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Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9

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Abstract

A non-sterile biosurfactant preparation (surfactin) was obtained from a 24-h culture of *Bacillus subtilis* O9 grown on sucrose and used to study its effect on the biodegradation of hydrocarbon wastes by an indigenous microbial community at the Erlenmeyer-flask scale. Crude biosurfactant was added to the cultures to obtain concentrations above and below the critical micelle concentration (CMC). Lower concentration affected neither biodegradation nor microbial growth. Higher concentration gave higher cell concentrations. Biodegradation of aliphatic hydrocarbons increased from 20.9 to 35.5% and in the case of aromatic hydrocarbons from nil to 41%, compared to the culture without biosurfactant. The enhancement effect of biosurfactant addition was more noticeable in the case of long chain alkanes. Pristane and phytane isoprenoids were degraded to the same extent as n-C17 and n-C18 alkanes and, consequently, no decrease in the ratios n-C17/pri and n-C18/phy was observed. Rapid production of surfactin crude preparation could make it practical for bioremediation of ship bilge wastes.

Abbreviations: CMC: Critical micelle concentration; UCM: Unresolved complex mixture

Introduction

Bioremediation of oil polluted ecosystems using microorganisms is an emerging environment-friendly technology, based on the fact that biological degradation is Nature's main process for the removal of non-volatile fractions of oil from the environment (Prince 1993). However, the limiting step seems to be the bioavailability of the particular hydrocarbons, due to low solubility in aqueous systems compatible with microbial life. Surface-active compounds may increase the bioavailability, either by increasing apparent hydrocarbon solubility in the aqueous system or by increasing the contact surface by means of stable emulsions (Desai & Banat 1997; Angelova & Schmauder 1999; Rosenberg & Ron 1999). Below the critical micelle concentration (CMC), surfactants in solution exist as monomers. Above the CMC, the formation of micelles occurs, and hydrocarbons can partition into the hydrophobic micellar core, increasing their apparent aqueous solubility. In some circumstances, partitioning into the micelle can make the hydrocarbon more accessible to the degrading microorganisms.

In most cases, the addition of synthetic surfactants inhibits biodegradation (Fietcher 1992; Rouse et al. 1994) by being toxic to the microorganisms or strongly partitioning the hydrocarbons. On the other hand, biologically produced surfactants (i.e., biosurfactants) do not have harmful effects on the environment, are not toxic to microorganisms, and may not sequester the hydrocarbons too strongly.

For bioremediation purposes, inoculation with biosurfactant producing microorganisms to enhance hydrocarbon biodegradation has also been considered, as this practice would offer the advantage of a continuous supply of a non-toxic and biodegradable surfactant at a low cost (Shreve et al. 1995). However, the microorganisms must adapt and be able to produce biosurfactants under the environmental conditions of the site to be remediated. Jain et al. (1992) found that the inoculation with rhamnolipid-producing *Pseudomonas aeruginosa* UG2 did not enhance biodegradation of a hydrocarbon mixture in soil. Alternatively, an exogenously produced biosurfactant can be used, and the literature describes many cases in which this procedure has resulted in biodegradation enhancement (Rouse et al. 1994; Banat 1995; Desai & Banat 1997).

We isolated *Bacillus subtilis* O9 from harbor sediments (San Antonio Oeste, Argentina) and selected it for its ability to produce surface-active compounds. Although it was screened among hydrocarbon-degrading isolates, previous studies have shown that soluble substrates, such as sucrose, resulted in higher biosurfactant production when compared to the production on hydrocarbons. The biosurfactant produced has been identified as surfactin (Morán et al. pers. comm.), a lipopeptide antibiotic produced by *Bacillus subtilis* strains and which already has been described (Peypoux et al. 1999).

In Puerto Madryn (Chubut, Argentina), ship waste containing a mixture of oil, grease, and water is collected from ship bilges and stored in outdoor dumping pools. This prevents the pollution of breeding areas of whales, seals, and other coastal fauna. However, accumulation of this highly polluting waste has led to a greater interest in studies to increase degradation rates. Naturally occurring microorganisms in the bilge waste have shown better hydrocarbon-biodegradation ability than other tested inocula (Olivera 1998, MSc. thesis). Therefore, the aim of this study was to evaluate the ability of crude preparations of the surfactin produced by *Bacillus subtilis* O9 to enhance hydrocarbon biodegradation of ship bilge waste.

Materials and methods

Microorganism and culture conditions

Bacillus subtilis O9 was routinely kept in agar slants of the production medium, which contained (in g l^{-1}): sucrose, 10; yeast extract, 5; $(NH_4)_2SO_4$, 1; Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 27; and $Mg_2SO_4 \cdot 7H_2O$, 0.6. Five ml l^{-1} of a trace element solution (Meyer & Fietcher 1985) were also added. Cultivation was performed at 30 °C in 250-ml Erlenmeyer flasks containing 75 ml of the production medium. They were inoculated with 1.5 ml of an overnight culture grown on the same culture medium and incubated for 24 hours in a gyrotory shaker at 160 rpm.

Biosurfactant crude extract

Cells were separated by centrifugation at 12,000 *g* for 30 min. at 4 °C. The supernatant was brought to pH 2.0 by the addition of 6 N HCl, left overnight at 4 °C, and then centrifuged at 12,000 *g* for 20 min at 4 °C. The pellet was resuspended in distilled water after adding 2.5 N NaOH to a final pH value of 8.0. Thirty ml of a non-sterile preparation containing 200 CMC (i.e., 200 × 20 mg l^{-1}) of biosurfactant were obtained from 1,200 ml of culture broth and used for the biodegradation experiment. The protein concentration in the extract, determined as described by Bradford (1979), was 1.96 g l^{-1}. Sucrose, determined by the dinitrosalycilic acid method (Miller 1959), was 1.28 g l^{-1}. Standards used for these determinations were bovine seroalbumin and sucrose, respectively.

Purification of the biosurfactant

In order to determine the CMC value in mg ml^{-1}, the biosurfactant was further purified according to Lin & Jiang (1997). Twelve ml of the crude extract were filtered trough a Nalgene Centrifuge Filter (MWCO = 10 kD) at 6,000 *g* until the minimal amount of retentate was achieved. The retentate was diluted in 50% methanol in order to dissociate the micelles and filtered at 6,000 *g* again. The filtrate was collected and, methanol was evaporated under vacuum in a rotatory evaporator at 65 °C, thus obtaining an aqueous solution of the biosurfactant, which was then frozen at −80 °C and lyophilised.

Determination of the CMC value

Measurements of surface tension were performed in different dilutions of a 3.6 g l^{-1} solution of purified surfactin. The value of CMC, expressed in mg l^{-1}, was obtained from the plot of surface tension versus the logarithm of the concentration. The CMC value was determined to be 20 mg l^{-1}.

Table 1. Amounts of inoculum, biosurfactant, and ship bilge waste added to the cultures.

Culture	Inoculum (ml l^{-1})	Biosurfactant (mg l^{-1})	Ship bilge waste (ml l^{-1})
Evaporation control	0	0	6
Biosurfactant control	0	80	6
Culture without biosurfactant	10	0	6
Culture with biosurfactant sub-CMC	10	10	6
Culture with biosurfactant supra-CMC	10	80	6

Biodegradation experiments

The culture medium consisted of seawater filtered through a Millipore membrane filter (0.45 μm) and then supplemented with the following (per liter of sea water): NH_4NO_3, 1 g; yeast extract, 0.2 g; and 4 ml of a phosphate solution containing 25 g $l^{-1}Na_2HPO_4{\cdot}12H_2O$ and 3.6 g $l^{-1}NaH_2PO_4$. An inoculum, consisting of the indigenous microbial community of the ship bilge waste, was prepared by adding one ml of non-sterile ship bilge waste to 100 ml of sterile medium supplemented with 0.5 g l^{-1} glucose; this was incubated in a 500-ml Erlenmeyer flask at 25 °C for 24 hours, and the medium was agitated and aerated by means of a continuous filtered air supply. The biodegradation experiment was conducted in 125-ml Erlenmeyer flasks containing 40 ml of the culture medium. Flasks were supplemented as shown in Table 1. Sterile controls were performed to determine remaining hydrocarbons after evaporation under the same experimental conditions. Taking into account that the biosurfactant preparation was not sterile and could contain spores of *Bacillus subtilis* O9, biosurfactant controls supplemented with the biosurfactant preparation at the highest concentration used in cultures were conducted. Cultures were incubated in a reciprocal shaker at 80 strokes min^{-1} and 25 °C during 7 days. Two replicates of each culture were left until the end of the cultivation period to determine residual hydrocarbons. A third replicate was periodically sampled for microbial growth and surface tension measurements.

Cultivation on glucose

The culture medium described above for the biodegradation experiment, but with 5 g l^{-1} glucose instead of the hydrocarbon waste, was used to cultivate the indigenous microbial community of the ship bilge waste in order to determine the effect of biosurfactant on microbial growth in the presence of a soluble carbon source. Replicate cultures were performed with and without 80 mg l^{-1} of biosurfactant, the highest biosurfactant used in the biodegradation experiment.

Analytical determinations

Estimation of microbial growth. Flasks were agitated for 1 min and left to stand for 30 s. Aliquots from the aqueous phase were taken, and absorbance at 450 nm (A_{450}) was determined.

Measurement of surface tension. Culture samples were centrifuged at 12,000 *g* for 20 min at 4 °C. The aqueous phase was carefully withdrawn and used to measure surface tension at 25 °C with a ring tensiometer (DuNuoy, Cat No 70545).

Surfactant concentration. Different dilutions of biosurfactant crude extract were prepared, and surface tension was measured. Surfactant concentration expressed in times of CMC was obtained from the plot of surface tension versus the logarithm of dilution. Surfactant concentration expressed in mg l^{-1} was calculated from the concentration expressed in times of CMC and the value of CMC determined as described above.

Hydrocarbon concentration. In order to recover hydrocarbons after the incubation period, flasks were extracted twice with 50 ml of hexane. Both extracts were pooled, adding fresh hexane to make exactly 100 ml. One ml of the solution was evaporated at room temperature to avoid volatile hydrocarbon loss. Following UNESCO guidelines (1982), the residue was redissolved in 0.2 ml hexane and passed through an aluminum oxide fractioning column. The column was first eluted with hexane to collect the aliphatic fraction, and then it was eluted with a 7 : 3 mixture of hexane and methylene chloride, followed by pure methylene chloride to collect the aromatic fraction. Aliphatic hydrocarbons were quantitated using a gas chromatograph (Konik 3000-HRGC) equipped with capillary column, flame ionization detector, and

splitless injector. The samples were analyzed for n-alkanes, pristane and phytane isoprenoids, unresolved complex mixture (UCM), and total aliphatics (resolved aliphatics + UCM). Ratios n-C17/pristane and n-C18/phytane, which were used as biodegradation indexes by some authors (Bragg et al. 1992; Marty & Martin 1996), were also calculated. Identification and quantification of hydrocarbons were made by comparison with external standard mixtures of alkanes. Total aromatic hydrocarbons were measured by fluorescence. Excitation and emission wavelengths were 310 and 360 nm, respectively. Calibration was performed with chrysene as a standard, and results were expressed in chrysene equivalents (UNESCO 1982; UNEP 1992).

Results and discussion

The greatest cell growth was observed in the presence of a supra-CMC biosurfactant concentration (Figure 1a). The indigenous microbial community of ship bilge waste caused a slight decrease in surface tension, evidencing that those microorganisms on their own were not able to produce significant amounts of biosurfactants (Figure 1b). Growth occurred in biosurfactant control flasks; therefore, germination of *Bacillus subtilis* O9 spores could be expected in all cultures containing biosurfactant. However, the culture with biosurfactant at sub-CMC concentrations did not show any decrease in surface tension (Figure 1b), indicating that *in situ* biosurfactant production by germinated spores was not noticeable. On the other hand, biosurfactant concentrations in biosurfactant controls and culture with biosurfactant supra-CMC were kept at a low value during the culture period, suggesting that the biosurfactant was not used significantly for growth.

Gas chromatography of the ship bilge waste showed that the aliphatic hydrocarbon fraction contained the homologous series of n-alkanes from n-C12 to n-C30, with a predominance of low molecular weight alkanes (n-alkanes $\leq$ C20) (Figure 2a). The UCM showed a bimodal distribution, which is characteristic of the presence of light and heavy fractions of hydrocarbons. The light fraction ranged from n-C12 to n-C19, with a maximum between n-C15 and n-C16, and the heavy fraction started with n-C23 and had a maximum between n-C27 and n-C29. Figure 2b shows the aliphatic fraction of the biodegraded waste, corresponding to the culture supplemented with biosurfactant at supra-CMC concentration. Resolved alkanes, isoprenoids, and both UCM fractions decreased.

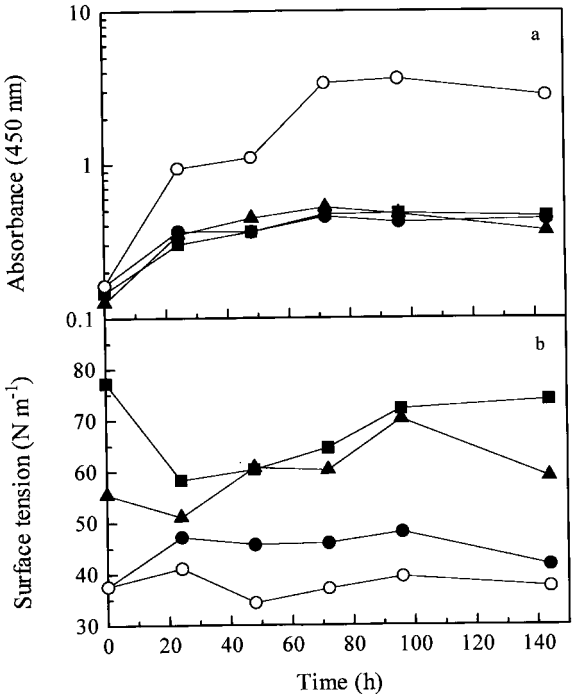

Figure 1. Growth measured as absorbance at 450 nm (a)and surface tension of supernatans (b) for the following cultures: biosurfactant control (●), culture without biosurfactant (■), culture with biosurfactant sub-CMC (▲) and culture with biosurfactant supra-CMC (○).

The residual aromatic and aliphatic hydrocarbon concentrations are shown in Table 2. The addition of a supra-CMC biosurfactant concentration to cultures of the indigenous microbial community of the ship bilge waste caused the sharpest decrease in aliphatic and aromatic hydrocarbons (35.5 and 41% respectively), and the highest biodegradation correlated with greatest cell growth. The addition of the biosurfactant in the absence of inoculum (biosurfactant controls) showed aliphatic and aromatic biodegradation percentages of 17.4 and 0% respectively, which were similar to those reached by the inoculated culture without biosurfactant. This is possibly due to the degrading activity of *Bacillus subtilis* O9, enhanced in the presence of supra-CMC biosurfactant concentration.

The nutrients supplied to the cultures by the addition of crude biosurfactant preparation consisted of 80 mg l^{-1} surfactin, 40 mg l^{-1} protein, and 26 mg l^{-1} carbohydrates. The difference in microbial growth between the culture without biosurfactant and the culture with 80 mg l^{-1} of biosurfactant was of 2.3 absorb-

Table 2. Residual hydrocarbons (mg per ml of culture medium). Values represent the arithmetic mean for duplicates ±1 standard deviation. The percentages of biodegradation are given in parenthesis.

	Resolved aliphatics	UCM	Total aliphatics	Total aromatics
Evaporation control	0.37 ± 0.06 (0.0)	1.36 ± 0.14 (0.0)	1.72 ± 0.19 (0.0)	0.17 ± 0.03 (0.0)
Biosurfactant control	0.32 ± 0.03 (13.5)	1.10 ± 0.11 (19.1)	1.43 ± 0.15 (17.4)	0.17 ± 0.02 (0.0)
Culture with biosurfactant	0.30 ± 0.02 (18.9)	1.06 ± 0.12 (22.1)	1.36 ± 0.09 (20.9)	0.18 ± 0.02 (0.0)
Culture with biosurfactant sub-CMC	0.39 ± 0.02 (0.0)	1.23 ± 0.00 (9.6)	1.61 ± 0.00 (6.4)	0.17 ± 0.02 (0.0)
Culture with biosurfactant supra-CMC	0.24 ± 0.03 (35.1)	0.87 ± 0.05 (36.0)	1.11 ± 0.08 (35.5)	0.10 ± 0.00 (41)

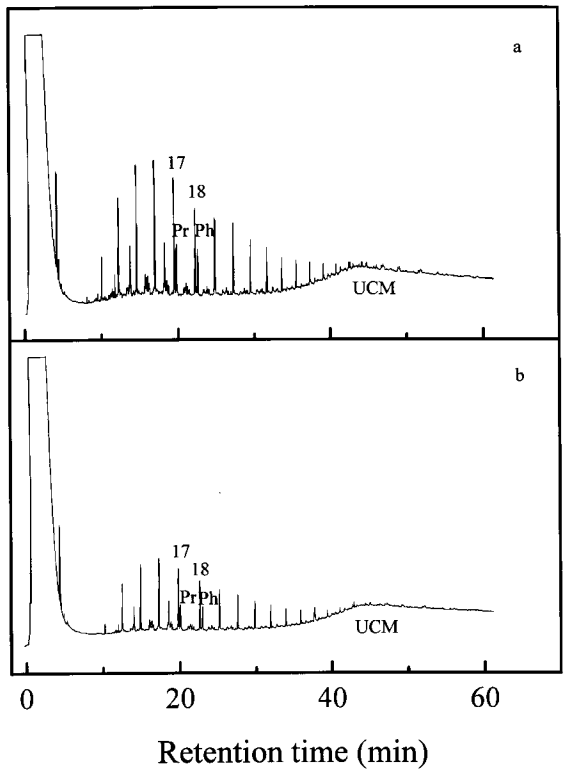

Figure 2. Gas chromatography of the aliphatic fraction of the ship bilge waste (a), and of the residual hydrocarbons in the culture with biosurfactant supra-CMC (b). Numbers 17 and 18 correspond to n-C17 and n-C18 alkanes. Pr and Ph correspond to pristane and phytane isoprenoids, respectively.

ance units, which corresponds to 0.37 g dry weight l^{-1} considering the relation between dry weight and absorbance in *Bacillus subtilis* O9 grown on production medium. Even though all the nutrients supplied in the biosurfactant preparation (carbohydrates, protein and surfactin) were used with a biomass yield of 60% (Pirt 1972), they would only account for 0.080 g dry biomass l^{-1}. In order to determine whether a growth factor was supplied to the cultures with the biosurfactant, the indigenous microbial community from the ship bilge waste was cultivated with glucose as carbon source, with and without 80 mg l^{-1} of biosurfactant. In this case, growth was not significantly stimulated by the supplementation of biosurfactant (data not shown). Therefore, the growth enhancement observed during growth on hydrocarbon would not be attributable to a nutrient from the biosurfactant preparation or to the utilization of the biosurfactant itself as a substrate. Thus, the biodegradation enhancement should be attributed to the presence of the biosurfactant, which probably increases hydrocarbon apparent availability.

None of the cultures produced significant variations of the n-C17/pristane and n-C18/phytane indexes (Table 3). This would suggest that the indigenous microbial community of the ship bilge waste and *Bacillus subtilis* O9 were able to degrade n-C17, n-C-18 alkanes, and the pristane and phytane isoprenoids to the same extent. The decrease of such indexes has been widely used to quantify biodegradation, because n-alkanes are considered more susceptible to biodegradation than isoprenoids (Atlas 1981; Marty & Martin 1996). However, Bragg et al. (1992) described that, in cases in which isoprenoids are degraded, the indexes become unreliable.

Figure 3 shows biodegradation percentages of different resolved alkanes, indicating the highest biodegradation enhancement for long-chain alkanes when the biosurfactant was present. Longer alkane chain have the lower aqueous solubility, resulting in its lower availability. Thus, the biosurfactant's effect on availability should be higher for longer alkanes.

Other microbial surfactants enhance hydrocarbon biodegradation. In the case of biosurfactant produced by *Torulopsis* strains, the enhancing effect was exerted for yeasts of the same genus, and this biosurfactant was considered a specific growth factor when hydro-

Table 3. Ratios n-C17/Pristane and N-C18/Phytane for the cultures.

Culture	n-C17/Pristane	n-C18/Phytane
Evaporation control	2.14	1.77
Biosurfactant control	2.14	1.77
Culture without biosurfactant	2.13	1.70
Culture with biosurfactant sub-CMC	2.08	1.74
Culture with biosurfactant supra-CMC	2.23	1.75

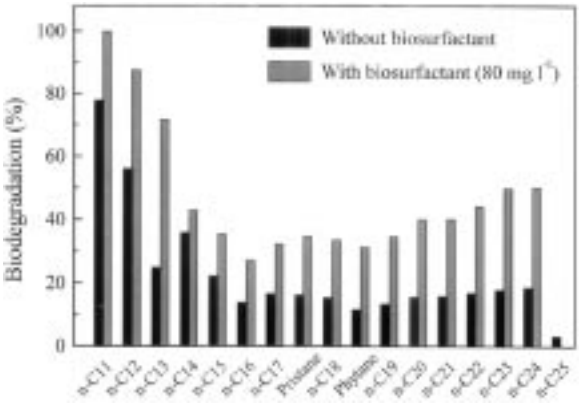

Figure 3. Percentages of biodegraded n-alkanes from n-C11 to n-C25 and pristane and phytane isoprenoids.

carbons are the carbon source (Hommel 1990; Inoue & Ito 1982). Addition of the rhamnolipid produced by *Pseudomonas aeruginosa* enhanced hydrocarbon degradation by the same organism (Itoh & Suzuki 1972; Shreve et al. 1995). Jain et al. (1992) found that a supra-CMC concentration of biosurfactants produced by *Pseudomonas aeruginosa* UG2 enhanced the biodegradation of different hydrocarbons by the native population of a soil sample, but the addition of sub-CMC concentration or the inoculation with this bacterium did not affect hydrocarbon degradation. This is similar to our results with the biosurfactant from *Bacillus subtilis* O9 in that only the addition of a supra-CMC concentration stimulated biodegradation. It also is consistent in that inoculation with *B. subtilis* O9, which indeed occurred due to the presence of spores in the biosurfactant preparation, did not result in biodegradation enhancement.

Despite the many advantages of surfactin over chemical surfactants for bioremediation and other diverse applications, its use has been limited on account of its high cost (Peypoux et al. 1999). If surfactin crude preparations can be produced on sucrose, in a short time, and in a relatively simple process, it represents a promising alternative for ship bilge waste treatment.

Conclusion

Surfactin from *Bacillus subtilis* O9 in a supra-CMC concentration, added in the form of a crude preparation, was able to enhance the biodegradation of ship bilge wastes and the growth of the indigenous microbial community. These results at laboratory scale represent a promising alternative for the biological treatment of hydrocarbon rich ship bilge wastes.

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References

Angelova B & Schmauder HP (1999) Lipophilic compounds in biotechnology- interactions with cells and technological problems. J. Biotechnol. 67: 13–32

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 45: 180–209

Banat, IM (1995) Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation. Bioresource Technol. 51: 1–12

Bradford, MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Bragg JR, Prince RC, Wilkinson JB & Atlas R (1992) Bioremediation for shoreline cleanup following the 1989 Alaskan oil spill. Exxon, Co. USA, Houston

Desai, JD & IM Banat (1997) Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61: 47–64

Fietcher, A (1992) Biosurfactants: moving towards industrial application. Trends Biotechnol. 10: 208–217

Hommel, RK (1990) Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. Biodegradation. 1: 107–119

Inoue S & S Ito (1982) Sophorolipids from *Torulopsis bombicola*: possible relation to alkane uptake. Appl. Environ. Microbiol. 43: 1278–1283

Itoh S & T Susuki (1972) Effects of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in n-paraffin-utilizing ability. Agric. Biol. Chem. 36: 2233–2235

Jain DK, H Lee & JT Trevors (1992) Effect of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil. J. Ind.Microbiol. 10: 87–93

Lin SC & Jiang HJ (1997) Recovery and purification of the lipopeptide biosurfactant of *Bacillus subtilis* by ultrafiltration. Biotechnol. Tech. 11: 413–416

Marty P & Y Martin (1996) Seed and feed strategy against oil spills in a marine environment: laboratory and simulated outdoor experiments with selected natural bacterial strains. J. Mar. Biotechnol. 4: 155–158

Meyer HP & Fietcher A (1985) Production of cloned human leukocyte interferon by *Bacillus subtilis*: optimal conditions are related with restrained growth. Appl. Environ. Microbiol. 50: 5023–5027

Miller, G.L. 1959. Use of dinitrosalycylic acid reagent for determination of reducing sugars. Anal. Chem. 13: 426–428

Peypoux F, Bonmatin JM & Wallach J (1999) Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotechnol. 51: 553–563

Pirt SJ (1975) Principles of microbe and cell cultivation. John Willey and Sons, New York

Prince RC. 1993. Petroleum spill bioremediation in marine environments. Crit. Rev. Microbiol. 19: 217–242

Rosenberg E & Ron E (1999) High- and low-molecular-mass microbial surfactants. Appl. Microbiol. Biotechnol. 52: 154–162

Rouse, DJ, Sabatini DA, Suflita JM & Harwell JH (1994) Influence of surfactants on microbial degradation of organic compounds. Crit. Rev. Environ. Sci. Technol. 24: 325–370

Shreve GS, Inguva S & Gunnam S (1995) Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *Pseudomonas aeruginosa*. Mol. Mar. Biol. Biotechnol. 4: 331–337

UNEP (1992) Determinations of petroleum hydrocarbons in sediments. Reference Methods for Marine Pollution Studies, No. 20

UNESCO (1982) Determinación de hidrocarburos del petróleo en los sedimentos. Manuales y Guías 11, p. 35