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Diesel degradation by emulsifying bacteria isolated from soils polluted with weathered petroleum hydrocarbons

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ABSTRACT

This study evaluated bacterial populations (total, lipolytic, P-solubilizers, N-fixing free, and hydrocarbonoclastic bacteria) from uncontaminated soils or soils contaminated with weathered petroleum hydrocarbons (WPH) and evaluated the emulsifying and diesel-degrading properties for each bacterial strain. Soils collected from Tabasco State (Mexico) included moderately contaminated (50,000 mg kg^{-1}), highly contaminated (150,000 mg kg^{-1}), or non-contaminated samples. Bacterial populations showed significant differences ($P < 0.05$) among the three soils, with highly contaminated soil containing the highest populations of lipolytic and phosphate (P) solubilizing bacteria. Thirteen bacterial strains were isolated from non-contaminated and highly contaminated soils. These bacteria were able to degrade diesel and to produce surfactants, as determined by four methods [emulsification index ($E_{24\%}$), cell surface hydrophobicity, drop collapse, and hydrocarbon displacement]. Bacteria with high emulsification activity were molecularly identified (16S rRNA) as *Serratia marcescens* C11S1, *S. marcescens* C7S3A, *Citrobacter freundii* CCC4DS3, *Raoultella ornithinolytica* C5S3, *Stenotrophomonas maltophilia* CCC10S1, and *St. pavanii* C5S3FN. The strain C7S3A showed the highest diesel emulsification (74.2%) and high cell hydrophobicity (173%) and diesel degradation (96%). A consortium containing the six bacterial strains also showed high diesel degradation (97%). The biochemical tests demonstrated that the bacterial strains were also able to produce indole acetic acid. Our results suggest that the emulsifying bacteria may useful for bioremediating soils chronically contaminated with weathered petroleum hydrocarbons.

1. Introduction

In Mexico, there are large surface areas contaminated with petroleum hydrocarbons, mainly due to accidental spills or leaks from underground deposits (Trindade et al., 2005; Chandankere et al., 2013, 2014). This has caused significant negative impacts and hazards for agroecosystems and human health (Basumatary et al., 2012; Mair et al., 2013; Masciandaro et al., 2013; Ojeda-Morales et al., 2013; Baldan et al., 2015).

Bioremediation is superior over physical and chemical methods of remediating contaminated soils (Jorfi et al., 2013; Lin et al., 2014; Souza et al., 2014). Thus, the inoculation of allochthonous microorganisms (bioaugmentation) with high capabilities to degrade hydrocarbons is one of the most important practices for bioremediating petroleum contaminated soils (Bento et al., 2005; Nikolopoulou and Kalogerakis, 2008; Hassanshahian et al., 2012; Lin et al., 2014; Quiao et al., 2014; Fodelianakis et al., 2015).

Biodegradation of petroleum hydrocarbons is complex and generally requires different microbial species or consortia with specific enzymatic capabilities that accelerate the rate of petroleum degradation

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(Mukherjee and Das, 2005; Mukherjee and Bordoloi, 2011; Aleer et al., 2014; Hassanshahian et al., 2014; Souza et al., 2014). Nevertheless, the efficacy of bioremediation through microbial action is generally limited by the low availability and solubility of petroleum hydrocarbons due to their hydrophobicity and adsorption into soil particles and to their weathering (Chia-Wei et al., 2013; Chandankere et al., 2014; Bezza et al., 2015). Weathered petroleum is characterized as being a solid material that shows no degree of fluidity at normal air temperature and is recalcitrant to microbial degradation (Gao, 2009; Tang et al., 2012; Riveroll-Larios et al., 2015).

Some soil bacteria are able to produce surfactants that favor hydrocarbon emulsification (Campos et al., 2014; Rao et al., 2014); thus, the adhesion of microbial cells to hydrophobic substrates allows them to be used as both co-substrate and carbon source to satisfy the microbial metabolism (Hassanshahian et al., 2012; Chandankere et al., 2014; Onur et al., 2015). Biosurfactants are part of a group of molecules and secondary metabolites with active surfaces that possess one hydrophobic part and one hydrophilic part, giving them the ability to act on the interface of different hydrophobic compounds (Nalini and Parthasarathi, 2013; Rao et al., 2014). Bacteria such as those in the *Acinetobacter* genus may produce substances with biosurfactant characteristics that degrade several hydrocarbons, including diesel (Gallego et al., 2001). Biosurfactants have several advantages over chemical surfactants, such as improved emulsification, biodegradability, and environmental compatibility, along with low toxicity. They can even continue their activity under extreme temperature, pH, and salinity conditions (Reddy et al., 2010; Hassanshahian et al., 2012; Souza et al., 2014; Chandankere et al., 2014).

There are few studies on the use of biosurfactant-producing bacteria for the recovery of chronically contaminated soils, such as those in western Tabasco (Mexico). Here, most petrochemical facilities are antiquated (> 50 years old), making them a risk for hydrocarbon spills due to rusted pipelines (Adams et al., 1999; Rivera-Cruz and Trujillo-Narcia, 2004). The use of emulsifying bacteria with potential physiological activities on plant growth in soils affected by weathered petroleum hydrocarbons may significantly contribute to mid- or long-term hydrocarbon degradation. Thus, the objective of this research was to isolate and select bacteria with emulsifying capabilities from soils chronically contaminated with weathered petroleum hydrocarbons in Tabasco State and to evaluate their capacity for degrading diesel.

2. Materials and methods

2.1. Sampling site

Two soils (Gleysol) contaminated with weathered petroleum were selected: A) 50,000 mg kg^{-1} (moderately contaminated) and B) 150,000 mg kg^{-1} (highly contaminated). These soils have been exposed for more than 50 years to chronic contamination due to accidental crude oil spills (Rodríguez-Rodríguez et al., 2016). Both soils were collected from the Jose Narciso Rovirosa ejido in the southern area of the La Venta Gas Processing Complex in Tabasco State (18° 4′39.83″ N, 94° 2′39.42″W and 18° 4′39.22″ N, 94° 2′38.46″ W); the distance between the sites was 35 m. A control soil (Gleysol) with no prior contamination impact (with 150 mg kg^{-1} biogenic hydrocarbons) was also collected from the Blasillo Huimanguillo ejido, Tabasco State (18° 3′9.35″ N, 93° 55′59.34″ W). The control site was located 12,000 m away from the contaminated sites. Surface soil samples (0–30 cm; 1 kg each) were collected from five random points in each site during January (2015, windy season) as indicated by the NOM-021-RECNAT-2000 (DOF, 2003). The samples were combined, dried, sieved (2 mm mesh), and used for analyzing microbiological profiles, soil properties, and petroleum hydrocarbon content in the laboratory.

The Koppeńs climate classification for the sampling locations is Afm, with an average annual rainfall of 2200 mm, 1200 mm of evaporation and a mean annual temperature of 26 °C (INEGI, 2001). Sampling sites are located in a coastal plain dominated by wetlands mainly used for foraging, with gleyed soils flooded up to 0.9 m depth. The vegetation consists of a meadow with grasses and rooted aquatic weeds. Along this land, there are underground pipelines (1 m depth) of 4, 6, and 24 inches in diameter; also, there are other pipelines transporting crude oil and natural gas to the Gas Petrochemical Complex of La Venta, Tabasco. In addition, there is also a capped oil well and a waste management dam (Rivera-Cruz et al., 2016). The soil pH varied from strongly to slightly acidic (5–5.5 for contaminated soils and 6.5 for control soil), and samples had a high total content of soil organic matter (> 8%), nitrogen (0.4–0.6%) and phosphorus (7–9 mg kg^{-1}). Cation exchange capacity was high, oscillating from 30 to 62 cmol (+) kg^{-1}, based on the NOM-SEMARNAT-2000 (DOF, 2002).

2.2. Microbiological analysis

The cultivable populations of total bacteria (TB), lipolytic bacteria (Blipol), phosphate solubilizing bacteria (PSB), nitrogen-fixing free-living bacteria (NFFLB), and NFFLB-hydrocarbonoclastic bacteria were evaluated. These bacterial groups were considered for their potential benefits to nutrient recycling and plant growth and for their contribution to hydrocarbon degradation.

The quantification of cultivable bacteria was performed by the dilution and plate counting technique (Ingraham and Ingraham, 1998), using specific culture media for TB (Baker® nutritive agar), PSB (Pikovskaya, 1948), and Blipol (Sierra, 1957). The NFFLB were quantified using the cultured medium proposed by Rennie (1981). In addition, this Renniés culture medium was supplemented with 300 µL diesel to evaluate the NFFLB-hydrocarbonoclastic bacteria (Ferrera-Cerrato et al., 2007). The plates of culture media for TB and NFFLB were incubated at 28 °C for 48 h, and those for Blipol and PSB were incubated at 28 °C for 72 h. The results were expressed in colony forming units per gram of dry soil (CFU g^{-1}).

2.3. Selection of emulsifying strains

The selection of the emulsifying bacteria was done using diesel as a source of hydrocarbons, as determined by the following tests. The emulsification index ($E_{24\%}$) was determined using protocols described by Cooper and Goldenberg (1987), in which 2 mL of diesel was added to a 4 mL bacterial culture propagated in Luria-Bertani medium (g L^{-1}: 10 Tryptone, 5 yeast extract, 5 NaCl, and 1 L-Tryptophan). The mixture was shaken in a vortex for 2 min, followed by a 24 h incubation at room temperature. The bacterial emulsifying effect was compared against a chemical surfactant (culture medium plus Tween 80, 2:1 v/v) as is standard. The $E_{24\%}$ was calculated with the following equation:

$$E_{24\%} = \frac{Height\ of\ the\ emulsified\ layer\ (mm)}{Total\ height\ of\ the\ liquid\ coloumn\ (mm)} \times 100$$

Cell surface hydrophobicity percentage (CSH%) was determined using the MATH (Microbial Adhesion Test Hydrocarbons) water-hydrocarbon biphasic method described by Rosenberg et al. (1980). The bacterial strains were propagated in the previously described Luria-Bertani medium. Bacterial samples were taken at the exponential phase (72 h) through three washings with the PUM-buffer solution [Phosphorus-Urea-Magnesium: (g L^{-1}): 19.7 K_2HPO_4, 7.26 KH_2PO_4, 1.8 $MgSO_4 \cdot 7H_2O$, pH 7.1] for 5 min at 8000 *g*. The cell population was adjusted to an optical density of 0.5 (A_{600}) in phosphate buffer solution. Two milliliters of the corresponding bacterial suspension (900–1200 × 10^6 cells mL^{-1}) were transferred to dilution tubes with 2 mL of diesel, shaken vigorously for 2 min in a vortex, and rested for 15 min to allow for the separation of phases. The optical density (A_{600}) was measured again in the aqueous phase. The CHS% was expressed as the diesel addition percentage through the following equation: CSH% = [1 − (A_{600} Final/A_{600} Initial)] * 100 (Blanco et al., 2010).