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Effects of *Bacillus subtilis* O9 biosurfactant on the bioremediation of crude oil-polluted soils

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Abstract

The application of a surfactant from *Bacillus subtilis* O9 (Bs) on the bioremediation of soils polluted with crude oil was assayed in soil microcosms under laboratory conditions. Three concentrations of biosurfactant were assayed (1.9, 19.5, and 39 mg kg^{-1} soil). Microcosms without biosurfactant were prepared as controls. During the experiment, the crude oil-degrading bacterial population, the aliphatic and aromatic hydrocarbons were monitored in each microcosm. The results indicated that applying Bs did not negatively affect the hydrocarbon-degrading microbial population Concentrations of 19 and 19.5 mg (Bs) per kilogram of soil stimulated the growth of the population involved in the crude oil degradation, and accelerated the biodegradation of the aliphatic hydrocarbons. However, none of the assayed Bs concentrations stimulated aromatic hydrocarbon degradation.

Abbreviations: CMC – critical micelle concentration; NALP – non-aqueous-phase liquid; RAH – resolved aliphatic hydrocarbons; UCM – Unresolved complex mixture

Introduction

Hydrocarbons have been released into the soil environment as a result of accidental spillage and improper disposal practices. These facts have led to environmental pollution, and constitute a serious threat to groundwater.

Among the many techniques employed to decontaminate affected sites, *in situ* bioremediation using indigenous microorganisms is the most widely used. One limitation of biodegradation is that many hydrocarbons are poorly accessible to bacteria due to their low solubility in aqueous systems compatible with microbial life (Reddy et al. 1982). Heavily contaminated soils contain a separate non-aqueous-phase liquid, which may be present as droplets or films on soil particle surfaces. Many hydrocarbons are insoluble in water and remain partitioned in the non-aqueous-phase liquid (Stelmack et al. 1999).

A possible way of enhancing the bioavailability of hydrophobic organic compounds is the application of surfactants, molecules that consist of a hydrophilic and hydrophobic parts. Surfactants increase the solubility of hydrocarbons by forming micelles. The surfactant molecules begin to

assemble into micelles above a certain concentration, known as the critical micelle concentration (CMC). Hydrocarbons can partition into the hydrophobic micellar core, increasing their apparent aqueous solubility (Edwards et al. 1991; Jafvet 1991). Some studies have indicated that surfactants enhance hydrocarbon biodegradation (Aronstein & Alexander 1992; Thiem 1994), others have found that applying surfactants inhibits biodegradation. The suggested mechanisms of inhibition include: (a) reduction in substrate bio-availability when bound into surfactant micelles (Volkering et al. 1995); (b) surfactant may alter the interactions between the cells and the substrate (Stelmack et al. 1999); and (c) surfactant toxicity may alter the composition of the microbial populations responsible for hydrocarbon mineralization (Colores et al. 2000; Tsomides et al. 1995).

Biologically produced surfactants have less toxicity to microorganisms and may not sequester the hydrocarbons too strongly (Siñeriz et al. 2001), Morán et al. (2000) isolated a strain of *Bacillus subtilis* O9 from sediments of San Antonio Oeste harbor (Argentina) and selected it for its ability to produce a surface-active compound. The biosurfactant produced by the isolated strain has been identified as surfactin by mass spectrometry (A.C. Morán pers. comm.), a lipopeptide antibiotic produced by *Bacillus subtilis* strains that has been described by Peypoux et al. (1999). The effect of the surfactin produced by the *Bacillus subtilis* O9 strain on hydrocarbon biodegradation of hydrocarbon liquid waste has been studied by Morán et al. (2000). This study concluded that the surfactin enhanced the biodegradation of hydrocarbon rich ship bilge wastes.

Bahía Blanca city (Buenos Aires Province, Argentina) has an important crude oil refinery and port facilities, destined to charge and discharge crude oil and its by-products. These activities generate hydrocarbon rich wastes and sometimes, accidental oil spills in water and soil. The most widely used method to decontaminate the affected soils is the land farming technique using the degradative potential of indigenous microorganisms. The goal of this study was to evaluate the effects of the surfactin from *Bacillus subtilis* O9 from San Antonio Oeste harbor on the bioremediation of a crude oil contaminated soil, in microcosms simulating a land farming treatment.

Materials and methods

Biodegradation experiment

The experiment was carried out in soil microcosms. The microcosms were prepared as follows: a sandy loam soil collected from Bahía Blanca district was amended with crude oil (type: 'Medanito'; origin: Neuquén, Argentina; density: 0.84) provided by a local refinery, for a final 5% w/w concentration. The mixture was thoroughly homogenized. Afterward, 2 kg portions of the mix were distributed in PVC boxes ($0.30 \times 0.30 \times 0.20$ m). Microcosms without crude oil were prepared as controls. To determine the C:N and C:P ratios, samples of the contaminated soil were analyzed at the Nitrogen 15 National Services Laboratory. Results indicated a C:N ratio of 20:1 and C:P ratio of 300:1, so we decided to avoid the use of an inorganic fertilizer. Nevertheless these parameters were controlled during the experiment. The content of organic carbon in the original soil and in the crude oil contaminated soil was 1.027% and 4.850%, respectively.

The experiment design was as follows: (1) soil microcosms without crude oil (control A); (2) soil microcosms with crude oil (control B), (3–5) soil microcosms with crude oil and 1.95, 19.5 and 39 mg of *Bacillus subtilis* O9 biosurfactant (Bs) per kg of soil, respectively. Each treatment was performed in triplicate. The Bs was obtained as indicated by Morán et al (2000), and the different Bs concentrations were prepared from a crude extract of surfactin 1300 CMC, where 1 CMC = $20\,\mathrm{mg\,l^{-1}}$. The value of CMC was obtained from the plot of surface tension versus the logarithm of concentration of purified surfactin (Morán et al. 2000). Surfactant concentration expressed in time of CMC was obtained from the plot of surface tension versus the logarithm of dilution of Bs crude extract. The different Bs concentrations were added to the microcosms with the first watering with tap water.

The soil in all microcosms was manually tilled at 15-day intervals to produce proper aeration. To ensure suitable watering, each microcosm was provided with a moisture sensor, which was regu-

lated to detect changes below 50% of the field capacity of the soil, The moisture sensor were designed and made by a technician in our laboratory. The microcosms were kept at room temperature with fluctuations between 15 and 25 °C, depending on the season.

Periodically, samples from all microcosms were taken with plastics tubes of 5 cm length and 1 cm diameter. Each sample consisted of five cores removed from different parts of the microcosm. The cores were mixed to obtain a representative sample from each microcosm. The following determinations were made:

Dry weight

Five grams of soil from each sample were dried at 105°C overnight in aluminum boxes until constant weight. The boxes and the soil were weighed on a precision balance (AND ER-180 A) and water content of the soil was calculated.

Hydrocarbon-degrading bacteria enumeration

The most probable number method (MPN) for enumerating hydrocarbon-degrading bacteria described by Mills et al. (1978) was used. The method was adapted to soil bacteria using Winogradsky solution (Pochon 1962), amended with 0.1% w/v NH_4NO_3 and 0.1% w/v KH_2PO_4 (WSN). As sole carbon and energy source, 25 μl of autoclaved crude oil was added to each tube with 5 ml of WSN (Mills et al. 1978). Three grams of soil sample were suspended in 27 ml of sterile phosphate buffer (pH 7.2), The suspension was dispersed for 5 min at 500 rpm on a shaker and allowed to settle for 1 min. Successive decimal dilutions in sterile Locke solution (1.5% NaCl, 0.04% $MgCl_2$, 0.01% KCl, pH 7–7.5) (Verna 1945) were prepared from the supernatant of the soil suspension. Three MPN tubes were inoculated with each dilution (three-row MPN). Control tubes without crude oil were inoculated with the first sample dilution. The tubes were incubated at 25 °C for 45 days and scored positive or negative on the basis of visible turbidity and changes in crude oil appearance and/or formation of membranes attached to crude oil film. To perform the statistical analysis, the MPN of crude oil-degrading bacteria were transformed to base-10 logarithm and expressed as log MPN g^{-1} dry soil.

Hydrocarbon concentration

Petroleum hydrocarbons from 10 g of soil were extracted with *n*-hexane (Merck) using a Soxhlet device, with a 125 ml extraction flask. The extraction was performed for 15 cycles. The extract was evaporated during 72 h at room temperature to avoid volatile hydrocarbon loss (Oudot 1984). After evaporation, the amount of recovered hydrocarbons was gravimetrically determined.

The residue was dissolved with 0.2 ml of hexane and loaded on a silica gel column to clean-up and fractionate the hydrocarbons. The residue was at first eluted with hexane to collect the aliphatic fraction, then with hexane:methylene chloride (7:3), and finally with methylene chloride to collect the aromatic fraction (Oudot 1984; UNEP 1992).

Aliphatic and aromatic hydrocarbons were quantified by gas chromatography. The chromatograph (Konik 3000 HRGC) was equipped with a flame ionization detector, split injection system and a capillary column (DB1, 30 m, 0.25 μm id). During analysis, the injector temperature was set at 250 °C, the detector temperature at 320 °C and the oven temperature was programmed to rise from 60 °C (2 min) to 290 °C (20 min) in 8 °C min^{-1} increments. The compounds present in both fractions were identified by matching the retention times against pure standards (Sigma-Aldrich).

The aliphatic hydrocarbon fraction was analyzed for individual *n*-alkanes, pristane and phytane isoprenoids, total resolved aliphatics (RAH), unresolved complex mixture (UCM), and total aliphatic concentrations (RAH + UCM). The UCM involves cycloalkanes, branched alkanes, and other compounds unresolved by the capillary column, which show as a 'hump' below the resolved compounds. The UCM shape corresponds to a unimodal form related to low, medium, and high molecular weight compounds, UCM is normally present in petrogenic hydrocarbon chromatographic profiles. In the aromatic fraction naphthalene, acenaphtylene, acenapthene, fluorene, phenanthrene, anthracene, 3-methyl phenanthrene, 2-methyl phenanthrene, 9-methyl phenanthrene, 1-methyl phenanthrene, 2,7-dimethyl phenanthrene, pyrene, fluoranthrene and benzo(*a*)pyrene were identified. These determinations were performed at days 0, 30, 210, 300 and

390 of the experiment. The results were expressed as mg of hydrocarbons g^{-1} dry soil.

To detect differences between the treatments and the controls during the experiment the obtained values were subjected to two-way analysis of variance (ANOVA). The values from each treatment along time were subjected to one-way ANOVA. Differences between times and treatments were determined using a *t*-test with 5% significance level.

Results and discussion

The $\log_{10}$ NMP of indigenous hydrocarbon-degrading bacteria in the different treatments during the experiment are shown in Figure 1. The presence of crude oil generated an important increase of the hydrocarbon-degrading bacterial population in all microcosms, compared with the controls without hydrocarbons (control A). During the first 90 days of the experiment no statistical differences were found between the treatments (at 150 and 210 days). The microcosms treated with 1.95 and 19.5 mg Bs kg^{-1} soil showed a significant increase ($p < 0.05$) in the bacterial population compared with the control microcosms without Bs (control B) at 150, 210 and 300 days. The soil amended with 39 mg Bs kg^{-1} exhibited hydrocarbon-degrading bacteria counts similar to the controls except at 300 days of treatment, when a statistically significant difference with no treated microcosms was detected ($p < 0.05$). At the end of the experiment a decrease in hydrocarbon-degrading bacteria number was observed in the microcosms with and without Bs, probably caused by the lower concentration and/or bioavailability of the hydrocarbons in the soil.

The assayed concentration of the biosurfactant from *Bacillus subtilis* O9 did not produce inhibitory effects on the indigenous hydrocarbon-degrading bacterial population. The observed increase in the microcosms amended with 1.95 and 19.5 mg Bs kg^{-1} soil might have been caused by enhanced hydrocarbons bioavailability. At the beginning of the experiment, there was a high concentration of available hydrocarbons that supported the crude oil-degrading population growth in all microcosms. After several months, most of the remaining crude oil might have been adsorbed on soil particles and limited the bioavailability of the substrate in the treatment without Bs. However, no significant stimulation of the crude oil-degrading bacterial populations was observed in the microcosms amended with 39 mg Bs kg^{-1} soil. This Bs concentration could have altered the interaction between the bacteria and the hydrocarbons. Volkering et al. (1995) have

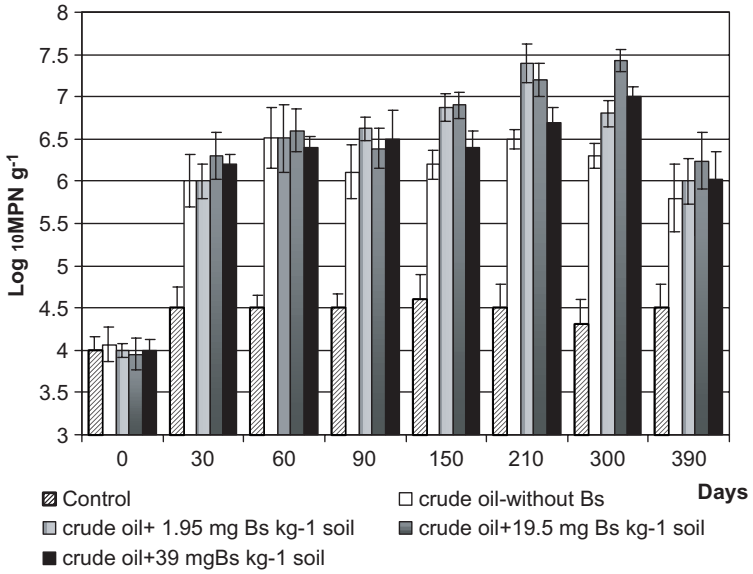

Figure 1. Log_{10} MPN of crude oil degrading bacteria per gram of dry soil in the different treatments during the experiment in soil microcosms. Values are arithmetic means for three microcosms per treatment. Bars represent standard deviations.

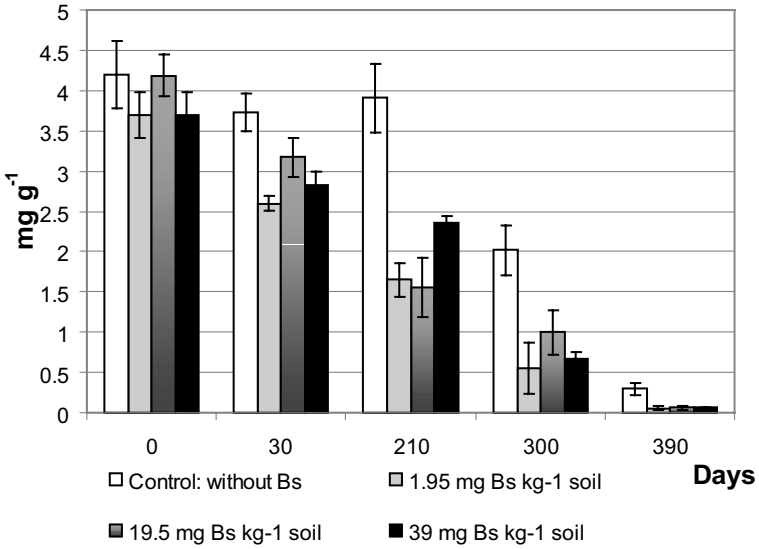

Figure 2. Concentration of resolved aliphatic hydrocarbons (RAH) per gram of dry soil during the experiment in soil microcosms. Values are arithmetic means for three microcosms per treatment. Bars represent standard deviations.

suggested that non-polar organic contaminants solubilized into surfactants micelles may be unavailable to microorganisms. Another explanation could be that this surfactant concentration in the soil inhibited the adhesion of the cells to the non-aqueous-phase liquid water interface (Foght et al. 1989; Stelmack et al. 1999).

The concentrations of RAH obtained from each treatment during the experiment are shown in Figure 2. No changes were detected in the microcosms without Bs until 300 days of treatment, when a significant decrease in RAH concentrations was observed. At 210 and 300 days of treatment, the differences between the microcosms without Bs and the treated ones were significant ($p < 0.05$). Even though at the end of the experience all microcosms reached similar concentrations, the addition of 1.95 and 19.5 mg Bs kg^{-1} soil resulted in a decrease of

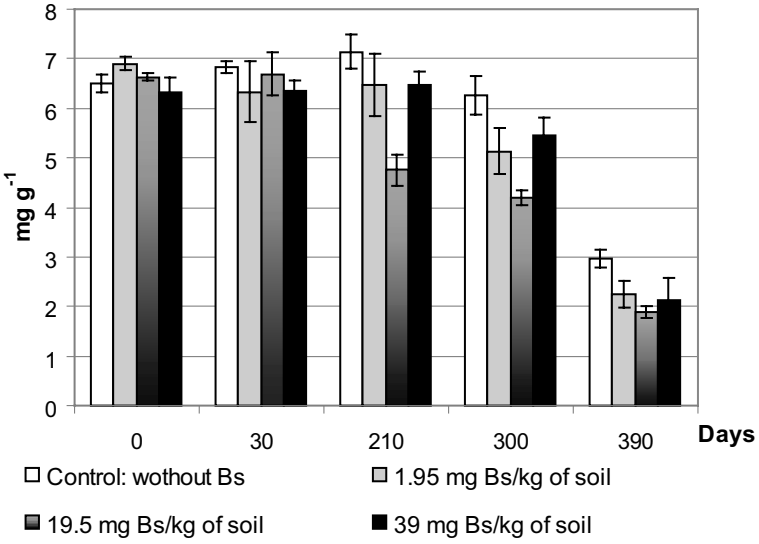

Figure 3. Aliphatic hydrocarbons: concentration of unresolved complex mixture (UCM) per gram of dry soil during the experiment in soil microcosms. Values are arithmetic means for three microcosms per treatment. Bars represent standard deviations.

between 58% and 40% in the RAH concentration at 210 days of treatment, 90 days before the microcosms without Bs.

UCM concentrations, from total aliphatic fraction at different times during the course of the experiment are shown in Figure 3. This fraction involves cycloalkanes and branched alkanes, which are more resistant to bacterial degradation. No differences were detected in the UCM concentration between the microcosms treated with Bs and the controls without Bs, except in the soil, amended with 19.5 mg Bs kg^{-1}. These microcosms showed a significant decrease of UCM concentration at 210 and 300 days of treatment compared with the controls without Bs. The microcosms with 19.5 mg Bs kg^{-1} showed an average biodegradation of 30% at 210 days when there was still no change observed in the controls.

No significant differences were detected in aromatic hydrocarbon degradation between microcosms with and without Bs at the end of the experiment.

Conclusions

From the results obtained in soil microcosms, we can conclude that the concentrations of surfactin from *Bacillus subtilis* O9 assayed in Bahia Blanca soil, did not cause a harmful effect on indigenous hydrocarbon-degrading bacteria. The concentrations 1.95 and 19.5 mg Bs kg^{-1} soil favored the growth of the bacterial population when the bioavailability of the substrate became limiting. Probably this effect accelerated the biodegradation rate of RAH. The concentration of 19.5 mg Bs kg^{-1} soil also showed a positive effect on the UCM fraction degradation rate. Although at the end of the experiment the Bs treated and control microcosms had similar hydrocarbons concentrations, the application of an adequate concentration of Bs diminished the concentration of some hydrocarbon fractions in less time. This fact suggests that the reduction of the toxicity of the pollutant to the biota (Pelletier et al. 2004), could accelerate the implementation of other stages in a remediation process, such as phytoremediation (Alexander 1999).

This work was a first step toward considering the application of this Bs in soil bioremediation; it was performed at laboratory scale and so it only considers some of all the biotic and abiotic factors involved in the natural environment.

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