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RESEARCH

Isolation & Characterization of Hydrocarbon Degrading Bacteria: A Bio-remedial Approach to Clean-up Oil Spills

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ABSTRACT

Hydrocarbon contamination is one of the major environmental problems due to oil spillage, automobile waste, and other industrial waste. Crude oil is the major source of energy for industrial, agricultural, and domestic use. Indian agriculture is largely dependent upon petroleum-driven technology for power generation, harvesting and post-harvest processing. Oil spillage occurs at oil wells and rigs during the drilling, production, refining, transport, and storage of petroleum. The release and accumulation of hydrocarbons in the environment is the main cause for concern due to the health hazards it poses to all forms of life and the environment. Commonly used approaches involve physical, biological, and chemical methods. Most of the technologies are expensive and not very efficient to deal with recalcitrant pollutants. The present study deals with the bioremediation of crude oil. The study involved the collection of surface soil of the spillage/contaminated area to isolate and identify the oil-degrading bacteria. Bacteria were isolated and grown on MSM-agar medium containing crude oil as a carbon source in Petri-dishes. The isolated strain of bacteria was effective in the biodegradation of oil in 28 days. The samples were analysed using GC-FID which demonstrated efficient degradation of oil by the isolated microbe. The hydrocarbon degraders were identified as Gram-negative cocci bacteria. The isolated bacteria could serve as a cost-effective and efficient alternative for microbial degradation of hydrocarbon pollutants in soil and water in an environmentally friendly and sustainable manner.

KEYWORDS

Oil spillage, bioremediation, cancer, gram negative bacteria.

INTRODUCTION

With the increasing use of petroleum-based hydrocarbons, their presence in the environment is an ever-increasing hazard and is the major contributor to environmental pollution. The quality of life on earth is inextricably associated with the overall quality and health of the environment. Fossil fuel-based products are the leading source of energy in various industries. Crude oil meets the world's maximum energy needs and the demand for raw materials in the chemical industry, thus playing a crucial role in the rapid development of the modern economy [**1**]. Crude oil is a complex mixture containing diverse range of hydrocarbons. Petroleum products contain thousands of individual hydrocarbons and related compounds. Their main components are usually subdivided into saturated (normal, branched-chain alkanes & cyclo-paraffins) and aromatic (monocyclic aromatic, and polynuclear/polycyclic aromatic hydrocarbons viz., MAH andPAHs respectively), containing alkyl side chain and or fused cyclo-paraffin rings) [**2**]. The less abundant resins and asphaltenes consist of more polar compounds containing heterocyclic, oxygenated hydrocarbons and aggregates with high molecular weight. Because hydrocarbons lack functional groups and low water solubility, they are considered to be unreactive.

The growing pollution hazards associated with the ever-increasing oil & gas exploration and exploitation activities have evoked a serious concern among the environmentalists world over [**3**]. During normal operations in oil field, crude oil spills do occur accidentally and are menace to the environment as they cause severe damage to surrounding ecosystem. Soil and water contamination is a very common occurrence in most oil exploration fields around the world. In the oil industry, oil spills during various drilling and production operations are a serious concern.

Bioremediation is an advanced biotechnological method used to degrade and detoxify hydrocarbon contaminants in a cost-effective manner with minimal threat to the environment [**4**].

Bioremediation is an efficient approach to degrading and removing oil-derived pollutants. Contrarily methods employing the physicochemical approach are cost-incurring and less efficient. Since most components of crude oil are biodegradable, oil-degrading microorganisms are ubiquitous. The population of hydrocarbon degraders is generally less than 1% of the total and accrues to 10% in polluted habitats and nutrient-depleted environs due to a lack of major nutrients like nitrogen or phosphorus [**5**]. Mixed cultures with diverse metabolic capabilities are commonly utilized as seeding inoculum for sowing in bio-remediable contaminated areas. Stimulating biological activity to move the polluted environment faster is an environmentally friendly strategy for the bioremediation of the crude oil-contaminated environment [**6**].

The current work aimed to explore and isolate microbial consortia or unique bacterial strains capable of effectively degrading hydrocarbons and thus mitigating the hazardous conditions in the environment. Hence, the chances for finding petroleum-based hydrocarbon degrading microbes are maximum at oil-spillage and oil-drilling locations. The soil composition of these locations is rich in microorganisms capable of degrading hydrocarbon constituents of petroleum and utilizing them as carbon sources. These microbes evolved in the course of evolution, especially during the period of the 'Cambrian explosion.' As the fossil organic matter trapped in rocks known as kerogen went through various transformation stages of diagenesis, catagenesis, and metagenesis, at the hydrocarbon-water interface microbial growth and development occurred. Thus it resulted in the evolutionary progression/development of hydrocarbon-degrading microbes spanning from the Ordovician to Jurassic and Cretaceous periods. During this entire course, microbes evolved and adapted to diverse conditions utilizing hydrocarbons as the source of energy.There is an obvious link between the microbes and hydrocarbons that have evolved and adapted to the environment during the geological periods of millions of years. Consequently, by means of their interactions with the environment,microbes have developed the potential to degrade hydrocarbons andare widely distributed among microbial populations. And the biodegradation is initiatednaturally whenthe environmental conditions are favourable. Our aim was to isolate and characterize such microbes and explore their ability to degrade petroleum based hydrocarbons from the sediments of crude oil spillage sites.

EXPERIMENTAL METHODS

Isolation of oil degrading bacteria

The soil samples were collected from hydrocarbon contaminated site. Surface soil of oil spilled area was excavated up to 30 cm depth and sufficient quantities of samples were collected from different places and different depths depending upon the spillage percolation. The samples were transferred into sterile polythene bags, following standard microbiological procedures and stored at 4°C. The samples were then mixed homogeneously to form a composite representative sample. This soil sample was used to isolate oil degrading microbes and isolated microbes were used to explore the hydrocarbon degrading potential using crude oil containing medium.

Culture and maintenance of isolated bacteria

For isolating hydrocarbon-degrading microbes, the culture was enriched by growing on oil-contaminated soil samples [**7**]. The medium used for culture was mineral salts medium (MSM) consisting of crude oil, agar, and essential nutrients. The microbial organisms were transferred from samples (50 µL) to the Petri dishes and cultured at 37°C. The oil degrading consortia were maintained in conical flasks and kept in a rotary shaker at 150-180 rpm until significant growth along with turbidity was observed. MSM that supports microbial growth was used with crude oil as the sole carbon source [**8**] for growing hydrocarbon-degrading bacterial cultures.

Oil degradation by the isolated bacteria

50 mL of minimal salt medium was added to the 250 mL Erlenmeyer flask and 1 g of crude oil was supplemented to each flask. 10 % (v/v) of inoculum was added to each sterilised flask and kept for four weeks in a shaker incubator at 37 ºC and 180 rpm. Samples were analyzed at regular intervals.

Growth and crude-oil removal assay

The flasks carrying the microbial cultures were incubated in a shaker incubator at 37 ºC and 180 rpm (**Fig. 1**). The microbial growth was confirmed with increase in the turbidity of the media and degradation of oil was analyzed by GC-FID [**9**]. For the sample preparation, dichloromethane was used to extract the degradation products from the oil sample used in culture medium. Dichloromethane extracted hydrophobic compounds, which were soluble in the organic solvent and formed a separate layer. After removal of the soluble organic layer aqueous layer containing insoluble precipitate of inorganic salts, soil etc. was left as discard.The organic layerof dichloromethane soluble part, which largely contained crude oil and its degradation products, was subjected to chromatography.

RESULTS AND DISCUSSION

Identification of microbes

To identify and characterize the bacterial isolates, Gram staining and visual observation under microscope was carried out. An aliquot of 50 µL of broth was plated on MSM-Agarose plates. For the enumeration of hydrocarbon oxidizers, the plates were incubated at 37°C for 10 days. The colonies thus grown on the plate (**Fig. 1**) were picked up and used to make the slides for characterization of microbes.

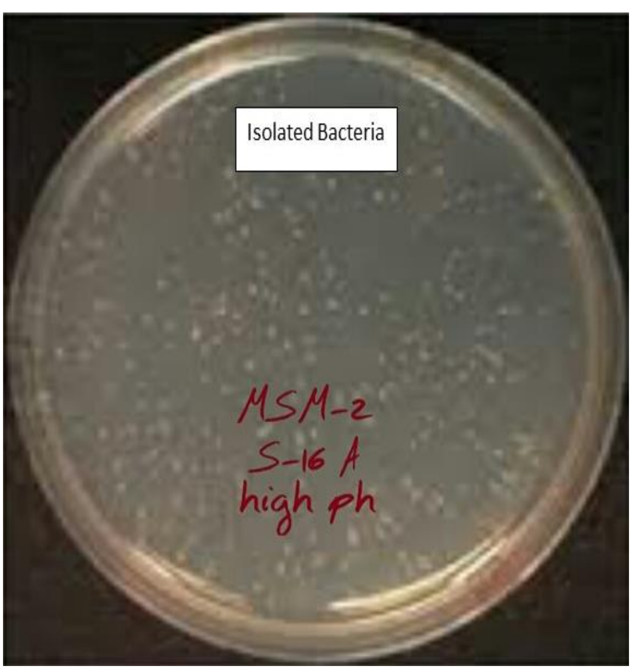

Fig. 1. The Petri plate showing hydrocarbon degraders growth.

The Gram's staining showed that bacteria were Gram's negative (**Fig. 2**).

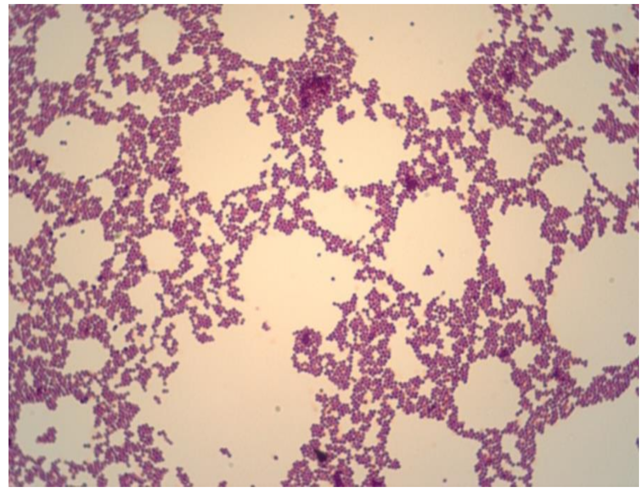

Fig. 2. Slide (under microscope) showing Gram negative bacteria.

Degradation of oil spillage

The isolated bacteria were grown in a Petri dish to explore their ability to degrade petroleum hydrocarbons. After ascertaining the biodegradation potential of the isolated bacterium, medium containing oil layer was inoculated with the culture in the Erlenmeyer flask and grown in a shaker incubator at 37 ºC at 180 rpm for 28 days (**Fig. 3**).

Fig. 3. Incubator shaker and the flasks showing microbial growth and a control.

After seven days the liquid broth appeared to be slightly turbid with oil on the surface which exhibited the presence of microbial growth. After fourteen days the size of the oil droplets was reduced and the broth appeared more turbid. After twenty-eight days the broth completely became turbid showing no oil droplets on the surface. No changes were observed in the contents of the control flask as the control was left un-inoculated.

GC analysis of oil enriched medium

The samples collected from previous step were subjected to GC analysis. Gas-Chromatography was used to determine the biodegradation potential and effectiveness of bacteria by analysing crude oil degradation products. The chromatogram of untreated and treated samples upon comparison showed differences in the peaks. The untreated sample showed numerous peaks indicating the presence of a diverse size range of hydrocarbons as compared to the treated samples. Fewer peaks are indicative of the reduced heterogeneity in the samples as a result of microbial degradation of crude oil.

Analysis of gas chromatography samples of oil exhibited the different stages in oil biodegradation (loss of n-paraffins followed by loss of acyclic isoprenoids) were observed (**Fig. 4**).

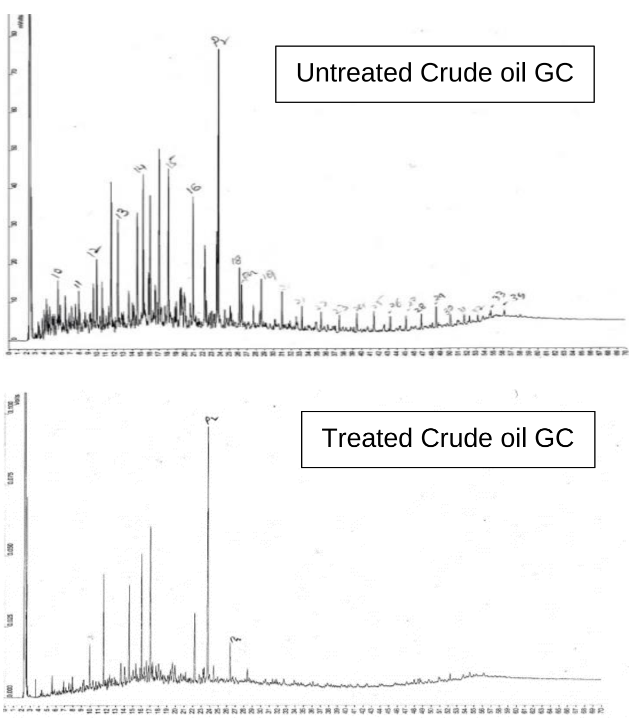

Fig. 4. Gas Chromatograms (i) Untreated & (ii) treated crude oil

In heavily biodegraded oil samples of late stages, GC analysis could not distinguish the differences in biodegradation due to interference produced by unresolved complex mixture (UCM or "hump") that dominates the GC traces of heavily degraded oils.

The cosmopolitan distribution of bacteria and their adaptability has given rise to highly diverse genetic forms that are capable of utilizing various nutrient sources for energy production. Thus, the collection of soil samples from the crude-oil spillage sites may already have a consortium of microbes enriched in hydrocarbon-degrading species. The results clearly indicate that the isolated bacterial cultures from the spillage sites are capable of efficiently degrading both the saturated and unsaturated MAHs/PAHs as indicated by the GC chromatograms (**Fig. 4**) of biodegraded hydrocarbons into smaller hydrocarbon chains which are easily utilized by microbes found in sub-soil ecosystems. These small-chain hydrocarbons are utilised by genetically diverse groups like *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Klebsiella*, *Mycobacterium*, *Corynebacterium*, *Nocardia Brevibacterium*, etc. [**10**]. Thus, the maximum challenge in bioremediation is posed by the lack of evidence and direction in the use of microbes for mitigation of the environmental levels of hydrocarbon pollutants [**11**]. Once the microbes are isolated, characterized, and enumerated for their efficiency in biodegradation and bioremediation of hydrocarbons they can be engineered, bio augmented and optimized to suit the requirements of bioremediation.

CONCLUSION

Microbial cultures were isolated and enriched from the petroleum-based hydrocarbon-polluted soil using a minimal salt medium. The enriched bacterial culture was characterized to determine their petroleum degrading potential. The effectiveness of isolated bacteria to degrade a diverse range of hydrocarbons found in petroleum was determined by analysing the biodegradation products by GC-FID. GC chromatograms demonstrate the biodegradation of crude oil hydrocarbons, thus suggesting that the isolated bacterial cultures could offer an effective and cheap alternative strategy for the bioremediation of soil and water samples. The current work possesses immense potential and could be scaled up to meet technological and commercial demands for bioremediation. The work presented here is part of continuous efforts being made to make technological refinements and advancements in the fast-developing field of bioremediation of petroleum hydrocarbons.

FUTURE PERSPECTIVES

Isolated bacterial cultures could serve as a cost-effective and sustainable alternative strategy for the bioremediation of soil and water.

Bioprocess optimization and the bioremediation process could utilize the bacterial isolates for up-scaling and commercialization of the technology.

The strategy of bacterial isolation and bio augmentation has the potential to tackle the problem of hydrocarbon contamination.

Microbial bioprospecting can lead to the discovery of relevant enzymes and genes involved in bioremediation.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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GRAPHICAL ABSTRACT