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Biodegradation of crude oil present in wastewaters: evaluation of biosurfactant production and catechol 2,3 dioxygenase activity

Biodegradación de petróleo crudo presente en aguas residuales: evaluación de la producción de biosurfactantes y actividad de catecol 2,3 dioxigenasa

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Abstract
Water demand is increasing because of demographic and urban development in the last decades. Crude oil is an essential energy resource for many anthropogenic activities. However, it is associated with the generation of environmental pollution. The degradation of crude oil by microorganisms involves a biochemical process of various enzymes such as dehydrogenases, hydroxylases, and oxygenase's for the degradation of aromatic and aliphatic hydrocarbons. A crucial enzyme involved in the degradation of aromatic hydrocarbons is catechol 2,3-dioxygenase. In addition, in adverse environmental conditions, various microorganisms produce biosurfactants, such as an adaptation and survival mechanism. In the present work, a native microbial consortium was used to study the hydrocarbon biodegradation of crude oil and its potential use to remove organic pollutants in wastewater. The kinetic degradation of crude oil was analyzed to determine the production of biosurfactants and the enzyme activity of catechol 2,3 dioxygenase. The degradation of the hydrocarbon was determined by aromatic-hydrocarbons (96.11%) and total-hydrocarbons (74.23%). The maximum values of the biosurfactant production were evaluated by oil displacement (206.95 mg/L) and emulsification capacity (DO_{600} 0.2895). The kinetic analysis showed that the complex mixture of hydrocarbons was responsible for generating the stress to synthesize biosurfactants through a native microbial consortium. However, the decrease in catechol 2,3 dioxygenase activity and biosurfactant production was related to the degradation of aromatic hydrocarbons. The microbial consortium could produce biosurfactants during crude oil degradation, and it has a great potential to remove aromatic hydrocarbons present in wastewater.
Keywords: Bioremediation, Enzyme activity, hydrocarbons, Bioemulsifiers, Wastewater.

Resumen
La demanda del agua esta en aumento debido al desarrollo urbano y demográfico en las últimas décadas. El petóleo crudo es una fuente de energía esencial para muchas actividades antropogénicas. Sin embargo, este es asociado con la generación de contaminación ambiental. La degradación del petróleo crudo por microorganismos involucra un proceso bioquimico de varias enzimas como las deshidrogenasas, hidroxilasas y oxigenasas para la degradacion de hidrocarburos alifáticos y aromáticos. Una enzima crucial involucrada en la degradación de los hidrocarburos aromáticos es la catecol 2,3 dioxigenasa. Adicionalmente, en condiciones ambientales adversas, algunos microorganismos producen biosurfactantes, como un mecanismo de adaptaión y superviviecia. En el presente trabajo, un consorcio nativo fue usado para estudiar la biodegradación de petróleo crudo y su potencial uso para remover contaminantes orgánicos en aguas residuales. La cinética de degradación del petróleo crudo fue analizado para determinar la producción de biosurfactantes y la actividad de la enzima catecol 2,3 dioigenasa. La degradación de los hidrocarburos fue determinado por aromáticos (96.11%) y totales (74.23%). El máximo valor de la producción de biosurfactantes fue evaluada por desplazamiento de petróleo (206.95 mg/L) y la capacidad de emulsificación (DO_{600} 0.2895). La análisis cinético mostró que la mezcla compleja de hidrocarburos fue el principal responsable de generar estrés para sintetizar biosurfactantes por el consorcio nativo microbiano. Sin embargo, la disminución en la actividad de catecol 2,3 diaxigenasa y la producción de biosurfactantes fue relacionado a la degradación de hidrocarburos aromáticos. El consorcio microbiano fue capaz de produccir biosurfactantes durante la degradación del petróleo crudo, y tuvo un potencial para remover hidrocarburos aromáticos presentes en aguas residuales.
Palabras clave: Biorremediación, Actividad enzimática, Hidrocarburos, Bioemulsificantes, Aguas Residuales.

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1 Introduction

Crude oil is an essential natural energy resource for many anthropogenic activities; however, it is associated with the generation of environmental pollution. This is caused by toxic compounds in crude oil (CO), and it has now become a target issue of concern (Dourado *et al*., 2017; Khatoon & Malik, 2019; Ubani *et al*., 2022).

Underground water and soil can be affected by the presence of CO, a complex mixture of hydrocarbons categorized into aliphatics, aromatics, resins (amides, sulfoxides, pyridines, quinolines, and carbazoles), and asphaltenes (phenols, ketones, esters, porphyrins and fatty acids) (Steliga, 2012; Yzquierdo-Ruíz *et al*., 2022). Aliphatic hydrocarbons usually comprise more than 75% of most crude oil, and they are biodegraded more rapidly than aromatic hydrocarbons (AHs) (Bacosa *et al*., 2011). AH have low hydrophobicity and remain in the environment, which makes them very toxic and carcinogenic to humans (Abdelhaleem *et al*., 2019; Hassan & Aly, 2018). The toxic AHs, particularly the polycyclic aromatic hydrocarbons (PAHs), with mutagenic and carcinogenic potential, are not easily degraded by microorganisms. The microbial degradation of monoaromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, and xylenes) has been extensively reported (Bacosa *et al*., 2011). AHs seriously threaten our environment by damaging ecosystems and affecting human health when they are accidentally released during exploration, drilling, extraction, storage, refining, and transportation (Houbron *et al*., 2021; Tao *et al*., 2020; Vázquez-Vélez *et al*., 2022).

Water demand is increasing because demographic and urban development in the last decades has become a source of conflict between countries. Water scarcity may be one of the most significant challenges in the coming years. Some authors have indicated that more than 50% of the global population will live in regions with moderate stress hydric by 2050 (Gam & Ben Rejeb, 2021). Therefore, physical, chemical, and biological approaches are necessary to remove hydrocarbons from contaminated environments. However, chemical and physical treatments are not effective when removing hydrocarbons. Then, there is a need for novel, eco-friendly, low-cost, simple, sustainable, and effective methods to remove CO and AH from contaminated water (Elumalai *et al*., 2021; Popoola & Yusuff, 2021). Bioremediation is an effective, affordable, and environmentally friendly technology because it uses microorganisms to degrade contaminants. Microbial degradation is the primary mechanism for removing hydrocarbon pollutants from the contaminated environment (Abdelhaleem *et al*., 2019; Canul-Chan *et al*., 2018; Olapade & Ronk, 2015; Xia *et al*., 2019).

During the biodegradation of CO hydrocarbons, microorganisms uptake energy from the degraded products, which helps them to survive in extreme and complicated environmental conditions. Bacteria are major contributors to hydrocarbon degradation; however, a diverse microbial population is usually required, along with aerobic conditions associated with different enzymes produced by the microorganisms in the presence of oxygen (Cisneros-de La Cueva *et al*., 2014; Elumalai *et al*., 2021; Muthu *et al*., 2018; Xia *et al*., 2019). For example, catechol 2,3 dioxygenase (C23O) can synergistically act upon hydrocarbon biodegradation in a contaminated environment.

The C23O enzyme causes the aromatic oxidation of AH, leading to the formation of intermediate degradation products (Xie *et al*., 2014). C23O is an enzyme indispensable for mineralizing monoaromatic hydrocarbons, including BTEX, biphenyl, chlorophenol, etc. This is a very important enzyme to the degradation of aromatic hydrocarbons because it catalyzes the critical and chemically difficult aromatic ring-cleavage reaction. It is considered a key enzyme involved in the biodegradation of aromatic compounds; C23O has been identified in various microorganisms, such as *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Gordonia*, *Thauera*, etc. (Táncsics *et al*., 2010; Zeng *et al*., 2020).

The low solubility and bioavailability of hydrophobic pollutants are the most important factors that limit the biodegradation efficiency of CO hydrocarbons (Sharma *et al*., 2018; Xia *et al*., 2019). Bacteria must produce biosurfactants (BS) to increase their solubility and bioavailability. This aptitude of many bacterial strains is a key factor during hydrocarbon biodegradation. In adverse environmental conditions, various microorganisms produce BS, such as an adaptation and survival mechanism. BS are metabolites associated with growth, allowing the hydrophobic solubilization by emulsifying hydrocarbons (Alvarado *et al*., 2022). The BS produced by oil-degrading bacteria promotes hydrocarbon uptake by bacterial cells (Ortega-de la Rosa *et al*., 2017; Sun *et al*., 2019). They contain both hydrophilic and hydrophobic groups, which could serve as solubilizers of hydrophobic organic compounds (Karlapudi *et al*., 2018; Sharma *et al*., 2018). BS has low toxicity, high biodegradability, and the ability to act in hydrocarbon-contaminated environments, which has broad applicability in bioremediation (Alvarado *et al*., 2022; El-Sheshtawy *et al*., 2022).

In a previous report on workgroup, the microbial population dynamics, taxonomy, and the catabolic capacity of the NMC exposed to crude and fuel oil were analyzed through metagenomics, where the most abundant genera were *Streptomyces* (6.83%), *Sphingomonas* (6.67%), *Limnobacter* (5.75%), while in fuel oil were *Pseudomonas* (4.89%) and *Burkholderia* (4.75%) (Canul-Chan *et al*., 2018).

In this work, a native microbial consortium (NMC) was used to study the biodegradation of crude oil hydrocarbons and specifically its potential use to remove aromatic hydrocarbons in wastewater. During the biodegradation study, C23O activity and BS production were evaluated. In

addition, a kinetic analysis of hydrocarbon biodegradation and BS production was studied using a non-structured model to obtain the kinetic parameters of the process.

2 Materials and methods

2.1 Media and culture conditions

The NMC employed in this research was previously isolated from oil-contaminated soil from Mérida, Yucatán, Mexico (Canul-Chan *et al*., 2018). The experiments were conducted in a 250-mL Erlenmeyer flask containing 100 mL of Bushnell-Hass mineral medium inoculated with 5% v/v (0.90 μg protein/mL) NMC, while 0.5% v/v CO (0.4892 g/L) was used as the sole carbon source. The possible losses of CO due to volatilization and adsorption by the indigenous consortium were monitored using abiotic and sterile controls.

2.2 Microbial growth analysis

The progress of microbial growth in the presence of CO was analyzed through cellular protein concentration and following the method by Peterson (1977). The experimental data obtained during the CO biodegradation process was fit using the logistic model. Which was used to fit the experimental biomass and biodegradation data; this model has been used and reported in another biodegradation process (Deive *et al*., 2010; Moscoso *et al*., 2012; Rosales *et al*., 2012).

$$X = \frac{X_{\max}}{1 + e^{\ln\left(\frac{X_{\max}}{X_0} - 1\right) - \mu t}} \tag{1}$$

$$D = \frac{D_{\max}}{1 + e^{\ln\left(\frac{D_{\max}}{D_0} - 1\right) - \mu_D t}} \tag{2}$$

where X and D, are the biomass (mg/mL) and hydrocarbon removal (%) at a specific time t (days) X_0 and D_0 are the initial biomass (mg/mL) and hydrocarbon removal (%), $X_{\max}$ and $D_{\max}$ are the maximum biomass (mg/mL) and hydrocarbon removal (%), and μ and μ_D represent the specific growth and biodegradation rate (h^{-1}).

2.3 Degradation of hydrocarbons

The remaining hydrocarbons from the media culture were obtained using 100 mL hexane in a Soxhlet extractor. The analysis was carried out using a gas chromatographer (Agilent Technologies 6890N) coupled to a mass detector (Agilent Technologies 5973N) with an automatic sampler and injector. The injection volume was 1 μL, helium gas was used as a carrier at 1mL/min, and an Equity^{TM} column (30 m x 0.25 mm, internal diameter 0.25 μm) was also employed. The analysis was carried out at 35°C for 15 min, increasing 5°C every minute until reaching 200°C; the final temperature was kept for 5 min. The amount of residual hydrocarbons was estimated using the total area obtained in the chromatograms of each sample. The biodegradation percentage of the hydrocarbons was calculated from the different amounts of total hydrocarbons and aromatic carbons according to the following formula:

$$Degradation(\%) = \frac{(Q_t - Q_i)}{Q_t} \times 100 \tag{3}$$

To investigate the efficiency of the microbial degradation of hydrocarbons, the biodegradation rates of total and aromatic hydrocarbon components were determined using the logistic model (Equation [2]).

2.4 Relationship between substrate and product

The BS production during the hydrocarbon biodegradation process was determined using two methodologies: emulsification capacity and oil displacement (Adetunji & Olaniran, 2019). The formation of BS was analyzed through the correlation between product and substrate, according to Mu *et al*. (2006).

$$\frac{dP}{dt} = -Y_P \frac{dS}{dt} \tag{4}$$

$$\int_0^P dP = -Y_P \int_{S_0}^S dS \tag{5}$$

$$P = Y_P(S_0 - S) \tag{6}$$

Where: P is the quantity of BS produced, S_0 and S are quantities of the substrate (hydrocarbons) to start and the end of the process, Y_P is the product yield expressed as mg of BS produced by g of total hydrocarbon or AH consumed.

2.5 C23O enzyme activity

The enzyme activity of C23O was determined using cell-free extracts, and the intermediate hydrocarbon degradation product, catechol Meta-cleavage dioxygenase activity, was assayed by monitoring the product with a UV-vis spectrophotometer. For this purpose, cells were harvested, washed with 50 mM sodium phosphate buffer (SPB, pH 7.0), and subjected to sonication (10 times, pulse of 20 s). Cell-free extracts were collected by centrifugation at 10,000 x g for 20 min (4ºC). The reaction mixture contained 20 μL catechol (10 mM) and 905 μL 50 mM SPB (pH 7.0). The activity of C23O was determined from the formation of 2-hydroxymuconic semialdehyde at 375 nm (λ_{375} = 36,000 M^{-1} cm^{-1}) using a UV-Vis spectrophotometer (Thermo Scientific^{TM} Evolution 300) (Hegeman, 1966). One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 pmol product/min at 30ºC (Lin & Milase, 2015). The protein concentration was determined with Peterson's method (Peterson, 1977).

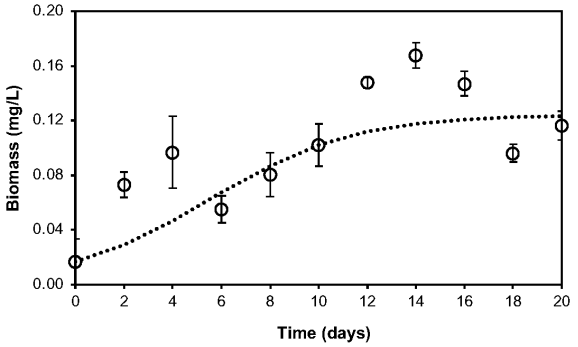

Figure 1. Kinetics of microbial growth (o) adjusted by logistic model (···) of native microbial consortium exposed to crude oil hydrocarbons.

2.6 *Statistical analysis*

Error bars in the plots represent the standard error for triplicate samples. The data was evaluated by analysis of variance (one-way ANOVA) with $p < 0.05$. The analyses were carried out using Microsoft Excel.

3 Results and discussion

3.1 *Crude oil degradation and microbial growth*

Parameters of kinetic growth and hydrocarbon degradation were determined when the native microbial consortium was cultured in Bushnell-Haas medium and exposed to 0.492 g/L CO (0.5 % v/v) as the sole carbon source. The logistic model was a valuable tool for studying dynamics and modeling experimental growth and degradation parameters, relevant to obtain a better knowledge of the bioremediation process.

Figure 1 shows the evolution of cell concentration in the NMC during the cultivation period (20 days). The application of the logistic model allows to observe that an exponential growth was detected between days 6 and 10, while the stationary phase started after day 16 days and lasted until day 22. The maximum growth was 0.147 ± 0.008 mg/L on day 14, and the specific growth rate was 0.3108 d^{-1}. This trend points to NMC's ability to use crude oil as a carbon and energy source.

TH and AH degradation percentages were 74.23 and 96.11%, respectively (Fig. 2). The logistic model was applied to TH and AH to obtain the specific degradation rate values of 0.5433 and 2.4908 d^{-1}, respectively. Interestingly, the degradation rate of AH was 4.58-fold that of TH. This could suggest the preference of some members of the NMC for AH.

The percentage of CO biodegradation reported in this work was higher (74.23%) than that reported by Ra *et al*. al (2018), where a mixed microbial consortium was capable to remove 47.57% of the CO hydrocarbons.

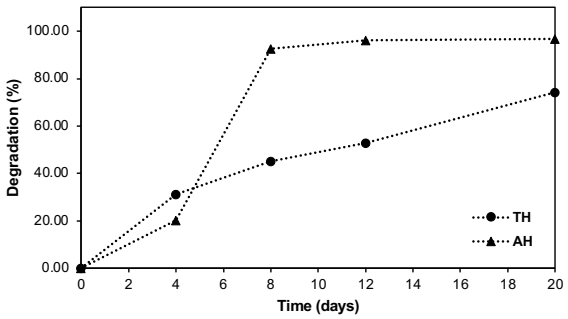

Figure 2. Evaluation of hydrocarbon degradation in crude oil by native microbial consortium. Total hydrocarbons (●) and aromatic hydrocarbons (▲).

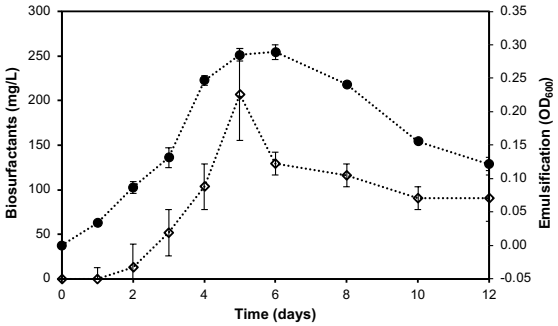

Figure 3. Biosurfactant production by native microbial consortium during crude oil degradation. Biosurfactant production by oil spread (◊); emulsification capacity expressed as tween 20 equivalents (●).

Kumari *et al*. (2018) reported a TPH biodegradation percentage of 76.67 % by a mixed microbial consortium. However, the percentage of AH reported in this work is better. The best results in the present work are a consequence of the employee of the NMC in comparison with the mixed microbial consortia employed in the previous reports (Kumari *et al*., 2018; Ra *et al*., 2018).

3.2 *Biosurfactant production*

The production of BS (Fig. 3) reached its peak value (206.96 mg/L) on day 4. Afterward, it decreased to an average value of 94.85 mg/L. A similar behavior was observed in the emulsification capacity: Its highest value of 0.2895 was observed on day and decreased in the days after until reaching an average value of 0.1358 (OD_{600}) on day 12.

The solution of Eq. [6] plotted in Figure 4 was used to predict the relationship between BS production and substrate consumption (TH and AH). The values obtained from the $Y_{p/s}$ coefficient were 63.554 mg BS/g TH and 248.92 mg of BS/g AH. Additionally, the correlation coefficient between TH and AH consumption and BS production showed that TH consumption and BS production obtained the highest value (0.9186).

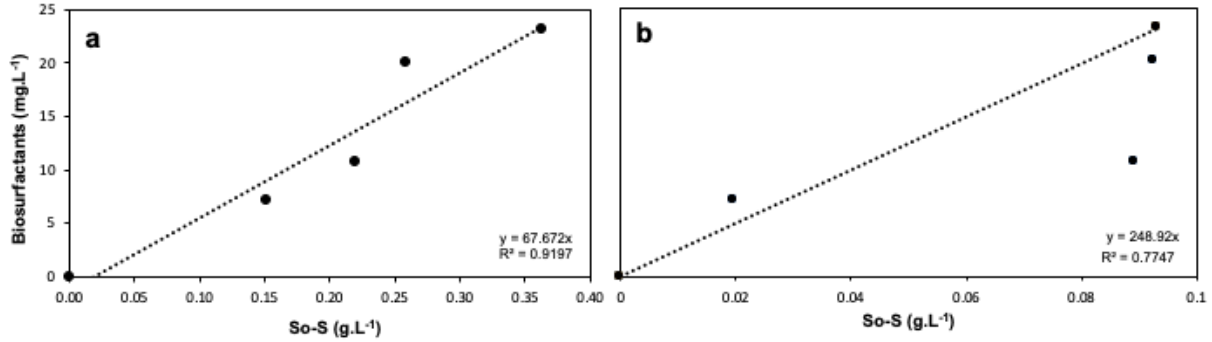

Figure 4. Kinetic analysis of the relationship between hydrocarbon consumption and biosurfactant production. Values of $Y_{P/S}$ and correlation coefficient are presented per group of hydrocarbons. Total hydrocarbons (a) and aromatic hydrocarbons (a) are adjusted by linear regression (⋯) in both cases.

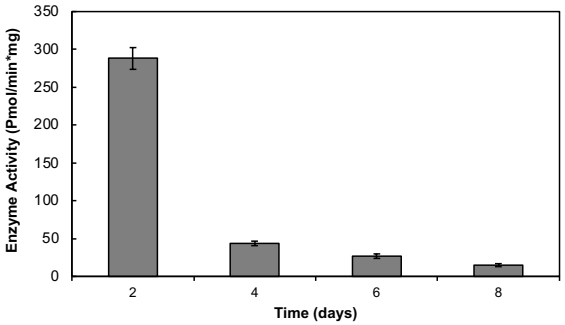

Figure 5. Analysis of enzymatic activity from native microbial consortium during crude oil biodegradation. Data shown represents the average of three independent trials. One-factor analysis of variance; p = 0.05.

Therefore, BS production has a better relationship with the presence and consumption of TH in CO, despite the values obtained from the $Y_{p/s}$ coefficient. However, the correlation coefficient between TH and AH consumption showed the lowest value (0.7747) because, usually, aliphatic hydrocarbons are biodegraded more rapidly than aromatic hydrocarbons (AHs) (Bacosa *et al*., 2011). Additionally, BS production is an adaptation and survival mechanism by the presence of hydrocarbons. BS are metabolites associated with growth, allowing the hydrophobic solubilization by emulsifying hydrocarbons in early stages of the biodegradation process (Alvarado *et al*., 2022).

3.3 Enzyme activity

The enzyme activity of C23O was analyzed in free cell extract obtained after CO biodegradation (Fig. 5). This trend points to NMC's ability to use crude oil as a carbon and energy source. As can be seen in Fig. 2, the C23O activity increased (285.13 ± 29.39 pmol min/mg protein) in the first two days of incubation; however, the activity decreased drastically by 80% for the following days of incubation. This increase in C23O enzymatic activity could suggest that the meta-cleavage pathway was induced for HA biodegradation. This coincides with the yellow color of the culture medium due to the production of the 2-hydroxy muconic semialdehyde metabolite catalyzed by the C23O enzyme (Chettri & Singh, 2019). On the other hand, the decrease in the enzymatic activity of C23O could be due to a decrease in the presence of aromatic hydrocarbons (mono and/or poly) under the conditions of the present investigation. However, this scenario needs to be deepened with more studies.

Many efforts are necessary to implement new technologies that guarantee clean water for the population. In this work, we present an alternative to remove hydrocarbons from wastewater and obtain byproducts with value added. During the biodegradation process of CO, the byproducts BS and enzyme (Catechol 2,3 dioxygenase) can be obtained from the free-cell crude extract. We analyzed the kinetic degradation of CO by an NMC to determine the relationship between BS production and the enzyme activity of C23O. According to the results, NMC degraded 74.24% of the TH in CO. However, the highest value was observed in AH degradation (96.88%). After analyzing microbial progress behavior, a specific growth rate of 0.3108 d^{-1} was identified.

In previous work, Padhi and Gokhale (2017) reported a specific growth rate of 0.912 d^{-1} when an indigenous mixed culture was tested for degraded benzene for a period of 72 h. This result is better than the value obtained in the present work since benzene is more easily degraded than CO. Another report indicated 48% efficiency when removing asphaltene using a mixed culture for 60 days at a growth rate of 0.310 d^{-1} (Tavassoli *et al*., 2012). This result agrees with this work, but the degradation percentage obtained in this work was superior (74.23 %). This can be explained by the observation present in other works, where aliphatic hydrocarbons in a petroleum mixture were biodegraded more rapidly than aromatic hydrocarbons (Zrafi-Nouira *et al*., 2009).

The specific growth rate obtained in this work agrees with previous reports, although some works have indicated

superior values. The behavior of the NMC is a consequence of the complex mixture of hydrocarbons; still, the NMC showed a preference for the AH in CO. The higher value obtained from AH degradation likely indicates a preference of the NMC for compounds like benzene, toluene, biphenyl, and naphthalene, among others detected in the GC-MS analysis. However, previous reports evaluating the capacity of *Rhodococcus* sp. to degrade a BTEX mixture show a specific growth rate of 20.73 and a specific degradation rate of 22.87 d^{-1} (You *et al*., 2018). In terms of bioremediation of petroleum contaminants, microbial consortia with the metabolic ability to degrade a wide range of hydrocarbons are preferred over a pure isolate (Gazali *et al*., 2004). This work demonstrated that NMC could degrade AH in a complex mixture of hydrocarbons like CO.

One of the key factors in CO degradation is a consequence of the lower solubility of petroleum hydrocarbons. Some reports have indicated an increase in the bioavailability of CO when microbial consortia produce BS or bioemulsifiers (Alvarado *et al*., 2022; El-Sheshtawy *et al*., 2022). The production and application of these molecules are of great interest in recent works (Tao *et al*., 2020). The solubility of polycyclic AH and other organic compounds in water improved with the presence of BS. The solubilization mechanism is attributed to the aliphatic characteristic of BS and their potential to create micelles (Lamichhane *et al*., 2017).

The solubility of AH compounds increases with the emulsifier concentration. Some reports have found a specific interaction between BS (rhamnolipids) and aromatic compounds in micelle formation. These interactions allow to improve the biodegradation process through changes in the enzyme activity (Chrzanowski *et al*., 2009). In this work, the BS production was directly related to the presence of total hydrocarbons and not that of AH, as proven by the kinetic analysis. Still, the BS production was not directly influenced by AH, as the kinetic analysis demonstrated.

The analysis of the relation between substrate consumption and BS production ($Y_{p/s}$) revealed values of 63.554 and 248.92, expressed as mg BS/g substrate consumed. Although the highest value was obtained with AH, the kinetic analysis revealed a major relation between TH with BS production. This means that BS production is a response of the NMC to the stress generated by the presence of the hydrocarbon mixture but is not related to a specific compound. In addition, the BS production and emulsification capacity decreased when AH was degraded, likely because of the possible encapsulation of AH into the micelles formed with BS. Therefore, according to previous reports, there can be changes in the enzyme activity due to the presence of BS (Chrzanowski *et al*., 2011).

Conclusions

The present work focused on the ability of an NMC to biodegrade CO and its capacity to remove hydrocarbons, especially AH, in wastewater. During the evaluation of the biodegradation process of CO, the production of BS and enzyme activity of C23O were observed. The decrease in enzyme activity and BS production was related to the reduction in the concentration of AH in wastewater. Finally. The NMC removes hydrocarbons in polluted water; additionally, byproducts with the capacity to act in hydrocarbon-contaminated environments. During the evaluation of crude oil's biodegradation process, biosurfactants' generation and the presence of the C23O enzyme were observed. The decrease in the activity of the latter and the production of biosurfactants could be related to the reduced concentration of aromatic hydrocarbons in wastewater. Finally, NMC can potentially remove hydrocarbons in contaminated wastewater and create products with applicability in bioremediation.

Nomenclature

CO	Crude oil
PAH	Polycyclic aromatic hydrocarbons
AH	Aromatic hydrocarbons
TH	Total hydrocarbons
C23O	Catechol 2,3 dioxygenase
BS	Biosurfactants
NMC	Native Microbial Consortium

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