BIOSURFACTANTS PRODUCED BY METAL-RESISTANT Pseudomonas aeruginosa ISOLATED FROM Zea mays Rhizosphere AND COMPOST

ABSTRACT

Contextualization: Pseudomonas aeruginosa is capable of producing biosurfactants which have many uses in bioremediation and the production of antiviral, antibacterial, antiparasitic, sporicidal and antifungal agents, among others.

Knowledge gap: This study describes the production of mono and di-rhamnolipid biosurfactants by P. aeruginosa strains isolated from Zea mays rhizosphere and composts in the state of Guerrero, Mexico.

Purpose: The overall aims were to investigate biosurfactant, pyocyanin production, and tolerance to heavy metals and antimicrobial activity capacity than biosurfactants produced from P. aeruginosa strains from corn rhizosphere and compost in Mexico.

Methodology: Biosurfactant production was determined based hemolysis on blood agar, blue halos in CTAB-Methylene blue agar, drop collapse test and production of foam on PPGAS.

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broth, the emulsion index (IE24) and antibacterial capacity. The strains were identified by sequence of the 16S rDNA gene and their resistance to heavy metals were also evaluated.

**Results and conclusions:** Two strains isolated from Zea mays rhizosphere (PAM8, PAM9) were the best biosurfactant producers and their extracts showed antimicrobial activity against Grampositive and Gramnegative bacteria. PAM8 and PAM9 showed >30% of cellular hydrophobicity to hydrocarbons, and were capable of emulsifying toluene, cyclohexane, petroleum, diesel and oils. All strains showed the same profile of heavy metal tolerance (As$^{5+}$ > As$^{3+}$ > Zn$^{2+}$ > Pb$^{2+}$ > Fe$^{3+}$ > Cd$^{2+}$ > Cu$^{2+}$ > Cr$^{6+}$ in concentrations of 20, 10, 10, 6, 4, 4, 2 and 2 mM., respectively). The isolation of biosurfactant-producing and heavy-metal tolerant bacteria from Zea mays rhizosphere and compost in Guerrero demonstrates the capacity for this region to harbor potentially important microbial strains for industrial or bioremediation applications.

**Keywords:** Rhamnolipids, hydrocarbons, bioremediation, biotechnological applications.

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**Propósito del estudio:** los objetivos generales fueron investigar la producción de biosurfactantes, piocianina y tolerancia a metales pesados y la capacidad de actividad antimicrobiana de los biosurfactantes producidos por cepas de *P. aeruginosa* aisladas de rizosfera y compost en México.

**Metodología:** Se determinó la producción de biosurfactantes con base en la hemólisis agar sangre, halos azules en agar CTAB-azul de metileno, ensayo de colapso de gotas, producción de espuma en medio PPGAS, índice de emulsión (IE24) y capacidad antibacterial, las cepas se identificaron por la secuencia del gen del 16S y también se evaluó la resistencia a metales pesados.

**Resultados y conclusiones:** Dos cepas aisladas de la rizosfera de Zea mays (PAM8, PAM9) fueron las mejores productoras de biosurfactantes, y sus extractos mostraron actividad antimicrobiana frente a bacterias Grampositivas y Gramnegativas. PAM8 y PAM9 mostraron >30% de hidrofobicidad a hidrocarburos y fueron capaces de emulsionar tolueno, ciclohexano, petróleo, diésel y aceites. Todas las cepas mostraron el mismo perfil de tolerancia a metales pesados (As$^{5+}$ > As$^{3+}$ > Zn$^{2+}$ > Pb$^{2+}$ > Fe$^{3+}$ > Cd$^{2+}$ > Cu$^{2+}$ > Cr$^{6+}$ en concentraciones de 20, 10, 10, 6, 4, 4, 2 y 2 mM., respectivamente). El aislamiento de bacterias productoras de biosurfactantes y tolerantes a metales pesados de la rizosfera y compost de Zea mays en Guerrero demuestra que la región alberga cepas microbianas potencialmente importantes para aplicaciones industriales o de biorremediación.

**Palabras clave:** Ramnolípidos, hidrocarburos, biorremediaciòn, aplicaciones biotecnológicas.
1. INTRODUCTION

Biosurfactants are molecules that lower the surface tension and increase the solubility between solutions that are non-miscible (To-ríbio Jimenez et al., 2014). Among their many applications, they may act as emulsifying, foaming agents and wetting agents (Pacwa-Płociniczak et al., 2011; Satpute et al., 2010). Biosurfactants are produced by microorganisms such as bacteria, yeast and filamentous fungi, and are often considered to be environmentally friendly compounds. They have been mostly studied in the context of microbial enhanced oil recovery (MEOR) and in the bioremediation of water and soils contaminated by hydrocarbon spills and heavy metals (Amani et al., 2013; Darvishi et al., 2011; Wu et al., 2008). Among the better-known biosurfactants are the rhamnolipids (RLs), mainly produced by the bacterium *P. aeruginosa*. 
Strains of *P. aeruginosa* can metabolize hexadecane and other polycyclic aromatic hydrocarbons to use them as a carbon source for growth, for synthesis of rhamnolipids (Beal and Betts, 2000; Müller et al., 2012; Nie et al., 2010) and to eliminate heavy metals from wastewater (El Zeftawy and Mulligan, 2011).

Rhamnolipids (the class glycolipids) from this species are known to function as antibacterial, antiviral, antiparasitic, sporicidal and antifungal agents (Abdel-Mawgoud et al., 2010; Bharali et al., 2013; Wang et al., 2005) alpha-L-rhamnopyranosyl-alpha-L-rhamnopyranosyl-beta-hydroxydecanoate (Rha-Rha-C(10). Another compound related to biosurfactants is pyocyanin, which is a blue, secondary metabolite with the ability to oxidize and reduce other molecules (Hassan et al., 1980). Biosurfactants and pyocyanin exhibited a peculiar correlation, as the culture supernatants containing both components emulsified hydrocarbons in a higher amount than that of the biosurfactant alone.

Enhancement in emulsification also occurred by mixing pyocyanin from PPGAS medium and biosurfactant (Das and Ma, 2013). *P. aeruginosa* is a free-living bacterium that has been isolated from soils, contaminated water, plant rhizospheres, animal skin, leachates, and human environments, including hospitals (Soberón-Chávez, 2001). Resistance of many *P. aeruginosa* strains to antibiotics, antiseptics, chemical disinfectants, heavy metals and metalloids suggest that the species is generally robust in nature and may be applied for human uses in certain cases. The overall objective was to investigate biosurfactant, pyocyanin production, and tolerance to heavy metals and antimicrobial activity capacity than biosurfactants produced from *P. aeruginosa* strains from corn rhizosphere and compost in Mexico.

### 2. MATERIALS AND METHODS
See Annex.

### 3. RESULTS AND DISCUSSION

**RESULTS**

**Selection and identification of biosurfactant-producing bacteria.**

A total of seven bacterial strains were characterized genetically and identified as the genus *Pseudomonas*. Four isolates were found in *Zea mays* rhizosphere (PAM7, PAM8, PAM9 and PAM10) and the rest were isolated from compost (PAB11, PAB12 and PAB13). The strains showed 99% identity to *P. aeruginosa* PAO1 (reference strain); however, PAM79 and PAM810 isolates are genetically related to strains of *Pseudomonas* sp (Fig 1). All strains exhibited the ability to produce biosurfactants, which was confirmed by beta hemolysis in blood agar plates and dark halos in CTAB-Methylene blue agar.
The surface activity of biosurfactants present in the cultures was positive for the tests of foaming activity in PPGAS broth, drop collapse test and oil displacement test (Fig. 2).

Figure 1. Phylogenetic analysis by neighbor-joining method on 16S rDNA gene sequences. Scale bar = 0.5 substitutions per nucleotide position. *Bacillus subtilis* YS52 (HQ202539) was used as an outgroup sequence.

Source: Authors

The biosurfactants produced can emulsify petroleum, diesel, toluene, xylene, cyclohexane and oils (mineral and vegetable) in different degrees, as shown on Table 1. The xylene hydrocarbon was mostly emulsified by the strains (average = 58%), while mineral oil did not exceed the 32% of average emulsification. *P. aeruginosa* strains with highest IE values were PAM8, PAM9, PAB11 and PAB13.

Source: Authors

*Emulsifying properties and cellular hydrophobicity associated with biosurfactants production*
The strain PAM8 has an average IE$_{24}$ of 64% and PAM9 emulsifies cyclohexane up to 70% in vegetable oil. PAB11 emulsifies petroleum to 79% and PAB13 emulsifies xylene to 67%. The Zea mays rhizosphere strains showed better emulsification in diesel compared to the compost strains, and did not present significant differences in the other substrates. The strongly hydrophobic (% Cellular Hydrophobicity or BATH) P. aeruginosa strains were PAM8 and PAM9 with 38% and 30% of BATH values, respectively (Table 1).

**Table 1.** Percent Index of emulsification (IE$_{24}$) and cellular hydrophobicity by biosurfactants of *P. aeruginosa.*

<table>
<thead>
<tr>
<th>Sample</th>
<th>P. aeruginosa</th>
<th>Diesel</th>
<th>Toluene</th>
<th>Xylene</th>
<th>Cyclohexane</th>
<th>Mineral Oil</th>
<th>Vegetable oil</th>
<th>Petroleum</th>
<th>% Cellular Hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize rhizosphere</td>
<td>PAM7</td>
<td>28</td>
<td>66</td>
<td>57</td>
<td>54</td>
<td>24</td>
<td>45</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>PAM8</td>
<td>60</td>
<td>24</td>
<td>61</td>
<td>64</td>
<td>33</td>
<td>52</td>
<td>26</td>
<td>38</td>
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<td></td>
<td>PAM9</td>
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</tr>
<tr>
<td></td>
<td>PAM10</td>
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<td>19</td>
<td>42</td>
<td>54</td>
<td>17</td>
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<td>65</td>
<td>45</td>
<td>42</td>
<td>54</td>
<td>73</td>
<td>32</td>
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</tbody>
</table>

*p value 0.04* 0.86 0.65 0.25 0.21 0.48 0.42 0.06

*Statistically different accorded to unpaired Student’s t-test (p ≤ 0.05)

**Source:** Authors

**Quantification and physicochemical characterization of biosurfactants.**

The *P. aeruginosa* strains with the highest biosurfactant titers were PAM8 (187± 5 mg/L), from Zea mays rhizosphere, and PAB13 (162±7 mg/L), from compost (Fig.3). The strain PAB12 showed poor performance, with 125±4 mg/L of biosurfactant. All biosurfactants obtained from the isolated *Pseudomonas* strains were negatively charged and retained their capacity to disperse and emulsify oil after treatment at ≥ 100 °C, salinity at 10% and ≥ pH10 (Fig. 3). Significant differences were observed between compost and Zea mays rhizosphere strains in terms of amount of biosurfactant produced but not in their physicochemical properties.
Surface activity of biosurfactants in presence of pyocyanin pigment.

The production of pigments was observed at the same time as formation of abundant foam in PPGAS culture so, due to its blue coloration, pyocyanin was quantified (Fig. 4). The highest production of pyocyanin was observed in Zea mays rhizosphere strains: PAM8 (11.9 ± 2.3 μL mL⁻¹), PAM9 (7.8 ± 1.3 μL mL⁻¹) and PAM10 (7.8 ± 1.0 μL mL⁻¹).

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**Source:** Authors

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**Table:**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Compost</th>
<th>Maize rhizosphere</th>
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<tbody>
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<td>PAM7</td>
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**Stability properties**

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<tr>
<th>Temperature</th>
<th>pH</th>
<th>Salinity (NaCl)</th>
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<td>10%</td>
</tr>
<tr>
<td>120°C</td>
<td>10</td>
<td>10%</td>
</tr>
</tbody>
</table>

**Source:** Authors

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**Figure 3.** Comparison of production and physicochemical properties of rhamnolipids. *Statistically different between compost and rhizosphere strains accorded to unpaired Student's t-test (p ≤ 0.05)

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**Figure 4.** Comparison of pyocyanin pigment between compost and rhizosphere strains. A) Quantification by colorimetric method. B) Intensity of pigment in PPGAS broh.

**Source:** Authors
**Antibacterial activity of biosurfactants.**

The produced rhamnolipids have antimicrobial activity against two Gram-positive and two Gram-negative bacteria evaluated (Table 2). Maize rhizosphere strains inhibit the bacterium *Bacillus subtilis* better than compost strains, and no significant differences in their antibacterial effect were observed for other bacteria. *P. aeruginosa* PAM8 showed the strongest activity against *B. subtilis*, *S. aureus*, *S. paucimobilis* and *K. pneumoniae* with inhibition halos of 26±6 mm, 22±4 mm, 32±5 mm and 24±3 mm, respectively.

**Table 2.** Comparison of antibacterial activity of biosurfactants produced by *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Inhibition zone diameter (mm)</th>
<th><em>P. aeruginosa</em></th>
<th><em>B. subtilis</em></th>
<th><em>S. aureus</em></th>
<th><em>S. paucimobilis</em></th>
<th><em>E. cloacae</em></th>
<th><em>P. vulgaris</em></th>
<th><em>K. pneumoniae</em></th>
<th><em>E. coli</em></th>
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<td>Maize rhizosphere</td>
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<tr>
<td>PAM7</td>
<td>22</td>
<td>20</td>
<td>21</td>
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<td>ND</td>
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<td>ND</td>
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<td>32</td>
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<td>24</td>
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<tr>
<td>PAM9</td>
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<td>Compost</td>
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<td>PAO-1</td>
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<td>30</td>
<td>ND</td>
<td>ND</td>
<td>23</td>
<td>ND</td>
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</tbody>
</table>

*p value 0.03* *0.94* *0.82* *-* *-* *0.84* *-

*Statistically different between compost and rhizosphere strains accorded to unaired Student’s t-test (p ≤ 0.05). ND=Not detected.

**Source:** Authors

**Heavy-metal resistance profile of *P. aeruginosa* strains.**

The petroleum and its derivatives contain traces of heavy metals and metalloids that give rise to the formation of organometallic compounds; therefore, we evaluated the heavy-metal resistance profile of biosurfactant-producing *Pseudomonas*. All the strains have similar MIC to heavy metals and metalloids in the following order: As$^{5+}$ (>20 mM), As$^{3+}$ (10 mM), Zn$^{2+}$ (10 mM), Pb$^{2+}$ (6 mM), Fe$^{3+}$ (4 mM), Cd$^{2+}$ (4 mM), Cu$^{2+}$ (2 mM) and Cr$^{6+}$ (2 mM). The colonies were hyper mucoid when grown in high concentrations of metals especially in Pb, Fe and Cu.

**DISCUSSION**

The bacterium *P. aeruginosa* is known for its ability to produce several bioactive compounds. In this study, report is made on seven strains isolated from the rhizosphere corn or compost; all isolates produced biosurfactants with emulsification indexes on the different hydrophobic substrates as shown on Table 1. Highlighted in the work by Pirôllo et al. (2008), a *P. aeruginosa* LBI strain was isolated from places that were contaminated by hydrocarbons. The biosurfactant produced maintained the physicochemical properties after treatment at 120 °C, 10% salinity and only PAM8 biosurfactant is active under alkaline conditions (pH 12).
Strains synthetized both mono-rhamnolipids and di-rhamnolipids, except PAB12 that only produces mono-rhamnolipid, probably lacks some gene responsible for incorporating rhamnose during the biosynthetic pathway. Our results of IE$_{24}$ agree with the results obtained by Janek et al., (2013), where it was reported that the rhamnolipid produced by a strain of *Pseudomonas* BD2 isolated in the Arctic, is capable of reducing the surface tension from 71 to 31 mN/m and emulsifies 70% olive oil, meanwhile for n-hexane, xylene, hexadecane and petroleum ether it emulsifies in a range from 51 to 65%.

We propose PAM8, PAM9 and PAM13 strains as candidates for use in bioremediation of marine environments and other systems by biosurfactants that are stable in hypersaline and alkaline conditions. The strains in this study are similar to those that the microbial consortium reported by Darvishi et al. (2011). That consortium produces a biosurfactant capable of resisting temperatures of over 70°C, salt concentrations higher than 15%, and a range of pH from 4 to 10. It also breaks down hydrocarbons, a fact that points it as a good candidate for the remediation of contaminated sites with these compounds.

It was observed that strains that synthesize more pigment also produce more biosurfactant and are better emulsifiers. Das and Ma (2013) report that the pyocyanin pigment enhances the biosurfactant-mediated hydrocarbon emulsification in clinical and environmental strains of *P. aeruginosa*. In the same way, the hydrocarbonoclastic activity (involved in the mineralization of hydrocarbon pollutants) is positively correlated with the pyocyanin production by *P. aeruginosa* strains isolated from gas stations, activated sludge and wastewater (Viana et al., 2018). Since the strains isolated in the present study are biosurfactant producers, they could potentially be used for Microbial Enhanced Oil Recovery (MEOR) in contaminated environments, in oil wells (Brown, 2010) or in agricultural soils (Sachdev and Cameotra, 2013). The production and concentrations the biosurfactants vary depending on the culture conditions and nutrients, especially the carbon source, as reported by Vanavil et al. (2013) It was reported that the concentration of rhamnolipids obtained when growing the strain in glucose was 88 mg/L, and 15-fold higher when they added Mg$^{2+}$ and Fe$^{2+}$ to the media. In the case of this study, it is still needed to find conditions for the improvement of the biosurfactant production.

The crude extracts of strain PAM8 showed high activity against *B. subtilis*, *S. aureus*, *S. paucimobilis* and *K. pneumoniae*. These results are consistent with Bharali et al. (2013) where the antibacterial activity of the *P. aeruginosa* OBP1 rhamnolipids on *S. aureus* and *K. pneumoniae* was shown. The rhamnolipids produced by *P. putida* 21BN against *B. subtilillus* (Tuleva et al., 2002). Another study showed the effect of the biosurfactant produced by *P. koreensis* M9b on Grampositive bacteria (Toribio et al.,

Biosurfactants produced by metal-resistant _Pseudomonas aeruginosa_ isolated from _Zea mays_ rhizosphere and compost

(2011). The antibacterial producers included in the CLSI (Clinical and Laboratory Standards Institute) to date are biosurfactants belonging to the class glycolipids and cyclic lipopeptides; for this reason, the biosurfactants produced by our isolates represent good candidates as antibacterial products.

The colonies showed fluorescence and were hyper-mucoid in presence of Pb, Zn and Fe. This was probably in response to the stress they underwent, since the exopolysaccharides have anionic functional groups as phosphates, sulfates, and carboxilic acids that bind to metals, sequestering them and avoiding the interactions of the metal inside the cell. Fluorescence was caused by the production of siderophores that could be related to the presence of specific metals in the soils that our isolates came from. Siderophores as the pio- verdine and piochelin are able to bind Fe, Cd, Cu and Zn, reducing their toxicity for bacteria (Dao et al., 1999; Dimkpa et al., 2009).

These data agree with Raja et al. (2006), who reported on a strain of _P. aeruginosa_ FC1 being capable of growing in 800 ppm of Pb, 700 ppm of Ni, 500 ppm of Cd and 400 ppm of Cr. On the other hand, studies conducted by Hassan et al. (2008) showed bacterial resistance to heavy metals in contaminated soils in Egypt. However, 89% of the isolates were inhibited by 3.5 mM of the metal, which represents a lower concentration than our isolates could resist. Bodour et al. (2003) This shows the production of associated biosurfactants with resistance to heavy metals, which is similar to the yield of this study.

4. CONCLUSIONS

Seven endemic strains of _P. aeruginosa_ were successfully isolated from the rhizosphere from pure breed _Zea mays_ and compost. The strains were able to produce rhamnolipid and has antimicrobial activity against Grampositive and Gramnegative bacteria. Moreover, they showed resistance to heavy metals. Strains PAM8 and PAM9, are the best candidates to use them for bioremediation and other biotechnological applications.

**AUTHOR’S CONTRIBUTION**

First author: Methodology, research, data analysis, conceptualization, writing, original draft. Second author: Research, conceptualization, data analysis, writing, review and editing. Third author: Logistics, review and edition. Fourth author: data analysis, review and editing. Fifth author: Resource acquisition, project manager, supervision, conceptualization, writing, review and editing.

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**LITERATURE CITED**


