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ISOLATION AND CHARACTERIZATION OF PHENANTHRENE DEGRADING *BACILLUS SUBTILIS* FROM HYDROCARBON POLLUTED SOIL IN ZARIA, KADUNA STATE

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are pollutants that accumulate in the soil and sediments due to their insolubility in water and lack of volatility and thus require eco-friendly remedial strategies. A bacterial species isolated and characterized from hydrocarbon polluted soil was identified as *Bacillus subtilis* and studied for the ability to degrade phenanthrene as a sole source of carbon and energy. The optimal conditions for phenanthrene biodegradation were determined to be 27℃ at a pH of 7.0. A significantly positive relationship was observed between microbial growth and the rate of phenanthrene degradation. The colour of the bacterial colonies on culture media changed to indigo on a plate with indole indicating the presence of the dioxygenase enzyme. The population density of *Bacillus subtilis* increased from 1.2×10^6 to 8.1×10^7cfu/ml on phenanthrene within 20 days, within the same period the percentage degradation of phenanthrene was 67.61%. The rate of degradation decreased from 5.15 mg L^{-1} day $^{-1}$in 10 days to 4.35 mg L^{-1} day $^{-1}$in 20 days at a degradation constant rate from 0.051K to 0.056K and the half-life was 13.58 days to 12.38 days respectively. This study revealed that a potential PAH strain was present in hydrocarbon polluted soil and can be useful in a detoxifying environment with toxic compounds effectively.

Keywords: *Phenanthrene, Biodegradation, Bacillus subtilis, Hydrocarbon polluted soil, Zaria.*

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are released into the environment as a result of the incomplete combustion of fossil fuels or by accidental discharge during the

transport, use, and disposal of petroleum products (1). Phenanthrene, a major pollutant is the smallest polycyclic aromatic hydrocarbon to have a 'bay and a kay' region that are highly reactive (1). The epoxides formed at the 'bay and kay' region are reactive as compared to the parent compound such as pyrene and anthracenes are suspected carcinogens (2). Phenanthrene is among the most widely transported PAHs in the hydrosphere because it is highly associated with industrial processes. Phenanthrene is degraded mainly by Nocardioforme Actinomycetes like *Mycobacterium*, *Bacillus*, and *Rhodococcus* species (2). The metabolic pathway is initiated by a ring hydroxylating dioxygenase to yield cis-3,4-dihydroxy-3,4-dihydrophenanthrene, which is subsequently metabolized to 1-hydroxy-2-naphthoic acid (1-H2NA). Ring-cleaving dioxygenases play an important role in the degradation of aromatic compounds; they catalyse the incorporation of two atoms of molecular oxygen into substrates. Based on the mode of ring-cleavage, they are grouped as extradiol dioxygenases, which require non-haem Fe(II) and cleave the aromatic ring proximal to one of the two hydroxylated carbon atoms, yielding semialdehyde (2) and intradiol dioxygenases, which require non-haem Fe(III) and cleave the aromatic ring between the two hydroxylated carbon atoms, yielding a muconic acid. Polycyclic aromatic hydrocarbon constitutes a group of priority environmental pollutants which are ubiquitous contaminants in soils and sediments which are of environmental concern because of their toxic, mutagenic, or carcinogenic effects (3). In recent years, the biodegradation of PAHs has received considerable attention and a variety of microorganisms have been reported to play important roles such as bacterial genera, including species of *Pseudomonas, Alcaligenes, Mycobacterium, Rhodococcus, Sphingomonas* (4,5). Polycyclic aromatic hydrocarbons released into the environment could be removed by many processes including volatilization on, adsorption of soil particles, however, the principal process for successful removal and elimination of PAHs from the environment is microbial transformation and degradation (6,7).

Recently, attention has been turned towards diverse PAHs metabolizing bacteria degrading mechanisms (8). To achieve an efficient bioremediation process, it is interesting to find new bacteria that can degrade PAHs in a new pathway without accumulating potentially toxic metabolites and also know which metabolites are

expected to accumulate so other strains that can degrade those metabolites are added if necessary (9). This present study aims to investigate the biodegradation potentials of indigenous bacterium isolated from hydrocarbon soil that can degrade phenanthrene and render it less toxic to the environment.

MATERIALS AND METHODS

Phenanthrene and Soil Sample Collection

The phenanthrene chemical was purchased from Sigma Aldrich (USA) through cardinal Scientific Supply Lagos, Nigeria. The properties of phenanthrene include Boiling point (332°C), Density (1.18g/cm^3), Melting point (101°C), and Solubility (1.6mg/l). The solution was prepared by weighing 0.1g of phenanthrene into a clean beaker, and twenty milliliter (20ml) of acetone.

The sampling site for this research was a mechanic workshop with soil having a long history of petroleum contamination in Samaru, Zaria, Kaduna State Nigeria. The coordinates of the site were latitude $5^o27'4''N$ and longitude $2^o10'5''E$. Composite soil sample was collected at 1-10cm at different sampling points using a sterile spoon after clearing debris from the soil surface (10). Soil samples for both physicochemical and microbiological analyses were collected in sterile polyethylene bags. The soil sample was sieved through a 2mm sieve for enrichment and biodegradation studies and kept at 4°C (11).

Physicochemical and Microbiological Parameters Polluted Soil Sample

The pH of the soil was measured using a pH meter (Jenway 3510 Staffordshire, England) This was done at the site and then confirmed in the laboratory. The moisture, organic and nitrogen content, available phosphorous, calcium, and magnesium were determined according to standard methods (12). Total heterotrophic bacteria were enumerated by plating aliquots 0.1ml of dilution factor (10^7) of soil sample on nutrient agar Oxoid Limited, Basingstoke, Hampshire, England) while the population of hydrocarbon utilizers was estimated on mineral salts medium (MSM) (13) that contained (1000ml) $NaHPO_4$ (2.2g), KH_2PO_4 (0.8g), NH_4NO_3 (3.0g), $CaCL_22H_2O$ (10g) which was supplemented with yeast extract (0.005g) as a source of growth factor and pH was adjusted using a buffer to 7.2. The mineral salts medium was fortified with nystatin (0.5g) (Sigma Aldrich USA) to suppress the growth of fungi. Sterile crude oil (Escravos Light) served as a source of carbon and energy. The colonies were counted after 5 days of incubation at room

temperature (27^{o}C) with a Stuart Scientific Colony Counter (Bibby Sterilin Stone Staffordshire 15 OSA, United Kingdom).

Enrichment, Isolation, and Characterization of Phenanthrene Degrader

Phenanthrene degrading bacteria were isolated on mineral salts medium (broth) supplemented with 0.025% phenanthrene as a source of carbon and energy. It was fortified with 0.5g of nystatin to suppress fungal growth. Air-dried contaminated soil sample (5g) was added to 50ml of mineral salts medium (broth) and the suspension was incubated with shaking at 150 rpm at room temperature (27°C ±2°C) using the rotary shaker in the dark until there was turbidity in thirty (30) days (14). One (1) millimetre of the enriched media was transferred into fresh mineral salts medium (broth) containing the phenanthrene crystals and incubation was done in the same manner. After five (5) consecutive transfers phenanthrene degraders were isolated by plating out dilutions from the final flask on Luria-bertani (LB) agar for purification. The most promising phenanthrene degrader based on the size of clear zone diameter was selected for further study (15).

The selected isolate was identified on the basics of its colonial morphology, cellular morphology, and biochemical characteristics according to the scheme of Cowan and Steels Manual (16). And further complemented with Analytical profile index phenotypic using API 12E V6.0 rapid test kit according to the manufacturer instructions.

Biodegradation of Phenanthrene by Isolate

Bacterial cells were washed in ten ml of sterile mineral salts medium and centrifuged at 3 revolutions per minute (rpm) for 15 minutes consecutively three times and decanted at each time (17). Ten flasks were set up containing 100 parts per million (ppm) of phenanthrene solution. Fifty mililitres of mineral salts medium was added to each flask and autoclaved at 121°C for 15 minutes. Each flask was inoculated with the inoculum (1ml) and incubated at room temperature 27°C for 24h (18).

Determination of Growth Profile of the Isolate

The flask was selected and shaken at 150rpm in the dark for 15 minutes in which 1ml of the suspension was taken and dissolved into 9ml of physiological saline and serial dilution was further done up to 10^{10} in sterile test tubes to reduce the microbial concentration. An aliquot of 0.1ml was taken at dilutions (10^{-7}) and was spread

out with a sterile bent glass rod aseptically into nutrient agar and this was done in duplicates and incubated at room temperature for 24h. The colonies were counted in colony-forming units per ml (Cfu/ml) (18). With a Stuart Scientific Colony Counter (Bibby Sterilin Stone Staffordshire St 15 OSA, United Kingdom).

Extraction of Residual Phenanthrene

Residual phenanthrene was extracted by the liquid extraction method according to the model described by (18). Twenty milliliter (20ml) of the culture selected for determination of growth profile on each day was extracted using a glass separating funnel with a stopper and a cork. Twenty milliliter (20ml) of hexane was added to the mixture and shaken vigorously with a rotary shaker for 30 minutes. The hydrocarbon layer was collected into a glass beaker and allowed to evaporate. The Gas Chromatography runs were carried out on the sample on days 0, 10, and day 20. The PAH data were fitted into the first-order kinetics model of (19).

$Y = ae^{-kt}$

Where y was the residual PAH in the culture
A is the initial PAH in culture
K is the degradation time and
T is the time of days
As calculated as
Half-life = Ln (2)/k

RESULTS

Physicochemical and Microbiological Properties of the Polluted Soil Sample

Table 1 revealed that the soil sample was acidic (pH 6.85), and the total organic carbon was 9.5 %. The table also showed that among the nutrient composition in the soil, calcium was recorded as the highest (4.6Cmol/kg) followed by magnesium (0.35Cmol/kg) with potassium being the least (0.013Cmol/kg). It was equally high in water content (22.0%).

Total hydrocarbon-degrading and heterotrophic bacteria in the soil were 4×10^{-6}cfu/g and 3×10^{-6}cfu/g respectively.

Table 1: Physicochemical properties of the polluted soil sample

Physicochemical Properties	Quantity
pH	6.85±0.106
Moisture Content (%)	22.0±0.707
Organic Carbon Content (%)	9.51±0.707
Available Phosphorous (%)	0.3770±0.036
Total Nitrogen (%)	0.140±0.007
Calcium (Cmol/Kg)	0.0466±0.283
Magnesium (Cmol/Kg)	0.35±0.021
Potassium (Cmol/Kg)	0.013±0.001
Sodium (Cmol/Kg)	0.063±0.001

Key: All values are expressed as Mean± SD-standard deviation, Cmol/kg-Centimole per kilogram, mg/kg-milligram per kilogram.

Morphologically, *Bacillus subtilis* presents as a Gram-positive bacterium, Rod-shaped with shiny pigmentation, the isolate was positive for motility and endospore formation which was located at the terminal position presented in Table 3. It tested positive for catalase and negative for oxidase. The growth rate and pattern kinetics of *Bacillus subtilis* supplemented with phenanthrene as a sole source of carbon and energy obtained from the biodegradation experiment showed initial slow growth between days 0 to day 4 but grew exponentially from day 8 to day 20 (Figure 1). Degradation kinetics showed that 67.61% was degraded in 20 days at the rate of $4.35mg1^{-1}d^{-1}$. The biodegradation constant (k) and half-life ($t_{1/2}$) were 0.056 d^{-1} and 12.38 d respectively. *Bacillus subtilis* shows a linear retrogressive curve indicating the exponential increase of cell biomass (Table 3) and reduction dynamics of the phenanthrene concentration within 20 days (Figure 1).

Table 2: Cultural, morphological and biochemical characteristics of *Bacillus subtilis*

Morphological and biochemical characterization	**Result**
Margin of colony	Coiled
Surface of colony	Smooth
Elevation of colony	Raised
Pigmentation of colony	Shiny cream
Cell arrangement	In clusters
Shape	Short rods
Urease	+
Gram staining	+
Spore staining	+
Motility	+
Citrate utilization	+
Catalase	+
Oxidase	-
Lactose	+
Glucose	-
Sucrose	-
Maltose	-
Fructose	-

Key: Negative (-), Positive (+)

Table 3: Percentage kinetics of degradation of phenanthrene by *Bacillus subtilis*

	Parameters			
Day	% phenanthrene degraded	Rate of degradation (mg L^{-1} day^{-1})	The degradation rate constant (K)	Half-life Days ($t^{1/2}$)
10	39.97	5.15	0.051	13.58
20	67.61	4.35	0.056	12.38

Key: Values are means of duplicate readings obtained from difference between the amount of residual phenanthrene on Day 0 of incubation of bacterial to Day 20, Half-life ($t^{1/2}$) Constant = 0.693, mg/L day-milligram per litre in a day.

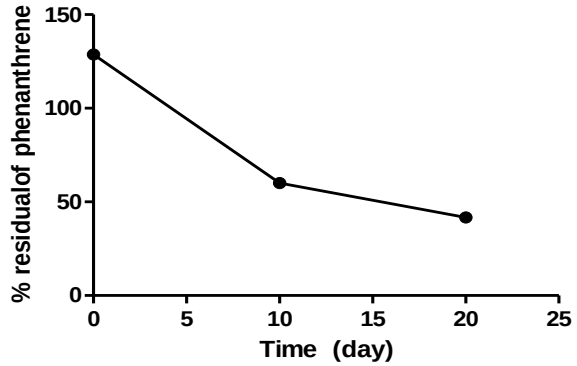

Figure 1: Reduction of phenanthrene by *Bacillus subtilis* in a microcosm experiment

DISCUSSION

Polycyclic aromatic hydrocarbons are a diverse group of priority pollutants containing two or more fused benzene rings that are arranged in linear, angular, and cluster conformations. Cleanup of environment priority pollutants is important due to their acute toxicity, low bioavailability, hydrophobicity, mutagenicity, and carcinogenicity (20). The biodegradation of PAHs is mainly through the activities of bacteria (20).

Physicochemical properties of the polluted soil sample used in this study indicated low concentrations of macronutrients such as nitrogen, phosphorous, sodium, potassium, and calcium. This may be attributed to the high demand for these inorganic nutrients by microorganisms for sugar phosphorylation, synthesis of amino acids, and nucleic acids such nucleotides and nucleosides increase in cell division, growth, biomass, and other cellular processes (21).

The pH of the polluted sample was slightly acidic (6.85) which indicates that phenanthrene degradation is accompanied by a lowering of the pH (22). This may be connected with the fact that phenanthrene degradation usually leads to the production of organic acids such as pyruvic and salicylic as their metabolites which invariably leads to a lowering the pH, however, the slight decrease in pH may be accounted for by the fact that the medium is buffered (22). The growth rate utilization of *Bacillus subtilis* on phenanthrene is (0.0156 h^{-1}) which is low compared to those reported in previous findings (20,15) who reported for (0.111, 0.082, and 0.067 h^{-1}).

In this study, *Bacillus subtilis* degraded 67.61% of the initial concentration of phenanthrene (100 mgI^{-1}) in 20 days. This degradation rate is lower than 87.92% (100 mg l^{-1}) in 30 days reported by Salam et al. (20) who discovered possibly for the first time, that a strain of *Proteus mirabilis* 10c degraded phenanthrene effectively at 99% (100 mg l^{-1}) in 16 days same as reported for *Diaphorobacter* respectively. It was, however, higher than 61.5%, 65.8%, and 33.7% reported for *Leclerciaade carboxylata*, *Bacillus cereus* py5, and

Bacillus megaterium py6 within 21 days respectively, in the same study. Degradation rates of 68%, 67%, and 47% within 30 days were reported for strains of *Pseudomonas putida* LP1, *Pseudomonas fluorescens* LP5, and *Pseudomonas cepacia* LP6 respectively (21, 15). The degradation rate is however slower than 72% (500 mg I^{-1}) recorded for *Rhodoccocus* sp. UWI within two weeks of the biodegradation experiment. However, the strain *Rhodoccocus* sp. UWI has always been effective for biodegradation purposes and has degraded various PAHs such as pyrene, naphthalene, and fluoranthene in high concentration as a result of the dioxygenase gene through the meta cleavage pathway before subsequently channeling the metabolites to the tricarboxylic pathway (21).

In this study, the population density of *Bacillus subtilis* increased from 2.5×10^7 to 8.11×10^8 CFU/ml on phenanthrene within 20 days, this is quite higher than the study reported by Yeung et al. (22) who reported a population density increase for *Bacillus subtilis* from 3.5×10^7 to 2.89×10^{10} within 8 days. The rate of degradation decreased from 5.15 mg L^{-1} day^{-1} within 10 days to 4.35 mg/L^{-1} day^{-1} in 20 days, at a constant degradation rate from 0.051 K to 0.056 K within the same period. A related study by Adeyemo and Obayori (23) reported an increase in degradation from 6.06 mg L^{-1} day^{-1} within 5 days to 7.05 mg L^{-1} day^{-1} within 10 days at a constant degradation rate from 0.058 K to 0.086 K, and half-life from 11.95 days to 8.058 days respectively, the increase in degradation rate and subsequent decrease in duration for the process could be as a result of the long incubation period during degradation and the concentration of phenanthrene.

CONCLUSION

The physicochemical properties of the polluted soil sample indicated a low amount of minerals such as magnesium (0.35 Cmol/kg), potassium (0.013 Cmol/kg), and calcium (0.046 Cmol/kg) and a high content of water (22.0%) with slightly acidic pH of 6.85.

Bacillus subtilis was enriched in mineral salts medium for 30 days and the medium was fortified with yeast extract for optimal growth and was identified based on cultural morphological, biochemical test, and analytical profile index.

Bacillus subtilis showed broad versatility in its action to degrade phenanthrene and utilized it as a sole source of carbon and energy.

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