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POLSKA AKADEMIA NAUK

Archives of Environmental Protection
Vol. 46 no. 4 pp. 59–69

PL ISSN 2083-4772
DOI 10.24425/aep.2020.135765

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Bacillus pumilus and *Paenibacillus lautus* effectivity in the process of biodegradation of diesel isolated from hydrocarbons contaminated agricultural soils

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Keywords: biodegradation; diesel; clandestine milking.

Abstract: In Mexico, one of the principal natural resources is oil, however, the activity related to it has generated hydrocarbon spills on agricultural soils. The aim of this study was to evaluate the biodegradability of diesel by means of indigenous bacteria isolated from agricultural soil contaminated with 68 900 mg kg^{-1} diesel. We examined indigenous bacterial strains in agricultural soils contaminated with diesel from Acatzingo, Puebla, Mexico. We performed a physicochemical soil characterization, and a bacterial population quantification favoring sporulated bacteria of the genera *Bacillus* and *Paenibacillus* taken from the study site. Six bacterial strains were isolated. The identification was made based on the 16S rRNA gene and API systems. The tolerance and biodegradation capacity in diesel were determined at 4 000 to 24 000 mg L^{-1} of diesel. Residual concentrations of diesel were determined by GC-FID. Soil contaminated with diesel alters the concentrations of organic matter, phosphorus and nitrogen. Analysis of soil samples showed heat resistant bacterial populations of 10^6 cfu g^{-1} dry soil. Six strains from soil pollution were identified – *Pseudomonas stutzeri* M1CH1, *Bacillus pumilus* M1CH1b, *Bacillus cereus* M1CH10, *Bacillus subtilis* M1CH15a, and *Paenibacillus lautus* strains M1CH19 and M1CH27. These bacteria showed different degradation behavior. *Bacillus pumilus* M1HC1b and *Paenibacillus lautus* M1CH27 use diesel oil as the sole carbon source. *Bacillus pumilus* degraded high concentrations of diesel (24 000 mg L^{-1}), while for *Paenibacillus lautus* it became toxic and the degradation was less.

Introduction

Oil is one of the main natural resources of wealth for Mexico, and Mexico is considered the 10th largest producer of oil in the world (Sanusi 2019). The oil production activity has led to the spillage of 93.32 million barrels of crude oil during the period which goes from 2000–2014 in Mexico, and the state Puebla ranked fourth in Mexico with regards to hydrocarbon spills (CNH 2015). The locality of Acatzingo, Puebla, Mexico contains isolated zones and planting lands that are crossed by pipelines and polyducts of Petroleos Mexicanos which have been seriously affected by clandestine milking which has led to spills of diesel, gasoline, fuel oil and crude oil. The petroleum spillage causes explosion risks and the contamination of ecosystems (water, air and soil), which may affect both soil structure and its agricultural fertility (Pawełczak et al. 2015), in consequence there is a decrease in crop yield and other alterations on biodiversity, irrigation systems, the economy and the health of the population (Ujowundu et al. 2011).

Diesel is one of the pollutants frequently reported to be found in these agricultural soils. It contains from 2 000 to 4 000 hydrocarbons and is a complex mixture of linear, branched and cyclic alkanes and polyaromatic compounds (PAHs) obtained from the middle fraction of the distillate during the oil separation process (Das and Chandran 2011).

These PAH compounds are of concern for human health because of their known genotoxic, mutagenic and carcinogenic effects (Schulte and Hauser 2012). Several treatment strategies have been developed to mitigate risks; these have included biological, physicochemical and thermal processes to remediate contaminated sites. Biological treatment such as bioremediation is a low cost and environmentally friendly possibility, since organic pollutants can be degraded to CO_2 and H_2O. Bioremediation involves the use of endemic or

artificially introduced autochthonous microorganisms to detoxify and degrade contaminants (Gillespie and Philp 2013); this is important locally but also has global implications. Hydrocarbon-degrading bacteria are widely distributed in different ecological habitats which explains the hydrocarbon depletion under native conditions. The occurrence of natural decontamination suggests the adaptation of microbes with the ability to degrade organic pollutants (Brito et al. 2015). It is possible to perform bioremediation with these bacteria using an economical and versatile procedure (Sharma 2012). Therefore, the efficiency of bioremediation correlates with the biodegradability, solubility and bioavailability of the hydrocarbons to bacteria and provides an optimal environment for testing (Ghafari et al. 2019). Different gram positive bacteria, such as *Bacillus cereus*, *Bacillus subtilis*, *Bacillus* sp., *Cellulosimicrobium cellulans*, *Corynebacterium* sp., *Gordonia* sp., *Paenibacillus ehimensis*, *Paenibacillus naphthalenovora*, *Rhodococcus* sp., among others, exhibit basic metabolic abilities to utilize various chemical compounds of the organic pollutant. At high concentrations diesel can become toxic due to the accumulation of PAHs and cyclic aromatic components into the lipid bilayer that increases its availability to the cell affecting the energy transduction across the biological membranes (Das and Chandran 2011, Jiang et al. 2016, Kebria et al. 2009, Shibulal et al. 2017).

The objective of the present study was to evaluate the diesel biodegradability by indigenous bacterial strains isolated from agricultural soils from Acatzingo, Puebla, Mexico, contaminated with diesel.

Materials and methods

Sampling

Agricultural soils accidentally contaminated with diesel and uncontaminated samples were collected from Acatzingo, Puebla, Mexico (18º 56´26´´N, 97º 41´53´´W and 18º 56´43.897´´N, 97º 41´54.79´´W respectively). Soil samples of 5 kg were collected from the top surface soil (0–15 cm) and stored at 4°C before analysis.

Physicochemical soil characterization

The physicochemical parameters pH, texture, moisture, total organic matter, total nitrogen, available phosphorus, and bulk density were determined in soil according to NOM-021--SEMARNAT (DOF, 2002). The diesel determination was quantified based on NOM-138-SEMARNAT/SSA1-2012 (DOF, 2013) and on the method NMX-AA-145-SCFI-2008 (DOF, 2008).

Quantification of diesel using gas GC-FID

Diesel in soil was quantified by gas chromatography with a flame ionization detector (GC-FID) based on EPA method 8015 C. At the same time, there were prepared calibration curves with 1 000, 2 000, 4 000, 5 000, 10 000, 15 000, 20 000 and 24 000 mg L^{-1} of diesel in dichloromethane. The extraction of diesel from bacterial culture samples was carried out by the EPA method 3510 C (separatory funnel liquid-liquid extraction) with dichloromethane (HPLC grade) in 1.25:1 (sample: solvent) ratio. Sample extracts were analyzed by gas chromatography with a DB-THP (30 m × 0.32 mm × 0.25 µm) capillary column, with the following parameters: the injector temperature was 250°C, the column was set at an initial temperature of 45°C for four minutes followed by a 20°C increment per minute to 275°C and the isothermal was held for one min. Carrier gas (N_2) flow was 10 mL/min for two min, and was used with a total run time of 16.5 min.

Isolation of bacteria

Bacteria were isolated from the diesel-contaminated soil samples under aerobic conditions. We used a 1:10 dilution in a phosphate buffer solution (g L^{-1}: KH_2PO_4 1.7008, K_2HPO_4 2.1772, Tween 80 1 mL at pH 7.0) and it was incubated at 60°C and 80°C for 2 h to favor sporulated bacteria of the genera *Bacillus* and *Paenibacillus*. Endospore producing strains survive longer in the soil under different environmental conditions, such as nutrient limitation or desiccation; this property will be useful when the soil is inoculated for a bioremediation process. Following this, we did serial dilutions from 10^{-1} to 10^{-6}, and inoculated 100 µl on nutrient agar plates by streaking. Then the plates were incubated at 30°C for 24 h. The total number of bacterial isolates was determined as colony-forming units (cfu) g^{-1} dry soil. Colonies of different morphology were selected from the soil, purified and stored at -70°C in a nutrient broth with glycerol to 50% for subsequent diesel tolerance assays.

Biochemical characterization and rRNA gene analysis

A bacterial suspension in 5 mL of saline solution (1% NaCl) was prepared from an isolated colony and then cultivated in the galleries. Biochemical characterization was carried out by the API 20 E and API 50 CH systems (references No. 20160 and 50300) and apiweb™ of bioMérieux®. Identification of bacteria was carried out by rDNA sequence; bacterial axenic cultures were grown on a liquid nutrient medium, 1.5 mL of liquid cultures were centrifuged and suspended with 150 µL of buffer solution (at pH=8 and 10 µg mL^{-1} lysozyme), and incubated at 37°C for 30 min. Subsequently, a volume of lauryl sarcosine (1%) and phenol-chloroform solution (Tris pH=8) were added, and gently mixed. The tubes were centrifuged at 12 396 × *g* by 10 min and an aqueous phase was recovered. Phenol extraction was repeated twice. DNA was precipitated with sodium acetate 0.3 M and two volumes of cold ethanol. DNA pellets were dissolved in 200 µl of Tris 10 mM-EDTA 1 mM (pH=8) with 0.01 µg mL^{-1} of RNase A and diluted with water to 0.01 µg mL^{-1}. PCR amplification of the 16S ribosomal RNA subunit gene was carried out using pUf and pUr primers. The amplification cycle consists of an initial denaturation at 94°C for 20 s followed by 30 cycles at 94°C for 10 s, 50°C for 20 s, and 72°C for 1 min a final extension at 72°C for 8 min. Amplicons were separated on an agarose gel (1.5% w/v). PCR amplicons were purified using the Promega® PCR Cleaning kit and sequenced by Sanger DNA synthesis. The resulting sequences were compared against reported sequences of the NCBI GenBank using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment of DNA sequences was carried out using the Clustal_Omega program (1.2.4 program, https://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was constructed using the Neighbor-Joining method with bootstrap analysis of 1 000 resampling using the MEGA X software package. Six 16S rRNA gene sequences were

obtained from the isolates and deposited on the NCBI GenBank with the following accession numbers: M1HC1 (MK256308), M1HC1b (MK256303), M1HC10 (MK256306), M1HC15a (MK256304), M1HC19 (MK256307), and M1HC27 (MK256305).

Preparation of inocula

The isolated bacteria (six) were grown overnight in 20 mL test tubes containing 5 mL of culture in a nutrient medium in a rotary shaker at 1 g at 30°C. Bacterial cells were harvested by centrifugation (2 152 × *g* for 10 min at 4°C), rinsed twice in phosphate buffer, and OD_{610} was adjusted to 0.05, corresponding to approximately 10^6 cells mL^{-1} (Bashan et al. 2011).

Diesel tolerance assays

The tolerance for diesel of each isolate was determined by the cell growth (OD_{610}) of the cultures at final diesel concentrations of 4 000, 8 000, 16 000, 24 000, 26 000, 40 000, 50 000 and 60 000 mg L^{-1} in a culture medium (g L^{-1}): NH_4NO_3 1.0 , KH_2PO_4 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.2, $CaCl_2$ 0.02, $FeCl_3$ 0.05, glucose 0.05, yeast extract 0.05 at pH 7.0. The tubes contained 5 mL of a culture medium as described above and were inoculated with 50 µl of a 10^6 cells mL^{-1} cellular suspension of the exponential phase of the bacterial growth and incubated aerobically (1 × *g*) for 96 h at 30°C; as a control 1.8016 g L^{-1} glucose was used. Bacterial growth was determined by turbidity (OD_{610}) at 96 h. All treatments were carried out in triplicate.

Biodegradation and kinetics assays

Based on the results of bacterial growth by turbidity (OD_{610}) in the tolerance assays, diesel concentrations of 4 000 to 24 000 mg L^{-1} were selected for the biodegradation assays. The bacterial isolates from cultures in a nutrient medium at the exponential phase of growth were transferred to 250 mL flasks with 1 000 µl of 10^6 cells mL^{-1}, each containing 100 mL of the sterile culture medium with 4 000, 8 000, 16 000, 20 000 and 24 000 mg L^{-1} diesel. The experiment was carried out in triplicate using uninoculated flasks as a control. All treatments were incubated at 30°C for determined intervals of time (0, 5, and 10 d). Residual concentrations of diesel were determined by GC-FID based on EPA method 3510 C (separatory funnel liquid-liquid extraction). The biodegradation was determined according to the following equation:

% Biodegradation = [(initial diesel concentration – final diesel concentration) / initial diesel concentration] * 100

- initial diesel concentration: initial diesel concentration
- final diesel concentration: residual diesel concentration in the treatment – residual diesel concentration in the control

Biodegradability of diesel is usually explained by first order kinetics, and the biological half-life ($t_{1/2}$) which is the time it takes for a substance to lose half of its amount was calculated according to Agarry et al. (2013). Mean generation times (*g*) and specific growth rates (µ) were calculated using nonlinear regression of bacterial growth curves from the first day during which growth rates were maximal.

Analysis of results

The minitab statistical package (version 17.3) was used to validate the differences between treatments, using a one-way ANOVA and a Tukey test. Probability was set at 0.05.

Results and discussion

Physicochemical characterization of soils

Contaminated soil contained 68 900 mg kg^{-1} of diesel; this elevated concentration can be unsatisfactory for plants and for the growth of some microbial populations. In addition, the presence of toxic materials such as cresol, phenols, and chlorine may inhibit the growth of hydrocarbon oxidizers (Ujowundu et al. 2011). The total organic matter, phosphate and electrical conductivity (EC) were high in the contaminated soil (33.5%, 0.0025%, 380 µS cm^{-1} respectively), while nitrogen was low (0.077%) (Table 1). The high amount of organic matter was due to the elevated concentrations of hydrocarbons adsorbed in soil particles. This, in turn, causes nitrogen lows with respect to the high organic matter concentration. In addition, we found low values of moisture (10%) and a decrease in pH (7.45) with respect to the non-impacted soil and it showed a sandy loam texture. These results were similar to those reported in other studies (Ujowundu et al. 2011); the low moisture may be due to the fact that hydrocarbons increased soil hydrophobicity in turn decreasing the moisture holding capacity. The decreased pH may be associated with growth and metabolic activity due to the production of carbon dioxide and organic acids in soils contaminated with hydrocarbons (diesel). In addition, the presence of ions in the soil such as carbonate, bicarbonate, hydrogen sulfide, elemental sulfur, iron oxides, free nitrogen and nitrous oxide can influence the increase in EC; such changes cause some minerals to become unstable, dissolve, mobilize and precipitate (Schumacher 1996). These results are related to what was mentioned by Pawełczak et al. (2015) who report that the release of petroleum products affects the physical and chemical properties of soils, these conditions lead to low soil fertility and thus low agricultural productivity and a reduction in the local economy.

Bacterial isolation and characterization

At the study site we quantified 1.16 × 10^6 cfu g^{-1} dry soil of viable indigenous bacterial populations that survived the contaminated soil conditions. Therefore, the size of the bacterial population reported in this study may be influenced by the physical and chemical characteristics of the soil, type of pollutant, and temperature according to Hamamura et al. (2013) and Liu et al. (2017). Bacterial populations establish and multiply among the soil particles (clays, silt and sand) forming ecological niches, these are attracted by affinity (ionic environment within the soil/water mix), size and surface area available (Oliver et al. 2007).

Six bacterial strains that differed in morphology were detected from agricultural soils contaminated with diesel. Their phylogenetic affiliation was determined through 16S rRNA gene sequence analysis. Of the bacterial indigenous isolates, 50% were affiliated with the *Bacillus* genus and 33% with *Paenibacillus*, which survive under inhospitable soil contamination conditions (68 900 mg kg^{-1} diesel). In the literature, it has been reported that sporulated bacteria, such

as the genus *Bacillus,* are one of the predominant bacterial strains of soils contaminated with hydrocarbons and provide a greater biodegradation capacity when compared with other bacteria from soil (Chaudhary et al. 2015, Ijah & Antai 2003, Ubani et al. 2016). In our study, we reported the occurrence of sporulated bacteria of the *Bacillus* and *Paenibacillus* genera isolated from agricultural contaminated soils with capacity to biodegrade 24 000 mg L^{-1} of diesel (Table 5).

The strains M1HC1b, M1HC10, and M1HC15a were related to *Bacillus pumilus* (98%), *B. cereus* (99%), and *B. subtilis* (96%) respectively. The strains M1HC19 and M1HC27 were related to *Paenibacillus lautus* with 99% and 90% of homology. The strain M1HC1 was closely related to *Pseudomonas stutzeri* (99%) (Table 2 and Figure 1). Different strains of *Bacillus cereus*, *Bacillus subtilis*, *Bacillus* sp., *Paenibacillus ehimensis*, *Paenibacillus naphthalenovora*, among others, have been reported to use different hydrocarbon compounds (Das and Chandran 2011, Kebria et al. 2009, Raju et al. 2017, Shibulal et al. 2017).

These bacteria have different survival mechanisms to tolerate organic pollutants, such as the use of hydrocarbons by membrane-bound oxygenases, changes that increase cell--surface hydrophobicity and consequently attach hydrocarbons (by altering their surface components as lipoteichoic acid, lipololysaccharide, proteins or porines of the outer membrane, surface fibrils, hydrophobic fimbriae, gramicidin S and prodigiosin) (Ron and Rosenberg 2014) and secrete bioemulsifiers (Xu et al. 2018).

In addition, these strains were biochemically characterized with the 50CH and 20E galleries of the API system. The API test provided us with aerobic metabolic information that complemented the 16S rRNA gene sequencing analysis of each study strain. *Bacillus* sp. (M1HC1b) was positive for Oxidase and Gelatine, and negative for Glycerol, D-Arabinose, L-Arabinose, Xyloside, D-Galactose, D-Mannitol, Amygdalin, D-Lactose, D-Sucrose, Amidon, Glycogen, Gentiobiose. The strains *Paenibacillus lautus* (M1HC19 and M1HC27) were positive for Glycerol, D-Arabinose, L-Arabinose, Xyloside, D-Galactose, D-Mannitol, Amygdalin, D-Lactose, D-Sucrose, Amidon, Glycogen, and Gentiobiose. However, they were negative for Gelatin hydrolysis, and they showed a differential metabolic pattern to Oxidase, D-Xylose, D-Mannose, D-Glucoside, D-Melibiose, D-Raffinose, D-Turanose, Urease, β-galactosidase, L-Lysine and L-Ornithine. The strains did not show differences for the rest of the metabolic patterns studied (data not shown). The strains M1HC1b, M1HC19 and M1HC27 were identified as *Bacillus* sp. (M1HC1b) and *Paenibacillus lautus* (M1HC19 and M1HC27) at a similar metabolic pattern as reported by Kebria et al. (2009) and Sharma et al. (2015) (Table 3). Other species of *Paenibacillus* as *P. larvae* using the API 50CH and BioLog Gen III systems were positive for glycerol, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, arbutin, esculin, salicin, D-maltose, D-trehalose and gelatin (Kilwinski et al. 2004, Rusenova and Parvanov 2014).

The tolerance to diesel of the isolates

Diesel tolerance was monitored for bacterial growth, the OD values of the strains *Pseudomonas stutzeri* M1HC1, *Bacillus pumilus* M1HC1b, *Bacillus cereus* M1HC10 and *Bacillus subtilis* M1HC15a decreased from 2.87 to 0.35 with increasing diesel concentration from zero to 60 000 mg L^{-1} after 96 h. *Paenibacillus lautus* M1HC19 has better growth than *Paenibacillus lautus* M1HC27 with OD values from 4.10 to 0.43 (Table 4). However, under treatments of 24 000 mg L^{-1} of diesel *Bacillus pumilus* M1HC1b, *Bacillus cereus* M1HC10

Table 1. Physicochemical characterization of unimpacted and impacted agricultural soils from Acatzingo, Puebla, Mexico

Parameters	Unimpacted soil	Impacted soil
pH	8.07	7.45
CE (µS cm^{-1})	128	380
Texture (%) (clay: silt: sand)	17.4: 18.0: 64.6	12.6: 36.6 : 50.8
Moisture (%)	18.29	10
Total organic matter (%)	1.30	33.5
Total nitrogen (%)	0.56	0.077
Available phosphorus (%)	0.0012	0.0025
Bulk density (g mL^{-1})	1.46	1.26
Diesel (mg kg^{-1})	Nd	68 900

Nd: not detected under the established methodology.

Table 2. Bacterial identification by 16S rRNA gene sequencing

Isolate	Phylogenetic affiliation	% Identities
M1HC1	*Pseudomonas stutzeri*	99
M1HC1b	*Bacillus pumilus*	98
M1HC10	*Bacillus cereus*	99
M1HC15a	*Bacillus subtilis*	96
M1HC19	*Paenibacillus lautus*	99
M1HC27	*Paenibacillus lautus*	90

and *Paenibacillus lautus* M1HC19 showed high OD values (0.75, 1.03 and 0.82 respectively); the rest of the bacterial strains were negatively affected in their growth. Furthermore, the bacterial growth of *Paenibacillus lautus* M1HC27 remained at approximately 0.35 in treatments of 8 000 to 24 000 mg L^{-1}. The growth observed might be due to hydrocarbon assimilating capabilities, as a result, to be adaptable to the previous exposure to hydrocarbons (Al-Disi et al. 2017). However, the isolated bacteria tolerated a lower concentration of diesel than that reported in the study site (68 900 mg kg^{-1} from diesel), due to the selection methodology used to isolate sporulated bacteria and exclude other bacterial groups with high tolerance that may be coexisting in the natural habitat of the soil. Also, the availability of the contaminant is probably lower in the soil compared to the conditions of the culture medium (Liu et al. 2017). Based on OD results, the strains selected for diesel degradation experiments were: *Bacillus pumilus* M1HC1b and *Paenibacillus lautus* M1HC19 due to the high OD values at 24 000 mg L^{-1} of diesel (0.75 and 0.82 respectively); and *Paenibacillus lautus* M1HC27 was selected because it showed constant growth of 8 000 to 24 000 mg L^{-1} (OD values of 0.38 to 0.31). Our results were higher than those reported by Nwinyi et al. (2014) where *Pseudomonas* sp. and *Bacillus* sp. were tolerant to diesel (2 000 mg L^{-1}) according to their growth in liquid cultures expressed as OD readings of 1.187 and 0.858 at 15 days of incubation.

Biodegradation kinetics

The biodegradation kinetics of diesel were explained by first-order kinetic models with a correlation coefficient (R^2) that lies between 0.814 and 0.995 (Table 5). The results for the kinetic biodegradation constants of *Bacillus pumilus* M1HC1b ranged from 0.079 to 0.828 degraded diesel day^{-1} and *Paenibacillus lautus* M1HC27 from 0.009 to 0.131 degraded diesel day^{-1}, with an inhibitory effect as a function of hydrocarbon concentration (Table 5). In addition, the half-life value increased when the diesel concentration was higher in the culture medium, presenting values of 0.837 to 8.743 days (*Bacillus pumilus* M1HC1b) and 5.288 to 75.991 days (*Paenibacillus lautus* M1HC27), therefore, a higher concentration of diesel requires more time to biodegrade it, while, the lowest rate of degradation was recorded in control groups with $\leq$ 0.018 degraded diesel day^{-1} (Table 5). *Bacillus pumilus* M1HC1b showed the highest capacity of biodegradation (100%) at 4 000 mg L^{-1} of diesel, and as the concentration of the contaminant increased, the biodegradation capacity of diesel decreased by 50%

Fig. 1. Phylogenetic tree of the partial sequences of the 16S rRNA gene of the bacterial isolates obtained using neighbor-joining analysis

significantly (with a probability value of 0.0001 (p-value) at a significance level of 0.05, and F-value = 294.822 of Fisher test) at the concentrations of 8 000 to 24 000 mg L^{-1} (Figure 2 and Table 5). So, this bacterial strain contains multienzyme systems for the degradation of the constituents of diesel as shown by different gas chromatograms (Figure 2); although its growth rate (μ) decreased from 0.41 to 0.24 as diesel concentration increased (Table 6), the degradation capacity remained at 50% at high concentrations of hydrocarbons (24 000 mg L^{-1}) (Table 5).

The higher growth rate (μ) and lower generation time (g) of evaluated strains (*Bacillus pumilus* M1HC1b μ= 0.41, g= 1.70; *Paenibacillus lautus* M1HC19 μ= 0.52, g= 1.32*;* and M1HC27 μ= 0.44, g=1.56) were at low concentrations of diesel (4 000 mg L^{-1}); this might be related to the higher breakdown and utilization of diesel. The gas chromatograms of diesel at different concentrations showed the comparison of content (Figure 2, Table 6). From these graphs we can see that *Bacillus pumilus* M1HC1b and *Paenibacillus lautus* M1HC27 behaved differently with respect to the degradation of diesel (Figure 2). *Bacillus pumilus* M1HC1b in 10 days managed to degrade 100% of diesel in the treatment with 4 000 mg L^{-1}, while in the treatment with 24 000 mg L^{-1} of diesel it only degraded 54.45% (Table 5). Growth rate was positively correlated to biodegradation in a significant way for both strains, i.e., high values of μ showed high biodegradation. *Bacillus pumilus* M1HC1b presented a significant correlation (r) of 0.8060, with an associated probability of 0.0000 (p-value) at a confidence level of 95%; and *Paenibacillus lautus* M1HC27 showed a significant correlation value (r = 0.4919), with an associated probability of 0.0006 (p-value). *Paenibacillus lautus* M1HC19 did not show a significant biodegradation of diesel (5.10 to 7.21%, data not shown), so its capacity for biodegradation was low and was not correlated to cell growth (r = -0.0656, p-value = 0.6685 at confidence level of 95%).

Different studies have been interested in the study of indigenous bacteria that degrade hydrocarbons since this guarantees their survival and the efficiency of degradation capacity (Kebria et al. 2009). The *Bacillus* genus has been reported to be one of the predominant bacterial strains for soils contaminated with hydrocarbons (Chaudhary et al. 2015, Ubani et al. 2016). This genus has greater biodegradation capacity when compared with other bacteria such as *Micrococcus varians*, *Pseudomonas aeruginosa*, *Vibrio* sp. and *Alcaligenes*

Table 3. Biochemical and physiological characteristics of *B. pumilus* and *P. lautus*

Feature	Strains		
	B. pumilus **(M1HC1b)**	***P. lautus*** **(M1HC19)**	***P. lautus*** **(M1HC27)**
Oxidase	+	–	+
Acid from:			
Glycerol	–	+	+
D-Arabinose	–	+	+
L-Arabinose	–	+	+
D-Xylose	–	+	–
Xyloside	–	+	+
D-Galactose	–	+	+
D-Mannose	–	+	–
D-Mannitol	–	+	+
D-Glucoside	–	+	–
Amygdalin	–	+	+
D-Lactose	–	+	+
D-Melibiose	–	+	–
D-Sucrose	–	+	+
D-Raffinose	–	+	–
Amidon	–	+	+
Glycogen	–	+	+
Gentiobiose	–	+	+
D-Turanose	–	+	–
Hydrolysis of:			
Gelatin	+	–	–
Urease	–	+	–
β-galactosidase	–	+	–
Voges-Proskauer	–	+	+
Utilization:			
L-Lysine	–	+	–
L-Ornithine	–	+	–

Table 4. Diesel tolerance by bacterial strains isolated from agricultural soils

Strain	Diesel concentration (mg L^{-1})									
	0	4 000	8 000	16 000	20 000	24 000	28 000	40 000	50 000	60 000
	Cell growth (OD610 nm)1									
P. stutzeri (M1HC1)	1.63 ±0.02	1.47 ±0.02	0.94 ±0.07	0.84 ±0.06	0.74 ±0.03	0.67 ±0.02	0.58 ±0.04	0.45 ±0.01	0.45 ±0.03	0.35 ±0.03
B. pumilus (M1HC1b)	1.57 ±0.02	1.36 ±0.12	1.20 ±0.02	1.10 ±0.03	0.71 ±0.09	0.75 ±0.01	0.62 ±0.02	0.56 ±0.01	0.45 ±0.01	0.36 ±0.15
B. cereus (M1HC10)	1.70 ±0.03	1.06 ±0.05	1.05 ±0.06	1.06 ±0.02	1.08 ±0.05	1.03 ±0.04	0.86 ±0.02	0.60 ±0.03	0.52 ±0.03	0.49 ±0.02
B. subtilis (M1HC15a)	2.87 ±0.06	1.30 ±0.05	1.27 ±0.02	0.90 ±0.01	0.80 ±0.08	0.62 ±0.07	0.62 ±0.05	0.61 ±0.02	0.59 ±0.03	0.59 ±0.02
P. lautus (M1HC19)	4.10 ±0.02	1.77 ±0.05	1.41 ±0.08	0.97 ±0.15	0.83 ±0.06	0.82 ±0.01	0.82 ±0.04	0.66 ±0.06	0.52 ±0.01	0.43 ±0.12
P. lautus (M1HC27)	1.34 ±0.04	0.59 ±0.01	0.38 ±0.02	0.36 ±0.03	0.36 ±0.01	0.31 ±0.05	0.24 ±0.02	0.24 ±0.03	Ng	Ng

[1] Bacterial growth determined by turbidity at 96 h. Average values of three repetitions ± standard deviation.
Ng: not growth.

Table 5. % Biodegradation, Biodegradation rate (k) of diesel and half-life ($t1/2$) by the different bacterial strains at different concentrations of diesel

Diesel (mg L^{-1})	Strains											
	Bacillus pumilus **(M1HC1b)**				***Paenibacillus lautus*** **(M1HC27)**				***Control Uninoculated***			
	% degradation[1]	R^2	k	$t_{1/2}$	% degradation	R^2	k	$t_{1/2}$	% degradation	R^2	k	$t_{1/2}$
4000	100.00 ± 0.10 b	0.828	0.828	0.837	71.72 ± 0.13 d	0.992	0.131	5.288	5.39 ± 0.99 a	0.943	0.018	38.426
8000	56.09 ± 7.84 a	0.972	0.087	7.992	47.77 ± 1.66 c	0.995	0.068	10.122	5.27 ± 0.20 a	0.792	0.016	43.199
16000	55.51 ± 5.36 a	0.871	0.084	8.237	31.22 ± 0.45 b	0.953	0.041	17.094	5.11 ± 0.43 a	0.987	0.014	48.956
20000	55.29 ± 0.04 a	0.814	0.079	8.793	17.42 ± 0.31 a	0.831	0.019	37.032	5.36 ± 0.57 a	0.557	0.009	75.288
24000	54.45 ± 1.59 a	0.990	0.079	8.743	17.03 ± 0.08 a	0.921	0.009	75.991	5.55 ± 0.20 a	0.983	0.005	139.838

[1] Values were the mean of triplicate treatments ± standard deviation incubated at 30°C by 10 days. Different letters in the column represent highly significant differences (p < 0.05) between treatments (Tukey's test).
k: biodegradation constant (day^{-1}); $t_{1/2}$: half-life (days); R^2: correlation coefficient

sp. (Ijah and Antai 2003). The biodegradation values reported in this work are higher than those found by other authors, since *Bacillus* and *Paenibacillus* degraded 13 068 mg L^{-1} of diesel (54.45% of 24 000 mg L^{-1}) and 4 087 mg L^{-1} of diesel (17.03% of 24 000 mg L^{-1}) in 10 days respectively, and they tolerated 60 000 mg L^{-1} diesel (Tables 4 and 5). Other researchers reported diesel degradations for *Bacillus cereus* of 8 250 mg L^{-1} at 15 days (82.5% of 10 000 mg L^{-1}) (Kebria et al. 2009), and 7 055 mg L^{-1} of diesel degraded at 30 days (83% of 8 500 mg L^{-1}) (Raju et al. 2017); while for *Paenibacillus* they reported 6 532.25 mg L^{-1} of degraded diesel at 20 days (76.85% of 8 500 mg L^{-1}) (Ganesh and Lin 2009).

The presence of gram-positive bacteria such as *Bacillus* and *Paenibacillus* in diesel-contaminated soils has been attributed to their tolerance, although they showed low values according to the contamination of the site. Their survival could be due to the fact that they act in consortium for the degradation of diesel; therefore, they are not the only ones that inhabit the soil and use the diesel as the source of carbon and energy. The search for bacteria with highly tolerant and biodegrading capacities will depend on the isolation conditions. As described by Akwukwaegbu et al. (2019), the degradation of a complex mixture such as diesel requires the participation of more than one bacteria or bacterial groups that may be acting in consortium to achieve a considerable biodegradation of the hydrocarbons and prevail in the contaminated site; this may be the case of the strains in our study. Other authors have reported the degradation and utilization of crude oil, diesel,

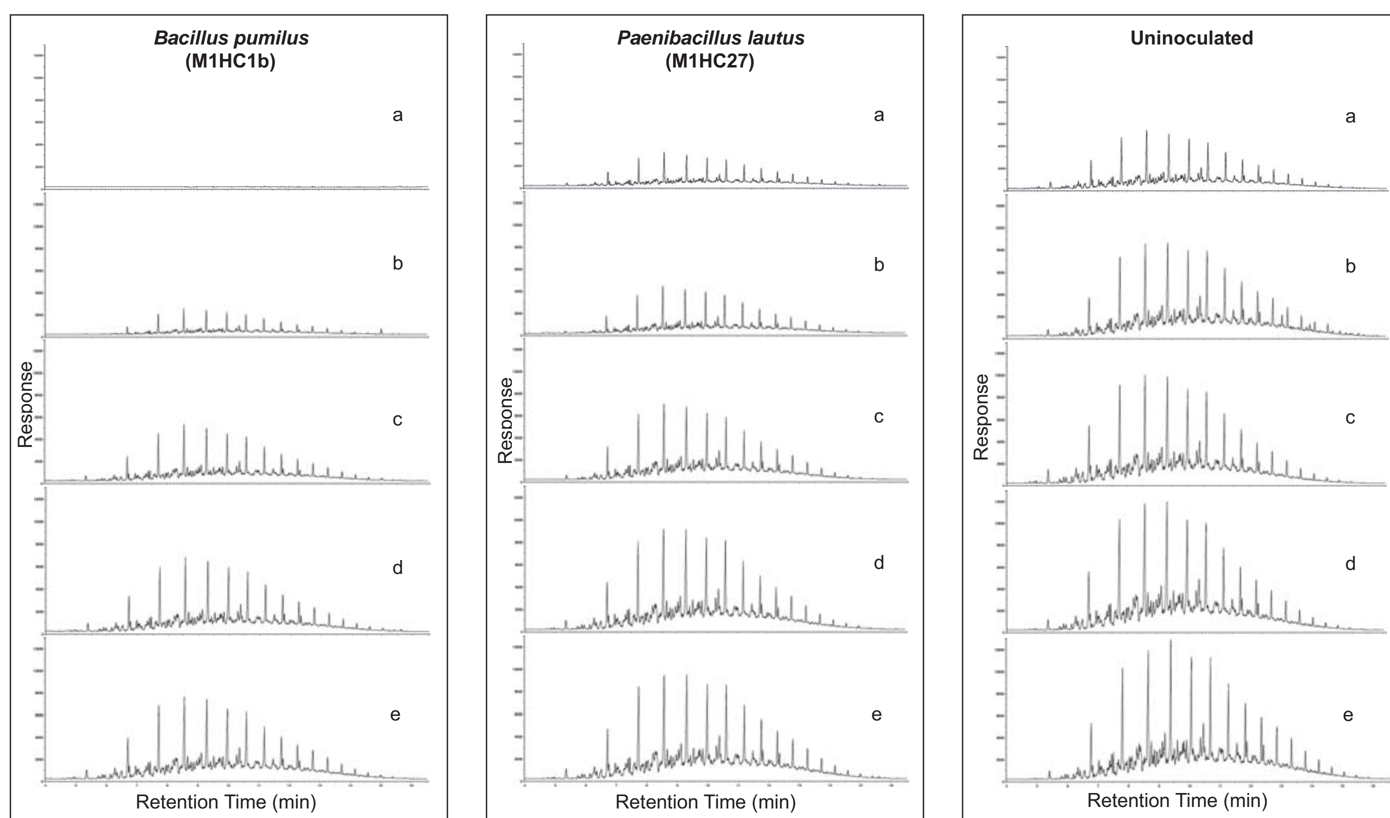

Fig. 2. GC-FID chromatograms showing the biodegradation of diesel at different concentrations 4 000 (a), 8 000 (b), 16 000 (c), 20 000 (d), and 24 000 mg L^{-1} (e) by the different bacterial strains after 10 days of incubation

Table 6. Growth rate bacterial at different concentrations of diesel

Diesel (mg L^{-1})	Strains					
	Bacillus pumilus (M1HC1b)		*Paenibacillus lautus* (M1HC27)		*Paenibacillus lautus* (M1HC19)	
	μ[1]	*g*	μ	*g*	μ	*g*
0	0.41 c	1.67 a	0.51 d	1.36 a	0.53 b	1.30 a
4000	0.41 c	1.70 a	0.44 c	1.56 b	0.52 b	1.32 a
8000	0.36 b	1.90 b	0.37 b	1.89 c	0.52 b	1.34 a
16000	0.36 b	1.91 b	0.37 b	1.90 c	0.51 b	1.37 a
20000	0.35 b	1.99 b	0.34 ab	2.01 cd	0.46 a	1.50 b
24000	0.24 a	2.92 c	0.32 a	2.17 d	0.44 a	1.56 b

[1] Values were the mean of triplicate treatments ± standard deviation incubated at 30°C by 10 days. Different letters in the column represent highly significant differences ($p < 0.05$) between treatments (Tukey's test).
NA: Not applicable; μ: specific growth rate (h^{-1}); *g*: generation time (h).

n-hexadecane, naphthalene as a carbon and energy source for the bacterial genera: *Acinetobacter*, *Achromobacter*, *Alcanivorax*, *Bacillus*, *Exiguobacterium*, *Halomonas*, *Microbacterium*, *Paenibacillus lautus, Pseudomonas*, *Rhodococcus*, *Streptomyces, Staphylococcus, Virgibacillus* (Cai et al. 2014, Doley and Barthakur 2017, Ismail et al. 2013, Kauppi et al. 2011, Ubani et al. 2016).

Since diesel is a complex hydrocarbon mixture, the susceptibility of hydrocarbons to microbial degradation is linear alkanes > branched alkanes > small aromatics > cyclic alkanes > PAH's (Das and Chandran 2011), given that *Bacillus pumilus* M1HC1b showed degradations of the constituents of diesel up to 100% in the treatment of 4 000 mg L^{-1} (Table 5, Figure 2). Generally, there are two factors that are effective in the biodegradation of *n*-alkanes: i) activation of metabolic enzymes, and ii) transportation of alkanes into the bacterial cell (Ghafari et al. 2019). Members of the *Bacillus* genus have been reported in diverse oil hydrocarbon contaminated environments (Mukherjee and Bordoloi 2012, Ramasamy et al. 2017). However, the loss in the biodiversity and microbial activity in diesel contaminated soil could be a result of the high concentration of diesel oil, due to its highly toxic PAHs components (approximately 25–30%) what affects microbial cell membranes (Ujowundu et al. 2011), cell viability and, consequently, the biodegradation capacity (Cisneros-de la Cueva 2014).

Paenibacillus strains isolated from the study site corroborated previous reports on the presence of this genus in different hydrocarbon-contaminated habitats ranging from petroleum contaminated sediment, plant rhizosphere (*Distichlis spicata*, *Juncus gerardi*, *Spartina altemiflora*, *Spartina airoides*), oil well and soil (Daane et al. 2001, Najafi et al. 2011). *Paenibacillus* sp. have been reported as efficient hydrocarbon-mineralizing organisms, which could utilize PAHs (as anthrone, biphenyl, naphthalene or phenanthrene) as the sole carbon source, and could also degrade different polychlorinated biphenyl, diesel, and crude oil (AL-Saleh and Obuekwe 2014, Daane et al. 2001, Ganesh and Lin 2009, Sakai et al. 2005).

Conclusions

In the present investigation, we studied bacterial strains with diesel degradation capacities that exist naturally in a contaminated soil environment. We show that both *Bacillus* and *Paenibacillus* strains isolated from hydrocarbon--contaminated agricultural soils degraded diesel, however, the concentration decreased by 100% and 71.72%, respectively, at 4 000 mg L^{-1}. In addition, *Bacillus pumilus* M1HC1b had a greater diesel degradation capacity (54.45% at 24 000 mg L^{-1}). Furthermore, we show a correlation between diesel degradation capacity and bacterial growth rate.

Acknowledgements

This work was partially supported by the Catedra-CONACYT program, SEP-PROMEP and Network: Microbial models of agrobiotechnological importance by SEP. Finally, we express our gratitude to James L. Fidelholts and Montes-Miró, R.G. for support in the English language version.

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