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Biorremediación de suelos contaminados con hidrocarburos mediante microorganismos nativos

Bioremediation of soils contaminated with hydrocarbons through native microorganisms

Biorremediação de solos contaminados com hidrocarbonetos através de microrganismos nativos

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Resumen

El presente trabajo de investigación estuvo dirigido al análisis de microorganismos presentes en los suelos que han sufrido derrames de petróleo en las cercanías de las estaciones productoras de petróleo, ubicadas en la zona de Dayuma, provincia de Orellana en el campo petrolero Auca, con el objetivo de evaluar la capacidad de remediación de suelos de dichos organismos unicelulares, que han sido expuestos a hidrocarburos, debido a accidentes operacionales en la zona; el propósito de este estudio fue verificar la presencia y el desempeño de la microbiota resistente a los carburos, observando su desempeño de remediación con estímulos naturales de atenuación, tales como; métodos redox y adición de glucosa para comparar el potencial de los microorganismos en la remoción de sus contaminantes en el suelo. Se realizaron técnicas de aislamiento de microorganismos cultivables, mediante el uso de medios de cultivo específicos luego de realizar diluciones seriadas hasta 10-5. Posteriormente, se clasificaron los grupos microbianos asociados, a través de diferentes métodos, como morfológicos, tinción de Gram y pruebas bioquímicas, con el fin de identificar el género bacteriano de los microorganismos. Además, se implementó un diseño de bloques al azar (DBA) con tres tratamientos TO (Suelo + Hidrocarburo + agua), T1 (Suelo + Hidrocarburo + agua + aireación) y T2 (suelo + hidrocarburo + agua + melaza). En el análisis estadístico de los resultados se utilizó un ANOVA con una prueba de Tukey al 5%. Obteniendo como resultados, la presencia de bacterias pertenecientes a la familia Enterobacteriaceae, siendo el género predominante Serratia Sp con un porcentaje de 39%. Otros géneros identificados fueron Shigella Sp con un 17%, Hafnia Sp con un 11%, Yersina Sp con un 11%, Enterocolitica Sp con un 6%, Citrobacter Sp Freundi Sp con un 6%, Proteus Sp con un 6%, Klebsiella Sp con un 6% y Enterobacter Sp con un 6%. Finalmente, se observó que de los tres tratamientos aplicados se logró degradar los hidrocarburos presentes en el suelo contaminado, siendo el tratamiento 3 el más efectivo con una tasa de degradación de hidrocarburos totales (TPHs) de 7484 mg/kg. Concluyendo que, si bien existen microgramos capaces de resistir los efectos de los hidrocarburos presentes en el suelo de la parroquia Dayuma, también son capaces de degradar en un 77% en suelos contaminados por hidrocarburos.

Palabras Clave: Diluciones; Medios de cultivo; Colonias bacterianas; TPHs; Metabolismo.

Abstract

This research was directed to the microorganisms analysis present in the soils, what have suffered oil spills near the petroleum production stations, located at the Dayuma´s area, Orellana province in the Auca oil field with the aim by assessing the soils remediation capacity of said unicellular organisms, which have been exposed to hydrocarbons, due to operational accidents in the area; the purpose this study was to verify the carbide resistant microbiota presence and performance, looking at its remediation performance with natural attenuation stimuli, such as; redox methods and glucosa addition to compare the microorganisms potential their contaminants removal in the soil. It was carried out cultivable microorganisms isolation techniques, through specific culture media use after making serial dilutions up 10-5. Subsequently, they were classified the associated microbial groups, through different methods, such as morphological, Gram staining and biochemical tests, the purpose was to identify the microorganisms bacterial genus. Further, it was implemented a randomized block design (DBA) with three treatments TO (Soil + Hydrocarbon + water), T1 (Soil + hydrocarbon + water + aeration) and T2 (soil + hydrocarbon + water + molasses). In the results statistical analysis, it was used an ANOVA with a 5% Tukey test. Getting as results, the bacteria presence belonging to the Enterobacteriaceae family, by being the predominant genus Serratia Sp with a 39% percentage. Other identified genera included Shigella Sp at 17%, Hafnia Sp at 11%, Yersina Sp at 11%, Enterocolitica Sp at 6%, Citrobacter Sp Freundi Sp at 6%, Proteus Sp at 6%, Klebsiella Sp with 6% and Enterobacter Sp with 6%. Finally, it was observed, which applied two from three treatments achieved to degrade the hydrocarbons present in the contaminated soil, with the treatment 3 was the most effective with a total hydrocarbons (TPHs) 7484 mg/kg degradation rate. Concluding that, if there are micrograms capable by resisting the hydrocarbons effects present in the soil from Dayuma parish, they also are capable by degrading 77% in soils contaminated by hydrocarbons.

Keywords: Dilutions; Culture media; Bacterial colonies; TPHs; Metabolism.

Resumo

Esta pesquisa foi direcionada para a análise dos microrganismos presentes nos solos, que sofreram derrames de petróleo junto às estações de produção de petróleo, localizadas na zona de Dayuma, província de Orellana, no campo petrolífero de Auca, com o objetivo de avaliar a capacidade de

remediação dos solos dos referidos organismos unicelulares, que foram expostos a hidrocarbonetos, devido a acidentes operacionais na área; o objetivo deste estudo foi verificar a presença e o desempenho da microbiota resistente ao carboneto, observando o seu desempenho na remediação com estímulos de atenuação natural, tais como; métodos redox e adição de glicose para comparar o potencial de remoção de contaminantes dos microrganismos no solo. Foram realizadas técnicas de isolamento de microrganismos cultiváveis, através da utilização de meios de cultura específicos após diluições seriadas até 10-5. Posteriormente, foram classificados os grupos microbianos associados, através de diferentes métodos, como morfológicos, coloração de Gram e testes bioquímicos, o objetivo foi identificar o género bacteriano dos microrganismos. Além disso, foi implementado um delineamento em blocos casualizados (DBA) com três tratamentos TO (Solo + Hidrocarboneto + água), T1 (Solo + hidrocarboneto + água + aeração) e T2 (solo + hidrocarboneto + água + melaço). Na análise estatística dos resultados foi utilizada a ANOVA com teste de Tukey a 5%. Obtendo como resultados a presença de bactérias pertencentes à família Enterobacteriaceae, sendo o género predominante Serratia Sp com uma percentagem de 39%. Outros géneros identificados incluíram Shigella Sp com 17%, Hafnia Sp com 11%, Yersina Sp com 11%, Enterocolitica Sp com 6%, Citrobacter Sp Freundi Sp com 6%, Proteus Sp com 6%, Klebsiella Sp com 6% e Enterobacter Sp . Por fim, observou-se que aplicou dois dos três tratamentos conseguidos para degradar os hidrocarbonetos presentes no solo contaminado, sendo que o tratamento 3 foi o mais eficaz com uma taxa de degradação de hidrocarbonetos totais (TPHs) de 7484 mg/kg. Concluindo que, se existem microgramas capazes de resistir aos efeitos dos hidrocarbonetos presentes no solo da freguesia de Dayuma, também são capazes de degradar 77% em solos contaminados por hidrocarbonetos.

Palavras-chave: Diluições; Meios de cultura; Colónias bacterianas; TPHs; Metabolismo.

Introduction

Soil contamination with hydrocarbons is a major environmental problem that affects numerous ecosystems worldwide (1). These toxic compounds, derived from industrial activities and accidental spills, pose a serious threat to soil quality and, consequently, to human health and biodiversity (2). Considering this issue, bioremediation has emerged as a promising environmental restoration strategy, utilizing native microorganisms that can naturally degrade and eliminate petroleum contaminants from the soil (3).

This study focuses on the bioremediation of hydrocarbon-contaminated soils through native microorganisms at the laboratory level (5). The central objective of this research is to evaluate the remediation capacity of soil that has been previously treated for hydrocarbon contamination, through the application of physical and microbiological treatments, in order to determine their effectiveness in restoring soil properties and health (6).

This investigation aimed to contribute to the advancement of bioremediation technology and the understanding of soil recovery processes affected by hydrocarbons, providing crucial information for environmental management and the protection of our surroundings (7). Throughout this study, we will explore the microbiological strategies employed in the laboratory to rehabilitate contaminated soils, as well as their viability in the effective remediation of these degraded ecosystems (8).

Methodology

Soil Sampling

To collect representative samples of contaminated soil, the systematic random method was used, ensuring an impartial and uniform selection of sampling points (9). A 10 m² plot was established, and sampling points were randomly distributed within this area. Then, a 30 cm deep excavation was carried out at each selected point (10).

For soil samples were collected, each weighing 5 kg, and placed in polyethylene bags with a pore diameter of 0.8 µm to prevent cross-contamination. Subsequently, they were mixed homogeneously to obtain four subsamples of 1 kg each (11). These subsamples were placed in properly labeled Ziploc bags with detailed information such as the collection site, date, sample type, responsible person, and corresponding sample number.

Control of Culture Media

In order to evaluate the proper functioning of the culture media used, a control of culture media was carried out. For this, thioglycolate medium, an enriched agar that facilitates the development of various microorganisms (12), was used. Additionally, MacConkey agar, a selective differential medium that inhibits the growth of Gram-positive bacteria and allows the distinction of Gram-

Pol. Con. (Edición núm. 96) Vol. 9, No 7, Julio 2024, pp. 3144-3160, ISSN: 2550 - 682X

negative bacteria, was used. In both culture media, an Enterococcus strain was inoculated to obtain a positive quality control and evaluate its appropriate growth.

Similarly, to establish a negative control, the same culture media were used without inoculating any microorganisms; this was done to ensure that the culture media were not contaminated at the time of seeding (13).

Serial Dilutions

For the isolation of native microorganisms, the serial dilution technique was used, which was also used to quantify the microorganisms on a culture plate. The soil sample was diluted 1 in 10. That is, 25g of soil in 125 ml of peptone water (10^{-1}), 1g of the previous solution in 9 mL of peptone water (10^{-2}), and so on until 10^{-5}.

Dilutions of 10^{-2}, 10^{-3}, and 10^{-4} were plated in duplicate, with 0.1 mL (100 µL) of each dilution being taken with a micropipette and plated on petri dishes with thioglycolate agar by spread plate method. Finally, they were incubated at 37 ±2 °C for 48 hours under optimal conditions for bacterial growth.

Quantification

To determine the number of Colony Forming Units per gram (CFU/g) in each of the samples, the number of colonies present in the countable dilution (10^{-3}) was counted. It was considered that one colony was equivalent to one CFU/g. This value represented the amount of CFU/g in the volume of the drop that was deposited by the replicator. Once the number of colonies in 1 ml (100 µL) was determined, the result was multiplied by the dilution factor used and divided by the volume in ml of the sample plated. In this way, the concentration of CFU in 1 ml (100 µL) of the sample was calculated

$$\text{CFU} = \frac{\text{N°}\frac{\text{CFU}}{\text{g}} \text{ x dilution factor}}{\text{sample plated volume mL}} \qquad \text{Ec1.}$$

Macroscopic Characterization

In the study of macroscopic characterization, the method proposed by MacFaddin in 2006 was employed (14). During this stage, the colonies formed on the surface of the culture medium were

Pol. Con. (Edición núm. 96) Vol. 9, No 7, Julio 2024, pp. 3144-3160, ISSN: 2550 - 682X

examined, analyzing both the top and bottom of the Petri dish. The objective was to identify the macroscopic characteristics of the colonies and distinguish those that are common and specific to each bacterial group. Through this approach, different morphotypes were identified and classified based on their macroscopic characteristics. Consequently, the following criteria were considered:

Axenic Bacterial Isolation

After obtaining a colony count result from the culture media, a new culture medium was inoculated to obtain axenic or pure cultures of the morphologically different colonies found. The isolation was carried out in thioglycolate medium, and a sterilized bacteriological loop was used to obtain a sample of the colonies and inoculate it using the streaking method. The plates with pure growth were placed in the incubator at a constant temperature of 37 ±2 °C for a period of 24 to 48 hours (15). Once the required time had elapsed, the plates were used for characterization, identification, and cryopreservation.

Microscopic Characterization (Gram Staining)

Gram staining is an effective and rapid technique that allowed us to distinguish between two categories of bacteria: Gram-positive and Gram-negative bacteria (16). The Gram staining technique is based on the structural differences of bacteria, such that Gram-positive bacteria acquire a purple color in Gram staining because the dye is retained in the dense peptidoglycan layer surrounding the cell, while Gram-negative bacteria do not retain the crystal violet during Gram staining due to their thinner peptidoglycan layer and therefore take on the color of the final counterstain safranin, appearing red or pink (17).

Gram Staining

For microscopic characterization, the following criteria were considered: Shape, Grouping, and Gram Staining (18).

Pol. Con. (Edición núm. 96) Vol. 9, No 7, Julio 2024, pp. 3144-3160, ISSN: 2550 - 682X

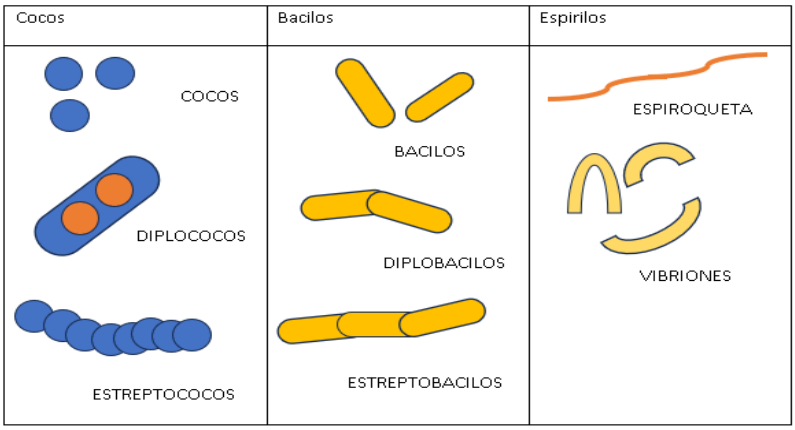

Figure1: *microscopic morphology of bacteria*

Biochemical Determination of Bacteria

In biochemical tests, various specific culture media were used to evaluate different metabolic aspects of microorganisms. These media included MacConkey Agar for the detection of lactose-fermenting gram-negative bacteria, Lysine Iron Agar to investigate the ability of some bacteria to break down lysine and produce sulfur reactions, triple sugar iron (TSI) Agar to analyze sugar fermentation and hydrogen sulfide production, SIM Agar to evaluate sulfur production, indole, and bacterial motility, Citrate Agar to determine the ability of bacteria to use citrate as the sole carbon source, and Urea Agar to verify urease enzyme activity(19).

Preparation of Cryopreservation Tubes with Glycerol and BHI Medium

An aqueous solution with glycerol preservative was developed to preserve bacteria in the logarithmic growth phase (24 hours of incubation) from the axenic medium (20).

Inoculation of Bacteria

The specific colony was identified and transferred to Eppendorf tubes using a previously sterilized metal loop. The tubes were subjected to vigorous shaking for 15 seconds in the vortex to ensure homogeneous distribution of the bacteria in the medium (21).

Storage of Prepared Tubes

Finally, the Eppendorf tubes were placed in a freezer at -20°C, ensuring future research.

Table 1: *Solution to preserve isolated bacteria*

Materials	Quantity
Liquid media (Tioglicolato)	5 mL
Distilled water	200 mL
Glicerin	2 drops pasteurs pipette

Results and discussion

Three treatments were carried out. Treatment 1 was considered the control group, in which a soil sample containing water and hydrocarbons was prepared. In Treatment 2, oxidation-reduction (redox) was applied, which is considered a passive volatilization technique for volatile contaminants. According to Manrique & Guisella (22), this method consists of promoting volatilization through periodic soil removal. In this case, soil removal was performed every 2 days for a month to promote the release of hydrocarbons present in volatile form. Subsequently, a TPHs test was conducted to evaluate the contamination levels in the treated soil.

In Treatment 3, molasses addition was applied, which is based on promoting the growth of microorganisms with the ability to decompose contaminating substances (bioremediation process). This can involve optimizing soil conditions to enhance the effectiveness of existing microorganisms or introducing new species. To promote biotic actions, specific soil conditions can be improved, such as adding nutrients, water, oxygen, and adjusting the pH (23). In this treatment, water with molasses was introduced to provide additional nutrients to the microorganisms present in the hydrocarbon-contaminated soil. Subsequently, TPHs tests were conducted to evaluate the degree of contamination in the treated soil. Finally, a comparison was made with the results obtained in the previous treatments to determine the effectiveness of the microbiological methodology in remediating contaminated soil.

Table 2 provides detailed information on the initial and final values of TPHs. In the initial phase, TPH levels were at 33,026 mg/kg, and by the end of the experiment, treatment 3 recorded a notable reduction, with 7,484 mg/kg of TPHs degraded. This decrease represents a 77% reduction in the total TPH content. This change is attributed to the positive influence of bacterial metabolites and the addition of extra nutrients, such as molasses, which played a crucial role in the hydrocarbon degradation process. In this context, the Enterobacteriaceae bacterial consortium, by multiplying more rapidly, significantly contributed to accelerating the decomposition of TPHs (24).

Pol. Con. (Edición núm. 96) Vol. 9, No 7, Julio 2024, pp. 3144-3160, ISSN: 2550 - 682X

Table 2: *Total Petroleum Hydrocarbons at the beginning and end of the treatment.*

Total Petroleum Hydrocarbons dry weight (mg/kg)	TPHs beginning	TPHs end	Allowed land use criteria		Degradation % TPHs	Methodology
TPHs	33026	7484	Farming land use <2500 mg/kg	Industrial land use <4000 mg/kg	77 %	M-GO-AM-62 IR

Analysis of Variance (ANOVA)

It is observed that the p-value in the ANOVA table is $<2e^{-16}$. Since it is less than 0.05, there is statistical evidence to reject Ho, meaning that there are differences in at least one of the treatments. Therefore, the TUKEY test is performed to determine which group means are different.

Table 3: *Analysis of Variance*

Analysis of Variance **(ANOVA)**						**Tukey (5 %)**	
F. V	**GL**	**SS**	**CM**	**F**	**P-value**	**Treatment**	**Average**
Treatments	2	1.085e+09	542263216	1238672	<2e-16	**T1**	33030
Error	6	2.627e+03	438			**T2**	27534
Total	8	1.09e+09				**T3**	7496

It is observed that the p-value in the ANOVA table is <2e-16. Since it is less than 0.05, there is statistical evidence to reject the alternative hypothesis, meaning that there are differences in at least one of the treatments. Therefore, the TUKEY test is performed to determine which group means are different (25).

In Figure 2, it is shown that there is a statistically significant difference between the average soil degradation of each treatment at the 0.05 significance level. Concluding that the best treatment is treatment 3, due to the greater hydrocarbon degradation in the soil and the validity of the data

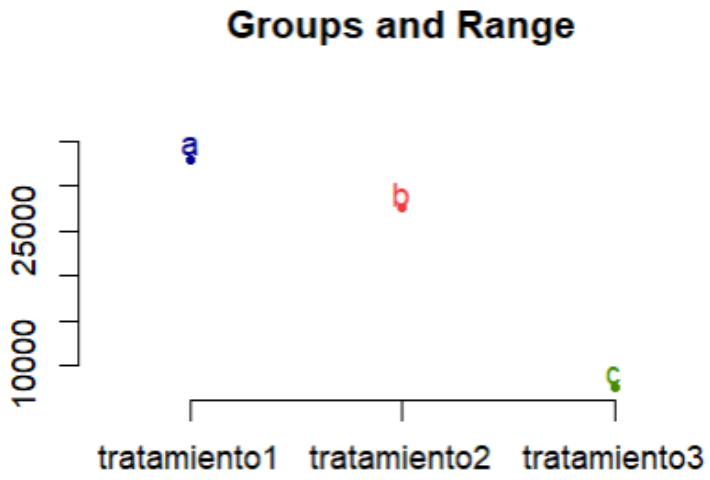

Figure 2: *Tukey 5% analysis of the treatments*

Analysis of Sample Variables

Moisture of the Hydrocarbon-Contaminated Sample

The following table describes the analysis of the moisture of the hydrocarbon-contaminated sample.

Table 3*: ANOVA and Tukey Analysis at 5% of the Sample Moisture*

ANOVA and Tukey Analysis						Tukey (5 %)	
F. V	GL	SS	CM	F	P- value	Tratamientos	Media
Treatments	8	1440.74	180.09	16.85	1.5394E-15	T1	52.22
Error	99	1058.33	10.69			T2	52.92
Total	107	2499.07	23.36			T3	60.14

Table 3 summarizes the average moisture levels in various treatments. The data reveals that treatment T1 has an average of 52.22%, closely followed by T2 with 52.92%, while T3 shows a higher average of 60.14%. It is important to emphasize that the recorded moisture levels are within the ideal ranges for the execution of bioremediation procedures. It is important to keep in mind that the optimal conditions for bacterial growth in bioremediation processes are usually between 30% and 90% of ambient moisture (26).

Pol. Con. (Edición núm. 96) Vol. 9, No 7, Julio 2024, pp. 3144-3160, ISSN: 2550 - 682X

Electrical Conductivity of the Hydrocarbon-Contaminated Sample

The following table describes the analysis of the electrical conductivity of the hydrocarbon-contaminated sample.

***Tabla 4**: Análisis de ANOVA y Tukey al 5% de la conductividad eléctrica de la muestra.*

Análisis de Varianza (ANOVA						**Tukey (5 %)**	
F. V	**GL**	**SS**	**CM**	**F**	**P- valor**	**Tratamientos**	**Media**
Tratamientos	8	3.93	0.49	2.968	0.005	**T1**	6.75
Error	99	16.40	0.17	2.968		**T2**	6.37
Total	107	20.33	0.19			**T3**	6.37

Table 4 analyzes the relationship between treatments and Electrical Conductivity (E.C.), a key measure of soil salinity that can vary due to various factors, such as the presence of hydrocarbons in the soil. In this context, the results indicate that treatments T1, T2, and T3 were evaluated in terms of their E.C. Treatment T1 shows the highest E.C., with a value of 6.75 dS/m, suggesting a higher concentration of saline compounds in the soil. Treatments T2 and T3 have similar E.C. values, both recording 6.37 dS/m. These Electrical Conductivity (E.C.) values indicate the influence of hydrocarbons on soil salinity (27).

pH of the Hydrocarbon-Contaminated Sample

The following table describes the analysis of the pH of the hydrocarbon-contaminated sample.

***Table 5:** ANOVA and Tukey Analysis at 5% of the Sample pH*

ANOVA and Tukey Analysis						**Tukey (5 %)**	
F. V	**GL**	**SS**	**CM**	**F**	**P-value**	**Treatments**	**Media**
Treatments	8	0.01	0.00080208	2.29	0.03	**T1**	8.16
Error	99	0.03	0.00035101			**T2**	8.18
Total	107	0.04	0.00038474			**T3**	8.18

Table 5 shows the average pH values for each treatment. The most notable results are those of T2 and T3, which have the highest pH in the process, recording 8.18, while T1 has the lowest pH with

8.16. These differences in pH are due to the addition of redox and molasses in these treatments, which has contributed to maintaining a similar pH among them (28).

Conusions

From three samples with 2 duplicates each, a total of 18 bacterial colonies were obtained. These colonies were classified as Gram-negative bacilli using the Gram staining technique. Subsequently, biochemical tests were carried out to characterize and determine the genus of the identified species. The cryopreservation of the bacteria was carried out at -20°C, proving to be a highly effective technique for the long-term preservation of bacterial strains. This ensures their viability and functionality, which is essential for their usefulness in future research. Treatment 3 achieved a remarkable efficiency by degrading 77% of the contamination present in the soil. These findings highlight the feasibility and potential of the remediation strategies used to mitigate the negative effects of hydrocarbon contamination in the soil. However, it is crucial to continue researching and refining these remediation techniques to further optimize their effectiveness and consider their large-scale application in similar environmental contamination situations.

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