# Simultaneous production of lipopeptide and rhamnolipid biosurfactants by *Pseudomonas aeruginosa*: A promising blend for biosurfactant-enhanced bioremediation

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## HIGHLIGHTS

- *Pseudomonas aeruginosa* MM and ML were isolated as diesel-degrading surfactant-producing strains.
- *P. aeruginosa* MM synthesizes rhamnolipids and lipopeptides simultaneously
- This surfactant blend enhances diesel degradation in soil microcosm assays

## ABSTRACT

Oil contamination is an environmental issue worldwide, and bioremediation has emerged as a preferred strategy to address this challenge. One of the limiting factors in oil biodegradation is its bioavailability, which can be solved by the addition of tensioactive compounds. Given the increasing demand for environmentally friendly surfactants, this study aimed to obtain cell-free biosurfactant extracts as additives in surfactant enhanced remediation (SER) protocols. Diesel-degrading, surfactant-producing strains were isolated to obtain biosurfactant extracts with minimal post-production purification cost. The most

promising extracts were tested for SER assays using diesel-contaminated microcosms. One of these showed a significant enhancement in diesel degradation compared with the controls. This extract was obtained from a *Pseudomonas aeruginosa* strain and consisted of a blend of rhamnolipids and lipopeptides. This is the first work reporting the co-production of both kinds of surfactants by a *P. aeruginosa* strain and its potential for application in surfactant-enhanced bioremediation strategies.

**Keywords: biosurfactants, microcosms, soil, bioremediation, biostimulation, diesel, *Pseudomonas aeruginosa***

## 1. INTRODUCTION

Hydrocarbon contamination is an environmental threat due to its impact on human and ecosystem health (Sakhaei and Riazi, 2022). Different remediation techniques have been proposed to mitigate the impact of oil and its derivatives on the environment. Among these, bioremediation—using living organisms to degrade contaminants—has emerged as one of the preferred strategies due to its cost-effectiveness and minimal environmental impact. But, despite the biodegradability of hydrocarbons, their limited bioavailability represents a challenge to bioremediation. To address this, the use of surfactant in biostimulation protocols was proposed, leading to the development of Surfactant Enhanced Remediation (SER) (Mulligan et al., 2001). Chemical surfactants, produced by industrial synthesis, have been shown to be effective in SER protocols, but also produce negative effects on the environment including the alteration of soil microbial community and soil enzymatic activity (Badmus et al., 2021). On the other hand, microbial surfactants or biosurfactants (BS), are less toxic, biodegradable, chemically versatile, and stable at a broad range of pH and temperature (Kashif et al., 2022). Biosurfactants are amphipathic molecules consisting of a hydrophobic moiety like fatty acid or their derivatives and a hydrophilic moiety that could be carboxylic acids, amino acids, carbohydrates, alcohols, or peptides (Thavarsi and Banat, 2019).

Among microbial surfactants, lipopeptides and glycolipids are the most commonly studied (Hayes et al., 2019). Bacterial lipopeptides surfactants are synthesized by nonribosomal peptide synthetases (NRPSs) - a multidomain enzyme that synthesizes oligopeptides without the ribosomal system - leading to the formation of a wide variety of lipopeptides (Hayes et al., 2019;  Yang et al., 2020; Chen et al., 2023). Those linear or cyclic oligopeptides are then acylated with different fatty acid moieties. These compounds are commonly secreted by bacterial genres such as *Bacillus (*surfactins, fengycins, and iturins), *Streptomyces* (daptomycin) and *Pseudomonas syringae and fluorecens* complex (Viscosin, Amphisin, Tolaasin, and Syringomycin) (Hayes et al., 2019; Chauhan et al., 2023; Zhang et al., 2023). LPs are known for their strong pseudo-solubilization properties and have found applications in environmental protection, industry, and medicine, including antimicrobial

and antitumor agents (Johnson et al., 2021). Between the glycolipids surfactants, the most studied group is rhamnolipids, particularly those produced by *Pseudomonas aeruginosa*. The biosynthesis of rhamnolipids involves three key enzymes: RhlA and RhlB, involved in mono-rhamnolipids synthesis, and RhlC which adds a second rhamnose to produce di-rhamnolipids. *rhlA* and *rhlB* genes are located on a single operon regulated by *rhlI* and *rhlR* genes. The third gene- *rhlC*- is also under the control of the *rhlI/R* system but is located in a different locus on the *P. aeruginosa* genome (Sarubbo et al., 2022).
In SER assays, biosurfactants have been shown to be more effective than synthetic ones, enhancing oil-in-water pseudo-solubilization while maintaining soil's biological functions and diversity (Zhuang et al., 2022). Nevertheless, BS production costs make them less competitive than synthetic surfactants, especially because of the costs of the used carbon sources like glucose and glycerol, and the purification process after fermentation (Eras-Muñoz et al., 2022). Surfactant post-fermentation production implies several purification steps, including cell-free supernatant acidification, crude product extraction, concentration, and several purification steps like ion exchange, adsorption-desorption, and preparative chromatographies (Invally et al., 2019; Varjani and Upasani, 2017). To avoid extra production costs, partially purified biosurfactants were used in several SER assays (Eras-Muñoz et al., 2022). Given this approach and the increasing demand for new biosurfactants (Jiang et al., 2020), the objective of this work was to obtain biosurfactant crude extracts composed of different compounds to be used in SER protocols.

## 2. Materials and methods

### 2.1. Strain isolation and cultivation

Diesel-degrading, surfactant-producing bacteria were isolated from an urban stream located in the neighborhood of an industrial waste treatment plant in Moreno district, Buenos Aires Province, Argentina (34º 34’ 50.9’’ S, 58º 49’ 25.6’’ W). Water samples (10 ml) were supplemented with 10% v/v filtered sterile diesel, 1% mM $MgSO_4.7H_2O$, and 1% MT solution (Lageeven et al., 1988). When necessary, yeast extract (0,2 or 0,02% w/v) was added. Samples were incubated in 100 ml capped bottles at 30°C and 250 rpm until

turbidity development, then 100 µl of each culture were inoculated in 10 ml of E2 minimum media (Lageeven et al., 1988) supplemented with 10% v/v diesel as the sole carbon source, and incubated at 30°C and 250 rpm during 7 days. Cultures that developed visible and stable emulsion at the diesel-medium interphase, were selected and plated onto Luria Broth (LB) agar plates. Isolated strains, showing different colony morphology were selected and stored at -80°C until use.

For biosurfactant production, 2 to 5 colonies were inoculated in 10 ml LB medium and incubated overnight at 30°C and 150 rpm. This seed culture was used to inoculate 100 ml of E2 medium supplemented with diesel (10%v/v), glucose (1% w/v), or raw sunflower oil (10% v/v) as the sole carbon source, at an $OD_{600}$ of 0.05. Cultures were incubated at 30°C and 250 rpm from 24hs to 7 days.

## 2.2. 16S rRNA gene analysis

Strain classification was performed by sequencing the *16S rRNA* gene. DNA from each pure culture was obtained using EasyPure Genomic DNA kit (TransGene Biotech) following the manufacturer's instructions. *16S rRNA* gene was sequenced (Macrogen-Korea) using 337F and 907R universal primers. To determine phylogenetic relationships, BLAST platform was used (Altschul et al., 1990).

A phylogenetic tree was made with seventeen type species of *Pseudomonas* genre and *Escherichia/Shigella coli ATCC 11775T X80725* was selected as an outgroup, utilizing Neighbor-Joining method and Bootstrap with 1000 repeats to estimate the tree (using the program MEGA 11 (Tamura et al., 2021).

## 2.3. Biosurfactant production

For biosurfactant crude extract (BCE) obtention, each strain was cultured as described, and cell-free supernatant was obtained by centrifugation at 10000 rpm for 5 min. The supernatant was collected, acidified to pH 2 with 6 N HCl, incubated at 4°C overnight, and then centrifuged at 10000 rpm for 10 minutes. The obtained pellet was resuspended in Tris HCl 0.1mM pH 8 buffer and extracted three times with one volume of ethyl acetate.

This BCE was then concentrated with a rotavap (Büchi 461 Water Bath) at 45°C and the remnant solid was resuspended in distilled water (2 ml for each 10 ml of initial cell-free supernatant) and conserved at -20 °C for posterior analysis.
As per rhamnolipids standards, a cell-free supernatant from *P. aeruginosa* PAO1 was extracted as described above.

## 2.4.Biosurfactant characterization

### 2.4.1. Emulsifying activity

The emulsification index (EI24) was determined according to Copper and Goldenberg (1987). Briefly, 5 ml of n-hexane (MERCK) was added to the same volume of cell-free supernatant, vigorously agitated by vortex for 2 minutes, and left to stand for 24 h. EI24 was defined as follow:

$$EI24\ (\%) = (H/T)*100\%$$

where H is the height of the emulsion phase, and T is the total height measured in cm. As a negative control, a culture medium without inoculation was used. All measurements were run in triplicate.

### 2.4.2. Drop collapse test

In the drop collapse test, 20 µl of cell-free supernatant was mixed with 1ul methylene blue 0,1% v/v. As a control, 20 µl of culture uncultured media was used. A 15 ul drop was placed on a hydrophobic surface (parafilm) and the contact angle was measured using ImageJ 1x software (Schneider et al., 2012). Relative contact angle (RCA) was defined as follows:

$$RCA\ (\%): (cai *100\%)/ cmca$$

where the contact angle of the isolates (cai) was relative to the contact angle of the culture media (cmca, as control), as the total percentage (100%).

### 2.4.3. Critical micelle concentration of the crude extract

To obtain CMC values, the compound mass present in 10 ml of BCE was calculated as dry weight after lyophilization (Duvnjak et al., 1982). To determine the surface tension (ST)+, a Du Nouy tensiometer (Cenco Du Nouy 70545) was used.

2.4.4 Analysis of the chemical nature of the putative surfactants preset in BCE

BCE was analyzed using thin-layer chromatography (TLC) using chloroform: methanol: acetic acid (65:15:2) as the mobile phase. TLCs were developed with Ninhydrin reagent to detect amino acids and peptides (Baranowska and Kozłowska, 1995) and Molisch reactive (α-naphthol and $H_2SO_4$) for detecting glycosidic moieties (Taki et al., 1985). ImageJ 1X software was used to analyze the TLC spots (Schneider et. al, 2012).

## 2.5. SER assays

Soil samples were obtained from the vicinity of the industrial waste treatment plant (34° 34' 50.7'' S, 58° 49' 27.2'' W). Soil was homogenized and kept in polyethylene black bags at 15°C until its posterior use. When necessary, part of the homogenized soil was sterilized by tyndallization as described previously (MacRae et al., 1967).

For microcosm construction, sterile (S) or non-sterile (NS) soil was supplemented with $KNO_3$ (2 g of N/kg of dried soil) and $K_2HPO_4$ (450 mg of P/kg of dried soil), adjusted to 60% field capacity, and artificially contaminated with 10% V/W diesel. Each experimental unit (ExU) consisted of the equivalent of 10 g of dried soil in a 45 mm Petri dish. Different sets of 5 ExUs each were designed using S or NS soils. When necessary, BCE was added as twice the CMC relative to the water present in the ExU.

Microcosms were incubated at 24°C for 30 days with weekly agitation to enhance aerobic diesel degradation. After this time, each experimental unit was transferred into a 100 ml glass bottle, extracted with 30 ml of n-hexane: acetone 1:1, and incubated for 4 hrs at room temperature and 180 rpm. Two ml of the organic phase were collected and centrifuged at 12.000 g for 10 min. The clear supernatant was analyzed in a gas chromatography (Agilent 7820A GC–FID with an automatic sampler ALS 7693) using an Agilent HP-5 capillary column (30 m, 0.25 µm de weight and 0.32 mm ID) as described

previously (Collonella et al., 2019). n-octane (Merck), with a retention time of approximately 6.7 minutes, was used as the internal control.

2.6. Genome annotation and bioinformatics analysis

The genome sequence was obtained by Illumina NovaSeq PE150 (Novegen inc). De Novo assembly was done using SOAP De Novo software, SPAdes software and Abyss software. The results of the three software were integrated with CISA software obtaining the 79 scaffolds. Draft genome sequence was deposited in Genebank AN: JAMWSM000000000
DNA-DNA in-silico hybridization was done using Type Strain Genome Server (https://tygs.dsmz.de) with the default setting. Biosurfactant synthesis-related genes were mined using the seed viewer platform (Overbeek et al., 2005), antismach 7.0 bacterial version with the default settings (at https://antismash.secondarymetabolites.org), and PRISM 4 (https://prism.adapsyn.com)

2.7. Statistical analysis

For the comparison between the two categories, t of Student was used. For comparison between more than two categories, ANOVA test was used. Models with and without interaction (additive) were analyzed and compared with the Akaike information criterion (AIC). The significance level used was 5%. If necessary, multiple comparisons were done with the Tukey test and Bonferroni correction. Statistical analyses were carried out using R software (R Development Core Team, 2024).

**3. RESULTS**

3.1. Characterization of the diesel-degrading, biosurfactant-producing bacteria.

Five strains were isolated as described and labeled as MM, ML, B, V, and R (Supplementary Material S1.). To analyze their capability to produce biosurfactants using different carbon sources, E2 medium supplemented with diesel (d), glucose (g), or

sunflower oil (so) was inoculated with each strain, and the cell-free supernatant was obtained. MM and ML strains showed the lowest reduction in the relative contact angle (31, 38, and 35 for MM, and 31, 33, and 47 for ML, for diesel, sunflower oil, and glucose as carbon sources, respectively). They also showed higher EI24 values for the three tested carbon sources (32, 47, and 45 for MM, and 65, 33, and 50 for ML, for diesel, sunflower oil, and glucose as carbon sources, respectively) (Fig. 1, Supplementary Material Fig. 2.). Therefore, both strains were selected for further studies.

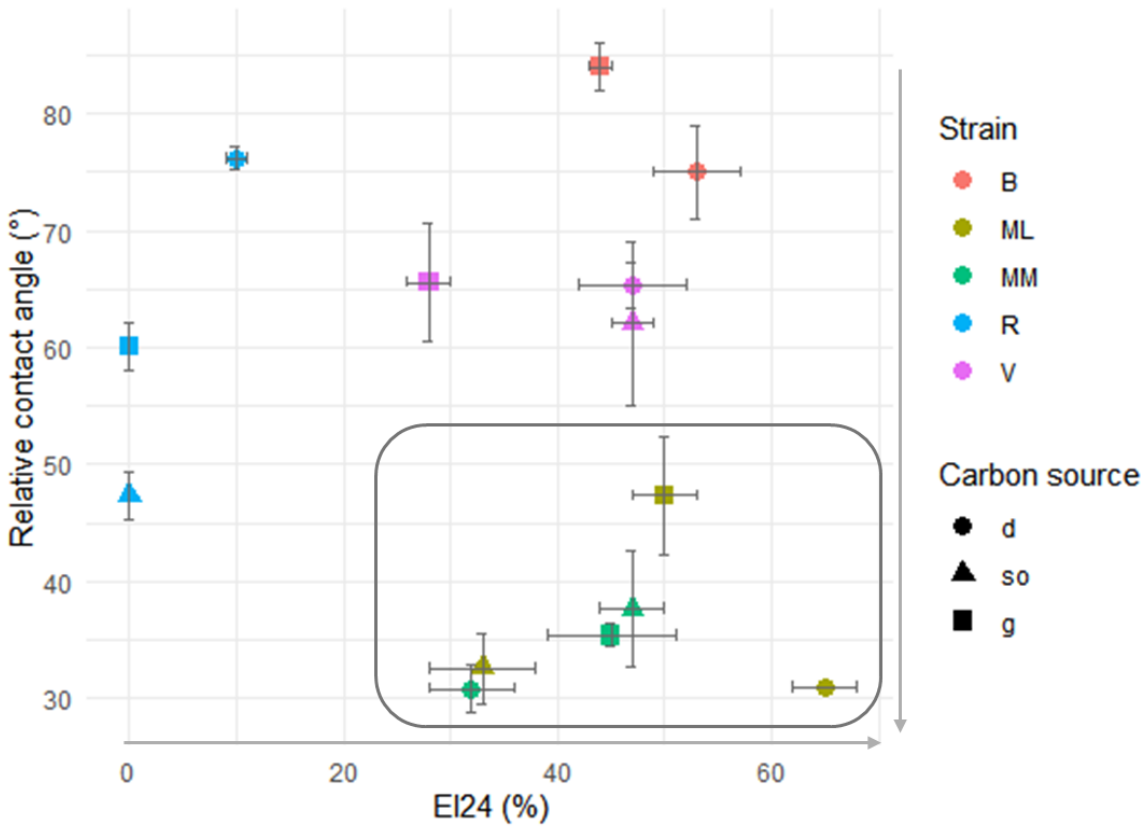



**Figure 1-** Relative contact angle values and IE24 (%) for the different isolates grown on three carbon sources (d: diesel, so: sunflower oil, g: glucose). Bars represent the standard error of three replicates. Arrows indicate the desired outcome for an effective surfactant, and the square highlights the crude extracts with the best properties.

MM and ML strains were Gram-negative, oxidase-positive, and catalase-positive. 16S rRNA gene sequencing and phylogenetic analysis showed that both are related to the *Pseudomonas aeruginosa* species (Fig 2). BLAST comparison revealed that MM presented 99.26% identity with *Pseudomonas aeruginosa* P2 (MF289196.1) while ML showed 98.98% with *Pseudomonas aeruginosa* DSE2 (H1457018.1).

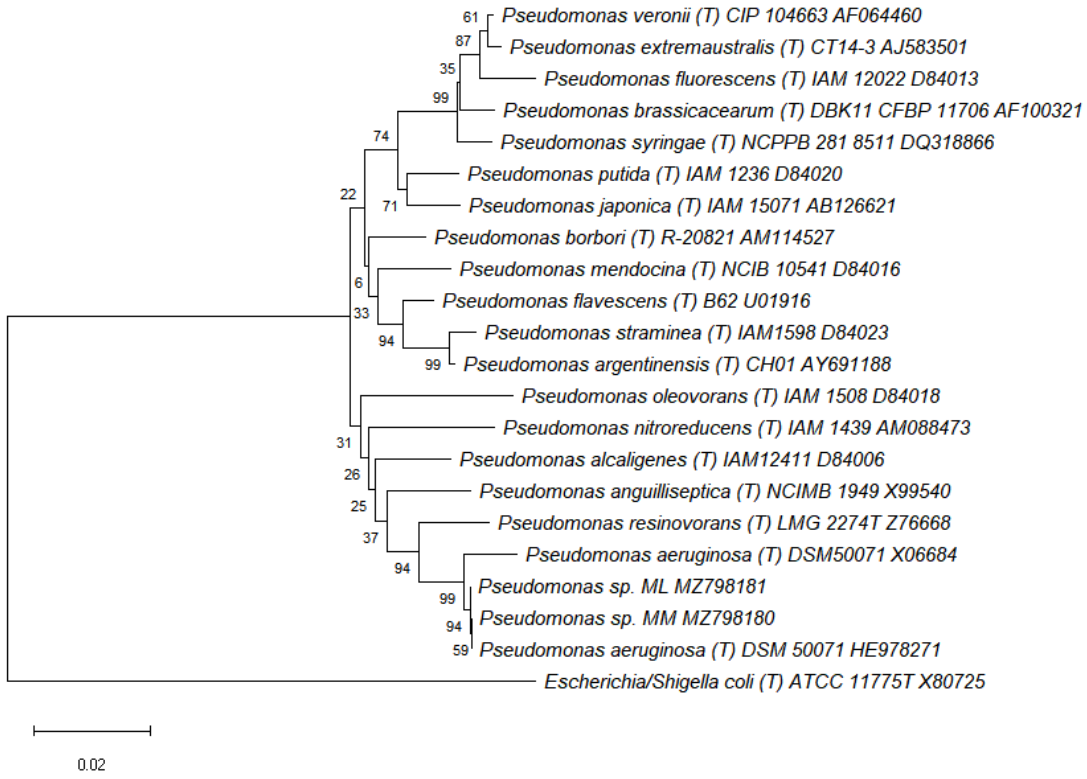



**Figure 2** - NJ phylogenetic tree consensus (Bootstrap 1000 iterations) of 16s rRNA sequences compared with type (T) *Pseudomonas* species. The sequence accession numbers are located on the right side.

## 3.2. Obtaining and characterization of Biosurfactant Crude Extract

As described in Fig. 1, and Supplementary material Fig. 1 and 2, as MM and ML strains were able to produce surface-active compounds using different carbon sources. For BCE production, raw sunflower oil was chosen.

To analyze the chemical nature of the produced biosurfactant, MM-BCE and ML-BCE were assayed by thin-layer chromatography. Both BCE showed two spots with similar RF to *P. aeruginosa* PAO1 mono- and di-rhamnolipids, having an Rf of 0.88 and 0.47, respectively. Interestingly, MM-BCE also showed spots with aminoacidic moieties characteristics (Fig. 3, Rf = 0.26). The di/mono rhamnolipid ratio (Drh/Mrh) obtained by TLC analysis were: MM-BCE= 2.21, ML-BCE= 0.88, PAO-BCE=5.12.

Critical micelle concentration (CMC) of MM-BCE and ML-BCE were assayed. MM-BCE showed a CMC of 317±11 ug/ml, reducing the surface tension from 72 dyn/cm to 33,5 ± 0,1 while The CMC of ML-BCE was 612±8 ug/ml producing an ST reduction from 72 dyn/cm to 33,7 ± 0,2.

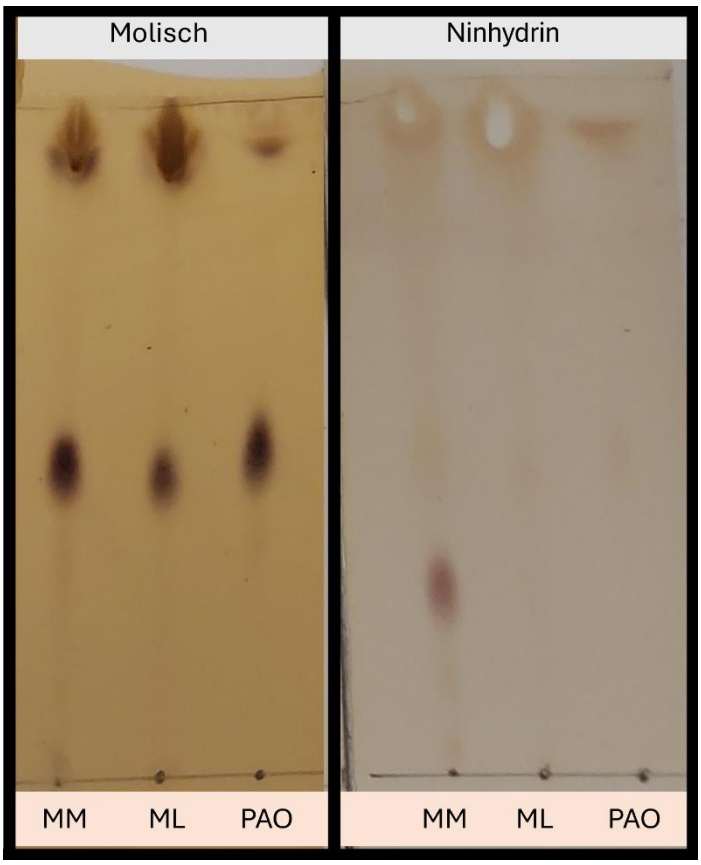



**Figure 3** - TLC for MM-BCE, ML-BCE, and PAO-BCE, developed with Molisch (glycolipid compounds) and Ninhydrin (peptidic compounds).

## 3.3. Surfactant-enhanced remediation assay

To analyze the effect of MM-BCE and ML-BCE in diesel degradation, 6 microcosm sets were used (Table 1).

**Table 1** - Description of the microcosm sets used to analyze the effect of MM and ML BCEs on diesel degradation. The table includes the tested characteristics, type of soil, and BCE added for each microcosm set.

| Microcosm set | Characteristic tested | Soil | BCE added |
|---|---|---|---|
| S1 | Abiotic degradation control | S | - |
| S2 | Effect of MM-BCE on abiotic degradation | S | MM |
| S3 | Effect of ML-BCE on abiotic degradation | S | ML |
| NS1 | Biotic degradation control | NS | - |
| NS2 | Effect of MM-BCE on biotic degradation | NS | MM |
| NS3 | Effect of ML-BCE on biotic degradation | NS | ML |

After 30 days, the remaining diesel in all S microcosms was higher than in NS microcosms (Fig 4). No significant differences were detected among the treatments in S microcosms. When MM-BCE was added to NS, the remaining hydrocarbon decreased by 47% compared with the control ($P < 0.0001$, Fig. 4). On the other hand, ML-BCE showed a slight but non-significant decrease in the remaining diesel (13%). As expected, no differences were observed when the crude extract was added to S microcosms.

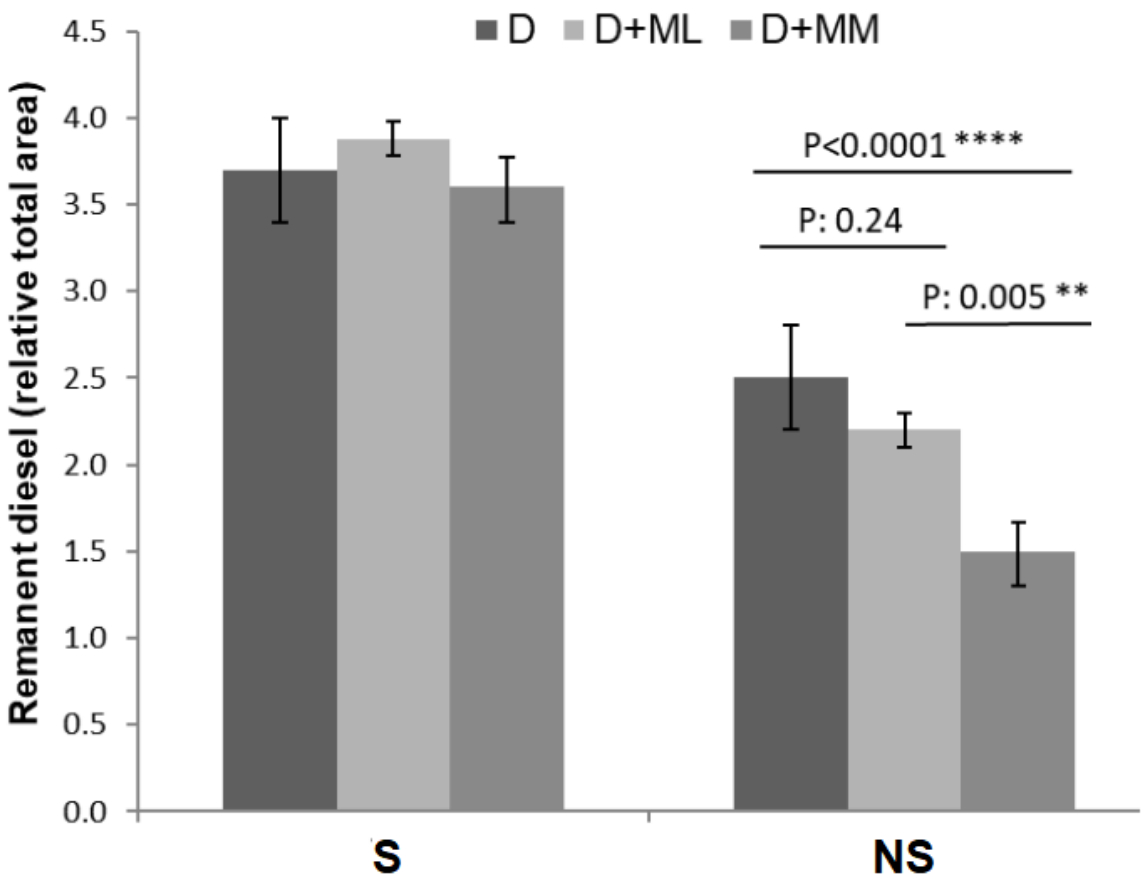



**Figure 4** - Remanent diesel (relative total area) in the S and NS microcosms with the three treatments: D for only diesel, D+MM for diesel with CEB MM, D+ML for diesel with CEB ML.

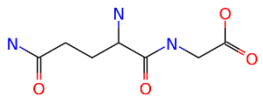
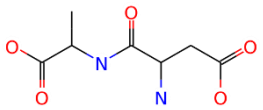
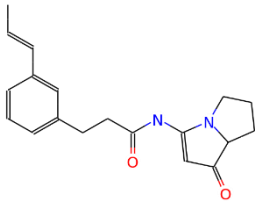
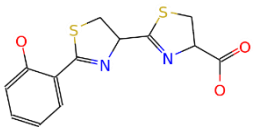
## 3.4. Bioinformatic analysis of MM strain

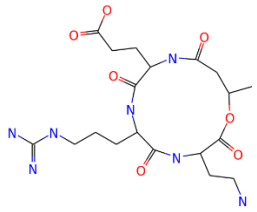
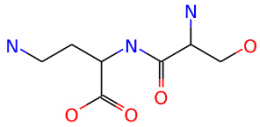
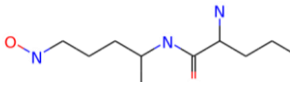
Due to the MM strain showing an unexpected blend of biosurfactants with promising use in SER approaches, its draft genome sequence was obtained. It showed that the MM strain has a dDDH value of 84.2 [CI: 80.4 - 87.3] with *P. aeruginosa* DSM 50071 confirming it belongs to *P. aeruginosa* group.

The genome analysis showed the presence of the *rhl*ABRI operon in scaffold 54 with an identity of 99% with *rhlABRI* operon of *P. aeruginosa* PAO1 and *rhl*C gene in scaffold 6 with an identity of 100% with rhlC of *P. aeruginosa* PAO1.

To search for putative NRPS that could be related to the synthesis of the lipopeptidic compound obtained in the CE, the draft genome sequence was also analyzed using Antismash and Prims 4 programs, detecting 8 putative NRPS in scaffolds 7, 15, 20, 31, 41,48, and 57 (Supplementary Material Antismash Results).

**Table 2** - Description of scaffolds found in MM strain. The table includes the putative compound, the MIBig similarity score and the predicted structure.

| Scaffold (orf/s) | Putative compound | MiBig similarity score | Predicted structure (prims 4) |
|---|---|---|---|
| 7 (137) | - | - | - |
| 15 (1) (partial) | Pseudomonine | 0.52 |  |
| 15 (99-102) | L-2-amino-4-methoxy-trans-3-butenoic acid | 0.82 |  |
| 20 (37) | Azetidomonamide | 0.89 |  |
| 33 (11-12) | Pyochelin | 1 |  |

| | | | |
|---|---|---|---|
| 41 (17) | Syringafactin | 0.28 |  |
| 48 (3) | Enterobactin | 0.27 |  |
| 57 (2) | Thermoactinoamide | 0.3 |  |

**4. DISCUSSION**

In this study, we obtained two diesel-degrading bacteria, out of 5 isolates, capable of synthesizing different biosurfactant compounds. It has been previously described that hydrophobic carbon source increases biosurfactant production yield (Eslami et al., 2020; de Oliveira Schmidt et al., 2021). Because of that, we used diesel or raw sunflower oil as carbon sources for biosurfactant production. However, we finally selected sunflower oil as the carbon source to avoid the downstream purification of undesired toxic compounds from the crude biosurfactant extract.

Both selected strains belonged to the *Pseudomonas aeruginosa* species assigned by 16S rRNA gene sequencing. TLC analysis of the BCEs obtained from *P. aeruginosa* MM and ML displayed the two characteristic spots corresponding to mono- and di-rhamnolipids congeners where the amount of di-rhamnolipids was always higher than that of mono-rhamnolipids. The difference in the RFs from the rhamnolipids spots of MM-BCE, ML-BCE, and PAO-BCE could be related to different compound congeners present in the mono- and di-rhamnolipids pool (El-Huesseiny et al., 2020). Our results also showed that MM-BCE

presented a higher di- to mono-rhamnolipids ratio than ML-BCE. Although it was described that di-rhamnolipids have stronger surface activity while mono-rhamnolipids have better emulsifying activity (Zhao et al., 2018), we observed that both MM-BCE and ML-BCE lowered the ST to very similar levels. Interestingly, MM-BCE also presented a spot detected by ninhydrin staining, suggesting a surfactant compound of a peptidic nature. Lipopeptides are scarcely documented in *Pseudomonas aeruginosa* strains, and in the few described cases (Hu et al., 2023; Liu et al., 2018; Bezza and Chirwa, 2016a, 2016b), species assignment was based on 16S rRNA gene sequencing. Here, we present for the first time a *Pseudomonas aeruginosa* strain confirmed by DNA-DNA hybridization that simultaneously produces rhamnolipids and a putative lipopeptide surfactant. The analysis of the draft genome of *P. aeruginosa* MM confirmed its capacity to produce mono- and di-rhamnolipids, as well as a putative syringafactin synthase codified in scaffold 41. Syringafactin is one of the 4 kinds of lipopeptides surfactants described in the *Pseudomonas* genre (Chauhan et al., 2023), however, to the best of our knowledge, this has not been previously described in *P. aeruginosa*.

The use of surfactants with diverse characteristics has demonstrated positive effects in previous works. For instance, Cheng et al. (2013) showed that a blend of glycolipid and lipopeptide produced by *B. subtilis* strain achieved 86% oil-washing efficiency. Their findings revealed that the lipopeptide primarily contributes to stability and oil-washing efficiency, while the glycolipid plays a greater role in enhancing emulsifying activity.

To compare the surfactant performance, one of the key parameters to analyze is the critical micelle concentration (CMC) (Kim and Vipulanandan, 2006). The CMC varies depending on the set of compounds produced, the carbon source, the bacterial species, and the degree of surfactant purification (De Oliveira Schmidt et al., 2021). It was described that in a mixture of surfactants, the CMC could be influenced by the synergism or antagonism between its compounds (Mandavi et al., 2008), being synergism the effect most frequently seen (Rosen and Kunjappu 2012). As a result, different types of surfactants are used in many industrial products and processes instead of pure components (Shah et al., 2021, Zhou et al., 2019 , Singh et al. 2023)

In this work, MM-BCE showed a lower CMC compared to ML-BCE. This difference could be attributed to several factors: the differences between mono- and di-rhamnolipids ratios and/or in biosurfactant composition, especially the presence of the putative lipopeptide surfactant detected. The obtained CMC values for MM-BCE and ML-BCE are consistent with the ones reported previously (Bordas et al., 2005, Pornsunthorntawee et al., 2008, Silva et al., 2010). When compared with synthetic surfactants the CMC of MM-BCE was approximately seven folds smaller than SDS (Valsaraj et al 1988). Finally, the EI24 of MM-BCE and ML-BCE were similar to those previously reported (Pourfadakari et al., 2021). Regarding surface tension reduction, both MM and ML achieved a value of 33 mN/m which is similar to what is found in the literature regarding different *P. aeruginosa* strains (Yin et al., 2009; Rikalovic et al., 2013; Costa et al., 2010)

Microcosm SER assay showed that MM-BCE and ML-BCE addition increased the rate of hydrocarbon degradation but with different effectiveness. It was previously described that different mixtures of surface-active compounds may have different capabilities to enhance the remediation of hydrophobic pollutants (Mendes et al., 2015). In fact, Szulc et al (2014) found no significant effect when commercial rhamnolipids or of 58.5-76.8 % (Olasanmi and Thring 2020) were used while Cameotra and Singh (2006) had a degradation of 95% using crude biosurfactants by *Pseudomonas aeruginosa* and *Rhodococcal* sp .

Regarding the use of lipopeptides surfactants for soil enhanced oil recovery (SER), Chaprão et al. (2015) reported a 10-20% increase in oil degradation when biosurfactants produced by *Bacillus* sp. and *C. sphaerica* UCP0995 were applied. Kavitha et al. (2014) showed that lipopeptidic surfactant from *Bacillus licheniformis* MTCC 5514 was found to remove over 85% of crude oil from contaminated soil. Cheng et al. (2013) reported that a blend of glycolipids and lipopeptides, including rhamnolipids, surfactin, and fengycin produced by *Bacillus subtilis* TU2, achieved 86% oil-washing efficiency and had excellent emulsification properties, highlighting its potential for SER.

The degradation rate also depends on the incubation time. For example, higher percentages (80-85%) were observed after 6 months (Szulc et. al 2014) while Akbari et al

(2021) reported about 10% of total hydrocarbon reduction after 30 incubation days and 20% after 80 days when rhamnolipids were added to microcosm assays. This suggests that the MM-BCE is effective and competitive, having a degradation rate of 47% after 30 days.

On the other hand, since diesel degradation in this study was conducted by autochthonous microbiota, the differences observed between the addition of MM-BCE and ML-BCE could be due not only to an increase in hydrocarbon bioavailability but also to how each BCE affected the bacterial community, especially the hydrocarbon-degrading taxa (Lu et al., 2019; Posada-Baquero et al., 2020; Wang et al., 2021a, Akbari et al., 2021 ).

## 5. CONCLUSION

In this work, we present for the first time a full sequenced *P. aeruginosa* strain capable of simultaneously synthesizing a blend of glycolipids and lipopeptides. A crude extract of this surfactant mix, with minimal post-production purification, resulted in significant diesel biodegradation compared to the controls in microcosm assays. These results represent a promising amendment to be use in the bioremediation of petroleum-contaminated sites and microbial-enhanced oil recovery (MEOR).

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Zhuang, X., Wang, Y., Wang, H., Dong, Y., Li, X., Wang, S., …, Wu, S., 2022. Comparison of the efficiency and microbial mechanisms of chemical-and bio-surfactants in remediation of petroleum hydrocarbon. Environ. Pollut. 314, 120198. https://doi.org/10.1016/j.envpol.2022.120198 **Simultaneous production of lipopeptide**

**and rhamnolipid biosurfactants by *Pseudomonas aeruginosa*: A promising blend for biosurfactant-enhanced bioremediation**

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**ABSTRACT**

Oil contamination is an environmental issue worldwide, and bioremediation has emerged as a preferred strategy to address this challenge. One of the limiting factors in oil biodegradation is its bioavailability, which can be solved by the addition of tensioactive compounds. Given the increasing demand for environmentally friendly surfactants, this study aimed to obtain cell-free biosurfactant extracts as additives in surfactant enhanced remediation (SER) protocols. Diesel-degrading, surfactant-producing strains were isolated to obtain biosurfactant extracts with minimal post-production purification cost. The most promising extracts were tested for SER assays using diesel-contaminated microcosms. One of these showed a significant enhancement in diesel degradation compared with the controls. This extract was obtained from a *Pseudomonas aeruginosa* strain and consisted of a blend of rhamnolipids and lipopeptides. This is the first work reporting the co-production of both kinds of surfactants by a *P. aeruginosa* strain and its potential for application in surfactant-enhanced bioremediation strategies.

**Keywords: biosurfactants, microcosms, soil, bioremediation, biostimulation, diesel, *Pseudomonas aeruginosa***

## 1. Introduction

Hydrocarbon contamination is an environmental threat due to its impact on human and ecosystem health (Sakhaei and Riazi, 2022). Different remediation techniques have been proposed to mitigate the impact of oil and its derivatives on the environment. Among these, bioremediation—using living organisms to degrade contaminants—has emerged as one of the preferred strategies due to its cost-effectiveness and minimal environmental impact. But, despite the biodegradability of hydrocarbons, their limited bioavailability represents a challenge to bioremediation. To address this, the use of surfactant in biostimulation protocols was proposed, leading to the development of Surfactant Enhanced Remediation (SER) (Mulligan et al., 2001). Chemical surfactants, produced by industrial synthesis, have been shown to be effective in SER protocols, but also produce negative effects on the environment including the alteration of soil microbial community and soil enzymatic activity (Badmus et al., 2021). On the other hand, microbial surfactants or biosurfactants (BS), are less toxic, biodegradable, chemically versatile, and stable at a broad range of pH and temperature (Kashif et al., 2022). Biosurfactants are amphipathic molecules consisting of a hydrophobic moiety like fatty acid or their derivatives and a hydrophilic moiety that could be carboxylic acids, amino acids, carbohydrates, alcohols, or peptides (Thavarsi and Banat, 2019).

Among microbial surfactants, lipopeptides and glycolipids are the most commonly studied (Hayes et al., 2019). Bacterial lipopeptides surfactants are synthesized by nonribosomal peptide synthetases (NRPSs) - a multidomain enzyme that synthesizes oligopeptides without the ribosomal system - leading to the formation of a wide variety of lipopeptides (Hayes et al., 2019;  Yang et al., 2020; Chen et al., 2023). Those linear or cyclic oligopeptides are then acylated with different fatty acid moieties. These compounds are commonly secreted by bacterial genres such as *Bacillus (*surfactins, fengycins, and iturins), *Streptomyces* (daptomycin) and *Pseudomonas syringae and fluorecens* complex (Viscosin, Amphisin, Tolaasin, and Syringomycin) (Hayes et al., 2019; Chauhan et al., 2023; Zhang et al., 2023). LPs are known for their strong pseudo-solubilization properties and have found applications in environmental protection, industry, and medicine, including antimicrobial

and antitumor agents (Johnson et al., 2021). Between the glycolipids surfactants, the most studied group is rhamnolipids, particularly those produced by *Pseudomonas aeruginosa*. The biosynthesis of rhamnolipids involves three key enzymes: RhlA and RhlB, involved in mono-rhamnolipids synthesis, and RhlC which adds a second rhamnose to produce di-rhamnolipids. *rhlA* and *rhlB* genes are located on a single operon regulated by *rhlI* and *rhlR* genes. The third gene- *rhlC*- is also under the control of the *rhlI/R* system but is located in a different locus on the *P. aeruginosa* genome (Sarubbo et al., 2022).
In SER assays, biosurfactants have been shown to be more effective than synthetic ones, enhancing oil-in-water pseudo-solubilization while maintaining soil's biological functions and diversity (Zhuang et al., 2022). Nevertheless, BS production costs make them less competitive than synthetic surfactants, especially because of the costs of the used carbon sources like glucose and glycerol, and the purification process after fermentation (Eras-Muñoz et al., 2022). Surfactant post-fermentation production implies several purification steps, including cell-free supernatant acidification, crude product extraction, concentration, and several purification steps like ion exchange, adsorption-desorption, and preparative chromatographies (Invally et al., 2019; Varjani and Upasani, 2017). To avoid extra production costs, partially purified biosurfactants were used in several SER assays (Eras-Muñoz et al., 2022). Given this approach and the increasing demand for new biosurfactants (Jiang et al., 2020), the objective of this work was to obtain biosurfactant crude extracts composed of different compounds to be used in SER protocols.

## 2. Materials and methods

### 2.1. Strain isolation and cultivation

Diesel-degrading, surfactant-producing bacteria were isolated from an urban stream located in the neighborhood of an industrial waste treatment plant in Moreno district, Buenos Aires Province, Argentina (34º 34’ 50.9’’ S, 58º 49’ 25.6’’ W). Water samples (10 ml) were supplemented with 10% v/v filtered sterile diesel, 1% mM $MgSO_4.7H_2O$, and 1% MT solution (Lageeven et al., 1988). When necessary, yeast extract (0,2 or 0,02% w/v) was added. Samples were incubated in 100 ml capped bottles at 30°C and 250 rpm until

turbidity development, then 100 µl of each culture were inoculated in 10 ml of E2 minimum media (Lageeven et al., 1988) supplemented with 10% v/v diesel as the sole carbon source, and incubated at 30°C and 250 rpm during 7 days. Cultures that developed visible and stable emulsion at the diesel-medium interphase, were selected and plated onto Luria Broth (LB) agar plates. Isolated strains, showing different colony morphology were selected and stored at -80°C until use.

For biosurfactant production, 2 to 5 colonies were inoculated in 10 ml LB medium and incubated overnight at 30°C and 150 rpm. This seed culture was used to inoculate 100 ml of E2 medium supplemented with diesel (10%v/v), glucose (1% w/v), or raw sunflower oil (10% v/v) as the sole carbon source, at an $OD_{600}$ of 0.05. Cultures were incubated at 30°C and 250 rpm from 24hs to 7 days.

## 2.2. 16S rRNA gene analysis

Strain classification was performed by sequencing the *16S rRNA* gene. DNA from each pure culture was obtained using EasyPure Genomic DNA kit (TransGene Biotech) following the manufacturer's instructions. *16S rRNA* gene was sequenced (Macrogen-Korea) using 337F and 907R universal primers. To determine phylogenetic relationships, BLAST platform was used (Altschul et al., 1990).

A phylogenetic tree was made with seventeen type species of *Pseudomonas* genre and *Escherichia/Shigella coli ATCC 11775T X80725* was selected as an outgroup, utilizing Neighbor-Joining method and Bootstrap with 1000 repeats to estimate the tree (using the program MEGA 11 (Tamura et al., 2021).

## 2.3. Biosurfactant production

For biosurfactant crude extract (BCE) obtention, each strain was cultured as described, and cell-free supernatant was obtained by centrifugation at 10000 rpm for 5 min. The supernatant was collected, acidified to pH 2 with 6 N HCl, incubated at 4°C overnight, and then centrifuged at 10000 rpm for 10 minutes. The obtained pellet was resuspended in Tris HCl 0.1mM pH 8 buffer and extracted three times with one volume of ethyl acetate.

This BCE was then concentrated with a rotavap (Büchi 461 Water Bath) at 45°C and the remnant solid was resuspended in distilled water (2 ml for each 10 ml of initial cell-free supernatant) and conserved at -20 °C for posterior analysis.
As per rhamnolipids standards, a cell-free supernatant from *P. aeruginosa* PAO1 was extracted as described above.

## 2.4.Biosurfactant characterization

### 2.4.1. Emulsifying activity

The emulsification index (EI24) was determined according to Copper and Goldenberg (1987). Briefly, 5 ml of n-hexane (MERCK) was added to the same volume of cell-free supernatant, vigorously agitated by vortex for 2 minutes, and left to stand for 24 h. EI24 was defined as follow:

$$EI24\ (\%) = (H/T)*100\%$$

where H is the height of the emulsion phase, and T is the total height measured in cm. As a negative control, a culture medium without inoculation was used. All measurements were run in triplicate.

### 2.4.2. Drop collapse test

In the drop collapse test, 20 µl of cell-free supernatant was mixed with 1ul methylene blue 0,1% v/v. As a control, 20 µl of culture uncultured media was used. A 15 ul drop was placed on a hydrophobic surface (parafilm) and the contact angle was measured using ImageJ 1x software (Schneider et al., 2012). Relative contact angle (RCA) was defined as follows:

$$RCA\ (\%): (cai *100\%)/ cmca$$

where the contact angle of the isolates (cai) was relative to the contact angle of the culture media (cmca, as control), as the total percentage (100%).

### 2.4.3. Critical micelle concentration of the crude extract

To obtain CMC values, the compound mass present in 10 ml of BCE was calculated as dry weight after lyophilization (Duvnjak et al., 1982). To determine the surface tension (ST)+, a Du Nouy tensiometer (Cenco Du Nouy 70545) was used.

2.4.4 Analysis of the chemical nature of the putative surfactants preset in BCE

BCE was analyzed using thin-layer chromatography (TLC) using chloroform: methanol: acetic acid (65:15:2) as the mobile phase. TLCs were developed with Ninhydrin reagent to detect amino acids and peptides (Baranowska and Kozłowska, 1995) and Molisch reactive (α-naphthol and $H_2SO_4$) for detecting glycosidic moieties (Taki et al., 1985). ImageJ 1X software was used to analyze the TLC spots (Schneider et. al, 2012).

## 2.5. SER assays

Soil samples were obtained from the vicinity of the industrial waste treatment plant (34° 34' 50.7'' S, 58° 49' 27.2'' W). Soil was homogenized and kept in polyethylene black bags at 15°C until its posterior use. When necessary, part of the homogenized soil was sterilized by tyndallization as described previously (MacRae et al., 1967).

For microcosm construction, sterile (S) or non-sterile (NS) soil was supplemented with $KNO_3$ (2 g of N/kg of dried soil) and $K_2HPO_4$ (450 mg of P/kg of dried soil), adjusted to 60% field capacity, and artificially contaminated with 10% V/W diesel. Each experimental unit (ExU) consisted of the equivalent of 10 g of dried soil in a 45 mm Petri dish. Different sets of 5 ExUs each were designed using S or NS soils. When necessary, BCE was added as twice the CMC relative to the water present in the ExU.

Microcosms were incubated at 24°C for 30 days with weekly agitation to enhance aerobic diesel degradation. After this time, each experimental unit was transferred into a 100 ml glass bottle, extracted with 30 ml of n-hexane: acetone 1:1, and incubated for 4 hrs at room temperature and 180 rpm. Two ml of the organic phase were collected and centrifuged at 12.000 g for 10 min. The clear supernatant was analyzed in a gas chromatography (Agilent 7820A GC–FID with an automatic sampler ALS 7693) using an Agilent HP-5 capillary column (30 m, 0.25 µm de weight and 0.32 mm ID) as described

previously (Collonella et al., 2019). n-octane (Merck), with a retention time of approximately 6.7 minutes, was used as the internal control.

### 2.6. Genome annotation and bioinformatics analysis

The genome sequence was obtained by Illumina NovaSeq PE150 (Novegen inc). De Novo assembly was done using SOAP De Novo software, SPAdes software and Abyss software. The results of the three software were integrated with CISA software obtaining the 79 scaffolds. Draft genome sequence was deposited in Genebank AN: JAMWSM000000000

DNA-DNA in-silico hybridization was done using Type Strain Genome Server (https://tygs.dsmz.de) with the default setting. Biosurfactant synthesis-related genes were mined using the seed viewer platform (Overbeek et al., 2005), antismach 7.0 bacterial version with the default settings (at https://antismash.secondarymetabolites.org), and PRISM 4 (https://prism.adapsyn.com)

### 2.7. Statistical analysis

For the comparison between the two categories, t of Student was used. For comparison between more than two categories, ANOVA test was used. Models with and without interaction (additive) were analyzed and compared with the Akaike information criterion (AIC). The significance level used was 5%. If necessary, multiple comparisons were done with the Tukey test and Bonferroni correction. Statistical analyses were carried out using R software (R Development Core Team, 2024).

## 3. RESULTS

### 3.1. Characterization of the diesel-degrading, biosurfactant-producing bacteria.

Five strains were isolated as described and labeled as MM, ML, B, V, and R (Supplementary Material S1.). To analyze their capability to produce biosurfactants using different carbon sources, E2 medium supplemented with diesel (d), glucose (g), or

sunflower oil (so) was inoculated with each strain, and the cell-free supernatant was obtained. MM and ML strains showed the lowest reduction in the relative contact angle (31, 38, and 35 for MM, and 31, 33, and 47 for ML, for diesel, sunflower oil, and glucose as carbon sources, respectively). They also showed higher EI24 values for the three tested carbon sources (32, 47, and 45 for MM, and 65, 33, and 50 for ML, for diesel, sunflower oil, and glucose as carbon sources, respectively) (Fig. 1, Supplementary Material Fig. 2.). Therefore, both strains were selected for further studies.

MM and ML strains were Gram-negative, oxidase-positive, and catalase-positive. 16S rRNA gene sequencing and phylogenetic analysis showed that both are related to the *Pseudomonas aeruginosa* species (Fig 2). BLAST comparison revealed that MM presented 99.26% identity with *Pseudomonas aeruginosa* P2 (MF289196.1) while ML showed 98.98% with *Pseudomonas aeruginosa* DSE2 (H1457018.1).

## 3.2. Biosurfactant Crude Extract obtention and characterization

As described in Fig. 1, and Supplementary material Fig. 1 and 2, as MM and ML strains were able to produce surface-active compounds using different carbon sources. For BCE production, raw sunflower oil was chosen.

To analyze the chemical nature of the produced biosurfactant, MM-BCE and ML-BCE were assayed by thin-layer chromatography. Both BCE showed 2 spots with similar RF to *P. aeruginosa* PAO1 mono- and di-rhamnolipids, having an Rf of 0.88 and 0.47, respectively. Interestingly, MM-BCE also showed spots with aminoacidic moieties characteristics (Fig. 3, Rf = 0.26). The di/mono rhamnolipid ratio (Drh/Mrh) obtained by TLC analysis were: MM-BCE= 2.21, ML-BCE= 0.88, PAO-BCE=5.12.

Critical micelle concentration (CMC) of MM-BCE and ML-BCE were assayed. MM-BCE showed a CMC of 317±11 ug/ml, reducing the surface tension from 72 dyn/cm to 33,5 ± 0,1 while The CMC of ML-BCE was 612±8 ug/ml producing an ST reduction from 72 dyn/cm to 33,7 ± 0,2.

## 3.3. Surfactant-enhanced remediation assay

To analyze the effect of MM-BCE and ML-BCE in diesel degradation, 6 microcosm sets were used (Table 1) .

Table 1 - Description of the microcosm sets used to analyze the effect of MM and ML BCEs on diesel degradation. The table includes the tested characteristics, type of soil, and BCE added for each microcosm set.

| **Microcosm set** | **Characteristic tested** | **Soil** | **BCE added** |
|---|---|---|---|
| S1 | Abiotic degradation control | S | - |
| S2 | Effect of MM-BCE on abiotic degradation | S | MM |
| S3 | Effect of ML-BCE on abiotic degradation | S | ML |
| NS1 | Biotic degradation control | NS | - |
| NS2 | Effect of MM-BCE on biotic degradation | NS | MM |
| NS3 | Effect of ML-BCE on biotic degradation | NS | ML |

After 30 days, the remaining diesel in all S microcosms was higher than in NS microcosms (Fig 4). No significant differences were detected among the treatments in S microcosms. When MM-BCE was added to NS, the remaining hydrocarbon decreased by 47% compared with the control ($P < 0.0001$, Fig. 4). On the other hand, ML-BCE showed a slight but non-significant decrease in the remaining diesel (13%). As expected, no differences were observed when the crude extract was added to S microcosms.

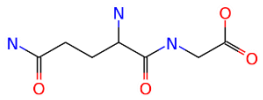
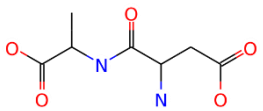
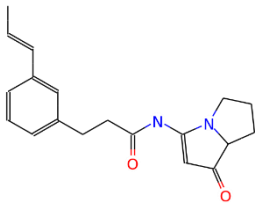
3.4. Bioinformatic analysis of MM strain

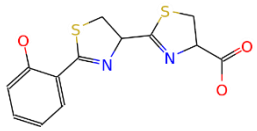
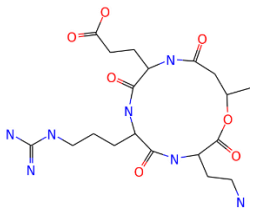
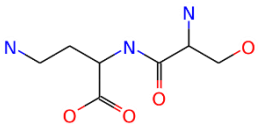
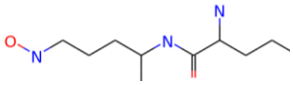
Due to the MM strain showing an unexpected blend of biosurfactants with promising use in SER approaches, its draft genome sequence was obtained. It showed that the MM strain has a dDDH value of 84.2 [CI: 80.4 - 87.3] with *P. aeruginosa* DSM 50071 confirming it belongs to *P. aeruginosa* group.

The genome analysis showed the presence of the *rhl*ABRI operon in scaffold 54 with an identity of 99% with *rhlABRI* operon of *P. aeruginosa* PAO1 and *rhl*C gene in scaffold 6 with an identity of 100% with rhlC of *P. aeruginosa* PAO1.

To search for putative NRPS that could be related to the synthesis of the lipopeptidic compound obtained in the CE, the draft genome sequence was also analyzed using Antismash and Prims 4 programs, detecting 8 putative NRPS in scaffolds 7, 15, 20, 31, 41,48, and 57 (Supplementary Material Antismash Results).

Table 2 - Description of scaffolds found in MM strain. The table includes the putative compound, the MIBig similarity score and the predicted structure.

| Scaffold (orf/s) | Putative compound | MiBig similarity score | Predicted structure (prims 4) |
|---|---|---|---|
| 7 (137) | - | - | - |
| 15 (1) (partial) | Pseudomonine | 0.52 |  |
| 15 (99-102) | L-2-amino-4-methoxy-trans-3-butenoic acid | 0.82 |  |
| 20 (37) | Azetidomonamide | 0.89 |  |

| | | | |
|---|---|---|---|
| 33 (11-12) | Pyochelin | 1 |  |
| 41 (17) | Syringafactin | 0.28 |  |
| 48 (3) | Enterobactin | 0.27 |  |
| 57 (2) | Thermoactinoamide | 0.3 |  |

**4. DISCUSSION**

In this study, we obtained two diesel-degrading bacteria, out of 5 isolates, capable of synthesizing different biosurfactant compounds. It has been previously described that hydrophobic carbon source increases biosurfactant production yield (Eslami et al., 2020; de Oliveira Schmidt et al., 2021). Because of that, we used diesel or raw sunflower oil as carbon sources for biosurfactant production. However, we finally selected sunflower oil as the carbon source to avoid the downstream purification of undesired toxic compounds from the crude biosurfactant extract.

Both selected strains belonged to the *Pseudomonas aeruginosa* species assigned by 16S rRNA gene sequencing. TLC analysis of the BCEs obtained from *P. aeruginosa* MM and ML displayed the two characteristic spots corresponding to mono- and di-rhamnolipids

congeners where the amount of di-rhamnolipids was always higher than that of mono-rhamnolipids. The difference in the RFs from the rhamnolipids spots of MM-BCE, ML-BCE, and PAO-BCE could be related to different compound congeners present in the mono- and di-rhamnolipids pool (El-Huesseiny et al., 2020). Our results also showed that MM-BCE presented a higher di- to mono-rhamnolipid ratio than ML-BCE. Although it was described that di-rhamnolipids have stronger surface activity while mono-rhamnolipids have better emulsifying activity (Zhao et al., 2018), we observed that both MM-BCE and ML-BCE lowered the ST to very similar levels. Interestingly, MM-BCE also presented a spot detected by ninhydrin staining, suggesting a surfactant compound of a peptidic nature. Lipopeptides are scarcely documented in *Pseudomonas aeruginosa* strains, and in the few described cases (Hu et al., 2023; Liu et al., 2018; Bezza and Chirwa, 2016a, 2016b), species assignment was based on 16S rRNA gene sequencing. Here, we present for the first time a *Pseudomonas aeruginosa* strain confirmed by DNA-DNA hybridization that simultaneously produces rhamnolipids and a putative lipopeptide surfactant. The analysis of the draft genome of *P. aeruginosa* MM confirmed its capacity to produce mono- and di-rhamnolipids, as well as a putative syringafactin synthase codified in scaffold 41. Syringafactin is one of the 4 kinds of lipopeptides surfactants described in the *Pseudomonas* genre (Chauhan et al., 2023), however, to the best of our knowledge, this has not been previously described in *P. aeruginosa*.

The use of surfactants with diverse characteristics has demonstrated positive effects in previous works. For instance, Cheng et al. (2013) showed that a blend of glycolipid and lipopeptide produced by *B. subtilis* strain achieved 86% oil-washing efficiency. Their findings revealed that the lipopeptide primarily contributes to stability and oil-washing efficiency, while the glycolipid plays a greater role in enhancing emulsifying activity.

To compare the surfactant performance, one of the key parameters to analyze is the critical micelle concentration (CMC) (Kim and Vipulanandan, 2006). The CMC varies depending on the set of compounds produced, the carbon source, the bacterial species, and the degree of surfactant purification (De Oliveira Schmidt et al., 2021). It was described that in a mixture of surfactants, the CMC could be influenced by the synergism

or antagonism between its compounds (Mandavi et al., 2008), being synergism the effect most frequently seen (Rosen and Kunjappu 2012). As a result, different types of surfactants are used in many industrial products and processes instead of pure components (Shah et al., 2021, Zhou et al., 2019 , Singh et al. 2023)

In this work, MM-BCE showed a lower CMC compared to ML-BCE. This difference could be attributed to several factors: the differences between mono- and di-rhamnolipids ratios and/or in biosurfactant composition, especially the presence of the putative lipopeptide surfactant detected. The obtained CMC values for MM-BCE and ML-BCE are consistent with the ones reported previously (Bordas et al., 2005, Pornsunthorntawee et al., 2008, Silva et al., 2010). When compared with synthetic surfactants the CMC of MM-BCE was approximately seven folds smaller than SDS (Valsaraj et al 1988). Finally, the EI24 of MM-BCE and ML-BCE were similar to those previously reported (Pourfadakari et al., 2021). Regarding surface tension reduction, both MM and ML achieved a value of 33 mN/m which is similar to what is found in the literature regarding different *P. aeruginosa* strains (Yin et al., 2009; Rikalovic et al., 2013; Costa et al., 2010)

Microcosm SER assay showed that MM-BCE and ML-BCE addition increased the rate of hydrocarbon degradation but with different effectiveness. It was previously described that different mixtures of surface-active compounds may have different capabilities to enhance the remediation of hydrophobic pollutants (Mendes et al., 2015). In fact, Szulc et al (2014) found no significant effect when commercial rhamnolipids or of 58.5-76.8 % (Olasanmi and Thring 2020) were used while Cameotra and Singh (2006) had a degradation of 95% using crude biosurfactants by *Pseudomonas aeruginosa* and *Rhodococcal* sp .

Regarding the use of lipopeptide surfactants for soil enhanced oil recovery (SER), Chaprão et al. (2015) reported a 10-20% increase in oil degradation when biosurfactants produced by *Bacillus* sp. and *C. sphaerica* UCP0995 were applied. Kavitha et al. (2014) showed that lipopeptidic surfactant from *Bacillus licheniformis* MTCC 5514 was found to remove over 85% of crude oil from contaminated soil. Cheng et al. (2013) reported that a blend of glycolipids and lipopeptides, including rhamnolipids, surfactin, and fengycin produced by

*Bacillus subtilis* TU2, achieved 86% oil-washing efficiency and had excellent emulsification properties, highlighting its potential for SER.

The degradation rate also depends on the incubation time. For example, higher percentages (80-85%) were observed after 6 months (Szulc et. al 2014) while Akbari et al (2021) reported about 10% of total hydrocarbon reduction after 30 incubation days and 20% after 80 days when rhamnolipids were added to microcosm assays. This suggests that the MM-BCE is effective and competitive, having a degradation rate of 47% after 30 days.

On the other hand, since diesel degradation in this study was conducted by autochthonous microbiota, the differences observed between the addition of MM-BCE and ML-BCE could be due not only to an increase in hydrocarbon bioavailability but also to how each BCE affected the bacterial community, especially the hydrocarbon-degrading taxa (Lu et al., 2019; Posada-Baquero et al., 2020; Wang et al., 2021a, Akbari et al., 2021 ).

## 5. CONCLUSION

In this work, we present for the first time a full sequenced *P. aeruginosa* strain capable of simultaneously synthesizing a blend of glycolipids and lipopeptides. A crude extract of this surfactant mix, with minimal post-production purification, resulted in significant diesel biodegradation compared to the controls in microcosm assays. These results represent a promising amendment to be use in the bioremediation of petroleum-contaminated sites and microbial-enhanced oil recovery (MEOR).

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