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Chemometric assessment of enhanced bioremediation of oil contaminated soils

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HIGHLIGHTS

- The CHEMSIC method is an accurate tool for assessing the bioremediation efficiency.
- Bacterial enrichment and addition of nutrients enhance removal of TPHs in soil.
- Bacterial enrichment increases the degradation of *n*-alkanes and some PACs.
- Chemometrics is a comprehensive approach for monitoring of the degradation of petroleum hydrocarbons.

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ABSTRACT

Bioremediation is a promising technique for reclamation of oil polluted soils. In this study, six methods for enhancing bioremediation were tested on oil contaminated soils from three refinery areas in Iran (Isfahan, Arak, and Tehran). The methods included bacterial enrichment, planting, and addition of nitrogen and phosphorous, molasses, hydrogen peroxide, and a surfactant (Tween 80). Total petroleum hydrocarbon (TPH) concentrations and CHEMometric analysis of Selected Ion Chromatograms (SIC) termed CHEMSIC method of petroleum biomarkers including terpanes, regular, diaromatic and triaromatic steranes were used for determining the level and type of hydrocarbon contamination. The same methods were used to study oil weathering of 2 to 6 ring polycyclic aromatic compounds (PACs). Results demonstrated that bacterial enrichment and addition of nutrients were most efficient with 50% to 62% removal of TPH. Furthermore, the CHEMSIC results demonstrated that the bacterial enrichment was more efficient in degradation of n-alkanes and low molecular weight PACs as well as alkylated PACs (e.g. C_3–C_4 naphthalenes, C_2 phenanthrenes and C_2–C_3 dibenzothiophenes), while nutrient addition led to a larger relative removal of isoprenoids (e.g. norpristane, pristane and phytane). It is concluded that the CHEMSIC method is a valuable tool for assessing bioremediation efficiency.

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1. Introduction

Oil pollution is a major environmental challenge in oil producing countries. Iran is the world's fourth largest producer of crude oil and oil pollution is therefore widespread in this region during production and transport activities. Biological methods such as enhanced microbial degradation and phytoremediation are promising green and cost effective tools for large scale remediation [1–3]. However, the time span for biological methods is often long and the techniques are less efficient on highly polluted sites and for remediation of heavier oil products [4–6]. Therefore, finding new approaches to enhance efficiency of bioremediation is desired.

Biostimulation (i.e. nutrient-enhanced bioremediation) is a promising approach, which has been shown to increase biodegradation rates of petroleum hydrocarbons among other organic pollutants by providing limiting nutrients (e.g. nitrogen and phosphorous) for activity of indigenous degrading microorganisms in soil [2,7,8]. Introduction of a group of natural microbial strains or a genetically engineered variant to treat contaminated soil or water, which is called bioaugmentation has also been shown to be an effective method for elimination of organic pollutants in contaminated media [2,8]. Zhang et al. [9] among others have demonstrated an

Abbreviations: CHEMSIC, CHEMometric analysis of Selected Ion Chromatograms; PACs, Polycyclic Aromatic Compounds; TPH, Total Petroleum Hydrocarbons; SICs, Selected Ion Chromatograms.

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increase in bioaugmentation efficiency of soils contaminant with oil products by addition of wheat straw. Other studies have demonstrated that the addition of surfactants and hydrogen peroxide as well as organic wastes into the oil-contaminated soils increases bioremediation efficiency [10–12].

While considerable studies have been carried out to show enhancement of bioremediation of oil-contaminated soils, they are mostly based on bulk properties such as total petroleum hydrocarbon (TPH) concentrations and gravimetric analysis. Furthermore, only a few selected aliphatics, and polycyclic aromatic compounds (PACs) are typically used as indicators for oil pollution. Gas chromatography with mass spectrometry detection (GC-MS) combined with multivariate statistics for oil hydrocarbon fingerprinting (chemometrics) can provide a more comprehensive and accurate tool for monitoring the changes in the oil hydrocarbon profiles during bioremediation [13–16]. Two-dimensional gas chromatography has been used as a powerful technique for characterization of biological and physical weathering processes of oil complex mixtures at a molecular level in surface waters and sediments as well as marine and land oil spills [17–19].

A detailed characterization and understanding of oil weathering at the molecular level can be considered as an essential part of tiered approaches for forensic oil spill identification, risk assessment of terrestrial and marine oil spills, and the evaluation of bioremediation efficiencies [20]. Chemometrics is the application of statistical and mathematical methods to chemistry that allows for a more advanced treatment of data derived from complex chemical mixtures [20] and can be used to estimate the importance of the contributing weathering processes including physical, biological and chemical weathering [15].

The aim of this study is to determine the most efficient short-term strategy for bioremediation of heavily oil contaminated soils from three refinery areas in Iran: Tehran, Arak and Isfahan. Six bioremediation strategies (both biostimulation and bioaugmentation) were tested in a two-month laboratory experiment. These included bacterial enrichment; planting; addition of nutrients, hydrogen peroxide, molasses and the surfactant Tween 80. The assessment of bioremediation efficiency was based on TPH concentrations; and more detailed oil hydrocarbon fingerprinting using the CHEMSIC (CHEMometric analysis of Selected Ion Chromatograms) method developed by Christensen et al. [20–22]. The CHEMSIC method consists of principal component analysis (PCA) of pre-processed and combined sections of GC-MS/SIM chromatograms [20]. In this study, we used 4 selected ion chromatograms (SICs) of petroleum biomarkers for source comparison; and 25 SICs of PACs for assessment of oil weathering.

2. Materials and methods

2.1. Soil sampling

Soil samples were collected from three locations: Tehran refinery, Isfahan refinery and Arak refinery located in the central part of Iran. The soils were contaminated with crude oil and heating oil due to transportation accidents, leakage from oil pipes and reservoirs. Tehran and Isfahan are referred to as the oldest refineries operating from 1968 and 1979, respectively), while Arak is one of the newest refineries in Iran beginning operation in 1993. Three composite samples made from five sub samples from each location were collected. Samples were taken down to 20 cm depth, after discarding the upper 3 cm of the soil surface which was heavily weathered due to evaporation and photooxidation processes. Each soil sample was crushed, thoroughly mixed, homogenized and then sieved through a 2 mm pore size sieve to remove large debris. Samples were stored at 4 °C.

Soil characteristics such as nitrogen, phosphors and organic carbon contents, pH, cation exchange capacity (CEC), the amounts of silt, clay and sand were measured [23] at the biotechnology facility at Isfahan Science & Technology Town, Iran.

2.2. Isolation, identification and selection of bacteria for bacterial enrichment

Soil bacteria were isolated according to the method of Saadoun [24]. Briefly, sub samples of 1 g soil were suspended in 100 ml of sterile distilled water, agitated in an incubator-shaker (Innova 4430, GMI, USA) at 100 rpm for 30 min, then serially diluted from 10^{-1} to 10^{-6}. Aliquots of 0.1 ml from each dilution were spread over the surface of nutrient agar plates and incubated in 30 °C for 24 h. Colonies of the bacterial isolates were transferred into 50 ml mineral salts medium, which was a modification of Leadbetter and Foster [25], supplemented with 0.05% (v/v) crude oil sterilized by filtration through 0.45 μm membranes and incubated at 30 °C in an incubator-shaker (Innova 4430, GMI, USA) at 200 rpm for 21 days. Bacterial growth was determined at a 7-day interval by the physical appearance (i.e. turbidity) and by measuring the optical density (OD) at 540 nm using a spectrophotometer (Milton Roy Spectronic 21D, Rochester, USA).

Growth on crude oil was also determined by the 'hole-plate diffusion method' that was reported by Saadoun [24]. The results were recorded daily by the physical appearance of the bacterial growth surrounding the holes during 6 days. Monooxygenase biodegradation pathway was also used to detect the biodegradation of oil by bacteria as described by Saadoun [24].

The morphological characterization including colour, size, and colony form as well as biochemical tests (e.g. Gram stain test, oxidase, catalase, indole formation and glucose fermentation) were used for bacterial identification. Bacterial strains were identified based on Bergey's manual of systematic bacteriology [26,27]. Finally, the bacteria that tolerate, and degrade oil were selected for using in the experiment according to the results of the "hole-plate diffusion method" and monooxygenase biodegradation test.

2.3. Experimental setup

The experimental setup consisted of 63 microcosms including 9 control samples (triplicates of each soil type–Tehran, Arak and Isfahan); and triplicates for each soil type and treatment ($3 \times 3 \times 6 = 54$ samples). Each sample contained 0.5 kg in a plastic pot. The samples were incubated for 2 months under controlled conditions (28 ± 2 °C, 12 h light, 75% water holding capacity ensured by gravimetric method and maintained by adding distilled water). Sub samples were collected after 2 months of incubation. Initial soil samples from Tehran, Arak and Isfahan (day 0) were kept at −20 °C until extraction. Soils in microcosms were homogenized before sub-sampling except for planted soils, which were sub-sampled from the rhizosphere. All types of amendments were added to the soil by spraying them into a thin layer of soil and mixing thoroughly by hand. This ensured proper homogenization of amendments with soil [28].

Treatments were as follows:

(i) Control: No treatment except for homogenization and wetting. The controls included samples from day 0 (CNT_0) and after 2 months (CNT).
(ii) Microbial enrichment (ENT): Nutrient broth (Merck, Germany) was used for preparing the inoculation of oil degrading bacteria. The isolated bacteria were grown in nutrient broth (having 20 $\mu L\,L^{-1}$ crude oil as a carbon source, which was sterilized by filtration through 0.45 μm membranes) in an incubator-shaker (Innova 4430, GMI, USA) for

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