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Bioremediating silty soil contaminated by phenanthrene, pyrene, benz(a)anthracene, benzo(a)pyrene using *Bacillus* sp. and *Pseudomonas* sp.: Biosurfactant/*Beta vulgaris* agrowaste effects

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Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant contaminants which are routinely found in numerous environmental matrices, contributing to ecological degradation. In this study, the removal of LMW and HMW PAHs with 4- and 5-benzene rings, by *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02 and *Pseudomonas aeruginosa* STK 03, was evaluated in silty soil for a period of 60 days. Subsequently, a biosurfactant produced from *Beta vulgaris* agrowaste was used to augment the removal of the aforementioned PAHs in mono- and co-cultures. The isolates proved to be proficient in removing the contaminants, with *B. licheniformis* STK01 cultures achieving the highest removal rates. Biosurfactant supplementation significantly enhanced the removal of benzo(a)pyrene- a 5-ring benzene HMW PAH. The highest removal rates achieved in biosurfactant-supplemented cultures were: 100% for phenanthrene, 95.32% for pyrene, 82.71% for benz(a)anthracene and 86.17% for benzo(a)pyrene. The kinetic data used to simulate removal rates were suitably described by first-order kinetics, with the rate constants showing that phenanthrene removal was rapid in cultures without biosurfactant (k = 0.0620 day^{-1}) as well as with biosurfactant (k = 0.0664 day^{-1}), while the removal rates for others followed in the order of their increasing molecular weight. The synergy of the bacterial isolates and the biosurfactant produced from *B. vulgaris* agrowaste could be used in environmental bioremediation of PAHs even in silty soil.

Key words: Benz(a)anthracene, benzo(a)pyrene, bioremediation, biosurfactant, *Beta vulgaris*, polycyclic aromatic hydrocarbons.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and recalcitrant contaminants, released into the environment through natural and anthropogenic sources (Sánchez et al., 2015). These sources are mainly biogenic, petrogenic and pyrolitic (Harvey, 1998). Owing to their hydrophobicity, they tend to fuse to non- and

porous particulate matter, making soil and sediment a suitable repository. Several PAHs have been identified as potential human mutagens and carcinogens (Grimmer, 1983). Chemical and biological methods have been used to remediate PAH-contaminated matrices, with the bioremediation approach being deemed suitable because it is environmentally benign and less invasive. Of the sixteen PAHs classified as priority pollutants by the United States Environmental Protection Agency (IRIS: EPA's Integrated Risk Information System, 1997), eight were identified as potential human carcinogens. These eight PAHs belong to the high molecular weight (HMW) class, a group associated with a higher tendency to bioaccumulate in environmental matrices.

Recent research studies have focused on the sequestration of these HMW PAHs by biologically-evolved microbial species and the exploration to enhance their bioavailability for subsequent removal from environmental matrices (Mishra and Singh, 2014; Wang et al., 2014; Moscoso et al., 2012; Lu et al., 2014; Chaudhary et al., 2011). Although several bacterial strains have shown an ability to remediate matrices contaminated by low molecular weight (LMW) PAHs, a few have demonstrated a similar ability for HMW PAHs with more than three benzene rings. Among these bacterial strains are many Gram-negative and a few Gram-positive species. These species include *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp. and *Acinetobacter* sp. (Mishra and Singh, 2014; Dandie et al., 2004; Boonchan et al., 2000), with degradation rates achieved ranging from 28 to 85% predominantly for 4-benzene ring PAHs such as pyrene and benzo(a)anthracene.

Moreover, a number of these studies often focused on the removal of PAHs in the aqueous phase as a secondary remediation process after extraction from solid matrices such as soil, that is, using washing as an extraction method prior to degradation due to the sequestration of these contaminants in soil. As a result of the small intraparticle pores in soil grains, Gram-negative bacteria appear to be better degraders of PAHs in such soil due to their thinner cellular membrane which may assist in higher PAH mass transfer across the cellular membrane, thereby facilitating sorption subsequent to intracellular degradation (Ma et al., 2013). Moreover, earlier studies had reported that most indigenous bacteria may be physically precluded from some intraparticle soil grain pores because of the mean diameter of the pores that the immobilized bacteria require to penetrate soil grains in order to access bound pollutants (Lawrence et al., 1979; Alexander, 1961).

This perhaps elucidates the limitations associated with the bioremediation of contaminated soil with a high fraction of clay and silt. Putatively, low bioavailability and mass transfer limitations are challenges to PAH bioremediation processes, particularly in soil. These limitations are often influenced by the molecular structure, weight and weathering of the contaminants including the soils' physicochemical characteristics. Several methods have been adopted to circumvent these challenges such as extraction (Lau et al., 2014; Silva et al., 2005; Song et al., 2011; Yap et al., 2012), adsorption/biosorption (Chang et al., 2004; Vidal et al., 2011; Kaya et al., 2013), co-metabolism (Reda, 2009) and biosurfactant application (Whang et al., 2008; Kang et al., 2009; Franzetti et al., 2010). Rather than utilizing these methods, an approach that is less intrusive and harmful to the environment is often preferred, such as the utilization of biosurfactants.

The application of biosurfactants to increase the bioavailability of pollutants in the environment seems to be a suitable method, considering that these surface chemistry modifying agents are benign. In addition, the availability of an array of suitable agrowaste for biosurfactant synthesis provides an alternative to mitigate the cost associated with their production. Several studies have reported the enhancement of PAH availability and subsequent biodegradation in the presence of biosurfactants (Jorfi et al., 2013; Husain, 2008). Naturally, PAH-degrading bacteria are able to access hydrophobic substrates in the environment through the synthesis of biosurfactants and their ability to directly attach to the hydrophobic substrate by modifying their cell membrane hydrophobicity (Das and Mukherjee, 2007). Biosurfactant-enhanced bioavailability often occurs via two mechanisms: 1) pre-micellar lowering of the surface tension, thereby enhancing the mobilization of the contaminants from particulate matrices resulting in increased sorption mass transfer and 2) micellar solubilization (Amodu et al., 2013).

Another less invasive approach for enhanced biodegradation of recalcitrant PAHs is by microbial co-cultivation using liquefied substrate with a lower surface tension. The biodegradation of phenanthrene, pyrene and benzo(a)anthracene by a bacterial consortium of *Staphylococcus warneri* and *Bacillus pumilus* increased from a maximum of 85% for mono-septic cultures obtained for each PAH, to biodegradation rates greater than 90% when co-cultivated cultures were used in a bioreactor system containing a mixture of PAHs, with a lowered surface tension aqueous phase (Moscoso et al., 2012). Although significant removal rates were achieved in these studies for some HMW PAHs, particularly those with less than 4-benzene rings, the degradation of 5- or more membered benzene ring PAHs, such as benzo(a)pyrene, is scarcely reported.

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In the authors' previous studies, novel bacterial strains were isolated that showed abilities for biosurfactant synthesis, with significant hydrocarbon emulsification indices and surface tension reduction under various environmental conditions (Amodu et al., 2014). In the present study, the effectiveness of these strains to remediate silty soil in which a mixture of PAHs is present, was investigated. The synergy of biosurfactant amendment with microbial co-culture cultivation on PAH-removal and the effect of contaminant co-metabolism were also studied. Finally, the dynamics of the bioremediation of the PAH contaminated soil was studied by monitoring the reduction kinetic rates for each of the PAH being studied.

MATERIALS AND METHODS

Microorganism and inoculum

Bacillus licheniformis STK 01 and *Bacillus subtilis* STK 02 were originally isolated from decaying wood chips and roadside coal tar respectively, both within the proximity of the Cape Peninsula University of Technology, Cape Town, South Africa. No sampling permission was required in these locations. However, *Pseudomonas aeruginosa* STK 03, which was isolated from a restricted location (an oil spill site at the Pipeline and Petroleum Product Marketing Company (PPMC) depot, Shagamu, Ogun State, Nigeria), and permission to sample was given by the Head of the depot (Area Manager). The bacterial strains were identified by morphological as well as 16S Ribosomal deoxyribonucleic acid (rDNA) sequence analysis. In addition, the *Bacilli* sp. were identified as Gram positive, while the *Pseudomonas* sp. was identified as a Gram negative strain. The sequences were registered in the NCBI with GenBank, with accession numbers KR011152, KR011153 and KR011154 for *B. licheniformis* STK 01, *B. subtilis* STK 02 and *P. aeruginosa* STK 03, respectively. They were maintained on nutrient agar slants at 4°C and subsequently subcultured every three weeks.

Chemical reagents

Phenanthrene (Phe), pyrene (Py), benz(a)anthracene (BaA) and benzo(a)pyrene (BaP) were all certified reference materials purchased from Sigma Aldrich Chemical Co. (Germany). Hexane (> 97%), dichloromethane (≥ 99.8%) and anhydrous sodium thiosulfate (> 98%) were also obtained from Sigma Aldrich, while a C-18 Solid Phase Extraction (SPE) 6 ml glass cartridge (0.5 g solid phase) was purchased from SUPELCO (Bellefonte, PA, USA) and utilized for the clean-up and concentration of the PAHs under evaluation.

Soil characterization

Uncontaminated soil sample was obtained within the vicinity of the university (CPUT). The soil was characterized using an American Society for Testing and Material method (ASTM method DIN-4188) coupled with a United Soil Classification system. The soil contained 30% clay, 20% silt (half passing through no. 200 sieve), 20% fine and 30% coarse sand (half being retained on no. 4 to 200 sieve). The soil was classified as a silty soil. It was collected, sterilized and stored at 4°C until use.

Bioremediation of PAHs in soil experiment-sample preparation

The bioremediation of PAHs containing soil was determined in the absence of indigenous microorganisms. Two hundred grams of soil was autoclaved twice at 121°C for 30 min, within a 12 h interval. The soil was spiked with a mixture of 40 mg PAH per kg of soil, using each of the following PAHs: Phe, Py, BaA, and BaP. The spiking process was carried out as described by Brinch et al. (2002), with a minor modification, that is, 20% of the soil was treated with the PAH mixture in acetonitrile. After the solvent has volatilized, the PAH-contaminated soil was mixed with 50% of the uncontaminated soil sample. After adequate mixing, the contaminated portion was then mixed with the remaining soil to ensure a uniform distribution of the contaminants in the soil. Ten grams of the contaminated soil was weighed into 100 mL Erlenmeyer flasks (covered with foil on the exterior), subsequent to incubation at 37°C in a dark, static incubator. The soil samples were inoculated with 8% (v/w) of overnight microbial cultures grown in nutrient broth. The concentration of the culture grown overnight was determined to be 10^8 CFU mL^{-1} by cell count using a Quebec Darkfield Colony Counter.

Different cultures were studied, viz the monocultures of each of the isolate (without supplementation with either biosurfactant or *B. vulgaris* agrowaste extract) and a co-culture of the isolates (consortium, without supplementation). The best performing culture was then supplemented with; 1) *B. vulgaris* waste (5%, w/w) agrowaste extract, and 2) the crude biosurfactant produced from *B. vulgaris* (5%, v/w) agrowaste extract.

Moreover, the concentration of PAHs in each flask was determined prior to incubation in order to assess the recovery efficiency of the extraction method used as well as the removal rate at the end of each experiment. The soil moisture content was maintained at 60% holding capacity as reported by Acevedo et al. (2011), by adding 5 mL of sterile water to each flask at 10 day intervals. Control experiments were prepared in a similar manner without an inoculum to account for the disappearance of PAHs due to abiotic factors. Each experiment was carried out in triplicate. Samples were incubated for 60 days; initially without periodic assessment of the degradation efficiency, but subsequently, the experiment was repeated with periodic sampling.

PAH extraction, clean-up and quantification

PAHs were extracted using an ultrasonication method. At the end of the experiment of 60 days, and during intermittent sampling, samples were transferred from Erlenmeyer flasks into 100 mL amber bottles and extracted with 20 mL of hexane for 20 min at 25°C in an ultrasonic bath. During the sonication, sample bottles were swirled intermittently to avoid the soil settling at the bottom. This step was repeated twice for each sample while the supernatants were pooled into another bottle and centrifuged at 5,000 rpm for 10 min, in preparation for the clean-up stage.

Clean-up procedure: Solid phase extraction

Solid phase extraction (SPE) column LC 18 cartridge was preconditioned with 30 mL of hexane (HEX) and dichloromethane (DCM). The order followed was: DCM – DCM/HEX – DCM, with a volume of 10 mL being loaded at a time for each preconditioning step. The DCM and HEX were mixed in a ratio 2:3 by volume. Sodium thiosulfate (1.0 g) was added to the top of the solid phase in the cartridge prior to conditioning. The supernatant collected from centrifugation was passed through the conditioned SPE cartridge followed by the elution of the PAH analytes with DCM and HEX, using a 7.5 mL of the eluent each time. The eluant collected was dialysed in a rotary evaporator; thereafter, the residue was

reconstituted in DCM to 1 mL in an amber vial, followed by analysis with a gas chromatography-flame ionisation detector (GC-FID).

GC-FID analysis – instrument operation

The GC-FID-analysis was performed using a 7890A Series GC-system (Agilent Technologies, CA, USA) equipped with a flame ionisation detector, an Agilent capillary column USB499114H (20 m x 180 µm x 0.14 µm) and an auto sampler. The oven programme was 170°C followed by ramping at 5°C min^{-1} up to 300°C with each ramping step being maintained for 3 min. Once a temperature of 300°C was reached, the temperature was increased to 310°C and held for 5 min. The total run time was 36 min. The post run time was used to wash the column with a DCM/HEX mixture, to remove any residual analytes before subsequent analyses. The carrier gas used was nitrogen, while a split mode injection was used with the injector temperature set to 250°C. A calibration curve was plotted using calibration standards, with a concentration in the range of 0.5 to 100 mg L^{-1} (R^2 = 0.9996), which was used to quantify the concentration of each analyte in order to evaluate the extraction method and to determine the level of abiotic disappearance of PAHs.

Bioremediation studies: Removal rate kinetics of PAHs from soil

Subsequent to the observation that biosurfactant supplementation resulted in increased removal efficiency, the kinetics of the reduction of PAHs and thus the effect of biosurfactant addition to the cultures on the bioremediation profile, was investigated. Hence, only the mono-cultures of *B. licheniformis* and with biosurfactant supplementation were used in this second stage of the study. The contaminated soil used contained an initial concentration of 50 mg per kg of soil for each of the PAHs, that is, Phe, Py and BaA and 25 mg of BaP. Culture preparation was done as described earlier. After soil spiking with PAHs, 50 g of the contaminated soil was transferred into a 250 mL Erlenmeyer flask covered with aluminium foil to prevent the disappearance of PAHs through UV irradiation. The flask was then incubated at 43 ± 2°C in a dark, shaking incubator at 180 rpm for 60 days. The temperature used here was the predetermined optimum temperature for *B. licheniformis* STK 01 growth and biosurfactant production in our previous report (Amodu et al., 2014). Samples were analyzed periodically and prior to sampling, the flasks were swirled thoroughly to ensure homogeneity. Two control experiments were used, one for each culture. All experiments were carried out in triplicates for this set of experiments. Samples were taken periodically to determine the concentration of PAHs in each flask, including uninoculated cultures (controls) and, by mass balance analysis, removal rates were evaluated. Extraction of analytes, clean-up and analysis using GC-FID were performed as described in earlier. The rate constant (*k*) was determined using a first-order decay rate expression (Equation 1):

$$-\frac{dC}{dt} = kC^n \quad (1)$$

where *C* is the concentration of PAH (mg L^{-1}), *t* is the time (day), *k* is the PAH removal rate constant (day^{-1}) and, *n* the reaction order, which is unity for first order kinetics (Kwon et al., 2009).

The above equation was integrated, while the logarithm of the ratio of PAH-concentration at the various sampling times to the initial concentration was plotted as a function of time. Hence, the disappearance rate, being the gradient of the plot, was determined.

RESULTS AND DISCUSSION

Bioremediation of PAHs from the soil

The biodegradation of phenanthrene, pyrene, benz(a)anthracene and benzo(a)pyrene by *B. licheniformis* STK 01, *B. subtilis* STK 02 and *P. aeruginosa* STK 03, is shown in Table 1. The cultures containing *B. licheniformis* were supplemented with biosurfactant and with an agrowaste (*B. vulgaris*) extract in order to investigate the effect of co-metabolic substrate utilization on the bioremediation of PAH contaminated soil. The biosurfactant used was produced by the *B. licheniformis* strain mentioned above from *B. vulgaris* waste extract, without supplementation with refined carbon sources or trace elements.

Table 1 shows the concentration of each PAH compound in the different cultures determined by the GC-FID prior to incubation as compared to the initial 40 mg kg^{-1} spiked into the soil. Hence, the recovery efficiency of the extraction method was evaluated using the experimental samples prior to incubation and it was found to be between 86 and 90% for Phe, Py, B(a)A and B(a)P. The PAH removal rates decreased with an increase in the molecular weight of the contaminants, for all the cultures studied with an exception being observed for the culture containing *P. aeruginosa*, whereby the degradation of the 5-benzene ring PAH (BaP) was higher than PAHs with 4-benzene rings (BaA and Py).

For all the experiments, the removal rate ranged from 73.97 to 96.88% for phenanthrene, 69.15 to 88.58% for pyrene, 62.21 to 83.30% for B(a)A, and 54.90 to 75.40% for B(a)P. The recalcitrance of PAHs to microbial sequestration often decreases with increasing molecular weight, as observed in this study and as reported elsewhere (Lors et al., 2012; Chaudhary et al., 2011). This phenomenon was expected to occur in the bioremediation of PAHs contaminated soil because, as the molecular weight increases, the tendency for the compound to sequester in a soil matrix and become non-bioavailable increases. Similarly, other researchers have also reported certain cases whereby HMW PAHs were degraded more than the LMW PAHs. Zhang et al. (2009), for instance, reported 97.7% degradation for pyrene and 82.1% for anthracene. Additionally, Acevedo et al. (2011), in a study of the biodegradation of some HMW PAHs, reported a degradation level of 60% for pyrene and 75% for B(a)P. Further research work may be required in this area to explicate the effects of structural symmetry of the pollutants on their biodegradation.

Comparing the removal rates in monoseptic cultures, it was observed that *B. licheniformis* achieved higher bioremediation levels than the other two isolates for all the PAHs. Generally, the microbial isolates used were all found to demonstrate a higher ability for the removal of the HMW PAHs. Few bacterial species have demonstrated a similar proficiency in soil environment. In

Table 1. Bioremediation of PAHs by: a) mono-septic cultures of *Bacillus licheniformis, Bacillus subtilis* and *Pseudomonas aeruginosa*; b) in co-cultures (consortium) using isolates, *Bacillus licheniformis* and *Bacillus subtilis,* including cultures augmented with *Beta vulgaris* waste extract and biosurfactant produced from the *B. vulgaris* waste extract. These samples were incubated for 60 days without periodic sampling.

PAH	*B. licheniformis*			*B. subtilis*			*P. aeruginosa*		
	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)
Mono-septic cultures									
Phe	38.20	3.28	91.43	34.03	5.16	84.83	34.21	5.79	83.97
Py	38.71	8.38	78.35	28.56	7.44	73.96	35.61	10.99	69.15
B(a)A	35.55	8.86	75.07	35.11	13.27	62.21	34.11	12.59	63.09
B(a)P	36.96	11.59	68.63	33.60	15.16	54.90	36.34	8.94	75.40*
Co- and augmented cultures									
PAHs	**Co-culture**			***B. licheniformis* and *B. vulgaris***			***B. licheniformis* and biosurfactant**		
	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)
Phe	34.56	3.34	90.34	37.18	3.69	90.07	38.84	1.21	96.88
Py	36.74	8.99	75.54	35.50	5.39	84.82	35.11	4.01	88.58
B(a)A	35.20	9.74	72.34	38.51	6.47	83.03	34.46	8.16	76.31
B(a)P	36.01	10.01	72.20	32.74	10.72	67.27	35.71	8.82	75.29

Phe– Phenanthrene, Py– pyrene, BaA– benz(a)anthracene, BaP– benzo(a)pyrene, %R*bd*- percentage biodremediation; Ci/f– initial and final concentration (mg/L). *Outlier- B(a)P samples had a higher margin of variability in terms of the quantified concentration.

a bioremediation study of BaP, it was found that *Pseudomonas aeruginosa* PSA5 and *Rhodococcus* sp. NJ2 reduced about 88 and 47% of the contaminant respectively, during a 25-day incubation period in a mineral salt medium (Mishra and Singh, 2014).

Considering the biosurfactant supplemented *B. licheniformis* cultures and the mono-culture without biosurfactant supplementation, it was observed that the reduction of pyrene and B(a)P increased from 78.35 to 88.58%, and 68.63 to 75.29%, respectively. Several other studies have reported biosurfactant-enhanced bioremediation of soil contaminated with PAHs. For example, Husain (2008) observed that a rhamnolipid emulsan produced by *Pseudomonas fluorescens* increased the removal of pyrene from 91 to 98% after 10 days of bioremediation studies. Similarly, the addition of biosurfactant synthesized by *P. aeruginosa* SP4 to a soil artificially contaminated with pyrene, was found to enhance removal rates by 25% (Jorfi et al., 2013).

The positive synergistic effects of *B. licheniformis* and *B. subtilis* in a co-culture, as well as the supplementation with the *B. vulgaris* agrowaste extract on the soil bioremediation process, was promising. It was observed that by supplementing the soil with the *B. vulgaris* agrowaste extract, could have served as a cometabolic substrate, thus enhancing the remediation process– an approach that has been reported for enhanced culture performance during the bioremediation of soil contaminated with organic compounds such as PAHs (Moscoso et al., 2012; Reda 2009). The presence of other easily metabolizable PAHs, that is, phenanthrene or other LMW PAHs, can reduce bioremediation rates. However, in such a situation, optimization of culture parameters may be required to control the microbial metabolic activity in order to avoid cell proliferation at the expense of bioremediation (Brinch et al., 2002). In a study of BaP degradation by *Lasiodiplodia theobromae* that lasted for 35 days, the presence of Phe in the culture was found to inhibit BaP degradation since phenanthrene, being a LMW PAH, is easier to metabolize (Wang et al., 2014). If the experiment had lasted longer, the microorganisms may adjust to the nutrient-limiting conditions and thus metabolize B(a)P. Usually, in a culture medium, the tendency is for the microorganisms to first metabolize a readily-accessible substrate and, under the deficiency of certain nutrient elements, such as nitrogen, the organisms can therefore manipulate their metabolic pathways by producing surface active agents extracellularly to solubilize the non-readily available substrate.

Bioremediation kinetic rates for PAHs in soil

As observed in the results shown in Table 1, *B. licheniformis* STK 01, as well as its supplementation with biosurfactant, demonstrated higher bioremediation levels for most of the PAHs studied than the other cultures. Hence, biosurfactant-supplemented cultures were used to study the reduction levels of PAHs with time, as shown in Table 2.

From the bioremediation profiles of the contaminants

Table 2. Reduction levels of PAHs with time by *B. licheniformis* STK 01 with and without biosurfactant supplementation, with periodic sampling.

Organism/days		Bioremediation (%)								
		3	8	15	21	28	35	42	50	60
Bacillus licheniformis STK 01	Phe	7.10	17.17	54.68	66.48	81.86	84.60	93.36	96.81	97.44
	Py	6.88	4.36	34.47	53.08	55.14	63.36	70.93	78.12	89.12
	BaA	7.45	8.25	51.84	56.05	66.40	67.54	73.23	74.02	76.03
	BaP	5.82	6.85	26.05	32.47	52.54	57.33	70.34	82.36	83.05
Bacillus licheniformis STK 01 with Biosurfactant	Phe	1.55	14.23	30.89	46.30	67.46	83.69	96.78	98.31	100
	Py	4.65	7.90	18.38	25.91	41.43	76.18	82.99	91.82	95.32
	BaA	9.85	12.24	29.86	56.58	65.81	70.76	78.80	81.90	82.71
	BaP	6.79	7.53	21.32	38.49	40.04	43.65	66.47	85.39	86.17

Phe– Phenanthrene, Py– pyrene, BaA– benz(a)anthracene, BaP– benzo(a)pyrene.

(Figures 1 and 2), a brief lag phase was noticeable up to day 7, prior to a decrease in concentration of the PAHs in the soil. Moreover, the profiles showed that most of the PAHs were sequestrated between days 7 and 40. For example, about 70% of Phe was reduced within the first 21 days (Figure 1a) while the BaA and BaP reached an equilibrium only after 50 days (Figures 1 and 2). As the concentration of the contaminants decreased, sorption into soil intrapores increased, causing the remaining fractions to become less bioavailable. This phenomenon is common in PAH-bioremediation studies; an observation particularly associated with HMW PAHs, as equally observed for B(a)A and B(a)P in this report. Furthermore, for B(a)P (Figure 2d), the bioremediation process seemed to undergo different phases– a seemingly stationary phase followed by a first-order decrease in PAH concentration between day 10 to 20 and between day 35 to 50, respectively. This may be attributed to the biosurfactant supplementation which seemed to increase the bioavailability of the contaminant from the soil matrix in a discrete form.

By mass balance analysis and also considering the periodic determination of the concentration of PAHs in the control experiment, the percentage removal rate for each of the contaminants in soil after the experiment after 60 days was 97.44, 89.12, 76.03 and 83.06% for Phe, Py, BaA and BaP respectively, for cultures without biosurfactant supplementation. Furthermore, the addition of biosurfactant slightly enhanced the degradation levels to 100, 95.32, 82.71 and 86.17% for Phe, Py, BaA and BaP, respectively. In all the studies, abiotic loss of PAHs, as determined in the uninoculated control flasks, was in the range of 4 to 10%. Hence, the overall concentration of PAHs obtained over the study period in the inoculated flasks was basically due to bioremediation by *B. licheniformis* strain STK 01. The effect of adsorption of PAHs to bacterial biomass on the overall loss may be considered to be negligible since the extraction solvent was added directly to the soil/culture samples. Therefore, the solvent was expected to solubilize both residual PAHs in solution and those adsorbed on to the biomass, as sonication is well known to effectively lyse biomass.

Previous studies on the kinetics of the bioremediation of PAHs in soil have reported results comparable to some of the results presented here. A study by Acevedo et al. (2011) on the rate kinetics of PAH reduction in soil for 60 days, reported that most PAH compounds studied were degraded within 14 to 35 days, while 75 and 60% degradation was achieved for Py and BaP respectively. Lors et al. (2012) also investigated the degradation kinetics of 16 PAHs in soil for 200 days and observed that the highest rates occurred in the first two months for most of the PAHs, with the LMWs being degraded within 7 to 34 days. In the same study, an average of 90% reduction was reported for most PAHs studied, with 85 and 35% being recorded for the 4- and 5- ring PAHs, respectively. For this study, the range was high (85%) for Phe and low (44%) for BaP, after 35 days. Table 3 shows the summary of some of the studies that have reported significant degradation of PAH compounds in soil environment.

It was also observed from the analyses that the rate kinetics showed that the soil containing phenanthrene was rapidly remediated both for cultures without biosurfactant (k = 0.0620 day^{-1}) and with biosurfactant (k = 0.0664 day^{-1}) as compared to the rate constants determined for Py, BaA and BaP (Figures 3 and Table 4). The rate constant values obtained in the culture with biosurfactant supplementation were in the order: $k_{phe} > k_{py} > k_{BaA} > k_{BaP}$, while for the culture without biosurfactant addition, the order observed was $k_{phe} > k_{py} > k_{BaA} = k_{BaP}$.

Conclusion

The bacterial isolates, *B. licheniformis* STK 01, *B. subtilis* STK 02 and *P. aeruginosa* STK 03 used in this study,

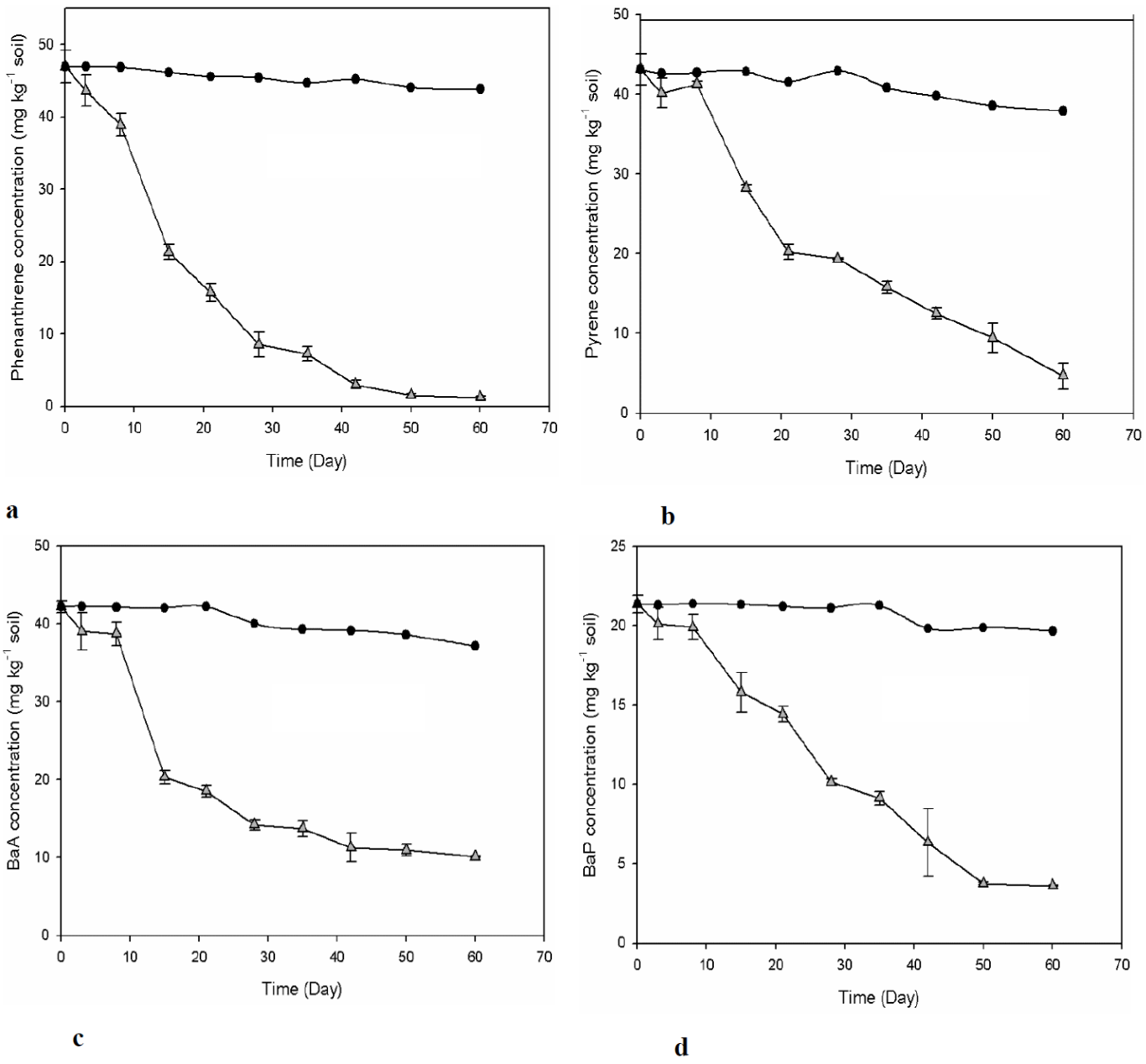

Figure 1. Concentration profile of (a) phenanthrene, (b) pyrene, (c) benz(a)anthracene and (d) benzo(a)pyrene for *Bacillus licheniformis* STK 01 cultures without biosurfactant supplementation. Error bar represents the standard deviation of three replicate determinations. Solid circles – control.

was able to remediate silty soil contaminated with PAHs (Figure 3). A high-remediation capability was observed for all the cultures studied, with the highest being 100% for phenanthrene, 95.32% for pyrene, 82.71% for benz(a)anthracene and 86.17% for benzo(a)pyrene. The results obtained showed that both Gram-positive and negative bacteria used were effective in remediating PAH contaminated soils particularly with enhanced mass transfer rates and bioavailability of the contaminants. Biosurfactant supplementation was found to significantly improve the remediation process of all the PAHs studied. On the other hand, culture supplementation with *B. vulgaris* agrowaste extract as a co-metabolic substrate in *B. licheniformis* STK 01 cultures enhanced removal rates for Phe, B(a)A and B(a)P. First-order reaction rate kinetics was found to fit the kinetic data well and analyses of the rate constant showed that phenanthrene degradation was the fastest both for cultures without biosurfactant (k = 0.0620 day^{-1}) and with biosurfactant (k = 0.0664 day^{-1}), in comparison with the rate constant

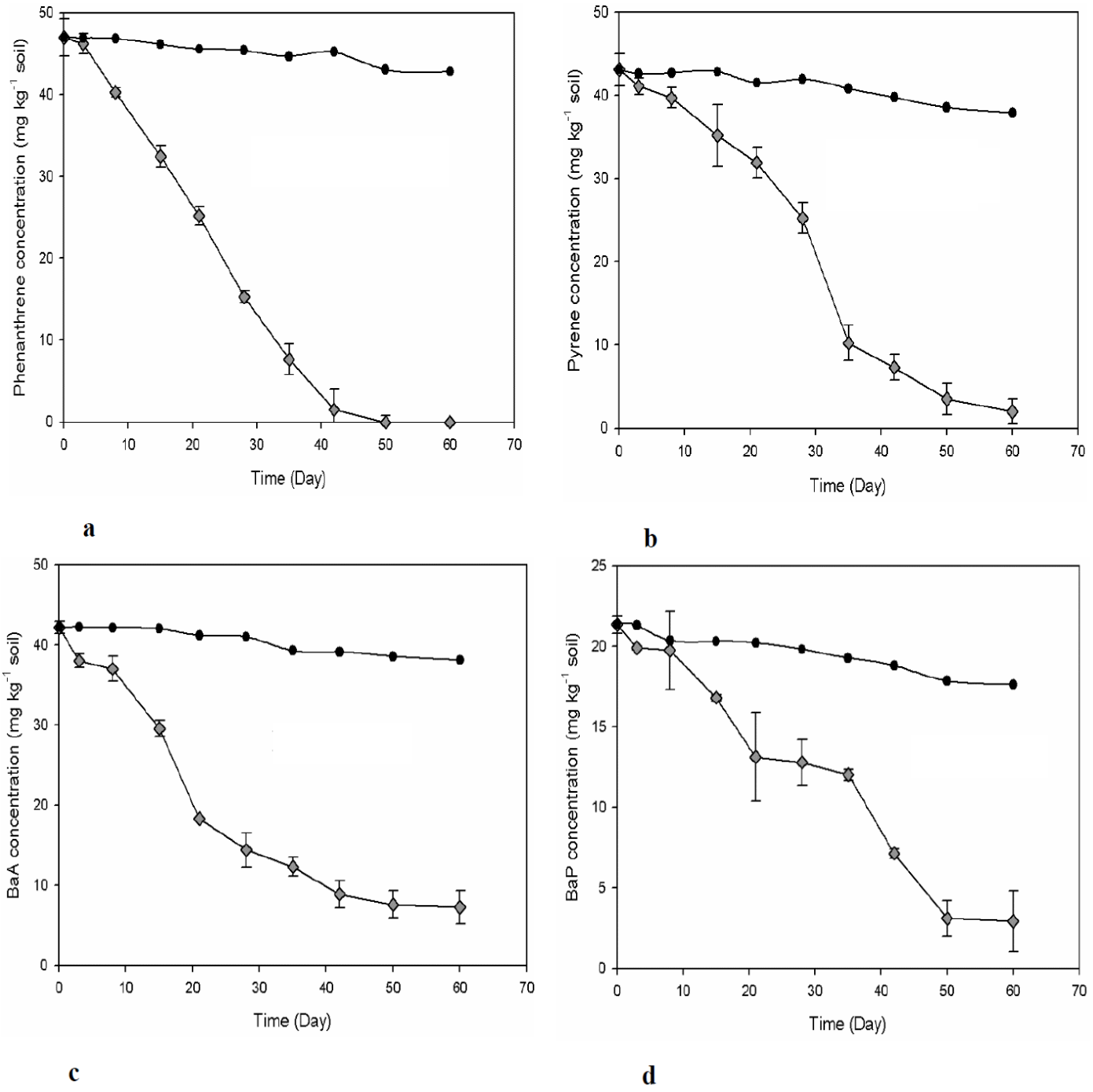

Figure 2. Concentration profile of (a) phenanthrene, (b) pyrene, (c) benz(a)anthracene and (d) benzo(a)pyrene for *Bacillus licheniformis* STK 01 cultures supplemented with a biosurfactant. Error bars represents the standard deviation of triplicate determinations. Solid circles – control.

determined for pyrene, benz(a)anthracene and benzo(a)pyrene. For further investigations, the effects of structural symmetry of PAHs on bioremediation together with the effects of micellar core solubilization and pre-micellar surface activity on the bioremediation kinetics of PAHs is recommended.

Abbreviations

PAHs, Polycyclic aromatic hydrocarbons; **BaP,** 5-benzene ring PAH; **LMW,** low molecular weight; **HMW,** high molecular weight; **rDNA,** ribosomal deoxyribonucleic acid; **SPE,** solid phase extraction; **HEX,** hexane; **DCM,**

Table 3. Summary of studies with significant degradation of PAHs in soil environment.

Microorganisms (with or without surfactant augmentation)	Soil media/initial concentration of PAHs	PAHs removed	Degradation level (%)	Experimental period (days)	References
B. licheniformis STK01	PAH contaminated soil	PHE, PY, BaA, BaP	100, 95.32, 82.71, and 86.17 respectively	60	This study
Peniophora incarnata KUC8836 *Mycoaciella bispora* KUC8201	Creosote-contaminated soil with an initial concentration of 229.49 mg kg^{-1} PAHs	PHE, FLUT, PY ANT	86.5, 77.4, 82.6, and 61.8 respectively	14	Lee et al. (2015)
Pseudomonas aeruginosa SP4	PAH contaminated soil (initial conc. 500 mg/kg soil)	PY	84.6	-	Jorfi et al. (2013)
Sphingobacteria and *Proteobacteria*	Soil contained 9362.1 µg kg^{-1} of USEPA priority PAHs	USEPA priority PAHs	20.2 - 35.8	56	Mao et al. (2012)
White-rot fungi *Trametes versicolor*	Contaminated soil (initial concentration - 1g of total PAHs/kg dry soil)	FLU, PHE, ANT, PY, BaA, CHRY	89	30	Sayara et al. (2011)
Acidovorax and *Sphingomonas* genera	PAH contaminated soil-contained 64% sand, 30% silt and 6% clay	NAPH, ACE, FLU, PHE, ANT, FLUT, PY, BaP, CHRY, BbF, BkF, BaP, DBahA, BghiP	76	140	Singleton et al. (2011)
Anthracophyllum discolor	PAH contaminated soil	PY BaP	60 75	60	Acevedo et al. (2011)
Enterobacteria and *Pseudomonas* genera	PAH contaminated soil	2-, 3- and 4-ring PAH concentrations	98, 97 and 82 respectively	180	Lors et al. (2010)
Pseudomonas fluorescens	soil contaminated with PAHs	PY	98	10	Husain (2008)

NAPH– Naphthalene, ACE– acenaphthene, FLU– fluorene, PHE– phenanthrene, ANT– anthracene, FLUT– fluoranthene, PY– pyrene, BaP benz[a]anthracene, CHRY– chrysene, BbF– benzo[b]fluoranthene, BkF– benzo[k]fluoranthene, BaP– benzo[a]pyrene, DBahA– dibenz[a,h]anthracene, BghiP– benzo[g,h,i]perylene.

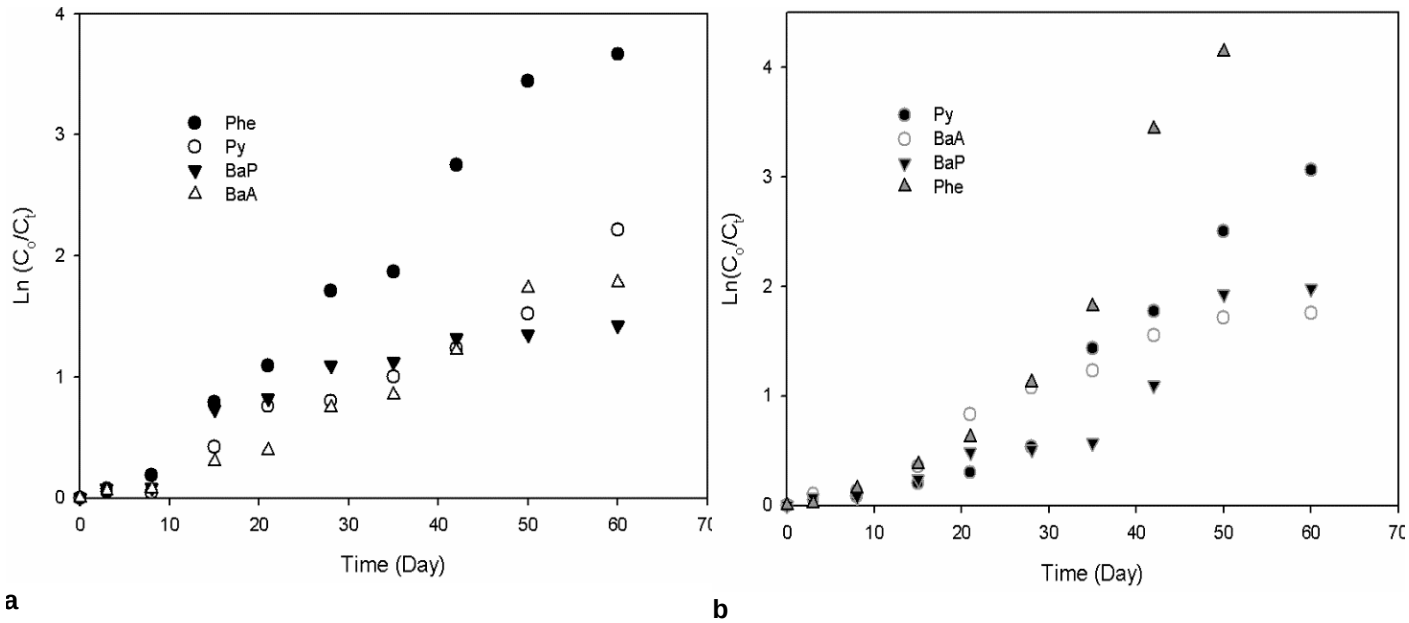

Figure 3. Linearized plot of first-order degradation kinetic model for (a) *B. licheniformis* STK 01 and (b) *B. licheniformis* STK 01 supplemented with biosurfactant.

Table 4. PAH-reduction rate constants and regression determining coefficients.

PAH compounds	*B. licheniformis* STK 01		*B. licheniformis* STK 01 supplemented with biosurfactant	
	k (day^{-1})	R^2	k (day^{-1})	R^2
Phenanthrene	0.0620	0.9759	0.0664	0.8382
Pyrene	0.0332	0.9602	0.0432	0.9208
Benz(a)anthracene	0.0290	0.8724	0.0292	0.8647
Benzo(a)pyrene	0.0291	0.9496	0.0272	0.8387

dichloromethane; **GC-FID,** gas chromatography-flame ionisation detector; **NCBI,** National Center for Biotechnology Information.

Conflict of Interests

The authors have not declared any conflict of interest.

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