



FUNGI ASSOCIATED WITH THE RHIZOSPHERE OF *Rhizophora mangle* AND THEIR RELATIONSHIP WITH THE NATURAL ATTENUATION OF PETROLEUM-CONTAMINATED SOILS †

[HONGOS ASOCIADOS A LA RIZOSFERA DE *Rhizophora mangle* Y SU RELACIÓN CON LA ATENUACIÓN NATURAL DE SUELOS CONTAMINADOS CON PETRÓLEO]

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SUMMARY

Background. Mangrove ecosystems in oil-producing areas in the south of Veracruz, Mexico are permanently threatened by oil spill contamination. In this context, *Rhizophora mangle* and the microbiota associated with its rhizosphere have adapted over time to processes of bioremediation and natural attenuation of petroleum hydrocarbons. **Objective.** To evaluate the hydrocarbonoclastic potential of filamentous fungi isolated from the rhizosphere of *R. mangle* present in a hydrocarbon-contaminated site in Veracruz. **Methodology.** A rhizosphere sample was characterized physicochemically and total petroleum hydrocarbons were quantified. Fungal strains with hydrocarbonoclastic capacity were isolated in Noble agar medium, salts, and paper impregnated with Maya crude oil. The strains were identified by cell morphology and rDNA ITS region sequencing, and the sequences were used to construct a phylogenetic tree using the maximum likelihood method. A liquid culture was developed in a mineral medium added with petroleum for 30 days and the saturated, polar, and aromatic fractions were quantified to determine the percentage of biodegradation. **Results.** Six strains of hydrocarbonoclastic fungi were isolated and identified in *R. mangle* rhizosphere contaminated with 109 916.5 mg kg⁻¹ of TPHs. Of these strains, *Aspergillus niger* and *A. flavus* showed the highest hydrocarbon biodegradation with 30.5 and 26.1%, respectively. The highest biodegradation of the saturated fraction was observed with *A. niger*, *A. flavus*, and *A.egyptiacus*; *Fusarium oxysporum* and *A. niger* preferred the polar fraction, while *A. niger* and *A. flavus* assimilated more of the aromatic fraction. **Implications.** These hydrocarbonoclastic strains may be potentially used in restoration strategies for hydrocarbon-contaminated mangroves. **Conclusion.** The microorganisms associated with contaminated mangroves are part of the natural attenuation of the studied site and may be useful for the treatment of sites affected by petroleum spills. **Key words:** contamination; hydrocarbonoclasts; mangrove; crude oil.

RESUMEN

Antecedentes. Los ecosistemas de manglar presentes en zonas petroleras del sur de Veracruz, México, se encuentran permanentemente amenazados por la contaminación por derrames de hidrocarburos. En este sentido, *Rhizophora mangle* y su microbiota asociada a la rizosfera se han adaptado a través del tiempo a procesos de biorremediación y atenuación natural de hidrocarburos del petróleo. **Objetivo.** Evaluar el potencial hidrocarbonoclasta de hongos filamentosos aislados de la rizosfera de *R. mangle* presentes en un sitio contaminado con hidrocarburos de Veracruz. **Metodología:** Se caracterizó fisicoquímicamente una muestra compuesta de rizosfera y se cuantificaron los hidrocarburos totales del petróleo. Se aislaron cepas fúngicas con capacidad hidrocarbonoclasta en medio agar noble,

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sales y con papel impregnado de petróleo crudo Maya. Las cepas fueron identificadas por morfología celular y por secuenciación de la región ITS del ADNr, se realizó un árbol filogenético con las secuencias por el método de máxima verosimilitud. Se realizó cultivo líquido en medio mineral adicionado con petróleo durante 30 días, se cuantificaron la fracción saturada, polar y de aromáticos determinando los porcentajes de biodegradación. **Resultados:** Se aislaron e identificaron seis cepas de hongos hidrocarbonoclastas a partir de rizosfera de *R. mangle* contaminado con 109 916.5 mg kg⁻¹ de HTP, de las cuales *Aspergillus niger* y *A. flavus* fueron los que presentaron una mayor biodegradación de hidrocarburos con un 30.5 y 26.1%, respectivamente. La mayor biodegradación de la fracción saturada fue con *A. niger*, *A. flavus* y *A. egyptiacus*; *Fusarium oxysporum* y *A. niger* prefirieron mejor la fracción polar; mientras que *A. niger* y *A. flavus* asimilaron más la fracción de aromáticos. **Implicaciones** Estas cepas hidrocarbonoclastas pueden tener un potencial para utilizarse en estrategias de restauración de manglares contaminados con hidrocarburos. **Conclusión** Los microorganismos asociados al manglar contaminado forman parte de la atenuación natural del sitio estudiado y podrían ser útiles en el tratamiento de sitios impactados por derrames de petróleo.

Palabras clave: contaminación; hidrocarbonoclastas; manglar; petróleo crudo.

INTRODUCTION

The coverage of mangrove ecosystems has suffered a decline worldwide mainly due to anthropogenic factors. In Mexico, important mangrove areas are found in the North, Central, and South Pacific, the Yucatán Peninsula, and the Gulf of Mexico (Rodríguez-Zuñiga *et al.*, 2013). In the latter, the littoral zone of the state of Veracruz represents 4.96% of the mangrove-covered surface area of Mexico (Cuevas-Díaz *et al.*, 2020). In the south of Veracruz, mangroves such as *Avicennia germinans*, *Laguncularia racemosa*, *Rhizophora mangle*, and *Conocarpus erectus* have been affected by petroleum contamination, mainly along the Coatzacoalcos River, due to spills caused by accidents in oil wells and storage tanks, as well as poor management, lack of maintenance of pipelines, and criminal activity. Mangrove areas in the Coatzacoalcos River are affected by oil spills because they are sites surrounded by large industrial complexes that belong to Petróleos Mexicanos (PEMEX) and private companies. Petroleum contamination in mangrove ecosystems is a serious environmental problem because there is a constant loss of diversity and ecosystem services are thus affected. There are native microorganisms that participate in the natural restoration of ecosystems, such as bacteria and fungi (Kumar and Gopal, 2015, Koshlaf and Ball, 2017). Some genera of fungi can grow in the presence of hydrocarbons (Chaurasia *et al.*, 2019, Al-Hawash *et al.*, 2018), the hydrocarbonoclastic activity allows these fungi to biotransform different hydrocarbon fractions. Some hydrocarbon biodegradation studies have used basidiomycetes such as *Chrysosporium*, *Phanerochaete*, *Pleurotus ostreatus* and *Trametes versicolor* (Yateem *et al.*, 1998, Mollea *et al.*, 2005). Similarly, filamentous fungi are used to treat petroleum-contaminated soils through remediation processes (Benguenab and Chibani, 2021). This bioremediation potential is mainly due to the characteristics of the enzyme systems and the growth of these fungi, which enables them to develop mycelia,

colonize different types of substrates, and access toxic compounds (D'Annibale *et al.*, 2006). The high cell surface area to cell volume ratio of filamentous fungi makes them efficient degraders in certain niches such as soil contaminated with toxic organic molecules (Martín *et al.*, 2004). In this context, there are reports of fungi such as *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani*, *Mucor racemosum*, *Penicillium notatum* and *Ulocladium atrum* that have been isolated from petroleum-contaminated soil in Saudi Arabia (Hashem, 2007). Furthermore, Mohsenzadeh *et al.* (2010) reported fungal species associated with the roots of *Polygonum aviculare* located in a petroleum-contaminated area in Iran, which included *Alternaria*, *Aspergillus*, *Bipolaris*, *Fusarium*, and *Rhizoctonia*. Considering the above, the objective of the present study was to isolate fungal strains associated with the rhizosphere of *Rhizophora mangle* contaminated with hydrocarbons and determine their hydrocarbonoclastic potential in culture media added with petroleum.

MATERIALS AND METHODS

Study area and sampling

Five 0.5-kg samples of the rhizosphere of *Rhizophora mangle*, known as red mangrove, were collected at a depth of 10 cm in a plot of 100 m² (Yang *et al.*, 2021; Mahmoudi *et al.*, 2022) in the locality of El Polvorín (Figure 1), located in the municipality of Cosoleacaque, Veracruz (18°3'42.42"N, -94.25'7.91"O). This area is affected by contamination from spills of Maya crude oil (API 21.85) caused by constant illegal tapping from Nuevo Teapa to Poza Rica, Veracruz, Mexico. The 0.5-kg soil samples were collected in three sterile containers in a systematized section of *R. mangle* monitored in a zigzag fashion. The samples were then dried and sieved with a mesh of size 2 and kept at 4 °C for subsequent physicochemical and microbiological analyses (Paez and Wilke, 2005).

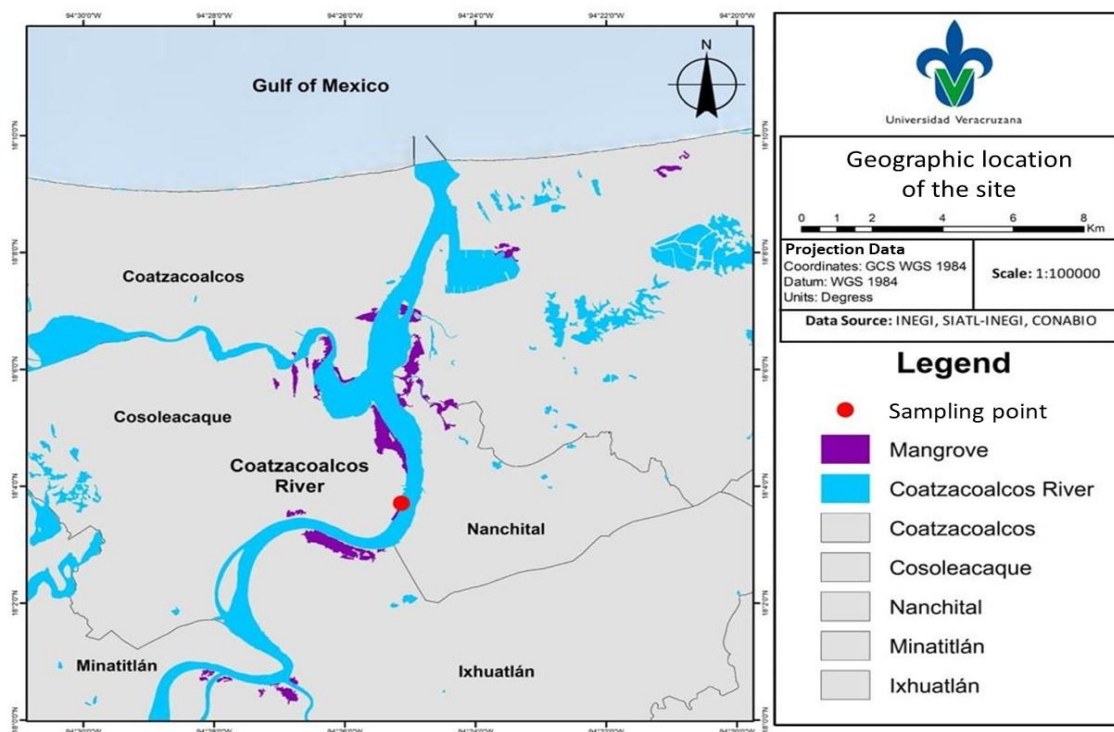


Figure 1. Sampling site where the mangrove affected by hydrocarbon spills is located.

Physicochemical characterization of the soil

A composite sample was made from the samples obtained using the shaking extraction method following the US EPA 3500B and US EPA 3540C (1996) methods and Schwab *et al.* (1999), with some modifications in shaking speed and solvent volume (Arce-Ortega *et al.*, 2004). Extraction was performed with 2 g of sample previously dried at 25 °C for 48 h, which were subsequently added with 3 g of anhydrous Na_2SO_4 and mixed in 15-mL tubes with a vortex mixer. The mix was then added with 5 mL of CH_2Cl_2 , shaken for 45 s, and then centrifuged at 6,000 rpm for 10 min. The supernatant was removed and placed in a round-bottom flask. This process was repeated three times. Finally, the CH_2Cl_2 was evaporated to dryness in a rotary evaporator under reduced pressure. The results are expressed in mg of TPH/kg of dry soil (total petroleum hydrocarbons, mg/kg of d.s.), which was calculated as follows: $\text{TPH} = (\text{RB} - \text{RA}) * (\text{CF}) / (\text{P} * \text{HF})$. Where: RA= weight of the empty container at constant weight (mg); RB= weight of the container with the concentrated organic extract (mg); P= quantity of extracted soil (g); HF= humidity correction factor (1 - (% humidity/100)); CF= correction factor to transform to kg of d.s. = 1000.

Isolation of hydrocarbonoclastic fungal strains

The strains were isolated by the plate dilution method (1×10^{-4}) from 10 g of sample. A 1-mL aliquot from

each dilution was placed on Petri plates, which were subsequently added with mineral agar (g/L): Noble agar, 15; KH_2PO_4 , 0.4; K_2HPO_4 , 1.6; NH_4Cl , 1.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.17; Na_2SO_4 , 0.609, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.045; acidified with lactic acid at a pH of 4.9 and placed on the lid of the Petri plate with paper impregnated with petroleum previously sterilized three times at 121 °C and 15 psi of pressure for one hour. Finally, they were incubated for 7 days at 25 ± 2 °C. The criteria for the selection and purification of the hydrocarbonoclastic fungal strains were their tolerance and capacity to develop in a microenvironment saturated with volatile petroleum hydrocarbons (Fernández *et al.*, 2006). The strains that developed mycelia in the presence of volatile hydrocarbons were then cultured in cellulose agar (g/L): crystalline cellulose, 5; $(\text{NH}_4)_2\text{SO}_4$, 5; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5; rose bengal (0.001); yeast extract (0.025), and bacteriological agar, 20. A 1.5 cm²-piece of filter paper impregnated with Maya crude oil was placed on each Petri plate containing cellulose agar. A hole punch was used to obtain one slice of 1 cm in diameter of each purified fungal strain, which was placed on the filter paper impregnated with Maya crude oil in the cellulose agar medium. Finally, they were incubated at 25 ± 2 °C for 7 days (Rivera *et al.*, 2004).

Identification of hydrocarbonoclastic strains

The fungal strains that showed the capacity to utilize hydrocarbons as a carbon source were identified by

their colony morphology and microscopic characteristics (Barnett and Hunter, 1998), and a molecular analysis was performed to corroborate the identity of the species. This was done by isolating the DNA following Yu *et al.* (2011), with some modifications. The rDNA ITS region was subsequently amplified using the primers ITS1 and ITS4. The amplified products were purified using the Wizard kit (Pro-mega) and nucleotides were sequenced in the Biotechnology Institute of UNAM using a sequencer (Applied Biosystems). Finally, the nucleotide sequences obtained were edited in BioEdit version 7.0.5.3 and compared with those in NCBI's GenBank (<http://www.ncbi.nlm.nih.gov/>) using BLAST to confirm the identity of the species.

Biodegradation capacity of the hydrocarbonoclastic strains

The biodegradation percentage (%B) of each hydrocarbonoclastic fungal strain was obtained following the methodology by Outdot *et al.* (1987). For this, a liquid culture was prepared in a mineral medium (MM) with the following composition (g/L): KCl, 0.25; NaH₂PO₄, 1; MgSO₄, 0.5; NO₃NH₄, 1; added with 0.1 g/L of chloramphenicol and with a pH adjusted to 6.2. We used 250 mL Erlenmeyer flasks with 70 mL of MM, 100 µL of sterile Maya crude oil, and 1x10⁶ conidia/mL. The abiotic control consisted of MM and Maya crude oil. They were subsequently incubated at 25 ± 2 °C for 30 days. After this time, each flask was extracted three times with 30 mL of CH₂Cl₂ in separatory funnels and the extracts were placed in 250-mL round-bottom flasks and subsequently evaporated to dryness in a rotary vacuum evaporator to continue monitoring each round-bottom flask at constant weight. All the assays were performed in triplicate. The biodegradation percentage (%B) of each strain was determined according to the following formula:

$$\%B = 100 (MC - M) / MC$$

Where: MC= extract mass in the control; M= mass in the culture. The extract obtained from the determination of the biodegradation (%B) of each fungal strain was used to determine the percentages of the saturated, aromatic, and polar hydrocarbon fractions by column chromatography using 100 mesh silica gel as the stationary phase and successive elutions of 60 mL of hexane, benzene, and methanol, respectively, as the mobile phase in chromatographic columns of 300 x 15 mm (Chafineau *et al.*, 1995).

RESULTS AND DISCUSSION

The physicochemical parameters of the composite rhizosphere sample are shown in Table 1. The soil was characterized as a silt loam soil with good water retention capacity, medium natural fertility, and a mildly acidic to neutral pH. It contained 109 916 mg kg⁻¹ of TPHs, which exceeds the maximum permissible limit (MPL) established by the standard NOM-138-SEMARNAT/SS-2012, which mentions that industrial soil can contain up to 6 000 mg kg⁻¹ of heavy fractions and 5 000 mg kg⁻¹ of medium fractions.

Table 1. Physicochemical characteristics of the composite sample of *R. mangle* rhizosphere.

Parameter	Results
pH	7.5
Humidity (%)	16.2
Organic matter (%)	6.7
Organic carbon (%)	3.9
Organic N (%) + Nitrates (%)	0.3303
Phosphorus (%)	1x10 ⁻⁴
C:N:P ratio	4:0.3:0
Texture (%)	Sand: 22.4 Clay: 5.9 Silt: 71.6
TPHs (mg kg ⁻¹)	109 916

Mangroves are known to show high productivity due to their abundant organic detritus, which allows the accumulation of hydrocarbons (Olguín *et al.*, 2007). According to Dragun and Barkack (2000), the recommended pH range for aerobic hydrocarbon degradation in soil is between 5 and 9, with an optimal value of 7. The content of organic nitrogen is below the required value for the composition of microbial cells, which is 14% according to Vidali (2001); however, hydrocarbon bioremediation processes show better results with C:N ratios of 100:10 to up to 100:1 (Van Hamme *et al.*, 2003). The C/N ratio (3.9/0.3) observed in our soil was 11.8, which is consistent with the C/N ratio of 5-17 required for hydrocarbon biodegradation in soil by microscopic fungi (Sternier and Elser, 2002).

Five morphologically different strains of microscopic fungi were isolated and purified, which were able to develop in and tolerate a microenvironment saturated with volatile petroleum hydrocarbons, as well as to reproduce while in direct contact with Maya crude oil (Figure 2). The hydrocarbonoclastic strains were characterized based on their colony morphology and microscopic characteristics, where four of them (H3S2, H4S1, H4S5, and H3S3) showed similarity to the genus *Aspergillus*.

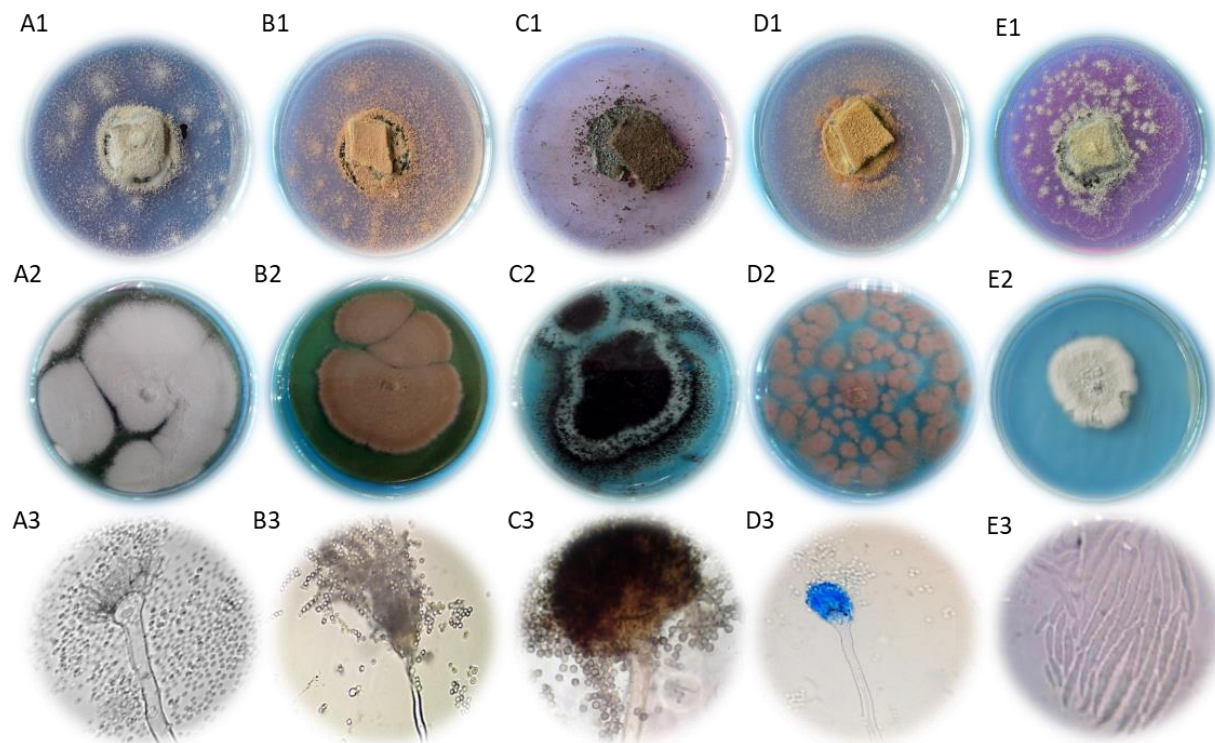


Figure 2. Strains of microscopic fungi with the capacity to tolerate and reproduce in the presence of total petroleum hydrocarbons. A1-A3 (H3S2, *Aspergillus flavus*), B1-B3 (H4S1, *Aspergillus terreus*), C1-C3 (H4S5, *Aspergillus niger*), D1-D3 (H3S3, *Aspergillus egyptiacus*), and E1-E3 (H3S6, *Fusarium oxysporum*).

The species *A. flavus* showed radial to columnar conidial heads, a generally hyaline conidiophore, and metulae that cover three fourths of the vesicle. *Aspergillus terreus* shows light brown conidial heads that form compact columns, spherical or pyriform vesicles, and globose or ellipsoidal conidia. *Aspergillus niger* is characterized by radiate conidial heads, conidiophores with a thick wall and a brown apex, spherical vesicles, metulae that cover the whole surface of the vesicle, and phialides with generally rough globose conidia. *Aspergillus egyptiacus* shows abundant conidial structures and conidiophores that are mostly not arranged in a typical *Aspergillus* head but in solitary phialides or small groups. Finally, the strain H3S6 is consistent with the description of *Fusarium oxysporum*, which has crescent-shaped, hyaline, septate macroconidia, as well as oval microconidia (Barnett and Hunter, 1998; Geiser *et al.*, 2007).

The morphological identification of the fungal isolates with hydrocarbonoclastic capacity was corroborated by sequencing the rDNA ITS region, whereby the amplification products of each fungal isolate were obtained at 600 bp. The following results were obtained from the nucleotide sequence analysis and their comparison in GenBank: the isolate H4S5 showed 100% similarity with and was grouped into the clade of *Aspergillus niger* Tiegh, the isolate H3S3 showed 99% similarity with and was grouped into the

clade of *Aspergillus egyptiacus* Moub. & Moustafa, and the isolate H3S6 showed 100% similarity with and was grouped into the clade of *Fusarium oxysporum* Schldl. The isolates H3S2 and H4S1 showed 99% similarity with *Aspergillus flavus* Link and *Aspergillus terreus* Thom, but the results of the cladogram were inconclusive and thus it was not possible to group them with these species (Figure 3).

These results agree with what has been reported by different authors, where the genera of hydrocarbonoclastic filamentous fungi that predominate in these sites are *Aspergillus*, *Penicillium*, and *Fusarium*. Strains of *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Emericella nidulans*, *Fusarium solani*, *Penicillium funiculosum*, *Rhizopus stolonifer*, and *Trichoderma harzianum* have been isolated from kerosene- and benzene-contaminated soil in Egypt (Hemida *et al.*, 1993). Colombo *et al.* (1996) isolated *Aspergillus terreus* and *Fusarium solani* from hydrocarbon-contaminated soil in Argentina and April *et al.* (2000) isolated 64 species of filamentous fungi from soil contaminated with crude oil in Canada, where only 6 genera showed the capacity to degrade hydrocarbons. Furthermore, Hashem (2007) reported the isolation of *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani*, *Mucor racemosus*, *Penicillium notatum*, and *Ulocladium atrum* from petroleum-

contaminated soil in Saudi Arabia. Similarly, Mohsenzadeh *et al.* (2010) reported fungal species associated with the roots of *Polygonum aviculare* in a petroleum-contaminated area in Iran, which included *Alternaria*, *Aspergillus*, *Bipolaris*, *Fusarium*, and *Rhizoctonia*. There is also a review of the isolation of fungi such as *Penicillium*, *Aspergillus*, *Fusarium*, and *Rhizopus* from hydrocarbon-contaminated soil and water (Pernía *et al.*, 2012). However, even though many fungal species have been isolated from this type of site, not all can degrade hydrocarbons.

The total petroleum hydrocarbon biodegradation observed is shown in Figure 4, where the strain H4S5 of *A. niger* showed the highest biodegradation percentage (30.5 %).

Once the biodegradation percentage of each fungal isolate was determined, the TPH composition (saturated, aromatic, and polar) was estimated to evaluate the capacity to biodegrade each TPH fraction (Figure 5). The results showed that the saturated fraction had the highest biodegradation percentages by all the fungal strains studied, followed by the polar fraction, and, to a lesser degree, the aromatic fraction. The strains that showed the highest consumption of the saturated fraction were *A. niger*, *A. flavus*, and *A. egyptiacus*, while *F. oxysporum* and *A. niger* consumed a higher percentage of the polar fraction than the other strains, and *A. niger* and *A. flavus* consumed a higher percentage of the aromatic fraction.

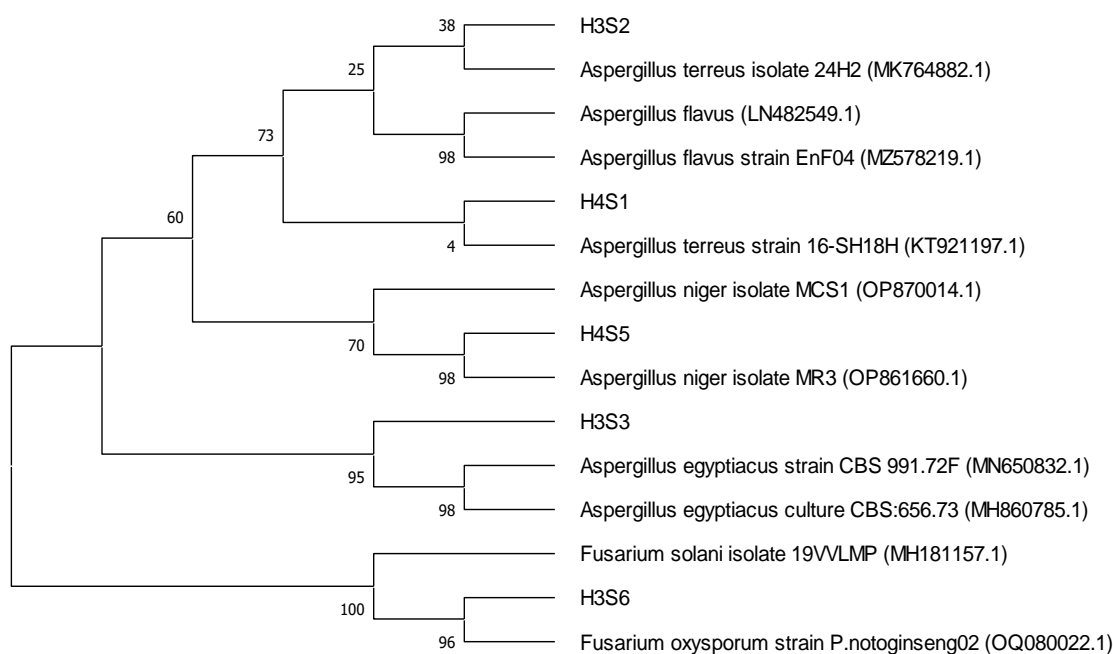


Figure 3. Maximum likelihood phylogenetic tree based on ITS sequences of hydrocarbonoclastic fungi from the rhizosphere of *R. mangle*. Numbers in the nodes are support values using 1000 replicates.

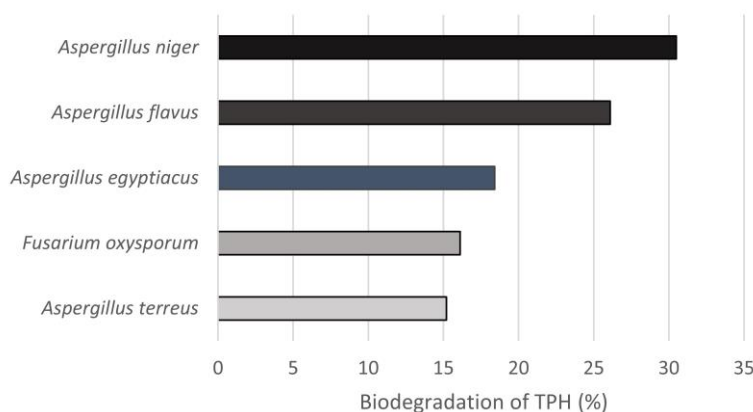


Figure 4. Total petroleum hydrocarbon (TPH) biodegradation percentage by the hydrocarbonoclastic fungi studied.

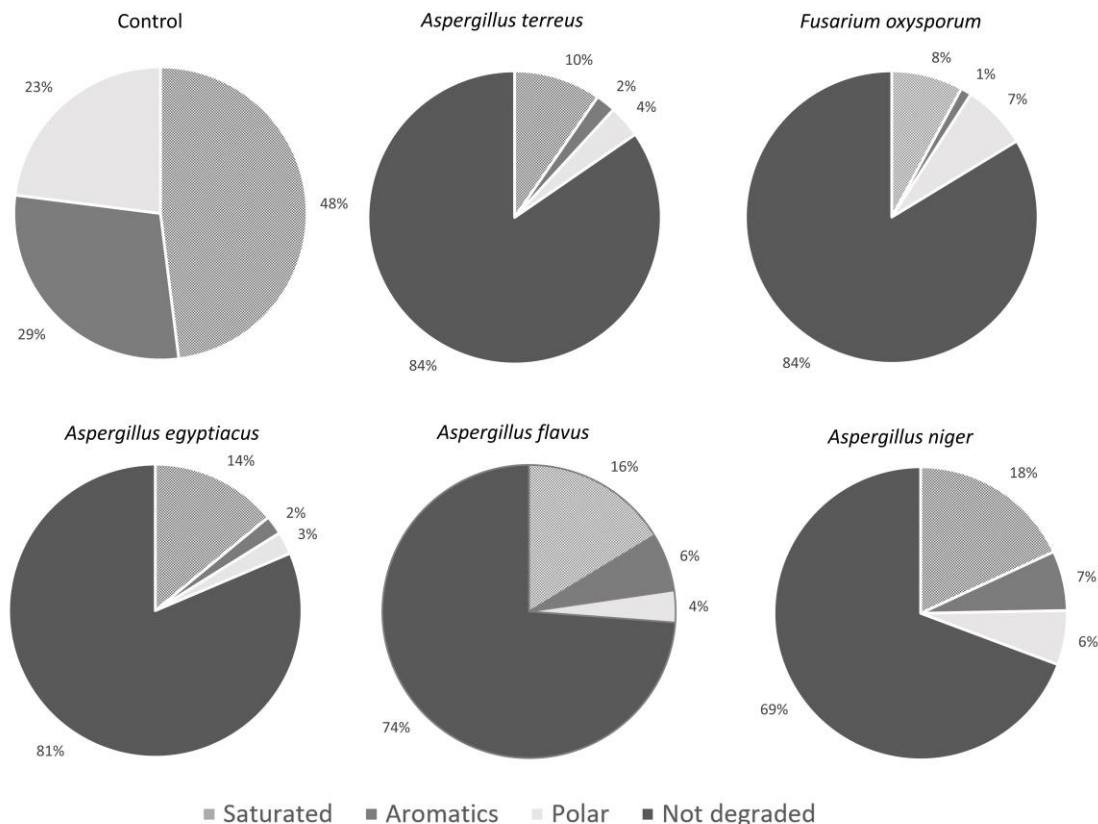


Figure 5. Consumption percentages of the saturated, aromatic, and polar TPH fractions by all the hydrocarbonoclastic fungal strains compared to the abiotic control.

The genera *Penicillium*, *Aspergillus*, and *Fusarium* are distinguished by their hydrocarbonoclastic capacity (Naranjo *et al.* 2007; Chaîneau *et al.*, 1999; Pernía *et al.*, 2012). The genus *Aspergillus* has been particularly widely described because of its capacity to metabolize TPHs (Oudot *et al.*, 1993; Chaîneau *et al.*, 1999; Benguenab and Chibani, 2021). The TPH biodegradation capacity of these strains showed values from 15.2 to 30.5%, which agrees with the results reported by Chaîneau *et al.* (1995) for strains of the genera *Penicillium*, *Aspergillus*, and *Fusarium*. The genus *Aspergillus* has been reported to grow rapidly in crude oil and, in some cases, can form very dense hyphal networks (April *et al.*, 2000). Moreover, Oudot *et al.* (1993) reported that this genus can degrade between 30 and 35% of saturated and aromatic hydrocarbons and 13% of resins and asphaltenes, which are constituents of crude oil. Hernández-Acosta *et al.* (2003) isolated *Trichoderma* sp., *Aspergillus* sp., and *Mucor* sp. from the rhizosphere of *Chamaecrista nictitans* and *Panicum* sp. from soils in Minatitlán, Veracruz. These microorganisms were able to degrade crude oil in contaminated soil and the rhizosphere of plants such as maize and beans when inoculated in soil contaminated with crude oil.

Saturated hydrocarbons showed the highest biodegradation percentages, which agrees with that observed by Chaîneau *et al.* (1999), who reported a higher degradation of this hydrocarbon fraction and demonstrated that the linear chains and alkane range were partially degraded by *Aspergillus niger*, *Penicillium resctrictum*, *Brevandimonas vesicularis*, and *Trichoderma harzianum*. The filamentous fungi identified in the present study showed an adaptive capacity to develop and metabolize TPHs as a carbon and energy source in plates with cellulose agar and petroleum, where mycelial growth was observed from the third day of incubation.

CONCLUSIONS

The native fungal isolates associated with the rhizosphere of *Rhizophora mangle* with the highest potential belong to the genera *Aspergillus* and *Fusarium*, since they were able to utilize hydrocarbons as a sole source of carbon and energy after 30 days in a liquid culture in mineral medium. These identified strains may be used in bioremediation or natural attenuation processes in contaminated sites, allowing the restoration of mangroves, which are of great

importance due to the ecosystem services they provide to the communities that live in this type of ecosystem.

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Conflict of interest. The authors declare that they have no conflicts of interest.

Compliance with ethical standards. Does not apply.

Data availability. Data are available upon request to the corresponding author (cespinoza@uv.mx).

Author contribution statement (CRediT). **O. Guzmán-López**-Conceptualization, Methodology, Visualization, and Writing - original draft, **A. Salinas-Castro**-Validation, Writing - original draft, Writing - review and editing, **G. Mendoza**-Validation, Writing, **A. Couttolenc**- Writing - original draft, Formal analysis, Visualization, **M.C. Cuevas-Díaz**- Formal Analysis, Validation, Writing - original draft, **C. Espinoza**-Project administration, Conceptualization, Formal Analysis, Validation, Writing - original draft.

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