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Biodegradation Potential of Petroleum Hydrocarbons by Bacterial Diversity in Soil

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Abstract: Due to widespread use of petroleum products, the number of petroleum-contaminated site has abounded. Natural attenuation, which relies on in situ biodegradation of pollutants, has received a large number of attentions, especially for petroleum contamination. Therefore in this research three type of samples, Diesel oil, Crude oil and Hexadecane, were chosen and oil degrading microorganisms were enriched by mineral media. The main strategies in bioremediation of oil spills, which include bio-stimulation, nutrient application, bio-augmentation, seeding with competent or adapted hydrocarbonoclastic bacteria or their consortium and genetically engineered microbes, are reviewed. Although the promise of bioremediation is yet to be realized, innovative areas in environmental biotechnology for oil spill clean-up are highlighted. By checking the biodegradative ability of oil-degrading microorganisms on individual hydrocarbon we showed that total bacterial could decompose intermediate chain length easily and very fast. Results shows that cultures amended with complex hydrocarbon mixtures (diesel and crude oil) never managed to remove more than 80% of the initial amount. In contrast, cultures with the individual pure compounds (hexadecane) at any of the tested concentrations removed more than 80% of the initial amount. The treatment achieved the maximum biodegradation of the pure compounds because abiotic losses of hydrocarbons in some treatments approached 20% of the initial compound added.

Key word: Biodegradation · Oil · Seepage · GC analysis

INTRODUCTION

Iran is the first country in the oil-rich Middle East region to start oil operations with current production capacities of over 4 million barrels of crude oil and 80,000 m^3/day of diesel fuel. There are up to 1,500,000 cubic meters of soil contaminated with crude oil in Iran. Soil and ground water are often contaminated with gasoline or diesel fuel from leaking underground storage tanks and also due to accidental spills and leakage from pipelines. Due to their mobility, these compounds may cause considerable damage not only in soils, but also in water intakes or ground water reservoirs [1].

The carbon number of diesel oil hydrocarbons, crude oils is between 11 and 25 (2000 to 4000 hydrocarbons) and the distillation range is between 180 to 380°C. They are a complex mixture of normal, branched and cyclic alkanes and aromatic compounds with the properties of low water solubility, high adsorption coefficient and high stability of the aromatic ring. Therefore, diesel fuel has been considered as priority pollutants which exert bio-hazardous effects on both human and other living organisms in the environment [2, 3]. Fortunately, this mixture represents an excellent substrate in the study of hydrocarbon biodegradation due to its composition [4]. Among several clean-up techniques available to remove petroleum hydrocarbons from the soil and groundwater, bioremediation processes are gaining ground due to their simplicity, higher efficiency and cost-effectiveness when compared to other technologies. The fate of petroleum hydrocarbons in the environment is largely controlled by abiotic factors which influence rates of microbial growth and enzymatic activities that determine the rates of petroleum hydrocarbon utilization.

Biodegradation by natural populations of microorganisms is the basic and the most reliable mechanism by which thousands of xenobiotic pollutants, including crude oil, are eliminated from the environment. The effects of environmental conditions on the microbial degradation of hydrocarbons and the effects of hydrocarbon contamination on microbial communities are areas of great interest. Bioremediation is a strategy to

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utilize biological activities as much as possible for quick elimination of environmental pollutants. Growth stimulation of indigenous microorganisms, biostimulation, along with inoculation of foreign oil-degrading bacteria is a promising means of accelerating detoxifying and degrading activities at a polluted site with minimum impact on the ecological systems.

Bioremediation of hydrocarbon-contaminated soils, which exploits microorganisms to degrade organic contaminants, has been established as an efficient, cost-effective and environmentally sound treatment [5, 6]. Although physical and chemical processes, such as dispersion, dilution, sorption, volatilization and abiotic transformations, are important means of hydrocarbon elimination, biodegradation is most often the primary mechanism for contaminant clean-up [6]. Microbial biotransformation is also considered a major environmental process affecting the fate of hydrocarbon compounds in both terrestrial and aquatic ecosystems [7, 8]. This suggests that the biodegradative contribution of the indigenous microorganisms is often significant [9, 10]. Degradation of crude oil by autochthonous microbial communities has been well documented [11, 12].

A substantial amount of researches has been conducted in the past century to demonstrate the significance of microorganisms for biodegrading crude and refined petroleums. It was estimated that from the time of the Exxon Valdez oil spill in 1989 until the fall of 1992, about 50% of the spilled oil was biodegraded either in the water column or in the intertidal sediments [13]. Methods that have been proved most effective in overcoming environmental limitations include oxygenation and nutrition. However, anaerobic hydrocarbon metabolisms have been seen in the past decade [14].

The use of large quantities of petroleum products has resulted in a high level of environmental pollution. Oil spills caused by blowouts, leakage from tanks and dumping of waste petroleum products, for example, lead to an elevated loading of petroleum hydrocarbons in soil, which results in significant decline in the quality of soil and makes it unfit for use. The persistence of petroleum pollution depends on the quantity and quality of the hydrocarbon mixture and on the properties of the affected ecosystem. Although light petroleum products, like gasoline are efficiently removed by many physio-chemical methods, heavier fuels like diesel often require other techniques because of their low volatility. Since many naturally occurring microorganisms have the ability to utilize hydrocarbons as the sole source of carbon and are widely distributed.

This research simulates one possible oil spill remediation measure was applied followed by microbe treatment. The aims of this study were to evaluate the biodegradation of petroleum hydrocarbons by inoculum addition to the soil where the indigenous population of hydrocarbon degrading microorganisms within the biodegradation period.

MATERIALS AND METHODS

Sampling: Soil samples were obtained from depths of 0.5 and 1 m as well as from ground surface in a contaminated area close to the Storage and Distribution Center of Oily Products in south of Iran (Ahvaz). Processing on soils began immediately upon arrival at the laboratory. Soil samples were sieved moist using a 2 mm mesh screen and thoroughly mixed.

Enrichment of Petroleum Hydrocarbon-degrading Bacteria: Five grams of samples from each soils were used as inocula. Serial sub-culturing was conducted with 150-mL mineral salt medium [14]. amended with crude oil from Oil-field in 250-mL Erlenmeyer flasks. Mineral salt medium contained 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g $CaCl_2 \cdot 2H_2O$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3 and 200 μL of 1.0 mol L^{-1} $FeCl_3$ per liter of distilled H_2O. The pH was adjusted to 7.0-7.2 with 0.1 mol L^{-1} NaOH. The amount of crude oil available at the beginning of each transfer into fresh media slowly increased from 0.5 to 4.0 mg mL^{-1} over the first 50 days of sub-culturing and remained at 4.0 mg mL^{-1} until the end of the enrichment period. The flask was incubated at 22 ± 2 °C. After 15 days, 2.5 ml of flask1 was transferred to a second flask (flask 2) with the same condition as flask 1.These incubating -transferring were repeated 4 times and at the final stage (fourth period) the amount of CFU (Colony Forming Units) in flask 4 was 20×10^8 per ml.

Optimization of Biodegradation Conditions: The soil samples were sieved by a 2 mm screen and were sterilized three times by autoclaving at 200°C for 30 min followed by incubation for 24 h at 37°C [15]. Six 250 ml Erlenmeyer flasks were prepared for each of the soils investigated. Additional flasks served as uninoculated controls. In each flask 200 g dw of soil was contaminated and mixed with 4 g of petroleum hydrocarbons.

Optimization of Biodegradation Conditions: The environmental conditions for the degradation of hydrocarbon by microbial diversity in soil were optimized

using an L16(45) orthogonal array design with a set of batch cultures. Orthogonal array design is based on the Taguchi method and used to determine optimal process parameters and analyses the effect and significance of different parameters through least experiments. Diesel oil (purchased from a gasoline station) was chosen as carbon source because it is an excellent model for studying biodegradation of hydrocarbon [4].

Mineral salt medium was modified as necessary to obtain the desired levels of variables. All the culture media were inoculated with 10 ml of cultures grown on MSM (amended with 3 mg mL^{-1} diesel oil as a carbon and energy source). Degradation experiments were conducted in 250-mL Erlenmeyer flasks.

Total Cell Counts: Total cell counts were determined during the degradation of hydrocarbons. The counting method was modified from that described by Margesin and Schinner [6]. Cell suspensions from the degradation experiment were centrifuged and resuspended to remove hydrocarbons and impurities, then quantitatively diluted, stained with 4, 6-diamidino-2-phenylindole (DAPI) and filtered on 0.2 μm Nuclepore polycarbonate filers. Blue DAPI fluorescence was observed using an epifluorescence microscope equipped with a ultraviolet light from a 100-W mercury arc lamp.

Detecting the Transformation of Diesel Oil, Crude Oil and N-alkanes: The abilities of all the individual and mixed bacterial cultures to transform diesel oil, crude oil and *n*-alkanes were assessed. The experiment was conducted in 150 ml of MSM in 250-mL Erlenmeyer flasks with 10 ml inoculum (optical density = 0.7 at 600 nm) for each flask. Each carbon source was provided at one of three concentrations (10, 20 and 30 mg mL^{-1} for diesel oil, crude oil and *n*-alkanes). The extent of degradation of diesel oil in the treatments was determined spectrophotometrically [16]. *n*-alkanes were measured using gas chromatography (GC). Removal of crude oil was determined gravimetrically [17].

GC Analysis: The collected samples were extracted twice by 50 ml dichloromethane (DCM) at ambient temperature. The extraction phase was concentrated in a vacuum evaporator to around 2 ml, then cold-dried by flow of nitrogen gas. The residue was diluted in 1 ml of DCM and analyzed by gas chromatography (GC. 0.5μl portion of the residue solution was injected into a GC (Philips Model PU4500) equipped with FID detector and a capillary column (BP1: 25 m × 0.53 mm ×1 μm) to determine the existing chemical compounds. The column was maintained at 50-250°C with increase rate of 8°C/min. The injection port and detector temperatures were 250°C. Helium was used as the carrier gas with flow rate of 1 ml/min.The column was isothermal at 50°C. The injection port and detector temperatures were 120 and 150°C, respectively. The column was maintained at 50-65°C with increase rate of 0.7°C/min. The injection port and detector temperatures were 170°C.

Statistical Analysis: Analysis of variance (SPSS version 11.0) was used to evaluate the significance of apparent differences between the extents of biodegradation of different hydrocarbons.

RESULTS AND DISCUSSION

Degradation of all substrates was monitored for a 14-day period. Because the total extent of substrate disappearance was the sum of abiotic elimination (presumably through volatilization) plus biodegradation, uninoculated control flasks were also prepared to distinguish physical removal of hydrocarbons from biodegradation.

Results shows that cultures amended with complex hydrocarbon mixtures (diesel and crude oil) never managed to remove more than 80% of the initial amount. In contrast, cultures with the individual pure compounds (hexadecane) at any of the tested concentrations removed more than 80% of the initial amount. The treatment achieved the maximum biodegradation of the pure compounds because abiotic losses of hydrocarbons in some treatments approached 20% of the initial compound added. This may reflect the presence of recalcitrant, biodegradation-resistant hydrocarbons in the crude and diesel oils, which survive to the end of the incubations with little or no biochemical alteration.

The extent of biodegradation of crude and diesel oil and hexadecane after 14 days of incubation with microbial community cultures and individual isolates. Naturally, the hexadecane-utilizing isolates degraded more hexadecane than either of the oils. The hexadecane degraders also removed a larger than oil.

Most of the studies on bioremediation of soils and sediments polluted by hydrocarbons have focused on the rates of contaminant degradation. However, less attention has been devoted to the effects of varying environmental conditions on those rates. Nonetheless, proper environmental conditions and amendment levels required for optimal hydrocarbon biodegradation have been determined under laboratory incubations [18].

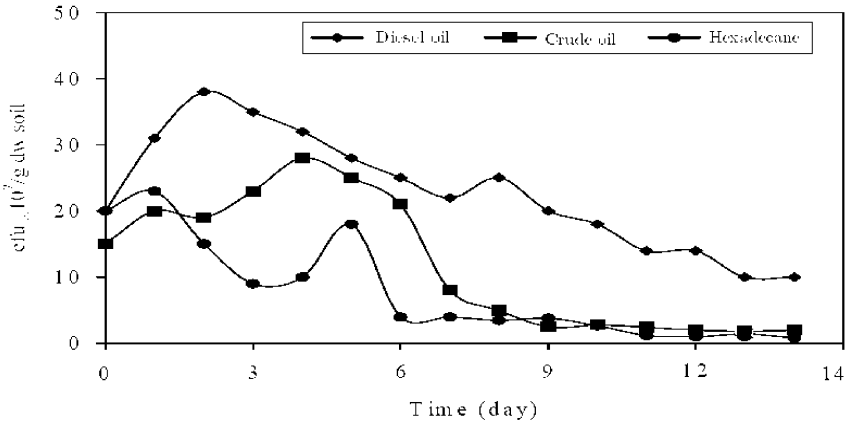

Fig 1: Time course of bacterial growth on petroleum hydrocarbon in 20% of the initial compound added

Table 1: Decontamination of petroleum hydrocarbons in soil

Petroleum fraction	Concentration	Initial concentration mg/g dw	Final concentration mg/g dw	Substrate biodegraded %
Diesel oil	10	24.9	5.45	78.1
	20		6.1	75.6
	30		6.9	72. 4
Crude oil	10	22.5	5.4	75.9
	20		4.9	78.1
	30		8.9	60.4
Hexadecane	10	24.4	2.6	89.3
	20		2.7	88.7
	30		2.8	88.3

Hydrocarbon loss by biological processes is the difference between the eliminated amounts of hydrocarbons in Poisoned control soils and non-poisoned soils. N-alkenes with the intermediate chain length (C_{10} - C_{24}) are degraded most rapidly. Short chain alkenes are toxic for many microorganisms but they generally evaporate rapidly. Very long chain alkenes are increasingly resistant to biodegradation [13].

Environmental biotechnology is an embodiment of several areas of research that is driven by service and regulation. The extensive utilization of crude oil as a major source of energy has increased the risks of accidental spills and hence pollution of the environment. Today, the need to reduce the negative impacts of oil pollution due to spills is motivating many researchers into innovations in various aspects of environmental biotechnology that will usher in sustainable development and sustainable environment. The integration of several of these technological advances for ameliorating the negative effects of oil spills in the environment will be most expedient and this review is aimed at highlighting many of these advances.

Biodegradation by indigenous bacteria is a feasible and promising treatment option for petroleum hydrocarbon-polluted soil. The levels of salinity and pH for optimal biodegradation by the indigenous hydrocarbon degraders are close to the natural conditions of the sample sites studied. Thus, successful bioremediation of oilfields might be accomplished by enhancing the activity of native bacteria through removing other environmental limitations on the growth and activities of hydrocarbon degraders. However, field pilot experiments on development of such effective methods for bioremediation of petroleum hydrocarbon-contaminated soil are necessary. Bioremediation of hydrocarbon-contaminated soils, which exploits microorganisms to degrade organic contaminants, has been established as an efficient, cost-effective and possible environmentally treatment [5, 6].

Although physical and chemical processes, such as dispersion, dilution, sorption, volatilization and abiotic transformations, are important means of hydrocarbon elimination, biodegradation is most often the primary mechanism for contaminant clean-up [6]. Thus, extensive degradation of crude oil probably requires metabolically diverse microbial communities [11,19-21].

The complex cooperatively between bacterial communities is possibly important during the disappearance of petroleum hydrocarbons, which might help to explain why individual isolates have lower biodegradation ability than consortia, especially for complex mixtures of compounds such as diesel oil and crude oil.

Bioremediation of oil-fields might be accomplished using these indigenous petroleum-degrading bacteria by enhancing their activity. Naturally, great care should be taken when results of laboratory experiments are extrapolated to field situations because liquid-culture studies in laboratory flasks do not reflect field conditions with a great accuracy. Field trials must be performed to corroborate conclusions drawn from laboratory experiments [18, 22].

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