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Degradation of Phenanthrene by *Corynebacterium urealyticum* in Liquid Culture and Sand Slurry

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ABSTRACT

Most studies on biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) evaluate the degradation potential of indigenous microorganisms in either liquid or solid media. There are limited studies on evaluation of the same microorganisms in degrading PAHs under non-indigenous condition in both liquid or solid media. This study investigated the potential of the bacteria, *Corynebacterium urealyticum* isolated from municipal sludge in degrading phenanthrene in both liquid and solid media. The study also evaluated the performance of the strain when subjected to low and high initial concentration of PAHs. Batch experiments were conducted over 20 days in reactors containing artificially contaminated phenanthrene minimal media and sand slurry inoculated with a bacterial culture. Phenanthrene degradation in liquid culture and sand slurry were found to be 82.15% and 27.71%, respectively. The degradation activity of bacteria in liquid culture remained active throughout the duration of the experiment, but this was not the case in the sand slurry. A significant difference was observed in the amount of phenanthrene remaining in the sand slurry when the bacteria was inoculated into the low and high phenanthrene concentrations. Percentages of phenanthrene remaining for both initial concentrations in liquid culture were not significant. From the bacteria growth curve plotted through viable count analysis, it was observed that the bacteria could immediately adapt to PAH-contaminated sand and had better capability to degrade phenanthrene in liquid culture compared to sand slurry.

Keywords: Biodegradation, *Corynebacterium urealyticum*, liquid cultures, phenanthrene, sand slurry

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread and are recalcitrant organic compounds that might pose a threat to mankind and the environment (Harvey 1991; Mrozik *et al*. 2003). Significant levels of PAHs have been detected in air, water, soils and sediments (Harvey 1991; Yang and Hildebrand 2006). PAHs pollution in soil differs from water. In water, PAHs may be diluted when

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a large volume of water is present. PAHs may be transported to other parts of the environment following the flow of water bodies. However, in soil, PAHs are generally adsorbed to soil particles and degradation of PAHs is limited (Madsen 2003). Phenanthrene is a three-ring PAH commonly found in water bodies and contaminated soil. This compound is normally utilized as a model compound in PAH biodegradation studies (Woo *et al*. 2004; Tang *et al*. 2005; Lors *et al.* 2010).

Biodegradation appears to be the main process responsible for the removal of PAHs from the natural environment (Wilson and Jones 1993; Mohan *et al.* 2006). Bacteria constitute one of the more frequently used microorganisms in biodegradation as they have the capability to transform PAHs to other organic or inorganic end products (Cerniglia 1984; Rakesh *et al*. 2005). Many bacteria are capable of consuming PAHs as energy and carbon sources or cometabolism, leading to the biodegradation and reduction processes (Atlas 1995; Rakesh *et al.* 2005). Various studies have identified these bacteria to be mainly from the genera *Pseudomonas sp., Rhodococcus sp., Mycobacterium sp., Sphingobacterium sp., Sphingomonas sp., Bacillus cereus, Flavobacterium sp., Beijerinckia sp*. and *Burkholderiacepacia* (Samanta *et al*. 1999; Moody *et al*. 2001; Abdul-Ghani 2008; Chauhan *et al.* 2008; Zhao *et al*. 2008; Janbandhu and Fulekar 2011).

Reports on use of bacteria strains in PAHs biodegradation in either liquid or solid media are available in the literature. Studies in liquid medium have been reported by Moody *et al.* (2001), Abdul-Ghani (2008), Zhao *et al.* (2008) and Janbandhu and Fulekar (2011). Bacteria used in these studies were capable of degrading more than 95% of PAHs in less than 14 days. However, for certain cases where the bacteria were exposed to high concentrations of PAH, less than 60% were degraded (Janbandhu and Fulekar 2011).

The capability of bacteria to degrade PAHs in solid medium was investigated by Kwok and Loh (2003), Sheng and Gong (2006), Gottfried *et al.* (2010) and Karamalidis *et al*. (2010). Incomplete degradation of PAHs was observed in most of these studies, that is, less than 85%. Moreover, a long duration was required to achieve a high percentage of removal (Karamalidis *et al.* 2010). Lower removal and longer duration are due to the complex processes involved such as solubility, physico-chemical sorption, concentration of PAH and low bioavailability of PAHs in solid medium (Boopathy 2000).

Reported degradation studies on the capability of bacteria under non-indigenous condition in both liquid and solid media are limited. Evaluation of PAHs biodegradation in different media by using similar bacteria could enhance our understanding of the performance of bacteria for the remediation of PAHs contaminated sites. This will lead to better implementation of effective remediation strategies.

The specific aim of this present work is to evaluate the potential of *Corynebacterium urealyticum* in degrading phenanthrene in liquid culture and in sand slurry. This study also compared the trend of degradation at different initial phenanthrene concentrations in both media. In addition, the survival of bacteria in sand slurry during the phenanthrene degradation was also investigated.

METHODOLOGY

Materials

All chemicals used for extraction, preparation of minimal media and bacteria culture were of analytical grade and supplied by Merck, Germany. Phenanthrene standard for Gas Chromatography Mass Spectrometer (GCMS) was obtained from Merck (Augsburg, Germany). For extraction in liquid culture, Solid Phase Micro Extraction (SPME) fibre holder assembly equipped with a 7 µm polydimethylsiloxane (PDMS) SPME fibre was purchased from Supelco, Sigma-Aldrich Chemie. Ultra-pure water (UPW) used in this study was purchased from Alga Purelab Ultra (18.2MΩ, United Kingdom).

Samples Preparation

Minimal media solution was prepared by dissolving 8.5 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 0.5 g $MgSO_4.7H_2O$, 0.0147 g $CaCl_2$, 0.0004 g $CuSO_4$, 0.001 g KI, 0.004 g $MnSO_4.H_2O$, 0.004 g $ZnSO_4$, 0.005 g H_3BO_3 and 0.002 g $FeCl_3$ in 1 L ultra pure water.

For the biodegradation study in liquid culture, 20 ml of minimal media solution and 30 ml of UPW was transferred into 250 ml conical flasks. For the biodegradation study in sand slurry, sand was sampled and washed in sand: water ratio of 1:2 (w: v) four times. Then it was dried in an oven at 60°C for 5 days. The cleaned and dried sand was sieved through 1.18 mm wire mesh. Then, 20 ml of minimal media solution and 40 g of cleaned sand was transferred into 500 ml conical flasks. The flasks for both tests were then subjected to steam sterilization for 15 min (Hirayama, HVE-50, Japan) to eliminate indigenous microorganisms.

After sterilization, both flasks were spiked with phenanthrene at different concentrations: 100 mg L^{-1} and 350 mg L^{-1} in minimal media solution while 100 mg kg^{-1} and 350 mg kg^{-1} in sand, respectively. Hexane was evaporated under continuous mixing to ensure homogenous distribution of phenanthrene in samples. Samples were stored under 4ºC until its use in biodegradation studies. The initial concentration of phenanthrene was verified in triplicate before being used for the biodegradation study.

Bacteria Strain Preparation

Corynebacterium urealyticum used in this study was isolated from municipal sludge by Othman et al. (2010) and preserved at -80°C in microbeads (microbankTM). The bacteria was revived by transferring a few of the frozen beads into universal bottles containing 20 mL of nutrient broth with the broth being incubated at 30ºC for three days. A series of dilution streaking was performed and the strain was sub-cultured three times to attain active bacteria before it was used in biodegradation studies.

Biodegradation Study

Flasks comprising artificially contaminated minimal media and artificially contaminated sand were inoculated with 5 ml bacterial inoculums. The inoculated inoculum in liquid culture and sand slurry would produce bacteria ranging in number from 10^7 - 10^8 colony forming unit (CFU) per mL or per gram of sample, respectively. All tests were conducted at an initial pH and temperature of 7.0 and 30°C, respectively. These conditions were adopted from optimisation studies by Othman *et al*. (2010).

After inoculation, all flasks were shaken in an incubation shaker at 150 rpm. Phenanthrene degradation was determined every day until the degradation was completed or the remaining concentrations stabilized over the 20-day study duration. This duration was determined from a preliminary study. Biodegradation studies were performed in triplicate. Three control reactor flasks without inoculum were also used during the study. Viable count analysis was conducted in all samples and no colony was found. In sand slurry, sterilized water was supplied at 2% remaining weight every day. Water was supplied to compensate for drying effects on samples during the experiment.

PAHs Extraction and Analysis

PAHs in liquid culture were extracted by SPME method optimised by Othman *et al.* (2010). A 20 mL sample was withdrawn and transferred into a 25 mL glass bottle with septum cap. The SPME fibre holder was immersed in the 25 mL glass bottle. Then, the sample was heated at 60ºC in a water bath for 60 min. The fibre was then retracted and transferred to a heated injection port of the GCMS (Perkin Elmer Clarus 600) for analysis. In this analysis, each sample was tested in triplicate with efficiency recovery from this method being 70%.

For sand slurries, 500 mg of the sand sample was dissolved in 25 mL of n-hexane and acetone 7:3 (v/v). The extractions were performed with the pressurized microwave extraction system (MAE) Multiwave 3000 (Anton Paar, Austria) at 10 bars for 40 minutes. When the extraction period was completed, the equipment was cooled to room temperature. Subsequently, the samples were filtered with Whatman fibre filter with pore size of 11 µm. The samples were concentrated by means of a rotary evaporator to about 1 mL. The extraction method was modified from Gfrerer and Lankmayr (2001).

Extracted supernatant was analyzed by using GCMS (Perkin Elmer Clarus 600). Elite Column 5MS with 30 m long, 0.25 mm internal diameter and 0.25 µm thickness was used to separate the compounds. The injector was operated at 250°C in the splitless mode with 2-min splitless period. Helium was used as the carrier gas with 1 mL min^{-1} constant flow rate. The column temperature was initially set at 50°C for 1 min, increased to 150°C at a rate of 15°C min^{-1} and held at 1 min, and finally ramped at 5°C min-1 to 300°C and held constant until the end of the 35 min total run time.

Survival of Bacteria in Sand Slurry

Concentration of bacteria from sand slurry was quantified by mixing 1 g of sand with 9 mL of sterile phosphate buffered saline and homogenized at high speed of 1min using a vortex mixer. Successive 1/10 dilutions were made by adding 1 mL of the sand suspension to 9 mL of phosphate buffered saline solution. An aliquot (0.1 mL) of each dilution was transferred to nutrient agar on petri dish. The dishes were incubated at 30°C at an inverted position. After four days, the number of bacterial colonies was counted using a plate counter. Plates with different dilutions were prepared and those with colonies in the range of 30 to 300 were used to estimate the number of bacteria. This number of colonies was then multiplied by the dilution factor to find the total number of bacteria per 1 g of the sand. The number of colonies was expressed as colony-forming units per gram of sand (CFU/g). All tests were conducted in triplicate.

Statistical Analysis and Biodegradation Kinetics

The difference in concentration of phenanthrene for both conditions was determined by using analysis of variance (ANOVA) test with time as co-variance. All statistical analyses were performed with Microsoft Excel software. The biodegradation kinetics of phenanthrene was described using the first order rate model (Equation 1):

$$S = S_0 e^{-kt} \qquad (1)$$

where S is the phenanthrene concentration in the medium at time, t, S_0 is the initial phenanthrene concentration and k is the first order rate constants of the degradation process. A linear plot of ln S against t is applied to provide the value of k from its slope (Equation. 2).

$$\text{Ln } S = -kt + ln\ So \qquad (2)$$

Based on Equation 1, values of first order rate constant (k) was determined. The half-life, $t_{1/2}$ is the time required for concentration of substrate to reach one-half of its initial value and calculated as Equation 3:

$$t_{1/2} = -\frac{\text{In }_{1/2}}{k} \qquad (3)$$

RESULTS AND DISCUSSION

Phenanthrene Degradation in Liquid Cultures and Sand Slurry

Fig. 1 shows phenanthrene degradation by *C. urealyticum* in liquid culture and sand slurry at an initial phenanthrene concentration of 350 mg L^{-1} and 350 mg kg^{-1} respectively. In both media, the degradation was observed in three phases with

Phase 1 being characterized by rapid degradation, Phase 2 with slow degradation and Phase 3 with very slow or almost no degradation. Rapid degradation of phenanthrene in Phase 1 showed that the bacteria easily adapted to the new environment, artificially phenanthrene-contaminated media. Degradation rates in liquid culture were determined to be 23.80 mg L^{-1} day^{-1}, 14.31 mg L^{-1} day^{-1} and 3.88 mg L^{-1} day-1 for phases 1, 2 and 3, respectively. These degradation rates were higher than the degradation rates in sand slurry which were 13.82 mg kg^{-1} day^{-1}, 1.45 mg kg^{-1} day^{-1} and 0.15 mg kg^{-1} day^{-1} for phase 1, phase 2 and phase 3, respectively.

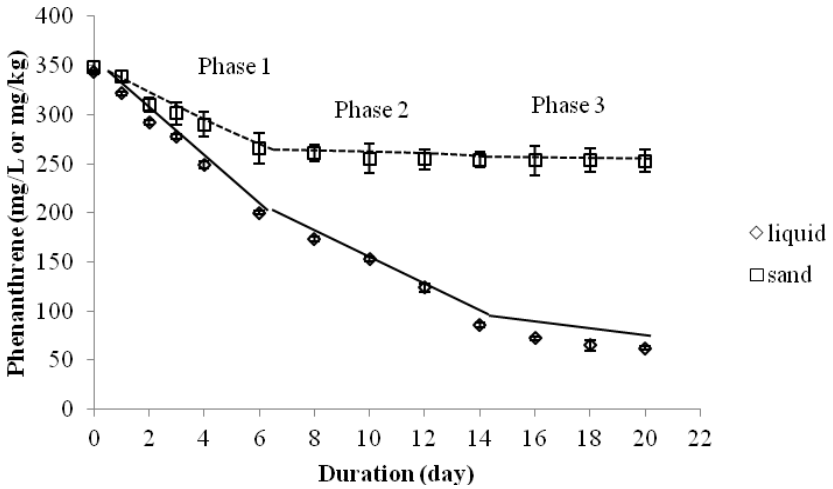

Fig. 1: Degradation of phenanthrene in liquid culture and sand slurry at initial phenanthrene concentration of 350 mg L^{-1} in liquid culture and 350 mg kg^{-1} in sand slurry. Data plotted are mean of three replicates; error bars represent SD

In this study, the bacteria were inoculated in nutrient broth, which is a liquid medium. In the liquid cultures, phenanthrene was dissolved in minimal media during sample preparation. As the liquid culture is a homogenous media, phenanthrene is readily available to the bacteria. Phenanthrene in sand slurry was entrapped and had strong adsorption to sand particles, contributing to low bioavailability of phenanthrene to the bacteria (Boopathy 2000).

PAHs degrading bacteria are able to degrade PAHs by the action of intracellular dioxygenases, meaning both PAHs and atoms of molecular oxygen must be transferred into the cell to begin PAHs degradation (Johnsen *et al*. 2005). Thus, the low bioavailability in sand slurry slowed and to some extent limited the transfer of phenanthrene into the cell and subsequently retarded the degradation process. PAHs form strong bonds with solid particles (Pizzul *et al.* 2007). Furthermore, PAHs are highly hydrophobic and have low solubility leading to their limited microbial degradation in solid media (Johnsen *et al*. 2005). In general, the results show that *C. urealyticum* was capable of degrading phenanthrene better in liquid culture than in sand slurry medium.

Effect of Different Phenanthrene Concentration in Both Media

Phenanthrene degradation at different initial concentrations in both media are shown in *Fig. 2a* and *2b*. In both media, similar trends of degradation were observed for both initial phenanthrene concentrations. More than 90.11% phenanthrene was degraded at 100 mg L^{-1}, while only 82.15% phenanthrene was degraded at 350 mg L^{-1} in liquid cultures. In sand slurry, 69.79% and 27.71% of phenanthrene was degraded at 100 mg kg^{-1} and 350 mg kg^{-1}, respectively. Overall, the remaining phenanthrene was found lower at low phenanthrene concentration (100 mg kg^{-1}) compared to a high concentration (350 mg kg^{-1}). These results show that bacteria performed better in degrading phenanthrene at low phenanthrene concentration compared to high concentration. In liquid culture, almost complete degradation was observed at both initial phenanthrene concentrations. On the other hand, no degradations were observed after day 6 in sand slurry.

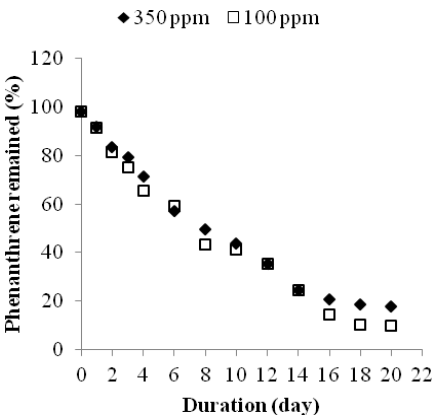

Fig. 2a: Phenanthrene degradation by Corynebacterium urealyticum in liquid cultures

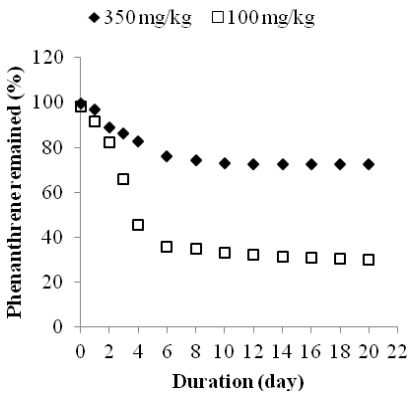

Fig. 2b: Phenanthrene degradation by Corynebacterium urealyticum in sand slurry

Generally, bacteria consume organic carbon as a substrate to produce energy during the metabolism process (Boopathy 2000). However, when the substrate is present in very high concentrations, the bacteria cell will be saturated with the substrate. The high concentration of substrate may become toxic to the bacteria and this reduces the efficiency of the degradation process. In a separate but related study, Othman *et al*. (2010) conducted a biodegradation study using the same bacteria at an initial phenanthrene concentration of less than 100 mg L^{-1} and found that the percentage of phenanthrene remaining was lower compared to the results of the present study. Thus, the degradation process of phenanthrene by the bacteria was optimum or attained steady state at 100 mg L^{-1}. At this point the strain has sufficient carbon sources for energy and growth. After the steady state, substrate saturation occurs and the degradation process starts decreasing, resulting in a lower percentage of degradation. Similar observations were reported by Bouchez *et al.* (1995) and Romero *et al.* (1998).

The degradation trend in liquid culture from this study was consistent with results from a study conducted by Abdul-Ghani (2008). Table 1a and 1b show the performances of PAHs degrading bacteria in liquid culture and solid media, respectively. Abdul-Ghani (2008) spiked his samples with mixed PAHs, whereas samples from this study were only spiked with individual PAHs. Irrespective of whether single or multi PAHs were used, the complete degradation of PAHs was observed in both studies. Meanwhile, the effect of different initial phenanthrene concentrations established in this study was consistent with the study conducted by Janbandhu and Fulekar (2011), which found that percentage degradation was reduced when initial phenanthrene concentrations exceeded 100 mg L^{-1}. In using a bacteria consortium, Janbandhu and Fulekar (2011) reported a degradation rate of 56.9% (from 250 mg L^{-1}) phenanthrene. In contrast, this study which used only single bacteria, namely, *C. urealyticum* degraded 82.15% of 350 mg L^{-1} in liquid media. The percentage degradation in this study was higher compared to that reported by Janbandhu and Fulekar (2011). Thus, this study showed that *C. urealyticum* is a better phenanthrene degrader. A study conducted by Zhao *et al.* (2008) also discovered a bacteria, namely *Sphingomonas sp*. that degraded 100% of 250 mg L^{-1} phenanthrene.

In sand slurry or solid medium, the degradation trend established in this study was consistent with the study reported by Sheng and Gong (2006), which also found that the degradation rate decreased with an increase in time. In addition, complete degradation was not achieved even over an extended duration. Similar findings have been reported by Kwok and Loh (2003), Gottfried *et al.* (2010) and Karamalidis *et al.* (2010). Results on effect of initial phenanthrene concentrations from this study concurred with the findings reported by Sheng and Gong (2006), which stated that as the initial concentration increased, the percentage of degradation was reduced. In general, it is observed that the percentage of degradation in studies conducted in solid media is lower compared to liquid culture studies. These studies show that complete degradation is not achievable in solid media.

Growth of C. urealyticum in Sand Slurry

Incomplete and slow degradation of PAH observed in sand slurry might be due to the inability of bacteria to attain and metabolise PAH as substrate for its growth. The inability of utilising a substrate is possibly due to the substrate being unavailable and/or toxic to the bacteria. Therefore, experiments on growth of the bacteria were conducted to determine its survival in sand slurry.

The growth curves of the bacteria in sand slurry at different initial phenanthrene concentrations are shown in *Fig. 3*. For the sample with an initial concentration of 100 mg kg^{-1}, the number of colonies increased from an average of 9.94 x 106 CFU g^{-1} at day 0 to 61-fold (6.13 x 108 CFU g^{-1}) at day 3. Furthermore, the colonies increased 14-fold (1.82 x 108 CFU g^{-1}) in the sample with initial concentration of 350 mg kg^{-1} at day 2. The bacteria continued to multiply for both concentrations until an early stationary phase was achieved at day 3 and day 2

for 100 mg kg^{-1} and 350 mg kg^{-1}, respectively. Then, the bacteria concentration began to decline until the end of the experiment. This reduction shows that the bacteria are unable to survive longer in sand slurries and subsequently retarded the degradation process.

TABLE 1a
Degradation of PAHs by different bacteria in liquid culture

References	Bacteria	PAHs	Initial conc. (mg L^{-1})	% degradation	Duration (day)
Abdul-Ghani (2008)	*Pseudomonas stutzeri bacterium* HS-D36 Uncultured *Pseudomonas sp.* clone 2-A *Pseudomonas stutzeri bacterium* LS401	dibenzothiophene phenanthrene anthracene (Mixed)	100	95	14
Zhao *et al.* (2008)	*Sphingomonas sp.*	phenanthrene	250	100	8
Janbandhu and Fulekar (2011)	*Sphingobacterium sp.*, *Bacillus cereus* *Achromobacterin solitus*	phenanthrene	100 250 500	100 56.9 25.8	14
This study	*Corynebacterium urealyticum*	phenanthrene	100 350	90 82	20

TABLE 1b
Degradation of PAHs by different bacteria in solid media

References	Bacteria	PAHs	Initial conc. (mg kg^{-1})	% degradation	Duration (day)
Kwok and Loh (2003)	*Pseudomonas putida* (ATCC 17484)	phenanthrene	300	70	20
Sheng and Gong (2006)	*Pseudomonas sp. GF3* + wheat (planted)	phenanthrene	100 200	84.8 70.2	80
Karamalidis *et al.* (2010)	*Pseudomonas aeruginosa*	16 PAHs (mixed)	58	70	191
Gottfried *et al.*(2010)	*Pseudomonas putida* (ATCC 17484)	phenanthrene	32	68	10
This study	*Corynebacterium urealyticum*	phenanthrene	100 350	70 27	20

Overall, *Fig. 3* shows that the bacteria can grow better in 100 mg kg^{-1} compared to 350 mg kg^{-1}, indicating that substrate concentrations can affect the growth of bacteria. Bacteria growth was optimum at a low substrate concentration of 100 mg kg^{-1}. Substrates at high concentrations (350 mg kg^{-1}) may be toxic to

the bacteria, slowing the bacteria replication rate. This result was also supported by Boopathy (2000), whose study found that one of the factors influencing the survival of bacteria in soil is the concentration of the substrates.

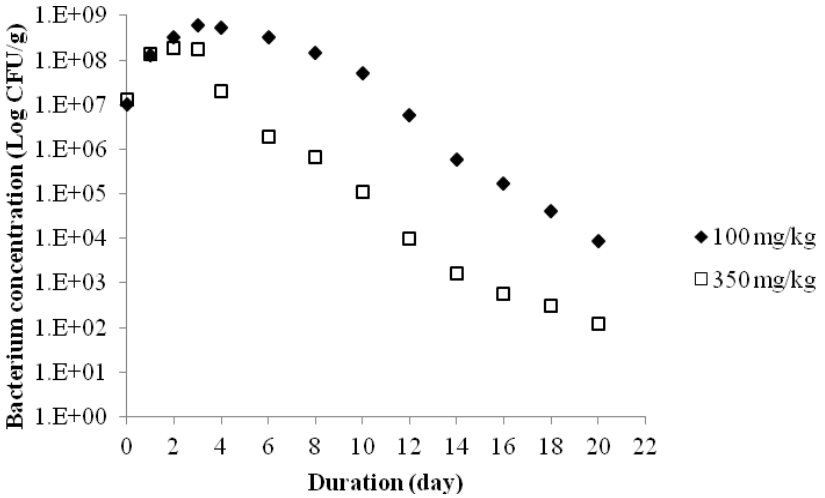

Fig. 3: The growth of the bacteria in sand slurry

For 100 mg kg^{-1} phenanthrene, the bacteria concentration began to reduce on day 4, that is, the day on which is no degradation was observed. On the other hand, the bacteria concentration began to reduce on day 3 for 350 mg kg^{-1} phenanthrene, which differed from the no degradation day, that is, day 8. After day 8, low bioavailability of phenanthrene may have contributed to the retardation of the biodegradation process. Therefore, both toxicity and bioavailability affected the degradation process at 350 mg kg^{-1}, whereas at 100 mg kg^{-1} only the bioavailability factor influenced the degradation process.

Findings on the growth of bacteria from this study contradicted observations made in liquid culture (Zhao *et al.* 2008; Janbandhu and Fulekar 2011). Janbandhu and Fulekar (2011) found that bacteria concentration increased within 14 days of experiment and remained constant towards the end of the experiment. Although samples were spiked with high initial phenanthrene concentrations, no reductions were detected in the bacteria concentration. Findings from Janbandhu and Fulekar (2011) were consistent with a study in liquid culture conducted by Zhao *et al.* (2008).

Statistical Analysis and Biodegradation Kinetic

Statistical analysis using ANOVA was conducted to establish the correlation between the degradation of phenanthrene at different initial phenanthrene concentrations, as shown in Table 2a and 2b. From ANOVA, at a 5% level of significance, it was found that the percentage of phenanthrene remaining for both concentrations in liquid culture was not significant (p>0.05). Thus, the result

confirms that the different concentrations of phenanthrene in liquid cultures from this study did not affect the degradation process. A significant correlation was observed (p<0.05) on percentage of phenanthrene degradation in sand slurry for both concentrations from the ANOVA analysis. This analysis showed that the degradation of phenanthrene by *C. urealyticum* in sand slurry was influenced by the initial phenanthrene concentration.

TABLE 2a
ANOVA analysis on the effect of different phenanthrene concentrations on the biodegradation process in liquid cultures

Source of variation	SS	df	MS	F	P-value	F crit
Between groups	69.97178	1	69.97178	0.077671	0.782866	4.259677
Within groups	21620.92	24	900.8715			
Total	21690.89	25				

*Groups: 350 mg L^{-1} and 100 mg L^{-1}

TABLE 2b
ANOVA analysis on the effect of different phenanthrene concentrations on the biodegradation process in sand slurry

Source of variation	SS	df	MS	F	P-value	F crit
Between groups	6069.522	1	6069.522	16.10301	0.00051	4.259677
Within groups	9046.043	24	376.9185			
Total	15115.56	25				

*Groups: 350 mg kg^{-1} and 100 mg kg^{-1}

In this study, the corelation between phenanthrene concentration of 100 mg kg^{-1} and 350 mg kg^{-1} to the bacteria concentration in sand slurry was also evaluated using ANOVA, as shown in Table 3a and 3b. At 5% level of significance, concentrations of phenanthrene and bacteria for both concentrations were found to be significant (p<0.05). As a result, it can be stated that bacteria concentration had influenced the degradation of phenanthrene.

Experiments in liquid culture fitted well to first order rate model ($R^2_{100\ mg\ L^{-1}}$= 0.97 and $R^2_{350\ mg\ L^{-1}}$ = 0.99). *Fig. 4* shows the plots of biodegradation data fit to the first-order kinetics model. First-order rate constant for low initial concentration, 100 mg L^{-1} (k = 0.118d^{-1}) was higher compared to high initial concentration, 350 mg L^{-1} (k= 0.092d^{-1}). The half-life values for low initial concentration, 100 mg L^{-1} ($t_{1/2}$ = 5.87 day) were significantly shorter than high initial concentration, 350 mg L^{-1} ($t_{1/2}$ = 7.53 day). Thus, more rapid degradation occurred at 100 mg L^{-1} compared to 350 mg L^{-1}.

Experiments in sand slurry were fitted well to first order rate model in the phase 1 with $R^2_{100\ mg\ kg^{-1}}$= 0.95 and R2350 mg kg^{-1}= 0.98, as shown in *Fig. 5*. For phases 2 and 3, degradation of phenanthrene in sand slurry was very slow, with almost no degradation occurring, and this trend did not fit any law on rate reaction.

TABLE 3a
ANOVA analysis on the effect of bacteria concentration grown in 100 mg kg^{-1} phenanthrene to biodegradation process in sand slurry

Source of variation	SS	df	MS	F	P-value	F crit
Between groups	69.97178	1	69.97178	0.077671	0.782866	4.259677
Within groups	21620.92	24	900.8715			
Total	21690.89	25				

*Groups: 350 mg L^{-1} and 100 mg L^{-1}

TABLE 3b
ANOVA analysis on the effect of bacteria concentration grown in 350 mg kg-1 phenanthrene to biodegradation process in sand slurry.

Source of variation	SS	df	MS	F	P-value	F crit
Between groups	6069.522	1	6069.522	16.10301	0.00051	4.259677
Within groups	9046.043	24	376.9185			
Total	15115.56	25				

*Groups: 350 mg kg^{-1} and 100 mg kg^{-1}

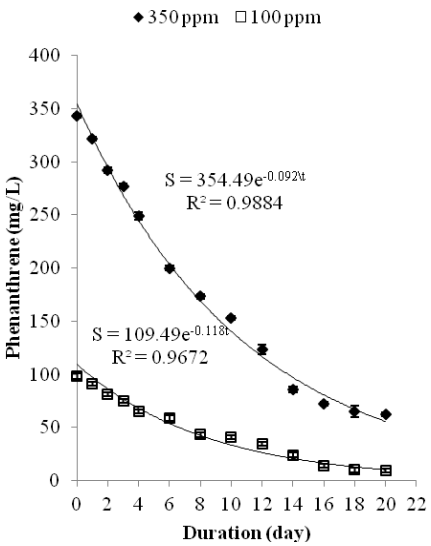

Fig. 4: First order biodegradation kinetic in liquid culture. Data plotted are mean of three replicates; error bars represent SD (±3%)

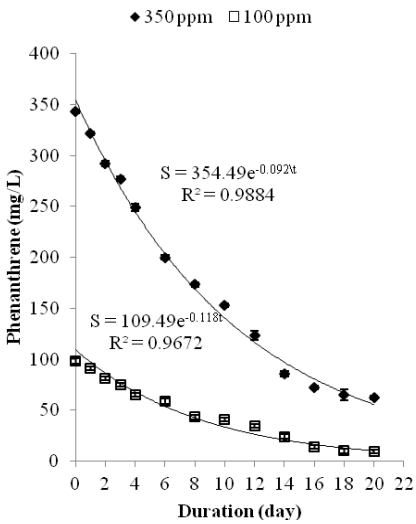

Fig. 5: First order biodegradation kinetic in sand slurry. Data plotted are mean of three replicates; error bars represent SD (±6%)

The reaction rate constant in sand slurry at 100 mg kg^{-1} (k = 0.184 d^{-1}) was higher compared to 350 mg kg^{-1} (k = 0.046 d^{-1}), as similarly observed in liquid culture. The phenanthrene was reduced to half of its initial concentration on day 3.76 for 100 mg kg^{-1}. Meanwhile, at 350 mg kg^{-1} of initial phenanthrene concentration, the bacteria was unable to degrade half of its initial concentration. Therefore, the half-life of 350 mg kg^{-1} phenanthrene cannot be determined by using first order rate model.

The degradation of phenanthrene in liquid culture fitted well with first-order rate model throughout the experiments. This was consistent with the findings made by Okpokwasili and Nweke (2005) and Kwon *et al*. (2009). Meanwhile, studies conducted in solid media such as in sediment slurry (Chen *et al.*, 2008) and PAHs contaminated soil (Yang *et al*. 2011) were consistent with the results on sand slurry from this study. The first order fitted well only for a certain period, such as 24 hours by Chen *et al*. (2008) and 8 days by Yang e*t al*. (2011). Subsequently, the degradation became very slow or no degradation was detected and in this period, it did not fit with the first order.

CONCLUSIONS

As a conclusion, the performance of *C. urealyticum* was better in liquid culture compared to sand slurry. It was observed that phenanthrene degradation at high initial concentration was lower compared to low initial concentration in both media. Degradation trends in both media established in this study were consistent with those reported in the literature. Complete degradation was observed in liquid culture, whereas in solid media, this was not the case. The bacteria were unable to survive longer in sand slurry, and this affected the degradation process. Percentages of phenanthrene remaining for both initial concentrations in liquid culture were not significant. This is in contrast with observations in sand slurry where the remaining phenanthrene was significantly higher. Bacteria concentration had influenced the degradation of phenanthrene in sand slurry at 5% level of significance. Phenanthrene degradation in liquid culture fitted well to the first-order rate model throughout the experiment, whereas in sand slurry, the first-order rate model only fitted well with phase 1.

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