Chapter 17

Petroleum Degradation: Promising Biotechnological Tools for Bioremediation

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Additional information is available at the end of the chapter

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Abstract

One of the most common chemicals involved in the soil contamination or soil pollution is petroleum hydrocarbons (PHs). As we know that PH-contaminated soil affects human health directly, such as (i) contact with soil, (ii) via inhalation of vaporized contaminants, and (iii) infiltration of soil contamination into groundwater aquifers used for human consumption. Microbiological processes play an important role in the removal of PHs and take advantage of the catabolic versatility of these organisms to degrade such compounds either partially or completely (mineralization). Thus, the present chapter moves around the relationship of microorganisms with PHs. Based on this concept, this chapter has been designed to address the following relevant issues: How to isolate PH-degrading microorganisms by co-enrichment and optimized enrichment methods? How to study the microbial community structure by high-throughput sequencing method? What are the metabolic versatilities of microorganisms for degrading PHs? How to treat the environmental problems through biological means? What are the available ecotoxicity studies for the analysis of residual PHs after the microbiological treatment at the PHs-contaminated sites? Thus, the aim of this chapter is to explain the importance of microorganisms in cleaning the oil-contaminated environments.

Keywords: biodegradation, bioremediation, ecotoxicology, microorganisms, petroleum hydrocarbons

1. Introduction

The most common contaminant in the environment is crude oil and its derivatives. Due to their wide spread occurrence and severe risks they pose to human health and water bodies (surface as well as ground), they require intense remediation practices at the contaminated sites. Strictly speaking, contamination is strongly correlated with the degree of industrialization and intensity of chemical usage. All hydrocarbon compounds derived from petroleum

sources are generally described as total petroleum hydrocarbons (TPHs). Fuels such as petrol, diesel, kerosene, and lubricating oils/greases all come under the category of TPHs. Soil pollution by petroleum hydrocarbons (PHs) is mainly due to oil drilling, waste disposal (oil and fuel dumping), and accidental spilling as may occur during activities. Crude oil and refined fuel spills from tanker ship accidents have caused severe damages to ecosystems in many parts of the world. The quantity of oil spilled during accidents has ranged from a few million gallons to several hundred thousand gallons [1].

Chemically, hydrocarbons seem to be simple organic substances (comprising only carbon and hydrogen). However, there are many kinds of compounds with different chemical and physical nature. Analysis of the components at the spilled sites gives lots of information about their diversity. For instance, at the contaminated sites, TPH compounds that have an aliphatic structure (i.e., straight or branched chains of carbon molecules) will behave differently from aromatic compounds (ringed chains of carbons). Similarly, TPH compounds that have less carbon molecules (short-chain compounds) will also act differently. Solubility, volatility, and organic partitioning coefficients are greatly influenced by the number of carbon atoms. For example, compounds with less than 16 carbon atoms tend to be more mobile at the spilled sites due to their greater solubility, volatility, and lower organic partitioning coefficients. On the other hand, lightweight aromatic compounds, for example, benzene is highly toxic chemical. Generally, heavy weight TPHs have opposing properties, which are readily adsorb into the organic fractions of soil. Another important form of PHs is polycyclic aromatic hydrocarbons (PAHs), which are a class of chemicals that occur naturally in coal, crude oil, and gasoline. Basically, PAHs are neutral, nonpolar, heavy weight substances and composed of multiple aromatic rings. More importantly, PAHs have higher toxicity and are typically more persistent in the environment. They are also produced when coal, oil, gas, wood, garbage, and tobacco are burned. PAHs generated from these sources can bind to or form small particles in the air. Thus, PAHs making them of greater concern if they are released into the environment. Another important aspect of PHs is their forms or phases at the spilled site. This greatly depends on original composition of the source of spilled TPHs, geological and hydrogeological conditions at the spilled sites, and the age since the spillage occurred. More often, upon the spillage, the majority of TPHs mass will be partitioned within the soil phase. In certain instances, TPHs are able to float on the surface of the water table. In this form, TPHs are encountered as a phase-separated liquid and are also called light nonaqueous phase liquid (LNAPL), which is principally due to their buoyancy. Other two important forms of TPHs at the spilled site are dissolved and vapor forms. A percentage of TPHs will also be dissolved into the groundwater or trapped as a vapor within the soil "pore-space" in the unsaturated zone. Fate of crude oil at the spilled site is shown in **Figure 1**.

Human health effects from environmental exposure to PHs are vary, principally depends on type and quantity of PHs. For instance, large amounts of naphthalene in air can irritate eyes and breathing passages. Moreover, blood and liver abnormalities were observed in workers who have been exposed (either skin contact or inhalation of vapors) to large amounts of naphthalene [2]. Several PAHs (pure or mixture) are considered to be cancer-causing chemicals. In well-established animal model studies, PAHs were linked to skin, lung, bladder, liver, and stomach cancers [3]. Adult exposure to PAHs has been linked to cardiovascular disease [4].

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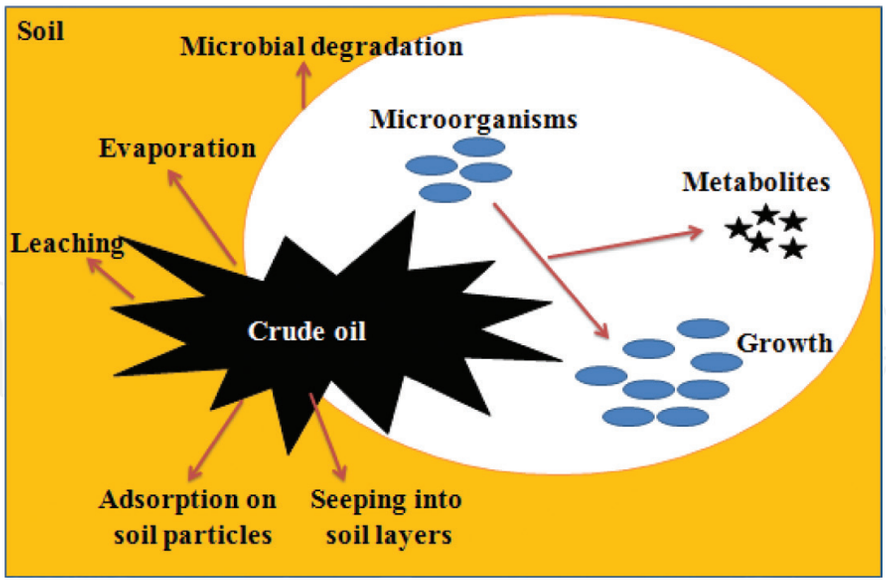

Figure 1. Fate of crude oil during land spillage.

Nevertheless, human health effects from environmental exposure to low levels of PAHs are unknown. Additionally, environmental impacts of PHs are numerable. There are many reports on the contamination of drinking water supplies by oil spillage [5]. Contamination can have an economic impact on tourism and marine resource extraction industries. More importantly, marine animals and birds exposed to oil spills are severely affected. Because of the health implications, less than 1% of oil-soaked birds survive, even after cleaning [6]. Additionally, fluctuations in body temperature, hypothermia, blindness, dehydration, impaired digestive process, and disorder of lungs and liver were observed in many heavily furred marine mammals exposed to oil spills.

By considering all the above facts, remediation and reclamation of PH-contaminated sites are essentially important to protect the health of ecosystem. There are two main remediation technologies, namely *ex situ* and *in situ* methods. *Ex situ* methods involve excavation of affected soils or extraction of contaminated groundwater and subsequent treatment at the surface. These methods consist of soil excavation and disposal to landfill and groundwater "pump and treat." In contrast, in *in situ* methods, the contaminated soils or groundwater are treated at the spill sites. These methods include but are not limited to solidification and stabilization, soil vapor extraction, permeable reactive barriers, monitored natural attenuation, bioremediation-phytoremediation, chemical oxidation, and steam-enhanced extraction and *in situ* thermal desorption. However, the further sections of this chapter give detailed information about microbial remediation of crude oil contaminated soil.

2. Enrichment and isolation of crude oil-degrading microorganisms

An enrichment culture is a medium with specific and known qualities that favors the growth of a particular microorganism. Enrichment cultures are used to increase the small number

of desired microorganism to the detectable level. Enrichment cultures are often used for soil sample. This type of technique is very useful for the detection and isolation of PH-degrading microorganisms from PH-contaminated soil. Brief description of enrichment method to be used for the isolation of crude oil-degrading bacteria is presented below.

Two 100-mL erlenmeyer flasks containing 25 mL of mineral salt medium (MSM) are prepared separately, one is for the isolation of crude oil-degrading bacteria and another is used for the isolation of crude oil-degrading fungi. Since bacteria and fungi have been reported as principal microorganisms of PH degradation, the information provided in this chapter is related to these two organisms only, unless otherwise it is stated. The composition of MSM is as follows (g L^{-1}): NaCl, 5.0; KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 1.0; $K_2HPO_4 \cdot 3H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.25; $NaNO_3$, 2.0; $FeCl_2 \cdot 4H_2O$, 0.02; and $CaCl_2$, 0.02. The pH of the medium in first and second flaks is adjusted to 7.2 and 5.5 for bacteria and fungi, respectively. After sterilizing the medium at 121°C for 30 min [7], 1.0% of crude oil contaminated soil is used as an inoculum to inoculate the medium separately. Then the medium is enriched for 7 days at respective temperatures (25°C for fungi and 30°C for bacteria) and 180 rpm on the rotary shaker. The culture is enriched by four consecutive inoculations of 1.5-mL inoculum to 100-mL Erlenmeyer flasks containing 25 mL of fresh MSM medium. Following enrichment, parts of the medium are plated onto the MSM medium containing 2.0% of agar and 1.0% of crude oil and incubated for 3–7 days separately for bacteria and fungi [8]. Finally, different pure colonies obtained from the plates are stored in the Luria-Bertani medium (bacteria) or Czapek Dox medium (fungi) with 15% of glycerol at −80°C until further use. Schematic representation of enrichment and isolation of crude oil-degrading bacteria is shown in **Figure 2**. However, microorganisms have also been enriched and isolated by using methods of various modifications. For instance, oil-degrading bacteria were isolated using sterile crude oil as the medium [9].

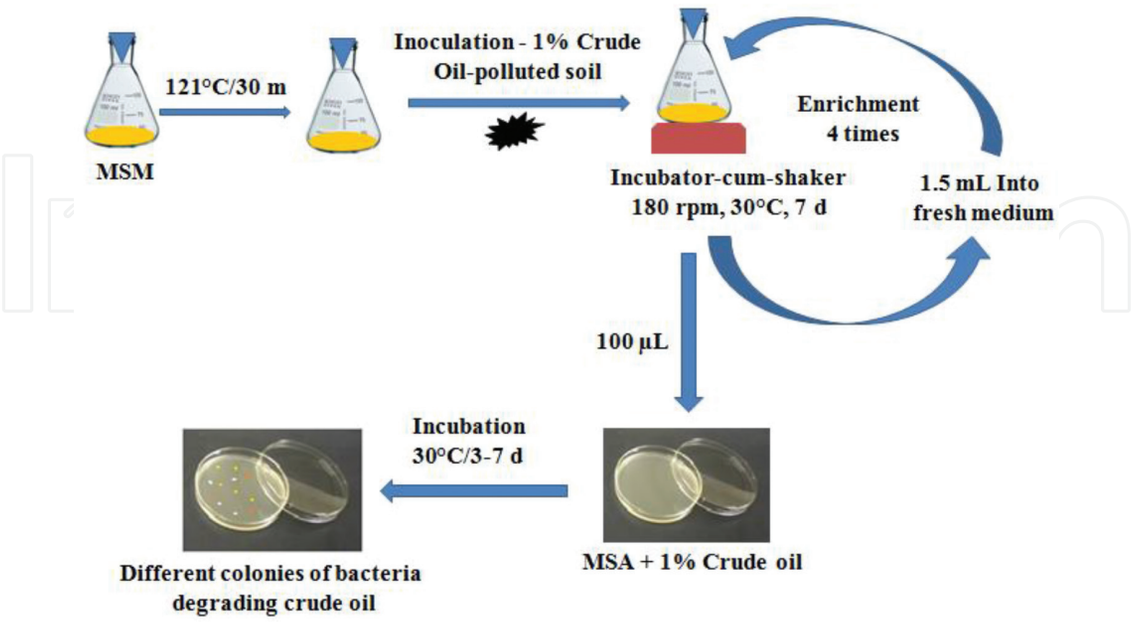

Figure 2. Enrichment and isolation of crude oil-degrading bacteria.

3. Microbial ecology of oil fields

Knowing which organisms are present in a particular habitat is critical to research in microbiology. Sequencing DNA enables researchers to determine which types of microbes may be present at the site of sample collection. Nowadays, DNA-based technologies playing a major role in the analysis of microbial communities at the PHs or organic compounds contaminated soils, water, and sediments [10]. A study of genetic material recovered directly from environmental samples is called "metagenomics." This field of science may also be referred to as "environmental genomics," "ecogenomics," or "community genomics." In early days, cultivated clonal cultures, early environmental gene sequencing cloned specific genes (16S or 18S rRNA genes) were used to produce a profile of diversity in a natural sample. A vast majority of microbial biodiversity had been missed by cultivation-based methods [11]. It is being frequently reported that the environmental samples contain more number of noncultivable microorganisms than cultivable one. Thus, recent studies focusing on either "shotgun" or Polymerase chain reaction (PCR) directed sequencing to get largely unbiased samples of all genes from all the members of the sampled communities [12]. Due to the ability of metagenomics to reveal the previously hidden diversity of microbial life and as the price of DNA sequencing continues to fall, metagenomics now allows microbial ecology to be investigated at a much greater scale and detail than before.

In the analysis of microbial community structure of PH-contaminated soil or sediment, the total chromosomal DNA is to be extracted by using one of several available commercial DNA kits. The extracted DNA is stored at −20°C until further use. In order to analyze the bacterial community structure, 16S rRNA genes are PCR amplified from the bulk DNA by using PCR reaction mixture. The volume of the PCR mixture is usually 20–50 μL, which contains template DNA, universal primers (e.g., 27F/1492R), each of four dNTPs, polymerase enzyme buffer, and polymerase enzyme (Taq or pfu polymerases). Likewise, the 16S rRNA region is amplified by PCR using the forward primer 27F (5-AGA GTT TGA TCC TGG CTC AG-3) and reverse primer 1492R (5-CGG CTA CCT TGT TAC GAC TT-3) [13]. Generally, DNA amplification is performed under the specified cycling conditions: 1 cycle of 2 min at 94°C, then 25 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final cycle of 10 min at 72°C. After amplification, PCR products, also called "amplicons," are tested by 2% agarose gel to confirm the specific length of DNA amplicons. Furthermore, these amplicons are purified for sequencing purpose. Nowadays, gel extraction and purifications combo kits are available commercially. These kits have ability to perform both a gel extraction and a purification of amplicons in a single step. In the case of fungal community structure analysis, ITS (internal transcribed spacer) regions of their whole DNA are amplified by using ITS1 and ITS2 primers. Finally, purified PCR products are used for sequencing purpose.

There are several high-throughput methods (formerly "next-generation") available for sequencing the genome. These methods include massively parallel signature sequencing (MPSS), polony sequencing, 454-Pyrosequencing, Illumina (Solexa) sequencing, SOLiD sequencing, Ion Torrent semiconductor sequencing, DNA nanoball sequencing, Heliscope single-molecule sequencing, single-molecule real-time (SMRT) sequencing, and nanopore DNA sequencing. Comparison of selective high-throughput sequencing methods [14, 15] is shown in **Table 1**.

Method	Read length	Accuracy	Time per run	Cost per 1 million base pairs in US$	Advantages	Disadvantages
Chain termination (Sanger)	400–900 bp	99.9%	20 min–3 h	2400	Long individual reads, useful for many applications	More expensive, time-consuming step of PCR
Pyro-sequencing	700 bp	99.9%	24 h	10	Long read size, fast	Runs are expensive, homopolymer errors
Ion semiconductor (Ion Torrent)	400 bp	98%	2 h	1	Less expensive equipment, fast	Homopolymer errors
Sequencing by synthesis (Illumina)	HiSeq 2500: 50–500 bp	99.9%	1–11 d	0.05–0.15	Potential for high sequence yield	Very expensive equipment requires high concentrations of DNA

Table 1. Comparison of selective high-throughput sequencing methods.

Once sequences are ready, they are analyzed using different bioinformatics tools (**Figure 3**). For instance, QUIIME software package (Quantitative insights into microbial ecology) [16] is used to analyze the sequences. More often, Basic Local Alignment Search Tool (BLAST) program of the National Centre for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) is used to search and identify the closest species. In-house Perl Scripts are used to analyze alpha- and beta-diversities within and among the samples, respectively. In addition to these, QUIIME software package (http://qiime.org/) and UPARSE pipeline (http://drive5.com-/uparse/) are used to analyze the reads and pick operational taxonomic units (OTUs). Then, sequences are assigned to OTUs at particular percent similarity. UPARSE is a method for generating clusters (OTUs) from next-generation sequencing reads of marker genes such as 16S rRNA, the fungal ITS region and the COI gene. Finally, representative sequence for each OUT is picked, and RDP (Ribosomal database project) classifier [17] is used to assign taxonomic data to each representative sequence. RDP provides quality-controlled, aligned, and annotated bacterial and archaeal 16S rRNA gene sequences and fungal 28S rRNA gene sequences and a suite of analysis tools to the scientific community. Finally, Simpsons Index of Diversity [18, 19] is used to define the community structure. Simpson's Index of Diversity = 1 − D:

$$D = \sum n(n-1)/N(N-1) \tag{1}$$

where n = total number of organisms of a particular species and N = total number of organisms of all species. More recently, in Ref. [20] researchers have studied and reported about the microbial community structure in crude oil-contaminated seawaters by using bioinformatics tools such as QUIIME, UPRASE, and RDP.

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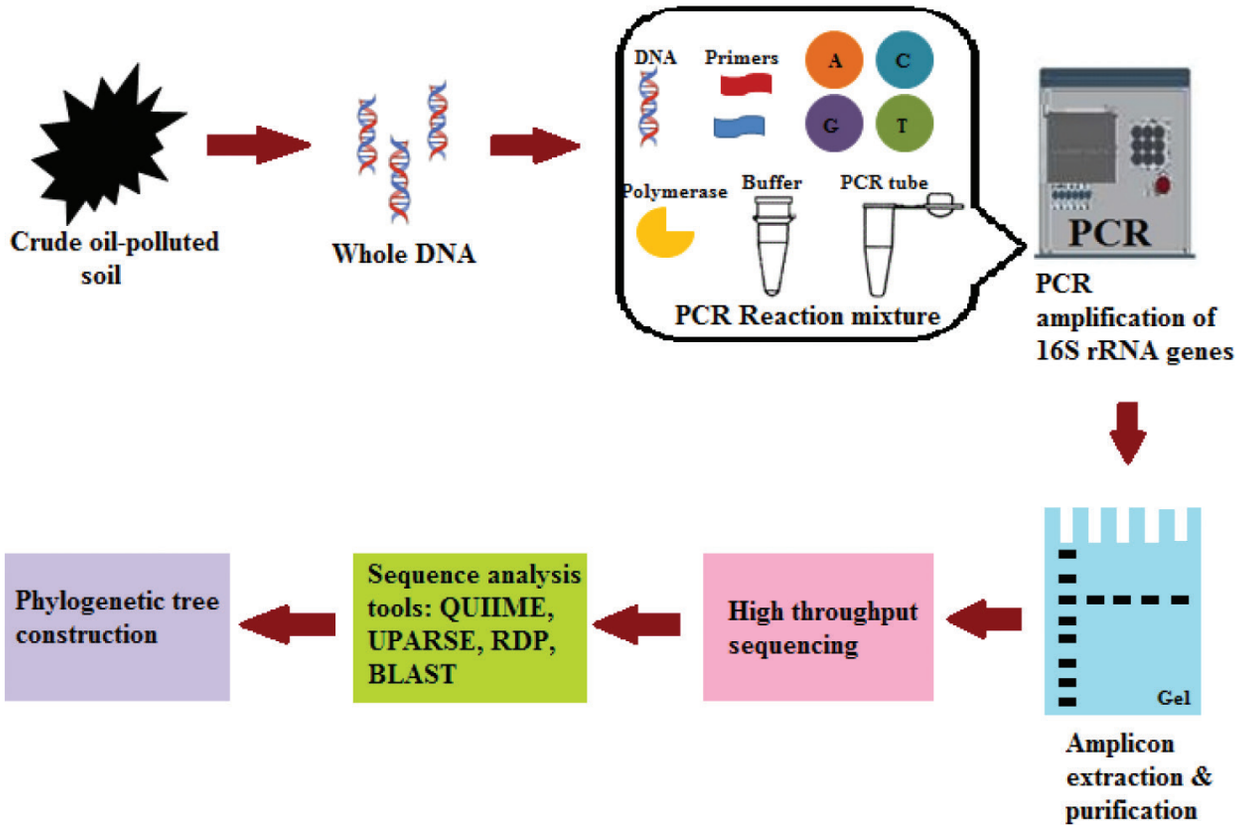

Figure 3. Schematic representation of microbial community analysis of crude oil-polluted soil by using high throughput sequencing methods.

4. Microbial degradation of PHs

Biodegradation is the disintegration of materials by bacteria, fungi, or other biological means. Substances to be degraded by microorganisms are generally organic materials. Materials in organic nature are degraded aerobically with oxygen or anaerobically without oxygen. More often, organic materials are the good nutrient sources for microorganisms. Since there is a large diversity in the microorganisms, a huge range of compounds are biodegraded, including hydrocarbons (e.g., oil), polychlorinated biphenyls (PCBs), PAHs, pharmaceutical substances, etc.

In most of the studies, microbial groups such as bacteria, yeast, and fungi have been identified as principal agents in the degradation of PHs, even though their degradation efficiencies are varying. However, bacteria are the most active and primary degraders of spilled oil in the environment [21], and some of them are known to grow exclusively on PHs as their sole carbon and energy source. In one of our recent investigations, we isolated two bacterial strains (*Bacillus thuringiensis* strain B3, *B. cereus* strain B6) from Ecuadorian oil fields, they grew exclusively in MSM containing 1% diesel as their carbon source [22]. So far, several bacterial genera, namely, *Acinetobacter*, *Aeromicrobium*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Burkholderia*, *Corynebacterium*, *Dietzia*, *Flavobacterium*, *Gordonia*, *Micrococcus*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas*, etc., isolated from petroleum contaminated soil proved to be the potential organisms for PHs' degradation [23, 24]. Similarly, several fungal genera isolated

from PH-contaminated soils, and reported as PH degraders. They include *Amorphoteca*, *Aspergillus*, *Candida*, *Cochliobolus*, *Fusarium*, *Graphium*, *Neosartorya*, *Penicillium*, *Phaenerochaete*, *Pichia*, *Pseudallescheria*, *Talaromyces*, and *Yarrowia* [24–27]. Recently, we found two indigenous fungal strains in Ecuadorian oil fields, they were belonging to the genus *Geomyces*, which could remove 77–80% of crude oil in medium and soil experiments, respectively [28]. In Ref. [29], researchers found 30% removal of crude oil by immobilized bacterial cells. In a more recent laboratory-based study, *Pseudomonas* sp. has removed 74% of PHs from the crude oil sludge in 7 days [30].

In the natural environment, biodegradation of crude oil involves a succession of species within the consortia of the present microbes. Impact of a microbial consortium on a contaminant is always much higher than by an individual organism. A single species can metabolize only a limited range of hydrocarbon substrates. Instead, a consortium of many different bacterial and/or fungal species, with broad enzymatic capacities, can degrade the maximum amount of contaminant. So far, several studies focused on the microbial degradation of PHs [8, 22, 31–34]; these studies reported that the microorganisms possess specific enzyme systems that enable them to degrade and utilize hydrocarbons as their sole carbon and energy sources [35]. Another important aspect is the production of biosurfactants by microorganisms during PHs degradation. Biosurfactants are the extracellular surfactants of the microorganisms, play major role in enhancing the bioavailability of contaminant to the microorganisms.

There are many environmental factors come into action during the degradation of PHs by microorganisms either *in vitro* or *in vivo*. Considering the physical factors, temperature plays an important role in biodegradation of hydrocarbons. It acts directly by affecting the chemistry of the pollutants, physiology, and diversity of the microbial flora. The highest degradation rates can be seen in the range of 30–40°C, 20–30°C, 15–20°C in soil, freshwater, and marine environments, respectively [36, 37]. Similar to above findings, members of *Geomyces* have shown optimum sporulation rates at 25°C on the medium containing either diesel or crude oil [28]. Another considerable factor that influences the microbial degradation of PHs is nutrients. Nitrogen and phosphorous are very important elements; they influence the rate of degradation greatly. Since PHs mainly contain carbon and hydrogen, microorganisms need additional elements for their growth on PHs. Additionally, pH, concentration, type and age of the contaminants also play major role in influencing the degradation of PHs by microorganisms.

With respect to the aerobic and anaerobic environments, nevertheless, the most rapid and complete degradation of the majority of organic pollutants is principally achieved under aerobic conditions. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes [38]. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all [39]. Several microbial enzymes have been identified as important agents in the degradation of PHs. For instance, oxygenases [40], monooxygenases [41], dioxygenases [42], and hydrolases [43] were among them.

The most widely used technique for the detection of residual PHs during microbial degradation is gas chromatography-flame ionizing detection (GC-FID). Helium or hydrogen or nitrogen is used as inert carrier gas in gas chromatography. Carrier gas carries the gaseous mixture

or aqueous liquids with boiling points < 400°C, which are to be analyzed. Analysis takes place in a capillary column onto a detector. This allows better resolution of components in complex mixtures. This method determines the content of TPHs in the range C_{10}—C_{40} (*n*-alkanes), from solids including soils and wastes. GC-FID is used for both quantitative and qualitative applications with detection limits of 10 mg TPHs per kg soil. There is another method in which GC is coupled with mass spectroscopy called GC-MS. MS is described as a universal detector because of its versatility in the measurement of TPHs and PAHs. Another analytical method is available for the characterization of PHs, called infrared spectroscopy (IR). In this method, a spectrum is produced with stretching and bending vibrations associated with a molecule when it absorbs energy in the IR region of the electromagnetic spectrum. The spectra of hydrocarbon derivatives originate mainly from combinations or overtones of the C—H stretching modes of saturated CH_2 and terminal —CH_3 or aromatic C—H functional groups. Thus, IR-based detection is very helpful the elucidation of functional groups of residual and parent PHs during microbial degradation. More recently, in Ref. [44], TPHs in a biopile system of crude oil-contaminated dessert soil were measured by using "in-house" gravimetric and Fourier transform infrared spectroscopy (FTIR) methods.

5. Bioremediation

Removal or neutralization of pollutants from a contaminated site by using organisms is called bioremediation. It is one type of waste management technique. Principally, hazardous substances are broken down to less toxic or nontoxic substances by organisms. Bioremediation technologies have different approaches. In one kind of approach, the bioremediation process can be either *in situ* or *ex situ*. The *in situ* approach involves treating the contaminated soil or water at the site of contamination, whereas the *ex situ* approach involves the removal of contaminated materials to be treated elsewhere. There is another kind of approach in the bioremediation process in which bioremediation can be achieved either by biostimulation or bioaugmentation. Biostimulation is a widely used approach, which involves stimulating naturally occurring microbial communities, either by nutrients or other needs (such as pH, moisture, aeration, electron donors, electron acceptors, etc.), to break down a contaminant. In bioaugmentation, organisms selected for high degradation efficiencies are used to inoculate the contaminated site. Most widely used bioremediation methods are shown in **Figure 4**.

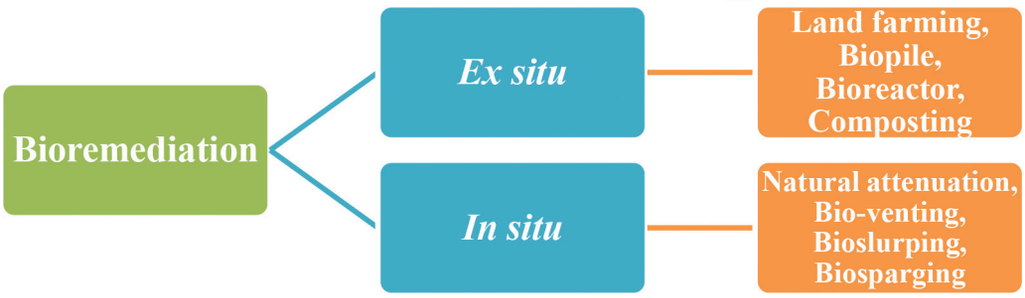

Figure 4. Types of bioremediation techniques.

5.1. Land farming

Land farming is the simplest method, which is inexpensive and requires less equipment (**Figure 5a**). It can be used either in the form of *ex situ* or *in situ* mode. The *in situ* or *ex situ* type of land farming method is applied when pollutant lies <1 or >1.7 m below the ground level, respectively [45]. Land farming consists of careful application of excavated polluted soil on a fixed layer of support above the ground surface. This allows the aerobic biodegradation of pollutant by autochthonous (indigenous) microorganisms. The major activities of land farming are soil tillage, which brings about aeration, addition of N-P-K fertilizers, and irrigation. Expectedly, all such operations greatly stimulate the indigenous microorganisms to enhance bioremediation during land farming. Practically, at the field level, this method is giving encouraging results. For instance, a land farming-based field trial experiment conducted in Canada for 3 years, where there was 80% removal of diesel contaminant from the soil [46].

5.2. Biopile

In this approach, there is above-ground piling of excavated polluted soil followed by amendments (nutrients and aeration) (**Figure 5b**). The remediated soil is placed in a liner to prevent further contamination of the soil, they may also be covered with plastic to control runoff, evaporation, and volatilization. This technique is widely used in nowadays due to easy controlling of nutrients, aeration, and temperature [47]. When the biopile system was combined with bioaugmentation and biostimulation approaches, >90% of TPHs were reduced in PH-contaminated soil in 94 days [48]. Nevertheless, the biopile system has its own disadvantages, such as conserve much space, robust engineering, cost or maintenance, and operation, lack of power supply at remote areas, heat generation resulted in the decreased microbial activities. Periodic turning (to enhance the aeration and subsequent hike in the biodegradation activities) of piled polluted soil is the principle of another bioremediation method called "windrows."

5.3. Bioreactor

A bioreactor is a vessel in which contaminated materials are converted to specific product(s) following series of biological reactions. There are different operating modes of bioreactor, such as batch, fed-batch, sequencing batch, continuous, and multistage. Polluted samples can be fed into a bioreactor either in the form of solid or slurry. One of the major advantages of bioreactor-based bioremediation is excellent control of bioprocess parameters such as temperature, pH, agitation and aeration rates, and substrate and inoculum concentrations. Another advantage of bioreactor is that it can be used for the treatment of either polluted water or soil. In a practical application of stirred tank bioreactor (2.5 L), 82–97% of TPHs were removed from crude oil-polluted sediment [49]. Yet, bioreactor-based bioremediation is not a full-scale practice due to several reasons. This approach is cost ineffective, because volume of polluted sample to be treated may be too large, requiring more manpower, capital, and safety measures for transporting the samples to the treatment site. Another disadvantage is due to several bioprocess parameters or variables of a bioreactor, if any parameter that is not properly controlled at optimum, this in turn will reduce microbial activities and will make process less effective. In addition to these, pollutants are likely to respond differently to different bioreactors. Thus, it is difficult to design a specific reactor for every pollutant.

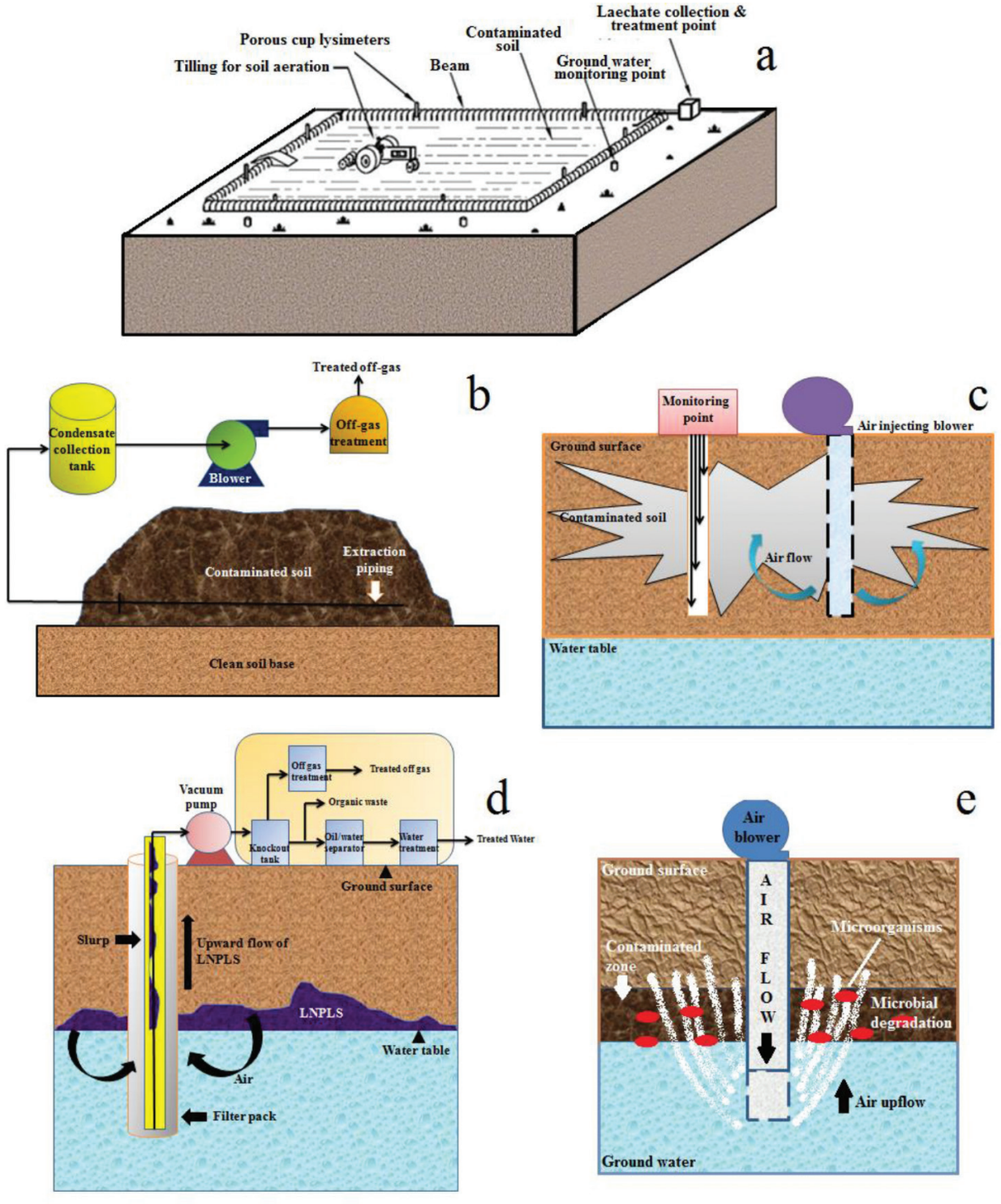

Figure 5. Bioremediation methods—(a) landfarming, (b) biopile, (c) bioventing, (d) bioslurping, and (e) biosparging.

5.4. Composting

Composting is a process of piling contaminated-soil along with organic substances such as manure, yard waste, or food-processing wastes. These are often added to supplement the amount of nutrients and readily degradable organic matter in soil. Stimulation of microbial

growth by added nutrients results in effective biodegradation in a relatively short period of time. Efficiency of compositing in the removal of PHs from soil has been tested practically by using several lab- and field-scale studies. For instance, 85% reduction in diesel content was reached when a soil spiked with diesel oil was mixed with biowaste (vegetable, fruit, and garden waste) at a 1:10 ratio (fresh weight) and composted in a monitored composting bin system for 12 weeks [50].

5.5. Natural attenuation

Reduction of concentration and amount of pollutants at contaminated sites by natural process is called "natural attenuation." It can also be termed as intrinsic remediation, bioattenuation, and intrinsic bioremediation. In the process of natural attenuation, contaminants are left on the site and the naturally occurring processes are left to clean up the site. Several processes are come into action during natural attenuation. For example, biological degradation, volatilization, dilution, dispersion, dilution of the contaminant and sorption of the contaminant onto the organic matter, and clay minerals in the soil. It is mainly used to remediate the contaminated aquifer when the contamination source has been removed. In particular, it is used for benzene, toluene, ethylbenzene, and xylene (BTEX) and more recently for chlorinated hydrocarbons. Other contaminants that could potentially be remediated by natural attenuation include pesticides and inorganic compounds. The success of natural attenuation greatly depends on the subsurface geology, hydrology, and microbiology. Major disadvantages of natural attenuation are as follows: (i) it is relatively very slow process, since it is nonengineered biodegradation process; (ii) long-term monitoring is an absolute necessity since there must be no risk to the environment and to humans.

5.6. Bioventing

Bioventing is an *in situ* remediation technology, it is used to treat the contaminated groundwater system (**Figure 5c**). However, recently, this technique has also been used to remediate contaminated soil. Bioventing enhances the activity of indigenous microorganisms and stimulates the natural *in situ* biodegradation of hydrocarbons by inducing air or oxygen flow (by direct air injection), and nutrients into the unsaturated zone. In a field-level application of bioventing process for cleaning the phenanthrene-contaminated soil, in Ref. [51], researchers observed 93% contaminant removal after 7 months.

5.7. Bioslurping

Bioslurping is a unique *in situ* technique, is a combination of bioventing and vacuum-enhanced pumping, and is used to bioremediate soils and water (**Figure 5d**). Principle of this method is pumping or separation of free-product that is lighter than water (light nonaqueous phase liquid or LNAPL) to recover free product from the groundwater and soil. The bioslurping system uses a "slurp" tube that extends into the free-product layer; the pump draws liquid (including free-product) and soil gas up the tube in the same process stream. Thus, slurp is much similar to a straw in a glass draws liquid. The pumping mechanism brings about upward movement of LNAPLs to the surface, where it becomes separated from water and

air. Once free products (contaminants) are separated, there is final treatment of contaminants by a conventional bioventing system to complete remediation process. This technique is cost effective because only a small amount of groundwater and soil vapor are pumped at a time, therefore the treatment plant used to treat the vapor and free product can be small.

5.8. Biosparging

It is also another *in situ* remediation technique. In biosparging, like bioventing, there is injection of air into soil subsurface to stimulate microbial activities in order to promote pollutant removal from polluted sites (**Figure 5e**). However, unlike bioventing, air is injected at the saturated zone. This causes upward movement of volatile organic compounds to the unsaturated zone to promote biodegradation. Biosparging has been widely used in treating aquifers contaminated with petroleum products, especially diesel and kerosene. This technique has shown effective results when applied to contaminated ground water. Practically, biosparging was used to clean benzene, toluene, ethylbenzene, and xylene (BTEX)-contaminated ground water, where they observed >70% reduction in BTEX [52].

5.9. Ecotoxicology

Ecotoxicology is the study of the effects of toxic chemicals on biological organisms, especially at the population, community, ecosystem levels. With regard to the present contest, ecotoxicity tests are conducted after the completion of bioremediation experiments. Toxicity of residual PHs and/or their products of microbial degradation present in the soil samples are tested though the survival, growth, behavior, and reproductions of organisms. Hence, bioassays can serve as a complementary tool in environmental risk assessment of bioremediated places, which help to determine whether the contaminant concentration at remediated sites is high enough to cause adverse effects on organisms. Frequently used toxicity tests are shown in **Figure 6**.

5.10. Earthworm survival tests

The common earthworm species, *Eisenia fetida*, is used to determine acute toxicity of the PH-contaminated soils before, during and after bioremediation. In this method, animals (~10) are placed into soil (~200 g) in 1-L wide-mouth jars with loose fitting lids. Lethal concentration-50

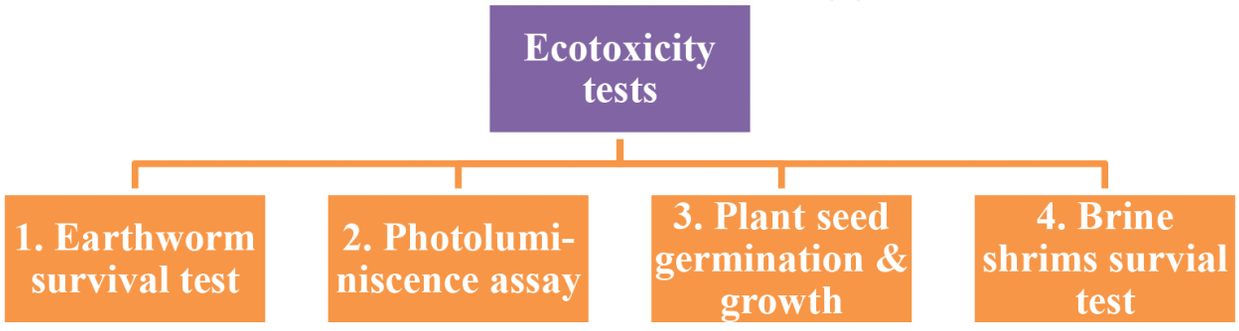

Figure 6. Types of ecotoxicity tests.

(LC50) for each soil is estimated using five concentrations of bioremediated soil (100, 50, 25, 12.5, 6.5, and 0%) prepared with control (contaminant free) soil. The soil water content is adjusted as per the requirement. Surviving earthworms are counted after 14 days of incubation at room temperature under constant fluorescent lighting conditions. Survival percent is inversely proportional to the toxicity of PHs. In a bioremediation experiment, it was found that the earthworm survival percentages were 28 and 100 after 4 and 12 weeks of treatment of heavy oil-contaminated soil [53].

5.11. Photo luminescence assay

In this method, there is a response of luminescent bacteria (*Photobacterium phosphoreum*) to residual PHs present in the treated contaminated-soils. However, this process needs special equipment and reagents such as Microtox analyzers and solid-phase test kits. Initially, soil dilutions are prepared (with Microtox diluent) and incubated for 20 min with reconstituted lyophilized bacteria. During the incubation, photoluminescence activity is induced in the bacteria (by kit reagents). Finally, activity of photoluminescence is detected by Microtox analyzer. Higher toxicity of PHs results in the lesser luminescence activity and vice versa. Photo luminescence assay is widely used in the bioremediation experiments. For instance, in Ref. [53], there was an observation of the loss of Microtox inhibiting activities by bioremediated soils, which were treated for 3 months.

5.12. Plant seed germination and growth

Plants depend on soil for germination and growth. Therefore, any alterations in the seed development may reflect the presence of toxic substances in the soil. Seed germination tests in ecotoxicological assays are considered short-term and evaluate acute toxicity effects. The effects of untreated and bioremediated oil soils are determined by using different plant species such as corn, wheat, oat, grass, cowpea, garden cress, etc. In this method, oily and oil-free soils are dispensed into wood or plastic containers having sufficient number of cells. Each cell should accommodate approximately 100 g soil. Then, 5–10 seeds are placed 1–1.5 cm below the soil surface. Generally, seed cultures are exposed to 12-h light/dark cycles at a soil surface light intensity of 310–350 lm with fluorescent lamps. Room temperature is maintained at 20–23°C and around 30% soil moisture capacity is maintained by spraying the soil surface with water. Time and germination percentages of seeds, plant growth (mg dry weight/plant) are determined before and after bioremediation. In one of our most recent investigations [54], we observed substantial improvement in germination time and percent germination of cowpea seeds in bioremediated soil over control soil.

6. Summary

Taken together, details provided in this chapter would seem to suggest that microbial processes are favorable tools for remediation of oil-contaminated sites. In this area, genome-based global studies are attracting widespread interest due to better understanding of metabolic and regulatory network, new information on the evolution of microbial degradation pathways and

molecular adaptability to environmental changes. Methods that we described in this chapter are essentially the same as we used previously in lab- and at field-based experiments in Ecuador. Our research underlined the importance of native microflora (*Bacillus cereus*, *Bacillus subtilis*, *Geomyces* sp., *Geomyces pannorum*) of Ecuadorian amazon rainforest in degrading petroleum hydrocarbons and metal biosorption. The most important limitation lies in "bioaugmentation," where adaptability of microorganisms to new environment is limited by multiple existing local environmental conditions. The findings of this study indicate that "biostimulation" is practically and economically more feasible than "bioaugmentation" for cleaning the oil-polluted sites. Future investigation focusing on "How to improve porosity and aeration of the contaminated soil?" is considerably important for biostimulation-based remediation techniques. Mixing of soils with rice hulls causes increased porosity and aeration. Additionally, soil treatment with hydrogen peroxide increases the oxygen content in the soil. Future studies on the current topic are therefore recommended in order to validate applicability of biostimulation for cleaning the petroleum hydrocarbons-contaminated soils on a large scale.

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References

[1] Oil spill. 2016. Available from: https://en.wikipedia.org/wiki/Oil_spill#cite_note-34 [Accessed: December 20, 2016]

[2] CDC. Polycyclic Aromatic Hydrocarbons (PAHs). 2009. Available from: https://www.epa.gov/sites/production/files/2014-03/documents/pahs_factsheet-_cdc_2013.pdf [Accessed: December 15, 2016]

[3] Bostrom CE, Gerde P, Hanberg A, Jernstrom B, Johansson C, Kyrklund T, Rannug A, Tornqvist M, Victorin K, Westerholm R. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. Environmental Health Perspectives. 2002;**110**(Suppl 3):451-488

[4] Korashy HM, El-Kadi AOS. The role of aryl hydrocarbon receptor in the pathogenesis of cardiovascular diseases. Drug Metabolism Reviews. 2006;**38**(3):411-450

[5] Oil Spill Disrupts Water Supply—Nation—The Star Online. 2012. Available from: http://www.thestar.com.my/news/nation/2012/03/05/oil-spill-disrupts-water-supply/ [Accessed: 20 April, 2015]

[6] Expert Recommends Killing Oil-Soaked Birds. Spiegel Online. 2010. Available from: http://www.spiegel.de/international/world/gulf-of-mexico-spill-expert-recommends-killing-oil-soaked-birds-a-693359.html [Accessed: 10 January, 2017]

[7] Liu YC, Li LZ, Wu Y, Tian W, Zhang LP, Xu L, Shen QR, Shen B. Isolation of an alkane-degrading *Alcanivorax* sp. strain 2B5 and cloning of the alkB gene. Bioresource Technology. 2010;**101**:310-316

[8] Wang XB, Chi CQ, Nie Y, Tang YQ, Tan Y, Wu G, Wu XL. Degradation of petroleum hydrocarbons (C6-C40) and crude oil by a novel *Dietzia* strain. Bioresource Technology. 2011;**102**(17):7755-7761

[9] Chai LJ, Jiang XW, Zhang F, Zheng BW, Shu FC, Wang ZL, Cui QF, Dong HP, Zhang ZZ, Hou DJ, She YH. Isolation and characterization of a crude oil degrading bacteria from formation water: Comparative genomic analysis of environmental *Ochrobactrum intermedium* isolate versus clinical strains. Journal of Zhejiang University Science B. 2015;**16**(10):865-874

[10] Paissé S, Coulon F, Goñi-Urriza M, Peperzak L, McGenity TJ, Duran R. Structure of bacterial communities along a hydrocarbon contamination gradient in a coastal sediment. FEMS Microbiology Ecology. 2008;**66**:295-305

[11] Hugenholtz P, Goebel BM, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology. 1998;**180**(18): 4765-4774

[12] Eisen JA. Environmental shotgun sequencing: Its potential and challenges for studying the hidden world of microbes. Public Library of Science Biology. 2007;**5**(3):e82

[13] Liu H, Yao J, Yuan Z, Shang Y, Chen H, Wang F, Masakorala K, Yu C, Cai M, Blake RE, Choi MF. Isolation and characterization of crude-oil-degrading bacteria from oil-water mixture in Dagang oilfield, China. International Biodeterioration & Biodegradation. 2014;**87**:52-59

[14] Liu L, Li Y, Li S, Hu N, He Y, Pong R, Lin D, Lu L, Law M. Comparison of next-generation sequencing systems. Journal of Biomedicine and Biotechnology. 2012;**2012**:1-11

[15] Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu Y. A tale of three next generation sequencing platforms: Comparison of Ion Torrent, Pacific biosciences and illuminaMiSeq sequencers. BMC Genomics. 2012;**13**(1):341

[16] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 2010;**7**(5):335-336

[17] Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;**26**(19):2460-2461

[18] Simpson EH. Measurement of diversity. Nature. 1949;**163**:688

[19] Bowman JS, Rasmussen S, Blom N, Deming JW, Rysgaard S, Sicheritz-Ponten T. Microbial community structure of Arctic multiyear sea ice and surface seawater by 454 sequencing of the 16S RNA gene. ISME Journal. 2012;**6**(1):11-20

[20] Meng L, Liu H, Bao M, Sunc P. Microbial community structure shifts are associated with temperature, dispersants and nutrients in crude oil-contaminated seawaters. Marine Pollution Bulletin. 2016;**111**(1-2):203-212

[21] Brooijmans RJ, Pastink MI, Siezen RJ. Hydrocarbon-degrading bacteria: The oil-spill clean-up crew. Microbial Biotechnology. 2009;**2**(6):587-594

[22] Maddela NR, Masabanda M, Leiva-Mora M. Novel diesel-oil degrading bacteria and fungi from Ecuadorian Amazon rainforest. Water Science and Technology. 2015;**71**(10): 1554-1561

[23] Daugulis AJ, McCracken CM. Microbial degradation of high and low molecular weight polyaromatic hydrocarbons in a two-phase partitioning bioreactor by two strains of *Sphingomonas* sp. Biotechnology Letters. 2003;**25**(17):1441-1444

[24] Chaillan F, Le Flèche A, Bury E, Phantavong YH, Grimont P, Saliot A, Oudot J. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. Research in Microbiology. 2004;**155**(7):587-595

[25] Kiran B, Rajender K, Narsi RB. Biodegradation of polycyclic aromatic hydrocarbons by white rot fungi *Phanerochaete chrysosporium* in sterile and unsterile soil. Journal of Scientific & Industrial Research. 2008;**67**:538-542

[26] Al-Nasrawi H. Biodegradation of crude oil by fungi isolated from Gulf of Mexico. Journal of Bioremediation & Biodegradation. 2012;**3**:147. DOI: 10.4172/2155-6199.1000147

[27] Covino S, D'Annibale A, Stazi SR, Cajthaml T, Cvancarová M, Stella T, Petruccioli M. Assessment of degradation potential of aliphatic hydrocarbons by autochthonous filamentous fungi from a historically polluted clay soil. Science of the Total Environment. 2015;**505**:545-554

[28] Maddela NR, Scalvenzi L, Pérez M, Montero C, Gooty JM. Efficiency of indigenous filamentous fungi for biodegradation of petroleum hydrocarbons in medium and soil: Laboratory study from Ecuador. Bulletin of Environmental Contamination and Toxicology. 2015;**95**(3):385-394

[29] Zhen-Yu W, Ying XU, Hao-Yun W, Jian Z, Dong-Mei G, Li FM, Xing B. Biodegradation of crude oil in contaminated soils by free and immobilized microorganisms. Pedosphere. 2012;**22**(5):717-725

[30] Obi LU, Atagana HI, Adeleke RA. Isolation and characterisation of crude oil sludge degrading bacteria. SpringerPlus. 2016;**5**(1):1946

[31] Maiti A, Das S, Bhattacharyya N. Isolation and characterization of a new bacterial strain from petroleum oil contaminated soil, India. Journal of Science. 2012;**2**(2):103-108

[32] Ahirwar S, Dehariya K. Isolation and characterization of hydrocarbon degrading microorganisms from petroleum oil contaminated soil sites. Bulletin of Environmental and Science Research. 2013;**2**(4):5-10

[33] El-Din SMB, Moussa TA, Moawad H, Sharaf OA. Isolation and characterization of polyaromatic hydrocarbons degrading bacteria from compost leachate. Journal of Advances in Biology. 2014;**5**(2):651-660

[34] Maddela NR, Scalvenzi L, Venkateswarlu K. (In Press) Microbial degradation of total petroleum hydrocarbons in crude oil: A field-scale study at the low-land rainforest of Ecuador. Environmental Technology. 2016. DOI: 10.1080/09593330.2016.1270356

[35] Panda S, Kar R, Panda C. Isolation and identification of petroleum hydrocarbon degrading microorganisms from oil contaminated environment. International Journal of Environmental Science Technology. 2013;**3**(5):1314-1321

[36] Bartha R, Bossert I. The treatment and disposal of petroleum wastes. In: Atlas RM, editor. Petroleum Microbiology. New York, NY, USA: MacMillan; 1984. pp. 553-578

[37] Cooney JJ. The fate of petroleum pollutants in fresh water ecosystems. In: Atlas RM, editor. Petroleum Microbiology. New York, NY, USA: MacMillan; 1984. pp. 399-434

[38] Ulrici W. Contaminant soil areas, different countries and contaminant monitoring of contaminants. In: Rehm H, Reed G, editors. Environmental Process II. Soil Decontamination Biotechnology. 2nd ed. Vol. 11. Chichester: Wiley; 2000. pp. 5-42

[39] Atlas R, Bragg J. Bioremediation of marine oil spills: When and when not—the Exxon Valdez experience. Microbial Biotechnology. 2009;**2**(2):213-221

[40] Van Beilen JB, Funhoff EG, Van Loon A, Just A, Kaysser L, Bouza M, Holtackers R, Rothlisberger M, Li Z, Witholt B. Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. Applied and Environmental Microbiology. 2006;**72**(1):59-65

[41] Mc Donald IR, Miguez CB, Rogge G, Bourque D, Wendlandt KD, Groleau D, Murrell J. Diversity of soluble methane monooxygenase-containing methanotrophs isolated from polluted environments. FEMS Microbiology Letters. 2006;**255**(2):225-232

[42] Maeng JHO, Sakai Y, Tani Y, Kato N. Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkane oxidation in *Acinetobacter* sp. strain M-1. Journal of Bacteriology. 1996;**178**(13):3695-3700

[43] Jan B, Beilen V, Neuenschwunder M, Suits THM, Roth C, Balada SB, Witholt B. Rubredoxins involved in alkane degradation. The Journal of Bacteriology. 2003;**184**(6):1722-1732

[44] Benyahia F, Embaby AS. Bioremediation of crude oil contaminated desert soil: Effect of biostimulation, bioaugmentation and bioavailability in biopile treatment systems. International Journal of Environmental Research and Public Health. 2016;**13**:1-11

[45] Nikolopoulou M, Pasadakis N, Norf H, Kalogerakis N. Enhanced ex situ bioremediation of crude oil contaminated beach sand by supplementation with nutrients and rhamnolipids. Marine Pollution Bulletin. 2013;**77**:37-44

[46] Paudyn K, Rutter A, Rowe RK, Poland JS. Remediation of hydrocarbon contaminated soils in the Canadian Arctic by landfarming. Cold Regions Science Technology. 2008;**53**:102-114

[47] Whelan MJ, Coulon F, Hince G, Rayner J, McWatters R, Spedding T, Snape I. Fate and transport of petroleum hydrocarbons in engineered biopiles in polar regions. Chemosphere. 2015;**131**:232-240

[48] Gomez F, Sartaj M. Optimization of field scale biopiles for bioremediation of petroleum hydrocarbon contaminated soil at low temperature conditions by response surface methodology (RSM). International Biodeterioration & Biodegradation. 2014;**89**:103-109

[49] Chikere CB, Okoye AU, Okpokwasili GC. Microbial community profiling of active oleophilic bacteria involved in bioreactor based crude-oil polluted sediment treatment. Journal of Applied & Environmental Microbiology. 2016;**4**:1-20

[50] Van Gestel K, Mergaert J, Swings J, Coosemans J, Ryckeboer J. Bioremediation of diesel oil-contaminated soil by composting with biowaste. Environmental Pollution. 2003;**125**:361-368

[51] Frutos FJG, Escolano O, Garcıa S, Mar Babın M, Fernandez MD. Bioventing remediation and ecotoxicity evaluation of phenanthrene-contaminated soil. Journal of Hazardous Materials. 2010;**183**:806-813

[52] Kao CM, Chen CY, Chen SC, Chien HY, Chen YL. Application of in situ biosparging to remediate a petroleum hydrocarbon spill site: Field and microbial evaluation. Chemosphere. 2008;**70**:1492-1499

[53] Salanitro JP, Dorn PB, Huesemann MH, Moore KO, Rhodes IA, Rice Jackson LM, Vipond TE, Western MM, Wisniewski HL. Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. Environmental Science & Technology. 1997;**31**:1769-1776

[54] Maddela NR, Burgos R, Kadiyala V, Banganegiri M, Carrión AR. Removal of crude oil from soil by using novel microorganisms of Ecuador soils: Solid and slurry phase methods. International Biodeterioration & Biodegradation. 2016;**108**:85-90

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