Optimization Kerosene Bio-degradation by a Local Soil Bacterium Isolate *Klebsiella pneumoniae* Sp. *pneumonia*

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**Abstract**

Isolated Bacteria from the roots of barley were studied; two stages of processes Isolated and screening were applied in order to find the best bacteria to remove kerosene from soil. The active bacteria are isolated for kerosene degradation process. It has been found that *Klebsiella pneumoniae* sp. have the highest kerosene degradation which is 88.5%. The optimum conditions of kerosene degradation by *Klebsiella pneumoniae* sp. are pH5, 48hr incubation period, 35°C temperature and 10000ppm the best kerosene concentration. The results 10000ppm showed that the maximum kerosene degradation can reach 99.58% after 48 h of incubation. Higher Kerosene degradation which was 99.83% was obtained at pH5. Kerosene degradation was found to be maximum at 35°C with 98.63%, where 10000ppm kerosene showed the highest degradation at 99.527%. The results indicate that the isolated *Klebsiella pneumoniae* sp. is extremely efficient in degrading kerosene hydrocarbons.

**Keywords:** *Klebsiella pneumoniae* sp., kerosene hydrocarbons degradation, optimum conditions, rhizodegradation.
INTRODUCTION

Plant enzymes, which are exuded from the roots can be used to degrade contaminants in the soil as a bioremediation technique. It is assumed that the result of bioremediation regarding petroleum-contaminated soil is based on the stimulation of degrading microorganisms in the rhizosphere, called phytostimulation or rhizodegradation. Biodegradation is usually considered a slow process because of the hydrophobic nature of the contaminants and consequent bioavailability limitations. Petroleum hydrocarbons, such as diesel with n-alkane markers which range in size from C8 to C25, are highly reduced organic molecules. They may be used as a carbon source and electron donors for microorganisms to back microbial metabolism. If the molecular weight increases, the biodegradation of the hydrocarbons decreases. Microorganisms may cause degradation of the hydrocarbons with a wide range of n-alkanes between C10–C35, considering the range C14–C19 as the preferred ones. For microbial respiration, electron acceptors other than oxygen are used when the conditions are anaerobic. During the process, hydrocarbons are oxidised to intermediate molecules and then to carbon dioxide, while terminal electron acceptors are reduced.

Rhizobacteria (RB) is defined as the bacteria that live in the surrounding area to the root or on root surface. Boosting the degradation of the hydrocarbon can be reached through a rhizosphere effect with plants that exude organic compounds through their roots which influence the abundance, diversity, or activity of potential hydrocarbon-degrading microorganisms in the zone surrounding the roots. The best attachment sites for microorganisms are the roots. They also provide nutrients in the form of exudates which consist of organic acids and amino acids, enzymes, sugars, and complex carbohydrates. Furthermore, Palmroth et al. stated that root exudates from plants surely help to degrade toxic organic chemicals and acts as substrates for soil microorganisms in order to increase biodegradation rate of the organic contaminant.

Biodegradation of hydrocarbon-contaminated soils which use the ability of microorganisms to degrade and/or detoxify organic contamination, has been considered as an efficient, economic, versatile and environmentally sound treatment for kerosene contaminated soils. There was no microorganism located to fully degrade petroleum hydrocarbon molecule. Yet, various species or strains of the same species may have the ability of degrading different groups of hydrocarbons which are found in kerosene. Pseudomonas, Serratia, Streptococcus, Micrococcus, Bacillus, Klebsiella, Proteus, Arthrobacter, Gordonia, Brevibacterium, Burkholderia and Mycobacterium species are located to degrade kerosene microorganisms that produce biosurfactant abound in nature. They are located in water-fresh water, groundwater, and sea- and land -soil, sediment and sludge. They can also be located in extreme environments, for example in oil reservoirs and thrive in a wide range of temperatures, pH values and salinity.

They may be isolated from undisturbed environments, where they have physiological functions which do not include the solubilisation of hydrophobic pollutants, such as antimicrobial activity, biofilm formation or processes of motility, and colonization of surfaces. Nevertheless, the most appropriate environment for widespread capability for biosurfactant production is hydrocarbon-degrading microbial communities. Some main genera which are Pseudomonas, Bacillus, Sphingomonas, Klebsiella and Actinobacteria in soils and sediments, and Pseudoalteromonas, Halomonas, Alcanivorax, and Acinetobacter in marine ecosystems generally dominated hydrocarbon-degrading bacterial populations.

Consequently, it is expected that many biosurfactant or bioemulsifier producers belong to these same genera. It is difficult to determine an estimate of the frequency of biosurfactant-producing strains within a microbial population because it depends on the experimental procedures used. In uncontaminated soils, 2-3% of screened populations are recorded to be biosurfactant-producing microorganisms.

In polluted soils, this figure increases to 25%. Furthermore, enrichment culture techniques specific for hydrocarbon-degrading bacteria can lead to a much higher detection of biosurfactant producers with estimates up to 80%. Biosurfactants which are produced by microorganisms are classified into two different
types depending on their chemical composition viz., low molecular weight surface-active agents called biosurfactants and high molecular weight biosurfactants referred to as bioemulsifiers.

The glycolipids, lipopeptides and lipoprotein, fatty acids, phospholipids, neutral lipids, particulate biosurfactants, and polymeric biosurfactant are examples of low molecular weight biosurfactants. Polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers are the compositions of the high molecular weight biosurfactants. The best examined bioemulsify are the bioemulsans which are produced by different species of Acinetobacter. Various industrial processes required different classes of biosurfactant. The consideration which was shown to the production of biosurfactants nowadays is chiefly because of their potential utilization in food processing, pharmacology, cosmetics, oil exploration and exploitation industries, environmental management, and agriculture. One application of biosurfactant which is of interest to environmentalist is in environmental management and bioremediation. In the crude oil-polluted sites, biosurfactants are being used successfully in the bioremediation. In the EXXON Valdex oil spill in Alaska, Pseudomonas aeruginosa SB30 (1%) was used to remove two times the oil on the water. The objective of this study is to determine the optimum conditions of kerosene degradation using local isolate of Klebsiella pneumonia sp. These parameters are pH, temperature, incubation period and concentration of kerosene or concentration of cadmium.

MATERIALS AND METHODS
Samples collection and bacterial isolation
Three isolated bacterial species, including Klebsiella pneumoniae sp., Enterobacter aerogenes sp. complex and Serratia fonticola were previously isolated from barley roots contaminated with kerosene for 15 days. The isolated procedures were done in the Biology and Biotechnology Department/College of Science/University of Baghdad, Iraq. The isolates were identified previously using Epi-test and biochemical tests, and maintained on Nutrient agar medium were used, these isolates were prepared for screening experiments.

The isolates were screened for kerosene (AL-Dorah Refinery) degradation according to method described by cultures from the kerosene enriched MS media. In which, kerosene were sprayed on the surface of minimal salt (MS) agar and the bacteria were isolated by plating (1ml) of enriched cultures. The plates were incubated (6 days) in the dark at 28-32°C. As culture appeared to obtain pure cultures, the colonies were intermittently transferred to fresh MS agar. Discrete bacterial colonies were isolated and sub-cultured onto separate agar plates on the basis of morphological observations. The pure cultures were incubated at 37°C for 18-24 hr.

Quantitative screening
Preparation of bacterial inoculum suspension
Isolates from a Nutrient agar stocks were inoculated into nutrient broth and incubated for 24hr at 30°C before using for tests. After incubation period, the cells in the suspension were counted by heamocytometer and the cell suspension was diluted using the same broth to obtain biomass concentration of 3×10⁸ cell/mL.

Optimum conditions for kerosene degradation
Optimum conditions were studied for kerosene degradation, including pH, temperature, incubation period and concentration of kerosene.

Effect of pH value
To determine the influence of the initial pH of the culture medium, a 250 ml Erlenmeyer flasks containing 50 ml of the selected degradation medium was adjusted using 0.1N HCl or 0.1N NaOH to give different pH values i.e. pH 4, 5, 6, 7 and 8 with 10000ppm kerosene. Then the culture medium was inoculated with the selected isolate and incubated in shaker incubator (Shaker Incubator, Jissy, Korea) 150 rpm at 30°C for 24 hr. After the incubation, supernatant was taken from each flask and the Kerosene

| Table 1. Correlation the removal of kerosene effect with variables |
|-----------------|-----------------|-----------------|-----------------|
| T               | Ci %            | pH              | Temp            |
| T               | 1               | -0.10679        | 1               |
| Ci %            | 0.10679         | 0.961806        | 0.619692        |
| pH              | 0.961806        | 0.619692        | 1               |
| Temp            | 0.794058        | 0.386405        | 0.887779        |
concentration was found by GC-FID analysis (GC System, Japan, type-5E-30).

**Effect of incubation time**

The best pH value obtained from the previous experiments was fixed to determine the optimum incubation time for the maximum Kerosene degradation, the time course for Kerosene degradation was analyzed at 24, 48, 72 and 96 hr\(^2\).

Degradation medium of the kerosene was prepared and inoculated with selected isolate and incubated in shaker incubator (150 rpm) at optimum pH.

### Table 2. Previous studies for bioremediation of hydrocarbon by bacteria sp.

| TPH                  | Condition | Bacteria sp.                          | Removal efficiency (%) | Ref. |
|----------------------|-----------|---------------------------------------|------------------------|------|
| Hydrocarbon          | 35°C, 48hr, pH 5.7 | *K. pneumoniae* | 60-70                  | 28   |
| Used engine oil (1-6%) | 25-45°C, pH 5-7 | *Ochrobactrum anthozi* HM1 and *Citrobacter freundii* HM2 | HM-1, HM-2, and their 29 mixture efficiently degraded, they achieved 65 ± 2.2, 58 ± 2.1, and 80 ± 1.9 %, respectively At optimum conditions pH=7.5, temp=37°C, UEO 2% and 150 rpm. more than 99% at pH 7, 35°C, and initial hydrocarbon concentration at 4%. degrade more than 99% of diesel oil at pH 7, 35°C and initial hydrocarbon concentration of 4%. | 29   |
| Diesel contaminated soil (1%-5%) | 25-45°C, pH 4-11 | *Acinetobacter baumannii* | 65 ± 2.2, 58 ± 2.1, and 80 ± 1.9 | 30   |
| Aromatic hydrocarbon Phenantrhene) Crude Oil | 20-45°C, pH 4-9 | *Klebsiella sp* | The degradation ratio at (400 ppm) was 84.9%, pH =7.0, (100-600ppm temperature =30°C | 31   |
| Hydrocarbon          | 37°C, upto 15 days, Petroleum hydrocarbon (petrol/octane/diesel) 1% | 10 types of bacterial species | The crude oil-degrading of Nitrosomonas sp. was (55.2 petrol degradative as Pseudomonas spp. (58%). The degradation ratio at (400 ppm) was 84.9%, pH =7.0, (100-600ppm temperature =30°C | 32   |
| Hydrocarbon          | 25 - 45°C, Diesel oil (V/V) 2% | *Bacillus cereus* DRDU1 on the basis of 16S rDNA | Efficiently degrading 96% of kerosene | 33   |
| Kerosene (5000ppm- 60000ppm) | 25 - 50°C, 24-96hr, pH4-8 | *Klebsiella pneumonia* sp. | Efficiently degrading 99.527% at pH5, 350C, study kerosene con.=10000ppm | 34   |
Effect of temperature
Kerosene degradation was achieved at different temperatures, i.e. 25°C, 35°C, 45°C and 50°C. After sterilization, the flasks were inoculated with 2% of overnight grown isolate and incubated in shaker incubator (150 rpm) at optimum pH and time.

Effect the concentration of kerosene
Maximum kerosene degradation was determined by using kerosene degradation medium containing different concentrations of kerosene 5000, 10000, 20000, 30000 and 60000ppm. The medium was inoculated with the selected isolate and in shaker incubator (150 rpm) at optimum temperature and time.

RESULTS
Quantitative screening
Three isolates were screened for their kerosene degradation by cultivating it in Nutrient agar. The kerosene was removed by Klebsiella pneumoniae sp., Enterobacter cloacae complex and Serratia fonticola were 0.883, 0.419 and 0.835, respectively, as in Figure 1.

To study the effect of the initial pH on kerosene degradation, Klebsiella pneumonia sp. were grown on kerosene degradation medium with different pH values 4-8. As it can be seen in Figure 2, higher kerosene degradation 0.998 was obtained at pH 5, while the kerosene degradation at 4, 6, 7, and 8 were 0.197, 0.993, 0.628, 0.946 respectively. Increasing or decreasing pH value above or below 6 leads to reduce in kerosene degradation.

Effect of incubation periods on kerosene degradation
Kerosene degradation reach to maximal at 48h of incubation, which recorded 0.995, while the Kerosene degradation at 24h, 72h and 96h were 0.45, 0.984 and 0.883 respectively as in Figure 3. Whereas, after 48h of incubation, the kerosene degradation was decreased with increasing the incubation time upto 48hr.

Fig. 1. Screening of isolates bacteria sp. for kerosene degradation.

The percentage of TPH removal on each sampling day was determined by using eq. 1

\[
\text{Removal efficiency} = \frac{\text{TPH}_0 - \text{TPH}_{SD}}{\text{TPH}_0} \times 100
\]

Where \( \text{TPH}_0 \) = total petroleum hydrocarbon on sampling day 0
\( \text{TPH}_{SD} \) = total petroleum hydrocarbon on sampling day

Fig. 2. Effect of initial pH on kerosene degradation by Klebsiella pneumonia sp.

Experimental parameter: after 24h, 30°C, rpm=150, kerosene con.=10000ppm

Fig. 3. Effect of incubation period on kerosene degradation from Klebsiella pneumonia sp.

Experimental parameter: pH 5, 30°C, 24hr, rpm=150, kerosene con.=10000ppm
Effect of temperatures on kerosene degradation
The results in Figure 4 showed the capability of isolate *Klebsiella pneumonia* sp. to grow and kerosene degradation at wide range of temperatures including 25°C, 35°C, 45°C and 50°C. Kerosene degradation by GC analysis was found to be maximum at 35°C with 0.992, while the kerosene degradation at 25°C, 45°C and 50°C were 0.975, 0.9853 and 0.979 respectively.

![Fig. 4. Effect of temperatures on kerosene degradation from Klebsiella pneumonia sp.](image)

Experimental parameter: pH 5, after 48 hr, rpm=150, kerosene con.=10000ppm

**Effect the concentrations of kerosene**

To determine the ability of *Klebsiella pneumonia* sp to degrade kerosene, the total petroleum hydrocarbons were estimated at 5000ppm, 10000ppm, 20000ppm, 30000ppm and 60000ppm kerosene degradation, where 10000ppm kerosene showed the highest degradation at 0.993, while the kerosene degradation at 5000ppm, 20000ppm, 30000ppm and 60000ppm were 0.973, 0.990, 0.492 and 0.103, respectively as shown in Figure 5.

![Fig. 5. Effect of concentrations of Kerosene on its degradation from Klebsiella pneumonia sp.](image)

Experimental parameter: pH 5, 35°C, 48h, kerosene con.=10000ppm, rpm=150

DISCUSSION
In the present study, the kerosene was removed by *Klebsiella pneumonia* sp., *Enterobacter cloacae* complex and *Serratia fonticola*. In accordance with these results, the isolate *Klebsiella pneumonia* sp. which had the higher kerosene degradation was selected for remaining studies. The variation between members of the same species in ability for kerosene degradation may be due to the genetic variation, the type and sources of isolates and the conditions of cultivation, such as media components, temperature, pH and aeration and stirring, which helped to increase the ability of *Klebsiella pneumonia* sp. isolate to produce the enzyme in a liquid medium.

Generally, the effect of pH in kerosene degradation is attributed to its role in the solubility of the medium nutritional substances, its influence on the substrate ionization and its availability for the microorganism, and reported maximum biosurfactant production at below pH 7. In present study, maximum kerosene degradation was observed at pH 5 and 48hr. This may be due to the change in the conditions of culture along the periods such as diminishing of oxygen, nutrients and accumulating of toxic metabolites which inhibit the bacterial growth. The incubation time plays an important role in the growth of microorganisms and kerosene degradation. It was the found the highest kerosene degradation produced by *Klebsiella pneumonia* sp. at 35°C.

The effect of temperature for growth of *A. baumannii* with diesel oil was evaluated. It was found that temperature is an important factor that affects the diesel degradation potential by bacteria. Mnif et al. found that 30°C was the optimum condition for the degradation of diesel by Bacillus subtilis SPB1. At the same time, the diesel oil-degrading ability of Pseudomonas sp. strain F4 was reported to be 37°C. The present study analyzed the optimum temperature for the degradation of diesel oil and it was found maximum at 35°C. The results indicate that the *Klebsiella pneumonia* sp. isolated in this study are extremely efficient in degrading kerosene hydrocarbons. Our results are accordance with previous reports. Some previous reports were also given in the Table 2.
CONCLUSIONS

It is concluded that Secondary screening for kerosene degradation has proved that the Klebsiella pneumonia sp. provide the highest level of kerosene degradation. The optimum pH for kerosene degradation was 5, optimum incubation periods were 48 hr whereas after 48 hr the kerosene degradation was decreased as the incubation time is increased. The optimum temperature for kerosene degradation was 35°C. The best kerosene concentration was 1%. The results indicate that the Klebsiella pneumonia sp. isolated in this study is extremely efficient in degrading kerosene hydrocarbons.

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**Supplementary Figure 1.** Gas chromatography of kerosene degradation analysis.

- a. TPH chromatograms for effect of different bacteria sp. in optimum treatment
- b. TPH chromatograms for effect of different pH in optimum treatment
- c. TPH chromatograms for effect of different incubation time in optimum treatment
- d. TPH chromatograms for effect of different temperature in optimum treatment
- e. TPH chromatograms for effect of different concentration of kerosene) in optimum treatment