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IMPROVING BIODEGRADATION OF BENZO(GHI)PERYLENE IN SOIL: EFFECTS OF BACTERIAL CO-CULTURE, AGROWASTE AND BIOSURFACTANT SUPPLEMENTATION

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Abstract: The feasibility of achieving high biodegradation of benzo(ghi)perylene (BghiP), one of the most recalcitrant and carcinogenic PAHs, was investigated in soil samples. Microorganisms used were *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03, with *Bacillus licheniformis* STK 01 being the primary B(ghi)P biodegrader. The effects of co-culturing the isolates, biosurfactant augmentation, and using phenanthrene (Phe) and *Beta vulgaris* as co-metabolic substrates were investigated in a 60 day trial experiment. B(ghi)P concentrations were determined by a GC-FID while degradation levels were estimated by mass balance analysis. At the end of the experiment, 52.70%, 40.50%, and 58.36% B(ghi)P were degraded by *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03 respectively, in mono-septic cultures without supplementation. However, the co-culturing of *B. licheniformis* and *B. subtilis* improved the degradation of B(ghi)P to 60.76%, *B. licheniformis* supplementation with *Beta vulgaris* waste increased the degradation to 58.36%, whereas biosurfactant addition to *B. licheniformis* increased the degradation to 60.90%. Moreover, B(ghi)P degradation kinetics observed for another 60 days, using *B. licheniformis* culture with biosurfactant supplementation, showed a further increased to 61.37%. Overall, the biological systems used, achieved a significant degradation efficiency of B(ghi)P in all the cultures studied, while first-order rate kinetics succinctly described the experimental kinetic data (R = 0.9878).

Keywords*:* Biodegradation, Biosurfactant, Environmental toxics, PAHs, soil.

1. INTRODUCTION

Persistent organic pollutants, such as high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAHs) and their derivatives, are often resistant to biodegradation. Their hydrophobicity restricts their bioavailability, reducing their biodegradation in the environment. The sources of these contaminants are natural as well as anthropogenic (Harvey 1998; Wick et al., 2011). Owing to the hydrophobic nature, soil and sediments are often their repositories in the environment, making them more resilient to biodegradation. The solubility of PAHs in water as well as in organic solvents varies depending on their molecular weight, structural orientation, type of solvent, and the octanol-water partition coefficient (Amodu et al., 2013). Sixteen of these contaminants had earlier been identified as priority and recalcitrant environmental contaminants by the Environmental Protection Agency (EPA), (USEPA, 1999) among which benzo(ghi)perylene (BghiP) has the highest number of clustered benzene rings, thus its classification as a Heavy Molecular Weight (HMW) PAH. Generally, as the number of benzene rings in a PAH compound increases, solubility decreases, causing increased sequestration and difficulty in obtaining significant biodegradation (Wick et al., 2011; Wild & Jones 1995). Consequently, few studies have reported significant findings on the biodegradation of B(ghi)P in soil, particularly, soil

with a high percentage of silt and clay.

Although several techniques have been used to treat PAHs contaminated samples, biodegradation is often considered environmentally benign and less invasive. Techniques such as extraction, surfactant washing, and adsorption/biosorption have been used, either alone or as pre-treatments to biodegradation (Chang et al., 2004; Kaya et al., 2013; Lau et al., 2014; Song et al., 2012; Wang et al., 2007; Yang et al., 2013). In circumstances whereby they are used inclusively, the contaminants are only transferred from one medium to another, instead of reducing them to innocuous end-products. On the other hand, rather than using soil washing as a pre-treatment process prior to biodegradation, a less invasive approach on the environment, which may be amenable to field application, is more promising.

Furthermore, numerous PAH-degrading microorganisms have been identified, particularly *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp., and *Acinetobacter* sp., (Boonchan et al., 2000; Dandie et al., 2004; Ghosh et al., 2014; Mishra & Singh 2014), with significant degradation being reported for 3-, 4- and 5-clustered benzene rings. Although earlier studies had indicated that most autochthonous bacteria, especially the gram-positives, may not be able to infiltrate the intraparticle pores of soil grains to access the sequestered contaminants (Alexander, 1977; Lawrence et al., 1979), novel microbial isolates and biological systems are now being developed to circumvent this challenge. These bacteria are considered relatively larger than the mean diameter of soil grain pores, which perhaps explains the reason for the increased accumulation of the contaminants in solid particulates. However, gram-negative bacteria, owing to their thin cellular membrane, are favoured to enhance the mass transfer of PAHs across the cellular membrane and degrade PAHs better in soil samples (Ma et al., 2013).

Generally, the rate of PAHs biodegradation is conceptually controlled by the following steps: desorption of the contaminants from the soil matrix to the aqueous phase; mass transfer of the desorbed contaminants to become microbially accessible; and microbial uptake and transformation (Reda, 2009; Reid et al., 2000; Semple et al., 2003). The overall rate can be limited by any of these steps, as none could be considered a rate determining step. Again, since only the fraction of PAHs that is available in aqueous phase is considered to be bioavailable for microbial uptake (Kwon et al., 2009; Yang et al., 2010), a number of methods have been used to enhance their desorption, migration and solubility. Prominent among the methods is the use of biosurfactants, coupled with identification of prolific and genetically evolved microbial species (Chaudhary et al., 2011; Lu et al., 2014; Mishra & Singh, 2014; Moscoso et al. 2012). In the environment, microorganisms putatively synthesize exogenous materials to enhance the solubility of hydrophobic contaminants through emulsification, under the limiting conditions of certain essential microelements, such as nitrogen (Fontes et al., 2012; Glick et al., 2010; Zhao et al., 2015). Another approach that has been applauded to enhance the biodegradation of PAHs and for its limited ecosystem disturbances is contaminant co-metabolism (Moscoso et al., 2012; Reda, 2009). With this approach, substantial degradation rates have been reported for some PAHs. Nonetheless, the degradation of B(ghi)P - a six benzene ring PAH, is barely reported.

In our previous study, prolific biosurfactant producing bacterial isolates were augmented with suitable agro waste to achieve significant biodegradation of pyrene, benz[a]anthracene and benzo[a]pyrene (Amodu et al., 2016). This study, therefore, investigates the ability of the isolates – *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03 to degrade B(ghi)P. In addition, the effects of biosurfactant supplementation, microbial co-culture and substrate/contaminant co-metabolism (using phenanthrene and *Beta vulgaris* waste) on the biodegradation of B(ghi)P was also conducted. Finally, the rate of degradation of B(ghi)P was evaluated by assuming first order kinetics.

2. MATERIALS AND METHODS

2.1. Microorganism and chemical reagents

The microorganisms used - *Bacillus licheniformis* STK 01 (KR011152), *Bacillus subtilis* STK 02 (KR011153) and *Pseudomonas aeruginosa* STK 03 (KR011154) were isolated from wood chips, coal tar, and an oil spill site, respectively, as reported in our previous work (Amodu et al., 2014). Phenanthrene (Phe) and B(ghi)P were obtained as certified reference materials; hexane, dichloromethane, acetonitrile, and anhydrous sodium thiosulfate as analytical grade chemicals (>98% purity), all from Sigma Aldrich (Germany). A C-18 Solid Phase Extraction (SPE) glass cartridge (0.5 g solid phase) was purchased from SUPELCO (Bellefonte, USA).

2.2. Sample preparation and PAHs biodegradation

All experiments were conducted with a model

soil composed of 30% clay, 20% silt, 20% fine, and 30% coarse sand, which was classified as silty soil based on the United Soil Classification system and the American Society for Testing and Material method (ASTM method DIN-4188). Two hundred grams of the soil was autoclaved at 121°C for 30 min, and left to cool to ambient temperature before spiking with Phe and B(ghi)P (40 mg PAH/Kg soil), as described by Brinch et al., (2002). The spiking was done aseptically to minimize microbial contamination of the soil. For each experiment, 10 g of contaminated soil was weighed into 100 mL Erlenmeyer flasks with a glass weighing boat, and seeded with overnight grown cultures of the bacterial species in nutrient broth (8%, v/w). The viable cell count was determined to be 10^8 CFU mL^{-1}, using a Quebec Darkfield Colony Counter. All flasks were incubated at 37°C in a dark static incubator. The inoculums comprised: cultures of each of the isolates without supplementation with neither biosurfactant nor *B. vulgaris* extract; a culture of *B. licheniformis* supplemented with crude biosurfactant; a culture of *B. licheniformis* augmented with dry milled *B. vulgaris* waste (5%, w/w); and a co-culture of the two *Bacillus* strains, i.e., *B. licheniformis* STK 01 and *B. subtilis* STK 02 (without augmentation).

In order to ascertain the degradation levels of B(ghi)P and Phe, and the recoverability efficiency of the extraction method, the concentration of the PAHs was quantified prior to and post biodegradation experimentation. A moisture holding capacity of 60% was maintained as previously described by Acevedo et al., (2011). The holding capacity was ensured by supplementing each experiment with 5 mL sterile distilled water per 200 g soil being supplemented in individual flask periodically at 10 days interval. Control experiments were prepared following similar procedures but without an inoculum, in order to account for the loss of PAHs due to abiotic factors. These experiments were monitored for 60 days. Each experimental set-up was carried out in triplicates.

2.3. PAHs extraction, clean-up and quantification

After 60 days of incubation, samples were extracted with 20 mL of hexane in 100 mL amber bottles using a sonicator. The sonication bath was operated at 25°C batchwise for 20 min, with periodic swirling to minimize sedimentation. Extraction aliquots were then pooled and centrifuged at 5000 rpm for 10 min. SPE cartridge was preconditioned, prior to sample clean-up, with sodium thiosulfate (10 g) as well as hexane and dichloromethane (30 mL of each). The reference method used was EPA's Method 610 (1984). The extracts obtained from the centrifugation were passed through the pre-conditioned SPE cartridges, while 7.5 mL of hexane and dichloromethane were used to elute the PAH analytes from the solid phase. The eluate collected was rotary evaporated, while the residue was reconstituted in dichloromethane (1 mL) in an amber vial. The PAHs were analysed using gas chromatography equipped with a flame ionization detector (GC-FID).

The method used for analysis was similar to that reported in Amodu et al., (2016). All samples were analysed using a GC (7890A Agilent Technologies, CA, USA) instrument with an auto sampler, equipped with a flame ionization detector, and a capillary column USB499114H (20 m x 180 μm x 0.14 μm). The oven temperature was maintained at 170°C, which was periodically ramped up (5°C min^{-1}), reaching 300°C, with each ramping step maintained for 3 min. Subsequently, the temperature was further increased to 310°C, and maintained for a further 5 min. The carrier gas used was nitrogen, while samples were injected split-wise at a temperature of 250°C. Additionally, a 6 min post run time was allowed to clean the column prior to subsequent injections, making a total run time of 36 min. The same extraction and clean-up procedure was carried out to quantify PAHs in flasks not inoculated, which served as the control experiment.

2.4. Kinetics of PAHs biodegradation in agitated cultures

Furthermore, of all the cultural set-ups mentioned earlier, *B. licheniformis* culture supplemented with biosurfactant gave the highest degradation level; hence, the degradation experiment was repeated for another 60 days, using this culture to evaluate the biodegradation kinetics. Similarly, the soil was spiked with 50 mg of Phe and 25 mg of B(ghi)P per kg soil, whereas microbial culture was prepared as described earlier. PAH contaminated soil (50 g) was transferred into 250 mL sealed Erlenmeyer flasks, and incubated in a dark shaken incubator at 43 ± 2°C and 180 rpm for 60 days. The optimum growth temperature for *B. licheniformis* STK 01 as well as for biosurfactant production was previously reported (Amodu et al., 2014). To generate a biodegradation kinetic profile, a mass balance analysis was adopted, following a periodic sampling regime. Prior to sampling, the flask was swirled gently to ensure homogeneity and to avoid PAHs sticking to the wall of the flask. Again, control experiments were set-up in a similar fashion, but without seeding the flasks with inoculum. All experiments were carried out in

triplicates. By assuming first order kinetics (Eq. 1), the rate of biodegradation can be determined;

$$-\frac{dC}{dt} = kC \qquad (1)$$

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

3. RESULTS AND DISCUSSION

3.1. B(ghi)P biodegradation

The results obtained for the degradation of B(ghi)P by *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03 are presented in table 1, whereas table 2 shows the degradation of B(ghi)P by *B. licheniformis* STK 01 with and without biosurfactant supplementation. In all the studied cultures, degradation ranged from 83.97 to 96.88% for Phe and from 40.50 to 60.90% for B(ghi)P.The results obtained show that *B. licheniformis* metabolized Phe (91.43%) more than the other two isolates in the mono-septic cultures, while *P. aeruginosa* performed better in degrading B(ghi)P. Meanwhile, the degradation level was slightly lower (90.34%) with the co-culture of *B. licheniformis* and *B. subtilis*, suggesting that *B. subtilis* may have repressed the metabolic pathway for Phe mineralization by *B. licheniformis*. Such repressive activities on metabolic pathways has been reported (Kuppusamy et al., 2016; Seo et al., 2009; Zhang et al., 2015). However, the co-culture led to a synergistic effect in the degradation of B(ghi)P, as shown in table 1. Similarly, the supplementation of *B. licheniformis* with biosurfactant enhanced the degradation of B(ghi)P from 52.73 to 60.90% compared to the mono-cultures without supplementation. Previous studies have reported on the applications of biosurfactant for enhanced degradation of PAHs in soil. Husain (2008), for example, reported that a rhamnolipid biosurfactant enhanced the biodegradation of pyrene, from 91 to 98%, after 10 days of bioremediation – one of the highest ever reported for pyrene. Also, Jorfi et al., (2013) observed that the supplementation of PAH degradation cultures with a biosurfactant, enhanced the biodegradation of pyrene from 59.8 to 84.6%, in an artificially contaminated soil.

Although co-metabolism had been reported as an enhancement for the biodegradation of PAHs (Moscoso et al., 2012; Reda, 2009), few studies have attempted to use solid agro waste. *B. vulgaris*, which had previously been identified as a suitable substrate for microbial growth and synthesis of biosurfactant (Amodu et al., 2014), was also found in this study to enhance the degradation of B(ghi)P from 52.73 to 58.36%. To the best of authors' knowledge, the use of *B. vulgaris* to enhance the degradation of PAHs, particularly the highly hydrophobic and recalcitrant B(ghi)P, is been reported for the first time in this study. Occasionally, with the availability of a more soluble co-substrate, a competitive substrate mineralization may occur which can lead to a low removal efficiency of the target contaminant.

The results obtained show that the availability of co-metabolic substrates has the capacity to influence PAHs biodegradation. In an experiment, which lasted for 35 days, Wang et al., (2014) reported that the availability of Phe reduced the biodegradation efficiency of benzo(a)pyrene (BaP).

In this study, Phe was easily metabolized, being a LMW PAH. However, microorganisms can alter their metabolic pathways by producing certain exogenous products to favour the degradation of the target contaminants, when the readily available substrate become exhausted or under the limiting conditions of certain essential elements (Fontes et al., 2012; Glick et al., 2010; Zhao et al., 2015). Moreover, under such conditions, surface active compounds are

Table 1. Bacterial degradation of PAHs in mono-septic cultures with agro-waste and biosurfactant supplementation

Mono-septic cultures									
PAHs	*B. licheniformis*			*B. subtilis*			*P. aeruginosa*		
	C_i	C_f	$\%R_{bd}$	C_i	C_f	$\%R_{bd}$	C_i	C_f	$\%R_{bd}$
Phe	38.20	3.28	91.43	34.03	5.16	84.83	34.21	5.79	83.97
B(ghi)P	32.44	15.34	52.73	32.52	19.35	40.50	26.59	11.06	58.42
Co- and augmented cultures									
PAHs	*B. licheniformis & B. subtilis*			*B. licheniformis & B. vulgaris*			*B. licheniformis &* 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
B(ghi)P	35.60	13.97	60.76	37.43	15.58	58.36	33.87	13.24	60.90

%R*bd*- percentage biodegradation, Ci – initial concentration (mg/L), Cf – initial and final concentration (mg/L)

produced to facilitate the desorption of sorbed contaminants from the particulate matrix to become available for microbial degradation. A similar phenomenon was observed in this study; the biodegradation of B(ghi)P became more significant after day 42 when Phe-limiting condition became noticeable.

3.2. Kinetic study of PAHs degradation

The degradation kinetic profiles for Phe and B(ghi)P by *B. licheniformis* STK 01 (Figs. 1 and 2) show the positive effect of biosurfactant supplementation. Also the positive effects of biosurfactant addition on the biodegradation of B(ghi)P degradation by *B. licheniformis* STK 01 can be observed in table 2.

For B(ghi)P biodegradation, a lag phase, for about 7 days, was noticeable, whereas such was not observed for Phe. This was due to the relatively higher solubility of Phe compared to that of B(ghi)P. Furthermore, the degradation profiles showed that a high percentage of the contaminants was degraded between day 7 and 40. For instance, within the first 21 days, about 70% of Phe was degraded (Fig. 1). For the non-supplemented cultures, degradation rates determined for Phe and B(ghi)P after the 60-day experiment were 97.44% and 51.58%, respectively. However, biosurfactant supplementation significantly enhanced the biodegradation of B(ghi)P to 61.37% (Table 2 & Fig. 2).

Comparable results have previously been reported for PAHs biodegradation in soil. In a 60 day trial experiment of PAHs biodegradation by Acevedo et al., (2011), it was discovered that most PAHs studied were degraded within 14 to 35 days; whereas 60% degradation was achieved for Py, 75% was reported for BaP.

Table 2. Percentage PAHs degradation by *B. licheniformis* STK 01 with and without biosurfactant augmentation

Microorganism	% Biodegradation									
	PAH/Day	3	8	15	21	28	35	42	50	60
B. licheniformis STK 01	Phe	7.10	17.17	54.68	66.48	81.86	84.60	93.36	96.81	97.44
	B(ghi)P	3.68	9.50	16.49	22.72	28.87	34.54	39.87	45.44	51.58
B. licheniformis STK 01 with biosurfactant	Phe	1.55	14.23	30.89	46.30	67.46	83.69	96.78	100	100
	B(ghi)P	1.28	7.45	20.40	27.60	34.86	41.33	47.26	57.63	61.37

Table 3. PAH degradation rate constants and regression determining coefficients

PAHs	***B. licheniformis* STK 01**		***B. licheniformis* STK 01 supplemented with biosurfactant**	
	k (day $^{-1}$)	R^2	k (day $^{-1}$)	R^2
Phe	$6.20\ 10^{-2}$	0.9759	$6.65\ 10^{-2}$	0.8382
B (ghi)P	$1.45\ 10^{-2}$	0.9502	$1.67\ 10^{-2}$	0.9878

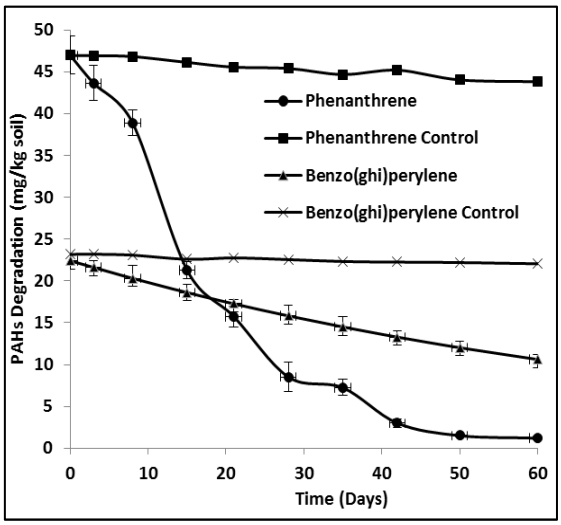


Figure 1. Biodegradation profile for Phe and B(ghi)P by *B. licheniformis* STK 01.

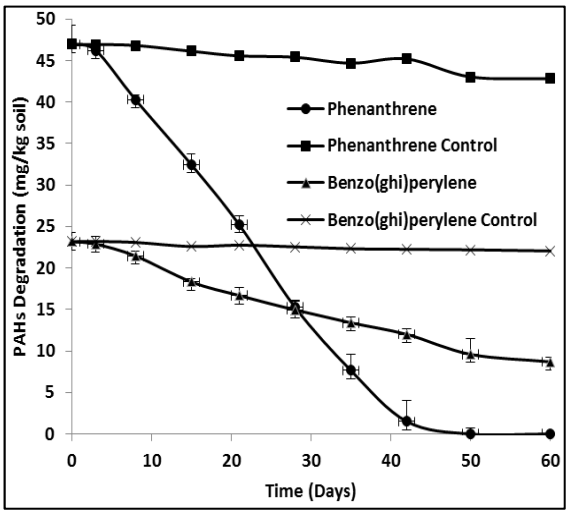
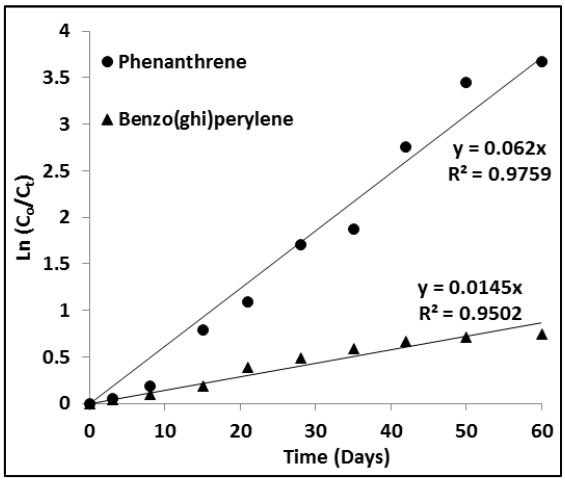


Figure 2. Biodegradation profile for Phe and B(ghi)P by *B.licheniformis* STK 01 with addition of biosurfactant.



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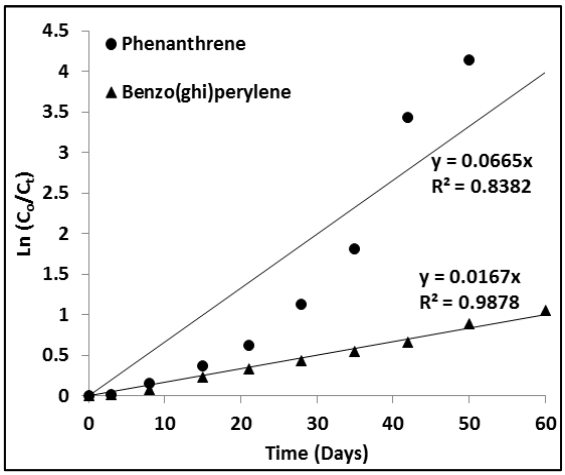

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Figure 3. First-order degradation kinetics for Phe and B(ghi)P by (a) *B. licheniformis* STK 01 and (b) *B. licheniformis* STK 01 and biosurfactant

Zhao et al., (2011) reported the positive effect of biosurfactants produced by *A. calcoaceticus* BU03 on the biodegradation of PAHs by *Bacillus subtilis* B-UM. Within the experimental period of 42 days, the degradation of Phe and BaP was significantly increased from 71.2 % and 16.4% to 83.8% and 68.3% respectively. Similarly, Lors et al., (2012) studied the biodegradation of 16 PAHs in soil, in a 200 day trial, and observed that most LMWs were degraded within 7 to 34 days. In addition, 85% and 35% degradation levels were recorded for the 4- and 5- ring PAHs. In all these studies, there was no reported attempt on the degradation of B(ghi)P. Hence, the biological systems deployed in this study proved to be novel in achieving, perhaps, one of the most significant degradation rates ever reported for B(ghi)P.

Furthermore, consequent on the assumptions that only the fraction of PAHs that is soluble in aqueous phase is available for biodegradation, first order kinetics (Eq. 1) becomes a suitable model to describe such a desorption-limited reaction rate (Kwon et al., 2009; Yang et al., 2010). By plotting the natural log of the ratio of initial concentration of PAH to its residual against time, the rate of biodegradation was evaluated, as shown in Figure 3 (a & b).

Expectedly, Phe biodegradation, being a LMW PAH and relatively less sorbed contaminant, was rapid in comparison to that observed for B(ghi)P.

The rate of degradation of PAHs, being governed by desorption and diffusion, had been reported to decrease with increasing molecular weight of the contaminants (Thiele-Bruhn & Brümmer, 2005; Wammer & Peters 2005). The degradation rates determined for Phe and B(ghi)P were 0.0620 and 0.0145 day^{-1} respectively (Table 3). However, the degradation became faster for both compounds – Phe (k = 0.0665 day^{-1}) and B(ghi)P (k = 0.0167 day^{-1}), when the culture was supplemented with biosurfactant.

4. CONCLUSION

The biodegradation of benzo(ghi)perylene, B(ghi)P, one of the most recalcitrant PAHs, was reported in this study. At the end of the 60 day trial experiment, 52.70%, 40.50%, and 58.36% B(ghi)P were degraded by *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03 respectively, in mono-septic cultures without supplementation. However, the co-culturing of *B. licheniformis* and *B. subtilis* improved the degradation of B(ghi)P to 60.76%, *B. licheniformis* supplementation with *Beta vulgaris* waste increased the degradation to 58.36%, while biosurfactant addition to *B. licheniformis* increased the degradation to 60.90%. But when the experiment was repeated for another 60 days, using *B. licheniformis* culture with biosurfactant supplementation, in order to assess the biodegradation kinetics, the level further increased to 61.37%.

Overall, the isolates showed novelty in achieving substantial degradation of B(ghi)P, whose degradation is rarely reported. Cultures supplementation with crude biosurfactants produced from a solid agro waste (*Beta vulgaris*) resulted into enhanced bioavailability and biodegradation of B(ghi)P. Also the co-culturing of the two *Bacilli* sp. showed a positive effect on the biodegradation of B(ghi)P. First-order rate kinetics were found to fit well the experimental kinetic data (R^2 = 0.9931) for B(ghi)P. Expectedly, the analyses of the rate constants showed that Phe degradation was faster than B(ghi)P's for all cultures.

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Declaration

Further, we declare that the work is a further extension of work published as: Amodu OS, Ojumu TV, Ntwampe SK (2016) Bioremediating silty soil contaminated by phenanthrene, pyrene, benz (a) anthracene, benzo (a) pyrene using *Bacillus* sp. and *Pseudomonas* sp.: Biosurfactant/*Beta vulgaris* agrowaste effects. Afr J Biotechnol 15:1058-1068.

Conflict of interest

The authors declare that there is no conflict of interest concerning this publication.

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