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# Bioremediation of hydrocarbon pollutants by *Pseudomonas putida* under optimal conditions

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## Abstract

**Background:** The extreme toxicity of petroleum products to human and environmental health, particularly when linked to large-scale unintentional spills, has made them one of the most serious and current environmental pollution issue. Petroleum products are a complex mixture of hydrocarbons.

**Methods:** Oil-contaminated soil samples were gathered and utilized to isolate hydrocarbon-degrading bacterial isolates. The bacterial isolates possessing bioremediation capabilities were identified using phenotypic determination and biochemical properties. The Optimal conditions including incubation period, temperature, different pH values and different carbon sources were studied for bioremediation of crude oil by bacterial isolates.

**Result:** In Wasit province of Iraq, *Pseudomonas putida, P. fluorescens,* and *P. aeruginosa* isolated from soil samples tainted with petroleum hydrocarbons. *Pseudomonas putida* have more ability for bioremediation of crude oil (68%) compared to *P. aeruginosa* and *P. fluorescens* (47%, 58%) respectively. The results of optical density (600nm), biomass and (E24%) index for *Pseudomonas putida* were (1.080, 1.98 and 60%) respectively. The Optimal conditions (incubation period, temperature, different pH values and different carbon sources) were studied for bioremediation of crude oil by *Pseudomonas putida* by using liquid BHM supplemented with 1% crude oil. The result of this study showed that the incubation period of 9 days was the optimum for bioremediation of hydrocarbons which was 88.33%. The optimum temperature and pH were 35 °C and 7 respectively. The carbon source (glucose and fructose) was optimal for hydrocarbon bioremediation.

**Conclusion:** This bacterial isolate *Pseudomonas putida* can be use in petroleum hydrocarbon bioremediation process.

## Introduction

Petroleum hydrocarbon pollutants are stubborn chemicals and are classified as priority pollutants. The toxicity of petroleum hydrocarbons to higher forms of life, including humans and microorganisms, has made them one of the most severe global issues [1]. Petroleum is an oily, combustible liquid that originates naturally beneath the earth's surface. It is made up of hydrocarbons combined with nitrogen, oxygen, and sulfur [2]. The most common organic contaminants of soil and aquatic environments are petroleum fuel spills from production, pipeline breaks, tank failure, storage and transportation. Because of the pollutants' cytotoxic, mutagenic, and carcinogenic impacts on people, they are categorized as hazardous wastes [3].

Traditional physical-chemical methods are expensive and can leave behind hazardous residues for the biota. Therefore, applying a lot of efficient and affordable bioremediation techniques is an incredibly significant method of cleaning up contaminated places among several other cleaning methods [4].

The biological breakdown of oil by microbes which function as metabolic machinery and use oil as carbon and energy sources is a contemporary strategy that is now under investigation [5]. Large amounts of oil-related organic pollutants, including aliphatic chemicals, n-alkanes, diesel fuel, monoaromatic compounds, toluene, benzene and polycyclic aromatic hydrocarbons (PA Hs) can be broken down by microorganisms [6].

## Methods

### Collection of soil samples and isolation of bacteria that degrade hydrocarbons

The study area, known as the Wasit oil depot, is situated in the southeast of Wasit province, Iraq, next to the Tigris River, encircled by agricultural areas, and is where the oil-contaminated soil samples were taken in March 2021. Fifty ml of Bushnell-Hass medium broth. The medium containing $KH_2PO_4$ (1g/L), $K_2HPO4$ (1g/L), $CaCl_2$ (0.02g/L), $(NH_4)_2SO_4$ (1g/L), $MgSO_4$ (0.2g/L) and $FeCl_3$ (0.05g/L) were put in Erlenmeyer flasks (250 ml) and enhanced with 1% crude oil, which was autoclaved at 121 ºC for 15 minutes to serve as a hydrocarbon source. Each Erlenmeyer flask contained 1 g of the initial soil sample after sterilization. Every Erlenmeyer flask was incubated for 14 days at 150 revolutions per minute at 30 ˚C in a shaker incubator [7]. Following the incubation period, each flask's optical density was measured using a spectrophotometer set to 600 nm. Samples and controls were cultured on BHM agar plates, and each flask had three duplicate plates made for it. The plates were then all incubated for 48 hours at 30 ˚C to isolate the most effective bacteria in the decomposition of hydrocarbon pollutants.

For the production of pure cultures of bacteria, a single colony of different isolates was selected, transferred many times from mixed culture plates onto Luria agar plates using the technique of streaking, then cultured for 24 hours at 30°C. [7]. Pure isolates were kept on Luria agar and nutrient slant was used for isolation and maintenance of pure strains.

### Screening of bacterial isolates that degrade crude oil

After reactivating pure bacterial isolates on nutritional agar and incubating for 24 hours at 30˚C, each isolate's bacterial inoculum was prepared using Luria-Bertani broth and incubated for 18 hours at 30˚C. The reactivated bacterial isolates were used to inoculate 50 ml of liquid BHM at pH 7 and 5 milliliters of 1% crude oil were added as a substrate. The Erlenmeyer flasks were then incubated in a shaker incubator for 7 days at 30˚C (150 rpm). The most effective bacterial isolates in decomposition were identified by measuring the following parameters after the incubation period: biomass, optical density, the emulsification index and the percentage of hydrocarbon decomposition using a gravimetric method [8].

### Measurement of bacterial growth

Using a UV-VIS spectrophotometer, the bacterial suspension's optical density was determined at 600 nm [9].

### Biomass Estimation

The dry weight method was used to estimate biomass. Following the incubation time, a 20 ml of culture broth was centrifuged for 20 minutes at 4000 rpm. After being resuspended in deionized water, the pellets were centrifuged once more. Pellets were washed out of the tube into pre-weighed filter paper after the supernatant was drained. After drying for 24 hours at 105 degrees Celsius, the filter paper was weighed again until a consistent dry weight was achieved [10].

### The emulsification index (E-24)

The emulsifying capacity of culture supernatant assessed according to [11]. 5 ml of culture supernatant and 1 ml of crude oil were combined in tube of test, vortexed violently for 2 minutes, and then allowed to stand for 24 hours in order to measure (E-24). The emulsification index percent was determined using this formula.

$$\text{E-24 Index \%} = \frac{\text{Height of emulsified layer}}{\text{Total height of solution}} \times 100$$

### Measurement of crude oil remediation by gravimetric method

It was measured depending on the method of [12].

### Identification of the bacterial isolates

A phenotypic identification of the bacterial isolates with bioremediation capabilities was determined by gram staining, colony size, shape, and texture. Biochemical features include urease test, indole test, test of catalase, methyl red test, starch hydrolysis, Voges Proskauer test, gelatin consumption test, motility test, Simmons citrate test and oxidase test.

### Optimal condition for bioremediation of hydrocarbon pollutants by *Pseudomonas putida*

#### a-Effect of incubation periods

Fifty ml of Bushnell-Hass medium broth was put in Erlenmeyer flasks and 1% crude oil was added as a hydrocarbon source. These flasks were inoculated with 5 ml of bacterial inoculum of *P. putida* (three replicates were made). Every flask was incubated in a shaker incubator at 30 ˚C, 150 rpm/min with different periods of incubation (3,6,9,12) days. After the end of each time period bacterial growth (O.D at 600nm) and the percentage of hydrocarbon degradation were measured for bacterial isolate *P. putida* and control [13].

#### b-Effect of temperatures

Fifty ml of liquid BHM was dispensed in 250ml Erlenmeyer flasks, pH adjusted at 7.0 and supplemented with 1% crude oil as a substrate, the media were inoculated with 5ml of bacterial inoculum of *P. putida* and then the flasks were incubated in varied temperatures 25, 30, 35, and 40˚C at 150 rpm for seven days. Following the incubation process, bacterial growth (O.D at 600nm) and the percentage of hydrocarbon degradation were measured for bacterial isolate *P. putida* and control [14].

#### c- The impact of varying pH values

The pH effect on bacterial decomposition of hydrocarbon compounds was determined by using a liquid BHM. The BH medium was distributed in the amount of 50 ml, After adjustments, the pH values of this medium became 6, 7, 8, and 9 using solutions of HCl (0.1N) and NaOH (0.1N) and the medium inoculated with 5 ml of bacterial inoculum *P. putida* and then incubation was done in shaker incubator at 150 rpm (30 ˚C for 7 days). Following the incubation process, bacterial growth (O.D at 600nm) and the percentage of hydrocarbon degradation were measured for bacterial culture *P. putida* and control [14].

#### d-Effect of different carbon sources

The liquid BH medium (50 ml) with its pH adjusted at 7.0 and supplemented with 1% crude oil as a substrate, the medium components were modified by adding (2%) from different carbon sources (sucrose, fructose, glucose and maltose) and by using separate flasks for each carbon source, five milliliters of *P. putida* bacterial inoculum were added to each flask, and it was subsequently incubated for seven days at 30˚C at 150 rpm. Following the interval of incubation bacterial growth (600nm) and the percentages of hydrocarbon degradation were measured for bacterial culture *P. putida* and control [9].

## Results

### Identification of bacterial isolates

*Pseudomonas putida , P. fluorescences* and *P. aeruginosa* and other bacterial species were identified according to morphological characteristics and the biochemical tests as shown in Table (1). These bacterial species have the ability to degrade heavy oil through using BHM media supplemented with crude oil as the sole energy and carbon source.

| Biochem-ical tests | Catalase test | Urease test | Motility test | Oxidase test | Starch hydrol ysis | Simon citrate test | Gram staining |
|---|---|---|---|---|---|---|---|
| HD (1) | + | - | + | + | - | + | - |
| HD (2) | + | - | + | + | - | + | - |
| HD (3) | + | + | + | + | - | - | - |

HD = Hydrocarbon Degradation, + : Positive , -: Negative
HD (1): *Pseudomonas putida*
HD (2): *P. fluorescens*
HD (3): *P. aeruginosa*

**Table 1:**-Biochemical properties of the bacterial isolates that have the ability to bioremediate hydrocarbon pollutants.

### Screening of bacterial isolates that degrade hydrocarbons

Four methods such as biomass, measurement of bacterial growth (optical density) emulsification index (E24%) and measurement of crude oil remediation by gravimetric method were used to assess bacterial isolates' capacity to break down heavy crude oil by using BHM media. Table (2) showed that *Pseudomonas putida* was the most effective bacterial isolate to remediate petroleum hydrocarbons compared with other species.

| Bacterial species | Optical density (600 nm) | Biomass | (E24%) index | Gravimetric analysis % |
|---|---|---|---|---|
| HD1: *Pseudomonas putida* | 1.080 | 1.98 | 60 | 68 |
| HD2: *P. fluorescens* | 0.168 | 0.983 | 22 | 47 |
| HD3: *P. aeruginosa* | 0.447 | 1.43 | 36 | 58 |

**Table 2:** Determination of microbial bioremediation of crude oil.

### Optimal condition for bioremediation of hydrocarbon pollutants by *Pseudomonas putida*

#### a-Incubation period and temperature

Different time periods (3,6,9,12) days were tested to determine optimal incubation time for bioremediation of hydrocarbon pollutants by *P. putida* , using BHM medium supplemented by 1% oil as the sole source of energy and carbon. The result of this study showed that

nine days was the optimal incubation period for hydrocarbon degradation. The percentage of hydrocarbon degradation of *P. putida* in 9 days of incubation was (88.33 %), while it was 40.66 %, 68.33 %, and 47.33 % for incubation period of 3, 6, and 12 days, respectively (Figure 1). Figure (1) showed that *P. putida* appeared highest optical density in (9 days) was (2.099) and the lower optical density appeared in (3 days) was (0.773).

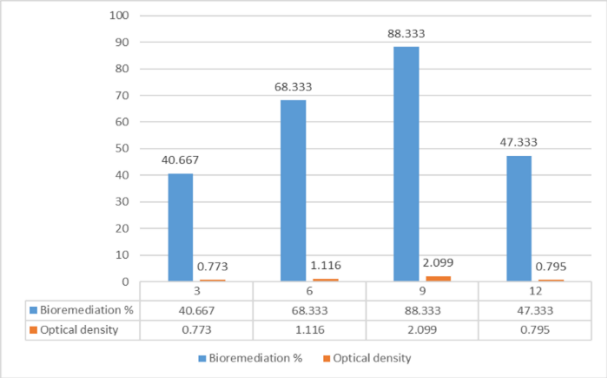


**Figure 1:** Percentage of oil bioremediation and optical density of *Pseudomonas putida* at different incubation periods.

By using BHM medium supplemented by 1% curd oil and inoculated with bacterial suspension *P. putida* in various temperatures (25°C, 30°C, 35°C, 40°C) to determine optimal temperature for bioremediation process. The result of this study showed that the highest value of hydrocarbon degradation of *P. putida* was (80.55 %) at 35 C while the lowest value was (50 %) at 25 C (Figure 2). *P. putida* appeared the highest optical density at temperature 35 ºC and the lower optical density was observed at 25 ºC. The optical density at 35 ºC was 0.777 and 0.327 at 25 °C respectively, compared to other temperatures (Fig 2).

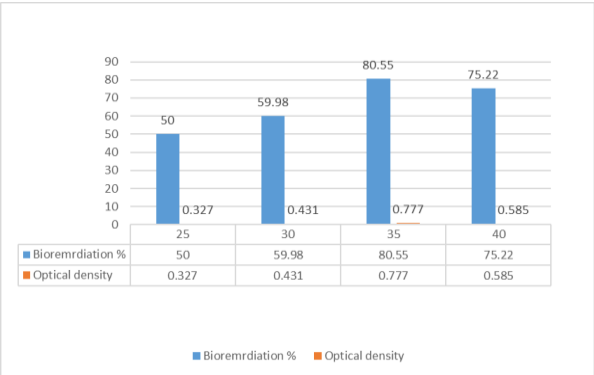


**Figure 2:** Percentage of oil bioremediation and optical density of *Pseudomonas putida* at various temperatures.

**b- pH and carbon sources**

This study showed that the optimal pH was 7 whereas, the percentage of hydrocarbon degradation for *P. putida* was 72% at pH 7 while it was 52 % , 44%, 39.3% at pH 6,8 and 9, respectively (Figure 3).The result of this study showed that the highest value of optical density (OD 600) for *P. putida* was 1.337 at pH 7, while the lowest value was 0.547 at pH 9 (Figure 3).

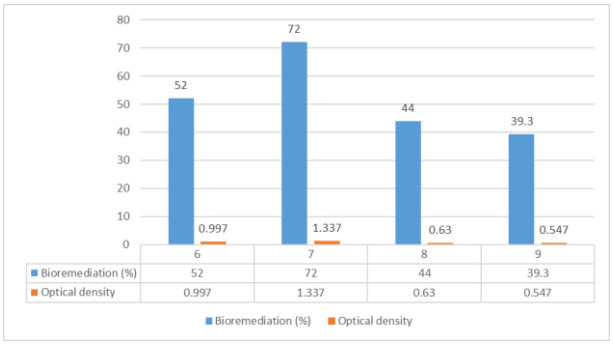


**Figure 3:** Percentage of oil bioremediation and optical density of *Pseudomonas putida* at various values of pH.

The percentage of hydrocarbon bioremediation of *P. putida* in BH medium modified by adding different carbon sources (2%) sucrose, fructose, glucose and maltose was different. These results showed that, glucose and fructose were favorable for the bacterial isolate *P. putida* whereas, the percentages of hydrocarbon bioremediation in the presence of glucose and fructose were 90% and 81.67% respectively compared to other carbon sources (Figure 4), while the highest values of optical density in the presence of glucose and fructose were 1.685 and 1.291 respectively (Figure 4).

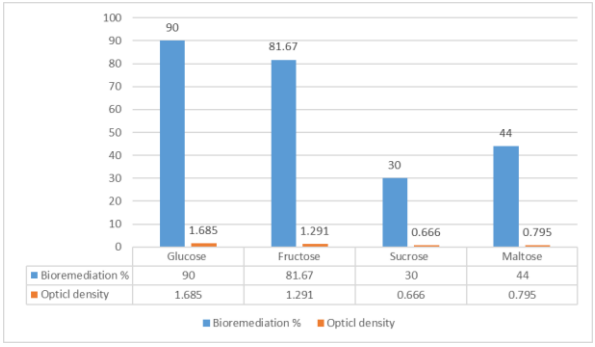


**Figure 4:** Oil degradation and optical density of *Pseudomonas putida* in the presence of various carbon sources.

## Discussion

In this research, soil samples were taken from Wasit oil depot /Iraq considered significant source for locally prevalent bacteria that can mineralize crude oil. The purpose of this study was the isolation of most active bacterial isolate in the biodegradation process. Microorganism growth is evidence that the biodegradation process is occurring and microorganisms can survive by utilizing the nutritional supplies in their living media and their population will rise [15]. The biodegradation of pollutants in

biodiesel/diesel blends by *P. putida* resulted in the production of biomass, carbon dioxide ($CO_2$), and water ($H_2O$) [16]. Optimal condition for bioremediation of hydrocarbon pollutants assessed in this study and the increase in optical density which denotes higher cell proliferation is evidence that the proportion of hydrocarbon elimination increased during the study period of (9 days). There are reports available that say that the highest hydrocarbon degradation was ranged from 74.04% to 90.09% in 10 to 14 days of incubation time in 1% oil by the bacterial isolate *Bacillus megaterium* [17]. It is also reported that in the incubation Period (10–14 days), the strain *Microbacterium esteraromaticum* destroyed around 60% of the PAHs provided as the only carbon source [18]. The highest value of hydrocarbon degradation by *P. putida* at 35 ◦ C is reported in this study. [19] discovered that the best temperatures for growing *Escherichia coli and Serratia marcescens* were 37°C, in order to utilize these bacteria in the bioremediation of hydrocarbon-polluted soils. pH is one of the factors that has the greatest influence on the growth of microbial cells in polluted environment. This study showed that the optimal pH was 7. These results are compatible with the results of [20] observed that bacterial isolates of *K. rosea* and *B. amyloliquefaciens* recorded the percentages of hydrocarbon degradation as 93.8% and 68.9% respectively at optimum conditions pH =7. It is recorded that the maximum degradation proportion of petroleum hydrocarbon was 65.4%. and maximum OD600 of the genus *Enterobacter* at the pH= 7.0 [21]. The result of this study showed that monosaccharides were more effective in petroleum hydrocarbon hydrolysis. Petroleum hydrocarbon degradation in the presence of glucose and fructose as an extra carbon source was more rapid than in the absence of glucose. The bacterial isolate *Microbacterium esteraromaticum* was able to degrade 98.7% of polycyclic aromatic hydrocarbon, benzo [a]pyrene and pyrene in mineral salts medium with glucose [18].

The results established that the bacterial isolate *Pseudomonas putida* identified from oil-polluted soil samples in Iraq was capable of consuming crude oil as its only source of carbon. The bacterial isolate can be used in petroleum hydrocarbon bioremediation process. The optimal pH for bacterial isolate *Pseudomonas putida* that decompose hydrocarbons was 7 and 9 days of incubation producing high levels of hydrocarbon bioremediation. The optimal temperature for bacterial isolates to decompose hydrocarbons was 35°C. This bacterial isolate preferred glucose and fructose to increase bioremediation percentage.

## Author Contributions

Fatima Kareem Shandookh
Responsible for collection of soil samples
Isolation of bacterial isolates
Measurement of variables of optical conditions
Melad Khalaf Mohammed
Ordering of results
Ahmed Darweesh Jabbar
Identification of bacterial isolates

## Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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