The use of Mixed Bacterial Culture to improve the Biodegradation of Diesel Pollution.

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Abstract. This study was conducted to evaluate the hydrocarbon biodegradation abilities of Enterobacter cloacae, Staphylococcus aureus, Sphingomonas paucimobilis, and Pentoae species which were isolated from different diesel-contaminated soil samples. The isolates were identified by the Vitek 2 system. Fourier-transform spectroscopy (FT-IR) tested the potential of these isolates to biodegrade the diesel according to the peak areas, a significant decrease in the area of the peaks at 2856-2928 cm⁻¹ corresponds to aliphatic hydrocarbons. The appearance of small peaks at 900-1032 cm⁻¹ refers to substituted benzene derivative compounds. An appearance of some new peaks at 3010-3030 cm⁻¹ which indicate the presence of alcohol (⁻OH) and ketones (RC=O). A sharp peak appeared at 1712 cm⁻¹ refers to the carbonyl group (C=O). The potential of biological surfactant production was tested using the Sigma 703D stand-alone tensiometer showed that these isolates were biological surfactant producers. The better results of the surface tension reduction test were obtained when using the mixed bacterial culture which reduced the surface tension of the medium from 66 mN/m to 35.15 mN/m. Single isolates and mixed bacterial culture have investigated their ability to degrade 3.0 % (v/v) of diesel as sole source of carbon and energy in Bushnell-Haas medium. The results demonstrated that the bacterial isolates could be effective in biodegradation of diesel spills individually and showed good biodegradation abilities when they are used together in the mixed bacterial culture.

Keywords: Biodegradation, Diesel, Mixed-culture, biological surfactant, 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]. Soil pollution with diesel fuel may be toxic to microorganisms and plants and acts as a
source of groundwater pollution [2]. Diesel fuels are complex mixtures of saturated hydrocarbons (primarily paraffins including n, iso, and cycloparaffins), and aromatic hydrocarbons (including naphthalenes and alkylbenzenes) obtained from the middle-distillate, gas-oil fraction during petroleum separation [3]. Remediation of petroleum contaminated sites could be achieved by either physicochemical or biological methods. Apart from the physicochemical treatment techniques, bioremediation is a clean and environmentally friendly treatment that is widely used to control environmental pollution [4]. Bioremediation is the use of microorganisms, mainly bacteria and fungi, or plants to utilize and break down environmental contaminants such as petroleum into less harmful substances [5]. It involves breakdown organic compounds through a biological transformation to less complex metabolites such as carbon dioxide and water. These techniques have a number of key advantages over traditional technologies including the fact that they are simple to implement, applicable over large areas, cost-effective and can lead to the complete removal of different contaminants [6; 7]. Due to the complexity of petroleum products, biodegradation caused by mixed cultures is more effective than that caused by pure culture since a broader enzymatic capability can be achieved [8]. Bacteria are considered to be efficient biodegrades due to the wide variety of species, their abundance, and their catabolic and anaerobic versatility, as well as their ability to adjust to adverse environmental conditions. Therefore, mixed bacterial culture of microbial community is required to complete biodegradation of petroleum pollutants [1].

The aim of this research was: studying the biodegradation of diesel by mixed bacterial cultures isolated from diesel-polluted soil for bioremediation process applications.

2. Materials and methods

2.1 Collection of samples

The soil samples were collected from different locations, including the local generators and Al Dura refinery in Baghdad. All samples were kept in sterile plastic bags and transported to laboratory for analysis [8].

2.2 Isolation and identification of Diesel degrading bacteria

Four grams of contaminated soil sample were suspended in 20 mL of sterile Bushnell-Haas medium (BHM) containing (g/l): MgSO$_4$.7H$_2$O, 1.0; K$_2$HPO$_4$, 1.0; KH$_2$PO$_4$, 1.0; FeCl$_3$, 0.05; NH$_4$NO$_3$, 1.0; CaCl$_2$, 0.02; pH to 7±0.2 which was sterilized at 121°C for 15 min. Bacteria were grown in 250 ml Erlenmeyer flasks for one week in a cool incubator shaker at 30 °C and 180 rpm. Isolation of pure cultures was carried out by streaking method on nutrient agar plates. The plates are placed in an incubator at 30 °C for 48 hours. Specific colonies were sub-culture on nutrient agar plates and incubated for another 48 hours. The pure cultures were preserved in the refrigerator at 4 °C for further study and identification. The isolates were classified based on their shape, color and biochemical properties than identified using VITEK 2 device [1].

2.3 Inoculum preparation

Bacterial isolates were cultured on nutrient agar at 30 °C for 48 h. Aqueous culture was then suspended, standardizing to the Macfarland scale tube number 8 [9]. Tube 8 represents turbidity of bacteria in the concentration (2.1 x 10$^9$ CFU/mL). The volume of inoculum was 4 ml.

2.4 Measuring Biodegradation of Diesel

Measuring the biodegradation of diesel by isolated bacteria was done with the following ways:

2.4.1 The use of FT-IR Spectrum technique

The four bacterial isolates individually were inoculated in of 93 ml BHM in Erlenmeyer flasks and 3.0 % (v/v) of diesel was added as the sole source of carbon. Flasks were incubated at 30°C in a cool incubator shaker at 180 rpm. Samples were withdrawn after 7 days during the experiment period.
Then 15 ml of medium was transferred into separating funnel containing chloroform (1:1; chloroform) with vigorous shaking. The chloroform layer which contains the remainder of diesel was tested using FT-IR spectrum [10].

2.4.2 The reduction of surface tension

The bacterial isolates were inoculated with 93 ml of BHM containing 3.0 % (v/v) of diesel in 250 ml Erlenmeyer flask and incubated at 30 °C in a cool shaker incubator at 180 rpm for 8 days. A medium was prepared by adding 10 ml of culture to 20 ml of carbon tetrachloride (CCL₄) to separate hydrocarbons from the liquid culture. Then the medium was poured in centrifuge tubes and centrifuged at 10000 rpm for 15 minutes at -5 °C to precipitate the cells [11]. The upper free cells layer that containing the diesel was used for surface tension test using a Sigma 703D Du-Nouy-Ring tensiometer [12].

2.4.3 The quantitative Loss of Diesel

Each of individual and mixed bacterial culture was suspended in 250 mL Erlenmeyer flasks which containing 93 mL of BHM. Diesel was added at concentration of 3.0 % (v/v) as a sole carbon source. Flasks were incubated at 30 °C in a cool incubator shaker at 180 rpm for 28 day. The experiment conducted in duplicate [13]. Then, to determine the remaining concentration of diesel at the end of incubation period, 15 ml of medium from each flask was taken. The acidity of the sample culture was adjusted to (pH <= 2) by adding HCl. The extraction was carried out by liquid-liquid extraction method by mixing equal volumes of sample and solvent (1:1 CCL₄). The amount of residual diesel was measured in the medium by using (Horiba oil analyzer ocma-350) device [14]. The degradation efficiency was calculated according to the following formula:

$$\text{Degradation rate } \% = \frac{\text{Initial conc. of diesel} - \text{Final conc. of diesel}}{\text{Initial conc. of diesel}} \times 100$$

3. Results and Discussion

3.1 FT-IR analysis of Diesel degradation

The biodegradation of diesel fractions was observed using Fourier-transform spectroscopy to identify the absorption of beams before and after exposure to bacterial isolates. In figure 1, the control sample showed the absorption bands locations of aliphatic compounds (C-H), (C-C) at 2856-2928 cm⁻¹ which appears in large and broad peaks, while a small peak at 720 cm⁻¹ refers to aromatic compounds (benzene ring) [15; 16]. Absorption bands at 1376- 1464 cm⁻¹ observed the symmetrical -CH₃, -CH2 of aliphatic compounds [17].
After 7 days of incubation, figures from 2 to 5 revealed that the spectrum reflected more absorption bands than the control sample under the effecting of bacterial isolates at concentration of 3 % (v/v) of diesel. Appearance of some new peaks at 3010-3030 cm\(^{-1}\) indicates the presence of alcohol (-OH) and ketones (RC=O). A sharp peak appeared at 1712 cm\(^{-1}\) refers to the carbonyl group (C=O). Moreover, the peak 1216 cm\(^{-1}\) in the wave range 1032-1320 cm\(^{-1}\) showed the presence of primary alcohol (C-OH) and carboxylic acid. A significant decrease in the area of the peaks at 2856-2928 cm\(^{-1}\) was corresponding to aliphatic hydrocarbons. The appearance of small peaks at 900-1032 cm\(^{-1}\) refers to substituted benzene derivative compounds [18]. A change in the absorption band at wave range 1360-1496 cm\(^{-1}\) refers to the transformation and consuming of hydrocarbons (C-H bond) in the diesel.

Figure 2. FT-IR spectrum of diesel at concentration 3.0 % treated by Enterobacter cloacae after 7 days, (at 30 °C, pH to 7±0.2)
Figure 3. FT-IR spectrum of diesel at concentration 3.0% treated by *Staphylococcus aureus* after 7 days, (at 30 °C, pH to 7±0.2)

Figure 4. FT-IR spectrum of diesel at concentration 3.0% treated by *Sphingomonas paucimobilis* after 7 days, (at 30 °C, pH to 7±0.2)
The presence of alcohol and ketone groups during biodegradation indicates the breaking down of aliphatic and aromatic compounds of diesel by bacterial isolates studied. Also, the changes in bands locations and area indicate the role of bacterial isolates in the degradation of hydrocarbons, which attributed to the fact that these compounds are naturally hydrophobic and have high molecular weights which have been converted by bacteria into low molecular weights and more hydrophilic compounds [19].

3.2 Determination of Surface Tension Reduction

The test of surface tension reduction by the individual isolates and by their mixed culture has been measured after 8 days of incubation. The results demonstrated that all isolates and the mixed culture were able to produce biological surfactants in BHM containing 3.0 % (v/v) of diesel as the only carbon source. The greatest surface tension reduction of the medium was achieved with the mixed culture which reached a value 35.15 mN/m compared to the control 66 mN/m. While the other data of the surface tension obtained from individual isolates still high and ranged 43.15 mN/m to59.01 mN/m, as shown in Table 1. These results correspond to the findings of Cerqueira et al. (2011) [20] who reported that a microbial consortium that included (B. cereus, P. aeruginosa, B. cibi, S. acidaminiphila and B. megaterium) was reduced the surface tension of the medium containing oily sludge from 60.4 mN/m to 36.6 mN/m. However, the other surface tension values as a result of the singular action for the isolates ranged from 41mN/m to 42.6mN/m.

The reduction in surface tension of the medium depends on the type of biological surfactants produced by bacterial isolates as well as the critical micelle concentration (CMC). The micelles are large molecules which when aggregated the surface tension begins to decrease [21]. The biodegradation process for petroleum hydrocarbons depends strongly on the hydrocarbons emulsifying. The biological surfactants which produced by bacterial species seems to be more potent than chemical surfactant, since they are environmentally friendly and non-toxic biodegradable compounds [12].
Table 1. Surface tension reduction by hydrocarbon-degrading bacterial isolates

| Bacterial Isolates            | Surface tension (m N/m) at concentration 3.0 % of diesel |
|-------------------------------|----------------------------------------------------------|
| E. cloacae                    | 59.01                                                    |
| S. aureus                     | 43.15                                                    |
| S. paucimobilis               | 46.55                                                    |
| Pentoae species               | 54.33                                                    |
| Mixed bacterial culture       | 35.15                                                    |

Surface tension of the control = 66 mN/m

3.3 Decomposition of Diesel

The biodegradation percentage of diesel at concentration of 3.0 % (v/v) by the individual isolates and their mixed culture after incubation at 30 °C for 28 days was demonstrated in figure 6. The efficiency of biodegradation varies between the isolates and ranged from 44 % to 70 % according to their ability to degrade diesel. At concentration 3.0 % (v/v) of diesel the mixed bacterial culture performed a maximum biodegradation up to (75.31 %) followed by S. paucimobilis, Pentoae sp., E. cloacae, and S. aureus (70 %, 67.6 %, 56 %, and 44 % respectively) as shown in figure 6 below. The individual isolates metabolize a limited range of hydrocarbon substrates and diesel 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 [1]. These results are consistent with Shahaby (2014) [22] who studied the biodegradation of complex mineral oil at concentrations 3 and 5% (v/v) by a mixed culture. The highest biodegradation rate occurred at 3 % (v/v) and reached 41.8%. Al-Wasify and Hamed (2014) [13] proved that after 28 days of incubation the mixed bacterial was degraded up to 88.5% of crude oil.
Figure 6. Biodegradation rate at concentration 3.0% of diesel by individual bacterial isolates and mixed culture after 28 days, (at 30°C, pH to 7±0.2)

4. Conclusions
The present study showed that the bioremediation of diesel with a mixed culture of Enterobacter cloacae, Staphylococcus aureus, Sphingomonas paucimobilis, and Pentoae species were more efficient than individual isolates after 7 days of incubation. Because of the ability of the mixed culture to produce the biological surfactants and reduced the surface tension of the medium from 66 mN/m to 35.15 mN/m. Also, FT-IR spectrum analysis confirmed the biodegradation of diesel by the four bacterial isolates when used individually. Biodegradation of diesel means the transformation of its complex, harmful components like polycyclic aromatic hydrocarbons, and long chain alkanes into simpler fractions. Accordingly, these bacteria could be useful in treating diesel polluted environment.

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