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# Biodegradation of Diesel Contaminated Soil Using Single Bacterial Strains and a Mixed Bacterial Consortium

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## Abstract

This study was conducted to assess the hydrocarbon degradation abilities of Sphingomonas paucimobilis, Pentoae species, Staphylococcus aureus, and Enterobacter cloacae, which isolated from diesel contaminated soil samples. Single strains and mixed bacterial consortia have been investigated their ability to degrade 1.0 % (v/v) of diesel oil in Bushnell- Haas medium as sole carbon and energy source. At temperature 30◦C, the individual bacterial isolates exhibited low growth and low degradation than did the mixed bacterial culture. After 28 days of incubation the combination of four isolates degraded an upper limit of diesel 88.4%. This was continued by 85.1% by S. paucimobilis, 84 % by Pentoae sp., 79% by S.aureus, and 74% by *E. cloacae*. For further evidence of the biodegradation effect of these isolates individually and as a mixed culture, which was supported by the use of technology chromatography confirming the occurrence of biodegradation. The results showed that the isolated bacteria are effective in biodegradation of diesel spills when used separately. It also confirmed the better biodegradability when used together in a mixed -culture.

***Keywords:*** *Bioremediation, Biodegradation, Diesel, Mixed-culture, Pollution.*

## 1. Introduction

Petroleum hydrocarbons are the most common environmental pollutants in the world, and oil spills show a great hazard to terrestrial and aquatic ecosystems. Oil pollution may arise either accidentally or operationally whenever oil is produced, transported, stored and treated, or when it is used on land or at sea. Oil spills are a major threat to the environment because they severely damage surrounding ecosystems [1].

Diesel is a medium distillate of petroleum hydrocarbons containing: normal alkanes, branched alkanes, olefins and small concentrations of polycyclic aromatic compounds [2]. As pollution of soil and groundwater through the uncontrolled release of petroleum products has become a major problem, a number of techniques have been tested to remediate contaminated sites[3].

Bioremediation has emerged as one of the promising alternative treatment options for removing petroleum hydrocarbons because of its low cost and effectiveness. Bioremediation is a method in which microorganisms are used for cleaning up process. They can be described as converting toxic and chemically complex organic compounds into non-toxic and simple inorganic compounds, such as $CO_2$ and $H_2O$ with the accumulation of microbial biomass, through oxidation under aerobic conditions [12]. Microorganisms are considered to be efficient

biodegrades due to the wide variety of species, their abundance, and their catabolic and anabolic versatility, as well as their ability to adjust to adverse environmental conditions [4]. The Biodegradation of petroleum hydrocarbon requires a complicated metabolic route that is not always present in one type of microorganism but can be observed in a microbial consortium. Numerous studies have been carried out on biodegradation of petroleum hydrocarbons using a mixed microorganism consortium [5]. In a mixed culture, the metabolic products of one type can be decomposed by another present in the culture and so on respectively, leading to a complete degradation of the substrate although there is no particular species of microorganisms in the community capable of destroying them alone[6]. In fact, since one species can metabolize only a limited range of hydrocarbon substrates, the group of many different bacteria, with wide enzymatic capacities, is usually involved in the degradation of petroleum hydrocarbons [7].

The aim of this study was to isolate local bacterial species that had the ability to decompose diesel and compare their biodegradability capabilities in single-use and mixed culture.

## 2. Materials and Methods

### 2.1. Collecting of Samples

The soil contaminated with diesel was collected at a depth of 0-15 cm [8] from different locations, including Al Dura oil refinery and the local generators in Baghdad city. All samples were kept in sterile nylon bags and transported to the laboratory to isolate and identify the bacteria. The Diesel (light oil) was used in the experiment work from Al Dura oil refinery.

### 2.2. Isolation of Bacteria from Diesel Contaminated Soil

Four grams of contaminated soil sample were suspended by a vortex mixer in 20 mL of sterile Bushnell-Haas medium containing (g/l): $MgSO_4.7H_2O$, 1.0; $K_2HPO_4$, 1.0; $KH_2PO_4$, 1.0; $FeCl_3$, 0.05; $NH_4NO_3$, 1.0; $CaCl_2$, 0.02; pH to 7±0.2 and sterilized at 121ºC for 15 min. Bacteria were grown in 250 ml Erlenmeyer flasks for one week in a cool shaker incubator at 30°C and 180 rpm. A loopful culture from the soil suspension was streaked on petri dishes containing nutrient agar. The plates are placed in an incubator at 30 ° C under aerobic conditions for 48 hours. Specific colonies were isolated and sub-culture on nutrient agar plates and incubated for another 48 hours.The plates are then placed in the refrigerator at 4 °C for further study and identification [9].

### 2.3. Bacterial Identification

The colonies that were developed on nutrient agar were classified according to their shape, color and biochemical properties. The microscopic examination of the Gram-stained slices is done to characterize gram-negative bacteria from others. Then the identification of bacterial isolates was carried out in Al-Nokhba medical laboratory using VITEK 2 device.

### 2.4. Inoculum Preparation

Bacterial strains were cultured on nutrient agar at 30 ° C for 48 h. Aqueous culture was then suspended, standardizing to the Macfarland scale tube number 7 [6]. Tube 7 represents turbidity of bacteria in the concentration ($2.1 \times 10^9$ CFU.mL$^1$).

### 2.5. Bacterial Biomass

To evaluate the produced bacterial biomass at the end of the incubation period a 10ml was taken from each flask and centrifugation at 10,000 rpm for 15 minutes. This biomass was washed with tetrachloride carbon ($CCL_4$) and then with distilled water to remove the remaining hydrocarbons, then the air was dried up to the fixed weight [10].

### 2.7. Account the Quantitative Loss of Diesel Oil

Each of single and mixed bacterial culture was taken from nutrient agar plate and suspended in 250mL conical flasks, each containing 100mL of sterile Bushnell-Haas medium with 1.0 (v/v) % diesel as the sole carbon source. Flasks were incubated in a cool shaker incubator at 180 rpm. The experiment conducted in duplicate and non-inoculated flasks forms controls. All flasks were incubated for 28 days at 30◦C. Then, to determine the remaining concentration of diesel oil after the end of incubation period, 15 ml of each flask was taken. Samples extraction was carried out using carbon tetrachloride ($CCL_4$). They were blended and put in a separating funnel with continuous shaking. The contents are then precipitated; two layers are formed: aqueous layer and $CCL_4$ layer containing the remaining diesel oil [10].

The last layer was poured and passed through the filter paper containing anhydrous sodium sulfates ($Na_2SO_4$). After $CCL_4$ evaporation, the amount of residual diesel oil was measured in the medium by using (Horiba oil analyzer ocma-350) device and the degradation efficiency was calculated according to equation (1):

$$\text{Degradation rete \%} = \frac{\text{Intial conc.of oil} - \text{Final conc.of oil}}{\text{Intial conc.of oil}} \times 100 \quad \ldots(1)$$

## 2.6. The use of Gas Chromatography Technique to Detect the Biodegradation of Diesel

The single and mixed bacterial culture were taken from a nutrient agar plate and suspended in 250mL conical flasks, each containing 100mL of sterile Bushnell-Haas medium with 1.0% (v/v) diesel as the sole carbon source. Flasks were incubated in a cool shaker incubator at 180 rpm 30$^{O}$C. The diesel was extracted from Bushnell-Haas medium by liquid- liquid extraction method as follows: (1 sample: 1chloroform). The sample was placed with chloroform in the separating funnel and shaking well after we allow the contents to be deposited. The bottom layer contains the residual concentration of diesel oil that will be withdrawn and tested using gas chromatography technology.

The Chromatographic Gas device (GC-Shimadzu Japanese Company, Model 2010) was fused silica capillary column SE30 a 30m length, and equipped with flame ionization detector. The column temperature is programmed to rising from 100 ° C to 300 ° C and pressure 100 kPa. The detector temperature was 300°C and injector temperature was maintained at 290$^{o}$C. The column temperature is programmed to rise from 100 ° C to 300 ° C at a rate of 3$^{o}$C / min and pressure 100 kPa. Nitrogen was used as the carrier gas[11].

## 3. Results and Discussion

### 3.1 Isolation and Identification of Hydrocarbons Utilizing Bacteria

Bacterial strains capable of decomposing diesel were isolated using Bushnell-Haas media to differentiate between hydrocarbons degrading bacteria and non-degraded bacteria. Four different strains were isolated from the contaminated soil, which was designated as NA-1, NA-2, NA-3, and NA-4. Morphological characteristics of isolated bacteria such as gram stain and biochemical tests were listed in Table1.

**Table 1,**
**Morphological characteristics, gram stain and biochemical tests**

| Characteristics | Isolated bacterial colonies | | | |
|---|---|---|---|---|
| | NA-1 | NA-2 | NA-3 | NA-4 |
| **Color of the colony** | **Creamy** | **Golden yellow** | **off white** | **Pale yellow** |
| **Shape of the cell** | Rod | round | rod | rod |
| **Gram's Staining** | - | + | - | - |
| **Motility** | Motile | Non-motile | Motile | Motile |
| **Spore forming** | - | - | - | - |
| **Catalase test** | + | + | + | + |
| **Oxidase test** | - | - | + | - |
| **Urease production test** | + | + | - | - |

(+) = positive result. (-) = negative result.

Colonies morphology of the isolates on nutrient agar and photomicrographs of staining of the isolates are shown in Figure 1. On the basis of the VITEK2 system, the bacteria were identified; NA-1 as an Enterobacter cloacae species, NA-2 as a Staphylococcus aureus, NA-3 as a Sphingomonas paucimobilis, NA-4 as a Pentoae species. The results of identification using the Vitek 2 system are tabulated in Table 2.

**Table 2,**
**Identified bacteria using VITEK 2 device.**

| No. | VITEK 2 |
|---|---|
| NA-1 | Enterobacter cloacae |
| NA-2 | Staphylococcus aureus |
| NA-3 | Sphingomonas paucimobilis |
| NA-4 | Pentoae sp. |

According to the previous studies of [12, 13] the strains of E.cloacae, S.aureus were reported to be isolated from oil contaminated soil and having the ability to utilize petroleum hydrocarbon as a sole source of carbon and energy. S. paucimobilis and Pentoae sp. are often isolated from oil-contaminated soil because of their unique ability to decompose polycyclic aromatic hydrocarbons

(PAHs), which are important in on-site bioremediation [14, 15, and 16]. In this study, all the isolated bacteria were able to grow in sterile Bushnell-Haas medium and degrade diesel oil. This finding supports the increased function of these bacterial genera in hydrocarbon biodegradation. Thus, these microorganisms could be effective in treatment of diesel spills in soil.

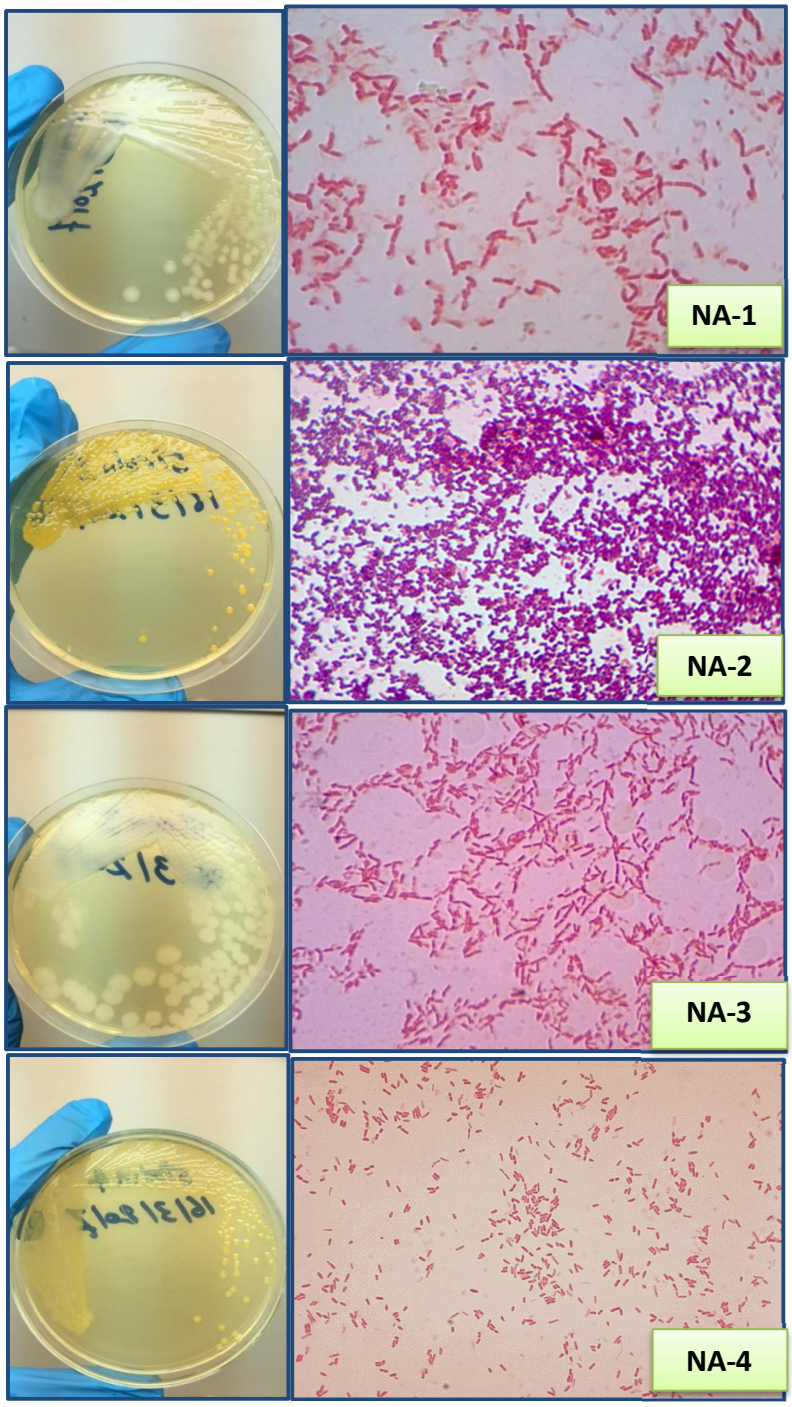


**Fig. 1. Colonies morphology and photomicrograph of isolated bacterial cultures NA-1: Enterobacter cloacae species, NA-2: Staphylococcus aureus, NA-3: Sphingomonas paucimobilis, NA-4: Pentoae sp.**

### 3.2. Dry Weight (Biomass)

Cell biomass quantities, in Table (3), show the growth of bacterial isolates of E. cloacae, S.aureus, S. paucimobilis, and Pentoae sp. in Bushnell-Haas medium .The results showed clearly that dry weight values at the end of incubation period (as a result of biodegradation of diesel oil) in each single strain and mixture (combination of the four strains) were high also,

we noted the resulted dry weight of the mixed culture was high and more than the dry weight of each singular strain. This indicates that microorganisms have benefited from existing organic matter as the sole source of carbon and energy and these bacteria are highly efficient in diesel degradation.

The results were agreed with precedent studies of [11] where the dry weight obtained was (3 g/l). Bacterial isolates break down diesel oil to small compounds of carbon that used to produce polysaccharides, proteins, and fatty acids need for proliferation and energy production. The Simple carbon compounds are merged into the new Cellular Ingredients, which means that they are used to make more microorganisms. Therefore, carbon was used to create further biomass.

**Table 3,**
**Cell biomass quantities.**

| Bacterial strain | Dry weight (g/100 ml) |
|---|---|
| E. cloacae | 0.2955 |
| S. aureus | 0.3685 |
| S. paucimobilis | 0.595 |
| Pentoae sp | 0.367 |
| Mixed culture | 1.226 |

### 3.3. The Percentages of Diesel Decomposition by Single and a Consortium of Isolates

The biodegradation percentage of diesel by the four isolates and their mixed culture after incubation at 30°C for 28 days was demonstrated in Table 4. The results showed that the maximum degradation rate was achieved through a mixed bacterial culture up to (88.4%). These results clearly showed that the mixed culture could carry out a maximum degradation for diesel followed by *S.* paucimobilis, Pentoae sp., S. aureus, and E. cloacae (85.1 %, 84 %, 79 %, and 74 % respectively).

It was noted that mixed bacterial culture gave the highest rate of biodegradation because there is not a pure strain of bacteria with limited metabolism capacity for the decomposition metabolic of all components within diesel and a variety of physiological and metabolic factors required for the biodegradation of Different compounds in diesel.

To put it more clearly the singular isolates metabolize a limited range of hydrocarbon substrates and diesel oil is made of a blend of compounds , so the biodegradation of it needs a mixture of different bacterial groups with broad enzymatic capabilities competent to degrade a wider range of hydrocarbons. In fact, a mixed-culture of the bacterial community is required to complete the biodegradation of petroleum contaminants because the hydrocarbon mixture varies markedly in the volatility, solubility, tendency to biodegradable and the certain enzymes cannot be gained in a single organism. These observations are consistent with [10] who proved that after 28 days of incubation the mixed bacterial degraded up to 88.5% of crude oil. Also, [16] concluded that the highest degree of degradation of diesel was observed in the treatment, when all three strains (Pantoea sp. ITSI10, Pantoea sp. BTRH79 and Pseudomonas sp.) were inoculated in combination into soil contaminated with 30% diesel and the maximum hydrocarbon reduction was reached (79%).

**Table 4,**
**Degradation of diesel oil.**

| Bacterial strain | % of diesel oil degradation |
|---|---|
| E. cloacae | 74 |
| S.aureus | 79 |
| S. paucimobilis | 85.1 |
| Pentoae sp. | 84 |
| Mixed culture | 88.4 |

Using Chromatographic Gas technology, it appears in the control sample (Figure 2) having 13 chemicals material ($C_9$ – $C_{21}$). After exposure diesel to *E. cloacae, S.aureus, S. paucimobilis, and Pentoae sp.* individually as it's clear from the charts in the first week of the experiment the low molecular weight compounds were degraded extensively where some ingredients of the diesel, especially C9 - C10 totally lost while C11 - C20 was reduced hinting that biodegradation of the hydrocarbons components has happened (Figure 3, 4, 5, 6). In the later days, and after two weeks, the visual checking of gas chromatography shows a significant demise of a number of peaks where the number of chemical ingredients decreased to 5, also lowered in the peak area of some components (Figure 7, 8, 9, 10). These results coincide with the fact that microorganisms first attack low molecular weight hydrocarbons while attacking upper and middle hydrocarbons later during incubation. The different potential of isolates may vary in the process of diesel metabolism because of their differences in the enzymatic degradation systems of hydrocarbons. Where some isolates have a strong affinity with the hydrocarbon components, some may be long-delayed due to the inhibitory components of diesel prior to the initiation of biodegradation. While In

contrast, GC analysis of the total extract of diesel (Figure 11) showed that the consortium caused a complete consumption of the major components. These results denote that the biodegradation rate of diesel has improved to a high level by added mixed bacterial culture. Also, suggest that these bacteria have the ability to attack all components of hydrocarbons when being mixed culture [17]. Therefore, the search of biodegradation by the bacterial consortium was essential because these mixed -cultures show metabolic diversity and superiority over pure-cultures.

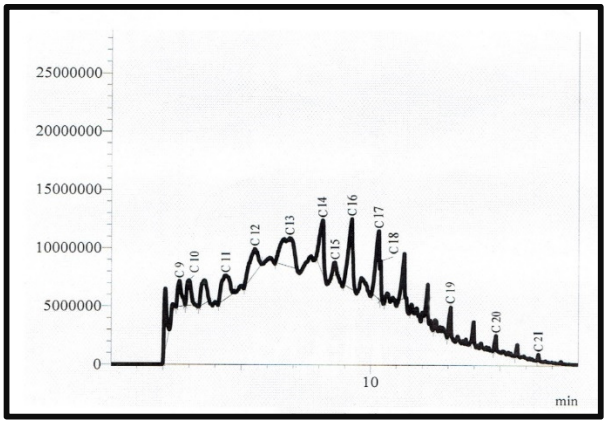


**Fig. 2. Chemical compounds present in the control sample of diesel using gas chromatography.**

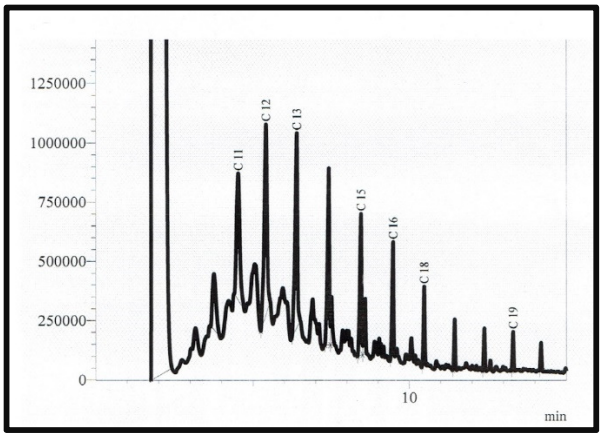


**Fig. 3. The residual chemical compounds in degraded diesel using *Enterobacter cloacae* species after 7 days.**

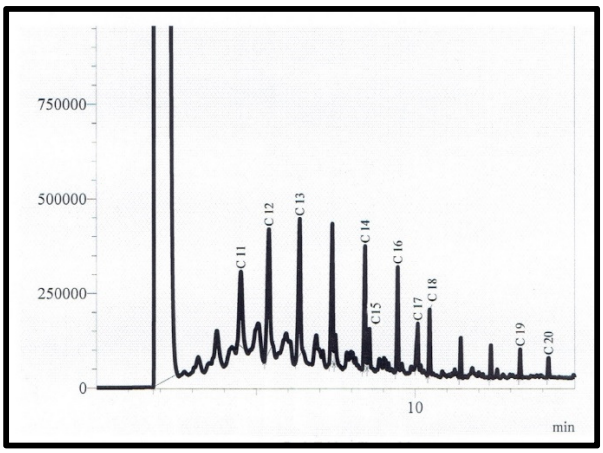


**Fig. 4. The residual chemical compounds in degraded diesel using *Staphylococcus aureus* species after 7 days.**

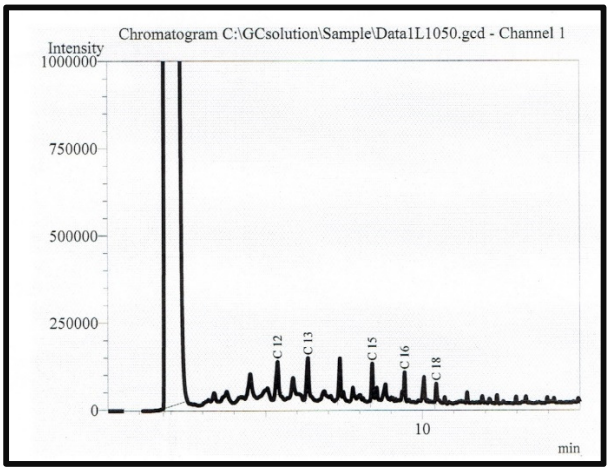


**Fig. 5. The residual chemical compounds in degraded diesel using *Sphingomonas paucimobilis* after 7 days.**

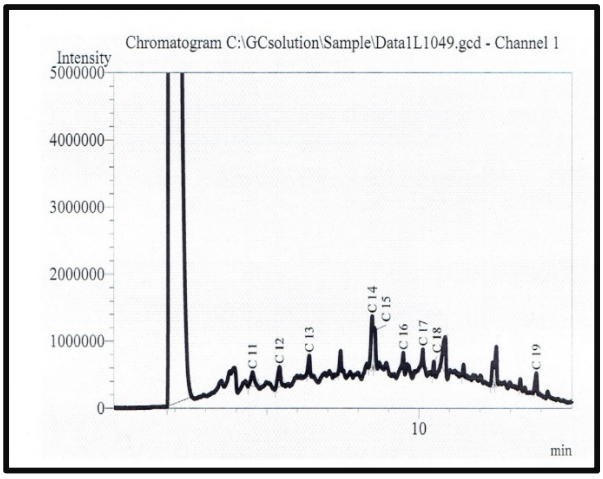


**Fig. 6. The residual chemical compounds in degraded diesel using *Pentoae species* after 7 days.**

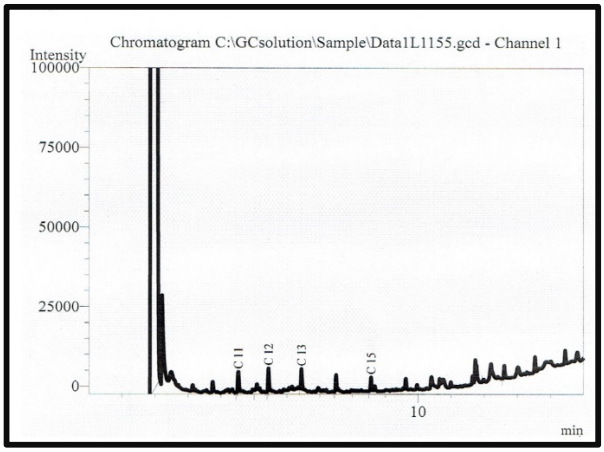


**Fig. 7. The residual chemical compounds in degraded diesel using *Enterobacter cloacae* species after 28 days.**

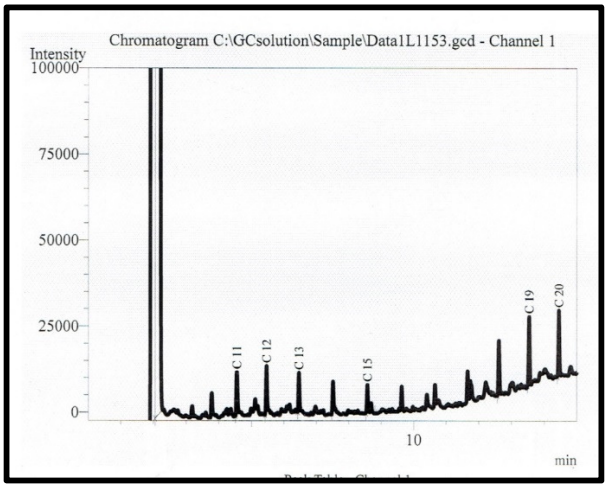


**Fig. 8. The residual chemical compounds in degraded diesel using Staphylococcus aureus after 28 days.**

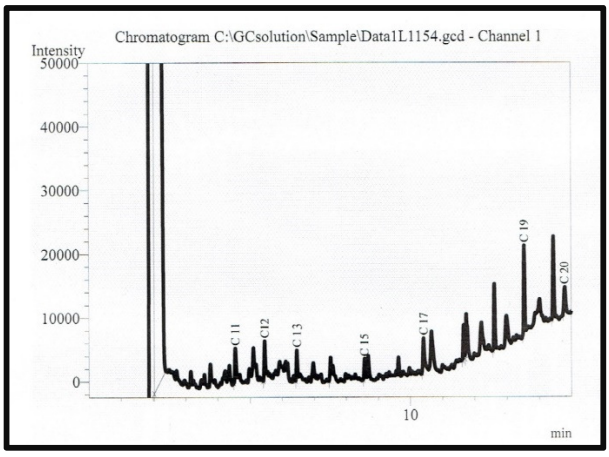


**Fig. 9. The residual chemical compounds in degraded diesel using *Sphingomonas paucimobilis* after 28 days.**

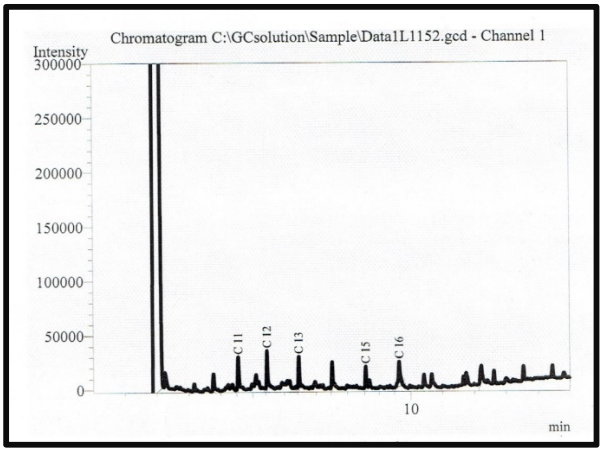


**Fig. 10. The residual chemical compounds in degraded diesel using Pentoae species after 28 days.**

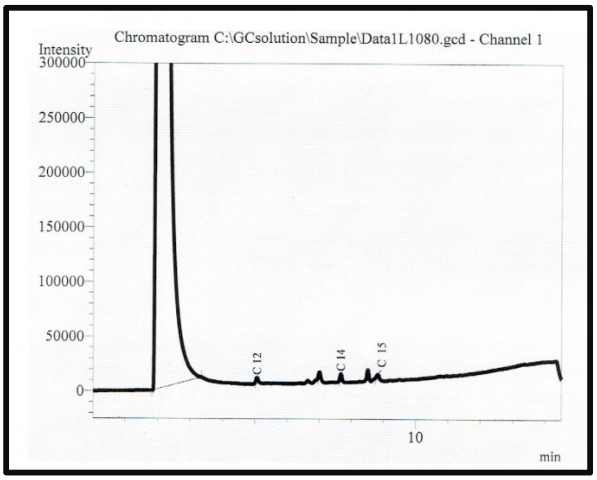


**Fig. 11. The residual chemical compounds in degraded diesel using bacterial consortium after 28 days.**

## 4. Conclusion

The present study showed that the bioremediation with a mixed culture of Enterobacter cloacae, Staphylococcus aureus, Sphingomonas paucimobilis, and Pentoae *species* (consortium) was more efficient than a singular culture. The mixed bacterial culture can achieve the maximum degradation (88.4%) of studied diesel at 30 ° C after four weeks of incubation followed up by: 85.1% by S. paucimobilis, 84 % by Pentoae sp., 79% by S.aureus, and 74% by E. cloacae. Biodegradation of diesel oil means the transformation of its complex, harmful components like polycyclic aromatic hydrocarbons, and long chain alkanes into simpler nonpolluting fractions.

It is evident from this investigation that all thoughtful isolates have a noted impact on the degradation of diesel oil. Thus, this research could help to lighten the way for bioremediation studies and provide information that allows the use of these isolates in biological treatment in environments contaminated with diesel oil.

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# التحلل البايولوجي للديزل المُلوث للتربة باستخدام سلالات بكتيرية بصورة منفردة وبشكل اتحاد بكتيري مختلط

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**الخلاصة**

أجُريت هذه الدراسة بهدف التعرف على مدى قابلية كل من Sphingomonas paucimobilis, Enterobacter cloacae Staphylococcus aureus و Pentoae species على التحلل البايولوجي للمركبات الهيدروكاربونية , حيث تم عزل هذه السلالات من عينات التربة الملوثة بزيت الديزل. تم التحقق من قابلية التحلل البايولوجي لكل من هذه العزلات بصورة منفردة وبشكل خليط بكتيري على تحليل ١% من زيت الديزل بوصفه مصدراً وحيداً للكاربون في وسط Bushnell- Haas medium.

عند درجة حرارة 30°C وبعد مدّة حضانة ٢٨ يوماً، اظهرت العزلات المنفردة نمو منخفض وكذلك معدل تحلل اقل مما تم الحصول عليه من حالة التأزر البكتيري حيث تم الوصول لأعلى معدل تحلل بايولوجي للمركبات الهيدروكاربونية بفعل الاتحاد البكتيري ٨٨,٤٪ متبوعاً بمعدل التحلل لباقي العزلات وعلى التوالي : ٨٥,١٪ بوساطة Sphingomonas paucimobilis و ٨٤٪ بوساطة Pentoae species و٧٩٪ بوساطة Staphylococcus aureus و ٧٤٪ بوساطة Enterobacter cloacae. وللحصول على مزيد من الأدلة على تأثير التحلل البيولوجي لهذه العزلات بشكل فردي وبشكل خليط بكتيري، تم استخدام تقنية كروموتوغرافيا الغاز و التي أكدت حدوث التحلل البيولوجي. أظهرت النتائج أن البكتيريا المعزولة فعالة في التحلل البايولوجي للانسكابات النفطية لزيت الديزل عند استخدامها بشكل منفصل و بينت افضل قابلية تحلل بايولوجي عند استخدامها معا بوصفه خليطاً بكتيرياً.