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Chapter 6

Phenanthrene Removal from Soil by a Strain of *Aspergillus niger* Producing Manganese Peroxidase of *Phanerochaete chrysosporium*

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Additional information is available at the end of the chapter

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1. Introduction

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 the environment [1,2]. Since the 1970s, research on the biological degradation of PAHs has demonstrated that bacteria, fungi and algae possess catabolic abilities that may be utilized for the remediation of PAH-contaminated soil and water. Phenanthrene (Phe) is one of several PAHs that are commonly found as pollutants in soils [3], estuarine waters, sediments and other terrestrial and aquatic sites [4] and has been shown to be toxic to marine diatoms, gastropods, mussels, and fish [5,6].

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 [7], among others. When small amounts of agroindustrial residues are added to contaminated soil they confer soil apparent bulk density and porosity, help to diffuse 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 [8]. Agroindustrial waste also contributes microorganisms with the ability to degrade toxic compounds; some studies, for example, have demonstrated that microbial biostimulation in a soil/sugarcane bagasse system at a ratio of 85:15 could remove 74% of total petroleum hydrocarbons (TPH) from the soil at 16 days [9, 10].

Several ligninolytic fungi have been grown on sugarcane bagasse and used as inoculum for the bioremediation of soil contaminated with polychlorinated biphenyls [10], phenanthrene [11], and benzo(a)pyrene [12]; such lignocellulosic materials are the natural habitat of the fungi. Previous work [13] has reported that non-ligninolytic filamentous fungi, such as *Aspergillus niger* and *Penicillium frequentans*, grown on sugarcane bagasse and added to a soil spiked with 400 ppm phenanthrene, achieved 54% removal of the pollutant from the soil after 7 days, while a mixed culture of *P. frequentans* and *Pseudomonas pickettii* achieved 73.6% removal at 18 days [11].

Filamentous fungi offer certain advantages over bacteria for bioremediation in solid culture because of their rapid colonization of solid substrates, 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 [14].

The most extensive studies have focused on white-rot basidiomycetes species such as *P. chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor*. These microorganisms degrade PAHs cometabolically. The removal of PAHs by ligninolytic fungi has been attributed mainly to their extracellular ligninolytic enzymes [15-18], 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 [19-22].

Also, non-ligninolytic fungi, 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 [13, 14, 23, 24]. These microorganisms carry out a mono-oxygenation of the PAH molecules by the intracellular cytochrome P-450 dependent oxidase system [25]. 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 1) [26-29].

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 1).

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 [30, 31]. 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 [32]. It is

therefore hypothesized 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 the soil (figure 1).

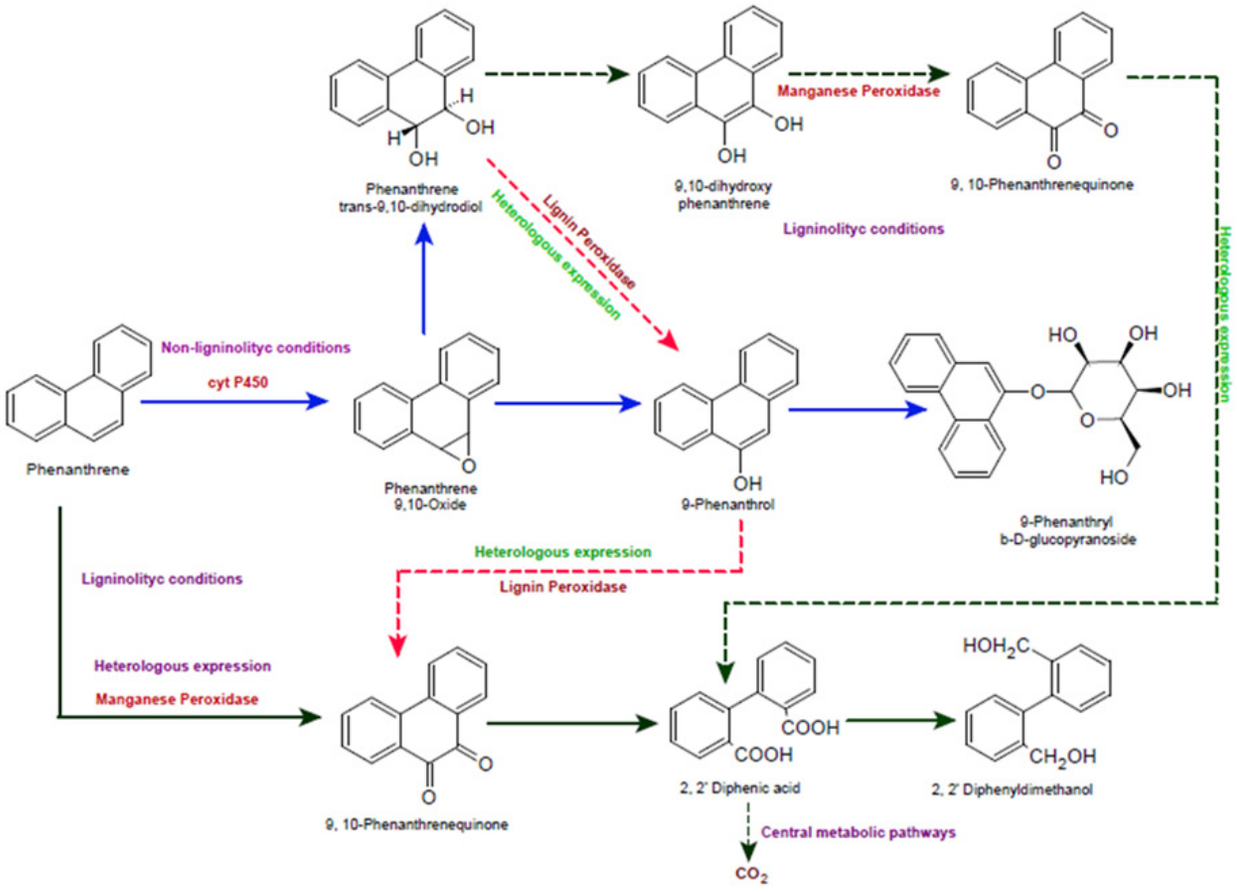

Figure 1. A proposed or hypothetical metabolic pathway for Phe degradation under ligninolytic and non-ligninolytic conditions and possible changes in the degradation pathway for the heterologous expression of genes encoding the production of peroxidase enzymes in non-ligninolytic fungi.

Some studies on the homologous expression of peroxidases in ligninolytic fungi in submerged culture, have shown that a transformed *Pleurotus ostreatus* was better at removing recalcitrant pollutant than the wild-type strain [33]. Other researchers reported the development of a homologous expression system for MnP and LiP in *P. chrysosporium*. The constitutively expressed *P. chrysosporium* glyceraldehyde phosphate dehydrogenase (*gpd*) promoter was used to drive the expression of the recombinant genes, now using nutrient-rich media in which the endogenous genes are not expressed. However, and despite the use of the strong promoter, production levels of the recombinant proteins remained at the same low level as is normally produced by the endogenous genes under starvation conditions [34, 35].

A study on the heterologous expression of these genes has been done on the baculovirus expression system [36]. In the *E. coli* expression system, LiPH8 was found to be expressed in

inactive inclusion bodies although activation was done in vitro [37], and the heterologous expression of MnP H4 isoenzyme in *E. coli* was also demonstrated [38]. The capacity of non-ligninolytic fungi to remove toxic compounds in solid culture may be used for the heterologous expression of peroxidase-encoding genes (manganese peroxidase, MnP and lignin peroxidase, LiP) from *P. chrysosporium*, thus increasing the degradation potential of PAHs in solid culture for soil bioremediation.

The expression of the lignin peroxidase gene of *Phlebia radiata* in *Trichoderma reesei*, failed to produce any extracellular LiP [39]. Overexpression of the *P. chrysosporium mnp*1 gene has been previously achieved in *Aspergillus oryzae* but at levels similar to that of the parental host in liquid culture [40]. Gene expression of *lip*A in *A. niger* F38 has also been studied, however LiP H8 activity was still detected at low levels [41]. Other researchers have used the protease-deficient *A. niger* strain for the expression of *mnp*1 and *lip*A [42]. Several factors have been identified which hamper the overproduction of recombinant proteins in filamentous fungi. In the case of heme-containing proteins, for example, limited heme availability has been indicated as a limiting factor [43, 44]. None of these studies focused on recombinant enzyme production in solid culture or its application in bioremediation systems.

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.

In our laboratory, one fungal strain was isolated from sugarcane bagasse using Mexican "Mayan" crude oil as carbon source [9]. This strain was identified by the sequence of ITS (Internal Transcription Spacer) fragments as: *Aspergillus niger* SCB2. The strain was able to tolerate (800 ppm) and to remove 45% of the initial Phe in solid culture, using sugarcane bagasse as texturizer with Phe-contaminated soil [13].

Aspergillus niger SCB2 was used to express a manganese peroxidase gene (*mnp*1) from *P. chrysosporium* using the inducible Taka amylase promoter and secretion signal from *A. oryzae* and the glucoamylase terminator of *A. awamori* [40], 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. No MnP activity was detected for the wild-type strain; however, the transformant strain of *A. niger* showed higher enzymatic activity in the presence of hemoglobin. The maximum specific activity of the SCB2-T3 strain was 3 U/l, whereas the control strains of *P. chrysosporium* reached 7.8 U/l. The maximum activity was obtained at 72 h for transformant and control strains. The transformants presented activity starting at 24 h, whereas the control strain presented maximum activity only at 72 h. In solid culture the recombinant *A. niger* SBC2-T3 strain 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. [32].

Although the transformant SCB2-T3 strain presented MnP enzymatic activity and production was maintained for 5 d, production levels of the recombinant proteins still remained lower than the control strain of *P. chrysosporium*, possibly because the promoter used for *mnp*1 expression was a maltose-inducible promoter. We, therefore, chose to analyze *mnp*1 expression regulated by a constitutive strong promoter (glyceraldehyde phosphate dehydrogenase, *gpd*A of *A. nidulans*) in *A. niger* SCB2 strain, and show the effect of heterologous expression of *mnp*1 gene in the transformant strain on the removal of Phe in solid culture using sugarcane bagasse as texturizer at microcosm level, and compare its degradation effectiveness with the wild-type strain.

In this study we obtained an effective heterologous expression of the mnp1 cassette controlled by the gpdA constitutive promoter in A. niger SCB2 strain. The MnP+7 transformant strain was selected due to its mayor MnP enzymatic activity after 48 h culture and up to 7 d, this important result shows that the new promoter favors protein production with catalytic activity from growth to idiophase, in comparison with SCB2-T3 strain, which shows only recombinant enzyme production while there was maltose in the culture medium, the compound that induces mnp1 gene expression in this strain. It is important for bioremediation systems that the oxidation involved PAHs enzymes are produced while stay in the soil. The longer the time in soil the increased enzyme production and higher removal of toxic compounds. On the other hand, the MnP+7 strain, was able to grow, tolerates and efficiently removed high Phe concentrations in contaminated soil as compared with the wild-type strain. After heterologous expression and the acquisition of these characteristics the MnP+7 strain, is a viable and important alternative for application in bioremediation of PAHs contaminated soils. This strain may have some potential as a bioaugmentation agent: is an efficient degrader of PAHs in high concentrations compared to other non-ligninolytic fungal strains which produce more toxic intermediaries than the original compound and tolerate lower PAH concentrations, and also compared to ligninolytic fungi not grown in soil and which are displaced by native soil microflora.

2. Methods

2.1. Heterologous expression of a *P. chrysosporium mnp*1 gene in *A. niger* SCB2

Aspergillus niger SCB2 [13] was used as the recipient in transformation experiments. pGMG-Hyg (Figure 2) was constructed by fusing the mature MnP cDNA to a 2326-bp fragment of the endogenous *gpd*A promoter and secretion signal from *A. oryzae* and to a 199-bp fragment containing the glucoamylase terminator of *A. awamori*. Fusions were created using T4 ligase (Fermentas). Plasmid pTAAMnP1 contains the secretion signal, MnP cDNA of *P. chrysosporium* and terminator, and was obtained from Dr. Daniel Cullen from the University of Wisconsin, USA [40]. Plasmid pDLAM89d contains the HygB resistance gene for fungi; this plasmid was donated by Dr. Jesus Aguirre of the Instituto de Fisiología Celular, UNAM, México. Plasmid pAN52.1 contains the *gpd*A promoter of *A. nidulans*.

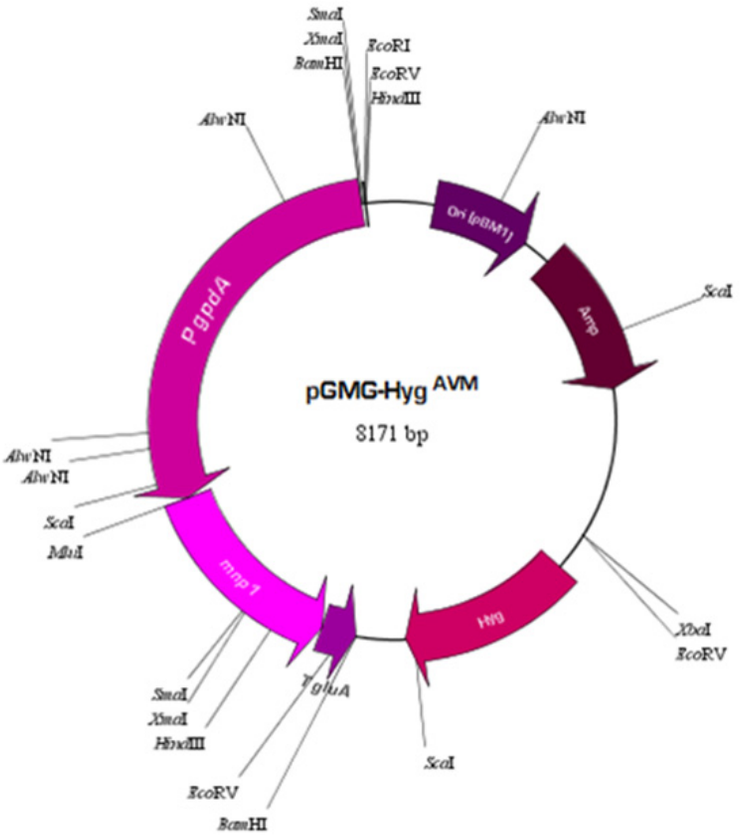

Figure 2. Expression vector pGMG-Hyg. The expression cassette contains *mnp*1 cDNA with constitutive *gpd*A promoter with the signal sequence for protein secretion and the glucoamylase gene from *A. awamori* as terminator. The plasmid contains the HygB resistance gene for the selection of fungal transformants.

Fungal transformation was done through a biolistic transformation protocol previously described for *A. nidulans* [45]. Intact conidia were inoculated into 15 ml of solid Czapek medium and then incubated for 6 h. Gold particles (1 mm diameter) were prepared and coated with plasmid pGMG-Hyg [46] and 8 μl loaded onto each of three macrocarrier discs for bombardment at 6 cm target distance, 28 in Hg vacuum and bombardment pressure of 1,200 psi. The plates were bombarded and incubated at 30°C for 2 hours, after which time a top dressing was applied of 10 ml Czapek-agar medium with 300 μg/ml hygromycin. Plates were incubated at 30°C until growth was observed. Control plates were bombarded with gold particles which were prepared as described above but not coated with plasmid.

2.2. Evaluation of enzymatic activity of recombinant MnP

Colony transformants were assayed for MnP activity using a modified plate assay method [34]. The spores of transformants obtained with HygB were inoculated onto disks (0.5 mm in diameter) of MM agar medium [47] supplemented with hemoglobin (1 g/l). The disks were incubated at 30°C for 2 d; when fungal growth began, the disks were inoculated in Petri dishes with MM agar medium, in addition to o-anisidine. The plates were incubated at 30°C for 24 h and then flooded with a solution of 50 mM Na-phosphate buffer (pH 4.5) and 0.04%

H_2O_2 on the surface of the plate and incubated at 30°C for 10 d in the dark. Positive controls were prepared with *P. chrysosporium* mycelia because this strain produces MnP; plates with the parental strain of *A. niger* were prepared for the negative control. Transformants which developed a purple halo were selected.

Incorporation of the recombinant *mnp*1 was checked through specific amplification in a PCR experiment using primers MnP1R_5173 (5′-GGATCCCTGTCTGGTCTTCTACAC-3′) and SS-MnP-MluI (5'- CGCGTATGATGGTCGCGTGGTGGTCTCT-3'). Genomic DNA was extracted from lyophilized mycelia of the wild-type and selected transformant strains, according to the described modified method [48]. After amplification, each PCR product was analyzed by agarose gel electrophoresis.

The MnP extracellular activity was determined spectrophotometrically by a modification of the method previously described using phenol red oxidation [49]. Absorbance was read at 610 nm. One unit of MnP activity was defined as 1 μmol product formed per minute.

The kinetics of wild-type and transformant strains of *A. niger* in liquid cultures were tested. Erlenmeyer flasks containing 50 ml of MM medium with hemoglobin were inoculated with 1×10^7 spores/ml and incubated at 30°C and 200 rpm for 7 d. Every 24 h, flasks were used to determine MnP activity in the supernatant. In addition, total protein concentration was quantified by Bradford reagent to determinate specific activity.

2.3. Phenanthrene removal by *A. niger* Mnp^{+7} and wild-type strain in solid culture

The ability of wild-type and transformant strains to remove Phe was determined at several times in the solid-state microcosm system, using the same culture conditions. Sugarcane bagasse was used as a fungal growth support and carbon source. The sterile material was moistened with MM medium and inoculated with 2×10^8 fungal spores/ml; all cultures were incubated for 2 d at 30°C. Uncontaminated soil obtained from a zone near a contaminated region in Coatzacoalcos, Veracruz, Mexico, was sterilized and contaminated with 600 ppm of Phe. The newly contaminated soil was mixed with the inoculated sugarcane bagasse and incubated at 30°C for 14 d, as well as a control (sterile bagasse and contaminated soil without fungi) to determine abiotic Phe removal. Evolution of CO_2 was measured daily to quantify the heterotrophic activity. After this period, Phe removal for both strains was determined by HPLC.

Heterotrophic activity was determined by Gas Chromatography (GC). Headspace samples were taken from the flasks and analyzed for CO_2 evolution. The headspace in each flask was flushed out daily for 15 min with sterile and moistened air. This allowed the preservation of aerobic conditions and avoided carbon dioxide accumulation. CO_2 quantification was reported as milligrams of CO_2 per gram of initial dry matter (IDM). Phenanthrene was extracted with microwave assisted extraction, according to EPA method 3546. Analysis of Phe was based on EPA method 3540 for the HPLC system.

3. Results

3.1. Heterologous expression of a *P. chrysosporium mnp*1 gene in *A. niger* SCB2

The heterologous expression of genes coding for different isoforms of MnP from *P. chrysosporium* has been reported in non-ligninolytic fungi, such as *A. oryzae*, *A. nidulans*, and *A. niger*. These enzymes were obtained extracellularly and with catalytic activity but have only been studied as expression systems for the production of heterologous proteins and have not been applied to the bioremediation of contaminated soils, as presented in this research [40,42,50,51].

Expression plasmid pGMG-Hyg was introduced into wild-type *A. niger* by biolistic transformation. A total of 8 transformants were isolated for their capacity to grow in Czapek plates with hygromycin B (HygB). After 5 d of incubation at 30°C and confirmed by PCR amplification, the result showed a single amplicon of 635 pb fragment observed in agarose gel electrophoresis (figure. 3) and no bands were observed for the wild-type stain. The positive control strain showed the expected fragment.

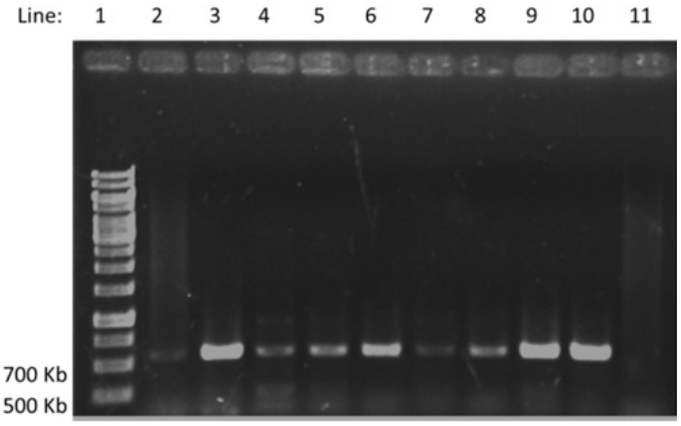

Figure 3. Agarose gel with PCR product obtained by *A. niger* transformants for expression plasmid pGMG-Hyg. Line 1: Gene Ruler 1 kb DNA ladder as a molecular marker. Line 2: MnP^{+1}. Line 3: MnP^{+2}. Line 4: MnP^{+3}. Line 5: MnP^{+4}. Line 6: MnP^{+5}. Line 7: MnP^{+6}. Line 8: MnP^{+7}. Line 9: MnP^{+8}. Line 10: *P. chrysosporium* as positive control. Line 11: *A. niger* SCB2 as negative control.

3.2. Evaluation of enzymatic activity of recombinant MnP

The transformants were evaluated for MnP activity by the o-anisidine coloration method in MM medium with hemoglobin plates. Transformants that developed a purple halo were selected. Four transformants (MnP^{+1}, MnP^{+4}, MnP^{+7} and MnP^{+8}) formed purple halos around the agar disk after 8 d of incubation, indicating extracellular peroxidase activity, as show in figure 4. The wild-type strain showed no coloration; however, the control strain of *P. chrysosporium* showed a greater purple halo. These results are similar to findings by other authors, who screened autochthonous or recombinant fungal strains for the formation of halos by o-anisidine oxidation on agar plates induced by manganese peroxidase activity [40, 42, 52].

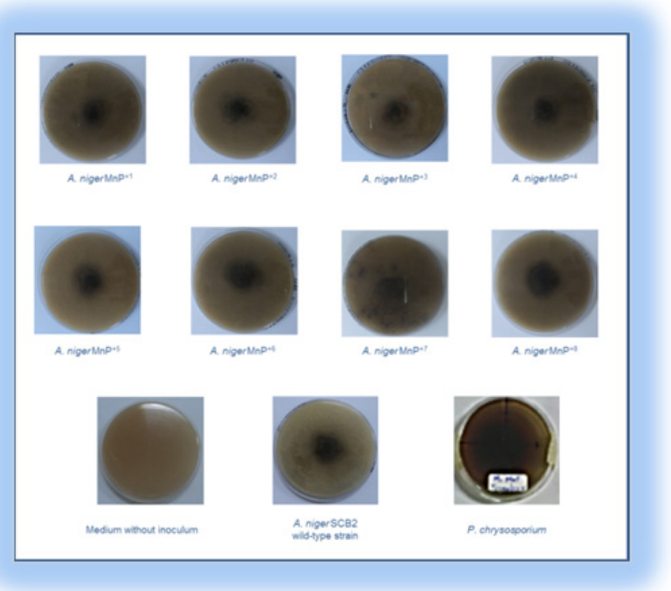

Figure 4. Qualitative determination of MnP activity produced by *A. niger* transformants in Petri dishes using o-anisidine as an indicator.

The MnP^{+7} transformant strain showed higher Phe tolerance than wild-type strain when inoculated into Cove's medium in Petri dishes at different Phe concentrations. At concentrations above 600 ppm, both strains showed a decrease in growth rate compared to their respective controls without Phe; however, the wild-type strain showed an inhibition in sporulation while the transformant strain was able to sporulate (figure 5). This coincides with the results in reference [53], which reported that fluorene at concentrations above 100 ppm caused growth inhibition of fungal strains isolated from a contaminated soil. In contrast, reference [54] reported that 100 ppm of anthracene had no inhibitory effect on the growth of fungi isolated from soil. This fact suggests that due to the production of MnP by the transformant strain for *mnp*1 gene expression, which is regulated by the *gpd*A constitutive promoter, there was an increased tolerance to high Phe concentrations.

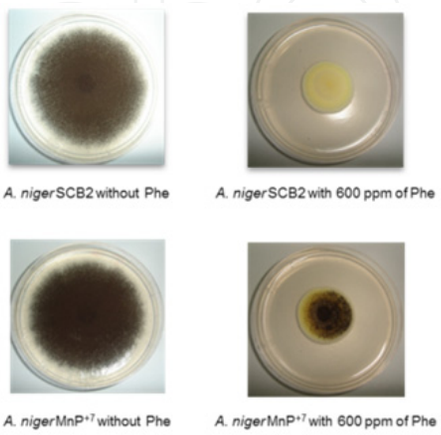

Figure 5. Effect of Phe on growth and sporulation of *A. niger* wild-type strain and transformant on superficial culture in Petri dishes with Cove's medium.

MnP productivity of the four selected strains was quantified in liquid culture using MM medium with hemoglobin. The activity was measured every 24 h for 7 days. As shown in figure 6, the wild-type strain did not present MnP activity. Although all transformant strains present different MnP activity, maximum activity was obtained by *A. niger* MnP^{+7} strain at 4 d (25.4 U/L); specific activity during this time was 3.67 U/mg of total protein, whereas the control strains of *P. chrysosporium* reached 7 U/L. These results demonstrate that the o-anisidine plate assay was consistent with the MnP production of the transformants in liquid culture. The results also confirmed that MnP production occurred in the transformants by introducing the *mnp*1 gene, since this activity was not detected in the wild-type strain. Differences observed between transformants are often explained by a differential integration of the heterologous gene within the fungal genome [33, 55].

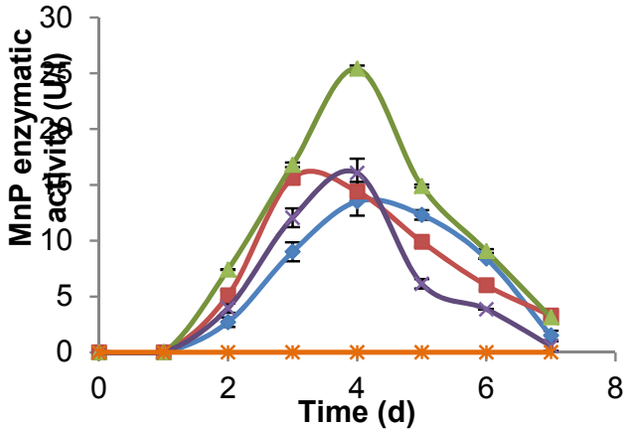

Figure 6. Enzymatic activity of MnP exerted by the wild-type strain and transformants of *A. niger* in liquid culture in MM medium supplemented with hemoglobin. (◆) *A. niger* MnP^{+1}; (■) *A. niger* MnP^{+4}; (▲) *A. niger* MnP^{+7}; (×) *A. niger* MnP^{+8}; and (∗) *A. niger* SCB2.

After 48 h, all transformant strains showed MnP activity in liquid medium. Because *mnp*1 expression is regulated by the constitutive *gpd*A promoter, which is involved in the glycolysis metabolic pathway forming part of the central metabolism, MnP production was started early on and maintained throughout the growth phase of the fungus [56].

The specific activity obtained by recombinant MnP with *A. niger* MnP^{+7} strain regulated by the constitutive *gpd*A promoter, and *A. niger* SCB2 T3 strain regulated by Taka-amylase promoter, showed similar results (3.67 and 3 U/L respectively); this may be caused by the culture media used for producing the recombinant enzymes since the medium used for SCB2 -T3 was COVE's medium, with maltose (50 g/L) as carbon source and inducer of *mnp*1 expression, supplemented with hemoglobin to increase MnP activity [32], and the medium used for MnP^{+7} was MM [47] also supplemented with hemoglobin but using glucose (10 g/L) as carbon source. It is well known that several factors should be considered in *mnp*1 expression, such as the carbon source, the culture medium, and the addition of a heme group. Recombinant MnP production by MnP^{+7} strain was increased by adding hemoglobin to the culture medium. Similar results have been obtained by other research groups in their studies on *mnp*1 expression in *Aspergillus* [40,42,51] and are explained by how the

recombinant protein is produced by *Aspergillus*, i.e., as an unstable apoprotein. This apoprotein requires the heme group to produce the active hemoprotein, which acquires a more stable conformation for proteolytic attack than the apoprotein. Due to low availability of heme provided by the heme biosynthetic pathway, this fact is considered a limiting factor for the production of heme proteins in different expression systems [41- 43, 52]. If the amount of heme produced by a microorganism is low in relation to the amount of apoprotein produced, the apoprotein will accumulate in the culture medium and undergo proteolytic degradation lowering the yield of the active hemoprotein. Other studies on manganese peroxidase expression in *Aspergillus* assume that hemoglobin may play a role not only in supplying heme but also in providing a protein excess in the culture medium, thereby protecting recombinant MnP from proteolytic degradation [42,51,57].

3.3. Phenanthrene removal by *A. niger* Mnp^{+7} and wild-type strain in solid culture

In order to evaluate the growth of the microorganism in solid culture, CO_2 evolution was quantified. Two tested strains showed different profiles and the ANOVA test indicated a significant ($p<0.05$) difference in the accumulated CO_2 production: the transformant strain produced more CO_2 than the wild-type, both in the presence and absence of Phe, and was around 15-18 mg CO_2 accumulated per gram of initial dry matter (IDM), whereas the wild-type strain produced only around 5-7 mg CO_2 accumulated/g IDM (figure 7A). This result demonstrated that *A. niger* MnP^{+7} strain was able to grow more on solid culture than *A. niger* SCB2; however, both strains presented a decrease in CO_2 production in the presence of Phe. This can be interpreted as a toxic effect of the compound on the growth of fungi and these results demonstrate that the plate assay with 600 ppm of Phe was consistent with a toxic effect on the growth. The highest microbial activity in all treatments analyzed was at 4 d (5 mg CO_2 instantaneous/g IDM) and a decrease in microbial activity was observed in all the inoculum treatments at 14 days (figure 7B). CO_2 production in the control without fungi was negligible.

The residual Phe extracted from treated soil was quantified by HPLC and the results of two strains are presented in figure 8. The wild-type strain had the lowest Phe removal capacity (approximately 7%) compared with the transformant MnP^{+7} strain which was able to remove approximately 44% of the initial Phe (0.6 mg/g IMD) in 14 d. The Phe extraction efficiency of the abiotic controls was 98%.

The increase in the removal percentage of Phe by the MnP^{+7} transformant strain in solid culture suggests that it is due to the production of MnP enzyme by the transformant strain which showed the ability to express the *mnp*1 gene regulated by the constitutive *gpd*A promoter. This fact has led to an increased tolerance in plate and solid culture and greater removal efficiency in high Phe concentrations in solid culture. This result is important because PAH degradation has only been studied in submerged culture by ligninolytic fungi isolated from contaminated soils and, since PAHs have low solubility in water, only low concentrations have been used; for example, a strain of *Aspergillus terreus* has been isolated

from a PAH-polluted soil and the metabolism of pyrene and benzo(a)pyrene by this fungus was investigated in liquid submerged culture supplemented with 50 and 25 ppm, respectively, of each compound [58]. *Penicillium chrysogenum* degraded 60% of fluorene (50 ppm initial) in the presence of Tween 80 after 2 days [53]. There are also reports of PAH removal by ligninolytic fungi in soils, but only concentrations below 200 ppm have been tested. *Fusarium sp*. E033 strain was isolated and able to survive in the presence of concentrations up to 300 ppm of benzo(a)pyrene and demonstrated that this strain was able to degrade 65 to 70% of the initial benzo(a)pyrene (using 100 ppm) provided within 30 d of incubation at 32°C [59]. Other authors report PAH degradation by some strains of the genus *Penicillium*, such as *P. frequentans*, capable of removing 52% of Phe in soil contaminated with 200 ppm in 17 d [60]. *P. janthinellum* degraded 50 ppm of benzo(a)pyrene after 48 d of incubation in soil in co-culture with bacteria [24].

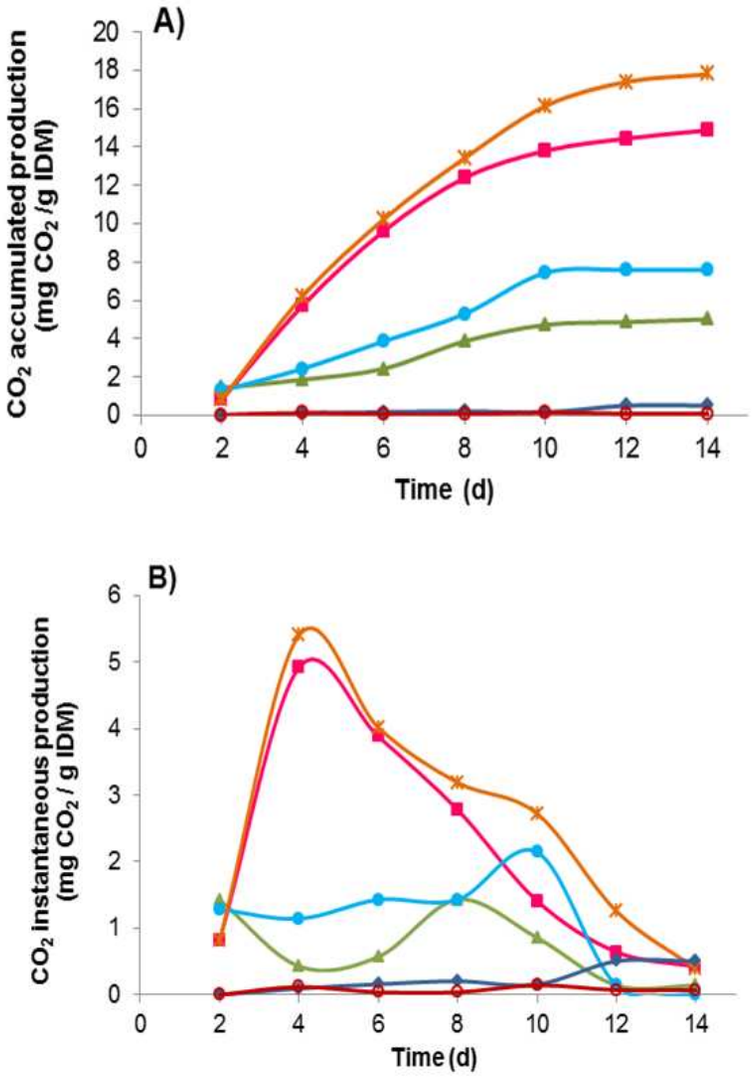

Figure 7. Microbial activity of both *A. niger* strains in solid culture in microcosm. A) CO_2 accumulated production and B) CO_2 instantaneous production. The different treatments were: (●) *A. niger* SCB2 without Phe; (▲) *A. niger* SCB2 with Phe; (∗) *A. niger* MnP^{+7} without Phe; (■) *A. niger* MnP^{+7} with Phe; (◆) abiotic control without Phe and (○) abiotic control with Phe.

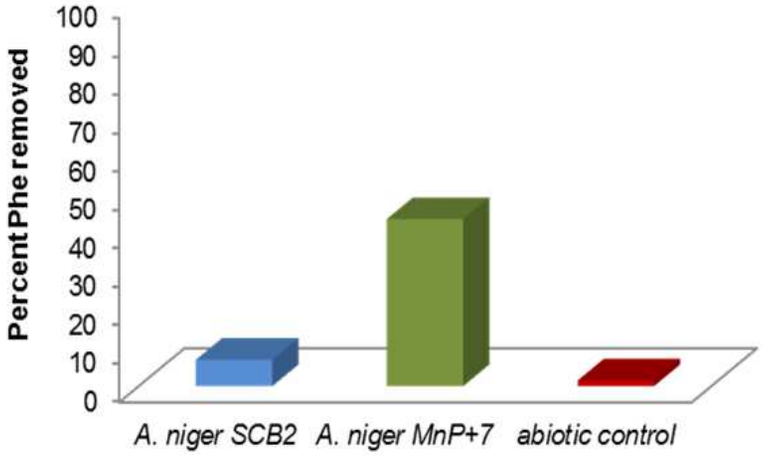

Figure 8. Phenanthrene removal percentage in solid culture by *A. niger* SCB2 and *A. niger* MnP^{+7} strains in soil contaminated with 600 ppm of Phe.

The increase in Phe concentration in solid culture showed a higher toxic effect on the wild-type strain. Compared to cultures carried out using soil contaminated with 0.4 mg/g IMD, the wild-type strain SCB2 was able to remove 75% of Phe, while *A. niger* SCB2-T3 strain removed 95% of Phe [32], so although the removal percentage obtained with MnP^{+7} strain was lower than *A. niger* SCB2-T3, MnP^{+7} strain is considered to be efficient in the removal of Phe in soil.

With respect to the intermediaries formed during Phe oxidation in solid culture for *A. niger* MnP^{+7}, preliminary results using polyurethane foam (PUF) as inert support have shown that the chromatographic profiles of the two strains in the presence of 600 ppm of Phe were different and the peaks observed in the chromatograms of the two strains in the presence of Phe were not observed in the respective controls (control inoculated without Phe and abiotic controls with Phe) (data not shown). This suggests that these peaks correspond to metabolites formed from Phe degradation; furthermore, the concentrations of residual Phe and intermediaries obtained from the transformant strain were lower when compared to those obtained from the wild-type strain. This result can be attributed to *mnp*1 expression since this is the only difference between the two strains, which were grown under the same culture conditions. These results allow us to infer that the differences in metabolism were caused by the presence of recombinant MnP enzyme, although we, have not yet identified the metabolites or intermediaries formed from degradation by transformant MnP^{+7} and wild-type strains.

1-phenanthrol, 2-phenanthrol, and phenanthrene trans-9,10-dihydrodiol have been reported as major metabolites from the metabolism of Phe by *A. niger* [61]. Other authors have reported that Phe was metabolized by *A. niger* into small amounts of 1- and 2-phenanthrol, and also 1-methoxyphenanthrene as a major ethyl acetate extractable metabolite; its retention time (RT) was of 36.7 min, indicating a less polar metabolite than Phe [62]. We have reported that the chromatographic profiles of *A. niger* SCB2-T3 in liquid culture in the presence of Phe were different to the wild-type strain. In the culture medium of the wild-type strain, a principal metabolite of Phe degradation with a RT of 1.7 min was detected. On the other

hand, two metabolites with a lower RT than Phe were extracted from the mycelium of the transformant strain; these are considered more polar compounds produced by Phe oxidation. No residual Phe was detected in the transformant strain's culture medium [32].

Bioaugmentation with an *Aspergillus* strain isolate did increase the extent of removal of benzo(a)anthracene and benzo(a) pyrene in soils significantly [65]. Bioaugmentation with *Cladosporium sphaerospermum* significantly stimulated PAH degradation, especially for high molecular weight PAH [66]. Other authors have reported a significantly improved degradation of high molecular weight PAH after bioaugmentation in PAH-contaminated soil [24, 29].

The results from this study also show that non-ligninolytic fungal strains are a viable alternative for application in bioremediation systems; moreover, bioaugmentation with genetically modified exogenous fungal strains for heterologous protein production in solid culture accelerates the process of removal and biodegradation of toxic compounds in contaminated soils.

4. Conclusion

The action of genetically modified non-ligninolytic fungal strains in bioremediation systems has not been reported, so that the results obtained in this investigation suggest that these microorganisms may have some potential as a bioaugmentation agent: they are efficient degraders of PAHs in high concentrations compared to other non-ligninolytic fungal strains which produce more toxic intermediaries than the original compound and tolerate lower PAH concentrations, and also compared to ligninolytic fungi not grown in soil and which are displaced by native soil microflora.

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