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FILAMENTOUS FUNGI REMOVE WEATHERED HYDROCARBONS FROM POLLUTED SOIL OF TROPICAL MÉXICO

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Key words: *Aspergillus*, bioaugmentation, *Cladosporium*, filamentous fungi, *Penicillium*, total petroleum hydrocarbons (TPH) removal, tropical Mexico

ABSTRACT

Weathered hydrocarbons from worldwide petrolic activities become more recalcitrant over time. The removal of petroleum hydrocarbons from a polluted soil [65,000 mg total petroleum hydrocarbons (TPH)/kg soil], which had been exposed to tropical environmental conditions for more than 20 years in southeast Mexico, was studied using filamentous fungi. Experiments were carried out in batch reactors (60 mL) containing a substrate consisting of polluted soil and sugar cane bagasse pith as bulk agent (80:20, w/w). Sterile and non-sterile batch reactors were inoculated with spore suspensions from *Aspergillus niger*, *Penicillium glabrum*, and *Cladosporium cladosporioides*. The TPH were determined at the beginning and at the end of the experiments, and the CO_2 production and accumulation were monitored by gas chromatography. All fungal species studied were associated to the removal of TPH, either on sterile or non-sterile treatments. A bioaugmentation process was observed due to the synergistic effect of *C. cladosporioides* and well-adapted indigenous microbial populations from the contaminated soil, as the highest removal of TPH (78.5%) and CO_2 accumulation (14.3%) were recorded in this non-sterile treatment. By contrast, the lowest TPH removal was recorded in the same species, but in the sterile treatment (62.3%) showing that the absence of adapted indigenous microbiota significantly reduced fungal metabolism (CO_2 accumulation: 9.1%), as well as the removal of TPH. Patterns of CO_2 accumulation and TPH removal in other treatments suggested interspecific competence between fungal species and the adapted indigenous microbiota.

Palabras clave: *Aspergillus*, bioaumentación, *Cladosporium*, hongos filamentosos, *Penicillium*, remoción de hidrocarburos totales del petroleo (HTP), trópico mexicano.

RESUMEN

Los hidrocarburos intemperizados de las actividades petroleras son más recalcitrantes a medida que transcurre el tiempo. Se estudió la remoción por hongos filamentosos de los hidrocarburos de petróleo de un suelo contaminado [65,000 mg de hidrocarburos totales de petróleo (HTPs)/kg de suelo], el cual había estado expuesto a condiciones ambientales tropicales por más de 20 años en el sureste de México. Los experimentos se llevaron a cabo en reactores de lote (60 mL), los cuales contenían un sustrato a base de suelo contaminado y bagacillo de caña de azúcar como agente texturizante (proporción 80:20, peso/peso). Se inocularon reactores de lote, estériles y no estériles, con suspensiones de esporas de *Aspergillus niger*, *Penicillium glabrum*, y *Cladosporium cladosporioides*. Los HTP se determinaron al principio y al final de los experimentos, mientras que la producción y acumulación de CO_2 se determinó por cromatografía de gases. Todas las especies fúngicas estudiadas estuvieron asociadas a la remoción de HTP, tanto en tratamientos estériles como no estériles. Se observó un proceso de bioaumentación debido al efecto sinérgico entre *C. cladosporioides* y la poblaciones microbianas nativas bien adaptadas del suelo contaminado, ya que la remoción más alta de HTP (78.5%) y mayor acumulación de CO_2 (14.3%) se registraron en este tratamiento no estéril. En cambio, la remoción más baja de HTP se observó en la misma especie, pero en el tratamiento estéril (62.3%), demostrando que la ausencia de microbiota nativa adaptada redujo significativamente el metabolismo fúngico (acumulación de CO_2: 9.1%), así como la remoción de HTP. Los patrones de acumulación de CO_2 y la remoción de HTP en los otros tratamientos pueden relacionarse con competencia interespecífica entre el hongo filamentoso y la microbiota nativa adaptada.

INTRODUCTION

Petrolic activities have generated extensive pollution of soils worldwide, mainly in those regions where petroleum is explored, extracted, and refined. The composition of hydrocarbons on polluted soil varies according to environmental conditions and natural degradation processes. Weathered hydrocarbons, which are predominantly saturated and aromatic, become more recalcitrant if polluted soils are not remediated, affecting underground water, food chains, and diverse human activities (e.g., agriculture, cattle raising, silviculture) (Atlas and Bartha 1998).

Bacteria and fungi have been used for degradation and removal of hydrocarbons, as they are capable of producing significant amounts of efficient enzymes (Saraswathy and Hallberg 2002, Chávez-Gómez et al. 2003, Moody et al. 2004, Genovese et al. 2008). Filamentous fungi, commonly found on lignocellulosic substrates, produce extracellular enzymes of low specificity, which can degrade differing recalcitrant compounds, such as hydrocarbons, resins and asphaltens (Tortella et al. 2005). Important enzymes used for bioremediation are lignin peroxidase, manganese-dependent peroxidase, and laccase (Evans and Hedger 2001).

Most fungal research work on bioremediation has been focused on the degradation of specific hydrocarbons using a variety of substrates, showing different levels of pollutant removal (Hammel et al. 1986, Gramss et al. 1999, Novotny et al. 1999, Cortés-Espinosa et al. 2006, Leonardi et al. 2007, Genovese et al. 2008). Mineralization rates by fungal enzymes ranging from 25-93% have been reported for polycyclic aromatic hydrocarbons (PAH) during a period of 8-15 weeks. In a previous research work, it was shown that the removal of weathered hydrocarbons from polluted soil can be improved synergistically by microorganisms from sugar cane bagasse pith, reaching up to 60% removal of total petroleum hydrocarbons (TPH) (Pérez-Armendáriz et al. 2004). In this study, it was assessed the effect of bioaugmentation using three species of filamentous fungi on the removal of hydrocarbons from real polluted soil exposed to tropical environmental conditions for more than 20 years in the State of Tabasco, southeast México.

MATERIALS AND METHODS

Fungal strains

The filamentous fungi studied were *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Penicillium glabrum* (Wehmer) Westling, and *Aspergillus niger* Tiegh. These species were previously isolated from sugar cane bagasse pith, and identified by molecular analysis (Cortés-Espinosa et al. 2006). Selected strains were grown on minimal medium: g/L: 7, $(NH_4)_2SO_4$; 5.7, K_2HPO_4; 2, $KHPO_4$; 2, $MgSO_4$; 15, agar; 1,000 ml distilled water, and preadapted to a petroleum environment using Mayan petroleum provided by the Mexican State company (PEMEX). Mayan petroleum was added on sterilized filter paper (3 cm^2; *ca.* 2 g petroleum) to every lid in order to develop an atmosphere of volatile hydrocarbons inside the petri dish. Bacterial growth was inhibited adding 3.5 ml/L of rose Bengal (Sigma-Aldrich, USA) and 40 μg/mL of streptomycin (Sigma-Aldrich, USA). Sterile petri dishes containing potato dextrose agar (PDA, Bioxon, México) as culture medium were inoculated with strains preadapted, and incubated for 5 days at 28 ºC for mycelial growth and spore production.

Batch reactors

A soil sample was obtained from a petroleum well located in the tropical State of Tabasco, southeast of México. The soil sample (ca. 20 kg) was placed in a dark glass container and kept at 4 ºC for transportation. In the laboratory, the soil was exposed to the sun for a few hours, homogenized manually for 15 min, and characterized as loamy sand (silt 4 %, sand 96 %) having a moisture content of 2 %, an organic matter content of 8.3 %, and a pH of 7.3. The concentration of total petroleum hydrocarbons (TPH) in the polluted soil was of 65 000 mg/kg, as the well from which the sample was taken had been exposed to diesel contamination for more than 20 years. Sugar cane bagasse pith (a lignocellulosic byproduct from the local sugar industry) was sterilized at 121 ºC for 20 min and it was used as solid support and carbon source in all experiments. This pith was characterized as follows: pH 4.5, density 0.8 g/cm^3, moisture content 80 %, total sugars 2.23 mg glucose/g (Rodríguez-Vázquez et al. 1999). Six different treatments were carried out as shown in **table I**. A non-sterile soil sample was used directly for part of the experiment, whereas the soil sample used in the other experimental part was autoclaved three times (every 24 h) at 121 ºC for 15 min, according to previous research (Pérez-Armendáriz et al. 2004, Cortés-Espinosa et al. 2006). Two controls, involving either sterile or non-sterile soil, were included. The batch reactors were glass serum bottles (60 ml) containing 16 g of soil, either sterile or non sterile, and 4 g of sterile sugar cane bagasse pith. The moisture content was adjusted to 60 %, using sterile distilled water. The C/N/P ratio was 100/10/1 using a sterile water solution of 1 N NH_4SO_4 (Sigma-Aldrich, USA) and 1 N K_2HPO_4 (Sigma-Aldrich, USA) as nitrogen and phosphorus sources. Fungal inocula from strains studied were prepared on PDA plates. Spores were harvested adding 2.5 ml of tween 80 (Sigma-Aldrich, USA) in order to obtain a sterile solution at a standardized concentration. Every reactor was inoculated with 1 mL of the spore suspension (inoculum at 2×10^7 spores/mL). Each bottle was capped with a Teflon septum. The atmosphere of reactors was exchanged every two days, injecting an air flow through a sterile filter (millipore 0.2 mm) for 15 min. All experiments were carried out in triplicate. Residual TPH were determined after 25 days of incubation at 25 ºC (Chávez-Gómez et al. 2003, Pérez-Armendáriz et al. 2004, Cortés-Espinosa et al. 2006).

Sample analysis

Microbial activity. It was monitored every two days, before each atmosphere exchange, taking an

TABLE I. EXPERIMENTAL DESIGN AND TREATMENTS STUDIED

Code	Soil treatment	Fungal species	Substrate (ratio 80:20, w/w)
T1	Non sterile	*Aspergillus niger*	Polluted soil + pith*
T2	Sterile	*A. niger*	Polluted soil + pith
T3	Non sterile	*Penicillium glabrum*	Polluted soil + pith
T4	Sterile	*P. glabrum*	Polluted soil + pith
T5	Non sterile	*Cladosporium cladosporioides*	Polluted soil + pith
T6	Sterile	*C. cladosporioides*	Polluted soil + pith
T7	Control 1 (non sterile)	-	Polluted soil + pith
T8	Control 2 (sterile)	-	Polluted soil + pith

* The sugar cane bagasse pith was sterile in all treatments and controls.

air sample of 5 µL from the headspace of batch reactors using a sterile syringe, which was injected into the gas chromatograph (Gow-Mac 580, USA) having a thermal conductivity detector. Data were expressed as percent of CO_2 produced and accumulated (Rodríguez-Vázquez et al. 1999).

Total petroleum hydrocarbons (TPH). The content of TPHs was determined in three samples at the beginning and at the end of the experiments, as previously described (Pérez-Armendáriz et al. 2004). Samples were taken for analysis at day 0 (initial soil sample) and at day 25. The total content of each batch reactor was homogenized by thorough manual mixing for 10 min. A sample (2 g) was taken from the mix, and dried at room temperature for 24 h. Hydrocarbons were extracted for 8 h with 80 mL of dichloromethane (Merck, USA) using Soxhlet. Dichloromethane phase was performed in a rotary evaporator, adding 2 g of anhydrous Na_2SO_4 (Sigma-Aldrich, USA). Dry samples were dissolved in 10 mL of dichloromethane, taking an aliquot of 500 µL to be dried at room temperature and dissolved in 5 mL of CCl_4 (Merck, USA). Residual hydrocarbons were determined following the modified EPA 418.1 method (USEPA 1979). Total petroleum hydrocarbons were assessed by infrared spectroscopy (Buck Scientific, model HC-404, USA), using 2930 cm^{-1} absorbance, specific for C-H bonds. Samples were analyzed by interpolation in a calibration plot.

Statistical analysis

Data were expressed as means ± standard deviations. One way analysis of variance and F test, at a level of significance of $P<0.05$, were carried out in order to determine significant differences among the means. Multiple comparisons among treatment means were made using the Tukey's test. All data were analyzed on Minitab Ltd. statistic software.

RESULTS AND DISCUSSION

The non-sterile control T7 showed the highest CO_2 production and accumulation (24.0 %) of all treatments, followed by the non-sterile treatment T5 (14.3 %) (**Table II**, **Fig. 1**). This indicated the presence of well-adapted indigenous microbial populations in the contaminated soil of T7, which were even biostimulated by the addition of nitrogen and phosphorus sources and thus they were responsible for the removal (61.5%) of TPH (**Table II**). In T5, it can be seen a bioaugmentation process due to the synergistic effect of the filamentous fungus *Cladosporium cladosporioides* and the adapted indigenous microbiota. This was confirmed by the highest TPH removal (78.5 %) of all treatments recorded in T5 (**Table II**). It is possible that a longer incubation of T5 would have led to further development of *C. cladosporioides* and the indigenous microbiota, and accordingly to a higher production of CO_2, biomass, and enzymes (D'Annibale et al. 2006). The lowest TPH removal of all treatments was recorded in the same species, but on the sterile treatment (T6, 62.3 %) showing that the absence of adapted indigenous microbiota significantly reduced CO_2 production and accumulation (9.1 %), as well as the removal of TPH. The bioaugmentation process is important considering the high level of initial pollution found in the soil studied (65 000 mg TPH/kg soil), which had been weathered for more than 20 years. The possible effect of soil sterilization on these results will be matter for further research.

Very low levels of CO_2 production and accumulation (1.5 %), as well as TPH removal (16.0 %), in the sterile control T8 may be associated to the presence of reproductive structures from adapted indigenous microbiota capable of resisting sterilization processes, which also affect substrate (polluted soil + sugar cane bagasse pith) structure and composition (Sylvia et al. 1999).

The comparison of sterilized and non-sterilized treatments showed variation according to each species (**Table II**, **Fig. 1**). In the case of *Aspergillus niger*, the CO_2 accumulation (T2, 9.9 %) and the TPH removal (T2, 76.3 %) on the sterile treatment were higher than those on the non-sterile treatment containing adapted indigenous microbiota (T1: CO_2, 3.4 %; TPH, 64.6 %). Similar results were obtained in *Penicillium glabrum* for CO_2 accumulation and TPHs removal, which were higher on the sterile treatment (T4: CO_2, 8.9 %; TPH, 72.5 %). Higher values for CO_2 accumulation and TPH removal on sterile treatments suggested some degree of interspecific competence between filamentous fungi studied (*A. niger*, *P. glabrum*) and adapted indigenous microbiota. Competence has also been reported for *Phanerochaete chrysosporium* and indigenous microorganisms (Fernández-Sánchez et al. 2001). By contrast, the removal of TPHs on the non-sterile treatment containing *Cladosporium cladosporioides* and adapted indigenous microbiota (T5, 78.5 %) was higher than that on the sterile treatment (T6, 62.3 %). In this case, the CO_2 production and accumulation were also higher on the non-sterile treatment (T5, 14.3 %) than on the sterile treatment (T6, 9.1 %), which indicated the presence of interspecific

TABLE II. THE REMOVAL OF TOTAL PETROLEUM HYDROCARBONS (TPH) AND CO_2 ACCUMULATION FROM DIFFERENT TREATMENTS STUDIED, USING POLLUTED SOIL AND SUGAR CANE BAGASSE PITH AS SUBSTRATE. THE INITIAL TPHs CONCENTRATION OF THE POLLUTED SOIL SAMPLE WAS 65 000 mg/kg

Code	Soil treatment	Fungal species	Residual TPH[1,2] (mg/kg)	SD (mg/kg)	Proportion of TPH removal (%)	Final CO_2[3] (%)	SD (%)
T1	Non sterile	*Aspergillus niger*	22,966.6 [b]	±2,282.7	64.6	3.4[e]	±0.58
T2	Sterile	*A. niger*	15,383.3 [a]	±2,089.4	76.3	9.9[c]	±0.15
T3	Non sterile	*Penicillium glabrum*	22,750.0 [b]	±650.0	65.0	5.4[d]	±0.24
T4	Sterile	*P. glabrum*	17,875.0 [a]	±2,298.1	72.5	8.9[c]	±0.14
T5	Non sterile	*Cladosporium cladosporioides*	13,975.0 [a]	±1,378.8	78.5	14.3[b]	±0.03
T6	Sterile	*C. cladosporioides*	24,483.3 [b]	±1,635.8	62.3	9.1[c]	±0.24
T7	Control 1 (non sterile)	-	25,025.0 [b]	±459.6	61.5	24.0[a]	±0.19
T8	Control 2 (sterile)	-	54,600.0 [c]	±919.2	16.0	1.5[f]	±0.49

[1] Residual TPH were assessed after 25 days of incubation
[2] TPH mean value (n=3). Means with different letters are significantly different (P<0.05)
[3] Final value for the CO_2 produced and accumulated (n=3). Means with different letters are significantly different (P<0.05)
SD= Standard deviation

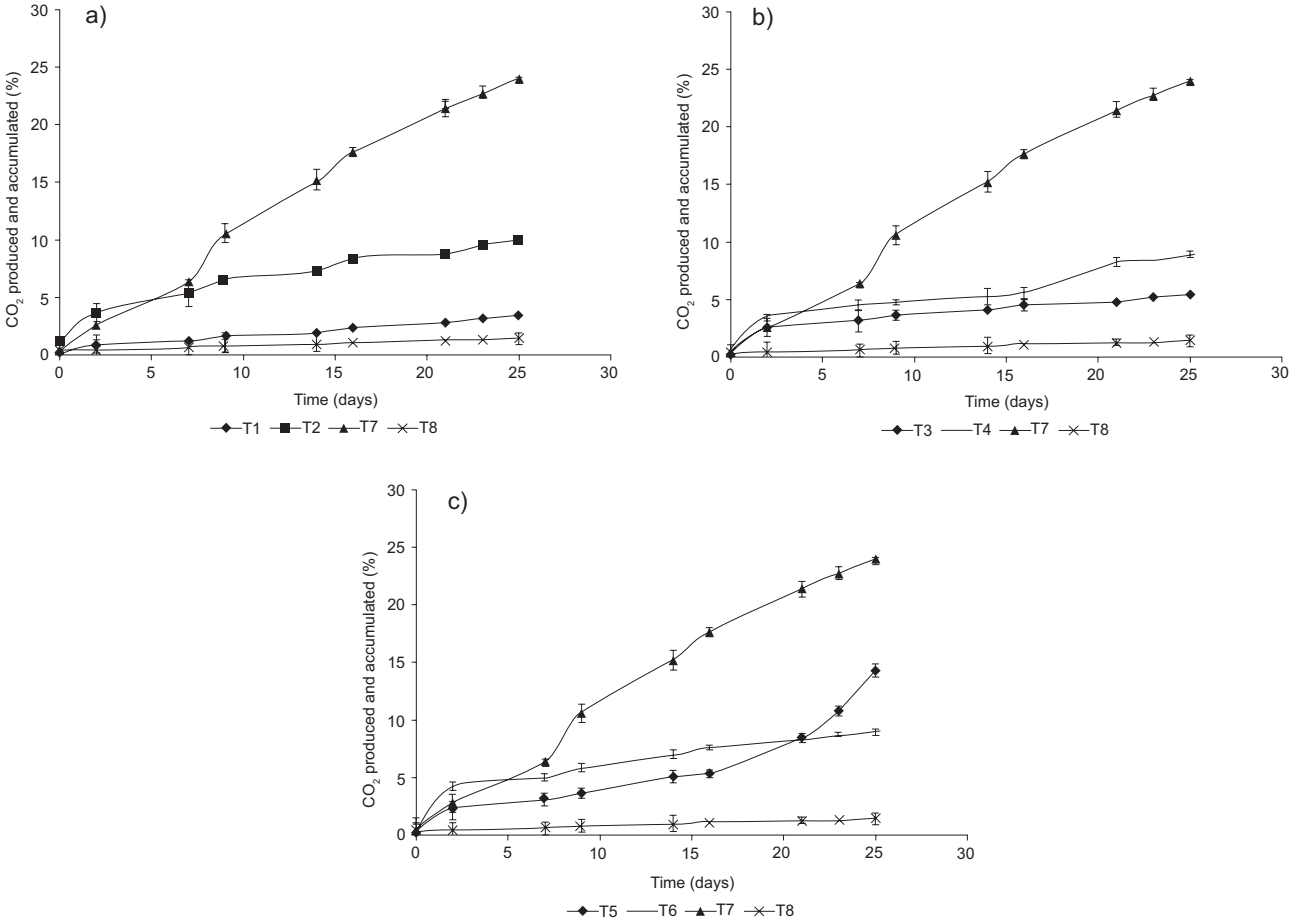

Fig. 1. Metabolic activity as CO_2 production and accumulation (%) in soil treatments studied, including non-sterile (T7) and sterile (T8) controls. A: *Aspergillus niger* on non-sterile soil (T1) and *A. niger* on sterilized soil (T2). B: *Penicillium glabrum* on non-sterile soil (T3) and *P. glabrum* on sterilized soil (T4). C: *Cladosporium cladosporioides* on non-sterile soil (T5) and *C. cladosporioides* on sterilized soil (T6)

co-metabolism between the filamentous fungus and adapted indigenous microbiota. Previous research has shown that a closely related species, *Cladosporium sphaerospermum*, which was isolated from aged soil of a gas manufacturing plant, was capable of degrading several PAH in four weeks using a non-sterile soil model (Potin et al. 2004). This ability to degrade hydrocarbons is due to the concerted action of extracellular and metabolic enzymes produced during *Cladosporium* growth.

An advantage of using experimental models based on real polluted soil is that the efficiency of a fungal species, such as *A. niger*, *P. glabrum* and *C. cladosporioides*, is directly tested against well-adapted indigenous microbial populations. This is in contrast with those species which are useful for bioremediation under laboratory conditions, but not efficient and poorly competitive under natural conditions where population development is affected by many factors, e.g., *Phanerochaete chrysosporium* (Lindley and Heydeman 1985, Meulenberg et al. 1997, Fernández-Sánchez et al. 2001, Potin et al. 2004). This research showed that *A. niger*, *P. glabrum* and *C. cladosporioides* are filamentous fungi with good potential for bioremediation processes in tropical regions of Latin America. They are capable of removing and degrading recalcitrant hydrocarbons through bioaugmentation and biostimulation, using sugar cane bagasse pith as bulking agent. However, they show differing responses when confronted with indigenous microbiota. Further studies are needed to understand the biological interactions between filamentous fungi and indigenous microbiota.

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