World Journal of Microbiology and Biotechnology, 8, 125–128

Biosurfactant production by two isolates of *Pseudomonas aeruginosa*

C. Rocha, F. San-Blas,* G. San-Blas and L. Vierma

Two strains of biosurfactant-producing bacteria, identified as *Pseudomonas aeruginosa*, were isolated from injection water and crude oil-associated water in Venezuelan oil fields. Both biosurfactants resembled rhamnolipids and produced stable emulsions of heavy and extra-heavy crude oils, reducing the surface tension of water from 72 to 28 dynes/cm. Tenso-active properties of the biosurfactants were not affected by pH, temperature, salinity or Ca^{2+} or Mg^{2+} at concentrations in excess of those found in many oil reservoirs in Venezuela.

Key words: Biosurfactants, microbially enhanced oil recovery, Pseudomonas aeruginosa, *rhamnolipids.*

Biosurfactants of microbial origin have been recognized as potential partial or total substitutes for synthetic surfactants for the oil industry, since they are biodegradable, often non-toxic (Kosaric *et al.* 1987) and can be produced by fermentation of low cost substrates (Reiling *et al.* 1986). Chemically, the commonest microbial surfactants so far described are glycolipids (for reviews, see Cooper & Zajic 1980; Lang & Wagner 1987), particularly lipids linked to disaccharides (trehalose and sophorose), monosaccharides (rhamnose), diglycosyl diglycerides or polysaccharides.

Bacteria able to synthesize surfactants constitutively seem to be a good choice for Microbial Enhanced Oil Recovery (MEOR), since addition of the appropriate nutrients and carbohydrate source into the oil reservoir would allow the production of biosurfactants *in situ*. Of these, *Pseudomonas aeruginosa* produces extracellular surface-active rhamnolipids (Lang & Wagner 1987) which differ in their rhamnose content and in the length and chemical nature of their lipid fraction (Itoh *et al.* 1971), depending on the strain, culture medium and growth conditions (Lang & Wagner 1987; Robert *et al.* 1989).

The purpose of this study was to isolate biosurfactant-producing microorganisms from different petroleum-related sources in Venezuela. The production of surfactants from two strains of *P. aeruginosa* isolated from injection and crude oil-associated waters is described. A partial characterization of the biosurfactants produced demonstrated their rhamnolipid nature.

C. Rocha, F. San-Blas and G. San-Blas are with the Instituto Venezolano de Investigaciones Cientificas (IVIC), Centro de Microbiologia y Biologia Celular, Apartado 21827, Caracas 1020A, Venezuela. L. Vierma is with INTEVEP S.A., Apartado 76343, Caracas 1070A, Venezuela. *Corresponding author.

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Materials and Methods

Crude Oils

A medium-heavy crude oil, 'Lagunillas LL3' (well LL3.Pb49; 23° API, American Petroleum Institute) and two extra-heavy crude oils, 'Hamaca' (well No. MFF5; 10.5° API) and 'Cerro Negro' (9° API), were used.

Enrichment and Selection of Biosurfactant-Producing Bacteria

Soil samples were continuously exposed to hydrocarbons, injection water (the water pumped into an oil reservoir in order to enhance residual crude oil recovery) or production water (the water recovered with crude oil) from an oil well subjected to secondary production. Samples were taken periodically, placed in sterile PYG medium (peptone, 5.0 g; yeast extract, 5.0 g; glucose, 15.0 g; distilled water, 1 litre) and held at 37°C on a reciprocating shaker at 180 strokes/min for 72 h. Strains able to degrade, change or emulsify crude oil were selected for further tests.

Taxonomic Identification of Selected Microorganisms

Bacteria were identified according to Doudoroff & Palleroni (1984), including specific tests to differentiate *Pseudomonas* species.

Qualitative and Quantitative Tests of Biosurfactants

Extracellular biosurfactant production was measured by culture broth tensiometry using an Autotensiomat ring detachment apparatus (Fisher, model 315, Pittsburgh, PA). Interfacial tension readings were made using a 10 ml overlay of LL3 crude oil. Those cultures producing biosurfactants that formed stable emulsions were then studied further.

To quantify biosurfactant activity, 1 ml of spent cell-free culture broth or purified biosurfactant (purified dry extract suspended in

100 ml of distilled water) from strains MEOR 171 or MEOR 172 (extracted as described below) were added to 15 ml 0.1 M Tris-$MgSO_4$ buffer, pH 7.0 and 0.2 ml of fuel-oil. After mixing for 30 min in a Vortex, mixtures were allowed to rest for 2 min and turbidity (Klett units, K.U.) was read in a Klett colorimeter against a blank of buffer and fuel-oil in the same proportion as above. An increase in turbidity was taken as a criterion of miscibility of the fuel-oil in the aqueous phase. Using this technique, the effects of temperature (0° to 100°C), pH (1 to 12), hardness ($CaCl_2$ and $MgSO_4$, 0.005 to 1%) and salinity (NaCl, 0.05 to 10%) on emulsifying activity, were measured. Critical micellar concentration (CMC) was determined by measuring the surface tensions of dilutions of cell-free spent medium, or purified biosurfactant in distilled water up to a constant value of surface tension.

Viscosity Measurements

Reductions in the viscosity of heavy and extra-heavy crude oils (10 g crude in 40 ml supernatant from either bacterial strain MEOR 171 or MEOR 172) were measured at room temperature with a Thomas Stoner viscometer (model 9730-F10, Thomas Scientific, USA).

Isolation of Biosurfactants from Strains MEOR 171 and MEOR 172

Surfactants were extracted from 100 ml of cell-free spent PYG broth, using chloroform/methanol/cell-free supernatant (2:1:3, by vol) according to Rambeloarisoa *et al.* (1984).

Thin Layer Chromatography (TLC)

Silica gel G (Merck) plates, 0.25 and 1 mm thick, were used. Solvents were (i) chloroform/methanol/acetic acid/water (170:25:25:4, by vol); (ii) chloroform/methanol/NH_4OH (70:25:3, by vol); (iii) propan-2-ol/acetone/0.1 M lactic acid (2:2:1, by vol). For visualization of lipids, phosphate, amino acids, glycolipids and sugars, conventional reagents were used (Chaplin & Kennedy 1987).

Quantitative Determination of Sugars

Samples were hydrolyzed with 1 M HCl at 100°C for 1 h. After neutralization, sugars were measured by the anthrone method (Chaplin & Kennedy 1987), using glucose or rhamnose (100 μg/ml) as a standard.

Results

Eight selected bacterial isolates induced significant changes to the three crude oils, when used as carbon sources for growth. These changes included break up of the crude oil into small spheres, colour changes, oil dispersion etc. Of this group of bacteria, only one was isolated from soil and was identified as belonging to the genus *Corynebacterium* (MEOR 158). Among the rest, four were isolated from production water and three from injection water. From these bacterial isolates, strains MEOR 171, from injection water, and MEOR 172, from production water, were selected for further studies, since uniform dispersion of the LL3 crude oil occurred throughout the aqueous culture medium. Both strains were identified as *Pseudomonas aeruginosa*. Dispersion

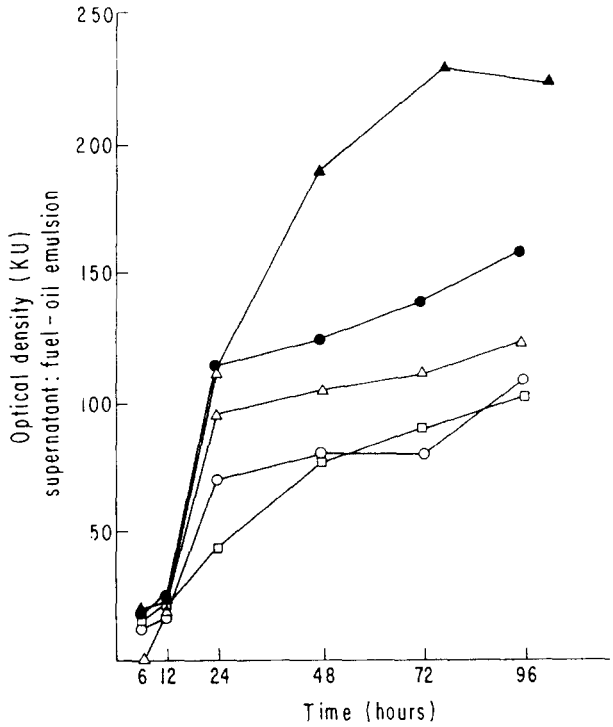

Figure 1. Effect of crude oil addition on biosurfactant production by strain MEOR 171: ●—PYG medium; ▲—PYG + LL3 crude oil added after 24 h of incubation; △—PYG + LL3 crude oil added before bacterial inoculation; ○—PY + LL3 crude oil; □—PY medium. (Glucose when present was at 15 g/l). Turbidity refers to spent medium:fuel-oil emulsions as described in the text.

was stable for several months; swirling the flask resulting in a uniform and immediate dispersion of the oil.

Biosurfactants were produced in culture media in which glucose was the only carbon source, although the addition of crude oil at the stationary phase of growth resulted in an increase of biosurfactant production (Figure 1). In cultures grown on glucose, addition of crude oil before bacterial inoculation decreased the amount of biosurfactant produced. In cultures lacking glucose, with or without crude oil, only a basal level of biosurfactant production was achieved (Figure 1). Although strains MEOR 171 and MEOR 172 only emulsified the lighter LL3 crude oil during growth, not Hamaca or Cerro Negro, cell-free spent broths derived from the cultures very efficiently emulsified the two extra-heavy crude oils. A higher emulsifying activity was found with the biosurfactant produced by strain MEOR 172 (hydrocarbon:biosurfactant ratio, 24:1, by vol) than with that produced by strain MEOR 171 (ratio 12:1, by vol). Emulsions were stable at temperatures ranging from 0 to 100°C. Using salt concentrations of 5,000 ppm or higher, Ca^{2+} and Mg^{2+} slightly increased (15 to 25%) the emulsifying activity of biosurfactant MEOR 172, while NaCl increased it by 50% with that from MEOR 171 and by 90% with that from MEOR 172.

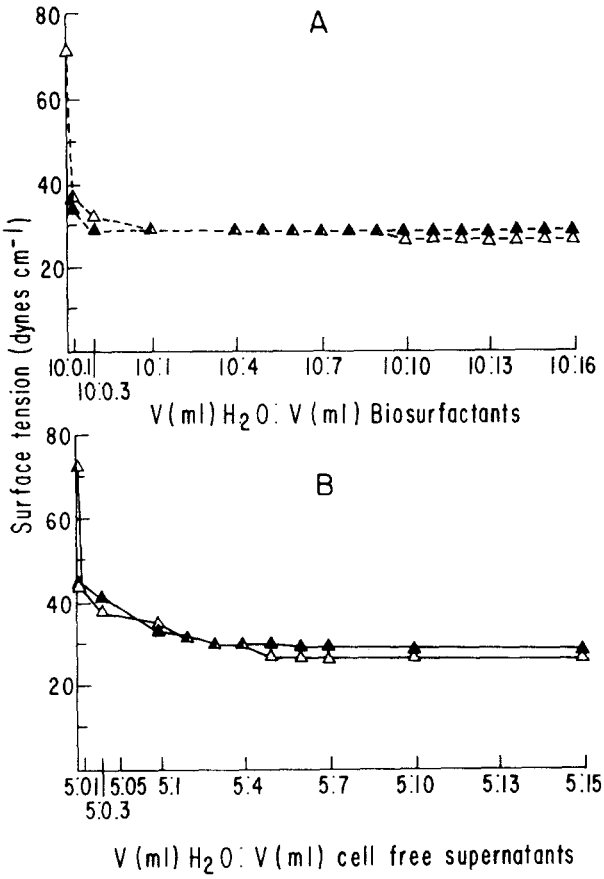

Figure 2. Surface tension reduction of distilled water by: (A) biosurfactants; (B) cell-free supernatants; ▲—strain MEOR 171; △—strain MEOR 172. Critical micellar concentrations were calculated on these results, as described in the text.

Surface tension of distilled water was reduced from 72 dynes/cm to 29 (MEOR 171) and to 27.5 (MEOR 172) dynes/cm, while interfacial tension decreased from 16 to 2.5 dynes/cm in both systems. These values were maintained over the range of temperature 4 to 100°C and with pH changes. Under the conditions used, CMC values were similar for cell-free spent media as well as for purified biosurfactants (Figure 2), values being 0.015% (w/v) and 0.045% (w/v) for MEOR 171 and MEOR 172, respectively. The viscosities of Cerro Negro, Hamaca or Lagunillas LL3 crude oil emulsions were reduced to 1.0 cpoise by cell-free spent broth of either bacterial strain.

TLC separated both chloroform/methanol extracted biosurfactants into four bands, of which band B (Rf = 0.46) contained the emulsifying activity. Both MEOR 171B and MEOR 172B samples were isolated for further chemical analysis. They reacted positively in TLC to α-naphthol, iodine and sulphuric acid, and negatively to phosphate and ninhydrin, indicating they were probably glycolipids. After acid hydrolysis, the only sugar identified by TLC was rhamnose (Rf = 0.80), comprising 13.5% (MEOR 171) and 21.3% (MEOR 172) of the whole structure.

Discussion

From the point of view of the oil industry, the ability of biosurfactants from strains MEOR 171 and MEOR 172 to emulsify Venezuelan heavy and extra-heavy crude oils compares favorably with previous reports (Rosenberg *et al.* 1979; Rambeloarisoa *et al.* 1984; Mattei & Bertrand 1985). Their optimal production at the stationary phase of growth may suggest their association to the cell during exponential growth, in functions probably unrelated to those of biosurfactants (Pines and Gutnick 1981).

The stimulation in emulsifying activity of cell-free spent culture medium following addition of LL3 crude oil 24 h after culture initiation (Figure 1) may be the result of an increase in biosurfactant production caused by initiation of a new growth cycle. In turn, surfactant accumulation would facilitate the use of crude oil as a carbon source. Alternatively, stimulation may be because of the production of a modified surfactant molecule with a higher emulsifying activity than that produced on glucose (Syldatk & Wagner 1987; Robert *et al.* 1989).

Biosurfactants from MEOR 171 and MEOR 172, besides being good emulsifiers, have excellent tenso-active properties, two characteristics which are not easily found together in other kinds of biosurfactants (Cooper & Zajic, 1980). Additionally, both surfactants decrease viscosity significantly, while forming highly stable emulsions, contrary to the instability reported for other biosurfactants on Boscan and Monagas crude oils, both Venezuelan extra-heavy oils (Gutnick 1984). From the results of the chemical analysis, it appears the surfactants are rhamnolipids, sample MEOR 172 having 1.58 times as much rhamnose as sample MEOR 171, analogous to rhamnolipids reported by Itoh *et al.* (1971) and Robert *et al.* (1989).

Biosurfactants from strains MEOR 171 and MEOR 172 have several properties that are desirable for enhanced oil recovery operations. They are not affected by temperature, pH or calcium and magnesium concentrations in the ranges found in many oil reservoirs, while their performance improves with increased salinity. They are produced constitutively in glucose and therefore may be produced on a large scale from low cost agro-industrial wastes. In conclusion, they constitute attractive choices for the Venezuelan oil industry to be considered in the processes of transportation, cleaning of tanks, decontamination of polluted areas and in microbial-enhanced oil recovery.

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(*Received in revised form* 18 *June* 1991; *accepted* 1 *July* 1991)