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Review Article

Role of endophytic fungi in bioremediation of petroleum hydrocarbon contaminated soil

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ABSTRACT

Continuous exploitation of petroleum hydrocarbons in various industries has led to their release as hazardous pollutants into the environment. Contamination by these polycyclic aromatic hydrocarbons had negatively shifted the structures of the rhizobial community, soil properties, ecological functions and human health. Hence, clean-up of hydrocarbon-contaminated soil by a low-cost and effective strategy is the need of the hour. Among the physical, chemical and biological methods, the biological method utilizing the efficiency of endophytes in reclamation of polluted soil is eco-friendly and cost-effective. Fungi (Endophytic fungi) are ubiquitous species that have the genetic potential to remediate the hydrocarbons in the soil in association with plants. This review highlights the hydrocarbon remediation mechanisms adopted by endophytic fungi which could be made used in the development of eco-friendly, sustainable-low-cost remediation technology to restore polluted soil.

INTRODUCTION

Biogenic and geological process results in the production of hydrocarbons which are used as fossil fuels. The successful economic growth and progress led by the heavy demand and commercialization of petroleum hydrocarbons and their products in various industries cannot be denied [1] have reported that the utilization of these petroleum hydrocarbons at the end of 2030 will reach 106.6 million barrels. They are of diverse nature that include small alkanes, cycloalkanes, alkynes, monoaromatic hydrocarbons (Toluene, Benzene, Ethyl benzene, Xylene), complex polycyclic aromatic hydrocarbons (naphthalene); asphaltenes (Phenols, Ketones, Fatty acids, Esters, Porphyrins), resins (Cardaxoles, Quinolines, Sulphonates, Amides, Pyridine), waxes, tar and other organic pollutants [2-5].

Pollution is caused by volatile and soluble small hydrocarbons, oil spills, and leakage from petro refinery industries during storage, loading, transportation and distribution. Unavoidably the complex composition, durability and stability [6] of the released waste products have made them to be a major pollutant affecting all living forms and a risk to human health. Their hydrophobic nature increases the affinity of the petroleum hydrocarbons toward air, water, and soil [7]. As they form a layer over the soil, they damage the soil structure, and alter the physical, chemical and biological properties of the soil making them phytotoxic/barren [8]. Expect few crops (rye, oat and wheat) most of the plant growth is affected by reduction of the germination rate, photosynthetic pigment, leaf morphology, oxidative stress and finally plant death [9]. The hydrocarbon pollutants affect human health as they exhibit toxicity on the eye, heart, liver, kidney, and nervous system and act as mutagen-causing cancer [10]. Considering the toxic effects on the environment and human health there is an urgent need to devise suitable technologies to alleviate the toxic effects of the petroleum hydrocarbons.

Remediation of the environment highly polluted by hydrocarbons was very difficult due to its highly toxic and less degrading nature. Treatment methods are laborious and huge which also depend on some factors like the nature, type and composition of the hydrocarbon pollutant, details of the polluted site, nature of the microbiome present or available and treatment procedures involved [11]. The types of reclamation methods based on mechanisms like containment, separation and remediation/destruction involve physical, chemical, physico-chemical, biological, thermal, electric, electromagnetic and ultrasonic treatment methods. If the reclamation method is carried out within the site i.e., in the original place, then it is called as in-situ and if not in the original place i.e., at the other site it is called as *ex-situ* [12,13].

Physical methods like the excavation of the polluted soil and then its treatment, surface capping, physical barriers to avoiding the transport of pollutants into the soil, ex-situ soil washing and heat desorption methods and microwave heating were adopted [7]. But constraints like transportation, potential health risk to humans caused by handling directly / indirectly, energy consumption, complexity of regulatory framework and technology and cost factors were observed in physical treatment [14]. Chemical methods include degradation of pollutants by hydrolysis, oxidation and reduction,

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ultraviolet and photocatalytic oxidation, adsorption of contaminants by activated carbon and remediation by utilizing electrochemical systems [7]. Biological methods include bioremediation utilising microorganisms and phytoremediation by plants [14,15].

Degradation of petroleum hydrocarbons by indigenous microorganisms with differing efficacies (bacteria, fungi, yeast) [16-18] is bioremediation. Rapid reproduction and metabolic pathways of bacteria made it possible to research primarily bacterial bioremediation [19,20]. When compared with bacteria fungi are found to be more potent in metabolizing and catabolizing hydrocarbons by the extracellular enzymes produced by them [21].

The efficacy of plants to degrade hydrocarbon pollutants is referred to as phytoremediation. It is an eco-friendly and cost-effective method when compared with conventional traditional methods. In phytoremediation, plants use mechanisms like phytostimulation, phytoextraction, phytovolatization, photodegradation and rhizofilteration to decontaminate pollutants [7]. Degradation by plants alone (Phytoremediation) was limited due to the slow growth and small size [22]. Remediation potential was further improved by the associated symbiotic and endophytic microbes [23-25]. Hence, the research hot-spot is exploring the plant-endophyte fungal association to find a remedy for hydrocarbon pollutants.

Fungi in bioremediation of hydrocarbon

Among the microbes, fungi were more preferred than bacteria in bioremediation [26]. The hyphae structure and metabolic enzymes of the fungi confers the ability to metabolize complex organic compounds into less complex compounds and incorporate them into its system which proves its undeniable potential for the degradation of hydrocarbons [27]. The cosmopolitan and abundant nature of endophytic fungi in contaminated regions overcomes the limitations through its symbiotic mutualistic relation with plants. The ability of some plants to withstand the stress caused by hydrocarbons was aided by endophytic fungi. Endophytic fungi colonize the intercellular spaces and symplast of the plant tissues either partly or throughout their life cycle without showing any distinct infection [28].

Endophytic fungi improve plant health and growth by conferring stress tolerance, disease resistance capacity, carbon sequestration, and growth stimulation [29].Their extra-matrical hyphae increase the surface area of absorption beyond the root hair zone facilitating the enhanced uptake of water and minerals leading to increased biomass and faster remediation [30].The resistance conferred to the plants towards harmful pollutants was due to the metabolism of harmful toxic compounds by extracellular enzymes released by fungi into the plant tissue or soil [31].

Endophytic fungi involved in bioremediation of hydrocarbon

The possession of the endosymbionts is known to improve the phytoremediation capacities of plants. The endophytic fungi play a more prominent role than bacteria in adapting the host plant to withstand the adverse effects of hydrocarbon contaminated soil due to its secondary metabolite production [32].The developmental plasticity and production of secondary metabolites and enzymes make the endophytic fungi, a suitable candidate to degrade pollutants [33,34] have observed the abilities of endophytic fungi in mangroves to degrade petroleum hydrocarbons (residual oil collected from motor vehicle service station) at Chorao, an island along Mandovi River, Goa, India. Among 57 fungal isolates, the highest degrading capacity was demonstrated by *Nigrospora* followed by *Aspergillus* sp., *Curvularia* sp., *Pestalotiopsis adusta*, *Fusarium* sp., *Aspergillus niger*, and *Cladosporium* sp [34]. Researchers identified endophytic fungi from the stem and leaf parts of 23 plants in Las Minas, a site contaminated by crude oil from natural springs in Ecuador, in which the majority of them belong angiosperm families followed by gymnosperm and bryophyte family. Nearly, 156 endophytic fungi were identified among which *Clonostachys* sp. Aspergillus sp., Verticillium sp., *Colletotrichum* sp, Phomopsis sp., and *Xylaria* sp., show a maximum of 90% hydrocarbon removal capacity, and *Clonostachys* sp, *Colletotrichum* sp., and *Saccharicola* sp. with 31% of degradation capacity when analysed by in-vitro colorimetric method [35].

Gas Chromatography analysis confirmed the complete removal of aliphatic compounds (Octane, Nonane, Undecane, Dodecane, and Tetradecane) and aromatics (1,2,3-trimethyl benzene, trans-decahydro naphthalene, 1,2,4,5-tetramethyl benzene, and pentamethylbenzene) by Aspergillus sp. and *Xylaria* sp. Whereas *Clonostachys* sp., *Verticillium* sp., and *Colletotrichum* sp. showed similar results except for partial degradation of n-nonane that showed partial degradation [35]. The polyaromatic hydrocarbons (PAHs) degradation efficacy of *Neotyphodium coenophialum* and *Neotyphodium uncinatum*, infecting *Festuca arundinacea* and Festuca pratensis respectively could degrade naphthalene, acenaphthene, acenaphthylene, benzo (a) anthracene, benzo (b) fluoranthene, benzo (k) fluoranthene, benzo (a) pyrene, perylene and indeno (1,2,3-c,d) [36]. *Cyperus laxus* Lam when inoculated with a microbial consortium that includes eight (08) known and two (2) unknown bacterial strains along with three fungal strains, *Penicillium janthinellum, Aspergillus carneus*, and *Aspergillus terreus* increased the phytoremediation of hydrocarbons thrice than non-inoculated plants[37].Inoculation of *Festuca arundinacea* by the filamentous fungus (*Lewia* sp.) has demonstrated 100% removal of phenanthrene, 70.3% of pyrene and 91.7% of hexadecane in perlite, and 100% removal of phenanthrene, 97.9% of pyrene and 95.1% of hexadecane in soil [38].

Researchers have identified the bioremediation potential of endophytic fungi *Phomopsis liquidambari* isolated from the inner bark of *Bischofia polycarpa* stems in utilizing 4-hydroxybenzoic acid (4-HBA) was found to be 94% and 99% in liquid culture and soil conditions, respectively [43]. Recently endophytic Fusarium sp. was isolated from the leaves of *Pterocarpus macrocarpus* Kurz was able to degrade benzo(a)pyrene (BAP), a five-ring polycyclic aromatic hydrocarbon produced by the incomplete combustion of organic materials[39,40] have identified the phenanthrene degrading ability (52.54%) of Phomopsis sp. (B3) when co-cultured with paddy by in-vitro culture method and further confirmation by field study shows the increase in degrading ability of phenanthrene was increased by 91.60% when compared with control groups. Several endophytic fungi can efficiently degrade plastic polyester polyurethane (PUR) in both solid and liquid suspensions. Aerobic and anaerobic utilization of plastic polyester polyurethane (PUR) as the sole carbon source by two *Pestalotiopsis microspora* isolates brings out complete degradation [41].

Polycyclic aromatic hydrocarbon (PAH), heterocyclic compound - indole [42] and phenolic 4- hydroxybenzoic acid [43] ferulic acid [44] cinnamic acid, [45] phenanthrene [46] and sinapic acid [47] and phytoestrogen luteolin [48] were efficiently degraded by *Phomopsis liquidambari* by secreting degradative enzymes and using them as sole carbon and energy source. The increased shoot and root biomass of *Festuca arundinacea* and *Festuca pratensi* (grass species) infected by endophytic fungi (*Neotyphodium*

coenophialum and *Neotyphodium uncinatum*), respectively were responsible for the removal of petroleum hydrocarbons from oil-contaminated soil [36].

The higher volume occupied by the highly branched fibrous root architecture of grass infected by *Neotyphodium* species was due to the additional effect of plant hormone induced by infection was reported to increase the rate of organic pollutants [49]. The two endophytic fungi Phomopsis sp. (B3) and *Ceratobasidum stevensii* (B6) isolated from *Bischofia polycarpam* stem's inner bark have shown 29.45 and 82.27% of phenanthrene removal capacity [46] in liquid culture method. Researchers have reported the colonization of the endophytic fungi *Phomopsis liquidambaris* in *Oryza sativa* L. and its phenanthrene degrading ability [50].

Mechanisms behind bioremediation

Enzymes

Endophytic fungi pave way for a novel approach to degrading toxic pollutants like hydrocarbons, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons, etc., by utilizing them as nutrition/energy source by their extracellular enzymes. Researchers [51,52] have reported the production of extracellular enzymes of low substrate specificity to degrade polyaromatic hydrocarbons. The endophytic fungi can produce extracellular enzymes- hydrolytic enzymes to degrade xylanases and cellulases and ligninolytic enzymes like laccases, ligninases, and peroxidases to degrade lignin and open phenyl rings [53] and intracellular enzyme-like P450 monooxygenase that could participate in the degradation of Polycyclic aromatic hydrocarbons (PAHs) [54].These ligninolytic enzymes could oxidize the phenolic and methoxyphenolic compounds, decarboxylase, and demethylate the organic pollutants [55].

Laccases, multicopper-enzymes, oxidize phenolic and non-phenolic compounds with concomitant reduction of oxygen to water playing a versatile role in bioremediation of polluted soil and polyaromatic hydrocarbon oxidation [56]. The oxidative exoenzyme slaccases and peroxidases non-specifically transform lignin by removing an electron and a proton from the hydroxyl / aromatic amine group of phenols to form free radicals [57]. The unstable free radical (phenoxy radical) undergoes both enzymatic and non-enzymatic reactions like hydration, disproportionation, or polymerization that led to Cα-oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage [58].

Laccases produced by *Pestalotiopsis palmarum* BM-04 efficiently remediate extra-heavy crude oil (up to 50000 ppm) and NaCl (2000mm) by using them as the sole carbon and energy source [59] Wang et al. have reported that laccases produced by *Monotospora* sp. strain W823 isolated from fresh stems of *Cynodon dactylon* are more active than white-rot fungi *Tramets* spp. The laccase activity could not only be induced by wheat bran but also by aromatic compounds related to lignin, phenol, and polyphenolic compounds. Researchers reported that the transformation of the polymeric compounds by oxidative exoenzymes will increase the availability of microorganisms [57]. Liu et al. have reported more amount of laccase production by the endophytic fungi *Monotospora* sp in *Cynodon dactylon*. *Pestalotiopsis* genus endophytic fungi of tropical plants possess the ability to produce laccase over lignocellulosic by-products [60]. and decompose by utilizing a wide range of polyaromatic hydrocarbons like pyrene, phenanthrene, dibenzothiophene and, naphthalene as carbon and energy source [43]. Naranjo et al. have studied the ability of the *Pestalotiopsis palmarum* BM-04 strain to produce laccase with wheat bran, lignin peroxidase with extra-heavy crude oil as the sole carbon and energy source [61,59]. The degradation of total petroleum hydrocarbons by two types of grass (*Festuca arundinacea* and Festuca pratensis) colonized by *Neotyphodium coenophialum* and *Neotyphodium uncinatum* was due to the increased root and shoot biomass, the release of water-soluble phenols and increased dehydrogenase activity in the soil [36].

Phomopsis sp. (B3) exhibits laccase activity and *Ceratobasidum stevensii* shows peroxidase [LiP] and manganese peroxidase [MnP] activities involved in the Degradation of PAH [62]. Enzymes located in the fresh fungal biomass of *Ceratobasidum stevensii* especially mycelium and the presence of Mn^{2+} ions are responsible for metabolizing phenanthrene and manganese peroxidase [MnP] oxidizes phenanthrene by using hydrogen peroxide as co-substrate [46]. The important step in degradation is the involvement of activated molecular oxygen to cleave the ring by oxygenase [63]. Increased activities of phenanthrene degrading enzymes like P450 monooxygenase, epoxide hydrolase, catechol 2, 3-dioxygenase, and dehydrogenase were identified by Fu et al. in *Phomopsis liquidambaris* in association with *Oryza sativa* L. [50].

Green Surfactants

Endophytic fungi have the potential to bioremediate the hydrocarbon contaminated sites by producing unique compounds called Green Surfactants. Surface and interfacial tension in the hydrocarbon mixtures were reduced by these biosurfactants which emulsify the hydrocarbon portion of the liquid contaminants, create microemulsion, lead to micelle formation and increase the solubility of the hydrocarbons in water [64]. These mechanisms facilitate their shifting from solid to liquid phase and enhance their availability for microbial degradation [65,66] has isolated 156 endophytic fungi and identified them underweight generaas *Pestalotiopsis* (33.3%), *Phomopsis* (25.0%), *Aspergillus* (11.5%), *Xylaria* (5.1%), *Penicillium* (2.5%), *Nectria* (1.9%), *Fusarium* (0.6%) and *Guignardia* (0.6%). Biosurfactant potential of the 8 endophytic fungi isolated from the roots and bark of *Myrcia guianensis* was analyzed and four strains (MgRe 2.3.1 – 64.93%; MgC 3.3.2 – 69. 52%; MgC 3.1.1 - 70.0%; and MgRe 1.3.1 – 75.75%) have shown good emulsifying potential (Emulsifying index – E24) when compared with Sodium dodecyl sulfate (Positive control – 78.45%) on kerosene [67]. This proves the biosurfactant producing ability of the four endophytic fungi in *Myrcia guianensis* efficient in the removal of petroleum hydrocarbons.

The *Phoma* sp. collected from *Eichhornia crassipes* (Mart.) Solms and *Cyperus ligularis* L., at the Negro River, Manaus, Brazil showed an emulsifier index of 52% for diesel oil [68,69] have isolated 35 different genera of endophytic fungi from leaves and branches of *Cananeia* and *Bertioga* in the mangrove forest, in Sao Paulo, Brazil. They were examined and out of these 35 genera, Martinho et al. have identified 15 endophytic fungi capable of producing emulsifiers. Endophytes belonging to genera *Diaporthe* sp and *Aspergillus* showed E24 values up to 62.8% when automobile engine oil was used. Emulsifying potential for commercial soybean oil by *Fusarium* sp. (50.0%) and *Aspergillus niger* (47.8%) was found to be above the positive control and similarly for hexane E24 was found to be 51.9%, emulsified by *Fusarium chlamydosporum* and 38.4% by *Aspergillus niger* [70] have also observed surfactant ability in 14 genera except for *Aspergillus niger* and Fusarium sp. *Xylaria enteroleuca* and *Trichoderma camerunense* with prominent activity among them.

On the peninsular Coast of India, 4 endosymbiotic fungi were isolated from the marine sponge *Fasciospongia cavernosa* [64].

Aspergillus ustus (MSF3) exhibited maximum biosurfactant production in Sabouraud dextrose broth and 42.8% of emulsification index for kerosene. The other strains exhibit varying emulsification potential as follows: MSF5 (17.5%), MSF1 (11.4%) and MSF8 (7.5%). It was reported that the increase in carbon content causes a simultaneous increase in biosurfactant production hence in the marine environment presence of this endosymbiotic fungi will bring out biodegradation of oil spillage sand thereby reducing the mortality rate of marine creatures, increase the dissolved oxygen level and light penetration ability [64].

Increased plant uptake

Our results suggest that *Lewia* sp. can be considered as a beneficial rhizo-remediator by stimulating hydrocarbon uptake. Plant uptake is one of the steps in the phytoremediation of soil contaminated with organic compounds. Information about the final plant PAH concentration is essential to predict the effectiveness of a phytoremediation operation [71]. *Festuca arundinacea* on infection with seed-borne filamentous fungus *Lewia* increases the uptake of hydrocarbons by the roots as evidenced by 6.8 fold pyrene accumulation in the roots in perlite after 45 days [38] as proved by Reynoso Cuevas et al. [72]. *Phomopsis liquidambaris* on endophyte relationship with Oriya sativa has increased its energy components like NADH and ATP which could supply energy to carry out the reactions concerned with phenanthrene absorption and degradation [50].

Increased enzyme activities and transcription rate

Xie and Dai have traced the pathway of ferulic acid degradation and found decarboxylation of ferulic acid to 4-vinyl guaiacol, then oxidized to vanillin and vanillic acid, followed by demethylation to protocatechuic acid and enters into the β-ketoadipate pathway for further degradation [44]. The presence of Ferulic acid has increased the activities and gene transcription levels of ferulic acid decarboxylase, laccase, and protocatechuate 3,4-dioxygenase. The P 450 monooxygenase converts phenanthrene to phenanthrene 9,10-oxide which was hydrolyzed by epoxide hydrolase to phenanthrene trans-9,10-dihydrodiol. Dehydrogenase then converts phenanthrene trans-9,10-dihydrodiol to 9,10-dihydroxyphenanthrene then by the action of catechol 2,3-dioxygenase, 9,10-phenanthrenequinone was produced. In another pathway, there occurs ring-opening in phenanthrene 9,10-oxide producing 9-phenanthrol which was oxidized to 9,10-phenanthrenequinone. After cleavage and oxidation, the formed 2,2'-diphenic acid enters into the TCA cycle [45]. *P. liquidambari* during phenanthrene degradation increases the transcription level of genes encoding for P450 monooxygenase, epoxide hydrolase, dehydrogenase, and catechol 2,3-dioxygenase [73].

Fu et al. [50] have explored by lab and pot studies that the presence of phenanthrene has induced the expression of the genes P450SMOB3 (Cytochrome P450 Monooxygenase), EHB3 (epoxide hydrolase), and DHB3 (dehydrogenase) involved in its degradation and the activity was more in roots when compared to leaves of *Oriza sativa* associated with *Phomopsis liquidambaris* proving its higher colonization in roots.

CONCLUSION

Indigenous and diversified endophytic fungi could bioremediate the hydrocarbon pollutants. Endophytic fungi in their association with the host plants improve their physiological growth traits and enhance the uptake and degradation of organic pollutants by producing secondary metabolite-like surfactants, increasing the activities of degrading enzymes and their gene expression. Knowledge about the mechanisms adopted by endophytic fungi will help to engineer the strategies adopted for bioremediation with better efficacy and also in designing microbial biosensors to monitor the remediation process.

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