Degradation of low molecular weight polycyclic aromatic hydrocarbons by microorganisms isolated from contaminated soil

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ABSTRACT

Degradation of low molecular weight PAHs by two bacterial species Micrococcus luteus and Kocuria rosea has been reported for the first time in the present study. Three fungal species of Aspergillus were also studied for their PAH-degradation potential. The microorganisms were isolated from oil contaminated soil and adapted with an exposure to high dose of PAH compounds for one year under laboratory conditions. The efficiency towards biodegradation increased from Kocuria rosea (55.4%) to Aspergillus ficuum (57.2%) to Aspergillus flavus (63.2%) to Micrococcus luteus (63.6%) to Aspergillus fumigatus (63.9%). Maximum degradation was observed for naphthalene followed by phenanthrene, fluoranthene, and pyrene for bacteria. M. luteus could degrade the compounds at a faster rate than K. rosea. A. flavus and A. fumigatus strains were better adapted to degradation. On the other hand, the removal efficiency of A. ficuum was considerably less, but it could remove fluoranthene efficiently. Observed trend of PAH biodegradation may be a function of melting point, aqueous solubility, structure of the PAH compound, and microbial growth. The microorganisms reported in the study can prove useful in remediation of PAH contaminated sites and may find an application in the design of novel bioremediation protocols.

Keywords: Biodegradation, PAHs, Biodegradation, Aspergillus, Micrococcus, Kocuria rosea.

1. Introduction

Incomplete combustion at high temperature (500-800°C) or subjection of organic material at low temperature (100-300°C) for long periods results in production of polycyclic aromatic hydrocarbons (PAHs). Low molecular weight PAHs (MW<250) occur in vapour phase and higher molecular weight PAHs remain associated with the particulate phase. Their fate in environment includes volatilization, photo-oxidation, chemical oxidation, adsorption on particulate (Kaushik et al. 2012), leaching and microbial degradation (Wild and Jones 1995). The hazards associated with the PAHs can be overcome by the use of conventional methods which involve removal, alteration, incineration or containment. These technologies are expensive, and in many cases transfer the pollutant from one phase to another. On the other hand, bioremediation transforms the compounds to less hazardous/non-hazardous forms with less input of chemicals, energy, and time (Providenti et al. 1993; Ward et al. 2003).

Although PAH may undergo adsorption, volatilization, photolysis, and chemical degradation, microbial degradation is the major degradation process (Bumpus 1989; Cerniglia 1992; Mannisto et al. 1996). Microbes are known for their catabolic activity in bioremediation, but changes in microbial communities are still unpredictable and the microbial community is still termed as a 'black box' (Dua et al. 2002). The PAH-degrading microorganism could be algae,
bacteria, and fungi. Biodegradation of a pollutant depends on the environmental conditions, number and type of the microorganisms, nature and chemical structure of the chemical compound being degraded (Tyagi et al. 2011). The rate of change in contaminant concentration is proportional to the concentration of contaminant in soil; and the time is dependent on the microorganisms, the contaminant type and its concentration (Cutright 1995). Thus, to devise a bioremediation system, a number of factors are to be counted for and have been extensively studied for their ability to degrade xenobiotics including PAHs (Haritash and Kaushik 2009). Several bacteria and fungi are known to degrade the persistent pollutants and have been extensively studied for their ability to degrade xenobiotics including PAHs. Most of the bacteria, representing biodegradation efficiency, are isolated from contaminated soil or sediments. Some of the bacteria studied for PAH degradation are Sphingomonas paucimobilis strain (Siddiqi et al 2002), Agrobacterium, Burkholderia, Rhodococcus sp., Mycobacterium (Sarma and Pakshirajan 2010), Pseudomonas and Flavobacterium species (Walter et al. 1991; Trzesicka-Mlynarz and Ward 1995). Romero et al. (1998) isolated Pseudomonas aeruginosa from a stream heavily polluted by a petroleum refinery. The species was found to be actively growing over high dosages of phenanthrene with complete removal of the pollutant in a period of thirty days. Rehmann et al. (1998) isolated a Mycobacterium spp., strain KR2 from a PAH contaminated soil of a gaswork plant, which was able to utilize pyrene as sole source of carbon and energy. The isolate metabolized up to 60% of the pyrene added (0.5 mg/ml) within 8 days at 20°C. Recently, soil fungi have also been studied regarding their ability to degrade polycyclic aromatic hydrocarbons (PAHs) and produce ligninolytic enzymes under microaerobic and very-low-oxygen conditions (Cajthaml et al. 2001; Silva et al. 2009). Such studies reported that fungi have a great capability to degrade a broad range of PAHs under low-oxygen conditions. The groups of fungi which include Aspergillus ochraceus, Cunninghamella elegans, Cunninghamella echinulata, Phanerochaete chrysosporium, Bjerkandera sp., Trametes versicolor and the yeast Saccharomyces cerevisiae have the ability to oxidize or transform polycyclic aromatic hydrocarbons and render them non-toxic (Muncnerova and Augustin 1994). Low-molecular-weight PAHs (2–4 rings) were found to be degraded most extensively by Aspergillus sp., Trichocladium canadense, and Fusarium oxysporum. The present study was undertaken in order to isolate PAH-degrading microorganisms from contaminated environment, and to study their adaptability under high concentration of PAHs. The potential of isolated microbes towards degradation of low molecular weight PAHs was also investigated.

2. Material and methods

2.1 Isolation and adaptation of microorganisms

In order to isolate the PAH-degrading microorganisms, soil contaminated by the spillage and leakage around the petroleum-storage tanks from Hisar city was obtained. Soil suspension was prepared in water (1: 10 w/v). The serial dilution of the order of $10^{-4}$ was inoculated on the Petri dishes having growth medium for bacteria and fungi. Different strains that grew on the plates were streaked on new plates in order to attain a pure culture. The pure cultures were, then, subjected to a dose of 10 mg/l each of naphthalene, fluoranthene, phenanthrene and pyrene for a period of one month; 25 mg/l for a period of next one month; 50 mg/l a period of next two months; 100 mg/l for a period of next two months; and a dose of 200 mg/l each of naphthalene, phenanthrene, fluoranthene, and pyrene for a period of next six months. In this manner, the isolated microorganisms were subjected to an increasing dose of the
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PAHs for a total period of one year for microbial adaptations under static and dark incubation conditions at 30°C over PAH-amended Nutrient Agar (for bacteria) and Rose Bengal Agar (for fungus) growth media. Finally, two strains of bacteria and three strains of fungi were selected to study the degradation of individual PAHs. The biochemical characterization of microorganisms was done in the Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.2 Degradation of PAHs

The degradation of naphthalene, phenanthrene, fluoranthene, and pyrene was studied in the laboratory. Liquid minimal salt medium [K₂HPO₄•3H₂O: 4.25; NaH₂PO₄•3H₂O: 1.00; NH₄Cl: 2.0; MgSO₄•7H₂O: 0.2; FeSO₄•7H₂O: 0.012; MnSO₄•7H₂O: 0.003; ZnSO₄•7H₂O: 0.003; and CoSO₄•7H₂O: 0.001 (each in gm/l)] was used for the degradation studies. Each experiment was performed using sterilized glass flasks (250ml) containing 150 ml of the sterilized liquid medium, 1.0 ml of respective culture solution (Optical Density of 0.10 at 600nm), and 0.6 ml of respective PAH solution (250 μg/ml) at a temperature of 30°C and 150 rpm under dark conditions for 15 days. All the experiments were run in three replicates along with blanks with no microbial inoculums. 10 ml of the medium was extracted from the flasks after 3, 6, 9, 12, and 15 days. The extracted volume (10 ml) was shaken in toluene on ultrasonic bath for 30 minutes. The extracts were eluted through silica-sodium sulphate column and reduced in volume using a rotary vacuum evaporator. The volume was further reduced by placing the extracts in a fume hood and final extracts were stored in refrigerator at 4°C, under dark conditions, till analyzed on HPLC.

Percentage of degradation was calculated using the following relation:

\[
\text{Percent degradation (%)} = \frac{(C_i - C_f) \times 100}{C_i}\]

Where, \(C_i\) is the final concentration of PAH compound in medium (1.0 μg/ml)

2.3 Analysis of samples

The extracts were analysed using High Pressure Liquid Chromatography (HPLC), Shimadzu LC 2010 CHT model with UV detector. The conditions for chromatographic analysis were injection volume: 20 μL; mobile phase: acetonitrile/water gradient at the rate of 1 ml/min; column: 2 x 250mm, reverse-phase, 5-μm C18; detector: UV at a wavelength of excitation 254 nm; emission 400 nm.

3. Results and discussion

The degradation of four different PAHs (Naphthalene, Phenanthrene, Fluoranthene, and Pyrene) was studied using two species of bacteria (Micrococcus luteus and Kocuria rosea) and three species of fungi, namely, Aspergillus ficuum (Reichardt) p. Hennings, Aspergillus flavus link, and Aspergillus fumigatus fresenius. All the strains were found to degrade PAHs to varying levels after a treatment period of 15 days.

3.1 Bacterial degradation

Micrococcus luteus was found to form yellow colonies and it grew at a rate faster than that of Kocuria rosea. The differential growth rate resulted in faster degradation by M. luteus.
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Degradation of different PAHs by *M. luteus* and *K. rosea* is given in Table 1. Percent degradation of naphthalene by *M. luteus* was observed to be 11.1, 29.7, 52.7, 66.4, and 68.7 after a period of 3, 6, 9, 12, and 15 days, respectively with an initial dose of 1 mg/l. The rate of degradation was almost constant throughout the period with a small initial lag phase. The lag phase was small since the growth of bacteria was fast and vigorous. The degradation curve started flattening after 12th day and it nearly stabilized on 15th day (Fig. 1a).

Degradation of Phenanthrene, too, depicted a similar trend. A small lag phase was observed initially and the rate of degradation was almost constant till the 9th day (Fig. 1b). Residual levels (mg/l) of 0.911, 0.735, 0.525, 0.406, and 0.371 were observed after a degradation period of 3, 6, 9, 12, and 15 days, respectively.

The rate of Phenanthrene degradation was found to be lower than the rate of degradation of naphthalene. The slow rate of degradation of Phenanthrene could be attributed to three benzene rings in Phenanthrene as compared to two of naphthalene. It has been observed that structural complexity as well as resistance to degradation increases with increase in number of rings or with an increase in molecular weight. The percent removal for fluoranthene was found to be 9.7, 28.8, 49.3, 57.6, and 61.4 after a period of 3, 6, 9, 12, and 15 days, respectively. A noticeable lag phase was seen in first 3 days (Fig. 1c). Similar differentiated lag phase, in first 3 days, was observed in degradation of Pyrene. The percent removal after 3 days was only 6.7% as against 11.1%, 8.9%, and 9.7% for naphthalene, Phenanthrene, and fluoranthene, respectively. Later, from day 3 to day 9, the rate of degradation improved substantially but was placed slightly lower than that of fluoranthene. The percent removal after 6, 9, 12, and 15 days was observed to be 25.9, 45.6, 57.4, and 61.3, respectively.

**Table 1:** Residual concentration (mg/ml) of different PAHs degraded by acclimatized microbial strains

<table>
<thead>
<tr>
<th>PAH</th>
<th>Species/Day</th>
<th>Residual concentration* (μg/ml)</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>15</th>
</tr>
</thead>
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<td>Naphthalene</td>
<td><em>M. luteus</em></td>
<td>1.000</td>
<td>0.889</td>
<td>0.703</td>
<td>0.473</td>
<td>0.336</td>
<td>0.313</td>
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<tr>
<td></td>
<td><em>K. rosea</em></td>
<td>1.000</td>
<td>0.925</td>
<td>0.782</td>
<td>0.553</td>
<td>0.427</td>
<td>0.402</td>
<td></td>
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<tr>
<td></td>
<td><em>A. ficuum</em></td>
<td>1.000</td>
<td>0.935</td>
<td>0.789</td>
<td>0.521</td>
<td>0.410</td>
<td>0.371</td>
<td></td>
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<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>1.000</td>
<td>0.875</td>
<td>0.715</td>
<td>0.464</td>
<td>0.332</td>
<td>0.312</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>1.000</td>
<td>0.932</td>
<td>0.786</td>
<td>0.453</td>
<td>0.298</td>
<td>0.263</td>
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<tr>
<td>Phenanthrene</td>
<td><em>M. luteus</em></td>
<td>1.000</td>
<td>0.911</td>
<td>0.735</td>
<td>0.525</td>
<td>0.406</td>
<td>0.371</td>
<td></td>
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<tr>
<td></td>
<td><em>K. rosea</em></td>
<td>1.000</td>
<td>0.921</td>
<td>0.810</td>
<td>0.586</td>
<td>0.475</td>
<td>0.454</td>
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<tr>
<td></td>
<td><em>A. ficuum</em></td>
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<td>0.936</td>
<td>0.815</td>
<td>0.694</td>
<td>0.558</td>
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<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>1.000</td>
<td>0.921</td>
<td>0.736</td>
<td>0.515</td>
<td>0.425</td>
<td>0.384</td>
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<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>1.000</td>
<td>0.924</td>
<td>0.721</td>
<td>0.594</td>
<td>0.441</td>
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<tr>
<td>Fluoranthene</td>
<td><em>M. luteus</em></td>
<td>1.000</td>
<td>0.903</td>
<td>0.712</td>
<td>0.507</td>
<td>0.424</td>
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<table>
<thead>
<tr>
<th></th>
<th>K. rosea</th>
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<tr>
<td></td>
<td>A. ficuum</td>
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<tr>
<td></td>
<td>A. flavus</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>A. fumigatus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrene</td>
<td>M. luteus</td>
<td>1.000</td>
<td>0.933</td>
<td>0.741</td>
<td>0.544</td>
<td>0.426</td>
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<tr>
<td></td>
<td>K. rosea</td>
<td>1.000</td>
<td>0.927</td>
<td>0.769</td>
<td>0.604</td>
<td>0.492</td>
</tr>
<tr>
<td></td>
<td>A. ficuum</td>
<td>1.000</td>
<td>0.946</td>
<td>0.842</td>
<td>0.694</td>
<td>0.505</td>
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<tr>
<td></td>
<td>A. flavus</td>
<td>1.000</td>
<td>0.941</td>
<td>0.733</td>
<td>0.556</td>
<td>0.451</td>
</tr>
<tr>
<td></td>
<td>A. fumigatus</td>
<td>1.000</td>
<td>0.929</td>
<td>0.742</td>
<td>0.534</td>
<td>0.449</td>
</tr>
</tbody>
</table>

*Residual concentration (µg/ml) values represent the mean of 3 replicates

*Kocuria rosea* or *Micrococcus roseus* was found to form red/pink colonies and its growth compared to *M. luteus* was less. It could degrade all four PAH compounds but at a degradation rate lower than *M. luteus*. Naphthalene was the PAH compound degraded most efficiently followed by the degradation of Phenanthrene. The lag phase in case of Phenanthrene was observed to be extended for about 6 days and the degradation rate increased after 6th day. Lower rate of degradation as compared to naphthalene could be attributed to the extra benzene ring in Phenanthrene. The percent degradation of fluoranthene was still lower. *K. rosea* could degrade 53.8% of fluoranthene in a period of 15 days. No specific lag phase was observed as the rate of degradation was almost the same from starting till the 9th day and it started dipping after it. Removal of Pyrene, too, was of the same order (53.3%) after a period of 15 days, but unlike fluoranthene, a lag phase in first three days was observed (Fig. 1d). Only 7.3% of Pyrene could be degraded in first three days as against 12.5% of fluoranthene.

3.2 Fungal Degradation

Degradation of Naphthalene by *A. ficuum* was 6.5%, 21.1%, 47.9%, 59.0%, and 62.9% after a period of 3, 6, 9, 12, and 15 days, respectively. The rate of degradation was slow for the first three days and it started increasing gradually till the 6th day. From 6th to 12th day, a sharp increase in removal/degradation rate of naphthalene was observed. From 12th day to 15th day, the removal rate dipped and approached near stabilization (Table 1; Fig. 2a). Sufficient growth of fungus had taken place in a period of about 2 weeks which lead to stabilization of number as well as degradation. In case of degradation by *A. flavus*, percentage degradation was 68.8% after 15 days. The trend of degradation was similar to the case of *A. ficuum*. Similarly, degradation by *A. fumigatus* followed a sigmoid curve where it picked a faster rate gradually depicted as 21.4%, 54.7%, 70.2%, and 73.7%. The slower rate may be attributed to the fact that the microbial biomass just started consuming /degrading the PAH and gradually increased to an optimum number and hence the rate of consumption / degradation started increasing accordingly.
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Degradation of phenanthrene too demonstrated similar trend for degradation for A. ficuum, A. flavus and A. fumigatus. In case of A. ficuum, the residual concentration left after 15 days was 0.503 ppm, respectively. The percentage degradation recorded was 49.7%. The results revealed that degradation of phenanthrene was slower than naphthalene. Nevertheless, the trend was more or less similar to that obtained in the previous case. The degradation of Phenanthrene by A. flavus and A. fumigatus was also similar. The percentage degradation observed was 61.6% and 61.9% after 15 days.

A. Ficuum degraded fluoranthene by 8.5%, 21.8%, 47.0%, 57.2%, and 61.6% on treatment exposure of 3, 6, 9, 12, and 15 days. Fluoranthene degradation by A. flavus represented a trend similar to that of A. ficuum with an overall removal rate slightly over the other (Fig. 2c). A residual concentration (mg/l) of 0.374 was observed after a period of 15 days. The corresponding percentage removal was 62.6%. The stabilization of removal near the end of 15 days might be due to stabilization of fungal biomass. The degradation by A. fumigatus represented the similar trend but the removal rate was slower compared to A. flavus and A. ficuum. Percent removal was observed to be 60.7% after an exposure of 15 days.

Figure 1: Degradation of (a)Naphthalene (b) phenanthrene (c) fluoranthene (d) pyrene by different lab acclimatized M luteus and K rosea
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Degradation of pyrene by A. ficuum was 54.6% during a period of 15 days. The rate of degradation was slow for the first three days and it started increasing gradually till the 6th day. From 6th to 12th day, a sharp increase in removal/degradation rate of naphthalene was observed. From 12th day to 15th day, the removal rate dipped and approached near stabilization (Table 1; Fig. 2d). In case of A. flavus, the residual concentration of pyrene was recorded as 0.402 mg/l after a period of 15 days. The corresponding percentage degradation was 59.8%. The trend of degradation was similar as observed in case of A. ficuum but the rate of degradation was substantially higher than A. ficuum from day 3 onwards. Similarly the degradation by A. fumigatus was slow during the first three days. The trend for degradation followed a sigmoid curve where it picked a faster rate as observed against 25.8%, 46.6%, 55.1%, and 59.6% degradation. About 55% of the degradation was attained till the 12th day. The rate of degradation of pyrene by A. fumigatus was placed slightly over A. ficuum, but less than the degradation by A. flavus.

Figure 2: Degradation of (a)Naphthalene (b) phenanthrene (c) fluoranthene (d) pyrene by different lab acclimatized strains of Aspergillus
Isolated microbial strains were capable of degrading Naphthalene, Phenanthrene, Fluoranthene, and Pyrene to a considerable degree in a period of 15 days. *M. luteus* could degrade the compounds at a faster rate than *K. rosea*. The growth of *M. luteus* and hence the rate of uptake of PAH compounds, as carbon source, was more. The bacterium *M. luteus* has recently gained the attention of researchers because of its potential role in degradation of hydrocarbons, olefinic compounds (Zhuang et al. 2003), biphenyls, and phthalates (Eaton 1982); and it has survived for at least 34,000 to 170,000 years on the basis of 16S rRNA analysis (Greenblat et al. 2004). *Kocuria rosea* has been shown to have keratinolytic activity and thus degrades feathers. It has also been isolated from hydrocarbon contaminated seawater (Harwati et al. 2007) and soil (Garcia-Rivero et al. 2007) and has the property to solubilize PAHs. Both the bacteria, therefore, can be promising tools in bioremediation of PAH contaminated soils.

Degradation of PAHs by fungus *Aspergillus* has also been reported in several other studies too. Bagy et al. (1992) screened *Aspergillus flavus* var. *columnaris* on naphthalene or anthracene modified medium for hydrocarbon-utilizing microorganisms. *Aspergillus* has also been reported to have maximum load (1.8 x 10^5 CFU/g dry weight) and number of species in organic compost with a property to degrade PAHs (Kastner and Mahro 1996; Anastasi et al. 2005). It produces β-glucosidase, xylanase, and endoglucanase enzymes with cotton-solubilizing activity (Parry et al. 1983). The β-glucosidases and endoglucanase produces glucose and a mixture of oligosaccharides from cellulose and are known to have hydrocarbon utilizing efficiency as well (Pandey and Mishra 1997; Iwashita et al. 1998).

In the present study, PAH-degradation by microorganisms may be a function of melting point and stability of the concerned PAH, aqueous solubility, and structure/shape of the PAH compound. The melting point and aqueous solubility of the PAHs are mentioned in Table 2. Naphthalene has least melting point and thus least stability and accordingly maximum microbial degradation. The percent degradation decreased with increase in melting point and decrease in solubility. Fungal degradation of fluoranthene and phenanthrene does not follow the melting point/stability and solubility pattern during degradation by *Aspergillus* strains which may be ascribed to the angular shape of phenanthrene and addition of a cyclopentane ring in fluoranthene. A relation between chemical stability and recalcitrance of PAH compound has also been mentioned by Tyagi et al. (2011).

### Table 2: Physico-chemical properties of polycyclic aromatic hydrocarbons

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name</th>
<th>M.F.</th>
<th>Molecular weight</th>
<th>B.Pt. # (°C)</th>
<th>M.Pt. # (°C)</th>
<th>V.P. #(Pa at 25 °C)</th>
<th>Aqueous solubility (mg/l)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naphthalene</td>
<td>C_{10}H_{8}</td>
<td>152</td>
<td>218</td>
<td>80.2</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Phenanthrene</td>
<td>C_{14}H_{10}</td>
<td>178</td>
<td>340</td>
<td>100.5</td>
<td>2 x 10^{-2}</td>
<td>1-2</td>
</tr>
<tr>
<td>3</td>
<td>Fluoranthene</td>
<td>C_{16}H_{10}</td>
<td>202</td>
<td>375</td>
<td>108.8</td>
<td>1.2 x 10^{-3}</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>Pyrene</td>
<td>C_{16}H_{10}</td>
<td>202</td>
<td>150.4</td>
<td>393</td>
<td>6.0 x 10^{-4}</td>
<td>0.12-0.18</td>
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</table>

*WGPAH, 2001; * Mackay et al., 1991
4. Conclusion

The degradation of four PAH compounds by *M. luteus*, after 15 days, revealed that maximum degradation was observed for naphthalene (68.7%) followed by Phenanthrene (62.9%), fluoranthene (61.4%), and Pyrene (61.3%). Minimum degradation was observed for *K. rosea*. *M. luteus* could degrade the compounds at a faster rate than *K. rosea* because the growth rate of *M. luteus* was more. The analysis of residual concentrations revealed that *M. luteus*, *A. flavus* and *A. fumigatus* strains were better adapted to degradation. These strains exhibited higher removal rates and an overall removal efficiency. The overall removal efficiency was higher for naphthalene as compared to other compounds. Maximum degradation of naphthalene was observed for *A. fumigatus* followed by *A. flavus*, *M. luteus*, *A. ficuum*, and *K. rosea*. The removal efficiency for Phenanthrene and fluoranthene was almost identical with marginal difference for *A. fumigatus* and *A. flavus*. Though the removal by *A. ficuum* was observed to be slow, it was useful in the removal of fluoranthene. The three strains of *Aspergillus*, *K. rosea*, and *M. luteus* can be used to remediate PAH-contaminated environments and the trend for biodegradation could be dependent on melting point and stability of the concerned PAH, aqueous solubility, structure of the PAH compound, and microbial growth and dynamics.

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5. References


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