Research, Society and Development, v. 10, n. 17, e220101724706, 2021
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v10i17.24706

Crude oil biodegradation by bacterial cells immobilized on corn starch-alginate beads

Biodegradação do petróleo por células bacterianas imobilizadas em esferas de amido de milho e alginato

Biodegradación del petróleo crudo por células bacterianas inmovilizadas sobre esferas de almidón de maíz y alginato

Received: 12/13/2021 | Reviewed: 12/19/2021 | Accept: 12/24/2021| Published: 12/27/2021

Carla Jaqueline Silva Sampaio
ORCID: https://orcid.org/0000-0003-1316-1084
Universidade Federal da Bahia, Brasil
E-mail: sampaiosjcarla@gmail.com
José Roberto Bispo de Souza
ORCID: https://orcid.org/0000-0002-8159-305X
Universidade Federal da Bahia, Brasil
E-mail: joserbs@ufba.br
Gilson Correia de Carvalho
ORCID: https://orcid.org/0000-0003-1800-888X
Universidade Federal da Bahia, Brasil
E-mail: biogilson@gmail.com
Cristina Maria Quintella
ORCID: https://orcid.org/0000-0002-3827-7625
Universidade Federal da Bahia, Brasil
E-mail: cris5000tina@gmail.com
Milton Ricardo de Abreu Roque
In memoriam
ORCID: https://orcid.org/0000-0002-9974-7731
Universidade Federal da Bahia, Brasil

Abstract
Immobilized cells have shown advantages in removing hydrocarbons from oil compared to the use of free cells. This work aimed to evaluate the potential of hydrocarbon degradation by bacteria immobilized in support matrices composed of commercial potassium alginate and corn starch. The polymeric immobilizing matrix was developed using dental alginate 2% and corn starch 0.5% and 1%. The matrices have macropores internally, good ability to immobilize cells, increasing baseline respiration (F2 and F3: 4 mg / l CO_2 60 days), bacterial biomass (F1: 1.5 x 10^6 CFU / g 60 days) and the removal of n- alkanes and PAHs of the sediment, when compared to free cells (0 mg / l CO2; 0.3 x 10^6 CFU / g 60 days). The aromatics hydrocarbons benzo (a) anthracene and anthracene were not degraded. Naphthalene and dibenzo (a) anthracene reached degradation rates of 60% and 80%, respectively. Therefore, the consortium tested and the polymer matrix developed are promising for use in bioremediation of environments contaminated by hydrocarbons.
Keywords: Biodegradation; Immobilization cell; Polymeric matrix; Hydrocarbon.

Resumo
As células imobilizadas apresentam vantagens na remoção de hidrocarbonetos do petróleo, quando comparadas ao uso de células livres. Este trabalho teve como objetivo avaliar o potencial de degradação de hidrocarbonetos por bactérias imobilizadas em matrizes de suporte compostas de alginato de potássio comercial e amido de milho. A matriz polimérica imobilizadora foi desenvolvida com alginato dental 2% e amido de milho 0,5% e 1%. As matrizes apresentam macroporos internamente, boa capacidade de imobilizar células, aumento da respiração basal (F2 e F3: 4 mg / l CO_2 60 dias), biomassa bacteriana (F1: 1,5 x 10^6 UFC / g 60 dias) e remoção de n-alcanos e HPAs do sedimento, quando comparados às células livres (0 mg / l CO_2; 0,3 x 10^6 UFC / g 60 dias). Os hidrocarbonetos aromáticos benzo (a) antraceno e antraceno não foram degradados. Naftaleno e dibenzo (a) antraceno atingiram taxas de degradação de 60% e 80%, respectivamente. Portanto, o consórcio testado e a matriz polimérica desenvolvida são promissores para uso na biorremediação de ambientes contaminados por hidrocarbonetos.
Palavras-chave: Biodegradação; Imobilização de célula; Matriz polimérica; Hidrocarbonetos.

Resumen

Las celdas inmovilizadas tienen ventajas en la eliminación de hidrocarburos del petróleo en comparación con el uso de celdas libres. Este trabajo tuvo como objetivo evaluar el potencial de degradación de hidrocarburos por bacterias inmovilizadas sobre matrices de soporte compuestas por alginato de potasio comercial y almidón de maíz. La matriz polimérica inmovilizadora se desarrolló con 2% de alginato dental y 0,5% y 1% de almidón de maíz. Las matrices tienen macroporos internos, buena capacidad para inmovilizar células, aumento de la respiración basal (F2 y F3: 4 mg / l de CO_2 60 días), biomasa bacteriana (F1: 1,5 x 10^6 UFC / g 60 días) y eliminación de n-alcanos y HAP del sedimento, en comparación con las células libres (0 mg / l CO_2; 0,3 x 10^6 UFC / g 60 días). Los hidrocarburos aromáticos benzo (a) antraceno y antraceno no se degradaron. El naftaleno y el dibenzo (a) antraceno alcanzaron tasas de degradación del 60% y el 80%, respectivamente. Por lo tanto, el consorcio probado y la matriz polimérica desarrollada son prometedores para su uso en la biorremediación de entornos contaminados por hidrocarburos.

Palabras clave: Biodegradación; Celda de inmovilización; Matriz polimérica; Hidrocarburos.

1. Introduction

The use of oil as an energy source presents a high risk to human health consequently resulting in environmental, economic and cultural damage (Kureel et al., 2017; Shen et al., 2015). Soil oil contamination can trigger the absorption and bioaccumulation of hydrocarbons by living organisms, also causing severe due to plant and animal exposure to hydrocarbons present in both fresh and weathered oil (Simons et al., 2013).

Petroleum hydrocarbon biodegradation is particularly limited by the low availability of these compounds, due to their reduced water solubility and strong tendency to adsorb to inorganic and organic soil components, reducing the efficiency of bioremediation processes (De la Cueva et al., 2016). Bioremediation is a slow process that depends, above all, on microorganisms that enzymatically transform pollutants into harmless products, such as CO_2 and H_2O. Bacteria demonstrate the ability to degrade oil, although a series of factors such as pH, temperature, oxygen, soil structure, moisture, nutrients, contaminant bioavailability and toxicity, and enzymatic apparatus, limit bacterial strain performance in biodegradation processes (Karigar; Rao, 2011).

The application of microorganisms entrapped in inert matrices is noteworthy as a tool of increasing interest in the decontamination of environments impacted by human activities linked to the oil production chain (Chen et al., 2016). The cell immobilization technique promotes cell protection and stability in the presence of contaminants, through the microenvironment provided by the employed matrix. Additionally, the microenvironment allows for cell multiplication and increases in biomass, aiding in pollutant degradation in contamination cases. This characteristic is essential for the use of immobilized cells in bioremediation, as it increases the efficiency of this process compared to the use of free cells (Chen et al., 2019; Li et al., 2019).

Materials used for cell immobilization must be similar to the extracellular matrix and suitable for cell survival, either from organic or inorganic origin. Materials of an organic origin are in general, more employed than inorganic materials due to specific characteristics that allow for greater cell viability. Most organic materials are composed of polymers that form hydrogels, such as cellulose, dextran, alginate, carrageenan, agar, agarose, chitosan and chitin. Inorganic origin matrices comprise silica, zeolite, clay and porous glass, among others (Bayat et al., 2015; Elnashar, 2010).

Among polymers of natural origin, alginate is noteworthy for its wide use in cell immobilization processes. Alginate, a polysaccharide obtained mainly from brown algae, makes up a family of copolymers made up of blocks of α-L-glucuronic acids (G) and β-D-manuronic acids (M) with 1-4 bonds (Xue et al., 2020); De Vos et al., 2014). Alginate exhibits certain physico-chemical properties, such as porosity and biocompatibility, making it an advantageous matrix for immobilization (Zhang et al., 2018).

The aim of the present study was to evaluate the potential of hydrocarbon degradation by immobilized bacteria in support matrices composed of commercial potassium alginate with or without corn starch, which present low production costs.

2 Methodology

2.1 Isolation, selection and bacterial identification

Strains were isolated from the gastrointestinal tract of Polychaeta worms obtained from hydrocarbon-contaminated sediment sampled by a corer device (40 cm depth) from Bahia de Todos Santos Bay, Bahia, Brazil, reported previously in research group work (Sampaio et al., 2019a).

For bacteria isolation from the gastrointestinal tract, the animals were submitted to a previous hygienization with sodium hypochlorite (2%) for 30 sec., alcohol (70%) for 30 sec. and subsequent washing with sterile distilled water for 3 times. The gastrointestinal tract was removed with the aid of a sterile needle, macerated and subjected to serial dilutions. Aliquots (100µL) from the serial dilution tubes were transferred to plates containing Zobell marine agar (Himedia) and incubated at 30 °C for 24h to 72h. The strains used to comprise the consortium were chosen after the evaluation of their isolated potential to degrade petroleum hydrocarbons based on crude oil tests. The bacteria were grown on nutrient agar (Himedia) at 30°C for 24h and inoculated into test tubes containing 9 mL of Büshnell-Haas mineral medium (BH) (K_2HPO_4 1.0g/L; KH_2PO_4 1.0 g/L; NH_4NO_3 1.0 g/L; $MgSO_4$ ($7H_2O$) 0.20 g/L; $FeCl_3$ ($2H_2O$) 0.05 g/L; $CaCl_2$ ($2H_2O$) 0.02 g/L; pH 7.0), 1% crude oil and 50 µL of the redox indicator 2,6-Dichlorphenol-indophenol (DCPIP), incubated at 30°C under orbital shaking at 120 rpm for 7 days (Hanson et al., 1993; Bučková et al., 2013). Tests were performed in triplicate, with an abiotic control.

For bacterial identification, genomic DNA was extracted and deposited at the GenBank Genetic Sequence Database (NCBI, USA) under accession number CCMICS 560 *Acinetobacter sp.* (GenBank accession number **MG926782)**, CCMICS 562 *Bacillus sp.* (GenBank accession number **MG926824)**, CCMICS 563 *Pantoea sp.* (GenBank accession number **MG928438)** and CCMICS 564 *Enterobacter sp.* (GenBank accession number **MG928498)**.

2.2 Bacterial cells immobilization in corn starch - alginate beads

The bacterial strains were previously grown in nutrient agar (Himedia) at 30°C for 24h. Following bacterial growth, bacterial cells were collected by centrifugation at 5000 rpm for 10 min. The bacterial cells were resuspended in sterile saline solution (0.9% NaCl) at an OD_{600} nm of 10^8 CFU/mL and mixed (1mL of cell suspension) with 20mL of solutions containing dental potassium alginate (Ezact Krom) and commercial corn starch (Table 1). This step was performed individually for each bacteria. Subsequently, the resulting mixture was extruded drop by drop into cold calcium chloride solution ($CaCl_2$ 0.1 M), alginate-cell solution was gelled forming uniform-sized (~0.25 cm) Ca - corn starch - alginate beads with entrapped bacterial cells. After being aged in $CaCl_2$ (0.1 M) solution for 1 h, the beads were washed three times with sterile distilled water to remove any excess $CaCl_2$. Finally, the beads were immersed in distilled water with glycerol (5% v/v) and stored at 4 °C (Gbassi et al., 2009). Patent deposit PI national BR102019013058-0, in secrecy phase.

Table 1. Composition of tested polymeric matrices.

Treatments	Composition
Control	Sediment + 1% crude oil
Free cells	Sediment + 1% crude oil + 1mL of each bacterial strain
F1	Sediment + 1% crude oil + 300mg of each bacterial immobilized beads
F2	Sediment + 1% crude oil + 300mg of each bacterial immobilized beads
F3	Sediment + 1% crude oil + 300mg of each bacterial immobilized beads

Source: Authors (2021).

2.3 Beads structure analysis by scanning electron microscopy (SEM)

Beads were fxed in Karnovsky's solution (70%) at pH 7.4 (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 mol L−1 sodium cacodylate buffer and distilled water) for 24 h. The samples were washed in 0.1 mol L^{-1} sodium cacodylate bufer and postfxed in osmium tetroxide 1%. After fxation, the samples were dehydrated in ethanol (30, 50, 70, 90 and 100%), dried on a critical point apparatus (Leica, model CPDO30) and metallized with a thin layer of gold (Deton Vacuum Desk IV Standard model) and analyzed by a JEOL Scanning Electron Microscope JSM, model 6390LV (Su et al., 2006).

2.4 Biodegradation test with immobilized cells beads

Biodegradation assay was conducted in a microcosm in amber flasks with capacity for 1L adapted with containers for evaluation of microbial activity through CO_2 uptake. The microscosm consisting of sterilized sediment, crude oil and bacterial immobilized beads and saline suspension (0,9% NaCl) containing free cells to control (OD_{600} nm of 10^8 CFU/mL), adapted from Wang et al. (2012). To maintain 50% of the sediment field capacity, medium BH was used. The compositions of microcosm for each treatment are shown in Table 2.

Table 2. Microcosm composition for each treatment.

Formulations	Potassium alginate (w/v)	Corn starch (w/v)
F1	2%	_
F2	2%	0,5%
F3	2%	1%

Source: Authors (2021).

Microcosms were placed at 30ºC, in the dark, for 60 days, and the aeration was performed mechanically. The microcosm tests were performed in triplicate.

Microorganism activity was evaluated by means of respirometric analyses. A plastic vessel containing 20 mL of NaOH (0,5 M) solution, prepared using distilled water, was inserted in each microcosm. The solution was removed, 2mL of $BaCl_2$

solution was added (10% w/v) and titrated with HCl (0,5 M) with phenolphthalein (1% w/v) . The flasks were titrated at times: 7, 15, 25, 30, 40, 50 and 60 days.

The captured CO_2 was quantified according to the following equation: mass of mg de mass of CO_2 = ((V_0 - V) x M x 6 x 1000)/Ws, where V_0 and V are the volumes (mL) of HCl used to titrate the abiotic control and the sample respectively, M is the molar concentration of the HCl solution and 6 is the conversion factor, Ws is dry sediment weight (Alef; Nannipieri, 1995).

2.5 Bacterial biomass assessment

Bacterial counting in the sediment was accomplished using the dilution plate-count method. An 1g sediment of each microcosm was added to tubes containing 9 mL of sterile saline solution (0.9% NaCl) and mechanically stirred. After a serial dilution, 100 µL of the suspensions were inoculated onto plates containing Nutrient Agar (Himedia) and incubated for 24h at 30 °C. After incubation, the replicate colonies were counted and the mean value calculated for each treatment. Counting was performed during the biodegradation test at 0, 7, 15, 25, 30, 40, 50 and 60 days. The results were expressed as log 10 CFU ml^{-1}, modified methodology of Varjani; Upasani (2020).

2.6 GC-MS

The sediment hydrocarbon extractions were performed according to US EPA Method 3550C (2007), in which 2g of the sediment was subjected to ultrasonic extraction using 10 mL of dichloromethane. The extracts were fractionated, where the n-alkanes fraction was eluted in 40 mL dichloromethane and the aromatic fraction (PAHs) was eluted in dichloromethane/n-hexane (1: 4, v/v) (US EPA, 1996). The crude oil was diluted with dichloromethane and analyzed, along with the sediment samples, using gas chromatography/mass spectrometry (GC–MS) (GC–MS QP5050A Shimadzu model).

For the hydrocarbons determinations, the separation column was a DB-5-HP (30 m × 0.25 mm × 0.25 µm; Agilent Technologies), and helium was the carrier gas at fow rates of 2.0 mL min−1. The n-alkanes, isoprenoids and PAHs were identified by the external standard method using an analytical curve prepared with Alkane Calibration std, 40147-U SUPELCO TCL and Polynuclear Aromatic Hydrocarbon Mix, 48905-U SUPELCO. The sediment marine standard certificate from the National Institute of Standards and Technology (NIST) SRM 1941b was used as an external standard for the quantifcation of the evaluated compounds. The percentage values of biodegradation were calculated according to equation (Isaac et al., 2015):

$$E_0\,(\%) = \frac{(H_i - H_f)}{H_i} \times 100,$$

where H_i is the initial concentration of hydrocarbons present in diesel oil and H_f is the residual concentration at the end of the experiment. This methodology was previously published by the research group (Sampaio et al., 2019b).

2.7 Analysis of hydrocarbon degradation genes

The presence n-alkanes and aromatic hydrocarbons degradation genes was verified through the PCR technique in the strains. For this purpose, the bacterial strains were cultivated in nutrient agar (Himedia) and incubated at 30°C/24h. After cultivation, DNA was extracted using the Wizard Genomic DNA Purification.Promega® kit. Amplification of the genes alkane monoxygenase alkBF(5'AAYACNGCNCAYGARCTNGGNCAYAA3'), alkBR(5'GCRTGRTGRTCNGARTGNCGYTG3'),

catechol 2,3 dioxygenase cat2,3F(5'AGGTGCTCGGTTTCTACCTGGCCGAAT5') and cat2,3R(5'ACGGTCATGAATCGTTCGTTGAG3') using the Taq DNA Polymerase kit, Brazil (Life TechnologiesTM). The amplified regions were visualized on a 1% agarose gel (m/v) (Ramadass et al., 2016; Karppinen et al., 2017).

2.8 Statistical analyses

The principal component analysis (PCA) using the CANOCO v5.10 package was applied to synthesize the general pattern of n-alkane and PAHs concentration responses throughout the applied treatments. The centroid of the scores of the three replicates was used to represent each treatment on the PCA biplot. An evaluation of the main components was performed by using the percentage of explainability of each component and by comparisons with the broken-stick model (Jackson, 1993; Legendre; Legendre, 2012), where only axes that presented explainability above the broken-stick model were considered interpretable. The significance between treatments, a permutational multivariate analysis of variance (PERMANOVA) was applied to the n-alkane and HPAs degradation data (alpha = 0.05). A Two-way analysis of variance (Two-way ANOVAs) followed by Tukey's Honestly Significant Difference (HSD) post hoc comparisons was used to test the effect of treatments on the hydrocarbons presenting the best degradation, biomass and CO_2 production (PAST v.3.17).

3 Results and Discussion

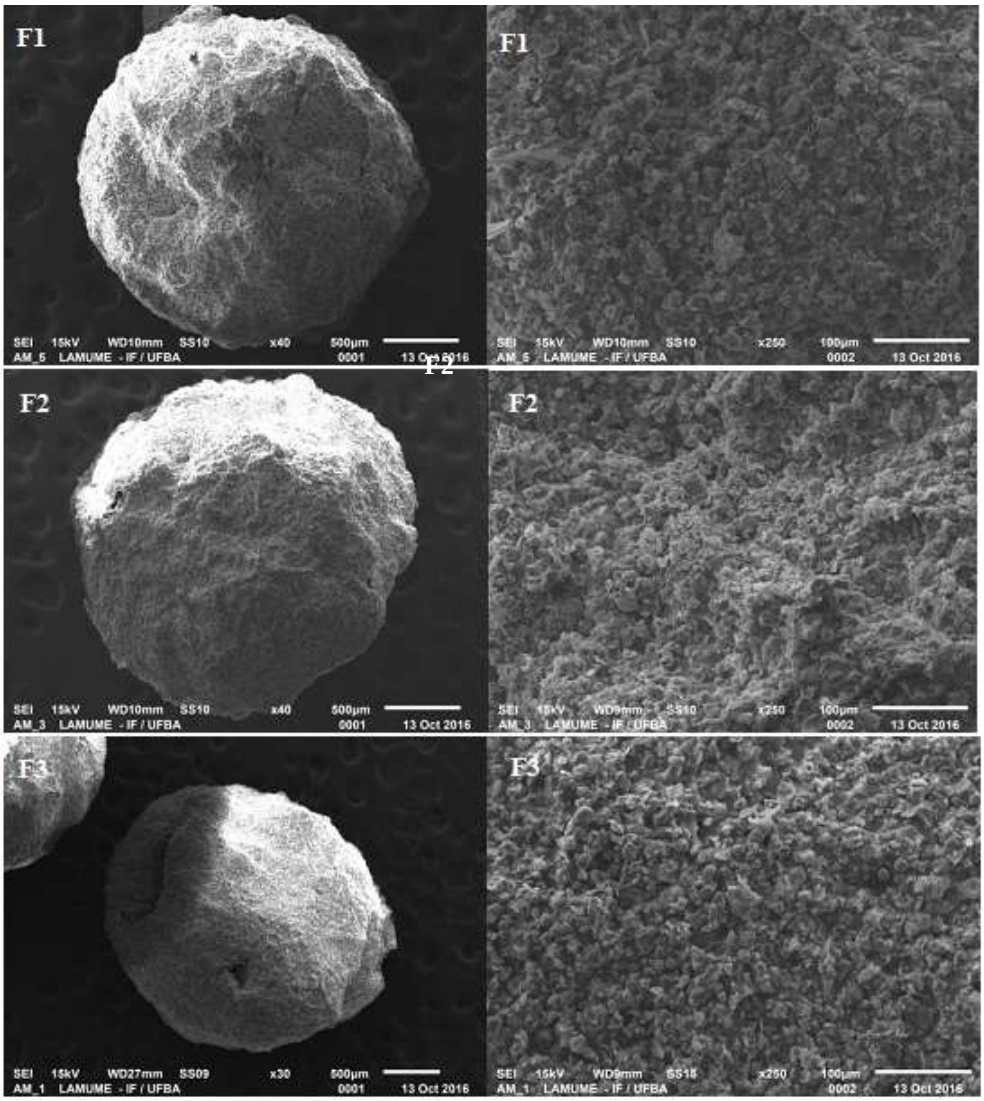
3.1 Corn starch - alginate beads structure

Initially, spheres without lineages were analyzed to verify the morphology originated from the different formulations. The SEM analyses indicated spheres with a spherical appearance and wall uniformity in the different formulations. All presented a similar appearance, massive, without the presence of external macropores (Figure 1), corroborating the smooth aspect and lack of pores in the calcium alginate spheres obtained by Banerjee et al. (2018).

Fareez et al. (2015) also verified the absence or reduction of porosity on alginate bead surfaces. The authors obtained reduced porosity and compacted the surface in formulations containing alginate and chitosan and alginate and xanthan gum. According to the authors, the absence of pores acts as a physical barrier, preventing the rapid diffusion of compounds into the beads, promoting cell protection and preventing their release into the environment. Thus, surface compacting prevents the immediate contact of oil with the employed cells and favors the increase of cell biomass, reducing toxicity and increasing the chances of degradation. However, the internal bead region still presents macroporosities that vary according to formulation (Figure 2).

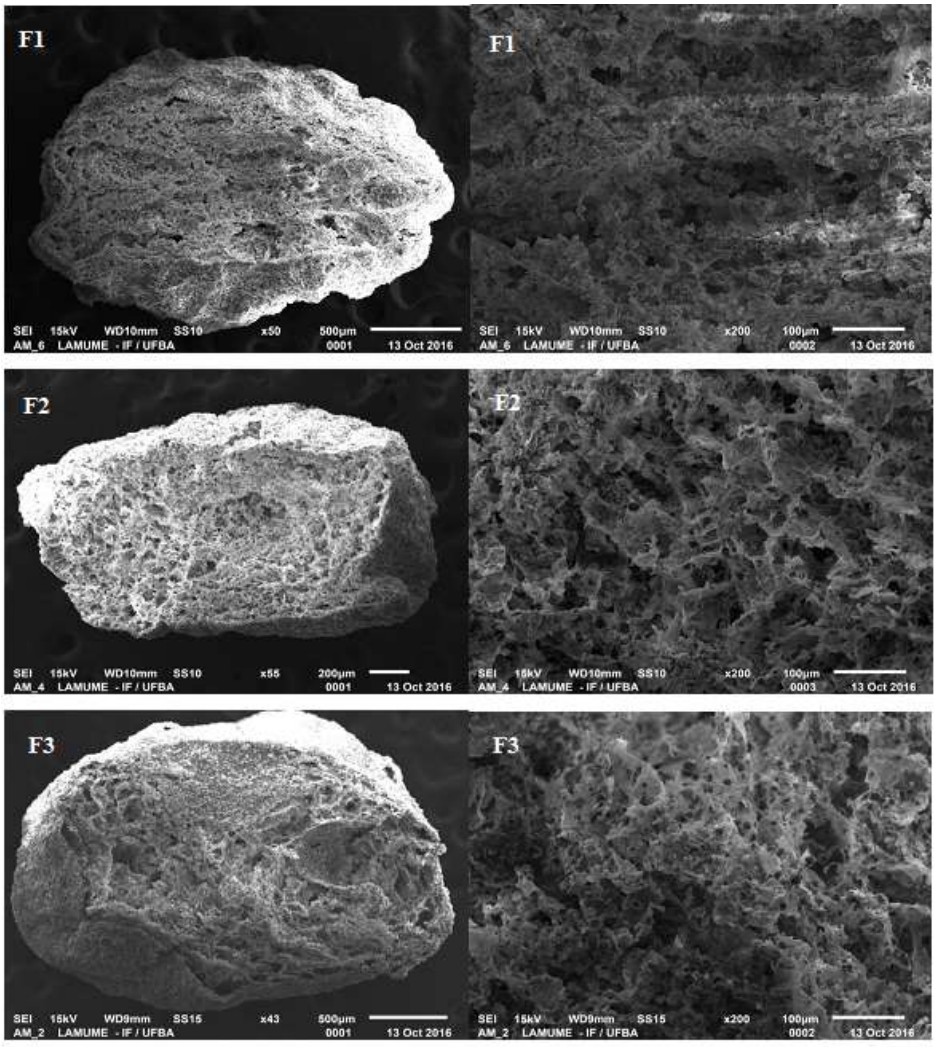
F1 presented a reduced number of macropores, while F2 exhibited greater porosity than the other formulations. The presence of corn starch seems to have promoted changes in the alginate gelation process, favoring pore formation, . Furthermore, corn starch delayed alginate polymerization during solution preparation and the extrusion process. Thus, the presence of starch in concentrations close to or higher than alginate impairs pore formation, as observed in F3. The presence of cells adhered to the polymeric matrix demonstrates the effectiveness of alginate and of its combination with corn starch in cell entrapment (Figure 3). Therefore, the tested support matrix offers favorable conditions for bacterial adhesion, thus comprising a vehicle for the immobilization of cells applied in bioremediation processes. Zommere; Nikolajeva (2017) demonstrated good bacterial cell adhesion and viability when immobilized on the sodium alginate support matrix with and without the addition of clay.

Figure 1 - SEM image indicating the structural aspect of the beads and superficial formulation morphology F1: alginate; F2: alginate + corn starch 0,5%; F3: alginate + corn starch 1%.



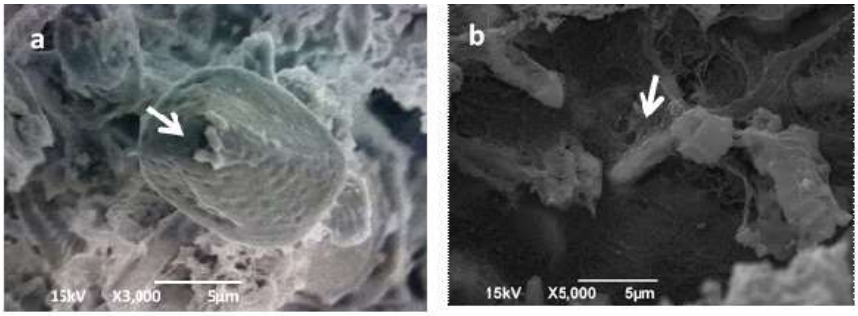
Source: Authors (2021).

Figure 2 - SEM of bead cross sections demonstrating the presence of pores. Formulations F1, F2 and F3.



Source: Authors (2021).

Figure 3 - SEM of bacteria adhered to the surface (a) and internal region (b) of the beads.



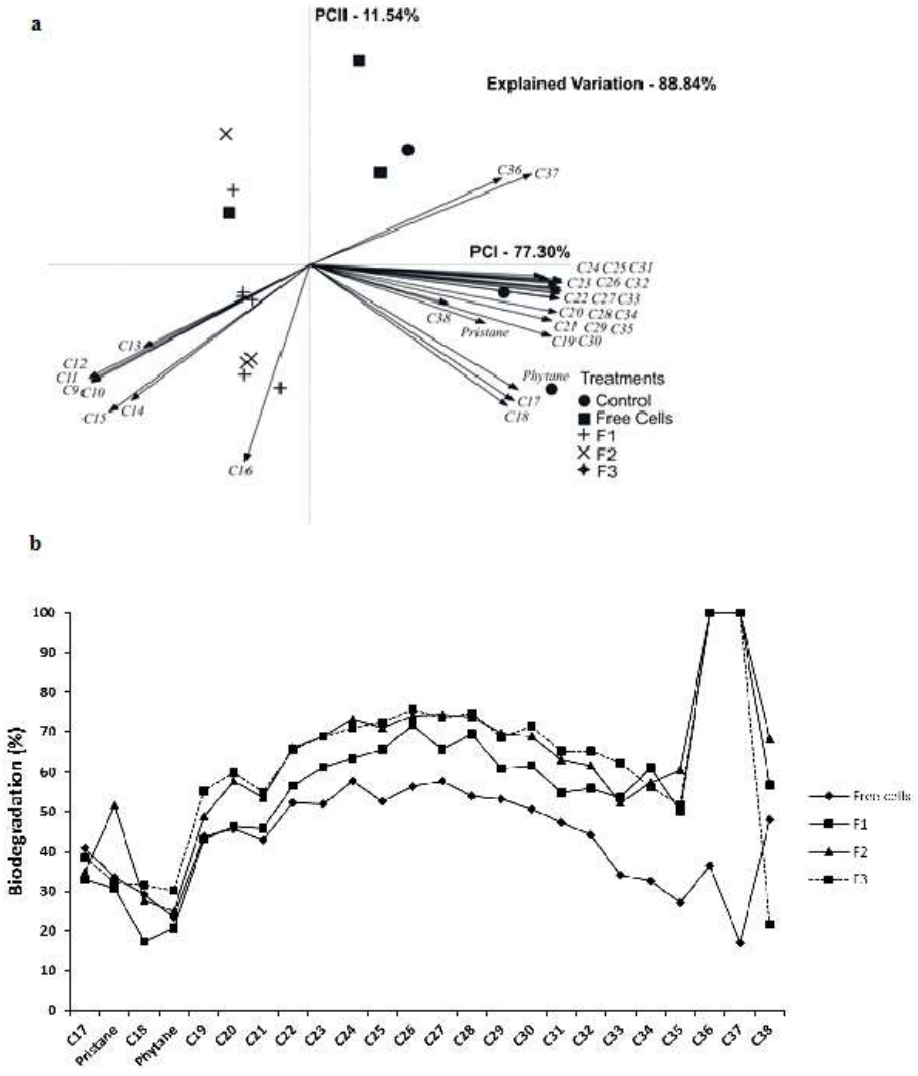
Source: Authors (2021).

3.2 Alkane biodegradation effects by bacterial immobilization beads

n-alkane analyses after 60 days of the experiment indicae the occurrence of marked degradation in long chain compounds C_{17} - C_{38} by immobilized bacteria (Figure 4). This was not observed in free cells, exhibited a higher correlation with the control in the PCA and, therefore, contain higher concentrations of long-chain n-alkanes. The correlation between free cells

and formulations with respect to degradation is explained by the PCI = 77.30%, which presents a pattern above the expected at random, when compared to the broken-stick model.

Figure 4 - Principal component analysis of n-alkane concentrations (a) and percentage of n-alkane biodegradation (b) after 60 days of microcosm incubation.



Source: Authors (2021).

Concerning the tested matrices, F2 and F3 demonstrated good ability to provide the consortium with favorable conditions for the metabolization of n-alkanes, optimizing the process. Therefore, the data suggest that spheres containing dental potassium alginate and corn starch are effective for use in the bioremediation of environments containing the compounds tested herein. n-alkane degradation by bacteria immobilized in the presence of carbohydrates, such as glucose and lactose, is optimized due to the co-metabolism carried out by the bacteria plus the biodegradability conferred by the molecular structure of n-alkanes (Shen et al., 2015).

The rate of n-alkane degradation generally decreased with increasing chain length (Lofthus et al., 2018). However, this study obtained higher degradation rates for higher molecular weight n-alkanes. Among these compounds, the best degradation

rates were obtained in C_{22} - C_{30} fractions in all tested treatments. The free bacteria presented degradation rates of around 50%, while immobilized bacteria obtained rates above 60%, thus demonstrating good oil degradability.

The data corroborate Zhang et al. (2018), who obtained biodegradation rates above 70% for C_{22} - C_{28} fractions using *Bacillus* sp. immobilized in a matrix extracted from algae after 21 days, while free cells resulted in low degradation of these compounds (63%). According to the authors, these degradation rates are the result of the material's ability to absorb oil combined with good bacteria adsorption to the matrix, similar to that observed in the present study.

Additionally, an increase in the concentration of low molecular weight C_9 - C_{16} n-alkanes in the three tested matrices, attributed to the intense catabolism of heavy chain n-alkanes, resulting in the formation of short and medium chain compounds. According to Rehm; Reiff (1981), the oxidation of the monoterminal, diterminal and subterminal long chain n-alkane types leads to the formation of by-products, such as $C_9 – C_{16}$ n-alkanes, alkenes and ketones. According to the authors, this process becomes intensified in the presence of several bacterial species, through compound co-oxidation.

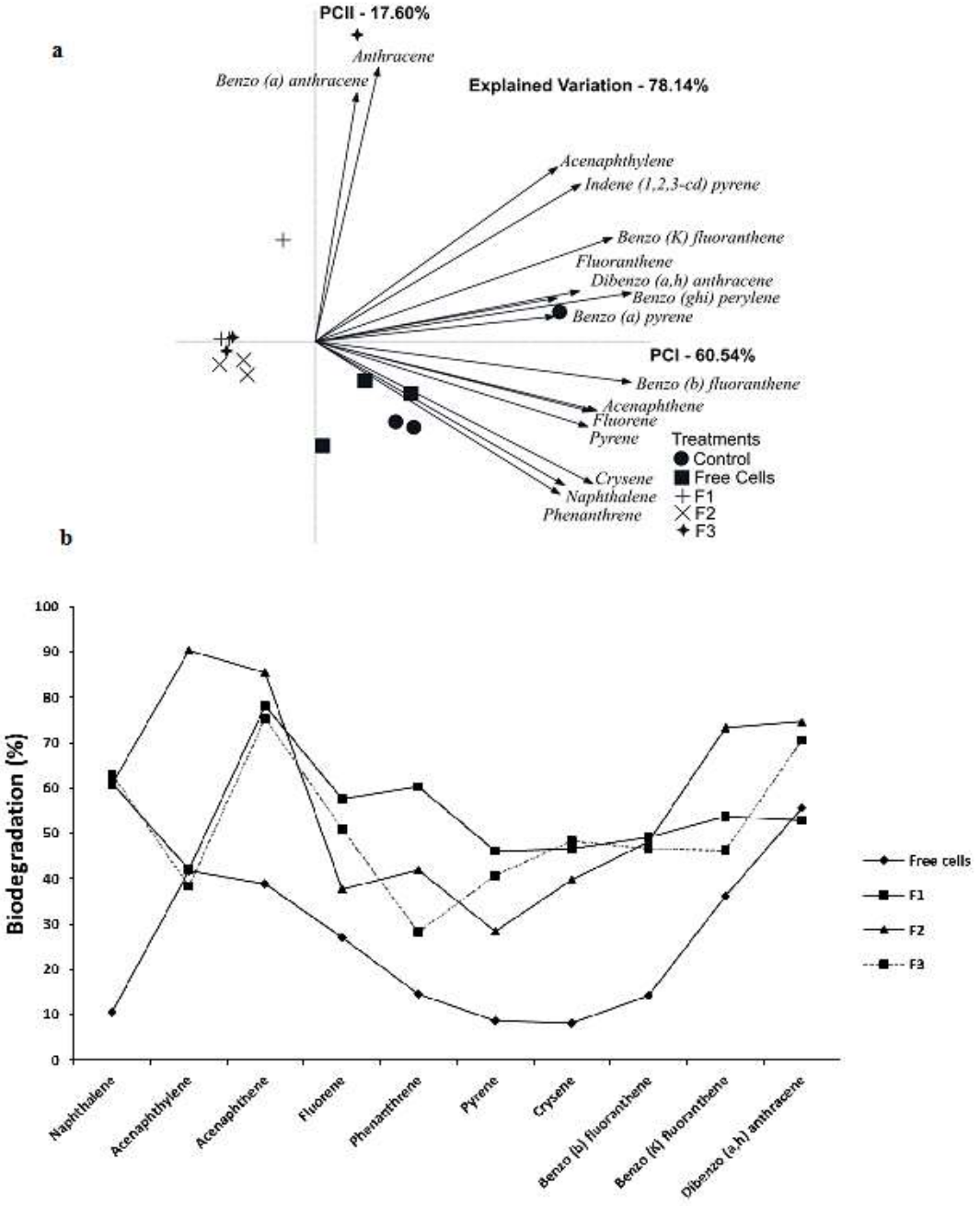
3.3 PAH biodegradation effects due to bacterial immobilization beads

The PAH analyses reveal a pattern similar to that observed for n-alkanes. , with decreases in 14 of the 16 analyzed PAH in the three tested matrices (PCI 60.54%) was observed. The same was not observed in free cells, which exhibited higher concentrations of 14 PAH, with the exception of anthracene and benzo (a) anthracene (PCII 17.60%) (Figure 5).

The investigated matrices demonstrated, in general, similar degradation patterns. For some compounds, such as acenaphthene, benzo (K) fluoranthene and dibenzo (a, h) anthracene, the F2 matrix presented higher reduction rates of 90%, 73%, 74%, respectively. Naphthalene removal by immobilized bacteria was of 60%, while free cells degraded only 10% of this compound, therefore demonstrating the effect of formulations in promoting bacterial metabolism. Cellular adherence to aromatic substrates is essential for good metabolic responses to the investigated compounds since, once adhered, the compound is degraded in the extracellular medium via exoenzymes or intracellularly metabolized by endoenzymes. The decreased levels of degradation reported for anthracene can be attributed to the difficulty of this three-ringed compound in adhering to bacterial cells, as reported by Jauhari et al. (2018). The low degradation observed for benzo (a) anthracene is related to solubility, bioavailability to microbial action and a complex structure formed by four fused aromatic rings (Deka; Lahkar, 2016).

The oil degradation rates observed in the immobilized cell treatments, are attributed to the protection effect against exposure to intermediate PAH consumption metabolites conferred by the polymeric matrix. In addition, the presence of the matrix may have allowed for the retention of extracellular biomolecules produced by bacteria, which benefits and increases PAH degradation (Liu et al., 2019). Similar results regarding PAH degradation were reported by Xu et al. (2019), who indicated an increase in the degradation of aromatic compounds by *Klebisiella* sp. immobilized on polyvinyl alcohol (PVA), sodium alginate and nano alumina.

Figure 5 - Principal component analysis (PCA) concerning PAH concentrations (a) and biodegradation percentages (b) after 60 days of microcosm incubation.



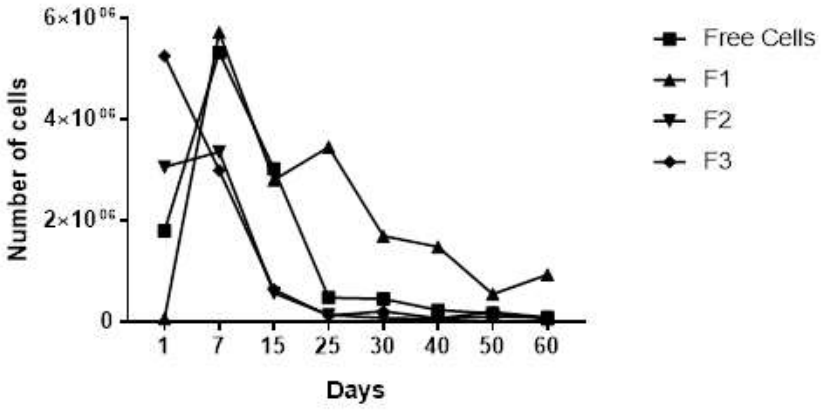
Source: Authors (2021).

Karabika et al. (2009) evaluated pyrene, fluoranthene, fluorene, anthracene and acenaphene degradation employing *Mycobacterium* sp. immobilized on a glass matrix, alginate/corn starch matrix and free cells, in both liquid media and in the soil, for 20 - 30 days. The authors obtained PAH removal rates of 100% for all treatments in the liquid media. In the soil, the findings indicate better degradation by free cells compared to immobilized cells. Therefore, no positive effect was observed concerning immobilization, only the excellent metabolizing capacity of these compounds by the tested strain, unlike the data obtained in the present study, in which the matrix increased bacterial strain abilities to remove PAHs.

3.4 Biomass determinations

Biomass evaluated at 24 h indicates good adsorption between the polymeric matrix and the cells, demonstrated by the increased number of cells observed during this period. Among the tested matrices, F3 presented the best performance (5 x 10^6 CFU / g) at this time. However, on the 7th day of incubation, declines in the number of cells attributed to the consortium interaction were noted for formulations F2 and F3. It is inferred that the bacterial populations were reduced due to the depletion of the substrate used for their metabolism (Figure 6).

Figure 6 - Bacterial biomass evaluated during the microcosm incubation period.



Source: Authors (2021).

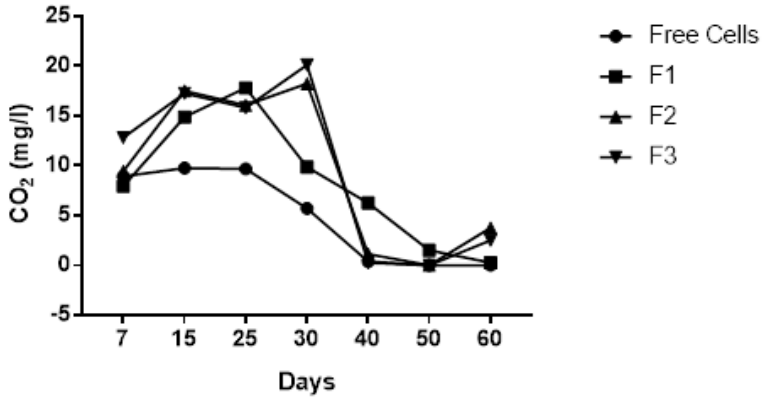
F1's performance was the best among the three formulations, allowing favorable cell growth conditions, similar to that of free cells, while also maintaining high cell concentrations for 60 days (106 CFU / g). However, only time point 1 (24 h) exhibited a statistically significant difference between treatments ($p = <0.0001$). In addition, the results of the analysis of variance for cell counts indicated a significant difference ($p = 0.0014$) for the interaction between treatments, especially for free cells x F3 ($p = 0.0393$) and F1 x F3 ($p = 0.0007$), for time point 1.

Biomass values lower than F1 have been reported by Maqbool et al. (2012) when employing rhizobacteria immobilized in sodium alginate 6% and diatomite 2%. Although the matrix constitution is similar to the formulations applied in the present study, the authors obtained cell concentrations around 10^4 UFC/g for bacteria immobilized for 60 days.

3.5 Respirometric analyses

Basal respiration analyses revealed increased CO_2 production rates (mg/L) until the 15th day by free cells (10 mg/L CO_2), F2 and F3, higher for the latter (17 mg/L CO_2) (Figure 7). F1 increased respiratory level until the 25th day. As of the 30th day, respiratory activity decreased in F1 and in free cells, with F2 and F3 presenting greater CO_2 production, decreasing only after the 40th day.

Figure 7 - Respirometric analyses conducted during the microcosm incubation period.



Source: Authors (2021).

The high CO_2 values observed in F2 and F3 corroborate the higher degradation rates observed and previously discussed for n-alkanes and some PAHs. Therefore, the presence of starch positively influenced bacterial metabolism. However, no differences regarding CO_2 concentrations in the tested matrices were observed.

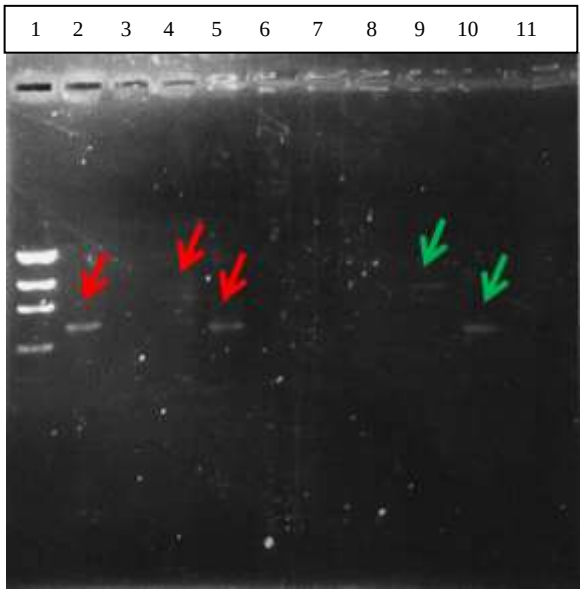
The increased CO_2 at 60 days verified in F2 and F3 reflects the increased in hydrocarbon or intermediate consumption rate by the two bacterial populations that remained viable until this experimental period. Hydrocarbons undergo different degradation rates within the bacterial consortium, due to the metabolic specificities of each microorganism (Ghazali et al., 2004). Therefore, the results demonstrate that immobilized bacteria displayed better adaptability to environmental conditions considering n-alkanes and PAHs degradation, CO_2 production and cell counts.

3.6 Identification of alkB and catechol 2,3 dioxygenase genes

The detection of genes responsible for hydrocarbon catabolism is paramount to assess the potential of the strains that make up the consortium. The alkB gene encoding the alkane monooxygenase enzyme was identified in *Acinetobacter* sp. CCMICS 560 and *Enterobacter* sp. CCMICS 564, while *Pantoea* sp. CCMICS 563 displayed unspecific bands, but with fragments detected in the alkB gene region (550bp). The alkB gene was not detected in *Bacillus* sp. CCMICS 562 (**Figure 8**).

The presence of the alkB gene confirms the metabolic capacity of the consortium, as alkane hydroxylases are important biocatalysts in the oxidation of saturated compounds such as n-alkane chains (Ramadass et al., 2016). The non-detection of the alkB gene in CCMICS 562 does not imply the lack of metabolic machinery to degrade alkanes, and it is important that analyses be performed to identify other genes associated to the synthesis of oxygenases, such as alkM, alkG and alkT. Cunha et al. (2006) obtained products resulting from the amplification of genes responsible for encoding alkane monooxygenases in bacteria belonging to the *Bacillus* genus isolated from an oil reservoir in Brazil, confirming this genus' ability to degrade oil. This is similar to that described by Elumalai et al. (2017), who observed C32 and C40 n-alkane degradation by bacteria belonging to the *Bacillus* genus, with a high production rate of alkane hydroxylase and alcohol dehydrogenase, both enzymes involved in the metabolic route of n-alkanes.

Figure 8 - AlkB gene amplification in *Acinetobacter* sp. CCMICS 560 (column 2) and *Enterobacter* sp. CCMICS564 (column 5) bacteria, with nonspecific bands noted for *Pantoea* sp. CCMICS 563 (column 4). Catechol 2,3 dioxygenase gene amplification displaying nonspecific bands for *Pantoea* sp. CCMICS 563 (column 9) and amplification for *Enterobacter* sp.CCMICS 564 (column 10).



Source: Authors (2021).

Among the strains tested for the cat 2,3 - catechol 2,3 dioxygenase gene, only *Enterobacter* sp. CCMICS 564 presented a fragment at an approximate height of 406 bp referring to the analyzed gene. Similar to what was observed for the alkB gene, *Pantoea* sp. CCMICS 563 also presented unspecific fragments, and the presence of the gene could not be confirmed. The absence of amplification of the cat 2,3 gene also does not mean the absence of enzymes able to oxidize aromatic compounds, as other genes linked to the metabolism pathways of these compounds may be present, such as nahAc for naphthalene and cat1,2 for catechol 1,2 dioxygenase, among others.

The detected genes reinforce the metabolic capacity of the analyzed bacteria, comprising an important factor in the synergism required by a microbial consortium and indicating biodegradation potentialization by the consortium against different groups of hydrocarbons (Abbasian et al., 2015).

4. Conclusion

Polymeric matrices made up of dental potassium alginate with or without corn starch were effective in cell immobilization, in addition to having macropores in their structure, combined with the low cost of production. The characteristics of the matrix and the good adsorption of the strains to the polymer allowed similar results in the removal of n-alkanes and PAHs from the sediment, this result being more expressive than when applying free cells. Additionally, the matrices provided favorable conditions for bacterial growth and metabolism, which led to high CO_2 and biomass values. Thus, the immobilization of bacteria in a support matrix composed of dental potassium alginate with or without corn starch is shown to be a good tool for application in bioremediation technique. Evaluating the effectiveness of oil degradation in other substrates, such as water and muddy sediments, using bacteria immobilized in the developed matrix, is a possibility for future studies.

Acknowledgments

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel - CAPES for the financial support in this study, as well as Petróleo Brasileiro S.A. – PETROBRAS for the oil used.

References

Abasian, F.; Lockington, R.; Mallavarapu, M.; & Naidu, R. (2015). A comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. *Applied Biochemistry and Biotechnology*, 176, 670-699.

Alef, K. & Nannipieri, P. (1995). Methods in applied soil microbiology and biochemistry. *London: Academic*.

Banerjee, S.; Banerjee, A.; & Sarkar, P. (2018). Statistical optimization of arsenic biosorption by microbial enzyme via Ca-alginate beads. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, 53 (5), 436–442. https://doi.org/10.1080/10934529.2017.1409009.

Bayat, Z.; Hassanshahian, M.; & Cappello, S. (2015). Immobilization of microbes for bioremediation of crude oil polluted environments: A Mini Review. *The Open Microbiology Journal*, 9, 48–54. https://doi.org/10.2174/1874285801509010048.

Bučková, M.; Puškarová, A.; Chovanová, K.; Kraková, L.; Ferianc, P.; & Pangallo, D. (2013). A simple strategy for investigating the diversity and hydrocarbon degradation abilities of cultivable bacteria from contaminated soil. *World Journal of Microbiology and Biotechnology*, 29 (6), 1085–1098. https://doi.org//10.1007/s11274-013-1277-5.

Chen, L.; Zhao, S.; Yang, Y.; Li, L.; & Wang, D. (2019). Study on degradation of oily wastewater by immobilized microorganisms with biodegradable polyacrylamide and sodium alginate mixture. *ACS Omega*, 4 (12), 15149–15157. https://doi.org/10.1021/acsomega.9b02045.

Chen, Y.; Yu, B.; Lin, J.; Naidu, R.; & Chen, Z. (2016). Simultaneous adsorption and biodegradation (SAB) of diesel oil using immobilized *Acinetobacter venetianus* on porous material. *Chemical Engineering Journal*, 289 (1), 463–470. https://doi.org/10.1016/j.cej.2016.01.010.

Cunha, C. D.; Rosado, A. S.; Sebastián, G.V.; Seldin, L.; & Weid, I. (2006). Oil biodegradation by *Bacillus* strains isolated from the rock of an oil reservoir located in a deep-water production basin in Brazil. *Applied Microbiology and Biotechnology*, 73, 949–959.

De la Cueva, S. C.; Rodríguez, C. H.; Cruz, N. O .S.; Contreras, J. A. R.; & Miranda, J. L. (2016). Changes in bacterial populations during bioremediation of soil contaminated with petroleum hydrocarbons. *Water, Air, and Soil Pollution*, 227 (91). https://doi.org/10.1007/s11270-016-2789-z.

De Vos, P.; Lazarjani, H. A.; Poncelet, D.; Faas, M. M. (2014). Polymers in cell encapsulation from an enveloped cell perspective. *Advanced Drug Delivery Reviews*, 67–68, 15–34. http://dx.doi.org/10.1016/j.addr.2013.11.005.

Deka, H. & Lahkar, J. (2016). Biodegradation of benzo(a)anthracene employing *Paenibacillus* sp. HD1PAH: A novel strain isolated from crude oil contaminated soil. *Polycyclic Aromatic Compounds*, 37 (2–3), 161–169. https://doi.org/10.1080/10406638.2016.1253593.

Elnashar, M. M. M. (2010). Review Article: Immobilized molecules using biomaterials and nanobiotechnology. *Journal of Biomaterials and Nanobiotechnology*, 1, 61–77. https://doi.org/10.4236/ jbnb.2010.11008.

Elumalai, P.; Parthipan, P.; Karthikeyan, O. P.; & Rajasekar, A. (2017). Enzyme-mediated biodegradation of long-chain n-alkanes (C32 and C40) by thermophilic bacteria. *3 Biotech*, 7, 116.

Fareez, I. M.; Lim, S. M.; Mishra, R.K.; & Ramasamy, K. (2015). Chitosan coated alginate-xanthan gum bead enhanced pH and thermotolerance of *Lactobacillus plantarum* LAB12. *International Journal of Biological Macromolecules*, 72, 1419–1428. https://doi.org/10.1016/j.ijbiomac.2014.10.054.

Gbassi, G. K.; Vandamme, T.; Ennahar, S.; & Marchioni, E. (2009). Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology*, 129 (1), 103–105. https://doi.org/10.1016/j.ijfoodmicro.2008.11.012.

Ghazali, F. M.; Rahman, R. Z. A.; Salleh, A. B.; & Basri, M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *International Biodeterioration and Biodegradation*, 54 (1), 61–67. https://doi.org/10.1016/j.ibiod.2004.02.002.

Hanson, K. G.; Desai, J. D.; & Desai, A .J. (1993). A rapid and simple screening technique for potential crude oil degrading microorganisms. *Biotechnology Techniques*, 7 (10), 745–748. https://doi.org/10.1007/BF00152624.

Isaac, P.; Martínez, F. L.; Bourguignon, N.; Sánchez, L .A.; & Ferrero, M.A . (2015). Improved PAHs removal performance by a defined bacterial consortium of indigenous *Pseudomonas* and actinobacteria from Patagonia, Argentina. *International Biodeterioration and Biodegradation*, 101, 23–31. https://doi.org/10.1016/j.ibiod.2015.03.014.

Jackson, D.A . (1993). Stopping rules in Principal Components Analysis: a comparison of heuristical and statistical approaches. *Ecology*, 74, 2204–2214. https://doi.org/10.2307/1939574.

Jauhari, N.; Mishra, S.; Kumari, B.; Singh, S.N.; Chauhan, P.S.; & Upreti, D.K. (2020). Bacteria induced degradation of anthracene mediated by catabolic enzymes. *Polycyclic Aromatic Compounds*, 40 (2), 313–325. https://doi.org/10.1080/10406638.2017.1420667.

Karabika, E.; Kallimanis, A.; Dados, A.; Pilidis, G.; Drainas, C.; & Koukkou, A. I. (2009). Taxonomic identification and use of free and entrapped cells of a new *Mycobacterium* sp., strain Spyr1 for degradation of polycyclic aromatic hydrocarbons (PAHs). *Applied Biochemistry and Biotechnology*, 159 (1), 155–167.

https://doi.org/10.1007/s12010-008-8463-1.

Karigar, C. S. & Rao, S. S.; (2011). Role of microbial enzymes in the bioremediation of pollutants: A review. *Enzyme Research*, 805187. https://doi.org/10.4061/2011/805187.

Kureel, M. K.; Geed, S. R.; Giri, B. S.; Rai, B. N.; & Singh, R. S. (2017). Biodegradation and kinetic study of benzene in bioreactor packed with PUF and alginate beads and immobilized with *Bacillus* sp. M3. *Bioresource Technology*, 242, 92–100. https://doi.org/10.1016/j.biortech.2017.03.167.

Legendre, P. & Legendre, L. (2012). Numerical Ecology, third ed., Elsevier, Amsterdam.

Li, N.; Xu, H.; Yang, Y.; Xu, X.; & Xue, J. (2019). Preparation, optimization and reusability of immobilized petroleum-degrading bacteria. *Environmental Technology* (United Kingdom), 1–11 https://doi.org/10.1080/09593330.2019.1703826.

Liu, S-H.; Zeng, Z-T.; Niu, Q-Y.; Xiao, R.; Zeng, G-M.; Liu, Y.; Cheng, M.; Hu, K.; Jiang, L-H.; Tan, X-F.; & Tao, J-J. (2019). Influence of immobilization on phenanthrene degradation by *Bacillus sp.* P1 in the presence of Cd(II). *Science of the Total Environment*, 655, 1279–1287. https://doi.org/10.1016/j.scitotenv.2018.11.272.

Lofthus, S.; Netzer, R.; Lewin, A.S.; Heggeset, T.M.B.; Haugen, T.; & Brakstad, O.G. (2018). Biodegradation of n-alkanes on oil–seawater interfaces at different temperatures and microbial communities associated with the degradation. *Biodegradation*, 29 (2), 141–157. https://doi.org/10.1007/s10532-018-9819-z.

Maqbool, F.; Wang, Z.; Xu, Y.; Zhao, J.; Gao, D.; Zhao, Y-G.; Bhatti, Z.A.; & Xing, B. (2012). Rhizodegradation of petroleum hydrocarbons by *Sesbania cannabina* in bioaugmented soil with free and immobilized consortium. *Journal of Hazardous Materials*, 237–238 (30), 262–269. https://doi.org/10.1016/j.jhazmat.2012.08.038.

Ramadass, K.; Megharaj, M.; Venkateswarlu, K.; & Naidu, R. (2016). Soil bacterial strains with heavy metal resistance and high potential in degrading diesel oil and n-alkanes. *International Journal of Environmental Science and Technology*, 13 (12), 2863-2874.

Rehm, H. J. & Reiff, I. (1981). Mechanisms and occurrence of microbial oxidation of long-chain alkanes. *Advances in Biochemical Engineering*, 19, 175–215. https://doi.org/10.1007/3-540-10464-X_18.

Sampaio, C. J. S.; Souza, J. R. B.; Carvalho, G. C.; Quintella, C. M.; & Roque, M. R. A. (2019a). Analysis of petroleum biodegradation by a bacterial consortium isolated from worms of the polychaeta class (Annelida): Implications for NPK fertilizer supplementation. *Journal of Environmental Management*, 246 (15), 617–624. https://doi.org/10.1016/j.jenvman.2019.06.018.

Sampaio, C. J. S.; Souza, J. R. B.; Damião, A. O.; Bahiense, T. C.; & Roque, M. R. A. (2019b). Biodegradation of polycyclic aromatic hydrocarbons (PAHs) in a diesel oil-contaminated mangrove by plant growth-promoting rhizobacteria. *3 Biotech*, 9 (4). https://doi.org/10.1007/s13205-019-1686-8.

Sampaio, C. J. S., Roque, M. R. A., Souza, J. R. B, Carvalho, G. C., & Vale, T. O. (2019) Patent deposit PI national BR102019013058-0, in secrecy phase.

Shen, T.; Pi, Y.; Xu, N.; Li, Y.; & Lu, J. (2015). Biodegradation of different petroleum hydrocarbons by free and immobilized microbial consortia. *Environmental Sciences: Processes and Impacts*, 17 (12), 2022–2033. https://doi.org/10.1039/C5EM00318K.

Simons, K. L.; Sheppard, P. J.; Adetutu, E. M.; Kadali, K.; Juhasz, A. L.; Manefield, M.; Sarma, P. M.; Lal, B.; & Ball, A.S . (2013). Carrier mounted bacterial consortium facilitates oil remediation in the marine environment. *Bioresource Technology*, 134, 107–116. https://doi.org/10.1016/j.biortech.2013.01.

Su, D.; Li, P .J.; Frank, S.; & Xiong, X. Z. (2006). Biodegradation of benzo[a]pyrene in soil by *Mucor sp.* SF06 and *Bacillus sp.* SB02 co-immobilized on vermiculite. *Journal of Environmental Sciences* (China), 18 (6), 1204–1209. https://doi.org/10.1016/S1001-0742(06)60063-6.

Varjani, S.; & Upasani, V. N. (2020). Soil microcosm study for bioremediation by a crude oil degrading *Pseudomonas aeruginosa* NCIM 5514. *Journal of Environmental Engineering* (United States), 146 (5). https://doi.org/10.1061/(asce)ee.1943-7870.0001687.

Wang, Z-Y.; Xu, Y.; Wang, H-Y.; Zhao, J.; Gao, D-M.; Li, F-M.; & Xing, B. (2012). Biodegradation of crude oil in contaminated soils by free and immobilized microorganisms. *Pedosphere*, 22 (5), 717–725. https://doi.org/10.1016/S1002-0160(12)60057-5.

Xu, X.; Zhou, H.; Chen, X.; Wang, B.; Jin, Z.; & Ji, F. (2019). Biodegradation potential of polycyclic aromatic hydrocarbons by immobilized *Klebsiella* sp. in soil washing effluent. *Chemosphere*, 223, 140–147. https://doi.org/10.1016/j.chemosphere.2019.01.196.

Xue, J.; Wu, Y.; Fu, X.; Li, N.; Sun, J.; & Qiao, Y. (2020). Study on degradation characteristics and bacterial community structure changes of immobilized cells in straw-alginate beads in marine environment. *Bioresource Technology Reports*, 10, 100402. https://doi.org/10.1016/j.biteb.2020.100402.

Zhang, Y.; Gao, W.; Lin, F.; Han, B.; He, C.; Li, Q.; Gao, X.; Cui, Z.; Sun, C.; & Zheng, L. (2018). Study on immobilization of marine oil-degrading bacteria by carrier of algae materials. *World Journal of Microbiology and Biotechnology*, 34 (6). https://doi.org/10.1007/s11274-018-2438-3.

Zommere, Ž. & Nikolajeva, V. (2017). Immobilization of bacterial association in alginate beads for bioremediation of oil-contaminated lands. *Environmental and Experimental Biology*, 15, 105-111. https://doi.org/10.22364/eeb.15.09.