International Journal of Biotechnology Applications
ISSN: 0975-2943 & E-ISSN: 0975-9123, Volume 5, Issue 1, 2013, pp.-147-154.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000218

APPLICATION OF MOLECULAR METHODS AS A BIOMARKER IN BIOREMEDIATION STUDIES

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Received: October 26, 2013; Accepted: November 27, 2013

Abstract- In contaminated soils, the efficiency of natural attenuation or engineered bioremediation largely depends on the biodegradation capacities of the total microflorae. In the present study, the biodegradation capacities of various bacteria towards petroleum-hydrocarbons were determined under laboratory conditions. The purpose of the study was to isolate and characterize petroleum-degrading bacteria from contaminated soil obtained from a refinery in Arzew, Algeria. A collection of 15 bacterial isolates were obtained by enrichment cultivation from oil-contaminated soil and an indigenous microbial consortium was developed by assembling four species of bacteria which could degrade different fractions of the petroleum hydrocarbons. 16S rRNA gene analysis was used to identify members of the consortium and oil biodegradability was analyzed by Thin Layer Chromatography (TLC) with Flame Ionization Detection (FID) that performs quantitative compositional analysis of oil samples. The Iatroscan TLC/FID system measured the relative percentages of the four major fractions of petroleum i.e. saturates aromatics, resins and asphaltenes. Results indicated that the constructed consortium which comprised the genera *Pseudomonas*, *Shewanella*, *Enterobacter* and *Serratia* used the hydrocarbons as sole sources of carbon where biodegradation was defined by an initial rapid decrease in the saturate and aromatic fractions from 56.44% and 34.72% to 51.77% and 27.77% respectively, coinciding with an increase in the asphaltene fraction. The resin content remained relatively constant throughout the project.

Alkane hydroxylase genes (*alkB)* were positively amplified in the *Pseudomonas* isolate by the polymerase chain reaction (PCR) method using degenerate primers. This functional gene was used as a marker to assess the catabolic potential of the bacteria for alkane pollutant biodegradation. The selected bacterial consortium looks promising for its application in bioremediation technologies.

Keywords- Bioremediation, petroleum-hydrocarbon, microbial consortium, 16S rRNA, *Pseudomonas*, TLC/FID, *alkB* genes

Citation: Guermouche M.A., Bensalah F. and Gray N. (2013) Application of Molecular Methods as a Biomarker in Bioremediation Studies. International Journal of Biotechnology Applications, ISSN: 0975-2943 & E-ISSN: 0975-9123, Volume 5, Issue 1, pp.-147-154.

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Introduction

Algeria is a major producer of petroleum. The production and refining industry, petroleum transportation and its end-use contributes to environmental pollution and produces serious ecological problems. The contamination of soil with petroleum hydrocarbons causes a significant decline in its quality and such soils become unusable [1]. Petroleum contains thousands of individual hydrocarbons and related compounds which can be ascribed to four major fractions. These main fractions are: saturates (*n*- and branched-chain alkanes, cycloparafins rings), aromatics (mono and polynuclear aromatic compounds (PAHs), resins and asphaltenes (heterocyclics, oxygenated hydrocarbons) [2]. Elimination of such oils from the marine environment and biological treatment of industrial wastes in refineries requires the intervention of different biotic and abiotic factors, among them biodegradation by microorganisms. Hydrocarbons, including (PAHs), have been categorized as priority pollutants by the US environmental Protection Agency (USEPA) and by many other environment and health organizations around the world [3].

To maximize the process of biodegradation in the application of bioremediation technologies, two main approaches have been explored: biostimulation, in which nutrients are added to stimulate the intrinsic hydrocarbon degraders and bioaugmentation, in which, microbial strains with specific degrading abilities are added to work cooperatively with normal indigenous soil micoorganisms. Depending on the complexity of oil products, a combination of bacterial strains as a consortium will be required to achieve extensive degradation. It is generally accepted that a single microorganism is not capable of degrading all compounds in such mixtures and that mixed cultures need to not only have broad substrate specificities but also to participate in cooxidation and commensalism to achieve degradation [4]. Many scientists have reported that mixed populations with overall broad enzymatic capacities are required to de-

grade complex mixtures of hydrocarbons such as crude oil in soil, fresh water, and marine environments. Bacteria are the most active agents in petroleum degradation where they work as primary degraders of spilled oil in the environment, and several bacteria are even known to feed exclusively on hydrocarbons [5]. 16S rRNA gene sequences of oil-utilizing bacteria have revealed that they were predominantly affiliated to the *Gammaproteobacteria* and the *Actinobacteria*. For instance, Christopher & Christopher [6], demonstrated the importance of *Pseudomonas* species in the early stage of degradation in a petroleum land treatment unit. Further, Gram-negative strains comprised of *Shewanella* and *Pseudomonas* genera among others, and isolated from an oily sludge were found to be tolerant of saturated, monoaromatic, and polyaromatic hydrocarbons indicating that Gram-negative bacteria are less sensitive to toxic compounds than Gram-positive bacteria [7]. Some *Enterobacteriacaea* including *Enterobacter* sp. and *Serratia* sp. isolated from a petroleum contaminated soil were also found to be potential degraders of crude oil hydrocarbons [8]. The adoption of nucleic acid-based technologies makes it possible to assess the biodegradation potential or microbial diversity in petroleum hydrocarbon contaminated soils by detecting the catabolic genes within indigenous microorganisms. The alkane hydroxylase system in Gram-negative bacteria encodes a three-component alkane hydroxylase complex, consisting of a particular non-heme integral-membrane di-iron alkane 1-monooxygenase (AlkB) and two soluble proteins, rubredoxin (AlkG) and rubredoxin reductase (AlkT) [9]. Alkane degradation is generally initiated by alkane hydroxylases which convert alkanes to alkanols. In terms of gross bacterial activity thin layer chromatography coupled with flame ionization detection (TLC/FID) has been effectively employed to evaluate the biodegradability of total petroleum including the non-volatile fractions in crude oil [10].

The main objective of the present work was to isolate and identify bacteria capable of degrading petroleum-hydrocarbons using phylogenetic analysis based on 16S rRNA gene sequences and to optimize the degradation process with a selected bacterial consortium. In addition, strains were tested for the presence of prokaryotic alkane hydroxylase genes (*alkB*). Furthermore, the extent of oil degradation by the consortium was determined by comparing specific fractions of hydrocarbons in the residual oils through TLC/FID hydrocarbon analysis.

Materials and Methods

Sampling Site

Petroleum-hydrocarbon contaminated soil samples were collected from a refinery in Arzew, Northern Algeria (Sonatrach, Algerian Petroleum Company). In total, five samples (obtained from the top 20 cm of the soil) were used from two sites contaminated by hydrocarbons [Fig-1]. Petroleum (P) and industrial rejection waste (RI) were provided by the refinery and these were used separately as the only source of carbon in MSM broth (Mineral Salt Medium) enrichments.

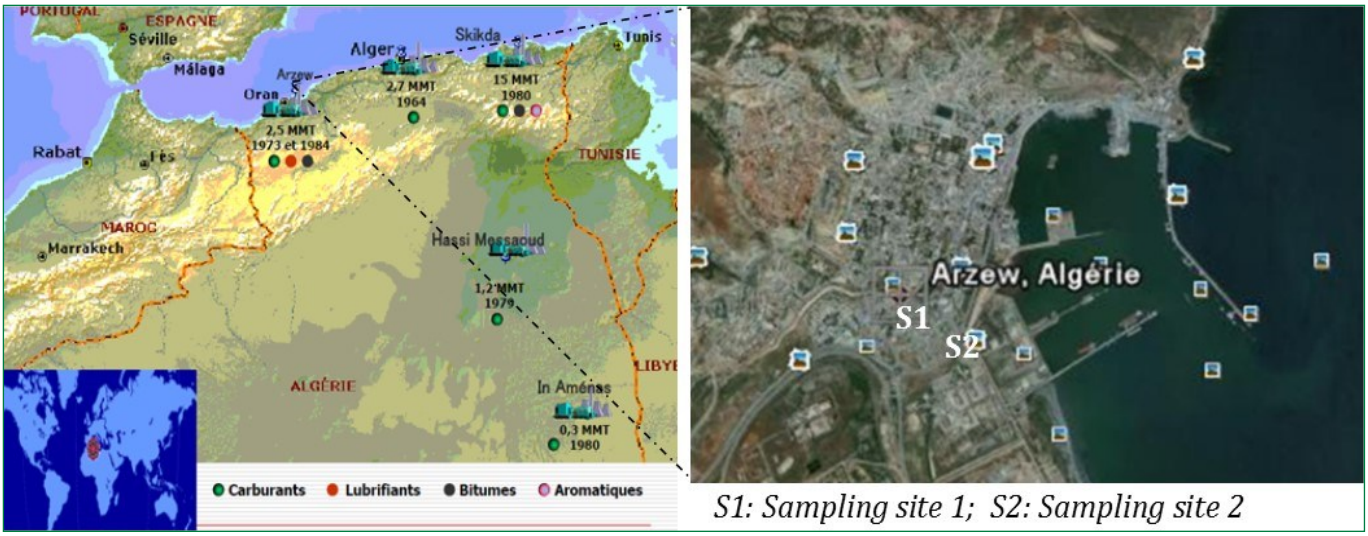

Fig. 1- Map of Algeria, showing where the contaminated soils were collected (Refinery of Arzew).

Enrichment and Isolation of Aerobic Hydrocarbon Degrading Bacteria

Bacteria able to degrade oil pollutants were isolated on Mineral Salt Medium (MSM) which contained per liter: 1.2g NH_4Cl; 1.6g K_2HPO_4; 0.4g KH_2PO_4; 0.1g NaCl, 1g KNO_3; 20g $MgSO_4.7H_2O$; 10g $CaCl_2.2H_2O$; 0.05g $FeCl_3$ at pH 7.1. The MSM also contained a trace elements solution (1ml) and vitamin solution (1ml) as described by Pfennig & Trüper [11] sterilized separately and added aseptically to the medium. For enrichment, 20g contaminated soil sample was added to 200 ml of MSM medium containing filtered oils (P or RI) at 0.025% (v/v) of each carbon source separately [12]. Cultures were carried out with intermittent shaking at room temperature (±28°C) on a rotary shaker (200 rpm) for 2 hours. Bacteria able to use oils were isolated from the MSM by a continual enrichment method whereby 20 ml of enriched culture was repeatedly re-inoculated into fresh MSM under similar incubation conditions. After four consecutive transfers (each including a short incubation interval 4 weeks), 1 ml of culture was serially diluted up to 10^8 fold and 100µl of all the dilutions were plated on Luria-Bertani (LB) agar and incubated at 30°C.

Screening and Maintenance of Isolated Strains

Ability to degrade petroleum hydrocarbons was confirmed by inoculating LB broth-grown pure cultures (18h) into a fresh MSM flask containing oil pollutants as sole carbon sources. Colonies were then transferred individually into MSM broth containing (P) or (RI) at 1% (v/v) and incubated at 30°C on a rotary shaker (200 rpm) for 2 weeks. Colonies which grew on LB agar using a significant low

percentage of filtered carbon sources (0.005%) were harvested with a sterile inoculation loop, pooled and transferred to the medium. Isolates were then maintained in glycerol nutrient broth (1:1, v/v). The mixture was vortexed and kept at -20°C [13].

Determination of Bacterial Biodegradative Activity by Turbidometry

Turbidometry was used to determine bacterial growth on the hydrocarbons P or RI provided as carbon sources in MSM broth enrichments. The degrading activity of each isolates was obtained from Mineral salt broth (MSB) experiments amended with 1% (w/v) of each hydrocarbon (P and RI) incubated at room temperature for 15 days. The growth of the bacterium was measured after inoculation by taking the Optical Density (O.D) readings at 600nm for 15 days at regular intervals of 2 days and compared with sterile mineral salt medium experiment as a blank.

Genomic DNA Extraction of Selected Bacterial Strains

The genomic DNA of selected bacteria was extracted using the modified method in [14]. Cells from an isolation plate were harvested and transferred into 1 ml of sterile distilled water, centrifuged and frozen for at least 1h at -20°C. The thawed pellet was washed in 1 ml TES buffer (6.7% (w/v) sucrose, 50 mM Trsi-Hcl (pH 8.0), 1 mM EDTA) and re-suspended in 300µl STET buffer (8% (w/v) sucrose, 5% Triton X-100, 50 mM Tris-HCl (pH 8.0), 50 mM EDAT). Lysis buffer 75µl (TES containing 1330 U/ml mutanolysin and 40 mg/ml lysozyme) was added and the suspension was incubated at 37°C. After addition of 40µl preheated (37°C) 20% SDS in TE buffer (10 mM Tris- HCl, 1 mM EDTA, pH 8.0) and glass beads, cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by 10 min incubation at 65°C. TE buffer 100µl was added and the lysate was extracted with 1 vol. phenol/chloroform/isoamylalcohol. The phases were separated by centrifugation (12,000x g, 10 min). The aqueous phase was carefully mixed with 70 µl 5 M NaCl and 1 ml isopropanol, and DNA was precipitated on ice for at least 15 min. DNA was collected by centrifugation (12,000x g, 10 min) and the pellet washed in cold 70% ethanol. The DNA pellet was finally dissolved in 50µl of distilled water and stored at -20°C. The DNA was verified on a 1% argarose gel after electrophoresis in Tris-acetate-EDTA buffer (TAE).

16S rRNA Amplification, Sequencing and Phylogenetic Analysis

The following universal primers were used for the PCR amplification of the 16S rRNA gene: 8UA: (5'-AGA GTT TGA TCC TGG CTC AG -3'), 1492r (5'-TAC GGG TAC CTT GTT ACG ACT T-3') [15]. The PCR mixture (50µl) contained 100 ng template DNA, Taq DNA polymerase (HotStar Taq Master Mix Qiagen GmbH, Germany) according to the manufacturer's instructions, 0.2 mM of dNTPs, 1.0 µM of each primer and 2.0 mM $MgCl_2$ and buffer (10X). Amplification was done using a PCR TECHNE TC-312 (Stone ST 15 OSA, UK) thermal cycler programmed as follows: 15 min at 95°C for initial heat activation and 35 cycles of 1 min at 94°C for denaturation, 1 min at 60°C for annealing and 1.5 min at 72°C for extension and 10 min at 72°C for a final extension. 5µl of the PCR products were examined in 1% agarose gel at 100 V. Sequencing reactions of 16S rRNA genes were done using the BigDye terminator sequencing kit on the 370A DNA analyser (Applied Biosystem). Sequences were then compared to those present in the Genbank database at the infobiogen website using BLAST (National Center for Biotechnology Information databases) and aligned in the MEGA5 software [16] using ClustalW. A phylogenetic tree was constructed using the MEGA5 software using the neighbor-joining method. In addition, 16S rRNA sequences from the isolates were classified using the RDP database Naïve Bayesian rRNA Classifier tool [17].

Polymerase Chain Reaction Detection of *alkB* Genes

For the detection of putative genes encoding alkane oxygenase, PCR was carried out with a pair of degenerate primers, monf (5'-TCAAYACMGSNCAYGARCT-3') and monr (5'-CCGTARTGYTCNA YRTARTT-3'), which were designed based on well-conserved motifs in the AlkB gene in the N-(NTXHELGHK) and C-terminal (INYIEHYGLL) domains [18]. This primer pair was expected to generate a PCR product of about 420 bp. The PCR mix of 50 µl contained the following: 5 µl 10× buffer (provided with Taq polymerase), 1.5 µl $MgCl_2$ (50 mM), 4-5 µl dNTP mix (2.5 mM each), 5-6 µl of each primer (10 µM), 10-15 ng of purified DNA from cultured strains, and 2.5 U Taq DNA polymerase (Invitrogen, Karlsruhe, Germany). Cycling was performed with an initial denaturation for 5 min at 94°C followed by 32 cycles of 30 s at 94°C, 30 s at 50-55°C, and 45 s at 72°C and a final elongation step for 5 min at 72°C. PCR products were separated in a 1.0% agarose gel. DNA smartladder 200 pb (Eurogentec) was used as molecular size marker.

Determination of Biodegradation of Petroleum-hydrocarbons by TLC/FID Analysis

Biodegradation was monitored in 1L flasks (in triplicate) containing 500 ml of MSM containing filtered oil (P or RI) at 1 % (w/v) as sole carbon source and 5 % (v/v) inoculum comprizing four selected isolates. Uninoculated controls for each pollutant were kept to monitor and control for the natural weathering of oils during incubation. Cultures were incubated on a rotary shaker (180 rpm) at 30°C for 4 weeks. Cultures and uninoculated controls were extracted sequentially with equal volumes of dichloromethane DCM, and then subjected to TLC/FID (Iatroscan type MK-5, Mitsubishi Kagaku Iatron, Tokyo, Japan) [19]. Extracts (3 µl) were manually spotted onto the end of silica-gel rods (Chromarods-SIII; Iatron) which were then placed in a development chamber and eluted in *n*-hexane for 30 min to separate the saturated hydrocarbons (~10 cm up the rod). The rods were then eluted with toluene to separate the aromatic hydrocarbons (~ 5cm up the rod) and finally the rods were placed for 2 minutes in a 95:5 DCM: methanol solution to separate the resins from the asphaltenes. After each development step the rods were left at room temperature for 5-10 min to dry. As soon as the rods were dried, they are placed in the Iatroscan system for analysis (hydrogen flow rate 160ml. min^{-1}, air flow rate 2 $L.min^{-1}$ and scan speed 30 s $scan^{-1}$). The resulting data was expressed as a percent fraction chromatograph of the total extractable material comprizing four peaks called the saturated, aromatic, resin and asphaltene fractions [20]. A standard oil (Std) of know composition was used to calibrate the overall Iatroscan analysis and monitor the responses of the individual fractions.

Results

Isolation and Screening of Isolated Strains

Through several independent enrichment and purification steps, using (P) and (RI) as source of carbon and energy, a total of 15 bacterial colonies with different morphologies were further purified on LB agar and strains were characterized for cellular and colony morphology (data not shown). The best potential degrading bacteria were: LGM106 which were Gram-negative rods which exhibited mucoid beige colonies on LB plates; LGM101 which were Gram-

negative cocci, which formed fast-growing and pink round colonies. LGM10 which were Gram-negative rods, which exhibited mucoid white colonies; and LGM104 which were Gram-negative rods, which formed round white colonies. These four isolates were cultured separately and mixed in equal proportion for use in culture interactions.

Assessment of Hydrocarbon Utilization by Turbidometry

OD changes with time for each of the isolates and the mixed cultures grown on hydrocarbons (petrol or RI) [Fig-2a], [Fig-2b]. These OD readings (turbidity) of the inoculated MSM broths measured at regular intervals (every 2 days) provide evidence of the hydrocarbon degrading activity of the bacteria. More specifically, linear increases in OD were observed between days 4 and 12 after which growth became limited. The results demonstrated that LGM101 and LGM106 have the greatest ability to degrade petroleum (P) while LGM106 demonstrated the greatest ability to degrade industrial rejection (RI).

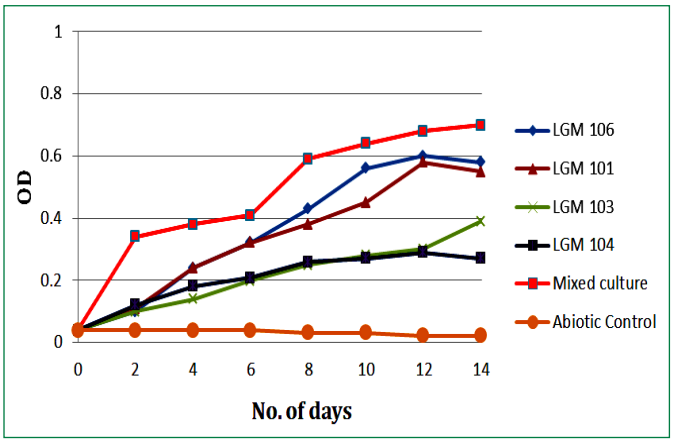

Fig. 2a- Growth curve (O.D values) of bacteria in hydrocarbon (P) degrading broth for a period of 15 days of incubation

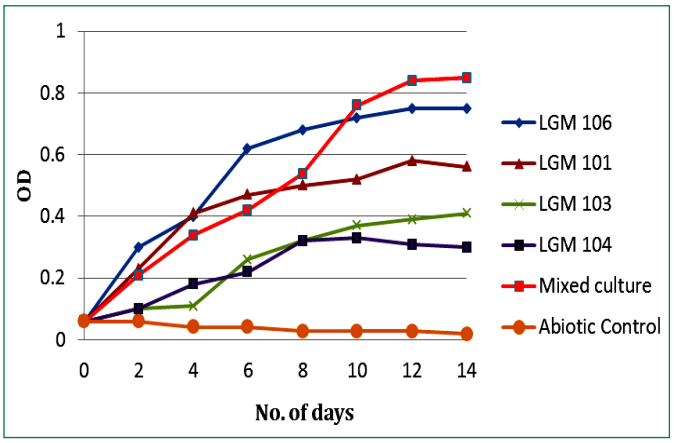

Fig. 2b- Growth curve (O.D values) of bacteria in hydrocarbon (RI) degrading broth for a period of 15 days of incubation

These data showed that while all the organisms utilized both the hydrocarbon substrates (P and RI) when supplied as the sole source of carbon and energy, the level of utilization differed from one bacterium to another (presumably due to differences in individual substrate utilization and growth characteristics). A mixed culture of the four strains showed the highest cell density. This may be ascribed to the fact that individual microorganisms may metabolize only a limited range of substrates, while the assemblage of different bacterial strains with broader enzymatic capability has a greater ability to degrade complex mixtures [21].

Molecular Characterization of Selected Bacteria

Based on nearly full length 16S rRNA gene sequence analysis [Fig-3] the isolates were found to be bacterial species which showed 95-100% homology with sequences in Genbank. Phylogenetic analysis [Fig-4] revealed that strain LGM106 formed a phyletic group with *Pseudomonas cedrina subsp. fulgida* DSM 14938 (99% sequence similarity) and *Pseudomonas cedrina* strain (99%). *Pseudomonas cedrina* strain has been placed in the *Pseudomonas fluorescence* group according to Anzai, et al [22]. Thus LGM106 was identified as a *Pseudomonas* sp. a conclusion strongly supported (95% confidence) by the RDP Naive Bayesian rRNA classifier tool. The LGM101 isolate was identified as a *Shewanella* sp. on the basis that it exhibited (98%) sequence similarity with *Shewanella hafniensis* strain P010 and its classification as the genus *Shewanella* (95% confidence, RDP classifier tool). LGM103 exhibited a (98%) sequence similarity with *Enterobacter hormaechei* ATCC 49162 strain CIP 103441 and was assigned (95% confidence, RDP classifier tool) to the family *Enterobacteriaceae*. LGM104 which also belongs to the family *Enterobacteriaceae* was found to be related (99%) to *Serratia proteamaculans* strain 4364 and was assigned (95% confidence, RDP classifier tool) to the genus *Serratia*.

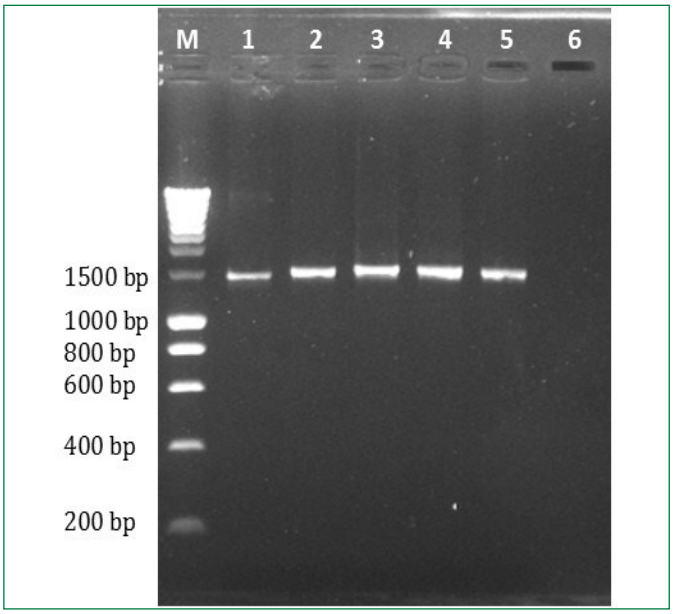

Fig. 3- Gel electrophoresis of PCR amplified 16S rRNA genes from selected consortium: lane M: SmartLadder 200 bp DNA, lane 1: LGM 101, Lane 2: LGM 103, Lane 3: LGM 104, Lane 4: LGM 106. Lane 5: positive control: *Pseudomonas aeruginosa* ATTC 27853, Lane 6: negative control without DNA.

Detection of Alkane Oxygenase Genes

Genomic DNAs isolated from the selected strains were used as templates for the amplification of *alkB* gene sequences. By using the degenerate primer pair monf and monr, the *alkB* gene fragment (420 bp) was successfully amplified [Fig-5] from *Pseudomonas* sp. (LGM106). However, no PCR product was observed with the other isolates i.e. *Shewanella* sp. (LGM101), *Enterobacter* sp. (LGM103) and *Serratia* sp. (LGM104) despite their growth on petroleum- hydrocarbon. Although these other isolates were negative for *alkB* genes, they may harbor other alkane monooxygenases.

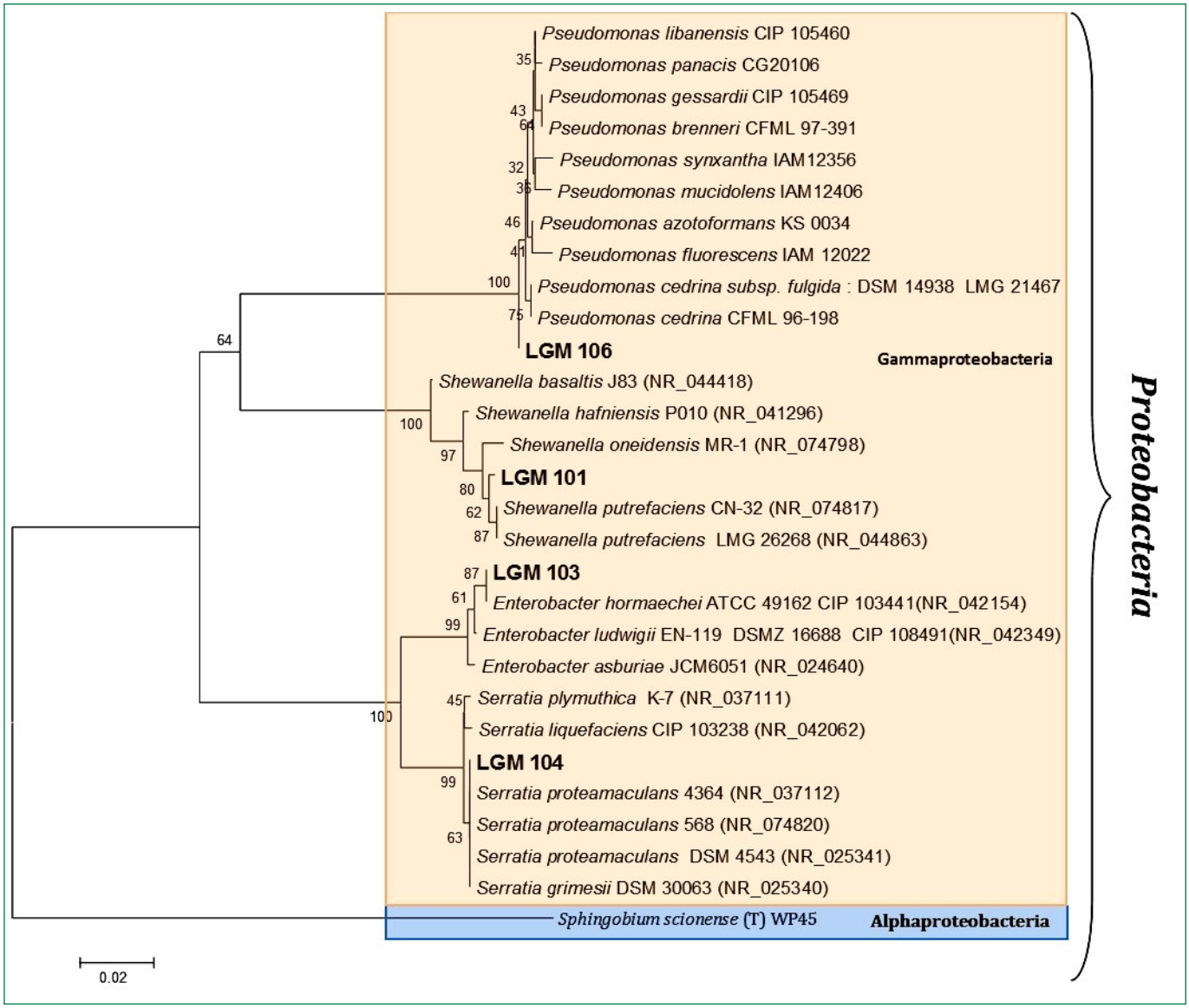

Fig. 4- Neighbor-joining phylogenetic tree based on 16S rRNA sequences of the strains within the consortium (LGM 106, LGM 101, LGM 103, LGM 104) and related species found by a BLASTn database search. The trees were generated with 1000 replicates and the number at the nodes represents values for bootstrap probabilities. Bar=0.02 nucleotide substitution per site.

Biodegradation of Petroleum-Hydrocarbon by the Selected Consortium

The data from the TLC/FID assay represent the % composition of the four major fractions of petroleum hydrocarbons with and without interaction with the bacterial consortium [Table-1] and [Fig-6]. The % composition of each component was calculated based on the ratio of peak areas for each identified group. The TLC/FID assay was calibrated using Standard oil which was found to contain 58.05% saturated hydrocarbons, 34.91% aromatics hydrocarbons, 5.33% resins, and 1.72% asphaltenes, which was in good agreement with the known compositions of that oil. To assess the extent of biodegradative hydrocarbon losses the composition of the abiotically incubated Algerian crude oil (P) and industrial rejection product (RI) were directly compared with the composition of the oils incubated in the presence of the mixture of bacterial isolates. This comparison identified a large decrease in the saturate component in the hydrocarbon substrate sources exposed to the bacterial isolates relative to the abiotic controls with a significant increase of the non-biodegradable fractions (asphaltenes). The marked compositional change in the saturates relative to the abiotic controls (P and RI) was likely due to their high susceptibility to microbial attack as has been pointed out in the literature [23]. There was no difference between the aromatic fraction in the Algerian crude oil abiotic control (P+c) and the matched biotic treatment which can be explained by the consortiums lower affinity for this component. Nevertheless, the static nature of the aromatic % composition in the data shown in [Table-1] relative to a decrease in saturate and increase in asphaltenes may indicate a moderate level of degradation of this component. Based on a comparison with the abiotic control the aromatic component in the biotic industrial rejection experiment (RI+c) appeared to be more accessible to the selected bacterial consortium. The composition of the resins was, however, the same in all cases and a there was a higher asphaltenes content in both of the biotic incubations [Fig-6C] and [Fig-6E]. This increase is consistent with the finding that resins and asphalthenes are resistant to biodegradation [24].

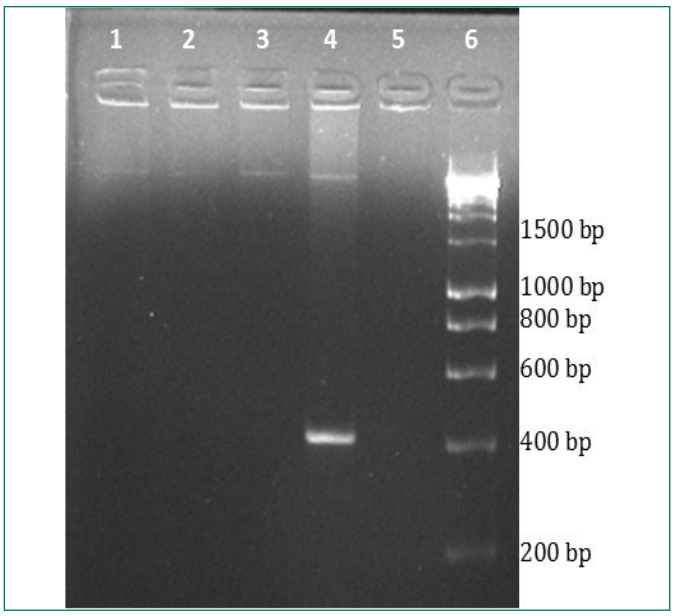

Fig. 5- Amplification patterns using *alkB* degenerate primers of the PCR products. Lanes 1, 2 and 3: PCR amplification from strains LGM (101, 103, 104) respectively; lane 4: AlkB detection in LGM 106; lane 5: negative control without DNA; lane 6: SmartLadder 200 bp DNA.

Table 1- Composition (%) of fractions in petroleum-hydrocarbon mixtures incubated in biotic (P+c and RI+c) and abiotic control experiments

Samples	Saturates	Aromatics	Resin	Ashphaltenes
Std	58.05	34.91	5.33	1.72
P	66.19	29.78	3.03	1.00
P+c	50.4	30.42	3.65	15.53
RI	56.44	34.72	5.15	3.68
RI+c	51.77	27.77	5.07	15.39

P: Crude oil, (P+c): Crude oil with consortium, RI: Industrial rejection, (RI+c): Industrial rejection with consortium.

Discussion

Biodegradation is one of the major means by which hydrocarbon pollutants can be removed from the environment and many species of aerobic bacteria can utilize hydrocarbons of crude oil and derived products released into the environment. The reported efficiency of biodegradation ranges from 0.13% to 50% for soil bacteria [25] and a wide range of organisms are involved in this process, often acting as consortia. It is frequently difficult to find organisms that will individually degrade all the fractions of crude oil (aliphatics, acyclics and aromatics). In this study, four bacterial isolates were used which have been confidently assigned to the *Proteobacteria*, class *Gamma-Proteobacteria*, and further assigned to the genera *Pseudomonas*, *Shewanella, Enterobacter* and *Serratia*. At this level of phylogenetic discrimination inferences on function are notoriously difficult to make, however, hydrocarbons degrading organisms with similar affiliations have been shown to be involved in hydrocarbon degradation under challenging conditions. For instance a microbial consortium with the ability to degrade crude oil at low temperatures was investigated by Deppe, et al [26] and *Shewanella* sp. and *Pseudomonas* sp. were the predominant phylotypes in these oil-treated microcosms. This mixed culture showed obvious advantages including: stability of the consortium, an adaptability for crude oil degradation by different combinations of the organisms and an ability to utilize various hydrocarbons, such as long-chain alkanes (*n*-C24 to *n*-C34), pristane, (methyl-) naphthalenes, and xylenes as sole carbon and energy sources.

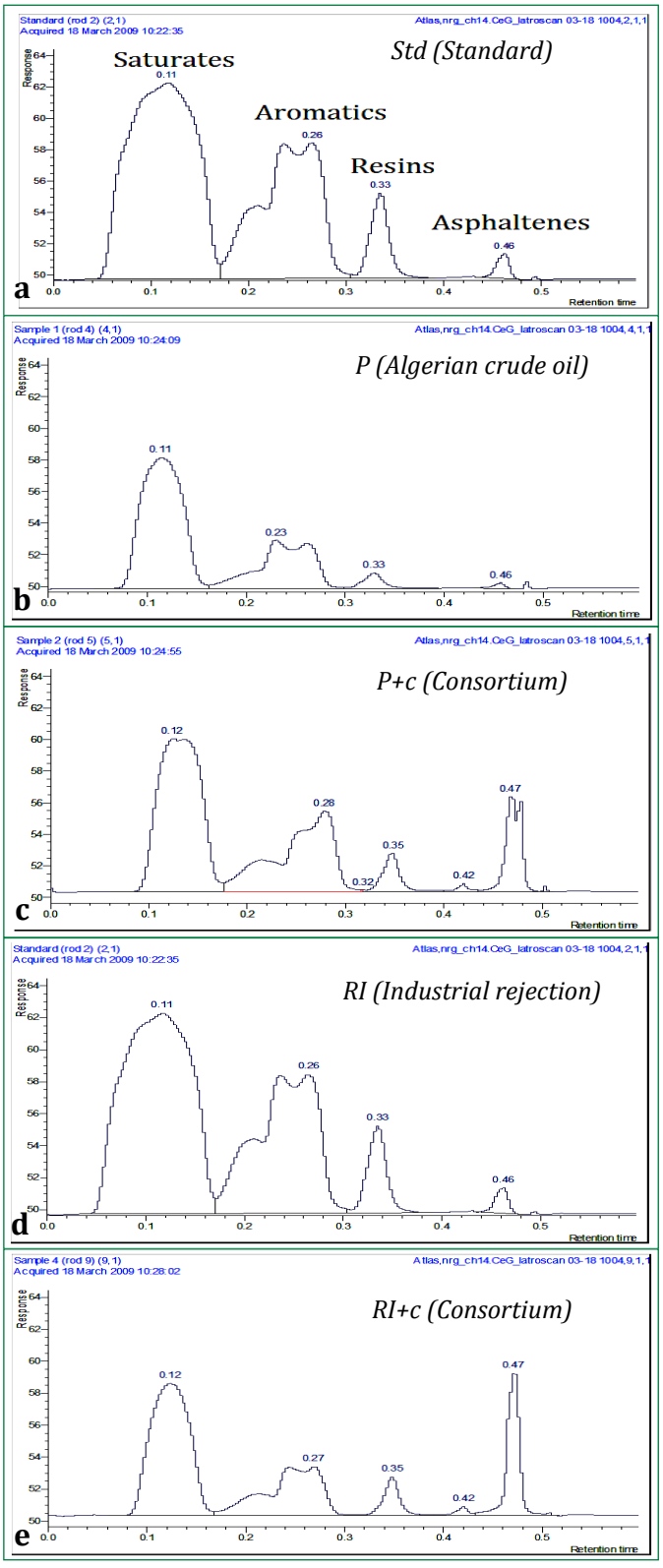

Fig. 6- Iatroscan Chromatographs showing **(a)** Standard (Std); **(b)** Crude oil (P); **(c)** P with Consortium; **(d)** industrial rejection (RI); and **(e)** RI with consortium. The horizontal axis represents the distance each peak has moved up the rod and the vertical axis is the FID response representing relative areas of each fraction.

Furthermore, Katsivela, et al [27] noted that the genera *Enterobacter* in a microbial community with other bacteria were detected in petroleum refinery waste sludge in land farmed soil and a depletion of *n*-alkanes of approximately 75-100% was observed. In addition, Chikere, et al [28] noted that strain CEE_131 (T) isolated from a sediment, degraded high-molecular-mass polycyclic aromatic hydrocarbons of four and five rings. This isolate exhibited a 91% sequences similarity with *Serratia proteomaculans* DSM4597 (T). Jin, et al [29] demonstrated that *Serratia* sp. NB2 in mixed cultivation could enhance the degradation of nitrobenzene compared with mono-cultivation. In this work, the consumption of hydrocarbons by a formulated consortium showed that the saturate and aromatic fractions were significantly degraded.

Major metabolic pathways for many petroleum components have been well studied and documented [30] which explain differences in susceptibility to biodegradation. *n*-alkanes are the most readily degraded components in a petroleum mixture and although the aromatics are generally more resistant to biodegradation, some low molecular weight aromatics such as naphthalene may be oxidized before many saturates [31]. According to Maki, et al [10] the decrease in the aromatic fraction in their study, could be explained by assuming aromatic compounds were converted to a polar form (i.e. moved to the resin or asphaltenes fraction). Different factors which influence hydrocarbon degradation have been reported [32] and a major one of these is their limited availability to microorganisms which may explain the more refractory nature of the aromatic fraction in interaction culture (P+c). Petroleum hydrocarbon constituents bind to soil components, and they are difficult to be removed or degraded [33]. As discussed above, hydrocarbons differ in their susceptibility to microbial attack and they can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all, and other factors affecting the rate of biodegradation, such as seeding size, available nutrients, the presence of inhibiting substrates, predation and insufficient movement of seed organisms within the soil have been suggested [23]. According to Warne Zouekia, et al [34], effective biodegradation depends on various parameters, in particular on the petroleum composition and on the ability of bacteria to adhere to and degrade the hydrocarbon phase. Much research has been performed to understand the physicochemical properties of various crude oil constituents. However, understanding of how the major components of crude oil can affect bacterial adhesion is still very limited. Cometabolism also plays an important role in oil biodegradation. Many complex branched, cyclic, and aromatic hydrocarbons, which otherwise would not be biodegraded individually, can be oxidized through such a mechanism in an oil mixture due to the abundance of other substrates that can be metabolized easily within the oil [35]. In the present study the alkane hydroxylase system of the *Pseudomonas* genus has been shown to differentiate independently from other genera, and we speculate that Pseudomonas might also differ from other closely related species in alkane catabolic activity. Many alkane hydroxylases are related to the integral membrane non-heme iron protein *alkB*, first discovered in a hexane-degrading fluorescent pseudomonad [36]. PCR with highly degenerate primers was used to show that genes encoding AlkB homologs are present in many alkane-degrading α-, ß- and ϒ-Proteobacteria included *Rhodocuccus erythropolis*, *Pseudomonas aeroginosa*, several *Acinetobacter* sp. and *Alcanivorax borkumensis* [37]. Bacterial strains contain multiple integral membrane AlkBs, and there are a number of possible explanations for this apparent redundancy; the enzymes might have different substrate ranges: some oxidize alkane from C_5 to C_{12}, whereas others oxidize C_{10}-C_{16} *n*-alkanes [38]. Different AlkBs might also be active during different growth phases, another explanation is that AlkBs could have different affinity constants for different alkanes or some AlkBs might preferentially oxidize non-linear, branched, cyclic aliphatic or aromatic compounds. The organic fraction associated with sands of the Algerian Sahara Desert was characterized with regards to n-alkanes, polynuclear aromatic hydrocarbons (PAH) and mono/dicarboxylic acids, n-alkanes homologues belonging to C_{16}-C_{35} interval, which were eluted with the first fraction [39].

To conclude, the results obtained suggest that a formulated oil degrading bacterial consortium has been identified and phylogenetic analysis has revealed that the consortium contains strains belonging to the genera *Pseudomonas, Enterobacter, Serratia* and *Shewanella*. This mixed bacterial culture could potentially be useful for bioremediation efforts since recently Gram-negative strains belonging to the *Shewanella* and *Pseudomonas* genera isolated from oily sludge were tolerant to saturated (n-hexane, n-heptane, n-decane, n-pentadecane, n-hexadecane, cyclohexane), monoaromatic (benzene, toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) and polyaromatic [7] hydrocarbons. In the engineered bioremediation domain, the selection of a strategy largely depends on the natural degradation capacities of local microflorae. In this respect, TLC/FID is a promising technique to assess in detail the bioremediation capacities of the bacterial microflorae. This method has advantages in measuring high-boiling-point hydrocarbons such as higher molecular weight saturates, aromatics, resins, and asphaltenes, some of which may not be detectable by GC or HPLC [31].

Finally, bacterial alkane hydroxylases are of high interest for bioremediation applications as they allow some bacteria like *Pseudomonas* sp. to grow in oil-contaminated environments. Furthermore, they have tremendous biotechnological potential as they catalyse the stereo- and regio-specific hydroxylation of chemically inert alkanes, which can then be used in the synthesis of pharmaceuticals and other high-cost chemicals [40]. From the foregoing, it is a reasonable conclusion that this mixed bacterial culture is a potential candidate for the bioremediation of hydrocarbon-polluted sites. This study provides a better understanding of the adaptation of bacteria inhabiting polluted environments to environmental fluctuations of pollutant nutrients, and for developing and implementing adequate bio-strategies in the future to enhance oil degradation in contaminated soil and the biotreatment of oily waste water in refineries.

Acknowledgements

This work was supported by the School of Civil Engineering and Geosciences, University of Newcastle, UK.

Conflicts of Interest: None declared.

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