Phenylobacterium Korensee Best Indigenous Petroleum Hydrocarbon Degrading Bacteria Isolated from Contaminated Soil of Bahror, Alwar Region, India.

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Abstract:

Contamination of soil, water and air due to hydrocarbons are a global issue and bioremediation provides probably the best way to remediate the contaminants. The current study shows the biodegradation of crude oil, diesel and used engine oil by a newly isolated Phenylobacterium korensee from contaminated soil of Bahror, Alwar, Rajasthan. Hydrocarbon degrading strain was screened on BHA (Bushnell Haas Agar) media supplemented with 2T engine oil as sole carbon source. The strain was found to be degrading at 1%, 4% and 10% of used 2T engine oil respectively after 14 days. Degradation was confirmed both gravimetrically and by Gas Chromatography Mass Spectroscopy analysis. The degradation was found very well at long term basis. The optimization of growth also studied at temperature and pH basis also. The significance of the study is that the percentage degradation of the complex petroleum supplements used in the study was found to be far higher than some of the previously reported values and this bacterial strain was firstly found from this contaminated site.

Key words: Phenylobacterium korensee, Complex hydrocarbon, Degradation of complex petroleum oil

Introduction:

Petroleum is a complex mixture of many thousands of compounds mainly consisting of carbon and hydrogen. Nowadays deliberate use of petroleum hydrocarbon products, such as diesel and engine oil increases the chance of soil pollution and gradually it is proving itself as a major environmental problem. Petroleum hydrocarbons can be introduced into the environment via oil spills, leaking or unplugged oil wells, the disposal ponds of waste petroleum products, abandoned oil refinery sites, pipe line ruptures, incomplete combustion of fossil fuels and accidental discharge during transport in tanks and ships failures. The spillage has severe health related impacts on human and aquatic animals. Disaster arising from such incidence result in the discharge of crude oil into the environment affecting soil, air and water bodies.

As industrialization expands, petroleum hydrocarbons become a greater potential source of soil contamination. Soil contamination with hydrocarbons causes extensive damage of local ecosystem since accumulation of pollutants in
animals and plant tissues may cause progeny’s death or mutation. It has become one of the major environmental pollution that is becoming more stringent and to be given a lot of attention. The elevated loading of petroleum hydrocarbons in soil cause a significant decline in soil quality and these soils have become unusable. To control the environmental risk caused by petroleum products, various new regulations have been introduced and, at the same time, research focusing on remediation (Alvare et al., 1991).

Biodegradation is being hydrocarbon degradation. Microorganisms used as a treatment option at many are endowed with metabolism machinery to hydrocarbons contaminated sites (Braddock et al., 1997), which exploits the ability of energy source. The extent of biodegradation microorganisms to degrade and/or detoxify hydrocarbons in contaminated sites is organic contaminants.

**Materials and Methods:**

Soil samples were collected from different motor workshop areas of Bahror industrial region. Petroleum oil used in this study was obtained from Bharat oil petroleum Ltd. Sitapura, Jaipur.

**Isolation, screening, morphology and biochemical characterization:**

Bacterial strain was isolated from petroleum contaminated soil from Bahror, Alwar region, Rajasthan, India. For their isolation selective media as Bushnell Hass Agar (BHA) containing 2T oil as sole source of carbon and energy was used. The bacterial strain was screened based on the ability of the bacterial species to degrade petroleum oil. The isolated bacterial colony was identified by morphological and biochemical characteristics.

The biochemical test was applied to identify bacterial isolates up to generic level which includes Lipase production test, Nitrate reduction test, Starch hydrolysis test, Carbohydrate fermentation test, Indole production test, Methyl Red-Voges Proskauer (MR-VP), Catalase test, Urea hydrolysis, Phenylalanine deaminase activity was done.

**Degradation Capability to Petroleum oil (2T Engine oil) by Phenyllobacterium korensee at different oil concentrations.** (1) By Gravimetric Analysis (2) By GCMS Method:

Bacterial degradation of petroleum oil was done using the protocol of Mittal and Singh (2000). Petroleum hydrocarbon degradation at different oil concentrations at 1%, 4% and 10%. First of all prepared the Luria Bertani (LB) Broth media and then inoculate the bacterial culture into the LB Broth media at 37°C for 48 hours. Secondly, prepared the Mineral Salt Media containing pH 5.6 ± 0.2. Then after inoculate the 1% 2T engine oil into the MS Media. At last inoculated 1% of the isolated inoculums from LB Broth into the respective flasks. Then proceed with the Gravimetric analysis on Day 0, Day 7 and Day 14.

1. **Gravimetric analysis** 1% 1N HCl: added in 25 ml media into each flask. 25 ml Acetone and Petroleum ether (in 1:1 ratio) was added and mixed properly. Then after 1ml Acetone was added and the funnel remain still for 15-20 minute. After 15-20 different layers (3 layers) was observed. The 1st and 2nd layers were discarded and the 3rd layer was collected in the weight beaker and kept at water bath at 100°C for 10-15 minutes for evaporation. After evaporation is complete, clean the beaker from outside properly to remove any water on the outer side and the again weight the beaker (final weight).

The amount of oil left in the beaker after evaporation was calculated as follows:

\[
\text{Amount of oil left} = \text{Final weight of beaker} \quad \text{– Initial weight of beaker}
\]

Percent Degradation was calculated by the following formula:

\[
\text{Degradation} = \frac{(\text{Initial weight} \quad \text{– Final weight})}{\text{Initial weight}} \times 100
\]

2. **By GCMS methods:**

**Principle of GC-MS:** GC/MS-a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures (Skoog et al., 2007). The GC-MS instrument consists of two main components. The gas chromatography portion separates different
compounds in the sample into pulses of pure chemicals based on their volatility by flowing an inert gas (mobile phase), which carries the sample, through a stationary phase fixed in the column (Skoog et al., 2007). Spectra of compounds are collected as they exit a chromatographic column by the mass spectrometer, which identifies and quantifies the chemicals according to their mass-to-charge ratio (m/z). These spectra can then be stored on the computer and analyzed.

**Procedure:**

The extract of each degraded oil samples was mixed in ethyl acetate and then these samples were filtrated. After filtration, remaining substance was ester. These samples were injected to the gas chromatography and mass spectroscopy. Then analyzed complex organic and biochemical mixtures.

**Results:**

Isolation, screening and biochemical characteristic of indigenous *Phenylobacterium korensee* from petroleum contaminated soil samples.

Selective media (BHA) containing 2T oil as sole source of carbon and energy was used for their isolation. Based on varied colony characteristic (margin, elevation, color etc.) bacterial isolate was screened.

But on the basis of cell morphology and biochemical characteristics, this bacterial isolate was not identified up to genus level. So, for the identification of this bacterial isolate 16S rRNA sequencing was done. Through 16S rRNA sequencing it was identified and was named *Phenylobacterium korensee*.

![Fig.1. Sample collection site and pure culture of *Phenylobacterium korensee*](image)

**Table A. Biochemical characteristics of the *Phenylobacterium korensee***

| Isolates | Catalase | Starch | Oxidase | Carbohydrate Fermentation | H2S | IMVIC | TSI Agar Test |
|----------|----------|--------|---------|---------------------------|-----|-------|--------------|
|          |          |        |         |                           |     |       | A  K  G  H2S |
| BSB 3    | +        | +      | -       | +                         | +   | -     | -            |

Here: + = Positive result; - = Negative result
Percentage Oil Degradation by *Phenylobacterium korensee* at different oil concentrations.

### Table: B1. Bacterial degradation of 1% 2T Engine Oil

| Bacterial Isolates          | Sample code | Weight of oil (gm) |
|-----------------------------|-------------|--------------------|
|                             |             | 0 Day | 7 Day | 10 Day | 14 Day |
| *Phenylobacterium korensee* | BS-B3       | 0.601 | 0.391 | 0.314  | 0.137  |

### Table: B2. Percent degradation of oil (At 1% oil concentration):

| Bacterial Isolates          | Sample code | 0 to 7 Day | 0 to 10 Day | 0 to 14 Day |
|-----------------------------|-------------|------------|-------------|-------------|
| *Phenylobacterium korensee* | BS-B3       | 34.9418    | 47.7537     | 77.2047     |

### Table: B3. Bacterial degradation of 4% 2T Engine Oil

| Bacterial Isolates          | Sample code | Weight of 2T oil (gm) |
|-----------------------------|-------------|-----------------------|
|                             |             | Day 0 | Day 7 | Day 10 | Day 14 |
| *Phenylobacterium korensee* | BS-B3       | 1.398 | 1.3   | 1.169  | 1.154  |

### Table: B4. Percentage of oil degradation at 4% 2T Engine oil

| Bacterial Isolates          | Sample code | Percent degradation |
|-----------------------------|-------------|---------------------|
|                             |             | Day 0 to 7 | Day 0 to 10 | Day 0 to 14 |
| *Phenylobacterium korensee* | BS-B3       | 70.1001     | 16.3805     | 17.4535     |

### Table: B5. Percentage of oil degradation: At 10% 2T Engine oil

| Bacterial Isolate           | Sample code | Percent degradation |
|-----------------------------|-------------|---------------------|
| *Phenylobacterium korensee* | BS-B3       | Day 0 to 7 | Day 0 to 10 | Day 0 to 14 |
|                             |             | 1.08       | 5.88       | 8.11        |
Best Indigenous Petroleum Hydrocarbon Degrading Bacteria Isolated from Contaminated Soil of Bahror, Alwar Region, India.

Fig.2: Degradation at 1 % oil concentration

Fig.3: Degradation at 4 % oil concentration

Fig.4: Degradation at 10 % oil concentration

Table C1: Percent Reduction of Peak Areas observed in GC-MS by *Phenylobacterium korensee* (BS-B3) indicating biodegradation due to these isolated bacteria

| S. No. | Sample | Peak/Retention time | Covered area of Retention time/Peak area | Difference of Peak area from control | Percent reduction of Peak area % |
|--------|--------|---------------------|-----------------------------------------|-------------------------------------|-------------------------------|
| 1      | Control | RT 6.34             | 13476280459                              |                                     |                               |
| 2      | 1% 2T oil | RT 6.50             | 4764225110                               |                                     |                               |
| 3      |        | RT 6.66             | 3683993298                               |                                     |                               |
| S. No. | Retention Time | BSB3(*Phenylobacterium korensee*) | Maximum Degradation Percentage (%) |
|--------|----------------|----------------------------------|-------------------------------------|
| 1      | RT 6.34/6.35   | 41.19                            |                                     |
| 2      | RT 6.49/6.50   | 70.33                            |                                     |
| 3      | RT 6.65/6.66   | 71.81                            | 71.81/BS-B3                        |
| 4      | RT 6.83/6.84   | 41.52                            |                                     |
| 5      | RT 6.94/6.95   | 41.45                            |                                     |
| 6      | RT 7.06/7.07   | 40.93                            |                                     |

Table C2: Chart of degradation of GC-MS Peaks at different Retention Time (RT) obtained from culture for the study which were cultured for 15 days on 1% 2T oil supplemented medium

Figure: 5 – 6 Degradation capability of the *Phenylobacterium korensee* (BS-B3) studied by GC-MS (In all the graphs Horizontal axis → Retention time and Vertical axis↑ represents Relative abundance).
Discussion:

Biodegradation by microorganisms is a primary mechanism by which petroleum hydrocarbons could be removed or destroyed from contaminated soil (Bartha R, 1986). Because microorganisms having metabolic capability to utilize such organic compound as their sole carbon source. Survival of microorganisms in a medium supplemented with petroleum hydrocarbon after their inoculation is a key deciding factor in the rate of biodegradation of hydrocarbon (Ramos et al., 1991). An increase in protein content signifies the increase in cell number and utilization of hydrocarbon supplement as a sole carbon source by the isolates (Mandri and Lin, 2007). The degradation potential and the cell growth were found to be decreasing with the increase in hydrocarbon supplements. This may be due to the increase in cytotoxicity in the medium with the increase in hydrocarbon supplements in the medium (Borah and Yadav, 2012; Borah and Yadav, 2014).

The present study focused on the degradation of petroleum hydrocarbon by a bacterial strains isolated from the petroleum contaminated site. According to Penet and Marchal, 2006 the pattern of degradation varies for different degrading microorganisms because different microorganisms posses different catabolizing enzymes. These enzymes play an important role in the hydrocarbon degradation and the respective genes that encode those enzymes were identified in some studies (Whyte et al., 2002; Hassanshahian et al., 2012).

The study was done to test the degradation capacity of petroleum oil by the bacterial species isolated from the soil sample of petroleum contaminated...
site. The bacterial isolate (*Phenylobacterium korensee*) showed different hydrocarbon degradation capabilities at different (1%, 4% and 10%) oil concentrations in the culture medium.

However, highest hydrocarbon degradation occurred at 1% 2T engine oil containing culture media. It ranged from 34.94% to 77.20% in 14 days, while in 14 days it showed growth in exponential phase. After 10 days of culture growth showing fast growth and degradation. It means, it was found best degrader in long term basis. So we concluded that it is best degrader of complex hydrocarbons because complex hydrocarbons take more time in degradation in nature.

When 4% 2T engine oil was supplemented then *Phenylobacterium korensee* showed upto 70.10% by 7th days. After 7th day its growth decreases and got stagnation.

When 10% of 2T engine oil was supplemented in the medium, this shows its slow and steady degradation process. Hence with time it may give better petroleum oil degradation and will not die out early. It’s true because of very high oil concentration the bacteria had to struggle hard for their survival and growth which resulted in poor degradation but continued.

However, by comparing the results of the complete period from the 0-14 days at 1% 2T engine oil and GC-MS on the 15th day, the *Phenylobacterium korensee* has been shown to be promising with very good percent degradation upto 71.81%. Here, also proved by Gass Chromatography Mass Spectroscopy (GCMS) that it is very good biodegrader in long term basis.

Conclusion:

Petroleum hydrocarbon contaminated soil affected the fertility of soil. In this study oil contaminated soil was remedied by the potent hydrocarbon degrader *Phenylobacterium korensee* isolate. It was firstly isolated from this contaminated site. This study can be a key options for the decontamination of oil polluted soils.

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