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ORIGINAL PAPER

In situ bioremediation using biosurfactant produced by solid state fermentation

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Abstract The discovery that certain microorganisms, living within a marine environment, can actually degrade components of oil, has made possible the utilization of biological methods for the treatment of oil spills. A biosurfactant accelerates the process of degradation of pollutant composites. The objective of this work was to study the bioremediation in situ of a diesel oil spill by utilizing a biosurfactant produced through fermentation and then compare it with chemical remediation. The quantification and identification of hydrocarbons were carried out by the process of gas chromatography. The soil indigenous microorganisms were monitored. The experiment with biosurfactant reached reductions of 99% of the aliphatic hydrocarbons, while that of the chemical disperser experiment reached a maximum of 90% reduction in 180 days. In 15 days the biosurfactant removed 77% of the aliphatic hydrocarbons, the diesel oil experiment 8.7% and the chemical disperser only 5%. The biosurfactant was 99% effective for the removal of aromatic polycyclic hydrocarbons, up to 3 rings.

Keywords Bioremediation · Biosurfactant · Hydrocarbons · Fungi

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Introduction

The contamination of soil and groundwater with petroleum hydrocarbon compounds raises critical issues regarding environmental and health concerns and focuses increased attention on the development of innovative and sound technologies for its remediation. Bioremediation of petroleum hydrocarbons has been proposed as an effective, economic, and environmentally friendly technology (Madigan et al. 2003). However, bioavailability of hydrophobic organic compounds (HOCs) to microorganisms could be a limiting factor during the biodegradation process (Guha and Jaffe 1996; Jonge et al. 1997).

The term "biosurfactant" has been widely used and refers to an isolated or non-isolated compound obtained from a microorganism that has the capacity to influence interfaces and to significantly reduce the amount of work required to overcome surface tension. This process allows one system to disperse into another. The main advantages of chemical surfactants are their high biodegradability and their ability to act in adverse conditions, such as extreme temperatures, pH levels and salinity degrees. Other advantages include their easy synthesis and low critical micelle concentration (CMC). In addition, they easily interact with lipids, proteins and carbohydrates (Garti 1999; Kosaric et al. 1987).

Studies with respect to enhanced bioremediation by surfactant addition have focused mainly on chemically synthetic surfactants. Yet, despite decades of research, successful bioremediation of soil contaminated with petroleum hydrocarbon remains a challenge (Das and Merkherjee 2007; Rahman et al. 2003; Santos et al. 2008; Whang et al. 2008).

The capacity of biosurfactants to emulsify hydrocarbon/water mixtures has been well documented. This emulsifying property is demonstrated by the significant increase of

hydrocarbon degradation, and for this purpose, is utilized for the bioremediation of contaminated soils and wellsprings (Crapez et al. 2002). According to Cameotra and Bollag (2003) biosurfactants may be used in situ to emulsify and increase the solubility of hydrophobic contaminants and thus facilitate the access of local microorganism to the contaminated area so that the degradation of hydrophobic compounds occurs.

Aromatic polycyclic hydrocarbons (APHs) are creosote components and are produced during the oil refining process, coker (gas oil) production and wood preservation. Many are suspect of being carcinogenic. The general form for the APHs is $C_{4n} + 2H_{2n+4}$, where 'n' is the number of rings. They are degraded in a form similar to a simple aromatic ring since the APHs are degraded one ring at a time (Mulligan 2005).

In comparison to synthetic surfactants, relatively little information is available for biologically produced surfactants (biosurfactants), but their application in bioremediation processes may be more acceptable from a social point of view because they require minimal interventions in the environment. Potential advantages of biosurfactants include their unusual structural diversity that may lead to unique properties, the possibility of cost effective production, and their biodegradability (Mulligan 2005; Mulligan et al. 2001; Volkering et al. 1998).

Biodegradation in soils contaminated with APHs is limited due to the low solubility of these components and the high capacity of adsorption in soil and sediments (Mcelroy et al. 1989; Mihelcic et al. 1993; Volkering et al. 1995). One way to increase the solubility of the APHs is by using biosurfactants as mobilizing agents (Ganeshalinghan et al. 1994). Several technologies have been developed and implemented for the remediation of soils and sediments in order to decrease financial costs. In situ soil treatment is a preferable method which has lower cost and is less destructive than ex situ bioremediation. However, there are many problems with the in situ process because it presents multiple variables that can not be fully controlled (Mulligan et al. 2001).

The information regarding the composition of microbial populations in contaminated soils is very limited (Trevors 1998). An analysis of the effects of APHs on the microbial communities can contribute to a better understanding of the process of remediation in contaminated soils, allowing for a more effective control of microorganisms in the process (Gentry et al. 2003).

The objective of this paper is to study the in situ bioremediation of a diesel oil spill by utilizing a biosurfactant produced by solid state fermentation and then to compare its efficiency to the chemical remediation process. In addition, the paper presents a study of the influences of these processes on the ecosystem.

Materials and methods

Microorganisms

The filamentous fungus used was *Aspergillus fumigatus*. In previous studies (Martins et al. 2006; Gabiatti et al. 2006) it proved to be a good biosurfactant producer. It was isolated from a site that was contaminated by hydrocarbons located in the state of São Paulo, Brazil. The strain utilized was provided by the Food Microbiology Laboratory of the Food Engineering Faculty State University (FEA/UNICAMP) located in Campinas, SP/Brazil. The microorganisms were kept in inclined test tubes with potato-dextrose agar at 4°C. The spore suspension was obtained from inoculum propagation in Roux flasks and the spore count was conducted in a Neubauer Chamber.

Solid state fermentation by using Erlenmeyer's as bioreactors

Fermentation was carried out in 1,000 ml Erlenmeyer flasks by using the *A. fumigatus*. The fermentative medium was composed of defatted rice bran and husk. The bran was ground in a mill and then sieved to standardize the granulometry between 0.420 and 0.500 mm, and the husk was utilized without being ground. In addition, a solution of nutrients composed by $MgSO_4{\cdot}7H_2O$, $NaNO_3$, K_3PO_4 were added. A yeast extract and peptone were also part of the fermentative medium, and 1% diesel oil was added as a source of additional carbon. Except for the diesel oil, the rest of the medium was sterilized in an autoclave at 121°C for 15 min.

The physical and chemical conditions used in the fermentation were studied before by Martins et al. (2006), then were defined, 50% moisture, 30°C, 4.5 pH and initial spore concentration of 4×10^6 spores g^{-1}. The process time was 144 h.

Analytical determinations

All the analyses were performed in triplicate. The pH determination during the means of preparation was directly performed in pH meter. The moisture level of the fermented samples was determined according to the AOAC (1995) methodology of measuring the weight loss after drying in an oven at 105°C until constant weight.

The production of biosurfactant was estimated through the emulsifying activity ($EA_{w/o}$). Biosurfactant was extracted from the liquid phase of the culture by adding 3 parts (w/v) of 90°C water and shaking the mixture at 160 rpm, 50°C, for 30 min in a shaker (BRAUN CERTOMAT BS-1, Melsungen, Germany). After extraction the biosurfactant solution was vacuum filtered and the extract

tested for emulsifying activity by adding 2 ml of soy oil to 3.5 ml of the extract and vortexing the mixture at 700 rev min^{-1} during 1 min and allowing the mixture to stand undisturbed for 24 h. The water-in-oil emulsifying activity ($EA_{w/o}$) was estimated after 24 h by calculating the ratio between the total height of oil and the height of emulsified oil (Broderick and Cooney 1982) and expressed as EU g^{-1}. The Eq. 1 was used to calculate the emulsifying activity:

$$EA_{w/o} = \frac{E \times D}{[m(1-U)]} \quad (1)$$

where $EA_{w/o}$ is the water-in-oil emulsifying activity (EU g^{-1}); E is the percentage ration between height of emulsion and total height; D is the dilution of the sample in water; m is the wet mass (g); and U is the water content of the fermented medium. The $EA_{w/o}$ is defined as the quantity of fermented bran needed to produce a 1% hydrophobic phase emulsion stable for 24 h, and is expressed in EU g^{-1} of fermented medium.

Bioremediation

Bioremediation was conducted on soil samples taken from the "Ilha dos Cavalos" (Horses Island) located in the city of Rio Grande, RS/Brazil, whose coordinates are south latitude 32°00′49" and west longitude 52°07′57". Four stainless steel containers measuring 1,000 × 1,000 × 500 mm were installed in the ground. With the permission of the Brazilian Institute of Environmental Studies (IBAMA), the experiments proceeded. The first container contained only local soil. This container was used as a control for all subsequent analyses. The second contained soil and diesel oil. The third container contained soil, diesel oil and a chemical disperser used for possible oil spills. The fourth contained soil, diesel oil, and a biosurfactant produced by solid state fermentation. One liter of diesel oil was used in each of the containers.

The determination of the biosurfactant concentration in the culture medium was done through the emulsifying activity. The quantity of biosurfactant used was calculated based on its emulsifying activity. For each 1 l of oil spread 875 g of biosurfactant medium and 400 ml of chemical disperser were added. These were diluted in water for a better homogenization. A dilution ratio of one part fermented bran to three parts water (1:3) was utilized. The amount of the chemical disperser and biosurfactant were also the same ratio of 1:3. The mixture of the fermented medium with biosurfactant and water or chemical disperser and water were spread on the ground without further homogenize. The biosurfactant was not extracted from the culture medium to be applied in the soil. All the medium was used in the bioremediation process.

Sampling

Bioremediation trials were evaluated for a period of 6 months with samples being collected on days 1, 2, 7, 14, 21, 28, 60, and 90. The microbiological and hydrocarbon analyses were performed on days 1, 2, 3, 4, 15, 30, 60, 90, 150, and 180. The determinations for sampling locations within the containers were conducted randomly and always at the same depth. The microorganism analysis samples were stored in sterilized flasks and processed on the same day. The hydrocarbon analysis samples were frozen and sent to another laboratory to be processed at a later date.

Physical–chemical and meteorological data

The soil environment and ambient temperature were verified for each sampling. The soil pH determination was conducted with a bayonet-type combined glass electrode with readings expressed in a scale of ±0.01. Pluviometric data was also evaluated by the Federal University of Rio Grande (FURG) meteorological station.

Hydrocarbon determination

The Hydrocarbon samples were defrosted at room temperature and then dried at a maximum temperature of 40°C. The hydrocarbon extractions were performed in a Soxhlet extractor for 8 h using a 1:1 ratio solvent of *N*-hexane/Dichloromethane. After the extraction, the extract was removed by a rotating evaporator connected to vacuum pump.

The methodology for the chromatographic column separation of aliphatic and aromatic hydrocarbons is described below. After the separation, the extracts were then re-concentrated via a rotating evaporator. The UNEP (1991) methodology was followed for the procedures performed in the aliphatic and aromatic hydrocarbon analyses.

The identification of the aromatic and aliphatic hydrocarbons in the sediment was performed by the coupling of gas chromatography with a Shimadzu® mass detection spectrometer model QP5050A with an OV-5 column (60 mm × 0.25 mm × 0.25 μm). The initial temperature was 120°C with minimum interval increases of 10°C min^{-1} up to 260°C, followed increasing 3°C min^{-1} up to 280°C remaining isothermal for 10 min. The temperature of the injector and detector was 280°C. A volume of 1 μl of sampled hydrocarbons was injected with a split of 1:32 and with a flux of 1 ml min^{-1} of helium gas. The energy of the detector was 1.5–2 Kev. The method detection limit was established in 0.50 ng g^{-1} of the compound.

The identification of the hydrocarbons was obtained through sampled injections, and then a comparison of mass spectrums was done with the partner sample or identification was done with the data from the library equipment.

The aromatic compound quantification was carried out by the internal standardization technique of utilizing a mixture of deuterated aromatic hydrocarbons. The extracts were diluted in 300 and 5,000 µl of dichlorine-methane which had an internal chromatographic pattern at 10 mg l^{-1}. Quantification comparison was done by area calculations.

C_{12} to C_{34} aliphatic hydrocarbons fractions were analyzed. The APHs investigated were the 16 substances classified as major pollutants according to the USEPA (The Environmental Protection Agency of the United States). The pollutants are as follows, acenaphthylene, acenaphthene, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo[b] fluoranthene, benzo(g,h,i)perylene, indeno(1,2,3,-cd)pyrene, benzo(k)fluoranthene, chrysene, diben[a,h]anthracene, phenanthrene, fluoranthene, fluorine, naphthalene and pyrene.

Mesophilic bacteria count

The bacteria were deeply inoculated in Petri dishes with agar plate count with a dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} performed in duplicate. These were incubated for 48 h at 37°C which revealed a colony count of between 20 and 250 colonies. The bacteria count calculations were performed according to the ABNT (1997; The Brazilian Associations of Standards and Techniques).

Fungi count

The fungi were inoculated by surface spreading in Petri dishes with Dichloran Rose Bengal Chloramphenicol agar in duplicate dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}: These were incubated between 96 and 120 h and the count was performed over a 48 h period at 30°C which showed results between 10 and 100 colonies. The fungi count calculations were performed according to the ABNT (1997; The Brazilian Associations of Standards and Techniques). A qualitative analysis on fungi was also performed based on observation and a comparison with the control container.

Results and discussion

The production of biosurfactant during the fermentative process according to the emulsifying activity was 6.38 UE g^{-1} and the productivity was 0.027 UE g^{-1} h^{-1}. The pH data are important parameters for the monitoring of marine systems. They provide significant data about global variations of geochemical environmental conditions. The pH soil was not adjusted because we wanted to simulate natural process. The data also provides information regarding the degradation tendency of oil and hydrocarbon components. The average pH values of each experiment on oil, oil and chemical disperser, oil and biosurfactant and of a control container are indicated in Table 1.

Table 1 Average pH values during bioremediation experiment "in situ"

Time (days)	Control	Diesel oil	Disperser	Biosurfactant
1	5.5 ± 0.1	5.6 ± 0.1	5.4 ± 0.1	5.3 ± 0.1
2	5.4 ± 0.1	5.6 ± 0.0	5.3 ± 0.2	5.3 ± 0.2
3	5.5 ± 0.0	5.7 ± 0.1	5.3 ± 0.1	5.4 ± 0.1
4	5.3 ± 0.1	5.9 ± 0.1	5.2 ± 0.2	5.4 ± 0.0
15	5.3 ± 0.0	4.9 ± 0.2	4.8 ± 0.2	5.2 ± 0.2
30	5.2 ± 0.1	5.4 ± 0.1	5.2 ± 0.1	5.3 ± 0.2
60	5.3 ± 0.2	5.5 ± 0.1	5.3 ± 0.0	5.2 ± 0.3
90	5.3 ± 0.1	5.5 ± 0.0	5.2 ± 0.2	5.3 ± 0.1
150	5.1 ± 0.1	5.0 ± 0.2	4.9 ± 0.1	5.3 ± 0.2
180	5.1 ± 0.1	3.4 ± 0.1	3.4 ± 0.0	4.0 ± 0.1

The results showed an acidic character for each sediment. According to Dibble and Bartha (1979) bioremediation occurs with more efficiency within in the 7.5–7.8 pH range. Delaune et al. (1981) and Hambrick et al. (1980) verified in their study that pH affects hydrocarbon degradation especially with APHs. The aromatic hydrocarbon biodegradation rates decreased when the original pH of 8.0 was altered to 5.0, 6.5 and 9.0. Throughout the duration of the experiment, the pH continually presented an acidic character possibly interfering with the action of the microorganism present in the soil and thus negatively influencing the rate of biodegradation.

Atmospheric temperature, soil, pluviometric precipitation rates and microbiological analysis were evaluated for 90 days during which time the local soil had already recovered from the environmental impact caused by the diesel oil. During this time the atmospheric temperature varied between 10.5 and 27°C with the average reading of 21.2°C. The average soil temperature was 18.2°C. The precipitation rate was 2.05 mm per day during this period. According to Semple et al. (2001) many factors govern the behavior of organic pollutants including soil characteristics, chemical properties, environmental factors, temperatures and precipitation rates.

Microbiological analysis

Table 2 represents the quantitative results of the fungi and bacteria counts during the 90 days of collection for each experiment. The quantitative data indicates how the distribution of fungi mould, yeast and bacteria occurred during the experiment; however, the qualitative data was utilized to better understand the condition of microbiota within the native sediment. In certain cases the fungi count remained constant; however, only one specific species was predominant.

Table 2 Bacteria and fungi count in each of the trials

Exper.	X (cfu g^{-1})	Time (days)							
		1	2	7	14	21	28	60	90
Con.	Mould	$2.5 \times 10^4 \pm 431$	$3.6 \times 10^4 \pm 658$	$3.2 \times 10^4 \pm 716$	$3.5 \times 10^4 \pm 492$	$3.3 \times 10^4 \pm 978$	$3.0 \times 10^4 \pm 762$	$3.1 \times 10^4 \pm 478$	$1.0 \times 10^5 \pm 394$
	Yeast	$1.3 \times 10^4 \pm 407$	$1.6 \times 10^4 \pm 519$	$1.0 \times 10^4 \pm 202$	$1.8 \times 10^4 \pm 620$	$9.0 \times 10^3 \pm 405$	$9.5 \times 10^3 \pm 356$	$7.0 \times 10^3 \pm 401$	$1.4 \times 10^4 \pm 963$
	Bacteria	$5.6 \times 10^5 \pm 2888$	$1.4 \times 10^6 \pm 4885$	$1.1 \times 10^6 \pm 2806$	$9.7 \times 10^5 \pm 6972$	$1.5 \times 10^5 \pm 5846$	$1.6 \times 10^5 \pm 3652$	$6.2 \times 10^4 \pm 1966$	$8.4 \times 10^4 \pm 6020$
Diesel oil	Fungi	$6.0 \times 10^4 \pm 454$	$3.2 \times 10^4 \pm 450$	$3.4 \times 10^4 \pm 887$	$3.6 \times 10^4 \pm 413$	$6.8 \times 10^4 \pm 290$	$1.2 \times 10^4 \pm 453$	$1.1 \times 10^4 \pm 917$	$9.0 \times 10^4 \pm 127$
	Bacteria	$1.0 \times 10^5 \pm 1166$	$1.2 \times 10^5 \pm 1476$	$1.1 \times 10^5 \pm 485$	$9.8 \times 10^4 \pm 1471$	$1.2 \times 10^5 \pm 931$	$1.2 \times 10^5 \pm 1338$	$1.8 \times 10^5 \pm 1343$	$1.9 \times 10^5 \pm 1747$
Bios.	Fungi	$4.5 \times 10^6 \pm 6051$	$8.9 \times 10^6 \pm 4567$	$5.7 \times 10^6 \pm 4700$	$2.0 \times 10^6 \pm 4109$	$8.2 \times 10^6 \pm 4698$	$3.1 \times 10^6 \pm 6993$	$8.5 \times 10^5 \pm 2966$	$3.5 \times 10^6 \pm 3040$
	Bacteria	$2.2 \times 10^6 \pm 4754$	$4.1 \times 10^6 \pm 5362$	$7.4 \times 10^6 \pm 5498$	$4.5 \times 10^6 \pm 398$	$2.5 \times 10^6 \pm 8081$	$1.4 \times 10^6 \pm 4397$	$1.1 \times 10^6 \pm 4740$	$1.2 \times 10^6 \pm 5124$
Disp.	Mould	$2.2 \times 10^4 \pm 321$	$3.6 \times 10^4 \pm 475$	$2.5 \times 10^4 \pm 420$	$1.6 \times 10^4 \pm 595$	$3.5 \times 10^4 \pm 781$	$3.7 \times 10^4 \pm 88$	$5.8 \times 10^4 \pm 758$	$1.9 \times 10^4 \pm 240$
	Yeast	$6.6 \times 10^4 \pm 178$	$1.3 \times 10^5 \pm 472$	$1.1 \times 10^5 \pm 283$	$3.1 \times 10^4 \pm 135$	$2.7 \times 10^4 \pm 47$	$8.7 \times 10^4 \pm 403$	$2.4 \times 10^4 \pm 197$	$8.6 \times 10^4 \pm 157$
	Bacteria	$4.2 \times 10^4 \pm 125$	$1.3 \times 10^4 \pm 151$	$4.5 \times 10^4 \pm 161$	$8.3 \times 10^4 \pm 239$	$2.3 \times 10^5 \pm 1263$	$8.9 \times 10^4 \pm 58$	$1.0 \times 10^5 \pm 420$	$1.1 \times 10^5 \pm 699$

Exper. experiments; *Con.* control; *Diesel* diesel oil; *Bios.* biosurfactant; *Disp.* disperser; *X* microorganism concentration

In the microbiological analysis the distribution of mould, yeast and bacteria in control and disperser experiments, fungi and bacteria were found in diesel oil and biosurfactant data, in these two last treatments it was not possible to distinguish between mould and yeast.

Experiment with diesel oil

We observed that the quantity of bacteria was affected within the first 30 days of the experiment. This impact was most likely caused by the toxicity that the diesel oil represented to these microorganisms. After this period, there was a reduction in the number of bacteria with the control experiment. This reduction was probably due to the fact of the climate changes in the region.

Evaluating only the data in a quantitative format, it was observed that the diesel oil did not have a major impact on the fungi microbiota; however, when performing a qualitative analysis, it was observed that a certain filamentous species of white fungus (not identified) was predominant during the first 30 days of the experiment, and within this period the soil was impacted more by the spill. After this 30 day period the soil began to regenerate, and the microbiota began to resemble the one in the control container.

Gentry et al. (2003) evaluated the influence of the pyrene and phenanthrene on the microbial population of a soil and observed that there was no significant impact on the bacteria ($P = 0.888$) and fungi ($P = 0.765$) during 21 weeks of incubation. However, a large portion of the bacterial population apparently self developed from the spores that may have been resistant to the contaminant.

Experiment with biosurfactant

In this experiment fungus bran along with biosurfactant was used. During the count we observed that the *A. fumigatus* had complete dominion over the other fungi. The rest of the local microbiota fungi were inhibited by the large concentration of this microorganism. The counts remained between 8×10^5 and 8.9×10^6 cfu g^{-1} for the experiment using biosurfactant, and counts remained at 4×10^4 for the control experiment.

Even after 90 days, the concentration of *A. fumigatus* remained constant demonstrating that this strain is resistant to hydrocarbons and to oscillations of environmental conditions. It was therefore observed that there were no regenerations of the natural microbiota and that the beneficial effect expected from the biosurfactant may have been suppressed by the impact of the fungus *A. fumigatus*.

For the experiment that utilized the biosurfactant, the count of mesophilic bacteria remained superior to that of the control. The fermentative medium where the biosurfactant production occurred was not sterile and therefore

other microorganisms could have developed in the medium besides the *A. fumigatus*. The bacteria count in the fungus bran was 1×10^7 cfu g^{-1} and presented the same morphological aspects of the ones found in the experiment with biosurfactant and were totally different from the ones found in the other experiments. This reveals that the bacteria naturally present in the environment were inhibited by the great concentration of this other specie.

Experiment with chemical disperser

For the experiment that contained diesel oil and chemical disperser, the behavior of each yeast, mould and bacteria are presented in Table 2. Moulds were only affected by the diesel oil and the chemical disperser for the first 30 days of the experiment. After this period, the natural microbiota started to regenerate and surpassed the control container count. According to Providenti et al. (1993) oil by-products are deficient in nitrogen and phosphorus and therefore hinder the growth of microorganisms.

The predominance of yeasts was verified when compared to the quantity of existing moulds in this trial. The quantity of yeasts were always at a higher level in comparison to the control experiment which readings kept at 1×10^4 cfu g^{-1} whereas with the disperser experiment the rate reached 1.4×10^5 cfu g^{-1}. This may be possibly related to the nutrients provided by the chemical disperser which would favor the growth of yeasts. One other factor to consider is that the environmental conditions within this container may have facilitated the development of certain species of yeasts.

Shortly after the spill, the bacteria were affected, and the impact on them was most likely caused by the diesel oil, for it was verified within the experiment with diesel oil that the bacteria behaved similarly. After 30 days, the bacteria count in the disperser trial began to resemble the control indicating a probable regeneration of the soil.

Aliphatic hydrocarbons

Only the fractions between C_{13} and C_{27} were considered for the biodegradation analysis of aliphatic hydrocarbons. During the 180 days of bioremediation, Fig. 1 reflects the total quantity of aliphatic hydrocarbons for the experiments which contained diesel oil, biosurfactant and chemical disperser.

It was observed that the effective degradation of the hydrocarbons occurred between 30 and 60 days. In 15 days the biosurfactant caused a reduction of 77% of aliphatic hydrocarbons, while within the same period, the container that contained only diesel oil represented a degradation of 8.7% of the hydrocarbons. The disperser container represented a reduction of 5% when compared to the first day of

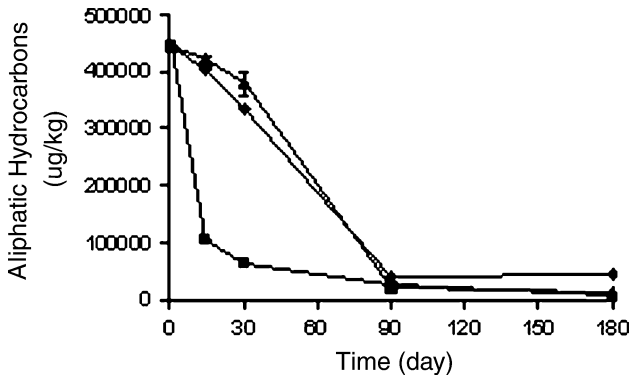

Fig. 1 Aliphatic hydrocarbons encountered during bioremediation. ■ Experiment with biosurfactant, ◆ Experiment with chemical disperser, ▲ Experiment with diesel oil

the experiment. By utilizing a biosurfactant produced by *Bacillus subtilis* 09, the surfactine was able to accentuate the degradation of hydrocarbon residuals in ship holds (Morán et al. 2000; Oliveira et al. 2000).

These rates show the viability of utilization of biosurfactant produced by solid state fermentation for diesel oil spills because it made microorganisms accessible to the hydrocarbons and obtained a faster degradation and consequent recovery of the native microorganisms. Because the microorganisms are the primary agents for the degradation of organic contaminants, the augmentation of microbial density may accelerate the degradation of contaminants (USEPA 1998). The microorganisms were also efficient in the biodegradation of aliphatic hydrocarbons within a short period of time.

Trials with the chemical disperser and diesel oil represented a higher degree of degradation of the aliphatic hydrocarbons after the 30 days of experiments. This may be related to the fact that these two experiments represented a greater impact on the natural microbiota environment, and only after 30 days did it began to recover itself. This greater impact may also have retarded the biodegradation of aliphatic hydrocarbons.

For the aliphatic hydrocarbons, it was observed that the diesel oil and biosurfactant experiments presented the best biodegradation reaching 98 and 99%, respectively, within a period of 180 days. Oberbremer et al. (1990) reported a significant increase in the degradation of hydrocarbons when sophorolipids were added to a system containing 10% soil and 1.35% of a hydrocarbon mixture in a mineral medium. In the absence of the surfactant, 81% of the hydrocarbon mixture was degraded in 114 h while in the presence of a biosurfactant more than 90% of it was degraded in 79 h.

The chemical disperser presented the lowest rate of biodegradation. It obtained a 90% reduction of the aliphatic hydrocarbons in a period of 180 days. After 90 days the rate stabilized.

Aromatic polycyclic hydrocarbons

Sixteen aromatic polycyclic hydrocarbons were quantified and identified by the U.S. Environmental Protection Agency (USEPA) as cancerous and mutagenic. Naphthalene, acenaphthylene and acenaphthene were not found in quantities enough to identify within any of the experiments.

The experiment that only contained diesel oil represented the greatest reduction of total aromatic polycyclic hydrocarbons, 76% in 180 days. The chemical disperser reached a 75% reduction of the total APHs and the biosurfactant reached a biodegradation of only 7% of the hydrocarbons. In general, there was a more effective degradation of the APHs up to 3 rings.

Biodegradation intensified after 30 days. This was probably due to the recuperation of the soil's natural microbiota which occurred after this period. Gentry et al. (2003) evaluated the influence of microbial population native to soil from the University of Arkansas. They evaluated the degradation of pyrene and observed that in 10 weeks that 97% of the pyrene added to the soil was still present within the environment. However, only 1% remained in the soil after 61 weeks.

The trial that contained the biosurfactant reflected the lowest biodegradation rate of aromatic polycyclic hydrocarbons (7%). This was probably due to the presence of (*A. fumigatus*) fungus and to bacteria predominant in the experiment. The presence of said fungus and bacteria may not be able to degrade these kinds of hydrocarbons of high molecular weight. Chaîneau et al. (1999) reported that in a solid medium containing salts and combustible oil, the *A. fumigatus* was able to degrade 29% of the total of hydrocarbons where 11% were aromatic and 47% were saturated.

For the experiment containing the biosurfactant, the degradation of aromatic polycyclic hydrocarbons up to 3 rings is illustrated in Fig. 2.

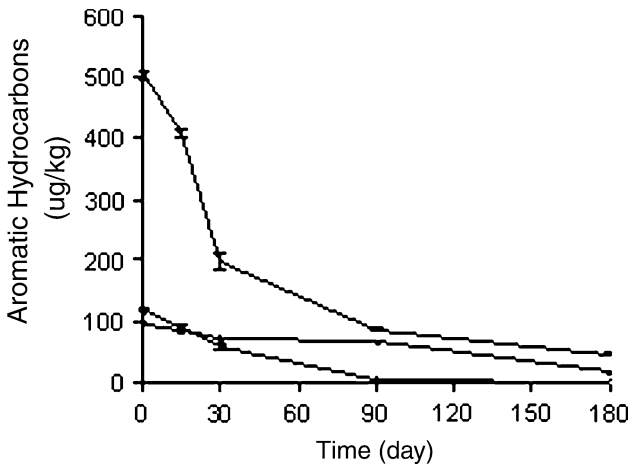

Fig. 2 Aromatic polycyclic hydrocarbons experiment with Biosurfactant with 3 rings. ■ Fluorene, ● Phenanthrene, ▲ Anthracene

We observed that the biosurfactant was effective in the reduction of aromatic polycyclic hydrocarbon of 3 rings presenting reductions of 99% for the fluorene, 91% for phenanthrene and 84% for anthracene. However, for the hydrocarbons with more than 4 rings, the biosurfactant did not perform at an effective biodegradation rate. Deschênes et al. (1996) reported that the rhamnolipids increased the solubilization of the APHs of 4 rings or more compared to APHs of 3 rings and that the biosurfactants were 5 times more effective than the synthetic surfactant (sodium dodecyl sulfate-SDS).

The treatment of samples contaminated by phenanthrene and naphthalene with biosurfactant resulted in an increase in mineralization and solubilization rates (Deziel et al. 1996). Zhang et al. (1997) studied the effects of biosurfactant on the dissolution, bioavailability and biodegradation of phenanthrene. The researchers evaluated two kinds of rhamnolipids and observed that both surfactants increased the solubility and the degradation rate of phenanthrene. The biosurfactant obtained a reduction of the phenanthrene rate (45.82 μg Kg^{-1}) below the acceptable concentration, and which according to Macleod and Semple (2000) and Reid et al. (2000) varied between 1 and 100 mg Kg^{-1}.

The effectiveness of the chemical disperser for the hydrocarbons with more than 3 rings as illustrated in Fig. 3.

We observed that for all the hydrocarbons with more than 3 rings, the chemical disperser represented reductions. The degradation for each substance is as follows, 75% for fluoranthene, 92% for pyrene, 79% for chrysene and 79% for benzo[b]fluoranthene. Lafrance and Lapointe (1998) reported that the biosurfactant produced by *Pseudomonas aeruginosa* (UG2) bacterium was much more efficient in the mobilization and co-transport of pyrene than the SDS

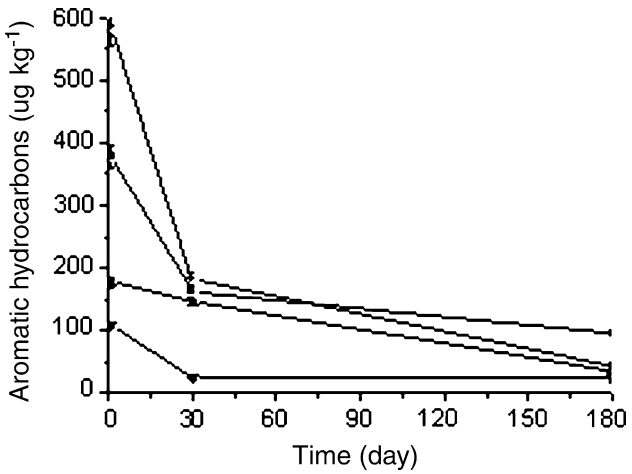

Fig. 3 Chemical disperser experiment for aromatic polycyclic hydrocarbons experiment with more than 3 rings. ■ Fluoranthene, ● Pyrene, ▲ Chrysene, ▼ Benzo[b] Fluoranthene

chemical surfactant. They also reported that the biosurfactant had a lower impact on the soil.

The chemical disperser was more efficient when compared to the biosurfactant in the aromatic compounds with a higher number of rings. For example indeno (1,2,3-cd) pyrene with a hydrocarbon of 6 rings represented a concentration of 66.59 μg Kg^{-1} on the 1st day and 2.48 μg Kg^{-1} on the 180th day. The success of the biosurfactant application in bioremediations requires that the biosurfactant system utilized be adequate to the physical and chemical conditions of the area affected by the contaminant.

Conclusion

The biosurfactant was efficient in the reduction of the aliphatic hydrocarbons. In 15 days there was a 77% reduction. Other reduction percentages are as follows: 8.7% for the experiment that contained only diesel oil, 5% for the experiment with chemical disperser.

The biosurfactant provided reductions that reached 99% for the APHs up to 3 rings. For the APHs with higher molecular weight (>3 rings), the chemical disperser was more effective reaching reductions up to 92%. However, when evaluating the bioremediation of the total APHs, it was verified that the experiments with chemical disperser and with diesel oil obtained reduction rates of 75 and 76%, respectively, during 180 days.

The environmental microbiota was affected in all experiments. However, in 30 days the native microorganisms had recovered. After this regeneration, the biodegradation process occurred at a faster rate either for the aliphatic or aromatic hydrocarbons. The biosurfactant experiment was the only one where no regeneration of native microbiota occurred even after 90 days.

This paper shows the viability for the utilization of biosurfactant produced by solid state fermentation in the case of a diesel oil spill on soil. It was efficient in the biodegradation of aliphatic and aromatics hydrocarbons up to 3 rings.

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