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Mejoramiento de la emulsificación del aceite residual automotriz con detergentes sintéticos y microbianos en la biorremediación del suelo

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Tesis

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Dedicatoria

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Contenido

i. RESUMEN

La contaminación de suelo por aceite residual automotriz (ARA) es un problema en Mexico y el mundo al sobrepasar la máxima concentración de 4,400 ppm permitida por la NOM-138-SEMARNAT/SSA1-2012 (NOM-138). Lo que requiere solución ecológica. El objetivo general de esta tesis fue biorremediar un suelo contaminado por ARA mediante la eficaz emulsificación con detergentes sintéticos y microbianos para la rápida eliminación de la mezcla de hidrocarburos por los microorganismos heterotróficos aerobios. Se realizaron experimentos de bioestimulación, bioaumentación y fitorremediación de suelo contaminado por ARA en matraces Bartha y jarras de Leonard medidos por la conversión del ARA en CO_2 y la desaparición por Soxhlet. Se concluyó por fitorremediación del suelo con *Phaseolus vulgaris* y *Xanthobacter autotrophicus* evaluado con la fenología y el peso fresco, y seco de la leguminosa comparado con el crecimiento *P. vulgaris* en el suelo contaminado por ARA sin bioestimular ni fitorremediar. Además, se realizó la optimización de biodetergentes, con la actividad lipolítica de géneros y especies de bacterias aisladas de suelo contaminado por ARA, cual fue usado para inducir la actividad lipolitica con ARA, en la bioestimulación de suelo contaminado por 60,000 ppm de ARA, para un rápido decremento a 2,000 ppm y biorremediarlo acorde con la NOM-138. En la biorecuperacion de suelo contaminado por ARA se demostró que al inicio de la bioestimulación debe ser por detergentes que emulsifican el ARA luego continuar con solución mineral para alcanzar la máxima mineralización del ARA. Mientras se demostró la biorecuperacion de suelo contaminado por 60,000 ppm de ARA, por bioestimulación con detergente, solución mineral, bioaumentación con *X. autotrophicus*, que decreció el ARA, concluir con la fitorremediación se usó *P. vulgaris* para eliminar el ARA a 190 ppm, valor equivalente a concentración natural de hidrocarburos.

Palabras clave: mezcla de hidrocarburos, actividad lipolitica, minerales esenciales, leguminosa, bacterias oxidantes de hidrocarburos.

ii. ABSTRACT

Soil contamination by waste motor oil (WMO) is a problem in Mexico and the world as it exceeds the maximum concentration of 4,400 ppm allowed by NOM-138-SEMARNAT/SSA1-2012 (NOM-138). This requires an ecological solution. The objective of this thesis was to bioremediate of WMO contaminated soil by effective emulsification with synthetic and microbial detergents for the rapid removal of the hydrocarbon mixture by aerobic heterotrophic microorganisms. Biostimulation, bioaugmentation and phytoremediation experiments of WMO contaminated soil in Bartha flasks and Leonard flasks measured by WMO conversion to CO_2 and disappearance by Soxhlet were performed. It was concluded by phytoremediation of soil with *Phaseolus vulgaris* and *Xanthobacter autotrophicus* evaluated with phenology and fresh and dry weight of legume compared to *P. vulgaris* growth in WMO contaminated soil without biostimulation and phytoremediation. In addition, the optimization of biodetergents, with the lipolytic activity of genera and species of bacteria isolated from soil contaminated by WMO, was performed, which was used to induce lipolytic activity with WMO, in the biostimulation of soil contaminated by 60,000 ppm of WMO, for a rapid decrease to 2,000 ppm and bioremediation in accordance with NOM-138. In the bioremediation of soil contaminated by WMO, it was demonstrated that the biostimulation should start with detergents that emulsify the WMO and then continue with mineral solution to reach the maximum mineralization of the WMO. While bioremediation of soil contaminated by 60,000 ppm of WMO was demonstrated by biostimulation with detergent, mineral solution, bioaugmentation with *X. autotrophicus*, which decreased the WMO, concluding with phytoremediation using *P. vulgaris* to eliminate the WMO at 190 ppm, a value equivalent to the natural concentration of hydrocarbons.

Key words: hydrocarbon mixture, lipolytic activity, essential minerals, legume, hydrocarbon oxidizing bacteria.

iii. RESUMEN GENERAL

La contaminación de suelo agrícola por hidrocarburos como los del aceite residual automotriz (ARA), es un problema ambiental en Mexico y el mundo. La toxicidad del ARA limita la producción agrícola, por ello la norma mexicana NOM-138-SEMARNAT/SSA1-2012 (NOM-138) establece el límite máximo permisible de 4,400 ppm de hidrocarburos en suelo. Cuando el ARA contamina el suelo, inhibe la vida microbiana y provoca pérdida de fertilidad. El ARA contiene hidrocarburos alifáticos, aromáticos y policíclicos insolubles, que no se mineralizan. La solución ecológica, es la bioestimulación del suelo con detergentes sintéticos y microbianos que emulsifiquen el ARA, luego oxidados por los microorganismos heterótrofos aerobios seguida de solución mineral según las propiedades químicas del suelo. El objetivo general de esta tesis fue biorremediar un suelo contaminado por ARA mediante la eficaz emulsificación con detergentes sintéticos y microbianos para la rápida eliminación de la mezcla de hidrocarburos por los microorganismos heterotróficos aerobios.

Se realizaron experimentos de bioestimulación, bioaumentación y fitorremediación de suelo contaminado por ARA en matraces Bartha, jarras de Leonard medidos por la conversión del ARA en CO_2 y la desaparición por Soxhlet. Luego se concluyó adecuadamente por fitorremediación con *Phaseolus vulgaris* y *Xanthobacter autotrophicus* evaluados mediante la fenología; el peso fresco y seco comparado con el crecimiento *P. vulgaris* en suelo contaminado por ARA sin bioestimular ni fitorremediar. Además, se logró la optimización de biodetergentes, y actividad lipolítica de géneros de especies de bacterias aisladas de suelo contaminado por ARA. Posteriormente la bioestimulación de suelo contaminado por 60,000 ppm de ARA, con extractos crudos de biodetergentes y lipasas decreció el ARA a 2,000 ppm, considerado suelo biorremediado según la NOM-138. Además, se demostró la biorecuperacion acelerada de suelo contaminado por 60,000 ppm de ARA, por bioestimulación con detergente y solución mineral, bioaumentación mediante *X. autotrophicus* oxidante de aromáticos, y fitorremediación con *P. vulgaris y X. autotrophicus* que decreció el ARA a 190 ppm, concentración natural de hidrocarburos en suelo adecuada para la reutilización agrícola.

iv. GENERAL ABSTRACT

Contamination of agricultural soil by hydrocarbons such as waste motor oil (ARA) is an environmental problem in Mexico and the world. The toxicity of WMO limits agricultural production, which is why Mexican standard NOM-138-SEMARNAT/SSA1-2012 (NOM-138) establishes a maximum permissible limit of 4,400 ppm of hydrocarbons in soil. When WMO contaminates soil, it inhibits microbial life and causes loss of fertility. WMO contains insoluble aliphatic, aromatic and polycyclic hydrocarbons, which do not mineralize. The ecological solution is soil biostimulation with synthetic and microbial detergents that emulsify the WMO, then oxidized by aerobic heterotrophic microorganisms followed by mineral solution according to the chemical properties of the soil. The objective of this thesis was to bioremediate of WMO contaminated soil by effective emulsification with synthetic and microbial detergents for the rapid removal of the hydrocarbon mixture by aerobic heterotrophic microorganisms.

Biostimulation, bioaugmentation and phytoremediation experiments of WMO contaminated soil were performed in Bartha flasks, Leonard flasks measured by WMO conversion to CO_2 and disappearance by Soxhlet. Then it was properly concluded by phytoremediation with *Phaseolus vulgaris* and *Xanthobacter autotrophicus* evaluated by phenology; fresh and dry weight compared to *P. vulgaris* growth in WMO contaminated soil without biostimulation and phytoremediation. In addition, optimization of biodetergents, and lipolytic activity of genera of bacterial species isolated from WMO-contaminated soil was achieved. Subsequently, biostimulation of soil contaminated by 60,000 ppm WMO, with crude extracts of biodetergents and lipases decreased WMO to 2,000 ppm, considered bioremediated soil according to NOM-138. In addition, accelerated bioremediation of soil contaminated by 60,000 ppm of WMO was demonstrated by biostimulation with detergent and mineral solution, bioaugmentation with X. autotrophicus, an aromatic oxidizer, and phytoremediation with *P. vulgaris* and *X. autotrophicus*, which decreased WMO to 190 ppm, a natural concentration of hydrocarbons in soil suitable for agricultural reuse.

v. CAPITULO I. INTRODUCCIÓN: Detergentes sintéticos y biológicos en la biorremediación de suelo: inconsistencias y evidencias.

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Detergentes y Biodetergentes en la Biorremediación de Suelo: Inconsistencias y Evidencias[1]

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Resumen

La contaminación del suelo por hidrocarburos (HICOS) es uno de los graves problemas ambientales. Cuando los HICOS contaminan el suelo, afectan la relación carbono: nitrógeno, causan la inhibición de la vida microbiana y pérdida de fertilidad. Una alternativa es la biorremediación, pero la baja solubilidad y la absorción de los HICOS en el suelo dificulta la eliminación. Por ello, al inicio de la biorremediación es necesaria la bioestimulación con un detergente (DEGE) o biodetergente (BIODET) para solubilizar los HICOS. Sin embargo, algunas investigaciones señalan que estos detergentes actúan directamente en la eliminación de los HICOS, las evidencias señalan que el DEGE y BIODET solo permite la desorción de los HICOS para solubilizarlos, por la formación de micelas.

Otros trabajos de biorremediación, aseguran que el tipo de DEGE y BIODET que utilizan solubiliza todos los HICOS de las mezclas contaminantes, pero de acuerdo a la composición del detergente, no pueden solubilizar toda la diversidad de HICOS.

1 1Artículo de Revisión derivado del proyecto de investigación 2.7 (2020), de la Universidad Michoacana de San Nicolás de Hidalgo, ejecutado entre 2018 y 2020; Financiado por Universidad de Harvard, Cambridge, Ma, EUA, Fundación Rockefeler 2021.

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Los autores declaran que no tienen conflicto de interés

Otras investigaciones afirman la síntesis "in situ" de BIODET por microorganismos inoculados en la bioaumentación de suelo impactado por HICOS, sin demostrarlo experimentalmente. El objetivo de esta revisión es analizar en la biorremediación de suelo contaminado por HICOS, la importancia de DEGE, BIODET y las inconsistencias generadas por su aplicación.

Palabras clave: biorremediación, contaminación, detergente, hidrocarburos, suelo.

Detergents and Detergents in Soil Bioremediation: Inconsistencies and Evidence

Abstract:

Soil pollution by hydrocarbons (HYCOS) is one of the serious environmental problems. When HYCOS contaminate the soil, they affect the carbon: nitrogen ratio, causing inhibition of microbial life and loss of fertility. An alternative is bioremediation, but the low solubility and absorption of HYCOS in the soil makes it difficult to eliminate. Therefore, at the beginning of the bioremediation, biostimulation with a detergent (DEGE) or biodetergent (BIODET) is necessary to solubilize the HYCOS. However, some research indicates that the detergents acts directly in the extraction of the HYCOS, the evidence indicates that the DEGE and BIODET only allows the desorption of the HYCOS to solubilize them, by the formation of mice. Other bioremediation works assure that the type of DEG and BIODET that it uses solubilizes all the HYCOS of the contaminating mixtures, but according to the composition of the detergent, it cannot emussify the entire range of HYCOS. Other investigations affirm the synthesis "in situ" of BIODET by microorganisms inoculated in the bioaugmentation of soil impacted by HYCOS, without demonstrating it experimentally. The objective of this review is to analyze in the bioremediation of the soil contaminated by HYCOS, the importance of DEGE, BIODET and the inconsistencies generated by their application.

Keywords: bioremediation, pollution, detergent, hydrocarbons, soil.

Detergentes e Detergentes na Bioremediação do Solo: Inconsistências e Evidências

Resumo:

A poluição por Óleo por Hidrocarbonetos (HICOS) é um dos graves problemas ambientais. Quando os HICOS poluem a água, eles afetam a relação carbono- nitrogênio, causando a inibição da vida microbiana e perda de fertilidade. Uma alternativa é a biorremediação, mas a baixa solubilidade e absorção do HICOS em sua eliminação dificulta sua eliminação. Portanto, no início da biorremediação, a bioestimulação com detergente (DEGE) ou biodetergente (BIODET) é necessária para solubilizar o HICOS. Porém, algumas pesquisas indicam que esses detergentes atuam diretamente na eliminação dos HICOS, as evidências indicam que o DEGE e o BIODET só permitem a dessorção dos HICOS para solubilizá-los, pela formação de micelas. Outros trabalhos de biorremediação garantem que o tipo de DEGE e BIODET usado solubilize todos os HICOS das misturas contaminantes, mas dependendo da composição do detergente, eles não podem solubilizar toda a diversidade de HICOS. Outras investigações afirmam a síntese "in situ" de BIODET por microrganismos inoculados na bioaumentação de solos impactados por HICOS, sem demonstrá-la experimentalmente. O objetivo desta revisão é analisar na biorremediação de solos contaminados por HICOS, a importância do DEGE, do BIODET e as inconsistências geradas por sua aplicação.

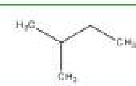
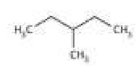
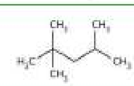
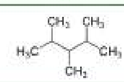
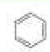
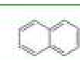
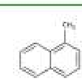
Palavras-chave: biorremediação, contaminação, detergente, hidrocarbonetos, solo.

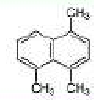
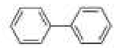
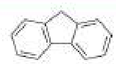
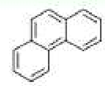
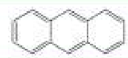
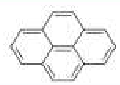
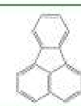
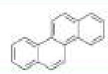
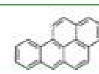
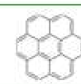
Introducción

Los hidrocarburos (HICOS) del petróleo están conformados por cadenas de 83 a 87 % de carbono (C) y de 11 a 14 % de hidrógeno (H), clasificados en alifáticos, aromáticos, asfáltenos y resinas (Hu *et al.*, 2013) que en la mayoría son insolubles o poco solubles, lo que representa un limitante en la eliminación en el suelo como se muestra en la Tabla N ◦ 1. Las fracciones alifáticas más sencillas tienen mayores valores de solubilidad como el n-pentano con 38.5 mg/L o 2-metil butano con 49.6 mg/L (Mackay & Shiu, 1981), mientras que disminuye cuando aumenta la complejidad como los aromáticos policíclicos, por ejemplo, benzo(a) pireno con 0.0038 mg/L y coroneno 0.00014 mg/L (Mackay & Shiu, 1977).

Los HICOS causan contaminación de suelo por derrames que han incrementado hasta en un 50 % en los últimos 15 años. Anualmente en el mundo, se estima que entre 1.7 y 8.8 millones de toneladas métricas de HICOS del petróleo, son liberados al ambiente (Sihag *et al.*, 2014). En los últimos 10 años en China se registraron más de 6000 suelos contaminados en diversas regiones de ese país (Zhang & Chen, 2017; Li *et al.*, 2019), mientras que, en Australia, esta cifra fue de 96,000 sitios en el 2015 (Duan *et al.*, 2015). Las cantidades de sitios contaminados por HICOS varían de país en país, posiblemente relacionadas con la cantidad de HICOS procedentes de la industria petroquímica o el grado de tecnificación. Por ejemplo, en 2013 en Alemania, se reportaron 474 sitios contaminados, mientras que, en Turquía en 2015, se identificaron solo 20 sitios (Aichner *et al.*, 2015; Karaca, 2016). En Estados Unidos, cerca del 90 % de sitios impactados, se relacionan a vertidos por HICO (Das & Chandran, 2011); de acuerdo a la USEPA, el número de sitios reportados fue alrededor de 600. En 2017 en México, particularmente en Tabasco, una de las mayores zonas petroquímicas en el país, se reportaron 114 incidentes ambientales de derrames y fugas de HICOS (Vargas *et al.*, 2017). En ese mismo año, la Procuraduría de Protección al Ambiente Mexicana (PROFEPA) recibió al menos 259 notificaciones de emergencias por derrames de HICOS tanto en agua como en suelo. En suelo, a nivel internacional, son considerados compuestos de alta prioridad para removerse de áreas contaminadas, sin embargo, las leyes sobre límites permisibles, son distintas de acuerdo a cada país, con base en volumen/peso. Por ejemplo, en Estados Unidos, la USEPA permite como límite máximo permisible de HICOS 3,308 ppm para las fracciones ligera, mediana y pesada (Tomlinson & Ruby, 2016). Mientras que la norma mexicana NOM-138-SEMARNAT/SSA1-2012 (NOM-138), permite una concentración mayor como límite máximo de 4,400 ppm.

Tabla N ° 1. Estructura química y solubilidad de algunos hidrocarburos contaminantes del suelo.

Tipo de hidrocarburo	Nombre	Estructura	Solubilidad (mg/L)	Referencia
Alifáticos	n-Pentano		38.5	Mackay & Shiu, 1981
	n-Hexano		9.5	Mackay & Shiu, 1981
	2-Metilbutano		49.6	Polak & Lu, 1973
	3-Metilpentano		17.9	Mackay & Shiu, 1981
	2,2,4-Trimetilpentano		2.05	Mackay & Shiu, 1981
	2,3,4-Trimetilpentano		2.30	Mackay & Shiu, 1981
Aromáticos	Benceno		1755	Mackay & Shiu, 1981
	Tolueno		526	Mackay & Shiu, 1981
	Naftaleno		31.7	Mackay & Shiu, 1981
	1-Metilnaftaleno		28.5	Mackay & Shiu, 1981

Tipo de hidrocarburo	Nombre	Estructura	Solubilidad (mg/L)	Referencia
	1,4,5-Trimetilnaftaleno		2.1	Mackay & Shiu, 1977
	Bifenilo		7	Mackay & Shiu, 1977
Policíclicos	Fluoreno		1.98	Mackay & Shiu, 1977
	Fenantreno		1.10	Mackay & Shiu, 1977
	Antraceno		0.073	Mackay & Shiu, 1977
	Pireno		0.135	Mackay & Shiu, 1977
	Fluorantreno		0.26	Mackay & Shiu, 1977
	Criseno		0.0020	Mackay & Shiu, 1977
	Benzo(a)pireno		0.0038	Mackay & Shiu, 1977
	Coroneno		0.00014	Mackay & Shiu, 1977

Fuente: Elaborada por el autor: Blanca Celeste Saucedo Martínez

La contaminación de suelo por HICOS en concentraciones excesivas, por lo que primero se hace una remoción mecánica y luego un lavado de suelo con un detergente (DEGE). Posteriormente se utilizan métodos biológicos como la biorremediación para eliminar los HICOS remanentes, ahí se utiliza primero un DEGE o biodetergente (BIODET) bajas concentraciones para emulsificar los HICOS y hacerlos disponibles para que los microorganismos los mineralicen.

Materiales y Métodos

La búsqueda de la literatura se realizó con fuentes primarias como lo son artículos científicos, de revisión, así como capítulos de libros. Para ello inicialmente se seleccionaron las bases de datos a emplear para obtener datos homogéneos recuperables actualmente por internet. Estas bases de datos fueron:

1. Scimago Journal & Country Rank
2. Scopus
3. Google académico
4. Springer Open

Posteriormente, para la estrategia de búsqueda, se seleccionaron las palabras clave y sinónimos en diferentes combinaciones en el idioma inglés, para identificar estudios relevantes relacionados al tema de investigación. Algunas de ellas fueron: hydrocarbons, bioremediation, soil, detergent, biodetergent, surfactant, emulsification detergent, emulsification surfactant hydrocarbon, bioremediation of soil hydrocarbons detergent, phytoremediation soil hydrocarbons detergent, synthesis biosurfactants soil hydrocarbon, biostimulation soil hydrocarbon detergent.

Se incluyeron referencias desde el año 1977 hasta el 2020, siendo en su mayoría del 2011 al actual.

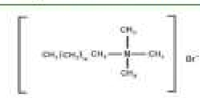
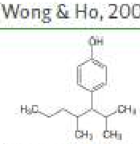
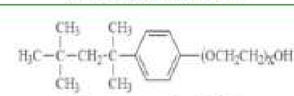
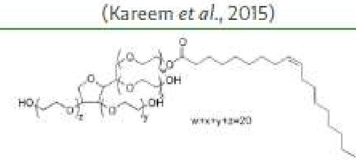
Composición Química de los Detergentes y Biodetergentes

Los microorganismos utilizan los HICOS como fuente de carbono y energía, por lo que requieren BIODET para emulsificarlos y que, en un suelo impactado por HICOS, la cantidad de BIODET sería insuficiente para la alta concentración de HICOS, lo que no permitiría la actividad microbiana, a diferencia de los DEGE que se aplican independientemente del nivel de concentración de HICOS después de la remoción mecánica y en la biorremediación. A continuación, se muestran algunos de los DEGE y BIODET más usados en la biorremediación.

Detergentes (DEGE)

Los detergentes (DEGE) son moléculas anfifílicas, con una parte hidrofílica y otra hidrofóbica, con la propiedad fundamental de formar micelas lo que facilita la humectación, dispersión y solubilización de moléculas hidrófobas en soluciones acuosas (Banat *et al.*, 2000; Koopal, 2012; Castiglione *et al.*, 2016). Existe una amplia variedad química de DEGE del tipo aniónicos, catiónicos, y no iónicos como los mostrados en la Tabla N ◦ 2, los iónicos y aniónicos son los más usados en la remediación y biorremediación (Lamichhane *et al.*, 2017).

Tabla N° 2. Estructura química de detergentes comúnmente empleados para la solubilización de hidrocarburos en el suelo.

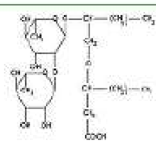
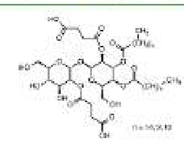
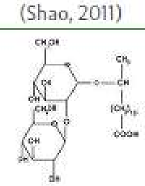
Detergentes	Estructura
Anionicos	
Dodecil sulfato de sodio	 (Bhardwaj *et al.*, 2014)
Catiónicos	
Bromuro de cetiltrimetilamonio	 (Karaca *et al.*, 2012)
Cloruro de cetilpiridinio	 (Khachane & Nagarsenker., 2011; Choi *et al.*, 2014)
Bromuro de dimetil dodecil amonio	 (Khachane& Nagarsenker., 2011)
No Iónicos	
Brij® 35	 (Wong & Ho, 2009)
Nonilfenol®	 (Hung *et al.*, 2010)
Tergitol® NP-10	 (Vicente *et al.*, 2017)
Triton® X-100	 (Kareem *et al.*, 2015)
Tween® 80	 (Souza *et al.*, 2012)

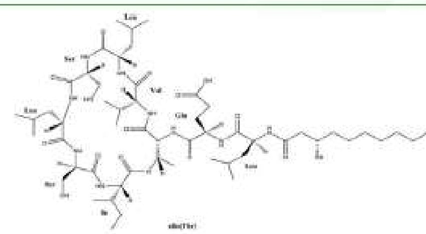
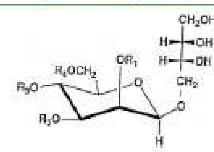
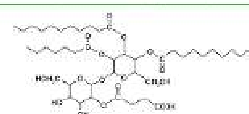
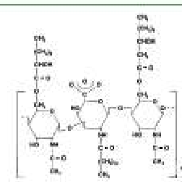
Fuente: elaborada por el autor: Blanca Celeste Saucedo Martínez

Biodetergentes

Los biodetergentes (BIODET) son metabolitos secundarios sintetizados por géneros de especies como *Bacillus* y *Pseudomonas* (Silva *et al*., 2014) y levaduras como *Candida bombicola*, *C. lipolytica* y *Saccharomyces cerevisiae* (Santos *et al*., 2016). Los BIODET contienen la fracción hidrófila de naturaleza polisacária con PO_4, y la hidrófoba con cadenas alifáticas (Pacwa-Plociniczak *et al*., 2011). En el suelo para la biorremediación, existe una amplia diversidad química de BIODET aplicados del tipo: glicolípidos, ramnolípidos, soforolípidos o trehalolipidos; lipopéptidos y lipoproteínas como el surfactin y el emulsan (Tabla N ◦ 3). Debido a la estructura anfifílica al igual que los DEGE, tienen la capacidad de aumentar el área de superficie de sustancias hidrófobas como los HICO para movilizarse cuando son absorbidos en el suelo (Lai *et al*., 2009; Whang *et al*., 2009; Kang *et al*., 2010; Mnif *et al*., 2017).

Tabla N ◦ 3. Estructura química de biodetergentes usados en la solubilización de hidrocarburos del suelo.

Biodetergente	Estructura
Glicolipidos	
Ramnolipidos	 (Plaza *et al*., 2014)
Trehalolipidos	 (Shao, 2011)
Soforolipidos	 (Plaza *et al*., 2014)

Biodetergente	Estructura
Lipopeptidos y lipopotreinas	
Viscosin	 (Khattari *et al*., 2015)
Surfactin	 (Plaza *et al*., 2014)
Mannosylerythritol lipidico	 (Salihu *et al*., 2009)
Lípido trehalosa	 (Teruel *et al*., 2014)
Polimericos	
Emulsan	 (Salihu *et al*., 2009)

Fuente: elaborada por el autor: Blanca Celeste Saucedo Martínez

En el suelo, de acuerdo a la literatura, tienen ventajas potenciales sobre los DEGE, como estabilidad en ambientes extremos de pH, temperatura y salinidad (Uzoigwe *et al*., 2015; Lamichhane *et al*., 2017; Gupta *et al*., 2019).

En la estructura química de los DEGE (Tabla N ° 2) y BIODET (Tabla N ° 3) se puede apreciar que tienen una parte similar como fracciones carbonadas y otra parte polar que permiten la solubilización de compuestos. En la biorremediación de suelo impactado por

HICOS, se puede bioestimular con mezclas de detergentes complementarios para acciones sinérgicas de ampliar el espectro de emulsificación, a diferencia de la aplicación individual como se reporta en la literatura (Gudiña *et al.*, 2015, de la Cueva *et al.*, 2016 o da Rocha *et al.*, 2019).

Biorremediación de Suelo Impactado por Hidrocarburos

Contaminación de suelo por hidrocarburos

En suelo cuando los HICOS se derraman, afectan drásticamente las propiedades fisicoquímicas. Inicialmente se forma una película en la superficie del suelo que disminuye la permeabilidad del suelo (Gordon *et al.*, 2018), limita la aireación, e infiltración de agua, donde los HICOS se adhieren por absorción a la materia orgánica y a las fracciones minerales, y lentamente se difunden en la columna de suelo. En consecuencia, se inhibe la mineralización de la materia orgánica y pérdida de fertilidad (Klamerus-Iwan *et al.*, 2015; Koshlaf & Ball, 2017).

La contaminación de suelo por HICOS es un problema complejo y por ello requiere de estrategias similares para reducir la concentración por debajo de los límites máximos permisibles por las regulaciones ambientales como la mexicana NOM-138 o la norteamericana Agencia de Protección Ambiental (EPA).

En suelo las soluciones comunes para resolver la contaminación por HICOS, involucra primero, una remoción mecánica para disminuir el exceso de HICOS; luego la remediación con agentes químicos, que oxidan la mayor parte de los HICOS, pero generan compuestos secundarios tóxicos al ambiente y agravan el problema (Bezza & Chirwa 2015; Effendi *et al.*, 2017).

Removido el exceso de HICOS, se aplican métodos biológicos como la biorremediación, por tres formas: bioestimulación, bioaumentación y fitorremediación (Das & Chandran, 2011; Azubuike *et al.*, 2016). La bioestimulación consiste en el enriquecimiento del suelo para la mineralización de los HICOS, la bioaumentación es la introducción de microorganismos individuales o consorcios con capacidad de oxidar HICOS (Herrero & Stuckey, 2015; Agnello *et al.*, 2016). Mientras que en FITO se usan plantas en conjunto con microorganismos de la rizosfera para degradar HICOS del suelo (Shaw & Burns, 2003; Akhundova & Atakishiyeva, 2015). En cualquiera de las tres formas se debe bioestimular el suelo con un DEGE o BIODET para solubilizar los HICOS remanentes (Sáenz-Marta *et al.*, 2015; Cheng *et al.*, 2017), para la posterior bioestimulación con soluciones minerales a base de nitrógeno (N), fósforo (P) y potasio (K), para reestablecer la relación C:N, derivada del exceso de C. Luego la bioestimulación complementaria con una fuente de O_2 que aceleran la mineralización y el contenido de humedad al 80 % de la capacidad de campo, ya que facilita la aireación e intercambio gaseoso para mantener el paso del O_2 e inducir la actividad oxidante de los HICOS por la microbiota nativa (Kumar, 2019).

Análisis de la biorremediación de suelo impactado por hidrocarburos: inconsistencias y evidencias del papel de los detergentes.

En la biorremediación de suelo impactado por HICOS que incluye: bioestimulación, bioaumentación y fitorremediación se deben emplean DEGE y BIODET, dado que las mezclas de HICOS son insolubles, lo que impide que se solubilicen y en consecuencia los microorganismos los mineralicen (Jimoh y Lin, 2020), sin embargo, en diversas investigaciones sobre biorremediación de suelo, se han detectado inconsistencias sobre el papel de DEGE y BIODET como se muestra a continuación.

Inconsistencia 1. Señalar que en el suelo el detergente empleado es responsable directo de la reducción parcial de los hidrocarburos.

a) Bioestimulación con detergente o biodetergente.

Existen algunos trabajos de bioestimulación de suelo impactado por HICOS que asumen que la reducción de la concentración de HICOS en suelo fue por el DEGE y/o BIODET, imposible pues solo los emulsifican y para que la microbiota nativa los oxide (Shi *et al*., 2020) Algunas investigaciones que muestran esta inconsistencia son las siguientes:

Moldes *et al*., 2011 bioestimularon un suelo impactado por 70,000 ppm de octano mediante BIODET, donde después de 45 días reportaron que lo redujo a 26, 040 ppm de octano.

Posteriormente Gudiña *et al*., 2015 bioestimularon un suelo contaminado por 10,000 ppm de petróleo donde asumieron que el BIODET redujo el petróleo hasta 4,500 ppm y que el DEGE Enordet a 5,500 ppm.

da Rocha *et al*., 2019 bioestimularon un suelo impactado por 40,000 ppm de HICOS por 42 días mediante BIODET ramnolipidos que reportaron los redujo hasta 28,020 ppm y que el Tween® 80 a 29,900 ppm de HICOS.

En los trabajos anteriores, se señala erróneamente que el DEGE o BIODET decreció los HICOS, aunque solo emulsificaron parte de los HICO al disminuir la tensión interfacial entre la fase no acuosa del suelo y la acuosa, de esta manera fueron parcialmente disponibles para que los microorganismos, estimulados por los minerales del suelo, los oxidaran de forma limitada, ante la ausencia de una solución rica en minerales de nitrógeno y fósforo (Li & Chen, 2009; Lamichhane *et al*., 2017; Ortega *et al*., 2018).

b) Bioestimulación con detergente, biodergente y solución mineral.

Mientras que existen investigaciones que aplican bioestimulación con solución mineral, para que los microorganismos oxiden los HICOS, en los resultados afirman que el DEGE y/o BIODET fueron responsables directos de la eliminación de los HICO, tales casos son los siguientes:

de la Cueva *et al*., 2016 bioestimularon un suelo contaminado por 36,616 ppm de petróleo mediante DEGE Tween® 80, solución mineral y después de 90 días reportaron una reducción a 16,176 ppm. Luego Bezza & Chirwa, 2017 bioestimularon un suelo impactado por 6,745 ppm de HICO aromáticos policíclicos, mediante BIODET y solución mineral con NH_4NO_3 y KH_2PO_4, que después de 64 días registraron 2,552 de concentración final de HICO. López-Miranda *et al.*, 2018 bioestimularon un suelo contaminado por 33,616 ppm mediante DEGE Tween® 80, y solución mineral con una reducción a 12,933 ppm.

El decremento en la concentración de HICO en los trabajos anteriores, se debe a que los DEGE o BIODET en la bioestimulación del suelo emulsificaron una parte de los HICO para que la microbiota los oxidara y para equilibrar la relación C: N derivada del exceso de C de los derivados del petróleo (Lawniczak *et al.*, 2020).

c) Bioaumentación y bioestimulación con detergente y biodetergente.

Del mismo modo que en las investigaciones anteriores, existen trabajos de bioaumentación donde se introducen géneros y especies de microorganismos que oxidan HICOS en el suelo (Lawniczak *et al.*, 2020), que se bioestimulan con DEGE o BIODET que asumen que oxidan los HICOS. A continuación, algunos ejemplos:

Jorfi *et al.*, 2013 bioaumentaron un suelo impactado por 10,0000 ppm de pireno mediante microorganismos no especificados y bioestimulación con BIODET glicolípido, donde afirmaron que después de 63 días de incubación redujo el pireno a 1,540 ppm. Lo que indica que el BIODET únicamente solubilizó la mayor parte del pireno para que en seguida los microorganismos lo mineralizara.

Similarmente Bezza & *Chirwa*, 2015, realizaron bioaumentación de lodo contaminado por 40,000 ppm de petróleo con *Ochrobactrum intermedium* CN3 y que señalan que bioestimulado mediante el BIODET glicolípido, redujo la concentración final de petróleo a 12,000 ppm después de 21 días.

En las investigaciones anteriores el papel de DEGE y BIODET fue únicamente de solubilizar los HICOS contaminantes del suelo, y lo que los eliminó fue la acción oxidante de los microorganismos introducidos en la bioaumentación, que fue inducida por los nutrientes naturales del suelo.

También se han documentado investigaciones de bioaumentación de suelos contaminados por HICOS, que, aunque realizan bioestimulación mediante DEGE o BIODET y solución mineral, siguen asegurando que el detergente es la razón principal de la reducción de los HICOS, tal es el caso de Xu *et al.*, 2018 que bioestimularon un suelo impactado por 50,000 ppm de petróleo mediante DEGE Tween® 80, SDS, solución nutritiva y bioaumentación con microorganismos de géneros no especificados. Después de 80 días asumieron que el DEGE influyó directamente para que el petróleo se redujera a 11,500 ppm, lo que no es posible, ya que la única función de DEGE fue la emulsificación parcial del petróleo. Mientras que los microorganismos estimulados por la solución nutritiva, que regula la relación C/ N / P, utilizaran el petróleo como fuente de carbono para su crecimiento (Shahi *et al.*, 2016).

d) Fitorremediación y bioestimulación con detergente o biodetergente.

Algunos autores que realizaron fitorremediación con diversas plantas cuyo sistema radical degrada HICO en conjunto con microorganismos y bioestimulación con DEGE y/o BIODET, señalan la misma inconsistencia sobre los detergentes como agentes directos del decremento de HICO, algunos ejemplos a continuación:

Akhundova & Atakishiyeva, en el 2015 fitorremediaron un suelo impactado por 10,000 ppm de petróleo mediante *Artemiza fragans*, con un consorcio microbiano y bioestimulación con BIODET, después de 56 días mostraron que el petróleo se redujo a 1,500 ppm en el suelo fitorremediado con *Artemiza fragans* sin el consorcio, afirmaron que el BIODET intervino en la eliminación del petróleo.

Más tarde Almansoory *et al.*, 2015 fitorremediaron un suelo impactado con 2,000 ppm de gasolina mediante *Ludwigia octovalvis* y bioestimulado con el DEGE dodecil sulfato de sodio que le atribuyen la reducción de la gasolina a 274 ppm.

Liao *et al.*, 2016 que fitorremediaron un suelo impactado por 5,000 ppm de petróleo mediante *Zea mays* y bioestimularon con DEGE Tween® 80, que después de 3 meses señalaron que la mayor parte del petróleo se redujo a 2,650 ppm por el DEGE.

Por su parte, Liduino *et al.*, 2018 que fitorremediaron un suelo impactado con 18,145 ppm de petróleo mediante *Helianthus annus* y bioestimularon con BIODET ramnolipidos, que indican que tuvo que ver directamente con la disminución del petróleo a 7,145 ppm.

Contrario a lo que afirman los estudios anteriores, la composición química de DEGE o BIODET empleado tuvo la única función de emulsificar una parte de los HICO, donde la planta utilizada para la fitorremediación los degradó y los microorganismos rizosféricos mineralizaron una parte de los HICOS en CO_2 y H_2O (González-Moscoso *et al.*, 2019).

Inconsistencia 2. El uso de un detergente o biodetergente emulsifica todos los tipos de hidrocarburos.

En la biorremediación de suelo impactado por HICO, para que los microorganismos del suelo oxiden los HICOS, deben ser emulsificados por DEGE o BIODET, sin embargo, las mezclas de HICOS tienen diferente composición química y grado de solubilidad (Tabla N ° 1), por lo que no se emulsifican totalmente con un solo tipo de DEGE o BIODET. El análisis de la literatura muestra que entre el 80 y 90 % de trabajos solo aplica un detergente:

de la Cueva *et al.*, 2016, realizaron bioestimulación de suelo impactado por 36,616 ppm de petróleo mediante DEGE Tween® 80 y solución mineral, donde asumieron que el DEGE emulsificó todos los HICO del petróleo, que los microorganismos estimulados por la solución mineral decrecieron el petróleo hasta 16,176 ppm después de 90 días. López-Miranda *et al.*, 2018 bioestimularon un suelo impactado por 33,616 ppm de hidrocarburos totales del petróleo, que fueron emulsificados en un 61.5 % con Tween® 80 y por bioestimulación con una solución mineral los microorganismos los disminuyeron hasta 12,933 ppm.

Los resultados anteriores muestran que la fracción remanente de HICO, no se emulsificó y por lo tanto no se mineralizó. Por ello es necesario conocer la composición química de la mezcla de HICOS contaminantes para seleccionar mezclas de DEGE y BIODET químicamente afines que emulsifiquen la mayor concentración posible de HICOS para que con la bioestimulación del suelo con una solución mineral los microorganismos del suelo los eliminen.

Inconsistencia 3. La síntesis "in situ" de biodetergentes por microorganismos inoculados en la bioaumentación de suelo impactado por hidrocarburos.

En la bioaumentación de suelo impactado por HICO, algunos autores que inoculan microorganismos que se supone sintetizan BIODET, asumen que en efecto lo hicieron, sin mostrar las evidencias, tales como:

Ángeles & Refugio en el 2013, bioaumentaron un suelo impactado por 32,421 ppm de petróleo mediante microorganismos productores de BIODET y bioestimularon con solución mineral, después de 10 días reportaron reducción del petróleo a 23,517 ppm en suelo.

Luego Mnif *et al.*, 2017 bioaumentaron un suelo contaminado por 50,000 ppm diésel con consorcio microbiano, dos bacterias productoras de BIODET y bioestimularon mediante BIODET y solución mineral. Después de 3 semanas registraron una concentración final de HICO 25,600 ppm de diesel.

Así como Vargas *et al.*, 2017 que bioaumentaron un suelo impactado con 120,000 ppm de HICO, mediante microorganismos productores de BIODET, donde después de 16 meses registraron 20,000 ppm de concentración final de HICO.

Estos estudios descritos anteriormente, señalan que los microorganismos inoculados en el suelo produjeron BIODET para emulsificar los HICOS, sin embargo, sin ninguna técnica de detección o cuantificación de los BIODET sintetizados en el suelo. Mientras que es complejo demostrar la síntesis de BIODET, ya que cuando los microorganismos se someten a pruebas a nivel de laboratorio, las condiciones son controladas y limitadas (Phulpoto *et al.*, 2020) a diferencia de lo que ocurre naturalmente en un suelo contaminado por HICO, donde existen múltiples interferencias (Cachada *et al.*, 2018).

Evidencias en la biorremediación de suelo, el detergente o biodetergente solubiliza los hidrocarburos contaminantes y la eliminación se debe a la actividad microbiana.

a) Bioestimulación con detergente o biodetergente.

Existen investigaciones de bioestimulación de suelo impactado por HICO que señalan que el papel de DEGE y BIODET, es emulsificar los HICO mientras que la bioestimulación por solución mineral de N, P, y K para los microorganismos del suelo, hace posible que los oxide como se señala a continuación:

Effendi *et al.*, 2017 bioestimularon un suelo impactado por 48,000 ppm de HICO donde el

DEGE Tween® 80 emulsificó parte de los HICO, para que con la bioestimulación con urea y triple superfosfato los microorganismos disminuyeran los HICOS hasta 14,400 ppm. Similarmente Ali *et al*., 2019 bioestimulación de suelo contaminado por 10,000 ppm de aceite residual automotriz con DEGE Triton® X-100 para emulsificar el aceite y posteriormente la bioestimulación de la microbiota con una solución mineral, fuera posible reducir el aceite residual automotriz a 3,150 ppm en 10 días.

Ambos trabajos anteriores muestran que el DEGE no interviene en la eliminación de los HICOS, puesto que solo podría emulsificar los HICOS, mientras que la microbiota nativa solo por bioestimulación con solución mineral podría mineralizarlos.

b) Bioaumentación y bioestimulación detergente o biodetergente.

Trabajos de bioaumentación y bioestimulación con BIODET y/o DEGE, también señalan que el papel del detergente es emulsificar los HICO y que la reducción de la concentración es derivada de los microorganismos inoculados como:

Szulc *et al*., 2014 bioaumentaron un suelo impactado por 10,000 ppm de diésel con un consorcio microbiano, y bioestimulación mediante BIODET ramnolipidos, que después de 365 días señalaron que los microorganismos lo redujeron a 937 ppm.

Yanto & Tachibana en el 2014 bioaumentaron un suelo estéril impactado por 30,000 ppm de HICO, con microorganismos no especificados y bioestimularon con DEGE Tween® 80 Después de 60 días mostraron que el DEGE emulsificó los HICO y los microorganismos oxidaron en un 80 % los HICO alifáticos, 19.2 % asfáltenos y 21.7 % de resinas.

c) Bioaumentación y bioestimulación con detergente o biodetergente y nutrientes.

En la literatura se encuentran trabajos de bioaumentación de suelos contaminados por HICOS, con bioestimulación por medio de DEGE o BIODET y solución mineral. Tales estudios reconocen que la única función del detergente es solubilizar los HICO, mientras que la mineralización de la concentración fue por los microorganismos inoculados estimulados por la solución mineral, como:

Yan *et al*., 2016 bioestimularon un suelo impactado por 13,000 ppm de diésel mediante DEGE Tween® 80, solución mineral y bioaumentación con un consorcio microbiano, que después de 50 días reportaron una reducción del diésel a 6,903 ppm. Otro ejemplo es Xue *et al*., 2020 que realizaron bioestimulación de suelo impactado con 35,000 ppm de HICO mediante BIODET ramnolipido, solución mineral con NH_4NO_3, KH_2PO_4 oxigenación y bioaumentación con un consorcio microbiano, después de 5 semanas registraron una reducción de HICO a 7,000 ppm. Los dos trabajos señalan que el DEGE y BIODET se aplicaron para solubilizar los contaminantes, para ser disponibles a la oxidación por la acción microbiana que solo es posible si se estimula con nutrientes esenciales como N y P.

Conclusiones

En este estudio se aclaró que, en la biorremediación de suelo impactado por HICOS, el DEGE o BIODET son necesarios al inicio únicamente para emulsificar los HICO y que los microorganismos estimulados por solución mineral los oxiden. Es necesario seleccionar las mezclas adecuadas de detergentes que sean químicamente afines a la composición química de los HICO que contaminan el suelo. Por su parte en la bioaumentación de suelo impactado por HICO, la única forma de comprobar la síntesis de BIODET en el suelo, es con técnicas que los detecten y cuantifiquen, como el marcaje molecular de los microorganismos. Es importante aclarar el papel de cada componente de la biorremediación, para evitar errores y malinterpretaciones en los ensayos, y obtener una recuperación de suelo eficiente.

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vi. HIPOTESIS

La biorremediación de un suelo contaminado por ARA mediante la eficaz emulsificación con detergentes sintéticos y microbianos logra la rápida eliminación de la mezcla de hidrocarburos por los microorganismos heterotróficos aerobios.

vii. OBJETIVO GENERAL

Biorremediar un suelo contaminado por ARA mediante la eficaz emulsificación con detergentes sintéticos y microbianos para la rápida eliminación de la mezcla de hidrocarburos por los microorganismos heterotróficos aerobios.

viii. OBJETIVOS

1. Analizar el rol del detergente Roma® en la bioestimulación del suelo impactado por ARA

2. Mejorar la bioestimulación de suelo impactado por ARA por emulsificación con Tween 80, Triton X-100 y dodecil sulfato de sodio, seguida de solución mineral.

3. Bioestimular, bioaumentar y fitorremediar un suelo impactado pro 60,000 ppm de ARA, para decrecer la concentración por debajo del límite máximo de la NOM-138.

4. Analizar que el ARA y el aceite lubricante son inductores de la síntesis de biodetergentes y de la actividad lipolitica.

5. Optimizar la síntesis de biodetergentes crudos y la actividad lipolitica de géneros y especies de bacterias oxidantes de ARA.

6. Caracterizar parcialmente el tipo de biodetergentes sintetizados por ese grupo de bacterias.

7. Analizar la bioestimulación de suelo impactado por ARA con detergentes comerciales y solución mineral.

8. Mejorar la bioestimulación de suelo impactado por ARA con Tween 80, Triton X-100, extractos crudos de biodetergentes, lipasas, plus solución mineral para reducir el ARA a una concentración inferior al límite máximo permitido de la NOM-130

ix. CAPITULO II. En la bioestimulación de suelo impactado por hidrocarburos, el detergente emulsifica, su eliminación depende de los microorganismos inducidos por la solución mineral

In the Biostimulation of Soil Impacted by Hydrocarbons the Detergent Emulsifies Them the Elimination Depends on Microorganisms Induced by Mineral Solution

Blanca Celeste Saucedo-Martínez [a],
Liliana Márquez-Benavides [b]
and Juan Manuel Sánchez-Yáñez [a*]

DOI:

ABSTRACT

The objective of this work was to analyze the role of Roma® detergent in the biostimulation of soil impacted by Waste motor oil (WMO). WMO is a soil contaminant that contains insoluble hydrocarbons, so it must necessarily be biostimulated with a detergent to emulsify it. However, some research erroneously assumes that the detergent, in addition to emulsifying the hydrocarbons, also helps to mineralize them. For this, the soil and sand were polluted by 60,000 ppm of WMO in respirometer-type microcosms, there biostimulated by Roma®, a mineral solution and hydrogen peroxide (H_2O_2) at 0.5%. Carbon dioxide (CO_2) and WMO concentration was quantified by Soxhlet and detergent by the methylene blue method. The experimental data were validated by Tukey HSD ANOVA. The biostimulated soil by Roma®, the mineral solution at 50 % and H_2O_2, generated the highest generation of 17.6 mg CO_2 100 g dry sand^{-1}, at 72 days and the maximum decrease of WMO up to 12,364 ppm, compared to the biostimulated soil only with Roma ® that registered 11.29 mg CO_2·100 g dry sand^{-1} and decreased WMO to 34,708 ppm. In the sterile sand polluted by WMO biostimulated by Roma®, the CO_2 registered a value of 1.1 mg · 100 g of dry sand^{-1}. In soil biostimulated by Roma® alone, 0.4 to 0.31 mL ·100 g of dry soil^{-1} was registered. These results demonstrate that Roma® detergent only emulsifies WMO for oxidation by aerobic heterotrophic microorganisms if biostimulated with a 50% mineral solution. This work shows that the detergent, according to its chemical

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composition, only emulsifies the WMO and maintains that it is the first obligatory action of the biostimulation and the oxidation of the WMO depends directly on the aerobic heterotrophic microorganism's native to the soil if they are biostimulated by a mineral solution.

Keywords: soil; sand; hydrocarbons; aerobic heterotrophic microorganisms; commercial surfactant; oxidation.

1. INTRODUCTION

Waste motor oil (WMO) is classified as a hazardous waste according to the General Law for Ecological Balance and Environmental Protection [1]. WMO is toxic to flora and fauna, which negatively affects soil diversity and density and, consequently, soil fertility is lost. WMO is a mixture of polycyclic, branched, cycloalkanes and linear aliphatic aromatic hydrocarbons [2], insoluble [3], so at the beginning of biostimulation a detergent is necessary to emulsify them in order to be oxidated [4]. In the literature, an inconsistency is reported that, in the biostimulation of WMO-impacted soils, the detergent is necessary for this mixture to mineralize [5,6]. However, based on the chemical composition of any detergent, this idea is incorrect. Mainly because the native aerobic heterotrophic soil microbiota can only oxidize WMO if it is biostimulated by essential minerals. The objective of this work was to analyze the role of Roma® detergent in the biostimulation of soil impacted by WMO and a mineral solution.

2. MATERIALS AND METHODS

2.1 Soil Sample Collection and Preparation

Soil collected from a site located at 19° 37' 10" north latitude 101° 16' 41.99" west longitude, at an altitude of 2013 m above the sea with a temperate climate in an agricultural zone called "Uruapilla" in the municipality of Morelia, Mich., on the Morelia-Pátzcuaro highway, Mexico. Subsequently, it was transferred to the Environmental Microbiology Laboratory of the Instituto de Investigaciones Químico-Biológicas (IIQB) of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH). Before starting the experiment, a physicochemical analysis of the soil was performed according to NOM-021-SEMARNAT-2000. For the sand and soil experiment they were impacted by 60,000 ppm WMO and biostimulated with detergent, mineral solution and H_2O_2. Two trials were prepared in triplicate, with a randomized block experimental design in microcosms, Bartha respirometer type: the first with 100 g of non-sterile and sterile sand to check when organic matter disappears, specifically heterotrophic soil microorganisms, the detergent only emulsifies the hydrocarbons, but does not mineralize the WMO; in the second on the soil, in both the particle size of uniform texture was adjusted by sieve number 20. The first experiment was in sterile sand, a component of the soil texture, without inorganic or organic nutrients, as well as without living microorganisms; chemical and biological factors necessary for the oxidation of WMO, under these conditions WMO does not emulsify due to the absence of detergent or the biodetergents of native microorganisms. Therefore, without biostimulation by mineral solution, the WMO is not mineralized. To eliminate aerobic heterotrophic microorganisms, the sand was sterilized at 121°C/1h in an autoclave. However, as is well known, WMO contains microorganisms due to contact with the environment and handling due to change of automotive oil, which, given its chemical composition, cannot be sterilized by moist heat or autoclaving, nor filtered with Millipore membranes due to its density. Therefore, ciprofloxacin, a broad-spectrum antibiotic, in addition to tecto 60, a fungicide, and the antiprotozoal: metronidazole, 1% (w/v) were added to inhibit heterotrophic microbial activity in the WMO. The experimental design of the first experiment consisted of: in a negative control 1 unsterilized sand impacted by WMO, negative control 2, sterile sand impacted by WMO, an absolute control or unsterilized sand not contaminated by WMO irrigated with water. Treatment 1 (T1) sterile sand impacted by WMO biostimulated with 50% mineral solution with the following chemical composition (g/L): NH_4NO_3 10, K_2HPO_4 2.5, KH_2PO_4 2, $MgSO_4$ 1, NaCl 0.1, $CaCl_2$ 0.1, $FeSO_4$ trace, with trace element solution (10 ml), pH 6.5-6.8. Treatment 2 (T2) sterile sand impacted by WMO and biostimulated with a commercial detergent called Roma® at 0.4% containing sodium alkylbenzene sulfonate, as active ingredient, processing aids and fragrance. In the second experiment, a complete soil with common physical, chemical and biological properties containing aerobic heterotrophic oxidizing OMM microorganisms was used. This assay was conducted to demonstrate that WMO mineralization depends on these microorganisms being biostimulated with a mineral solution. The experimental design of the second experiment was: an absolute control of soil without WMO irrigated only with water, a soil impacted by WMO without biostimulation or negative control. Treatment 1 (T1) soil impacted by WMO, biostimulated with 0.4% Roma®. Treatment 2 (T2) soil impacted by WMO, biostimulated with 100% mineral solution. Treatment 3 (T3) soil impacted by WMO, biostimulated with 50% mineral solution, Treatment 4 (T4) soil impacted by WMO, biostimulated with 0.4% Roma®, 50% mineral solution and 0.5% H_2O_2 as an O_2 source. In both experiments, WMO mineralization was indirectly measured by CO_2 quantification every third day for 72 days [7], the change in WMO concentration was quantified by Soxhlet at the beginning and end of the experiment [8].

2.2 Statistical Analysis

The experimental data were analyzed by Tukey HSD at 0.05 % and using the Statgraphics Centurion XVI.II program [9].

3. RESULTS AND DISCUSSION

Table 1 shows the physicochemical properties of the soil before the WMO was added, there it is observed that the total nitrogen (N) content was 0.32% and phosphorus (P) with 219.34. From what the mineral solution was adjusted to 50% to ensure after the biostimulation with the detergent it was feasible to eliminate the WMO [10].

Table 1. Physicochemical properties of soil without waste motor oil

Parameter	Value	Interpretation
pH	5.67	Moderately acid
Organic matter (%)	10.44	Very high
Texture (%)	31.8 (clay), 26.92 (sand), 42.0 (silt)	Silty-clay
Total, Nitrogen (%)	0.32	Medium
Phosphorus ppm	219.34	Very high
Sodium (Na+) ppm	153.38	High
Potassium (K+) ppm	168.61	High
Microelements (ppm):		
Iron (Fe2+)	13.91	High
Zinc (ZN 2+)	0.37	Low
Copper (Cu 2+)	0.54	Low
Manganese (Mn 2+)	4.62	Low

Fig. 1 shows the production of CO_2 in sterile sand impacted by WMO biostimulated with Roma® after 72 days where a low numerical CO2 value was recorded at 0.95 mg CO2 100 g dry sand^{1}. The sand sterilization eliminated the native heterotrophic aerobic microorganisms and prevented the mineralization of the WMO, although the Roma ® detergent emulsified a part of the WMO by the microorganisms that contaminated this hydrocarbon mixture and that the added antimicrobials did not inhibit [11]. While production of CO_2 in the sterile sand impacted by WMO biostimulated with the 50% mineral solution without ROMA detergent registered 1.39 mg CO_2 100 g dry sand^{-1}, which supports that the lack of WMO emulsification limited mineralization. In the non-sterile sand impacted by WMO, used as a negative control, 1.54 mg CO_2 100 g dry sand^{-1} was recorded by the native aerobic heterotrophic microorganisms that contaminated the WMO and that the nature of the mixture prevented the activity of the added inhibitors. The chemical composition of Roma® detergent only allowed to emulsify the WMO, but not the mineralization of the WMO, which is dependent on native aerobic heterotrophic microorganisms biostimulated by the mineral solution.

Fig. 2 shows that the soil impacted by WMO biostimulated with the commercial detergent Roma®, 50% mineral solution and H_2O_2, registered the maximum CO_2 production with 17.6 mg CO_2 100 g of dry soil^{-1}, this Roma® detergent emulsified the WMO, so that the native aerobic heterotrophic microbes biostimulated by the mineral solution and the H_2O_2 which ensured a sufficient supply of O_2 to obtain maximum oxidation of the WMO. The numerical value of 17.6 mg had a statistical difference compared to the soil biostimulated only with Roma® detergent, which registered 11.29 mg CO_2/100 g dry soil^{-1}, which showed that the detergent alone emulsifies the WMO. It is not possible for WMO to be consumed, in principle, due to its chemical composition, as well as insufficient mineral concentration for the aerobic heterotrophic microbiota to oxidize WMO [12]. In the uncontaminated soil by the WMO irrigated only with water or absolute control, 4.4 mg CO_2 /100 g dry soil^{-1} were registered, due to mineralization of the organic matter of the soil by the heterotrophic aerobic microbiota; this CO_2 value was subtracted from the soil impacted by WMO to adjust for the CO_2 from WMO oxidation. Unlike the soil impacted by WMO irrigated only with water, without biostimulation, which reached 11 mg CO_2 /100 g of dry soil^{-1}. These results support that the generation of CO_2 in WMO impacted soil biostimulated by ROMA detergent or native aerobic heterotrophic microorganisms biostimulated by the 50% mineral solution favored the maximum oxidation of the WMO with the greatest release of CO_2 [13].

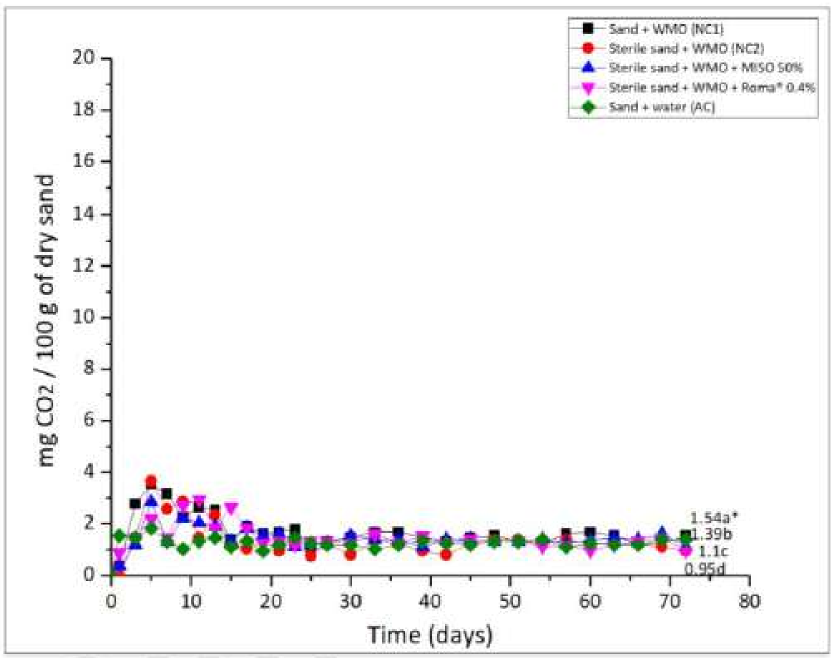

Fig. 1. CO_2 production in sand impacted by waste motor oil biostimulated by Roma® and mineral solution

** Different letters indicate statistical difference. ANOVA Tukey 0.025 WMO: Waste Motor Oil, AC: Absolute Control, NC: Negative Control, MISO: Mineral Solution*

Table 2 shows the maximum decrease of WMO concentration in the impacted soil that after 72 days of biostimulation with Roma® detergent, 50% mineral solution and H_2O_2; there it was recorded that it decreased from 60,000 to 12,364 ppm of WMO, which is equivalent to 79.4% mineralization. The numerical values of WMO concentration from 60,000 to 12,364 ppm were statistically different from the numerical value registered in the biostimulated soil only with Roma® detergent, that decreased to 34,708 ppm of WMO, equivalent to 42.1% mineralization. In the soil impacted by WMO no biostimulated, or negative control, a total of 46,565 ppm of WMO that is equivalent to 22.4% of WMO mineralization due to the natural attenuation effect with respect to biodetergents and lipases by native soil microorganisms. In the soil impacted by WMO, the results recorded agree with the amount of CO_2 released, its shows that Roma® detergent contains sodium alkyl benzenesulfonate as an active ingredient [14], which emulsified the WMO, so that native aerobic heterotrophic microorganisms biostimulated by the 50% mineral solution were able to oxidize the maximum concentration of WMO [15]. In contrast, in the WMO-impacted soil biostimulated by 100% mineral solution, it inhibited WMO oxidation, since soil nitrogen and phosphorus were added by the mineral solution to the existing one in the soil [16]. All WMO concentration values were higher than the limit allowed by NOM-138, so the experiment will continue for a few weeks until the soil is registered according to that standard for a value lower than 4,440 ppm.

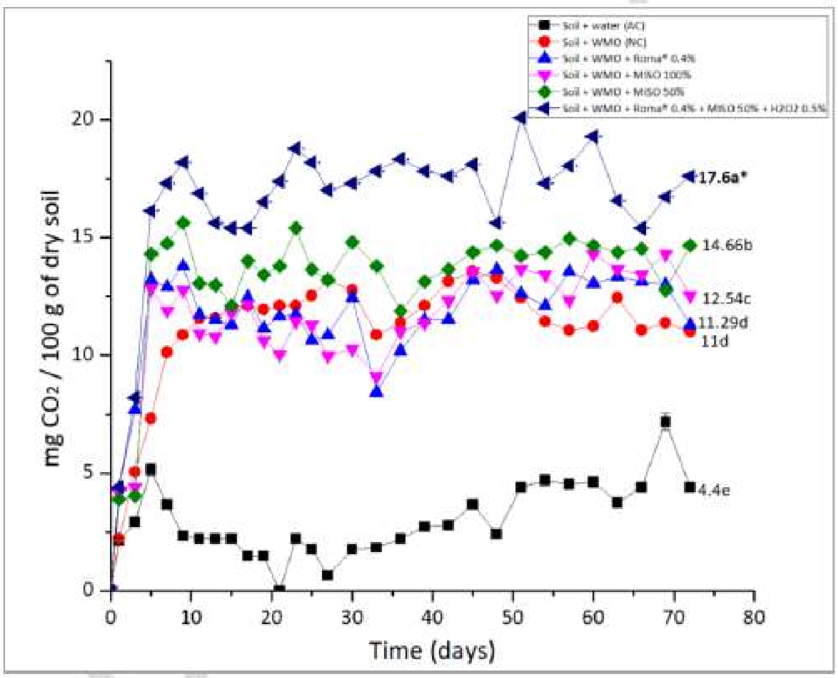

Fig. 2. CO_2 production in soil impacted by waste motor oil biostimulated with Roma® 0.5%, mineral solution and H_2O_2

** Different letters indicate statistical difference. ANOVA Tukey 0.025 WMO:Waste Motor Oil, AC: Absolute Control, NC: Negative Control, MISO: Mineral Solution*

Table 3 shows the detection of the detergent sodium alkyl benzene sulfonate in the soil impacted by WMO biostimulated with the detergent, mineral solution and H_2O_2, after 72 days of bioremediation at 654 nm and an absorbance of 0.627. These results show that the detergent at the end of the experiment was only partially degraded. Its shows that it did not participate in the mineralization of WMO, but only emulsify it. In the impacted soil biostimulated by mineral solution at 50% and

100% and no biostimulation with detergent, a minimal amount of anionic detergent was detected, suggesting that it was due to the biodetergents synthesized by the soil microorganisms [17,18-21].

Table 2. Waste motor oil concentration in soil impacted after 72 days of biostimulation

Soil	Waste motor oil concentration after 72 days (ppm)	Percentage of mineralization
WMO (negative control)	46,565^{d}	22.4^{d*}
WMO + detergent	34,708^{c}	42.1^{c}
WMO + mineral solution 50%	16,256^{b}	72.9^{b}
WMO + mineral solution 100%	34,372^{c}	42.7^{c}
WMO + detergent + mineral solution 50% + H_2O_2	12,364^{a}	79.4^{a}

** Different letters indicate statistical difference. ANOVA Tukey 0.025 WMO: Waste Motor Oil*

Table 3. Detergent concentration in soil impacted by 60,000 ppm waste motor oil before and after 72 days of biostimulation

Soil	Detergent concentration (ml/ 100 g soil)
Initial:	
WMO + detergente	0.4^{a*}
Final:	
WMO + detergent	0.31^{b}
WMO + mineral solution 50%	0.09^{c}
WMO + mineral solution 100%	0.08^{c}
WMO + detergent + mineral solution 50% + H_2O_2	0.29^{b}

** Different letters indicate statistical difference. ANOVA Tukey 0.025 WMO: Waste Motor Oil*

4. CONCLUSIONS

This work supports that the precise function of the detergent is to emulsify the WMO at the beginning of the biostimulation while oxidation dependent on the soil microorganisms induced by mineral solution as well as other activities from these microorganisms like lipases to eliminate WMO

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Research projects at the Second Meeting of Young Researchers of the State of Michoacán 2013. She was selected as a participant in the III workshop on Professional Skills for Young Scientists in 2016. She is a specialist in Bioremediation of soils impacted by hydrocarbons, solid waste and plant growth promoting bacteria. She is author and co-author of 28 Scientific Papers in recognized international scientific journals. She is speaker at national and international scientific congresses and evaluator of national projects. She is professor in the career of Biochemical Engineering at the Instituto Tecnológico de Morelia.
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Special Award (If any): She was received Dr. Santiago Cendejas Huerta Award for Master's Thesis in Engineering in 2017.

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x. CAPITULO III. Remoción biológica de aceite residual automotriz de un suelo agrícola por detergentes y solución mineral

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Wings to the Research

Research Article

Biological Removal of Waste Motor Oil from an Agricultural Soil by Detergents and Mineral Solution

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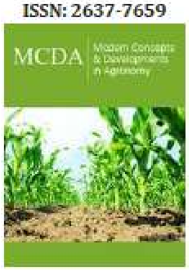

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Abstract

Agricultural soil polluted by Waste Motor Oil (WMO) due to machinery used for plant production, causes loss of its productivity. WMO is chemical composed of an insoluble mixture of aliphatic, aromatic and polycyclic hydrocarbons. To solve this problem, it is necessary to start biostimulation with a mixture of detergents chemically compatible with WMO hydrocarbons, following by biostimulation applying mineral solution according to the physicochemical properties of the agricultural soil. Therefore, the aim of this work was to improve biostimulation of WMO polluted soil by emulsification with Tween 80®, Triton X-100® and sodium dodecyl sulfate, followed by a mineral solution. In that sense, the agricultural soil polluted by 60,000ppm of WMO in Bartha flasks was first biostimulated applied Tween 80®, Triton X-100® and sodium dodecyl sulfate, and then with a mineral solution. The response variables were carbon dioxide (CO2) production and final WMO concentration. Gas chromatography was used to determine the hydrocarbon type of the WMO before and after biostimulation of soil polluted by WMO. The experimental data were validated by Tukey HSD ANOVA. The results showed that the agricultural soil impacted by WMO biostimulated applied Tween 80®, Triton X-100® and mineral solution, after 45 days, registered 12.76mg of CO2/100g of dry soil corresponding a WMO decreasing from 60,000 to 11,000ppm, numerical values statistically different compared to the soil impacted by WMO not biostimulated with detergents and no biostimulated with mineral solution, used as negative control registered 6.96mg of CO2/100g of dry soil, corresponding to WMO decreased from 60,000 to 56,000ppm. The chromatogram of soil impacted by 60, 000ppm WMO after biostimulation with Tween 80®, Triton X-100® and mineral solution showed the disappearance of short and long chain aliphatic hydrocarbons up to 35 carbons, as well as low and high molecular weight aromatics up to 26 carbons. These results support that in agricultural soil contaminated by WMO biostimulation has to start with a detergent mixture for maximum emulsification that facilitates biostimulation with a mineral solution designed according to the chemical properties of the soil.

Keywords: Soil; Hydrocarbons; Amphoteric; Heterotrophic aerobic microorganisms; Mineralization

Introduction

Waste Motor Oil (WMO) is one of the most common agricultural soil contaminants due to machinery used during plant cultivation. WMO are chemicals composed of an insoluble mixture of aliphatic, aromatic, and polycyclic hydrocarbons [1]. For WMO to be efficiently emulsified, it is necessary to initiate biostimulation with a mixture of detergents chemically compatible with WNO hydrocarbons. Subsequently the WMO can be mineralized by biostimulation of the soil with a mineral solution depending on the physicochemical properties of the soil related to the Carbon: Nitrogen (C: N) ratio as well as other essential minerals used by heterotrophic aerobic microorganisms native to this soil [2]. In the literature review report, between 80-90% of the related investigations use a single type of detergent, such as Tween 80®, whose composition is insufficient to emulsify most of the variety of hydrocarbons [3-5]. Therefore, the aim of this work was to improve biostimulation of soil polluted by WMO with Tween 80®, Triton X-100® and sodium dodecyl sulfate, followed a mineral solution.

Materials and Methods

Soil sample collection and preparation

Soil sampling was carried out based in NOM-021- SEMARNAT-2000 (NOM-021) [6] using the zigzag method at adepth of 20cm, from an agricultural area in the municipality of Morelia, Michoacán, Mexico, called "Uruapilla". To avoid pests and diseases, the soil was solarized for 48 hours and sieved with a No. 10 mesh. Then, a physicochemical analysis of the soil was carried out according to NOM-021 before polluting agricultural soil by WMO.

Biostimulation experiment of soil polluted by 60,000 ppm WMO with Tween 80®, Triton X-100®, SDS® and with 50% mineral solution

To demonstrate that detergent mixing is necessary for WMO emulsification in soil biostimulation, a triplicate randomized block experiment was conducted in Bartha flasks with 100g of soil. Then the soil was contaminated by 60, 000 ppm WMO from an automobile mechanical workshop in the city of Morelia, Mich, Mexico. The volume of the WMO was measured with a glass pipette and poured into the soil to contaminate it. To start the soil was biostimulated with Tween 80®, Triton X-100®, SDS® and 50% mineral solution. The mineral solution had the following composition (g/L): NH_4NO_3 10, K_2HPO_4 2.5, KH_2PO_4 2, $MgSO_4$ 1, NaCl 0.1, $CaCl_2$ 0.1, $FeSO_4$ traces, trace elements solution 10 and pH 6.5-6.8 according to the physicochemical agricultural soil proprieties based in organic matter, total nitrogen and phosphates concentration. In Bartha flasks with the contaminated soil, 9ml of 0.1N NaOH was introduced into the flask reservoir. The flasks were kept in incubation at 30 °C for 45 days. The indirect response variable of WMO metabolism was the released carbon dioxide (CO_2) derived from biological activity and trapped by NaOH [7]. The mineralization of the WMO in the agricultural soil was complemented with the determination of the concentration of its hydrocarbons that disappeared by Soxhlet technique [8]. Experimental design is shown in Table 1.

Table 1: Experimental design of biostimulation of soil impacted by 60,000ppm WMO applied detergents and mineral solution.

Soil	Waste Motor Oil	Tween 80® (0.5%)	Tritón X-100® (0.5%)	SDS® (0.5%)	Mineral Solution (50%)
Absolute control	-	-	-	-	-
Negative control	+	-	-	-	-
T1	+	+	-	-	+
T2	+	-	+	-	+
T3	+	-	-	+	+
T4	+	+	+	-	+
T5	+	+	-	+	+
T6	+	-	+	+	+
T7	+	+	+	+	+

Added= (+) no added= (-)

Identification of WMO hydrocarbons before and after biostimulation of contaminated soil

Soil samples impacted by WMO before and after biostimulation with detergents and mineral solution were analyzed in an Agilent Technologies 7890A series gas chromatograph coupled to 5975C series masses, 1.0µL of sample was injected in Spitless mode, helium (99.995% purity) was used as carrier gas in a Zebron capillary column. 5 MS 30.0m long, with an internal diameter of 0.25mm and a film thickness of 0.25mm. The injector temperature was 250 °C and the initial oven temperature was 50 °C with a ramp of 30-150 °C, ramp 2 of 10 °C c/min up to 310 °C/2min. The temperature detector was 280 °C with an equilibration time of 3 min and a maximum temperature of 320 °C (modified method of Peng et al. [9]).

Statistical Analysis

Differences in experimental data between groups were confirmed by one-way Analysis of Variance (ANOVA) and the Tukey HSD test ($\alpha = 0.05$) with the Statgraphics Centurion XVI.II program [10].

Results and Discussion

The physicochemical analysis of the soil proprieties is shown in Table 2, where total nitrogen (N) had a mean value of 0.32 % and phosphates (PO_4^{-3}) with a high value of 219.34ppm, (NOM-021-SEMARNAT-2000), these properties indicated that the soil required to be biostimulated with mineral solution at 50 %, to induce native heterotrophic aerobic microorganisms to eliminate WMO [11]. According to Figure 1, in agricultural soil polluted by WMO before biostimulation by detergents and mineral solution it shows that it was a heterogeneous mixture of linear and branched aliphatic HCs from 12 to 35 carbons; were also detected aromatics such as benzene, phenols and naphthalene.

Table 2: Physicochemical properties of soil not polluted by waste motor oil.

Parameter	Value	Interpretation
pH	5.67	Moderately acidic
Organic matter (%)	10.44	Very high
Texture (%)	31.8 (clay), 26.92 (sand), 42.0 (silt)	Clayey silt
Total nitrogen (%)	0.32	Medium
Phosphorus ppm	219.34	Very high
Sodium (Na^+) ppm	153.38	High
Potassium (K^+) ppm	168.61	High
Microelements (ppm):		
Iron (Fe^{2+})	13.91	High
Zinc (Zn^{2+})	0.37	Low
Copper (Cu^{2+})	0.54	Low
Manganese (Mn^{2+})	4.62	Low1

1NOM-021-SEMARNAT-2000

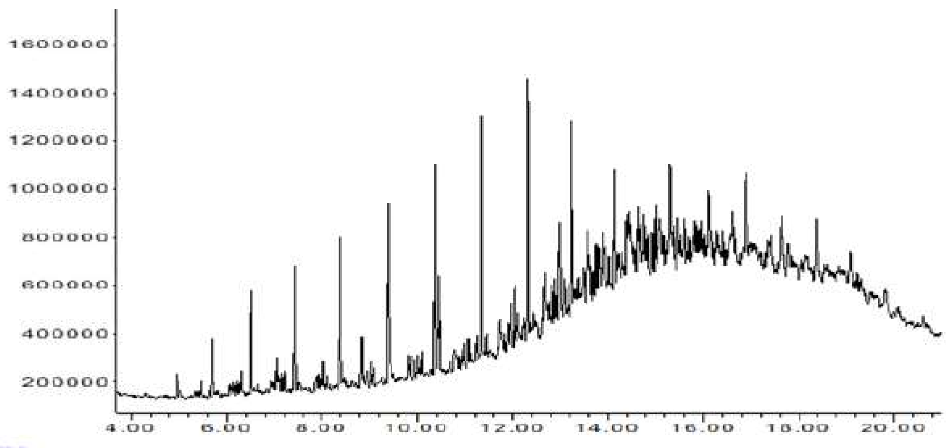

Figure 1: Chromatogram of soil impacted by 60, 000 ppm of waste motor oil before bio stimulation applied detergents and mineral solution at 50%.

Figure 2 shows biostimulation of agricultural soil polluted by WMO applied Tween 80, Triton X-100 and 50% mineral solution, by the amount of CO_2 from WMO mineralization. While the same soil not polluted by WMO irrigated only with water or absolute control registered 7.55mg CO_2/100g dry soil on day 45, derived from the mineralization of native organic matter due to aerobic heterotrophic microorganisms in any agricultural soil that, according to the physicochemical analysis, was high before the biostimulation by detergents and mineral solution. The value of CO_2 released by the mineralization of organic matter in the uncontaminated agricultural soil used as an absolute control was subtracted from the value of CO_2 in the soil polluted by WMO biostimulated by detergents and mineral solution to obtain only the CO_2 released by WMO mineralization.

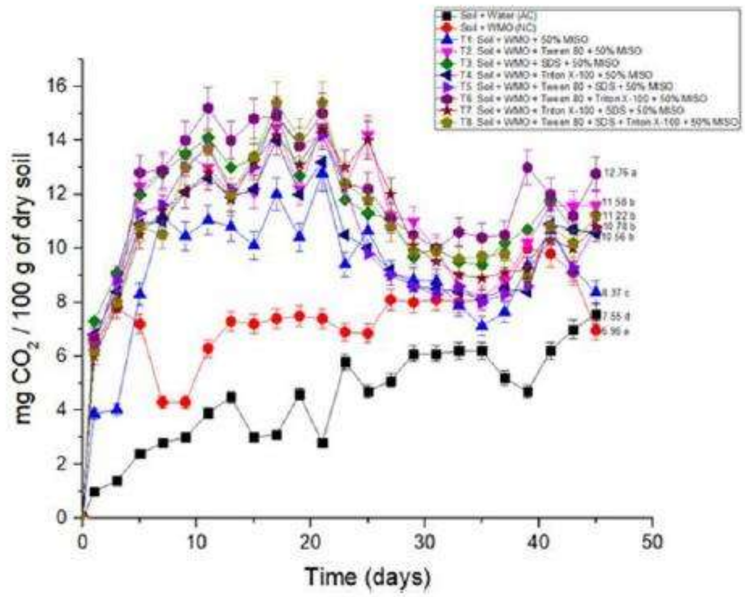

Figure 2: CO_2 production during biostimulation of soil impacted by 60,000 ppm of waste motor oil applied Tween 80®, Triton X-100®, SDS® and biostimulation with mineral solution at 50%. (WMO: waste motor oil, AC: absolute control, NC: negative control, MISO: mineral solution. Different letters indicate statistical difference).

Biostimulation soil impacted by WMO applied Tween 80®, Triton X-100® and mineral solution at 50% released 12. 76mg CO_2/100g dry soil, numerical value statistically different compared to soil polluted by WMO not biostimulated with detergents, neither mineral solution used as negative control registered 6.96mg CO_2/100g dry soil, derived from natural attenuation; compared to soil polluted by WMO biostimulated only SDS® and mineral solution at 50% that generated 11.22mg CO_2/100g dry soil.

The results support that biostimulation of agricultural soil polluted by WMO the chemical composition of Tween 80® [12] and Triton X-100® [13], were chemically compatible to emulsify WMO and improve soil biostimulation with 50% mineral solution to induced heterotrophic aerobic native microorganisms to mineralize most of the WMO as a carbon and energy source for maximum CO_2 released. At the beginning of biostimulation of soil polluted by WMO its lightest fraction of hydrocarbons was rapidly oxidized [14].

Table 3 shows during biostimulation of soil polluted by WMO applied Tween 80® / Triton X-100® and mineral solution at 50%, reached 81.6 % mineralization corresponding to maximum oxidation of WMO from 60, 000ppm to 11, 000ppm. Biostimulation of soil contaminated by WMO with detergents and mineral solution according to the chromatogram in Figure 3 shows that short, long and linear aliphatic hydrocarbons, as well as some low and high molecular weight aromatics from WMO that were compatible with Tween 80® and Triton X-100® that achieved its maximum emulsification, to improve biostimulation applied mineral solution at 50 % to enhance the ability of native heterotrophic aerobic microorganisms to mineralize WMO.

Table 3: Concentration of waste motor oil in impacted soil biostimulated applied Tween 80®, Triton X-100®, SDS® and biostimulation with mineral solution at 50%.

Soil	WMO Concentration after 45 Days (ppm)	Percentage of Mineralization (%)
WMO (Negativo control)	56,000^{f}	6.6^{e}
T1: WMO + Tween 80® + 50% MISO	18,000^{d}	70^{c}
T2: WMO + Triton X-100® + 50% MISO	19,500^{e}	67.5^{d}
T3: WMO + SDS® + 50% MISO	18,000^{d}	70^{c}

T4: WMO + Tween 80®/Triton X-100® + 50% MISO	11,000[a]	81.6[a*]
T5: WMO + Tween 80®/SDS® + 50% MISO	15,400[b]	74.3[b]
T6: WMO + Triton X-100®/SDS® + 50% MISO	17,600[c]	70.6[c]
T7: WMO + Tween 80®/Triton X-100®/SDS® + 50% MISO	15,500[b]	74.1[b]

*Different letters indicate statistical difference according to ANOVA-Tukey ($p<0.05$)

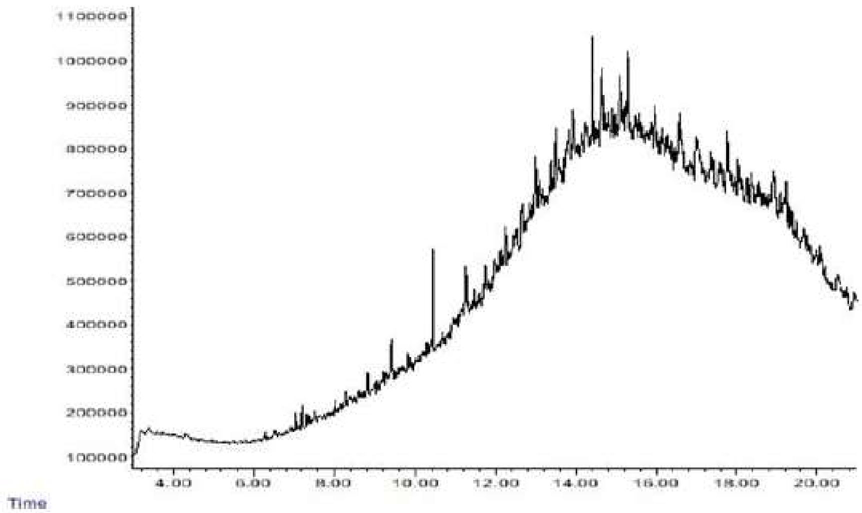

Figure 3: Chromatogram of soil impacted by 60, 000ppm waste motor oil after biostimulation applied Tween 80®, Triton X-100® and mineral solution at 50%.

The results of the soil impacted by WMO biostimulated applied Tween 80® / SDS® and then biostimulation with mineral solution at 50 %, indicating due to its chemical composition [15-16], emulsified short chain linear hydrocarbons of WMO and the biostimulation applied mineral solution at 50% induced heterotrophic aerobic microorganisms to mineralize and decrease WMO from 60,000 to 15,400ppm equivalente at 74.3% of WMO. In agricultural soil impacted by WMO biostimulated applied Tween 80® / Triton X-100® / SDS® partially emulsified its hydrocarbons then biostimulation with mineral solution at 50% to improve the effective mineralization of WMO from 60,000 to 15,500 ppm equivalent to a 74.1% reduction in its concentration. The WMO reduction values, were statistically different compared to soil impacted by WMO biostimulated only with SDS®, that has linear carbon structure Ali et al. [17] to emulsify some hydrocarbons of WMO, then biostimulation by mineral solution at 50% registered decreased from 60,000 to 18,000ppm corresponding 70% elimination of WMO. In soil impacted by WMO biostimulated only with Triton X-100® having a short carbon chain and aromatic ring, partially emulsified short chain aliphatic hydrocarbons and some low molecular weight aromatics. Followed by biostimulation of heterotrophic aerobic microorganisms applied mineral solution at 50%, that effectively used them as only carbon source and energy decrease to reduce the WMO concentration from 60,000 to 19,500ppm or 65.7 or reduction [18].

Figure 3 shows the chromatogram of soil impacted by 60, 000ppm WMO after biostimulation with Tween 80®, Triton X-100® and mineral solution at 50%, shown the disappearance of short and long chain aliphatic hydrocarbons up to 35 carbons, as well as low and high molecular weight aromatics up to 26 carbons. These facts support the results of Table 3, that demonstrated the chemical compatibility of the detergents in soil biostimulation to emulsify WMO hydrocarbons, that biostimulated by mineral solution induced a rapid mineralization of WMO by the native aerobic heterotrophic microorganisms of agricultural soil to use those WMO hydrocarbons as the only source of carbon and energy [19].

Conclusion

This research showed that in any agricultural soil polluted by a mixture of hydrocarbons of the WMO type, biostimulation has to start with detergents according to the chemical properties of these hydrocarbons to ensure that the biostimulation with mineral solution is adequate to the chemical properties of the soil for efficient and fast mineralization of WMO in order to recover its health for reuse in the production of safe food.

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xi. CAPITULO IV. Biorrecuperación de suelo agrícola impactado por aceite residual automotriz con *Phaseolus vulgaris* y *Xanthobacter autotrophicus*

plants

Article

Biorecovery of Agricultural Soil Impacted by Waste Motor Oil with *Phaseolus vulgaris* and *Xanthobacter autotrophicus*

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Abstract: Agricultural soil contamination by waste motor oil (WMO) is a worldwide environmental problem. The phytotoxicity of WMO hydrocarbons limits agricultural production; therefore, Mexican standard NOM-138-SEMARNAT/SSA1-2012 (NOM-138) establishes a maximum permissible limit of 4400 ppm for hydrocarbons in soil. The objectives of this study are to (a) biostimulate, (b) bioaugment, and (c) phytoremediate soil impacted by 60,000 ppm of WMO, to decrease it to a concentration lower than the maximum allowed by NOM-138. Soil contaminated with WMO was biostimulated, bioaugmented, and phytoremediated, and the response variables were WMO concentration, germination, phenology, and biomass of *Phaseolus vulgaris*. The experimental data were validated by Tukey HSD ANOVA. The maximum decrease in WMO was recorded in the soil biostimulated, bioaugmented, and phytoremediated by *P. vulgaris* from 60,000 ppm to 190 ppm, which was considerably lower than the maximum allowable limit of 4400 ppm of NOM-138 after five months. Biostimulation of WMO-impacted soil by detergent, mineral solution and bioaugmentation with *Xanthobacter autotrophicus* accelerated the reduction in WMO concentration, which allowed phytoremediation with *P. vulgaris* to oxidize aromatic hydrocarbons and recover WMO-impacted agricultural soil faster than other bioremediation strategies.

Keywords: soil; hydrocarbons; cometabolism; phytotoxicity; legume; endophytic bacteria

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1. Introduction

Agricultural soils are affected by mixtures of aliphatic and aromatic hydrocarbons from waste motor oil (WMO) because of spills that limit plant growth and health [1–3]. The maximum recognized environmental standard in Mexico, NOM-138 SEMARNAT/SSA1-2012 (NOM-138) [4], establishes a maximum permissible limit of 4400 ppm for hydrocarbons in soil.

Globally, agricultural soils are polluted with high levels of WMO [5]. When agricultural soil is contaminated, plants can absorb hydrocarbons, inhibit plant growth, and decrease yield over long periods. These agricultural products pose a risk to human health [6]. An ecological alternative to solve this problem is biostimulation with detergent solutions such as Tween® 80 (Sigma-Aldrich, Darmstadt, Germany) and Triton® X-100 (Sigma-Aldrich, Darmstadt, Germany) to emulsify WMO, inducing heterotrophic soil microbiota by enrichment with mineral solution (MISO) containing essential elements to decrease WMO concentration [7].

Subsequent bioaugmentation occurs when the soil is inoculated with *Xanthobacter autotrophicus*, a facultative endophytic bacteria that uses WMO as a source of carbon and energy to remove WMO [8–16]. Biostimulation and bioaugmentation are strategies for reducing WMO and phytoremediation by *Phaseolus vulgaris* and *X. autotrophicus* [17] to ensure that the final WMO concentration is below the limit accepted by NOM-138 [4]. The objectives of this study were (a) biostimulation, (b) bioaugmentation, and (c) phytoremediation of soil impacted by 60,000 ppm of WMO to decrease it to a concentration lower than the maximum allowed by NOM-138 [4].

2. Results

2.1. Physicochemical Analysis of the Agricultural Soil

Table 1 shows the physicochemical analysis of the agricultural soil.

Table 1. Physicochemical properties of agricultural soil not contaminated by waste motor oil.

Parameter	Value	Interpretation
pH	5.67	Moderately acidic (5.1–6.5)
Organic matter (%)	10.44	Very high (>6.0)
Texture (%)	31.8 (clay), 26.92 (sand), 42.0 (silt)	Clayey silt
Total nitrogen (ppm)	3200	Very high (>0.25)
Phosphorus (ppm)	219.34	Very high
Sodium (Na^+) (ppm)	153.38	High
Potassium (K^+) (ppm)	168.61	High
Microelements (ppm):		
Iron (Fe^{2+})	13.91	Appropriate (>4.5)
Zinc (Zn^{2+})	0.37	Deficient (<0.5)
Copper (Cu^{2+})	0.54	Appropriate (>0.2)
Manganese (Mn^{2+})	4.62	Appropriate [1] (>1)

[1] NOM-021-SEMARNAT-2000.

2.2. Reduction in Waste Motor Oil after Biostimulation and Soil Bioaugmentation

Table 2 shows the decreasing concentration of WMO from 60,000 to 17,599 ppm in agricultural soil (70.6% WMO mineralization) when biostimulated with Tween® 80, Triton® X-100, and 50% of MISO followed by bioaugmentation with *X. autotrophicus*. Results of Tukey HSD test ($p < 0.05$) confirmed that these values were significantly lower than those for the biostimulation of WMO-polluted soil from 60,000 to 23,088 ppm (61.52% of WMO mineralization). These values were also significantly lower than those for the agricultural soil contaminated by WMO not subjected to biostimulation or bioaugmentation (negative control) with 39,215 ppm of WMO from the initial 60,000 ppm (34.65% of WMO mineralization).

Table 2. Concentration of waste motor oil in agricultural soil after biostimulation and bioaugmentation with *Xanthobacter autotrophicus*.

Soil	WMO Concentration after 3 Months (ppm)	Mineralization Percentage (%)
Negative control soil impacted by WMO *	39,215 ± 0.06 ^{c,**}	34.65 ± 0.01 ^{c}
T1: Soil impacted by WMO biostimulated with Tween® 80 + Triton® X-100 and 50% MISO	23,088 ± 0.06 ^{b}	61.62 ± 0.03 ^{b}
T2: Soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and bioaugmented with *X. autotrophicus*	17,599 ± 0.12 ^{a}	70.67 ± 0.24 ^{a}

* WMO: waste motor oil; MISO: mineral solution. ANOVA Tukey $p < 0.05$ ** Different letters indicate significant difference, $n = 6$.

2.3. Germination of *P. vulgaris* with *X. autotrophicus* in Phytoremediation of Soil

Table 3 shows the 92% maximum germination percentage of *P. vulgaris* with *X. autotrophicus* after five days of phytoremediation of agricultural soil polluted by WMO. This percentage numerical value was significantly higher than the 83% germination of *P. vulgaris* not inoculated with *X. autotrophicus* compared to 100% germination of *P. vulgaris* in soil not polluted by WMO, fed with 100% MISO used as a relative control with 80% germination.

Table 3. Germination percentage and days to emergency of *Phaseolus vulgaris* enhanced with *Xanthobacter autotrophicus* during phytoremediation of agricultural soil impacted by waste motor oil.

Phaseolus vulgaris	Emergency Days	Germination Percentage (%)
Absolute control *P. vulgaris* without WMO * soil irrigated only water	6 ± 0.13 [b,**]	75 ± 0.15 [c]
Relative control *P. vulgaris* without WMO fed 100% MISO	6 ± 0.02 [b]	80 ± 0.10 [b]
T1: *P. vulgaris* in soil impacted by WMO + biostimulated with Tween® 80/Triton® X-100 and 50% MISO	5 ± 0.03 [a]	75 ± 0.01 [c]
T2: *P. vulgaris* in soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and bioaugmented with X. autotrophicus	5 ± 0.08 [a]	83 ± 0.03 [b]
T3: *P. vulgaris* enhanced with *X. autotrophicus* in soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and bioaugmented with *X. autotrophicus*	5 ± 0.16 [a]	92 ± 0.13 [a]

* WMO: waste motor oil; MISO: mineral solution. ANOVA Tukey $p < 0.05$ ** Different letters indicate significant difference, $n = 6$.

2.4. Phenology and Biomass of P. vulgaris Enhanced with X. autotrophicus in Soil Phytoremediation at the Seedling Stage

Table 4 shows the phenology and biomass of *P. vulgaris* at the seedling stage during the phytoremediation of agricultural soil affected by WMO. *P. vulgaris* reached the greatest plant height (PHE) of 36.42 cm, with an aerial fresh weight (AFW) of 2.13 cm, and aerial dry weight (ADW) of 0.219 cm when planted in soil previously biostimulated and bioaugmented. The above numerical values were significantly higher than those of *P. vulgaris* cultured in soil not contaminated by WMO, fed with 100% MISO, and used as a relative control with 31.27 cm of PHE, 1.82 g of AFW, and 0.16 g of ADW and *P. vulgaris* grown in agricultural soil not contaminated by WMO irrigated only with water used as an absolute control with a root length (RL) of 7.16 cm, root fresh weight (RFW) of 0.1 g, and root dry weight (RDW) of 0.016 g. The above numerical values were significantly higher than those of *P. vulgaris* grown in agricultural soil contaminated by WMO and uninoculated with *X. autotrophicus*, previously biostimulated and bioaugmented with 5.85 cm of RL, 0.04 g RFW, and 0.011 g RDW.

2.5. Phenology and Biomass of P. vulgaris Enhanced with X. autotrophicus in Soil Phytoremediation at the Physiological Maturity Stage

Table 5 shows the phenology and biomass of *P. vulgaris* at the physiological maturity stage during phytoremediation of agricultural soil polluted by WMO, as can be seen in Figure 1. *P. vulgaris* showed PHE of 108.57 cm, RL of 20.28 cm, AFW of 8.37 g, RFW of 3.61 g, ADW of 1.12 g, and RDW of 0.21 g. The above numerical values were significantly higher than those of *P. vulgaris* fed with 100% MISO growing in soil uncontaminated by WMO (relative control) with 70.56 of PHA, 18.25 cm of RL, 3.78 g of AFW, 1.24 g of RFW, 0.72 g of ADW, 0.08 g RDW. Similarly, *P. vulgaris* enhanced with *X. autotrophicus* showed 93.38 cm of PH, 17.33 cm of RL, 4.58 g AFW, 1.51 g of RFW, 0.63 g ADW, and 0.1 g of RDW.

Table 4. Phenology and biomass of *Phaseolus vulgaris* enhanced with *Xanthobacter autotrophicus* at seedling level during phytoremediation of soil impacted by remainder waste motor oil.

Phaseolus vulgaris	Plant Height (cm)	Root Length (cm)	Aerial Fresh Weight (g)	Root Fresh Weight (g)	Aerial Dry Weight (g)	Root Dry Weight (g)
Absolute control *P. vulgaris* without WMO * soil irrigated only water	$27.25 \pm 0.02^{c**}$	7.16 ± 0.06^{a}	1.46 ± 0.18^{d}	0.1 ± 0.14^{a}	0.161 ± 0.14^{c}	0.016 ± 0.10^{a}
Relative control *P. vulgaris* without WMO fed 100% MISO	31.27 ± 0.10^{b}	6.5 ± 0.23^{b}	1.82 ± 0.27^{b}	0.09 ± 0.05^{b}	0.16 ± 0.05^{c}	0.012 ± 0.18^{c}
T1: *P. vulgaris* in soil impacted by WMO + biostimulated with Tween® 80/Triton® X-100 and 50% MISO	30 ± 0.15^{b}	4.14 ± 0.27^{d}	1.39 ± 0.18^{d}	0.04 ± 0.30^{d}	0.169 ± 0.30^{c}	0.009 ± 0.15^{e}
T2: *P. vulgaris* in soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and bioaugmented with *X. autotrophicus*	36.42 ± 0.16^{a}	5.85 ± 0.05^{c}	2.13 ± 0.15^{a}	0.04 ± 0.18^{d}	0.219 ± 0.23^{a}	0.011 ± 0.06^{d}
T3: *P. vulgaris* enhanced with *X. autotrophicus* in soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and bioaugmented with *X. autotrophicus*	27.25 ± 0.18^{c}	6.58 ± 0.20^{b}	1.62 ± 0.10^{c}	0.06 ± 0.22^{c}	0.184 ± 0.10^{b}	0.014 ± 0.16^{b}

* WMO: waste motor oil; MISO: mineral solution. ANOVA Tukey $p < 0.05$ ** Different letters indicate significant difference, $n = 6$.

Table 5. Phenology and biomass of *Phaseolus vulgaris* enhanced with *Xanthobacter autotrophicus* at physiological maturity level during phytoremediation of soil impacted by waste motor oil.

Phaseolus vulgaris	Plant Height (cm)	Root Length (cm)	Aerial Fresh Weight (g)	Root Fresh Weight (g)	Aerial Dry Weight (g)	Root Dry Weight (g)
Absolute control *P. vulgaris* without WMO * soil irrigated only with water	$76.42 \pm 0.07^{d**}$	20.42 ± 0.02^{a}	2.4 ± 0.15^{e}	1.38 ± 0.23^{d}	0.71 ± 0.03^{c}	0.14 ± 0.15^{b}
Relative control *P. vulgaris* without WMO fed 100% MISO	70.56 ± 0.12^{e}	18.25 ± 0.18^{b}	3.78 ± 0.23^{d}	1.24 ± 0.11^{e}	0.72 ± 0.13^{c}	0.08 ± 0.22^{d}
T1: *P. vulgaris* in soil impacted by WMO + biostimulated with Tween® 80/Triton® X-100 and 50% MISO	84.85 ± 0.1^{c}	17.28 ± 0.12^{c}	4.18 ± 0.15^{c}	1.79 ± 0.08^{d}	0.91 ± 0.24^{b}	0.12 ± 0.14^{c}
T2: *P. vulgaris* in soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and bioaugmented with *X. autotrophicus*	108.57 ± 0.13^{a}	20.28 ± 0.35^{a}	8.37 ± 0.06^{a}	3.61 ± 0.15^{a}	1.12 ± 0.31^{a}	0.21 ± 0.04^{a}
T3: *P. vulgaris* enhanced with *X. autotrophicus* in soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and bioaugmented with *X. autotrophicus*	93.38 ± 0.12^{b}	17.33 ± 0.40^{c}	4.58 ± 0.01^{b}	1.51 ± 0.02^{c}	0.63 ± 0.10^{d}	0.1 ± 0.14^{c}

* WMO: waste motor oil; MISO: mineral solution. ANOVA Tukey $p < 0.05$ ** Different letters indicate significant difference $n = 6$.

(a) (b) (c) (d) (e)

Figure 1. Growth of *Phaseolus vulgaris* enhanced with *Xanthobacter autotrophicus* at physiological maturity during phytoremediation of agricultural soil impacted by waste motor oil. **(a)** Absolute control *P. vulgaris* irrigated with water in agricultural soil devoid of waste motor oil (WMO) contamination. **(b)** Relative control *P. vulgaris* in agricultural soil devoid of WMO contamination, fed by 100% mineral solution (MISO). **(c)** T1 *P. vulgaris* in agricultural soil impacted by WMO biostimulated with Tween® 80/ Triton® X-100 and 50% MISO. **(d)** T2 *P. vulgaris* in agricultural soil impacted by WMO biostimulated with Tween® 80/ Triton® X-100 and 50% MISO and bioaugmented with *X. autotrophicus*. **(e)** T3 *P. vulgaris* enhanced with *X. autotrophicus* in agricultural soil impacted by WMO biostimulated with Tween® 80/ Triton® X-100, 50% MISO and bioaugmented with *X. autotrophicus*.

2.6. *Final WMO Concentration in Agricultural Soil after Biostimulation, Bioaugmentation, and Phytoremediation*

Table 6 shows the final WMO concentration in agricultural soil after three months of biostimulation and bioaugmentation, after two months of phytoremediation. The maximum decrease in WMO concentration was detected in the soil biostimulated, bioaugmented, and phytoremediated with *P. vulgaris* where WMO decreased from 60,000 to 190 ppm, a value that was significantly lower than that for soil contaminated by WMO. Biostimulation, bioaugmentation, and phytoremediation with *P. vulgaris* enhanced with *X. autotrophicus* improved the WMO reduction from 60,000 to 680 ppm; the agricultural soil affected by WMO only biostimulated and phytoremediated with *P. vulgaris* showed a final concentration of 3040 ppm from an initial of 60,000 ppm. All WMO numerical concentration values were lower than the accepted maximum limit of 4400 ppm of NOM 138, and at the same time were significantly lower than that for the 35,210 ppm WMO of the negative control soil.

2.7. *Residual Hydrocarbons in WMO after Biostimulation, Bioaugmentation, and Phytoremediation*

The chromatogram (Figure 2) shows that after three months of biostimulation and bioaugmentation of soil impacted by WMO, the simplest aliphatic hydrocarbons, linear, branched short- and long-chain hydrocarbons of 12 and 14 carbons, linear hydrocarbons consisting of up to 35 carbons, and aromatics such as (1-methyl-4-(1-methylpropyl)-benzene, 2-nitro-tertiary-butanol, and 2-isopropyl-5-methyl-1 heptanol were eliminated. Aliphatics such as nonadecane, eneicosane, eicosane, and heptacosane were also eliminated, while aromatics, naphthalene, and 1-cyclohex-1-1-enyl-1-phenyl-ethanol were oxidized. The remaining hydrocarbons were some of the more complex 14-carbon and 36-carbon aromatics as well as some 17-carbon aliphatics.

Table 6. Final concentration of waste motor oil in agricultural soil after three months of biostimulation, bioaugmentation, and two months of phytoremediation.

Soil	WMO Final Concentration (ppm)
Negative control soil impacted by WMO *	35,210 ± 0.12 [d]
T1: Soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO and phytoremediated with *P. vulgaris*	3040 ± 0.01 [c]
T2: Soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO, bioaugmented with *X. autotrophicus* and phytoremediated with *P. vulgaris*	190 ± 0.03 [a,**]
T3: Soil impacted by WMO biostimulated with Tween® 80/Triton® X-100, 50% MISO, bioaugmented with *X. autotrophicus*, and phytoremediated with *P. vulgaris* inoculated with *X. autotrophicus*	680 ± 0.09 [b]

* WMO: waste motor oil; MISO: mineral solution. ANOVA Tukey $p < 0.05$ ** Different letters indicate significant difference, $n = 6$.

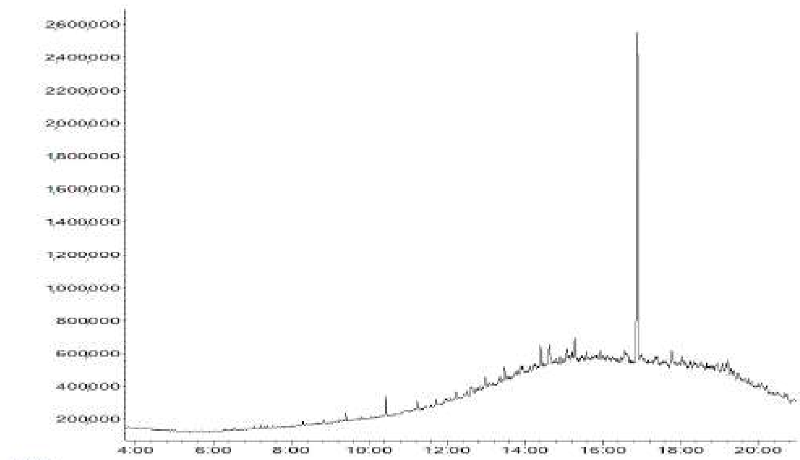


Figure 2. Chromatogram of 14 C, 36 C aromatic and 17 C aliphatic hydrocarbons in WMO after biostimulation, bioaugmentation, and phytoremediation.

3. Discussion

The physicochemical properties of the soil (Table 1) showed that the soil required biostimulation with 50% MISO to ensure that the native soil microorganisms received the maximum concentration of basic minerals to induce WMO mineralization [18].

The decrease in WMO after biostimulation and bioaugmentation (Table 2) indicates that biostimulation and bioaugmentation accelerated mineralization of WMO, as has been reported in another research [19]. First, biostimulation with a mix of detergents emulsified the WMO and 50% MISO to enable degradation of WMO by the native heterotrophic aerobic microorganisms. WMO removal was then improved by bioaugmentation with *X. autotrophicus* to decrease the concentration of WMO and facilitate phytoremediation of soil with *P. vulgaris* and *X. autotrophicus* to reduce the concentration of WMO to a value below the maximum accepted value of NOM-138 [4].

The germination percentage (Table 3) results suggested that *P. vulgaris* improved with *X. autotrophicus* increased tolerance to WMO and, therefore, provided maximum germination. While *P. vulgaris* not inoculated with *X. autotrophicus* showed symptoms of phytotoxicity to WMO and, therefore, showed a reduction in germination percentage, similar to observations reported in previous research where excess hydrocarbons inhibited seed germination for soil phytoremediation [20–22].

The growth of *P. vulgaris* at seedling level (Table 4) showed its sensitivity to WMO phytotoxicity; this response of *P. vulgaris* is used as an indicator of the degree of recovery or contamination of the soil to the concentration of WMO [15,23]. Thus, if the WMO concentrations in soil decrease, *P. vulgaris* starts growth with a pattern analogous to that observed when the soil is not contaminated with WMO in part because bioaugmentation with *X. autotrophicus* cimulation, bioaugmentation, and phytoremediaauses oxidation of aromatic hydrocarbons and, subsequently, reduction of the concentration of WMO as reported in the literature [17,19,24].

The biomass values of *P. vulgaris* at physiological maturity (Table 5) indicate healthy growth only in agricultural soils without hydrocarbons (Figure 1). This result supports the fact that phytoremediation of any agricultural soil could be applied to soils contaminated with relatively high concentration of hydrocarbons such as those of WMO, if its biorecovery begins with biostimulation followed by bioaugmentation, where *X. autotrophicus* can decrease WMO concentration and finally when *P. vulgaris* is enhanced with *X. autotrophicus*, a facultative endophyte able to invade roots to transform organic compounds of photosynthesis into phytohormones [15,25]. *X. autotrophicus* induces a root system in *P. vulgaris* with a greater capacity for the phytodegradation of WMO and simultaneously optimizes the absorption of minerals [26,27]. The above facilitates the biorecovery of agricultural soil and allows it to be safely implemented in agricultural cultivation, while ensuring that the produce obtained is safe for human and animal consumption due to a decrease in WMO equivalent to any soil free of WMO contamination [21,28].

The response of *P. vulgaris* with and without *X. autotrophicus* shows that it is possible to biorecover agricultural soil contaminated by a relatively high concentration of WMO (Table 6) [12,22,26,27]. However, it was evident that biostimulation of this soil was affected by WMO without bioaugmentation [29], followed by phytoremediation with only *P. vulgaris*. Although it reduced the concentration of WMO to a value of 3040 ppm, which was lower than the value limit recognized by NOM-138 [4], the treatment does not fully render the use of the agricultural soil for plant production because of the risk of absorption of the WMO by agricultural crops [1,2,30]. In clear contrast to the biostimulation of the soil impacted by WMO, followed by bioaugmentation with *X. autotrophicus*, which has the ability to degrade aromatics from the WMO [14,16] to continue with the phytoremediation of the WMO with *P. vulgaris*, which was housed by *X. autotrophicus* [20,25], a decrease of 190 ppm in the WMO was achieved [12,31], a concentration equivalent to that observed in soil devoid of contamination by hydrocarbon mixtures that ensures agricultural production without risk to the human or animal consumer [2,18,30]. The decrease in the concentration of WMO in the agricultural soil when biostimulated, followed by bioaugmentation with *X. autotrophicus* and then inoculation of *P. vulgaris* in phytoremediation, resulted in a final concentration of 680 ppm of WMO [32]. These concentrations are still in the concentration range detected in soil not contaminated with hydrocarbon mixtures; thus, agricultural soil is safe for agricultural production without the risk arising from consumption of produce [1]. It also indicates that the presence of an excess of *X. autotrophicus* in the soil, as in *P. vulgaris*, does not improve its ability to remove WMO [12,33]. In the agricultural soil used as a negative control, the WMO decrease was due to natural attenuation caused by soil microorganisms able to mineralize WMO according to environmental conditions depending on time [9].

The remaining hydrocarbons detected in the chromatogram (Figure 2) indicate that soil biostimulation and bioaugmentation accelerated the phytoremediation process of the impacted soil to remove most of the remaining WMO hydrocarbons by *P. vulgaris*, indicating that the use of *P. vulgaris* in association with native microorganisms and the endophyte *X. autotrophicus* removed not only aliphatic hydrocarbons but also many aromatics in the soil bioaugmentation, which maximized the removal of WMO and shortened the soil recovery time.

4. Materials and Methods

4.1. Agricultural Soil Sample Collection and Preparation

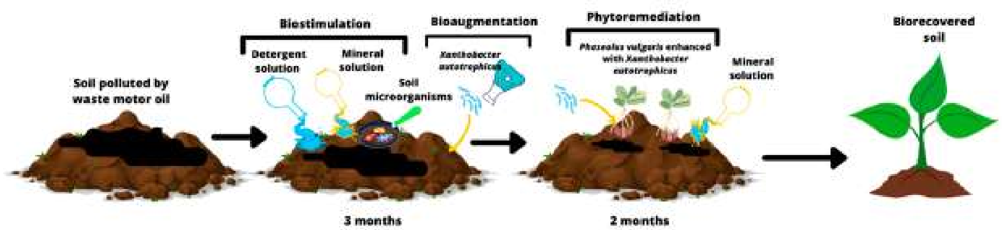
Soil was collected from a mild weather site in the municipality of Salvador Escalante, Mich, Mexico. Physicochemical analysis of the soil was performed according to the NOM-021-SEMARNAT-2000 standard [34]. The soil was solarized at 70 °C/48 h to minimize the problem of pests and diseases and sieved with No. 20 mesh.

4.2. Biostimulation and Bioaugmentation of Soil Impacted by WMO with X. autotrophicus

A sextuplicate, randomized block experiment was conducted in Leonard jars with 1 kg of soil. The soil was contaminated with 60,000 ppm of the WMO trademark Mobil® ExxonMobil, Irving, TX, USA from a mechanical workshop. The initial WMO concentration was corroborated by Soxhlet analysis, which is widely used in the literature [27]. The first step of biostimulation began with a 0.5% solution of detergents mixed with Tween® 80 and Triton® X-100, both Sigma Aldrich® (Darmstadt, Germany) and laboratory grade. The solution of these detergents at low concentrations, such as 0.5%, does not inhibit soil microbiota and promotes WMO hydrocarbon emulsification [35,36]. Simultaneously, the soil was biostimulated with 50% MISO, where the nutrient amendment was made to achieve an N:P ratio of 10:1, which is the recommended ratio range to promote and enhance hydrocarbon mineralization [37], with the following composition (g/L): NH_4NO_3 10, K_2HPO_4 2.5, KH_2PO_4 2, $MgSO_4$ 1, NaCl 0.1, $CaCl_2$ 0.1, $FeSO_4$ traces, element solution 10, and pH 6.5–6.8. The second step for soil polluted by WMO was bioaugmentation with *X. autotrophicus* that was activated on agar without nitrate and sucrose with the following composition (g/L): K_2HPO_4 1, $MgSO_4$-H_2O 0.5, KCl 0.5, $FeSO_4.7H_2O$, pH 7.2 [25], and incubated at 30 °C for 48 h. *Xanthobacter autotrophicus* was obtained from Collection of Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, Cambridge, MA, USA, which had previously been isolated from polluted soil with hydrocarbons. The microbial cells of *X. autotrophicus* were suspended in detergent saline solution (NaCl 0.85% and detergent 0.01%) at a McFarland standard level of 0.5 containing approximately 1.5×10^8 colony-forming units (CFU)/mL of *X. autotrophicus*. Then, the soil polluted by WMO was inoculated with 5 mL of *X. autotrophicus*/kg of soil, suspended in detergent saline solution for bioaugmentation according to the experimental design for three months with: absolute control of uncontaminated soil with only irrigated with water, a negative control of soil impacted by 60,000 ppm WMO, Treatment 1 (T1) soil + WMO, biostimulated with 50% of MISO, Tween® 80, and 0.5% Triton® X-100; Treatment 2 (T2) soil + WMO, biostimulated with 50% MISO, Tween® 80, and 0.5% Triton® X-100 and bioaugmented with *X. autotrophicus* (Figure 2). Measurement of the decrease in the WMO hydrocarbon concentration in the soil after biostimulation and bioaugmentation was performed as a total petroleum hydrocarbon decrease analysis using the Soxhlet extraction method. Soxhlet extraction is a classical method for the extraction of thermally stable and recalcitrant organic compounds from complex matrices [38–40].

4.3. Phytoremediation of Soil Impacted by WMO with P. vulgaris Enhanced with X. autotrophicus

Phytoremediation with *P. vulgaris* was enhanced with *X. autotrophicus* to obtain a maximum decrease of WMO still available after biostimulation and bioaugmentation in a sextuplicate in a randomized block experiment. *X. autotrophicus* was grown on agar without nitrate or sucrose [24]. Further, six uninoculated seeds of *P. vulgaris* were sown in each Leonard's jar containing 1 kg of soil with WMO that was biostimulated with 50% MISO during phytoremediation (Figure 3), and 36 seeds of *P. vulgaris* were inoculated with 5.0 mL of *X. autotrophicus*. Its concentration was adjusted to the McFarland standard tube CFU 1 equivalent to 3×10^8 colony-forming units/mL. The experimental design for two months was as follows: *P. vulgaris* in absolute control of uncontaminated soil only irrigated with water; *P. vulgaris* in relative control soil fed with 100% MISO; Treatment 1 (T1) *P. vulgaris* in soil + WMO, previously biostimulated with 50% MISO, Tween® 80, and 0.5% Triton® X-100; Treatment 2 (T2) *P. vulgaris* in soil + WMO, previously biostimulated with 50% MISO, Tween® 80, and 0.5% Triton® X-100 and bioaugmented with *X. autotrophicus*, and (T3) *P. vulgaris* inoculated with *X. autotrophicus* + WMO, previously biostimulated with 50% MISO, Tween® 80, and 0.5% Triton® X-100 and bioaugmented with *X. autotrophicus*. Germination percentage was measured at six days, and phenology and biomass were measured at the seedling and physiological maturity stages as indicators. After two months of phytoremediation, the final concentration of WMO was determined by total petroleum hydrocarbon decrease analysis using the Soxhlet extraction method [27,38–40]. Finally, soil samples with WMO were analyzed using an Agilent Technologies 7890A series gas chromatograph coupled to 5975C inert mass spectrometer (Conquer Scientific, Poway, CA, USA); 1.0 µL of the sample was injected in splitless mode, helium (99.995% purity) was used as the carrier gas in a Zebron-5MS capillary column with 30.0 m length, 0.25 mm internal diameter, and 0.25 mm film thickness. The injector temperature was 250 °C, and the initial oven temperature was 50 °C. The temperature detector was 280 °C with an equilibrium time of 3 min and a maximum temperature of 320 °C [41].



(a) (b)

Figure 3. Bioremediation of soil impacted by waste motor oil, (**a**) biostimulation: induces mineralization of WMO by soil microorganisms/ bioaugmentation: increase WMO mineralization, (**b**) phytoremediation: end of the elimination of remaining WMO.

4.4. Statistical Analysis

The statistical estimation was carried out using Statgraphics Centurion XVI.II software. ANOVA was done with six replications for each combination of the nominal variables. In the case of statistically significant data ($p < 0.05$) a post hoc Tukey honest significant difference (HSD) was used [42,43].

5. Conclusions

Bioremediation of agricultural soils impacted by WMO, through biostimulation with MISO, detergent solution, and bioaugmentation with *X. autotrophicus*, accelerated the reduction of WMO concentration, which allowed phytoremediation with *P. vulgaris* enabling oxidation of aromatic hydrocarbons. This makes it an effective soil remediation strategy that ensures the reduction of WMO to a concentration similar to that detected in non-contaminated soils, allowing sustainable and safe agricultural production for humans and animal use in addition to showing rapid results compared to other bioremediation strategies.

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xii. CAPITULO V. Optimización bacteriana de síntesis de biodetergentes y actividad lipolitica inducida por aceite residual automotriz

CAPÍTULO 9

BACTERIAL OPTIMIZATION OF BIODETERGENT SYNTHESIS AND LIPOLYTIC ACTIVITY INDUCED BY WASTE RESIDUAL OIL

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ABSTRACT: In soil contaminated by waste motor oil. It´s possible to isolate genera and species of bacteria with the capacity to synthesize biodetergents and lipolytic activity to emulsify and hydrolyze WMO. Of interesting potential value for use in the bioremediation of environments impacted by hydrocarbons. The objectives of this work were: The objectives of this work were: 1) to analyze that WMO and lubricating oil are inducers of biodetergent synthesis and lipolytic activity 2) to optimize the synthesis of crude biodetergents and lipolytic activity in these genera and bacterial species 3) to partially characterize the type of biodetergent synthesized by this bacterial group. In this sense, *Achromobacter denitrificans, Bacillus horneckiae, B. safensis, B. subtilis* and *Gordonia amicalis* isolated from soil impacted by WMO were inoculated in a mineral medium with lubricating oil and WMO as a carbon source, cultivated at 30°C and 200 rpm for 144 h compared to *Xanthobacter autotrophicus*. To detect and partially characterize anionic biodetergents and lipolytic activity, in a mineral medium with WMO. The optimization of the synthesis of biodetergents and lipolytic activity were used two sources of inorganic nitrogen NH_4NO_3, and $NaNO_3$ under agitation. For which, every 48 h / 6 days, the anionic glycolipid biodetergents, their emulsification capacity, lipolytic activity and quantification of WMO consumption as an inducer of biodetergents and lipolytic activity were detected in the crude extracts, all experimental data were analyzed by ANOVA Tukey. The results showed that *A. denitrificans* reached the highest lipolytic activity of 280.3 U/mL in mineral medium with WMO and 210.07 3 U/mL in mineral medium with lubricating oil.

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Compared with *X. autotrophicus,* that reached 275.53 U/mL induced by WMO and 165.6 U/mL induced by lubricating oil. *G. amicalis* reached the maximum production of anionic biodetergent with 1.13 ml/100 ml of mineral medium and WMO. The WMO was the best inducer for the optimization of biodetergent synthesis and lipolytic activity, thus in *A. denitrificans* with $NaNO_3$, at pH 7 and 250 rpm reached 0.3147 mg of crude biodetergents/mL, and 0.654 mL of anionics/100 mL of mineral medium compared to *X. autotrophicus* that induced by WMO, NaNO3, pH 6.5 and 250 rpm registered the highest concentration of crude biodetergent with 0.352 mg/mL, 0.7156 mL of anionic biodetergent/100 mL of mineral medium at 144 h of incubation. *A. denitrificans* induced WMO with $NaNO_3$, pH 6.5 and 250 rpm reached 94.23 U/ml. 197.63 U/mL of lipolytic activity compared to *X. autotrophicus* with and 45.16 U/mL of lipolytic activity. The highest emulsification rate of 100% was registered in the crude biodetergents of *A. denitrificans* with $NaNO_3$, at pH 6 and 250 rpm. Qualitatively, the glycolipids were the most synthesized for those genera. The WMO with NH_4NO_3 pH 6, 250 rpm, was the greatest inducer of biodetergents synthesis and lipolytic activity by *A. denitrificans*. This research showed the potential capacity of genera and species isolated from soil impacted by WMO to synthesize biodetergents and induction of lipolytic activity for an efficient application in the bioremediation of soil impacted by hydrocarbon mixtures.

KEYWORDS: Soil. Heterotrophic aerobic bacteria. Emulsification. Hydrolytic enzimes. Hydrocarbons metabolism.

1 INTRODUCTION

Biology explains that heterotrophic cells require biodetergents to emulsify nonpolar compounds like waste motor oil and then lipases to hydrolyze them for use as a carbon and energy source. Hydrocarbon mixtures are one of the main agricultural soil contaminants, such as used motor oil (WMO), that can select genera and bacterial heterotrophic species capable of emulsifying and hydrolyzing WMO. Consequently, genera and heterotrophic species of bacteria such as *Achromobacter denitrificans, Bacillus horneckiae, B. safensis, B. subtilis, G. amicalis* isolated from soil impacted by WMO have the potential ability to synthesize biodetergents to emulsify and lipase activity to hydrolyze WMO, as essential mechanisms to mineralize it (Rabbani et al., 2013; Karlapudi et al., 2018: Soumeya et al., 2022).. This implies that the synthesis of biodetergents and lipolytic activity could be artificially induced with lubricating oil or with WMO (Alam et al., 2018). The objectives of this work were: 1) to analyze that WMO and lubricating oil are inducers of biodetergent synthesis and lipolytic activity 2) to optimize the synthesis of crude biodetergents and lipolytic activity in these genera and bacterial species 3) to partially characterize the type of biodetergent synthesized by this bacterial group.

2 MATERIALS AND METHODS

2.1 ANALYSIS OF SYNTHESIS OF BIODETERGENTS AND ACTIVITY LIPOLYTIC IN GENERA AND SPECIES ISOLATED FROM SOIL IMPACTED BY WMO

Genera and species of bacteria classified as: *Achromobacter* sp, *B. horneckiae, B. safensis, B. subtilis, G. amicalis*, were growth in 5 ml of mineral medium with WMO and lubricating oil at 1% v/v as sole carbon and energy sources for the synthesis of biodetergents and lipolytic activity compared to *X. autotrophicus* isolated from agricultural soil no polluted by WMO. The WMO was obtained from a mechanical workshop in the city of Morelia, Michoacán, Mexico, while the lubricating oil was Mobil Super 15w40 Multigrade Trc-pro 946 Ml. All strain were activated on the following chemical composition (g/L): WMO or lubricating oil 10.0 5 g/L of NH_4NO_3 or $NaNO_3$; 1 g/L of K_2HPO_4, KH_2PO_4, $MgSO_4$, NaCl, 0.5 g/L of $FeSO_4$ and $CuSO_4$. Bacterial cultures were incubated at 30 °C for 48 h at 250 rpm, then 1 ml aliquots were taken and filtered on 0.22-micron membranes to obtain the cell-free supernatant in that the crude secreted enzymes were found. Crude extracellular lipases were quantified spectrophotometrically, using p-nitrophenyl palmitate (p-NPP) (Sigma-Aldrich) as substrate according to Li *et al*., 2013. In this method, the hydrolysis of p-NPP to release p-nitrophenol (p-NP) was measured. Thirty mg of p-NPP was dissolved in 10 ml of isopropanol and 90 ml of 100 mM Tris-HCl at pH 8.0. From this mixture 150 µL was taken and the reaction was initiated by adding 100 µL of the cell-free filtrate grown in mineral medium with WMO and lubricating oil at 1% v/v. After 1 h incubation at 37°C, absorbance was measured at 405 nm in a spectrophotometer against a control containing the same components except the cell-free filtrate. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per minute under the assay conditions (Pedroza-Padilla *et al*., 2017). While anionic biodetergents were measured by the spectrophotometric method with methylene blue (Selberg *et al*., 2007).

2.2 OPTIMIZATION OF THE SYNTHESIS OF BIODETERGENTS AND LIPOLYTIC ACTIVITY BY *A. DENITRIFICANS* COMPARED TO *XANTHOBACTER AUTOTROPHICUS*

To test the enhanced synthesis of biodetergents, an experiment was performed in 250 ml flasks in triplicate with mineral medium, previously, WMO was selected as the best source of carbon and energy and inducer of biodetergents and lipases activity. As a source of nitrogen, 5 g/L of NH_4NO_3 or $NaNO_3$, of yeast extract as a growth factor that was kept for all treatments, as well as 1 g/L of K_2HPO_4, KH_2PO_4, $MgSO_4$, NaCl, 0.5 g/L of $FeSO_4$ and $CuSO_4$. Then the mineral mediums were inoculated with 10 ml of *A.*

denitrificans to compare with *X. autotrophicus* in detergent saline solution individually with an inoculum density according to McFarland's number 4. The incubation temperature was 30°C, the pH used were: 6.5 and 7, in agitation at 200 and 250 rpm. To analyze the maximum concentration of biodetergents, samples were taken at 48, 94 and 144 h in plastic Falcon-type tubes with lids. Then, the possible biodetergents and lipases were extracted by centrifugation of the mineral medium at 8000 rpm for 15 minutes at 4 ° C. From the extracted supernatants, 3 ml were taken to perform the quantification of lipolytic activity by spectrophotometry, described by Li *et al*., 2013, as well as the measurement of anionic biodetergents by the spectrophotometric method by Selberg *et al*., 2007. The remaining supernatants were acidified to pH 2 with 6 M HCl and kept overnight for precipitation of biodetergents (Bharali *et al*., 2011). Final extraction of the biodetergents was performed with 2: 1 (v/v) organic solvents of chloroform and methanol according to Satpute *et al*., 2010. The solvents were evaporated at room temperature and the resulting residue was the crude biodetergents. The weight or dry weigh of the biodetergents was obtained in mg/ml (Khan *et al*., 2017).

2.3 EMULSIFICATION INDEX

The emulsification index (E24) of the crude biodetergents extracted at 144 h was determined by the method of Alvarez *et al*., 2015 modified, the cell-free supernatant was added in WMO with a ratio of 3:2 (crude biodetergents: WMO). The mixture was gently agitated and the height in cm of the emulsion layer and the total solution height after 24 h were calculated. The emulsion index was calculated using the equation:

$$\boldsymbol{Emulsification\ index\ (E24) = \frac{Emulsified\ height}{Total\ solution\ height} X100}$$

2.4 DETECTION OF GLYCOLIPID BIODETERGENTS

To 1 ml of cell-free supernatant, 1 ml of 5% (w/v) phenol was added. To this mixture, 2-5 ml of concentrated sulfuric acid was added dropwise until the characteristic color developed. The development of orange color indicated the presence of glycolipids according to Ellaiah *et al*., 2002.

2.5 BIURET TEST FOR DETECTION OF LIPOPEPTIDES AND GLYCOLIPIDS

The Biuret qualitative test was used to detect the presence of biodetergents lipopeptides and glycolipids. 2 ml of biosurfactant crude extract solution were first heated to 70 °C before mixing with 2 ml of 1M NaOH solution. Then, a drop of two milliliters of 1% $CuSO_4$ was slowly added to observe any color change; green color for glycolipid and violet color for lipopeptide (Kadhum & Haydar, 2020).

2.6 STATISTICAL ANALYSIS

The experimental data were analyzed by ANOVA Tukey HSD at 0.05 % with the program Statgraphics Centurion XVI.II (Walpole *et al.*, 2007).

3 RESULTS AND DISCUSSION

3.1 ANALYSIS OF THE SYNTHESIS OF BIODETERGENTS AND LIPASES ACTIVITY IN BACTERIAL GENERA AND SPECIES ISOLATED FROM SOIL IMPACTED BY WMO

Table 1 shows the potential capacity of biodetergents and lipases activity synthesis induced by WMO and lubricating oil. All five genera and species of bacteria synthesized biodetergents of anionic nature. *B. safensis* registered the highest value of 1.18 ml of anionic biodetergents/100 ml of medium, compared to *X. autotrophicus* registered 0.62 ml of anionic biodetergents/100 ml of medium and *A. denitrificans* with 0.65 ml of anionic biodetergents/100 ml of medium. These results show that *X. autotrophicus* synthesizes biodetergents of anionic nature compared to *G. amicalis* and *A. denitrificans* that synthesize biodetergents of the glycolipid type which are anionic kind that could be rhamnolipids or sophorolipids, composed of a hydrophobic part of lipids and a hydrophilic part composed of carbohydrates (Fenibo *et al.*, 2019; Joy *et al.*, 2019; Zargar *et al.*, 2022). While in the case of *B. subtilis* it is suggested that it synthesized Surfactin type biodetergent that is an anionic cyclic lipopeptide composed of an amino acid chain linked to a fatty acid chain (Moutinho *et al.*, 2021), that has been reported as one of the most common biodetergents synthesized by this genus and species of bacteria (Zhao *et al.*, 2020). In the case of lipolytic activity *A. denitrificans* reached the highest activity of 280.3 U/mL induced by WMO and 210.07 3 U/mL in mineral medium induced by lubricating oil, while *G. amicalis* registered lipolytic activity of 205.2 U/mL induced by WMO, and 195.6 U/mL with lubricating oil. Compared to, *X. autotrophicus* that registered a lipolytic activity of 275.53 U/mL in mineral medium induced by WMO and 165.7 U/mL in mineral medium induced by lubricating oil, numerical values statistically different from the uninoculated mineral medium where no lipolytic activity was registered.

The results show that these genera and species of bacteria synthesized a greater diversity of biodetergents and specific lipolytic activity in demand for the emulsification and hydrolysis of the wide diversity of WMO hydrocarbons such as aliphatic and aromatic hydrocarbons from 15 to 50 carbons of WMO, as well as 34 to 90 times more aromatic hydrocarbons than lubricating oil (Dominguez Rosado and Pichtel, 2003), that had registered an average hydrocarbon composition of 7 to 26 aliphatic and aromatic carbons (Yao *et al.* , 2020). These results confirm that *A. denitrificans, B. horneckiae, B. safensis, B. subtilis, G. amicalis* compared to *X. autotrophicus*, could synthesize biodetergents and lipases activity induced by WMO including an adequate temperature and oxygenation level. The results showed that these genera and species could have a potential to synthesis and application of crude extracts of biodetergents and lipases for environmental impacted by hydrocarbons mixtures to emulsify and hydrolyze them (Noro *et al.*, 2020). In that sense extract crude of biodetergents and lipases as part of the strategies for soil bioremediation polluted by WMO. They are a promising action to solve this type of problem of environmental contamination caused by mixtures of hydrocarbons (Jacob *et al.*, 2022).

Table 1. Concentration of biodetergents and lipolytic activity from *Achromobacter denitrificans, Bacillus horneckiae, B. safensis, B. subtilis, Gordonia amicalis* compared to *Xanthobacter autotrophicus* induced by lubricating oil and waste motor oil.

Bacteria	Mineral medium with lubricating oil		Mineral medium with waste motor oil	
	Concentration of anionic biodetergent (mL/100 ml of medium)	Lipolytic activity (U/ml)	Concentration of anionic biodetergent (mL/100 ml of medium)	Lipolytic activity (U/ml)
Control	0^{e*}	0^{c}	0^{e}	0^{c}
A. denitrificans	0.55^{b}	210.07^{b}	0.62^{b*}	**280.3^{a}**
B. horneckiae	0.39^{d}	124.1^{f}	0.42^{d}	190.4^{e}
B. safensis	0.44^{c}	**223.5^{a}**	**1.18^{a}**	255.4^{b}
B. subtilis	0.41^{d}	179.2^{d}	0.45^{c}	243.3^{c}
G. amicalis	**1.11^{a}**	195.6^{c}	**1.13^{a}**	205.2^{d}
X. autotrophicus	0.55^{b}	165.7^{e}	0.65^{b}	**275.53^{a}**

*Different letters are statistically different according to ANOVA-Tukey HSD at 0.05 %.

Table 2 shows the selection of the best results of the optimization of the synthesis of biodetergents and specific lipolytic activity of *A. denitrificans* at 48 h in mineral medium induced by WMO, with $NaNO_3$, pH 6.5 and 250 rpm reached 0.227 mg of crude biodetergents /mL, 0.436 mL of anionic biodetergents /100 mL of mineral medium and 194.23 U/mL of lipolytic activity. Those numerical values were statistically different compared to A. *denitrificans* using NH_4NO_3 at pH 7 at 200 rpm, that reached 0.018 mg crude biodetergents /mL, 1.044 mL anionic biodetergents /100 mL mineral medium and 132.44 U/mL lipolytic activity.

Table 3 shows the optimization of synthesis of biodetergents and lipases activity of *A. denitrificans* at 96 h in mineral medium induced by WMO, using $NaNO_3$, pH 6.5

and 250 rpm that reached 0.2804 mg of crude biodetergents /mL, 0.610 mL of anionic biodetergents /100 mL of mineral medium and 127.03 U/mL of lipolytic activity. Those numerical values were statistically different compared to those of *A. denitrificans* with $NaNO_3$, pH 7 at 200 rpm, that registered 0.2624 mg of crude biodetergents /mL, 0.5174 mL of anionic biodetergents /100 mL of mineral medium and 48.415 U/mL of lipolytic activity.

Table 4, shows the optimization of synthesis of biodetergents and lipases activity of *A.denitrificans* at 144 h in mineral medium WMO, using $NaNO_3$, pH 7 and 250 rpm registered 0.3147 mg of crude biodetergents /mL, 0.6548 mL of anionic biodetergents /100 mL of medium and 45. 165 U/mL of lipolytic activity, these numerical values had not statistical difference compared to NH_4NO_3, pH 7 and 250 rpm, when *A. denitrificans* reached 0.2977 mg of crude biodetergents /mL, 0.8425 mL of anionic biodetergents /100 mL of mineral medium and 42.695 U/mL of lipolytic activity. The results were similar when hydrocarbon mixtures were used as carbon source for the synthesis of biodetergents (Wang *et al.,* 2014; Jimoh & Lin, 2018). While for inducing lipases activity vegetable oils were very usefully such as olive oil it has been reported by Llesanmi et al., 2020. The synthesis of biodetergents by *A. denitrificans* had maximum at 144 h, in opposite way that reported in other research with *Paenibacillus* sp D9 that registered the highest synthesis after 72h of incubation (Jimoh & Lin, 2018). In that sense the highest lipases activity was detected at 48 h, then decreased, other reports also point out the maximum lipase activity in the first 12 h until 48h. These data suggest that after this time lipases activity decreases due to nutrient consumption of hydrocarbons. In the case of inorganic and organic of compounds of nitrogen source, are necessary for cell growth as well as biodetergent synthesis (Chakraborty *et al.*, 2015). In this work inorganic source of nitrogen as well as $NaNO_3$ was better one for the synthesis of biodetergents and lipases activity than NH_4NO_3, as other research reported indicated that $NaNO_3$ is one of the best inorganic compounds for the synthesis of biodetergents (Onwosi & Odibo, 2012; Amin *et al.,* 2021). While of pH, the highest lipolytic activity was detected in a pH narrow range of 6.5-7, that coincides with other studies that neutral pH 7 could be optimal for synthesis of biodetergents and lipases activity, compared to alkaline or acidic pH values, that cause drastic decrease in synthesis of biodetergents and lipases activity (Jimoh & Lin, 2018; Llesanmi *et al.,* 2020). Finally, agitation of 250 rpm affecting O_2 distribution inside of mineral medium due that biodetergents and lipases depends on aerobic metabolism, compared to lower levels of agitation values, that reduces the concentration of oxygen which inhibits the synthesis of biodetergents and lipases activity (A'yuni *et al.,* 2021; Zambry *et al*., 2021).

Table 2. Optimization of the synthesis of biodetergents and lipolytic activity from *Achromobacter denitrificans*, at 30°C at 48 h.

Run	Source inorganic nitrogen	pH	Agitation (rpm)	48 h		
				Biodetergent weight(mg/ml)	Anionic biodetergent (ml/100 ml of medium)	Lipolytic activity (U/ml)
1	NH_4NO_3	7	200	0.0187g*	**1.0441a**	132.446e
2	$NaNO_3$	7	200	0.1336e	0.5882b	116.198f
4	$NaNO_3$	6.5	200	0.2054b	0.4558e	160.346c
9	NH_4NO_3	6.5	0	0.0039h	0.5617c	153.781d
11	NH_4NO_3	6	0	0.1668d	0.58b	53.5036g
15	NH_4NO_3	6.5	250	0.125f	0.5219d	178.263b
16	$NaNO_3$	6.5	250	**0.2277a**	0.436f	**194.236a**
18	$NaNO_3$	6	250	0.1802c	0.5126d	155.356d

*Different letters are statistically different according to ANOVA-Tukey HSD at 0.05 %.

Table 3. Optimization of biodetergent synthesis and lipolytic activity from *Achromobacter denitrificans*, in mineral medium and waste motor oil at 30°C at 96 h.

Run	Inorganic source of nitrogen	pH	Agitation (rpm)	96 h		
				Biodetergent weight(mg/ml)	Anionic biodetergent (ml/100 ml of medium)	Lipolytic activity (U/ml)
2	$NaNO_3$	7	200	**0.2624a***	0.5174c	48.415f
4	$NaNO_3$	6.5	200	**0.2682a**	0.4776d	42.671g
13	NH_4NO_3	7	250	0.2022c	0.5705b	**165.762a**
14	$NaNO_3$	7	250	**0.2685a**	**0.6369a**	155.91b
15	NH_4NO_3	6.5	250	0.2475b	**0.6192a**	74.65d
16	$NaNO_3$	6.5	250	**0.2804a**	**0.6104a**	127.03c
17	NH_4NO_3	6	250	0.2287b	0.579b	73.854d
18	$NaNO_3$	6	250	0.2354b	0.5201c	68.612e

*Different letters are statistically different according to ANOVA-Tukey HSD at 0.05 %.

Table 4. Optimization of biodetergent synthesis and lipolytic activity from *Achromobacter denitrificans*, in mineral medium waste motor oil at 30°C at 144 h

Run	Inorganic source of nitrogen	pH	Agitation (rpm)	144 h		
				Biodetergent weight(mg/ml)	Anionic biodetergent (mL/100 ml of medium)	Lipolytic activity (U/ml)
1	NH_4NO_3	7	200	**0.2826a***	0.4865c	76.644b
2	$NaNO_3$	7	200	**0.2855a**	0.4776d	54.16d
4	$NaNO_3$	6.5	200	0.2742b	0.4953c	65.812c
6	$NaNO_3$	6	200	0.2122c	0.497c	**110.522a**
13	NH_4NO_3	7	250	**0.2977a**	**0.8425a**	42.695g

Run	Inorganic source of nitrogen	pH	Agitation (rpm)	144 h		
				Biodetergent weight(mg/ml)	Anionic biodetergent (mL/100 ml of medium)	Lipolytic activity (U/ml)
14	$NaNO_3$	7	250	**0.3147a**	0.6548b	45.165f
16	$NaNO_3$	6.5	250	**0.2998a**	0.6538b	46.285f
17	NH_4NO_3	6	250	0.2714b	0.6712b	51.256e

*Different letters are statistically different according to ANOVA- Tukey HSD at 0.05 %.

Table 5 shows the selection of the best results of the optimization of synthesis of biodetergents and lipolytic activity of *X. autotrophicus* at 48 h in mineral medium using WMO, NH_4NO_3, pH 6 and 250 rpm reached 0.1452 mg of crude biodetergents / mL, 0.7869 mL of anionic biodetergents /100 mL of mineral medium and 174.233 U/ mL of lipolytic activity. These numerical values had statistically different from those of *X. autotrophicus* growing in NH_4NO_3, pH 6 at 200 rpm, that reached 0.0923 mg crude biodetergents /mL, 0.4466 mL anionic biodetergents /100 mL mineral medium and 154.43 U/mL of lipolytic activity.

Table 6 shows the optimization of synthesis of biodetergents and lipolytic activity of X. *autotrophicus* at 96 h in mineral medium with WMO, using $NaNO_3$, pH 6.5 and 250 rpm reached 0.2755 mg of crude biodetergents /mL, 0.8945 mL of anionic biodetergents /100 mL of mineral medium and 115.699 U/mL of lipolytic activity. Those numerical values were statistically different compared to *X. autotrophicus* grown in $NaNO_3$, pH 7 at 200 rpm, that reached 0.2351 mg crude biodetergents /mL, 0.7269 mL anionic biodetergents /100 mL of mineral medium and 82.489 U/mL lipolytic activity.

Table 7, shows the optimization of synthesis of biodetergents and lipases activity of *X. autotrophicus* at 144 h in mineral medium plus WMO, with NH_4NO_3, pH 6 and 250 rpm reached 0.2986 mg of biodetergents /ml, 0.7569 mL of anionic biodetergents /100 ml of mineral medium and 245. 742 U/mL of lipolytic activity, these numerical values had statistical difference when grown in NH_4NO_3, pH 7 and 250 rpm, there *X. autotrophicus* reached 0.3466 mg of biodetergents /ml, 0.6426 ml of anionic biodetergents /100 ml of mineral medium and 108.544 U/mL of lipolytic activity. The synthesis of biodetergents and lipolytic activity by *X. autotrophicus* reached the maximum at 144 h. These values were similar to those reported by Khademolhosseini *et al.*, 2019, when *Pseudomonas aeruginosa* release highest biodetergent synthesis at 144 h of growth. In the case $NaNO_3$ induced the maximum synthesis of biodetergents and lipolytic activity, same as well as other studies reported that $NaNO_3$ is the best inorganic source of nitrogen to increase biodetergents and lipolytic activity (Cheirsilp *et al.,* 2021; Krishna & Chandran, 2022). While the effect of pH on the highest lipolytic activity was detected in at pH range between 6-6.5, similar data to those reported by Nema *et al.*, 2019, when *Aspergillus niger* reached the highest lipolytic activity at pH 6. In biodetergent synthesis most research point to pH 7 as the optimum (Kumar *et al.*, 2021). While in terms of agitation at 250 rpm *A. denitrificans*, *X. autotrophicus* reached the highest synthesis of biodetergents, that with higher agitation there is greater oxygenation, that allows aerobic synthesis of biodetergents and lipolytic activity (Adetunji *et al.*, 2021; Li *et al.*, 2022).

Table 5. Optimization of biodetergent synthesis and lipolytic activity from *Xanthobacter autotrophicus* in mineral medium and waste motor oil at 30°C at 48 h.

Run	Inorganic source of nitrogen	pH	Agitation (rpm)	48 h		
				Biodetergent weight(mg/ml)	**Anionic biodetergent (ml/100 ml of medium)**	**Lipolytic activity (U/ml)**
5	NH_4NO_3	6	200	0.0923b*	0.4466d	154.43b
13	NH_4NO_3	7	250	0.08562c	0.6348c	69.485e
14	$NaNO_3$	7	250	**0.1422a**	0.7458b	68.478e
15	NH_4NO_3	6.5	250	**0.1365a**	0.7655b	78.463d
16	$NaNO_3$	6.5	250	0.0952b	**0.8756a**	88.478c
17	NH_4NO_3	6	250	**0.1452a**	0.7869b	**174.233a**
18	$NaNO_3$	6	250	0.0753d	0.6421c	66.662f

*Different letters are statistically different according to ANOVA-Tukey HSD at 0.05 %.

Table 6. Optimization of the synthesis of biodetergents and lipolytic activity from *Xanthobacter autotrophicus* in waste motor oil at 30°C at 96h.

Run	Inorganic source of nitrogen	pH	Agitation (rpm)	96 h		
				Biodetergent weight(mg/ml)	**Anionic biodetergent (ml/100 ml of medium)**	**Lipolytic activity (U/ml)**
4	$NaNO_3$	6.5	200	0.2156c*	0.4555e	101.755e
5	NH_4NO_3	6	200	0.2016d	0.4326e	164.361b
13	NH_4NO_3	7	250	0.2485b	0.7129c	84.157g
14	$NaNO_3$	7	250	0.2351b	0.7269c	82.489g
15	NH_4NO_3	6.5	250	0.2214c	0.7556b	108.236d
16	$NaNO_3$	6.5	250	**0.2755a**	**0.8945a**	115.699c
17	NH_4NO_3	6	250	**0.2662a**	0.7645b	**201.455a**
18	$NaNO_3$	6	250	0.2365b	0.6148d	89.266f

*Different letters are statistically different according to ANOVA Tukey HSD at 0.05 %.

Table 7. Optimization of biodetergent synthesis and lipolytic activity from *Xanthobacter autotrophicus* in mineral medium and waste motor oil at 30°C at 144 h.

Run	Inorganic source of nitrogen	pH	Agitation (rpm)	144 h		
				Biodetergent weight(mg/ml)	Anionic biodetergent (mL/100 ml of medium)	Lipolytic activity (U/ml)
1	NH_4NO_3	7	200	0.3165c*	0.5086e	94.369g
4	$NaNO_3$	6.5	200	**0.3563a**	0.377g	89.938h
5	NH_4NO_3	6	200	0.3248c	0.4126f	178.452d
13	NH_4NO_3	7	250	0.3466b	0.6426d	108.544f
14	$NaNO_3$	7	250	0.3356b	0.6849c	145.336e
15	NH_4NO_3	6.5	250	0.2799d	0.6452d	186.266c
16	$NaNO_3$	6.5	250	**0.3526a**	0.7156b	197.636b
17	NH_4NO_3	6	250	0.2986d	**0.7569a**	**245.742a**

*Different letters are statistically different according to ANOVA Tukey HSD at 0.05 %.

Table 8 shows the best results of the emulsification index of the crude biodetergents of *A. denitrificans*, in mineral medium and waste motor oil at 30°C at 144 h. There, *A. denitrificans* grown with $NaNO_3$, at pH 6 and 250 rpm, the crude biodetergents reached 100% emulsification of WMO. Numerical per cent values were statistically different compared to crude biodetergents of *A. denitrificans* using NH_4NO_3 at pH 6 and 250 rpm reached 95% emulsification of WMO. These numerical values were statistically different compared to those detected of the control tube of water and WMO, registered 0% emulsification of WMO.

Table 9 shows the best results of the emulsification index of the crude biodetergents of *X. autotrophicus*, using $NaNO_3$ as inorganic source of nitrogen, pH 6 and 7, 200 rpm, as well as with $NaNO_3$ pH 7 and 250 rpm, the crude biodetergents registered 100% emulsification of WMO. These values were statistically higher than the WMO water control tube, with 0% WMO emulsification. These results support the results of the optimization of the synthesis of crude biodetergents due to high speed of agitation, maximum production and therefore the best rate of WMO emulsification.

Table 8. Emulsification index of *Achromobacter denitrificans* biodetergents in mineral medium and waste motor oil at 30°C at 144 h.

Run	Source of inorganic nitrogen	pH	Agitation (rpm)	Emulsification index (%)
Control (water)	-	-	-	0d*
3	NH_4NO_3	6.5	200	91c
4	$NaNO_3$	6.5	200	91c
6	$NaNO_3$	6	200	90c

Run	Source of inorganic nitrogen	pH	Agitation (rpm)	Emulsification index (%)
13	NH_4NO_3	7	250	95b
14	$NaNO_3$	7	250	90c
17	NH_4NO_3	6	250	95b
18	$NaNO_3$	6	250	**100a**

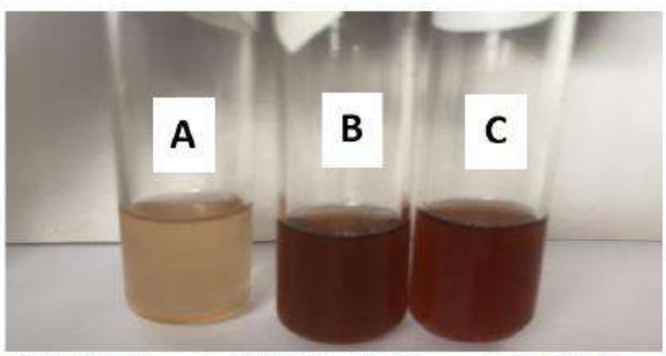
*Different letters are statistically different according to ANOVA-Tukey HSD at 0.05.

Table 9. Emulsification index of *Xanthobacter autotrophicus* biodetergents in mineral medium and waste motor oil at 30°C at 144 h.

Run+	Inorganic source of nitrogen	pH	Emulsification index (%)
Control (water)	-	-	0c*
2	$NaNO_3$	7	**100a**
6	$NaNO_3$	6	**100a**
13	NH_4NO_3	7	95b
14	$NaNO_3$	7	**100a**
15	NH_4NO_3	6.5	98a
17	NH_4NO_3	6	95b
18	$NaNO_3$	6	95b

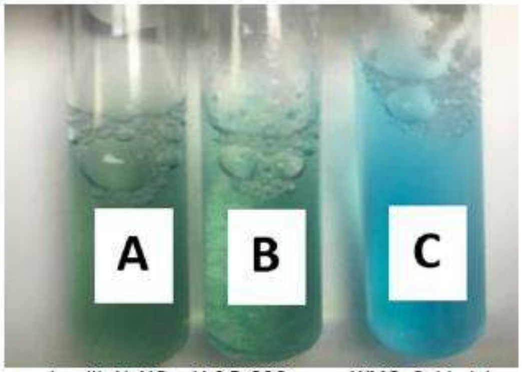
+= agitation 250 rpm *Different letters are statistically different according to ANOVA Tukey HSD at 0.05 %.

Figure 3. Detection of glycolipid biodetergents from *A. denitrificans*, in mineral medium and waste motor oil at 30°C at 144 h.



A: Control (Water + WMO), **B:** biodetergents with NH_4NO_3, pH 6, 250 rpm+ WMO, **C:** biodetergents with $NaNO_3$, pH 6, 250 rpm + WMO.

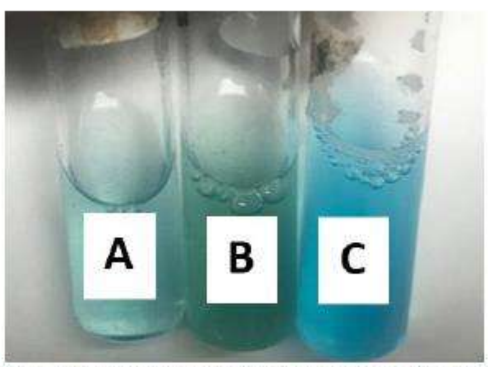
Figure 5. Biuret test for detection of glycolipids and lipopetides of *X. autotrophicus*, in mineral medium plus waste motor oil at 30°C at 144 h.



A: water + WMO, **B:** biodetergents with $NaNO_3$ pH 6.5, 200 rpm + WMO, **C:** biodetergents with $NaNO_3$ pH 6, 250 rpm + WMO.

Figure 3 shows the detection of glycolipid-type biodetergents of *A. denitrificans*, in mineral medium plus WMO at 30°C at 144 h, in all the runs of the mineral medium plus WMO *A. denitrificans* releasing glycolipids due to the dark orange coloration, in the mineral medium with WMO (B) *Achromobacter* grown with NH_4NO_3, pH 6, 250 rpm, and in the mineral medium with WMO (C) *A. denitrificans* grown on $NaNO_3$, pH 6, 250 rpm, compared with the control tube (A it was not detected. In Figure 4, the detection of glycolipids was also evident that in mineral medium with WMO (B) *X. autotrophicus* grown in $NaNO_3$, pH 6.5, 200 rpm, and in mineral medium with WMO (C) X. *autotrophicus* grown with $NaNO_3$, pH 6, 200 rpm, compared to control or water, there the presence of glycolipids was not detected. These results indicated that *X. autotrophicus* synthesizes biodetergents of glycolipid, induced by WMO. Also *A. denitrificans* synthesize biodetergents of the glycolipid type that are anionic composed of a hydrophobic lipid part and a hydrophilic part composed of a carbohydrate (Fenibo et al., 2019; Joy et al., 2019; Zargar et al., 2022).

Figure 5. Biuret test for detection of glycolipids and lipopetides by *X. autotrophicus*, in mineral medium and waste motor oil at 30°C at 144 h.



A: biodetergents on $NaNO_3$ pH 6, 250 rpm + WMO. **B:** biodetergents on NH_4NO_3 pH 6, 250 rpm + WMO. **C:** Control (water + WMO).

A. denitrificans in mineral medium with WMO, at 30°C at 144 h, released glycolipids due the green coloration, except in the mineral induced by WMO (A) *A. denitrificans* using $NaNO_3$, pH 6, at 250 rpm the light blue coloration was light blue, compared to WMO mineral medium (B) A. *denitrificans* using NH_4NO_3, pH 6, at 250 rpm green coloration was detected. In that sense Kadhum & Haydar in 2020, isolated 6 bacterial genera that were positive for the synthesis of glycolipid biodetergents by Biuret assay. These results support the ability of these bacteria to synthesize glycolipid-type biodetergents induced by WMO. The consumption of WMO in ml, after the synthesis of biodetergents and lipolytic activity of *Achromobacter* sp, in mineral medium induced by WMO, at 30°C after 144 h. The highest WMO consumption was registered in mineral medium with NH_4NO_3 pH 6, 250 rpm, *A. denitrificans* was able to consume 7. 35 ml of WMO out of the initial 10 ml, this support that these conditions inducing to synthesize the maximum amount of biodetergents and lipolytic activity to mineralize of WMO, which numerical values were statistically different compared to the consumption of 0.55 ml of WMO out of the initial 10 ml, in mineral medium with WMO, using $NaNO_3$ pH 6.5, at 250 rpm.

4 CONCLUSIONS

This research demonstrated the potential ability of genera and species isolated from soil polluted by WMO, to synthesize biodetergents and lipolytic activity induced by WMO for its mineralization.

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xiii. CAPITULO VI. Comparación de detergentes, sus aditivos, extractos crudos de biodetergentes y lipasas plus solución mineral en la biorremediación de suelo impactado por hidrocarburos.

Chapter 9

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Comparison of Detergents, Their Additives, Crude Extracts of Biodetergents and Lipases Plus Mineral Solution in the Bioremediation of Soil Impacted by Hydrocarbons

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ABSTRACT

This chapter highlight (a) to analyze biostimulation of soil impacted by WMO with Eficaz®, Salvo® and Savia Gel® plus mineral solution; (b) to study in soil impacted by WMO biostimulation with polyethylene glycol, sodium tripolyphosphate plus mineral solution (c) to improve in soil impacted by WMO biostimulation with Tween 80, Triton X-100, with crude extracts of biodetergents and lipases, plus a mineral solution to reduce the WMO to a value below the maximum of 4,400 ppm of NOM-138 SEMANART/SSA1-2012 (NOM-138) to regard as bioremediated. In biostimulation of agricultural soil impacted by 60,000 ppm of waste motor oil (WMO), it is neither completely solubilized with a detergent nor removed as expected with a mineral solution. For this reason, in the biostimulation of soil impacted by WMO, pure detergents are applied: Tween-80 and Tritton-X 100 or commercial detergents: Eficaz®, Salvo® and Savia Gel®, ignoring the effect of their additives: polyethylene glycol or sodium tripolyphosphate, in the emulsification of WMO emulsification. Nor if the application of combining crude extracts of biodetergents and lipases plus a mineral solution is more effective in the bioremediation of that soil. In this sense, the production of CO_2 by WMO oxidation and its concentration by Soxhlet were measured. The experimental data were validated by Tukey HSD ANOVA. The results showed that the soil impacted with 60,000 ppm of WMO biostimulated with Salvo®, Eficaz® plus mineral solution, had maximum CO_2 production on day

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45 with 10.42 mg CO_2/100 g and WMO decreased from 60,000 ppm to 21,700 ppm. In soil impacted by WMO biostimulated with sodium tripolyphosphate and mineral solution, in 40 days it reached 9.2 mg CO_2/100 g of soil. In soil impacted by WMO biostimulated with Tween 80, Triton X-100, crude extracts of biodetergent and lipases plus mineral solution, the maximum CO_2 production was reached and the highest of WMO decrease from 60,000 ppm to 1,600 ppm. In soil impacted by WMO biostimulated by crude extracts of biodetergents and lipases plus mineral solution, not biostimulated by Tween 80 or Triton X-100, WMO decreased from 60,000 to 2,000 ppm. This result shows in biostimulation of soil polluted by WMO applying crude extracts of biodetergents and lipases are better than the use of commercial with its additives and pure detergents plus mineral solution in the removal of WMO for soil bioremediation according to NOM-138.

Keywords: Soil; hydrocarbon mixture; biostimulation; surfactants; nutritional balance; bioremediation.

1. INTRODUCTION

In agricultural soil contaminated by waste motor oil (WMO), an insoluble mixture of aliphatic, aromatic and polycyclic hydrocarbons, require chemically pure or commercial detergents that contain additives followed by a mineral solution to reduce WMO to a value below 4,400 ppm, the maximum accepted by the Mexican norm NOM-138 SEMANART/SSA1-2012 (NOM-138) to consider that this soil is bioremediated. In this sense, when commercial additives are used, it is unknown how much they influence the emulsification of the WMO, so that they are effectively eliminated with the mineral solution and comply with NOM-138. Although there is information based on the ability of native soil heterotrophic aerobic microorganisms to synthesize biodetergents that emulsify WMO as well as the induction of their lipases to hydrolyze them and then oxidize them by biostimulation with the mineral solution [1-5].

The bioremediation strategy in agricultural soil impacted by WMO, has to initiate biostimulation with a crude extract of biodetergent microorganisms isolated from soil polluted by WMO [6,7-9], simultaneously with an extract of the lipases induced in these microorganisms capable of oxidizing WMO hydrocarbons [10,11-14]. Since these extracellular enzymes that hydrolyze aromatic and aliphatic hydrocarbons are stable in the agricultural soil environment [15,16-18], specifically if the biostimulation is continued with a mineral solution in balance with the chemical properties of the soil [19,20,21]. The objectives of this work were a) to analyze the biostimulation of soil impacted by WMO with Eficaz®, Salvo® and Savia Gel® plus mineral solution, b) to study the biostimulation of soil contaminated by WMO with polyethylene glycol, tripolyphosphate and a mineral solution, c) To improve the biostimulation of soil contaminated by WMO with tween 80, triton X-100, crude extracts of biodetergents and lipases, plus a mineral solution to reduce the WMO concentration to a value below the limit permissible by NOM-138.

2. MATERIALS AND METHODS

2.1 Soil sample Collection and Preparation

Soil sampling was carried out according to NOM-021 SEMARNAT-2000 [5,22] or (NOM-021) using the zig zag method at a depth of 20 cm, from an agricultural area in the municipality of Morelia, Michoacán, Mexico, called "Uruapilla". To avoid pests and diseases, the soil was solarized for 48 hours and sieved with No. 10 mesh. Subsequently, a physicochemical analysis of the soil was performed according to NOM-021 before contaminating the agricultural soil with WMO.

Biostimulation of soil impacted by 60,000 ppm WMO with Eficaz®, Salvo®, Savia Gel® mineral solution and H_2O_2

A randomized block experiment was carried out in triplicate in Bartha flasks with 100 g of soil impacted by WMO bio stimulated with commercial detergents: Eficaz®, Salvo®, Savia Gel® at 0.5% [23] individually or mixtures and a mineral solution. The soil was impacted with 60,000 ppm WMO, and a 50% mineral solution was biostimulated with detergents based on the concentration of organic matter, total nitrogen and phosphates from the physicochemical analysis of the soil, with the following composition (g/l): NH_4NO_3 10, K_2HPO_4 2.5, KH_2PO_4 2, $MgSO_4$ 1, NaCl 0.1, $CaCl_2$ 0.1, $FeSO_4$ traces, trace elements solution 10 and pH 6.5-6. 8 [24]. it was also biostimulated with 0.5% H_2O_2 an inorganic source of O_2. As an indirect measure of WMO solubilization and mineralization, CO_2 was quantified every third day for 45 days, including WMO concentration by Soxhlet at the beginning and end of the experiment [4,25].

Biostimulation of soil impacted by 60,000 ppm WMO with detergent additives: polyethylene glycol, sodium tripolyphosphate and mineral solution

In Bartha flasks, soil contaminated by WMO biostimulated with detergent additives was added [26]: 0.5% polyethylene glycol, 0.5% sodium tripolyphosphate and 50% mineral solution. As a response variable, CO_2 produced by the oxidation of the biostimulated WMO with the mineral solution was quantified every fifth day for 45 days, and the concentration of WMO by Soxhlet at the beginning and end of the experiment [4].

Biostimulation of soil impacted by 60,000 ppm WMO with Tween 80®, Triton X-100®, crude extracts of biodetergents and lipases plus mineral solution.

In Bartha flasks with soil contaminated by 60,000 ppm of WMO biostimulated with Tween 80®, 0.5% Triton X-100®, a crude extract of biodetergent and lipases, plus 50% mineral solution for 45. For this step were cultured *Achromobacter* sp and *Xanthobacter autotrophicus* isolated from soil polluted by hydrocarbons both were able to grow in waste residual oil, or soybean oil (g/L): WMO or soybean oil 10.0ml $NaNO_3$ 5, yeast extract 3.5, K_2HPO_4 1, KH_2PO_4 1,

$MgSO_4$ 1, NaCl 1, $FeSO_4$ 0.5 and $CuSO_4$ 0.5 [26], culture media were sterilized at 121 °C/15 min This culture media were inoculated with 10 ml/100ml of *Achromobacter* sp and *X. autotrophicus* suspended in detergent saline solution individually with a density according to McFarland's number 4. The inoculated liquid cultures were in agitation at 250 rpm. 30°C, and 250 rpm for 144 hours. Subsequently, the crude biodetergent and lipase extracts were separated from the cells by centrifugation of the cultured broth at 8000 rpm for 15 min at 4°C and kept at 5°C before be applied to agricultural soil. As a response variable, CO_2 produced by the oxidation of the biostimulated WMO with the mineral solution was quantified every fifth day for 45 days, and the concentration of WMO by Soxhlet at the beginning and end of the experiment [4,26].

Biostimulation of soil impacted by 60,000 ppm WMO w, Tween 80® and Triton X-100® at 0.5%, and crude extract of biodetergents and lipases plus mineral solution

The experiment was also conducted in Bartha flasks each containing 100 grams of soil contaminated by 60,000 ppm of WMO. Subsequently, it was biostimulated for 45 days with 6 ml of detergent solution of Tween 80® and Triton X-100® at 0.5%, 6 ml of crude extract of biodetergents and lipases, and 6 ml of solution mineral at 50%, per 100 grams of soil. For the production of the crude extract of biodetergents and lipases, liquid cultures inoculated with *Achromobacter* sp and *Xanthobacter autotrophicus* were used. To obtain crude extracts of biodetergents and lipases, liquid cultures were made with the following composition (g/L): WMO 10 (ml/L), NaNO3 5, yeast extract 3.5, K_2HPO_4 1, KH_2PO_4 1, $MgSO_4$ 1, NaCl 1, $FeSO_4$ 0.5 and $CuSO_4$ 0.5 was sterilized at 121 °C for 15 min. Then the culture broths were inoculated with 10 ml of *Achromobacter* sp and *Xanthobacter autotrophicus* in detergent saline solution individually with an inoculum density according to McFarland's number 4. The inoculated liquid cultures were kept in agitation at 30°C, pH 7 and 250 rpm for 144 hours. Subsequently, the biodetergent and lipase extracts were separated from the cells by centrifugation of the cultured broth at 8000 rpm for 15 min at 4°C. The first indirect response variable of WMO mineralization was the CO_2 released, as well as quantification of WMO by Soxhlet at the beginning and at the end of the experiment [4,5,27].

2.2 Statistical Analysis

Differences in experimental data between groups were confirmed by one-way analysis of variance (ANOVA) and analyzed by Tukey HSD test (α = 0.05) with Statgraphics Centurion XVI.II software [6].

3. RESULTS AND DISCUSSION

Table 1 shows the physicochemical analysis of the soil that contained total nitrogen (N) of 0.32% average value, soluble phosphates (P) with high value of 219.34 ppm, also high concentration of organic matter of 10.44%, an acid pH of 5.67; silt-clay texture. Concentration of N and P support that this soil has favorable conditions for biostimulation with mineral solution at 50 % while soil organic matter allow a wide diversity of heterotrophic microorganisms to mineralize WMO [7,25,28].

Table 1. Physico-chemical properties of uncontaminated agricultural soil by waste motor oil

Parameter	Value	Interpretation
pH	5.67	Moderately acid
Organic matter (%)	10.44	Very high
Texture (%)	31.8 (clay), 26.92 (sand), 42.0 (silt)	Silty-clay
Total, Nitrogen (%)	0.32	Medium
Phosphorus ppm	219.34	Very high
Sodium (Na+) ppm	153.38	High
Potassium (K+) ppm	168.61	High
Microelements (ppm):		
Iron (Fe2+)	13.91	High
Zinc (ZN 2+)	0.37	Low
Copper (Cu 2+)	0.54	Low
Manganese (Mn 2+)	4.62	Low

Reference [22]

Biostimulation of soil impacted by 60,000 ppm WMO with Eficaz®, Salvo® and Savia Gel®, mineral solution and H_2O_2

In Fig. 1 in soil impacted by 60,000 ppm WMO no biostimulated only irrigated with water or absolute control, registered 4.45 mg CO_2/100 g dry soil, due to the mineralization of native organic matter, a value that was subtracted in the soil impacted by WMO to adjust to CO_2 from WMO mineralization. While soil polluted by WMO biostimulated with Salvo® and Eficaz® plus MISO and H_2O_2 reached 10.42 mg CO_2/100 g dry soil this numerical value was statistically different than the same numerical value with 1.47 mg CO_2/100 g soil, at the soil impacted by WMO no biostimulated or negative control also compared to soil impacted by WMO biostimulated with Eficaz®, plus mineral solution and H_2O_2 that reached 8.93 mg CO_2/100 g soil [29,30]. These results support that the mixture of Salvo® and Eficaz®, solubilized more WMO to be oxidized by biostimulation with mineral solution and H_2O_2 [31,32,33]. In the soil polluted by WMO biostimulated with Eficaz®, Salvo® and Savia Gel® applied individually, solubilized less WMO [34,35,36]. In the soil polluted by WMO biostimulated with the mixture of Salvo®, Eficaz® and Savia Gel®, decreased CO_2 production, due that Savia Gel®, contains fragrances acting as an antimicrobial aromatic that inhibited WMO mineralization [15,37,38,39]. Finally, as was expected in soil impacted by WMO no biostimulated or negative control, natural attenuation solubilized and mineralized a minimal amount of WMO [19,40,41].

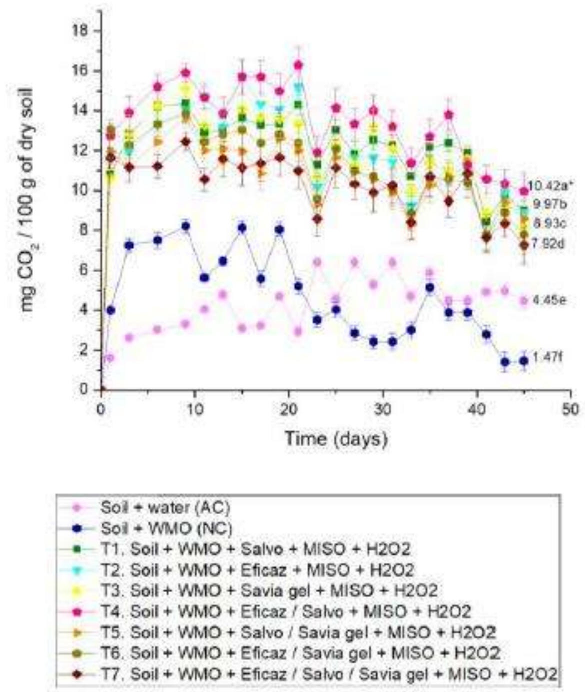

Fig. 1. CO_2 production in soil impacted by 60,000 ppm of waste motor oil biostimulated by commercial detergents: Eficaz®, Salvo®, Savia gel® plus mineral solution and H_2O_2

*WMO: waste motor oil, MISO: mineral solution. *Different letters indicate statistical difference ANOVA-Tukey HSD test ($\alpha = 0.05$)*

Table 2 shows soil contaminated by WMO, biostimulated with Eficaz®-Salvo® mixture followed by mineral solution and H_2O_2, as well as soil pollutes by WMO biostimulated with Salvo® mineral solution and H_2O_2, registered the highest decrease in WMO, both with a reduction from 60,000 ppm to 21,700 ppm. These results indicated that biostimulated by Salvo® or Salvo®/Eficaz® mixture solubilized the same amount of WMO, due to the active ingredient dodecyl benzenesulfonic acid in Eficaz® and alkyl ethoxy sulfate in Salvo®, besides amine oxide in Salvo® that is a foaming agent that increased WMO solubilization [7,8,9,20]. Then biostimulation by mineral solution and H_2O_2, induced its highest mineralization of WMO. Eficaz® detergent has cleaning adjuvants as well as polycarboxylates or polyphosphates [21,42,43] to sequester divalent cations from hard water do not participate in WMO solubilization [16-18]. In opposite way compared to soil contaminated by WMO biostimulated with Savia gel® mineral solution and H_2O_2 concentration was reduced from 60,000 ppm to 24,200 ppm. In the soil impacted by WMO non biostimulation used as negative control WMO decreased from 60,000 to 48,400 ppm, due to natural attenuation, in this case synthesis of biodetergents by the h native microbiota that solubilized a minimum part of the WMO and therefore the microorganisms mineralized them biostimulated with the soil nutrients [25,27,28,44]. These results indicated that the inconsistency in biostimulation of soil impacted by WMO, the detergent mixture increases solubilization, is possibly only applicable for biostimulation with pure detergents, since commercial detergents contain additives [7,28], that could increase or inhibit the solubilization and later mineralization of WMO when biostimulation is follow by mineral solution [8,9,11-14]

Table 2. Concentration of waste motor oil in soil impacted by 60,000 ppm of waste motor oil after biostimulation by commercial detergents Eficaz®, Salvo® and Savia Gel®

Soil	Waste motor oil concentration after 45 days (ppm)
WMO (Negative control)	48,400[c*]
T1: WMO + Salvo® + mineral solution+ H_2O_2	21,700[a]
T2: WMO + Eficaz® + mineral solution + H_2O_2	23,500[b]
T3: WMO + Savia gel® + mineral solution + H_2O_2	24,200[b]
T4: WMO + Eficaz®/Salvo® + mineral solution + H_2O_2	21, 700[a]
T5: WMO + Salvo®/Savia gel® + mineral solution + H_2O_2	23,400[b]
T6: WMO + Eficaz®/Savia gel® + mineral solution + H_2O_2	24,900[b]
T7: WMO + Eficaz®/Salvo®/Savia gel® + mineral solution + H_2O_2	24,200[b]

*WMO: waste motor oil, * Different letters indicate statistical difference ANOVA-Tukey HSD test (α = 0.05)*

Biostimulation of soil impacted by 60,000 ppm WMO with Polyethylene glycol and Sodium Tripolyphosphate detergent additives and mineral solution

Fig. 2 shows that the highest production of CO_2 was registered in the soil by WMO biostimulated with Polyethylene glycol and 50% mineral solution at day 17 with 15.5 mg CO_2/ 100 g soil, at day 45 it was 7.2 mg CO_2/ 100 g soil, statistically different values compared to those registered in the soil impacted by WMO biostimulated with Sodium Tripolyphosphate and 50% mineral solution, with 14. 2 mg CO_2/ 100 g soil at day 7 and 9.2 mg CO_2/ 100 g soil at day 45, these numerical values had no statistical difference compared to those reported in the soil impacted by WMO no biostimulated, used as negative control with 11.5 mg CO_2/ 100 g soil at day 7 and 6.91 mg CO_2/ 100 g soil at day 45 due to natural attenuation. This results support that in the soil polluted by WMO biostimulated with Polyethylene glycol and Sodium Tripolyphosphate, CO2 production was due to partial oxidation of WMO by the native heterotrophic aerobic microbiota biostimulated by mineral solution at 50%, equivalent to the action of natural attenuation as was detected in soil polluted by WMO no biostimulated used as negative control, there the CO_2 released was similar. In soil polluted by WMO biostimulated of Polyethylene glycol, a polyether also known as polyethylene oxide [8,21,42,43] has high polarity and solubility in water [9,11-14], supporting that enhanced WMO solubilization in soil. While Sodium Tripolyphosphate, although known to be water soluble and have emulsifying property, did not enhance WMO emulsification [11,36,37], since CO_2 production values had no statistical difference compared to the natural attenuation in soil polluted by WMO no biostimulated or negative control

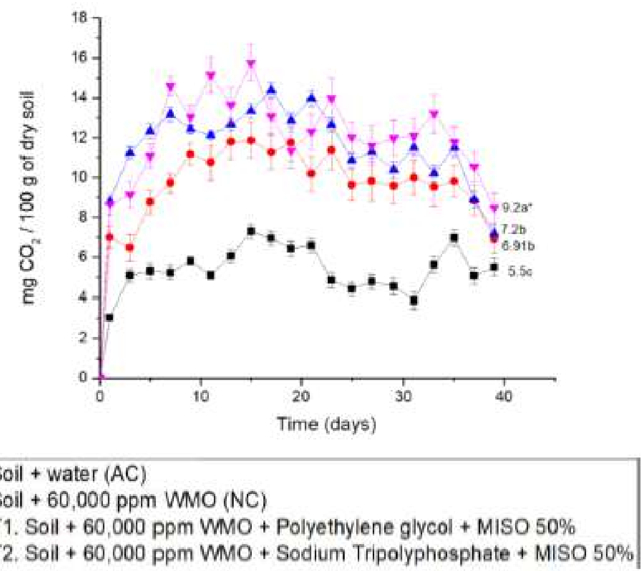

Fig. 2. CO_2 production in soil impacted by 60,000 ppm of waste motor oil biostimulated by polyethylene glycol, sodium tripolyphosphate and mineral solution

* Different letters indicate statistical difference according to ANOVA-Tukey HSD test (α = 0.05) WMO= waste motor oil, AC= absolute control, NC= negative control, MISO=mineral solution.

Biostimulation of the soil impacted by 60,000 ppm of WMO Tween 80® and Triton X-100® at 0.5%, crude extract of biodetergents and lipases with mineral solution.

Fig. 3 shows that initially, between days 0 and 3, CO_2 production due to mineralization of WMO was limited, due the adaptation phase of the native heterotrophic aerobic microorganisms to the WMO, then CO_2 decreases on day 7 increases again, since the first fractions of WMO that is mineralized corresponds to low molecular weight aliphatics are solubilized and eliminated then the complex HICO with high molecular weight aromatics and polycyclic in the days after day 12 [12-14]. The highest CO_2 production was in soil impacted by 60,000 ppm WMO biostimulated with biodetergent crude extract + 50% mineral solution with maximum amount of 20.7 mg CO_2 at day 11, and 19.3 mg CO_2 at end of experiment time, day 45. These numerical values had no statistical difference compared to registered in the soil impacted by 60,000 ppm WMO biostimulated with biodetergent and lipase and mineral solution at 50% with a maximum of 18.62 mg of CO_2 detected at day 23 at end of experiment was 17.9 mg of CO_2 on day 45. This result indicated that biodetergent crude extract enhanced the emulsification of WMO [7,8,9,11,20,33], that subsequently a rapid hydrolysis of aromatic fractions with lipase crude extract [14,36,37], while biostimulation of mineral solution at 50% enriched soil with N, P and K inorganic salts to induce of WMO mineralization by the native heterotrophic aerobic microorganisms [16,17,45]. The numerical values 18.62 and 17.9 mg CO_2 had statistical difference compared to these registered in the soil impacted by WMO non biostimulated, used as negative control with 9.8 mg CO_2 at day 45 due to natural attenuation [18, 25, 44].

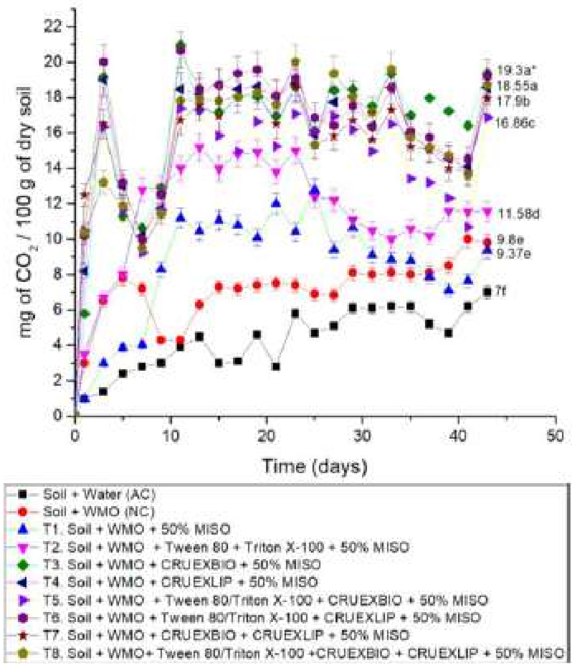

Fig. 3. CO_2 production in soil impacted by 60,000 ppm of waste motor oil biostimulated by Tween 80®, Triton X-100®, biodetergent and lipase crude extract and 50% mineral solution

*WMO: waste motor oil, AC: absolute control, NC negative control: CRUEXBIO=crude extract of biodetergents, CRUEXLIP= crude extract lipases, MISO: mineral solution. * Different letters: indicates statistical difference ANOVA-Tukey HSD test (α = 0.05)*

Table 3. Remaining waste motor oil after 45 days of biostimulation of soil impacted by 60,000 ppm of waste motor oil, with Tween 80, Triton X-100, biodetergent and lipase crude extracts plus 50% mineral solution

Soil	Waste motor oil concentration after 45 days (ppm)
WMO (Negative control)	48,400[h*]
T1: WMO + 50% mineral solution	26,300[f]
T2: WMO + Tween 80 + Triton X-100 + 50% mineral solution	20,100[c]
T3: WMO + crude extract of biodetergents + 50% mineral solution	35,000[g]
T4: WMO + crude extract of lipases + 50% mineral solution	25,700[e]
T5: WMO + Tween 80 + Triton X-100 + crude extract of biodetergents + 50% mineral solution	12,100[b]
T6: WMO + Tween 80 + Triton X-100 + crude extract of lipases + 50% mineral solution	22,000[d]
T7: WMO + crude extracts of biodetergents and lipase + 50% mineral solution	2,000[a]
T8: WMO + Tween 80 + Triton X-100 + crude extract of biodetergents and lipase + 50% mineral solution	1, 600[a]

*WMO= waste motor oil, *different letters indicated values statistically different according to ANOVA-Tukey HSD test (α = 0.05)*

Table 3 shows the final WMO concentration in the soil after biostimulation. The highest reduction of WMO was registered in impacted soil biostimulated by biodetergent and lipase crude extract + Tween 80 + Triton X-100 + mineral solution at 50%. When WMO concentration went from 60,000 to 1,600 ppm WMO, a numerical value had no statistical difference to soil impacted by WMO biostimulated by biodetergent and lipase crude extract + 50% MISO and no detergents with a decrease from 60,000 to 2,000 ppm WMO. These results demonstrated of a soil impacted by a relatively high concentration of WMO by biostimulation using a crude extract of biodetergents and lipases, unpurified, in contrast to most related works with soil contaminated by relatively low concentrations of hydrocarbons is biostimulated with chemically pure biodetergents and lipases [27,33,35,46], seldom were registered positive results in soil recovering polluted by hydrocarbons, according to environmental standards. In that sense Gudina et al. [21] in a soil contaminated by 10,000 ppm of WMO biostimulated by pure biodetergent and mineral solution WMO was reduced to 4,500 ppm. The same way, da Rocha et al. [42] in soil impacted by 40,000 ppm hydrocarbons biostimulated with rhamnolipid an biodetergent and the activity of natural attenuation these hydrocarbons were reduced until 28,020 ppm. While Bezza & Chirwa [43] in soil impacted by 6,745 ppm polycyclic aromatic hydrocarbons was biostimulated with a biodetergent and mineral solution achieving a decreased until 2,552 ppm after 64 days. In the soil impacted by WMO biostimulated with Tween 80 and Triton X-100, because these pure and biological detergents are similar to WMO hydrocarbons to be hydrolyzed by biostimulation with crude extract of biodetergents [27,31,34,46] and lipases that generated hydrocarbon fractions facilitated to the aerobic heterotrophic microbiota biostimulated by mineral solution [36,37,45]. While in the soil impacted by WMO biostimulated only with a 50% mineral solution, a reduction of WMO from 60,000 ppm to 26,300 ppm was detected due to the absence of the biodetergent crude extract and lipases that prevented the availability of WMO hydrocarbons to be oxidized [29,47-49] by the heterotrophic native microbiota of the soil [31,32], consequently there was no decrease in the concentration expected, at least for the limit established by NOM-138. These result supports that the application of crude extracts of biodetergents and lipases are a novel and effective alternative for the bioremediation of soil polluted by WMO in less time than common bioremediation technic applying pure detergents [30,38,39,50,51]. It's reported that the high the percentage of compounds of organic as like as lipases and biodetergents in the soil, the lower the retention of hydrocarbons, since both these types of enzimes and biological surfactant are able to reduce the sorption capacity in the soil, and therefore the maximum removal of WMO [40,52].

4. CONCLUSIONS

This research demonstrated that in agricultural soil polluted by WMO can be biostimulated with crude extracts of biodetergents and lipases as long as biostimulation apply mineral solution adjusted to chemical proprieties of soil, to achieve effective bioremediation according to environmental regulations in approximately 30% less time compared to the use of commercial detergents in common bioremediation processes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Research and Academic Experience: She is a PhD Candidate in Biological Sciences, Master of Science in Environmental Engineering and Biochemical Engineering. She is the recipient of an economic stimulus for having presented one of the best research projects at the Second Meeting of Young Researchers of the State of Michoacán. She got research projects at the Second Meeting of Young Researchers of the State of Michoacán 2013. She has Selected as a participant in the III Workshop on Professional Skills for Young Scientists 2016. She is a specialist in Bioremediation of soils impacted by hydrocarbons, solid waste and plant growth promoting bacteria.

Research Area: Her area of research mainly focused on environmental Sciences.

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xiv. CONCLUSIONES

El trabajo demostró la biorecuperacion de suelo contaminado por 60,000 ppm de ARA, por bioestimulación con detergente, solución mineral, bioaumentación con *X. autotrophicus* y fitorremediación se usó *P. vulgaris*, así como la bioestimulación del suelo impactado, mediante extractos crudos de biodetergentes y lipasas y solución mineral, que lograron el rápido decremento del ARA comparado con otras estrategias de biorremediaión acorde a la NOM-138 para la reutilización del suelo con fines agrícolas.

xv. ANEXOS

ARTÍCULO

Efectos del huachicoleo en México

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https://pixabay.com/es/photos/gasolina-diesel-gas-automotor-996617/

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El huachicoleo **es el robo de combustibles** —como la gasolina—, el cual se realiza mediante la perforación de las tuberías que lo transportan. Es un problema muy común en México que ha generado problemas a la salud del ser humano, económicos, sociales y ambientales. Para remediar el problema del huachicoleo y evitar que siga ocurriendo, es necesario **realizar acciones en conjunto** entre los propietarios de las áreas afectadas, las autoridades y la parte técnica.

Huachicol, huachicoleo y huachicolero

Los medios de transporte que utilizamos a diario necesitan gasolina para su funcionamiento; sin embargo, de acuerdo con la situación económica del país y que cada vez es más cara y difícil distribuirla, muchas personas han decidido robarla, haciendo perforaciones en las tuberías que la transportan para evitar pagarla. Esta acción comúnmente es conocida como **«huachicoleo»**.

La palabra **«huachicol»**, de acuerdo a la Academia Mexicana de la Lengua (2019), se usa para referirse a un ladrón, mientras que en la definición de **«huachicolero»** el sufijo *-ero* indica ocupación o cargo, por lo tanto, «huachicolero» se le conoce a la persona que roba gasolina. Como huachicol también se conoce a una bebida alcohólica adulterada con alcohol de caña y otros compuestos; además, sirve para nombrar el combustible robado que puede estar adulterado.

Como mencionamos al principio, el huachicoleo consiste en la perforación de las tuberías para extraer la gasolina que es utilizada para uso propio o para **venderla de manera ilegal a un precio más bajo**. En el país hay una red de 8 mil 390 kilómetros de poliductos que van desde el lugar donde se fabrica hasta los espacios de almacenamiento.

Se informa que en el primer semestre de 2020, las Fuerzas Armadas en México detectaron 1,948 tomas clandestinas en Hidalgo, 909 en Puebla, 724 en el Estado de México, 317 en Guanajuato y 278 en Veracruz. Para combatir este problema y evitar que siga este robo, el gobierno ha decidido **cerrar varios de los ductos** para reparar las perforaciones y transportar la gasolina en pipas a través de carreteras. El problema de esto, es que algunos estados del país fueron perjudicados por la **escasez y retraso en el abasto** a las gasolineras.

Problemática del huachicoleo

El huachicoleo ha provocado varios tipos de problemas sociales, económicos, de salud y ambientales. Por ejemplo, entre los económicos, podemos mencionar que durante el primer trimestre del año 2021, las pérdidas por contrabando de combustibles fueron de 20 mil millones de pesos

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anuales, cifra similar a la que el presidente Andrés Manuel López Obrador indicó que se destinaría para la compra de las vacunas contra el COVID-19 a nivel nacional en el 2020.

En cuanto a los **daños ambientales,** se ha detectado que esta acción causa contaminación de suelo, aire y agua; sin embargo, escasamente se reportan, por lo que poco se sabe sobre cifras de sitios afectados y mucho menos se hace algo para solucionarlo. Por ejemplo, en localidades de Hidalgo y en la zona de Tlahuelilpan, especialistas del área insisten en la restauración ambiental, además existen cientos de lugares con registro de tomas clandestinas que han causado contaminación, cuya biorremediación tiene un costo de 175 mil pesos por hectárea, monto que difícilmente pueden costear los agricultores.

De acuerdo a los datos sobre sustentabilidad de Petróleos Mexicanos (PEMEX), el huachicoleo provocó el **crecimiento de 13.5% de las zonas contaminadas** por PEMEX de 2012 a 2015. Según información de la Dirección General de Inspección de Fuentes de Contaminación de la PROFEPA, durante el sexenio del presidente Enrique Peña Nieto, hubo en todo el país 4,386 emergencias causadas por «huachicoleo», pero solo se reportó daño ambiental en unas 300 hectáreas de terreno. Esto se debió a que 2,615 de estas fugas no reportaron públicamente la extensión de suelo afectado. Algunos de los datos más actuales señalan que a finales de 2021, en Mazatlán, había al menos 10 tomas clandestinas, lo que es un peligro latente ya que las tuberías pasan por debajo de casas y comercios, además de que podrían repercutir en la flora y fauna del mar de esa zona. Mientras que Hidalgo terminó el 2021 con 1,383 denuncias por robo de combustibles, equivalente a un aumento del 33.8% con respecto al 2020.

Las instituciones encargadas de atender los problemas del robo de combustibles son la Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) y la Procuraduría Federal de Protección al Ambiente (PROFEPA), las cuales **no han divulgado acciones de atención a los ecosistemas** afectados por esta situación. El tiempo estimado para remediar las áreas dañadas es de aproximadamente cinco años con un costo de 200,000 pesos por hectárea.

Propuestas de acción

Para solucionar el problema se requiere la participación activa de los dueños de los terrenos afectados, de las autoridades correspondientes y de la parte técnica. En cuanto a los **propietarios de los terrenos,** a ellos corresponde asesorarse legalmente para proteger adecuadamente sus propiedades y capacitarse en materia legal para la prevención y remediación de suelos contaminados, así como en derechos y obligaciones como propietarios de terrenos.

Por su parte, **las autoridades** necesitan implementar leyes y reglamentos de protección ambiental estrictos para la prevención y recuperación de sitios contaminados para que, por medio de ello, se responsabilice a los culpables de la contaminación para la remediación de sitios y obligarlos a que paguen una indemnización a los propietarios afectados.

Para solucionar el problema de contaminación de suelos por combustibles hay métodos, siendo el más común el uso de compuestos químicos, los cuales son muy costosos y generan más contaminantes en lugar de arreglar el problema. Por otra parte, están los **métodos biológicos**, donde se usan microorganismos y plantas para eliminar los combustibles del suelo, los cuales son de bajo costo y amigables con el ambiente. Es de importancia extrema eliminar estos contaminantes del ambiente, siendo el responsable de estas acciones la SEMARNAT y no PEMEX.

El huachicoleo es una práctica altamente peligrosa y que causa graves daños, por lo que es de vital importancia tomar medidas para que esto deje de ocurrir. Una alternativa sería dejar de usar gasolina y **preferir combustibles ecológicos** como el bioetanol y el gas natural, los cuales son de bajo costo y bajo riesgo para el ambiente y para la salud del ser humano.

sabermás

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