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DEGRADATION OF PYRENE IN SOIL AND *IN VITRO* BY A *BACILLUS LENTUS* STRAIN ISOLATED FROM AN ASPHALT PLANT SOIL SITE IN LAGOS, NIGERIA

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ABSTRACT

A bacterium isolated from an asphalt plant soil and identified as a strain of *Bacillus lentus* was tested *in vitro* and in sterilized and native soils for ability to survive and sustain pyrene degradation over a period of 63 days. The exponential growth rate *in vitro* was 0.049 d^{-1} and the doubling time 2.65 d. In the control flask without pyrene, organism density remained virtually constant. In sterilized soil seeded with *Bacillus lentus* LP32 pyrene concentration declined from 335.0 mg/kg to 8.56 mg/kg at the rate of 10.88 mg/kg/day and half-life of 5.67 days. Similarly, in the native soil seeded with organism there was a decline from 305.2 mg/kg to 8.58 mg/kg in 30 days at the rate of 9.89 mg/kg/day and half-life 5.82. The percentage pyrene degraded in both sterilized and native soils were similar, at 97.45% and 97.19% respectively. This study demonstrated the potential of *Bacillus lentus* LP32 to serve as seed for enhanced bioremediation of pyrene polluted soil.

Keywords : *Bacillus,* Biodegradation, Bioremediation, Gas chromatography, Pyrene

INTRODUCTION

Pyrene is a peri-condensed four-ring polycyclic aromatic hydrocarbon which is naturally found in petroleum and resulting from incomplete combustion of organic compounds. Its high molecular weight, clustered structure and consequent hydrophobicity and aqueous solubility of 0.135 mg/l make it poorly bioavailable, resistant to biodegradation and persistent in the environment (ATSDR, 1995; Cerniglia and Shuttleworth, 2002). Although not a carcinogen, it is a mutagenic and toxic compound used as a model for the study of metabolism of such first-class carcinogens as benzo(a) pyrene with which it shares molecular structure. It is equally a regulated contaminant at sites polluted with petroleum hydrocarbons (Kazunga and Aitken, 2000). Reports have shown that presence of pyrene in soil leads to increased uptake of sodium and calcium and decreased yield of some vegetable crops amongst other deleterious effects (Krzebietke and Sienkiewicz, 2010).

Previously documented to be degraded mainly by nocardioforme actinomycetes such as *Mycobacterium* species and *Rhodococcus* (Cerniglia, 1992), in the last decade and a half, pyrene has been shown to be degraded by bacteria cutting across Gram-positive and Gram-negative genera, including *Bacillus, Corynebacterium, Micrococcus, Pseudomonas, Sphingomonas, Stenotrophomonas, Alcaligenes, Achromobacter, Leclercia, Cycloclasticus and Burkholderia and Proteus* (Kanaly and Harayama, 2010; Obayori *et al.,* 2013b).

Bioremediation is a preferred method of reclamation of polluted sites compared with physical and chemical methods because of its environmental friendliness and relative cost-effectiveness (Vidali, 2001). Whereas most bioremediation techniques focus on biostimulation of autochthonous strains, studies have shown that at times such indigenous strains may lack the necessary gene battery and cocktail of enzymes for complete mineralization or carry out the process at unsatisfactory rates. Therefore, bioaugmentation approach using carefully selected single strains or consortium has been advocated. Reports have however shown disparities in the performance of bacterial isolates *in vitro* and in soil. Such disparities have been attributed in part to unfavorable interaction between the allochthonous strain and the autochthonous populations, and poor adaptation to inclement environmental conditions (Johnsen *et al.*, 2005). Survival of the inoculum in soil is a key

consideration, just as inoculation protocols and effect of indigenous community have come under scrutiny (Kastner *et al*., 1998).

We had previously shown that *Pseudomonas* strains isolated from hydrocarbon polluted Nigerian soils survived and degraded pyrene effectively when seeded into pyrene spiked agricultural soil (Obayori *et al*., 2013b). In this study, a *Bacillus* organism isolated based on its ability to degrade pyrene was assayed for survival and pyrene degradation in unsterilized (native) and sterilized soils.

METHODOLOGY

Chemicals

The pyrene used in this study was 99% pure, and product of Sigma-Aldrich Inc. St. Louis, Mo, USA. All other chemical reagents, except otherwise stated, were of the highest purity and product of BDH Poole, England. Nutrient agar and agar technical were products of Lab M Limited, United Kingdom.

Microorganism and culture condition

The isolation and characterization of the organism used in this study, *Bacillus lentus* strain LP32, had been described previously (Obayori, 2008; Opere *et al*., 2013). Axenic culture of the isolate was maintained on glycerol: nutrient broth (50:50) at -20 ºC. The purity of the isolate was checked by plating out on nutrient agar for colony morphology and staining for Gram reaction and gross cellular morphology. Ability to grow on pyrene was checked by plating out on pyrene coated mineral salt agar plate and observing for clearing zones around the colonies upon incubation (Kiyohara *et al*., 1982).

Stock culture was prepared by collecting several colonies growing on Luria Bertani agar with a sterile wire loop and dispersing the clump of cells in 5 ml of physiological saline (0.85%). This was then inoculated into a mineral salt medium containing pyrene (100 mg/L) as sole source of carbon and energy and grown with shaking (175 rpm) at ambient temperature (27 ± 2 ºC) to log phase. The mineral salt medium (MSM) described by Kastner *et al*. (1994) was used. It contained per litre: Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g and $MgSO_4$. $7H_2O$, 0.20 g. The pH of the medium for bacteria was adjusted to 7.2 and fortified with nystatin (50 μg/ml) to suppress fungal growth. Trace elements solution (1 ml per litre) described by Bauchop and Elsden (1960) was sterilized separately and added aseptically to the medium. The cells were harvested by centrifugation (7000 g; 10 minutes), washed in decreasing concentration of MSM twice and re-suspended in the same MSM to achieve approximately 10^8 cfu/ml cell population.

Growth of isolate in vitro

The kinetics of growth of *Bacillus* sp. LP32 on pyrene was evaluated in liquid medium by inoculation of 0.5 ml of suspension into 50 ml of MSM supplemented 100 ppm of pyrene as sole source of carbon and energy. Degradation of pyrene was monitored by growth and multiplication of cells, assessed by visual observation for turbidity and by plating out aliquots of dilutions from the flasks on nutrient agar at intervals of three days

Soil sample collection

Pristine soil samples were collected from an uncontaminated farmland at the Science Faculty, Lagos State University with oven sterilized mason's trowel. Soil was collected at a depth of 5 to 12 cm after clearing the soil surface of twigs, dried leaves, roots and other debris. The soil samples were collected in two glass jars each containing about 1 kg of soil; one of the samples was sterilized by autoclaving for 30 minutes followed by 24-hour incubation at 37 ºC three consecutive times.

Determination of soil physicochemical parameters

The soil physicochemistry was evaluated using standard analytical protocols described previously (Nelson and Sommers, 1982; AOAC, 1995). Parameters include pH, moisture, phosphorus, ammonium nitrogen, nitrate, organic carbon, and total hydrocarbon. Potassium and sodium were determined using flame atomic absorption spectrophotometry. Water holding capacity (WHC) of the soil was determined using the modified container capacity described by Jones *et al*. (2008).

Soil microcosm experiment

A method modified from Kastner *et al.* (1998) was used. Soil samples were divided into unsterilized (Native soil), and sterilized soil (SS). Each experimental set up consisted of 500 ml metal cup containing 100 g of soil. The experimental set up made use of four different types of soil and pyrene combinations, namely: Native soil (NS); Sterilized soil (SS); Native soil + pyrene (NSP); and sterilized soil + pyrene (SSP).

Spiking of soil sample

Pyrene (100 mg) was dissolved in 10 ml of acetone in a 100-ml conical flask. The acetone was vented off. After complete removal of acetone, 20 ml of distilled water was added to the flask and brought to boiling, to desorb the pyrene from the flask. The pyrene suspension was then divided into three equal portions and poured onto triplicate soils in the metal cup and thoroughly mixed using a sterilized glass rod to give approximately 33.3 mg/100 g of soil (equivalent to between 305 mg/kg and 335 mg/ kg. The exact amount in each cup was obtained after extraction and gas chromatographic determination as described below.

Inoculation and evaluation of pyrene degradation in soil

Evaluation of pyrene degradation in soil microcosm was carried out by inoculating triplicate 500 ml metal cups containing 100 g of soil sample each, with suspension of washed cells. The metal cups were covered with aluminum foil and incubated at room temperature (27±2 ºC) in the dark for 62 days. Thus NS + P + organism and SS + P + organism were obtained. In the soil microcosm experiment set up, determination of the total viable count of bacterial cells were carried out every 7 days by weighing out 1g of soil sample from each of the triplicate metal cups representing a set up with one isolate. A serial dilution was done to 10^{-7} on each 1g of soil sample. Aliquots of the 7th dilution were plated out in triplicates and incubated at 27±2 ºC, in the dark (Kastner *et al.,* 1998). Samples were taken from microcosm set up on day 0, day 30 and day 60 to determine residual pyrene.

Extraction and determination of residual pyrene by gas chromatography

Extraction of residual pyrene was carried out by using 10 ml of dichloromethane as solvent with 10 g of soil as described below. Soil sample (10 g) measured into a conical flask and 10 ml of dichloromethane was added into the flask. The flask was shaken on a rotary shaker at 200 rpm for 30 minutes to desorb the pyrene from soil particles. After allowing the flask to stand for another 15 minutes, for soil particles to sediment, the sample was filtered afterward into a glass beaker. The sample was dried in a preheated oven, and then reconstituted to 1 ml in hexane.

Concentrated sample (1 µg) was injected into the Gas Chromatograph apparatus (Hewlett Packard 5890-series II) with flame ionization detector (GC-FID). The column was OV 101; thickness and width p80/100 mesh, stationary phase WHP 5%. Carrier gas was nitrogen; combustion gases were air/hydrogen, oven temperature was kept at 230 ºC, injector temperature was 200 ºC, detector temperature 200 ºC, holding time 2 minutes, and ramping rate 10 ºC/minute. Initial temperature was 70 ºC final temperature 320 ºC. The concentration of the standard and the peak area of the profile of the standard were then compared to that of the sample to get concentration of the hydrocarbons in the sample. The polycyclic aromatic hydrocarbon (PAH) data were fitted to the first order kinetics model of Yeung *et al.* (1997).

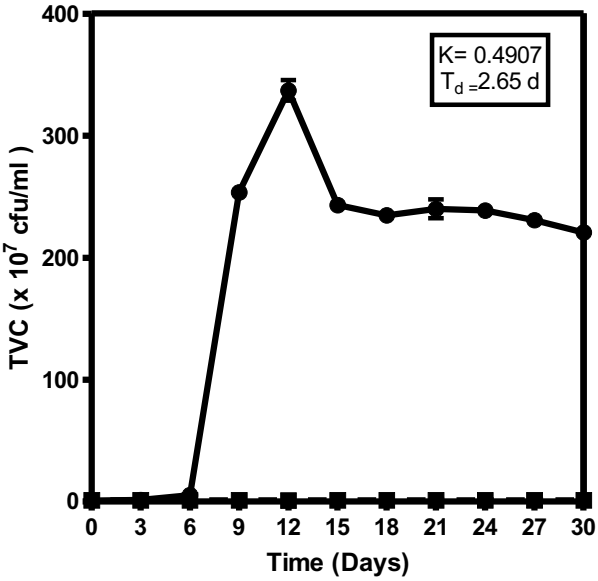

Figure 1: Growth profile of *Bacillus lentus* LP32 on MSM supplemented with pyrene as sole carbon and energy source. Experimental (●); Control (■); Growth rate (K); Doubling time (T_d)

Growth and survival of isolate in native and sterilized soils

The organism exhibited similar growth patterns on both native and sterilized soil microcosms (Figure 2). After an initial lag phase and slow growth within the first 7 days, from population density averaging x 10^7, peaks of organism densities of 1.22 x 10^9 and 1.21 x 10^9 were recorded in native and sterilized soils respectively on day 21. Organism densities dropped slightly in both treatments between day 21 and 28, then proceeded on a free fall to densities close to initial day 0 values.

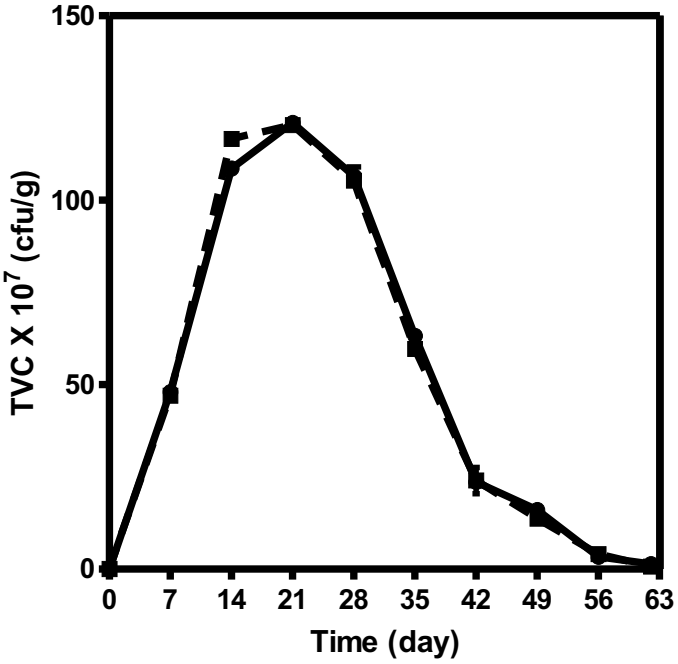

Figure 2: growth profile of *Bacillus lentus* in sterilized soil with pyrene (●) and native soil with pyrene (■)

Table 1: Physico-chemical parameters of soil used for microcosm study

Soil Parameter	Value
pH	7.02
WHC (%)	23
Potassium (mg/kg)	12.14
Sodium (mg/kg)	6.4
Phosphorus (mg/kg)	41.01
Nitrate (mg/kg)	27
Total organic carbon (%)	8.0
Total hydrocarbon (mg/kg)	54.29

Table 2: Kinetics of degradation of pyrene in soil

Treatment	Pyrene concentration (mg/kg)			*Percentage degradation (%)	Rate of degradation (mg/kg/day)	Degradation rate constant (/day)	Half life (day)
	Day 0	Day 30	Day 60				
SSP	335.30	310.86	305.07	7.26	0.84	0.0025	277.25
NSP	305.20	233.12	178.45	23.61	2.40	0.0090	77.00
SSPO	335.00	8.56	ND	97.45	10.88	0.1222	5.67
NSPO	305.20	8.58	ND	97.19	9.89	0.1191	5.82

SSP: Sterilised Soil +Pyrene; Native Soil+Pyrene; Sterilised Soil+Pyrene+Organism; Native Soil+Pyrene+Organism

Kinetics of degradation of pyrene in soil microcosms

The kinetics of degradation showed that in the sterilized soil with pyrene there was reduction in pyrene concentration from 335.30 mg/kg to 310.86 mg/kg (7.26% degradation), giving rates of degradation, degradation rate constant and half-life of 0.84 mg/kg/day, 0.00025/day and 227.25 days respectively within 30 days. Similar trend was observed in the native soil with pyrene alone, except that the corresponding values were 23.61%, 2.40 mg/kg/day, 0.0090/day and 77.00 days respectively. In the compartments seeded with organisms, there was rapid decline in pyrene concentration within 30 days. In sterilized soil with organism pyrene concentration declined from 335.0 mg/kg to 8.56 mg/kg at the rate of 10.88 mg/kg/day and half-life of 5.67 days, while in the native soil with organism there was a decline from 305.2 mg/kg to 8.58 mg/kg in 30 days with rate of degradation 9.89 mg/kg/day and half-life 5.82. The percentages degraded were similar, 97.45 % and 97.19% respectively.

DISCUSSION

The *Bacillus lentus* species strain LP32 used in this study is one of the few *Bacillus* species strains known to degrade pyrene. Most of the bacteria earlier known to degrade pyrene were high G+C Gram positive, especially nocardioforme

actinomycetes (Kanaly and Harayama, 2000). In the last one decade, however, there has been an increase in the number of low G+C Gram positive pyrene degraders, many of which are bacilli (Gaskin and Bentham, 2005; Das and Mukherjee, 2007; Khanna *et al*., 2011; Kumari *et al.,* 2013). Yuliani *et al.* (2012) isolated four *Bacillus* strains capable of degrading 99% of pyrene supplied in 30 days. Strain LP32 which was originally isolated on pyrene (Obayori, 2008) has been shown to show specificity for diverse hydrocarbons including cyclohexane and cyclohexanone both of which it degraded in pure culture up to 90 percent within 18 days (Opere *et al.,* 2013).

The increase in cell density of greater than two orders of magnitude observed for *Bacillus lentus* LP32 compares well with those previously reported in the literature. For instance, Klankeo *et al.* (2009) reported increases of 6.32 – 7.55 log cfu/ml and 6.32 – 8.05 log cfu/ml for *Diaphorobacter* sp. KOTLB and *Pseudoxanthomonas* sp. RN402 respectively when supplied with 100 ppm of pyrene in mineral salt medium, values that fell short of ours even when higher degradation of 90% was recorded by these authors. Similarly, strain LP32 exhibited greater increase in cell density than all the three pyrene-degrading *Pseudomonas* strains earlier reported from hydrocarbon polluted soils in Lagos, Nigeria (Obayori *et al.,* 2008), which showed increase in cell densities of less than two orders of magnitude. This finding suggests that not only is strain LP32 an efficient degrader of pyrene, it is also very efficient in conversion of the substrate to biomass using it as source of carbon.

In both sterilized and native soils spiked with pyrene population of *Bacillus lentus* sp. LP32 ranged between 10^7 and 10^9. This is in consonance with earlier findings which had established that organism concentrations of $10^7 – 10^8$ are sufficient to establish degradation activity in soil (Kastner *et al*., 1998). The fact that there was no significant difference between degradation rates in native and sterilized soils is attributable to lack of pyrene metabolic capability by the individual indigenous flora and lack of production of pyrene degradation promoting factors that could be of benefit to the seeded organism by the indigenous bacteria. This is further buttressed by lack of growth of individual indigenous flora on pyrene coated plates. Thus it would seem that the seeded organism was very efficient in colonizing the new environment and overwhelming the indigenous strains and rendering them irrelevant to the process of degradation. This is at variance with the report of Obayori *et al.* (2013a) in which higher degradation rate was observed in native soil seeded with consortium of pyrene degrading *Pseudomonas* spp. (87.65%) than in sterilized soil seeded with similar consortium (37.34% -50.30 %). Similarly, Kumari *et al.* (2013) reported 44% degradation of supplied pyrene (100 ppm) by native microbes within 28 days, but when the soil was seeded with pyrene degraders higher degradation percentages ranging from 75 % to 96% were recorded, while Ghaly *et al.* (2013) reported a mere 57.86% degradation in soil spiked with pyrene in a bioreactor after seeding with *Mycobacterium* species. However, our result in this instance is not without similar outcomes from previous studies. For instance, Cheung and Kinkle (2001) reported higher levels of mineralisation when soil was sterilized and inoculated with a pyrene degrading *Mycobacterium* strain 135, than when inoculation was carried out without prior sterilization. Furthermore, the decrease in concentration of pyrene in sterilized soils without inoculum can be attributed to abiotic loss occasioned by sequestering of pyrene into soil microspore (Kastner *et al.,* 1998).

Population of degrader increased concomitantly with decreasing pyrene concentration showing direct linkage between observed growth and utilization. The observed decline in microbial population after 30 days, which represent end-point of degradation, further buttresses complete dependence of pyrene degrader on pyrene as sole carbon and energy sole source. What is surprising, however, is that the organism, being spore former, would not have died but rather form spores and still exist in the soil at its optimal population. The only plausible explanation for this may be that other conditions for spore formation failed to prevail.

Conclusively, LP32 could grow both in laboratory

culture and soil microcosm with significant degradation of pyrene. Its ability to survive and maintain a high population density in both native and sterilized soils demonstrated the potential of *Bacillus lentus* LP32 to serve as seed for enhanced bioremediation of pyrene polluted soil.

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