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NOTA CIENTÍFICA

Petroleum hydrocarbon degradation by isolated mangrove bacteria

Degradación de hidrocarburos de petróleo por bacterias aisladas de manglares

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Abstract

The petroleum hydrocarbon contamination represents a worldwide problem, since its accumulation promotes a serious environmental impact. Thereby, the use of microorganisms, such as those from mangrove micro biota, as degrading agents of various carbon sources is poorly exploited in environmental remediation processes. Thus, this *in vitro* study evaluated the degrading potential of isolated bacteria from mangrove sediments in the degradation of petroleum hydrocarbons. Analysis of the genetic diversity using the 16S rRNA marker revealed closely related (99%) sequences with *Proteobacterium*, *Pseudomonas* and *Exiguobacterium*. Results showed the bacterial growth in the mineral saline medium (MSM) containing 1% petroleum or diesel, as carbon sources. This growth was determinated by optical density at 595 nm for 15 days, with sample withdrawal every 48 h. Bacterial growth indicated the hydrocarbon metabolization. However, bacteria were more efficient at degrading petroleum. Overall, experimental data displayed the potential application of these bacteria in bioremediation processes, due to their metabolic and adaptive capacities to grow in a rich hydrocarbon medium.

Keywords: microorganisms; metabolism; Hydrocarbons; pollution; bioremediation.

Resumen

Los hidrocarburos de petróleo representan un problema mundial, pues su acumulación promueve un serio impacto ambiental. Así, el uso de microorganismos, por ejemplo los de la microbiota de manglares, como agentes degradadores de diversas fuentes de carbono, es poco explotado en procesos de remediación ambiental. Así, este estudio evaluó *in vitro* el potencial degradador de bacterias aisladas de sedimento de manglar en la degradación de hidrocarburos. El análisis genético usando el marcador 16S rRNA reveló secuencias íntimamente relacionadas (99%) con *Proteobacterium*, *Pseudomonas* y *Exiguobacterium*. Los resultados mostraron el crecimiento de bacterias en medio salino mineral (MSM) conteniendo petróleo o diesel al 1%, como fuentes de carbono. Este crecimiento, determinado por densidad óptica (DO) a 595 nm durante 15 días, con toma de muestras a cada 48 h, indicó la matabolización de hidrocarburos. Sin embargo, las bacterias fueron más eficientes en degradarlos. Por lo tanto, los resultados muestran la potencial aplicación de las bacterias en procesos de biorremediación por su capacidad metabólica y adaptativa de crecimiento usando hidrocarburos.

Palabras clave: microorganismos; metabolismo; hidrocarburos; contaminación; biorremediación.

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Introduction

Petroleum and its derivatives are a source of aliphatic, alicyclic and aromatic hydrocarbons, representing an energy source as well as one of the main pollutants worldwide, due to spills of urban, industrial wastes and gas emissions (Khan et al. 2018, Logeshwaran et al. 2018).

Petroleum is a hydrophobic compound complex mixture (*e.g.* alkanes, aromatic substances, asphaltenes, and resins). Most of these components (between 60% and 90%) are biodegradable, while 10 to 40% of these components are in crude or refined oil to recalcitrant molecules (Yang et al. 2017). Although it is a small rate, therecalcitrant portion represents tons of pollutants impacts on ecosystems due to bioaccumulation and bioaugmentation in the food chain (Posada-Baquero & Ortega-Calvo 2011).

Thus, hydrocarbon accumulation and exposure represent a risk to health and the environment, causing adverse effects (Tormoehlen et al. 2014, Ahmed & Fakhruddin 2018). Thereby, the hydrocarbon removal is crucial, leading to apply several procedures, such as soil washing, chemical oxidation, electrokinetics, phytoremediation (Gitipour et al. 2018). Moreover, microorganisms also play a key role in the degradation of these pollutants into simple and less toxic compounds by the process called bioremediation (Singh & Chandra 2014).

However, the petroleum fraction compound aqueous low solubility hinders the microbial action in degrading them. Therefore, the microbial degradation first stage implies an efficient interaction between the cell surface and oil in order to facilitate the compound transport across cell membranes (Seo et al. 2009).

Despite studies in this area, oil transport and conversion mechanism by the bacterial biochemical machinery is not fully elucidated (Devpura et al. 2017). However, the petroleum hydrocarbon biodegradation by natural microbial communities is one of the primary mechanisms for its elimination or control (Varjani 2017). Based on its low energy cost and the compound persistence decreasing regarding other processes, the bioremediation is an ecological and economically feasible waste treatment route to minimize environmental impact (Al-Hawash et al. 2018).

Regarding bioremediation, mangrove soils are a microorganism source to be prospected with respect to its ability to degrade hydrocarbons. The microbial diversity in this biome is due its environmental conditions, with high salinity and organic matter levels (Santos et al. 2011). Under these stress conditions, it is possible to isolate microorganisms capable of degrading different carbon sources, playing a key role in the functioning and maintenance of this ecosystem. However, this microbial diversity is still poorly explored regarding its biotechnological potential (Sahoo & Dhal 2009, Tiralerdpanich et al. 2018).

Based on the role of these microorganisms in the transformation of several carbon sources, participating in biogeochemical cycles, there is a scientific interest in prospecting them as hydrocarbon degrading agents. Therefore, mangrove microbial communities should be analyzed as a biological tool applicable in bioremediation processes (Pucci et al. 2010, Friesen et al. 2018).

Thereby, this study assessed the *in vitro* capacity of bacteria isolated from mangrove sediments to metabolize petroleum and diesel, used as carbon source.

Material and methods

Sampling area, and bacterial isolation and identification.- Mangrove sediment samples were collected in geo-referenced areas, influenced by Vasa-Barris and Piauí-Real Rivers, State of Sergipe, Brazil, under the State Environmental Administration (ADEMA) guidance. Samples were placed in plastic bags, transported and stored at 4 °C for microbiological evaluation. For the hydrocarbon tolerant bacteria isolation, mangrove soil (1 g) was suspended in 9 mL of 0.85% NaCl and stirred in an orbital shaker (180 rpm/50 min/room temperature). Thereafter, 10-fold serial dilution bacterial suspension was spread on plates containing MSM-agar coated with fluoranthene, phenanthrene and pyrene surface layer diluted in acetone at 10 and 50 mg/L^{-1}, and incubated at 28 °C for 72 h (Tam et al. 2003). Bacterial isolates were identified by analyzing color, morphology and Gram staining by optical microscopy (×1000) (Axisoskop, Zeiss, Germany). Isolates were kept in Luria Bertani medium (tryptone, 10 g/L, yeast extract, 5 g/L and NaCl, 5 g/L) and agar at 4 °C and transferred at 2-week intervals (Liu et al. 2016).

Isolated strains were then identified by PCR amplification of the 16S rDNA gene sequence, using primers 68F (5'AAC GCG AAC CTT AC 3') 1401 and R (5'-CGG TGT GTA CAA GAC CC 3') (Fox et al. 1977). Amplification was performed under following conditions: 3 min at 95 ºC, followed by 35 cycles at 40 s at 94 ºC, 40 s at 56 ºC, 40 sat 72 ºC and 3 min at 72 ºC. After sequencing, DNA 16S rDNA amplicons from each isolate were identified by comparison using BLASTN at http://www.ncbi.nlm.nih.gov/BLAST.

Biodegradability assay.- This assay was performed in 100 mL Erlenmeyer flasks containing 40 mL MSM (KH_2PO_4 0.5g/L, K_2HPO_4 0.5 g/L, $CaCl_2$ 1g/L, NaCl 2.0 g/L, $MgSO_4$ $7H_20$ 0.5 g/L, $MnSO_4$ $7H_2O$ 0.01 g/L, $FeSO_4$ $7H_2O$ 0.01 g/L, NH_4NO_3 1.0 g/L) supplemented with 1% crude petroleum or diesel, as carbon sources (Prathyusha et al. 2016). After adjusting the culture medium pH to 7.0, and autoclaving them, bacteria were inoculated separately into each vial and incubated at 27 ºC for 15 days. Samples were collected at 48 h intervals to evaluate bacterial growth by turbidometry (density (OD) at 595 nm).

Isolated mangrove soil bacteria *Bacillus aerophilus*, *Exiguobacterium profundum*, *Pseudomonas xanthomarina*, *Proteobacterium*, *Bacillus aerophilus*, *Pseudomonas* sp. and *Bacillus* sp. were used in this assay for their ability to degrade hydrocarbons.

Results and discussion

Mangrove hydrocarbon-degrading bacteria: isolation and identification.- Microbial growth using a pyrene, fluoranthene and phenanthrene as carbon source allowed to isolate 10 colonies capable of metabolizing hydrocarbons. The microscopic analysis identified the isolates as gram-negative bacteria, morphologically related to coccus, bacilli and coccobacilli (Table 1).

From these colonies, six isolates showed a hydrocarbon biodegradation efficiency. The 16S rRNA gene fragment sequencing indicated sequences closely related to *Proteobacterium*, *Pseudomonas* and *Exiguobacterium*, showing identity levels ranging from 90 to 99%, as depicted in Table 1.

Bacteria belonging to *Pseudomonase*, *Bacillus*, *Exiguobacterium* and *Proteobacterium* genus have been reported with

hydrocarbon-degrading capacities (Prathyusha et al. 2016, Begum & Rahul 2017).

The accumulation and changes in the composition of in this ecosystem results in a diverse indigenous microbiota still poorly understood (Andreote et al. 2012). These aspects, therefore, stimulate the exploration of hydrocarbon-degrading microorganisms for the development and management of oil bioremediation strategies (Angel 2018).

Hydrocarbon-degrading bacterium screening.- To evaluate the bacterium potential, the screening was performed in mineral saline supplemented with 1% of petroleum and diesel, used separately. Although required as a carbon source, these hydrocarbons may be toxic to bacteria due to their solvent effect on the lipid structures of cell membranes (Denich et al. 2013). However, the results showed the ability of these microorganisms to metabolize petroleum or diesel, observed by the culture medium turbidometric determination. Based on the MSM broth turbidity, assessed at 24 h regular intervals, it was determined the catabolic ability bacteria to use hydrocarbons provided by the petroleum and diesel addition as energy substrates (Figs. 1 – 2).

With respect to petroleum used as carbon source (Fig. 1), results displayed higher growth peaks of the microorganisms between 6 and 10 days of culture with *P. xanthomarina*, *Proteobacterium* and *B. aerophilus* exhibiting the highest OD values, ranging from 0, 48 and 0.90, respectively, compared to *E. profundum*, *Pseudomonas* sp. and *Bacillus* sp. (D.O. mean value = 0.350).

Also, it is worth mentioning that *P. xanthomarine*, *Proteobacterium*, *Pseudomonas* sp. and *E. profundum* exhibited a varied increase regarding their turbidity upon reaching the 10th day growth, after the decline phase (Das & Chandran 2011). This behaviour may indicate a bacterial assimilation process of metabolites, resulting from the petroleum hydrocarbon degradation. This metabolic aspect provides important information regarding microorganisms used as consortia to increase pollutant degradation and improve the environmental recovery process efficiency (Patowary et al. 2016).

Regarding the diesel biodegradation, bacteria exhibited growth, monitored by optical density with maximum values between 0.2 and 0.6, as depicted in Figure 2. This result was similar to that described by Nwinyi et al (2014), using bacteria belonging to the same genus displayed the ability to degrade diesel. However, the bacterial species growth decrease in diesel compared to the use of petroleum the carbon source can be attributed to the hydrocarbon stress on cell membranes, affecting the permeability and, consequently, the energy production. However, all bacteria exhibited idiophasic growth behavior over the experimental period, indicating a microbial metabolic adaptation related to the use of diesel degradation metabolites (Palanisamy et al. 2014).

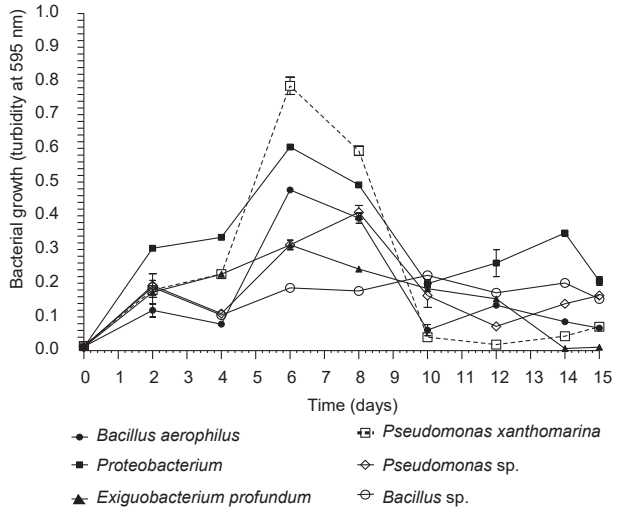

Figure 1. Hydrocarbon degradation potential of mangrove sediment bacterial isolates by evaluating their growth in MSM broth supplemented with 1% petroleum as carbon source.

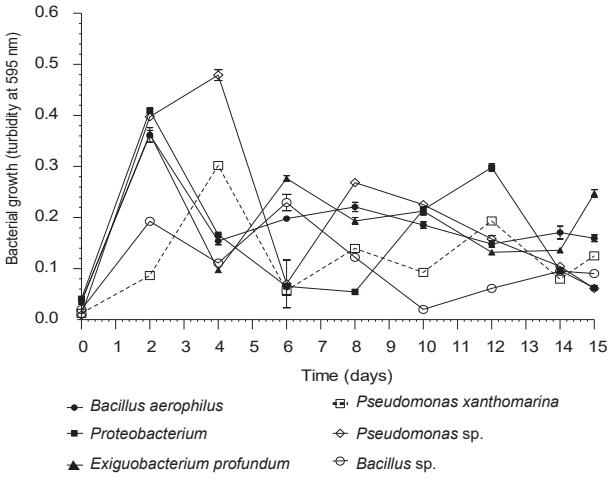

Figure 2. Hydrocarbon degradation potential of mangrove sediment bacterial isolates by evaluating their growth in MSM broth supplemented with 1% diesel as carbon source.

Table 2. Molecular identification of bacterial isolates of mangrove sediments by the maximum likelihood method.

Bacterium	Identity level (%)	Genbank access number*	Morphology
Bacillus aerophilus	90	KT758426.1	coccobacilli
Exiguobacterium profundum	99	AB752299.1	coccus
Pseudomonas xanthomarina	99	AB176954.1	coccobacilli
Proteobacterium	100	KC887933.1	coccobacilli
Pseudomonas sp.	92	KJ126972.1	coccus
Bacillus sp.	94	Z26929.1	bacilli

Overall, it concludes that results show the potential of bacterial species isolated from mangrove soils. Although preliminary, the bacteria were able to degrade hydrocarbons from petroleum and diesel, used as carbon sources for their growth. However, further studies are required in order to optimize parameters relating to physical, chemical and biological conditions, aiming to develop bioremediation processes.

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