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Degradation of Phenanthrene and some selected Petroleum Hydrocarbons by *Lysinibacillus fusiformis* (ALSL 5)

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are universal environmental contaminants of great concern with regard to their potential exposure and deleterious effect on human health. This study was conducted to determine phenanthrene and other PAHs degradation activity of *Lysinibacillus fusiformis*. The biodegradation study was analyzed by Gas Chromatography-Flame Ionization Detector (GC-FID) and the result showed that the isolate was able to degrade 57 %, the rate of degradation was 6.126 mg/l/day, the rate of degradation constant was 0.0085/day and the half-life was 8.0975 days in the selective medium within days 10. *Lysinibacillus fusiformis*, ALSL 5 also showed activities on the other PAHs it was tested on. The results revealed that *Lysinibacillus fusiformis*, ALSL 5 was able to degrade phenanthrene significantly and it could prove to be promising bacteria for bioremediation of PAH-containing pollutant from the contaminated site.

KEYWORDS: *Lysinibacillus fusiformis* ALSL 5, phenanthrene, Polycyclic Aromatic Hydrocarbons (PAHs), environmental contaminants, bioremediation

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INTRODUCTION

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Accidental releases of petroleum products are of particular concern in the environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants. Polycyclic aromatic hydrocarbs (PAHs), also known as arenes, are large family of non-functionalized aromatic compounds containing either two or more fused benzene rings. They are derived from natural and anthropogenic sources, like combustion and pyrolysis, making them abundant in the environment. They are created when substances such as coal, oil, gas and organic matter/waste are burned incompletely. At high temperature, organic compounds are partially cracked to smaller unstable fragments, mostly radicals that recombine to give relatively stable PAHs. Also, aromatization at lower temperatures (100–150 °C) requiring much more time produces large quantities of alkylated PAHs [1]. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants. Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products [2].

The contamination of the environment by polycyclic aromatic hydrocarbons (PAHs) is becoming a rising environmental concern. They have a widespread distribution in the environment and the carcinogenicity and mutagenicity of several of these compounds have been proven [3]. In 2001 PAHs were ranked the ninth most threatening compounds to human health [4]. Several epidemiological studies on PAHs especially among workers exposed to these compounds in a number of countries have been carried out [5]. PAHs comprise the largest class of chemical compound known to be cancer-causing agents and are included in the European Union and United States Environmental Protection Agency (EPA) priority pollutant list due to their mutagenic and carcinogenic properties.

The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dispersion, and washing. However, these technologies are expensive and can

lead to incomplete decomposition of contaminants. The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry [6].

Degradation of Polycyclic Aromatic Hydrocarbons (PAHs), e.g Phenanthrene, by microorganisms such as *Lysinibacillus fusiformis* (ALSL5) has been studied over the years but not much work has been done in determining if *Lysinibacillus fusiformis* (ALSL5) has the ability to degrade other substrates. The aim of this study is to determine the ability of *Lysinibacillus fusiformis* strain ALSL5 to degrade phenanthrene and other petroleum hydrocarbons.

MATERIALS AND METHODS

Chemical Reagents

The microbiological media such as Agar-Agar, peptone, Nutrient Agar, Yeast extract were all products of HIMEDIA laboratories. The chemical regents used in this study were of analytical grade and were products of United Kingdom, England and India such as Sodium hydroxide (NaOH) from Fisher Scientific Company USA, Sodium chloride (NaCl) from Klincent Kib, Mumbai India, Ferocyonate from Surechem products Hd, England, 4-Aminoantipyrine from Burgoyne, Burbidge's and co, Mumbai India. The substrate used phenanthrene was a product obtained from Sigma Life Science, Japan.

Equipment and Materials

The equipment used include used include: Microscope, Incubator, Hot air oven, Fridge, Weighing balance, pH meter, autoclave, shaker. Materials include: cotton wool, test tubes, conical flasks, bijou bottles, micropipette, petri dishes, universal bottle, inoculating loop, tape, aluminum foil, spirit lamp.

Sterilization of Equipment and Materials

Glassware such as test-tube, conical flask, bijou bottle was washed and sterilized in the oven for 1 hour at 160°c. The media was sterilized in the autoclave for 15 minutes at 121°c. The work bench was swabbed with cotton wool and ethanol. The equipment used were properly cleaned before and after use.

Preparation of media

Manufacturers' specification for the preparation of media were used in this research work which includes: Luria Bertani agar, Mineral salt medium, Nutrient agar.

Nutrient agar

Nutrient agar (28 g) was added to 1000 mL of distilled water in a conical flask. The solution was swirled and heated to 100°C to ensure suspended solid was dissolved. The mixture was then autoclaved at 121°C for 15 minutes.

Luria bertani agar

Luria Bertani agar contains 18 g of agar-agar, 10 g of peptone bacteriological, 10 g of Sodium Chloride and 5 g of yeast extract and the salts were dissolved in 1000 mL of distilled water in a clean conical flask. It was swirled and boiled to dissolve suspended solids. The media was then autoclaved at 121°C for 15 minutes.

Mineral salt medium

The mineral salt medium contains 2.13 g Na_2HPO_4 (Disodium phosphate), 1.3 KH_2PO_4 (Monopotassium phosphate), 0.50 g NH_4Cl (Ammonium chloride), 0.2 g $MgSO_4.7H_2O$ (Magnesium Sulphate Heptahydrate), all dissolved in 800mL of distilled water and pH adjusted to 7.0 by the addition of concentrated HCl (Acidic) or NaOH then distilled water was added to make up to 1000 mL. The medium was then autoclaved at 121°C for 15 minutes.

Bacterial Strain

Isolate used for this study, *Lysinibacillus fusiformis* strain ALSL5 (Genebank accession no: KY0399021. The organism was obtained from a mechanical village at Alakuko Lagos and preserved on Luria Bertani glycerol (50:50) at -20°c.

Biochemical test

The tests carried out in this study include: Gram staining, Catalase test, Oxidase test, Colonial morphology, Endospore staining, Capsule staining, and Motility test.

Colonial morphology

Nutrient agar plate was prepared and the test organism was sub-cultured on it and then incubated for 24 hours. Then the shape, texture, growth pattern, elevation, margin and form of the colonies were carefully observed.

Gram staining

Gram staining was carried out to observe the characteristics of the organism with the aid of a microscope. A clean glass slide was used, disinfected and labelled properly, and a smear of the organism was made on a clean glass slide and then heated. A drop of crystal violet (Primary stain) was placed on the smear for 60 seconds and was rinsed off. A drop of Lugol's iodine was next for 30 seconds, then the slide was rinsed. A drop of Ethanol (decolourizer) was applied for 10 seconds and was rinsed off afterwards. Another drop of Safranin o. (Secondary stain) was placed on the slide for 30 seconds and the slide was rinsed gently afterwards and was allowed to dry before examined under a microscope.

Capsule staining

A smear of the test organism was made on a clean glass slide and it was allowed to air-dry. Then a drop if crystal violet was applied

and left for 2 minutes, after which it was gently rinsed off. The slide was blotted dry and was observed using the microscope with oil immersion.

Endospore staining

Endospore test was carried out to determine if the organism is a spore former. A 48 hours old culture was used to prepare a smear on a clean glass slide and was heat fixed by moving it around gently and carefully over a spirit lamp flame. The smear was flooded with malachite green stain and was heated gently over a beaker of boiling water for 10 minutes, and malachite green stain was continuously added to prevent it from drying on the slide while being heated. The slide was rinsed and drops of safranin o. was added for counter staining about for 20 seconds thereafter the slide rinsed and allowed to dry, and it was examined under the compound microscope.

Motility test

Motility indole urease (MIU) test is used to determine Motility, Indole formation and Urease test. Sulfide indole Motility medium (SIM) was prepared in a test tube which is a semisolid agar used to determine hydrogen sulfide production, indole formation and motility, the isolate was inoculated with a straight wire loop, making a single stab down the center of the tube to about half the depth of the medium, then incubate at 37℃.

Catalase test

Most aerobic microorganism are capable of producing the enzyme catalase although to different extents. This test is based on the principle that when organism containing catalase enzyme are mixed with hydrogen peroxide (H_2O_2), gaseous oxygen is released.

A suspension of 18-hour old culture of the test organisms was made with sterile distilled water on a clean glass microscope slide. A few drops of hydrogen peroxide were added using a dropping pipette. The evolution of gas bubbles caused by liberation of the free oxygen indicated the presence of catalase enzyme.

Oxidase test

Oxidase test was carried out to determine if the organism produces certain cytochrome C oxidase. A disk which is also known as a redox indicator was used to carry out the test. Bactident oxidase test strips were inserted to the overnight broth culture of isolates. The strips were withdrawn at once and left for 10 minutes for color change from yellow to purple confirmed the presence of oxidase. The oxidase test strips were impacted with 1% tetramethyl-p-phenyldiamine solution.

Detection of Ring-hydroxylation and Metabolite

Aromatic ring dioxygenase activity detection using indole

Phenanthrene degrading ALSL5 was pre-grown on yeast-extract peptone agar plates, thereafter indole crystals were then placed in the lids of the petri dishes and were then incubated for at 20ºC for a day.

Detection of cis-dihydrodiols

A flask containing 5 mL of MSM-phenanthrene and also containing the degrader was dispensed in a test tube and centrifuged at 3500 rpm for 10 minutes. 1 mL of culture supernatant was dispensed in another sterile test tube and acidified with 200 μl of 1 M Hydrochloric acid (HCl) before heating for 10 minutes at 100ºC. Thereafter, 200 μl of 1M Sodium hydroxide (NaOH) was added followed by the addition of 0.85% of 4-aminoantipyrine was also added.

Detection of phenolic compounds

A flask containing 5 mL of MSM-phenanthrene and the degrader was dispensed in a sterile test tube and centrifuged at 3500rpm for 10 minutes. 1 mL of the culture supernatant was then transferred into another sterile test tube and 200 μl of 0.85% 4-aminoantipyrine was added, afterwards, 200 μl of 1.4% Sodium bicarbonate ($NaHCO_3$), and 100μl of 5.4% Potassium ferricyanide ($K_3Fe(CN)_3$)was added to the culture supernatant and left for 10 minutes to detect color change.

Substrate Specificity Test

The substrates used in this experiment were of two types: Liquids and crystals, the liquids include: crude oil, kerosene, diesel, biphenyl, o-phthalic acid, engine oil, naphthalene acid, α- naphthol and that of the crystals include: dibenzothiophene, 4-chloroaniline, succinic acid, cinnamic acid. The crystals firstly were dissolved in acetone then 1 mL was dispensed into a conical flask and allowed to vent off, then 50 mL of MSM was introduced in it and then sterilized. After sterilization, 1 mL of the inoculum was introduced and then shook for 15 days. 1 mL of the liquids was dispensed in a conical flask and allowed to vent off, then 50 mL of MSM was dispensed and sterilized after which 1 mL of the inoculum was introduced and shook for 15 days.

Chromatographic Analysis of Residual Phenanthrene

To extract residual phenanthrene, 20 mL of sample was dispensed into a gas separation funnel with a stopper and cork. To this, 20 mL of Hexane was added as the solvent and the mixture was shook vigorously for 30 minutes while occasionally releasing the cork to let out pressure that has been built within the funnel. After which the hydrocarbon layer was collected into a glass beaker and allowed to evaporate in air to 1 mL. The concentrated 1 mL extract was transferred to a sample vial and kept in a refrigerator awaiting analysis.

The standard chromatogram was used to standardize the Gas Chromatography in preparation of the sample analysis. Afterwards, 1μl of the extract was injected into the GC and a corresponding chromatogram was generated also. The chromatogram of both the standard and the sample are then compared with respect to the concentration of standard. Hexane extracts (1.0 μl) of residual

Table 1: Colonial morphology of bacteria isolate

Bacteria isolate	Colony size	Colony shape	Colony color	Colony elevation	Colony margin	Colony surface	Colony texture	Colony Opacity
ALSL 5	Small	Irregular	Cream	Convex	Undulate	Rough	Watery	Opaque

PAH were analysed with Hewlett Packard 5890 Series II gas chromatograph equipped with flame ionization detector (FID). The column was OV-3 with length of 30 m, thickness 3.2 μm, and internal diameter of 5.3 μm. The carrier gas was nitrogen. The injector and detector temperatures were maintained at 220° C and 270° C respectively. The column temperature was programmed at an initial temperature of 50° C; this was held for 2 minutes and then ramped at 10° C/min to 250° C and held for 5 min. The GC analysis were carried out on the sample at day 0 and day 10. The PAH data were fitted to a first order kinetics model.

RESULTS

Morphological and Biochemical Characterization

Macroscopic and microscopic observation

The colonial morphology of the isolate, strain ALSL5 was assessed on Luria bertani agar plates (Figure 1) and is indicated in Table 1. After gram staining, the organism was viewed with a compound microscope using x100 oil immersion objective lens. The organism stained blue is said to be gram positive, rod shaped, arranged in singles.

Biochemical characteristics

The results of the biochemical tests carried out were taken properly according to specifications and are indicated in Table 2.

Detection of Ring-hydroxylation and Metabolite

Aromatic ring dioxygenase detection

After incubation with indole crystals, *L. fusiformis* ALSL5 gave a blue color indicating the presence of the aromatic ring dioxygenase enzyme responsible for dihydroxylating phenanthrene (Figure 2).

Detection of cis-dihydrodiols

A yellow color was observed after testing at different intervals indicating a presumptive positive color for the presence of cis-dihyrodiols in plate Figure 3 while the blank test tube showed a reddish brown to light brown color.

Detection of phenolic compounds

No color changes were detected after testing at different intervals.

Substrate Specificity of the Isolate

After 15 days of incubation and thoroughly shake, the activity of the organism on the substrates tested was determined by visual turbidity as shown in Table 3 in comparison with the control flasks for each substrate.

Table 2: Biochemical characterization of strain ALSL 5

TEST	RESULT
Capsule	Negative
Endospore	Positive
Motility	Negative
Indole	Negative
Urease	Positive
Catalase	Positive
Oxidase	Positive

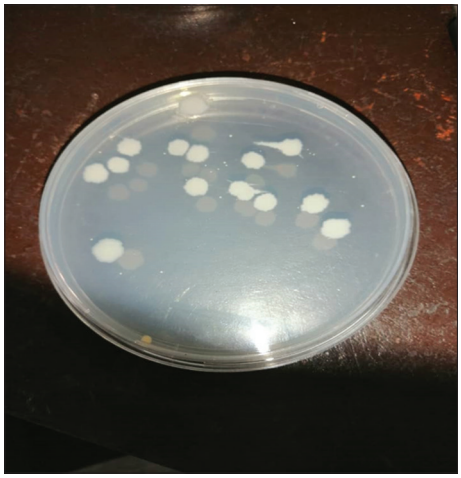

Figure 1: *Lysinibacillus fusiformis*, strain ALSL 5 colonies grown on Luria-Bertani agar plates

Growth Profile of the Isolate on Phenanthrene

The growth profile of *Lysinibacillus fusiformis* strain ALSL5 on phenanthrene was monitored at day 0, the population density (CFU/mL) of strain ALSL5 in mineral salt medium containing phenanthrene was 3.1 x 10^8, 6.3 x 10^8, 1.0 x 10^5 on the 6th day and 1.0 x 10^9 on the 10th day.

Kinetics of Phenanthrene Degradation

Determination of residual phenanthrene showed that 57 % was degraded by the organism, a degradation rate of 6.126 mg/l/day and rate constant of 0.085 with half-life of 8.1 days shown in Table 4.

DISCUSSION

PAHs can be formed both during biological processes and as products of incomplete combustion from either natural combustion sources (forest and brush fires) or man-made combustion sources (automobile emissions and cigarette smoke). Thus, PAHs are commonly detected in air, soil, and

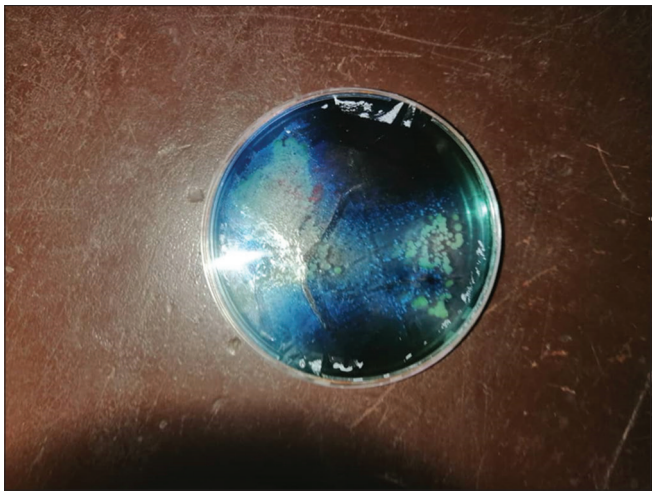

Figure 2: Appearance of blue colonies, which indicates a positive result for the presence of aromatic ring dioxygenase.

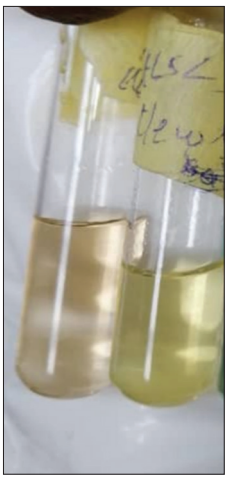

Figure 3: A yellow color indicating a positive result and a light brown color indicating a negative result for cis-dihydrodiols.

Table 3: Substrate specificity of *Lysinibacillus fusiformis, ALSL 5*

Substrates	Results
Crude oil	+++
Kerosene	++
Diesel	+++
Biphenyl	++
Ophthalmic	++
Engine oil	+++
Naphthalene	—
α– naphthol	+
Dibenzothiophene	++
4-chloroaniline	+++
Succinic	+
Cinnamic	++

- - No growth
+ - Poor growth
+ + Moderate growth
+ + + Luxuriant growth

water. Therefore, PAHs are considered ubiquitous in the environment [7].

Polycyclic aromatic hydrocarbons, also known as arenes, are hydrocarbons that have at least one aromatic ring. Emissions from anthropogenic activities predominate; nevertheless, some PAHs in the environment originate from natural sources such as open burning, natural losses or seepage of petroleum or coal deposits, and volcanic activities. Major anthropogenic sources of PAHs include residential heating, coal gasification and liquefying plants, carbon black, coal-tar pitch and asphalt production, coke and aluminum production, catalytic cracking towers and related activities in petroleum refineries as well as and motor vehicle exhaust [8]. PAHs are found in the ambient air in gas phase and as sorbet to aerosols. Atmospheric partitioning of PAH compounds between the particulate and the gaseous phases strongly influences their fate and transport in the atmosphere and the way they enter into the human body. The removal of PAHs from the atmosphere by dry and wet deposition processes are strongly influenced by their gas/particle partitioning. Atmospheric deposition is a major source for PAHs in soil. Clean-up of environments polluted with PAHs is important due to their acute toxicity, low bioavailability, hydrophobicity, mutagenicity and carcinogenicity [9].

In this study, we report the degradation of phenanthrene and other hydrocarbon substrates by *Lysinibacillus fusiformis* strain ALSL5 as it has not been fully studied. In another research, by Abd-Elsalam, Hassan E *et, al.*[10] *Burkholderia* species VUN10013 was found to utilize and degrade phenanthrene and anthracene but it couldn't degrade other PAHs such as pyrene, fluoranthrene, chrysene or benzo(α)pyrene. However, when present in a mixture with phenanthrene or anthracene, some PAHs such as pyrene and fluoranthene could be degraded by strain VUN10013.A similar occurrence was reported in this study as the growth rate on other substrates such was measured and no growth was observed on Naphthalene which shows *Lysinibacillus fusiformis* strain ALSL5 couldn't degrade Naphthalene.

After 10 days of cultivation, the degradation percentage was 57.5, which was determined by gas chromatography analysis. Compared to *Acinetobacter sp. P3d* which showed a maximum degradation rate of 93.58% after 28 days cultivation according to Fazilah *et al.*, 2018[11].

Activities of ring hydroxylating enzyme such as presence of aromatic ring dioxygenase on *Lysinibacillus fursiformi* was detected by using indole and cis-hyrodiols color indicator as compared to Vamsee-Krishna *et al.*, 2006[12] which showed P4,5DO activity on Phenanthrene grown cells, whereas phthalic acid-grown cells showed protocatechuate 3,4-dioxygenase (P3,4DO) activity.

Lysinibacillus fusiformis can utilize these substrates on phenanthrene, crude oil, engine oil, diesel, 4- chloroaniline which showed more abundant activities and moderate growth with biphenyl, kerosene, ophthalic, while naphthalene acid showed no activities as compared to *Pseudomonas sp. strain PPD* which can utilize phenanthrene, hydroxybenzoic acids and phthalic acid isomers according to Jaigeeth *et al.*, 2009[13].

This genus *Lysinibacillus* has been tested for potential bioremediation use in research, it was reported that *L. sphaericus*

Table 4: Kinetics of degradation of phenanthrene by *Lysinibacillus fusiformis*, ALSL 5

Bacteria isolate	Phenanthrene concentration (mg/l)			Percentage degradation (%)	Rate of degradation (mg/l/day)	Degradation rate constant (/day)	Half-life ($t^{1/2}$)
ALSL 5	Day 0	Day 5	Day 10	57 %	6.126 mg/l/day	0.0856 /day	8.1 days
	100	69.13	43				

G1 and *L. fusiformis* are able to clean up industrial effluent and areas contaminated with mercury [14].*L. fusiformis* produces biosurfactants which were characterized on the basis of their emulsifying properties with petrol, diesel, mobile oil and petrol engine oil [15]. Besides all features discussing up on molecule biosynthesis, bioremediation and biocatalysts, the genus *Lysinibacillus* often regarded as environmental contaminants was isolated in the clinical microbiology laboratory. Although Clinical relevant infections with *Lysinibacillus* sp. are still uncommon, a rare case of severe sepsis due to *L. fusiformis* and *L.sphaericus* was reported [16].

CONCLUSIONS

Based on this study, *Lysininbacillus fusiformis*, strain ALSL 5 was found to be a potent degrader of phenanthrene. Its activity on crude oil, kerosene, diesel, biphenyl, o-phthalic acid, engine oil, α- naphthol, dibenzothiophene, 4-chloroaniline, succinic acid, cinnamic acid is a great indicator that *Lysinibacillus fusiformis* can also degrade other hydrocarbons.

Lysiniibacillus fusiformis holds a promising future for use in clean-up of hydrocarbon polluted environment, this will reduce environmental risks and hazard caused by petroleum and its derivatives.

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