PYRENE BIODEGRADATION POTENTIALS OF AN ACTINOMYCETE, *MICROBACTERIUM ESTERAROMATICUM* ISOLATED FROM TROPICAL HYDROCARBON-CONTAMINATED SOIL

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ABSTRACT

A novel pyrene-degrading actinomycete, phylogenetically identified as *Microbacterium esteraromaticum* strain SL9 was isolated from a polluted hydrocarbon-contaminated soil in Lagos, Nigeria. Growth of the isolate on pyrene was assayed using total viable counts, pyrene degradation was monitored using gas chromatography (GC-FID) while UV-Vis spectrophotometry was used to detect metabolites of pyrene degradation. The isolate tolerated salt concentration of up to 6%, grew luxuriantly on crude oil and exhibited weak utilization of fluorene, acenaphthene and engine oil. It resisted cefotaxime, ciprofloxacin and amoxicilin, but was susceptible to meropenem, linezolid and vancomycin. It also resisted elevated concentrations of heavy metals such as 1-5 mM lead and nickel. On pyrene, the isolate exhibited growth rate and doubling time of 0.023 h^{-1} and 1.25 h, respectively. It degraded 55.16 (27.58 mg L^{-1}) and 89.28% (44.64 mg L^{-1}) of pyrene (50 mg L^{-1}) within 12 and 21 days respectively, while the rate of pyrene utilization was 0.09 mg L^{-1}h^{-1}. Catechol dioxygenase assay using UV-Vis spectrophotometry revealed the detection of *meta* cleavage compound, 2-hydroxymuconic semialdehyde in the crude cell lysate. The results of this study showed the catabolic versatility of *Microbacterium* species on hydrocarbon substrates and their potential as seeds for bioremediation of environments co-contaminated with polycyclic aromatic hydrocarbons and heavy metals.

Keywords: Biodegradation; pyrene; Hydrocarbon-contaminated soils; *Microbacterium esteraromaticum*

INTRODUCTION

Pyrene is a peri-condensed four-ring polycyclic aromatic hydrocarbon (PAH). It belongs to the class of non-alternant high molecular weight (HMW) PAH. It is a regulated pollutant at sites contaminated with petroleum. Pyrene equally results from incomplete combustion of organic materials and other processes of pyrolysis and pyrosynthesis (**Kanaly and Harayama, 2010**). Like all HMW PAHs, pyrene exhibits high molecular stability, low solubility in aqueous phase and is highly hydrophobic, thus accounting for its persistence in the environment. Although not known to be carcinogenic, its mutagenicity has been demonstrated in laboratory animals and it is equally known to be highly toxic with evidence of respiratory function impairment (**USDHS, 1990**). It is a signature compound for the study of carcinogenic high molecular weight polycyclic aromatic hydrocarbons degradation such as benzo(a)pyrene with which it shares structure (**Obayori et al., 2013**)

The first pyrene degrading bacterium to be isolated was *Mycobacterium* sp. Strain PYR1 (**Heitkamp et al., 1988a**) later renamed correctly as *Mycobacterium vanbaalenii* strain PYR1 by **Khan et al. (2002)**. Subsequently, more pyrene degraders, mainly norcadioform actinomycetes with remarkable metabolic versatility were reported (**Walter et al., 1991; Boldrin et al., 1993; Schneider et al., 1996; Dean-Ross and Cerniglia, 1996; Churchill et al., 1999; Bastiaens et al., 2000**). But today, the literature is replete with pyrene degraders spanning low G+C Gram-positive and Gram-negative genera such as *Bacillus, Corynebacterium, Micrococcus, Pseudomonas, Sphingomonas, Stenotrophomonas, Alcaligenes, Achromobacter, Leclercia, Cycloclasticus, Burkholderia* and *Proteus* (**Sarma et al., 2004; Gaskin and Bentham, 2005; Lin and Cai, 2008; Wang et al., 2008; Ceyhan, 2012**). Notwithstanding the rich literature on pyrene degradation globally, reports of HMW PAH degraders from sub-Sahara Africa are virtually absent. However, **Obayori et al. (2008)** reported the isolation of three pyrene degrading *Pseudomonas* strains from hydrocarbon-contaminated soils in Lagos, Nigeria.

Actinomycetes are a group of diverse bacteria that are Gram positive with high guanine plus cytosine in the DNA (> 55 mol %). They are good candidates for bioremediation of polluted soils due to their capablility to produce extracellular enzymes that degrade a wide range of complex organic compounds, production of spores that are impervious to desiccation and filamentous growth that favours colonization of soil particles (**Ensign, 1992; Salam et al., 2014**). Particularly, actinomycetes are good candidates for degradation of hydrophobic compounds such as PAHs due to their surfactant-producing activity. The biosurfactant may be extracellular such as glycolipids (trehalose lipid and lipopeptide produced by *Rhodococcus* and *Arthrobacter* species) or cellular biosurfactants such as mycolic acid, which allow adherence of the microbial cells to hydrophobic phases in two-phase systems (**Singer and Finnerty, 1990; Morikawa et al., 1993; Neu, 1996**).

The initial reactions in the classic aerobic degradation of pyreneby bacteria are catalysed by a multi-component enzyme system and involve the incorporation of other atoms of oxygen molecules at the 4, 5-carbon positions to produce a dihydrodiol. The dihydrodiol is re-aromatised and the dihydroxylated product is cleaved to products, which are processed through the phenanthrene pathway into the tricarboxylic acid (TCA) cycle (**Heitkamp et al., 1988b**). However, a plethora of other routes exist, some leading to complete degradation, while others merely lead to dead-end products (**Kim et al., 2007**). The significance of pyrene in the bioremediation of petroleum-polluted sites and the effect of products of incomplete degradation of pyrene on the fate of other PAHs in the environment has further compelled research on pyrene degraders (**Kazunga and Aitken, 2000**).

Heavy metals are introduced into soil compartments through various anthropogenic activities such as petroleum exploration, waste disposals, and corrosion of metals in use among others. Toxicity of heavy metals to autochthonous microbial community in soil has been reported to also inhibit biodegradation of pollutants in co-contaminated sites (**Sandrin et al., 2000**). To tolerate heavy metals stress, microorganisms have evolved various resistance mechanisms such as efflux pumps, enzymatic detoxification, permeability barriers, intra- and extracellular sequestration, and reduction (**Nies, 1999**).

Notwithstanding recent shift in focus from isolation to demonstration of degraders in the environment in recent years, considerable attention continues to be directed to search for novel culturable organisms. Such isolates are continuously relevant in discerning relationship between phylogeny and specific metabolic function and also for the purpose of bio-augmentation (**Hilyard et al., 2008**). Previously, we had reported the degradation of anthracene by a *Microbacterium* species (**Salam et al., 2014**). Here we report the isolation and characterization of a *Microbacterium esteraromaticum* strain able to use pyrene as sole source of carbon and energy. To the best of our knowledge, this is the first report of isolation and characterization of pyrene-degrading *Microbacterium esteraromaticum* strain.

MATERIALS AND METHODS

Sampling site

Soil samples for this study were collected from an automobile workshop at Okokomaiko, Lagos, Nigeria. The coordinates of the sampling site were latitude 6° 28' 23″ N and longitude 3° 11' 14″ E, respectively. Soil samples were collected at a depth of 10–12 cm using sterile trowel after clearing debris from the soil surface. Samples for physicochemical analysis were collected in clean black polythene bags, while samples for microbiological analysis were collected in sterile screw-capped bottles. Immediate analysis of the samples were carried out within 5 h of collection or stored at 4 °C. Physicochemical properties of the soil sample, which has been described elsewhere showed a weakly acidic pH of 6.10, an unusually high hydrocarbon content (157,068 mg/kg) and a plethora of heavy metals such as lead (0.11 mg/kg), zinc (3.31 mg/kg) and nickel (4.34 mg/kg) (**Salam et al., 2014**).

Enrichment and isolation of pyrene-degrading bacteria

Bacteria with capacity for pyrene degradation were isolated on pyrene mineral salts medium (MSM) by continual enrichment method. Mineral salts medium described by **Mills et al. (1978)** and modified by **Okpokwasili and Amanchukwu (1988)** was used. The medium was supplemented with yeast extract (0.005 g/l) as source of growth factors. After adjusting the pH to 7.0, the medium was fortified with 50 µg/ml and 20 µg/ml of nystatin and nalidixic acid respectively for selective isolation of actinomycetes. Air-dried contaminated soil (5 g) was added to 45 ml of MSM containing 50 mg/l of pyrene. Enrichment was carried out by incubation with shaking (180 rpm) at room temperature (29 ± 2 °C) in the dark for 4–5 weeks until there was turbidity. After five consecutive transfers, pyrene degraders were isolated by plating out dilutions from the final flasks on Luria–Bertani (LB) agar. The colonies that appeared were further purified by subculturing once onto LB agar. Ability to degrade pyrene was confirmed by inoculating phosphate buffer-washed LB broth grown culture in fresh MSM flask supplemented with 50 mg/l pyrene as sole carbon source. One isolate designated strain SL9 out of the six screened was selected for further study based on its extensive degradative ability.

Maintenance and identification of isolates

The pure pyrene-degrading isolate was maintained in glycerol/LB broth medium (1:1, v/v). Pure colonies subcultured on LB agar supplemented with low percentage of pyrene (0.005%) were harvested with sterile inoculating loop, pooled and transferred to the medium. The mixture was shaken to homogenize and kept at -20°C.

Identification and characterization of isolate

Pure culture of bacterial isolate was identified based on their colonial morphology, cellular morphology and biochemical characteristics according to the identification scheme of Bergey's Manual of Determinative Bacteriology (**Holt et al., 1994**). Antibiotic susceptibility of the isolate was carried out using multidiscs. The antibiotics tested include streptomycin (30 µg), erythromycin (15 µg), vancomycin (30µg), doxycycline (30µg), meropenem (10µg), linezolid (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), amoxicillin (30 µg) and pefloxacin (30 µg). Salt tolerance was tested in LB broth supplemented with NaCl ranging from 1 - 10% (w/v). Incubation was carried out at room temperature (29 ± 2°C) for one week with shaking and daily observation for growth. PCR amplification of the 16S rRNA gene from genomic DNA of strain SL9 was performed using the universal primers 27f (5'-AGAGTTTGATC{A/C}TGGCTCAG-3') and 1378r (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (**Heuer et al., 1997**). The reaction mix contained 20 pmol each of universal primers, 10 µl of Ex *Taq* buffer (Mg^{2+} plus), 2.5 mM of each dNTPs, 2.5 U (0.5 µl) of Ex *Taq* polymerase (Takara) and 1.0 µl of purified genomic DNA in a total volume of 100 microlitres. Amplification conditions consisted of an initial denaturation step at 95°C for 3 min, 30 amplification cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 2 min with the final extension at 72°C for 7 min before cooling to 4°C.

The amplicon was analyzed on 1% agarose gel in 1xTAE, run at 100V for 30-35 min. The PCR product (~1500 bp DNA fragment) was cloned into the plasmid vector pT7Blue® (Novagen, USA) after purification by centrifugation using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, Wis.). The purified PCR product was transformed into *Escherichia coli* strain DH5α (Toyobo). After the transformation of *E. coli* DH5α-competent cells, clones were picked. Selected clones were then sequenced using the ABI Prism 3730xl DNA Sequencer (Applied Biosystems, UK) according to the manufacturer's instructions. The 16S rRNA nucleotide sequence obtained from both strands was aligned (CLUSTAL W) and the homology search for 16S rRNA was performed in the DDBJ/EMBL/GenBank database using the Basic Local Alignment Search Tool (BLAST) program (**Altschul et al., 1990**). Strain SL9 sequence had been deposited in the DDBJ/EMBL/GenBank database under the accession number AB646581.2. Phylogenetic tree showing evolutionary relationship of strain SL9 with reference sequences from the database was constructed using neighbour joining algorithm within the program MEGA 5.1 (The Biodesign Institute) and bootstrapped with 100 repetitions.

Substrate Specificity of strain SL9

Substrates utilization pattern of strain SL9 on different hydrocarbons was evaluated in MSM containing the respective hydrocarbons as sole carbon and energy source at a concentration of 100 ppm. Stock solution of each hydrocarbon substrate was prepared by dissolving 1 g of it in 10 ml dimethyl sulfoxide (DMSO) and filter-sterilizing the solution using hydrophobic filter (Advantec, JP020AN). Sterile MSM (5 ml) was prepared in test tubes and 5 µl of stock solution of the respective hydrocarbons was added. Strain SL9 was added at 1% (v/v). Incubation was carried out at room temperature in the dark for 14 days. Test tubes prepared as above but without carbon source served as control. Degradation was monitored by visual observation for turbidity. The hydrocarbons tested include naphthalene, fluorene, acenaphthene, pyrene, dibenzothiophene and dibenzofuran. Liquid hydrocarbons like crude oil and engine oil were autoclaved and added separately to the sterile MSM at 0.1% (v/v).

Metal tolerance assay

Strain SL9 was grown in LB broth for 18 h at room temperature. Cells were harvested by centrifugation (7,000×g; 10 min), washed twice with sterile phosphate buffer, and resuspended in the same buffer solution. The cell concentration of bacterial suspensions was determined by measuring the optical density of the samples at 600 nm and relating the value to a calibration curve (10^{10}cfu L^{-1}=1 ODunit). Stock solutions (1 M) of metal salts namely, HgCl$_2$, NiSO$_4$, and Pb(NO$_3$)$_2$ were prepared in distilled water, filter sterilized using 0.22-µm membrane filters, and stored in sterile bottles in the dark at 4 °C. Dilutions to 1, 5, 10, and 15mM of Hg^{2+}, Ni^{2+}, and Pb^{2+}were made from the stock solutions into LB broth. The media were dispensed in 5-ml aliquots and inoculated with 50 µl (1%, v/v) inoculum. Each of the experiment was conducted in triplicates. LB broth not supplemented with heavy metals and inocula serves as controls. Growth of the inocula was measured by absorbance at 600 nm and occasional viable count assay. Resistance was assayed by determining the maximum tolerance concentrations (MTCs) for the isolates after 10 days of incubation. MTC is defined as the highest concentration of metal, which do not affect the viable counts of organisms.

Evaluation of pyrene biodegradation

Pyrene degradation potentials of the pure isolate was assayed by inoculating 250-ml replicate flasks containing 50 ml of MSM supplemented with 50 mg/l of pyrene as sole carbon and energy source respectively. Flasks were inoculated with 0.5 ml of MSM-washed 18 h-24 h LB agar-grown cells and subsequently incubated at 180 rpm in the dark for 21 days at room temperature. Flask containing heat-killed cells sterilized at 121°C for 15 min and supplemented with pyrene as described above were used as controls. Samples were withdrawn from each flask at 3 days interval and aliquots of appropriate dilutions were plated (in triplicates) onto nutrient agar for total viable counts (TVC).

Extraction of residual pyrene

Residual pyrene was extracted by liquid-liquid extraction. Briefly, broth culture (50 ml) was extracted twice with an equal volume of hexane. After removing the aqueous phase with separating funnel, the organic fraction was concentrated to 1 ml and the residual pyrene concentration was determined by gas chromatography. Control flasks were also extracted similarly.

Analytical method

Hexane extracts (1.0 µl) of residual pyrene were analyzed with Hewlett Packard 5890 Series II gas chromatograph equipped with flame ionization detector (FID) and 30 m long HP-5 column (internal diameter, 0.25 mm; film thickness, 0.25 µm). The carrier gas was nitrogen. The injector and detector temperatures were

maintained at 300°C and 320°C, respectively. The column temperature was programmed from 60°C to 500°C for 27 min. The gas chromatograph column was programmed at an initial temperature of 60°C; this was held for 2 min, and then ramped at 12°C/min to 205°C and held for 16 min. nitrogen column pressure was 37 psi, the hydrogen pressure was 9 psi and compressed air pressure was 13 psi. The software was Chem Station. Rev. A. 05. 01.

Catechol dioxygenase assay

Two milliliters of strain SL9 was harvested by centrifugation at the late logarithmic phase from MSM medium containing pyrene and was suspended in1-ml MSM. Cells were lysed by the addition of 20 μl toluene and after vigorous mixing, unbroken cells and cell debris were removed by centrifugation at 16,000×g for 30 s. The clear supernatants were immediately used for the assay or placed on ice for not more than 10 min. Activity assays were performed using GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific, USA). The reaction was initiated by the addition of 100 μl catechol solution (100 μM) to a reaction mixture in a 1-cm light path quartz cuvette containing 800 μl phosphate buffer and 100 μl of crude lysate. The blank cuvette contained the same amount of enzyme in the same buffer with the exception of catechol. Activities of catechol 1,2-dioxygenase and 2,3-dioxygenase were monitored at 260 and 375 nm, respectively (**Wang *et al*., 2008**).

Statistical analysis

Mean generation times (T_d) and specific growth rates (μ) of the isolate on pyrene was calculated using non-linear regression of growth curves for the period when growth rates were maximal using Prism version 5.0 (Graphpad software, San Diego, CA).

RESULTS

Identification and characterization of pyrene-degrading isolate

Continuous enrichment resulted in the isolation of several pyrene degraders. However, only one isolate designated strain SL9 out of the six isolates, displayed strong degradative ability. Phenotypic characterization of the isolate showed that it was obligately aerobic, Gram-positive, non-spore forming, irregular rods occurring singly or in clusters. On LB agar, strain SL9 was circular, smooth, translucent, yellow-pigmented, opaque, low-convex, moist colonies with entire margins. Strain SL9 colonies were catalase positive but showed negative reaction for oxidase, methyl red, Voges-Proskauer, indole, gelatinase and H_2S production. It was positive for starch hydrolysis and is unable to utilize all the sugars tested with exception of mannitol, maltitol and salicin. The isolate tolerated salt concentration of up to 6% while growth cease above this concentration. It resisted cefotaxime, ciprofloxacin and amoxicillin, but was susceptible to meropenem, linezolid and vancomycin.

However, comparisons of the 16S rRNA partial fragments of strain SL9 (1374 bp) with the nucleotide sequences in the DDBJ/EMBL/GenBank database indicated significant alignments of the strain with *Microbacterium esteraromaticum*. Nucleotide sequence analysis of the partial 16S rRNA gene showed that Strain SL9 exhibited 99% identity with other *Microbacterium esteraromaticum* strains such as *M. esteraromaticum* strain SL6 (AB646579.2), and *M. esteraromaticum* strain S29 (AB099658.1), respectively. The relationship between strain SL9 (AB646581.2) and the nearest phylogenetic relatives is shown in Figure 1.
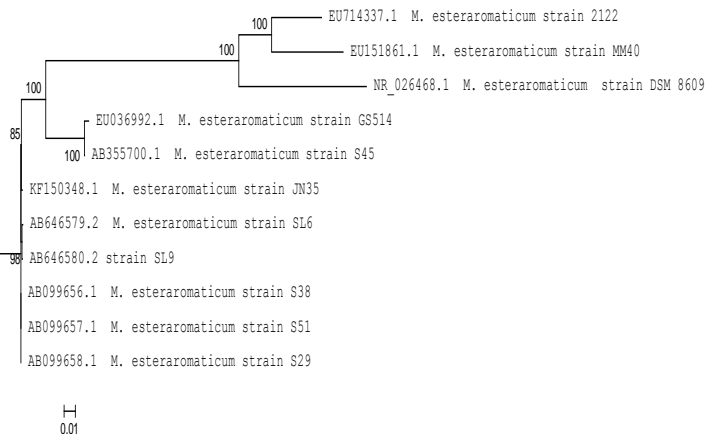
Figure 1 Phylogenetic tree resulting from neighbour joining (NJ) analysis of 16S rRNA showing the phylogenetic positions of pyrene-degrading *Microbacterium esteraromaticum* strain SL9 (AB646580.2) and related strains of *M. esteraromaticum* retrieved from NCBI GenBank. Accession number of each microorganism used in the analysis is shown before the species name. The scale

bar indicates the numbers of nucleotide substitutions per position. Bootstrap values (expressed as percentage of 100 replicons) are shown at the branch.

Substrate specificity of pyrene-degrading isolate

Substrate spectrum analysis of strain SL9 on various aromatic and heteroaromatic hydrocarbon substrates was conducted to ascertain the substrate utilization pattern of the isolate. The analysis revealed different utilization patterns. Strain SL9 failed to grow on naphthalene and dibenzofuran. It grew luxuriantly on pyrene and crude oil but exhibited weak growth on fluorene, acenaphthene and engine oil, respectively (Table 1).

Table 1 Substrate specificity test

| Substrate	Isolate (SL
Naphthalene	-
Fluorene	++
Acenaphthene	++
Pyrene	+++
Dibenzofuran	-
Dibenzothiophene	+
Crude oil	+++
Engine oil	++

+++ Luxuriant growth (>10^6 cfu/ml after 5 days of incubation); ++ Weak growth (>10^6 cfu/ml after 1 week of incubation); + Poor growth (>10^6 cfu/ml after 2 weeks of incubation); - No growth (<10^6 cfu/ml after 2 weeks of incubation)

Metal tolerance of pyrene-degrading isolate

Metal tolerance assay of strain SL9 on various heavy metals was conducted to determine the tolerance limit of the isolate to various concentrations of heavy metals. The assay revealed different resistance patterns of the isolate to each of the heavy metal. Cessations of growth were observed in the presence of 5, 10, and 15 mM mercury as well as 10 and 15 mM lead and nickel. However, the isolate showed resistance to 1 mM mercury and also resisted 1–5 mM of nickel and lead, respectively.

Kinetics of pyrene degradation

The utilization of pyrene as a sole source of carbon and energy by strain SL9 was confirmed by an increase in cell population with a concomitant loss of pyrene. The growth kinetics of strain SL9 on pyrene is depicted in Figure 2 and Table 2. The strain exhibited a short lag phase followed by consistent increase in cell density with corresponding decrease in pyrene concentration. Strain SL9 grew exponentially from an initial cell density of 1.85 x 10^6 cfu/ml to 2.81 x 10^8 cfu/ml in 9 days. The growth decline to 2.76 x 10^8 cfu/ml at day 12 but peaked slightly at day 15 to 2.79 x 10^8 cfu/ml. It thereafter maintained a decreasing trend. During the exponential growth of the isolate on pyrene, it exhibited a growth rate and doubling time of 0.023 h^{-1} and 1.25 h, respectively.
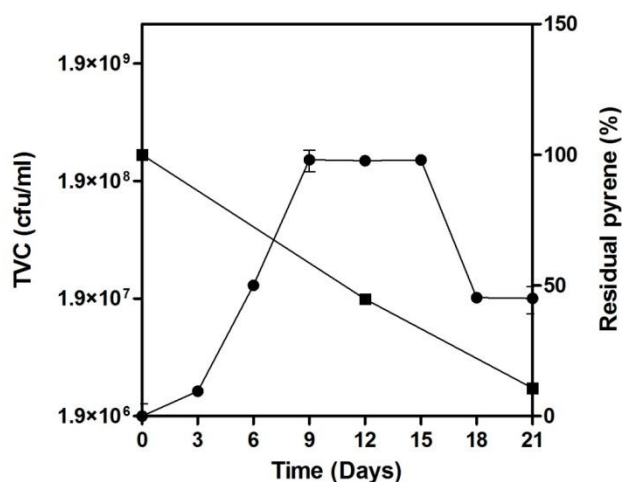
Figure 2 Growth dynamics of strain SL9 in minimal medium amended with 50 mg/l pyrene showing total viable count, TVC (●) and residual pyrene (■). Data points represent the mean of three replicate flasks. In the case of population counts, error bars represent standard deviation. Residual pyrene was determined with reference to pyrene recovered from heat-killed controls.

Pyrene transformation by *M. esteraromaticum* strain SL9 was studied at 72-h intervals in MSM containing 50 mg/L pyrene. Gas chromatographic analysis of residual pyrene showed that after 12 days of incubation, pyrene content decreased to 44.84% (22.42 mg/L) corresponding to removal of 55.16% (27.58 mg/L) pyrene. At the end of 21 days incubation, the residual pyrene content decreased further to 10.72% (5.36 mg/L) corresponding to uptake of 89.28% (44.64 mg/L)

pyrene. However, in the heat-killed control flasks, no apparent decrease of the substrate was observed, thus confirming that pyrene depletion from the MSM was due to biodegradation by strain SL9 rather than to non-specific abiotic losses such as substrate volatility or absorption to the glass tubes.

Table 2 Growth kinetics of pyrene-degrading isolate

Isolate	Growth rate, K (h^{-1})	Mean generation time, ΔT_d (h)	% degradation[1] (day 12)	% degradation[1] (day 21)	Degradation rate (%/h)	Rate of degradation (mg $L^{-1}h^{-1}$)
Microbacterium esteraromaticum strain SL9	0.023	30.0	55.16	89.3	0.18	0.09

[1]Percent degradation values represent the net decrease (FID area counts) calculated with reference to the amount recovered from heat-killed control flasks

Detection of ring fission enzyme

Strain SL9 degraded catechol via the *meta* pathway as reflected in increase activity (increase in absorbance spectra values) at 375 nm when monitored using UV-Vis spectrophotometer. This indicate the formation of 2-hydroxymuconic semialdehyde via catechol 2,3-dioxygenase activity (Figure 3). At absorbance value of 260 nm, there is consistent decrease in absorbance spectra values, indicating that the isolate lacks the ability to degrade catechol using the *ortho* pathway.
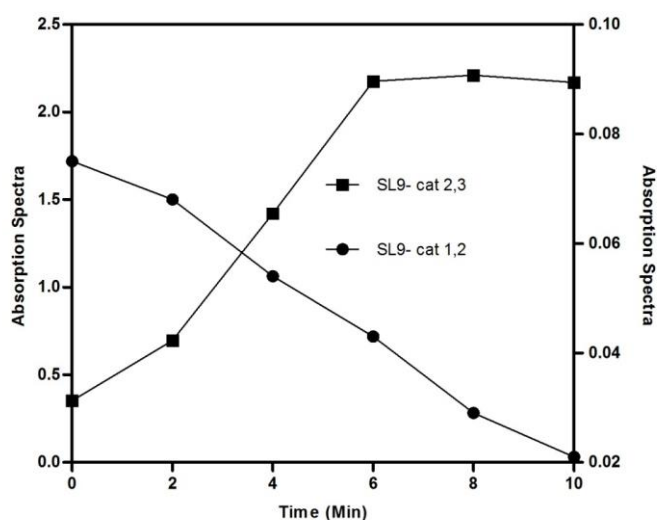
Figure 3 Enzymatic transformation of catechol to 2-hydroxymuconic semialdehyde by lysate of pyrene-grown cells. The reaction was started in a sample cuvette containing 100 ml of cell lysate in 800 ml of phosphate buffer, pH 7.5, by the addition of 100 mM catechol. Optical absorption spectra were recorded at periodic intervals of 0, 2, 4, 6, 8, and 10 min. Increase in absorption spectra at 375 nm indicate conversion of catechol to 2-hydroxymuconic semialdehyde by strain SL9. Consistent decreases in absorption spectra were observed at 260 nm.

DISCUSSION

PAHs are ubiquitous environmental pollutants renowned for their hydrophobicity, low bioavailability and bioaccumulation potential (**Nkansah et al., 2011**). A good knowledge of biodegradative potentials of novel strains isolated from contaminated sites is pivotal in designing an enduring bioremediation strategy. In this study, pyrene, a peri-condensed PAH was degraded by a Gram-positive, high G + C actinomycete, *Microbacterium esteraromaticum* sp. strain SL9, isolated from a heavily polluted hydrocarbon contaminated soil. Previously, **Obayori et al. (2008)** isolated three pyrene-degrading *Pseudomonas* spp. strains LP1, LP5 and LP6 from hydrocarbon-contaminated soil in Lagos, Nigeria.

The isolate tolerated salt concentration of up to 6%. This physiological property qualifies strain SL9 as a possible candidate for bioaugmentation purposes. It has been demonstrated that salinity of the inoculating medium could be crucial in the survival and proliferation of allochthonous strains during bioremediation process (**Kastner et al., 1998**). Microorganisms produce antibiotics as a survival strategy and for competitive edge in an oligotropic, highly compacted and diverse contaminated soil environment. Therefore, adequate understanding of antibiotic sensitivity and resistant patterns of isolates with potentials as seed for bioremediation is crucial. Resistance of strain SL9 to amoxicilin, cefotaxime and ciprofloxacin may be attributed to acquisition of resistant genes to these antibiotics through gene transfer, as soil environments are replete with these antibiotics, which could allow evolution of resistance by indigenous strains.

The substrate utilization pattern of strain SL9 on various aromatic and heteroaromatic hydrocarbon substrates revealed different utilization patterns. This may be attributed to the varied composition of the substrates and the diverse nature of hydrocarbon products present at the site where the isolate was recovered.The luxuriant growth of the isolates observed on crude oil as compared to sparse growth on engine oil may be attributed to two factors. First, crude oil, a complex mixture of different chemical composition may favorably support growth of microorganisms better than refined petroleum product such as engine oil due to diverse nutrient options available in crude oil as source of carbon and energy. Second, at the MWO site where the isolate was recovered, different types of oil products may have been indiscriminate disposed. These pollutants inevitably found their way into the soil thereby resulting in adaptation of autochthonous organisms to the pollutants due to selective pressure and acquisition of degradative abilities (**Salam et al., 2011**).

In this study, the isolate tolerated 1 mM mercury and 1-5 mM nickel and lead, respectively. This is not surprising considering the detection of iron, lead, zinc and nickel in the soil as revealed in the soil physicochemistry. The presence of these heavy metals at the sampling site indicate gross pollution as heavy oils and spent oils rich in heavy metals are indiscriminately disposed at the sampling site. Isolation of a pyrene degrader from MWO sampling site in spite of the high heavy metals presence may be due to the possibility of the degrader harboring genes for heavy metals resistance and the high hydrocarbon and organic carbon contents of the site. Heavy metals are persistent environmental pollutants that are introduced into the environment through anthropogenic activities and other sources of industrial wastes. In low concentrations, heavy metals are micronutrients, which play significant roles in cell growth and metabolic functions. However, at high concentrations, heavy metals induce oxidative stress, interfere with protein folding, and function (**Nies, 1999**).

Strain SL9 grew on pyrene with a growth rate and doubling time of 0.023 h^{-1} and 1.25 h, respectively. This growth rate is lower than 0.056 h^{-1} reported by **Boldrin et al. (1993)** for *Mycobacterium* BB1. However, this value is higher than 0.014 and 0.013 h^{-1} reported by **Thibault et al. (1996)** for *Pseudomonas* spp. K-12 and B-24, respectively. It is equally higher than 0.018 and 0.017 reported by **Obayori et al. (2008)** for *Pseudomonas* spp. LP1 and LP6, respectively. In addition, pyrene utilization rate of strain SL9 (0.09 mg l^{-1} h^{-1}) is lower than 0.56 mg l^{-1} h^{-1} reported by **Dean-Ross and Cerniglia (1996)** for *Mycobacterium flavescens* and 0.111 mg l^{-1} h^{-1} reported by **Obayori et al. (2008)** for *Pseudomonas* sp. LP1. It is however higher than 0.082 and 0.067 mg l^{-1} h^{-1} reported by the latter for *Pseudomonas* spp. LP5 and LP6, respectively. Earlier observations have indicated that growth rate of isolates on substrates and their rate of utilization is not entirely determined by the intrinsic properties of the isolates but also culture conditions such as crystal size of substrates and variations in physicochemical parameters such as pH (**Grosser et al., 1991; Boldrin et al., 1993**).

In this study, strain SL9 degraded 55.16 and 89.24% of the initial concentration of pyrene (50 mg l^{-1}) in 12 and 21 days. This degradation rate is lower than 72% (500 mg l^{-1}) in two weeks and 62% in 24 h reported for *M. flavescens* and *Rhodococcus* strain UW1, respectively (**Walter et al., 1991; Dean-Ross and Cerniglia, 1996**). It is however higher than 61.5% reported for *Leclercia adecarboxylata* (Sarma *et al.*, 2004). It is also higher than 65.8% and 33.7% (50 μg ml⁻¹) within 21 days reported for *Bacillus cereus* Py5 and *Bacillus megaterium* Py6 as well as 68, 67 and 47% within 30 days reported for *Pseudomonas* spp. Strains LP1, LP5 and LP6, respectively (**Lin and Cai, 2008; Obayori et al., 2008**).

Aerobic degradation of PAHs normally proceeds via the upper pathway characterized by ring destabilization and cleavage through dioxygenation and dehydrogenation, resulting in a catecholic intermediate (**Mason and Cammack, 1992; Diaz, 2004; Vaillancourt et al., 2006**). In the lower pathway, ring fission of the catecholic intermediate through the *ortho* (catechol 1,2- dioxygenase) or *meta* cleavage (catechol 2,3 dioxygenase) occurs, which lead to intermediates of the tricarboxylic acid cycle. In this study, catechol is *meta* cleaved by catechol 2,3dioxygenase (C23O) produced by strain SL9 resulting in the formation of 2-hydroxymuconic semialdehyde. Though **Obayori et al. (2008)** reported the detection of catechol 1,2 dioxygenase in three pyrene-degrading *Pseudomonas* strains isolated from contaminated soils in Lagos, Nigeria, several researchers have reported the ubiquity of C23O in most PAH degradation pathway (**Meyer et al., 1999; Zhao et al., 2011**). This is partly due to their versatility and their

preponderance in many catabolic and biosynthetic pathways (**Vaillancourt *et al*., 2006**).

CONCLUSION

This study described, for the first time, pyrene biodegradation potential of a *Microbacterium esteraromaticum* strain from tropical hydrocarbon-contaminated soil and also showed the degradative ability of the isolate on other aromatics and complex hydrocarbon mixtures. This study also demonstrate for the first time the ability a pyrene-degrading *Microbacterium esteraromaticum* strain to resist some antibiotics and tolerate elevated concentrations of some heavy metals. This showed its potential as seed for bioremediation of hydrocarbon-impacted soils co-contaminated with heavy metals. Further research works will focus on the metabolic pathway employed by strain SL9 for pyrene degradation and the degradative genes involved in the process.

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