Comparison of phenanthrene removal by Aspergillus niger ATC 16404 (filamentous fungi) and Pseudomonas putida KT2442 (bacteria) in enriched nutrient-liquid medium

N Hamzah ¹, N A F M Kamil ², N Singhal ³, L Padhye ³, S Swift ⁴
¹ Department of Water Resource and Environmental System, Faculty of Civil Engineering, Universiti Teknologi MARA, Selangor, Malaysia
² Faculty of Civil and Environmental Engineering, Universiti Tun Hussein Onn Malaysia, 86400 Batu Pahat, Johor, Malaysia
³ Department of Environmental Engineering, Faculty of Civil and Environmental Engineering, University of Auckland, Auckland, New Zealand
⁴ Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand

E-mail: nurhidayah0527@salam.uitm.edu.my

Abstract: Polycyclic Aromatic Hydrocarbons (PAHs) is one of the persistent and carcinogenic pollutants that needs to be eliminated from the environment. The study on degradation of PAHs by bacteria is thoroughly discussed in literature. Many strains of bacteria were chosen in order to eliminate the PAHs compound in the environment. However, there are less study on the filamentous fungi although fungi appears to be an abundant population and as dominant group in PAHs contaminated soil habitats [1], [2]. This study was conducted to determine and compare the Phenanthrene (PHE) removal by fungi and bacteria in excessive nutrient-liquid culture. Then, the survival for both strains was investigated in the presence of PHE and finally, the analysis on the fungi-PHE interaction was carried out. In condition of excessive nutrient, the removal of PHE was evaluated for fungi and bacteria in batch experiment for 5 days. PHE removal for A. niger and P. putida were found to be 97% and 20% respectively after 5 days. The presence of PHE was negatively inhibits the grow of the bacteria and the fungus. The PHE uptake mechanism for A. niger was observed to be a passive transport mechanism with 45 µg per g fungus dry weight within 24 hr of incubation. As a conclusion, filamentous fungi have the potent role in the removal of PHE as well as bacteria but depending on the strains and the condition of the environment. Fungi is known to co-metabolize the PHE meanwhile, PHE can be used as sole carbon for bacteria. This preliminary result is significant in understanding the bacteria-fungi-PHE interaction to enhance the degradation of PAHs for co-culture study in the future.

1. Introduction
Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) by using bacteria has been widely studied [3], [4]. It is broadly accepted that the type of bacteria plays an important role in degrading this pollutant especially in soil. Studies show that the degradation of PAHs will be longer in soil compare to aqueous. This is because; the PAHs are not directly bioavailable to bacteria as PAHs tend to adsorb on soil particles whereby bacteria prefer to be in aqueous phase [5]. Besides bacteria, fungi also share same habitats in most hydrocarbon contaminated sites. Therefore, fungi are able to degrade the pollutants as effective as bacteria and unlike bacteria, fungi are more tolerant to the toxic compound like PAHs.
Fungi use their extracellular and intracellular enzymes to metabolize pollutants depending on the type of fungi and which part of their body interacts with the pollutants. Incubation with mycelia as a PAHs degrader will increase the degradation rate as compared to spore inoculation. This may be due to high surface area and high production of degradation enzymes [6], [7]. Besides, mycelium is also proven to degrade both hydrophilic and hydrophobic pollutants [8].

The aim of this study is to evaluate the removal of Phenanthrene (PHE) by fungus strain (Aspergillus niger ATC 16404) and bacteria strain (Pseudomonas putida KT2442), to determine the growth rate of both strains during the removal study and finally analyse the interaction between fungi and PHE. Pseudomonas sp are considered as hydrophilic strains and use their flagella to move in aqueous solution. They are also known as naphthalene, phenantherene and anthracene degrader [9]. Hydrophobic Aspergillus niger is the most popular fungus in any studies involving fungi and the most commonly found fungus in soil site [10], [11], wastewater [12] and drinking water [13]. This is due to the fact that, this fungus is able to produce the glucoamylase (GLA) enzyme to produce glucose and fructose syrup which is excessively used in food industry [14].

2. Materials and Methods

2.1 PHE Standard Solution Preparation

In this research, phenanthrene which was purchased from Sigma-Aldrich, Germany, was used throughout the experimental work as a target pollutant. Before any analysis can be made, a standard curve needs to be plotted using the following concentrations: 100, 80, 60, 20 and 10 ppm respectively. 100 mg of phenanthrene will be dissolved in 1000 ml of acetone to make final concentration of 100 ppm standard stock before diluted to 80, 60, 20 and 10 ppm concentration to obtain a standard curve for HPLC analysis.

2.2 Bacteria preparation

A loopful Pseudomonas putida KT2442 which has been coloured by green fluorescense protein (gfp) was taken out from the -80°C seed stock vial and was plated onto R2A agar. The agar plate was stored in a refrigerator in a laboratory until it is to be used. A single colony from the plate then was streaked to other fresh R2A agar plate and kept for at least 3 weeks. In order to prepare bacterial culture for experiment, a colony of P.putida KT224 was taken out from the agar plate using a sterilized 1µL inoculating loop and placed in 10 ml of aqueous Bushnell Hass (BH) broth with 10 % glucose (w/v) as the sole carbon source in a capped flask. 1 ml of the broth culture was transferred to 100 ml of fresh BH broth with 10% glucose in a flask. The bacteria was consequently grown in 28°C incubator (Innova 4330, New Brunswick Scientific) at 200 rpm for 24 hours. The culture was grown in a shaker incubator at 200 rpm and 28°C for 12 hours until the stationary phase was reached. The stationary phase of P.putida KT2442 was previously determined by previous student.

2.3 Fungi Preparation

Aspergillus niger was provided from PC2 laboratory at Faculty of Medical and Health Science, University of Auckland, New Zealand. This study was conducted entirely in The University of Auckland, New Zealand. 10 ml of saline solution was poured over the A.niger plate and was streaked to lift-off the spores. For re-growth of the mould, 1 ml of fungus spore solution was spread evenly over Potato Dextrose Agar (PDA) plate and incubated at 28°C for 1-3 days for mycelia growth and more than 3 - 4 days for spore. For experiment purpose, 1 ml of the spore solution was transferred into 1ml of saline solution. The solution was then adjusted to an absorbance of 0.9 to 1.0 at 600 nm to get at least 50 × 10^6 – 100 × 10^6 spores/ml.

For producing living mycelia, 5µL of spore suspension was grown in the middle PD agar and incubated at 28°C for 3 days. The mycelium pellet was produced and gently removed from the agar using tweezer. It is important to wash the pellet with Potassium Buffer Saline (PBS) three times before use for the removal of phenanthrene experiments.
2.4 Phenanthrene Removal Study

For phenanthrene removal experiment, stock solution of PHE was prepared and poured into the empty 40 ml amber glass bottle to get the initial concentration of PHE in bottle of 100 ppm. After that 10 ml of sterilised PD broth was used as liquid medium for both strains and poured into the glass bottle. The liquid medium was adjusted to pH 7 as an initial pH. Bacteria suspension and fungus mycelium pellet were inoculated into the mixture individually and the bottle mouth was covered with cotton ball and aluminium foil for air circulation. Mixture of medium without stains and with strains but without PHE were prepared as control. The bottles were then incubated in the incubation shaker (Innova 4330, New Brunswick Scientific), 200 rpm at 28°C for 5 days. All experiments were carried out triplicate and each bottle was sacrificed every day with Na2O2 to stop the removal process. Most importantly, the fungus was then washed with Dichloromethane (DCM) three times to remove PHE adsorption and the wash is pooled with the solution to get total PHE removal.

For determining the PHE removal, the solution was extracted with Dichloromethane (DCM) with equal volume of medium three times (3) and the residue was resuspended with acetonitrile (ACN) before being transferred into vial and analysed using HPLC. The recovery of this extraction method was 90%.

The percentage of PHE removal was calculated using this equation:

$$\frac{C_i - C_o}{C_i} \times 100\%$$

where $C_i$ is initial concentration of PHE in mg/L and $C_o$ is final concentration of PHE in mg/L.

2.5 Bacteria and fungi growth

After each incubation day, 1ml of bacteria suspension was calculated using plate count method. For fungi, by using tweezer, the mycelium was taken out and frozen to dry for 24 hrs before the dry weight was recorded and used for uptake study.

2.6 Phenanthrene uptake by fungi

In order to evaluate the interaction between PHE and fungi, the fungus was treated with sodium azide (NaN₃) to identify the transport of PHE through fungal cell wall and amount of PHE uptake. After 1 hr incubation, the fungus was inoculated to the mixture and went through the process as mention above (Phenanthrene Removal Study). After freeze dried, the mycelium was digested with 15 ml of 5 N NaOH for three days [15]. After the incubation period end, the suspension was extracted three times using DCM and the residue was resuspended with acetonitrile (ACN) before transfer into vial and analyse using HPLC.

3. Result and Discussion

3.1 The removal of Phenanthrene and Bacteria, Fungi Growth

The percentage removal of PHE within 5 days of incubation period was illustrated in Figure 1. From the figure, A.niger shows higher removal compared to P.putida with difference of 79% (P < 0.05). It is known that fungi especially A.niger is able to degrade 70 % of PHE in solid agar medium [16]. The higher removal by A.niger is probably due to the dry weight of mycelium pellet which was increased by 30% at day 1. According to Figure 1 and Figure 2b, there are linear relationship between fungus grow and PHE percentage removal; as the fungus growing, the percentage of removal also increases ($r^2 = 0.908$) (graph not shown). It is known that fungus do not use PHE as sole carbon instead co-metabolize the PHE in the presence of nutrient. The excessive nutrient condition in this study seems to be an advantage condition for A.niger. Unlike fungi, in addition of co-substrate, the removal of PHE by P.putida was decreased after day 3 with reduction of 66% but the bacteria growth was maintained at above 6.7 × 10⁹ cfu/ml. This situation is common in the degradation of PAHs with co-substrate by bacteria. The competition existed between PHE and co-substrate. Therefore, most of the biodegradation study in-situ has failed because in the contaminated site, there would be plenty of nutrients for bacteria to consume as carbon sources which is easier to be accessed and digested compared to PAHs. However, this result indicates the potential of fungi over bacteria in removing PAHs in-situ. Moreover, the high
concentration of PHE (100 ppm) that was used in this study demonstrates the high tolerance of *A. niger* to PHE.

Meanwhile, *A. niger* treated with NaN₃ shows remarkable PHE removal with 84% within 24 hr and 87% after 5 days. Most of fungal biomass is successful to remove dyes and heavy metals in previous study due to high biosorption rate [17], [18].

![Figure 1](image1.png)

**Figure 1.** The percentage removal of PHE by *A. niger* and *P. putida* within 5 days. No significant difference of the PHE removal between *A. niger* with and without NaN₃ (P > 0.05) but there is a significant difference of the PHE removal between *A. niger* and *P. putida* (P < 0.05) Error bars show standard deviation for triplicate sample.

![Figure 2](image2.png)

**Figure 2.** (a) Number of bacteria count without addition of PHE and with PHE for 5 days. With PHE, the growth was increased by 50% at day 1 and maintain linear until 5 days. No significant difference in addition of PHE (P > 0.05) to the growth of bacteria; (b) the growth rate of *A. niger* within 5 days. *A. niger* without PHE was used as control meanwhile *A. niger* was treated with NaN₃ before PHE removal experiment used for fungi-PHE interaction analysis. There is significant difference between 3 groups (P < 0.05). Error bars show standard deviation for triplicate sample.

### 3.2 Interaction of Fungi and PHE

The idea of treating the fungus with NaN₃ was to identify the mechanism of PHE transport into fungal cell. Treatment with NaN₃ was to inhibit the fungi grow by shielding the fungal cell wall membrane with NaN₃ solution from the uptake of nutrient. This can be clearly seen in Figure 2b whereby there was no grow for *A. niger* after treated with NaN₃. However, based on the uptake result (Figure 3), it
shows that the transport of PHE into fungal cell wall is passive transport which means, PHE can easily pass through the fungal cell wall. The uptake of PHE by untreated \textit{A.niger} was increased by 87\% after 5 days of incubation. Meanwhile, for treated \textit{A.niger}, the uptake of PHE was reduced by 33\% after 5 days. The reduction of uptake by treated \textit{A.niger} is probably due to the less amount of PHE remaining in the mixture sample. The amount of PHE uptake was not much as compared to previous study which for \textit{Fusarium solani}, the uptake of PHE was 200 µg/g of fungus [19]. However, that study used fungus biomass whereas in this study, active mycelium pellet was chosen.

Although the fungus did not grow within the incubation periods (Figure 2b), the removal of PHE was high most probably due to the continuous uptake of PHE and the intracellular enzymes which is embedded in the cell that can still degrade the PHE. However, this study did not measure amount of intracellular enzyme and metabolites produced. The uptake of untreated \textit{A.niger} was lower maybe because of the high amount of intracellular enzyme as the fungus grow and immediately degrades the PHE. Treating the fungus with NaN\textsubscript{3} will not be statistically significant in removing the PHE but significant to influence the uptake of PHE.

![Figure 3](image)

\textbf{Figure 3.} This figure shows the concentration of PHE uptake by \textit{A.niger} in unit µg /g fungus dry weight within 5 days. There is a statistical difference of the PHE uptake between the groups (P < 0.05). Error bars show standard deviation for triplicate sample.

\section*{4. Conclusion}

The growth of fungi is important in removing PHE as fungus co-metabolizes the PHE as they grow. However, the situation is not the same for bacteria as the higher amount of bacteria colony do not promise higher removal due to the competition between PHE and co-substrate. This study suggests that \textit{A.niger} is suitable for the removal of PHE in nutrient enriched water/wastewater as compared to \textit{P.putida} owing to their tolerance to PAHs toxicity and adaptation to the environment. The passive transport of PHE through \textit{A.niger} fungal cell wall is beneficial in PAHs bioremediation because PAHs mode of attacked by filamentous fungi is intracellularly which uses the intracellular enzyme to degrade the PAHs. In the future, co-culture between \textit{A.niger} and \textit{P.putida} will be carried out to identify their interactions towards the removal of PHE and other higher molecular ring.
Acknowledgement
Special thanks to Malaysia Ministry of High Education (MoHE) for funding the study under SLAB/SLAI scholarship.

References
[1] Gadd G M 2007 Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation Mycol. Res., vol 111 no 1, pp 3.
[2] Salvo V S, Gallizia L, Moreno M and Fabiano M 2005 Fungal communities in PAH-impacted sediments of Genoa-Voltri Harbour (NW Mediterranean, Italy) Mar. Pollut. Bull., vol 50 no 5 pp 553.
[3] Wu M, Chen L, Tian Y, Ding Y, and a Dick W 2013 Degradation of polycyclic aromatic hydrocarbons by microbial consortia enriched from three soils using two different culture media Environ. Pollut., vol 178 pp 152.
[4] Jimenez-Sanchez C, Wick L Y, Cantos M, and Ortega-Calvo J J 2015 Impact of dissolved organic matter on bacterial tactic motility, attachment, and transport Environ. Sci. Technol., vol 49 no 7 pp 4498.
[5] Johnsen A R, Wick L Y, and Harms H 2005 Principles of microbial PAH-degradation in soil Environ. Pollut. vol 133 no 1 pp 71.
[6] Potin O, Rafin C, and Veignie E 2004 Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil Int. Biodeterior. Biodegradation, vol 54 no 1 pp 45.
[7] Syed K and Yadav J S 2012 P450 monooxygenases (P450ome) of the model white rot fungus Phanerochaete chrysosporium Crit. Rev. Microbiol., vol 38 no 4 pp 339.
[8] Elkær B, Ellegaard-jensen L, Nyrop C, Rosendahl S, and Aamand J 2013 Fungal hyphae stimulate bacterial degradation of Environ. Pollut., vol 181 pp 122.
[9] Kahng H Y, Nam K, Kukor J J, Yoon B J, Lee D H, Oh D C, Kam S K and Oh K H 2002 PAH utilization by Pseudomonas rhodesiae KK1 isolated from a former manufactured-gas plant site Appl. Microbiol. Biotechnol., vol. 60 no 4 pp 475.
[10] Chaîneau C H, Morel J, Dupont J, Bury E and Oudot J 1999 Comparison of the fuel oil biodegradation potential of hydrocarbon-assimilating microorganisms isolated from a temperate agricultural soil Sci. Total Environ., vol 227 no 2–3 pp 237.
[11] Vasco M F, Cepero M C, Restrepo S and Vives-Florez M J 2011 Recovery of mitosporic fungi actively growing in soils after bacterial bioremediation of oily sludge and their potential for removing recalcitrant hydrocarbons Int. Biodeterior. Biodegradation, vol 65 no 4 pp 649.
[12] Siqueira V and Lima N 2012 Surface hydrophobicity of culture and water biofilm of Penicillium spp., Curr. Microbiol., vol 64 no 2 pp 93.
[13] Sauer J, Sigurskjold B W, Christensen U, Frandsen T P, Mirgorodskaya E, Harrison M, Roepstorff P and Svensson B 2000 Glucoamylase: structure/function relationships, and protein engineering. Biochim. Biophys. Acta, vol 1543 no 2 pp 275.
[14] Verdin A, Louné-Hadj Sahraoui A, Newsam R, Robinson G and Durand R 2005 Polycyclic aromatic hydrocarbons storage by Fusarium solani in intracellular lipid vesicles. Environ. Pollut., vol 133 no 2 pp 283.
[16] Cortés-Espinosa D V, Fernández-Perrino F J, Arana-Cuenca A, Esparza-García F, Loera O and Rodríguez-Vázquez R 2006 Selection and identification of fungi isolated from sugarcane bagasse and their application for phenanthrene removal from soil. *J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng.*, vol 41 no 3 pp 475.

[17] Maurya N S, A. K. Mittal, Cornel P and Rother E 2006 Biosorption of dyes using dead macro fungi: Effect of dye structure, ionic strength and pH *Bioresour. Technol.*, vol 97 pp 512.

[18] Chaudhry M T and Zohaib M 2014 Biosorption characteristics of Aspergillus fumigatus for the decolorization of triphenylmethane dye acid violet 49 pp 3133.

[19] Fayeulle A, Veignie E, Slomianny C, Dewailly E, Munch J C and Rafin C 2014 Energy-dependent uptake of benzo[a]pyrene and its cytoskeleton-dependent intracellular transport by the telluric fungus Fusarium solani. *Environ. Sci. Pollut. Res. Int.*, vol 21 no 5 pp 3515.