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Bioremediation alternatives for total petroleum hydrocarbon removal in agricultural soil

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ABSTRACT

Objective: The purpose of the present study is to highlight the importance of assessing bioremediation and total petroleum hydrocarbon removal by bioaugmentation and biostimulation on the rhizosphere.

Design/methodology/approach: An 89-day experiment was established with treatments considering plant (corn) establishment crude petroleum (25,000 mg kg^{-1}) bacteria and hydrocarbonoclastic fungi adding nitrogen and phosphorus to agricultural soil. At the end of the experiment, hydrocarbonoclastic fungal and bacterial populations and total petroleum hydrocarbon removal were assessed.

Results: Both microbial groups increased in number and time. The treatment with 120 kg nitrogen ha^{-1} and 12.5 kg phosphorus ha^{-1} allowed the highest population (227×10^3 g^{-1} of colony forming units (CFU) of hydrocarbonoclastic bacteria). A total of 83% petroleum hydrocarbon removal was obtained as established in 89 days.

Limitations on study/implications: The effectiveness of bioremediation can vary significantly in real environments due to factors, such as soil variability, climate.

Findings/conclusions: The previous results highlight the importance of using these bioremediation techniques to eliminate hydrocarbons in contaminated agricultural soils.

Keywords: Environmental impact, contaminated soils, biodegradation, microorganisms, nutrients.

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INTRODUCTION

At global level, the growth of the petroleum industry involves a constant increase in activities, such as exploration, exploitation, refinery, transport, and consumption of the products derived from petroleum (Meyer, 2023; Ortuño, 2021). However, petroleum products generate environmental impact, such as soil, water, and air global warming, causing serious public health besides economic and social problems (Martins *et al.*, 2019).

In many affected sites, the population and natural resources are exposed to continuous risks (Okonofua *et al.*, 2023; Ukhurebor *et al.*, 2021). Agricultural soils contaminated by hydrocarbons show problems in quality and cultivation production, which are due to the loss of beneficial microflora, as well as to the decrease of air and water availability and contaminant toxicity among other factors (Devi *et al.*, 2022).

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The alternatives proposed for cleaning the contaminated sites are bioremediation technologies, which by means of the microbial activity convert the contaminants into innocuous compounds, such as carbon dioxide and water Bioremediation implies soil and water decontamination by the interaction of microorganism-soil/water-contaminant (Bhat & Hakeem, 2020; Singh *et al.*, 2020). The biodegradation speed of diverse soil hydrocarbons depends on factors, such as texture, structure, temperature, pH, oxide-reduction potential, oxygen, nutrients, organic matter, salinity, water content, quantity and bioavailability of the contaminants (Cambarieri *et al.*, 2021).

When agricultural hydrocarbon contaminated soils contain few nutrients, the biodegradation processes are significantly reduced because the plants require such elements for their development, above all for their radical system growth, the habitat where microorganisms transform the contaminants (Panwar, R., & Mathur, 2023). Therefore, incorporating essential nutrients in soil is necessary (Martínez *et al.*, 2019). Given the crucial importance of these topics in the framework of the present research, two bioremediation alternatives (bioaugmentation and biostimulation) were carried out to determine the total petroleum hydrocarbon (TPH) removal in agricultural soil with the future goal of restoring their functions in the ecosystem.

It is hypothesized that bioaugmentation and biostimulation will be effective in removing total petroleum hydrocarbons in an agricultural soil and that both alternatives will lead to a significant improvement in soil remediation.

MATERIALS AND METHODS

For the experiment, Mayan crude petroleum was used provided by the Mexican Petroleum Institute (IMP, Instituto Mexicano del Petróleo), characterized as heavy (21° to 22° API, American Petroleum Institute, U.S.A.), sour and with sulfur content (3.4-3.8% in weight) according to the scheme in accordance with the security data page (PEMEX, 2023) and the Mexican regulation NOM-018-STPS-2015. Petroleum is viscous liquid, black, water insoluble, with a boiling point from 71 to 538 °C, flammable, vapor pressure 6.0 lb/plg^2 and a relative density of 0.9269 15.5°C.

Preparation of fungal and hydrocarbonoclastic bacterial inoculum

Two types of inoculums were prepared: one composed of hydrocarbonoclastic bacteria (HC) *Agrobacterium radiobacter* and *Pseudomonas* sp. and the other one by fungus genera *Trichoderma* sp., *Aspergillus* sp. and *Mucor* sp. Both inocula were cultivated in six-250 mL assay flasks of which three of them contained 50 mL of combined Rennie (1981) mineral carbon culture medium, modified by Hernández-Acosta *et al.*, (2003) for HC bacterial growth. The other three flasks used Eggins and Punhg (Leander and Curl, 1972) for fungi. Besides the culture media, 2 mL of petroleum were added to each flask, so the microorganisms used them as a carbon source.

For the HC bacteria, an incubation time of 22 h was considered and eight days for fungi. Subsequently, the culture medium in the flasks were collected to be used as inoculum in the experiment under greenhouse conditions. The inoculum from fungi and bacteria were quantified in colony forming units (CFU) mL^{-1}.

Establishment of the greenhouse experiment

The sterile agricultural soil used previously determined the following properties: texture, organic material, pH, total nitrogen (N), phosphorus (P), potassium (K), and the cationic exchange by the methods established in the Mexican norm NOM-021-SEMARNAT-2000 (Semarnat, 2002). Petroleum (25,000 mg kg^{-1}) was homogenized previously to be transferred into 940-mL amber glass flasks. Each flask had a quantity of 700 g of crude soil petroleum.

On soil petroleum was used as a reactive degree source of nitrogen to ammonium sulfate [$(NH_4)_2SO_4$] in dosage of 0, 120, 240, and 360 kg N ha^{-1}. The criterium for using these doses was due to the result of the fertility analysis indicating poor N content. Thus, a low dosage was considered and a higher one than that recommended since the plant has the disadvantage of developing adequately due to the contaminated soil. Creole corn (MV06) was used as plant indicator. With respect to P, only one dosage (12.5 kg of P ha^{-1}) of reactive degree monobasic potassium phosphate (KH_2PO_4) was used to cover a minimum demand since corn cultivation requires reduced applications of this nutrient.

Subsequently, two corn seeds were placed at the center of each flask; 5 mL of the HC bacterial inoculum were applied with a charge of 5.0×10^{10} CFU mL^{-1} and 4.0×10^{8} CFU mL^{-1} were used for the HC fungal inoculum. The experimental units within the greenhouse were maintained at 50% humidity with distilled water, the first soil sampling was performed at 65 days, the second one at 79 days, and the last one at 89 days. Sampling intervals were considered to observe significant changes in the soil and soil plant interaction during the growth cycle of corn, allowing for the evaluation of bioremediation techniques.

The experiment was established under a complete randomized design with a factorial arrangement of 4×3 with 4 replicates where the factors were the treatments and incubation days: T1: Plant+25,000 mg kg^{-1} of petroleum+HC bacteria+HC fungi+0 kg N ha^{-1}+12.5 kg P ha^{-1}; T2. Plant+25,000 mg kg^{-1} of petroleum+HC bacteria+HC fungi+120 kg N ha^{-1}+12.5 kg P ha^{-1}; T3: Plant+25,000 mg kg^{-1} of petroleum+HC bacteria+HC fungi+240 kg N ha^{-1}+12.5 kg P ha^{-1}; T4: Plant+25,000 mg kg^{-1} of petroleum+HC bacteria+HC fungi+360 kg N ha^{-1}+12.5 kg P ha^{-1}.

In each sampling, the CFU of the fungal and bacterial HC were determined according to Hernández-Acosta *et al.* (2003), as well as the TPH elimination percentage according to the United States Environmental Protection Agency (EPA) 418.1 method and quantified by infrared spectrophotometry modified by EPA (1986) method.

Data analysis

Normality tests (Shapiro Wilks) and homogeneity of variance (Levene's test) were applied to the data obtained. Once the assumptions were met ($p\geq0,05$), an analysis of variance and multiple comparison of means (ANOVA) was performed by Tukey's method ($p\leq0.05$). To find significant differences among the treatments, a statistical model was used:

$$Y_{ijk}=\mu+T_i+Dj+T_iD_j+\varepsilon_{ijk}$$

Where: Y_{ijk}=obtained response; μ=general media; T_i=treatment i effect; D_j=effect by days; D_jT_i=interaction; ε_{ijk}=observation associated randomized error Y_{ijk}. All the previous was made using InfoStat statistical software, free version (Di Rienzo *et al.*, 2017).

RESULTS AND DISCUSSION

The chemical and physical analyses performed on the soil used in the experiment and its comparison with the reference values established in NOM-021-SEMARNAT-2000 showed the following results: sandy loam texture, moderately alkaline pH (7.8), extremely poor organic matter percentage (0.459), poor total N (0.040%) content, high phosphorus concentration (11.04 mg kg^{-1}), low potassium concentration (0.340 Cmol (+) kg^{-1}) and cationic exchange capacity (9.15 Cmol (+) kg^{-1}).

After performing the statistical analyses, significant differences ($p<0.05$) were observed per treatment, days and interaction (Table 1). By analyzing the results per treatments, T1 did not show significant ($p>0.05$) differences in the bacterial CFU per day. The HC bacterial CFU were significantly greater ($p<0.05$) in soil with T2 (120 kg N ha^{-1} and 12.5 kg P ha^{-1}) at 89 days, which also occurred with the fungal CFU. T3 and T4 showed greater bacterial population at 89 days, but not in fungi whose greater populations showed at 79 days.

Table 1. Hydrocarbonoclastic populations and total petroleum hydrocarbon removal at 65, 79, and 89 days.

Treatments	Days	Bacteria HC 1×10^3 CFU g^{-1} soil	Fungi HC 1×10^5 CFU g^{-1} soil	Concentration/Elimination TPH	
				(mg kg^{-1})	(%)*
T1	65	155 a	14 a	15,470 a	38
	79	127 a	5 c	13,875 b	44
	89	147 a	13 b	11,239 c	55
T2	65	93 b	9 b	12,638 a	49
	79	85 c	7 c	12,417 b	50
	89	227 a	14 a	10,442 c	58
T3	65	156 b	18 b	14,625 a	41
	79	84 c	9 c	12,667 b	49
	89	188 a	24 a	5,756 c	77
T4	65	72 c	6 c	9,650 b	61
	79	80 b	35 a	11,833 a	53
	89	104 a	17 b	4,334 c	83
Factors					
Treatments		0.0001	0.0001	0.0001	
Days		0.0001	0.0001	0.0001	
Treatment×Days		0.0001	0.0001	0.0001	

Different letters in the column per treatment indicate that a statistically significant difference exists. Tukey's test for multiple comparison of means ($p<0.05$). * Percentage with respect to the initial petroleum concentration. TPH: total petroleum hydrocarbons, HC: Hydrocarboboclast.

Hydrocarbon degrading microorganisms have different capacities in the contaminated ecosystems to reduce the potential of the dangerous chemicals released in the environment (Domínguez-Sánchez *et al.*, 2018). In general, the bacterial and fungal HC populations in soil increase significantly in time. As observed, this microbial behavior is defined as adaptation or acclimation when the microorganisms adapt to the contaminated medium characteristics, which could trigger their populations, and species selection that degrade the contaminants to which they are exposed, besides adding their microbial composition and physiological capacity to achieve success in bioremediation (Brzeszcz *et al.*, 2023; Atlas & Bartha, 2002).

It should be noted that the treatments where inorganic sources were applied and the corn plant was present, greater hydrocarbonoclastic bacterial and fungal populations were observed when compared to the treatment without fertilizer.

In this respect, Rivera *et al.* (2018) mentioned that in biostimulation in hydrocarbon contaminated soils, nutrient application allows increasing the native microbial activity and new cells. In respect to the plant presence, Shah y Daverey (2020), contaminant degradation on soil suggest increases in the rhizosphere area due to the existing synergism between the microorganisms and the area (Hernández-Valencia *et al.*, 2017). In the present research, the rhizosphere biostimulation allowed the microbial populations to act with greater efficiency in degrading petroleum.

The TPH removal was greater in T4 (360 kg N ha^{-1} and 12.5 kg P ha^{-1}) at 89 days, observing that as the N dosage increased and time passed by, the hydrocarbon concentration decreased (Table 1). The previous result confirmed that the corn rhizosphere biostimulation increases HC bacterial and fungal populations. In this respect, Rodríguez-Gonzales *et al.* (2022) mentioned that applying nutrients as N and P in a contaminated site is important to favor microorganism growth, which are in charge of biodegrading and help in plant growth and development.

On the other hand, Hernández-Valencia *et al.* (2019) investigated biostimulation with nitrogen, phosphorus, detergent, microorganisms and worm compost to mineralize automotive waste oil in soil. After 130 days, the soil had 1,532 mg kg^{-1} of the contaminant, with 98% removal. In an agricultural soil contaminated with motor oil, Juárez-Cisneros *et al.* (2023) applied biostimulation with fungal extract and green manure, reducing the hydrocarbon concentration from 34,500 to 2,066 ppm in 60 days. Subsequently, by phytoremediation, they achieved a final concentration of 86.9 ppm in 120 days.

In contrast to previous work, this research focused on the use of nitrogen and phosphorus as the primary nutrients in an 89 days experiment. Despite these more simplified conditions, a contaminant removal rate of 83% was achieved, which represented considerable savings in time and money.

This study reveals important benefits for the soil, thus contributing to its restoration and health. The high percentage of TPH removal suggests that the addition of hydrocarbonoclast microorganisms, which use hydrocarbons as a carbon source to break down oil molecules, together with the incorporation of nutrients as an energy source, can promote more efficient degradation of hydrocarbons. This strategy, supported by previous

studies such as Atlas & Bartha (2002) suggests that microbial biodegradation can be an effective tool to mitigate soil contamination.

CONCLUSIONS

The combined application of bioaugmentation and biostimulation on the corn rhizosphere was efficient in agricultural soil remediation contaminated by removing up to 83% of total petroleum hydrocarbon in a period of 89 days. With these bioremediation alternatives, some soil functions within the ecosystem, such as the increase in microbial populations, soil fertility improvement has a bearing in plant growth development, as Poaceae or Gramineae.

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