Bio-degradation of crude oil using local bacterial isolates

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Abstract. Twenty seven hydrocarbon degrading bacterial isolates were isolated from five hydrocarbon contaminated sites. The study revealed a high efficiency of bacteria adapted to the biodegradation of hydrocarbons (petroleum) isolated from soil contaminated with oil residues. The isolates were examined for their hydrocarbon degradation in media supplemented with crude oil at five different concentrations 2%, 3% 5%, 7% and 10% incubated for 5 different time intervals 5, 10, 15, 20, and 30 days. The results indicated that all the isolates possessed potential to degrade the wide variety of hydrocarbons. The most efficient among them was SD1 which degraded most tested hydrocarbon (98%) showing maximum growth at 3.3 gm/l of biomass concentration and 15 days incubation. SD1 isolate was identified on the basis of morphological and biochemical characteristics and confirmed with 16s rRNA sequencing. GCMS Analysis showed significant differences in the composition of hydrocarbons in Crude oil. It could be concluded that native flora of hydrocarbon contaminated site adapt to the environmental condition and could be implicated to remove hydrocarbons.

1. Introduction
Petroleum hydrocarbons are the most widespread contaminants within the marine environment. Pollution by hydrocarbons in marine environments may be the consequence of various natural (natural seepages) and/or anthropogenic activities (discharge during tanks and/or ships transportation and/or pipeline failures) as well as the chronic pollution (ships, harbours, oil terminals, freshwater run-off, rivers and sewage) [1].

The "fate" of petroleum in the sea water largely depends on mechanical (wave, wind), physical (temperature, UV) and chemical (pH, dissolved oxygen and nutrient concentration) factors which may differently influence its natural transformation (oil weathering) and bio-degradation [2]. On the above mentioned basis, bioremediation techniques have been developed and improved for cleaning up oil-polluted marine environments as an alternative to chemical and physical techniques [3]. As reported in different studies, a wide variety of marine bacteria are known to degrade petroleum hydrocarbons, and those, distributed over several (sub) phyla (α-, β-, and γ-Proteobacteria; Bacteroidetes / Chlorobi group) have been described so far [4;5]. Genera Pseudomonas, Rhodococcus, Bacillus, Micrococcus, Staphylococcus, Acinetobacter and Serratia; also, some colonies resembled Actinomycetes. Besides being typical soil microorganisms, all the genera have been reported to be present in hydrocarbon-contaminated sites, and have also been reported as hydrocarbon degraders [6;
Hydrocarbon molecules that are released into the environment are hard to remove, since they adsorb to surfaces and are trapped by capillarity in a water-immiscible phase. Bioremediation has proven to be an alternative to diminish the effects caused by hydrocarbon contamination of soil and water, using the metabolic capacities of microorganisms that can use hydrocarbons as source of carbon and energy, or that can modify them by cometabolism. The efficiency of removal is directly related to the compounds' chemical structure, to its bioavailability (concentration, toxicity, mobility and access) and to the physicochemical conditions present in the environment [8]. Bioremediation can be described as the conversion of pollutants (hydrocarbons) by micro-organisms (bacteria) into energy, cell mass and biological waste products [9]. It is also necessary to study the microbial degradation of crude oil as an environmentally friendly way of cleaning up oil-polluted areas.

2. Material and methods

2.1. Oil sample
Crude oil samples were obtained from Doura refinery in Baghdad

2.2. Soil sample
Samples were collected randomly from soil surface which heavily contaminated with oil, (automobile workshop and electric generators), at a depth of 5cm, in sterile bags and tightly packed, then carefully transferred to the laboratory and stored at 4°C. They were used to isolate the bacteria.

2.3. Isolation of bacteria
Isolation of bacteria were performed by soil dilution, one gram of dried soil was dissolved in 9ml of distilled water , aqueous dilutions, of the suspension were applied in nutrient agar , the plates were incubated at 37°C for 24 hrs. Different isolates were carried out then selected colonies of bacteria were transferred from mixed culture plates onto respective agar plates and incubated at 37°C for 24hrs plates containing pure cultures were stored at 4°C until the examination [2].

2.4. Preparation of Inocula.
Inocula of 0.1 mL aliquots of four overnight nutrient broth cultures for each strain individually and 1 for mixed consortium) was washed twice in physiological saline solution (0.87% NaCl, pH 7.2) and suspended in the same to optical density of 0.1 (OD$_{600}$) (2).

2.5. Primary screening for oil degradation
The individual from overnight culture at the log phase of growth were transferred to 250 mL conical flasks, each containing 50mL of sterile mineral salts medium (table 1) with (0.2% v/v) crude oil [7]. The experiment was carried out in duplicate and uninoculated flasks constituted the controls, accounting for abiotic losses. All flasks were incubated at 30°C for seven day. Residual concentrations of crude oil were determined by gas chromatography.[5].

| Minerals         | g/l  |
|------------------|------|
| NaCl             | 1.0  |
| CaCl$_2$         | 0.02 |
| KH$_2$PO$_4$     | 1.0  |
| K$_2$HPO$_4$     | 1.0  |
| NH$_3$NO$_2$     | 1.0  |
| FeCl$_3$         | 0.002|
| MnSO$_4$.2H$_2$O | 0.002|
| Yeast extract    | 1.0  |
2.6. Identification of bacterial isolates
The colony characteristics and cellular morphology of the isolated, their pigmentation, staining reactions, were done.

2.7. Isolation of DNA
A wizard genomic DNA extraction kit (Promega) used.

2.8. Amplification of 16S rRNA gene
Amplification of 16S rRNA gene was performed using GoTaq Green Master Mix (Promega, USA) according to the manufacturer’s recommendations. The synthesized primers were used for amplification. Temperature–time profile of PCR was the following:
- 30 cycles of 95 °C for 0.5 min, 65 °C for 2 min and 72 °C for 2 min, all steps at maximal ramp rate (approximately 3 °C s⁻¹ in heating mode and 1.5 °C s⁻¹ in cooling mode).
- Analysis of PCR products was performed using electrophoresis in 2% gel, containing ethidium bromide. A marker contains DNA fragments of known size were used to know the expected product size. Then gel was visualized under ultraviolet (UV) light.

2.9. 16S rRNA gene sequences
DNA coding for 16S RNA was PCR amplified by using chromosomal DNA as a template and oligonucleotides Ribo- For (5′AGTTTGATCCTGGCTCAG- 3′; and Ribo- Rev (5′CCTACGTATTACCGCGGC- 3′). Those two oligonucleotides were designed to amplify a 540 bp DNA fragment. The nucleotide sequences were used for the analysis of sequence similarity through Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The percentage differences of the resultant partial 16S rRNA gene sequences among different species in the same group/genus of species were determined by pairwise alignment using MEGA 6 [10].

2.10. Oil Degradation
For examining the oil degradation, mineral salt medium was used one about 50ml medium was dispended in 250ml conical flasks. The media was inoculated with bacteria and incubated at 30°C for 27 days in shaking incubator at 150 rpm.

2.11. Biomass estimation
For biomass estimation after incubation period dry weight method used, centrifuge at 4000 rpm for 30 min to precipitate the cells, extracted by acetone and hexane mixture 3:1 then dried at 105 °C for 24 hrs, biomass weighted [11].

Removal percentage estimation
Removal percentage was estimated by use equation
\[
\text{Removal percentage} = \left( \frac{\text{total peak area(control)} - \text{total peak area(test)}}{\text{total peak area(control)}} \right) \times 100
\] [12].

2.12. DCPIP method for assessing the potential of bacteria
Efficacy of oil degrading bacteria was assessed using DCPIP technique. The mixture of 0.1 ml of microbial culture, 3 ml BH medium containing oil sample and 0.3ml of DCPIP was incubated at 30°C. Bacteria capable of altering the oil components produce electrons which can take part in oxidation and reduction reactions. The oxidized (blue) and reduced (colourless) conditions of DCPIP will help in identifying the activity of oil degrading microbes [12]. Change in colour was observed at 660 nm against uninoculated blank tube periodically to assess the potential of consortia.

2.13. Crude oil extraction from test flask
Cells and crude oil was centrifuged at a speed of 5000 rpm for 35 min such that the biomass settled at the bottom and the supernatant aqueous phase containing bulk of the oil separated biomass pellet was extracted by adding 2 ml of hexane.
2.14. Crude oil extraction from control flask
Flask containing 50ml medium with 2% crude oil was acidified with HCL. Equal volume of hexane was added and flask was placed on shaker at 120 rpm for 20 min. This solution was then transferred in separating funnel, mixed well and then the aqueous the hexane phases were allowed to separate. The lower layer of H$_2$O was drained and the extraction was repeated with 10 ml of solvent at 72°C.

2.15. Gas Chromatography
The analysis of oil degradation were done using gas chromatography (Dani) equipped with flame ionization detector (GC-FID). The carrier gas was nitrogen and the column used for separation was DN5 column, three m long and 0.23mm diameter. The operating conditions were as follows: initial oven temperature 100°C for 0.5 min and temperature ramp at a rate of 10°C min$^{-1}$ to a final temperature of 280 °C. The injector temperature was 250 °C; the FID detector was at 320 °C; the flow rate was 1 ml/min. This method was modified from [13; 14].

3. Results and Discussion
3.1. Isolation and Screening of Hydrocarbons-Degrading Microorganisms
To isolate different hydrocarbon-degrading bacteria, enrichment culture methods were used according to protocol described in Material and Methods. The enrichment procedure for obtaining contaminated soil hydrocarbon-degrading microbes was performed in multiple cycles to ensure that the microbes which were obtained at the end of the enrichment cycle were capable of utilizing the petroleum compounds rather than just tolerating it. Twenty seven bacterial isolates were isolated from enrichment cultures that were established at 30°C for 4 weeks. Five of the isolated strains that showed higher growth rate on crude oil were selected from the twenty seven isolates for further study. These isolates showed a varying degradation profile for the total petroleum hydrocarbon [TPH] of the petroleum contaminated soil. In our present work, we report that SD1 isolate could efficiently degrade total petroleum hydrocarbon removal about 98.245%, over a period of 14 days with 3.3 g/l as dry weight (Fig 1 and 2). According to many authors, bacteria have been described as being more efficient hydrocarbon degraders than other microorganisms or at least that bacteria are more commonly used as a test microorganism [4; 15]. Many other investigators have reported the involvement of bacteria and yeast in crude oil biodegradation [7; 8]. On the contrary, there is scanty information that bacteria are better hydrocarbon degraders than others [11; 12]. For that reason, growth ability of selected strain (SD1) was tested in an enrichment liquid medium.

![Figure 1. Biomass measurement of active isolate](image)

**Figure 1.** Biomass measurement of active isolate
Figure 2. Hydrocarbons removal of active Bacillus isolates.

The 2.6-DCPIP screening test was used to detect the hydrocarbon-degrading properties of the bacteria. All the five selected isolated found to have hydrocarbon-degrading capability by the 2.6-DCPIP assay. SD1 isolate exhibited the highest hydrocarbon-degrading ability (Fig 3).

Figure 3. Estimation of hydrocarbon-degrading ability of soil bacterial isolates using the 2, 6-DCPIP assay.

Morphological characteristics: Isolated bacterial colony was white, the shape of the cell was bacillus and gram staining was positive. Sequence comparison demonstrated the affiliation of the strain SD1 to Bacillus cereus (Fig. 4). The naturally present microorganisms in the soil were capable of degrading the pollutant as much as the samples enriched with Bacillus. Several studies have reported on the roles of Bacillus cereus more tolerant to high level of hydrocarbon in soil due to their
resistance endospore [16, 17] showed that of 73 aerobic bacteria ability to degraded petroleum hydrocarbon.

![Figure 4](image_url)

Figure 4. Agarose gel electrophoresis analysis of PCR reaction for DNA extracted from pure culture of SD1 strain.

For confirmation of hydrocarbon degrading activity in MSM GC-MS analysis of control (oil) (without bacteria) was done which showed it was a mixture of different hydrocarbons and further it was compared with GCMS results of oil extracts from inoculated medium. The evaluation of the oil biodegradation by SD1 strain was carried out during the process of 14 days using gas chromatography with mass spectrometry GC/MS. After 14 days of incubation, the undegraded oil hydrocarbon residue was extracted twice with equal volumes of hexane. The results showed appearance of new compounds through bio-degradation with less molecular weight and less complex such as carboxylic acids and alcohols.

| Retention time(min) | Compound                        | Formula     |
|---------------------|--------------------------------|-------------|
| 2.567               | 3-Methyl-3-pentanol             | C8H14O3     |
| 4.758               | 2-Nitrohexane                   | C8H13NO2    |
| 5.808               | Cyclopentane, 1-acetyl-1,2-epoxy-| C7H10O3    |
| 9.692               | 2-Methylundecane                | C12H26      |
| 11.217              | 4,8-Dimethyltridecane           | C13H32      |
| 12.342              | 3-Ethyl-2,7-dimethyloctane      | C12H26      |
| 14.008              | 4,8-Dimethyltridecane           | C13H32      |
| 15.277              | 2-Methylnonadecane              | C19H42      |
| 20.733              | 2-Methyleicosane                | C21H44      |
| 21.677              | 5-Butylnonane                   | C14H28      |
| 22.583              | 9-Octylheptadecane              | C25H52      |
Oil containing \(n\)-alkanes, branched alkanes, and small concentrations of aromatic polycyclic compounds. DS1 isolate was able of utilizing a wide range of hydrocarbons, with a preference for alkanes with intermediate carbon chain lengths as shown in (table 1and 2). Bacterial micro flora was found actively able to degrade total mixture of hydrocarbons present in oil contaminated soil samples collected from motor workshop area\[18\]. The result was confirmed by almost total disappearance of the corresponding peak of each compound. It is expected that the hydrocarbon assimilating capabilities in the liquid medium is due to adaptation of isolate due to previous exposure to hydrocarbons. It may indicate the ability of the emulsification of hydrocarbons, which is a major factor for hydrocarbon uptake and assimilation.

Table 3. The major molecular fragmentation of crude oil compounds after degradation

| Retention time(min) | Compound                             | Formula              |
|---------------------|--------------------------------------|----------------------|
| 3.017               | 1-Hydroxy-1 methylcyclopentane        | C\(_8\)H\(_{12}\)O \_  |
| 3.292               | Acetic acid, isobutyl ester           | C\(_6\)H\(_{12}\)O\(_2\) |
| 4.775               | -Nitrohexane                         | C\(_6\)H\(_{13}\)NO\(_2\) |
| 5.417               | 2-Hexanecarboxylic acid              | C\(_{11}\)H\(_{14}\)O\(_2\) |
| 5.817               | rans-4,4-Dimethyl-2-Pentene           | C\(_{16}\)H\(_{14}\) |
| 5.983               | 4-Methyl-3-pentenoic acid            | C\(_{9}\)H\(_{10}\)O\(_2\) |
| 8.533               | alpha-Hydroxyisobutanoic acid        | C\(_{7}\)H\(_{12}\)O\(_3\) |
| 18.717              | n-Eicosane                           | C\(_{20}\)H\(_{42}\) |

4. Conclusions
Our study focused on bacterial isolate isolated from oil contaminated soil from Al –Dora petroleum refinery, identified as Bacillus cereus. A direct relationship was found between both cell growth of the bacterial isolate and crude oil biodegradation. These strains have high levels of crude oil degradation and sufficient growth on mineral medium supplemented with hydrocarbons. In this study, the isolated bacteria have been shown to degrade a wide range of hydrocarbons and completely metabolize \(n\)-alkanes. From the data presented in this study, it can be concluded that the investigated strain Bacillus cereus could be considered as good prospects for their application in bioremediation of hydrocarbon contaminated environment and improvement of hydrocarbon removing treatment of contaminated soil.

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