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Review Article

ENHANCED BIOREMEDIATION OF PETROLEUM CONTAMINATED SITES: AN INTEGRATIVE APPROACH THROUGH MICROBIAL TECHNOLOGY

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Abstract: Degradation of petroleum hydrocarbons by the means of microorganisms is a favourable approach over physical removal methods owing to their ubiquitous nature. Enhanced bioremediation, *i.e*., the use of a patented combination of microorganisms, surfactants, and emulsifiers, break the contaminant down into tiny pieces, which can then be surrounded by enzymes and quickly digested. *In situ* bioremediation techniques involving processes like biostimulation, bioaugmentation and intrinsic bioremediation do not require excavation of the contaminated soils, so is less expensive, create less dust, and cause less release of contaminants than *ex situ* techniques. Abiotic factors such as structure of the hydrocarbons, temperature, physical state of the pollutant, salinity and pressure and oxygen content are known to affect the rate of degradation. Also, many biotic factors *viz*., chemotactic attraction of microorganisms towards pollutants, production of biosurfactants, formation of biofilms have been affirmed to augment the process of degradation. Hydrocarbons interact with the soil matrix and the microorganisms present in the vicinity, determining the fate of the contaminant relative to its chemical nature and microbial degradative capabilities. Degradation can be monitored by measuring the changes occurred over time in concentration of the hydrocarbons and by the increase/decrease in the number of microorganisms present in the vicinity. This review presents an overview of techniques employed in treatment of petroleum hydrocarbon contaminated sites by the means of bioremediation and the factors affecting it.

Keywords: *Bioremediation, Petroleum Hydrocarbons, Biostimulation, Bioaugmentation, Biosurfactants*

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Introduction

Increase of human needs towards petrochemical products such as natural gas, diesel, gasoline, and asphalts has resulted in significant contamination of several terrestrial and marine sites with petroleum or petroleum by-products [1]. The hydrocarbon sector worldwide has been undergoing radical changes leading to increased industrial activity in hydrocarbon processing like exploration, drilling, processing, and refining process. This has also led to increase in generation of oily wastes (sludge), contaminated sites and wastewater. Besides this, industries concerned with oil exploration and drilling, storage terminals and oil depots also face the problem of sludge generation and disposal [2]. Gasoline which is a lighter petroleum product can be effectually treated by certain well-defined physiochemical methods. However, fuels which has heavier densities such as diesel oil rely on vicarious techniques owing to their low volatility. High density of diesel oil is contributed by presence of n-paraffin, branched paraffin, cyclo-paraffin and aromatic hydrocarbons; apart from presence of high number of alkanes which is also common in gasoline [3].

India, US EPA (United States Environmental Protection Agency) and OECD (Organization for Economic Co-operation and Development) countries have designated oily wastes as hazardous wastes (Ministry of Environment and Forest, Government of India, 2000). Petroleum storage facilities are frequently the source of pollution due to leaks and spills during fuel transfer and storage [4].

Presence of petroleum hydrocarbons in the environment are a matter of concern due to:

1) Volatility, which poses a fire/explosion hazard.

2) Toxicity to living organisms; there are evidences of mutagenic effects to bacteria cells and carcinogenic effects on animal cells. PAHs and alkanes (n-C9 to n-C14) have been found to be more toxic to plants than heavier hydrocarbon compounds above n-C15.

3) Mobility. Lighter hydrocarbons are mobile and can be a problem at considerable distances from their point of release due to transport in groundwater or air.

4) Persistence in the environment.

5) Potential interference with water retention and transmission and with nutrient supplies in soils [1].

Oil contamination has severe impact on the plant as well as animal ecosystem including human health [2]. There have been various reports on diversification of physical and chemical nature of natural habitats, deleterious effects on marine life and its ecosystem. Many lethal and sub-lethal toxic effects are described for polycyclic aromatic hydrocarbons (PAHs) such as cancer of skin, gastrointestinal pipe, and bladder. BTEX (Benzene, Toluene, Ethyl benzene and xylene) are known to cause birth defects, still birth, mutations, cancer, and liver diseases [5,6]. Oil contaminated soil lose its fertility and affects seed germination [7,8]. Commercially available diesel fuel is composed of ~64% saturated aliphatic hydrocarbons (alkanes), ~1 to 2% unsaturated aliphatic hydrocarbons, and ~35% aromatic hydrocarbons (including polycyclic aromatic hydrocarbons). Compared to other medium distillate fuels, diesel has the highest content of environmentally persistent hydrocarbons, which are often highly toxic and are regulated due to their mutagenicity and carcinogenicity [9]. Hence disposal of the same in an improper manner may cause a serious environmental problem. A range of *in situ* and *ex situ* remediation methods including natural attenuation, chemical, physical, and mechanical engineering approaches as well as the application of microorganisms have been implemented to reduce the petroleum hydrocarbon contamination.

Each of these methods has advantages and disadvantages regarding its costs and capacity to remediate the contaminant. For example, physical removal of contaminated soil and washing with solvents are expensive and need facilities that transfer the contaminated soil to the clean-up location. Methods such as land filling, incineration, air spurging which are termed conventional, have been applied since early times for remediation of oily waste [2,10]. Physical and chemical processes such as excavation can be as simple as hauling the contaminated soil to a regulated landfill but can also involve aerating the excavated material in the case of volatile organic compounds (VOCs).

The Surfactant Enhanced Aquifer Remediation (SEAR) is a process which has been used for desorption of fuels. It involves injection of hydrocarbon mitigation agents and/or surfactants which binds to non-aqueous phase liquid. Another process, termed pumping, includes pumping out contaminated ground water by a vacuum pump and purifies this ground water by passing through a series of vessels containing materials designed to adsorb the contaminants. Activated carbon in granular form is usually used for petroleum contaminated sites for this purpose Other methods inculpate employing flocculants accompanied by usage of sand filters; and air stripping for volatile pollutants such as BTEX of gasoline. Stabilization/solidification (S/S) is a remediation/treatment technology that relies on the reaction between a binder and soil to stop/prevent or reduce the mobility of contaminants. Stabilization involves the addition of reagents to a contaminated material (e.g. soil or sludge) to produce more chemically stable constituents; whereas solidification involves the addition of reagents to a contaminated material to impart physical/dimensional stability to contain contaminants in a solid product and reduce access by external agents (e.g. air, rainfall). However, the uptake of S/S technologies has been relatively modest, and several barriers have been identified. Remediation by chemical oxidation involves the injection of strong oxidants such as hydrogen peroxide, ozone gas, potassium permanganate or persulfates. While filtration, extraction and adsorption on resins separate unwanted compounds (but do not destroy them), advanced oxidation processes using oxidizing agents like H_2O_2 generate toxic intermediates and involve enormous cost [11]. Similarly, estimated cost of (i) pump and treat technology is $20-50 million per MT, (ii) biological treatment for landfarming is $39-88 per MT, slurry treatment is $88-165 per MT, and (iii) composting is $404-467 per m3 of soil [12]. The conventional methods are considered neither environment friendly nor cost effective [13]. The common drawback is that they are not the permanent solution for the environmental pollution and sometimes they are not cost effective [2].

It is an established fact that virtually all types of hydrocarbons are susceptible to microbial degradation and hence the relevance of using the biotechnological approach using the microbial capability for bioremediation of the hazardous waste is justified [14,15]. Bioremediation has emerged as one of the most promising treatment options for oil contamination [16]. Bioremediation has been defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes". This technology is based on the premise that a large percentage of oil components are readily biodegradable in nature [17]. Bioremediation is a process that uses naturally occurring microorganisms to transform harmful substances to nontoxic compounds [18]. There are innumerable strains of microbes under basic categories of bacteria, yeast or fungi, which degrade oily sludge and waste water effluent sludge through digestion of harmful chemicals and compounds present in oily sludge and waste water effluent sludge into simpler, less toxic or non-toxic substances [19]. Petroleum hydrocarbons have been used as energy source by many species of soil bacteria, which would transform the hydrocarbons to final products such as carbon dioxide, water, and fatty acids [19]. Bioremediation exploits this natural process by promoting the growth of microbes that can effectively degrade specific contaminants and convert them to nontoxic by-products. *There are two basic types of bioremediation:*

(a) "Biostimulation" provides nutrients to the indigenous microbial populations and promotes growth and increases metabolic activity that is used to degrade contaminants.

(b) "Bioaugmentation" introduces specific blends of microorganisms into a contaminated environment or into a bioreactor to initiate the bioremediation process, which increases the population of the fit to handle the biodegradative process in the contaminated area [10].

Laboratory studies and field tests have shown that bioremediation can enhance oil biodegradation. The success of bioremediation depends on having the appropriate microorganisms in place under suitable environmental conditions. Its operational use can be limited by the composition of the contaminant.

Advantages of Bioremediation

Minimal exposure of onsite workers to the contaminant
Long term protection of public health
The cheapest of all methods of pollutant removal
The process can be done on site with a minimum amount of space and equipment
Eliminates the need to transport of hazardous material
Uses natural process
Transform pollutants instead of simply moving them from one media to another
Perform the degradation in an acceptable time frame [20,21].

Disadvantages: Potential Problems

Poor management
Unable to estimate the length of time it's going to take; it may vary from site. It can take a few months to as long as a few years.
Not all organic compounds are biodegradable [22,23,24].

Bioremediation Strategies

In conventional bioremediation, enzymes released by the microbes can only attack one surface of the contaminant, this leads to slower, less effective remediation, whereas in enhanced bioremediation, a patented combination of surfactants and emulsifiers break the contaminant down into tiny pieces, which can then be surrounded by enzymes and quickly digested [25]. Bioremediation accelerates what would occur naturally by "biodegradation" or the conversion of spilled oil by naturally occurring oil-degrading microbes into carbon dioxide, water, and biomass. This process can be enhanced by biostimulation and bioaugmentation. Bioremediation can be performed at the site of contamination (*in situ*) or on contamination removed from the original site (*ex situ*) [26,27]. *In situ* techniques involves processes like biostimulation, bioaugmentation and intrinsic bioremediation and do not require of the contaminated soils so it is less expensive, create less dust, and cause less release of contaminants than *ex situ* techniques [20,21].

Ex situ bioremediation involves removing the contaminated soil or water and treating it with microbes and nutrients to mineralize the contaminant. *Ex situ* techniques can be faster, easier to control, and used for treating a wider range of contaminants and soil types than *in situ* techniques. Excavation is however needed for completing the bioremediation step. Both slurry phase and solid phase bioremediation are included in *Ex situ* techniques [28].

***In- situ* Bioremediation**

In situ are preferred over *ex situ* techniques as they do not require excavation, are less expensive and release less dust and contaminants [20,21]. However, the process is slow and difficult to manage [21]. Aerobic *in situ* bioremediation supplies oxygen and nutrients to the microorganisms to metabolize the xenobiotics. Air is pumped into the soil above the water table in liquid form as hydrogen peroxide, a mechanism also known as bioventing. *In situ* bioremediation may not work well in clays or in highly layered subsurface environments because oxygen cannot be evenly distributed throughout the treatment area. This process often requires years to reach cleanup goals, depending mainly on how biodegradable specific contaminants are [26].

Biostimulation

It involves the modification of the soil environment to stimulate existing bacteria capable of bioremediation. This can be done by addition of various forms of rate limiting nutrients such as phosphorus, nitrogen, oxygen or carbon (e.g. in the form of molasses) and electron acceptors/donors (acetate, nitrate, sulphate, glutamate, *etc.*) and gaseous formulations to contaminated environment [29,30].

Alternatively, pollutants removal rates have also been stimulated by generating an optimal balance of physical factors such as aeration, temperature and buffering of environmental pH by altering the redox state and electrokinetics state of contaminated samples. The additives are added to the sites through injection wells. This process, overall, is referred to as bioremediation and is an EPA-approved method for reversing the presence of oil or gas spills. Biostimulation is more effective if it is used in combination with bioaugmentation methods [31]. To evaluate the performance of the biostimulation methods in comparison of bioaugmentation and natural attenuation, the kinetic efficiency of biostimulation has been found to be relatively slow as compared to bioaugmentation process [32].

Bioaugmentation

Bioaugmentation is a rational rearrangement of microbial richness leading to dominion of microbial group(s) with specific catabolic traits which are necessary for cleaning up of pollutants [33]. The important advantage of bioaugmentation is that it enhances the performance of indigenous bacteria through the addition of bacterial strains with specific activities for several folds over a relatively short time scale. Conventionally, bioagumentation studies have focused on exogenous introduction of efficient pollutant degrading strain(s) or bacterial consortium to contaminated site for decontamination purpose. Interestingly, few studies have attempted to evaluate the *in-situ* biodegradation performance [34].

For effective *in situ* biodegradation, bioaugmentation is necessary. Ultimately, the bioaugmentation strategy may depend on the degree of contamination and the time frame available for remediation. Apart from the above methods, quite a few other variants of bioagumentation have also been developed and implemented successfully for remediation purpose [35]. "Co-bioaugmentation" is one such variant wherein the process is rendered effectively by exogenous introduction of multiple microbial strains with different metabolic potentials [36]. Bioaugmentation is a straightforward approach; yet the use of this technology has not become common largely due the limited success with isolation of efficient pollutant degrading microorganisms. Further the bioaugmentation process need to address several other issues such as optimization of the process before its application [35].

Mechanism of *in-situ* bioremediation

Petroleum is an extremely complex mixture of hydrocarbons. From the hundreds of individual components, several classes, based on related structures can be recognized. Several studies have been performed to determine the metabolic pathways for degradation of these compounds, and there have been several reviews on this subject [22,23,24].

Hydrocarbon Structure and Biodegradation

Hydrocarbon compound structure is important in bio-degradability. The n-alkanes (straight chain) and n-alkyl-aromatics (substituted aromatic) in the C10 to C22 range are of low toxicity and the most biodegradable [19,24]. Those above C22 are considered lesser toxic, but are on tough on their biodegradation process because of low water solubility and their ability to attain solid to semi solid state at a temperature of 35°C. Hydrocarbons ranging from C1 to C4 are gaseous in nature and easily biodegradable, however, C5 to C9 range hydrocarbons are described as having highest solvent-membrane toxicity to microorganisms [37]. The ß-oxidation step required for degradation of branched n-alkanes and cyclo-alkanes is inhibited due to the presence of tertiary and quaternary carbon atoms in them. Aromatic hydro-carbons are biodegradable, but the bioavailability of high molecular weight compounds such as PAH's decreases dramatically as the number of condensed rings increases. These compounds exhibiting lower biodegradability due to surface absorption and low solubility are commonly referred to as recal citrants or xenobiotics [38].

Pathways of degradation of petroleum hydrocarbons

Aliphatic hydrocarbons

There are two biodegradation pathways for the alkanes. The initial step in the degradation of aliphatic hydrocarbons involves the enzyme monooxygenases or dioxygenases [39]. The monooxygenase attacks the terminal methyl group and a primary alcohol is formed [24]. The alcohol is further oxidized to the corresponding aldehyde and fatty acid. Whereas, in the second pathway a dioxygenase enzyme acts on the terminal methyl group of an n-alkane resulting in the addition of two oxygen atoms which in turn results in the formation of a peroxide that is converted to a fatty acid. The carboxylic acid groups in the fatty acids are then further metabolized via the β-oxidation pathway to form acetyl CoA or propionyl CoA [depending on the number of carbon atoms (even or odd) in the n-alkane]. These compounds are then subsequently metabolized via the tricarboxylic acid cycle (TCA cycle) to CO_2 and H_2O [23,24,40].

Aromatic hydrocarbons

The low molecular weight PAHs (<3 rings) are more susceptible to microbial degradation than the high molecular weight PAHs (>4 rings) [40,41]. The process of degradation requires the presence of molecular oxygen to initiate the enzymatic attack of PAH rings. At the initial, dioxygenase-catalyzed oxidation of arenes yields vicinal cis-dihydrodiols as early intermediates. These dihdroxylated byproducts may then be cleaved by intradiol or extradiol ringcleaving dioxygenases through either an ortho-cleavage pathway or meta-cleavage pathway, leading to intermediates such as protocatechuate and catechols [42]. The catechol aromatic ring is then cleaved, again with the help of a dioxygenase. Catechol catabolism can subsequently follow one of two pathways. Either the ortho-cleavage pathway, in which the ring is cleaved between the two carbon atoms with hydroxyl groups or the meta-cleavage pathway, in which the ring splits between adjacent carbon atoms with or without a hydroxyl group. Although some studies have reported microbial degradation of hydrocarbons at high rates under optimal conditions [22,23,27,43-45] high molecular weight aromatics exhibit only slow rates of biodegradation [23,24,39].

Monitoring *in-situ* bioremediation

The efficiency of most of the *in-situ* bioremediation process has been evacuated in the form of time dependent and endpoint measurement of complete disappearance of the target pollutant(s) [27]. Approaches in monitoring and verifying enhanced *in situ* biodegradation includes measurement of changes over time in the (a) concentration of hydrocarbons, (b) temperature, (c) number of hydrocarbon degrading microorganisms, (d) ratio of fast degrading hydrocarbons, and (e) metabolic intermediates [46]. Measurements of oxygen consumption over time and elevated carbon dioxide concentrations in soil gas have been used as indicators of hydrocarbon degradation [46]. One method to substantiate biodegradation is to measure stable carbon isotope ratios in soil gas carbon dioxide [47,48,49]. A measurement of the stable carbon isotope ratios (δ13C) of microbial metabolic end products presents a promising method for monitoring *in situ* bioremediation of petroleum hydrocarbons. Differences between the δ13C values of hydrocarbons and indigenous carbon sources (e.g., plant matter, soil carbonates) can be exploited to trace the origins of metabolic end products. However, in zones of methanogenesis and/or where the δ13C values of endogenous plant matter overlap those of hydrocarbons, δ13C measurements can produce ambiguous results. In such cases, simultaneous measurement of the radiocarbon (14C) contents of metabolic end products can be used to determine their sources [49].

Organisms which can utilize benzene, toluene, ethylbenzene, xylene, alkanes and polycyclic aromatic hydrocarbons have been studied extensively and their mechanism of compounds degradation has been elucidated [23,40]. Knowledge of the metabolic pathways used by anaerobic bacteria to break down hydrocarbon has allowed identifying unique intermediate compounds that can be used as biomarkers for *in situ* activity. One of these intermediates is 2-methlybenzylsuccinate, the product of fumarate addition to o-xylene by the enzyme responsible for toluene utilization. This compound has been found to be reliable indicator of anaerobic toluene degradation. Field studies have confirmed that the biomarker is detectable in field samples and its distribution corresponds to areas where active biodegradation is predicted. For naphthalene, three biomarkers have been identified [2-naphthoic acid (2-NA), tetrahydro-2-NA, and hexahydro-2-NA] that can be used in the field to identify areas of active *in situ* degradation [48].

The potential of functional gene array analysis to monitor changes in the amount of functional marker genes as indicators of PAH's biodegradation has also been investigated. A prototype functional gene array has been developed for targeting key functions in the biodegradation of naphthalene, toluene, and xylene. To aid correlation between multiple samples, the process of internal standard probe-based normalization was imported. Coupled with one-colour hybridization, the signal normalization improved the consistency among replicate hybridizations resulting in better discrimination for the differences in the amount of target DNA. During the naphthalene biodegradation in a PAH-contaminated soil slurry microcosm, the normalized hybridization signals in naphthalene catabolic gene probes were shown to be in good agreement with the number of naphthalene-degradation genes and the production of $14CO_2$. Gene arrays provide efficient means for monitoring of contaminant biodegradation in the environment [50].

One more technique that has proved to be a good predictor of monitoring the rate of biodegradation is push- pull test. In a push- pull test reactants along with a conservative tracer are injected or "pushed" into a well (petroleum reservoir) and allowed to incubate for a prescribed length of time. Samples are then withdrawn or "pulled" from the same well as a function of time. Analytes are measured in the extracted water to determine breakthrough curves for each compound under investigation and interpretations are made relative to the unreactive tracer. Breakthrough curves for the reactants, products, and the tracer can then be used to calculate mass balances and ultimately the biotransformation rates. In quantifying the loss of parent hydrocarbon and formation of the daughter metabolite, a conservative *in situ* rate constant can be delineated [51].

The kinetics based *in-situ* hydrocarbon bioremediation have been considerably aided by advancements in different analytical methods such as atomic absorption spectroscopy (AAS), colorimetry, inductively coupled plasma atomic emission spectroscopy (ICP-AES), polarography, selective ion electrodes, X-ray fluorescence, energy dispersive analysis via X-rays (EDAX), and electron microprobe analysis *etc*. to simple field portable instruments [52]. The current TPH detection systems are generally limited to spectrophotometric chemical test kits. These offer the convenience of field analysis but have several important limitations; such as speciation is not achieved, only total content is determined, chemical and physical interferences to measured signal can be dramatic, still require a high degree of user competence Thus, data are poor to determine trends in pollution and to contribute to a proper risk assessment. A more attractive approach should accomplish for a proper solution of the above limitations to implement the advance analytical techniques to monitor the *in-situ* bioremediation of hydrocarbon [52].

The effective monitoring of microbial bioremediation under *in-situ* conditions is rather poor because in many cases the decrease in pollutant concentration may be observed as an outcome of absorbance of the pollutant to the environment matrix [53]. Most of the methods for pollutants extraction from the environmental samples are based on the chemical nature of the target pollutant(s). Based on the chemical nature of the extraction treatment, the methods may be classified as organic solvent extraction, chemical-oxidation extraction, super fluid extraction and aqueous sample extraction [54]. These workers have recently provided a comprehensive data of the different extraction methods indicating their functional classification, target contamination, working principle, weaknesses, and strengths. The recent studies are indicating that efficient pollutant extraction is targeting the physio-chemical nature (soil adsorption, hydrophobic, water solubility *etc*.) of the pollutant for developing efficient extraction methods [53]. Advancement in the technological methods for qualitative and quantitative estimation of chemical pollutants and their speciation, along with improvement in the extraction methods, has led to efficient and accurate pollutants degradation kinetics. Further development in these techniques, would lead to increased success of *in-situ* bioremediation.

Ex-situ bioremediation

The *ex-situ* approach to hydrocarbon bioremediation is carried out above ground by physically extracting the impacted medium. It is commonly applied to dissolved-phase contamination via pumping and treatment with above ground bioreactors. Soils are treated above ground via land-farming, biopiling and composting.

The primary advantage to these *ex-situ* approaches is the degree of control that can be exerted over the processes being used to manipulate the system [55]. Generally, the primary disadvantage is the expense and disruption associated with removal, treatment, and disposal or replacement of the impacted medium. Ex- situ bioremediation of petroleum contaminated sites involves various treatments *viz*.-

Slurry-phase bioremediation

Contaminated soil is made as slurry when combined with water and other additives inside large tanks termed as 'bioreactors'. This slurry maintains the activity of microorganism in their optimum phase. Inside the bioreactor, oxygen and nutrients are added, and an optimum environment is created for complete degradation of contaminants. Upon completion of the treatment, the water is removed from the solids, which are disposed of or treated further if they still contain pollutants [56]. Compared to other treatment processes, slurry-phase biological treatment is better for contaminated clays. The success of the process is highly dependent on the specific soil and chemical properties of the contaminated material. This technology is particularly useful where rapid remediation is a high priority [57].

Solid-phase bioremediation

In solid-phase bioremediation, soils in the above ground treatment areas are attended to by creating a collection system which will prevent the contaminant from escaping the treatment. Moisture, heat, nutrients, or oxygen are controlled to enhance biodegradation for the application of this treatment [58]. Solid-phase systems are relatively simple to operate and maintain, require a large amount of space, and cleanups require more time to complete than with slurry-phase processes. Solid phase soil treatment includes landfarming, soil biopiles, and composting [59].

Landfarming

In this relatively simple treatment method, contaminated soils are excavated and spread on a pad with a built-in system to collect any "leachate" or contaminated liquids that seep out of contaminant-soaked soil. In landfarming, the contaminated soil undergoes systematic turning over for proper mixing of air in it. Bioremediation slows down if there is no availability of nutrients, and ambient oxygen and temperature. In some cases, reduction of contaminant concentrations may be attributed more to volatilization than biodegradation. When the process is conducted in enclosures controlling escaping volatile contaminants, volatilization losses are minimized [60].

Soil biopiles

Contaminated soil is piled in heaps several meters high over an air distribution system. Aeration is performed with a vacuum pump. Ambient moisture and nutrient levels are retained for optimum bioremediation. The soil heaps can be placed in enclosures. Volatile contaminants are easily controlled since they are usually part of the air stream being pulled through the pile [61].

Composting

Biodegradable waste is mixed with a bulking agent such as straw, hay, or corn cobs to make it easier to deliver the optimum levels of air and water to the microorganisms. Three common designs are static pile composting (compost is formed into piles and aerated with blowers or vacuum pumps), mechanically agitated in vessel composting (compost is placed in a treatment vessel where it is mixed and aerated), and windrow composting (compost is placed in long piles known as windrows and periodically mixed by tractors or similar equipment) [62].

Biotic and abiotic factors for enhancement of bioremediation

Various biotic and abiotic factors which influence the biodegradation of oil, rates of microbial growth and enzymatic activities, affects the rate of petroleum hydrocarbon degradation. The persistence of petroleum pollutants depends on the quantity and quality of the hydrocarbon mixture and on the properties of the affected ecosystem [14,63].

Biotic factors

Bacterial Chemotaxis

Microorganisms exhibit wide behavioral adoptions that can be of great significance for *in-situ* bioremediation purposes. Chemotaxis is one of the most important adoptions, because it allows increased bioavailability of the pollutants and thereby, helps in maximization of pollutants degradation [64]. A few ranges of bacteria belonging to diverse group have been identified to exhibit chemo taxis towards environmental pollutants such as petroleum associated hydrocarbons, explosive, nitroaromatic compounds, polycyclic aromatic hydrocarbons [65]. Common soil bacteria such as *Rhizobium* sp., *Bradyrhizobium* sp., *Pseudomonas* sp., and *Azospirillum* sp. have been shown to be chemotactically attracted toward different aromatic hydrocarbons. Many of these compounds are present in soils, sediments, and rhizosphere, and they serve as sources of carbon and energy for the microorganisms. Aromatic acids such as benzoate, p-hydroxybenzoate (PHB), methylbenzoates, o-, m-, and p- toluates, salicylate, DL-mandelate, ß-phenylpyruvate, and benzoylformate have been reported to be attractants for *Pseudomonas putida* PRS2000 [64]. Based on metabolism chemotaxis is divided in to two subcategories. Metabolism-dependent and another metabolism-independent chemotaxis. The first one is associated with pollutants that are used as a source of metabolic energy. The second one is associated with pollutants that are co-metabolically transformed to generate lesser toxic product [64,66]. Electron donors/acceptors act as important components of the metabolic machinery. Therefore, bacterial moments towards electron acceptors/donors may be called metabolism-dependent chemotaxis. Generally, microbiologists consider positive chemotaxis for detoxification of hydrocarbons [67].

Negative chemotaxis phenomenon, therefore, could be utilized to explore the alternative methods for control of the microbial fouling by coating surfaces with less universal toxic chemical at non-lethal concentrations which prevent fouling by repelling a potential primary microbial film [68]. However, negative chemotaxis may be utilized for developing new approaches for controlling the non-specific microbial fouling. Bacterial chemotaxis behavior has focused on the phenotypic characterization of chemotactic responses in an in vitro environment based on assays (drop plate, swarm plate and capillary assay) that have been used successfully for qualitative determination of chemotaxis [69,70,71]. Further, it has been suggested that development of assays for quantitation of chemotactic response may bring a significant improvement in the determination of chemotactic behavior as well as development of bioremediation technology [72]. The development of assays for qualitative and quantitative chemotactic responses is expected to improve the *in-situ* bioremediation methods significantly. It is being realized that distribution of chemotaxis-related genetic elements is much wider than initially expected [73].

Biofilm

Formation of a 'biofilm' is one of the adaptive responses that can be successfully implemented for *in-situ* bioremediation process [74]. Most of the organisms release a slimy material, which gets adhered to a matrix or substrate. This slimy coating is identified as 'microbial biofilm'. Initial studies have indicated the role of microbial biofilms in microbial pathogenesis, wherein biofilms were reported to act for bacterial survival against the host's defense mechanism [75]. However, later, this phenomenon was also found to be associated with *in-situ* microbial activities [76]. Microbes adhere to the environmental surfaces and their association with these surfaces involves the synthesis of extra cellular homo- or hetero polymers of sugars called exo-polysaccharides (EPS). These extracellular polymers are fabricated and extended from the cells to the surfaces in a very organized manner forming a tangled and channelized network of the polymeric fibres [69]. The whole assemblage including the resident microbes and the channelized network of the EPS is termed as Biofilm. Because of its polyelectrolyte nature the microbial biofilms are highly absorptive and can collect significant quantities of silt, clay hydrocarbons and other detritus from their immediate environment [76]. Thus, their unique physical, biological, and chemical properties make them a very useful tool for environment cleanliness. Hence the microbial biofilms are implicated in the natural and modulated environmental cleanliness systems for purification of the drinking water, detoxification of the oil spills, removal of heavy metals and biodegradation of the hazardous xenobiotics in the contaminated waters and the soils, as well as environmental monitoring biosensor fabrications [77].

Biosurfactants

Hydrocarbons and alkanes have low solubility in water. This fact, coupled to the fact that the first step in hydrocarbon degradation involves a membrane-bound oxygenase, makes it essential for bacteria to come in direct contact with the hydrocarbon substrates. Emulsification enhances interaction between bacteria and hydrocarbons, most of the petroleum degrading bacteria are therefore potent emulsifiers. These surfactants help to disperse the oil, increase the surface area for growth, and help detach the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted [78,79,80]. In general, glycolipids constitute the low molecular weight biosurfactants. Rhamnolipids, trehalose lipids and sophorolipids are the types of glycolipid bioemulsifiers which are acylated with long chain fatty acids or hydroxyl fatty acids [80]. Emulsification occurs when cultures reach stationary phase of growth. This regulatory feature appears to be general, and probably applies to the production of both low and high molecular weight emulsifiers, as in all cases emulsifier production is concurrent with the increase in cell density and the onset of the stationary phase of growth [80].

Since, a very limited range of hydrocarbons are recognized by bacteria for utilization, the oil degrading bacteria become nutrient-starved as soon as hydrocarbons are starts depleting from the oil droplet. For example, the cell surface hydrophobicity of *Pseudomonas aeruginosa* was greatly increased by the presence of cell-bound rhamnolipid [81], whereas the cell-surface hydrophobicity of Acinetobacter strains was reduced by the presence of its cell-bound emulsifier [78]. These data suggest that microorganisms can use their biosurfactants to regulate their cell-surface properties to attach or detach from surfaces according to need. This has been demonstrated for *A. calcoaceticus* RAG-1 growing on crude oil [78]. During exponential growth, emulsan is cell bound in the form of a minicapsule. This bacterium utilizes only relatively long chain n-alkanes for growth. After these compounds are utilized, RAG-1 becomes starved, although it is still attached to the oil droplet, which is enriched in aromatics and cyclic paraffins. Starvation of RAG-1 causes release of the minicapsule of emulsan. It was shown that this released emulsan forms a polymeric film on the n-alkane depleted oil droplet, thereby desorbing the starved cell [78]. In effect, the 'emulsifier' frees the cell to find fresh substrate. At the same time, the depleted oil droplet has been 'marked' as used, because it now has a hydrophilic outer surface to which the bacterium cannot attach. The detachment of bacteria from the depleted oil drop enables them to move to other drops where they metabolize the specific group of utilizable hydrocarbons. Therefore, detachment of bacteria from oil drops results in a more efficient bioremediation.

Abiotic factors

Abiotic factors play important role in most environmental phenomena [82]. The metabolic reaction involved in the bioremediation of hydrocarbons also follows the principal of 'enzyme catalysis' and therefore they exhibit optimal performance only over a very narrow range of physio-chemical parameters.

Temperature

Biodegradation of hydrocarbons can occur over a wide range of temperatures. Many psychrotropic, mesophilic, and thermophilic hydrocarbon-utilizing microorganisms have been isolated [83]. In an earlier study, Zhao *et al*. (2017) [84], Brzeszcz and Kaszycki (2018) [85], and Arulazhagan *et al*. (2017) [86] have reported hydrocarbon degradation at 0°C. On the other hand, Zhang *et al*. (2012) [87], Das and Tiwary (2013) [88] have reported on hydrocarbon degradation up to 50-70°C. Atlas (1981) [17] and Van Hong *et al*. (2018) [83] found that the effects of temperature differ, depending on the hydrocarbon composition of a petroleum mixture. Low temperature retards the rates of volatilization of low molecular weight hydrocarbons, some of which are toxic to microorganisms. The presence of such toxic components was found to delay the onset of oil biodegradation at low temperatures. At 20°C, lighter oils had greater abiotic losses and were more susceptible to biodegradation than heavier oils; rates of oil mineralization for the heavier oils were significantly lower at 20°C than for the lighter ones.

Nutrients

When considering an oil slick, there is a mass of carbon available for microbial growth within a limited area. Since microorganisms require nitrogen and phosphorus for incorporation into biomass, the availability of these nutrients within the same area as the hydrocarbon is critical [83]. Rates of diffusion may be inadequate to supply sufficient nitrogen and phosphorus to establish optimal C/N and C/P ratios for microbial growth and metabolism. Researchers examining the fate of large oil spills have thus concluded in many cases that concentrations of N and P are limiting with respect to rates of hydrocarbon biodegradation [90]. Atlas (1981) [17] has described an oleophilic nitrogen and phosphorus fertilizer which could overcome limitations of nitrogen and phosphorus in the contaminated soil thus stimulating petroleum biodegradation. The fertilizer consisting of paraffinized urea and octylphosphate supported degradation of oil. Optimal C/N and C/P ratios were 10:1 and 100:1, respectively. This study indicated that rates of nutrient replenishment are generally inadequate to support rapid biodegradation of large quantities of oil. The nitrogen and phosphorus containing fertilizers can be used to stimulate microbial hydrocarbon degradation.

Physical state of oil pollutants

The physical state of petroleum hydrocarbons has a marked effect on their biodegradation. The degree of spreading of the oil spill determines in parts the surface area of oil available for microbial colonization by hydrocarbon degrading microorganisms [91]. Liquid aromatic hydrocarbons are utilized by bacteria at the water- hydrocarbon surface whereas solid aromatic hydrocarbons are not metabolized. At 30°C diphenylmethane is a liquid and can be degraded easily but at 20°C, its solid form cannot be utilized by *Pseudomonas* sp. Similarly, naphthalene cannot be utilized in the solid form but can be utilized if dissolved in liquid hydrocarbon [29]. Availability of increased surface area accelerates biodegradation [92]. Hydrocarbon degrading microorganisms can be observed growing over the entire surface of an oil droplet; growth does not appear to occur within oil droplets.

Salinity and Pressure

These two factors are specific features of ecosystems such as saline lakes and deep seas (high hydrostatic pressure), which represent specialized environment where petroleum contamination can occur [93]. Walworth *et al*. (2013) [94] have examined hydrocarbon degradation in hypersaline environments. When hydrocarbons were added to natural samples of various salinities (ranging from 3.3 to 28.4%) rates of metabolism of hydrocarbons decreased with increase in the salinity. Microorganisms in saline waters containing more than 20% of salinity could not use mineral oil as sole source of carbon and energy. Thus, proving that biodegradation is not possible in hyper-saline environment.

Oxygen

In hydrocarbon aerobic bioremediation, oxygen availability is a critical factor [95]. Bacterial activity proceeds more rapidly if sufficient oxygen is provided. During aerobic biodegradation, molecular oxygen is reduced to water while petroleum hydrocarbon is oxidized to create energy, cell mass, and carbon dioxide. The supply of oxygen to the scene of microbial activity is controlled by soil saturation and conduction. Abayneh *et al*. (2018) [95] reported that the requirement of oxygen to degrade hydrocarbon is 3.1 g of oxygen for 1.0 g of hydrocarbon. The largest amount of oxygen required is approximately 200,000 ppm in a well aerated soil and 8 ppm in a saturated soil. Soil venting is a method that provides oxygen to the contaminated area by introducing air into the contaminated zone to increase the activity of native bacteria and allow them to degrade the contaminants. Abayneh *et al*. (2018) [95] reported three bioventing projects in southern California. They treated the test zone with ammonia and air which resulted in increase of the microbial counts and in the number of degraded hydrocarbons.

Conclusion

Oil or fuel can harm the marine environment by smothering marine life or acting as a toxin to both marine and coastal flora and fauna. Several remediation alternatives have been in use for the restoration of polluted systems. Certain strains of microorganisms can digest these petroleum hydrocarbons. Bioremediation has successfully employed these microbes to eliminate petroleum contaminants and degrade them into carbon dioxide, water, and salt. Biodegradation can be indirectly assessed by measuring the microbial numbers, biomass and/or activity.

A wide range of *in-situ* and *ex-situ* methods of bioremediation are available to treat such contaminated sites. Although *in-situ* bioremediation is slower process than *ex-situ*, but the degree of restoration is much enhanced. The process of bioremediation is influenced by various biotic and abiotic factors which determine the biodegradability of hydrocarbons. These factors assisted by techniques and strategies help in monitoring the remediation process. However, these methods are not sufficient alone and quest for new microbial driven process are nevertheless a pre-requisite in modern research to enhance petroleum hydrocarbon remediation.

Application of review

Bioremediation, which exploits the biodegradative abilities of live organisms and/or their products have proven to be the preferred alternative in the long-term restoration of petroleum hydrocarbon polluted niche, with the added advantage of cost efficiency and environmental friendliness. Scientists have extended the scope of bioremediation by developing and cultivating microbes for use under varied conditions.

Research Category: Bioremediation

Abbreviations:

US EPA: United States Environmental Protection Agency
OECD: Organization for Economic Co-operation and Development
PAH: Polycyclic Aromatic Hydrocarbons
BTEX: Benzene, Toluene, Ethyl benzene and Xylene
SEAR: Surfactant Enhanced Aquifer Remediation
VOCs: Volatile Organic Compounds
AAS: Atomic Absorption Spectroscopy
ICP-AES: Inductively Coupled Plasma Atomic Emission Spectroscopy
EDAX: Energy Dispersive Analysis via X-rays, PHB: p-hydroxybenzoate

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