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Isolation, characterization and identification of hydrocarbonoclastic *Pseudomonas* species inhabiting the rhizosphere of *Crotalaria micans* Link

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ABSTRACT

The activities of the oil industry have caused hydrocarbon pollution in many areas, whose biological removal (bioremediation), categorized as ecological and cheap use microorganism's metabolism. The genus Pseudomonas is a heterogeneous and environmentally significant group in the rhizosphere of plants, related to bioremediation process. Thus, this research includes the isolation, characterization and identification of the Pseudomonas species existing in the rhizosphere of a legume present in a savanna soil polluted by an oil spill from the pipeline Caripito-Puerto La Cruz, 100 meters from the entrance of the Amana del Tamarindo village, Monagas state. The legume was identified with the register of the Herbarium UOJ (UDO, Maturín), keys, and the TROPICOS database. Pseudomonas species were isolated in Bushnell-Haas liquid medium + 1% (w/v) of crude oil. The dilutions (5%, v/v) were streaked on plates with Pseudomonas agar base supplemented with Cetrimide (10g), Fucidin (10g) and Cephalosporin (50gL^{-1}) from which colonies were purified. The phenotypic characteristics, Gram stain test, catalase and cytochrome oxidase enzymes activities, pyocyanin and fluorescein pigments production, RaPIDTm NF Plus and API 20 NE tests and MicroScan AutoScan4 identified P. fluorescens, P. putida and P. aeruginosa in the rhizosphere of Crotalaria micans. These species could sustain this plant growth through reduction of the toxic effects of spilled oil. In addition, this study could be a step towards the development of a bioremediation strategy.

Key words: *Pseudomonas*, *Crotalaria micans*, savanna, Venezuela.

INTRODUCTION

The oil industry is the main source of income for Venezuela, whose activities involving handling and extraction have resulted in severe contamination of soil, water and air; some of these environments with agricultural vocation, livestock and fisheries, even more it has been damaged drinking water sources, with direct threat to public health [6, 12, 54].

Petroleum is a mixture of aliphatic hydrocarbons, aromatic hydrocarbons, resins and asphaltenes, which cause huge disorders of ecosystems biotic and abiotic components, some of them being carcinogenics and neurotoxins [22, 43, 48].

A variety of technologies are currently available to treat the soil and water contaminated with hazardous materials, including excavation and containment in secured landfills, extraction, stabilization and solidification of vapor, soil washing, oil washing, solvent extraction, thermal desorption, vitrification and incineration [50]. In addition to the physical and chemical alternatives, there are biological methods (Bioremediation), which are considered ecological and cheaper options [26, 51]. Bioremediation is a means of cleaning contaminated environments throughout the microorganism's metabolic abilities to turn contaminants into harmless products by mineralization and carbon dioxide and water generation, or by conversion into microbial biomass. This is the main mechanism for the removal of hydrocarbons by microorganism's natural populations in contaminated sites [28, 36, 45].

Bacteria that have the ability to degrade hydrocarbons compounds are known as hydrocarbonoclastics bacteria [11], which naturally can emulsify, transport and degrade hydrocarbons. Hydrocarbonoclastics bacteria degrade hydrocarbons by suppressing the hydrocarbon chain to make it shorter, mechanism where operate specific enzymes encoded by bacterial plasmid [2, 4].

The association between the contaminated area plants and microorganisms is the key factor in the bioremediation process. The roots increase the diversity and number of microorganisms in the rhizosphere by providing carbon and nitrogen through radical exudates (sugars, organic acids and other high molecular weight organic compounds); this rhizosphere effect can be exploited and used in bioremediation; process specifically defined as Rhizoremediation [23, 31].

The genus *Pseudomonas* is the most heterogeneous and environmentally significant known bacterial group and includes mobile gram-negative aerobic rods, extended in all nature and characterized by its high metabolic versatility given by a complex enzymatic system. In addition, *Pseudomonas* species tend to be predominant among the bacteria associated with plants rhizosphere. Jaharamma *et al*. [20]; Opasola *et al*. [35]; Tripathy *et al*. [47] and Uğur *et al*. [49] found several species of this genus in some species of leguminous plants, which exhibited biocontrol and bioremediation activities.

In some species of *Pseudomonas*, able to degrade hydrocarbons, the production of surfactants is considered as the first stage of the hydrocarbonoclastic process, these compounds have been described as the glycolipic nature, among them the rhamnolipids. They have been recognized in P. *aeruginosa* [6, 38], *P. putida* [10, 25], *P. fluorescens* [34, 39] and *P. stutzeri* [21, 46].

Leguminous plants have been reported as effective in the rhizoremediation process, probably for its ability to increase soil nitrogen, with a high C:N ratio; on this basis these plants are used for revegetation of contaminated sites [31, 13]. The use of the association savanna native legumes-*Pseudomonas* spp., for the rhizoremediation of contaminated savanna soils of Monagas state could be the great value in the recovery of these areas. In this context the objective of the present study includes the isolation, characterization and identification of *Pseudomonas* species existing in the rhizosphere of a legume present (colonizing or survivor) in a savanna soil contaminated with oil spill occurred in the year 2011, with a view to explain how these bacterial species naturally support the growth of this leguminous plant through the reduction of the toxicity of spilled crude oil (hydrocarbonoclastic effects); at the same time it would support the recommendation of revegetation of the contaminated area with this species.

MATERIALS AND METHODS

Soil sampling

It was sampled an area of 50 m^2 of a contaminated site (9°38'52" N, 63°7'20" E, 46m asl) by an spill of crude oil from the pipeline (16 inch) Caripito-Puerto La Cruz, at 100 m from the entrance of Amana del Tamarindo village, Monagas State, Venezuela (Figures 1A and B).

Identification of the collected leguminous plant

Specimens with flowers and fruits of a leguminous plant present in the contaminated area were pressed by traditional methods for subsequent identification. For identification it was used the registries of the UOJ Herbarium (UDO, Maturín, Monagas state), as well as the taxonomic revision of the genus *Crotalaria* by Avendaño [5]. The scientific name was updated according to the TROPICOS database of the Missouri Botanical Garden. The exsiccates were deposited in the herbarium UOJ .

Isolation, characterization and identification of hydrocarbonoclastics species of *Pseudomonas*

A soil area of 20 cm^3/plant around the roots was used to isolate the *Pseudomonas* species associated with the rhizosphere of the legume collected. Roots were transferred to the laboratory in a sterile plastic bag under refrigeration on ice. It was obtained soil rhizosphere by light shaking of the root, from which, once mixed in aseptic conditions were taken 5g, this sample was suspended in 100 ml of liquid medium of Bushnell-Haas: HB [9] with 1% (w/v) of crude oil as sole source of carbon, to favor the multiplication of microorganisms hydrocarbons degrading. The samples were incubated at room temperature on an orbital shaker at 180 rpm for 7 days. Thereafter, they were diluted in liquid medium HB (5%, v/v) with crude oil (1% w/v) to get five replicates, each one was streaked on *Pseudomonas* agar base plates supplemented with Cetrimide (C: 10g), Fucidin (F: 10g) and Cephalosporin (C: 50gL^{-1}) (CFC Agar). The isolated colonies were newly streaked on CFC agar for purification; thus, following the Mead and Adams [30] recommendations, who suggested this combination (CFC) of agents to produce a more specific medium for isolating pseudomonads, and allows the growth of pigmented and non pigmented species. Incubation was carried out at 30° C for 48 hours. Subcultures of the colonies on plates of nutrient agar (Difco) for the observation of the phenotypic characteristics were made. The Gram stain test was used to confirm the negativity of the colonies for this stain (Gram-), to this group belongs the *Pseudomonas* species. Also, catalase activity assay by the addition of hydrogen peroxide (3%) to strain pure cultures and the enzyme cytochrome oxidase test (Pathotec ® CO diagnostic test) were made to differentiate *Pseudomonas* spp. (oxidase +) from other species of *Pseudomonas* and other Gram-negative bacilli (oxidase -). Species characterization in terms of the use of carbohydrates was made through RaPIDTm NF Plus (Remel) and API 20 NZ (BioMérieux). The production of pyocyanin and fluorescein pigments was observed on *Pseudomonas* agar P and *Pseudomonas* agar F media, respectively. The qualitative micromethods RaPIDTm NF Plus (Remel) and API 20 NZ (BioMérieux), also allowed the identification of species, because they incorporate conventional tests and monosubstrates chromogenics tests. The results were compared with those obtained by Uğur *et al.* [49], who widely characterized several *Pseudomonas* species, and those reported in the Bergey's Manual of Systematic Bacteriology [8]. In addition, it was made the confirmation of species with MicroScan AutoScan4 of Dade Behring, following the methodology indicated by the manufacturer and using panels for quick identification.

Figure 1. Areas (A, B) of the sampled zone where can be observed crude oil contamination

RESULTS AND DISCUSSION

Soil sampling

The sampling area were located very few plants, belonging to the Families Fabaceae and Poaceae; it may be considered a razed site (Figures 1A and 1B). It argues that the death of the plants in petroleum-contaminated sites is due to the negative effects of hydrocarbons in the physiology of the plant. Adoki and Orugbani [1] and Eze *et al*. [14] reported crude oil as a hydrophobic material, which reduces the breathing and the permeability of cell membranes of the affected parts; therefore, decreases nutrient absorption, metabolism and growth and eventually leading to death.

From the collected legumes, it was identified the species described below and from whose rhizospheric soil were isolated, characterized and identified the *Pseudomonas* species reported in this paper.

Identification of the collected leguminous plant

In accordance with the specifications in the consulted literature [5] and the comparison with exsiccates in the Herbarium UOJ (cited below), the legume collected was identified as *Crotalaria micans* Link (Figure 2). The exsiccate deposited in the herbarium UOJ is shown in Figure 3.

Figure 2. *Crotalaria micans* Link. A inflorescences, B. fruits

Avendaño [5] details the characteristics that distinguish *C. micans*: *Herb* or shrub of 0.6-3 m tall. *Stems* striates, pubescents. *Leaves* trifoliate; leaflets elliptic, elliptic-obovates, apex 4-10 cm long, 1.4-3 cm wide; stipules 0.1-0.5 cm long, free, setaceous, pubescent; petioles *2*.7-8 cm long, petiolules 0.2-0.5 cm long*;* apex acute-mucronate; base attenuated, margins entires, adaxial surface glabrous; abaxial surface puberulous or velutine. *Inflorescence* terminal, multiflowered; bracts 1-1.5 cm long, linears, pubescents, caducous; pedicels 0.5-0.9 cm long; bracteoles linears, 0.6-1 cm long, pubescents, inserted just above the middle part of the pedicel, caducous. *Flowers* 1.2-1.8 cm long; calyx campanulate, 0.7-1.2 cm long, 5-lobed, lobules velutine; corolla papilionacea, yellow, standard 1.1-2 cm long, 1.1-2.4 cm wide, keel 1.1-1.5 cm long, 0.4-0.8 cm wide, non-twisted beak, upper margins densely pubescent. Stamens filaments with lengthened anthers 0.3-0.7 cm long or with rounded anthers 0.4-0.9 long. Ovary tomentose; estile 0.8-1 cm long, curve, persistent, pubescent. *Fruit* legume 0.2-0.5 cm long, 0.9-1.5 cm wide, oblong or globose, puberulous, velutine, tomentose. *Seeds* 0.4-0.6 cm long, 0.3-0.5 wide, glimmers.

Figure 2. Exsiccate of *C. micans* Link, deposited in the herbarium UOJ
One hundred meters from the entrance to Amana del Tamarindo Village,
9°38'52" N, 63°7'20" E, 46m asl, 11/12/2012, J. Mayz & A. Lárez 4607 (UOJ 11705).

Crotalaria micans Link has the following synonyms *Crotalaria anagyroides* Kunth, *Crotalaria brachystachya* Benth., *Crotalaria dombeyana* DC., *Crotalaria stipulata* Vell. and *Crotalaria triphylla* Vell. [39]. It is found in Argentina, Bolivia, Brazil, China, Colombia, Guyana, Madagascar, Uruguay, Paraguay and Venezuela [33].

Revised exsiccates
- La Morocoymera hill, family Leopardi ranch, Caripe of the Oilbird, 10°04′10″ N 63°11′59″ W, 1200 m asl, 03/04/2000, A. Lárez, A. Brown & R. Gonzalez 2986.
- Field behind the machinery workshop, UDO, Jusepín, 9°45′00″ N 63°31′00″ W, 100 m asl, 21/04/1971, L. Leonett s/n (UOJ 2569).

The presence of *Crotalaria micans* in the disturbed area by the crude oil spill can responds to several aspects:

(a) Its ability to colonize problematic areas has been linked, as Villalobos and Ramirez [45] indicated, to features highly conserved in its reproductive system, among these, mainly its autogamous behavior.

(b) The germination capacity of seeds of this species under these polluted conditions, probably linked to an innate resistance of seeds coat, which does not allow damage to the embryo. Harper [19] considered that the germination and initial plant growth are key stages for plant establishment in a vegetal community. For example, Amakiri and Onofeghara [3] showed that *Capsicum frutescens* seeds retained nearly 100% of its viability after 32 weeks of soaking in crude oil; this capacity was attributed to the resistance of the seeds cover. In addition, Robson *et al*. [37] found in his essays that the legumes *Psoralea esculenta* and *Melilotus officinalis* showed the most promising potential of phytoremediation based on its good germination in soils contaminated with oil.

(c) Its ability to fix atmospheric nitrogen. Gudin and Syratt [18] reported that when a soil is contaminated with oil hydrocarbons, carbon stimulates microorganisms growth, but causes an imbalance in the C: N proportion. This can give rise to the immobilization of soil nitrogen by the microbial biomass, leaving the soil without available N for plant growth; if C. *micans* can fix nitrogen and use it in growth, it could be a hit to grow this plant in soils contaminated with petroleum hydrocarbons.

Isolation, characterization and identification of hydrocarbons degrading *Pseudomonas* species

From the CFC agar plates were isolated 35 presumptive P*seudomonas* strains, whose Gram stain test allowed the observation of bacilli and confirmed the negative reaction (Gram -) to this dye, group to which belong the *Pseudomonas* species. In nutrient agar, the colonies were circular, convex, entire margin, without or with pigmentation, which varied between brown and pale yellow, some shinny. In addition, the test of the enzymes cytochrome oxidase and catalase, allowed discarding five isolates, which resulted catalase and oxidase negative.

The results of the biochemical characterization and the production of the pigment pyocyanin and fluorescein (Figures 4 and 5), observed on plates with *Pseudomonas* agar P and *Pseudomonas* agar F, respectively, allowed the comparison with the species descriptions given in the Bergey's Manual of Systematic Bacteriology [8] and Uğur *et al*. [49]. Seventeen strains were identified as *P. fluorescen*s, 8 as *P. putida* and 5 as *P. aeruginosa* (Table 1). The named species identification was confirmed through the rapid identification panels used with MicroScan AutoScan 4 de Dade Behring equipment.

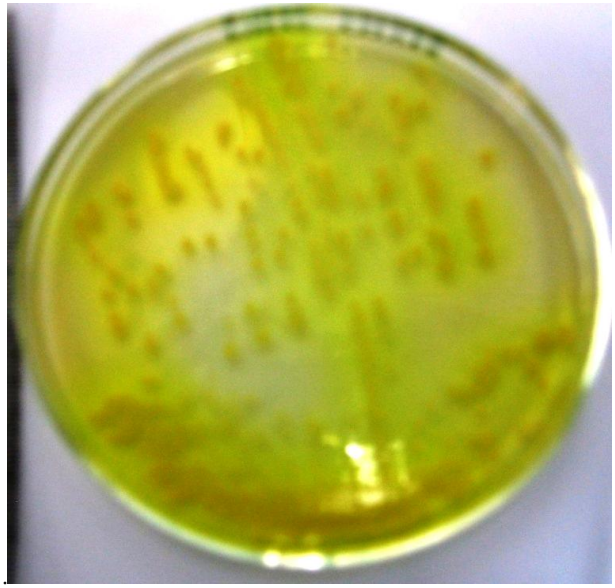

Figure 4. Isolates of *P. aeruginosa* in *Pseudomonas* agar P.
The production of pyocyanine is seen as an area of blue-green color that surrounds the colonies.

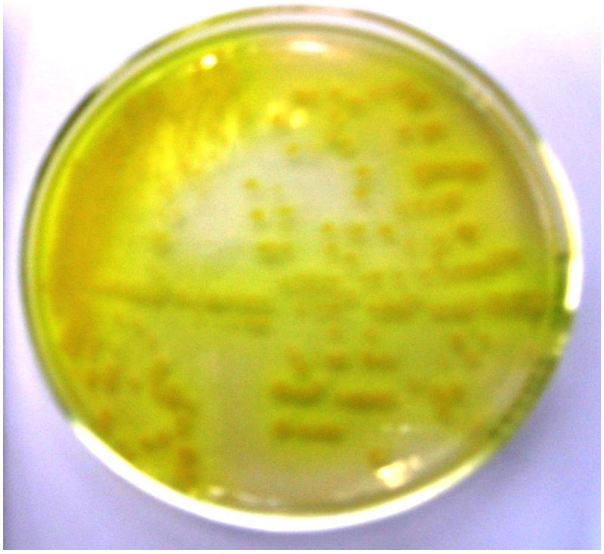

Figure 5. Colonies of *P. aeruginosa* in *Pseudomonas* agar F.
The production of fluorescein is seen as an area of fluorescent greenish-yellow color that surrounds the colonies.

Table 1. Differential characteristics of *Pseudomonas* species

Characteristics	***Pseudomonas* species**		
	P. aeruginosa	*P. fluorescens*	*P. putida*
Oxidase Reaction	+	+	+
Catalase Reaction	+	+	+
Growth at 41°C	+	-	-
Pyocyanin Production	+	-	-
Fluorescein Production	+	+	-
Indol Production	-	-	-
Gelatin Hydrolisis	+	-	-
β-Galactosidase Hydrolisis	-	-	-
Ureasa Reaction	+	-	-
Carbon Sources Utilization			
D-Glucose	+	+	+
L-Arabinose	-	-	-
D-Mannose	-	+	-
D-Mannitol	+	+	-
N-Acetyl-D-Glucosamine	+	-	-
Maltose	-	-	-
Citrate	+	+	+
Gluconate	+	+	+
L-Malate	+	+	+
DL-Arginine	+	+	+
Fenylacetate	-	-	-
Adipate	+	-	-
Caprate	+	+	+

The petroleum hydrocarbons resistance of hydrocarbonoclastics *Pseudomonas* species could be based on similar mechanisms to those presented against antibiotics, called Resistance Mediated by Impermeability, whose bases are the resistance of the little permeable outer membrane, given by the porins; the appearance of a β - lactamase AmpC inducible; and an active pumps system of antimicrobials expulsion [41].

More specifically; the studies of Lăzăroaie [27] and Weber *et al.* [53], revealed several defensive mechanisms developed by *P. aeruginosa* in toxicity hydrocarbons contamination conditions; among them:

a) Mineralization of saturated and aromatic hydrocarbons.

b) Reduction of the hydrophobicity, which prevents the accumulation of hydrocarbons in cell membrane.

c) Changes in the main groups of phospholipids of the membranes to maintain its fluidity and impermeability restore its integrity and reduce degradation of the hydrocarbons in it.

d) Strong induction of some proteins synthesis and reordering of the vesicles to remove hydrocarbons from inside the cells.

e) Modification of cell membrane polysaccharides.

f) flow of hydrocarbons in a dependent energetic process together with a system of flagellar transport, which allows the transfer of some proteins involved with hydrocarbons, out of the periplasmic space or the outer membrane.

In addition, the production of biofilms by identified *Pseudomonas* species [42], can account to bacterial resistance to hydrocarbons. Flemming and Wingender [16] consider that biofilms could reduce the accessibility of the hydrocarbons to bacterial cells throughout the production of extracellular material that builds an adhesive gel or matrix, which surrounds the cells and protects them from difficult conditions.

The colonizing action of the identified hydrocarbonoclastics species of the genus *Pseudomonas* (*P. aeruginosa*, *P. fluorescens* and *P. putida*) requires coordination and specific action of certain enzymes and proteins as Fernández *et al*. [15] points out. These species besides its remedial action by their ability to use hydrocarbons (biodegradation)

may also act as plant growth promoters, making available phosphorus and releasing IAA phytohormone [24, 40, 44]; thus, they are a great potential for cleaning hydrocarbons contaminated soils.

There are several investigations showing the beneficial effects of hydrocarbonoclastics species of the genus *Pseudomonas* in plants growth, through hydrocarbons degradation and toxic effects reduction. For example, Benedek *et al*. [7] found that *Pseudomonas fluorescens* BBN1 and *Rhodococcus qingshengii* BBG1 together, reached degradation rates of 95% (n-dodecane), 66% (toluene) and 70% (naphthalene) of the contaminants initial concentration at 42 days, and Gofar [17] found an increase in roots dry weight of *Lepironia mucronata*, cultivated in a petroleum-contaminated soil and inoculated with the hydrocarbonoclastics bacteria *Alcaligenes faecalis* and *Pseudomonas alcaligenes*.

CONCLUSION

This study could be an important step towards the development of a bioremediation strategy.

The plant species *Crotalaria micans* (Fabaceae) was identified in the area contaminated with petroleum, close to Amana del Tamarindo village (Monagas state).

Based on biochemical characterization and production of pyocyanin and fluorescein pigments with support of rapid identification techniques and specialized literature were identified three hydrocarbonoclastics species of the genus *Pseudomonas* in the rhizosphere of *Crotalaria micans*: *P. fluorescens*, *P. putida* and *P. aeruginosa*.

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