Biodegradation of Kerosene by Soil Bacterial Species from Contaminated Site

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Abstract**:** *In this study, we investigated the use of indigenous bacterial species from contaminated site to degrade kerosene. The method of continual enrichment on kerosene, yielded bacterial isolates with potential of utilizing kerosene as growth substrates. The time course studies monitored by the optical density (OD) and pH fluxes of the pure cultures in varying concentrations of kerosene resulted in exponential increase in cell numbers and decline in the pH values. From the morphological and biochemical characterization and comparison with respect to the standard references, the isolates were presumably the members of the genera Rhodococcus Bacillus* and *Aerobacter species. All the strains readily utilized the kerosene as sole sources of carbon and energy.*

Key Words**:** kerosene, degradation, enrichment, optical density, pH

Introduction

Petroleum-based products are the major source of energy for industry and daily life. Petroleum products consist of extremely complex mixture of aliphatic and aromatic hydrocarbons. The kerosene fractions, have been described as one of the greatest pollution problems in the environment (21). Kerosene is a colorless, flammable hydrocarbon liquid derived from fractional distillation of petroleum at 150 -275 °C. It consists of a characteristic odor and taste. Kerosene is insoluble in water, but is miscible with most organic solvents. Structurally, it is composed mostly of saturated hydrocarbon molecules containing twelve to fifteen carbon

atoms. Kerosene possesses moderate to high acute toxicity to biota, with product-specific toxicity related to the type and concentration of aromatic compounds (1). Numerous applications of kerosene include: aircraft gas turbine, as jet fuel for commercial airlines and military services (10). Kerosene serves as spray oil to combat insects on citrus plants. It also has application as a solvent in paints, cleaners and pesticides. Due to its wide application in several forms of transportation, there is increase in its production demand for transport, stockpiling and distribution.

In animals, kerosene could be mild producing transient ocular irritation that may result to conjunctivitis, hyperaemia and lacrimation (12). Some rare complications of kerosene intoxication include cardiac arrhythmia and ventricular fibrillation, attributed to increased myocardial sensitivity to endogenous catecholamines (2). The insolubility of kerosene in water poses the greatest pollution problem, where the petrol fraction rapidly evaporates and the insoluble "tar balls" of the alsphaltene residue either sink in ocean beds or are located along the shoreline around the beaches. This causes difficulty during the cleaning up of such sites.

Biodegradation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic, versatile and environmentally sound treatment for kerosene contaminated soils (13, 14, 22). No single microorganism has been found to completely degrade petroleum hydrocarbon molecule. However, different species or strains of the same species may be capable of degrading different groups of hydrocarbons, found in kerosene (7). *Pseudomonas, Serratia, Streptococcus, Micrococcus, Bacillus, Klebsiella, Proteus, Arthrobacter, Gordonia, Brevibacterium, Burkholderia* and *Mycobacterium* species have been found to degrade kerosene (3). The degradation of kerosene is a sequential process in which n-alkanes are generally removed first followed by the degradation of iso-alkanes ,cycloalkanes, 1-3 ring aromatics and finally poly-aromatics (9). In this study, we investigated the use of pure cultures of indigenous bacterial species from contaminated site to degrade kerosene.

Material and Methods

Chemicals and Reagents

Nutrient agar, nutrient broth were obtained from Micro master, India. Urease base agar, starch agar, methyl red and Voges Proskauer medium, peptone water were obtained from the Microbiology laboratory of Covenant University, Ota. The reagents $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$, K_2HPO_4, KH_2PO_4, and NaCl of analytical grade were obtained from Merck, Germany.

Kerosene was purchased from a gas station in Oju-Ore, Ogun State, Nigeria.

Stock Solutions and Media Preparation

Chloride-free minimal salts (MS) medium as described by (11) with slight modification were used for all the enrichment and degradation experiments. The medium consisted of (g) 0.5 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.076 $Ca(NO_3)_2.4H_2O$ and 40mM phosphate buffer (pH 7.25). Solid MS medium was prepared by the addition of 1.8% Bacto–agar (Difco Laboratories, Detriot, MI USA). The MS medium was supplemented with the kerosene oil.

Enrichment of microbial communities

Kerosene contaminated soil samples were collected from spilled surroundings of kerosene tank at Oju-Ore at Sango Ota, Ogun State, Nigeria. Kerosene degrading bacteria were initially isolated by traditional enrichment culture methods. For this, 5.0g of the soil samples were used to inoculate MS medium contained in a conical flask (200ml). The medium was amended with kerosene (0.3% v/v) as the primary carbon source. The flask containing the enrichment cultures were incubated on a shaker (Model H2Q-X 300) at 65 rpm at 30^{o}C for 12 days. Subsequent transfers from these enrichments were made fortnightly by using the same methods and conditions. After about 1 month, the enrichment cultures were transferred to a fresh medium using 18% inoculums and continued cultivation under the same conditions. Subsequent transfers were carried out using 2% inoculum and the procedure repeated for six successive times.

Determination of physicochemical parameters of soil

The soil samples were collected randomly at the site of study using a clean container. The stones were sieved and the soil samples thoroughly mixed and stored in 20ml McCartney bottles. The sample were analyzed for PAH, heavy metals and pH.

Inoculum preparation

The obtained cultures were incubated on a freshly prepared sterile nutrient broth in cotton wool stoppered Balch tubes and incubated at 30^{o}C for 120h. The cells were harvested by centrifugation at 35 x 100 rpm for 50 minutes, washed two times in phosphate buffer saline at pH of 7.25, transferred into a sterile 2.0 ml eppendorf tubes and re-suspended in MS medium as previously described by (15,16) to an optical density of 0.4 at 600 nm. The inocula were used for the growth and degradation studies.

Isolation, purification and characterization of hydrocarbon-oclastic bacterial species

Axenic cultures from the kerosene–enriched MS media were isolated by plating out 1.0 ml of the enriched cultures onto minimal salt (MS) agar, sprayed with kerosene on the surface. The plates were incubated in the dark at 28 - 32^{o}C for 6 days.

Colonies were periodically transferred to fresh MS agar as they appeared to obtain pure cultures. Using morphological observations, discrete bacterial colonies were isolated and sub-cultured onto separate agar plates. The pure cultures were incubated at 37°C for 18-24 h. The pure cultures were provisionally named as A ,B, D E G classified using standard cultural and morphological techniques and comparison with standard reference organisms (5,18). The following tests were carried out coagulase, citrate, Gram stain, morphology, catalase, oxidase, colony motility, methyl red, Voges Proskauer, indole, nitrate reduction, gelatin hydrolysis, spore test, starch hydrolysis, and sugar utilization.

Growth on kerosene as carbon and energy source

The axenic cultures of the tentatively named bacterial species A, B, D, E and G were tested for their ability to utilize kerosene. The tests were performed in bioreactors (Balch tubes) containing MS medium (10 mL) supplemented with the kerosene, which were inoculated by the pure cultures at 10^5 cells/ml. Tubes were labeled, stoppered with sterile cotton and incubated in shaker (Model H2Q-X 300) at 65 rpm at 30^oC for 12 days. An abiotic control was set up with the inoculated control (MS medium and kerosene oil devoid of organism) incubated at the same conditions as samples. Measurements of optical density (OD) and pH were carried out at 1, 3, 6, 9 and 12 days interval. Optical density was measured at 600 nm using Genesys 10 UVS Spectrophotometer.

Statistical analysis

Statistical tests (mean and standard deviation) were performed using the graph pad prism 4.0 computer software programme.

Results and Discussion

Five bacterial species were isolated and characterized; however two were selected for further biodegradation studies because of their better degradation activity during the preliminary studies. The isolates A, B and D are non-motile, catalase positive, Gram positive rod, capable of fermenting sucrose and maltose. The morphology and biochemical characterization suggested that they are similar to the members of the genus *Rhodococcus* sp. The isolates C and E were non-motile, catalase positive, capable of fermenting glucose. The data of Table 2 showed that isolates (C, E)were similar to members of the genus *Bacillus* and *Aerobacter sp*.

The concentrations of the poly-aromatic hydrocarbons and heavy metals in the contaminated soil are as shown in Table 1 included: fluorene 2.18ppm, Indeno (1, 2, 3-cd) pyrene 27.79 ppm; acenapthalene, fluoranthene and Nickel were not detected. The pH of the polluted soil was 7.18.

Table 1: Hydrocarbons detected at the selected site.

(PAH Hydrocarbons)	Selected soil
Physical	Light brown (silt, sand appearance)
Acenapthalene	Not detected
Fluoranthene	Not detected
Fluorene	2.18 ppm
Phenanthrene	Not detected
Indeno(1,2,3-cd)pyrene	27.79ppm
Lead	3.59
Nickel	Not detected

Table 2: Morphological and biochemical characteristics of pure bacterial species capable of degrading Kerosene

	A	B	C	D	E
Gram's Reaction	+	+	+	+	+
Motility Test	-	-	-	-	-
Acid-Fast Stain	-	-	-	-	-
Spore Stain	+	-	+	-	-
Catalase Test	+	+	+	+	+
Urease Test	+	+	+	+	+
Indole Test	-	-	-	-	-
MR Test	-	-	-	-	-
VP Test	-	-	-	-	-
Growth at 35^{o}C	+	+	+	+	+
Growth at pH at 6.0	+	+	+	+	+

Maltose	+	+	+	+	+
Sucrose	+	+	+	+	+
*Glucose	-	+	+	+	+
*Lactose	+	+	-	+	-
	Rhodococcus sp.	*Rhodococcuss* sp.	*Bacillus* sp.	*Rhodococcus*.	*Aerobacter* p.

+: Positive Reaction, -: Negative Reaction, *: Sugar Fermentation Tests

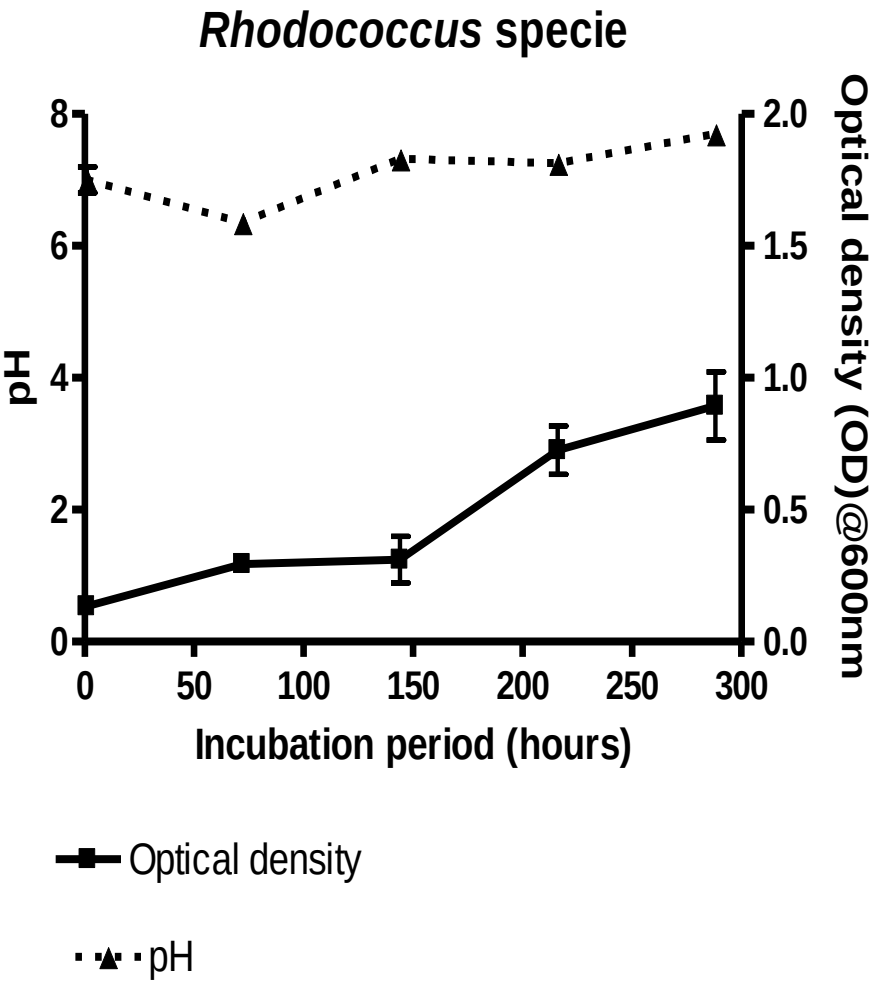

Figure 1. Degradation of Kerosene by *Rhodococcus* specie (strain A) cells. Data represent the mean and standard deviation of duplicate determinations of the gradient fluxes in the pH and Optical density OD at 600nm. The large error bars were due to differential responses of the cells in the duplicate tubes

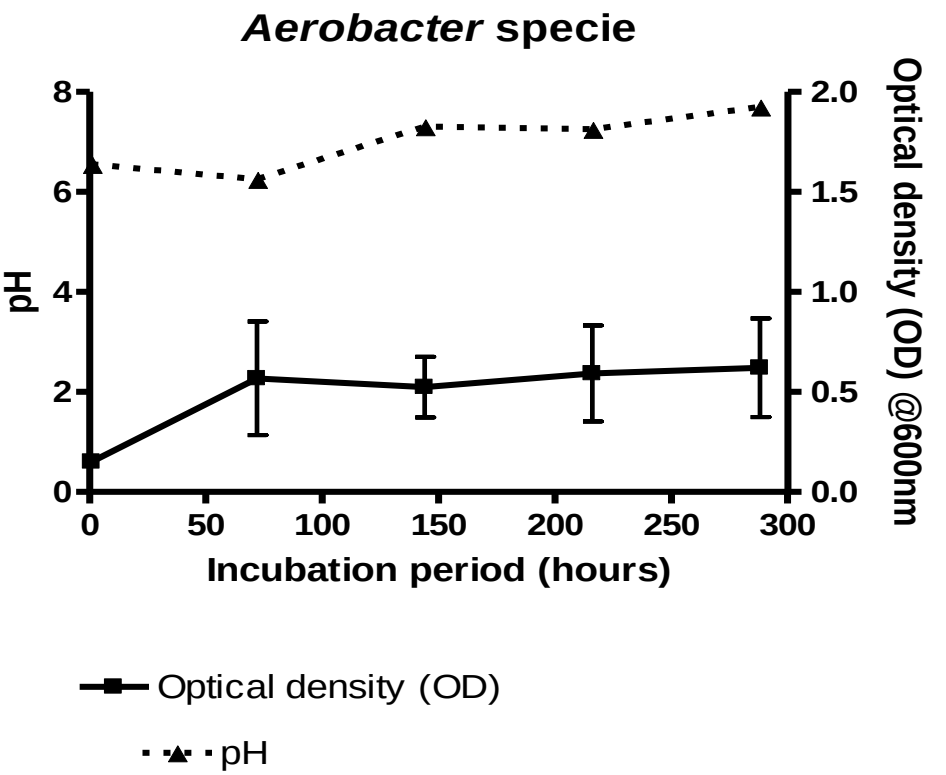

Figure 2. Degradation of Kerosene by *Aerobacter* specie cells. Data represent the mean and standard deviation of duplicate determinations of the gradient fluxes in the pH and Optical density OD at 600nm. The large error bars were due to differential responses of the cells in the duplicate tubes.

One of the critical ways to achieve the isolation of microorganisms with rare metabolic ability is by enrichment with substrates of target compound. This approach has been effective for the isolation and characterizing of the needed bacterial species. In this study, this approach yielded five organisms that could utilize kerosene. The morphological and biochemical characterization revealed they were all Gram-positive bacteria. These include species of *Rhodococcus* sp. of strains (A, B and

E), *Aerobacter* sp. (D), *and Bacillus* sp. (G). *Pseudomonas fluorescens* and *Bacillus subtilis* have been reported to use kerosene as carbon source (17). In the report of (18) hydrocarbon-utilizing bacterial genera isolated from most oil contaminated soil include: *Pseudomonas, Bacillus, Micrococcus, Flavobacterium* and *Klebsiella* species. In addition, the study of (3) reported isolating similar hydrocarbon utilizing bacteria from the Niger Delta aquatic systems and petroleum effluents. In their reports, it has been observed that some microorganisms are more abundant in areas of high concentration of hydrocarbons. These microflora are actively oxidizing the hydrocarbons and this is considered as another source of carbon for use in the ecosystem.

It has been noted that the availability of a compound to the microorganisms dictates its biodegradability (20). Thus, a highly water-soluble compound is more amenable to bacterial degrading enzymes and paramount when evaluating its toxicity to microorganisms. Thus, biodegradation is interaction between the aqueous solubility of the compound and its toxicity.

In this study, it was observed that the selected bacterial species were able to utilize the kerosene, where the pH values ranged from slightly acidic to alkaline. In addition, there was increase in the optical densities of the two selected bacterial species.

The *Rhodococcus* specie strain A was able to utilize the kerosene in the MS medium as its carbon source with OD readings increased from 0.134 - 0.894 after 12 days of incubation, the pH values obtained were 7.0 – 7.7. The *Aerobacter* sp OD readings increased from 0.151-0.621 and pH values of 6.3- 7.8 within the same incubation period. These results supported the report of (6) that had similar trend in their OD results. According to the report, the total viable counts increased significantly as the days of incubation progressed while there was significant decrease in pH as microbial isolates utilized the oil contaminated soil. However, the pH readings in our study increased slightly.

In this study, it was evident that the obtained pH was within the optimum pH for soil bacterial species. This further shows that our bacterial species may have followed the pathway that results to alkaline end products. It is conceivable to assert that it may have contributed to the increase in the alkaline state of the prevailing soil condition.

In conclusion, the bacterial isolates from kerosene contaminated site were able to utilize the kerosene as carbon and energy source. This showed that kerosene-degrading bacteria are abundant in the soil and these obtained bacterial species

might be exploited for use in the cleanup of kerosene polluted sites.

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