Plant growth promoting characterization of soil bacteria isolated from petroleum contaminated soil

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Abstract— *Contaminant-degrading bacteria can be included among the plant-growth promoting bacteria; because the presence of contaminants, in general produce negatively effects on plant's growth; thus, the elimination of the inhibiting contaminants will benefit them. Although contaminant-degrading strains have been traditionally isolated from various environments; the number of studies that reported the isolation and identification of soil bacteria with contaminant-degrading abilities have increased. The aim of this study was to characterized microbial strains isolated from petroleum contaminated soil by plant growth promotion traits to recommend them as potential bioinoculants. In this work, five of the six soil isolates were classified as Indole Acetic Acid higher producers and only one of them as lower producer. Sporosarcina aquimarina strain -Q3 and Bacillus cereus strain +F2 tested in Axonopus affinis plantlets bioassay, showed that these isolates were the most effective promoters of this plant species; therefore, these soil bacteria with possible hydrocarbon degradation ability could be considered as potential bioinoculants and can be recommended with a practical importance for the rhizoremediation of petroleum contaminated sites and plant growth promotion.*

Keywords— Soil bacteria, Hydrocarbons, Indole acetic acid, Plant growth promoters.

I. INTRODUCTION

Even petroleum is one of the main components of modern industrial society; an increasing number of sites are seriously contaminated by hydrocarbons (HCs) [1, 2, 3, 4]. It has been reported that the use of plants in conjunction with HC degrading and/or plant growth-promoting bacteria (PGPB) offers much more potential for the remediation of HC contaminated soils [5, 6, 7, 8]. There are knowing bacteria that possess HC-degradation pathways and metabolic activities not only improve plant tolerance to HC pollutants by degrading these organic compounds [9, 10, 11], additionally if they are PGPB, they could mitigate plant stress and also enhance plant growth and development [12, 13, 14, 15]. Some authors [5, 12, 13, 16, 17, 18, 19, 20] mentioned that rhizobacteria (RB) and endophytic bacteria (EB), contributed to biodegradation of toxic organic compounds in polluted soil and could have potential for improving phytoremediation. Particularly, the RB colonize the root environment and participate in the degradation of HCs [6, 20, 21, 22, 23]. Vilchez and Manzanera [24] mention that the presence and characterization of beneficial PGPB in petroleum contaminated soils is limited; even there have been reports about adequate plant species to enhance the rhizoremediation of this kind of organic contaminants [25, 26, 27]. The aim of this study was to characterized microbial strains isolated from petroleum contaminated soil by plant growth promotion traits to recommend them as potential bioinoculants.

II. MATERIAL AND METHOD

2.1 Isolation of soil bacteria from petroleum contaminated soils

Several rhizobacteria were isolated from contaminated soils from an abandoned refinery; four composite sampled soils were collected from two sites according to contamination grade (light hydrocarbon fraction (LHF: C5–C10) 90ppm, middle hydrocarbon fraction (MHF: C10–C28, 100ppm) and heavy hydrocarbon fraction (HHF: C28–C40, 450ppm), divided in two: Sample "A" and Sample "B". The bacteria were isolated according to Melo et al., [28], placing 1g of each contaminated soil samples, in a 50mL Erlenmeyer flasks containing five of 0.1cm diameter glass beads and 10 mL of sterile phosphate saline buffer (1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.20 g KCl, 8 g NaCl / L, pH= 7.4) and shaking the flasks for 30 minutes at room temperature. After agitation, 0.1 mL of appropriate soil extracts of each flask were placed onto agar Luria-Bertani (LB), separately supplemented with phenanthrene (LB+F, 100mg/10mL), phenanthrol (LB+OH, 100mg/10mL) and phenanthrenequinone (LB+Q, 100mg/10mL), to isolate specific bacteria employing these carbon sources. Plates were incubated at 28ºC for 48h and the isolated soil bacteria strains were maintained and preserved on LB medium plates for the conventional bacterial analyses and identified by the determination of gene 16S rRNA sequences. Colony PCR was

performed from live cell cultured on agar LB medium plates. Cells were harvested after 24h and processed for DNA isolation using the Allers and Linchen [29] procedure. Using the purified genomic DNA, the molecular target gene 16S rRNA was amplified using universal primer set fD1 and rD1 designed by Weisburg et al., [30]. Aliquots of PCR reaction products were electrophoresed in 1% agarose gel and then stained with ethidium bromide. These PCR products were purified and sequenced by the Unidad de Biotecnología y Prototipos of FES-Iztacala (UNAM). The sequences were then compared to similar sequences in the databases using BLAST analysis (Basic Logical Alignment Search Tool, BLAST at NCBI).

2.2 Evaluation of the IAA production of the isolated soil bacteria

The isolated soil bacteria, were analyzed by their Indole Acetic Acid (IAA) production [31, 32] using the Salkowski reagent according to the method of Melo et al., [28]. Auxin production by the soil bacteria strains was analyzed in the presence and absence of L-Tryptophan and determined by colorimetry. The assays were done taking 4.9 mL of sterile LB liquid media, added to culture tubes (10 x 15cm) and supplemented with L-Trp at final concentrations of 1, 2 and 5 mg/L. The culture tubes were inoculated with 0.1 mL of each soil bacteria inoculum (5 x 10^7 cells/mL) in sterile distilled water. The culture tubes were incubated at 28ºC for 120h. After the incubation, the cultures were centrifuged at 3,500 rpm, at 25ºC for 45 minutes to discard the bacteria pellets and to recover the supernatant where the auxins were excreted; 2 mL of each supernatants were mixed with 2 mL of Salkowski's coloring reagent and the development of a pink color indicates IAA production, quantified reading its absorbance at 535 nm and the concentration was estimated by a standard IAA curve. The assays with and without L-Trp were performed by triplicate.

2.3 Plant growth promoting bioassay of *Axonopus affinis* plantlets inoculated with the isolated soil bacteria

Commercially obtained certified seeds of *A. affinis* (Chase) were surface-sterilized with 10% sodium hypochlorite, rinsed with sterile distilled water. These seeds were bacterized with each selected soil bacteria as inoculum, submerging them for 30 minutes; this suspension inoculum was prepared from cell cultures on Agar-LB medium plates, an inoculum was taken with sterile calibrated inoculation loop (1/100cells) and resuspended in nephelometric flasks containing 25mL of BHI liquid medium, the bacteria inoculum was adjusted to 7 x 10^8 cells/mL and the flasks were shaken at room temperature for 24h, after this time, the inoculum was finally adjusted to 18 x 10^8 cells/mL and employed to imbibed *A. affinis* seeds. Control seeds were imbibed in sterile distilled water also for 30 minutes at room temperature. Finally, bacterized seeds and control seeds were deposited on sterile plastic pots of 50mL capacity (5 seeds/pot) filled with 28g of sterile vermiculite. Pots were wet with 15 mL of mineral solution according to Labra-Cardón et al., [33]; all the experiments were performed by quintuplicate and plantlets of *A. affinis* were cultured and maintained at 28°C in a growth chamber with a 12:12 photoperiod for 10 days.

2.4 Plant Growth Index of *A. affinis* plantlets inoculated with the selected soil bacteria

Plantlets of *A. affinis* were collected at the end of the bioassays, weighted as fresh biomass and then dried at 70°C for 24h to obtain their dry weight. Plant Growth Index (PGI, %) was calculated based in dry weight expressed in grams, by the formula: PGI = Pin / Pcont x 100; where, Pin: is the mean dry biomass of plantlets inoculated with each selected soil bacteria and Pcont: is the mean dry biomass of control plantlets (not inoculated).

2.5 Statistical analysis

All data obtained were analysed by ANOVA test, and Tukey-Kramer Method using the statistics program Graph Pad Instat Ver. 2.03.

III. RESULTS AND DISCUSSION

3.1 Characteristics of the soil bacteria isolates

There were isolated six selected soil bacteria from the two samples of petroleum contaminated soil, with different colonial morphology (Table 1). Five of them were isolated from soil sample "A"; two isolated in LB+Q (–Q1 and –Q3 strains), two isolated in LB+F (-F6(2-1) and –F6(2-2) strains) and one isolated in LB+OH (-OH4 strain). There was only one strain isolated from soil sample "B" in LB+F medium (+F2 strain). Gram behavior of the isolates was diverse; two isolates were Gram positive Cocci and the rest of the isolates were 50% Gram positive Bacilli and 50% Gram negative Bacilli, all the isolated bacteria were identified based on its DNA sequence homology analysis (99%), as follows: *Sporosarcina aquimarina* strain -Q3, *Staphylococcus* sp. strain –F6 (2-2), *Achromobacter* sp. strain –Q1, *Peanibacillus* sp. strain –OH4, *Staphylococcus sciuri* strain –F6 (2-1) and *Bacillus cereus* strain +F2.

TABLE 1
COLONY AND MICROSCOPY MORPHOLOGICAL CHARACTERISTICS OF THE SIX ISOLATED SOIL BACTERIA FROM PETROLEUM CONTAMINATED SOIL

Soil bacteria	Soil Sample	Form	Size	Color	Edge	Surface	Aspect	Consistence	Elevation	Reflect light	Gram behavior
-F-6(2-1)	A	Round	0.3 cm	White	Undulate	Smooth	Wet	Butyrous	Flat	Brilliant	Cocci Gram +
-F-6(2-2)	A	Rhizoid	0.4 cm	Colorless	Filamentous	Smooth	Wet	Butyrous	Flat	Brilliant	Cocci Gram +
-OH4	A	Round	0.5 cm	White	Lobate	Smooth	Wet	Butyrous	Convex	Brilliant	Bacilli Gram +
-Q1	A	Round	0.2 cm	White	Complete	Smooth	Wet	Butyrous	Convex	Brilliant	Bacilli Gram -
-Q3	A	Round	0.3 cm	Pink	Complete	Smooth	Wet	Butyrous	Convex	Brilliant	Bacilli Gram -
+F2	B	Irregular	0.7 cm	White	Undulate	Smooth	Dry	Dry	Flat	Mate	Bacilli Gram +

Salinas et al., [34] noted a list of the principal genera of hydrocarbon degradators: *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Nocardia*, *Pseudomonas*, *Micrococcus* and *Sphingomonas*. The colonial and microscopically morphology characteristics of the isolated soil bacteria were similar to those characteristics that the mentioned genera possess. It may suggest that the isolated strains belong to genera that are related to petroleum hydrocarbons degradation.

3.2 Characterization of the indol acetic acid producers

The results of the physiological plant growth promoting trait, the IAA production, are present in Fig.1; the six isolated soil bacteria were screened for their ability to produce plant growth regulator the IAA, recording with different concentrations of tryptophan (0, 1, 2 and 5 mg/L) that induce or not the IAA concentration. The IAA production of the isolates without tryptophan was 10 to 46 µg/mL. According to Khalid et al., [35] categorization of *in vitro* production of IAA by rhizobacteria in three principal groups (lower producers: 1 to 10 µg/mL IAA, medium producers: 11 to 20 µg/mL IAA and higher producers: 21 to 30 µg/mL IAA); the isolated soil bacteria were classified as follows: *Bacillus cereus* strain +F2 as lower producer (9.9µg/mL) and the rest of the isolates were higher producers: *Staphylococcus sciuri* strain –F6(2-1), 28.2µg/mL, *Peanibacillus* sp. strain –OH4, 39.1µg/mL, *Achromobacter* sp. strain –Q1, 39.2µg/mL, *Staphylococcus* sp. strain –F6(2-2) 43.3µg/mL and *Sporosarcina aquimarina* strain -Q3, 46.3µg/mL.

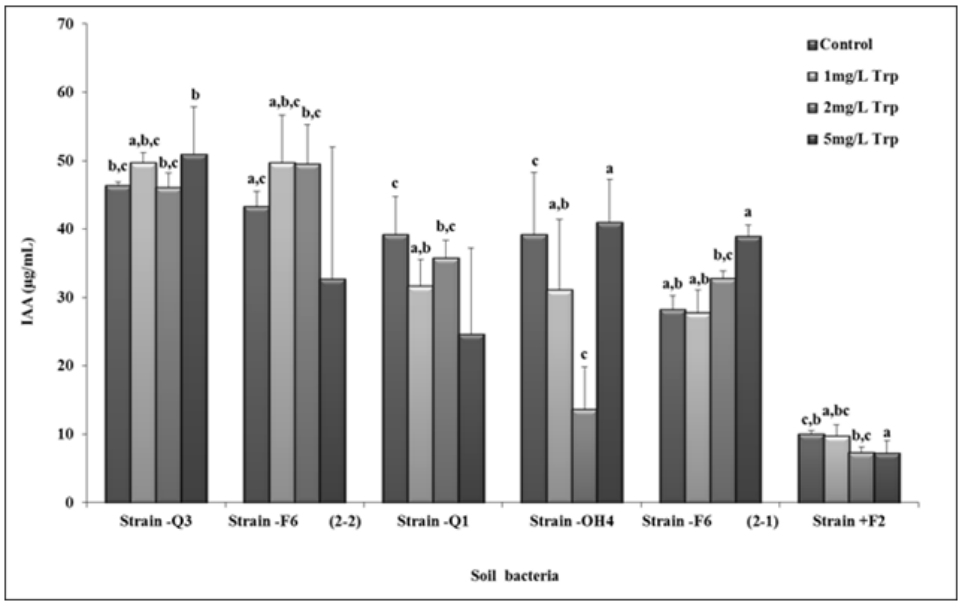

FIG.1. INDOLE ACETIC ACID PRODUCTION BY THE SIX ISOLATED SOIL BACTERIA WITH AND WITHOUT TRYPTOPHAN. MEAN VALUES + S.D. FROM THREE REPLICATES FOR IAA PRODUCTION. THE DIFFERENT LOWER-CASE LETTERS SHOWS THE SIGNIFICANT DIFFERENCES BETWEEN EXPERIMENTS (a: p< 0.05, b: p< 0.01 and c: p< 0.001).

Ahmad et al., [36, 37] mentioned that there are numerous soil bacteria involved in culture and soil auxins biosynthesis [38]; some of them produce these phytohormones in the presence of their precursor the amino acid L-Tryptophan. The IAA

production of the isolated soil bacteria with the presence of Trp: *Sporosarcina aquimarina* strain -Q3 and *Bacillus cereus* strain +F2, do not showed an increase of IAA concentration with the addition of the amino acid to the medium; strains *Staphylococcus* sp. strain –F6(2-2) and *Achromobacter* sp. strain –Q1 showed that the IAA concentration decrease as the Trp concentration increased, only *Staphylococcus sciuri* strain –F6(2-1) showed an increase of IAA production as Trp concentration increased: 27.7, 32.7 and 38.8µg/mL with 1, 2 and 5mg/L Trp, respectively. The soil bacteria *Peanibacillus* sp. strain –OH4 showed a decrease in IAA production with 1 and 2mg/L Trp (31.3 and 13.6µg/mL, respectively) and the addition of 5mg/L Trp increased the IAA production (40.9µg/mL).

Interactions among microbes and plants have received great consideration because of the possible role of microbes on plant growth promotion and degradation of HCs in contaminated soil [12, 39, 40]. Recently, Cowie et al., [41] reported that PGPB enhanced HCs removal from the soil, mainly due to improving plant growth, and bacterial population and activities. Similarly HC-degrading bacteria enhanced plant biomass production and HC degradation [8, 15, 42]. Ho et al., [43]; Gurska et al., [44] and Afzal et al., [15] mentioned that the synergistic action between plants and inoculated bacteria, allows HCs rhizodegradation, efficiently, comparing it to solely microbial remediation or phytoremediation. Glick [45] recommended the use of bacteria having both pollutants degrading and plant growth promoting activities; because they are superior to only bacteria that have only one of these activities. Rojas et al., [46] have reported that some rhizobacteria possess a great IAA production (around 35.5µg/mL) and are recommended as an excellent plant growth promoters; in this study all of the isolated soil bacteria could be a suitable candidates for IAA producers, and some of the isolated genera founded in this work have been reported by other authors as plant growth promoters as *Bacillus* and *Peanibacillus* [47].

3.3 Growth promotion of *Axonopus affinis* plantlets

Five of the isolated soil bacteria tested as plant growth promoters showed an *A. affinis* plantlets promotion (Fig. 2); and only *Peanibacillus* sp. strain –OH4 a decrease in it. PGI of *A. affinis* plantlets from inoculated seeds showed that these grown more than control plantlets, as follows: inoculated with *Staphylococcus* sp. strain –F6(2-2) 2%, *Staphylococcus sciuri* strain –F6(2-1) 4%, *Achromobacter* sp. strain –Q1 23%, *Sporosarcina aquimarina* strain -Q3 51% and *Bacillus cereus* strain +F2 76%.

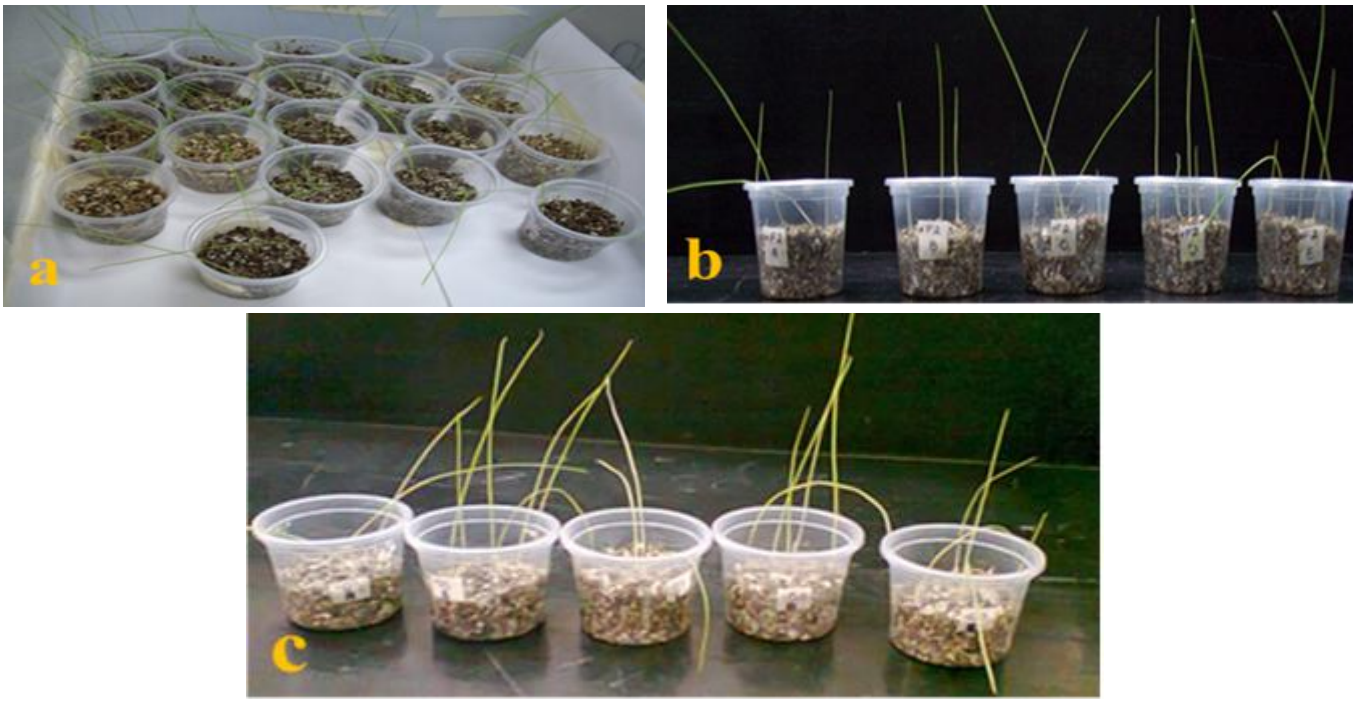

FIG. 2. *AXONOPUS AFFINIS* PLANTLETS BIOASSAY. A) GENERAL VIEW, B) INOCULATED WITH *BACILLUS CEREUS* STRAIN +F2 AND C) INOCULATED WITH *SPOROSARCINA AQUIMARINA* STRAIN -Q3.

In this study, *Bacillus cereus* strain +F2 was the only isolate considered as IAA lower producer; although it, this isolate was the best plant growth promoter followed by the most IAA higher producer: *Sporosarcina aquimarina* strain -Q3. *A. affinis* plantlets may have more affinity to *B. cereus* strain +F2 and culture conditions of plants also favored the IAA production by this bacterium and been adequate to promote the plantlets' response. Dry biomass of *A. affinis* plantlets (Fig. 3) showed that

there was an evident increase of growth in plantlets inoculated with three of the isolated soil bacteria; two of them with IAA higher production and one isolate with the lowest IAA production.

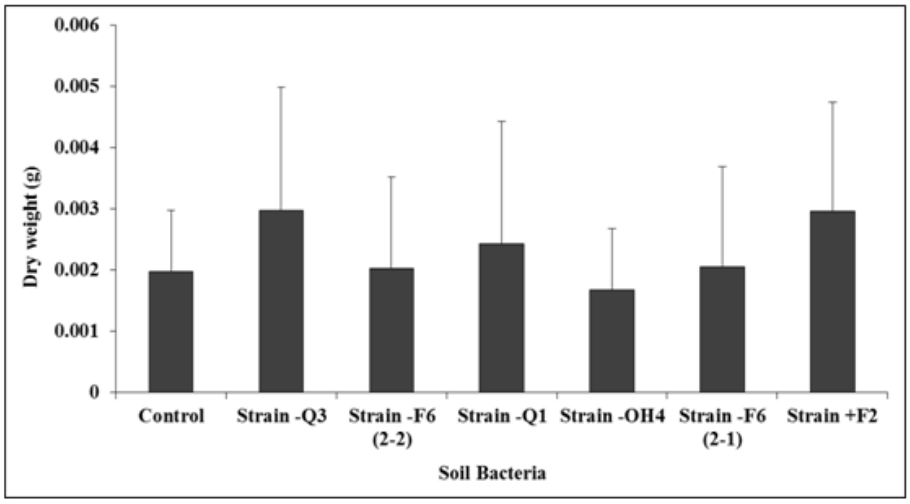

FIG. 3. *AXONOPUS AFFINIS* PLANTLETS BIOMASS. DRY BIOMASS OF CONTROL AND INOCULATED WITH THE SIX ISOLATED SOIL BACTERIA PLANTLETS. MEAN VALUES ±S.D. FROM 25 REPLICATES FOR IAA PRODUCTION. NO SIGNIFICANT DIFFERENCES WERE FOUND BETWEEN EXPERIMENTS ($p < 0.05$).

It is important to note as Khan et al. [20] mention, that the association between plant and the inoculated bacteria plays an important role not only in the rhizosphere colonization, it also depends on their associated bacteria to make an efficient degradation of contaminants as some authors noted [6, 8, 48]. Rylo et al., [49] mention that *Sporosarcina aquimarina* strains solubilize phosphate and are N_2 fixing bacteria; and also were reported as IAA and siderophores producers.

IV. CONCLUSION

In this work, the six isolated bacteria from two contaminated soil samples with petroleum hydrocarbons were characterized by one of the principal plant growth promoting traits, the Indole Acetic Acid production; five of the six isolates were classified as IAA higher producers and only one of them as lower producer. Four of them were affected in the IAA production by the presence and concentration of tryptophan and only two of them were not affected by the amino acid at any concentration. *Sporosarcina aquimarina* strain -Q3 and *Bacillus cereus* strain +F2 tested in *A. affinis* plantlets bioassay to analyze their activity as plant growth promoters, showed that these isolates were the most effective promoters of this plant species; even these strains have the highest and the lowest IAA production, respectively. Therefore, these soil bacteria with possible hydrocarbon degradation ability could be considered as potential bioinoculants and can be recommended with a practical importance for the rhizoremediation of petroleum contaminated sites and plant growth promotion.

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