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**RESEARCH ARTICLE**

# MOLECULAR CHARACTERIZATION OF KEROSENE DEGRADING BACTERIA ISOLATED FROM KEROSENE POLLUTED SOIL

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## *Abstract*

Bacillus thuringiensis was identified has high potential degradation of kerosene. The organism was identified from the kerosene polluted soil sample. 8 different colonies were isolated from the contaminated soil. Colonies were subjected to morphological and biochemical characterization. Then the bacterial isolated were inoculated in the Bushnell Hass medium with 1% of Kerosene which acts as the sole carbon source for their growth. KC1, KC6 and KC8 different isolates were identified which utilizes the Kerosene and grows. Then the isolates were studied for their degradation by turbidometric method. Antibiotic sensitivity test showed all the isolates KC1 to KC8 were Ampicillin resistant, sensitive to Chloramphenicol, Kanamycin and Gentamycin and KC7 was resistant to streptomycin. By spectrometric method the KC1 isolate has high degrading potential of kerosene. The potential organism can be used further for removing of the contamination from the polluted area.



## INTRODUCTION

Lamp oil (Kerosene) is complex mixtures of hydrocarbons consist of paraffin, aromatic, and hydrocarbons with carbon numbers predominantly in the C9 and C16 range. Kerosene contamination affects soil physicochemical properties such as pH, conductivity, total phosphorus, microbial biomass and heavy metal content which are important indicators for assessing soil quality, birth rate and productivity The parts of kerosene at a fraction of petroleum could pose grave environmental problems when it instantly or indirectly enters into the environment because of their chemical nature.

Hydrocarbon degrading microorganisms require an environmental habitat that takes in a sufficient and preferably sustainable source of foods, water, air, mild ambient temperature, and a moderate pH. (Leahy & Colwell, 1990). Many microorganisms have the power to use hydrocarbons as sole sources of carbon as energy for metabolic activities and these microorganisms are ubiquitous and widely spread in the nature. (Jyothi et al., 2012).

The utilization of microorganisms of degrading the pollutant was safe to our surroundings to protect from contamination. So this study conducted to find the efficient kerosene degrading bacteria from kerosene polluted soil.

## MATERIAL AND METHODS

### SAMPLE COLLECTION

The kerosene polluted soil samples were collected aseptically from Sankagiri (T.k) Salem (D.t). Tamil Nadu, India.

**PHYSIOCHEMICAL CHARACTERIZATION**

The kerosene polluted soil samples were analysed for pH (Potentiometric method), electrical conductivity (EC meter), nitrogen (Alkaline Permanganate method, (Subbiah and Asija, 1956)), phosphorous (Olsen's method (Olsen et al., 1954 and Watanable and Olsen 1965))**,** potassium (Boiling Nitric acid method using Flame Photometer). M**icronutrients by** atomic absorption spectrophotometer.

**ISOLATION OF MICROORGANISM FROM SOIL SAMPLE**

The 1 gm of kerosene polluted soil sample were taken and serially diluted from $10^{-1}$ to $10^{-8}$ dilutions. The colonies were obtained by spread plate method. The microbial population were calculated as follows

Population of microorganism present in 1gm of soil sample = Average no. of colonies X Plate detection factor.

**IDENTIFICATION OF MICROORGANISM**

The cultures were morphologically and biochemically identified by staining and biochemical tests like indole test, methyl red test, voges-proskauer test, citrate utilization test, triple sugar iron-agar test, catalase test, oxidase test, nitrate reduction test, litmus milk reaction test, urease test, carbohydrate fermentation test, starch hydrolysis test, gelatin hydrolysis test. The microorganisms were pure cultured by specific medium like blood agar for a Citrobacter species, Macconkey agar for an Klebsiella species, Cystine Lactose Electrolyte Deficient (CLED) agar for an proteus species to isolating pure culture.

**ANTIBIOTIC SENSITIVITY TEST**

The isolated microorganisms were performed Antibiotic Sensitivity test. The antibiotic discs used are Ampicillin, Streptomycin, Gentamycin, Kanamycin and Chloramphenicol.

**HYDROCARBON DEGRADATION**

**ISOLATION OF HYDROCARBON DEGRADING BACTERIA**

The bacteria were isolated by inoculating the soil samples on enrichment medium that contains the autoclaved Bushnell-Haas agar supplemented with single hydrocarbon compound as sole carbon source (1% kerosene). The plates were incubated at 37°C for 10-15 days and observed.

**DETERMINATION OF BACTERIAL BIODEGRADATIVE ACTIVITY BY TURBIDOMETRY METHOD**

Turbidometry is to determine the bacterial growth by utilizing the hydrocarbons (1% kerosene) given as carbon source in MSM broth. The medium contains $K_2HPO_4$ (1.8g/l); $NH_4Cl$ (4g/l); $MgSo_4.7H_2O$ (0.2g/l); NaCl (0.1g/l); $Na_2So_4.7H_2O$ (0.01g/l); Carbon source (1% kerosene); and distilled water (1L) with $P^H$ 7.2. The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The degrading activities of each isolates were obtained by using mineral salt broth (MSB) in which 1% of hydrocarbon (kerosene) was added and incubated at room temperature for 15 days. The growth of the bacterium was measured by taking the O.D readings at 595 nm from 0 hrs – 15 days at regular intervals of 2 days against mineral salt medium as blank.

**16S rRNA sequencing**

Genomic DNA of the isolate was extracted with a GenElute DNA extraction kit from Sigma. The 16S rRNA gene of isolate was amplified using the universal primers 8F (5′- AGAGTTTGATCCTGGCTCAG) and 1541R (50-AAGGAGGTGATCCAGCCGCA-3′) (Kebria et al., 2009).

## RESULT

**PHYSICOCHEMICAL ANALYSIS**

The physicochemical characteristics of the soil influenced by the impact of kerosene as shown in table 1 are substantiated below, the pH value of control soil sample was 7.7 and KS1, KS3, and KS4 was 7.1 and KS2 was 6.8. The electric conductivity of the Control sample was 0.5 µs/cm, KS1 and KS4 was 3.8µs/cm, KS2 was 0.3µs/cm, and KS3 was 0.4µs/cm. The kerosene soil sample and Control sample doesn't contain lime.

**Macronutrients of the Polluted Soil**

The soil containing macronutrients were Nitrogen, Phosphorus and Potassium. The high amount of Nitrogen was present in the Soil sample KS2 with 73 kg/ac, which is Sandy Loam (SL) soil. The Control sample nitrogen was 70 kg/ac. In other soil samples Loamy Sand (LS) contain 64kg/ac in KS1 and KS4, 62kg/ac in KS3 sample. Phosphorus content of the Control, KS1, KS2 and KS4 contain 6kg/ac and KS3 contain 4kg/ac. Potassium content of the Control sample was 104kg/ac, KS1 and KS4 contained 100kg/ac, KS2 contained 93kg/ac and KS3 contained 86kg/ac. (TABLE 1)

**Micronutrients of the Polluted Soil**

The micronutrient content of the Kerosene polluted Soil (Control, KS1, KS2, KS3, and KS4) studied was Ferric, Manganese, Zinc and Copper. Ferric Content was 5.6ppm, 6.0ppm, 4.8ppm, 6.8ppm and 7.4ppm. Manganese content was 2.6ppm, 2.8ppm, 2.6ppm, 3.0ppm and 2.2ppm. Zinc content was Control, KS1, KS3 and KS4 contained 1.0ppm, 0.8ppm and 1.0ppm. Copper content was 1.0ppm, 0.6ppm, 0.8ppm, 1.0ppm and 1.2ppm. (TABLE 1)

**ISOLATION OF MICROORGANISMS**

The result of the bacterial count show that kerosene polluted soil had the highest count of $296x10^{-4}$ CFU/ml, $248x10^{-5}$ CFU/ml and $224x10^{-6}$ CFU/ml. (TABLE 2)

**MORPHOLOGICAL CHARACTERISATION OF MICROORGANISMS**

The Morphological characterization of the all the isolates shows gram negative rod shaped bacteria. (TABLE 2)

## DENTIFICATION OF ISOLATED MICROORGANISMS

The biochemical characterisation results were listed in Table. 3. Isolated microorganisms are KC1 Bacillus, KC2, KC5, KC6 Klepsiella pneumoniae, KC3 Citrobacter sedlakii, KC4 Klebsiella ozaenae, KC7 Citrobacter intermedius and KC8 Proteus mirabilis. (Table.4)

**TABLE 1: SOIL SAMPLE ANALYSIS**

| SOIL SAMPLE | | KS1 | KS2 | KS3 | KS4 |
|---|---|---|---|---|---|
| WEIGHT (gm) | | 305 | 205 | 475 | 215 |
| TEXTURE | | LS | SL | LS | LS |
| LIME STATUS | | NO | NO | NO | NO |
| $P^H$ | | 7.1 | 6.8 | 7.1 | 7.1 |
| ELECTRIC CONDUCTIVITY | | 3.8 | 0.3 | 0.4 | 3.8 |
| MACRO NUTRIENTS kg/ac | NITROGEN | 64 | 73 | 62 | 64 |
| | PHOSPHORUS | 6 | 6 | 4 | 6 |
| | POTASSIUM | 100 | 93 | 86 | 100 |
| MICRO NUTRIENTS (PPM) | FEROUS | 6.0 | 4.8 | 6.8 | 7.4 |
| | MANGANESE | 2.8 | 2.6 | 3.0 | 2.2 |
| | ZINC | 0.8 | 1.0 | 0.8 | 0.8 |
| | COPPER | 0.6 | 0.8 | 1.0 | 1.2 |

**TABLE 2: COLONY COUNTING**

| S.NO | DILUTION | COLONIES | TOTAL PLATE COUNT |
|---|---|---|---|
| 1. | $10^{-4}$ | 74×4 | $296\times10^{-4}$ |
| 2. | $10^{-5}$ | 62×4 | $248\times10^{-5}$ |
| 3. | $10^{-6}$ | 56×4 | $224\times10^{-6}$ |

**TABLE 3: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION**

| **ISOLATES** | | **KC1** | **KC2** | **KC3** | **KC4** | **KC5** | **KC6** | **KC7** | **KC8** |
|---|---|---|---|---|---|---|---|---|---|
| **SIMPLE STAINING** | | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod |
| **GRAM STAINING** | | +ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| **INDOLE** | | - | - | + | - | - | - | + | - |
| **MR** | | + | - | + | + | - | - | - | + |
| **VP** | | - | - | + | + | + | + | - | - |
| **CITRATE** | | + | + | + | - | + | + | + | + |
| **UREASE** | | + | + | + | + | + | + | - | + |
| **CATALASE** | | + | + | + | - | + | + | + | + |
| **OXIDASE** | | + | + | + | + | + | + | - | - |
| **TSI** | | + | + | + | - | + | + | + | - |
| **$NO_3$ REDUCTION TEST** | | - | + | ± | - | + | ± | + | + |
| **LITMUS MILK REACTION** | | Alkaine | Acid, gas, curd | Alkaline | Acid, gas, curd | Acid, gas, curd | Acid, gas, curd | Alkaline | Alkaline |
| **GELATIN** | | - | - | - | - | - | - | - | - |
| **STARCH HYDROLYSIS** | | - | - | - | - | - | - | - | - |
| **CARBOHYDRATE** | **GLUCOSE** | + | + | + | + | + | + | + | + |
| | **LACTOSE** | + | + | + | + | + | + | + | - |
| | **SUCROSE** | + | + | + | + | + | + | + | + |
| | **FRUCTOSE** | + | - | + | + | + | + | + | + |

**TABLE 4: LIST OF IDENTIFIED ISOLATES KC1 TO KC8**

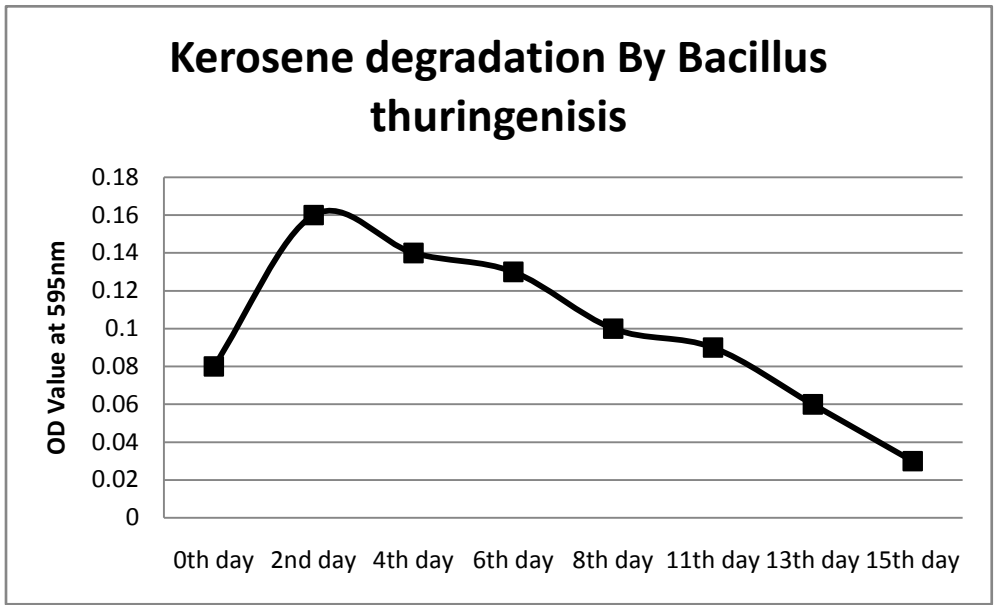
| S.NO | ISOLATED COLONIES | ORGANISMS |
|---|---|---|
| 1 | KC1 | *Bacillus thuringiensis* |
| 2 | KC2, KC5, KC6 | *Klebsiella pneumoniae* |
| 3 | KC3 | *Citrobacter sp* |
| 4 | KC4 | *Klebsiella sp* |
| 5 | KC7 | *Citrobacter sp* |
| 6 | KC8 | *Proteus mirabilis* |

**TABLE 5 GROWTH CURVE READINGS AT 595nm FOR 15 DAYS OF INCUBATION**

| ORGANISMS | GROWTH CURVE READING AT 595 nm FOR 15 DAYS INCUBATION (O.D) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $0^{th}$ day | $2^{nd}$ day | $4^{th}$ day | $6^{th}$ day | $8^{th}$ day | $11^{th}$ day | $13^{th}$ day | $15^{th}$ day |
| **Control + kerosene** | 0.05 | 0.03 | 0.08 | 0.06 | 0.04 | 0.03 | 0.11 | 0.07 |
| **KC1** | 0.08 | 0.16 | 0.14 | 0.13 | 0.1 | 0.09 | 0.06 | 0.03 |
| **KC2** | 0.11 | 0.06 | 0.05 | 0.07 | 0.03 | 0.08 | 0.05 | 0.08 |
| **KC3** | 0.15 | 0.07 | 0.04 | 0.10 | 0.04 | 0.07 | 0.09 | 0.14 |
| **KC4** | 0.07 | 0.13 | 0.08 | 0.05 | 0.04 | 0.10 | 0.04 | 0.06 |
| **KC5** | 0.09 | 0.22 | 0.05 | 0.09 | 0.04 | 0.02 | 0.05 | 0.09 |
| **KC6** | 0.08 | 0.15 | 0.16 | 0.08 | 0.06 | 0.08 | 0.06 | 0.04 |
| **KC7** | 0.06 | 0.08 | 0.12 | 0.07 | 0.04 | 0.02 | 0.05 | 0.10 |
| **KC8** | 0.04 | 0.12 | 0.06 | 0.08 | 0.09 | 0.06 | 0.04 | 0.11 |

**TABLE 6 DEGRADATION OF pH VALUE FOR 15 DAYS OF INCUBATION**

| ORGANISMS | $P^H$ VALUE | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $0^{th}$ day | $2^{nd}$ day | $4^{th}$ day | $6^{th}$ day | $8^{th}$ day | $11^{th}$ day | $13^{th}$ day | $15^{th}$ day |
| **Control + kerosene** | 8.06 | 7.09 | 7.00 | 6.92 | 7.54 | 7.84 | 7.33 | 7.58 |
| **KC1** | 8.09 | 7.05 | 7.03 | 7.02 | 8.26 | 8.10 | 7.35 | 7.50 |
| **KC2** | 8.37 | 7.19 | 6.98 | 6.96 | 8.19 | 8.06 | 7.34 | 7.18 |
| **KC3** | 8.58 | 6.88 | 6.79 | 7.37 | 8.17 | 8.00 | 7.35 | 7.42 |
| **KC4** | 8.41 | 7.21 | 7.17 | 7.40 | 7.96 | 7.98 | 7.31 | 7.45 |
| **KC5** | 8.45 | 7.19 | 7.23 | 7.35 | 8.02 | 7.98 | 7.14 | 7.41 |
| **KC6** | 8.25 | 7.23 | 7.14 | 7.39 | 7.97 | 7.91 | 7.20 | 6.95 |
| **KC7** | 8.17 | 6.84 | 7.17 | 7.37 | 7.91 | 7.86 | 7.15 | 6.88 |
| **KC8** | 8.23 | 6.95 | 7.09 | 7.33 | 7.87 | 7.84 | 7.12 | 6.96 |




**ANTIBIOTIC SENCITIVITY TEST**

Ampicillin resistant organisms were KC1 to KC8. Streptomycin resistant organisms were KC7 and sensitive organisms were KC1 to KC6 and KC8. Gentamycin, Kanamycin and Chloramphenicol sensitive organisms were KC1 to KC8.

**HYDROCARBON DEGRADATION**

Isolates KC1, KC6 and KC8 formed clear zone around the organisms in Bushnell-Haas medium

**HYDROCARBON BY TURBIDOMETRY**

The table shows the OD readings of biodegrading activity of each isolates on hydrocarbon (Kerosene). The OD readings based on the turbidity of MSM broth at regular intervals of 2 days the degradation activity on hydrocarbons by bacteria. The results demonstrated that KC1 and KC6 have the greatest ability to degrade kerosene. (TABLE 5)

The highly degrading microorganism isolated from kerosene polluted soil. From the 8 isolates KC1 shows the higher degradation potential. The organism was biochemically characterized, 16S rRNA sequenced and identified as Bacillus thuringiensis gram positive bacteria with 1414bp and the sequence was submitted to NCBI.

**DISCUSSION**

Kerosene is the type hydrocarbon, the impact of these in the soil leads to change in physiochemical characteristics compared to the Sandy loam and loamy sand, soil the pH of the Sandy loam compared low in the loamy sand soil. But both show the neutral pH. Reduction in pH, conductivity and total phosphorus were observed in simulations of the soil with kerosene from 6.8 to 7.1.

The pH of the unpolluted soil fell within the pH range of between 5-7 which is suitable for most good agricultural soils, since Rotter (2006) reported that most good agricultural soils have a pH between 5 and 7. Electrical conductivity is a criterion of the presence of salts. The electrical conductivity of kerosene polluted sandy loam soil 0.3 µS/cm and loamy sand soil 0.4 and 3.8 µS/cm.

The non polar environment for the soil ions, retarding their movement and immobilizing them, resulting in reduced ionic mobility, velocity and consequently bringing about reducing conductivity (Akpoveta et al., 2011). Hydrocarbon in the soil reduces pH and conductivity Osuji and Nwoye (2007). Kerosene is a product of crude oil, which is highly carbonated and contains some proportion of nitrogen from crude oil contains varying proportions of nitrogenous substances, thus accounting for the increased levels of carbon and nitrogen in the kerosene simulated soil (Akpoveta et al., 2011).

In marine and freshwater environments, the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally became the determining factor for oil degradation. Several authors have reported the negative effects of high NPK levels on the biodegradation of hydrocarbons, especially on aromatic (Das et al., 2011).

The bacterial count of the kerosene polluted soil of our study has the microbial count of 296x10-4 colonies in the sandy loam soil. 1.4x106 to 3.1 x106 of bacterial count and 0 – 1.6x10 2 colonies of fungal count were observed in the Nigerian country (Akpor et al., 2007).

The kerosene degrading microorganism was isolated has the Bacillus sp. Citrobacter species from the kerosene polluted soil. There are different researchers who have found the kerosene degrading microorganism from the polluted soil. The organism such as Alcaligen sp., Bacillus sp., Chromobacterium sp., Corynebacterium sp., Pseudomonas Sp., Aeromonas sp., Seratia sp., Flavobacterium sp., Micrococcus sp., Proteus sp., Cellulomonas sp., were isolated as the kerosene degrading microorganism isolated from the Kerosene polluted soil in the Nigeria (Akpoveta et al., 2011). Bacillus sp., and Pseudomonas sp., was identified as kerosene degrading microorganism (Akpor et al., 2007, Alekhina et al., 2007, Makut et al., 2010 and Khan et al., 2011.)

Our results indicated that all the organisms maximally utilized all the hydrocarbon substrate (kerosene) when furnished as the only origin of carbon and energy, although, the level of utilization differs from one bug to another (due to conflicts in their growth) and from one hydrocarbon substrate to the others, due to the obvious deviations in their molecular sizes. The bacterium with the least degrading activities on kerosene was KC2 and KC4 respectively. These degrading capabilities on different hydrocarbons revealed that the microorganisms isolated from the soil and water samples were able to degrade hydrocarbons. The cells were able to multiply within the days of study, indicating that they were able to degrade and utilize the oil for their growth and development, hence the concomitant increase in the concentration of the broth (turbidity). This gradual growth in the absorption of the broth indicates bacterial growth, hence degradation of hydrocarbons, mostly between days 5 and 12 and gradual decline in the compactness of the broth suggests a reduction in the bacterial population and that the hydrocarbon has been degraded, mostly between days 13 and 15.

Bacillus thuringiensis was a gram negative rod shaped bacteria. It is used as commercial bio insecticide. It produce hydrolytic enzyme, proteases (Egorov et al., 1978; Epremyan et al., 1981), amylases (Tobey et al., 1977), esterases (Yongmei et al., 1981), nucleases (Priest et al., 1977), chitinases (Barboza-Corona et al., 1999) and chitosanases (Cruz et al 2004)

Diesel degrading microorganism from the diesel polluted region of Iranian, the 16s RNA sequence strain has the close relationship Bacillus Cereus and Bacillus thuringiensis (Kebria et al., 2009). Bacillus thuringiensis have a potential to degrade the hydrocarbons. Thus biological method of degrading the pollutant was safe to our environment.

**CONCLUSION**

Soil is the natural material which consists of both organic and inorganic compounds which support the characteristics of the soil to maintain equality in all conditions. But due to the man-made pollution, such as oil spills, hydrocarbon contamination and all the xenobiotic there is a change in the alterations of the nutrients present in the soil and use of microbial colonies has been affected. Alliteration makes the soil to release of more CO2 from them it leads to the cause of environmental pollution such as climate change and global warming etc. The biological method of degradation is useful to degrade the toxic pollutant to non toxic chemical. Therefore the present work was studied

to identify kerosene degrading microorganism from the kerosene polluted soil. In which the following species Bacillus species, Klebsiella species and Proteus species were identified from the kerosene contaminated soil. One of the species was 16s RNA sequenced and identified as Bacillus thuringiensis. The identified organisms were resistant to Ampicillin and sensitive to Chloramphenicol, Kanamycin and Gentamycin. Effective kerosene hydrocarbon degrading bacteria from the study is Bacillus thuringiensis.

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