and 1.88 g, respectively. The immobilized mycelia culture degraded 88% of phenanthrene, 93% of anthracene and 85% of pyrene within 20 days. In comparison, free mycelia culture rapidly degraded 42% of phenanthrene, 92% of anthracene and 87% of pyrene at the end of 20 days incubation period. The high correlation between the amounts of polycyclic aromatic hydrocarbons degraded and laccase activity of both immobilized and free mycelia culture indicated possible involvement of laccase in polycyclic aromatic hydrocarbons degradation.

Key words: Mycelia culture, free mycelia, laccase, PAHs degradation

INTRODUCTION

Ligninolytic fungi which, colonizes wood and other ligninocellulosic materials, are abundant in nature and have gained considerable attention for their bioremediation potential. The enzymes that are involved in lignin breakdown can also degrade a wide range of pollutants (Pointing, 2001). Ligninolytic fungi which includes *Phanerochaete chrysosporium* (Brodkorb and Legge, 1992), *Trametes versicolor* (Field *et al*., 1992) and *Pleurotus ostreatus* (Bezalel *et al*., 1996) have been extensively studied for their ability to degrade PAHs. These species are producers of ligninolytic enzymes such as Lignin Peroxidase (LiP), manganese-dependent peroxidase (MnP) and manganese-independent peroxidase (Brodkorb and Legge, 1992; Pickard *et al*., 1999). These extracellular enzymes have low substrate specificity and diffuse into the soil matrix where the polycyclic aromatic hydrocarbons (PAHs) are entrapped. Laccase (benzenediol: oxygen oxidoreductase; *p*-diphenol oxidase, urishiol oxidase) are polyphenol oxidases that catalyzes the oxidation of phenolic compounds with a concomitant reduction of oxygen to water. Due to the remarkable high oxidative ability, the basidiomycete, *Pycnoporus sanguineus*, an excellent producer of laccase in submerged liquid culture (Pointing *et al*., 2000) and solid substrate fermentation (Vikineswary *et al*., 2006) is a potential candidate for bioremediation of PAHs.

Mycelia immobilization has been used for the production of ligninolytic enzymes and bioremediation of pollutants such as chlorophenols (Sedarati *et al*., 2003) and textile dyes (Ziegenhagen and Hofrichter, 2000). To our knowledge, it has not been applied to the biodegradation of PAHs. Thus, the aims of this study were to evaluate the advantages of Ecomat immobilized *Pycnoporus sanguineus* mycelia for laccase productivity and PAHs degradation compared to free mycelia culture.

MATERIALS AND METHODS

The study of the effect of *P. sanguineus* immobilization on mycelial biomass yield, laccase production and PAHs degradation was conducted in 2003.

Fungus: *Pycnoporus sanguineus* (Linn. ex Fr.) Murrill strain CY788 provided by Prof. E.B.G. Jones (National Center for Genetic Engineering and Biotechnology, Thailand) was maintained on potato dextrose agar plates (PDA) at 29±2°C for seven days. The fungus was grown in growth medium, GYMP medium (glucose-yeast-malt-peptone) at pH 7.2, containing, gram per litre, $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.46; K_2HPO_4, 1.0; glucose, 20.0; peptone, 2.0; yeast extract, 2.0 and malt extract, 2.0.

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Immobilization matrix: The ligninocellulose fibres of empty fruit bunches from oil palm, Ecomat, was obtained from Ecofibre Technology, Malaysia. Ecomat was cut into 1 cm^3 cubes, oven dried overnight at 65°C and cooled in the desiccator before the dry weight was taken. Three cubes each of similar weight (ca 2.0-2.5 g) were added into each 250 mL Erlenmeyer flask.

Immobilization procedure: The mycelial suspension of 7 days old *P. sanguineus* culture was inoculated into 100 mL GYMP medium containing three cubes of Ecomat in 250 mL Erlenmeyer flasks. Immobilization of *P. sanguineus* on the Ecomat was carried out for three days at 29±2°C in a rotary shaker at 150 rpm. The free mycelial culture was prepared by inoculating seven days old mycelial suspension of *P. sanguineus* into 100 mL GYMP in 250 mL Erlenmeyer flasks and incubated for three days at 29±2°C in a rotary shaker at 150 rpm.

Incubation of immobilized and free mycelia with PAHs: The three-days old immobilized and free mycelia of *P. sanguineus* cultures were then transferred to GYMP medium containing PAHs (anthracene, phenanthrene and pyrene) each at 10 ppm final concentrations. GYMP medium (100 mL) and GYMP medium (100 mL) containing Ecomat spiked with 10 ppm of PAHs mix standard were prepared as controls. Both free and immobilized cultures and controls were then incubated at 29±2°C and agitated at 150 rpm in complete darkness for 20 days.

Effect of PAHs on the mycelial growth of *P. sanguineus*: Dry weight of free mycelia was estimated by filtering cultures through pre-weighed oven-dried Whatman filter paper (No. 1), rinsed thoroughly with distilled water and dried at 65°C until constant weight (Morgan *et al*., 1991). The fungal biomass of immobilized mycelia was estimated by subtracting the initial weight of dried Ecomat from the colonized material. The initial mycelial dry weight of free mycelia and immobilized mycelia were determined at 2, 5, 7, 10, 15 and 20 days which were utilized for PAHs degradation.

Laccase activity during mycelia growth in the growth medium: Laccase activity in the growth medium of immobilized and free mycelia cultures were assayed at regular intervals. Laccase activity was measured spectrophotometrically at 525 nm (Harkin and Obst, 1973). One unit (U) of enzyme activity was defined as the amount of enzyme producing one OD unit in one milliliter of the growth medium after 1 min.

Optimization of mobile phase for detection of PAHs: PAHs were analyzed using a Spectra-Physics HPLC system equipped with a pumping station, a Spectra-Physics UV2000 detector and integrated with a Data Jet integrator. Determination of PAHs using HPLC was modified by Field *et al.* (1992). The column (150×4.6 mm ID) packed with Prosphere 300A PAH (5 μm particles) coupled with guard column was used for PAHs separation. The flow rate was set at 1.5 mL min^{-1}. The compositions of elution solvents (acetonitrile and water) as mobile phase was determined for optimized separation of each PAH compound in PAHs mix standard. The compositions of water and acetonitrile tested were 20:80 (v/v%), 25:75 (v/v%), 38:62 (v/v%) and 40:60 (v/v%), respectively, The selection for optimized mobile phase was based on the maximum PAHs separation and optimum peak height response. The PAHs were detected at 254 nm using UV detector. Precision of detection method using HPLC was evaluated by injecting 20 μL aliquots of PAHs mix standard having concentrations of 10 ppm of PAHs mix standard over a one-month period.

Extraction and analysis of PAHs: PAHs were extracted from the growth medium and analyzed at days 2, 5, 7, 10, 15 and 20. Residual PAHs in the medium and adsorbed to both the free mycelia and immobilized mycelia were extracted with 20 mL of dichloromethane, shaken vigorously for five minutes and evaporated to dryness. The dried extracts were then re-dissolved in 1 mL of acetonitrile and analyzed. Twenty microliter of samples were injected into the injector and allowed to run for 12 min. Samples were eluted isocratically with an eluent consisting 38/62 (v/v%) water/acetonitrile. The PAHs were detected at 254 nm with a UV detector. The amount of PAHs degraded was determined relative to the controls as described as follow:

$$\text{PAHs degraded (\%)} = \frac{[\text{PAHs (control)} - \text{PAHs (absorbed)} - \text{PAHS (medium)}]}{\text{PAHs (control)}} \times 100\%$$

Statistical analysis: Mean values (triplicates) of the laccase activity in growth medium from both free and immobilized mycelial culture were obtained and subjected to one-way analysis of variance (ANOVA). The mean values were tested for significance using the multiple range tests at 95% at Least Significant Difference (LSD). The least significant difference analysis was conducted to detect any significant difference in laccase activity of *P. sanguineus* in the presence of PAH compounds.

RESULTS AND DISCUSSION

Effect of PAHs on the mycelial growth of *Pycnoporus sanguineus*: *Pycnoporus sanguineus* free mycelia exposed to PAHS showed poor growth compared to immobilized culture during the whole period of incubation (Fig. 1). The initial fungal biomass at day two was 0.4 g

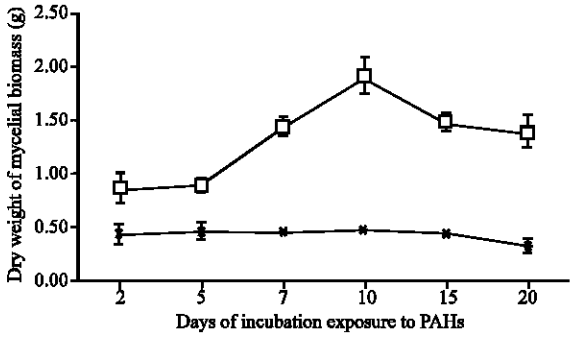

Fig. 1: Dry weight increase of mycelial biomass of free mycelia (-×-) and immobilized mycelia (□) in growth medium artificially contaminated with 10 mg L^{-1} PAHs. Results are expressed as the mean and standard deviation of triplicate values, n = 3

and steadily increased up to 15% (0.46 g) on day 10 of incubation. The fungal biomass, then decreased by 31% (0.33 g) at the cessation of incubation. Immobilized mycelia, however, showed good growth compared to free mycelia throughout the whole incubation period (Fig. 1). Dry weight of mycelial biomass of 0.83 g was obtained on day two of incubation, approximately two fold higher compared to free mycelia culture. Rapid growth was recorded from day five of incubation and peaked at day 10 of incubation. Maximum mycelial biomass of 1.88 g was obtained on day 10, thereafter, a reduction in mycelial biomass occurred with a reduction of about 20% of its maximum biomass by day 20 of incubation. The dry weight increase of fungal biomass in immobilized mycelia was over four folds higher than free mycelia at day 10 of incubation. There were advantages of selecting natural materials as support for immobilization of mycelia as these were compostable and inexpensive. Ecomat is a waste product of palm oil industry, whereby Ecomat could be composted once they are no longer useful as an immobilization support (Shin *et al*., 2002). Overall, the immobilized mycelia of *P. sanguineus* showed better tolerance towards PAHs than the free mycelia. Comparatively, higher biomass growth and laccase activity was obtained in immobilized mycelia when exposed to 10 ppm of PAHs. Immobilization of *P. sanguineus* on Ecomat, a natural support material, supported higher mycelial growth of over four folds compared to the free mycelia culture. Ecomat made from 100% natural oil palm residues contained a higher proportion of easily accessible and biodegradable plant material such as cellulose or hemicellulose. Therefore, Ecomat provides exogenous nutrients (carbon source from the breakdown of cellulose and lignin) to encourage fungal biomass proliferation.

Table 1: Comparison of laccase activity of *P. sanguineus* immobilized and free mycelia

	Laccase activity* (U mL^{-1})	
Incubation (days)	Immobilized mycelia	Free mycelia
2	0.318±0.044[a]	0.092±0.008[a]
5	1.387±0.083[a]	0.117±0.006[b]
7	1.483±0.045[a]	0.650±0.130[b]
10	1.453±0.076[a]	1.032±0.074[b]
15	1.613±0.075[a]	1.132±0.107[b]
20	1.528±0.025[a]	1.007±0.008[b]

±Refers to standard deviation; results are expressed as the mean of triplicate values, n = 3 *Same letter from the same row denotes statistically not significant ($p>0.05$)

Laccase activity of immobilized and free mycelia culture: Extracellular laccase was monitored when both immobilized and free mycelia of *P. sanguineus* were incubated in the growth medium supplemented with 10 ppm of PAHs (Table 1). Laccase activity of both immobilized and free mycelia culture were detected on day two and reached a maximum on day 15. There was a rapid increase in laccase activity observed in the *P. sanguineus* free mycelia throughout the whole incubation and reached its maximum activity of 1.13 U mL^{-1} at day 15 of incubation. However, *P. sanguineus* supported on Ecomat proved to produce significantly higher ($p<0.05$) laccase activity than free mycelia. This implies the advantages shown in the utilization of Ecomat to facilitate fungal colonization and enhance laccase activity in the presence of PAHs. Furthermore, this simplicity of the surface immobilization of *P. sanguineus* on Ecomat provides a potential method for a bioreactor design. In addition, immobilized culture on Ecomat was able to sustain high laccase activity for longer incubation period compared to free mycelia culture. This could be due to the ability of *P. sanguineus* to metabolize the lignocellulose components in Ecomat, making the whole process much more economical. Comparatively, the maximum laccase activity of immobilized mycelia of *P. sanguineus* on Ecomat was 2.0 and 2.9 folds higher than other species of white rot fungi cultivated in submerged liquid culture such as *Trametes* sp. (Rodriguez Cuoto *et al*., 2004) and *Pleurotus ostreatus* (Medeiros *et al*., 1999), respectively, Thus, this revealed the high potential of Ecomat to improve laccase production by *P. sanguineus*.

Analysis of PAHs: The chromatogram shown in Fig. 2a-c shows the resolution of 10 ppm PAHs mix standard eluted at 38% water and 62% acetonitrile (v/v).

The aim of this study was to determine the optimized chromatographic conditions for optimized separation or referred as resolution of individual PAH compound. It was found that highest peak height response was

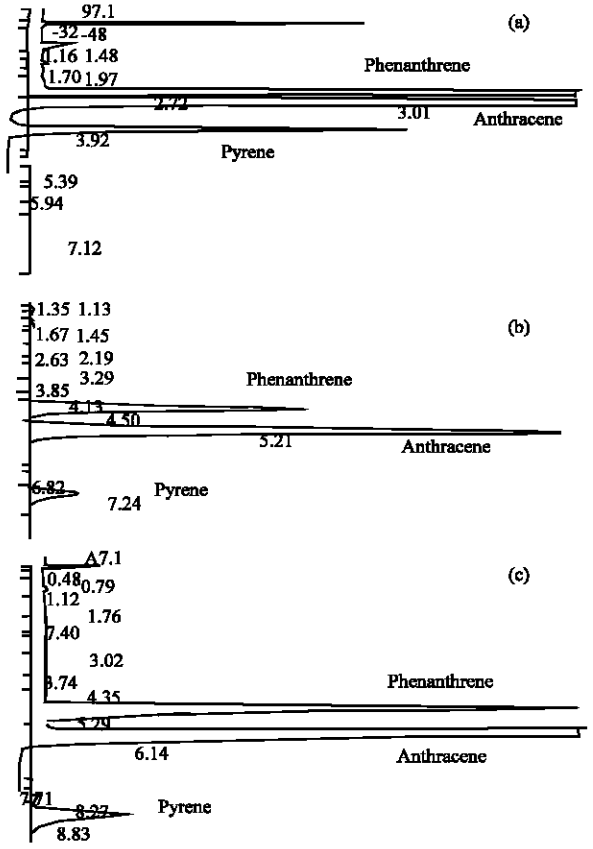

Fig. 2: Resolution of phenanthrene, anthracene and pyrene in a standard mixture at 10 ppm eluted by water and acetonitrile at ratio (a) 25:75 (%v/v); (b) 38:62 (%v/v) and (c) 40:60 (%v/v)

obtained for each PAH where 75% of acetonitrile (v/v) was employed (Fig. 2a). However, baseline separation of phenanthrene, anthracene and pyrene in PAHs mix standard was not achieved. At 62% of acetonitrile, higher resolution of PAH compounds was observed while maintaining considerably high sensitivity of peak height detection (Fig. 2b). Lowering acetonitrile to 60% in the mobile phase compositions showed better resolution of the PAH compounds but the peak height response of each PAH was found to be reduced by 40% water and 60% acetonitrile (Fig. 2c). Thus, the optimized mobile phase composition, which consisted 38% water and 62% acetonitrile (v/v) was employed in this study for the analysis of PAHs. The analysis of phenanthrene, anthracene and pyrene in the mix standard mix was completed in 12 min at the flow-rate of 1.5 mL min. Baseline resolution was obtained between all PAH compounds indicating that contaminants were not present in the standard solution. Phenanthrene was detected at retention time of 4.52±0.06 min, while

Table 2: Retention times and peak height reproducibility for phenanthrene, anthracene and pyrene by HPLC with coefficient of variation

	Statistics			
PAHs	Retention time (min)	Coefficient of variation (%)	Peak height (AU)	Coefficient of variation (%)
Phenanthrene	4.57±0.09	1.97	230309±12183	5.29
Anthracene	5.30±0.12	2.26	444963±21124	4.75
Pyrene	7.39±0.17	2.30	41458±2119	5.11

Mean value of retention time with standard deviation based on ten successive injection, (n = 10) of 20 μL aliquots of mixed PAHs standard

anthracene was detected at an average retention time of 5.23±0.08 min. The average retention time for pyrene was obtained at 7.29±0.13 min. In recent years, the HPLC method has been used for determination of PAHs in food (Simko, 2002), water (Pino *et al*., 2002; Chen *et al*., 2004) and soils or sediments (Song *et al*., 2002). According to Simko (2002), HPLC has advantages compared to gas chromatography in PAH analysis such as good resolution of PAH compounds with high sensitivity and specificity under ultraviolet and fluorescence detection and analysis are usually carried out at ambient temperature. The composition of water and acetonitrile of 38:62 (% v/v) used in isocratic condition was able to separate phenanthrene, anthracene and pyrene in mixture standard solution with sufficient sensitivity using ultraviolet detection.

Reproducibility of the retention times and peak height measurements was good as shown in Table 2. The random error of retention times for phenanthrene, anthracene and pyrene estimated from the calculation of the coefficient variation were 1.97, 2.26 and 2.30%, respectively.

The random error of retention times for phenanthrene, anthracene and pyrene estimated from the calculation of the coefficient variation were 1.97, 2.26 and 2.30%, respective likewise, reproducibility of the peak height measurements was good for phenanthrene, anthracene and pyrene, with coefficients of variation of 5.29, 4.75 and 5.11%, respectively, Thus, it was found that the combined errors of HPLC resolution, injection and detection were minimal. In addition, the reproducibility of the peak heights achieved in every injection for a period of one month indicated that the PAH compounds dissolved in acetonitrile were stable. Hence, storage of PAHs standard solution dissolved in acetonitrile was found to be stable over a long period of time. In this, study, good resolution and high reproducibility of PAHs detection were achieved using the chromatographic method employed. Therefore, there is no risk of thermal decomposition of analytes. High peak height reproducibility of PAHs showed that PAH compounds were not degraded during the analysis at ambient temperature. Likewise, high reproducibility of both retention times and peak height responses observed after

repeated injections of PAHs standards solutions also indicated no change in the PAH column efficiency.

Biodegradation of PAHs by *Pycnoporus sanguineus*: The biodegradation of 10 ppm phenanthrene, anthracene and pyrene by free mycelia and immobilized mycelia of *P. sanguineus* was monitored for 20 days (Fig. 3). It was found that phenanthrene, anthracene and pyrene were efficiently degraded by free mycelia, with relative removals of over 13, 64 and 58%, to give final concentrations of 8.62, 3.55 and 4.15 ppm, respectively, compared to control after two days of incubation. Anthracene was degraded rapidly by the free mycelia culture of *P. sanguineus* with relative removal of over 92%, closely followed by pyrene and phenanthrene, with relative removal of over 87 and 42%, respectively, compared to control within 20 days of incubation. Their corresponding final concentrations were 5.74, 0.74 and 1.28 ppm for phenanthrene, anthracene and pyrene, respectively, at the cessation of incubation. Concomitant to the rapid PAHs degradation, laccase activity in free mycelia culture increased proportionately during the whole incubation period.

The immobilized *P. sanguineus* culture proved to be more efficient in PAHs degradation as the degradation rate was more rapid compared to free mycelia culture. The PAHs degraded by immobilized culture on day two was over 65, 89 and 80%, with corresponding concentrations of 6.57, 8.91 and 8.03 ppm for phenanthrene, anthracene and pyrene, respectively. At the cessation of the incubation, only 11.7, 6.3 and 14.9% of phenanthrene, anthracene and pyrene, respectively was recovered from the culture medium. Their corresponding final concentrations were 0.12, 0.06 and 0.15 ppm for phenanthrene, anthracene and pyrene, respectively Initially, removal of PAHs from the liquid culture was more rapid in the immobilized mycelia culture compared to the free mycelia culture. Degradation efficiency of phenanthrene, anthracene and pyrene in immobilized culture was 4.8, 1.4 and 1.4 folds higher than the free mycelia culture on day 2 of incubation respectively. Subsequently, degradation efficiency of PAHs was reduced in the immobilized mycelia culture. Degradation efficiency of phenanthrene in immobilized mycelia culture was over 2 folds higher than the free mycelia culture at the cessation of incubation. Degradation efficiency of anthracene was comparable between immobilized mycelia and free mycelia cultures of *P. sanguineus* at the end of the incubation. Degradation efficiency of pyrene in immobilized mycelia culture was 2.4% lower than the free mycelia culture at the cessation of incubation.

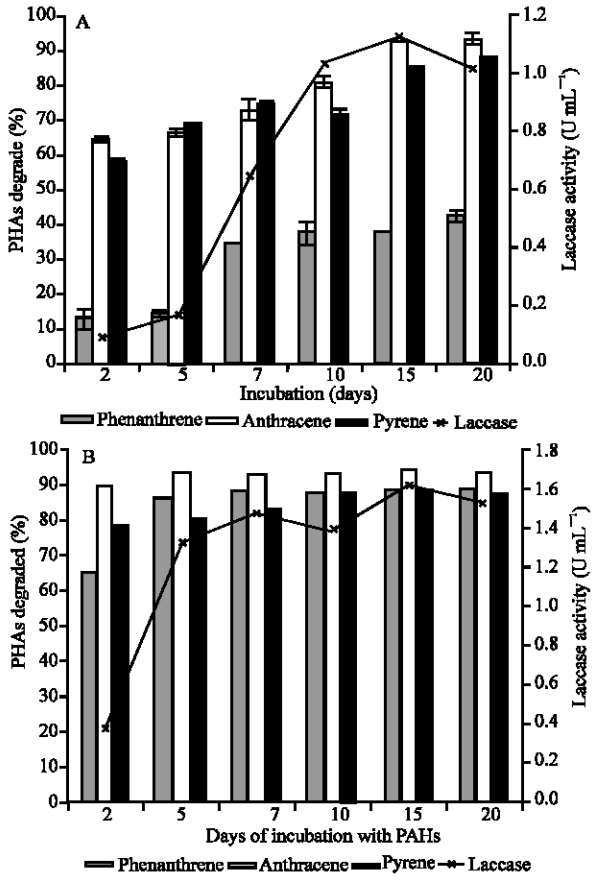

Fig. 3: Percentage of PAHs degraded and laccase activity of *P. sanguineus* (A) free mycelia and (B) immobilized mycelia during 20 days of incubation. Results are expressed as the mean and standard deviation of triplicate values

The removal of PAHs in the incubation medium was due to the enzymatic degradation of PAHs. The amount of PAHs adsorbed to the mycelial biomass was found to be insignificant ($p>0.05$). PAHs extracted from the mycelial biomass showed that minimal amount of phenanthrene, anthracene and pyrene was adsorbed to the mycelial biomass in both immobilized and free mycelia culture.

The correlation coefficient (R^2) of 0.87 and 0.99 between degradation of these PAHs by free mycelia and immobilized mycelia, respectively, and laccase activity indicated that the increase in PAHs degradation was proportionate with the increase in laccase activity of *P. sanguineus* (Fig. 4). Therefore, laccase produced by *P. sanguineus* may be responsible for degradation of the PAH compounds.

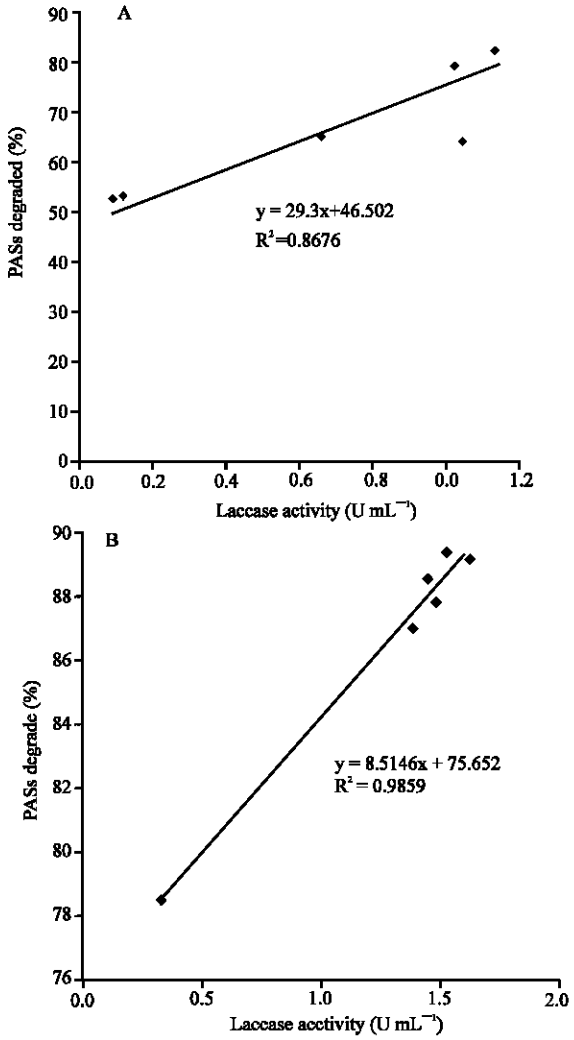

Fig. 4: Correlation between PAHs degradation and laccase production by (A) free mycelia and (B) immobilized mycelia of *P. sanguineus*. The correlation is based on the correlation coefficient (R^2)

The degradation of PAHs was rapid while laccase activity of the free mycelia culture was found to be low in the early stage of incubation. However, the capacity of *P. sanguineus* immobilized on Ecomat to remove anthracene, phenanthrene and pyrene exceeded the efficiency of the free mycelia culture. The rapid degradation of these compounds correlated with the increasing laccase activity during the whole incubation period. The direct involvement of laccase in PAHs degradation was further confirmed by the high correlation coefficients between the degree of PAHs degradation and laccase activity. Gramss *et al.* (1999) had reported PAHs degradation was correlated to laccase activity in wood-degrading fungi with correlation coefficient (R^2) equivalent to 0.85. Thus, laccase proved to be directly involved in PAHs degradation. According to Gramss *et al.* (1999), they had tested 58 fungal isolates from different physio-ecological groups and found these basidiomycetes were able to remove 40-60% of phenanthrene, 72-82% of anthracene and 58-70% of pyrene in a mixture of five PAHs (total 10 ppm PAHs) in 14 days of incubation. In this study, *P. sanguineus* in both immobilized and free mycelia culture were able to remove 51-81% of phenanthrene, 93-94% of anthracene and 83-85% of pyrene in 15 days of incubation. Therefore, *P. sanguineus* tested in this study was found to be an efficient PAHs degrader compared to some wood-degrading fungi that included *Bjerkandera adusta*, *Hypholoma frowardii* and *Pleurotus ostreatus* reported by Gramss *et al.* (1999). Furthermore, Gramss *et al.* (1999) found the role of lignin-modifying enzymes (LMEs) which include laccase was correlated to PAHs degradation. In this study, laccase was the dominant enzyme detected throughout the incubation period (Fig. 2). In addition, Pointing *et al.* (2000) had reported that *P. sanguineus* was an excellent producer of laccase in submerged liquid culture and solid substrate fermentation. In their study, it was found that laccase was the dominant enzyme produced by *P. sanguineus*. Therefore, the possible role of laccase in PAHs degradation is illustrated in this study.

The degradation of each PAH by *P. sanguineus* in both free mycelia and immobilized culture in descending order was anthracene > pyrene > phenanthrene. Although phenanthrene and anthracene shared the same molecular weight (178.23 g mol^{-1}), the structure of phenanthrene is more compact and angular compared to anthracene. This may be a contributory factor in its higher resistance to fungal enzyme attack. Furthermore, the ionization potential (IP) of anthracene (7.44 eV) is lower than phenanthrene (7.90 eV), indicating its higher susceptibility to enzymatic reactions. Pyrene, on the other hand, was readily degraded by *P. sanguineus* free mycelia culture and immobilized culture compared to phenanthrene during the 20 days incubation period. The IP of pyrene (7.42 eV) was lower than both anthracene (7.44 eV) and phenanthrene (7.90 eV), which renders it liable to attack by ligninolytic enzymes (Dasbetani and Ivanov, 1999). Collins *et al.* (1996) had reported on laccases of *Trametes versicolor*, which were able to oxidize PAHs with IP lower than 7.45 eV. Thus, this study proved the direct involvement and role of laccase produced by *P. sanguineus* for rapid degradation of anthracene, phenanthrene and pyrene.

CONCLUSIONS

In recent years, the widespread distribution of PAHs in the environment has caused considerable and worldwide concern. The rapid extension and national development in many countries had contributed to the PAHs pollution. In addition, the increasing cases of forest fires and emission through automobile became the significant sources of atmospheric and sedimentary hydrocarbons. Thus, in order to minimize the negative impact on environment, utilization of microorganisms to bioremediate PAHs have received significant attention in recent years.

In the present study in laboratory scale, sub-tropical basidiomycete, *Pycnoporus sanguineus* was an efficient laccase producer and PAHs degrader. Further, immobilization of *P. sanguineus* on a lignocellulolytic material, Ecomat enhanced biomass productivity and sustained high laccase activity in prolonged incubation period, hence, degraded over 85% PAHs in 20 days of incubation. Thus, the future prospects for large-scale laccase production and PAHs degradation by immobilized *P. sanguineus* need to be studied with a multi dimensional approach. Further studies on PAHs degradation pathways by laccase are important to understand the breakdown metabolites and their toxicity potentials.

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