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AEROBIC DEGRADATION OF DIESEL BY A PURE CULTURE OF *Aspergillus terreus* KP862582

DEGRADACIÓN AERÓBICA DE DIÉSEL POR UN CULTIVO PURO DE *Aspergillus terreus* KP862582

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Abstract

This study was conducted with a fungal strain isolated from a mining soil contaminated with total petroleum hydrocarbons (TPH) and properly identified by polymerase chain reaction (PCR) technique as *Aspergillus terreus* KP862582. The biodegradation potential of this pure culture was evaluated at laboratory scale; a wide diesel concentration range, from 10,000 to 50,000 mg diesel/kg soil (ppm), was tested using sterile soil microcosm over a 90-day period. Aerobic biodegradation of diesel by *Aspergillus terreus* KP862582 was significantly greater ($p < 0.05$) for 10,000, 20,000, and 30,000 ppm, with rate constant values of 0.025, 0.023, and 0.012 1/day, respectively. Cell viability at these concentrations was favored because it showed a significant increase during the first period of biodegradation (0-30 days), from this time onwards efficiency removal and cell viability decreased considerably. This pattern was observed as concentration of diesel increased, resulting in a much lower biodegradation rate for 40,000 ppm (0.005 1/day) and 50,000 ppm (0.002 1/day). Based on the results of this study it is concluded that the strain of *Aspergillus terreus* KP862582 can be used in the bioremediation of soils contaminated with petroleum hydrocarbons at concentrations of 10,000 and 20,000 ppm, and comply with the MPL established by the Mexican regulation.

Keywords: aerobic degradation, TPH contaminated soils, kinetics, mining industry.

Resumen

Este estudio se realizó con una cepa fúngica aislada de un suelo minero, contaminado con hidrocarburos totales de petróleo (HTP), e identificada como *Aspergillus terreus* KP862582 mediante la técnica de reacción en cadena de la polimerasa. El potencial de biodegradación de este cultivo puro se evaluó a nivel laboratorio a concentraciones de 10,000 a 50,000 mg de diésel/kg de suelo (ppm), usando microcosmos con suelo estéril durante 90 días. La capacidad de biodegradación aeróbica del *Aspergillus terreus* KP862582 fue significativamente mayor ($p < 0.05$) para 10,000, 20,000 y 30,000 ppm, con constantes de velocidad de biodegradación del diésel de 0.025, 0.023 y 0.012 1/día, respectivamente. La viabilidad celular del *Aspergillus terreus* KP862582 en estas concentraciones mostró un incremento significativo durante los primeros 30 días, a partir de este tiempo la eficiencia de remoción y la viabilidad celular disminuyeron considerablemente. Este comportamiento se observó a medida que aumentó la concentración del diésel, resultando en una menor tasa de degradación para 40,000 ppm (0.005 1/día) y 50,000 ppm (0.002 1/día). Con base en los resultados se concluye que la cepa de *Aspergillus terreus* KP862582 puede ser usada en la biorremediación de suelos contaminados con HTP, con concentraciones de 10,000 y 20,000 ppm, y cumplir con el LMP establecido por la regulación Mexicana.

Palabras clave: cinética, degradación aeróbica, industria minera, suelos contaminados con HTP.

1 Introduction

Diesel oil spills have increased considerably and they are one the main problems of environmental pollution. The main causes of these spills in soil and groundwater are due to their massive production as fuels for transportation, accidental spills while transported, and by leakage of pipeline and underground storage tanks (UST) (Lee *et al.,* 2006; Zanaroli *et al.,* 2010). Diesel oil is a medium

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distillate of petroleum which contains up to 4,000 hydrocarbons, it is a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from fractional distillation of petroleum between 250-360°C (Patil *et al.,* 2012). The polycyclic aromatic hydrocarbons (PAHs) in diesel include benzene, formaldehyde, 1,3-butadiene, ethylene dibromide, benzo[a]pyrene, benz[a]anthracene, and dibenz[a,h]anthracene; they are adsorbed on the exhaust particles and according to several studies are classified as possible carcinogens to humans. The conventional methods of cleanup have been successful in meeting up with the regulatory standards, but these techniques have limitations such as incomplete neutralization of pollutant, transfer of pollutants from one place to the other, and they are expensive (Abdulsalam, 2011). One of the most important methods of decontamination fate of pollutants in both terrestrial and aquatic environments is the microbial degradation, well known as bioremediation. Researchers have found that bioremediation is an effective method for removing petroleum hydrocarbons from contaminated soils, marine environments, and underwater sinkholes (Hollaway *et al.,* 1980; Floodgate, 1984; Atlas, 1985; Brook, 2001; Martínez-Prado *et al.,* 2014; Cisneros-de La Cueva *et al.,* 2014; Medina-Moreno *et al.,* 2014; Dhar *et al.,* 2014). Biodegradation of petroleum and other hydrocarbons in the environment is a complex process and aspects such as nature and amount of oil or hydrocarbons present, environmental conditions (moisture is required to support growth of soil biota and in degradation of pollutants as a consequence), and composition of the autochthonous microbial community must be considered (Chaudhry *et al.,* 2012). Bioremediation comprise two stages: (1) biostimulation, that involves identifying and adjusting certain physicochemical factors and (2) bioaugmentation, which consists in the addition of specialized microbial population into a contaminated site to enhance the rate of contaminant biodegradation in the affected soil (Abdulsalam *et al.,* 2011). Over the last few years, bioaugmentation has been successfully used for the remediation of sites contaminated with petroleum compounds and a number of toxic organic chemicals (Alisi *et al.,* 2009).

Numerous microorganisms are known for their ability to degrade hydrocarbons; both, bacteria (Cisneros-de La Cueva *et al.,* 2014; Medina-Moreno *et al.,* 2014) and fungi (Cerniglia, 1984; Fan and Krishnamurthy, 1995; Pinedo-Rivilla *et al.,* 2009; Gheyrati and Gunale, 2010) play important roles in the biotransformation of petroleum hydrocarbons. More studies with bacteria have been reported; however, fungal metabolism is a key for the degradation of aromatic compounds and showed to be more effective than bacterial metabolism, under the experimental conditions tested; suggesting the use of fungi is highly recommended in bioremediation. It is well documented that hydrocarbons spilled in the environment can be biodegraded mainly by bacteria, fungi, and yeast. According to an overview by Das and Chandran (2011) biodegradation efficiencies reported are higher for soil fungi (6 to 82%) as compared to soil bacteria (29 to 50%) (Jones *et al.,* 1970; Pinholt *et al.,* 1979; Gheyrati and Gunale, 2010). Fungi have been the subject of recent research due to their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin degradation, which are capable of degrading high molecular weight, complex or more recalcitrant compounds, including aromatic structures. Several studies have reported the use of eukaryotic organisms as a bioremediation method to remove a wide variety of environmental pollutants (Pinedo-Rivilla *et al.,* 2009). Studies on PAHs biodegradation by white-rot fungi, belonging to the genera *Phanerochaete, Polyporus, Stereum, Lentinus, Bjerkandera, Irpex, Pleurotus*, and *Phlebia* to remediate contaminated soils is well known. Studies conducted with fungus *Cladosporium* showed ability to degrade aromatic compounds in aqueous solution polluted with diesel. Fungi belonging to the genera *Aspergillus, Penicillium, Paecilomyces, Coriolus, Pycnoporus, Pleurotus, Fomitopsis,* and *Daedalea*, have been found to be responsible for degrading PHAs in soil and aquatic environments.

It is important to mention that microorganisms in TPH contaminated soil are present as mixed cultures (bacteria, fungi, or both) rather than pure cultures, which are limited in their metabolic activity by factors such as the type and concentration of petroleum hydrocarbons. Diesel oil consists of alkanes, cycloalkanes, and aromatic compounds, and several of these compounds, especially linear alkanes, are known to be easily biodegradable. Many metabolites or biotransformation products have been reported depending on the parent compound used as carbon source, via metabolism and co-metabolism, by pure or mixed cultures (Pinedo-Rivilla *et al.,* 2009). Recent studies have been conducted to test the ability of axenic cultures (consists of a single microbial species from a single cell) of soil fungi to degrade petroleum hydrocarbons with excellent results and even higher efficiencies as compared with

mixed cultures. Fungal isolates tested were *Aspergillus niger* (axenic) with a 95% degradation efficiency, mixed culture of *Aspergillus niger* and *Aspergillus fumigatus* degraded 90% of hydrocarbons, and the lowest degradation rate of 70% was obtained with a mixed culture of *A. niger, A. fumigatus, Fusarium solani*, and *Penicllium funiculosu* (AI-Jawhari, 2014).

Microbiological kinetic studies based on biological kinetic equations include parameters that describe the growth of biological solids, substrate utilization rates, food-microorganisms ratio (F/M), and the mean cell residence time. Among biokinetic parameters used include specific growth rate, substrate consumption rate, half velocity constant, maximum cell yield, and endogenous decay coefficient (Al-Malack, 2006). A wide variety of kinetic models have been used to describe the interaction between the growth of microorganisms and utilization of the growth-limiting substrate in activated sludge processes. Among the main models is the Monod and Andrew-Haldane equations (Agarry *et al.,* 2010) and other models have been developed for the case of a limiting nutrient such as the equation of Contois, Moser and Tessier (Omstead, 1989). The aim of this study was to test the ability of *Aspergillus terreus* KP862582, a filamentous fungus isolated from a mining soil facility contaminated with total petroleum hydrocarbons (TPH), to aerobically (oxygen used as electron acceptor) degrade high concentrations of diesel as sole carbon-electron donor source, and kinetic parameters were determined.

2 Materials and methods

2.1 Soil

Isolation of hydrocarbonoclastic filamentous fungi was conducted using a TPH contaminated soil (33,616 ± 1,097 mg/kg), provided by Goldcorp mining facility located in San Dimas Tayoltita, Durango, Mexico. Sand was used to conduct the biodegradation experiments; it was treated in an oven at 200°C for 48 h to remove native microbial populations present and organic matter content.

2.2 Isolation and identification of the indigenous hydrocarbonoclastic filamentous fungi

Fungal strain was isolated following the sequential dilution method by adding 1 g soil sample in 99 mL of sterile physiological solution and magnetically stirred for 60 min. Then aliquots were taken and diluted serially and spread on Petri dishes which contained (g/L): Agar-Agar (16.0), dye rose bengal (0.02), chloramphenicol (0.1), streptomycin sulfate (0.55), and mineral salt solution (Wu *et al.,* 2009) containing: NaCl (12.3), $(NH_4)_2SO_4$ (2.0), K_2HPO_4 (1.55), $NaH_2PO_4{\cdot}2H_2O$ (0.85), $MgCl_2{\cdot}6H_2O$ (2.53), $CaCl_2{\cdot}2H_2O$ (0.73), KCl (0.33), $MgSO_4{\cdot}7H_2O$ (3.15), $NaHCO_3$ (0.09), $ZnSO_4{\cdot}7H_2O$ (0.002), $FeSO_4{\cdot}7H_2O$ (0.005), $Na_2MoO_4{\cdot}2H_2O$ (0.0002), $CuSO_4{\cdot}5H_2O$ (0.0002), $CoCl_2{\cdot}6H_2O$ (0.0004), $MnCl_2{\cdot}2H_2O$ (0.001), H_3BO_3 (0.001), KI (0.0005), $KAl(SO_4)_2{\cdot}12H_2O$ (0.0005), $NiCl_2{\cdot}6H_2O$ (0.0005); at a final pH of 5.5 ± 0.1. A sterile filter paper (Whatman No. 1) saturated with diesel was placed in the cover of the Petri dish according to the technique described by Thijsse and van der Linden (1961). Petri dishes were incubated at 28°C for 14 days. Individual fungal colonies with different macroscopic morphological characteristics were purified transferring slants on potato dextrose agar (PDA) supplemented with 0.5 mg streptomycin per liter (Etuk *et al.,* 2012).

2.3 Molecular identification

2.3.1 DNA extraction and PCR

Fungal isolate selected was grown on 5 mL of sabouraud dextroxe agar in a 50 mL flask at 37°C for 2 days (Fredricks *et al.,* 2005). The DNA extraction was performed by duplicate according to bead beating method (Yeates *et al.,* 1998). The DNA was amplified by PCR using a set primers of the 18S rRNA ribosomal gene that target highly conserved regions NS1 (5-GTAGTCATATGCTTGTCTC-3) and GCfung (5-GCATTCCCCGTTACCCGTTG-3) (White *et al.,* 1990; May *et al.,* 2001). In each reaction mixture a total volume of 25 μL was used containing: 0.25 μL Taq DNA polymerase (5 u/μL) (PROMEGA Bio, EUA), 1 μL Deoxynucleoside triphosphate (10 mM), 1 μL $MgCl_2$ (25 mM), 0.5 μL of each primer, 5 μL Buffer 5X, 0.5 μL of template DNA (38.7 ng/μL) and 16.25 μL of nuclease free water. Reaction conditions for PCR were as follows: initial DNA denaturation at 95°C for 1 min followed by 10 cycles of denaturation at 95°C for 30 s annealing 65 to 60°C for 30 s, a reduction in temperature of 0.5°C each cycle, and followed by an extension at 72°C for 1 min. Additionally, 20 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 7 min was performed with a gradient thermal

cycler T (Techne TC-5000). The DNA extracted and PCR amplification was analyzed by agarose gel electrophoresis and ethidium bromide staining. The PCR products were purified using ZR DNA Clean up Kit Zymo Research; thereafter these products were sequenced by Institute of Biotechnology (UNAM, Cuernavaca-Morelos, Mexico).

2.3.2 Phylogenetic analysis

Sequences were analyzed with the BioEdit (2013) software and compared with sequences of homologs and orthologs using the online BLAST program of National Center for Biotechnology Information (NCBI, 2015). The analysis of phylogenetic relationships was derived by neighbor joining method using software MEGA version 6.0 (Tamura *et al.,* 2013).

2.4 Experimental phase

2.4.1 Inoculum preparation and fungal growth

The fungal isolates were cultivated on PDA plates at 28°C for 5-7 days; spores were collected in sterile distilled water and subsequently counted with a Neubauer chamber to prepare a conidial suspension at a concentration of 10^7 conidia/mL.

Enumeration of fungal population in experimental batch reactors was determined at 15, 30, 45, 60, 75, and 90 days with the serial dilution method (Krüger *et al.,* 2009). One gram of soil, from sacrificed batch reactors, was suspended in 9 mL of 0.85% NaCl sterile solution and serial dilutions were conducted up to 10^{-7}. One mL of each dilution was transferred to Petri plates containing potato dextrose agar (PDA) with 0.5 mg streptomycin per liter and incubated at 28°C for 5-7 days. After incubation, the developed colonies on the PDA plates were counted and recorded as counts of total viable heterotrophic fungi and expressed as colony forming units per gram of soil. This method estimates the number of viable fungal propagules, as number of colony forming units (CFU) present, capable of growing per gram of soil (equation 1).

$$CFU/g_{soil} = (\text{Number of colonies on plate}) \times (\text{Reciprocal of sample dilution}) \quad (1)$$

2.4.2 TPH and CO_2 analysis

Concentration of TPH as a function of time was determined by following EPA 821-B-94-004 and EPA 3540C methods (US EPA, 1995; US EPA, 1996). Samples of 10 g of soil, from sacrificed reactors by duplicate, were taken at 0, 10, 15, 30, 45, 60, 75, and 90 days for extraction and quantification of hydrocarbons with a detection limit of 30 mg/kg dry soil. Results were expressed as mg of hydrocarbon /kg of dry soil (ppm).

On the other hand, carbon dioxide (CO_2) produced was determined by the Isenmeyer method (Alef and Nannipieri, 1995). CO_2 evolved from diesel degradation was captured in an alkali trap containing 25 mL of NaOH solution (0.5 N). Trap was removed every 3 days and resulting solution containing absorbed CO_2 was titrated with HCl (1 N). Collected data were used to calculate the production of CO_2 by means of equation 2.

$$\frac{\frac{CO_2(mg)}{SW}}{t} = \frac{(V_o - V \times 1.1)}{dwt} \quad (2)$$

Where SW is the amount of soil dry weight (g), t is the incubation time (h), V_o is the average volume of HCl used to titrate the initial NaOH solution (mL), V is the average volume of HCl spent on titration of the sample at time t, dwt is the dry weight of 1 g of soil sample, and 1.1 is the conversion factor (1 mL of NaOH 0.5 N is equivalent to 1.1 mg of CO_2).

2.4.3 Diesel biodegradation

To test the ability of diesel biodegradation by the isolated fungus different concentrations of diesel were prepared (10,000, 20,000, 30,000, 40,000, and 50,000 mg/kg soil); required diesel amounts were filtered using Millipore filter 0.45 μm. Diesel was added to 100 g uncontaminated soil (sand) in glass bottles of 250 mL capacity with a two-bored screw cap; one bore for air flow input (6.27 mL/h) to maintain aerobic conditions and the other bore for capturing CO_2. Ammonium nitrate (NH_4NO_3) and potassium phosphate dibasic (K_2HPO_4) were added at a ratio of 100:15:1(C:N:P) as the most recommended by Yang *et al.* (2009). Inoculum was added in a concentration of 3×10^6 conidia/g soil, which is in the range of the minimum amount needed to start fermentation in solid medium and used by some authors (Raimbault and Alazard, 1980; Roussos and Perraud-Gaime, 1996; Gheyrati and Gunale, 2010; Lemos *et al., 2002*; Volke-Sepulveda *et al*., 2006). Viable counts were determined to all batch reactors as a function of time (0, 15, 30, 45, 60, 75, and 90 days). Controls without fungal inoculations were used to follow the behavior of the microbial activity and losses as abiotic control. Moisture in all treatments was maintained at 70% of

field capacity for the extent of the experiment (90 days) at 28°C in the dark. Hydrocarbon extraction from samples was carried out by using the modified EPA 3550B method to measure the loss of TPH.

2.4.4 Batch reactor mass balance

Mass balance was conducted in all batch reactors (Figure 1) considering diesel added (D), residual hydrocarbons H_R, carbon dioxide produced (CO_2), and initial (X_o) and final (X) fungal population. Mass of hydrocarbons degraded was estimated based on diesel mass added and residual TPH at the end of the experiment in each concentration tested; carbon dioxide values were normalized with respect to total CO_2 evolved to compare its tendency.

2.5 Calculation of biodegradation rate constant and half-life time

The biodegradation first-order rate constant (k) was estimated evaluating the slope of the best fit line on a plot of concentration vs time, using equation 3.

$$C = C_o e^{(-kt)} \tag{3}$$

Where C_o is the initial concentration of diesel (mg/kg soil), C is the concentration of diesel at a time (t) (Abbassi and Shquirat, 2008). The half-life time ($t_{1/2}$) was estimated from the derivative of equation 4 (Suarez and Rifai, 2010).

$$t_{1/2} = \frac{\ln 2}{k} \tag{4}$$

2.6 Estimation of biokinetic parameters

The equations to model the interaction between the use of diesel as a substrate-carbon source-electron donor and the kinetics of cell growth of *Aspergillus terreus* KP862582 were based on the mathematical model established by Haldane as shown on equation 5.

$$\mu = \frac{\mu_{max} S}{K_s + S + \left(\frac{S^2}{K_i}\right)} \tag{5}$$

Where μ is the specific growth rate (1/day), μ_{max} is the maximum specific growth rate (1/day), S is the amount of substrate (mg/kg), K_s is the saturation constant (mg/kg), and K_i is the growth inhibition constant (mg/kg). Since high concentrations (10,000 to 50,000 ppm) of diesel were tested, equation 5 is reduced to equation 6; the model parameters, μ_{max} and K_i, were fitted to the experimental data with Excel solver (Microsoft) using the Levenberg-Marquardt method (Kumar *et al.,* 2005).

$$\mu = \frac{\mu_{max} S}{S + \left(\frac{S^2}{K_i}\right)} \tag{6}$$

The generation or doubling time (τ_d) required for cell division during the exponential phase was calculated by equation 7 (Singh *et al.,* 2008).

$$\tau_d = \frac{\ln 2}{\mu} \tag{7}$$

The maintenance energy coefficient (m) was estimated using equation 8, according to the model proposed by Pirt; where Y is the observed yield coefficient and Y_G is the true growth yield coefficient (Van, 2007; Martonosi, 2012).

$$\frac{1}{Y} = \frac{1}{Y_G} + \frac{m}{\mu} \tag{8}$$

Endogenous decay rate k_d (1/day) during the stationary phase was estimated using equation 9 (Peled *et al.,* 1977).

$$X = X_{S_o} e^{-k_d t} \tag{9}$$

X is the initial biomass concentration (CFU), X_{S_o} is the biomass concentration at the beginning of stationary phase (CFU), and t is time (day).

2.7 Statistical analysis

Results were analyzed statistically using analysis of variance (ANOVA) by comparisons of means between groups using tests such as Student-Fischer PLSD (Protected Least Significant Difference) and Tuckey-Kramer. Differences were considered as significant at $p < 0.05$ (Guiraud *et al.,* 2003).

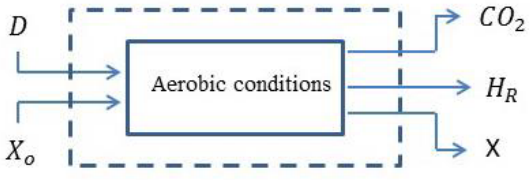

Fig. 1. Mass balance in the batch reactor: Diesel added (D), Initial fungal population (X_o), Residual hydrocarbons (H_R), Carbon dioxide produced (CO_2), and Final fungal population (X).

3 Results and discussion

3.1 Identification of hydrocarbonoclastic autochthonous fungi

Hydrocarbonoclastic fungal strains were isolated from a mining TPH contaminated soil. The isolated fungus was selected by its fast reproduction from among four strains (*Aspergillus terreus, Aspergillus niger, Aspergillus oryzae*, and *Aspergillus fumigatus*), with no further analysis for the other 3 strains. Phylogenetic analysis was based on sequence comparison; Figure 2 shows that the partial sequence of 18S RNA gene of the fungal strain was 99% identical to *Aspergillus terreus* accession number KP862582. Macroscopic analysis of the *Aspergillus terreus* KP862582 strain on PDA agar showed a brown colony with white halo on its surface and soft texture. The microscopic characteristics showed a conidial head formed by chains of conidia globose to ellipsoidal with unbranched conidiophores of smooth walls (Figure 3). Both molecular and morphological characterization supports that this specie belongs to *Aspergillus terreus* KP862582.

3.2 Diesel biodegradation

Table 1 shows biodegradability of diesel by *Aspergillus terreus* KP862582 expressed as percentage. A greater significant reduction of TPH was reached in 10,000 and 20,000 mg/kg soil concentrations with higher constant rate values and significantly lower half-life time values, as compared to the other concentrations. It was observed that as diesel concentration increased (30,000, 40000, and 50,000 mg/kg) the rate constant values decreased substantially to 0.012 ± 0.0002, 0.005 ± 0, and 0.002 ± 7.07E-05 (1/day) respectively; resulting in a significantly low TPH biodegradation. The inhibition effect of biodegradation by *Aspergillus terreus* KP862582 as concentration of diesel increased can be attributed to the fact that high concentrations of diesel cause a decrease in urease activity, which plays an important role in the transformation of urea to ammonia and carbon dioxide (Hui *et al.,* 2006). Several negative effects on the cell are induced by high diesel concentrations, as reported by Sikkema *et al.* (1995); McIntosh *et al.* (1980), and White *et al.* (1981): mechanical stress and increase in temperature caused by cellular accumulation of petroleum hydrocarbons in the area of the acyl chains of the phospholipid monolayers of the cell membrane, therefore the structure of the cell membrane is affected causing alterations as increased permeability with an increase in the flow of protons. Time is a factor that had a significant effect in diesel biodegradation by *Aspergillus terreus* KP862582, maximum biodegradation took place during the first 15 days; as the bioremediation process continued, the TPH biodegradation gradually decreased. A possible explanation for this behavior might be that during biodegradation the incomplete TPH metabolism tends to produce more toxic metabolites for microorganisms (Hu *et al.,* 2012); other studies conducted by Andersson *et al.* (2009) showed that during the biodegradation process the TPH bioavailability decreases due to formation of native toxins.

3.3 Microbial growth of isolated fungi

Evaluation of microbial growth was used to investigate *Aspergillus terreus* KP862582 cell growth on diesel at different concentrations. During the first 30 days of experimentation the total fungal counts reached its maximum, afterwards as time increased a gradual drop was observed up to 90 days (Figure 4). An increment in the number of viable cells is used as an indicator of bioremediation process feasibility; in this case the consumption of diesel as sole carbon source-electron donor by the fungal strain, in the presence of oxygen as electron acceptor. Counts of *Aspergillus terreus* KP862582 at 10,000, 20,000, and 30,000 mg/kg concentrations were significantly higher ($p < 0.05$) as compared to 40,000 and 50,000 ppm concentrations. The stimulating effect on cell viability on the first three concentrations can be attributed to an increase in cellular metabolic activity of microorganisms (Edwards, 1970).

3.4 Biokinetic parameters

The Haldane's model inhibition by substrate was used to represent microbial cell growth kinetics of *Aspergillus terreus* KP862582. Higher growth values were obtained at 10,000 and 20,000 ppm concentrations, as compared to the other three concentrations. Time required for doubling the biomass concentration (τ_d) was significantly greater for 30,000, 40,000, and 50,000 ppm concentrations; meaning that at low TPH concentrations *Aspergillus terreus* KP862582 required less time to double cellular material indicating inhibition was caused by higher concentrations. Endogenous decay coefficient k_d describes the capacity of the microorganisms to

survive during some periods in the absence of nutrients (Singh *et al.,* 2008). In this study k_d coefficient was estimated from values comprised during the period of cell reduction considering death phase; values obtained for 30,000, 40,000, and 50,000 ppm were greater than those of 10,000 and 20,000 concentrations (Table 2). An inhibition effect by increased concentration of diesel became evident since higher concentrations (40,000 and 50,000) showed larger values in m coefficient (Table 2), so that the energy spent at these concentrations to maintain cell activity of *Aspergillus terreus* KP862582 was higher. In this study high K_i values were obtained at lower concentrations of diesel indicating that it was less toxic for *Aspergillus terreus* KP862582 to metabolize it, which agrees to the fact that K_i indicates the degree of toxicity of the substrate medium to the microorganisms (Singh *et al.,* 2008).

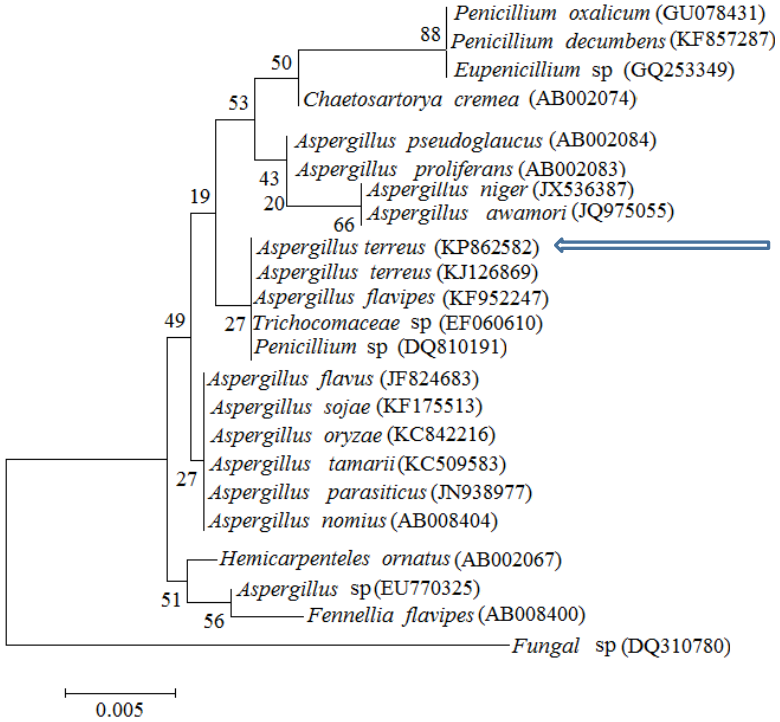

Fig. 2. Phylogenetic tree showing the branches currently targeted by upper group-level oligonucleotide probes.

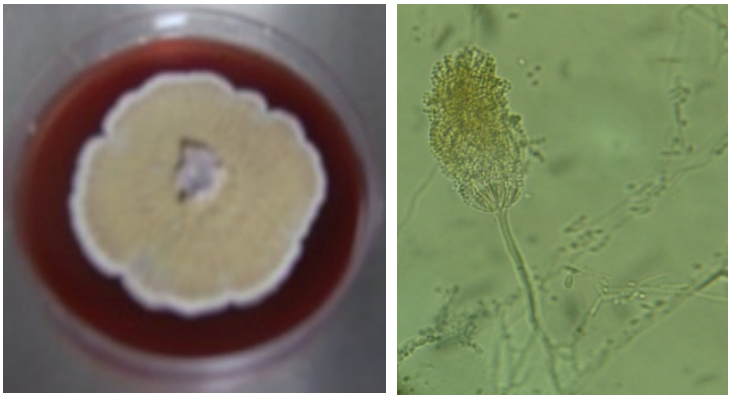

Fig. 3. *Aspergillus terreus* KP862582. Left: In PDA supplemented with 0.5 mg streptomycin/L at 28°C for 7 days. Right: Microscopic characteristics.

Table 1. Experimental results at different diesel concentrations by *Arspergillus terreus* KP862582 for 90 days of experimentation

[Diesel] (mg/kg)	Diesel removal (%)	Biodegradation rate constants k (1/day)	R^2	$t_{1/2}$ (day)
10,000	90.08 ± 0.408	0.025± 0.0013	0.873	26.9 ± 1.41
20,000	87.68 ± 0.420	0.023 ± 0.0003	0.895	29.4 ± 0.44
30,000	69.87 ± 0.219	0.012 ± 0.0002	0.867	57.0 ± 0.99
40,000	36.58 ± 0.188	0.005 ± 0	0.885	135.9 ± 0
50,000	18.17 ± 0.368	0.002 ± 7.07E-05	0.948	308.2 ± 9.69
Control	2.97 ± 0 .075	0	0	0

Table 2. Biokinetic parameters for *Aspergillus terreus* KP862582 obtained in the biodegradation of diesel at different concentrations

[Diesel] (mg/kg)	μ_{max} (1/day)	K_i (mg/kg)	m (mg substrate /CFU-day)	k_d (1/day)	τ_d (day)
10,000	0.76	26,598	0.001	0.117	0.912
20,000	0.80	11,923	0.004	0.134	0.862
30,000	0.43	19,567	0.010	0.189	1.612
40,000	0.24	696	0.020	0.196	2.896
50,000	0.18	609	0.022	0.217	3.707

Table 3. Mass balance of TPH degraded by *Aspergillus terreus* KP862582 at different diesel concentrations (mg/kg). Same average inoculum of 3.1×10^6 CFU/kg soil was added in all batch reactors

[Diesel] (mg/kg)	Diesel removal (%)	Mass added (mg)	Mass degraded (mg)	$[H_R]$ (mg/kg)
10,000	90 ± 0.41	1,000	901 ± 41	992 ± 41
20,000	88 ± 0.42	2,000	1,754 ± 84	2,464 ± 84
30,000	70 ± 0.22	3,000	2,096 ± 66	9,040 ± 66
40,000	36 ± 0.19	4,000	1,463 ± 75	25,369 ± 75
50,000	18 ± 0.37	5,000	908 ± 184	40,917 ± 184

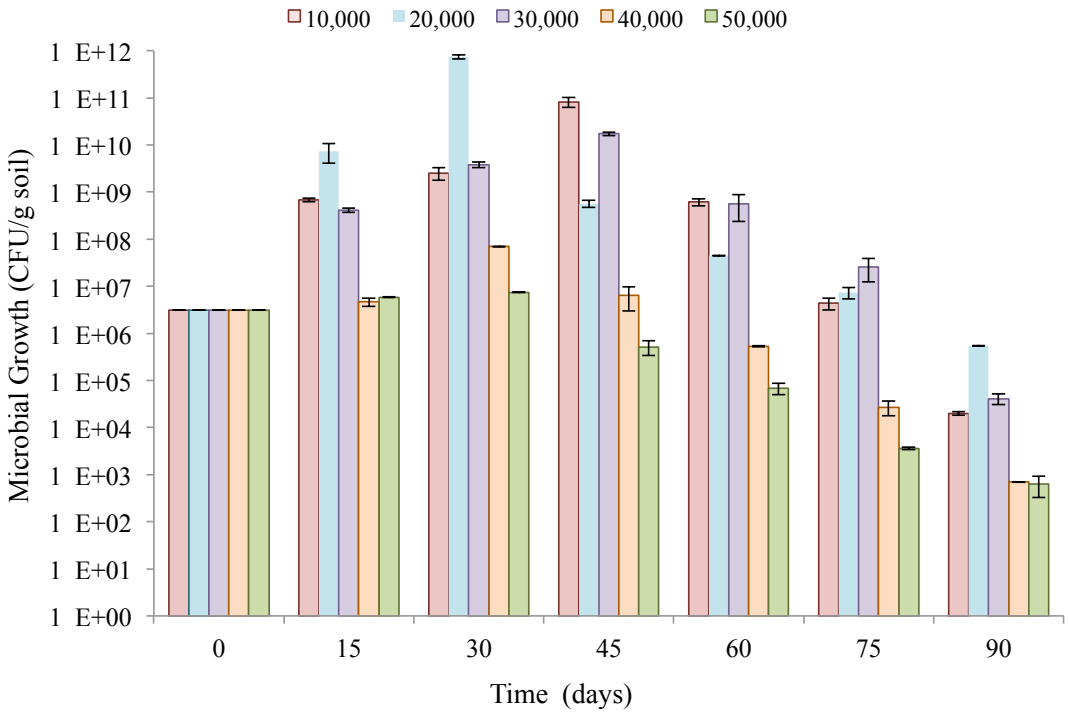

Fig. 4. Cell growth of *Aspergillus terreus* KP862582 at different diesel concentrations (mg/kg).

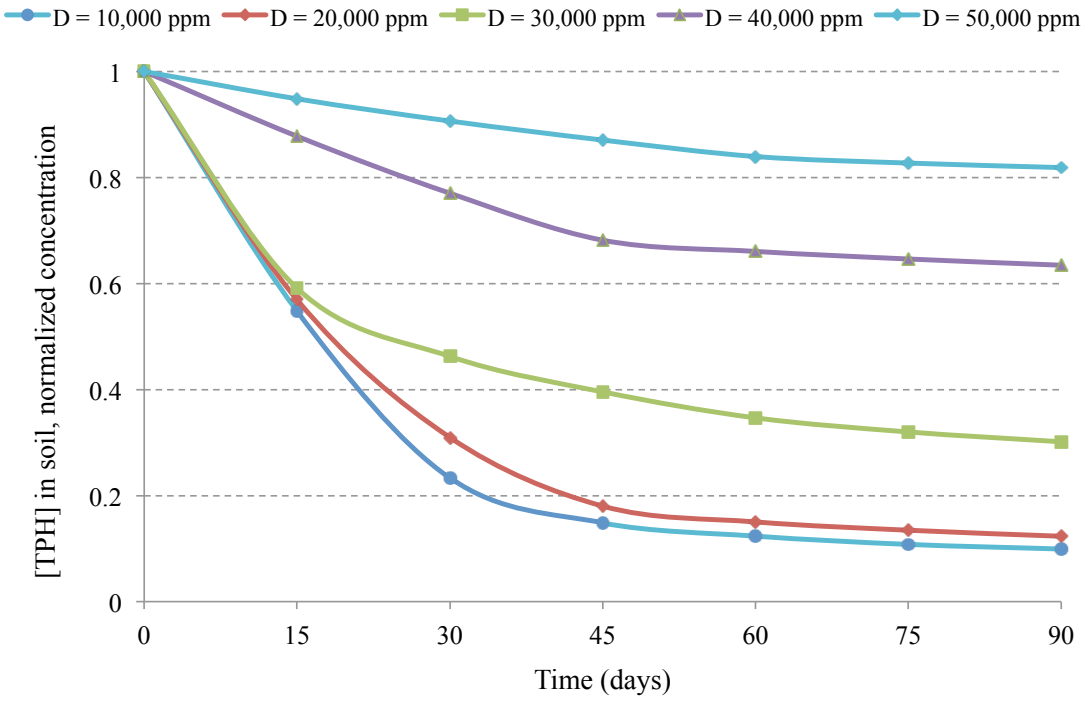

Fig. 5. Tendency of petroleum hydrocarbons normalized concentration in soil.

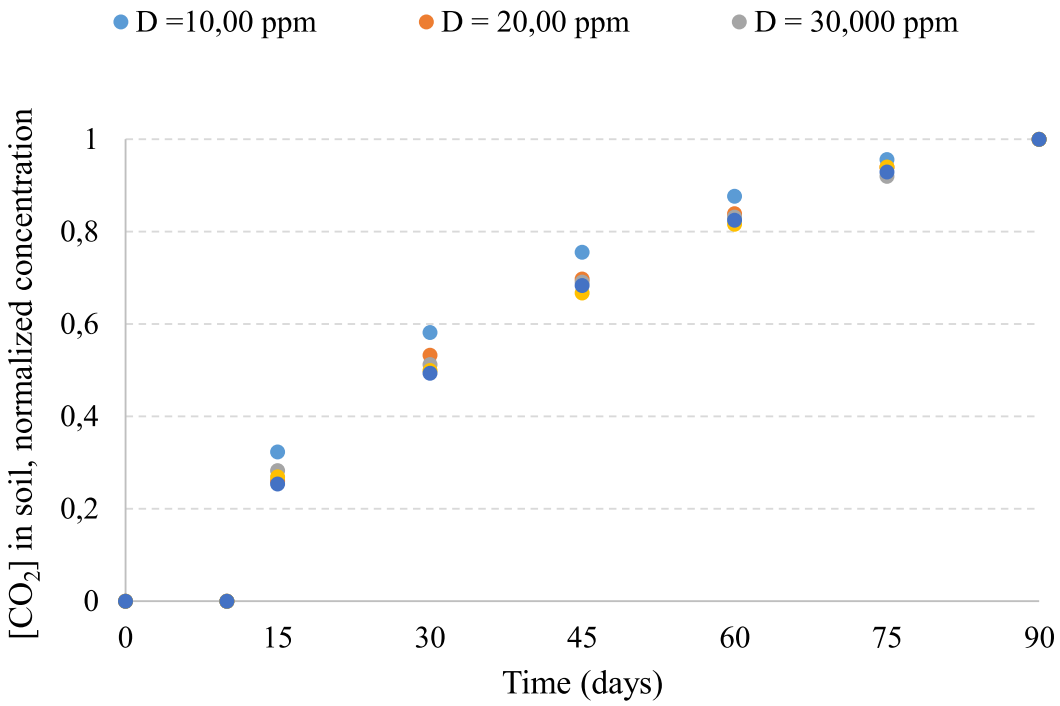

Fig. 6. Mass balance of CO_2 by *Aspergillus terreus* KP862582 at different diesel concentrations. Normalized values: $\frac{(CO_2)_t}{(CO_2)_{90\ days}}$ as CO_2 produced at any given time and total CO_2 at the end of the experiment (90 days).

3.5 Mass balance of TPH, CO_2 and fungal biomass

TPH consumption in all treatments is shown in Figure 5, based on normalized values, and its trend was to decrease as diesel concentration increased. Mass balance (Table 3) showed that the highest mass degraded (2,096 ± 66 mg) was reached at 30,000 ppm followed by 20,000 ppm (1,754 ± 84 mg) and 40,000 ppm (1,463 ± 75 mg), and about the same for 50,000 ppm (908 ± 184 mg) and 10,000 ppm (901 ± 41 mg); however, only 10,000 and 20,000 ppm batch reactors met levels (between 5,000 to 6,000 mg TPH/kg soil) established by NOM-138-SEMARNAT/SS-2003 in Mexican regulations (SEMARNAT, 2003). It is important to recall that the same fungal population (CFU/kg) was added in all cases, which indicates that *Aspergillus terreus* KP862582 is capable of tolerating high TPH concentrations. In order to evaluate mineralization of carbon source by *Aspergillus terreus* KP862582 concentration of CO_2 was also normalized and same tendency was observed for all tested concentrations (Figure 6). The trend of these results is consistent with the biodegradation of diesel by fungal activity; Vanishree *et al.* (2014) evaluated the amount of CO_2 released during biodegradation of petroleum hydrocarbons as an indicator of the activity of *Aspergillus* sp. Lastly, an approximation of fungal biomass produced (X_p) might be indirectly calculated with a mass balance (Figure 1) according to equation 10; maintaining aerobic conditions to ensure the presence of oxygen (electron acceptor). However, the viable count (CFU/kg) gives a better representation of the microbial participation on the bioremediation process, because there are not many methods to measure the mass of mycelial growth.

$$X_p = (X - X_o) = D - (CO_2 + H_R) \qquad (10)$$

Conclusions

Kinetic experimental research was conducted with a fungal strain isolated from a TPH contaminated soil of a local mining industry. This pure culture was properly identified as *Aspergillus terreus* KP862582

showing ability to degrade diesel as sole carbon and electron donor source under aerobic conditions. The importance of this research was focused on studying a pure culture of *Aspergillus terreus* KP862582 to test its ability to degrade high diesel concentrations and identify its contribution in the bioremediation process. Hydrocarbon consumption and carbon dioxide evolved from carbon source degradation were monitored along the experiment; metabolites were not measured or identified. *Aspergillus terreus* KP862582 strain biodegraded 88 ± 0.42% and 70 ± 0.22% of diesel at 20,000 and 30,000 ppm, respectively; reaching a maximum degradation of 90 ± 0.41% for the 10,000 ppm concentration and much lower degradation at 40,000 (36 ± 0.19%) and 50,000 (18 ± 0.37%) ppm concentrations, respectively; caused by the inhibitory effect on microbial growth and consequently on diesel mineralization. Batch reactors for 10,000 and 20,000 ppm reached 992 ± 41 and 2,464 ± 84 mg TPH/kg soil, respectively; a much lower concentration than the maximum permissible limit (MPL) set by Mexican regulation; MPL ranges between 5,000 (medium fraction) to 6,000 (heavy fraction) mg TPH/kg soil. These results support the fact that this autochthonous pure culture was able to degrade petroleum hydrocarbons under these experimental conditions; percentage degradation and cell viability suggests that *Aspergillus terreus* KP862582 is a potential candidate to be used in the treatment of soil contaminated with complex organic chemical compounds and induce removal at high concentrations by bioaugmentation; as in the case of TPH-contaminated soil at Goldcorp mining facility. Based on recent reported results with axenic cultures it is recommended to conduct research with *Aspergillus terreus* KP862582 to confirm if higher efficiencies can be achieved, to prove its technical feasibility to implement a bioremediation process at a larger scale.

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Nomenclature

C_o	initial concentration of diesel, mg/kg soil
C	concentration of diesel at a time, mg/kg soil
CO_2	carbon dioxide produced, mg
D	mass of diesel at time = 0, mg
H_R	residual hydrocarbons at the end of the experiment, mg
k	biodegradation first-order rate constant, 1/day
k_d	endogenous decay rate, 1/day
K_i	growth inhibition constant, mg/kg
K_s	saturation constant, mg/kg
m	maintenance energy coefficient, mg substrate /CFU-day
S	amount of substrate, mg/kg
$t_{1/2}$	half-life time of biodegradation, day
X	initial biomass concentration, CFU
X_p	fungal biomass produced, mg
X_{S_o}	biomass concentration at the beginning of stationary phase, CFU
Y	observed yield coefficient, CFU/mg substrate
Y_G	true growth coefficient, CFU/mg substrate

Greek symbols

μ	specific growth rate, 1/day
μ_{max}	maximum specific growth rate, 1/day
τ_d	generation or doubling time, day

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