

## Identification of Bacteria from the Rhizosphere of *Jatropha curcas* with Characteristics of Biotechnological Interest

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In the present study *Pseudomonas* sp. S18 (PMA-76), *Pseudomonas aeruginosa* N7B1 (PMA-83), *Pseudomonas* sp. (PMA-88), *Pseudomonas nitroreducens* TX1 (PMA-89), *Pseudomonas strains* sp. bf1 (PHx -97), *Pseudomonas aeruginosa* F23 (PHx -98), *Pseudomonas aeruginosa* F23 (PHx -101) strains were isolated from the rhizosphere of physic nut. The PMA-83 PMA-88 PMA-89, PHx -97, PHx -98, PHx -101 strains grew up in the presence of phenol 0.05% and benzene 1.0%, as a sole carbon source, as well as in the presence of zinc, copper and cobalt in concentrations of 0.1 and 5 mg/ml. The *Pseudomonas* sp. S18 (PMA-76) strain was the one that had the highest capacity to solubilize phosphate (9.6 mm). Moreover indole production in 6 strains were detected and while biosurfactants production was detected in 5 strains. Furthermore, the presence of *nifH* genes in PMA-83, PHx -97, PHx-98 and PHx P-101 strains were also detected, indicating the ability to fix nitrogen. PMA 83 is the strain in which the growth in phenol, benzene and metals were detected, thus indole production, the presence of biosurfactants and *nifH* genes which are characteristics of biotechnological interest.

**Key words:** Phosphate solubilization, *Jatropha curcas*, indoles, biosurfactants.

*Jatropha curcas* L., is a drought tolerant plant and grows well on marginal soils (Berchmans and Hirata 2008; Abou-Kheira *et al.* 2009). Moreover, it has been demonstrated that this species has the ability to control soil erosion and potential to phytoremediation (Kumar *et al.* 2008; Mangkoedihardjo *et al.* 2008; Behera *et al.* 2010; Reubens *et al.* 2011). In Mexico, *J. curcas* is known

as Piñón (physic nut) and can be found as living fences in several states, both in the Pacific slope and along the Gulf of Mexico (Makkar *et al.* 1998). The seeds of this plant are toxic due to its high content of phorbol esters, but in Mexico there are some non-toxic varieties used as food in some rural áreas (Ovando *et al.* 2009; Martínez-Herrera *et al.* 2010).

The rhizosphere microbiota includes the greatest diversity of organisms that interact directly with a particular plant; therefore it has a huge impact and adaptation capacity of the plant.

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Bacterial and fungal communities in the rhizosphere affect plant immunity (Van Wees *et al.* 2008; Berendsen *et al.* 2012; Ronald and Shirasu 2012), pathogens abundance, nutrient acquisition (Jones *et al.* 2009; Richardson *et al.* 2009) and stress tolerance (Doubkova *et al.* 2012; Marasco *et al.* 2012). The soil microbial community is an important part of the plant symbiotic network. Soil is the largest microorganisms reservoir that affect plant growth, vigor, fertility and stress tolerance (Buée *et al.* 2009; Faure *et al.* 2009; Lambers *et al.* 2009; Lugtenberg and Kamilova 2009; Chaparro *et al.* 2012; Doornbos *et al.* 2012; Bakker *et al.* 2013). All plants have a direct interaction with soil microorganisms in the rhizosphere, which is the floor area immediately surrounding the root, where root exudates plant directly influence the structure and function of the microbial soil community. Microorganisms growing in the rhizosphere nutrient-rich produce molecular signals that promote the plants adaptation and growth (hormones) and can disrupt communication between plants on natural systems (Faure *et al.* 2009; Sanon *et al.* 2009).

Rhizosphere microorganisms can provide a shortcut to the limiting nutrients (symbiotic N<sub>2</sub> fixing) or increase the total surface area of the root system (mycorrhiza). Many reviews have described the positive effects of beneficial symbiotic root rhizosphere (Buée *et al.* 2009; Bakker *et al.* 2013), factors affecting the rhizosphere microbial communities (Philippot *et al.* 2013), and microbial effects on plant health (Berendsen *et al.* 2012; Berlec 2012; Bever *et al.* 2012) and stress tolerance (Rodriguez *et al.* 2008). Therefore, the crop importance of the physic nut as well as of microorganisms in the rhizosphere, in this study has been conducted bacteria with biotechnological characteristics associated to physic nut rhizosphere.

## MATERIALS AND METHODS

### Isolation of rhizosphere bacteria

Rhizosphere bacteria physic nut (*Jatropha curcas L.*) were isolated, plants were collected in Mazatán, Tapachula, Huixtla, Huehuetán, Mapastepec, Pijijiapan and Tonalá, cities of the State of Chiapas, Mexico. Approximately one gram of the plant root was

placed into tubes containing 9 ml of 10 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, which were shaken vigorously for a solution where the microorganisms of rhizosphere. 200 µl of this solution was inoculated on BAZ semisolid medium (0.2% Azelaic acid, 0.02% L-citrulline, 0.04% K<sub>2</sub>HPO<sub>4</sub>, 0.04% KH<sub>2</sub>PO<sub>4</sub>, y 0.02%, MgSO<sub>4</sub>·7H<sub>2</sub>O), incubated at 28 °C for one week, the bacterial growth tubes were reseeded twice. After this reseeded were planted on solid culture medium supplemented with cycloheximide BAC (100 mg/ml) and incubated at 28 °C for 72 h. Colonies that showed different morphology were purified in BAC medium, the purity of isolated was verified in PY medium, pure colonies were stored in 70% glycerol at - 70 °C, for further characterization.

### Bacterial growth in phenol and benzene

Isolates were grown in liquid medium BSE for 14 h at 29 °C with agitation 250 rpm, the bacterial cultures were centrifuged and adjusted to an optical density of 0.2 at 600 nm, 100 µl of these cultures were inoculated in duplicate in SAAC medium had the following composition (in grams/liter): K<sub>2</sub>HPO<sub>4</sub>, 0.4; KH<sub>2</sub>PO<sub>4</sub>, 0.4; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; CaCl<sub>2</sub>, 0.02; Na<sub>2</sub>MoO<sub>4</sub>, 0.002; FeCl<sub>2</sub>, 0.01; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5; bromothymol blue, 0.075; Agar, 18. Containing phenol as carbon source 0.1% and 0.05 % benzene<sup>11</sup>. Petri plates were incubated at 29 °C for 4 days.

### Metal resistance

To evaluate the resistance to different metals, isolates were grown on medium containing 0.1, 5.0 and 10.0 mg/ml of the following metals: copper, cobalt and zinc, as CuSO<sub>4</sub>·5H<sub>2</sub>O CoCl<sub>2</sub>·6H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, respectively. The stock solution from each metal salt was as follow: 0.5 g/ml for CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.5 g/ml and for CoCl<sub>2</sub>·6H<sub>2</sub>O 0.5 g/ml for ZnSO<sub>4</sub>·7H<sub>2</sub>O. Bacteria were inoculated into liquid medium BSE for 14 h at 29 °C at 250 rpm. The cultures were adjusted to OD 0.2 at 600 nm, 100 µl were inoculated in PY medium with different concentrations of metals in duplicate, they were incubated at 29 °C for 4 days.

### Phosphate solubilization

To determine the ability to solubilize phosphate was determined by inoculating the strains in liquid medium PY and incubated at 29 °C for 24 h at 200 rpm, the bacterial cultures were centrifuged and adjusted to an optical density of 0.2 at 600 nm, were seeded in triplicate in NBRIP culture medium had the following composition (in

grams/liter): Glucose, 10;  $\text{Ca}_3(\text{PO}_4)_2$ , 5;  $(\text{NH}_4)_2\text{SO}_4$ , 0.1;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25; KCl, 0.2;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 5; Rojo Congo, 0.025; Agar, 18 and incubated at 28 °C for 7 days, then the size of the halos around the colonies were measured (Caballero *et al.* 2007).

Production determination of AIA (Qualitative) Strains were evaluated to detect the production of indoles which were grown in liquid medium NFB had the following composition (in grams/liter): Malic acid, 5;  $\text{K}_2\text{HPO}_4$ , 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2; NaCl, 0.1;  $\text{CaCl}_2$ , 0.02;  $\text{FeSO}_4$ , 0.015;  $\text{Na}_2\text{MoO}_4$ , 0.0025;  $\text{MnSO}_4$ , 0.01; KOH, 4.8;  $\text{NH}_4\text{Cl}$ , 0.2; yeasts extract, 0.3;  $\text{H}_3\text{BO}_3$ , 0.01; the bacterial cultures were incubated for 18 h at 200 rpm, after that, were they adjusted to an optical density of 0.2 at 600 nm, 100  $\mu\text{l}$  of culture were inoculated into medium Jain and Patriquin, with and without tryptophan, incubated 30 °C for 24 and 48 h at 200 rpm. 600  $\mu\text{l}$  aliquots of the cultures were taken and centrifuged for 5 min, at 5000 g. The presence of indole in the supernatant was detected according to the modified method by Rahman *et al.* (2010), in which the hormone in the sample reacts with the reagent Salkowski.

#### **Biosurfactants production assay (BS) agar hexadecyltrimethylammonium bromide (CTAB)**

Isolates were grown in liquid medium PY 24 h at 29 °C with shaking at 250 rpm, the bacterial cultures were centrifuged and adjusted to an optical density of 0.2 at 600 nm and inoculated in triplicate in the culture medium of mineral salts and carbon source of low solubility (glycerol), methylene blue and CTAB, incubated at 28 °C for 48 h. CTAB is a cation which reacts with the BS, it forms a CTAB-BS methylene blue complex. Strains in the presence of a translucent halo around the colony growth in agar are considered positive for the biosurfactants production (Chandankere *et al.* 2013).

#### **BOX-PCR**

The bacterial isolates were amplified by PCR- BOX technique using oligonucleotides BOXA1R. The conditions used were: 95 °C for 5 min and 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min, and final elongation cycle of 10 min, for 10 min at 72 °C (Versalovic *et al.* 1994).

#### **PCR *nifH* genes amplification**

PolF and PolR oligonucleotides were used for amplification of *nifH* genes, using the PCR conditions described by Poly *et al.* (2001). The reaction amplifies a 360 bp fragment comprising a fragment of *nifH* gene.

#### **16S rRNA gene Sequencing**

The gene 16S was amplified using the oligonucleotides rD1 and fD1 under the conditions described by Weisburg *et al.*, (1991), the amplification products were purified from the gel using the GeneJET kit (Thermo Scientific), the purified products were sent for sequencing to the sequencing unit of the Instituto de Biotecnología, Universidad Nacional Autónoma de México. The 16S rRNA sequences of the isolates were compared with the 16S rRNA genes of the GenBank database. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987), based on 800 nucleotides, according to the distance matrix of Jukes and Cantor (1969), using the program MEGA 5 (Kumar *et al.* 2001)

## **RESULTS**

#### **Isolation**

The pH of the soil from the roots of plants physic nut (*Jatropha curcas*) collected in the 7 cities in Chiapas were analyzed, the values obtained are between 4.4-9.9. 250 isolated based on the morphology of the colonies were selected.

#### **Bacterial growth in phenol and benzene**

Selected 250 strains were grown in culture media containing 1.0% benzene and 0.05% phenol, as only carbon source. PMA-83, PMA-89, PHx-97, PHx 98 and PHx-101 strains grew in the presence of benzene and phenol, except PMA-88 strain that didn't grow in phenol those strains that grew in benzene to phenol also increased except PMA-88 strain (Table 1).

#### **Metal tolerance**

Evidence of growth in the presence of different metals in three concentrations of copper salts, zinc and cobalt 7 selected strains were performed, all strains grew in the presence of Zn, Cu and Co at a concentration of 0.1 mg/ml (Table 1), however in concentrations of 5 mg/ml and 10 mg/ml no strain growth was observed.

#### **Phosphate solubilization**

Phosphate solubilizing ability was varied between isolates strains of plant rhizosphere physic nut. PMA-76 and PMA-89 strains exhibited the remarkable ability to solubilize phosphate, this because they were the two strains in which halos larger diameter 9.6 and 9.0 mm respectively were observed in the medium with  $\text{Ca}_3(\text{PO}_4)_2$  as the

sole P source, after 72 h incubation (Table 2). Also PMA-83 PMA-88, PHx-97 and PHx-101 strains were able to solubilize phosphate; however, halos formed were smaller in diameter than the PMA-76 and PMA-89 strains. The PHx-98 strain did not show the formation of halos in the medium with  $\text{Ca}_3(\text{PO}_4)_2$  as the sole source of P (Table 2).

#### Indole production

Salkowski test revealed that the PHx-97 strain observed greater color intensity after 24 h, indicating the presence of indoles in the culture

medium containing tryptophan. Strains PMA-83, PMA-88, PMA-89 and 101-PHx coloration was also observed but less intense than the PHx-97 strain, while the PMA-76 and PHx-97 strains did not present any coloration observed indicating the presence of indoles (Table 2).

#### Biosurfactants production

7 Selected strains were grown with minimal mineral salts medium and glycerin as a carbon source in agar CTAB. After 48 h of incubation, the PMA-76, PMA-83, PMA-88, PMA-

**Table 1.** Growth of the strains isolated from the rhizosphere of physic nut in aromatics and metals compounds

Strains	Growth in the presence of metalsResistance (mg / ml)			Aromatics growth	
	Zn	Co	Cu	Benzene	Phenol
PMA-76	0.1,5.0	0.1	0.1	-	-
PMA-83	0.1	0.1	0.1	+	+
PMA-88	0.1,5.0	0.1	0.1	+	-
PMA-89	0.1	0.1	0.1	+	+
PHx-97	0.1,5.0	0.1	0.1	+	+
PHx-98	0.1,5.0	0.1	0.1	+	+
PHx-101	0.1	0.1	0.1	+	+

**Table 2.** Biotechnological characteristics of strains isolated from the rhizosphere of physic nut

Strains	Phosphate solubilization Halo size (mm)	Biosurfactants Production	Indole acetic acid production
PMA-76	9.6	+	-
PMA-83	6.8	+	+
PMA-88	7.4	+	+
PMA-89	9.0	+	+
PHx-97	7.0	+	+
PHx-98	0.0	-	+
PHx-101	4.5	-	+

**Table 3.** Characteristics biotechnological reportd strains isolated from rhizosphere of physic nut

Strains	Identity of the gene 16S	Characteristics biotechnology
PMA-76	<i>Pseudomonas sp.</i> S18	unreported
PMA-83	<i>Pseudomonas aeruginosa</i> N7B1	Degradation of polycyclic aromatic hydrocarbons
PMA-88	<i>Pseudomonas sp.</i>	Adsorption in soils contaminated with cadmium
PMA-89	<i>Pseudomonas nitroreducens</i> TX1	Degradation of long-chain alkylphenols
PHx-97	<i>Pseudomonas sp.</i> bf1	Phenol degradation
PHx-98	<i>Pseudomonas aeruginosa</i> F23	unreported
PHx-101	<i>Pseudomonas aeruginosa</i> F23	unreported

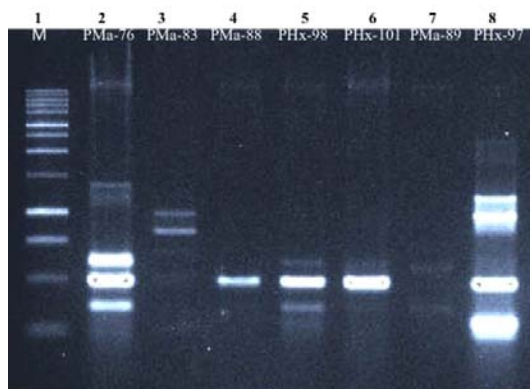
89, PHx-97 and PHx-101 strains showed a translucent halo around the colony, indicating the presence of production of biosurfactants (Table 2), while the PHx-98 strain did not show halo.

### BOX PCR

7 strains that showed different biotechnology traits were grouped by genomic fingerprints were obtained with oligonucleotides BOXA1, were different genomic footprints were found (Figure 1).

### PCR genes *nifH* amplification

Of the 7 strains selected, they were amplified with the oligonucleotides polyF and polyR, the PMA-83, PHx-97 PHx-98 and PHx-101 strains amplified a PCR product of 360 bp corresponding to fragment *nifH* gene, with this



**Fig. 1.** BOX - PCR products of strains isolated from the rhizosphere of *Jatropha curcas*, lane 1 molecular weight marker, lane 2 PMA-76, lane 3 PMA-83, lane 4 PMA-88, lane 5 PHx 98, lane 6 PHx - 101, lane 7 PMA-89, lane 8 PHx-97

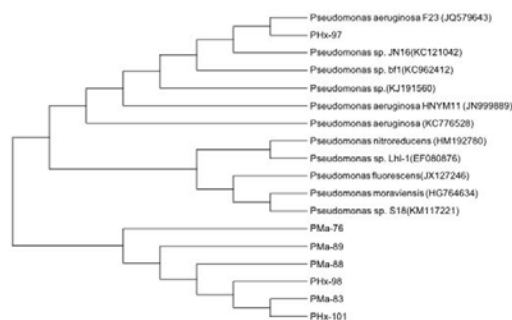
### DISCUSSION

Of the 250 strains isolated from the rhizosphere of plants physic nut collected in the state of Chiapas, they were detected characteristics of biotechnological interest in 7 strains for applications in agriculture, the PMA-83 strains (*Pseudomonas aeruginosa* N7B1), PMA-88 (*Pseudomonas* sp.), PMA-89 (*Pseudomonas nitroreducens* TX1), PHx-97 (*Pseudomonas* sp. bf1), PHx-98 (*Pseudomonas aeruginosa* F23), PHx-101 (*Pseudomonas aeruginosa* F23) grew in the presence of 0.05% phenol and benzene 0.1 % as sole carbon source (Table 2), these results are very similar to those reported by Caballero-Mellado *et*

result it is confirmed the presence of this gene, which suggests its capacity to fix nitrogen.

### Sequencing gene 16S rARN

16S rDNA sequences analyzed GenBank were identified as *Pseudomonas aeruginosa* N7B1 (PMA-83), *Pseudomonas* sp. (PMA-88), *Pseudomonas nitroreducens* TX1 (PMA-89), *Pseudomonas nitroreducens* TX1 (PMA-89), *Pseudomonas* sp. bf1 (PHx-97), *Pseudomonas aeruginosa* F23 (PHx-98), *Pseudomonas aeruginosa* F23 (PHx-101), in which they were detected characteristics of biotechnological interest (Table 3). In the phylogenetic tree constructed using 16S gene sequences you can see the relationship of the strains identified the rhizosphere of *Jatropha curcas* with the strains reported in GenBank (Fig. 2).



**Fig. 2.** Phylogenetic tree based on 16S rRNA gene sequences in which one can observe *Pseudomonas* species associated with the rhizosphere of plants *Jatropha curcas*

*al.* 2007, where strains of *Burkholderia* species *Burkholderia xenovorans* unamae and grow in the presence of these compounds. Although all of them had the ability to grow in these compounds, the PMA-89 (*Pseudomonas nitroreducens* TX1) strain has been reported to degrade long-chain alkylphenols, due to the interest of this biotechnology capacity the genome of this strain is sequenced, which can be used to search for genes involved in the degradation of phenol and benzene (Chen *et al.* 2006), however not only was it detected the ability to grow in the presence of xenobiotics, but also it was detected in this study, the ability of this strain to produce indoles, phosphate solubilization and growth in the



presence of metals such as Zn, Co, Cu in concentrations of 0.1 mg/ml (Table 2,3). Eventhough, in the PMA-76 (*Pseudomonas* sp. S18) strain did not grow in the presence of phenol and benzene, it was the strain that had the highest ability to solubilizing phosphate 9.6 mm (Table 3) these results are very similar to those reported by Caballero-Mellado *et al.* 2007; with strains of *Burkholderia tropica*, while in other studies with *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* they reported a reduced ability to solubilize phosphate. Although it has been reported indoles production in *Pseudomonas fluorescens* and *Pseudomonas plecoglossicida* (Kaur and Reddy 2013), this work reportes in this capacity species PMA-83 (*Pseudomonas aeruginosa* N7B1), PMA-88 (*Pseudomonas* sp.), PMA-89 (*Pseudomonas nitroreducens* TX1), PHX-97 (*Pseudomonas* sp. bf1), PHX-98 (*Pseudomonas aeruginosa* F23), PHX-101 (*Pseudomonas aeruginosa* F23) that are species of the genus *Pseudomonas* which have been studied for their ability to degrade hydrocarbons. In this work, biosurfactants production was also evident in a specific medium using glycerol as a carbon source. The biosurfactants production was detected in PMA-76 (*Pseudomonas* sp. S18), PMA-83 (*Pseudomonas aeruginosa* N7B1), PMA-88 (*Pseudomonas* sp.), PMA-89 (*Pseudomonas nitroreducens* TX1), PHX-97 strains (*Pseudomonas* sp. bf1) (Table 3), although these characteristics were reported in *Pseudomonas fluorescens* (Yañez and Wong 2013), strains isolated from the rhizosphere of physic nut had not been reported specifically for biosurfactants production which is of great interest for biotechnology applications in bioremediation of heavy metals, hydrocarbons and pesticides. It is reasonably likely that bacterial production strains that showed BS, do to solubilize phosphates, grow in insoluble carbon sources such as benzene and phenol and, at the same times to desorb heavy metals in soil (Lee *et al.* 2008; Arutchelvi *et al.* 2011). Which is of great interest, not only because they also produce biosurfactants and solubilize phosphate produce indole acetic acid. The ability of this strains to grow in the presence of zinc, cobalt and copper at concentrations of 0.1 and 5.0 mg/ml (Table 2), is a characteristic of rhizobacteria. That in other studies they have demonstrated that metals resistant

bacteria can promote plant growth as well as increase the adsorption of these metals by hyperaccumulator or not hyperaccumulator plants. Jiang *et al.* 2008 reported that in corn and tomato plants inoculated with *Burkholderia* sp. J62 there was a significant increase in the adsorption of Pb and Cd, which opens the possibility for the use of these strains isolated from the rhizosphere of physic nut tolerant to metals as an alternative in bioremediation and phytoremediation process and as growth promoters in plants (Piotrowska-Seget *et al.* 2005). Furthermore, the presence of the *nif H* gene was also detected in the strains PMA-83 (*Pseudomonas aeruginosa* N7B1), PHX-97 (*Pseudomonas* sp. bf1), PHX-98 (*Pseudomonas aeruginosa* F23) and PHX-101 (*Pseudomonas aeruginosa* F23) which indicates that these strains have the ability to fix nitrogen, this capability has been reported in *Pseudomonas stutzeri* isolated in crop fields of *A. sativa* and *H. vulgare* in Greece (Venieraki *et al.* 2011). The characteristics of biotechnological interest detected in different species genus *Pseudomonas* isolated from the rhizosphere of physic nut make these strains can be an alternative for use in processes for promoting growth crop plants of agricultural interest, it can also be employed in processes of bioremediation of soils contaminated with hydrocarbons, pesticides and metals.

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