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Original Research

Evaluate the removal of Phenanthrene (PHE) by Fungus and Screening of Extracellular Enzyme Activities during Biodegradation

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Abstract:

Biodegradation is the degradation of the materials into environmentally acceptable products such as water, carbon dioxide, and biomass by the action of naturally available microorganisms under normal environmental conditions. Phenanthrene, as a widespread polycyclic aromatic hydrocarbons (PAHs) contaminant in vitro and in vivo of plant, has the characteristics of carcinogenicity, teratogenicity and mutagenicity.

Environmental pollution by petroleum hydrocarbons from contaminatedgroundwater and soils is a serious threat to human health. Microbial fuelcells (MFCs) could be employed in the treatment of these recalcitrant pollutants with concomitant bioelectricity generation. Microbial degradation of Phenanthrene with several fungi screened from nature was conducted to select fungi for the bioremediation of Phenanthrene. *Thrichoderma sp.* S019, a fungus collected from soil, had the highest rateof degradation on the agar medium containing Phenanthrene. Maximal degradation (72%) was obtained when *Trichoderma sp.* S019 was incubated for 30 days after the addition of 0.1 mM of Phenanthrene to the liquid medium. Furthermore, the degradation of Phenanthrene was affected by the addition of a carbon source, the addition of a nitrogen source and agitation. Also, 1,2-Dioxygenase and 2,3-Dioxygenase were produced by *Trichoderma sp. S*019 in a liquid medium. These enzymes play an important role in the metabolism of substrates, revealing a high stereoselectivity for initial dioxygenase and enzymatic hydration since the K-region of phenanthrene was the major site of metabolism.

Keywords: Phenanthrene, Enzyme, Biodegradation, Fungus.

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Introduction:

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemicals thatoccur naturally in coal, crude oil, and gasoline. They result from burning coal,oil, gas, wood, garbage, and tobacco. PAHs can bind to or form small particlesin the air. High heat when cooking meat and other foods will form PAHs. Naphthalene is a manmade PAH used in the United States to make other chemicals and mothballs. Cigarette smoke contains many PAHs [1]. PAHs are uncharged, non-polar molecules, with distinctive properties due in part to the delocalized electrons in their aromatic rings. Many of them are found in coal and in oil deposits, and are also produced by the combustion oforganic matter—for example, in engines and incinerators or when biomass burns in forest fires [2]. Polycyclic aromatic hydrocarbons are discussed as possible starting materials for abiotic syntheses of materials required by the earliest forms of life. [3,4]

Phenanthrene is a polycyclic aromatic hydrocarbon (PAH) derived from coal tar. There is no commercial production of or use for this compound. Phenanthrene is environmentally ubiquitous as a by-product of incomplete fossil fuel and wood combustion and is present in ambient air, surface and drinking water, and food products. The toxicity and carcinogenicity of PAHs are abundantly published, which is primarily benzo[a]pyrene; however, toxicity data for phenanthrene are limited [5,6].

Data from structurally related PAHs suggest that phenanthrene is absorbed readily from the gut and lungs. In general, PAHs are highly lipid-soluble and pass across epithelial membranes. Data regarding subchronic, chronic, developmental, or reproductive toxicity of phenanthrene in experimental animals by any route of exposure was not located from the available literature. Toxicity Reference Values (TRVs) for the oral or dermal route of phenanthrene exposure cannot be derived. Data is unavailable to derive TRVs for avian, amphibian, and reptilian species [7].

Bioremediation is a process used to treat contaminated media, including water, soil and subsurface material, by altering environmental conditions to stimulategrowth of microorganisms that degrade the target pollutants. [8,9] Most bioremediation is inadvertent, involving native organisms. Research on bioremediation is heavily focused on stimulating the process by inoculation of a polluted site with organisms or supplying nutrients to promote the growth. In principle, bioremediation could be used to reduce the impact of byproducts created from anthropogenic activities, such as industrialization and agricultural processes [10] Bioremediation could prove less expensive and more sustainable than other remediation alternatives. [11]. Biodegradation is the breakdown of organic matter by microorganisms, such as bacteria and fungi.[12] It is generally assumed to be a natural process, which differentiatesit from composting. Composting is a human-driven process in which biodegradation occurs under a specific set of circumstances.

The process of biodegradation is threefold: first an object undergoes biodeterioration, which is the mechanical weakening of its structure; then follows bio fragmentation, which is the breakdown of materials by microorganisms; and finally, assimilation, which is the incorporation of the old material into new cells.

Fungi share same habitats in most hydrocarbon contaminated sites. Therefore, fungi are able to degrade the pollutants as effective as bacteria and unlike bacteria, fungi are more tolerant to the toxic compound like PAHs. Many isolated fungal species have been reported to be capable of biodegrading PAHs. As a main group of microorganisms, filamentous fungi (including white rot fungi (WRF). Fungi use their extracellular and intracellular enzymesto metabolize pollutants depending on the type of fungi and which part of theirbody interacts with the pollutants. Incubation with mycelia as a PAHs degrader will increase the degradation rate as compared to spore inoculation. This may be due to high surface area and high production of degradation enzymes [13], [14]. Besides, mycelium is also proven to degrade both hydrophilic and hydrophobic pollutants.

The aim of this study is to evaluate the removal of Phenanthrene (PHE) by fungus isolate, to

determine the growth rate of both strains during the removalstudy and finally analyse the interaction between fungi. Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic compounds that have accumulated in the environment due to a variety of anthropogenic activities and their persistence is chiefly due their low water solubility. PAHs are often mutagenic and carcinogenic which emphasizes the importance of their removal from theenvironment [15,16].

Phenanthrene

Phenanthrene is a polycyclic aromatic hydrocarbon (PAH) with formula C14H10, consisting of three fused benzene rings. It is a colorless, crystal-like solid, but can also appear yellow. Phenanthrene is used to make dyes, plastics and pesticides, explosives and drugs. It has also been used to make bile acids, cholesterol and steroids [17]. Phenanthrene occurs naturally and also is a man-made chemical. Commonly,humans are exposed to phenanthrene through inhalation of cigarette smoke but there are many routes of exposure. Animal studies have shown that phenanthrene is a potential carcinogen [18]. However, according to IARC, it is not identified as a probable, possible or confirmed human carcinogen[19].

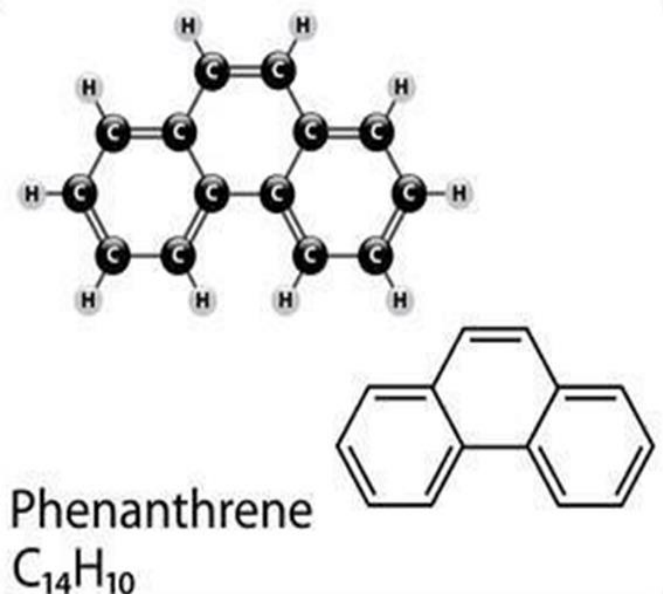

Fig 1. Formula structure of Phenanthrene.

Biodegradation

Biodegradation is the breakdown of organic matter by microorganisms, such as bacteria and fungi [20]. It is generally assumed to be a natural process,which differentiates it from composting. Composting is a human-driven process in which biodegradation occurs under a specific set of circumstances [21].

The process of biodegradation is threefold: first an object undergoes biodeterioration, which is the mechanical weakening of its structure; then follows biofragmentation, which is the breakdown of materials by microorganisms; and finally assimilation, which is the incorporation of the old material into new cells [22].

In practice, almost all chemical compounds and materials are subject to biodegradation, the key element being time. Things like vegetables may degrade within days, while glass and some plastics are on the other end of the scale, taking many millennia to finally decompose. A standard for biodegradability used by the European Union is that greater than 90% of the original material must be converted into CO_2, water and minerals by biologicalprocesses within 6 months[23].

Determination of PAHs degradation by fungal isolates

The medium used for the degradation investigation was the same as that for the enrichment procedure. The selected fungal isolates were first sub cultured in MSM agar plate supplemented with ANT or BAA to activate the mycelial growth. Six agar plugs (5 mm in diameter) with active mycelia from the periphery of the fungal colony were aseptically inoculated into 125 ml conical flasks with 50 ml of culture medium with ANT or BAA. The medium without inoculum was set up as the blank control to detect abiotic degradation.Triplicate flasks of each isolate and control were established for analysis. Allthe flasks were incubated at 25 ± 1 °C on a rotary shaker at 150 rpm in dark. At every 10 day interval, ethyl acetate extraction was undertaken by using three flasks of mycelia with culture medium for each treatment according to the method from [24,25] Three other flasks were used to determine the growth and analysis of metabolites.

Screening of extracellular enzyme activities during biodegradation

Enzyme activities were assayed every 5 days

throughout the total 40 days incubation period. Extracellular cultures were harvested by the centrifugationunder 14,000 rpm at 4 °C to discard the fungal cells and pellets, and the enzymatic activities in the extracellular fluids were spectrophotometerically determined. Lac- case activity was determined by the oxidation of 2,2-azino- bis-3- ethyl-benzthiazoline-6-sulfonic acid (ABTS) based on the method of [26,27]. by using 0.1 mM ABTS in the reaction buffer of 0.1 M sodium tartrate(pH 4.5) with 50 ll culture filtrate. One unit (U) of laccase activity was definedas the production of 1 mol of product per minute under the condition of 30 °C and pH 4.5. Manganese-dependent peroxidase (MnP) activity and lignin peroxidase (LiP) was detected using phenol red and veratryl alcohol, respectively, according to reported studies [28]. The concentration of total extracellular protein was also measured spectrophotometerically at 750 nm.

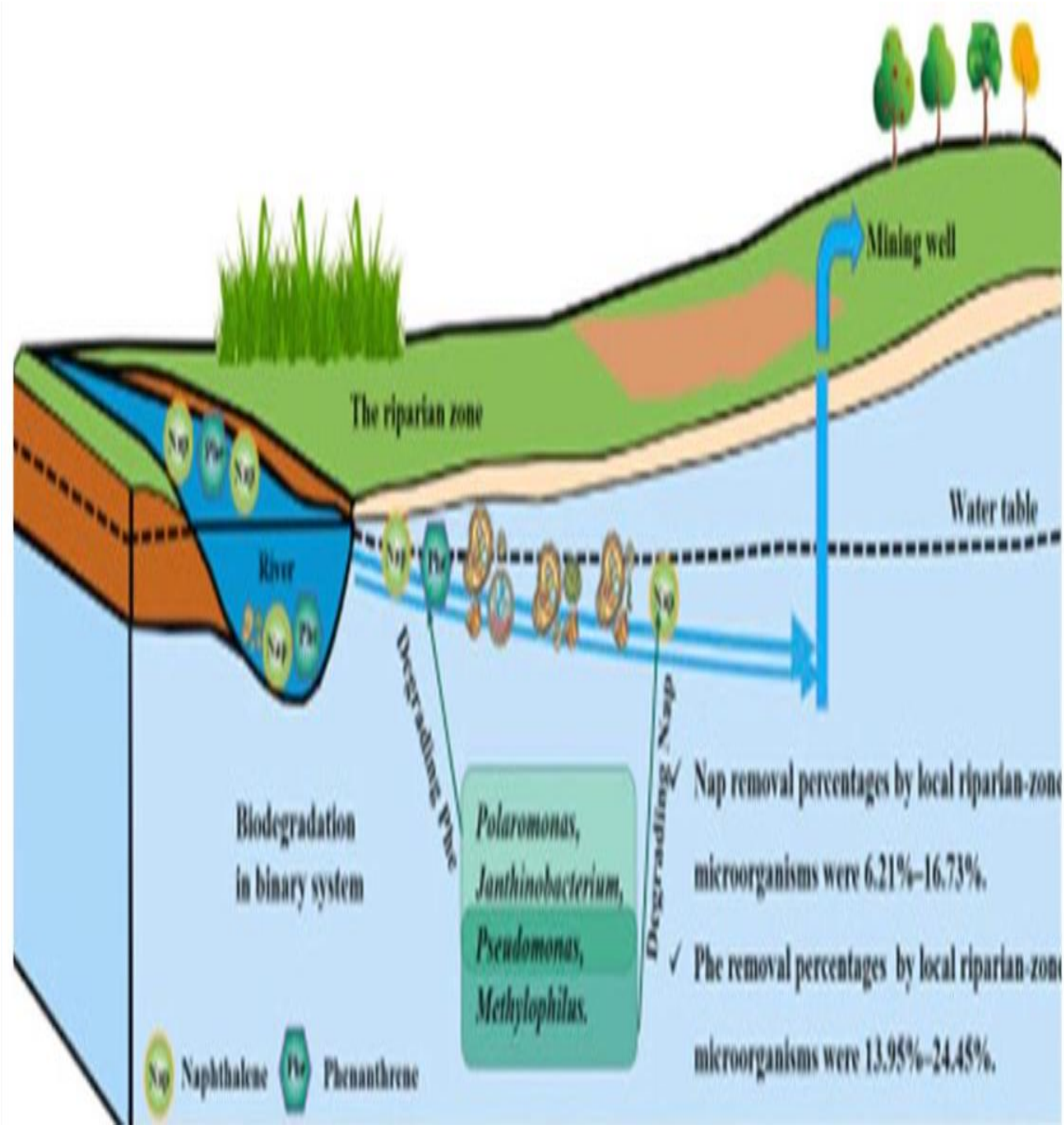

Fig 2. Biodegradation process of Phenanthrene.

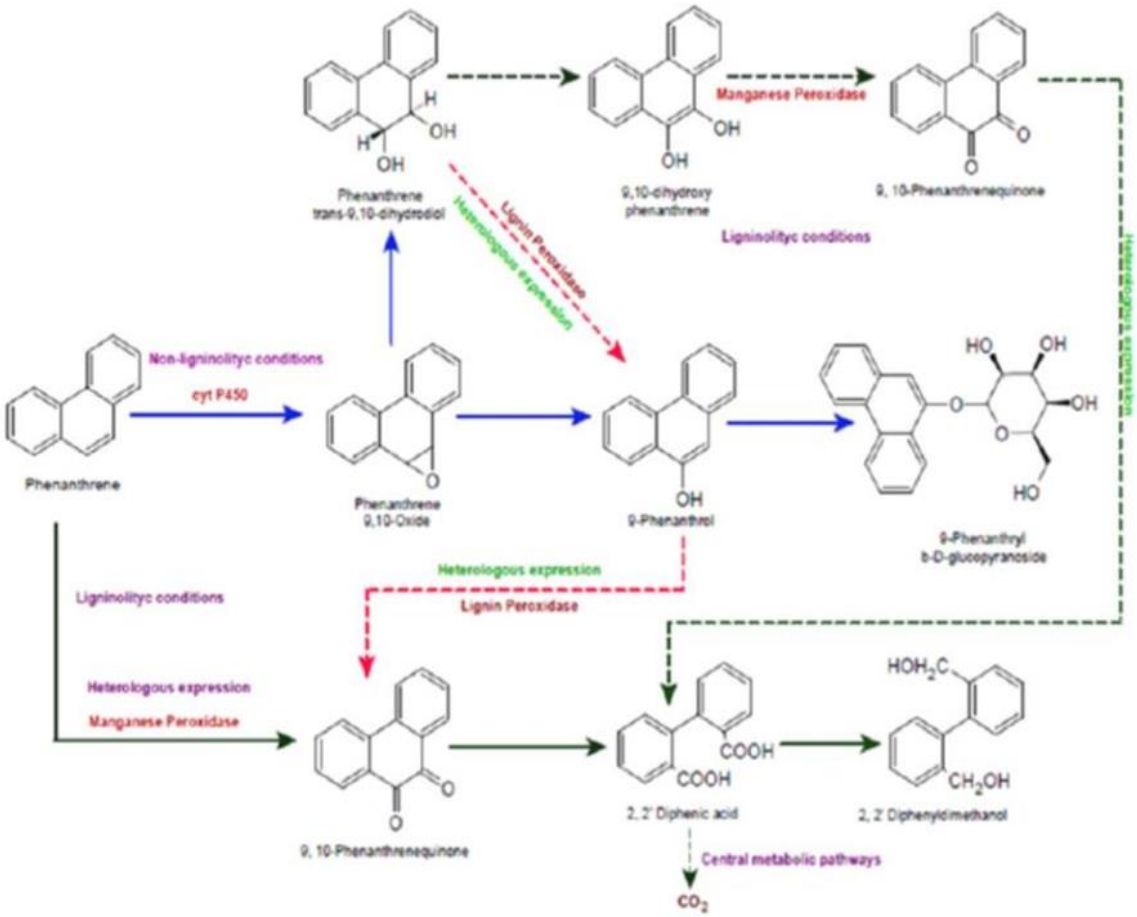

Fig 3:- A proposed or hypothetical metabolic pathway for Phe degradation under ligninolytic and non-ligninolytic conditions and possible changes in the degradation pathway for the heterologous expression of genes encoding the production of peroxidase enzymes in non-ligninolytic fungi.

Phenanthrene biodegradation by *Aspergillus niger*

Since the 1970s, research on the biological degradation of PAHs has demonstrated that bacteria, fungi and algae possess catabolic abilities that maybe utilized for the remediation of PAH-contaminated soil and water[29]. Phenanthrene (Phe) is one of several PAHs that are commonly found as pollutants in soils, estuarine waters, sediments and other terrestrial and aquaticsites [30]. Solid culture systems have shown great effectiveness in the removal of toxic compounds from soil. In this method, agroindustrial wastes are used such as wheat straw, corn stalks, sugarcane bagasse and pine wood chips [31]. Among others. When small amounts of agroindustrial residues are added to contaminated soil they confer soil apparent bulk density and porosity, help todiffuse oxygen between the particles and increase water retention. They are also used to support the growth of exogenous microorganisms, which are bioaugmented in soil to accelerate the degradation process, and, because of their nature, serve as carbon, phosphorus and nitrogen sources which are potentially important for the growth of organic pollutant degrading microorganisms [32]. Filamentous fungi offer certain advantages over bacteriafor bioremediation in solid culture because of their rapid colonization of solidsubstrates, such as soil or agroindustrial residues. In addition, they secrete large numbers of extracellular enzymes in solid culture and tolerate high concentrations of toxic compounds [33]. The most extensive studies have focused on white-rot basidiomycetes species such as *P. chrysosporium, Pleurotus ostreatus*, and *Trametes versicolor*. These microorganisms degradePAHs cometabolically [34].

The removal of PAHs by ligninolytic fungi has been attributed mainly to theirextracellular ligninolytic enzymes [35], but their preference to colonize compact wood is a clear disadvantage since it limits their capability to grow in a completely different

environment such as soil [35]. Also, non-ligninolyticfungi, such as *Cunninghamella elegans*, *Penicillium janthinellum*, *Aspergillus niger* and *Syncephalastrum sp*., are able to transform a variety of PAHs, including chrysene and benzo(a)pyrene, to polar metabolites [36]. These microorganisms carry out a mono-oxygenation of the PAH molecules by the intracellular cytochrome P-450 dependent oxidase system [37]. These fungi do not produce extracellular peroxidases, however, they do produce cytochrome P450 monooxygenase which can oxidize PAHs to epoxides and dihydrodiols: highly potent carcinogens that accumulate in soil Figure (3). The efficient application in bioremediation of contaminated soils is dependent, then, on having fungal strains with the ability to grow in contaminated soil without being displaced by indigenous microflora and which also produce efficient PAH-degrading enzymes such as lignin and manganese peroxidases or phenoloxidases which allow the mineralization of toxic compounds (figure 3). To achieve this goal, genetic engineering has been an important tool to generate genetically modified microorganisms(GEMs) through the expression of gene clusters encoding the degradation of a wide variety of pollutants. For example, simple aromatics, nitro aromatics, chloroaromatics, polycyclic aromatics, biphenyls, polychlorinated biphenyls,oil components etc., have been cloned and characterized for an increased degradation potential compared to their naturally occurring counterparts. Studies have focused primarily on bacteria and obtained good results for bioremediation systems [40, 41].

Knowledge of similar activities in fungi is limited to some white-rot fungi and a few species of non-ligninolytic fungi; however some studies have focused on toxic compound degradation, where recombinant strains were more efficient in the removal of PAHs from soil than wild-type strain [42]. Hypothesised that heterologous expression of genes codifying MnP and LiP in non-ligninolytic fungi will complement the degradation pathway of cytochrome P450 to obtain complete mineralization of the hydrocarbon without leaving more toxic intermediary compounds which accumulate in thesoil (figure 3)[43].

We have studied the possibility of producing these peroxidases in non-ligninolytic fungi isolated from contaminated soil because of their capacity to remove PAHs in soil; a number of filamentous fungal species are capable of secreting large amounts of proteins into the medium[44]. In our laboratory one fungi was isolated from sugarcane bagasse using Mexican “Mayan” crudeoil as carbon source [45]. *Aspergillus niger* SCB2 was used to express a manganese peroxidase gene (mnp1) from *P.chrysosporium* using the inducibleTaka amylase promoter and secretion signal from *A.oryzae* and the glucoamylase terminator of *A.awamori* [46], aiming at increasing its PAH degradation capacity. Transformants were selected based on their resistance to hygromycin B and the discoloration induced on Poly R-478 dye by peroxidase activity. The kinetics of *A. niger* SCB2-T3 were measured in complete medium supplemented with hemoglobin to increase the MnP activity[47]. however, the transformant isolate of *A. niger* showed higher enzymatic activity in the presence of hemoglobin. The maximum specific activity of the SCB2-T3 isolate was 3 U/l, whereas the control isolates of *P. chrysosporium* reached 7.8 U/l. The maximum activity was obtained at 72 h for transformant and control isolates[48]. The transformants presented activity starting at 24 h, whereas the control isolate presented maximum activity only at 72 h. In solid culture the recombinant *A. niger* SBC2-T3 isolate was able to remove 95% of the initial Phe (400 ppm) from a microcosm soil system after 17 days, whereas the wild strain removed 72% under the same conditions. [49-50].

Fusarium avenaceum play important role in phenanthrene, pyrene as well as fluoranthene degradation and their mechanisms were also investigated [51]. One of causes by their efficient production of ligninolytic enzymes(peroxidase enzymes)Phanerochaete chrysosporium could degrade ANT and phenanthrene by producing lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) [52]

However, white-rot fungi belong to the species

with slow growth rate, and need relatively high concentrations of oxygen to increase their activities [53].

[54] Have also reported that the mycelial aging of white-rot fungi as well as the contamination by bacteria under the non-sterile condition can affect their application in waste water treatment. In addition, another limitation of white-rot fungi is their sensitivity to shear stress, which could hinder the extracellularenzyme production, such as MnP [55]. [56,57] have also used *Bjerkandera sp.*And established a soil slurry reactor to reduce the shear stress for improvementof fungal cell growth and ligninolytic enzymatic secretion. So in order to overcome the above disadvantages of white-rot fungi, attempts should be taken to receive more fast growing non-white-rot fungal isolates in the field of biodegradation. Actually, only limited information is available on the degradation of PAHs by non-white-rot fungi.For example, *Cunninghamella sp*. and *Aspergillus sp*. were reported for their potential in the transformation of benzo[a]pyrene and the conjugation mechanisms during the degradation [58]. [59,60] also investigated the co-metabolism between the bacterial consortia and *Penicillium janthinellum* isolated from contaminated soils on pyrene and chrysene to demonstrate their efficient mineralization ability, andthe consequent reduction in mutagenicity, when compared to axenic inoculum.Another report indicates that *Fusarium sp*. isolated from PAHs-contaminated soil could degrade anthracene, phenanthrene and pyrene with a high initial concentration (250mg l-1), when growing with five different bacterial species. Many studies on *Fusarium spp*. have shown their capability to degrade high- molecular-weight organic compounds such as coal cellulose, xylan, pectin.

Conclusion:

The biodegradation of PAH compounds by non-white-rot fungi isolated from mangrove sediments. *F. solani* MAS2 and MBS1 were found with the capability of degrading ANT and BAA as sole carbon source of up 40% and 60% of the initial added amount, respectively. Six metabolic products, including two new intermediates from the ANT degradation, detected by SMPE-GC/MS, showed the metabolic mechanism ofnon-white-rot fungi is partially similar to that of white-rot fungi. Production of laccase indicates its involvement for the transformation of PAHs substrates,and further in vitro assessment for direct enzymatic degradation will be performed to investigate the correlation between the PAH removal and enzyme production. In addition, studies on its purification, characterization and direct enzymatic degradation are also under investigation for a better understanding on its function for PAHs degradation.

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