Biodegradation of Petroleum Compound Using *Pseudomonas aeruginosa*

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Abstract

The present study was conducted to determine the biodegradation of petrol by *Pseudomonas aeruginosa* isolated from petrol contaminated soil. The isolated strain was able to grow in minimal broth along with 2.5%, 5%, 7.5% and 10% concentrations of petrol which indicated the capability of the organism in degrading petrol and utilizing it as a source for growth. The isolated strain’s efficiency was determined by analyzing the parameters pH, optical density and CO₂ released during petrol degradation. High Performance Liquid Chromatography (HPLC) analysis also confirmed the degradation of petrol by *Pseudomonas aeruginosa*.

Keywords

Biodegradation, Hydrocarbons, *Pseudomonas aeruginosa*, Petrol

Subject Areas: Environmental Sciences, Microbiology

1. Introduction

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. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year [1]. Polycyclic Aromatic Hydrocarbons (PAHS) are important pollutants which are introduced into the environment through different ways such as anthropogenic activities, combustion, undesirable discharging of oil tankers, spills around petroleum refineries and gas plant facilities [2]-[4]. Hydrocarbons are considered to be of biological origin, since short and long chain hydrocarbons (alkanes: C10 - C20, C20 - C40) appear to be exclusively the origin of biological processes [5]. These compounds have toxic, carcinogenic and mutagenic properties [6] [7] and are considered as a serious hazard to human health and environment [8]-[12]. The use of microorganisms to reduce petroleum pollution through bioremediation has
been shown to be a viable, relatively low cost, low-tech approach and is more widespread than chemical and physical treatments [13].

There has been extensive evidence on microbial degradation of petroleum hydrocarbons [14]. Since the biodegradation of petroleum hydrocarbons is a natural process controlled by temperature, pH and the scarcity of nutrients such as N and P [15] [16], bioremediation is a viable and promising method for clean-up and remedy of hydrocarbon polluted environment [17].

Bacteria are the most active agents in petroleum biodegradation and there is evidence of their fundamental role as primary degraders of spilled oil [18]-[20]. Effect of various nutrients on the degradation of crude oil by different bacteria was investigated by several scientists [21]-[23]. Several factors, both physico-chemical and biological, affect the rate of microbial degradation of hydrocarbons in soil. Recently, growing interest in the use of several Pseudomonads during degradation of crude oil have been reported [24]-[28]. However, application of statistical experimental design for optimization of crude oil degradation with *Pseudomonas* sp. was rarely investigated. Recently, medium optimization for a novel crude-oil degrading lipase from *Pseudomonas aeruginosa* SL-72 using statistical approaches for bioremediation of crude-oil was reported [29].

ZoBell [30] reported that nearly hundred species of bacteria, representing thirty microbial genera, had hydrocarbon oxidizing properties. Many species and genera had been found to have this ability [31]. The heterotrophic microorganisms found in the soil include naturally occurring populations that have the ability to degrade petroleum products species of *Pseudomonas*, *Arthrobacter*, *Alcaligenes*, *Corynebacterium*, *Flavobacterium*, *Achromobacter*, *Micrococcus*, *Nocardia*, and *Mycobacterium* appear to be the most consistently isolated hydrocarbon degrading bacteria from soil [32]. The present work is designed to isolate bacterial strain capable of degrading oil contaminants and to test its efficiency of petrol degradation.

### 2. Materials and Methods

For isolating efficient petrol degrading bacterial strain, soil samples were collected from petrol bunks and automobile workshops around Madurai in sterile containers and brought to the laboratory immediately [33]. Such soil samples were subjected to serial dilution up to $10^{-7}$ dilution [34]. From $10^{-5}$ and $10^{-6}$ dilution, 0.1 ml was taken and spread plated on Bushnell Hass mineral salt medium (Magnesium sulphate 0.2 g, Calcium chloride 0.02 g, Monopotassium phosphate 1 g, Dipotassium phosphate 1 g, Ammonium nitrate 1 g and Ferric chloride 0.05 g in one litre) containing 2.5% of petrol [35]. These petri plates were incubated at 37˚C for 24 hours. Among the developed colonies, one bacterial colony was selected for further studies.

The selected bacterial strain was subjected to Gram’s staining and other biochemical tests like MR, VP, Indole, Catalase, Citrate, Gelatin liquefaction, Cellulbiose, Lactose, Maltose, Sucrose, D-xylose, Trehalose, Sorbitol, Malonate, D-Arabinose and Glycerol utilization for identification adopting Bergey’s Manual [36].

For testing the biodegradation efficiency of the isolated bacterial strain, 100 ml of minimal broth (Dextrose 1 g, Ammonium sulphate 1 g, Dipotassium phosphate 0.7 g, monopotassium phosphate 2 g, Sodium citrate 0.5 g, and Magnesium sulphate 0.1 g) containing 2.5%, 5%, 7.5% and 10% petrol concentrations separately in 250 ml Erlenmeyer flasks were prepared. To each flask, one ml inoculum from the pure culture of the isolated strain during the logarithmic phase was added. The culture flasks were incubated in a shaker at 30˚C at 100 rpm. After 4, 8, 12 and 16 days of treatment, pH, Optical density (OD) and CO₂ were determined for each petrol concentration [37] [38].

The pH of the medium was estimated using a pH meter after every four days of treatment. The optical density of the culture medium was determined at 600 nm after 0, 4, 8, 12 and 16 days of treatment. For CO₂ estimation one ml the culture medium was taken after 4, 8, 12 and 16 days of treatment from each petrol concentration and treated against 0.05 N NaOH solution, the indicator used was phenolphthalein and the appearance of pink colour was the endpoint. Using the following formula the amount of CO₂ was calculated [33].

$$
\text{CO}_2 \text{ (mg/L)} = \frac{\text{Titre value} \times \text{Normality of NaOH} \times 1000 \times 44}{\text{Volume of the sample}}
$$

For HPLC analysis, fermented broth of the bacterial strain from 10% petrol concentration and the control were taken after 16 days of treatment and were administered for HPLC analysis at CECRI, Karaikudi (Model-Shimadzn, Pump-LC-20 AD, PDA detector—SPDM 20A and Injection—20 μl) [39]. The parameters pH, optical density and CO₂ were subjected to two way analysis of variance (ANOVA) using MS Excel. Variations were
considered statistically significant only when the calculated F value was greater than the tabulated F value at P is less than or equal the 0.05 [40].

3. Results and Discussion

Based on the biochemical tests, the isolated bacterial strain was identified as *Pseudomonas aeruginosa*. It was a gram negative rod exhibiting positive results to Citrate, Catalase, Gelatin liquefaction, Malonate and Glycerol tests. Negative results were observed for Gram reaction, Indole, Methyl Red (MR), Voges Proskauer (VP), Cellobiose, Lactose, Maltose, Sucrose, D-xylose, Trehalose, Sorbitol and D-Arabinose tests (Table 1).

*Figure 1* divulges the changes in pH recorded after 4, 8, 12 and 16 days of treatment with *Pseudomonas aeruginosa* upto 8 days of treatment, decline in pH was observed in all the petrol concentration except 7.5% indicating the formation of organic acids after petrol degradation. After 8 days of treatment pH level was increased in all the petrol concentrations was noticed due to petrol degradation.

Changes in optical density during the treatment of petrol by *Pseudomonas aeruginosa* are illustrated in *Figure 2*. Increase in optical density values during the initial period of treatment was noticed. But afterwards, there was a decline and the maximum optical density was observed at 7.5% petrol concentration after eight days of treatment.

*Figure 3* shows the changes in the CO₂ level of the culture medium during the treatment of petrol by *Pseudomonas aeruginosa*. Petrol degradation resulted in the production of CO₂ which showed a linear increase with the increase in petrol concentration. Highest level of CO₂ was observed for 10% petrol after eight days of treatment. CO₂ release showed on increase during initial period and later remained in the asymptote level except 5% petrol concentration.

### Table 1. Biochemical tests used for the identification of the isolated organism.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Biochemical Tests</th>
<th><em>Pseudomonas aeruginosa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Colony character</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Colony size Medium</td>
<td>Rod</td>
</tr>
<tr>
<td>3</td>
<td>Cell type</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Gram reaction –</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MR test –</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>VP test –</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Indole test –</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Catalase test +</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Citrate test +</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Gelatin Liquefaction +</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Cellobiose –</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Lactose –</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Maltose –</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Sucrose –</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>D-xylose –</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Trehalose –</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Sorbitol –</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Malonate +</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>D-Arabinose –</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Glycerol +</td>
<td></td>
</tr>
</tbody>
</table>

Note: + Positive; – Negative.
High Performance Liquid Chromatographic analysis report for control having 10% petrol concentration without inoculum is exhibited in Figure 4. In this, only two peaks were seen with the retention time of 1.933 and 2.247 minutes. Figure 5 illustrates the HPLC analysis report for 10% petrol concentration treated with *Pseudomonas aeruginosa* for sixteen days. In this, both the peaks observed in the control were missing but several new peaks with different retention time were appearing which indicated the degradation of petrol into several intermediates.

Table 2 divulges the two way analysis of variance for the parameters, pH, optical density and CO₂ with the variables treatment period and petrol concentration. Variations due to petrol concentration were statistically
Table 2. Two way analysis of variance for the factors with the variables, treatment period and petrol concentration for *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Source of variation</th>
<th>df</th>
<th>MS</th>
<th>Calculated F value</th>
<th>Table F value</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Treatment period</td>
<td>3</td>
<td>0.257</td>
<td>13.056</td>
<td>3.862548</td>
<td>Significant</td>
</tr>
<tr>
<td></td>
<td>Petrol concentration</td>
<td>3</td>
<td>0.425</td>
<td>2.154</td>
<td>3.862548</td>
<td>Not significant</td>
</tr>
<tr>
<td>Optical density</td>
<td>Treatment period</td>
<td>3</td>
<td>0.006</td>
<td>2.390</td>
<td>3.259167</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>Petrol concentration</td>
<td>3</td>
<td>0.013</td>
<td>5.448</td>
<td>3.862548</td>
<td>Significant</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>Treatment period</td>
<td>3</td>
<td>68566.67</td>
<td>4.636</td>
<td>3.862548</td>
<td>Significant</td>
</tr>
<tr>
<td></td>
<td>Petrol concentration</td>
<td>3</td>
<td>237966.7</td>
<td>3.863</td>
<td>3.862548</td>
<td>Significant</td>
</tr>
</tbody>
</table>

significant for optical density and CO₂ while they were not statistically significant due to treatment period. Variations due to treatment period were significant for pH, but they were not significant due to petrol concentration.
The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions [41]. The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate and pyruvate. Sugars required for various biosyntheses and growth are synthesized by gluconeogenesis.

The microorganisms implicated in oil degradation are widely distributed in nature and have been isolated from soil and water with their oil degrading potentials [42]. The microbes capable of utilizing oil and oil products as a sole source of carbon and energy occur practically everywhere in air, water and soil [43]. It is estimated that in one gram of unpolluted soil, there are only 100 to 1,000 cells of hydrocarbon degrading microorganisms, whereas, in one gram of soil polluted by oil, their number increases to $1 \times 10^6$ to $5 \times 10^7$ cells, especially if pollution occurred repeatedly and for a long time [44]. Taxonomic characteristics of these isolates identified them as *Pseudomonas* sp., *Staphylococcus* sp., *Micrococcus* sp., *Psychrobacter* sp., and *Alcaligenes faecalis* [45]. *Micrococcus, Staphylococcus, Pseudomonas putida* and *Alcaligenes* were also reported to degrade diesel oil [46].

The rate of crude oil biodegradation in the soil was rapid and it might be due to the microorganisms in the soil having efficiency in utilizing the residual crude oil as a source of carbon and energy [47]. Crude oil contains hydrocarbon and does not resist attack by microorganisms. The hydrocarbon utilizing microorganisms isolated from the soil were species of *Bacillus, Lactobacter, Arthrobacter, Pseudomonas, Micrococcus, Zoopage, and Articulosporium*. *Bacillus* sp. predominated, especially in the crude oil polluted soil. This may be due to the ability of these organisms to produce spores, which may shield them from the toxic effects of the hydrocarbons [48].

However, bacterial degradation of oil is more significant (80%) than fungi (20%) [49] [50]. The genus *Pseudomonas* and *Bacillus* in particular have been the subject of numerous studies. The *Pseudomonas* sp is one of the best crude oil degraders. *Pseudomonas* is termed as Oil Eating Bug in crude oil industry. An interesting and useful characteristic of many *Pseudomonas* sp. is their ability to utilize a wide variety of organic substrates for growth. The bacterium invades the crude oil which consists of rich source of organic compounds suitable for the growth of this bacterium and produces biosurfactants that clean up the crude oil [51].

*Pseudomonas aeruginosa* exhibited the most excellent hexadecane-degrading ability at pH 7.0. Difference in the ability of *Pseudomonas aeruginosa* to biodegrade n-hexadecane at different pH values might be related not only to its role in the process of hydrocarbon metabolism, but also to its microbial diversity and activity under the acidic, neutral or alkaline conditions [52]. In this study pH showed an increase in all the concentrations and the highest pH was observed for 7.5% petrol concentration.

Microorganisms are considered to be efficient biodegraders due to their abundance, the wide variety of species, and their catabolic and anabolic versatility, as well as their ability to adapt to adverse environmental conditions. Through the metabolic activity of these microorganisms, organic pollutants can be transformed into water, CO$_2$ and biomass [53].

The complete degradation of a compound to its mineral components, in which the organic carbon of the compound is converted to CO$_2$ by respiration, is called mineralization. When the aerobic conditions are maintained, CO$_2$ evolution can be used as a measure of microbial metabolic activity with reasonable accuracy [54]. Studies involving the measurement of the rate of mineralization can provide important information about the biodegradability of industrial waste compounds, including oil residues. In this study changes in pH, CO$_2$ and cell growth indirectly indicate the degradation of petrol.

The initial decline in pH may indicate the formation of organic acids as a result of petrol degradation by *P. aeruginosa*. The higher concentrations of petrol, during initial period of treatment exhibited an increase in biomass confirming the isolate being capable of exhibiting growth by breaking down petroleum hydrocarbons. This is also confirmed by the increased release of carbon dioxide which may be a product of petrol degradation. HPLC analysis also offers further confirmation exhibiting new peaks which represent the metabolites of petrol degradation.

The pattern of degradation showed that the microorganisms first attacked the lower and higher hydrocarbon chains and those of middle length were attacked later in the course of incubation. Considerable information on the microbial degradation is available in the literature, but less is known on the biodegradability of some petro-
leum commercial products such as kerosene [55]. The dominant mechanism that breaks down these petroleum products is biodegradation, which is carried out by natural microbial population [56]. In the present study *Pseudomonas aeruginosa* is able to degrade petrol which is used as carbon and energy source.

4. Conclusion

The degradation of petrol by the isolate, *Pseudomonas aeruginosa* was found to be more efficient. This is evidenced by the changes in pH, increase in optical density and CO₂ released. Appearance of new peaks in the HPLC analysis confirms the degradation process. Thus, the isolate has the ability to tolerate the petrol concentrations and grow on them. Hence this strain can be employed as bioremediation agent and used in restoring the ecosystem contaminated with oil.

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References


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