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Halotolerant Biofilm in Coffee Beans for Phenanthrene Degradation Under Selected Culture Conditions through a Plackett-Burman Experimental Design

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Abstract

A halotolerant biofilm was developed on green coffee beans by sequential enrichment in the presence of diesel in order to degrade phenanthrene. Experiments were performed under several combined culture conditions using a Plackett-Burman experimental design. The microorganisms composing the biofilm were identified. These included three fungi, *Aspergillus niger, Fusarium solani* and *Fusarium oxysporum*, identified by the Fungal Biodiversity Centre of Holland by macroscopic and microscopic morphological evaluation and by molecular techniques (18s-ITS1-5.8S-ITS2-28s rRNA); two yeasts, *Candida orthopsilosis* and *Rhodotorula mucilaginosa*; and two bacteria, *Pseudomonas putida* and *Klebsiella variicola,* identified by the Mexican Collection of Microbial Cultures (Colección Mexicana de Cultivos Microbianos, CINVESTAV) according to colony morphology and biochemical [API 20C AUX and API20 (NE and E), respectively] and molecular (fragments ITS1-5.8S-16s-26s and 16s rRNA, respectively) tests. Each microorganism and biofilm were tested for their tolerance to diesel and salinity conditions and their capacity to degrade phenanthrene; degradation capacity was affected significantly ($\alpha<0.05$) at low concentrations of $NaNO_3$ and KH_2PO_4 and at high concentrations of peptone, $CaCO_3$ and $FeSO_4$ ($R^2=0.95$; C=24.97). Two of the tested conditions, T2 and T5, degraded 91.92% and 89.33%, respectively, of 180 mg/L of phenanthrene. Thus, the biofilms developed from microorganisms of green coffee beans preadapted to salinity conditions presented the metabolic capability to remove phenanthrene from seawater under selective culture conditions.

Keywords: Plackett-Burman; Biofilm; Salinity; Green coffee beans; Phenanthrene; Polynuclear aromatic hydrocarbons

Introduction

Polyaromatic hydrocarbons (PAHs) are of environmental and toxicological significance because they do not burn easily and can persist in the environment for long periods of time. Some studies attribute the toxicity of crude oil to two- and three-ring PAHs [1]. The most abundant are fused with one to four alkyl substituents, wherein toxicity increases with increasing number of substituents [2]. In general, PAHs are poorly soluble in water and highly lipophilic, although their behavior varies individually.

Environmental contamination by PAHs occurs as a result of natural processes and anthropogenic activities. For instance, oil leaks and spills as well as activities related to the extraction, refinement, storage, transportation and distribution of PAHs can lead to contamination. For various reasons, approximately 3.2 million tons of oil is estimated to be annually discharged to marine environments. Of this amount, 15% is the result of accidents on ships or tanks in addition to pipeline ruptures or explosions on oil rigs. An oil spill of 1 m^3 can form a slick of 100 m in diameter and 0.1 mm thick. Much of the oil evaporates (50%), in particular short-chain aliphatic alkanes (with 12 carbon atoms or less) and lighter monoaromatic compounds (benzene, toluene and xylenes), and decomposes in the atmosphere by photo-oxidation [3,4], resulting in an increase of the polar fraction of the remaining oil. Furthermore, dispersed oil has a large surface area. By other natural processes, oil may slowly biodegrade in water, while remaining compounds form emulsions [3].

From the 1940s to 2010, oil spills have occurred worldwide, mainly in the Gulf of Mexico and notably during the Ixtoc-I and the Deepwater Horizon/Macondo well blowouts/explosions. Table 1 details of several oil spills that have occurred in the last 50 years, describing their diverse causes and spill volumes. The cleaning methodologies have included pressure injection of cold and hot water, burning, addition of dispersants, natural attenuation and bioremediation. The oil released by the Deepwater Horizons rig at the Macondo well flowed from an approximate depth of 1,500 m below the sea surface, creating both underwater plumes in the ocean and oil slicks on the sea surface. The depth of the well enabled a longer exposure time of crude oil to the water column, creating underwater plumes composed of soluble fractions of hydrocarbons (C_1-C_3) and PAHs. The soluble components and PAHs of this spill were demonstrated to cause defects in the cardiovascular and craniofacial development of zebrafish (*Danio rerio*) and alterations in programmed cell death, including embryogenic disorders [5].

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Spill	Amoco Cadiz [7]	Ixtoc-I [7]	Exxon Valdez [8]	Prestige [9,10]	DeepWater Horizons [8]
Incident	Stranding of crude carrier ship	Blowout during drilling of oil well	Stranding of ship and fracture of 11 oil cargo tanks	Hull breach of oil ship	Macondo well blowout and explosion on the Deepwater Horizon oil rig
Location	Coast of Brittany, France	Gulf of Mexico	Prince William Sound, Alaska	Northwestern Spanish seaboard	Mexican Gulf
Date	March 1978	June 3, 1979 to March 20, 1980	March 24, 1989	November 19, 2002	April 20 to July 15, 2010
Affected area	322 km	2,800 km^2	2,087 km of insular coasts	18 beaches and coasts of Galician and Cantabrian seaboard	6,475 km^2
Oil type	Arabian and Iranian light oil (~1:1)	Ixtoc-I light oil	Alaskan North Slope heavy oil	Russian heavy oil	Light Louisiana oil with high amounts of methane
Volume (tons)	223,000	476,000 tons 600,000	37,000	77,000	779,000
Density (g/cm^3)	0.85	0.84		0.99	0.82[e]
API			29.0		35.2
Treatment technology(s)	Physical and chemical methods, natural bioremediation	Incineration, chemical dispersants	Injection of pressurized water, use of fertilizers	Injection of pressurized water, bioremediation	Intensive use of chemical dispersants, incineration
Chemical composition					
Saturate	39%	50%		22%	74%[e]
Aromatic				50%	16%[e]
Resin and asphaltene	27%	18%		30%	10%[e]

Table 1: Details of several oil spills that have occurred in the last 50 years, describing their diverse causes and spill volumes.

Some fractions of crude oil, such as PAHs with two to three rings, are extremely toxic to marine organisms (including microorganisms capable of degrading other petroleum fractions). Thus, the ecological impact of PAHs on marine environments can be significant according to mass balance determinations [6].

Some of the major emerging technologies include the use of natural, physical and biological remediation processes to remove oil from marine waters. The use of microorganisms has represented one of the best alternatives for the bioremediation of different environments, although their use in marine environments needs to be further explored [11,12]. Naturally occurring biological processes have long been recognized for their ability to degrade chemical compounds. However, since the Exxon Valdez spill in 1989, several alternative means of exploiting the metabolic capabilities of microorganisms in order to degrade hydrocarbons by natural processes have been researched with the ultimate goal of creating affordable and large-scale technological applications that are capable of effectively restoring contaminated environments [11].

Many microorganisms have the enzymatic ability to metabolize some components of crude oil; however, a consortia or mixed culture of microorganisms is often required to significantly degrade toxic compounds [13]. This method has certain advantages over biostimulation, especially in cases of high pollutant toxicity or when the necessary quantity or quality of endogenous microorganisms with appropriate enzymatic capabilities is not available [12]. When bioaugmentation is used as a bioremediation strategy, it is necessary to immobilize exogenous microorganisms under suitable culture conditions and to minimize the impact of different biotic and abiotic factors. Different measures can contribute to the development and survival of microorganisms by increasing their catalytic activity, forming a greater number of microenvironments, lowering susceptibility to abiotic factors and increasing resistance to high concentrations of contaminants [14].

Microorganisms can also be immobilized on agro-industrial wastes, which are rich sources of nutrients that can promote the microbial growth of organisms [15,16], thereby preventing environmental problems associated with the disposal of materials. In particular, green coffee beans are rich in carbohydrates, fatty acids, nitrogen, minerals, aliphatic acids and phenolic compounds [17]. Furthermore, many cosmopolitan organisms may coexist on green coffee beans and exert symbiotic effects with native organisms [18] acting together, for example, to decontaminate seawater affected by hydrocarbon spills. This technology may also be used in cases where other technologies that affect the environment, such as surfactants or fertilizers, have already been applied [8].

The working group of CINVESTAV has conducted research on the application of coffee beans and the microorganisms associated with them for the bioremediation of soil and water contaminated with pesticides, hydrocarbons or other recalcitrant compounds [19-22]. As previously mentioned, coffee beans are rich in nutrients that promote the growth of a variety of microorganisms. In fact, several associated microorganisms capable of degrading recalcitrant compounds have been identified, including the following bacteria: *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Stenotrophomonas maltophilia, Flavimonas oryzihabitans* and *Morganella morganii* [23]. In addition, biosurfactants produced by bacteria, such as *Bacillus cereus* [24], and fungi of the genera *Aspergillus*, *Penicillium* and *Absidia* that have the ability to degrade hydrocarbons [21] have been isolated.

Coffee beans and lignocellulosic residues, such as straw, bagasse and agave fibers have also been used in soil and water bioremediation and have been shown to reduce high concentrations of medium-fraction hydrocarbons (MFH), heavy-fraction hydrocarbons (HFH) and polyaromatic hydrocarbons (PAHs) [19,21]. This technology [18] commercially applied to soil, groundwater and shallow water contaminated with diesel or fats and in several contract operations with Pemex, the state-owned Mexican oil and gas company [25].

In addition to the metabolic capabilities of microorganisms, the success of bioremediation depends on the knowledge of the factors that limit or promote the degradation of pollutants, particularly in the

case of complex mixtures such as crude oil. For instance, knowledge of the environmental conditions that favor the degradation of pollutants would enable more efficient treatment technologies to be developed and successfully applied. Furthermore, a growing interest exists in the development of cost-effective inoculum for large scale applications.

The development of biofilms on inoculum, preferably organic materials, could represent an inexpensive and environmentally harmless technique [26,27]. Another practical consideration is that specialized technology or highly qualified personnel should not be required in order to facilitate the implementation of such techniques. In selecting organic material for use as an inoculum, several characteristics should be considered, including the porosity of the material as well as its surface area, cell affinity, long-term biodegradability and nutrient content. The porosity and surface area influence the circulation of gases, metabolites, enzymes and pollutants and may enhance the survival of microorganisms [26,28]. In the present study, green coffee beans of low quality and spent coffee grounds were used since they have a high content of nutrients and other components [17,23], that allow for microbial growth.

The objective of this study was to produce a biofilm capable of degrading phenanthrene in distilled and sea water by using microorganisms isolated from low-quality green coffee beans in the presence of diesel.

Materials and Methods

Biofilm development

A total of 1.9 kg of green coffee beans (GCB) from the states of Veracruz and Oaxaca, Mexico, was ground in a Waring blender and sieved with ASTM sieves, no. 10, 20 and 40, to obtain particles of three sizes (<0.42 mm, 0.42-0.86 mm and 0.86-2.0 mm). Moisture content, ash and water activity were determined. The biofilm was prepared by adding 40 mL of the culture medium and the equivalent of 1% of the dry weight biomass of the selected GCB treatment (particle size of 0.42-0.86 mm at 40% water holding capacity) to 125 mL Erlenmeyer flasks; microbial growth was performed at 28°C and 125 rpm. The increase in biomass was determined by calculating the dry weight increase per day.

Biofilm production from green coffee beans

Four g of ground GCB of three particle sizes (PS) (<0.42, 0.86-2.0, and 0.86-2.0 mm) and three water holding capacities (WHC) (20%, 30% and 40%) were incubated at 25°C for 5 days. The biofilms produced by nine culture conditions were examined using a 3^2 factorial design, considering three values for particle size and three for moisture content (Table 2); to determine the microbial activity (MA) was determined by the respirometric method [19].

Biofilms	Moisture (%WHC)	Particle Size (mm)
I1	20	<0.42
I2	20	0.42-0.86
I3	20	0.86-2.0
I4	30	<0.42
I5	30	0.42-0.86
I6	30	0.86-2.0
I7	40	<0.42
I8	40	0.42-0.86
I9	40	0.86-2.0

Table 2: Values for particle size and moisture content.

Isolation and identification of biofilm microorganisms

One g of colonized coffee bean particles in 125 mL Erlenmeyer flasks containing 40 mL of modified Wunder culture medium [16,29], was inoculated with 0.1% (w/v) glucose (1 g/L) and 1.0% (v/v) diesel sterilized by filtration through 0.22 µm Whatman filter paper; the mixture was then incubated for 5 days at 28°C and 120 rpm. Following six cycles of enrichment, the solid particles were allowed to sediment for 1 h, and an aliquot of 4 mL was transferred to 36 mL of fresh culture medium (FD 1:10) with 1.0% (w/v) of diesel without glucose and then incubated under the same conditions (Figure 1).

During the seventh cycle of enrichment, serial dilutions were made: 1 mL of the resulting culture was added to 9 mL of sterile saline solution (0.90% w/v). During the eighth and final cycle, additional serial dilutions (up to 10^{-6}) were performed using a sterile saline solution (0.85% w/v). Fungi were isolated by adding 100 µL of the first three dilutions to three separate Petri dishes with malt-yeast sucrose agar. For yeast isolation, dichloran rose-bengal chloramphenicol agar was used. Inoculated cultures were incubated at 28°C for 7 days. Bacteria isolation was carried out in Petri dishes with nutrient agar and nystatin (0.005% w/v), to which 100 µL of the final three dilutions was added. Inoculated cultures were incubated at 37°C for 48 h (Figure 2). Microorganism counts were performed for the serial dilutions; plate counts were expressed in terms of colony forming units (CFU/mL).

Identification of microorganisms

Fungi were identified by the Fungal Biodiversity Centre of Holland by macroscopic and microscopic morphological evaluation and by molecular techniques (18s-ITS1-5.8S-ITS2-28s rRNA). Yeasts and bacteria were identified according to colony morphology and biochemical [API 20C AUX and API20 (NE and E), respectively] and molecular (fragments ITS1-5.8S-16s-26s and 16s rRNA, respectively) tests and by consulting the Mexican Collection of Microbial Cultures (Colección Mexicana de Cultivos Microbianos, CINVESTAV).

Determination of microorganisms and biofilm tolerance to salinity

Microorganisms and GCB biofilms were cultured in both distilled water (DW) and seawater (SW), the latter from Salina Cruz, Oaxaca (pH 7.4, EC=53.75 ± 0.06 mS/cm and salinity=35.31 ± 0.1 PSU). Microorganisms were previously cultivated in a modified Wunder culture broth consisting of (g/L): glucose (1), malt extract (1), polypeptone (1), $NaNO_3$ (1), KH_2PO_4 (0.875), $MgSO_4{\cdot}7H_2O$ (0.5), $CaCl_2$ (0.1), NaCl (0.1), $MnSO_4{\cdot}H_2O$ (0.02) and $FeSO_4{\cdot}7H_2O$ (0.001) [4]. For each condition, 1 g of ground GCB and distilled water was added to serological bottles of 125 mL. These experimental units (EU) were capped with plastic plugs and left to rest at room temperature for 6 d. The water activity of GCB was also measured under isothermal conditions using a Novasina Thermoconstanter (HUMIDAT-TH1) equipped with temperature and humidity sensors [30].

Growth on Bushnell-Hass solid plate medium with diesel

Pseudomonas putida, *Klebsiella va riicola*, *Candida orthopsilosis*, *Rhodotorula mucilaginous*, *Fusarium oxysporum*, *Fusarium solani* and *Aspergillus niger* were plated with Bushnell-Haas growth medium, [31] supplemented with 1% (v/v) sterile diesel (filter sterilized with a membrane filter of 0.22 µm nylon and fiberglass) as

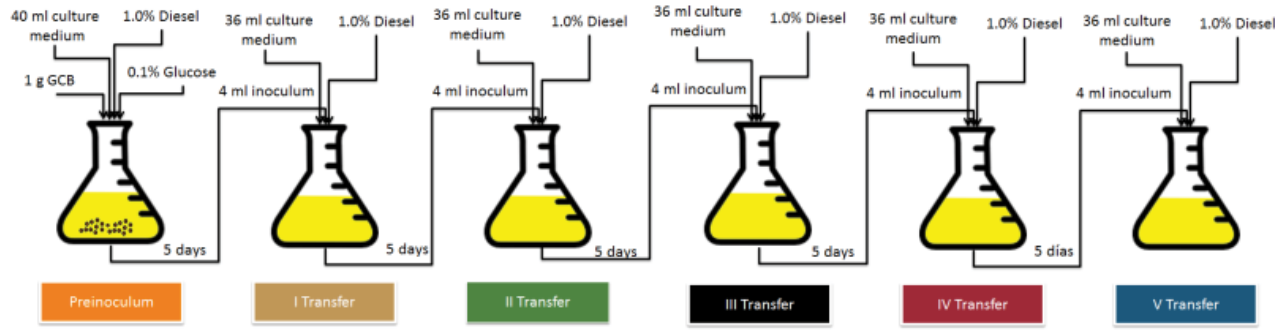

Figure 1: Isolation and identification of biofilm microorganisms.

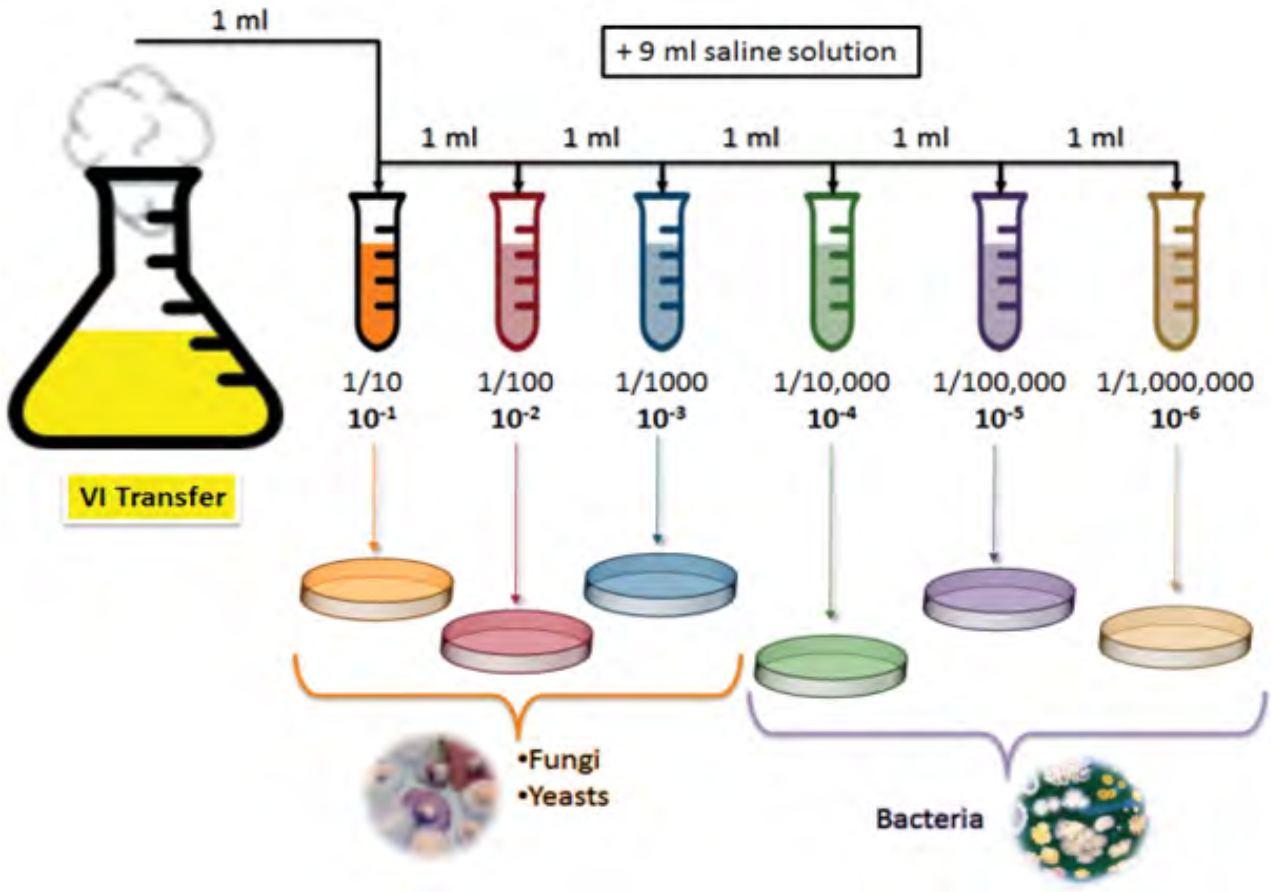

Figure 2: Microorganism counts were performed for the serial dilutions; plate counts.

the sole carbon source (BH-D). The control cultures were grown in Petri dishes with Bushnell-Hass glucose (BH-G) 1% (w/v) medium.

Culture parameters for phenanthene degradation by bioflims in distilled water using a Plackett-Burman experimental design

The culture conditions to evaluate phenanthrene (Phe) degradation by biofilms were determined using a Plackett-Burman experimental design, which is a variant of a two-level fractional factorial design [32]. This method can rapidly assess the effects of various parameters of the culture medium and provide indications of how each component affects contaminant degradation. The equivalent of 1% of the dry weight biomass of previously colonized coffee beans (0.42-0.86 mm particle size) was added to each experimental unit in addition to 40 mL of culture medium and 180 ppm of Phe. Based on this quantity of carbon (Phe) and C:N:P ratios of 100:20:2 and 100:5:1, sodium nitrate ($NaNO_3$) and peptone were added as inorganic and organic nitrogen sources, respectively, in addition to potassium phosphate monobasic (KH_2PO_4) as an inorganic phosphorus source.

Other nutrients were also added: magnesium ($MgSO_4$ $7H_2O$), calcium ($CaCO_3$) and iron ($FeSO_4$ $7H_2O$). To make Phe available to microorganisms, the nonionic surfactant Tween 80 was used at 0.5% and 1.5% (v/v). To determine the experimental error, 4 central points (PC) were defined. Table 3 presents the matrix with the natural and coded values of the variables. Cultures were incubated for 4 to 9 days at 28°C and 120 rpm. To assess removal of Phe resulting from abiotic photo-oxidation, three tubes with distilled water and 180 mg/L Phe were settled.

Phenanthrene residual extraction from liquid medium (based on EPA 3510-C and 8310)

Residual Phe was quantified in a cell-free liquid medium and filtered in a vacuum flask; the culture medium was filtered with an average pore filter paper. Per 40 mL of culture medium, 30 mL of HPLC-grade dichloromethane was added to the Erlenmeyer flask with the sample. Then, the solvent was transferred to porcelain funnel to rinse the biomass and the filter paper; this operation was repeated twice. The dichloromethane was concentrated in a rotary evaporator at 40°C without completely drying, leaving a 1 to 2 mL sample to analyze by Florisil column purification. Florisil was previously activated by heating at 600°C for 8 h and moistened with hexane.

Removal of adsorbed phenanthrene from residual biomass and coffee beans

After filtering the samples in the vacuum, the biomass and coffee bean particles were placed in test tubes of 7.5 to 10 mL with a screw cap. One g of anhydrous sodium sulfate was added for each gram of wet biomass weight and mixed with a spatula. Five mL of 1:1 hexane:acetone solvent was added to the test tube and sonicated for 20 min. The sample was recovered in round, flat-bottomed flasks. Two further extractions using 5 mL of the solvent mixture were performed, and extracts were pooled in the same flat-bottomed flask. The sample was concentrated by rotovaporation at 50°C without reaching dryness, leaving 1 to 2 mL. Subsequently, the sample was transferred directly to packed Florisil cartridges using a Pasteur pipette column. The surface of the flask was rinsed with a 1:1 hexane:acetone solvent to resuspend the sample and then passed through the Florisil column, previously activated and moistened with hexane. The solvent was evaporated in a water bath at 50°C to near dryness and then completely evaporated at room temperature. It was gauged with acetone in the same amber bottle.

Quantification of phenanthrene

Phe was quantified by a high-performance liquid chromatography (HPLC) system using an UV detector. During the mobile phase, a 75:25 acetonitrile:water solution was used. Water flow was set at 1 mL/min, and Vydac stationary phase ion exchange column C18 was used. The detector was set at 254 nm wavelength for detection of HPA. Twenty µL of the samples was were injected. Phe concentration in the samples was calculated from a calibration curve of the contaminant.

Phenanthrene degradation assays

Four mL of a 500 mg/L phenanthrene (Phe) solution dissolved in hexane was added to each experimental unit for a final concentration of 50 mg/L. The hexane was allowed to evaporate, and then 40 mL of the modified Wunder culture broth, containing either free cells or organisms immobilized on loofah fibers, were added. The cultures were incubated for 15 days at 28°C and 125 rpm. The variants in this phase of the experiment were distilled water (DW) or seawater (SW), free (F) or immobilized (I) cells on loofah fibers and presence (G) or absence (wG) of glucose (6 g/L) as a primary carbon source.

Two types of controls were also defined; the first (C1) was used to assess contaminant loss due to physical and chemical processes, while the second (C2) represented an abiotic control of sterilized biomass in order to assess potential adsorption of contaminants by the loofah fibres. In both controls and biological treatments, a dry weight equivalent of 1% of colonized GCB was added.

Statistics

All experiments were performed in triplicate. Experimental errors were estimated and depicted with standard error bar plots. The Least Significant Difference (LSD) test was used to compare the means of the different treatments; analyses were performed in SAS version 9.0. Design Expert version 7.0 was used to perform the analyses of variance of the calculated Response-Surface models, and Origin Pro 8 was used for the growth models.

Results and Discussion

T	$NaNO_3$ (mg/L)	Peptone (g/L)	KH_2PO_4 (mg/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	$CaCO_3$ (mg/L)	$FeSO_4 \cdot 7H_2O$ (mg/L)	Tween 80 4% (mL/L)
T1	229 (+1)	3 (+1)	16.6 (+1)	0.50 (-1)	200 +1)	5 (-1)	5 (-1)
T2	57 (-1)	3 (+1)	16.6 (+1)	1.00 (+1)	150 (-1)	15 (+1)	5 (-1)
T3	57 (-1)	1 (-1)	16.6 (+1)	1.00 (+1)	200 (+1)	5 (-1)	15 (+1)
T4	229 (+1)	1 (-1)	8.3 (-1)	1.00 (+1)	200 (+1)	15 (+1)	5 (-1)
T5	57 (-1)	3 (+1)	8.3 (-1)	0.50 (-1)	200 (+1)	15 (+1)	15 (+1)
T6	229 (+1)	1 (-1)	16.6 (+1)	0.50 (-1)	150 (-1)	15 (+1)	15 (+1)
T7	229 (+1)	3 (+1)	8.3 (-1)	1.00 (+1)	150 (-1)	5 (-1)	15 (+1)
T8	57 (-1)	1 (-1)	8.3 (-1)	0.50 (-1)	150 (-1)	5 (-1)	5 (-1)
TC	143 (0)	2 (0)	12.4 (0)	0.75 (0)	175 (0)	10 (0)	10 (0)
TC	143 (0)	2 (0)	12.4 (0)	0.75 (0)	175 (0)	10 (0)	10 (0)
TC	143 (0)	2 (0)	12.4 (0)	0.75 (0)	175 (0)	10 (0)	10 (0)
TC	143 (0)	2 (0)	12.4 (0)	0.75 (0)	175 (0)	10 (0)	10 (0)

Table 3: Matrix with the natural and coded values of the variables.

Particle Size (PS) (mm)	PS Distribution GCB weight (%)	Moisture (%)	SD	Ash Content (%)	SD
<0.42	13.12	7.01	0.12	0.0461	0.0029
0.42-0.86	15.59	5.37	0.39	0.0418	0.0033
0.86-2.0	69.69	2.99	0.39	0.0384	0.0014
>2.0	1.59				

SD=Standard deviation.

Table 4: The characteristics of the green coffee bean.

Characteristics of coffee bean particles

In Table 4 the characteristics of the green coffee bean (GCB) particles are presented. As shown, 70% of the particle fractions have a diameter of 0.86-2.0 mm. In general, smaller particle size corresponds with higher humidity (Table 2). The smaller particle size could be more porous and absorb more moisture in the air or during the grinding process. Additionally, each particle size corresponds with a different ratio of external hard endosperm to internal soft endosperm; each type of endosperm absorbs or desorbs humidity differentially. The Least Significant Difference (LSD) mean comparison test showed no statistically significant differences in moisture content between the two larger particle sizes.

The largest characteristic differences occurred between the smallest and largest particle sizes (Table 4); however, the comparison of the ash content indicated no statistically significant differences between contiguous particle sizes. The measurement of ash content is useful in food analyses since it measures the total amount of minerals in a sample. Minerals also form part of the chemical composition of lipids and proteins. In this case, no large differences in the mineral content according to particle size were found.

Microbial count

The results of the viable cell counts are shown in Table 5. The treatment of spent coffee (CTP) from Veracruz corresponded with a greater number of colony forming units (CFU) of yeasts, molds and fungi on the selective media. Meanwhile, the treatment of GCB from Veracruz and Oaxaca corresponded with the highest number of bacterial CFU on selective media. Since a balance among populations of fungi, yeasts, molds and bacteria was sought, GCBs were used in subsequent experiments, as its difference between the bacterial and fungal counts is lower.

Isolation and identification of microorganisms

A total of seven species were isolated from coffee beans after their sequential enrichment in the presence of diesel, including three filamentous fungi. Using the taxonomic keys [33] one of the fungi was identified as *Aspergillus niger*, while the remaining two were identified as belonging to the genus *Fusarium*. The latter were sent for identification at the species level to the Fungal Biodiversity Centre in the Netherlands and were subsequently identified as *Fusarium solani* and *Fusarium oxysporum* using molecular techniques (fragments 18s-ITS1-5.8s-ITS2-28s rRNA). Yeasts and bacteria were identified according to colony morphology by consulting the Mexican Microbial Culture Collection in addition to biochemical (API 20C AUX and API20 [NE and E], respectively) and molecular (fragments 16s-ITS1-5.8s-26s and 16s rRNA, respectivelly) tests. Two yeast species were identified, corresponding to *Candida orthopsilosis* [34] and *Rhodotorula mucilaginosa*, and two bacteria were identified as *Klebsiella* and *Pseudomonas putida variicola*. To distinguish the species of *Klebsiella pneumoniae*, an adonitol assimilation test [35] was conducted. All genera reported in this paper (*Fusarium, Aspergillus, Candida, Rhodotorula, Pseudomonas* and *Klebsiella*) have been found associated with seedlings and coffee cherries either during ripening, fermentation or storage of grains [36,37].

Filamentous fungi

Aspergillus niger. Belongs to the *Deuteromycota* division, class *Hyphomycetes*, order *Hyphomycetales* and family *Moniliaceae* [33]. Macroscopic features: Colonies formed in rapid growth potato dextrose agar (PDA) medium. Growing front of colonies initially white to slightly yellowish in color, begins to appear black as sporulation advances. Light yellow color on the reverse of the colony. Granular and almost dusty texture with a flat topography.

Microscopic features: Well-developed and branched hyphae and abundant conidiophores. Conidiophores long and erect. Subspherical terminated vesicles. Large number of phialides observed on surface as conidial heads radiate. Metula present and twice the size of phialides. Globose and brown conidia. Abundantly produced conidiophores and conidia with predominantly black or brown pigmentation.

Genus Fusarium. Belongs to class *Ascomycetes*, order *Hypocreales* and family Hypocreaceae [33]. *Fusarium* species produce two types of conidia from phialides: macroconidia and microconidia [4]. The former are long and curved, multiseptate and crescent-shaped, with a more or less pointed apical cell and in many species with foot-shaped basal cells. Microconidia are generally unicellular, oval-shaped or spheroidal, similar in width to the macroconidia, with a rounded or truncated base. Both spores are not always produced [38].

Fusarium solani. Macroscopic features: Mycelium sparse and velvety like felt with flat topography. Circular colony shape. PDA culture medium shows colony front with concentric zones of different color: pale pink fades toward a black central ring; outer ring with pinkish tones. Center of reverse colony peach and brown in color, fading to an outer amber color.

Microscopic features: Conidiophores with phialides that taper toward the tip; poorly defined phialide necklaces, solitary or gathered. Multiseptate macroconidia divided by 2 to 5 partitions, crescent-shaped and wider at the upper half. Less abundant and oval-shaped microconidia, mixed with a greater amount of macroconidia. Microconidia isolated in heads, occasionally containing a partition.

Fusarium oxysporum. Macroscopic features: Circular colony initially develops with a cottony mycelium texture and ends as felt texture. Flat topography. On PDA, colony presents homogeneous pale pink to salmon colour.

Microscopic features: Conidiophores present phialides similar to *F. solani*, tapering toward the tip; necklaces poorly defined. Phialides occur as solitary or in branched clusters. Crescent-shaped multiseptate macroconidia; microconidia less abundant and oval

Media	GCB		CTP	
	CFU (E+04)	Std. Dev. (E+04)	CFU (E+05)	Std. Dev. (E+05)
RB	8.35	0.21	1.99	2.28
VM	4.40	2.26	0.46	0.51
LMS	4.00	0.42	2.21	1.39
	CFU (E+08)	Std. Dev. (E+08)	CFU (E+06)	Std. Dev. (E+05)
PCA	0.65	0.86	0.42	0.42
AN	1.44	1.99	3.80	2.83

Table 5: The results of the Plackett-Burman design.

Trat.	Phe degradation (mg/L)	
	4d	9d
T1	33.34	23.25
T2	25.82	91.92
T3	27.04	21.16
T4	26.38	29.60
T5	36.38	89.33
T6	16.52	15.61
T7	79.03	66.70
T8	0	70.18
TC	82.00	40.07
TC	83.39	34.93
TC	83.37	29.33
TC	83.69	90.95

Table 6: Phe degradation results.

with one or two septa. Distinguishable from *F. solani* by the shorter microconidia produced by phialides.

Yeasts

Candida orthopsilosis. Belongs to the class *Blastomycetes*, order *Moniliales* and family *Cryptocicaceae*. Widely distributed in the environment; colonizes humans and other mammals. Dimorphic fungus in yeast form during saprophytic state. Forms filaments of varying lengths during parasitic state [38]. Colonial morphology: convex with slight elevation; entirely circular margins, 5 mm diameter at 24 h; smooth surface, white or cream in colour.

Rhodotorula mucilaginosa. Belongs to the phylum *Basidiomycota*, class *Urediniomycetes*, order *Sporidiales* and family *Sporidiobolaceae*. Common inhabitant of the environment; found in various environmental matrices such as air, undergrown and ocean water. Part of commensal microflora of skin, nails and mucous membranes in humans; can colonize other plants and mammals. Mucoid and encapsulated cells; ferments sugar. Distinguishable by production of carotenoid pigments, giving colonies a rose or reddish tint that blocks certain wavelengths (620-750 nm), preventing cell damage. Also found in fresh or salt water reservoirs, permanent or seasonal, as well as in rivers and streams; frequently associated with human infections [38].

Colonial morphology: red coral colonies; smooth, mucoid and bright in color; convex shape with slight elevation; entirely circular margins, 5 mm diameter at 24 h.

Bacteria

Klebsiella variicola. Belongs to the class *Gamma proteobacteria*, order *Enterobacteriales* and family *Enterobacteriaceae*, containing common human pathogens. *Klebsiella* is a genus of Gram-negative, facultative anaerobic, nonmotile bacteria. Outermost layer formed by a polysaccharide capsule, distinguishing this organism from other genera of the family [39] Nitrogen-fixing ability; often used as a positive for biochemically evaluating enzyme nitrogenase activity of control isolates. Colonial and cellular morphology: circular colonies 2-3 mm; entirely circular margins at 24 h; flat, brown surface; non-diffusible mucosa pigmentation with consistent distribution; short, non-mobile bacillary cells without presence of spores.

Pseudomonas putida. Belongs to the class *Gamma proteobacteria*, order *Pseudomonadales* and family *Pseudomonadaceae*. The genus *Pseudomonas* is composed of Gram-negative, motility positive and scourge non-spore-forming bacteria. Curved or straight bacillus found isolated, in pairs or forming short chains. Catalase positive; catalyzes decomposition of hydrogen peroxide into oxygen and water. Some species synthesize exopolysaccharides. Capsule facilitates cell adhesion and biofilm formation.

This bacterium, found in most soil and water habitats with presence of oxygen, is one of the main opportunistic bacteria causing nosocomial infections [39]. It is of great industrial interest among bacteria of the genus *Pseudomonas* for its ability to colonize root systems of plants, form biofilms and be manageable from a genetic perspective in addition to its potential for degrading aromatic and xenobiotic compounds. Colonial and cellular morphology: circular colonies 2-3 mm; irregular edges at 24 h and cream-colored flat surface; non-diffusible pigment and mucosa consistency; solitary and mobile bacillary cells without presence of spores.

Biofilms formation

The best biofilms were produced from medium-sized green coffee bean (GCB) particles with a high moisture content (WHC) and an incubation time of one day. In this case, the microorganisms had colonized GCB before their harvest and persisted during the storage of the beans. However, due to low water activity of the GCB (0.275 ± 0.006), these microorganisms did not proliferate until the moisture content of the beans increased [40,41]. The WHC of GCB of low and medium PS (3.12 ± 0.08 and 3.20 ± 0.10 mL/g GCB, respectively) is higher than the WHC of large PS coffee beans (1.74 ± 0.03 mL/g GCB). This explains the higher microbial activity in biofilms of low and medium PS and high WHC during the first few days of incubation. However, neither the PS nor WHC were significantly influencing microbial growth after three days of incubation. This may be related to other factors that affect microbial succession, such as the competition for substrates and the enzymatic capacity of the colonizing species [36]. In operational terms, biofilms that promote the highest microbial activities at the lowest incubation times are more suitable for remediation since these imply lower operating expenses.

Increases in salinity are toxic to microorganisms without the capacity to adapt to high salt concentrations [42,43]. However, the results of this study are similar to those obtained elsewhere [44,45]; for example, it was observed that microorganisms of activated sludge may adapt to high salinity environments after a certain period without any inhibition of biomass production or floc formation. The results of this study also suggest that halotolerant organisms were present in the biofilms of GCB analyzed in this study. The diversity of species found on GCB could lead to different adaptation strategies by which cells adjust to changes in salt concentration. Furthermore, biofilms of mixed cultures contain numerous microorganisms and thus have a higher potential to survive in extreme environments due to the mutually beneficial physiological interactions among different groups of organisms.

Both halotolerant and halophilic microorganisms can be used to degrade contaminants. Their ability to degrade polyaromatic hydrocarbons, as well as other compounds, such as textile dyes [46-48], in environments of varying salinity has been proven [49,50]. While microorganisms of GCB are tolerant to diesel, their enzymatic mechanisms are not as specialized as other microorganisms isolated from soils and water contaminated with hydrocarbons. For example, a mixed culture was used to degrade mixtures of polyaromatic hydrocarbons (dibenzofuran [33 mg/L], dibenzothiophene [40 mg/L], fluorene [11 mg/L], phenanthrene [175 mg/L], anthracene [10 mg/L], fluoranthene [60 mg/L] and pyrene [50 mg/L]) using free and immobilized cells on granular clay. The authors report Phe and anthracene degradation percentages of 77% and 61%, respectively, after degradation assays were incubated for 6.5 days in 100 mL Erlenmeyer flasks [51]. Previous experiments in which diesel was used as the sole carbon source during the isolation of GCB microorganisms and their growth on solid culture mediums (before inoculation into liquid cultures) have shown similar results: GCB microorganisms are able to degrade hydrocarbon mixtures of petrol, diesel and other fuels with short hydrocarbon chains.

Plackett-Burman experimental design to determine optimal culture conditions for the degradation of phenanthrene

Table 5 presents the results of the Plackett-Burman design. Importantly, treatments with an asterisk (*) formed a strong emulsion during liquid-liquid extraction. All treatments that formed strong emulsions had a low concentration of Phe, suggesting the contaminant was lost during the extraction process and not due to biotic degradation processes. This was corroborated by examining the central points of the ninth day of culture. The PCD samples that showed strong emulsion had a very low concentration of Phe, while the remaining three did not. No relationship between samples and emulsified surfactant concentration is observed, suggesting that mechanical mixing during contaminant removal favored emulsification.

Treatments with greater removal of Phe that did not form considerable emulsions were T5 and T8. After day 4 of incubation, T5 showed a Phe reduction of 29% (53 ppm), while at 9 day of culture, there was a reduction of over 80% (146 ppm). Meanwhile, T8 did not show Phe degradation (Table 6) on the day 4 of cultivation, yet by the ninth day, there was a reduction of 67% (121 ppm); this result could be attributed to an adsorption-desorption process. Analyses of variance and regression determined that degradation at day 4 was not affected by any of the factors, and therefore, the model did not fit the experimental data (R^2=0.35, CV=87.52). This could be due the interference of the formed emulsions. In contrast, the ANOVA showed on day 9 a significant (α<0.05) reduction in concentration of $NaNO_3$ and KH_2PO_4 as well as high values of peptone and $FeSO_4$ (R^2=0.95 and CV=24.97), indicating a type III error. Only $MgSO_4$ $7H_2O$ and the surfactant Tween had no significant effect, representing a type I error, as shown in the regression analysis at following:

$$Phe(\%) = +43.82 - 20.93 * NaNO_3 + 13.08 * Peptone - 16.74 KH_2PO_4 + 5.13 * MgSO_4 7H_2O - 15.02 * CaCO_3 + 9.40 * FeSO_4 + 0.99 * Tween80$$

To increase degradation of Phe, the concentrations of KH_2PO_4 and $NaNO_3$ should be reduced and the concentration of peptone should be increased, as evidenced in assays T2 and T5. These conditions may be appropriate for hydrocarbon degradation and support the metabolic degradation capabilities of microorganisms pre-adapted to salty conditions. For instance, the degradation rate of microorganisms in pristine marine environments is 0.03 g/m^3d, while degradation of adapted microbial communities ranges from 0.5 to 50 g/m^3d [52,53].

Low levels of fixed forms of phosphate and nitrogen are often limiting factors. A C:N:P ratio of 100:10:1 is generally employed for this type of study. However, laboratory studies indicate that some subpopulations of microorganisms require more suitable N:P ratios to optimally degrade the different types of hydrocarbons that comprise crude oil [54]. One study evaluated the effect of the addition of 34 different N:P ratios on the degradation of two model hydrocarbons (hexane and Phe) in soils. The highest rates of hexane biodegradation were obtained with a N:P ratio of 7:1. In contrast, the biodegradation of Phe has a bimodal response with two optimal N:P ratios of 5:1 and a 20:1. This is possible if metabolically distinct microbial populations are consuming the substrate. Theoretically, to convert 1 g of hydrocarbon to cellular material, about 150 mg of N and 30 mg of P are required [53].

Other nutrients such as potassium and iron are also considered important during processes of hydrocarbon biodegradation [8]. In general, hydrocarbon degraders in pristine marine environments make up 1% of total microflora, this value increases to 10% in areas with occasional oil spills, such as the Gulf of Mexico (Atlas and Hazen). In a study previously developed [55] to determine the factors affecting the dispersion of crude oil in seawater, higher rates of dispersion were achieved with final concentrations of potassium phosphate and ammonium sulfate of 0.029 mM (10 mg/L) and 7.6 mM (2 g/L), respectively, implying a N:P ratio of 200:1. Although appropriate nutrients are necessary in sea water to achieve a suitable ratio and to enhance biodegradation of hydrocarbons, excessive concentration of nutrients can inhibit microbial growth. Some authors have reported negative effects of high levels of N, P and K on the biodegradation of hydrocarbons in soils and sediments [56,57], especially aromatic hydrocarbons [58]. Nevertheless, in seashore environments specific nutrient ratios are impossible to maintain as a consequence of nutrient leaching and wave and tidal action.

Other culture conditions were not evaluated in this study. For example, oxygen is usually not a limiting factor in well-aerated (high energy) marine environments, but in low-energy environments, such as lakes and marshes [8,53], there is a theoretical oxygen demand of 3.5 mg O_2/mg HC [59]. Wetlands and marshes have aerobic sediments zones several millimeters in thickness, yet sediments impacted with hydrocarbons usually create anaerobic zones. Notably, the composition of crude oil is the most important determining factor of biodegradability. Physical factors, such as temperature, also play an important role and affect contaminant chemistry and physiology as well as the metabolism and diversity of microorganisms. Although oil can be degraded from temperatures below 0°C and up to 80°C [8], the highest rates of degradation in soils occur from 30 to 40°C, in fresh water from 20 to 30°C and in marine environments from 15 to 20°C.

Conclusions

The use of medium-sized green coffee bean (GCB) particles with a high moisture content (water holding capacity) and an incubation time of one day provided the best conditions for producing biofilms. In this case, microorganisms had colonized GCB before their harvest and persisted during the storage of the beans. These microorganisms composing the biofilm are capable of adapting to sea water and degrading Phe without any inhibition of biomass production or floc formation. Therefore, the use of mixed GCB cultures represents a sustainable technology to remediate both fresh and marine water and would provide environmental and economic benefits due to the

low cost of this procedure. A period of 48-54 hours was sufficient to prepare cultures of microorganisms, and a total of 72-78 h are required to immobilize the inoculum and the biofilm on the support structure. It is important to consider a suitable N:P ratio for microorganisms pre-adapted to salty, contaminated water in order to enhance their metabolic capacity to degrade hydrocarbons, mainly polyaromatic hydrocarbons.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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