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Isolation of Indigenous Hydrocarbon Transforming Bacteria from Oil Contaminated Soils in Libya: Selection for Use as Potential Inocula for Soil Bioremediation

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Abstract The Libyan oil industry has left a significant legacy of contamination and methods are required to remediate oil-contaminated soils in the area. In this work hydrocarbon utilizing microorganisms were isolated and identified from contaminated soil samples obtained from an oil Refinery (Zawia, Libya). After initial screening of eleven isolates capable of growth on hexadecane, the five most promising hydrocarbon–utilizing bacteria were isolated and tested for biosurfactant production and emulsification activity. They were identified (using 16S rRNA sequence analysis) as *Pseudomonas putida*, *Pseudomonas species*, *Betaproteobacterium*, *Actinomyces species*, and *Bacillus species*. Among the five species tested, *Pseudomonas putida* showed superior performance in terms of growth on hydrocarbons (1.0×10^{10} CFU (ml)), E24 emulsifying activity (86%) and ability to transform hydrocarbons in pure culture. Interestingly, gas chromatographic analysis of crude oil treated with *P. putida* showed a decrease in heavy hydrocarbon fractions demonstrating a clear potential for this microbe to be used as a soil inoculant in bioremediation.

Keywords: *Biosurfactant, Emulsifying Activity, hydrocarbon–utilizing bacteria, Pseudomonas putida, Betaproteo bacterium, Actinomyces species, Bacillus species*

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1. Introduction

Pollution with petroleum and its products is considered a major problem worldwide due to its impact on human health and the environment. Petroleum hydrocarbons are considered to be the most common group of persistent organic contaminants and are known to be toxic to many organisms (e.g. [16,40]). Moreover it has been shown that petroleum hydrocarbons cause significant losses in soil quality due to their toxicity towards biological processes catalyzed by soil microorganisms as in [35].

Reddy et al. [40] discussed a variety of biological processes which are affected by petroleum products due to their carcinogenicity, pointing out that the toxic compounds of petroleum hydrocarbons are accumulated in tissues, "causing genetic mutations and cell atrophy. The purpose of remediating contaminated sites is not to remove every last drop of contamination for the sake of it, but to protect human health and the environment and eventually to attain sustainable development as in [24]. During the last century hydrocarbon contamination has become a major environmental problem as a result of the manufacture, transportation, and distribution of petroleum products [9]. Since the activities of the oil associated industry in Libya have expanded, high levels of hydrocarbon contamination have been recorded at several sites**.** In particular the Libyan coast, where oil industries are located are affected by serious oil pollution and for the last 40-50 years in the Mediterranean ecosystem, the levels of petroleum hydrocarbon have increased [1]. Soil and groundwater contamination was recorded in the western part of Libya caused by pipeline corrosion (Libyan Petroleum Institute reports). Additional studies have observed high levels of TPHs in the Gulf of Sirte in the North of Libya and 35000 mg/kg of petroleum hydrocarbons were recorded in soil contaminated by crude oil and petroleum products in the area surrounding the Zawia refinery in the west of Libya [2,15].

Despite the level of oil contaminated soil known to be present in Libyan soils relatively little work has been carried out on the potential bioremediation of these soils. These sandy soils may lack the appropriate hydrocarbon degraders and nutrients required for the removal of hydrocarbons by bioremediation. For example, McMillen [27] compared biodegradation rates between sandy soils and clay soils; and found that sandy soils have lower biodegradation potential than clay and loam soils. The reasons for the low biodegradation of sandy soils maybe due to low numbers of hydrocarbon degraders present, low water holding capacity, low total organic carbon

content, and/or low surface area available for growth. To address some of these issues this work aimed to isolate hydrocarbon-transforming bacteria from oil contaminated sandy soil from Libya and to assess their potential to be used as inocula to assist the bioremediation of such soils.

2. Materials and Methods

2.1. Physical and Chemical Characterization of the Soil and Contaminants

2.1.1. Collection of Samples

The site used in this study was the Zawia Oil Refinery, where different fuels including naphtha, gasoline, kerosene, light vacuum gas oil (VGO), fuel oil, base lubricating oils, and asphalt are processed. Twelve hydrocarbon contaminated soil samples of approximately 1kg (0-15cm depth) were collected from around four oil storage tanks located at Zawia Oil Refinery, Libya, in June 2008. Soil samples were collected to reflect a range of hydrocarbon concentrations and to represent various potential sources of contamination. Samples were placed in plastic bags and cooled for transportation. After the soil samples were received at the LPI (Libyan Petroleum Institute) microbiological laboratory, the soil was air dried for 48 hours and all samples mixed to obtain one homogenous sample. One homogenous sample was used as any future potential large scale bioremediation would be carried out on such a mixed soil and so that a manageable number of laboratory manipulations could be carried out. Soils were sieved to 2mm and subsamples of 500g were set aside for soil analysis.

2.1.2. Soil Analysis

Soil texture: Particle size was determined using a Master Sizer 2000 (Malner International) at a chemical analysis laboratory, namely the Libyan Petroleum Institute (LPI). The petroleum products that contaminated the soil samples were extracted using a Soxhlet Extraction System (SES). Dichloromethane was used for the extraction.

2.1.3. Soil pH

Soil pH was determined using a pH meter (Jenway 3020) on soil suspensions in water in triplicates as described in [10]. Twenty five grams of soil was stirred continuously with 50ml of d-eionised water d.d.H_2O for 15 minutes and allowed to equilibrate for another 15 minutes. The pH meter was immersed in the supernatant and was rotated gently before recording pH.

2.1.4. Moisture Content and Water Holding Capacity (WHC)

Moisture content was determined by drying 10g of the soil sample in an oven at 105℃. 10g of oven dried soil was placed on filter papers (Whatman No. 42) and fitted into Buchner funnels. De-ionised water was added slowly (at a rate of 1cm hr^{-1}) until the water level was just above the soil surface, he soil was saturated and dripping into the flask below. The funnel was then removed and left to drain overnight until no further drainage occurred. The soil was left for 24 hours, rewetted to saturation and the whole apparatus was reweighed. The percentage of moisture content of the soil in triplicate was then determined. Water holding capacity (100%) was then calculated.

2.2. Isolation and Purification of Hydrocarbon Degrading Bacteria from Contaminated Soil (Using Hexadecane)

Two grams of the homogenized hydrocarbon contaminated soil samples were added to 50ml of Enrichment Medium (EM) $(NH_4)_2SO_4$ (1.5); $NaH_2PO_4.H_2O$(1.5); K_2HPO_4 (4.65); KCl (0.1); $MgSO_4.7H_2O$ (0.2); Yeast Extract (0.5); Peptone (0.5); Casamino Acid (0.5); Trace Elements (2.0), (pH=7.1), and varying hydrocarbon (hexadecane) concentrations 0.1%, 1.0%, and 5.0% (v/v) were added. Cultures were grown in 250ml Erlenmeyer flasks and incubated with shaking at 150 rpm for 7d at 37℃, and 30℃ for the purpose of isolating hydrocarbon utilizing bacteria from contaminated soil samples, as well as to evaluate their optimum growth temperature. Eleven bacteria were isolated and purified using two types of solid growth media. Solid mineral salt media was used and n-hexadecane was added on a disc of sterilized filter paper and placed in the lid on the plate as the carbon and energy source. Solid Bushnell Hass medium was also inoculated with 1ml of the same culture with a layer of filter paper supplemented, with either 1ml of n-hexadecane or diesel oil, onto the agar in order to develop the embedded colonies [17] following a similar method to that of Supaphol et al. [47]. Isolated bacterial species were evaluated using different temperature levels and hydrocarbon concentration to determine their ability to grow on hydrocarbons (using hexadecane as a sole carbon source) and their optimum growth temperatures. In addition, bacterial isolates were grown on phenol red dextrose (PRD) medium which is designed for general aerobic and facultative anaerobic bacteria for seven days at 30℃, and then isolates were gram stained. After this five bacterial species showing the best growth on hexadecane were selected, and their ability to grow on crude or diesel oil as a sole carbon source was determined (see below).

2.2.1. Ability of Pure Cultures of Hydrocarbon Degrading Bacteria to Grow on Crude Oil or Diesel Oil as Sole Carbon and Energy Sources

Five pure cultures of hydrocarbon degrading bacteria (isolated as above – section 2.2) were grown in enrichment medium containing 0.1% (v/v) of n-hexadecane. Cultures were grown in 250ml Erlenmeyer flasks and incubated with shaking at 150rpm for 7d at 30℃. Then each of the cultures grown were transferred to two sets of mineral medium containing (g/L) $NaNO_3$ (4); Na_2HPO_4(0.5); KH_2PO_4 (1.5); KCl (0.1); $MgSO_4.7H2O$ (0.2); $CaCl_2$ (0.01); $FeSO_4.7H_2O$, (0.0011); and yeast extract (0.1).The medium was supplemented with 1mL of the trace elements solution (Gerhardt, 1981; Plaza, 2008) (mg/L): $ZnSO_4.7H_2O$ (50); $MnCl_2.4H_2O$ (400); $CoCl_2.6H_2O$ (1); $CuSO_4.5H_2O$ (0.4); H_3BO_2 (2); and $Na_2MoO_4.2H_2O$ (500). One set contained 1.0% (v/v) of diesel oil and the other set contained 1.0% (v/v) of crude oil. All cultures were incubated at 30℃ for 15 days. The five bacterial isolates with the ability to grow on target hydrocarbons were assessed further using growth rate,

emulsification index, and biosurfactant production as assessment parameters. The purpose of this was to reduce the number of isolates studied to a manageable level i.e. select the bacteria that grew best on diesel oil and crude oil as a sole carbon source.

2.2.2. Growth Condition Optimization

Growth of the five isolates was monitored by measuring the optical density (OD) at a wavelength of using a spectrophotometer (Jenway Series 6105 UV–Vis) at 540nm. Doubling time (Td) and the maximum specific growth rate (μ_{max}) of the five isolates as a pure culture were calculated.

2.2.3. Emulsification Activity Measurement

The hydrocarbon degraders isolated were grown on emulsified crude oil and n-hexadecane. The emulsification activity (E24) was assessed using the Kerosene test as in [11]. Test oil substrates (2ml) of kerosene and diesel were used and added to an equal volume of the overnight culture broth in a test tube, and then mixed for 2 minutes (vortexed). The emulsion stability was determined after 24 hours by dividing the measured height of the emulsion layer by the mixture's total height, multiplied by 100. The equation used to determine the emulsion index E24 (%) is as follows: E24 (%) = the height of the emulsion layer / the height of the total solution ×100% (e.g. [4,20]). The bacteria with the best abilities to produce biosurfactant and grow on oils were then selected and studied further for their ability to degrade hydrocarbons.

2.2.4. Assessment of Hydrocarbon Transformation Ability

In this section, two pure isolates (LPI HKB and LPI HKW) with maximum specific growth rates and the best emulsifying activity on crude oil as a carbon source were selected for further investigation so as to determine their ability to transform HCs. Their growth rates under different conditions (hydrocarbon concentrations 0.1% and 1.0% (v/v), as well as temperatures 37℃ and 30℃ were assessed. The inocula of two species were prepared by growth on Nutrient Broth (OXOID) at 30℃ for 18 hours in an orbital shaker at 150rpm. The culture broth was centrifuged (8.000rpm for 10 min), and the supernatant medium discharged. The cell pellets obtained were rinsed in sterile saline. The cell mass was collected and suspended in 20ml of mineral salt medium (used as inoculum). The suspensions containing 10^4–10^5 cells mL^{-1} of pure cultures were used as inocula in a large chemostat culture vessel for the biodegradation experiment (see below – section 2.3).

2.3. Biodegradation of Crude Oil and Diesel Oil Experiments

To assess the ability of the isolated strains LPI HKB and LPI HKW to grow in crude oil and diesel oil as sole carbon sources and degrade long-chain crude oil and petroleum hydrocarbons, cells of 10-ml from two overnight pure cultures (10^4–10^5 cells/ml) were used to inoculate 1800 ml of mineral salt medium (MSM, supplemented with 1.0% (v/v) of commercial diesel fuel and crude oil in a large chemostat culture vessel. The system was set up by calibrating the pH between 7.0 - 7.3. The temperature was set at 30℃ and the stirring rate was set at an upper limit of 250rpm. Bacterial growth was estimated by two parameters: colony forming units (CFU/ml –see section 2.3.1) and optical density of the cultures at 540nm using a Jenway Series 6105 UV–Visspectrophotometer. Residual TPH values were assessed after 21 days of incubation on diesel oil, and crude oil by gas chromatography (section 2.3.3). Sterile controls were also set up for comparison purposes.

2.3.1. Microbiological Studies: Determination of Microbial Population by Plate-Count Method

The estimation of total bacterial number in liquid culture samples were evaluated by spread plate methods from chemostat culture samples taken on days 0, 7 and 21. Culture (1mL) was suspended in 9ml of ¼ strength ringers solution and shaken for 10 min (with a vortex mixer) to achieve a homogenized suspension. Following suspension, the supernatant containing bacterial cells were serially diluted with Ringers solution. A series of dilutions was then carried out, up to 10^{-7} at the start of the experiment. A sample (0.1ml) of the appropriate dilution was then inoculated onto sterile, 20-ml Petri dishes containing Nutrient Agar (DIFCO). Colonies on the plates were counted after incubation for 7days at 30°C. The number of colony forming units per milliliter culture (CFU/mL) was calculated.

2.3.2. Crude Oil and Diesel Oil Extraction

The crude oil was extracted from MSM after 21d of incubation using liquid–liquid extraction techniques by a mixture of n-hexane and dichloromethane (1:1). After extraction the solvent was evaporated. Crude oil extracts were subject to distillation to obtain the boiling distribution of the petroleum components. Diesel oil was extracted using the same organic solvent system n-hexane and dichloromethane. The total remaining content of the culture vessel (1500 mL, as some of the volume was taking for chemical and microbiological analyses) was transferred to a liquid-liquid extractor and 250 mL of solvent mixture added. The extracted solution was then dried through solvent washed anhydrous sodium sulfate using Whatman filter paper number 50. Extracted crude oil and diesel oil samples were analysed using gas chromatography.

2.3.3. Gas Chromatograph Analysis

Analyses of n-alkanes and total petroleum hydrocarbons were performed at the beginning and end (21 days) of the chemostat incubation using a Chrompack Model 439 capillary gas chromatograph flame ionization detector (FID).The gas chromatographic analyses were conducted with a 300℃ detector, 300℃ injector, split ratio on 100:1 and samples of 0.1µl injections. A column temperature of 40℃ was held for 2 min and then ramped at a rate of 5℃/min to a final temperature of 300℃ and held for 30 min. Degradation in the liquid chemostat culture was estimated as the difference between the initial and final concentrations of total hydrocarbons [38].

2.4. Identification of Isolates by 16S rRNA Analysis

DNA was extracted from the bacterial colonies according to the protocol described by Sigma's GenElute Bacterial Genomic Kit. Cell harvest; 1.5 mL of an overnight bacterial broth (NB) culture was centrifuged for 2 minutes at 12,000-16,000×g, and the supernatant discarded. The cell pellet was re-suspended thoroughly in 200µl of Lysozyme solution prepared previously and incubated for 30 minutes at 37℃. Twenty µl of the proteinase K solution was added to the sample. This was followed by 200µl of Lysis Solution C, and the solution mixed thoroughly by vortexing at 15 seconds. The sample was then incubated at 55℃ for 10 minutes.

Five hundred µl of Column Preparation Solution were added to each pre-assembled GenEluteMinprep binding column seated in a 2mL collection tube. Centrifugation at 12,000 ×g for 1 minute was carried out and then the eluate was discarded. Two hundred µl of the ethanol (95 - 100%) was added to the lysate and mixed thoroughly by vortexing to 5-10 seconds to obtain a homogeneous mixture. The contents of the tube were transferred to the binding column, and then centrifuged at 6500 ×g for 1 minute. The collection tube containing the eluate was discarded and then the column was placed in a new 2mL collection tube. In the first wash, 500µl of wash solution was added to the column and centrifuged for 1 minute at 6500 ×g, discarding the collection tube containing the eluate and placing the column in a new 2mL collection tube. Another 500µl of wash solution was added to the column and centrifuged for 3 minutes at maximum speed (12,000-16,000 ×g) to dry the column. The column had to be free of ethanol before eluting DNA, so the column was centrifuged at maximum speed for an extra 1 minute. The collection tube containing the eluate was discarded and then the column was placed in a new 2mL collection tube. Two hundred µl of elution solution was pipetted onto the center of the column and centrifuged at 6500 ×g for 1 minute to elute the DNA.

2.4.1. Checking DNA Integrity by Electrophoresis

Four µl of DNA extract with 2 µl loading dye was combined and then loaded onto 1% (w/v) agarose gel with ethidium bromide, 0.5 x TBE loading buffer (0.5 µl / ml). Two µl 100bp ladder markers were loaded and the gel was run at 100V for 30 minutes. Extracted DNA was diluted with PCR grade water to 1:10, 1:25, and 1:50 for PCR reaction.

2.4.2. PCR Amplification of 16S rRNA Gene

PCR Conditions and Optimization: Genomic DNA from hydrocarbon degraders were amplified by polymerase chain reaction (PCR) using the specific bacterial primers 27f and 1525r (see Table 1). These are universal primers that bind at the conserved 5` and 3`ends of 16S rRNA of the eubacteria. PCR amplification was performed using 50µl of PCR reaction mixture containing 5.0µl of 10 X NH_4.Reaction buffer was added to 0.8 µl dNTP (Bioline), mix (12.5 mM) each 1.0 µl Forward primer 27 f (20 µM), 1.0 µl Reverse primer 1525 r (20 µM), (Table 1) 3.0 µl $MgCl_2$, 38.0 µl Sterile Milli – Q water, 0.5 µl Taq polymerase (Bioline). Forty-nine µl of PCR reaction mixture solution was transferred to each of the seven microfuge tubes. The first tube contained 49µl of solution with 1µl of distilled water, which formed the negative (-ve) control; the second tube contained 49µl with 1µl of genomic DNA (E. coli), which formed the positive (+ve) control; and five tubes contained 49µl + 1µl of template DNA. The preparation was mixed and centrifuged for 5 seconds. All reagents used were from Bioline, London, UK. PCR was performed with a Biometra Thermal Cycler. The programme consisted of an initial step of 5 minutes at 95℃, followed by 30 cycles, including a denaturing step at 95℃ for 30 seconds, an annealing step at 55℃ for 30 seconds, and an elongation step at 72℃ for 1 minute. The last cycle was followed by a final extension at 72℃ for 10 minutes. In order check the sizes of the PCR products, 4µl of the PCR reactions were analyzed by 1.0% agarose gel electrophoresis in TAE (Tris acetic acid EDTA buffer, 1% w/v; 30 minutes at 100 V, 0.5 × TBE), stained with ethidium bromide, and visualized under UV light using image analysis software (Bio-Rad Gel Doc, Flour-S multi imager) as in [49]. The size of the amplified fragments was estimated by comparing with a 100bp molecular size marker.

2.4.3. Sequencing of PCR Products

The PCR products were cleaned up using ExoSAP-IT PCR clean-up kit, the protocol consists of a single pipetting step (enzyme mixture addition), a 15-min incubation at 37℃ followed by a further 15-min incubation at 80℃ for enzyme inactivation, then the PCR for products were sequenced by Geneius Laboratories Ltd (www.geneiuslabs.co.uk)) using Sanger sequencing based on a big dye terminator using an ABI 3730xl DNA sequencer. Sequences were viewed using MEGA software version 4.0. A contiguous sequence using forward and reverse sequences was made and the contiguous sequences were submitted to BLAST (https://www.ncbi.nlm.nih.gov/blast) for comparison with known data bases.

2.4.4. Statistical Analysis

Analysis of variance was used to compare data between soils in all treatments. All statistical analyses were performed using SPSS version 14 STATISTICA for Windows release 5.1, in order determine whether or not the plate counts and biodegradation process resulted in statistically significant changes in PH transformation and microbiological parameters at a confidence level of 95% or P> 0.05. Results were analysed by one way ANOVA followed by the Least Significant Difference (post hoc) test to determine individual differences between samples.

3. Results

3.1. Soil Chemical and Physical Analysis – the Zawia Refinery

The characteristics of the combined contaminated soil sample taken are presented in Table 2. A range of physical and chemical analyses carried out showed that soil texture was sandy and nutrient levels were low. Soil pH was neutral to slightly alkaline. Total petroleum hydrocarbon (TPH) content was 26532 mg per kg, 0.0694 ppm nitrogen,

9.6 ppm phosphorus, and 3.17% moisture, grain size distribution (sand 76.8%, silt 15.3% clay 7.9%).

3.2. Isolation and Study of Hydrocarbon-Degrading Bacteria Overview

Firstly soil samples were added to enrichment medium (section 2.2) containing hexadecane as a sole C source and bacteria growing were plated out onto solid agar media containing either n-hexadecane or diesel as sole carbon sources. In order to reduce the number of isolates to a manageable level they were cultured at different temperatures and substrate (hexadecane) concentrations and the bacteria that grew best (5 cultures at this stage) selected for identification (by morphological and molecular methods) and further assessment i.e., ability to grow on crude or diesel oil as a carbon source and emulsification ability. The two best performing bacteria were then selected and assessed for their ability to grow on and transform (or degrade) crude oil and diesel. The main aim of this work was to isolate bacteria capable of transforming crude oil and diesel oil, and assess their suitability as potential microbial soil inoculants for bioremediation. To make the results more accessible only data relating to the selected isolates is presented below.

3.2.1. Evaluation of Bacterial Isolates Capable of Growing on Hexadecane

Altogether eleven bacterial isolates were isolated from the enrichment procedure (section 2.2.1). Selection of the most promising isolates was achieved by assessing their growth (spectrophotometry) on hexadecane in liquid culture (results not shown for purposes of clarity). The 5 bacteria showing the best growth (and labeled as LPI HKB, HKS, HKY, HKW and HKP) were identified. The isolates were gram-stained and examined under a microscope: see below for morphological descriptions and images (Figure 1). Isolate LPI HKW is gram-positive rod shaped bacterium and forms endospores. Cells appear as individuals and in chains; Isolate LPI HKY is a rod-shaped gram-positive strain. The cells were filamentous similar in morphology to *Actinomyces species* and formed spores external to the cell, Isolate LPI HKS is a long rod shaped gram positive bacteria and appeared in aggregates, Isolate LPI HKB is a gram-positive rod shaped cell, Isolate LPI HKP forms large colonies that have regular edges and are cells are gram-negative, All bacterial strains were aerobes; except for LPI HKS, which was a facultative anaerobe. *Microscopy and photomicroscopy:* DIAPHOT 300/DIAPHOT200 epifluorescence microscope was used for observation the size and morphology of the bacterial colonies. A Lucia G/F system with an image resolution of 97 ×716 pixels was attached to the microscope for photomicroscopy with 100× magnification objective; one pixel typically corresponds to 0.05-0.1 um. The 5 bacterial isolates were then identified using molecular techniques.

3.2.2. Molecular-Based Identification of Bacterial Isolates

The 5 bacterial isolates were characterized to genus level based on the complete sequence of the 16S ribosomal RNA gene. The 16S rRNA products were purified and sequenced (Section 2.4.3), the sequences of 16S rRNA were submitted to BLAST under accession numbers FM211694.1, EF599311.1, AF282889.1, FM211694.1, and AF526907 respectively and the bacteria identified as in Table 3 (below). It was not possible to obtain full identification to species level of all the bacterial isolates.

3.3. Effect of Temperature and Crude Oil Concentrations on Growth of the 5 Bacterial Isolates

Pure cultures of the 5 selected bacteria were supplemented with different concentrations of crude oil - 0.1, 1.0, and 5.0% (v/v) and incubated for 21 days. Growth of the isolated bacterial strains on different concentrations of crude oil was evaluated by measuring culture absorbance at 540nm using a spectrophotometer. Two sets of incubation temperature, 30℃ and 37℃, were used and all isolates showed better growth at 30℃ and 1% hydrocarbon concentration in the batch culture test system (see Table 4). Results obtained at 37^{o}C are not shown for clarity). The optimum growth rates of the different isolates on 1.0% (v/v) of crude oil were calculated and showed that the *Bacillus* species and *Pseudomonas putida* grew quicker than other isolates on hydrocarbons of 1.0% (v/v) at 30℃. The maximum specific growth rates (μmax) for the *Pseudomonas species* were 0.301 h^{-1} (*P. putida*) and 0.436 h^{-1}, for *Bacillus species and* 0.358 h^{-11} for the *Nocardiacyria cigeorgici* (Table 4). Overall, *Pseudomonas putida and the Bacillus* species gave the highest growth rate of the 5 isolates.

Table 1. Target Sites, Sequences and Specificity of Primers Targeting Small Subunit Ribosomal RNA Used for PCR Analysis [28]

Primer	Target Site	Sequence (5´to3´)	Specificity
f27	137-156	AGAGTTTGATCCTGGCTCAG	Bacteria
r1525	1640-1658	AAGGAGGTGATCCAGCC	Bacteria

Table 2. General Physical and Chemical Properties of Contaminated Soil Samples Taken From the Oil Refinery Site. 12 Soil Samples Were Taken and Mixed to Homogeneity. The Bellow Results Reflect Values Obtained From the Homogenized Sample

Contaminated Soil Property	
Sampling location	The Oil Refinery
Texture	Sand
pH	7.64
Water holding capacity (%)	3.17
Moisture (%) @105℃	3.51
AvailableC(ppm)	6.79%
Available N (ppm)	
NH_4-N	0.0694ppm
Available P (ppm)	9.6 ppm
Available K (ppm)	32.4 ppm
Total Petroleum Hydrocarbon mg/kg	26532 mg/kg
CFU	3.7×10^{7} CFU g^{-1}

Table 3. Identification of Selected Hydrocarbon-Degrading Bacterial Isolates by 16S rDNA Gene Sequence

Isolates	16S rDNA sequence comparison		
	Species as close relatives	Accession no.	Similarity (%)
LPI HKB	*Pseudomonas putida*	FM211694.1	100.0
LPI HKS	*Beta proteobacterium*	EF599311.1	97.0
LPI HKY	*Nocardiacyriacigeorgici*	AF282889.1	99.1
LPI HKP	*Pseudomona* ssp.	FM211694.1	100.0
LPI HKW	*Bacillu* ssp.	AF526907	99.1

3.3.1. Emulsification Activity Measurement

The E24 (emulsification index) was measured at different stages of growth of the 5 bacterial isolates. Both of the *Pseudomonas species* and the *Bacillus species* showed a maximum E24 value after 48 hours of incubation (E24= 86% for *P. putida*, 61% for *Pseudomonas* species and 66% for the *Bacillus* isolate), Table 5 shows the emulsification activity (E24) of all five isolates on kerosene. The emulsifying activity of all isolated species was highest during the stationary phase. Given that *Pseudomonas putida* and the *Bacillus* species performed better than all the other isolates (based on growth on oil and emulsification ability) these 2 isolates were used in all subsequent studies.

3.3.2. Crude Oil Removal from Liquid Culture by *Pseudomonas putida* and *Bacillus species*

The residual oil content was determined using soxhlet extraction of the culture by the procedure described in section 2.3.2. Reductions in TPH concentrations during growth on crude oil by both isolates are presented in Table 6. During the log phase, crude oil-containing medium became emulsified. This indicates production of emulsifying agent by *Pseudomonas putida* and *Bacillus species*. Microbial growth resulted in a significant reduction ($p<0.05$) in light hydrocarbon fractions (C9-C14). Degradation of intermediate (C15- C20) crude oil was above 70% after incubation of 21 days for *Bacillus species*, while it was 49% with *Pseudomonas putida*.

3.3.3. Transformation of Diesel Oil by *P. putida* and *Bacillus species*

Changes of TPH concentration during diesel oil degradation by both isolates (*Pseudomonas putida* and *Bacillus species*) are presented in Figure 2. Gas chromatographic analysis of diesel oil 1.0% (v/v) treated with *Pseudomonas putida* and *Bacillus species* indicated that the TPH removal of diesel oil (1.0% w/v) by *Pseudomonas putida* was higher than the biodegradation obtained by *Bacillus species*. The control samples, which had no bacteria added, showed losses of the total petroleum hydrocarbon to a certain extent but these losses were mainly in the shorter fraction and were significantly ($p<0.05$) less than observed in the bacterial treated cultures. During the experiment it was also observed that the transformation of different molecular weight compounds varied between the two bacteria. The addition of *Pseudomonas putida* species enhanced degradation of middle and long chain compounds compared with the control that was not supplemented with any microbial species, whereas *Bacillus species* resulted in significantly ($p<0.05$) greater degradation of middle-chain compounds than the control. Overall *Pseudomonas putida* was more effective in transforming the diesel oil than crude oil, while *Bacillus species* was more promising to grow and transform the crude oil. However transformation of the hydrocarbon compounds was higher with diesel oil compared to crude oil.

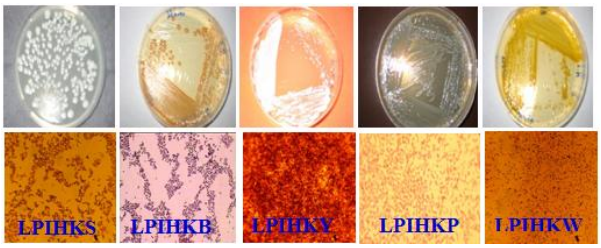

Figure 1. Colony Morphology and Gram Stain of The 5 Bacterial Isolates (labelled as LPI HKB, HKS, HKY, HKW and HKP) x 100 Magnification Was Used

Table 4. Optimum Growth rate of 5 Isolated Bacterial Strains on 0.1%, 1.0% and 5.0% Concentrations of Crude Oil at 30°C

PH Conc. by vol	Optimum Growth Rate μ hr^{-1} of isolates				
	Bacillus species	*P. species*	*Nocardiacyri-acigeorgici*	*Beta proteobacterium*	*P. putida*
0.1	0.134	0.0587	0.066	0.0445	0.078
1.0	0.358	0.301	0.171	0.024	0.436
5.0	0.158	0.176	0.067	0.044	0.136

Table 5. Emulsification Activity on Kerosene of Five Hydrocarbon Degraders Isolates

Strains	E_{24}%
Pseudomonas putida	86
Beta proteobacterium	32
Nocardiacyriacigeorgici	53
Pseudomonas species	61
Bacillus species	66

Table 6. Illustrates the removal of short/long chain alkanes (from C9 to C14, and C15- C20) during crude oil degradation

Fraction	TPH Removal from crude oil	
	Bacillus species	*Pseudomonas putida*
Light (C9 - C14)	73%	63%
Intermediate (C15- C20)	73%	49%
Heavy (C21 - C26)	42%	37%

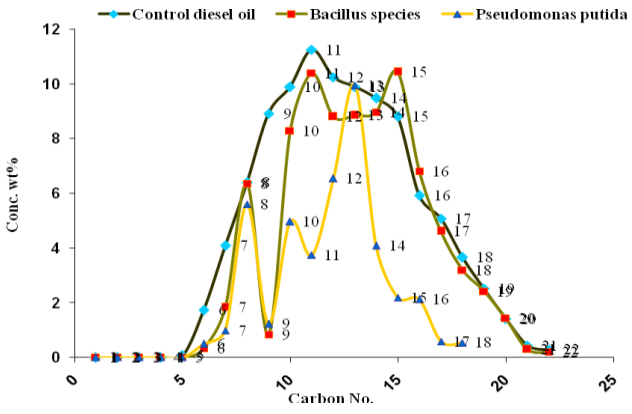

Figure 2. Transformation of Different Molecular Weight Compounds in Diesel Oil by *P. putida* and *Bacillus species*

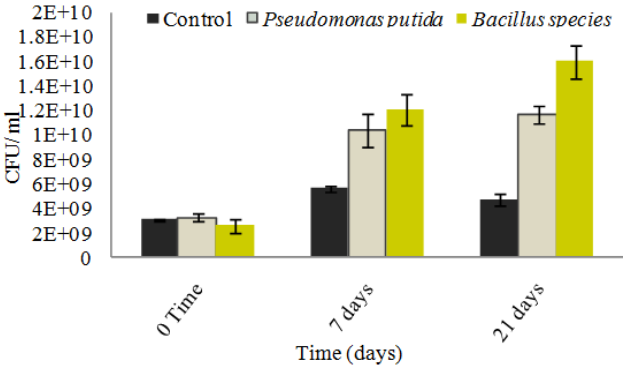

Figure 3. Changes in Hydrocarbon Degraders Counts (CFU/ml) on Crude Oil at the Initial and Final Time of Treatment

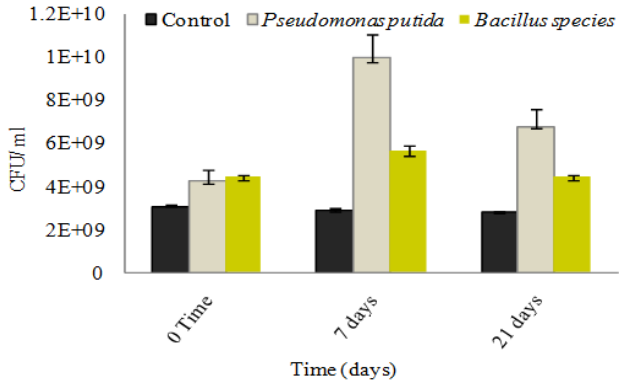

Figure 4. Changes in Hydrocarbon Degraders Counts (CFU /ml) on Diesel Oil at the Initial and Final Time of Treatment

3.3.4. Change in Microbial Populations during Biodegradation of Oil (Diesel and Crude Oil)

Results obtained from incubated cultures on different hydrocarbon sources are reported in Figure 3 and Figure 4. Growth with diesel oil started from day 3, while growth with crude oil started from day 7. The incubated culture on diesel oil showed that bacterial counts increased during the experiment. Also, the addition of diesel oil was favorable for *Pseudomonas species*, whose counts reached a maximum of 1.0×10^{10} CFU (ml) by the middle of the biodegradation experiment. *Bacillus species* counts were generally higher in culture with crude oil than diesel oil. The initial count of *Bacillus species* in the incubated culture was found to be 2.5×10^{9} colony-forming units CFU (ml). The number thus increased about 10-fold after 7 days of preparation of the culture (Figure 3).

4. Discussion

4.1. Soil Properties

It was very important to analyze the suitability of the site for a bioremediation operation, and determine the levels of contaminants, nutrients, soil type, and the presence of biodegrading microorganisms. Soil samples used in this study were sandy soils, which are low in nutrient levels, and this could limit the metabolism of the existing microorganisms capable of degrading hydrocarbons in the soil. For example, soils considered being high in N and P contain 9.80 mg P and 300.00 mg total N respectively per kg as in [14], while the soils in this study only contained 0.0694 mg N and 9.6 mg P per kg. The importance of nutrients to microbial processes has long been known. Nitrogen (N) is required in amino acids, and phosphorus (P) is involved in energy transport as adenosine triphosphate. Both N and P are significant factors in supporting microbial growth and frequently added to soils to enhance bioremediation [12], [30] and given the low N and P status of the soils in this study (coupled with the very high carbon content due to pollution) this means that these nutrients will be required to promote bioremediation of the soils. The level of hydrocarbons found in the soil samples was very high (approx 26 000 mg TPH /kg soil).The levels of hydrocarbons found in other contaminated site studies are similar and such levels have been subject to bioremediation. Hence, the soil in this study is expected to be amenable to bioremediation not withstanding the nutrient limitations.

4.1.1. Bioremediation of Sandy Soils

Bioremediation of sandy soils has received rather less attention than other soil types and there are a number of issues (in addition to nutrient limitation) that may affect microbial transformation of hydrocarbon pollutants. Sandy soils are known to reduce the transportation of phosphate and make it unavailable for biological activity [21]. Ghazali et al. [13] discussed how limited microbial growth and subsequent pollutant transformation in a fine sand was due to a lack of oxygen. The soil became waterlogged as a result of the addition of liquid. One possible reason is that the pores between sand particles are small, thus they allow less of a path for water and gas flow. Another study by Rowland et al. [41] investigated bioremediation of oily beach sand dunes on the British coast, demonstrating that when the sand became saturated with water the breakdown process appeared to stop. Thus during any bioremediation process of sandy soil it is important to ensure that the sand does not become saturated and any work should be carried out below the water holding capacity of the soil.

4.2. Bacterial Isolation and Identification

One particular issue with bioremediation of sandy soils may be an insufficient number of microbes and presence of microbes capable of transforming pollutants. This study was able to easily isolate bacteria from contaminated sandy soil capable of growing on hydrocarbons as a sole source of carbon and energy and five isolates in particular, were found to grow on a variety of hydrocarbon types including hexadecane, crude oil and diesel oil. They were identified by 16S rRNA sequences as *Pseudomonas putida*, *Pseudomonas species*, *Beta proteobacterium*, *Bacillus species*, and *Nocardiacyriacigeorgici*. Use of 16S rRNA is currently considered to be the 'gold standard' for bacterial identification but despite this the technique does have limitations in delineating species due to similarity in 16S rRNA sequence in some genera and lack of sequence information in available searchable databases. For example, different *Bacillus species* are known to have similar 16S rRNA sequences and obtaining a species name can be problematic in this genus. For example, Plaza et al. [36] discussed that 16S rRNA gene sequencing could not

clearly assign *Bacillus sp*. (T-1), *Bacillus sp*. (T′-1) isolated from petroleum-contaminated soils to any species in the genus *Bacillus* as both isolates showed 99% similarity to two distinct species of the genus (*B. subtilis* and *B. licheniformis* for T-1 and *B. subtilis* and *B. amyloliquefaciens* for T′-1). *Pseudomonas species* and *Bacillus species* are two of the most widely studied industrial microorganisms. Isolates similar to those we obtained were used by other authors to degrade hydrocarbons. Widada et al. [51] reported that the majority of hydrocarbon degrading bacteria belong to the *Pseudomonas* genus and Bento et al. [6] demonstrated that *Bacillus species* are one of the most widely studied hydrocarbon degrading bacteria. Plaza et al. [36] also discussed the ability of hydrocarbon degrader bacteria - such as *Pesudomonas sp., Actinobacter sp., Bacillus sp., Rhodococcus sp.*, and *Arthobacter sp*. in terms of biodegradation and biosurfactant production. In a Kuwaiti desert soil, researchers found that the predominant indigenous oil-utilizing bacteria belong to *Micrococcus*, *Pseudomonas*, *Bacillus*, *Arthrobacter*, and the group of *nocardioforms*, particularly the genus *Rhodococcus* [37]. This previous work shows that the isolates found in this study belong to known hydrocarbon transforming bacterial genera. The five isolates were then examined further for their ability to emulsify hydrocarbons and transform different oil types.

4.3. Emulsification Activity of Bacterial Isolates

Biosurfactants reduce surface and interfacial tensions in both aqueous solution and hydrocarbon mixtures, making them potential agents for bioremediation as in [4]. In this study, emulsification activity was used as an indicator to measure the production of biosurfactant as this method has been used in several previous studies [11,48]. Measuring the emulsifying activity of the culture broth during different stages of growth showed that activity was highest at the end of the exponential growth phase and stationary phase. Nayak et al. [29] found that in the initial 48 h the production of rhamnolipid was low, but that after 120 h levels increased and reached 280 mg 1^{-1}, clearly showing that to the production of biosurfactant is growth associated. Both *Pseudomonas species* studied here were shown to have high emulsification activity (up to 86% and 61%), *Bacillus species* had 66% activity, while *Nocardiacyria cigeorgici* showed 53% emulsification activity. The unidentified *Beta proteobacterium* isolate had only 32% activity demonstrating a wide variety of potential biosurfactant production between the isolates. Banat et al. [5] revealed that *B. subtilis* and *B. pumilus* are the only two *Bacillus species* that have been identified and reported as biosurfactant producer. In our study, the *Bacillus species* increased emulsification to 66% and therefore likely produced biosurfactant. Plaza et al. [36] stated that many microorganisms which biodegrade crude and refined oils produce surface active compounds (biosurfactants) and so the production of biosurfactants by the isolates is to be expected. Estimation of the emulsification index (E_{24}) has been considered one of the essential approaches for screening potential biosurfactant-producing microorganisms. Most published E_{24} values in the literature have been reported to be around 65% [7]. However, we obtained 86% E_{24} values with *Pseudomonas putida* species, thus another promising microbial candidate for biosurfactant production has been identified in this research. High levels of biosurfactant production have also been found in previous work e.g. a *Bacillus subtilis* strain displayed E24 values close to 90% [5]. Overall, the results demonstrate that the bacteria isolated in this study have a high emulsification activity (despite the fact that emulsification activity was not optimised in this study) and these bacteria could be potentially used as inoculants to enhance bioremediation of oil contamination. All five isolates were used for further study to examine their ability to grow on various types and levels of pollutants.

4.4. Effect of Carbon Source (Pollutant) Concentration and Temperature on Bacterial Growth

Many studies have discussed how *Pseudomonas* and *Bacillus species* can tolerate high concentrations of hydrocarbons and have a high degradation capability. It has been suggested that *Bacillus species* are more tolerant to high levels of hydrocarbons in soil as *Bacillus species* forms endospores which are stress resistant [13,44]. Numerous genera of hydrocarbon degraders bacteria tolerate high concentrations of the hydrocarbons and have a high degradation capability. Most of them belong to *Pseudomonas, Sphingomonas, Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Brevibacterium, Xanthomonas, Mycobacterium, Rhodococcus* and *Bacillus [36]*.

The Pseudomonas putida used in this work had the highest growth rates of all isolates with the 1.0 % (vol) concentration of crude oil used.

All the isolated petroleum hydrocarbon degraders showed favorable growth on hydrocarbons at a temperature of 30℃. This is supported by previous work showing that the optimum temperature for enhancing diesel degradation was 30℃ [43]. Bossert el al. [8] suggest that the volatility and toxicity of oil increases at higher temperatures, therefore the slower growth of isolates observed in this work could be explained by an increase in temperature related toxicity. In addition, diesel oil was found to be toxic to microorganisms due to the solvent effect of diesel which destroy bacterial cell membranes, the growth of DRY11bacterial strain started to decline when the concentration of diesel was above 4% (v/v) [43]. The two isolates (*P. putida* and *Bacillus species*) that showed the highest growth rates on the hydrocarbon sources were then examined for their ability to transform hydrocarbons (diesel and crude oil) using gas chromatography analysis.

4.5. Effect of Pseudomonas and Bacillus Species on Diesel and Crude Oil Transformation

The total residual total petroleum hydrocarbon analysis of diesel oil showed that *P. putida* had higher transformation abilities than the *Bacillus* isolate under the experimental conditions used. The more detailed gas chromatographic analysis of diesel oil treated with *P.*

putida species, in particular, showed a decrease in heavy hydrocarbon fractions as opposed to the *Bacillus* sp. which did not transform the higher molecular weight compounds. Most of the light parts of the intermediate weight fractions initially present in the extracted oil were degraded by both species.

The results indicate a clear difference in biodegradation potential between the 2 isolates and the ability of the *P. putida* species to remove the higher molecular oil components is of interest as these components are typically harder to transform and persist in soil bioremediation. For example, Gogoi et al. [14] demonstrated that contaminated soils are more dominated by alkane degrading microorganisms than aromatic and polyaromatic hydrocarbon degraders. The higher molecular weight hydrocarbons are more resistant to microbial degradation, and may accumulate in soil.

The levels of oil removal in this study are similar to others reported previously. For example, other studies reached high transformation, whether by increasing the temperature or expanding the incubation time. Ghazalai et al. [13] studied two strains of *B. stearothermophilus* and achieved 80–89% degradation of crude oil (5 g/l) within 5 days at 60℃. In other work, hydrocarbon-degrading bacteria were identified as *Pseudomonas aeruginosa AP02*. This strain showed ability to utilize a range of hydrocarbons on C5–C19 n-alkanes and polycyclic aromatic hydrocarbons as sole carbon source. The isolate degraded 99% of crude oil 1% (v/v) and diesel oil 2% (v/v) when added to a basal mineral medium and reached optimum growth within 7 days of incubation [34].

4.6. Effect of Diesel Oil and Crude Oil on Growth of *Pseudomonas putida* and Bacillus

In the current experiment, *Pseudomonas putida* was found to grow better on diesel oil compared to crude oil, and counts increased from 3.2×10^{9} CFU/ (ml) to a maximum count of 1.0×10^{10} CFU / (ml) over the 21d incubation. Similar increases in numbers have been observed previously [50]. In the current experiment counts of *Bacillus species* increased was high when crude oil used as carbon source.

The results from this work suggest that both species (*Pseudomonas putida* and *Bacillus species)* have the ability to remove hydrocarbons of different carbon chain lengths.

5. Summary & Conclusions

The soil samples from the refinery were found to have low nutrient levels, have a sandy texture and to be highly contaminated with petroleum hydrocarbons. Hydrocarbon utilizing microorganisms were isolated and identified from contaminated soil samples. After initial screening of eleven isolates capable of growth on hexadecane the five most promising hydrocarbon–utilizing bacteria were isolated and tested for emulsification activity. They were identified by the 16S rRNA gene as *Pseudomonas putida* species, *Pseudomonas species, Beta proteobacterium, Nocardia cyriacigeorgici*, and *Bacillus species*. Emulsifying capacity was evaluated using the E24 emulsification index. The isolated species exhibited high emulsification activity. Among the five species tested, *Pseudomonas putida* showed superior performance in terms of growth on hydrocarbons, emulsifying activity and ability to transform hydrocarbons in pure culture.

Interestingly, the gas chromatographic analysis of crude oil treated with *P. putida* showed a decrease in heavy hydrocarbon fractions demonstrating a clear potential for this microbe to be used as a soil inoculant in bioremediation. As the *Bacillus* isolate also grew well on crude oil and also appeared to transform high molecular weight HCs this organism would be a potential useful inoculant for bioremediation purposes and the two could be used in a multiple inoculation approach.

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