



Fungal Bioremediation of Soils Contaminated by Petroleum Hydrocarbons

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8.1 Introduction

Irrespective of the regional barrier, environmental pollution is considered one of the major challenges faced by living beings. The repercussion of environmental pollution was highly alarming, which are reflected by threatening more than 1141 species globally (International Union for Nature conservation). Due to the instant and direct effect of air and water pollution, more emphasis was given by both scientific and nonscientific communities. Apart from nuclear disasters such as Chernobyl in 1986 and Fukushima Daiichi in 2011, a predominant focus on soil pollution was not given globally (Friedman 2011). On the other hand, the experience from the toxicity effect on more than 20,000 people in and around sites of worst soil polluted lands, such as the dumpsite for the e-waste processing in Ghanaian capital Accra, Dzershinsk, Russia for dioxins, Hazaribagh, Bangladesh for tannery waste, and Niger river delta for petroleum hydrocarbons, diverted the several scientists to address the problem. Acting as a sink for several pollutants, the hazardous effects of soil pollution is very immense which include several health-related issues such as cancer, premature birth,

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mental retardation, impotency, and miscarriage. In spite of several remediations of the pollutants, the diversified properties of the soil along with their unique interactions with the pollutants difficult to devise a universal remediation strategy for soil treatment.

To understand the transport, distribution, and fate of pollutants in the soil, it is mandatory to have a comprehensive knowledge of the intricate functionality of the soil (Van Breemen and Buurman 2002). In general terminology, soil can be termed as a dynamic naturally occurring compound on the earth's surface, which is formed from the earth crust by the action of living organisms and weather agents. Depending on the accessibility of the parent rock and climatic conditions, the duration for the formation of soil ranges (Van Breemen and Buurman 2002). On the other hand, the living habitat of great diverse groups of organisms majorly dictates the physical, chemical, and nutrient profile of the soil, which results in the variation of the soil composition from place to place. Although the soil properties are very diverse with more than 50% of solid fraction from parental earth crust, the base composition of the soil is dictated by the mineral content, organic matter, water, and air. The combination of these compounds decides the chemical profile, color, porosity, and texture of the soil. The large constituent of soil-mineral fracture consists of silicate minerals, quartz, and feldspars with particle size ≥ 0.05 mm along with secondary minerals (0.05–0.002 mm), which results from the weathering of primary minerals. The sand particles from 0.05 to 2 mm have a negligible role in the interaction with pollutants however the secondary minerals silt (0.05–0.002 mm) and lower than 0.002 mm clay minerals have a prominent role in the soil–pollutant interaction (Van Breemen and Buurman 2002).

On the other hand, the organic fraction due to their negative charge sequesters key positively charged compounds, such as potassium, sulfur, nitrogen, and carbon, and acts as a major sink for the plant macronutrients. The source of soil organic matter (SOM) fraction includes detritus stages of decomposed living organisms such as microorganisms, plants, and animals (Senesi and Loffredo 2018). However, the reversible masking of the pollutant by the SOM due to hydrophobic interactions limits the utilization of the conventional treatment process. The majority of the pollutants adsorb on the surface of the soil organic matter by hydrophobic interaction, pi–pi interaction, and are largely dependent on the soil's physiological conditions such as pH, temperature, redox condition, surface area, and available chemical species (Lehmann and Kleber 2015).

The humic substance present in the SOM attributes an overall negative surface charge due to the presence of –OH, –COOH, –SH, and –C=O– functional groups and acts as a key player in sequestering the pollutants in soil by exhibiting high affinity for cationic pollutants in soil (Rivero et al. 2004). Apart from the SOM, the presence of inorganic ions such as NO_3^- , Cl^- , HPO_4 , and SO_4^{2-} along with both natural and synthetic chelating agents such as citric acid, fulvic acid, gluconic acid, oxalate, and ethylene diamino tetracetic acid (EDTA) affect the remediation

efficiency in soil. Hence, despite the lower percentage of SOM in the soil, the presence of SOM claimed to have a substantial influence on pollutant interaction with soil, making them a prime target in the soil remediation.

8.2 Pollutants in Soil and their Classification

The vast classes of the soil pollutants can be grouped into two major groups, the organic pollutants and inorganic pollutants. Irrespective of the grouping both the groups have both natural and anthropogenic origins with diverse chemical properties (Bernes 1998). In spite of the ability of the indigenous microorganism for the degradation of pollutants, the hydrophobic nature, and structural stability of a few pollutants elevates the toxicity of the soil and paralyze the natural remediation efficiency of the soil microbiota. The majority of organic pollutants consist of carbon and hydrogen backbone and can be categorized into petroleum hydrocarbons (PHC), pharmaceutical active compounds and its derivatives, pesticides, and chlorinated compounds (Zuloaga et al. 2012). On the other hand, inorganic compounds include heavy metals, radioactive compounds, salts, and nutrients. Among the inorganic pollutants, the presence of heavy metals and metalloids are of greatest concern due to their potential toxicity to living organisms. Being resistant to degradation, the heavy metals can be remediated by biotransferring to lesser toxic compounds rather than degradation. Although these two categories of pollutants differ in structural and chemical properties, their interaction with soil due to their hydrophobic nature and their persistence nature along with hazardous toxicity properties make them unique and of greatest concern.

Depending on the persistence nature and the severity of toxicity in terms of teratogenicity, carcinogenic, and mutagenicity, environmental protection agency (EPA), Clean water act 1977 grouped 126 pollutants as priority pollutants (Stout et al. 2015). However, a recent increase in the number of anthropogenic pollutants in an environment, having the properties of disrupting the reproductive and endocrine system in micro concentrations, initiated EPA to categorize the compounds under a special category of “contaminants of emerging concern” (Vidal-Dorsch et al. 2012). These compounds are not completely understood due to their non-monotonic toxicity nature with similar properties to that of priority pollutants. Most pharmaceutical compounds and pesticides come under this category.

8.2.1 Petroleum Hydrocarbons

Petroleum hydrocarbons are a group of several hundreds of compounds consisting of crude oil or derivative of crude oil (Riser-Roberts 1998). Depending on the source of the occurrence the physical and chemical properties of crude oil vary. Due to this diversity, crude oil is generally classified based on the density or sulfur content or the geographical location of the production. However, conventional crude oil is classified either as light crude oil (low density) or heavy crude oil (high density) (Yasin

Table 8.1 Classification of total petroleum hydrocarbons (TPH) according to the equivalent carbon chain length (EC) [(a) according to ATSDR and (b) according to US-EPA]

Primary fraction	Secondary fractions	Examples	Surrogate
Aliphatic	EC5 to EC8 (low carbon range)	<i>n</i> -hexane	<i>n</i> -hexane ^{a,b}
	EC >9–EC16 (medium carbon range)	Kerosene, dearomatized petroleum stream, JP-7	JP-7, dearomatized petroleum ^a
	EC >16–EC 35 (high carbon range)	White mineral oil	Mineral oil ^{a,b}
Aromatic	EC 6–EC 9 (low carbon range)	Benzene, toluene, ethylbenzene, xylene	Toluene ^{a,b}
	EC >9–EC 16 (middle carbon range)	Isopropyl benzene, naphthalene, 3-methylnaphthalene.	Naphthalene ^a
	EC >16–EC 35 (high carbon range)	Fluorene, fluoranthene, benzo (a) pyrene	Pyrene ^{a,b}

et al. 2013). In terms of the chemical structure, crude oil represents a complex mixture of carbon and hydrogen backbone along with small quantities of sulfur, nitrogen, oxygen, and other metals (Yasin et al. 2013).

These combinations and unique chemical constituents in crude oil makes them one of the most complex naturally occurring compounds so far analyzed. Due to the practical difficulty in analyzing the properties and toxicity of every compound present in the soil-contaminated crude oil, environmentalist considered the usage of total petroleum hydrocarbons (TPHs) as the best indicator to define the levels of toxicity present in the petroleum-contaminated soil (Kuppusamy et al. 2020a). However, the quantification of TPH resulted in the gross toxicity of the soil rather than defining the individual contribution of the compounds in elucidating the toxicity. As a result for a better understanding based on the carbon chain length, scientists divided the TPH into fractions and designated them as EC (Equivalent Carbon number index) (Yang et al. 2015). Further, the toxicity levels of each fraction were assessed with the reference to the presence of a selected component in the fractions called “surrogate” in accordance with ATSDR (Agency for Toxic Substances and Disease Registry) (Table 8.1).

8.2.2 Toxicity of Petroleum Hydrocarbons

The toxicity of petroleum hydrocarbons present in soil depends mainly on their type and concentrations. Besides, environmental effects and the presence of co-contaminants such as heavy metals sometimes decide the toxicity of petroleum hydrocarbons (Khan et al. 2018). Exposure to these contaminants results in short-term health effects such as eye irritation, skin allergy, nausea, and diarrhea (Kuppusamy et al. 2020b); however, continuous exposure leads to long-term chronic health issues. Being lipophilic, most TPH compounds specifically aromatic

hydrocarbons tend to adsorption in the adipose tissue and cause damage to the internal organs. Irrespective of their presence in soil or water, the TPH and their epoxides exhibit high toxicity to mammals, amphibians, reptiles, and microorganisms. It has been reported that these compounds are commonly carcinogenic and mutagenic, also, recent discovery holds their responsibility for being immunotoxin, endocrine disruptor, genotoxic, and teratogenic. Exposure to polycyclic aromatic hydrocarbons during pregnancy reported to affect the progeny's mental ability and reproductive system (Agarwal et al. 2018). Despite wide research, the toxicity data are limited to either whole petroleum hydrocarbons or by considering key components in them, which are relatively heterogeneous on applying to actual petroleum hydrocarbon exposures. In addition, variability in the composition of crude oil due to variation in the refining process and the presence of impurities makes it difficult in assessing the eco-toxicity levels of petroleum hydrocarbons.

8.2.3 Fate of Hydrocarbons in Soil

Remediation of TPHs from the environment can be achieved either by removing or completing degradation of the TPHs (Kuppusamy et al. 2020c). However, the hydrophobic nature of the TPHs along with their structural stability, complete removal or degradation of these compounds is considered to be a major challenging task (Aguelmous et al. 2019). The fate of the TPHs in soil depends on several factors including the chemical and physical properties of the soil, aging of soil, and the presence of co-contaminants (Fig. 8.1) (Adams et al. 2015). Majority of low molecular weight TPH either evaporates or oxidize or is degraded by the action of microorganisms. However, persistent compounds tend to be adsorbed onto the soil organic matter or leached into water bodies. In nature, TPHs are removed from the environment by various processes such as photooxidation and chemical oxidation (Adams et al. 2015). On the other hand, the overall contribution of natural

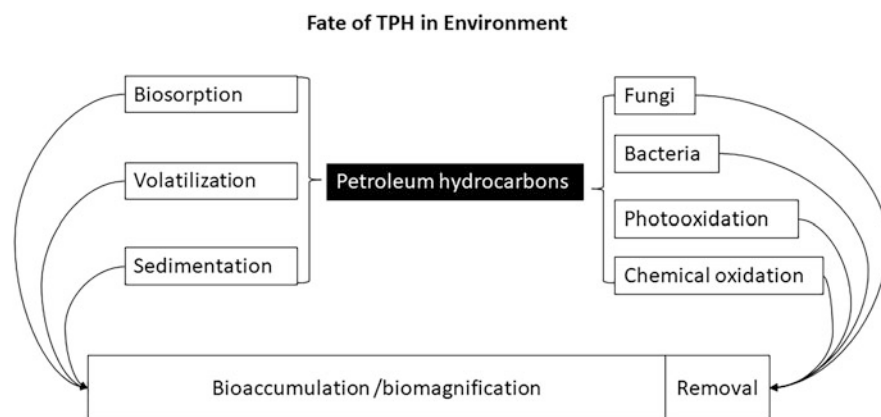


Fig. 8.1 Fate of total petroleum hydrocarbons in soil

remediation for the removal of the TPHs was limited to low molecular weight TPHs, which urges for the development of technologies for the degradation of persistent petroleum hydrocarbons.

8.3 Remediation of Hydrocarbons

With the advancement in technology, various treatment strategies were introduced for the effective treatment of TPHs in the soil. To generalize the treatment technologies were divided into physical, chemical, and biological methods. In addition, the utilization of chemicals for remediation such as surfactants was found to be non-ecofriendly and in turn act as a toxic compound to the soil microbiota. In this context, the utilization of green technologies for pollutant removal by the usage of bacteria or fungi as a bioagent is considered the most cost-effective and eco-friendly efficiency method (Shah et al. 2019).

The term remediation refers to the degradation, removal, or transformation of contaminants to less toxic or harmless substances. The method includes restricting the mobilization of pollutants and preventing spreading to uncontaminated sites or degradation. Bioremediation is a process, where the goals of remediation were achieved with biological agents (Microbial or enzymatic). This widely used technique for the treatment of TPHs contaminated soil is believed to be invasive and cost-effective. Bioremediation involves either conversion of contaminants to less toxic compounds or mineralization of the contaminants to inorganic compounds such as carbon dioxide (CO₂) or water (H₂O) (Saha et al. 2019). The bioremediation process is classified into two strategies in situ and ex situ methods (Fig. 8.2). The former strategy involves remediation of the TPH at the site of contamination whereas ex situ involves the excavation of soil and treating the soil elsewhere (Adams et al. 2015). In situ treatment is considered as low cost and low maintenance along with environment-friendly process for the remediation of soil. On the other hand, ex situ treatment was reported to be an efficient remediation strategy in less time compared

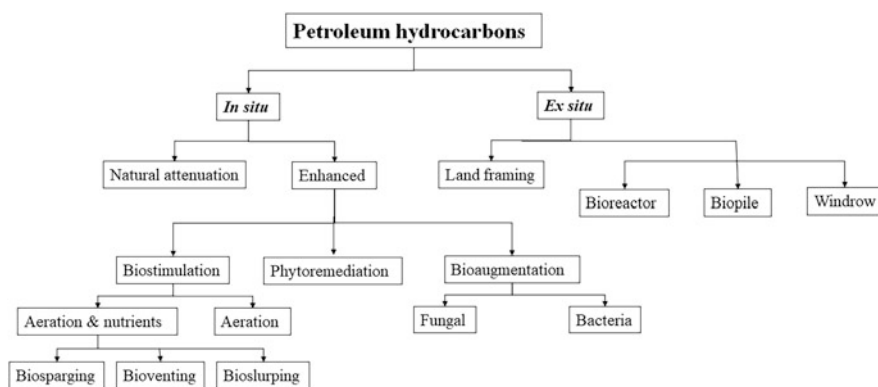


Fig. 8.2 Various strategies for cleanup of soil contaminated with petroleum hydrocarbons

to in situ, which is considered a major advantage (Shahsavari et al. 2017). However, the involvement of excavation and transport of soil makes the ex situ treatment costly. In addition, a variation of soil properties along with the influence of co-contaminants present in the soil makes the in situ treatment inconsistent inefficiency and outcome. On the other hand, with the advancement in the soil chemistry and biology, several in situ technologies were reported, which include biostimulation, bioaugmentation, and phytoremediation. Bioaugmentation and phytoremediation are of great interest due to their efficacy and easy handling.

8.3.1 Bioaugmentation

Since the early 2000s addition of microorganisms as a supplement to the contaminated soil has been proven to be an alternative strategy for the bioremediation of the TPHs in soil (Wu et al. 2016). This strategy is commonly used when indigenous microorganisms failed to degrade the contaminants or the soil become abiotic in condition due to the high toxicity of the contaminants (Adams et al. 2015). Other conditions for bioaugmentation are also considered when the hydrocarbon-degrading microbial population is low. The success of the strategy depends on the ability of the microorganism to survive in a hostile and foreign environment by overcoming the competition of indigenous microorganisms. In addition, the presence of the contaminants and their concentrations along with several environmental factors play a decisive role in judging the outcome of bioaugmentation (Cunningham and Philp 2000).

Microbial degradation of the TPHs in the soil is considered an ultimate natural mechanism by which the cleanup of soil can be done in an environmentally friendly way. The commonly used bacteria include *Bacillus sp.* (Das and Mukherjee 2007), *Acinetobacter sp.* (Mishra et al. 2004), *Burkholderia sp.* (Jasmine and Mukherji 2013), *Arthrobacter sp.* (Poi et al. 2017), *Mycobacterium sp.*, *Rhodococcus sp.*, *Pseudomonas sp.* (Das and Mukherjee 2007), and *Sphingomonas sp.* (Tyagi et al. 2011), which were isolated from the contaminated sites with TPHs. Different microorganisms have different mechanisms for degrading the TPHs in the soil. Some microorganisms depending on their metabolism prefer aromatic or alkane hydrocarbons, while others jointly degrade both.

Although protozoa and algae are important contributors to the microbial community in both terrestrial and aquatic ecosystems, reports on their involvement in the degradation of TPHs are limited (Adams et al. 2015). On the other hand, bacteria are the commonly preferred microorganism that is used as a bioaugmenting agent for the remediation of contaminants. However, lack of broad contaminant range alone makes fungi a suitable alternative, specifically for treating the TPHs contaminated soil. Further, easy adaptation to extreme conditions and their ability to grow in a wide range of pH made fungi suitable bioremediation agent (Schadt et al. 2003, Rousk et al. 2010).

8.3.2 Fungal Bioaugmentation

The fungal biomass is unique and diverse, having the ability to survive in a range of heterogeneous environments. The ability to utilize soil organic matter by colonizing on both biotic and abiotic surface act as an additional advantage for mycoremediation. The mycelia formation of the ability of fungi other than yeast aids them in easy colonization of soil and helps in the translocation of key nutrients and water (Pérez-Armendáriz et al. 2010). Also, the ability of the fungi to grow in petroleum or crude oil spill site, by utilizing them as a sole carbon source makes them potential organisms for hydrocarbon degradation. Apart from the several filamentous fungi genera, *Aspergillus* and *Trichoderma* were the commonly used Ascomycetes and white-rot fungi in basidiomycetes.

The most studied fungi for bioremediation are basidiomycetes, specifically, that are saprotrophic and biotrophic fungi. Saprotrophic basidiomycetes are the fungi that grow on dead organic matter, among which white-rot fungi exhibit a high potential for bioremediation of TPHs (Bosco and Mollea 2019). These fungi can utilize phenol backbone lignin compounds present in the dead plant matter as a sole carbon source until complete mineralization. On the other hand, biotrophic basidiomycetes (Ectomycorrhizal fungi) obtain the nutrient by having a mutualistic plant partner, where fungal mycelia envelopes the hair root and penetrate the cells of the cortex.

8.3.3 White-Rot Fungi

White-rot fungi (WRF) are one of the major naturally available bioremediation agents in nature. This Hymenomycetes class fungi grow on the woody plant, having the ability to degrade lignin or lignin-like compounds (Pointing 2001). WRF degrades the lignin molecules in the woody plant, less affecting the cellulose and hemicellulose. These degraders are specifically active under nitrogen starvation by activating their secondary metabolism for the production of a variety of ligninolytic enzymes (Esterhuizen-Londt et al. 2019). The secretion of the ligninolytic enzymes (lignin peroxidase, manganese peroxidase, aryl-alcohol oxidase) along with laccase, make them a potential bioremediation agent (Manavalan et al. 2015). The genera of *Trametes* sp., *Phanerochaete chrysosporium*, *Pleurotus* sp., and *Bjerkandera* are the four common WRFs used for bioremediation studies. The enzymes secreted by the white-rot fungi have the ability to transform a variety of organic pollutants such as PAHs, pesticides, and pharmaceuticals in wastewater and soil (Ellouze and Sayadi 2016).

The ability of WRT to sustain highly toxic compounds such as organic pollutants and hydrocarbons makes them a suitable bioaugmentation agent in comparison with bacteria (Table 8.2). The nontarget specific nature of the ligninolytic enzymes makes this WRT ideal for the remediation of a wide range of contaminants that include dyes, polycyclic aromatic hydrocarbons Pharmaceutical active compounds (PhAC), and pesticides (Mir-Tutusaus et al. 2018). Significant degradation of the contaminants can be achieved in combination with biostimulation; however, the

Table 8.2 Bioremediation of petroleum components present in soil by basidiomycetes fungi

Contaminants	Basidiomycetes	Conditions	Degradation (%)	References
Diesel fuel	<i>Pleurotus sp.</i>	5% level of contamination	55.53%	Ogbo et al. (2010)
		10% level of contamination	35.53%	
Benzo(a)pyrene	Ozonation + Spent Mushroom Compost	10 min pre-ozonation treatment	>75%	Russo et al. (2012)
			82%	
Bunker C fuel oil—C10 alkane	<i>Trametes versicolor</i>	180 days of growth on pine media	98.10%	Young et al. (2015)
Bunker C fuel oil—C14 alkane			48.60%	
Bunker C fuel oil—Phenanthrene			76.40%	
Bunker C fuel oil—C10 alkane	<i>P. Strigosozonata</i>	20 days of growth	99%	Young et al. (2015)
Anthracene	<i>Pleurotus ostreatus</i>	110 days incubation	96.00%	Acevedo-Sandoval et al. (2018)
Pyrene			86.00%	
Chrysene			98.00%	
Benzo(a)anthracene			98.00%	
Diesel's F2 fraction (C10-C16)	<i>Pleurotus ostreatus, Salix planifolia</i>		69–73%	Robichaud et al. (2019)
Hydrocarbons	<i>A. bisporus</i>	Soils:25–28 degrees Celsius; 60% water holding capacity	71.50%	Mohammadi-Sichani et al. (2019)
Hydrocarbons	<i>Pleurotus pulmonarius</i>	Soil amended with 150 g of sawdust	90.12%	Stanley et al. (2017)
Crude oil	<i>Lentinus subnudus</i>	Incubation for 3 months	20%	Adenipekun and Fasidi (2005)
Aromatic weathered hydrocarbon contaminated soil	<i>Phanerochaete chrysosporium</i> NRRL 6361	Incubation for 30 days	94.46%	D'Annibale et al. (2005)
PAHs in soil	<i>Phanerochaete chrysosporium</i>	19 days incubation	72.77–25.50%	Wang et al. (2009)
High molecular weight polycyclic aromatic hydrocarbons	<i>Trametes versicolor</i>	180 days of incubation	71%	Lladó et al. (2013)
	<i>Lentinus tigrinus</i>		61.2%	
PAHs in soil	<i>Bjerkandera sp. BOS55</i>	30 days of incubation	30 mg/kg of soil	Valentin et al. (2007)
Nitrobenzene and anthracene	<i>Trametes trogii</i>	12–24 days	90%	Levin et al. (2003)

(continued)

Table 8.2 (continued)

Contaminants	Basidiomycetes	Conditions	Degradation (%)	References
PAHs	<i>Pleurotus ostreatus</i>	80 days	80–95%	Norton (2012)
Total aliphatic hydrocarbon (TAH)	<i>Pleurotus ostreatus</i>	60 days	86.80%	Covino et al. (2016)

possibility of promoting indigenous microbes and subsequently creating competition for the augmented WRT makes this approach under wide scrutiny (Wu et al. 2016). As an alternative by considering the ability of WRT to degrade lignocellulosic material, bulking agents such as rice husk are used (Adewole and Olanrewaju 2017; Meysami and Baheri 2003). The commonly used substrates include rice straw, corn cob, straw bales, sugar beet pulps, cotton waste, wheat bran, rice bran, fragmented wood, sawdust, and coffee pulp (Young et al. 2015, Harry-Asobara et al. 2018). Furthermore, these agents were coated with animal manures or nutrients to enhance effective growth. By considering the significance of extracellular enzymes produced by white-rot fungi and their role in the degradation of hydrocarbons in soil, attempts have been made to increase the activity of the enzyme cocktail by growing in solid-state fermentation in the presence of various bulking agents such as orange peels (Rosales et al. 2007).

8.3.4 Other Fungi

Apart from the white-rot fungi, several ascomycete fungi exhibit a high ability to remediate contaminants mainly by the intracellular metabolism of xenobiotic mediated by cytochrome P450 enzymes, by producing secondary metabolites such as biosurfactants, lignin modifying enzymes, or polyunsaturated fatty acids. Ascomycetes such as *Trichoderma sp.* exhibit the ability to degrade the PAHs by the secretion of laccase to a limited extent (Nazifa et al. 2018). Ascomycetes are well adapted to polluted environments and are more frequent than basidiomycetes in these environments. Marine fungus is one example. By exploiting the ability of the marine fungus to adapt to high saline conditions and pH, utilization of these fungi as bioaugmenting agent have an advantage over terrestrial fungi in degrading hydrocarbons in extreme soil conditions (Table 8.3). Besides, their ability to tolerate heavy metals, such as lead and copper, adds an additional advantage.

8.3.5 Factors Affecting the Fungal Bioremediation

Bioremediation of hydrocarbons by fungi is affected by various factors which include physical-chemical properties of soil, the presence of co-contaminants, and their interaction with contaminants (Magan et al. 2010). The major factors that affect bioremediation are mentioned below.

Table 8.3 Bioremediation of petroleum components present in soil by Ascomycetes fungi

Contaminants	Ascomycetes	Condition	Degradation (%)	References
TPH	<i>Scedosporium</i>	182 days	91.20%	Adetutu et al. (2015)
TPH	<i>Penicillium funiculosum</i>	Acidic soil	30%	Mancera-López et al. (2008)
	<i>Rhizopus sp</i>		36%	
	<i>Aspergillus sydowii</i>		17%	
Diesel oil	<i>Fusarium solani EH</i>	Growing concentration (4 mL/L); diesel oil (7.2 mg/100 mL)	90.28% (static); 93.05% (shaking)	Mohamed and El-Kassas (2010)
Motor oil	<i>Bionectria sp.</i>	8% (v/w) of inoculum; 60% moisture content; 10% (v/w) used motor oil	91%	Kota (2010)
Petroleum chain hydrocarbon	<i>Mucor circinelloides f. circinelloides</i>		61.80%	HongBo et al. (2011)
Crude oil	<i>A. niger</i>	28 days of treatment	95.00%	Al-Jawhari (2015)
Aliphatic hydrocarbons	<i>Pseudallescheria sp. 18A</i>	60 days mycoaugmentation	79.70%	Covino et al. (2015)
Diesel spiked soil: C12–40 HC fraction	<i>Trichoderma longibrachiatum</i>	Inoculum conc.: 1×10^{10} conidia/mL; 96 days inoculation	54.2 ± 1.6%	Andreolli et al. (2016)
	<i>Trichoderma harzianum CCECH-Te1</i>		47–69.1%	
Diesel oil: TPHs	<i>Trichoderma reesei H002</i>	40 days; Temp-25° C	94.78%	Nazifa et al. (2018)
TPH	<i>Verticillium sp.</i>		99.60%	Marín et al. (2018)
Crude oil n-Alkane	<i>Penicillium citrinum</i>		77.00%	Barnes et al. (2018)
			95.37%	
TPH	<i>Lambertella</i>	2 months incubation	47.60%	Becarelli et al. (2019)
Crude oil	<i>A. flavus</i>	15 days	60.00%	Al-Dossary et al. (2019)

8.3.5.1 Oxygen and Nutrient Requirements

The growth and acceleration of the bioaugmented fungi depend on the availability of carbon and nitrogen source in the soil (Quintella et al. 2019). The growth of the fungi is generally hindered if there is a shortage of organic and inorganic compounds. On the other hand, being aerobic, lignin-degrading fungi require a continuous supply of aeration and other key nutrients (Boopathy 2000). Fungi require a continuous supply of oxygen, in addition, molecular oxygen is needed for the activation of key

degrading enzymes, which include laccase and oxygenase. In addition, while degrading the hydrocarbons in the dead soil, additional minerals such as calcium, sulfur, and magnesium along with other nutrients are essentially supplied to support the growth of fungi.

8.3.5.2 pH

The growth of fungi and their stability depend on the pH of the soil (Tortella et al. 2015). Most of the soil pH range from 5.0 to 9.0, which makes the indigenous microbes to sustain. Most of the microbes tolerate pH 4 to pH 8 but preferably pH 6.5 to pH 7.5. However, fungal optimum pH varies from pH 4.0 to pH 7.0, making them a perfect choice for bioremediation of oil spills in acidic soil (pH 4.0) (Asif et al. 2017). In general, before bioaugmenting the fungi, the pH of the soil has to be adjusted either with calcium carbonate or with organic acids to bring it to neutral.

8.3.5.3 Temperature

Temperature plays a major role in affecting the outcome of the TPHs degradation in the soil by fungi. In terms of the hydrocarbons, with the increase in the temperature, a gradual reduction in the surface tension properties leads to an increase in the bioavailability of the TPHs in water (Magan et al. 2010). On the other hand, fungi are sensitive to modulation in the temperature and the optimum temperature required for these fungi ranges from 20 °C to 40 °C (Fukasawa 2018). Depending on the nature of the fungi, during bioaugmentation, a slight variation in temperature gradually reduce the degradation ability and may aid indigenous microbes to propagate. However, in tropical and temperate zones, the temperature does not act as a major limiting factor.

8.3.5.4 Water Availability

Water content plays a crucial role in bioremediation processes by influencing fungal growth and their metabolic activities (Bastos and Magan 2009). Sufficient moisture content is required both for transport of gases and contaminants and for movement and growth of microorganisms. Optimal microbial growth occurs when the moisture content is between 30% and 80% (Sangeetha et al. 2004). When the moisture content is below 10% WRF becomes less active. However, during the waterlogging condition, the moisture content reaches the above optimal limit leading to the development of the anoxic condition, which the bioremediation rates.

8.3.5.5 Other Parameters

Texture, hydraulic conductivity, and permeability are the three interrelated properties of the soil that affect the availability of nutrients, contaminants, and oxygen in the soil. Low permeability soils are ineffective in soil flushing techniques (Magan et al. 2010). The conductivity in the range of 10–4 cm/s supports the transport of nutrients and pollutants within the soil thereby increasing the efficiency of the bioremediation. The presence of organic matter in greater quantities affect the bioavailability of the TPHs and enzymes. On the other hand, the salinity of the soil

has a positive impact on the degradation ability of WRF. It was noted that an increase in the salinity along with temperature reduces the growth of WRF (Tortella et al. 2015).

8.4 Types of Bioaugmentation

Apart from the fungal selection, bioaugmentation can be classified into three types depending on the external supplements. With the advancement, several organic and inorganic supplements were reported to be included with bioaugmentation, making a huge list. To simplify the types of bioaugmentation were broadly classified into

1. Bioaugmentation and biostimulation with organic nutrients.
2. Bioaugmentation and biostimulation with inorganic nutrients.
3. Bioaugmentation with surfactants.

Among the above three strategies, bioaugmentation with surfactants for the remediation of soil, TPH is considered to enhance the efficiency of the degradation process by reducing the overall time. Surfactants are compounds that reduce the surface tension between two interfaces (Mao et al. 2015). In the soil, utilization of the surfactants reduces the interfacial tension that holds TPHs with soil organic matter and makes them release into the aqueous phase. In the aqueous, the surfactant traps the TPHs in their micelles (Goddard 2017). The micelles are surfactant aggregates that are formed in liquid colloid, with either hydrophobic “tail” or hydrophilic “head” facing the aqueous phase. The concentrations that are required for the surfactants to form micelles are called critical micellar concentration (CMC).

The most common type of surfactant that is used is an anionic surfactant or nonionic surfactant (Table 8.4). Apart from the advantage of using surfactants, surfactants exhibit the ability to adsorb to soil particles and exhibit toxicity, which is more prominent in cationic surfactants (Mao et al. 2015). On the other hand, the surfactants are complex in structure and resist degradation which further complicates the process. As an alternative, several biosurfactants were introduced, which are produced by microorganisms. These compounds are easy to degrade and exhibit

Table 8.4 Common surfactants used for the bioremediation of hydrocarbons

	Anionic	Cationic	Non-ionic
Surfactant	<ul style="list-style-type: none"> • Sodium dodecyl sulfate (SDS) • Perfluorooctanoic acid • Sodium dodecyl • Benzene sulfonate 	<ul style="list-style-type: none"> • 1-dodecylpyridinium Chloride • Didecyl dimethyl ammonium chloride 	<ul style="list-style-type: none"> • Tween 80 • Triton X-100 • Triton X-20 • Cocamide • Brij-35
Biosurfactant	<ul style="list-style-type: none"> • Rhamnolipids 		<ul style="list-style-type: none"> • Sophorolipid • Fructose lipid • Surfactin • Guar gum

similar properties to that of chemical surfactants (Lang and Wagner 2017). The commonly utilized biosurfactant is rhamnolipids, which is a widely used anionic biosurfactant in the remediation of PAHs in soil (Zhen et al. 2019).

In addition, surfactants can increase the secretion of ligninolytic enzymes by removing the enzymes that are trapped in the mycelium or hyphae of the white-rot fungi (Singh and Singh 2017). On the other hand, surfactants have the ability to ease transport of vesicles that could carry enzymes. Despite several advantages, utilization of the surfactants beyond certain concentrations causes toxicity towards the WRF (Lechuga et al. 2016).

8.5 Mechanisms of Petroleum Fungal Hydrocarbon Degradation

In most organic pollutants, complete degradation was achieved in the aerobic conditions. Oxidation of organic pollutants is considered as an initial step in the degradation process by WRF, which is followed by a series of catalytic actions of oxygenases and peroxidases leading to mineralization of the organic pollutant to either carbon dioxide or water. On the other hand, peripheral degradation pathways convert these pollutants into several central intermediate metabolism compounds.

8.5.1 Fungal Degradation of PHCs Polycyclic Hydrocarbons

Mycoremediation suitably occurs either through adsorption of the hydrocarbon or degradation of the compound by the fungi. The petroleum hydrocarbon contaminants sustain proportionate molecular structures that enable effective fungal strains to act upon them. Fungi follow two main mechanisms for breaking down recalcitrant petroleum hydrocarbons.

1. *Intracellular attack via cytochrome P450 (CYP 450)*, utilizing oxidation, reduction, hydrolysis, and dehalogenation processes. The recalcitrant compounds adsorbed into the fungal system and degraded by cytochrome P450 monooxygenase in microcosms have been reported (Das and Chandran 2011). The CYP system first catalyzes the aromatic structured contaminants to form arene oxide, which is highly reactive and carcinogenic. Further, these arene oxides are catalyzed to trans-dihydrodiols by epoxide hydrolase or rearrange nonenzymatically to phenols. Moreover, hydroxylation products undergo the detoxification process to form various intermediates, which are excreted (Prenafeta-Boldú et al. 2018).
2. *Extracellular oxidation* of petroleum hydrocarbons occurs through the secretion of extracellular enzymes by the fungi for the oxidation of the recalcitrant compound mediated by oxidative and ligninolytic enzymes.

8.5.2 Ligninolytic Enzymes

Laccases (EC 1.10. 3.2) are multicopper enzymes, which catalyze oxidation reactions coupled to the four-electron reduction of molecular oxygen to water. These high redox potential enzymes have the ability to oxidize the phenolic compounds including TPH to carbon dioxide (Prenafeta-Boldú et al. 2018). This substrate nonspecific enzyme is commonly produced by fungal ascomycetes such as *Trichoderma* and Basidiomycetes—white-rot fungi. In addition, the requirement of oxygen as co-substrate for the initial degradation makes the laccase a more popular choice for the degradation of TPH. The key intermediate product in oxidation of aromatic hydrocarbons is phenoxy radicals, which couple to quinone followed by ring fission leading to the formation of carbon dioxide and water as end products (Hwang et al. 2007).

Tyrosinase (EC 1.14. 18.1) is derived from *Streptomyces glaucescens* and the fungi *Neurospora crassa* and *Agaricus bisporus*. Recent studies suggest the ability of white-rot fungi to produce tyrosinase (Seo et al. 2003). Contrary to laccases, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine (Madhavi and Lele 2009). Tyrosinases are metalloenzymes belonging to the type-3 copper protein family which contain two copper ions in the active site. Tyrosinases perform two sequential enzymatic reactions: hydroxylation of monophenols and oxidation of diphenols to form quinones which polymerize spontaneously to melanin. Tyrosinase is capable of oxidizing the phenols into insoluble substances that can be eliminated by precipitating or filtering them.

Lignin peroxidase (EC 1.11. 1.14) is also referred to as diaryl propane oxygenase. Lignin peroxidase is a heme-containing enzyme that catalyzes hydrogen peroxide-dependent oxidative degradation of lignin. Lignin peroxidase catalyzes the biodegradation of lignin using hydrogen peroxide. Lignin peroxidase oxidizes phenols to phenoxy radicals and nonphenolic aromatics to radical cations.

Compared to lignin peroxidase, manganese peroxidase follows a different mechanism of action. Manganese peroxidase (EC 1.11. 1.13) is an extracellular heme enzyme that catalyzes the peroxide-dependent oxidation of Mn (II) to Mn (III). Manganese peroxidase catalyzes the conversion of Mn^{+2} to Mn^{+3} using hydrogen peroxide. Aryl-alcohol oxidase is the third class of ligninolytic enzymes. Aryl-alcohol oxidase (EC 1.1. 3.7) belongs to the family of oxidoreductases specifically those acting on the CH–OH group of donors with oxygen as acceptor. Aryl-alcohol oxidase oxidizes a variety of aromatic benzyl (and some aliphatic polyunsaturated) alcohols to the corresponding aldehydes. In addition, aryl-alcohol oxidase participates in the oxidation of aromatic aldehydes to the corresponding acids and also has activity on furfural derivatives.

Ligninolytic enzymes exhibit a mutualistic working mechanism. Among them, laccase and AAO work through direct oxidation, by attaching to the hydroxyl functional groups and thereby breaking down large phenolic polymers. On the other hand, lignin peroxidase and manganese peroxidase act by producing reactive peroxide species, which aid in the degradation of hydrocarbon compounds. Overall, relating the by-products produced by these enzymes confirms the mutualistic role of

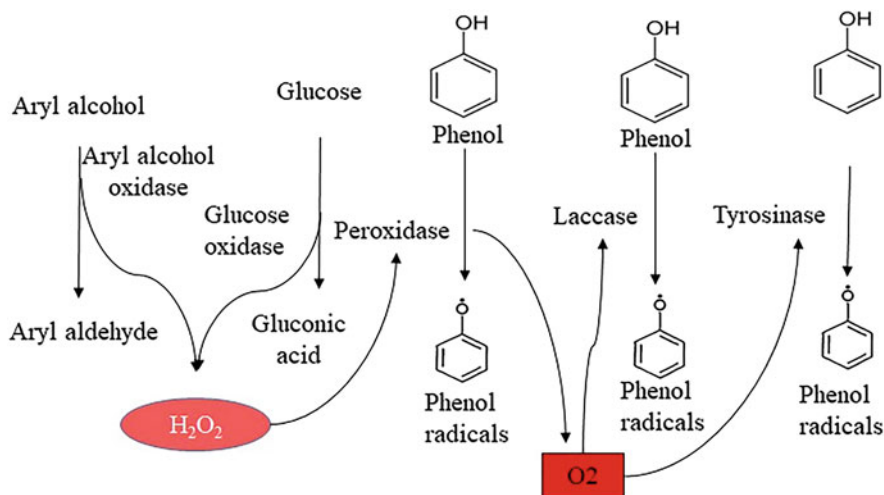


Fig. 8.3 Mutualistic mechanism of ligninolytic and oxidative enzymes in degradation of phenolic compounds

ligninolytic and oxidative enzymes in the degradation of hydrocarbons (Fig. 8.3). Being controversial on the degradation debate of pollutants by ligninolytic enzymes or laccase as a cumulative and as sole, the current results clearly shows the cumulative effect of the enzymes in the degradation of pollutants (Agrawal and Shahi 2017).

8.6 Conclusion

Bioremediation of the subsurface oil spills or the presence of petroleum hydrocarbons in the soil is of major concern due to its potential toxicity. Bioremediation processes by fungi, specifically by white-rot fungi, involve multiple dimensional studies by considering various factors that affect the efficiency. Despite various techniques to enhance the remediation, including surfactants and bulking agents to address the problem, there is still a gap for innovation needed to design industrially feasible processes. With current knowledge on the bioremediation of petroleum hydrocarbons degrading pathway and the enzymes involved, it may be concluded that white-rot fungi with surfactants can be considered as a key strategy for the cleanup of soil contaminated with petroleum hydrocarbons.

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