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Bioremediation Potential of Hydrocarbon-Degrading Fungi from Select Soil Niches of India

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Abstract

The global facing serious environmental problems environmental pollution and contamination, resulting from petroleum hydrocarbons from human activities in the area of oil and gas sectors responsible for hydrocarbon contamination in soil. Developing countries, including India biggest environmental problem caused by hydrocarbon contamination in soil. Various techniques, including mechanical and chemical methods used for the bioremediation and degradation of hydrocarbon pollutants from the environment. Among the microorganisms, fungi are efficient, reliable, cost-effective, and environmentally friendly use in the bioremediation technology for the cleanup and detoxification of hydrocarbon contamination from the environment VIZ, soil. The fungi ensure the complete degradation of hydrocarbon contaminants into carbon dioxide, water, inorganic compounds and cell biomass. The findings showed that fungi are more efficient and effective in the removal of hydrocarbon contamination from soil. The advancement of bioremediation of hydrocarbon degradation involves fungal enzymes; genetically modified fungi reduce time and cost.

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Introduction

Bioremediation is an alternative technique that can be used to solve hydrocarbon pollution. Bioremediation has been described as a process that uses microorganisms or their enzymes to return the natural environments altered by contaminants to its original stages, Cerniglia, et al.,2020. Fungal biodegradation of petroleum hydrocarbons is nowadays, a cost-effective and environmentally friendly process that can be used to clean-up and detoxify hydrocarbon pollutants.

Fungi is one of the microorganisms previously discovered from sediments, water, and soil that have been polluted by hydrocarbons. In recent years various fungi have been isolated from petroleum hydrocarbon contaminated sites, Gnavi, et al. 2017. Furthermore, according to some recent investigation, various fungi can be able to degrade petroleum mixtures, including the polycyclic aromatic hydrocarbons (PAHs), transform hydrocarbon into energy and biomass as well as biological waste products, these activities result in detoxification and elimination of hydrocarbon pollutants from the environment, including soil, water, sediments and industrial waste, presence of hydrocarbons pollutants in these aforementioned environments are serious threat to public health Park, et al., 2019. The ability to degrade high molecular weight hydrocarbon pollutants depends on some advantages that were found in fungi. These include: (i) secretion of low substrate-specific enzymes (ii) ability to grow in an

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extreme environment than bacteria (iii) more access to hydrocarbon contaminants due to the formation of the mycelial network Taylor, et al., 2016.

Fungi has been reported to produce biosurfactants which serve as a mechanism for achieving biodegradation of hydrocarbons; however, this is not open to all but rather some specific fungal organisms Hassanshahian, et al., 2013. Objective of study To isolate & identify PAHs degrading fungi from soil. To screen the biodegradation of PAHs in isolated fungi. To determine the biodegradability of isolates in varying concentrations of PAHs. To perform qualitative analysis of PAHs degradation based on fungal biomass.

Materials and Methods

Collection of soil samples

The samples were collected from of Sagar, Madhya Pradesh, India, total Six oil-contaminated soil samples used for isolation of fungi ,were from six different sites. Each polybag was well labelled with the site of collection. Petrol Pump Soil From Railway Station, Sagar (M.P.), Garage Soil, Makroniya, Kabulapur Bus Stand Soil , Civil Lines Garage Soil, Makroniya Garage Soil – 1, Makroniya Petrol Pump Soil

Isolation and screening of indigenous fungi

Direct plate Method has been used for the isolation of fungi from soil , Pinch of each fine soil sample was sprinkled on solid agar plates having hydrocarbon as a sole carbon source ,with the help of sterilized spatula. Media and Chemicals, for isolation of

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fungi, Bacto Bushnell – Haas agar media has been used. Special About Bacto Bushnell – Haas Media, Chemically Defined, and Contain no any carbon source. The composition of Bacto Bushnell – Haas Agar Media: $MgSO_4$ -0.2 gm L^{-1}, $CaCl_2$ -0.02 gm L^{-1}, KH_2PO_4-1 gm L^{-1}, Fe Cl -0.05 gm L^{-1}, NH_4NO -1 gm L^{-1}, Agar – agar - 5 gm L^{-1}, Distilled water -1 L, pH -7.0. Preparation of Stock Solution: Naphthalene – 5 gm of naphthalene was dissolved in 10 ml of acetonitrile and heat for a while until crystals disappeared. Acenaphthene -5 gm of acenaphthene was dissolved in 10 ml of acetone and heated for a while until the crystals disappeared. Anthracene – 5 gm of acenaphthene was dissolved in 10 ml of acetone and heat for a while until crystals disappeared. 0.5% each of , Naphthalene, Acenaphthene & Anthracene from stock solution were added in the medium as a sole carbon source. 0.1% of Tween 80 was added to the medium. Mediums were sterilized at 121˚C ,15 lbs pressure for 20 minutes. After sterilization, the media was poured in sterilized petri plates aseptically, and all the plates were left for overnight for solidification.Pinch of each fine soil sample was sprinkled on the media with the help of sterilized spatula. All the plates were incubated at 28 ^oC temperature, for 20 – 25 days, Pure isolates were maintained on Potato Dextrose Agar (PDA) slants.

Results and Discussion

Identification of isolated fungi

The spore-bearing mycelia were then carefully sectioned, teased out ,and

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stained on a slide using lactophenol cotton blue stain and then observed with a light microscope.

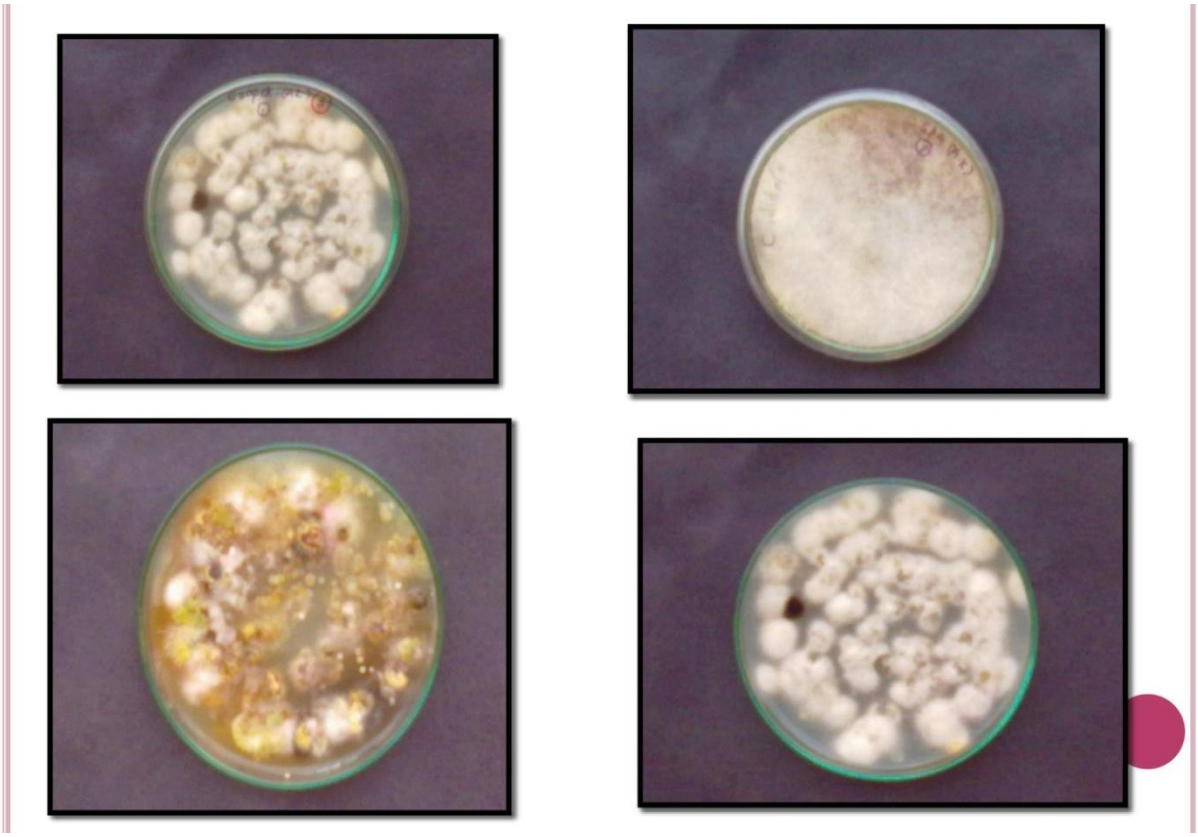

Fig-12

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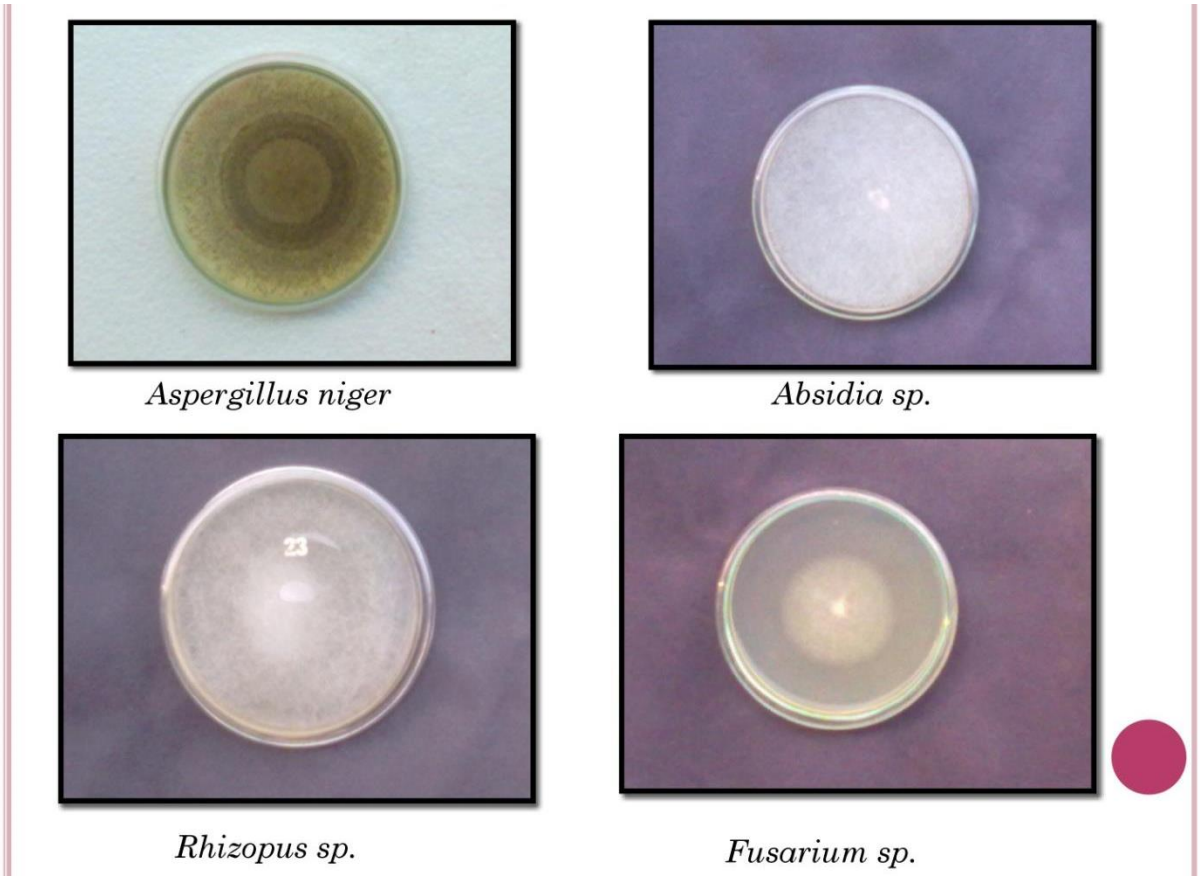

Fig-14

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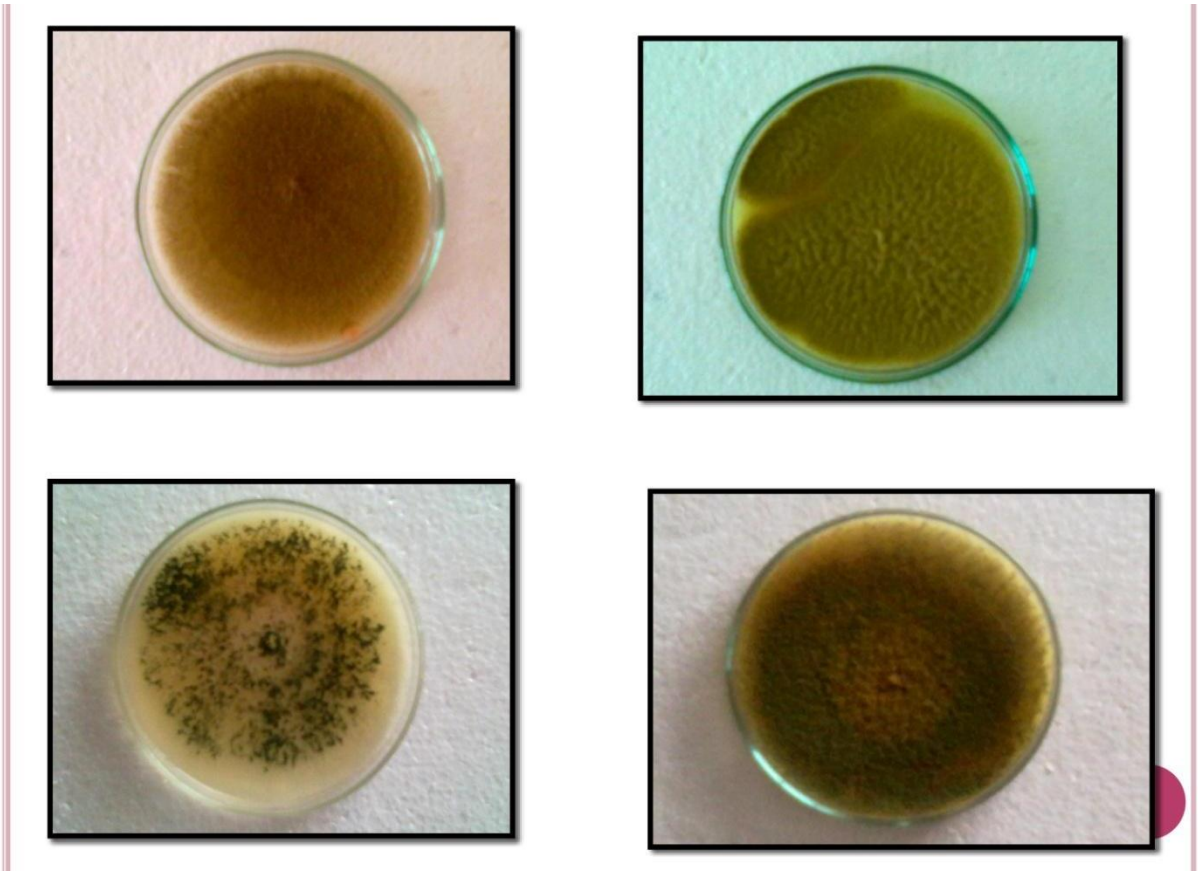

Fig-15

Screening in solid agar plate

Bacto Bushnell – Haas agar media of varying concentration of each hydrocarbon was prepared. Four different concentration of hydrocarbon are as follows :- 1.0% - 10 µg/ml - 1.00 gm dissolved in 100 ml distilled water, 0.75% - 7.5 µg/ml - 0.75 gm dissolved in 100 ml distilled water, 0.5% - 5 µg/ml - 0.50 gm dissolved in 100 ml distilled water Bacto Bushnell – Haas agar media of four different concentration for each carbon source was prepared separately , incorporated with 0.1% Tween

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80. The medium was sterilized at 121˚C temperature,15 lbs pressure for 20 minutes. All fungal isolates were point inoculated at all plates of different concentration of each hydrocarbon.

All the plates were incubated at 28˚C for 12 - 15 days. After incubation all plates were observed for fungal growth. **Screening in broth** On the basis of results obtained from Screening in solid agar plates, screening in broth (Bacto Bushnell - Haas) was carried out. Based on this, specific concentration of carbon source for screening of isolates in broth for all three compounds were fixed to be 0.5% (5µg/ml).

Preparation of Methylene blue solution (Redox indicator)

The 2% ($^{w}/_{v}$) methylene blue solution as a redox indicator was prepared and 1 gm of methylene blue was dissolved in 50 ml of distilled water. The prepared solution was sterilized separately at temperature 121˚C , 15 lbs pressure for 20 minutes.

Media for Screening Bacto Bushnell – Haas broth was used for the screening test. Medium were sterilized at 121˚C , 15 lbs pressure for 20 minutes.Three agar discs of a pure culture of each isolate were inoculated into 50 ml of sterilized Bacto Bushnell – Haas broth in well labelled 150 ml Erlenmeyer

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flask. 0.5% of each hydrocarbon (Naphthalene, Acenaphthene & Anthracene separately),& Tween 80 (0.1%) along with 0.1% Tween – 80 and 0.05 ml of methylene blue solution was also transferred to media. Control flask was also prepared having no organism.All the flask were incubated at temperature 28˚C with constant shaking at 120 rev/min for 7 days. The aliquots in the flask was monitored daily for colour change from deep blue to colourless.

Percentage of Methylene blue reduction After incubation, broth in flask were subjected to filteration by filter paper to separate biomass & other, followed by centrifugation at 8000 rpm for 15 minutes. Supernatant thus obtained were analyzed spectrophotometrically at 609 nm (for methylene blue). Percentage of biodegradation was calculated by a formula – *% of Degradation= [1- Absorbance of treated sample/Absorbance of control] × 100*

Biodegradation assay of the isolated cultures

Three agar discs of fresh pure cultures of each isolates were inoculated into the Bacto Bushnell – Haas broth (50ml/150ml Erlenmeyer flask) containing 0.1% ($^{v}/_{v}$) Tween 80 & 0.5% ($^{v}/_{v}$). Each hydrocarbon (Naphthalene, Acenaphthene & Anthracene) was added to different well labelled flask separately.

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A total of eight fungal isolates were obtained from Bacto Bushnell – Haas medium identified as - *Fusarium sp., Rhizopus sp., Absidia sp., Aspergillus niger, Aspergillus terreus, Aspergillus versicolor,Trichoderma harzianum & Aspergillus sp.* Among these biodegraders, few organism showed best potential for biodegradation e.g *Fusarium spp., Rhizopus sp., Trichoderma harzianum,* & *Aspergillus versicolor* . It was observed during screening in agar plates, as well as in methylene blue reduction test for all three hydrocarbon compounds. The ability of these isolates to produce a colour change in the medium is presumably due to the reduction of the indicator by the oxidized products of hydrocarbon degradation or due to the fungal growth which utilizes oxygen for their metabolism. Among the eight fungal isolates *Fusarium sp., Rhizopus sp., Trichoderma harzianum & Aspergillus versicolor* displayed the fastest onset of biodegradation.

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Figur-14: Methylene blue reduction

Table-1: Reduction of methylene blue by fungal isolates after 7 days of incubation in different hydrocarbon.

S. No.	Organism	Naphthalene	Acenaphthene	Anthracene
1	Control	1.023	0.722	0.843
2	*Fusarium sp.*	**0.671**	0.**303**	0.**504**
3	*Rhizopus sp.*	0.817	0.524	0.621
4	*Absidia sp.*	0.732	0.618	0.611
5	*Aspergillus niger*	0.824	0.603	0.599
6	*Aspergillus terreus*	0.864	0.706	0.792
7	*Aspergillus versicolor*	0.702	0.507	0.667
8	*Trichoderma harzianum*	0.**658**	0.583	0.**498**
9	*Aspergillus sp.*	0.857	0.**318**	0.653

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Table 2: Percentage of methylene blue reduction.

S. No.	Organism	Naphthalene	Acenaphthene	Anthracene
1	Control	100%	100%	100%
2	*Fusarium sp.*	**34.5%**	**58.04%**	**40.22%**
3	*Rhizopus sp.*	20.17%	27.42%	26.33%
4	*Absidia sp.*	28.44%	14.40%	27.52%
5	*Aspergillus niger*	19.43%	16.48%	28.94%
6	*Aspergillus terreus*	15.54%	02.21%	05.93%
7	*Aspergillus versicolor*	**31.37%**	29.77%	20.87%
8	*Trichoderma harzianum*	**35.68%**	19.26%	**40.93 %**
9	*Aspergillus sp.*	16.23 %	**55.96 %**	22.54%

Table 3: Dry weight of fungal biomass

S. No.	Organism	Dry weight of fungal biomass (in gram)		
		Naphthalene	Acenaphthene	Anthracene
1	*Fusarium sp.*	**0.488**	**0.766**	**2.187**
2	*Rhizopus sp.*	**0.562**	0.515	0.243
3	*Absidia sp.*	0.191	0.351	0.292
4	*Aspergillus niger*	0.179	0.284	0.350
5	*Aspergillus terreus*	0.247	**0.589**	0.872
6	*Aspergillus versicolor*	0.152	0.369	0.242
7	*Trichoderma harzianum*	0.148	**0.665**	**1.244**
8	*Aspergillus sp.*	**0.395**	0.366	1.015

For Naphthalene

Fusarium sp., Aspergillus versicolor, and *Trichoderma harzianum* gave best result as compared to other isolates. **34.5 % , 31.37% and 35.68%** of biodegradation were noted. Rhizopus sp. also gave good results in terms of biodegradation,which was estimated by their bulk biomass of .562 gm followed with *Fusarium sp.* with a biomass of .488 gm. Least amount of degradation was showed by ***Aspergillus terreus & Aspergillus niger*** with percentage of methylene blue reduction **15.54 %** and **19.43 %**.

For Acenaphthene *Fusarium sp.*, and

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Aspergillus sp. showed best degradation potential . Reduction of methylene blue by **58.04%** and **55.96%** were recorded after incubation of 7 days at 28˚C in shaking condition. In terms of biomass, ***Fusarium sp. , Aspergillus terreus & Trichoderma harzianum*** having the bulk biomass of **0.766 gm, 0.589 gm, 0.665 gm** respectively. **For Anthracene** On the basis of methylene blue reduction test and dry weight of fungal biomass, *Fusarium sp. , Rhizopus sp. , Trichoderma harzianum and Aspergillus sp.* gave best result of degrading anthracene. **40.22% , 26.33% , 40.93% and 22.54%** of methylene blue was reduced by these isolates respectively. *Rhizopus sp. , Trichoderma harzianum and Aspergillus sp.* gave the biomass of **2.187 gm , 1.244 gm and 1.015 gm** respectively.

Field application of the isolates

Oil spillage, accidental leakage of petroleum products , oil refineries , discharge of spent oil from garages and others are the major sources of pollution. . These microbes can remove or reduce to atleast non – toxic level. One approach is to attach or stick these microorganism to some carriers or extenders in the form of powdered or liquid form using Tween 80 which also enhances the degradation process. In order to prevent run – off, 'inert' stickers or adhesives (such as molasses, corn syrup, latexes) may be incorporated into the formulation. A good sticker combined with charcoal can serve as a protectant. This will reduce the effects of ultra – violet light, dessication other

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environmental factors. The advantage associated with fungal remediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies (such as incineration, thermal desorption, extraction). The use of fungi is expected to relatively economical as they can be grown on a no. of inexpensive forest or agricultural wastes such as corncobs, sawdust. More so, their utilization is a gentle non – aggressive approach.

Current Trends In Mycoremediation Of PAHs

Fungal bioreactors for the removal of PAHs is one of the contributions of the biotechnological approach. During the last decades, fungal bioreactors for the degradation of PAHs have been developed. These bioreactors are still in the developmental phase; some of them are Immobilized bioreactors, closed batch feed bioreactors, compost bioreactors, and others. Immobilized bioreactors are best known forthe removal of naphthalene. Good removal of naphthalene was attributed to the maintenance of immobilized cells of *Phanerochaete chrysosporium* in bioreactors. The system is a closed batch feed design equipped with gas scrubbers, thus preventing toxic metabolites from escaping in the environment. This system allows simultaneous determination of mineralization of PAHs and mass recovery. Composting is one of the most promising reactor system for hazardous soil treatment. Composting can reduce the amount of extractable PAHs by stimulating biodegradation or

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binding of intermediates to organic matter in soil.

Similar results showed hetetrophilic fungi are 3.6x105, 7.1x105, and 9.51x106.Cfu/g, *Penicillium spp , A. niger, Mucor spp, Rhodotorulla spp, Rhizopus, Phanerochaete spp,Alternaria alternate, Fusarium spp* are strains of fungi that have been discovered by different scholars to degrade different components of petroleum. This study has revealed that oil polluted soil in the Niger Delta is habited by hydrocarbon-degrading fungi, which can be biostimulated to enhance oil pollution cleanup in the area (Egbo et al., 2018). Mangrove fungus #NIOSNM126 (*Penicillium citrinum*) was found to be highly efficient in biodegradation of crude oil, reducing the total crude oil content by 77% and the individual *n*-alkane fraction by an average of 95.37%, indicating it to be a potential candidate for the development into a bioremediation agent (Natasha et al, 2018) and Higher TPH and HMWPAHs biodegradation levels in bioaugmented microcosms were also associated to a significant decrease in acute ecotoxicity (EC50) by Vibrio fischeri bioluminescence inhibition assays. Molecular profiling and counting of viable hydrocarbon-degrading bacteria from soil microcosms revealed that fungal bioaugmentation promoted thegrowth of autochthonous active hydrocarbon-degrading bacteria. The implementation of such an approach to enhance hydrocarbon biodegradation should be considered as a novel bioremediation strategy for the treatment of the most recalcitrant and highly genotoxic hydrocarbons in

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aged industrially polluted soils (Medaura et al., 2021). The filamentous fungi used in pollutant removal, including widely studied species of Aspergillus, Penicillium, Fusarium, Verticillium, Phanerochaete and other species of Basidiomycota and Zygomycota are summarized. The removal efficiency of filamentous fungi and time of elimination of a wide variety of pollutant compounds and their easy handling make them excellent tools for the bioremediation of emerging contaminants. Various types of beneficial byproducts made by filamentous fungi, such as raw material for feed and food production, chitosan, ethanol, lignocellulolytic enzymes, organic acids, as well as nanoparticles, are discussed. Finally, challenges faced, future prospects, and how innovative technologies can be used to further exploit and enhance the abilities of fungi in wastewater remediation, are mentioned (Ghosh et al., 2023), Among the fungi, *R. mulciginosa* showed most utilization potential of spent oil from petrol driven vehicle, while *C. terreus* was the least. The spent oil from diesel vehicle, *A. fumigatus* exhibits highest utilization capacity, whereas *R. oryzae* was the least. The results imply that consortium of the isolates could be effective for remediation of hydrocarbon contaminated soil (Essien et al., 2023). The current study suggested the possible oil-biodegradation activities induced by three Fusarium isolates from Riyadh, Saudi Arabia. The produced biosurfactants are not toxic against tomato seed germination, emphasizing their environmental sustainability (Al-Otibi et al., 2023).

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Conclusion

In this study, the dominant fungal strains isolated from two crude oil-polluted sites were identified as *Aspergillus oryzae* and *Mucor irregularis*. The two fungi showed high tolerance to varying concentrations of a complex hydrocarbon mixture (used engine oil) and demonstrated hydrocarbon degradation abilities. The different enzyme expressions and activities shown by the analyzed fungi could be helpful for their survival in contaminated environments, by allowing them to utilize the hydrocarbons present in the substrate as nutrients. Therefore, these two strains are potential candidates for the remediation of hydrocarbon polluted soils. However, further studies are needed to understand, from genetic and biochemical points of view, the hydrocarbon degradation mechanisms of these fungi, and therefore enhance their degradation performance. This can be achieved through the use of the traditional recombinant DNA technology and advanced gene manipulation tools such as the CRIPR-Cas systems, George-Okafor, 2009. Efforts are ongoing in our laboratory to obtain more information on the chemical processes and pathways used by these fungi in degrading hydrocarbons. We are also aiming to characterize possible biosynthetic gene clusters/genes associated with laccase expression in these two fungi and attempt to further enhance their expressions in heterologous yeast hosts.

In general, fungi have demonstrated their ability to degrade low molecular weight PAHs faster in soil but higher

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molecular weight PAHs, degradation is limited. Thus it is important to screen isolate and apply fungi that can degrade high molecular weight PAHs efficiently. The use of white rot fungi in soil bioremediation of PAHs is an exciting and promising technology, because of their ability to degrade highly condensed PAHs its high tolerance to substrate & a large capacity to compete with indigenous soil microflora. Fungal degradation of PAHs has been achieved successfully in bioreactors. . An understanding of metabolic pathways and metabolite is crucial to the development of successfully strategies for mycoremediation in soil. Extensive research on enzymes involved in metabolic pathways is necessary to optimize the process of biodegradation by fungi. Genes encoding enzymes involving degradation oh high molecular weight PAHs must be cloned, sequenced & characterized. The development of selective bioengineered fungi can open a new era in PAH environment.

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