



Increase in Total Petroleum Hydrocarbons Removal Rate in Contaminated Mining Soil Through Bioaugmentation with Autochthonous Fungi During the Slow Bioremediation Stage

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Abstract The removal of total petroleum hydrocarbons (TPHs) from contaminated mining soil was carried out under in vitro conditions. The aerobic consumption of TPH in the slow bioremediation stage via biostimulation with native microorganisms and biostimulation-bioaugmentation with autochthonous fungal isolates was evaluated. The initial TPH concentration was $70,880 \pm 975$ mg TPH/kg soil, soil was amended with nutrients at a C:N:P ratio of 100:15:1, the water content was adjusted to the soil field capacity, and batch microcosm reactors were incubated at room temperature ($20.5 \pm 3.1^\circ\text{C}$) for 90 days. The bioaugmentation process was tested using four hydrocarbonoclastic fungal strains isolated from the same contaminated mining soil individually and a mixed culture of the four isolates. The molecular characterization of the isolated fungi was based on sequence analysis of 18S rRNA, and the fungi were identified as *Aspergillus niger* MT786339.1, *Aspergillus fumigatus* MT786338.1, *Aspergillus terreus*

MT786341.1, and *Aspergillus flavus* MT786340.1. The best TPH removal was achieved by inoculation with the fungal consortium ($57 \pm 1.97\%$) at 45 days (slow stage) after initiating the biostimulation process, followed by inoculation with *Aspergillus niger* ($49 \pm 1.2\%$), *Aspergillus terreus* ($44 \pm 0.67\%$), *Aspergillus fumigatus* ($35 \pm 0.98\%$), and *Aspergillus flavus* ($32 \pm 0.38\%$), while the degradation rate achieved with native microorganisms was only $21.6 \pm 1.5\%$; statistical analysis of the results showed significant differences.

Keywords Autochthonous fungal microorganisms · Biostimulation · Bioaugmentation · Fast remediation stage · Slow remediation stage · TPH

1 Introduction

The importance of the mining industry in Mexico is such that it is positioned as the world leader in silver production, the ninth largest producer of gold, and the seventh in copper production, according to 2018 statistics (ITA 2020). On the other hand, the mining industry negatively impacts the environment; it contributes to noise pollution, soil compaction and loss of fertility, habitat destruction, contamination of aquifers, and visual deterioration of the landscape. Among the contaminants discharged by mining activities that negatively impact soils are TPHs from accidental or intentional spills of crude oil, gasoline, oil, and lubricants.

TPH is a term used to describe a family of thousands of compounds that come from crude oil, among which

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aliphatic and aromatic compounds stand out. Specifically, the high hydrophobicity and low solubility of these compounds confer their recalcitrance to the soil matrix, which affects ecosystems (flora and wildlife). These compounds usually have C6 to C35 carbon chains like gasoline, diesel, and oil, and as there are many compounds present in crude oil, it is recommended that they be quantified as TPHs (ATSDR 2020). There are many examples that highlight the presence of TPHs in the environment; those caused by anthropogenic activities can be emphasized as the most prevalent, such as vehicle fleet and machinery maintenance, accidental spills and leakage from underground storage tanks (UST), and spills during the extraction and transport of hydrocarbons (Lee et al. 2007), causing soil and groundwater pollution. Hence, it is necessary to have adequate methods for handling this type of compound to avoid spillage into the environment as well as practical solutions for recovery and removal of these contaminants (Suppen et al. 2006). It is important to mention that contamination of the soil in the facilities of Mexican mining companies, and many other cases worldwide, has resulted from accidental TPH spills, mainly of diesel, gasoline, and residual oils. When spills occur, the soil must be confined for subsequent treatment; hence, it is essential to offer treatment alternatives to comply with the maximum permissible limits (MPL) set by the current Mexican legislation (DOF 2013).

Recovery of soils can be performed using bioremediation technologies, a well-known and commonly used biological process that is sustainable, environmentally friendly, inexpensive, and easy to apply, using indigenous organisms of the contaminated site or other sites (exogenous) organisms (Aniefiok and Udo 2019). Bioremediation is a biological technology that can be performed by different microorganisms (mostly fungi and bacteria) under aerobic (O_2 as an electron acceptor, ea^-) or anaerobic (without O_2 ; different ea^- is utilized) conditions where the substrate-carbon source (TPH) is oxidized, degraded, and/or mineralized to CO_2 , H_2O , and biomass. The efficiency in the removal of pollutants is a function of different factors, such as the substrate availability, electron donor (ed^-), carbon source, ea^- , micro- and macronutrients, inoculum concentration, moisture, and environmental conditions (Kauppi et al. 2011; Kensa 2011).

Bioremediation can be carried out through three different processes: bioattenuation or natural degradation, biostimulation, and bioaugmentation. In natural

attenuation, the degradation of pollutants (substrate, carbon source, and ed^-) takes place in the presence of an electron acceptor (ea^-) due to the activity of indigenous microorganisms. In biostimulation, micro- and macronutrients and ea^- are required in the system to accelerate the biodegradation of the substrate (carbon source and ed^-), and a greater removal efficiency is achieved than that with natural attenuation. For bioaugmentation, degradation of contaminants occurs at an even greater rate because of the inoculation of microorganisms into the system; these added microorganisms must be as efficient as the natives and capable of degrading the target contaminants (Abena et al. 2019; Essabri et al. 2019; Safdari et al. 2018; Mrozik and Piotrowska-Seget 2010; Aleer et al. 2010). This involves the removal and/or microbial transformation of hydrocarbons into safe or less toxic metabolic compounds to the ecosystem through aerobic or anaerobic metabolic pathways, which depend on the chemical composition of the pollutant and the microbial species present in the contaminated soil (Prenafeta-Boldú et al. 2018; Etuk et al. 2012; Abdulsalam et al. 2011; Alisi et al. 2009).

Some studies have evaluated the ability of axenic cultures (only one type of microorganism from a single cell) of soil fungi to remove TPHs with outstanding results and even higher efficiencies than mixed cultures. Some of the fungal isolates (pure or mixed) reported were as follows: a mixed culture of *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium funiculosum*, and *Fusarium solani* showed the lowest removal efficiency of 70%; a mixed culture of *Aspergillus fumigatus* and *Aspergillus niger* removed 90% of hydrocarbons; and *Aspergillus niger* (axenic) had the highest degradation rate of 95% (Al-Jawhari 2014). Bioremediation may use indigenous organisms typical of the contaminated site with the required nutrients (biostimulation) or exogenous and native isolated organisms (bioaugmentation) and can be performed *in situ* or *ex situ* (Kaczyńska et al. 2015; Moliterni et al. 2011; Xu and Lu 2010; Bento et al. 2005). Studies conducted by Essabri et al. (2019) reported TPH removal via bioaugmentation and biostimulation by three hydrocarbonoclastic fungal strains: *Penicillium ochrochloron*, *Aspergillus niger*, and *Trichoderma viride*. These eukaryotic microorganisms were isolated from olive oil effluent and used to degrade the hydrocarbons—as the sole carbon source—present in contaminated soil in *in vitro* experiments.

The selection of technologies for the recovery of contaminated soils depends on the pollutant composition, concentration, physicochemical characteristics, and the risk that the pollutant poses to ecosystem health (Kaczyńska et al. 2015; Semple et al. 2001; Brohon and Gourdon 2000). Usually, the degradation of hydrocarbons requires the incorporation of molecular oxygen into its molecules through the contribution of monooxygenase enzymes, while the degradation of aromatic pollutants by the addition of two oxygen molecules in the ring is catalyzed by dioxygenases (Madigan et al. 2015; Parales and Ju 2011; Arora et al. 2010).

The degradation of hydrocarbons requires the synergistic participation of microbial consortia (Wackett et al. 2001), from which two removal stages are distinguished, fast (F_S) and slow (S_S), assuming that in the first stage (F_S), the hydrocarbons are easily degraded and mainly consumed, while in the second stage (S_S), the degradation is slower. It is also considered that the degradation rate is linked not only to the growth of microorganisms present in the contaminated soil but also to the contaminant concentration, and in some cases, the biodegradation of TPH by indigenous bacterial populations in mining soil takes place in the presence of a surfactant (López-Miranda et al. 2018; De la Cueva et al. 2016; Walter et al. 1997). According to early considerations, the aim of this work was to demonstrate that autochthonous hydrocarbonoclastic fungal strains, *Aspergillus niger* MT786339.1, *Aspergillus terreus* MT786341.1, *Aspergillus fumigatus* MT786338.1, and *Aspergillus flavus* MT786340.1, isolated from the same contaminated mining soil and a mixed culture formed by the four strains, may favor aerobic TPH degradation via bioaugmentation when they are inoculated at the beginning of the slow bioremediation stage (S_S).

2 Materials and Methods

2.1 Reactants

The following chemicals were purchased from the Jalmek Company (Monterrey, Mexico): $(\text{NH}_4)_2\text{SO}_4$ (batch 16-0703-11A57) and K_2HPO_4 (batch 15-1510-06P51); H_2SO_4 (batch 733341) was procured from the Monterrey Chemical Products Fermont Company (Monterrey, Mexico); and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (batch 46) was obtained from CIVEQ Company (Guadalajara, Mexico).

2.2 Soil

The contaminated mining soil used was provided by a mining facility located in San Dimas Tayoltita, Durango, Mexico, and its concentration was $70,880 \pm 975$ ppm TPH (mg TPH/kg soil). Actual contaminated soil was used to isolate hydrocarbonoclastic filamentous fungi and to conduct biodegradation experiments via biostimulation and biostimulation-bioaugmentation.

2.3 Experimental Procedure

2.3.1 Isolation and Identification of Autochthonous Hydrocarbonoclastic Eukaryotic Microorganisms

Fungal strains were isolated according to the sequential dilution method: A mixture of 1 g of soil sample and 99 mL of sterile physiological solution was magnetically stirred for 1 h (Cisneros-de la Cueva et al. 2016). Aliquots were taken and diluted serially on Petri dishes containing agar-agar (16 mg/mL), streptomycin sulfate (550 mg/L), rose bengal dye (200 mg/L), chloramphenicol (100 mg/L), and mineral salt solution containing (g/L) NaCl (12.3), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3.15), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (2.53), $(\text{NH}_4)_2\text{SO}_4$ (2.0), K_2HPO_4 (1.55), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.85), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.73), KCl (0.33), NaHCO_3 (0.09), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.002), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005), $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ (0.001), H_3BO_3 (0.001), KI (0.0005), $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (0.0005), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.0005), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.0004), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.0002), and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.0002) at a final pH of 5.5 ± 0.1 . A sterile filter paper (Whatman No. 1) saturated with diesel, used as the carbon source and electron donor (*ed*⁻), 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 2 weeks. Individual fungal colonies with different macroscopic morphological characteristics were purified, and colonies were transferred to potato dextrose agar (PDA) supplemented with 0.5 mg/L streptomycin (Etuk et al. 2012).

The isolated fungal species were grown in 5 mL of Sabouraud dextrose agar in a 50 mL flask at 37°C for 48 h (Fredricks et al. 2005). DNA extraction was performed in duplicate according to the bead-beating method (Yeates et al. 1998). The amplification of DNA was conducted by PCR using a set of primers that target highly conserved regions of the 18S rRNA ribosomal gene, NS1 (5-GTAGTCATATGCTTGCTC-3) and GCfung (5-

GCATTCCCCGTTACCCGTTG-3) (May et al. 2001; White et al. 1990). In each reaction mixture, a total volume of 25 μL was used, containing 1 μL of deoxynucleoside triphosphate (10 mM), 0.25 μL of Taq DNA polymerase (5 u/ μL) (PROMEGA Bio, USA), 0.5 μL of each primer, 5 μL of Buffer 5X, 0.5 μL of template DNA (38.7 ng/ μL), 1 μL of MgCl_2 (25 mM), and 16.25 mL of nuclease-free water. Conditions for the PCR were as follows: initial DNA denaturation at 95°C for 1 min followed by 10 denaturation cycles at 95°C for 30 s, annealing at 65 to 60°C for 30 s, a reduction in temperature of 0.5°C per cycle, and 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, were performed with a gradient PCR/Techne Thermal Cycler (Model TC-5000, USA). The extracted DNA and PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The PCR products were purified using the DNA Clean-up and Concentrator Kits (Zymo Research, USA); subsequently, these products were sequenced by Biotechnology Institute (UNAM, Cuernavaca-Morelos, Mexico).

2.3.2 TPH Analysis

The concentration of residual total petroleum hydrocarbons in contaminated soil [TPH]_R was measured by the EPA 821-B-94-004 and EPA 3540C methods (US EPA 1995; US EPA 1996). Samples of 10 g of soil from sacrificed reactors were taken in duplicate at 0, 15, 30, 45, 60, 75, and 90 days for extraction and quantification of hydrocarbons with a detection limit of 30 mg/kg dry soil. The results were expressed as mg of TPH/kg of dry soil (ppm), while the viable microorganism count, expressed as colony forming units contained in a gram of soil (CFU/g soil), was quantified by the serial dilution method (Krüger et al. 2009) using potato dextrose agar (PDA) as the culture medium. One gram of soil was suspended in 9 mL of sterile distilled water, and then 1 mL of the first dilution was taken and transferred to a new dilution tube. The serial dilution was continued until the desired dilution was obtained (10^{-8}); plates were inoculated by surface extension and incubated at 28°C for 7 days.

2.3.3 Inoculum Preparation and Fungal Growth

This stage was conducted using axenic cultures. Fungal microorganisms were inoculated by surface

extension in inclined 50 mL tubes containing 25 mL of PDA as a culture medium and were incubated for 7 days at 28°C. Conidia were harvested with 15 mL of sterile water, and their concentration was determined in a Neubauer chamber; this concentration was used to calculate the volume needed to produce inoculum with a concentration of 10^6 conidia/g soil.

2.3.4 Soil Preparation for In Vitro Experiments

Mining-contaminated soil was sieved, and the soil that passed through a US standard sieve number 10 was collected, while the soil was retained in sieve number 12 to achieve a particle size of 2 mm. The concentration of C:N:P was adjusted to a 100:15:1 ratio using the Tchobanoglous & Vigil method for nutrient balance with a mineral solution (10.63 g $(\text{NH}_4)_2\text{SO}_4$ /kg soil and 0.70 g K_2HPO_4 /kg soil); the soil moisture was adjusted to 70% of its field capacity (185 mL H_2O /kg soil) (Eweis et al. 1998). For all treatments evaluated, experiments were carried out using sacrificed batch reactors in duplicate: P1 (14), P2 (56), and P3 (70) (Tchobanoglous and Vigil 1993).

2.3.5 TPH Consumption by Microorganisms via Biostimulation and Biostimulation-Bioaugmentation

A series of three treatments were established to evaluate the behavior of native microorganisms vs isolated fungal strains (each of the 4 isolated fungi and a mixed culture of the four) in terms of TPH consumption. Natural attenuation-biostimulation (P1) was used as a control, and only nutrients were added; biostimulation-bioaugmentation (P2) was performed using inoculation of single fungal strains at the beginning of the process where fast degradation takes place ($F_s = 0$ d); and biostimulation-bioaugmentation (P3) was performed by inoculation at the beginning of the slow bioremediation ($S_s = 45$ d) stage. According to the above, the experimental design, formulation, and ID of the four fungal strains used are summarized in Table 1.

P1: Biostimulation with Indigenous Microorganisms. The biostimulation experiments were carried out in duplicate in 220-mL loose-lid flasks with the following conditions: 100 g of contaminated soil (TPH

Table 1 Experimental design: formulation of evaluated processes and fungal strains used

Component	Processes		
	P1	P2 ($t = 0$ day)	P3 ($t = 45$ days)
Native soil (g)	+	-	+
Sterile soil (g)	-	+	-
Nutrients: $(\text{NH}_4)_2\text{SO}_4$ & K_2HPO_4	+	+	+
Water	+	+	+
Microorganisms			
<i>Aspergillus niger</i> MT786339.1	-	+	+
<i>Aspergillus fumigatus</i> MT786338.1	-	+	+
<i>Aspergillus terreus</i> MT786341.1	-	+	+
<i>Aspergillus flavus</i> MT786340.1	-	+	+
Fungal mixed culture (FMC)	-	-	+

as carbon source-substrate-electron donor), 18.5 mL of mineral solution as source of micro- and macronutrients, and the field capacity of the soil was adjusted to 70%. The flasks were incubated at room temperature ($20.5 \pm 3.12^\circ\text{C}$) for 90 days. Changes in the concentration of TPH were recorded, reactors were sacrificed every 15 days ($t = 0, 15, 30, 45, 60, 75,$ and 90 days) taking samples of 10 g of soil for analysis of the total residual hydrocarbon content and the viable count of microorganisms as colony forming units (CFU)/g soil.

P2: *Bioaugmentation with Inoculum Added at 0 days.* Bioaugmentation experiments in the fast degradation stage (F_s) were carried out in duplicate in 220-mL loose-lid flasks with the following conditions: 100 g of sterilized contaminated soil (UV rays for 1 h in an ESCO AVC-D2 laminar flow hood), 18.5 mL of mineral solution as source of micro- and macronutrients, and the field capacity of the soil was adjusted to 70%. Batch reactors were inoculated with each of the four isolated strains individually at a concentration of 1×10^6 conidia/g soil (Table 1); flasks were incubated at room temperature ($20.5 \pm 3.12^\circ\text{C}$) for 90 days. The change in the concentration of TPH was measured, and duplicate reactors were sacrificed every 15 days ($t = 0, 15, 30, 45, 60, 75,$ and 90 days). Samples of 10 g of soil were taken for analysis of the residual TPH content, and

the viable count of microorganisms was recorded as colony forming units (CFU)/g soil.

P3: *Bioaugmentation with Fungal Inoculum Added at 45 days.* Experiments in the slow degradation stage (S_s) were conducted to evaluate the influence of the isolated fungal microorganisms on the degradation of TPH as the sole carbon source-substrate-electron donor. Duplicate 220-mL loose-lid flasks were established containing 100 g of contaminated soil and 18.5 mL of mineral solution, and the field capacity of the soil was adjusted to 70%; flasks were incubated at room temperature ($20.5 \pm 3.12^\circ\text{C}$) for 45 days (bioaugmentation of native microorganisms); at this time, flasks were inoculated with 1×10^6 conidia/g soil for each single pure culture and with the consortium (mixed fungal culture, each of the four fungal strains was in equal proportion) (bioaugmentation). The change in the concentration of TPH was measured and duplicate reactors were sacrificed every 15 days ($t = 45, 60, 75,$ and 90 days). Samples of 10 g of soil were taken for analysis of the residual TPH content and the viable count of microorganisms (CFU/g soil).

2.3.6 TPH Removal Efficiency by Hydrocarbonoclastic Microorganisms

To determine the removal efficiency of each process (R), TPH was consumed by the fungal strains as an electron donor-substrate-carbon source in an aerobic process where the electron acceptor O_2 was not limited throughout the entire process. This efficiency was

estimated as the difference in the TPH content at time zero (TPH_0) and at 90 days (TPH_t), as shown in Eq. (1).

$$R = \left(\frac{TPH_0 - TPH_t}{TPH_0} \right) * 100 \quad (1)$$

2.3.7 Evaluation of the Biodegradation Rate Constant and Half-Life Time

The first-order biodegradation rate constant (k) was determined by evaluating the slope of the best fit line on a plot of concentration vs time using Eq. (2).

$$C = C_o e^{-kt} \quad (2)$$

where C_o is the initial concentration of hydrocarbons (mg TPH/kg soil) and C is the concentration of hydrocarbons at time (t) (Abbassi and Shquirat 2008). On the other hand, the half-life time ($t_{1/2}$) was estimated from the derivative of Eq. (3) (Suarez and Rifai 2010).

$$t_{1/2} = \frac{\ln 2}{k} \quad (3)$$

2.4 Statistical Analysis

The experimental results were analyzed by one-way analysis of variance (ANOVA) to validate the differences by comparisons of means between groups using Tukey HSD (honest significant difference) with Statistica 12 software. The experimental design was a single factor with 3 levels and two replicates, having as a response variable the change in TPH concentration at a significance level (α) value of 0.05.

3 Results and Discussion

3.1 Molecular Characterization of Isolated Autochthonous Hydrocarbonoclastic Fungi

Four hydrocarbonoclastic fungal strains were isolated from TPH-contaminated mining soil and selected on the basis of their fast reproduction. Molecular identification was based on sequence comparison of the 18S rRNA gene, and the sequences of the fungal strains were deposited in GenBank, with the following accession numbers: *Aspergillus niger* accession number MT786339.1, *Aspergillus fumigatus* accession number

MT786338.1, *Aspergillus terreus* accession number MT786341.1, and *Aspergillus flavus* accession number MT786340.1.

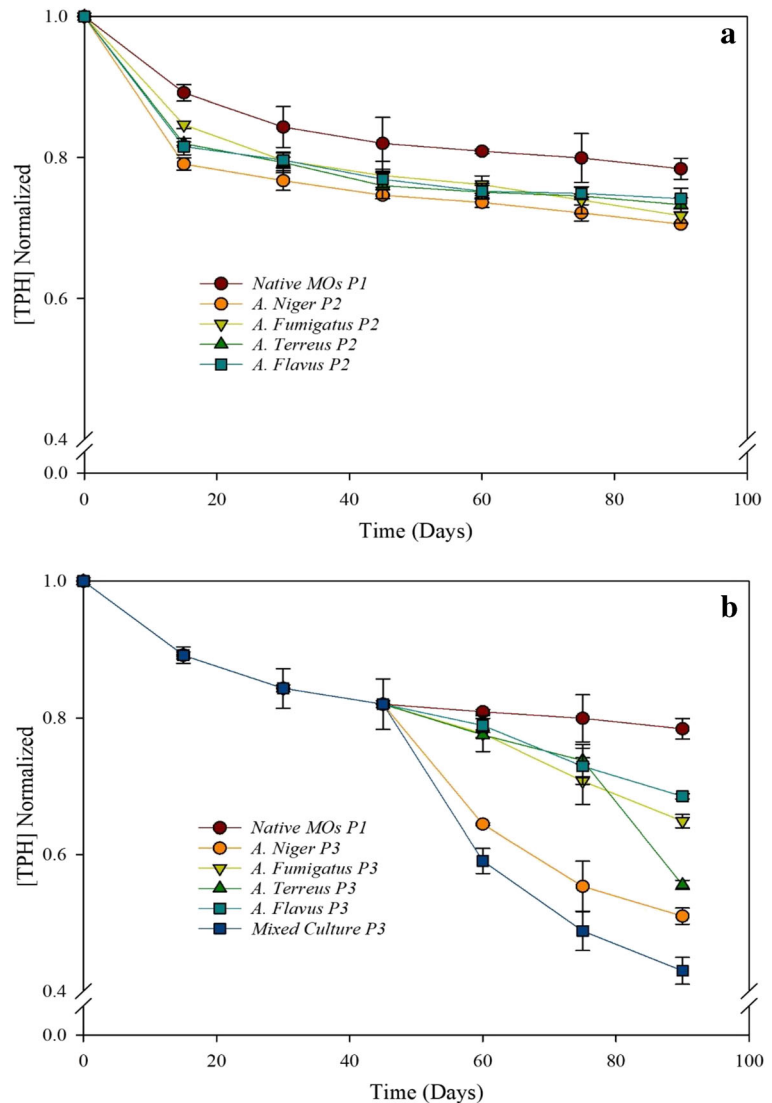
3.2 Degradation of TPH by Native Consortium and Isolated Fungal Microorganisms

P1: Consumption of TPH by Biostimulation with Native Microorganisms. Figure 1 depicts the kinetics of TPH biodegradation for all three processes evaluated in the present research based on normalized concentration values, and its trend was expected to decrease with time. In P1, rapid degradation took place (F_S) from 0 to 45 days; after that, TPH consumption slowed down (S_S) and remained almost constant until the end of the experiment. In this treatment (P1), a $21.58 \pm 1.50\%$ removal of TPH was achieved for the 90-day period. This result is even higher than that reported by Benyahia and Shams Embaby (2016), who achieved 23% consumption of TPH in a period of 156 days in a biostimulation process with indigenous microorganisms in a biopile system of desert soil, in which the average TPH initial concentration was similar to the concentration in the present study.

Based on the results observed in the P1 process, 45 days was chosen as the time point to conduct further tests with inoculation of the 4 isolated fungal strains (pure and mixed culture) under bioaugmentation conditions (P3, Fig. 1b). Several authors have pointed out that successful bioremediation of TPH-contaminated soils by biostimulation (addition of appropriate nutrients, N and/or P) results in better metabolic activity of native microorganisms and, therefore, accelerates the degradation of hydrocarbons (Ghaly et al. 2013; Suja et al. 2014; Smith et al. 2015).

P2: Bioaugmentation in Sterile Soil with Isolated Fungal Strains (Inoculation at 0 day). The kinetic profiles of TPH degradation carried out in sterile soil by the isolated hydrocarbonoclastic fungal microorganisms added at time zero are also shown in Fig. 1a. As shown, the degradation rates for the P2 process under the treatments were close for all fungal strains tested but higher than those for P1; values ranged from 1.14- to 1.33-fold those of P1. However, statistical analysis showed that there were significant differences ($p < 0.05$) only for *Aspergillus niger* ($p = 0.01695$) and *Aspergillus flavus* ($p = 0.04701$) but not for the other two strains. These results clearly show that there was little influence of the inoculum when added to sterile soil at time zero, probably

Fig. 1 TPH degradation kinetics of native microorganisms vs isolated fungal strains for the different processes evaluated. (a) Biostimulation of native MOs (P1) and pure individual fungal MOs (P2). (b) Biostimulation of native MOs (P1) vs bioaugmentation with isolated fungal strains, individual and mixed culture, inoculated at 45 days (P3)



because enzyme activity was not triggered at the early stage of the process (Fig. 2).

P3: Biostimulation with Native MOs vs Bioaugmentation with Isolated Fungal Strains (Inoculation at 45 days). Although an increase in the TPH removal rate was observed in the fast degradation stage, a bioaugmentation experiment was conducted at the slow degradation stage, wherein contaminated native soil was inoculated with the isolated fungal strains (single and mixed) at day 45. The untreated soil was only biostimulated with micro- and macronutrients present in the mineral solution. Bioremediation by the native

microorganisms was allowed for 45 days; at this time, the soil was inoculated, in separate experiments, with the four strains of fungi individually and with a mixture of the four strains in equal proportions, and the experiment was continued for another 45 days (Fig. 1b).

As shown, bioaugmentation offered a much higher degradation rate than the biostimulation with native microorganisms (Fig. 3). TPH removal increased from 1.6- to 3.3-fold with the addition of each fungal strain individually and 4.2-fold with the consortium, with the mixed fungal culture offered the best degradation rate. Accordingly, statistical analysis showed that there were significant differences ($p < 0.05$) in all cases as follows:

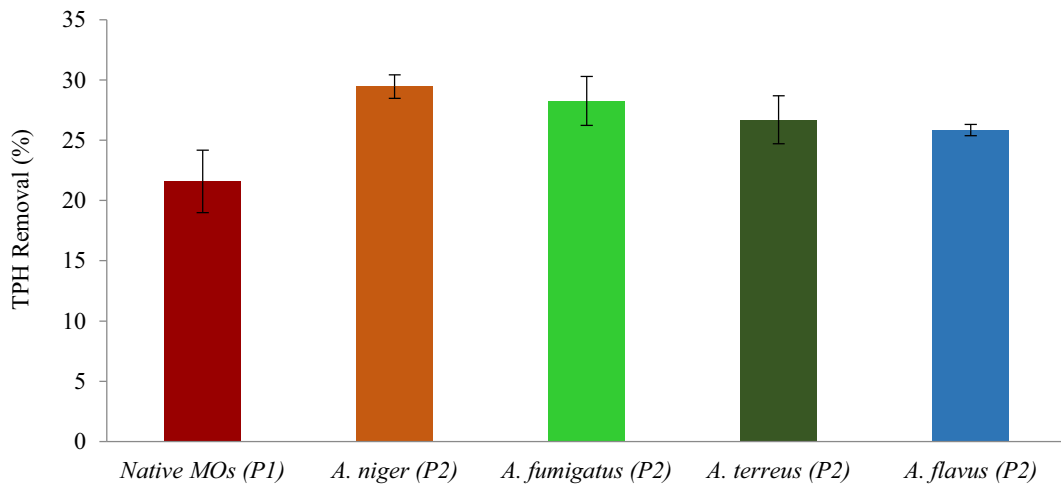


Fig. 2 TPH degradation removal (%) at 90 days for native microorganisms (P1) vs each of the four isolated fungal strains evaluated (P2)

Aspergillus niger ($p = 0.000225$), *Aspergillus flavus* ($p = 0.003413$), *Aspergillus terreus* ($p = 0.000225$), *Aspergillus fumigatus* ($p = 0.000431$), and the mixed fungal culture (0.000225). These results undoubtedly show that there was a significant influence of the inoculum when added at 45 days, supporting the fact that enzyme activity was properly exhibited at this stage of the process, showing that the native microflora favors the removal of contaminants, due to the fact that they release mono- and dioxygenase enzymes, responsible for the reduction of aliphatic and aromatic compounds (Alvarez and Illman 2006). The highest percentage of degradation was obtained with the consortium, achieving a TPH degradation efficiency of $57 \pm 1.97\%$, and the rates ranged from 35 ± 0.98 to $49 \pm 1.19\%$ for the individual fungal strains when compared to that of P1 (Tables 2 and 3).

Table 2 summarizes the results for the aerobic degradation of TPH for all processes conducted, and degradation rates are presented from highest to lowest. It is clear that the bioaugmentation process (P3) offered the best removal, with higher degradation constants and significantly lower half-life times: fungal mixed culture ($9.76 \times 10^{-3} \pm 7.07 \times 10^{-6}$ 1/d and 71.06 ± 0.05 days, respectively) offered the best degradation followed by *A. niger* MT786339.1, *A. terreus* MT786341.1, *A. fumigatus* MT786338.1, and *A. flavus* MT786340.1 as compared to P2 and P1; native microorganisms (P1) yielded the lowest degradation constant rate ($2.35 \times 10^{-3} \pm 7.07 \times 10^{-5}$ 1/day) and longest half-life time (295.09 ± 8.88 days). Several studies report that the participation of microbial consortia has advantages over

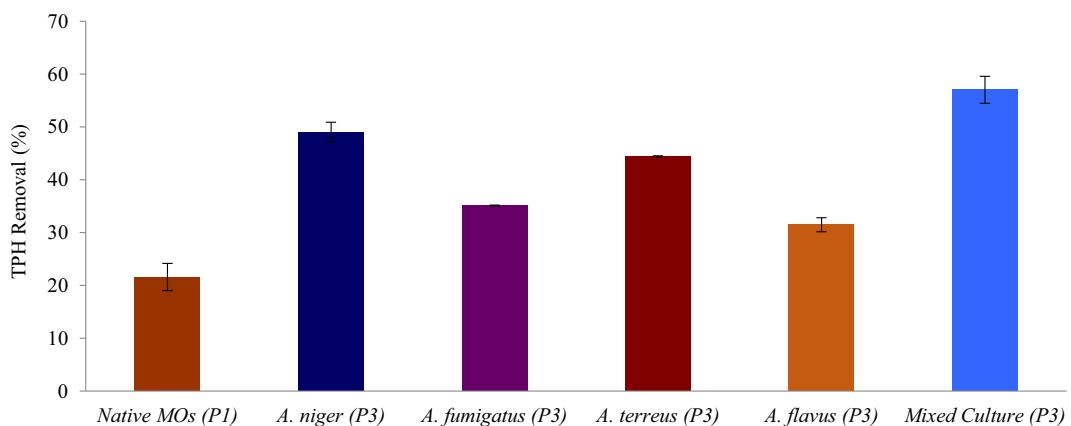


Fig. 3 TPH consumption (%) by isolated fungal strains at 90 days; inoculum (individual strains or consortium) was added at the slow remediation stage (45 days)

Table 2 Summarized results for all three processes: aerobic degradation of TPHs present in mining-contaminated soil after 90 days of experimentation

Process: microorganism	TPH removal (%)	Biodegradation rate k (1/day)	R ²	t _{1/2} (day)
P3: Mixed Fungal Culture	57 ± 1.97	9.76×10 ⁻³ ± 7.07×10 ⁻⁶	0.93	71.06 ± 0.05
P3: <i>Aspergillus niger</i> MT786339.1	49 ± 1.2	7.73×10 ⁻³ ± 1.41×10 ⁻⁴	0.95	89.68 ± 1.64
P3: <i>Aspergillus terreus</i> MT786341.1	44 ± 0.67	5.31×10 ⁻³ ± 2.05×10 ⁻⁴	0.86	130.76 ± 5.05
P3: <i>Aspergillus fumigatus</i> MT786338.1	35 ± 0.98	4.39×10 ⁻³ ± 3.96×10 ⁻⁴	0.98	158.54 ± 14.30
P3: <i>Aspergillus flavus</i> MT786340.1	32 ± 0.38	3.82×10 ⁻³ ± 8.49×10 ⁻⁵	0.97	181.50 ± 4.03
P2: <i>Aspergillus fumigatus</i> MT786338.1	28 ± 1.05	3.13×10 ⁻³ ± 7.07×10 ⁻⁶	0.82	221.81 ± 0.50
P2: <i>Aspergillus niger</i> MT786339.1	29 ± 0.01	3.03×10 ⁻³ ± 0.00	0.71	228.76 ± 0.00
P2: <i>Aspergillus terreus</i> MT786341.1	27 ± 0.99	2.80×10 ⁻³ ± 1.41×10 ⁻⁴	0.72	259.45 ± 13.03
P2: <i>Aspergillus flavus</i> MT786340.1	26 ± 1.49	2.68×10 ⁻³ ± 1.34×10 ⁻⁴	0.71	247.87 ± 12.52
P1 native MOs	22 ± 1.50	2.35×10 ⁻³ ± 7.07×10 ⁻⁵	0.82	295.09 ± 8.88

P1: Biostimulation of native microorganisms

P2: Biostimulation-bioaugmentation in sterile soil with isolated fungal strains, inoculation at 0 day

P3: Biostimulation-bioaugmentation with isolated fungal strains, inoculation at 45 days

individual species in efficiently degrading hydrocarbon mixtures, because the microbial communities evolved together to survive in contaminated soil (Zafra et al. 2017).

Table 3 shows the comparison among all processes (P1, P2, and P3) and microorganisms (native, isolated fungi, and mixed fungal culture) for the aerobic

degradation of TPH in mining-contaminated soil; experiments were conducted at the same initial TPH concentration and under the same conditions for a period of 90 days. Values can be read by row and column to compare 2 microorganisms under different processes. For instance, the last row shows that the mixed fungal culture (P3) produced a higher degradation rate of TPH in all cases

Table 3 TPH biodegradation rate comparison (increase or decrease) among all processes and microorganisms evaluated after 90 days of experimentation

Process: microorganism	P1 Native MOs	P2 <i>A. niger</i>	P3 <i>A. niger</i>	P2 <i>A. fumigatus</i>	P3 <i>A. fumigatus</i>	P2 <i>A. terreus</i>	P3 <i>A. terreus</i>	P2 <i>A. flavus</i>	P3 <i>A. flavus</i>	P3 Mixed Fungal Culture
P1: Native MOs	1.00	0.78	0.30	0.75	0.54	0.84	0.44	0.88	0.62	0.24
P2: <i>A. niger</i>	1.29	1.00	0.39	0.97	0.69	1.08	0.57	1.13	0.79	0.31
P3: <i>A. niger</i>	3.29	2.55	1.00	2.47	1.76	2.76	1.46	2.89	2.02	0.79
P2: <i>A. fumigatus</i>	1.33	1.03	0.40	1.00	0.71	1.12	0.59	1.17	0.82	0.32
P3: <i>A. fumigatus</i>	1.87	1.45	0.57	1.40	1.00	1.57	0.83	1.64	1.15	0.45
P2: <i>A. terreus</i>	1.19	0.92	0.36	0.90	0.64	1.00	0.53	1.05	0.73	0.29
P3: <i>A. terreus</i>	2.26	1.75	0.69	1.70	1.21	1.89	1.00	1.98	1.39	0.54
P2: <i>A. flavus</i>	1.14	0.88	0.35	0.86	0.61	0.96	0.50	1.00	0.70	0.27
P3: <i>A. flavus</i>	1.63	1.26	0.49	1.22	0.87	1.36	0.72	1.43	1.00	0.39
P3: Mixed fungal culture	4.15	3.22	1.26	3.12	2.22	3.48	1.84	3.65	2.55	1.00

P1: Biostimulation of native microorganisms

P2: Biostimulation-bioaugmentation in sterile soil with isolated fungal strains, inoculation at 0 day

P3: Biostimulation-bioaugmentation with isolated fungal strains, inoculation at 45 days

when compared to any other microorganism treatment (columns), e.g., a 4.2-fold greater degradation than native microorganisms (MOs) (Sakineh et al. 2012).

Several authors have reported negative effects on the cell induced by high hydrocarbon concentrations, mechanical stress, and an increase in temperature caused by cellular accumulation of TPHs in the acyl chains of the phospholipid monolayers of the cell membrane, which is why the structure of the cell membrane is affected and changes such as increased permeability with increased proton flux are observed (Sikkema et al. 1995; White et al. 1981; McIntosh et al. 1980).

The results in this research can be compared to those reported by Benyahia and Shams Embaby (2016). The desert soil biopile system was bioaugmented with Amnrite P-300 (containing 10 strains belonging predominantly to the *Pseudomonas* genus, including both aerobic and facultative organisms, and the total viable count was not less than 5.0×10^8 CFU/g) (Cbio 2020). The results showed a 77% TPH reduction over a period of 156 days, whereas the system with polyoxyethylene (20) sorbitan monooleate (Tween 80) gave a TPH removal rate of 56%, while in the present research, a $57.01 \pm 1.97\%$ removal was achieved in 90 days.

Different studies report the use of either bacteria or fungi to degrade several petroleum hydrocarbons in biostimulation or bioaugmentation processes. Abena et al. (2019) conducted studies with a consortium of five exogenous bacteria under natural attenuation with nutrients (biostimulation) and bioaugmentation with nutrients (biostimulation) to degrade TPH in highly contaminated soils. They found that the bioaugmentation treatment showed better degradation of TPH (48.10%) than the natural attenuation-biostimulation process. Suja et al. (2014) worked with crude oil-contaminated soil in a microcosm tank experiment (2 kg soil samples), and the combination of bioaugmentation (*Acinetobacter* sp. and *Pseudomonas* sp.) and biostimulation (mineral salt solution was added) gave the highest degradation rate, with 79% removal of TPH. Iheanacho et al. (2014) isolated indigenous fungal species from an oil exploration zone and tested their hydrocarbon degradation potentials. *Yarrowia lipolytica* ATCC 9773 (> 82%) and *Zygorrhinchus* sp. (2) (>83%) achieved removal via biodegradation within 14 days, demonstrating that these fungal species are potential degraders to be used in the treatment of oil-polluted farmlands (soil).

Crude oil is made from a mixture of different compounds, and individual microorganisms metabolize only

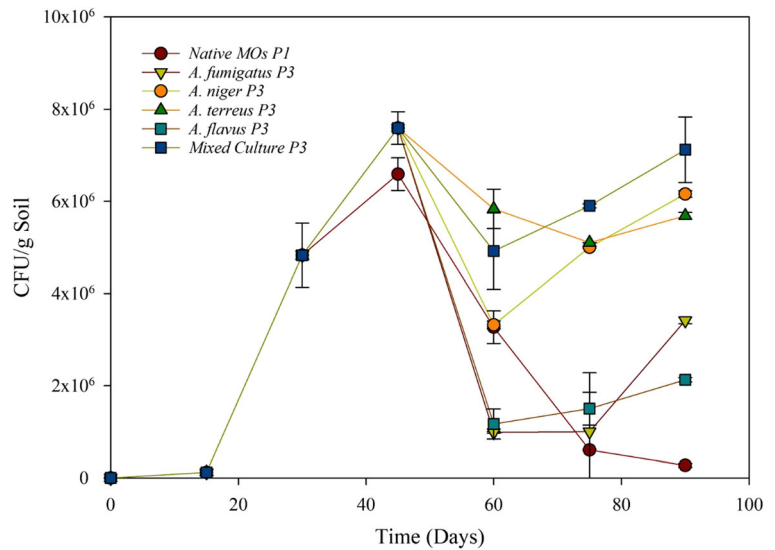
a limited range of hydrocarbon substrates; therefore, biodegradation of petroleum hydrocarbons requires a mixture or consortia of different bacterial and fungal strains (Al-Saleh et al. 2009; Bordenave et al. 2007). Microorganisms have the necessary enzymes to achieve optimal results and degrade a wide range of hydrocarbons (Leahy and Colwell 1990). D'Annibale et al. (2006) carried out the bioremediation of contaminated soil with aromatic hydrocarbons and confirmed that the isolation of nine indigenous fungi (*Allescheriella* sp. strain DABAC 1, *Stachybotrys* sp. strain DABAC 3, and *Phlebia* sp. strain DABAC 9, among others) followed by their reinoculation at the same source could be a beneficial remediation strategy.

3.3 Fungal Growth and TPH Mass balance

According to the microbial kinetics of the native microorganisms (biostimulation), the four individual fungal strains isolated, and the mixed fungal culture, at 90 days, the mixed fungal culture had the greatest growth, followed by *A. niger*, *A. terreus*, *A. fumigatus*, *A. flavus*, and the native microorganisms; this corresponded to the degradation (%) of TPHs, since reproduction of microorganisms or their biosynthesis (anabolism) is directly related to the consumption of substrate-*ed*-carbon source (TPH). The initial average microbial count in all treatments corresponded to 1×10^3 CFU/g soil, and the addition of micro- and macronutrients biostimulated the growth of the native microorganisms during the first 45 days (fast stage, Fs). Afterwards, at the beginning of the slow degradation stage (day 45), 1×10^6 conidia/g soil of each of the four fungal strains and their mixture were inoculated in separate treatments. Growth was measured at all sampling points, and it was observed that the microbial counts peaked at day 45 of the bioremediation process and decreased to their lowest value at day 60; this behavior explains the presence of the slow bioremediation stage. However, in all treatments for the P3 process, ascendant growth behavior was observed during the final 30 days of experimentation, as seen in Fig. 4. We suggest that a longer experimental period should be examined to confirm whether better results can be achieved.

The mass balances for all three processes evaluated are summarized in Table 4 in terms of TPH removal (%), TPH mass degraded (mg), and residual TPH (H_R) mass (mg). Inoculation in the slow rate stage (at 45 d

Fig. 4 Viable microbial count per gram of soil for the P3 process where fungal strains were inoculated at 45 days after the biostimulation of native microorganisms was started. The initial average microbial count in all treatments corresponded to 1×10^3 CFU/g soil, and at day 45, 1×10^6 conidia/g soil was added



gave the best results; the highest degradation removal ($57 \pm 1.97\%$) was achieved in P3 with the mixed fungal culture, and the lowest corresponded to P1 with the native microorganisms (22 ± 1.50).

4 Overall Discussion and Conclusions

Hydrocarbonoclastic fungal strains were isolated from TPH-contaminated mining soil, four of which were selected on the basis of their fast growth and identified based on sequence comparison of their 18S rRNA.

Native microorganisms were tested for the removal of TPH (*ed*) under aerobic conditions (*ea*: O_2) via biostimulation and compared to biostimulation-bioaugmentation systems by inoculation with the 4 isolated fungal strains (in pure and mixed culture). The inoculation of fungal strains at the beginning of the slow bioremediation stage (S_S) significantly improved the removal efficiency of TPHs from the soil. This phenomenon is attributed to the increased concentration of microorganisms and the presence of enzymes, just at the time where the indigenous microbial activity was decreasing considerably. Extended exposure of viable

Table 4 Mass balance of degraded and residual TPH under the treatments with native microorganisms and isolated fungal strains. The same average inoculum was added in all batch reactors

Process	TPH removal (%)	Mass added (mg)	Mass degraded (mg)	H _R (mg)
Biostimulation of Native Microorganisms				
P1	22 ± 2	7088	1529 ± 85	5559 ± 183
Biostimulation-bioaugmentation in sterile soil with isolated fungal strains, inoculation at 0 day				
P2 (<i>A. niger</i>)	29 ± 0.01	7088	2088 ± 28	5000 ± 69
P2 (<i>A. terreus</i>)	27 ± 1		1892 ± 44	5196 ± 142
P2 (<i>A. flavus</i>)	26 ± 2		1832 ± 130	5256 ± 33
P2 (<i>A. fumigatus</i>)	28 ± 1		2003 ± 470	5085 ± 144
Biostimulation-bioaugmentation with isolated fungal strains, inoculation at 45 days				
P3 (<i>A. niger</i>)	49 ± 1	7088	3473 ± 37	3615 ± 134
P3 (<i>A. terreus</i>)	44 ± 1		3150 ± 91	3939 ± 7
P3 (<i>A. flavus</i>)	31 ± 0.4		2232 ± 4	4856 ± 94
P3 (<i>A. fumigatus</i>)	35 ± 1		2490 ± 104	4598 ± 6
P3 Mixed culture	57 ± 2		4040 ± 84	3048 ± 181

microorganisms to a strongly nonpolar environment modifies the permeability of their cell membranes and decreases the concentration of viable microorganisms. As a result, inoculation with fresh fungal cells accelerates the breakdown of the remaining complex hydrocarbons. Regarding the four isolated fungal strains tested in this study and inoculated individually, *Aspergillus niger* MT786339.1 exhibited the greatest degradation of TPH ($49 \pm 1.2\%$), followed by *Aspergillus terreus* MT786341.1 ($44 \pm 0.67\%$), *Aspergillus fumigatus* MT786338.1 ($35 \pm 0.98\%$), and *Aspergillus flavus* MT786340.1 ($32 \pm 0.38\%$); however, the best results were achieved when the mixed fungal consortium (all four fungal strains in equal proportion) was used for inoculation ($57 \pm 1.97\%$). George-Okafor et al. (2009) conducted isolation studies and found that 8 strains showed potential to biodegrade hydrocarbons: *Aspergillus versicolor*, *Aspergillus niger*, *Aspergillus flavus*, *Syncephalastrum* spp., *Trichoderma* spp., *Neurospora sitophila*, *Rhizopus arrhizus*, and *Mucor* spp. Of these eight strains, *A. niger* and *A. versicolor* presented the highest biodegradation efficiency, which agrees with the results obtained in the present work. Adekunle and Adeniyi (2015) studied the biodegradation of petroleum diesel by bioaugmentation with fungi, where *Aspergillus niger* showed a 20.93% removal rate. In the present work, isolated *A. niger* achieved a TPH degradation rate of $49 \pm 1.2\%$, a 2.34-fold increase for the same strain. It is worth mentioning that in a similar study on the removal of diesel by *Aspergillus terreus*, reported by Cisneros-de la Cueva et al. (2016), for an initial diesel concentration of 50,000 mg diesel/kg soil with inoculation at time zero (fast degradation rate), the reaction rate constant was $0.002 \pm 7.07E-05$ 1/day, and the half-life time was 308.2 ± 9.69 days, close to the values found in the present study. However, when inoculation was performed at 45 days, the rate constant showed a 2.66-fold degradation rate, even when the initial concentration of TPH was 1.42 times higher ($70,880 \pm 975$ mg TPH/kg), confirming the influence that bioaugmentation has when inoculation is performed in the slow degradation stage. Other authors have shown that the addition of exogenous microorganisms promotes significant changes in the composition of the soil microbial community (Festa et al. 2016), so better efficiency is accomplished in the bioremediation process (Agnello et al. 2016; Kadali et al. 2012; Li et al. 2012; Tang et al. 2010). On the other hand, it has been demonstrated that the microbial count and type of microorganisms present in the system

influence the concentration and type of hydrocarbons removed, and a strong association exists between TPH chemical composition and microorganisms associated with degradation (López-Miranda et al. 2018; De la Cueva et al. 2016). Fungi, for the most part, can break down some of the largest molecules present in nature (Fernández-Luqueño et al. 2011); therefore, fungi have been shown to degrade hydrocarbons better than bacteria. The overall results in the present study support that isolated fungal strains were able to degrade TPHs at a faster rate, with a better performance, when fresh cells were reinoculated as a consortium at the slow remediation stage; further experiments are suggested to determine the maximum degradation rate in biostimulation-bioaugmentation experiments at a larger scale.

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Declarations

Competing Interests The authors declare no competing interests.

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