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SELECTION OF BIOSURFACTAN/BIOEMULSIFIER-PRODUCING BACTERIA FROM HYDROCARBON-CONTAMINATED SOIL

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ABSTRACT

Petroleum-derived hydrocarbons are among the most persistent soil contaminants, and some hydrocarbon-degrading microorganisms can produce biosurfactants to increase bioavailability and degradation. The aim of this work was to identify biosurfactant-producing bacterial strains isolated from hydrocarbon-contaminated sites, and to evaluate their biosurfactant properties. The drop-collapse method and minimal agar added with a layer of combustoleo were used for screening, and positive strains were grown in liquid medium, and surface tension and emulsification index were determined in cell-free supernantant and cell suspension. A total of 324 bacterial strains were tested, and 17 were positive for the drop-collapse and hydrocarbon-layer agar methods. Most of the strains were *Pseudomonas*, except for three strains (*Acinetobacter, Bacillus, Rhodococcus*). Surface tension was similar in cell-free and cell suspension measurements, with values in the range of 58 to 26 (mN/m), and all formed stable emulsions with motor oil (76-93% E_{24}). Considering the variety of molecular structures among microbial biosurfactants, they have different chemical properties that can be exploited commercially, for applications as diverse as bioremediation or degradable detergents.

Key words: biosurfactants, bioemulsifiers, soil microbiota, hydrocarbon contamination.

INTRODUCTION

The release of contaminants to the environment, including petroleum and petroleum-derived products, is one of the main causes of global contamination (29). It is also a risk for human and animal health, since many of these contaminants have demonstrated to be toxic and carcinogenic (27). Hydrocarbon molecules that are released into the environment are hard to remove, since they adsorb to surfaces and are trapped by capillarity in a water–immiscible phase. Bioremediation has proven to be an alternative to diminish the effects caused by hydrocarbon contamination of soil and water, using the metabolic capacities of microorganisms that can use hydrocarbons as source of carbon and energy, or that can modify them by cometabolism. The efficiency of removal is directly related to the compounds' chemical structure, to its bioavailabity (concentration, toxicity, mobility and access) and to the physicochemical conditions present in the environment (10).

Biosurfactants are produced by many organisms, in order

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to metabolize water-immiscible substrates, allowing its adsorption, emulsification or dispersion. For the microorganisms, production of biosurfactants is an advantage in soil, giving them advantages in specific conditions (30). Microbial biosurfactants are mainly produced by aerobic microorganisms, using as carbon sources carbohydrates, hydrocarbons, animal or vegetal oils or a mixture of them (6, 14). Biosurfactants can be intracellular (remain attached to the cell wall) and/or can be excreted to the media (1). When the biosurfactants are intracellular, their structure include membrane lipids, and promote the transport of insoluble substrates through the membrane; when they are extracellular, the biosurfactants help on the substrate solubilization and are usually a complex structure of lipids, proteins and carbohydrates (27). The main difference in the chemical nature of the different biosurfactant molecules is in the hydrophilic head; allowing for a wide range of variation in their physical and biological properties (20).

In the literature, the terms biosurfactant and bioemulsifier are considered interchangeable, but although all bioemulsifiers are considered biosurfactants, not all the biosurfactants produce stable emulsions. Biosurfactants can reduce the surface tension between two liquids, and bioemulsifiers induce a dispersion of undissolved material throughout the liquid, by formation and stabilization of droplets of the dispersed phase (13, 14). Microbial biosurfactants are classified by its chemical composition and its microbial origin (13). Low molecular weight (glycolipidis or lypopeptides) can diminish surface tension, but does not form stable emulsions (10). On the other hand, biopolymers are less effective on lowering the surface tension, but are highly effective on the production of emulsions, and have a considerable specificity for the substrate (30).

The search for biosurfactant-producing microbial strains is still an interesting area of research (3), because of the diversity of the molecules and the wide variety of uses that those molecules have. There are several methods used for screening and isolation of interesting bacterial strains; an easy and common method is the drop-collapse test (5), and it has been described that the carbon source used to grow the microorganisms is important on the identification of biosurfactat producing strains (6). Therefore, the aim of this work was to identify biosurfactant and/or bioemulsifier producing bacteria from a collection of soil bacterial isolates obtained from hydrocarbon-contaminated sites.

METHODS

Bacterial strains

Bacterial strains were isolated from soil contaminated with either combustoleo (Fuel Oil No. 6) or used mobile oil, which was used on laboratory-scale bioreactors to study different bioremediation strategies. Remediation strategies studied included biostimulation (fertilizer solution N 0.03%, P 0.01%) and bioaugmentation (bacterial consortium obtained by selective enrichment with gasoline vapors), as well as a control bioreactor where only water was added (24). Microbial activity in the bioreactors was followed for six months in the mobile oil bioreactors and for one year in the combustoleo reactors. From the bioreactors, soil samples were taken periodically (every two weeks) and screened for hydrocarbon-degrading bacteria by inoculation into M9 mineral salt agar (Na_2HPO_4 6g, KH_2PO_4 3g, NaCl 0.5g, NH_4Cl .1 g, $MgSO_4 \cdot 7H_2O$ 0.24g and $CaCl_2$ 0.01g, Bacto-Agar 15 g, distilled water 1 l) without carbon source added to the plates, and were incubated at 25°C in a chamber saturated with gasoline vapors from 5-7 days. Colonies with different colonial morphology were selected and inoculated into Tryptic Soy Agar (TSA) (Bioxon, Mexico) to obtain pure cultures, and strains were maintained by periodical transfer into TSA. Selected strains were characterized by Gram stain and biochemical tests, including carbohydrate fermentation (sucrose, trehalose, glucose, arabinose, xylose, galactose, maltose, ribose, mannose, lactose, fructose) and catalase and oxidase reactions (17). All inorganic salts were JTBaker (Mexico City, Mexico) and carbohydrates for biochemical test were purchased form Sigma (Mexico City, Mexico).

Growth conditions

Pure cultures were grown on M9 minimal salt broth added with glucose (2% w/v) olive oil, paraffin or sucrose (1% w/v), and incubated at 120 rpm for five days, according to Bodour and Miller (4). After growth, biosurfactant production was tested. Cells were recovered by centrifugation (12,000 x *g*, 5 min) and resuspended in 1 ml of M9 broth. Cell suspension and supernatant were used in the biosurfactant activity assay.

Qualitative evaluation of biosurfactant production

The drop-collapse method was used for initial identification of biosurfactant-producing bacteria (4). Briefly, 1.8 µl of 10W 40 Pennzoil (Pennsylvania, USA) oil was added to each well of a 96 well microtiter plate lid. The lid was equilibrated for 24h at room temperature, and then 5 µl of the culture were added to the surface of oil. The shape of the drop was inspected after 1 min; if the drop remained beaded, the result was scored as negative. If the drop collapsed, the result was scored as positive. Tests were carried by triplicate, using culture supernatant and cell suspensions. Also, biosurfactant production was monitored in M9 Agar plates added with a layer of combustoleo (fuel oil No. 6), according to the method proposed by Kiyohara *et al.* (16). Also, a set of M9 Agar plates added with 10mM of glucose were used to test the effect of an additional carbon source on biosurfactant production. Plates were inoculated by aseptically transferring a bacterial colony with sterile toothpicks onto the M9 Agar plate. Plates were incubated at 28°C for 7 days.

Determination of Surface Tension

Bacterial strains that were positive in the drop-collapse test were also evaluated for surface tension and for stable emulsion formation. Strains were grown in M9 broth added with glucose (2% w/v for *Pseudomonas*) or sucrose (1% w/v for *Bacillus*, *Acinetobacter*, *Rhodococcus*) and incubated at 120 rpm for five days. For surface tension measurements, 5 ml of broth supernatant were transferred to a glass tube that was submerged in a water bath at a constant temperature (28°C). Surface tension was calculated by measuring the height reached by the liquid when freely ascended trough a capillary tube (23). As control, non-inoculated broth was used, and the surface tension was calculated according to the following formula:

$$\gamma = \frac{\mathrm{rh}\delta\mathrm{g}}{2} \qquad (1)$$

γ = Surface tension (mN/m); δ = Density (g/mL); g = gravity (980 cm/s^2); r = capillary radius (0.05 cm); h = height of the liquid column (cm).

Determination of emulsification index

For determination of the emulsification index, 2 ml of supernatant or cell suspension and 3 ml of a selected hydrocarbon were mixed in a test tube and vortexed for 2 min. The test tubes were maintained at 25°C and the height of emulsion layer was measured after 24 h to determine the emulsification index (11). The equation used to determine the emulsification index (E_{24}) is as follows:

$$E_{24} = \frac{height\ of\ emulsion\ layer}{height\ of\ total\ solution} X100\% \qquad (2)$$

Statistical analysis

The data analysis was carried out with Minitab Inc. software (version14.13). One-way ANOVA was used to determine whether significant ($p < 0.05$) variation occurred among emulsification index and surface tension measurements of bacterial isolates. Tukey's-test was used to perform multiple comparisons between means.

RESULTS AND DISCUSSION

Screening of biosurfactant producing bacteria

Bacterial strains were isolated from two different soils contaminated with hydrocarbons (fuel oil No. 6 and used motor oil) that were subjected to different bioremediation procedures in laboratory-scale bioreactors and were isolated during the period that the bioreactors were followed (24). A total of 324 microbial isolates were selected for test of biosurfactant production based on their growth on M9 agar plates without carbon source, and incubated in a gasoline-saturated

atmosphere as well as on the basis of their colonial morphologies. Of all isolates, 102 were obtained from a site contaminated with used mobile oil and 222 from a site contaminated with fuel oil No. 6 (combustoleo). According to the initial characterization (colonial morphology, Gram stain and biochemical tests), bacterial isolates belonged to the genera *Pseudomonas*, *Rhodococcus*, *Bacillus*, *Micrococcus*, *Staphylococcus*, *Acinetobacter* and *Serratia*; also, some colonies resembled Actinomycetes. Besides being typical soil microorganisms, all the genera have been reported to be present in hydrocarbon-contaminated sites, and have also been reported as hydrocarbon degraders (7, 29).

One of the factors that is important in biosurfactant production is the carbon source present, since some microorganisms produce biosurfactants in water insoluble substrates, such as vegetable or mineral oils, and other microorganisms can produce this metabolites when carbohydrates are present as carbon sources (6). Therefore, all 324 bacterial strains were grown in M9 added with glucose, sucrose or olive oil, and the supernatant was used to select for biosurfactant-production bacteria by the drop-collapse test. Seventeen isolates were positive and of those, 11 strains were growing in glucose, 6 in olive oil and only 3 in sucrose; of those, 3 strains produced biosurfactant in olive oil as well as in glucose as substrates (Table 1). The substrates used have been reported to promote biosurfactant production (4, 26, 32). Although the biodegradability capacity of the 324 strains has not been tested by reduction of hydrocarbons in pure culture, many are likely to degrade hydrocarbons since they were isolated in M9 Agar plates, incubated in a hydrocarbon-saturated atmosphere as carbon source. However, only a small proportion were biosurfactant producers, which support the concept of bacterial community, whereas the biosurfactant producers will liberate the surface-tension-active molecules, that will help other microorganisms to degrade the wide variety of hydrocarbons found at the site (28, 30).

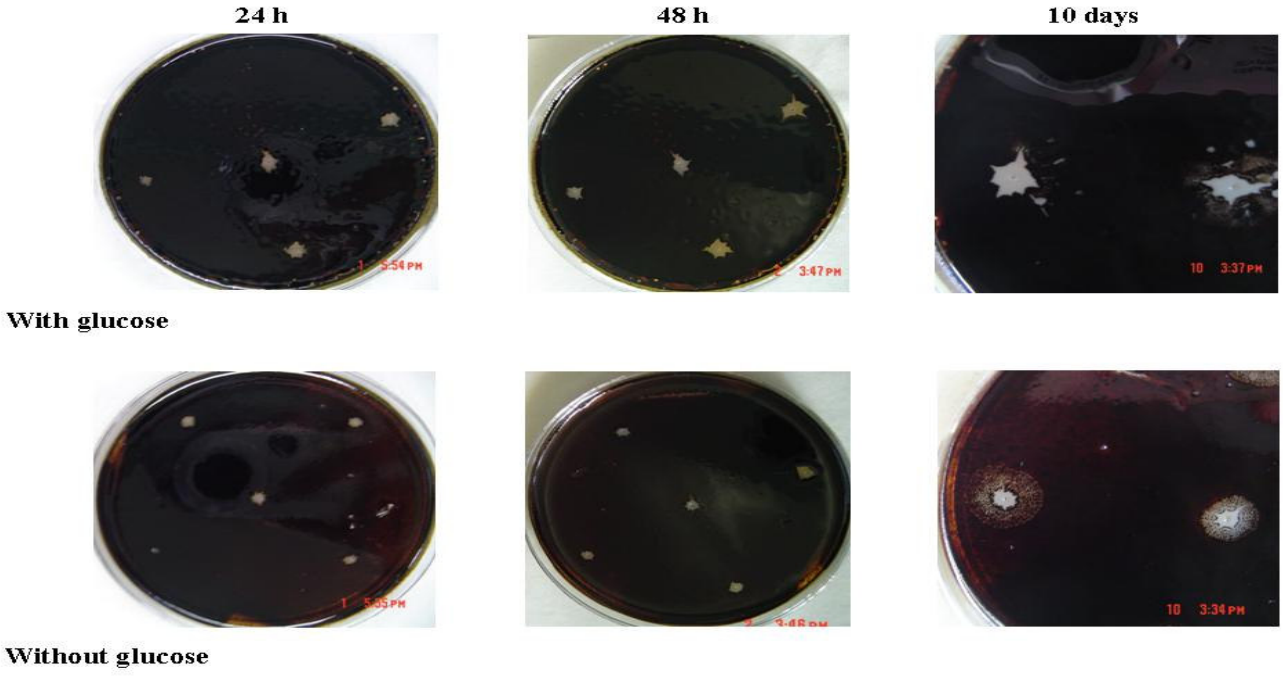
Table 1. Biosurfactant production and preliminary identification of isolates growing in glucose, sucrose and olive oil as the carbon source (Gram stain: P, Gram-positive; N, Gram-negative. Biosurfactant production: +, positive response; -, negative response)

Isolate	Origin	Gram stain	Genera	Glucose	Olive oil	Sucrose
R1M5	Combustoleo	P	*Bacillus*	-	-	+
R2Ib	Mobile oil	N	*Pseudomonas*	+	-	-
R36b	Mobile oil	N	*Pseudomonas*	+	-	-
R3Ia	Mobile oil	N	*Pseudomonas*	+	-	-
R3Ib	Mobile oil	N	*Pseudomonas*	+	-	-
R3M2a	Combustoleo	N	*Pseudomonas*	+	-	-
R3M2b	Combustoleo	N	*Pseudomonas*	-	+	-
R3M5	Combustoleo	N	*Pseudomonas*	+	+	-
R3Ma	Mobile oil	N	*Pseudomonas*	+	-	-
R3Mb	Mobile oil	N	*Pseudomonas*	+	+	-
R4M20	Combustoleo	P	*Rhodococcus*	-	-	+
R4M2a	Combustoleo	N	*Pseudomonas*	+	-	-
R4M2b	Combustoleo	N	*Pseudomonas*	-	+	-
R4M4	Combustoleo	N	*Acinetobacter*	-	-	+
RIMa	Mobile oil	N	*Pseudomonas*	-	+	-
Rn – 13	Mobile oil	N	*Pseudomonas*	+	-	-
Rn – 19	Mobile oil	N	*Pseudomonas*	-	+	-

The drop-collapse test was considered positive when within one minute, the drop expanded on the oily surface of the microplate lid. Besides the 17 positive isolates, another 21 strains (18 in olive oil, 1 in maize starch, 1 in sucrose and 1 in paraffin) showed a partially collapsed drop after one minute, suggesting that those microorganisms produced only a small amount of biosurfactant, or that it remained intracellular (4). Biosurfactant-producing strains had a turbid and pigmented growth in broth when growing in glucose, with a foamy aspect; those that grew in olive oil had a milky aspect, with growth attached to the substrate; while the strains positive in sucrose did not had a characteristic growth. Most of the positive strains were identified as *Pseudomonas* spp., but identification could not be obtained up to species level. The *Pseudomonas* genera is one of the most reported for biosurfactant production, and it has also been reported that the most known biosurfactant that they produce, is a rhamnolipid (22, 33). Although most of the reports on rhamnolipid production are attributed to *Pseudomonas aeruginosa* strains, there are reports of other *Pseudomonas* related microorganisms, such as *Burholderia plantarii* (2) and *Ps. chlororaphis* (15) that produce biosurfactants.

When the 17 isolates described in Table 1 were inoculated in an M9 agar plate covered with a layer of combustoleo, a clear zone around the colonies was observed in the glucose-free plates after 24 h of incubation. After 4 days of incubation, emulsification zones were observed and increased after longer incubation times (Figure 1). It has been suggested that the biosurfactant remains attached to the cell surface until a saturation point is reached; then the biosurfactant is liberated to the surroundings and emulsification occurs (8) as the main mechanism to introduce water-insoluble substrates to the cell interior (7). Southam *et al.* (31) demonstrated by transmission electron microscopy, the interphase between hydrocarbon-degrading bacteria and small oil droplets that were encapsulated by the biosurfactant. This phenomenon can explain the clear area and further emulsion observed in glucose-free medium. On the other hand, Das and Mukherjee (12) found that supplementing the medium with glucose as a co-carbon source enhanced the rate of PAH degradation in selected bacteria. With the use of glucose, in the M9 Agar, the microorganisms only increased the clear zone around the colonies, but no emulsion was observed (Figure 1). The presence of a water-soluble substrate such as glucose, can lead to a hydrophobic cell membrane, and the production of a biosurfactant with no emulsification activity (27).

Figure 1. Growth of bacterial strain R2Ib in M9 Agar plates with a top layer of combustoleo and with or without glucose (10 mM) at different incubation periods.



Tensioactive properties of biosurfactants

Surface tension measurement showed that in order to give a positive drop collapse test, a surface tension lower than 45 mN/m was necessary. Surface tension of M9 minimal broth was 69.97 mN/m. No differences were observed in the surface tension of cell-free supernantant and cell suspensions for all bacterial strains tested ($p>0.05$, data no shown), opposing results obtained by Batista *et al.* (3). Glycolipids produced by *Pseudomonas* are low molecular weight compounds, which can lower the medium surface tension below 30 mN/m (25). The lower surface tension values, both with or without cells, was reached by *Pseudomonas* strains (26.7 mN/m), and were closely followed by the *Bacillus* strain (33.32 mN/m) (Table 2). Even though lypopeptides produced by *Bacillus* are known as one of the most powerful microbial biosurfactants (20), *Pseudomonas* rhamnolipids are also effective, and both of them are extracellular (21). Isolates that liberate biosurfactants into the culture medium are interesting from an industrial point of view, because the product can be easily removed from the culture media (19).

Table 2. Surface tension and emulsification index of bacterial strains isolated from hydrocarbon-contaminated soils

		Emulsification Index			
Name	**SURFACE TENSION (mN/m)**	**Diesel**	**Decane**	**Kerosene**	**Motor oil**
R1M5	33.3 ^{b} ± 2.9	0.8^{d} ± 1.3	10.3 ^{a} ± 0.9	78.0 ^{ab} ±1.6	80.3 ^{ab} ± 4.6
R2Ib	28.3 ^{b} ± 2.9	33.6^{bcd} ± 0.8	8.9 ^{a} ± 1.6	100.0 ^{a}	90.3 ^{a} ± 1.4
R36b	33.3 ^{b} ± 7.6	56.5^{abc} ± 3.9	0.0 ^{a}	67.1 ^{b} ± 1.7	91.6 ^{a} ± 0.8
R3Ia	58.3^{a} ± 2.9	6.3^{cd} ± 2.4	2.5 ^{a} ± 4.3	5.27 ^{c} ± 1.3	90.4 ^{a} ± 9.2
R3Ib	43.3 ^{ab} ± 18	100^{a}	1.5 ^{a} ± 0.6	100.0 ^{a}	85.4 ^{ab} ± 5.1
R3M2a	56.6 ^{a} ± 5.8	0.7^{d} ± 1.2	2.4 ^{a} ± 4.1	61.7 ^{b} ± 8.1	82.5 ^{ab} ± 4.7
R3M2b	31.7 ^{b} ± 2.9	88^{ab} ± 17.0	4.1 ^{a} ± 0.1	100.0 ^{a}	92.8 ^{a} ± 6.8
R3M5	35.0 ^{ab} ± 5.0	0.0 ^{d}	3.4 ^{a} ± 0.1	0.0 ^{c}	87.7 ^{ab} ± 5.5
R3Ma	26.7 ^{b} ± 2.9	100^{a}	6.5 ^{a} ± 3.2	100.0 ^{a}	87.2 ^{ab} ± 0.8
R3Mb	30.00 ^{b} ± 0.0	51.5^{bcd} ± 24	7.0 ^{a} ± 4.7	90.3 ^{a} ± 16.8	90.1 ^{ab} ± 3.1
R4M20	56.6 ^{a} ± 7.6	87.8^{ab} ± 0.5	0.0 ^{a}	0.0 ^{c}	76.2 ^{b} ± 5.2
R4M2a	46.7 ^{ab} ± 17	55.9^{abc} ± 21	5.3 ^{a} ±3.0	0.0 ^{c}	85.0 ^{ab} ± 0.7
R4M2b	28.3 ^{b} ± 5.8	100^{a}	5.8 ^{a} ± 0.1	100.0 ^{a}	85.5 ^{ab} ± 7.5
R4M4	58.3 ^{a} ± 7.6	6.5^{cd} ± 2.1	9.2 ^{a} ± 1.0	0.0 ^{c}	86.1 ^{ab} ± 2.2
RIMa	33.3 ^{b} ± 2.0	100^{a}	5.0 ^{a} ± 2.4	100.0 ^{a}	86.5 ^{ab} ± 4.1
Rn-13	26.7 ^{b} ± 2.9	90.2^{a} ± 16.9	0.0 ^{a}	66.3 ^{b} ± 2.3	86.2 ^{ab} ± 1.3
Rn-19	31.7 ^{b} ± 7.6	65.6^{abc} ± 21	5.6 ^{a} ± 0.0	100.0 ^{a}	85.7 ^{ab} ± 0.7

Results are shown as average and standard deviation of three replicates. In a column, different letters represent different statistical groups ($p<0.05$).

The terms biosurfactant and bioemulsifier are used as synonyms in scientific literature. However, while the molecular structure of the surfactant is well defined (a surfactant has both hydrophilic and hydrophobic moieties present within the same molecule), the term emulsifier is often used in an application-oriented manner to describe the combination of all the surface active compounds that constitute the emulsion secreted by the cell to facilitate the assimilation of an insoluble substrate (14). The debate is if a surfactant that reduces the surface tension of water, can form stable emulsions (3, 9). As described in Table 2, most of the strains that had the lower surface tension values were also the ones that formed the largest and more stable

emulsions. Also, there was no significant difference between the emulsions formed by the released biosurfactant or the cell suspension ($p>0.05$). Negative control was the M9 minimal medium mixed with the different hydrophobic substrates. For all water-insoluble compounds tested, emulsions were more stable in the hydrocarbonated portion of the oil-water mixture. When a supernatant without cells was used, emulsification index (after 24 hours) ranged from 0 to 100% for diesel, from 0.0 to 100% for kerosene, and from 76.2 to 92.8% for motor oil (Table 2), with high statistical differences between strains within each compound ($F=15.55$ for diesel, $F=100.60$ for kerosene and $F=2.35$ for motor oil respectively; $p<0.01$). For decane, emulsion index was in the range of 0-10% and there were no differences among strains ($p>0.05$).

Emulsification index can vary with bacterial growth phase, bacterial interactions and hydrophobic compound tested (18). The highest emulsification index values of diesel, kerosene and motor oil were detected for *Pseudomonas* strains. Monteiro *et al*. (22) reported an emulsification index of 70% after 30 days of incubation, demonstrating that emulsions produced by *P. aeruginosa* rhamnolipids are stable, and can be used in the control of environmental contamination. Only *Bacillus* and *Acinetobacter* formed stable emulsions with decane. Emulsions formed by *Acinetobacter* were small, but optically clear, probably due to vesicles rich in phosphatidiletanolamine that are formed, as observed by Desai & Banat (13), and the emulsion formed by *Rhodococcus* cells incorporated air in the emulsion, giving a column height higher than the controls.

Hydrocarbon contaminated sites can be considered as enrichment environments for selection of hydrocarbon-degrading and/or biosurfactant producing microbial strains. Production of biosurfactants and bioemulsifiers by soil microorganisms provide them with an advantage in contaminated sited, since they can use water insoluble carbon sources for growth. Identification and selection of microbial strains with those capacities, can lead to the identification and functional characterization of their biosurfactants. Considering that there is a wide variety of molecular structures among microbial biosurfactants, they also have different chemical properties that can be exploited commercially, for applications as diverse as bioremediation or degradable detergents.

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