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Biosurfactant-enhanced degradation of residual hydrocarbons from ship bilge wastes

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The use of *Bacillus subtilis* O9 biosurfactant (surfactin) and of bioaugmentation to improve the treatment of residual hydrocarbons from ship bilge wastes was studied. A biodegradation experiment was conducted in aquaria placed outdoors under non-aseptic conditions. Three treatments were examined: culture medium plus bilge wastes, bioaugmentation with microorganisms from bilge wastes, and bioaugmentation plus biosurfactant. Samples were analyzed for viable counts, aliphatic and aromatic hydrocarbon concentrations, *n*-C17/pristane and *n*-C18/phytane ratios. While the addition of biosurfactant stimulated hydrocarbon degradation, bioaugmentation did not produce any remarkable effect. At day 10, the remaining percentages of aliphatic and aromatic hydrocarbons in aquaria, which received biosurfactant, were 6.8 and 7.2, respectively, while it took 20 days to reach comparable results with the other treatments. The biosurfactant did not affect the preferential biodegradation of *n*-C17/pristane and *n*-C18/phytane. This biosurfactant, which can be produced in a relatively simple and inexpensive process, is a promising alternative in the optimization of hydrocarbon waste treatment. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 70–73.

Keywords: biodegradation; hydrocarbon; biosurfactant; surfactin; *Bacillus*

Introduction

Several areas along the Patagonian coast (Argentina) are exposed to hydrocarbon pollution. The main oil pollution sources include petroleum exploitation and its transportation in tankers, bilge washing and ballast discharge. High levels of anthropogenic hydrocarbons were associated with crude oil exploitation and harbour areas [6]. One of the most serious problems resulting from port activities is the inadequate handling of liquid waste from ships. Bilge waste, which consists of oil, grease and seawater, may be the most damaging to the environment. Studies leading to higher degradation rates of this potential contaminant are of interest, especially so when considering pollution risks to coasts that are breeding areas for whales, seals, seabirds, and other coastal fauna.

Numerous studies have proved the ability of microorganisms to degrade a wide range of hydrocarbons, including aliphatics [10,15], polycyclic aromatic hydrocarbons [5], and resins [21]. Naturally occurring microorganisms in the bilge waste have shown high hydrocarbon degradation capacity [16]. However, the low solubility of hydrocarbons in water limits their biodegradation. Surfactants of biological or chemical origin have been used to improve bioavailability of water-immiscible compounds [2,19]. It has been proposed that microbial surfactants can increase the surface area of hydrophobic substances, increase the apparent solubility of hydrophobic substrates or desorb them from surfaces, and regulate the attachment and detachment of microorganisms to and from surfaces [18]. Among the biosurfactants that can enhance hydrocarbon biodegradation are heteropolysaccharides, fatty acids, phospholipids, and glycolipids such as rhamnolipids and sophorolipids [8,18,19]. Surfactin, an antibiotic lipopeptide produced by *Bacillus subtilis* strains [17], has been effective in releasing hydrocarbons from oily surfaces and has been used for microbially enhanced oil recovery (MEOR) technology [3].

B. subtilis O9, isolated from polluted sediments from the Patagonian coast, produces the biosurfactant surfactin when grown on sucrose [14]. This biosurfactant, in the form of a crude preparation, enhanced hydrocarbon biodegradation and microbial growth under laboratory conditions [13]. Our objective in this study was to evaluate, in outdoor experiments, the usefulness of both supplementation with *B. subtilis* O9 biosurfactant and bioaugmentation with native microorganisms, for the treatment of residual hydrocarbons from ship bilge waste. The use of autochthonous microorganisms also adds to the very limited knowledge of them and their potential for future environmental applications.

Materials and methods

Experimental design

Bilge wastes were collected from dumping pools in Puerto Madryn City. Six aquaria (20 l capacity), placed outdoors under non-aseptic conditions, were used. Each aquarium was filled with 8 l of filtered (0.45 μm) seawater supplemented with (in g l^{-1}): NH_4NO_3, 1; yeast extract, 0.2; 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. The experiment consisted of three treatments, with two aquaria for each treatment. To each aquarium of the first treatment (control), 50 ml of the hydrocarbon waste was added. The second treatment (bioaugmented) contained 50 ml of hydrocarbon waste and 400 ml of inoculum, and the third (biosurfactant) 50 ml of hydrocarbon waste, 400 ml of inoculum, and 16 ml of a biosurfactant preparation containing 4 g l^{-1} of surfactin. During the experiment, an air pump

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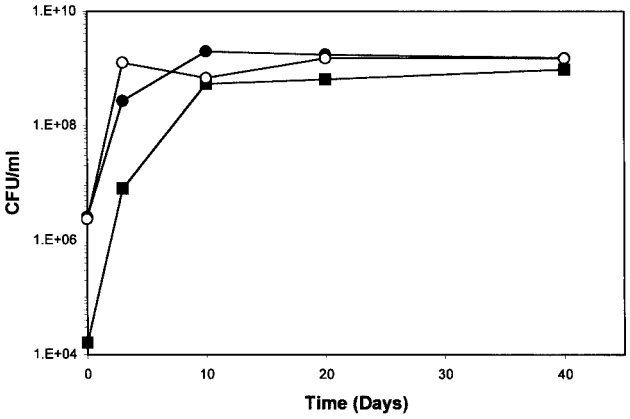

Figure 1 Viable counts in control (■), bioaugmented (•), and biosurfactant (○) treatments. Symbols indicate mean values of two independent replicas. Coefficients of variation were between 2.8% and 14.1%.

connected by a delivery system to air diffusers in each aquarium was used to maintain an aerobic environment.

During the experiment, the temperature was between 33.2°C and 2.2°C, and the mean wind speed was 17.3 km h^{-1} (J.C. Labraga, Area Fısica Ambiental CENPAT, personal communication).

Inoculum preparation

The inoculum contained native microorganisms from the bilge waste dumping pool, which has shown better hydrocarbon biodegradation ability than other inocula tested (Olivera 1998, MSc Thesis). The inoculum was prepared in 2 l Erlenmeyer flasks containing 700 ml of the medium described previously, supplemented with 0.5 g l^{-1} glucose and 10 ml l^{-1} of ship bilge waste. Flasks were incubated for 24 h at 25°C and aerated by continuous filtered air. The inoculum viable count was 8×10^7 cfu ml^{-1}.

Biosurfactant production

B. subtilis O9 was preserved on agar slants of the production medium. The production medium contained (in g l^{-1}): sucrose, 10; yeast extract, 5.0; $(NH_4)_2SO_4$, 1; Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NaCl, 27 and $Mg_2SO_4{\cdot}7H_2O$, 0.6. An mount of 5 ml l^{-1} of a trace element solution [12] was also added. The cultures were performed in 250 ml Erlenmeyer flasks containing 75 ml of culture medium. They were inoculated at 20 ml l^{-1} with an overnight culture, grown on the same culture medium, and incubated in a gyratory shaker at 160 rpm and 30°C for 24 h.

Cells were separated by centrifugation at 12,000×*g* at 4°C for 30 min. The supernatant was brought to pH 2 by the addition of 6 N HCl, left overnight at 4°C, and then centrifuged at 12,000×*g* at 4°C for 20 min. The pellet was resuspended in distilled water, adding 2.5 N NaOH to give a final pH of 8. This crude preparation contained 200 CMC (critical micelle concentration) of biosurfactant.

In order to determine the CMC value in milligrams per milliliter, the biosurfactant was further purified [9]. Twelve milliliters of the crude extract was filtered trough a Nalgene Centrifuge Filter (MWCO=10 kDa) at 6000×*g* until the minimal amount of retentate was achieved. The retentate was diluted in 50% methanol in order to dissociate the micelles and filtered again at 6000×*g*. The filtrate was collected and methanol was evaporated by applying vacuum in a rotatory evaporator at 65°C, yielding an

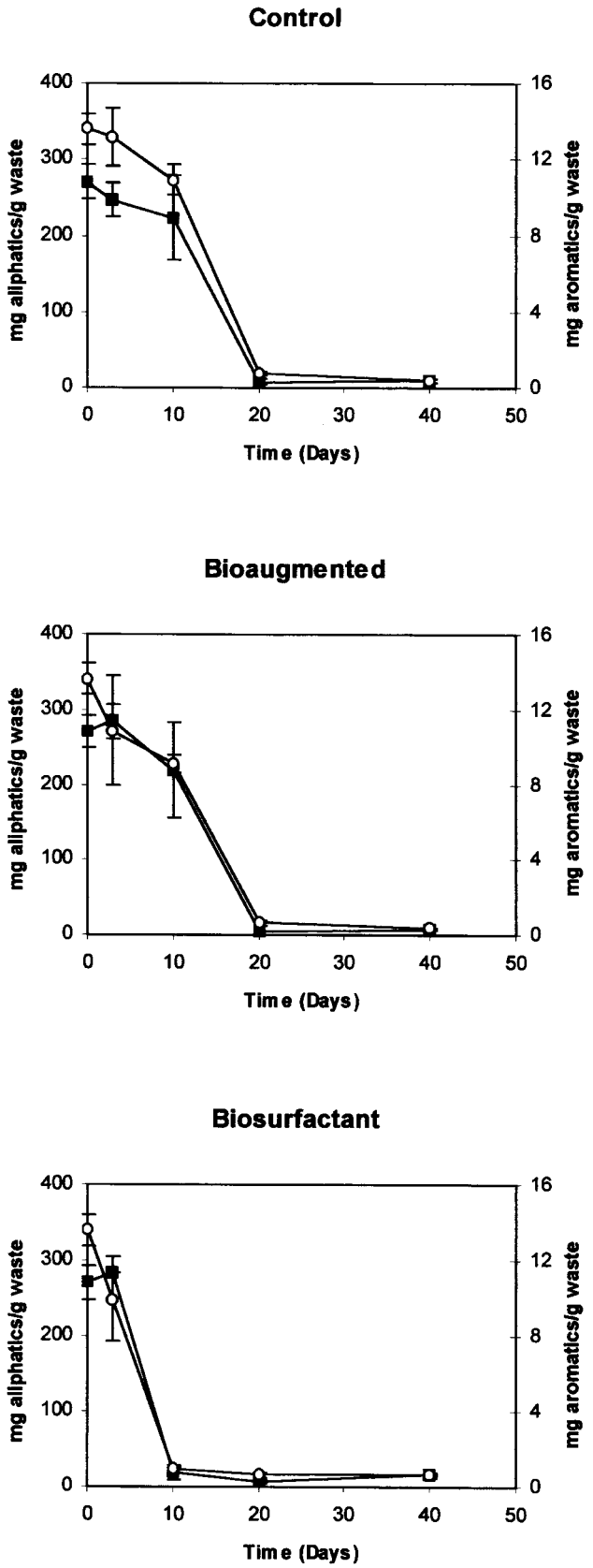

Figure 2 Total aliphatic (■) and total aromatic (○) hydrocarbon concentrations in control, bioaugmented, and biosurfactant aquaria. Symbols indicate mean values of two independent replicas. Bars correspond to ± 1 SD.

aqueous solution of the biosurfactant, which was then frozen at $-80^{\circ}C$ and lyophilised.

Measurements of surface tension were performed using different dilutions of a 3.6 g l^{-1} solution of purified surfactin. The value of CMC, expressed in milligrams per liter, 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} of surfactin.

Microbiological analysis

Samples were periodically collected from the aqueous phase, which remained mixed by air bubbling, and viable counts were determined by the spread plate method on natural seawater medium supplemented with (g l^{-1}): glucose, 5.0; $NaNO_3$, 2.97; NH_4Cl, 3.74; yeast extract, 3; and agar, 20. Colonies on the plates were counted after 5 days of incubation at 22°C.

Chemical analysis

Samples from the oil phase of each aquarium were collected after 3, 10, 20, and 40 days. Each composite sample consisted of five subsamples of about 200 mg taken at random from the oil phase using a stainless steel spatula. Subsamples were combined and mixed, and then about 150 mg was weighed and dissolved in 4 ml of high-purity hexane, shaken well and allowed to settle [7]. Two milliliters of the supernatant was transferred to a glass vial and left to evaporate at room temperature. The residue was redissolved in 0.2 ml hexane. Clean up, isolation of hydrocarbon fractions (aliphatic and aromatic), and hydrocarbon analysis were described previously [6]. The aliphatic fraction was measured by high-resolution gas chromatography (KONIK-3000). 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 (*n*-C17/Pri) and *n*-C18/phytane (*n*-C18/Phy), which were used as biodegradation indices [4,11], were calculated. Total aromatic hydrocarbons were measured by fluorescence. Calibration was performed with chrysene as a standard, and results were expressed in chrysene equivalents. To determine the content of hydrocarbons in the aqueous phase of the most contrasting treatments (control and biosurfactant), aliquots of 100 ml were taken at the same time as oil samples, extracted twice with 50 ml of hexane, concentrated, and analyzed as described above.

Statistical analysis

Bivariate ANOVAs with replications were conducted to compare decline in concentrations of total aromatic and total aliphatic hydrocarbons, and the evolution of biodegradation rates *n*-C17/Pri and *n*-C18/Phy [20]. When these analyses indicated significant differences, univariate ANOVAs were run to test the statistical similarity of data between treatments at each time point. For all statistical tests, the significance level was $\alpha=0.05$.

Results

Until day 10, viable counts in the bioaugmented treatment, as well as with biosurfactant, remained higher than in the control treatment. Afterwards, the three treatments tended to reach comparable concentrations (Figure 1).

Bivariate ANOVAs showed statistically significant differences among the three treatments for the concentrations of total aliphatic and total aromatic hydrocarbons. Moreover, treatments showed differential effects on the concentrations of these compounds during the course of the experiment.

Univariate ANOVAs run on data at each sample time (3, 10, 20, and 40 days) indicated significant differences due to treatments in the concentrations of total aliphatics and total aromatics at day 10, and of total aliphatics at day 40. At day 10, the hydrocarbon concentration in aquaria treated with biosurfactant was much lower than those observed in other aquaria (Figure 2). In the second case (day 40), the lowest aliphatic concentration was detected in the bioaugmented aquaria. No significant differences were detected among treatments at any other sampling time.

Gas chromatograms of bilge waste aliphatic fractions showed the continuous series of *n*-alkanes from *n*-C11 to *n*-C30, the presence of pristane and phytane isoprenoids, and an UCM. Low-molecular-weight *n*-alkanes (*n*-C$\leq$20) predominated. The UCM showed a bimodal distribution with maxima at C15–C16 and C27–C29, which are characteristic of light and heavy hydrocarbon fractions, respectively. The biosurfactant stimulated biodegradation in both components of the aliphatic fraction, resolved aliphatics (*n*-alkanes plus pristane and phytane) and UCM, especially between days 3 and 10.

Table 1 Decrease in the *n*-C17/pristane and *n*-C18/phytane ratios at different treatments (control, bioaugmented, and biosurfactant)

Treatment	Time (days)	*n*-C17/pristane	*n*-C18/phytane
Control	0	1.86±0.042	1.68±0.120
	3	1.88±0.028	1.70±0.056
	10	1.03±0.183	0.88±0.113
	20	0.29±0.035	0.23±0.021
	40	0.29±0.113	0.21±0.007
Bioaugmented	0	1.86±0.042	1.68±0.120
	3	1.88±0.014	1.75±0.035
	10	0.78±0.155	0.67±0.120
	20	0.40±0.155	0.16±0.198
	40	0.08±0.000	0.09±0.000
Biosurfactant	0	1.86±0.042	1.68±0.120
	3	1.91±0.021	1.64±0.021
	10	0.75±0.120	0.70±0.205
	20	0.26±0.127	0.12±0.021
	40	0.29±0.074	0.14±0.007

The ratios *n*-C17/Pri and *n*-C18/Phy decreased with time, indicating a biodegradation process in which *n*-alkanes were degraded faster than isoprenoids (Table 1). No statistically significant differences among treatments were found.

At day 10, when the most significant difference among treatments was observed, total aromatic and aliphatic hydrocarbon concentrations in the aqueous phase of the biosurfactant-treated aquaria represented only 3.9±1.4% and 2.8±0.6%, respectively, of hydrocarbon decrease in the oil phase. A similar relationship was observed for controls.

Discussion

In the control and bioaugmented treatments, the period of most rapid aliphatic and aromatic concentration decrease was between days 10 and 20, while in the biosurfactant-treated systems, it was between days 3 and 10. Both periods corresponded to the early stationary phase of growth and a high viable microbial concentration.

Control and bioaugmented aquaria showed a similar hydrocarbon concentration decline, indicating that bioaugmentation with native bilge waste microorganisms did not produce a significant effect. On the other hand, the addition of biosurfactant stimulated hydrocarbon degradation.

Biosurfactants exert a highly specific influence on different microorganisms growing on hydrocarbons [8]. Treatments with different biosurfactants have shown a variety of effects on hydrocarbon biodegration, ranging from enhancement to inhibition [19]. In the present study, the biosurfactant stimulated hydrocarbon degradation activity of a natural microbial community already adapted to hydrocarbons, shortening the time required for hydrocarbon degradation. After 20 days, the remaining concentrations of aliphatic and aromatic hydrocarbons in controls were 3.0% and 5.8% of the initial amount, and in bioaugmented aquaria, 2.1% and 5.1%, respectively. It took only 10 days to reach comparable percentages in biosurfactant-treated aquaria (aliphatics 6.8%, aromatics 7.2%). Moreover, in the latter case, hydrocarbon concentration in the aqueous phase was very low compared to the hydrocarbon decrease in the oil phase. This indicates that the enhancement of hydrocarbon bioavailability, caused by the biosurfactant which is associated with interfacial tension reduction between aqueous and oil phases, is accompanied by a process of active biodegradation of these compounds.

The addition of *B. subtilis* O9 biosurfactant did not affect the preferential biodegradation of *n*-C17/pristane and *n*-C18/phytane ratios. It should be noted that the concentrations of these hydrocarbons were very much lower in biosurfactant-treated aquaria than in the rest (e.g. day 10); this indicates that the biosurfactant increases bioavailability of hydrocarbons but does not modify their preferential biodegradation. In spite of the faster biodegradation of *n*-alkanes, microorganisms from bilge wastes also degraded pristane and phytane. At day 20, the remaining percentages of heptadecane and octadecane were about 0.3–0.7%, while pristane and phytane percentages were about 2–5%. This is in accordance with the results from previous laboratory experiments, where after a 12-day incubation period, 75% of the initial concentration of these isoprenoids had disappeared [16].

Bacillus biosurfactants have been effective in MEOR applications [1,3]. In this study, a different feature of the usefulness of surfactin for environmental purposes was tested. Surfactin in the form of a crude extract increased hydrocarbon bioavailability and shortened the time required for its natural biodegradation. Surfactin also has the advantage of being produced in a relatively simple and inexpensive process.

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