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TREATMENT OF PETROLEUM HYDROCARBON CONTAMINATED SITES WITH BIOREMEDIATION AGENTS

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Abstract

Petroleum spills and the incomplete combustion of fossil fuels lead to accumulation of petroleum hydrocarbons in the environment. Many polycyclic aromatic hydrocarbons (PAHs), released as a result of incomplete combustion of organic matter, are highly toxic, mutagenic and carcinogenic. All the conventional methods that have been used to remove these compounds from the environment, suffer from various limitations. Bioremediation is the promising technology for the treatment of hydrocarbon-contaminated sites as it is cost effective and will lead to complete mineralization. Bioremediation tends to bring about mineralization of organic contaminants into carbon dioxide, water, or transformation of complex organic contaminants to other simple organic compounds by employing different microorganisms that are capable of degrading hydrocarbon contaminants. Metabolic engineering might help to improve the efficiency of degradation of contaminants by micro-organisms. However, the efficiency of indigenous microbes for field bioremediation can be, significantly, improved by optimizing factors such as bioavailability, adsorption and mass transfer. This paper reviews the efficiency of different bioremediation agents, agriculture fertilizers and genetically modified bacteria for bioremediation of hydrocarbon contaminated sites and also briefly focuses on factors influencing bioremediation process.

Introduction

Bioremediation, defined as the use of microorganisms to detoxify or remove pollutants, owing to their diverse metabolic capability, is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry [1]. The term petroleum refers to a highly complex mixture of a wide variety of low and high molecular weight hydrocarbons. This complex mixture contains saturated alkanes, branched alkanes, alkenes, napthenes (homo-cyclic and hetero-cyclic), aromatics like resins, asphaltenes, naptheno-aromatics and hydrocarbons containing different functional groups like carboxylic acids, ethers, etc [2]. Petroleum-based products are the major source of energy for industry and life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport and storage of petroleum products [3]. It is estimated that between 1.7 and 8.8 million metric tons of oil is released into the world`s water every year [4]. Biological processes are also involved in the clean-up of contaminated marine environments, although, the addition of nitrogen and phosphorus nutrients has been shown to accelerate the speed of biodegradation [5].The biodegradation of many components of petroleum hydrocarbons has been reported in a variety of terrestrial and marine cold ecosystem, including Arctic, Alpine and Antarctic seawater and sediments [6, 7, 8]. Oil degrading or oil-emulsifying bacteria would be useful for the petroleum industry not only for assessing the fate and effects of the spilled oil, but also for isolating novel oil-degrading bacteria [9]. Numerous diesel-degrading bacteria have been isolated from Antarctica, one of the largest sources of hydrocarbon contaminants in polar region [10, 11]. There are about 22 most popular genera of bacteria that can metabolize petroleum hydrocarbons. Among these *Pseudomonas, Aeromonas, Bacillus, Flavobacterium, Corynebacterium, Micrococcus* are most important. Based on crude oil degradation capacity, *Pseudomonas aeruginosa* most actively utilize hydrocarbons in the crude oil. Previous observations have identified that *Pseudomonas* genus possesses the most efficient hydrocarbon degrading microorganisms [12, 13, 14, 15]. Bioremediation has several potential advantages over conventional technologies, such as being less costly, less intrusive to the contaminated site, and more environmental friendly being in terms of its end products [16].Metabolic engineering might help to improve the efficiency of degradation of toxic compounds by microorganisms [17]. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentration of nutrients and oxygen are present and that pH is between 6 and 9. The physical and chemical characteristics of the oil and oil surface area are also important determinants of bioremediation success.

There are two main approaches to oil spill bioremediation: (I) Bioaugmentation, in which known oil-degrading bacteria are added to supplement the existing microbial population, and (II) Biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting co-substrates[3,16]. Temperature also influences petroleum biodegradation by its effect on the physical and chemical properties of the oil, rate of hydrocarbon metabolism by microorganism and composition of the microbial community [18]. Extreme conditions are expected to have a negative influence on the ability of microbial population to degrade hydrocarbons. This is because the fate of petroleum hydrocarbons in the environment is largely determined by local environmental conditions, which influence the rate of microbial growth and enzymatic activities [19]. Microbiological culture, nutrient additives and enzyme additives that significantly increase the rate of biodegradation to mitigate the effect of the discharge were defied as bioremediation agents by U.S.EPA (Environmental Protection Agency) [20]. This article provides updated information about bioremediation agents which are beneficial for biodegradation of petroleum hydrocarbon contaminated environments.

Bioremediation Agents and their Effectiveness

In recent years, various non-biodegradable components present in oil have been successfully used as biomarkers to distinguish between biodegradation and the physical/chemical loss of oil. This also help to mitigate the high variability associated with field studies [21, 22, 23]. The U.S.EPA has defined bioremediation agents as "microbiological cultures, enzyme additives or nutrient additives that significantly increase the rate of biodegradation to mitigate the effect of the discharge" [20]. Compared to laboratory investigation, few tests have been carried out to evaluate the effectiveness of bioremediation products in the field because such trials are both difficult and expensive to conduct [24]. Bioremediation of hydrocarbon contaminated petrol bunk soil is greatly affected by mixed consortium with organic amendments. A combination of red soil, petrol spilled soil, mixed consortium, poultry litter, coirpith and biosurfactant was noticed as a better combination for the biodegradation of hydrocarbon [25]. The effect of heating was greater than that of forced aeration under field condition because of the porous structure and shallow depth of the treated soil material which resulted in rapid diffusion of oxygen into soil. Maximum hydrocarbon biodegradation occurred between 25 to 37^{o}C [26]. The maximum degradation range for environments such as soil, marine water and fresh water are 30-40^{o}C, 15-20^{o}C and 20-30^{o}C respectively [27, 28]. Bioremediation agents are classified as bioaugmentation and biostimulation agents, based on the two main approaches to oil spill bioremediation. Numerous bioremediation products had been proposed and promoted by their venders, especially during early 1990s, when bioremediation was popularized as "the ultimate solution" to oil spills [29].

Bioaugmentation-

Bioaugmentation is a process of open heimer biotechnology that save money, reduce the length of remediation and at the same time gives successful result. Bioaugmentation with microorganism has been shown to increase degradation of numerous compounds including chlorinated solvents, methyl tert-butyl ether, nitrophenols, oil, pentachlorophenol, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and several pesticides such as altrazine, dicamba, and carbofuran [30, 31, 32, 33, 34]. The ability to distribute the inoculants depends on what organism is being used. Fungi which are larger than bacteria are usually restricted to surface application while bacteria are more adaptable to surface or subsurface applications [35]. A field study in Prince William Sound following the Exxom valdez spill to investigate the effectiveness of two commercial microbial products vis-à-vis natural attenuation and nutrient addition alone. This field trial failed to demonstrate enhanced oil biodegradation by these products, no biostimulation occurred in the nutrient control plots either. There were no significant differences between any of the treatment and control plots during the 27-day trial period [36]. Bioaugmentation was performed with *Penicillium funiculosurr* and *Aspergillus sydowi*, strains isolated from two aged soil contaminated with 60,600 and 500,000 mg of total petroleum hydrocarbon per kilogram of dried soil [37]. Various strategies are being experimented to make bioaugmentation a successful technology in sites that lack significant population of biodegrading microorganisms [38].

Biostimulation-

Biostimulation is employed for the acceleration of the biodegradation process by the addition of rate limiting nutrients acting as limiting factor in remediation of shoreline ecosystem that are heavily contaminated with hydrocarbon. However, the wetlands are an exception, because if oil gets penetrated into the wetlands to any appreciable extent, the impact zone becomes anaerobic and due to oxygen limitation the effective treatment of the site is prevented [16]. Most commonly used water-soluble nutrients products include mineral nutrient salt e.g. KNO_3, $NaNO_3$, NH_4O_3, K_2HPO_4, $MgNH_4PO_4$ and many commercial inorganic fertilizers. They are usually applied in the field through the spraying of nutrient solution or spreading of dry granules. This approach has been effective in enhancing oil biodegradation in many field trials [21, 39]. To promote oil removal, biocarrier for immobilization of indigenous hydrocarbon degrading bacteria was developed using peanut hull powder [40]. Additives are usually added to the subsurface through injection wells. Subsurface characteristics such as a groundwater velocity, hydraulic conductivity of the subsurface, and lithology of the subsurface are important in developing a biostimulation system. The indigenous microorganisms present in the soil are responsible for degradation of the pollutant, but biostimulation can be improved by bioaugmentation [41, 42].

Table1: **Bioremediation agents in NCP product schedule (Adapted from USEPA, 2002) (43)**

Name of Trademark	Product type	Manufacture
BET BIOPETRO	MC	Bio Enviro Tech, Tomball TX
BILGEPRO	NA	International Environmental Products, LLC, Conshohocken, PA
INIPOL EPA 22	NA	Societe, CECA S.A., France
LAND AND SEA RESTORATION	NA	Land and Sea Restoration LLC, San Antonio, TX

MICRO-BLAZE	MC	Verde Environmental, Inc., Houston, TX
OIL SPILL EATHER II	NA/EA	Oil Spill Eater International, Corporation Dallas, TX
OPPENHEIMER FORMULA	MC	Oppenheimer Biotechnology, Inc., Austin, TX
PRISTINE SEA II	MC	Marine system, Baton Rouge, LA
STEP ONE	MC	B & S Research, Inc., Embarrass, MN
SYSTEM E.T.20	MC	Quantum Environmental Technologies, Inc., (QET), La Jolla, CA
VB591^{TM}WATER, VB997TMSOIL,AND BINUTRIX	NA	BioNutra Tech, Inc., Houston, TX
WMI-2000	MC	WMI International, Inc., Houston, TX

Abbreviation of product type:
MC—Microbial Culture
EA—Enzyme Additive
NA—Nutrient Additive

Generic Agriculture Fertilizers

Fertilizers help in accelerating the biodegradation process, thus they can be efficiently used as biostimulation agents. Common agricultural fertilizers are a good choice as nutrient additives since they are inexpensive and readily available. Different water-soluble and inorganic fertilizers have been used for this purpose. The most commonly used water-soluble fertilizers include mineral nutrient salts such as KNO_3, $NaNO_3$, NH_4O_3, K_2HPO_4, and MgNH4PO4. However, the water-soluble nutrients may get wash-out easily, thus attempts have been made to design nutrient delivery systems that overcome the washout problems and also provide enhanced nutrient availability for oil biodegradation.

In 1976, common fertilizers were used for the first field trial in Spitsbergen, Norway. Forcados unweathered crude oil was released at a rate of 10 L/m^2 on two 10 m^2 plots. It was observed that the respiration rate of micro-organisms was higher in the fertilized plotas compared to the control plot, suggesting that the addition of fertilizers enhanced the oil degradation process [44]. A series of tests were also conducted in Fisheries and Oceans, Canada, to investigate the effect of different types of fertilizers and delivery strategies in a low energy, sandy beach or in a salt marsh. These tests demonstrated that periodic addition of inorganic fertilizers such as ammonium nitrate and triple super phosphate increased the rate of oil removal from beaches [45]. However, no enhancement of the degradation process was achieved at the lower level of oil contamination in sandy beach. For the salt marsh, the results turned out to be completely opposite as the enhancement by fertilizers occurred at the 0.3% contamination level but no enhancement was observed at 3% oil contamination.

In 1995, Lee *et al*conducted another field study to compare the potential of inorganic nutrients with organic fish bone-meal fertilizers. They concluded that organic fertilizers had the greatest effect on microbial growth and activity as the bone-meal contains readily biodegradable form of carbon, whereas the inorganic nutrients were found to be more effective in stimulating crude oil biodegradation [46]. A recent study carried out by Chorom *et al* 2010, showed that the application of fertilizers at 2 ton/ha rate in oil-contaminated soil lead to greater rates of biodegradation after 5 weeks indicating the feasibility of bioremediation [47]. Certain studies conducted in wetland environments have concluded that biostimulation using fertilizers was ineffective in treating some oil-contaminated salt marshes or freshwater wetlands due to oxygen limitation [48, 49, 50].

The success of biostimulation using different fertilizers is highly case specific as it depends on oil properties, the composition of the fertilizers and the characteristics of the contaminated environments. Slow release fertilizers are one of the newly developed approaches that are used to overcome nutrient-washout problems. Slow release fertilizers provide continuous sources of nutrients to oil contaminated areas. These fertilizers are solid in nature and consist of either relatively insoluble nutrients or water-soluble nutrients coated with hydrophobic materials such as paraffin or vegetables oil [44]. Several field studies have been carried out to study the effectiveness of slow release fertilizers on enhancing oil degradation [51, 52, 53, 54].The major challenge during the use of slow release fertilizers is the control on nutrients release rates. If the nutrients are released too quickly, they will subject to rapid washout and will not be lasting for long. And if they are released too slowly, the concentration will never build up to a sufficient level to support rapid biodegradation rates. Slow release fertilizers are readily available, cost effective and less labour intensive, thus this approach might be a boon for treating low-energy, oil-contaminated shorelines.

Hydrocarbon degradation by genetically modified bacteria

Environmental contamination due to hydrocarbons is a very serious problem whether the source of hydrocarbon is petroleum, pesticides or other toxic organic matter. Petroleum hydrocarbons are highly toxic to all forms of life as they can turbulently migrate from the site of contamination and adversely affect terrestrial and aquatic ecosystems as well as humans [2]. There are vast numbers of studies available on the microbial degradation of hydrocarbons. Genetically modified microorganisms (GMMs), especially bacteria, find a considerable number of applications in bioremediation of hazardous petroleum hydrocarbons.

In general, GM technology is widely used to alter or improve the existing catabolic pathways used by micro-organisms to degrade the pollutants [55]. GM technology also possesses the potential to extend such pathways to include additional target compounds that may otherwise not be degraded [56, 57]. Extension of the scope of the catabolic pathways is usually brought about by either introducing additional genetic sequences or by altering the existing genes [58, 59]. Since each stage of any catabolic pathway is mediated by enzymes that are formed as a result of transcription and translation of specific genes, thus the genes are also needed to be arranged in operons ensuring that transcription occurs in the correct sequence toproduce the required enzymes [60].

In a study carried out by Devos *et al* (1992) it was demonstrated that insertion of a gene encoding toluate 1,2-dioxygenase, enabled the bacterium *Pseudomonas* sp B13 to degrade 4-Chlorobenzoate (4CB) and 3,5-dichlorobenzoate (3, 5 DCB) [61]. Bioremediation of hydrocarbons has been focused generally on two major groups that include- Polycyclic Aromatic Hydrocarbons (PAHs) and BTEX (Benzene, toluene, ethylbenzene and xylene) compounds [62]. Lee *et al* (1995) reported that biodegradation of benzene, toluene, and p-xylene (BTX) in the environment does not result in the mineralization of the three hydrocarbons. However, the genetic modification of *Pseudomonas putida* by the insertion of the *todC1C2BA* genes (encoding toluene dioxygenase), enabled the complete mineralization of BTX without the accumulation of intermediate metabolites [63]. Sayer *et al* (2000) used bioluminescent *Pseudomonas fluorescens* strain HK44 for the biodegradation of PAH naphthalene, the strain had been genetically modified to express the naphthalene catabolic plasmid *puTK21* which had itself been mutagenized by a tranposon-inserted *lux* gene [64]. This system had the advantages that it had increased expression of naphthalene catabolic genes and also caused the microorganism to bioluminesce. This aided the monitoring of bioremediation levels by monitoring the level of bioluminescence. Bioluminescence monitoring being non-invasive, non-destructive, rapid and population specific is much more favorable [65].

There are still many other reports on the degradation of hydrocarbons using genetically modified bacteria; Table 2 shows some of the examples. Genetic modification has got the potential to enable both the construction of bacteria with multiple degradative pathways and the creation of micro-organisms that are able to degrade previously non-biodegradable compounds. The applications of genetically modified bacteria had been successfully applied to different stages of bioremediation processes, viz- process monitoring, strain monitoring, stress response, end-point analysis and toxicity assessment [3]. Examples of these applications are listed in Table 3.

The concept of releasing genetically modified bacteria into the environment requires a clear understanding of their behavior, dispersal, survival and the ability to detect and monitor the fate of genes and organisms within a microbial community [17]. For the development of successful bioremediation techniques using genetically modified bacteria, the amalgamation of microbiological and ecological knowledge, biochemical mechanism and field engineering designs is quite essential.

Table 2: **Genetic engineering for the biodegradation of contaminants [3]**

Microorganisms	**Modification**	**Contaminants**	**Reference No.**
Pseudomonas putida	pathway	4-ethylbenzoate	[66]
P. putida KT2442	pathway	Toluene/benzoate	[67]
Pseudomonas sp.FRI	pathway	Cholro-, methylbenzoate	[68]
Comamonas testosterone VP44	Substrate specificity	o-, p-, monochlorobiphenyls	[69]
Pseudomonas sp. LB400	Substrate specificity	PCB	[70]
P. pseudoalcaligenes KF707-D2	Substrate specificity	TCE, toluene, benzene	[71]

Table 3: **Application of genetically modified bacteria for assessing the biodegradation process efficiency [65]**

Microorganisms	**Application**	**Contaminants**	**Reference No.**
A. eutrophus H850Lr	Process monitoring	PCB	[72]
P.putida TVA8	Process monitoring	TCE, BTEX	[73]
B. cepacia BRI6001L	Strain monitoring	2, 4-D	[74]
*P. fluorescens*1058/pUCD607	Stress response	BTEX	[75]
Pseudomonas strain Shk1	Toxicity assessment	2, 4-dinitrophenol hydoquinone	[76]
A. eutrophus 2050	End-point analysis	Non-polar narcotics	[77]

Bioremediation Controlling Factors

The susceptibility of bioremediation process depends largely on different controlling factors. The primary requirement for bioremediation is the existence of a microbial population capable to degrade the target contaminants. The outcome of each degradation process depend on microbial (biomass concentration, population diversity, enzyme activity), substrate (physio-chemical characteristics, molecular structure and concentration) and a range of environmental factor (pH, temperature, moisture content, availability of electron acceptors and carbon or carbon sources)[78]. The physio-chemical property of PAHs such as water solubility is an important factor during degradation of PAHs. It is believed that microorganisms can only degrade the PAHs fraction which is dissolve in the treatment matrix solution. The dissolved fraction of PAHs is known as bioavailable fraction. The water solubility of PAHs decreases with the increase in number of fused benzene rings and with angularity. Thus, high molecular weight PAHs are less degradable compared with low molecular weight PAHs [79].

The favourable range of environmental factors like temperature, pH, moisture contents and nutrients etc., for efficient degradation of contaminants are summarized in Table 4.

Table 4: Favourable ranges of environmental factors for efficient bioremediation [41]

Parameter	Optimum value
pH	6.5-8
Oxygen content	10-18%
Nutrient content	C:N:P = 100:10:1
Temperature ^{o}C	20-30
Moisture content	40-60%
Contaminants	5-10% dry weight of soil
Soil type	Low clay or silt content
Heavy metals	700ppm

Health and Environmental anxiety

The toxicity of hydrocarbon molecules and their availability for microbial metabolism depend on their chemical and physical nature. Petroleum hydrocarbon molecules possessing a wide range of molecular weights and boiling points, cause diverse amount of toxicity to the environment. Petroleum hydrocarbon can be lethal based upon the nature of the petroleum fraction, the way of exposure to it and the duration of exposure. Hydrocarbon can cause a variety of health effects in humans and wildlife. They can even damage any organ system in human body including nervous system, respiratory system, immune system, liver, kidney endocrine system etc. [80]. Polycyclic aromatic hydrocarbons (PAHs) are responsible for several adverse effects such as immune-toxicity, genotoxicity and reproductive toxicity [81]. Consequently, contamination of environment with such contaminants poses a big threat for several life aspects and leads to the encouragement of comprehensive strategies for environmental remedial action.

Conclusion and Future Prospects

Hydrocarbon contamination is a worldwide threat to environment and bioremediation is the promising technology for the treatment of hydrocarbon contaminated sites. Also, bioremediation could be used as a preventive measure for the future, after the post-contamination treatment. The process of bioremediation depends on the ability of indigenous microbes to transform or mineralize the organic contaminants. Microorganisms possess various enzyme systems to bring about degradation of hydrocarbons as a major source of carbon and energy. It is of great ecological significance to understand the mechanism of biodegradation by these microorganisms.

The rate and efficiency of biodegradation by indigenous microbes can be enhanced not only by the addition of fertilizers or nutrients under controlled conditions but also by introducing additional hydrocarbon degrading microorganisms to the contaminated sites. The former approach requires the detailed study of factors affecting bioremediation process while the latter one demands the isolation of highly efficient hydrocarbon degrading strains of microorganisms.

The use of Genetically Engineered Microorganisms (GEMs) might be advantageous for treatment of heavily contaminated sites for bioremediation processes. Enhanced biodegradation rates could be achieved through cloning of genes of biodegradation pathway(s) with broader substrate specificities. However, it is quite necessary to evaluate the stability of any GEMs before their field application.

Bioremediation is a multi-disciplinary technique that requires the proper knowledge of the natural habitat as well as the mechanism of biodegradation of the microbes before developing cost-effective, ecologically safe and environmentally sound bioremediation plans.

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