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Ex Situ Treatment of Hydrocarbon-Contaminated Soil Using Biosurfactants from *Lactobacillus pentosus*

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ABSTRACT: The utilization of biosurfactants for the bioremediation of contaminated soil is not yet well established, because of the high production cost of biosurfactants. Consequently, it is interesting to look for new biosurfactants that can be produced at a large scale, and it can be employed for the bioremediation of contaminated sites. In this work, biosurfactants from *Lactobacillus pentosus* growing in hemicellulosic sugars solutions, with a similar composition of sugars found in trimming vine shoot hydrolysates, were employed in the bioremediation of soil contaminated with octane. It was observed that the presence of biosurfactant from *L. pentosus* accelerated the biodegradation of octane in soil. After 15 days of treatment, biosurfactants from *L. pentosus* reduced the concentration of octane in the soil to 58.6 and 62.8%, for soil charged with 700 and 70000 mg/kg of hydrocarbon, respectively, whereas after 30 days of treatment, 76% of octane in soil was biodegraded in both cases. In the absence of biosurfactant and after 15 days of incubation, only 1.2 and 24% of octane was biodegraded in soil charged with 700 and 70000 mg/kg of octane, respectively. Thus, the use of biosurfactants from *L. pentosus*, as part of a well-designed bioremediation process, can provide mechanisms to mobilize the target contaminants from the soil surface to make them more available to the microbial population.

KEYWORDS: Biosurfactant, *L. pentosus*, soil, octane, bioremediation

INTRODUCTION

Remediation of soil contaminated with petroleum and its derived products is a major environmental concern due to their toxic, mutagenic, and carcinogenic properties. These products are introduced in to the environment due to various anthropogenic activities, such as accidental spills from transportation processes, leaking underground storage tanks, and poor waste disposal practices.

Surfactants create emulsions by enabling the suspension of hydrophobic compounds (oil, hydrocarbons) in water. They act as dispersants or flocculants, enabling the suspension of solids, such as paint pigments, in liquid. These useful properties have fueled the growth of the surfactant market, mainly in the bioremediation of contaminated sites by hydrophobic compounds. The surfactant market has annual global sales of $23.9 billion,[1] whereas the annual global production of surfactants is about 13 million metric tons.[2,3]

On the other hand, related with the petrochemical consumption for the production of surfactants, Patel et al.[4] found that 0.57 tons of petrochemical intermediates were consumed per each ton of surfactant produced. Assuming that these ratios apply to global surfactant manufacturing today, surfactant production consumes about 7.4 Mt of petrochemical intermediates annually.[5] Life cycle analysis has estimated that each ton of petrochemical intermediate used for surfactant production generates 4270 kg of emitted CO_2.[4] Thus, the use of petrochemicals to produce surfactants generates annual emission of 31.6 billion kg of CO_2. According to the U.S. Environmental Protection Agency, combustion of 1 gallon of gasoline produces 8.8 kg of CO_2. Thus, Reznik et al.[5] estimated that annual worldwide use of petrochemicals for surfactant production emits CO_2 equivalent to the combustion of 3.6 billion gallons of gasoline.

In comparison with chemical surfactants, biosurfactants are biological compounds that exhibit high surface-active properties. Microorganisms, plants, and animals, including humans, produce them. Biosurfactants are significantly less toxic than synthetic petroleum-based surfactants.[3]

Moreover, biosurfactants can be obtained by fermenting agroindustrial residues using different microorganisms.[6−8] For instance, Bustos et al.[6] carried out continuous production of lactic acid and biosurfactants from hemicellulosic sugars of trimming vine shoots. Moreover, Portilla et al.[7] found that there is an optimum carbon source ratio (glucose/xylose) to produce biosurfactants from *Lactobacillus pentosus*. They found that trimming vine shoots, after hydrolysis with sulfuric acid, produced hemicellulosic sugars with an adequate xylose/glucose ratio to obtain biosurfactants using *L. pentosus*.

The commercial success of biosurfactants is still limited by their high production cost. Optimized growth conditions using cheap renewable substrates (agro-industrial wastes) and novel, efficient methods for isolation and purification of biosurfactants could make their production more economically feasible.[10]

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In this work, biosurfactants obtained after fermentation of hemicellulosic sugars with *L. pentosus* were added to soil containing octane, at different doses, and incubated for 45 days. Moreover, incubations with soil in the absence of biosurfactant, and contaminated with octane, were employed as a control.

MATERIALS AND METHODS

Soil Characterization. Soil samples employed in this study were sieved by 2 mm before analyses. The water content was estimated by soil drying at 105 °C until constant weight, as per Guitián and Carballas.[11] The pH was determined either in water or 0.1 N KCl in a 1:1.5 soil: solution ratio and was measured after 10 min and 2 h, respectively. The total organic carbon (TOC) and organic matter (OM) were determined by oxidation with a mixture of $K_2Cr_2O_7$ and H_2SO_4 and titration with Mohr salt, following the method proposed by Sauerlandt and modified by Guitián and Carballas.[11] The grain size distribution was classified as coarse sand (0.2−2 mm), fine sand (0.05−0.2 mm), coarse silt (0.02−0.05 mm), fine silt (0.002−0.02 mm), and clay (<0.002 mm) by wet sieving followed by the Robinson pipet as per Guitián and Carballas.[1] The nitrogen (N) content was determined by wet digestion with H_2SO_4, by using the Kjeldhal method as described in Guitián and Carballas.[11] The dehydrogenase activity (DHA) was measured by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF), following the method described by Tabatabai.[12] The octane in soil was analyzed, by triplicate, using head space gas chromatography.

Microorganism. *L. pentosus* CECT-4023 T (ATCC-8041) was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown on plates using the complete media MRS agar. Inocula were prepared by solubilization of cells from plates with 5 mL of sterilized MRS broth.

Fermentation of Hemicellulosic Sugars by *L. pentosus*. The clarified hydrolysates were supplemented with nutrients (10 g/L of yeast extract and 10 g/L of corn steep liquid), sterilized, and used directly as fermentation media. The chemostat fermentation was carried out in a 2 L Applikon fermentor at 200 rpm with a 1.6 working volume at 31 °C, and the pH was controlled at 5.85 for 48 h. Once the fermentation was finished, the *L. pentosus* biomass was separated from the fermentation medium by centrifugation to biosurfactant extraction.

Extraction of Biosurfactants. Cells were recovered by centrifugation, washed twice in demineralized water, and resuspended in 50 mL of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCL with the pH adjusted to 7.0). The bacteria were left a room temperature up to 2 h with gentle stirring for biosurfactant release. Biosurfactants were obtained in the PBS, and bacteria were removed by centrifugation. The remaining supernatant liquid was tested for surface activity.

Surface Activity Determination. The surface activity of biosurfactants produced by the bacterial strains was determined by measuring the surface tension of the samples with the ring method. The surface tension of PBS extract containing the biosurfactants from *L. pentosus* was measured using a KRUSS K6 Tensiometer equipped with a 1.9 cm Du Noüy platinum ring. To increase the accuracy, an average of triplicates was used for this study.

Incubation Experiments. Soil contaminated with 700 and 70000 mg/kg of octane (one of the main hydrocarbons of gasoline) was incubated in the presence and absence of biosurfactants from *L. pentosus*. The liquid/solid ratio of biosurfactant/soil was 1:5, and biosurfactant was added onto soil at its critical micellar concentration (CMC). Incubations of soil-biosurfactants were carried out at 25 °C in Erlenmeyer flasks, without shaking by triplicate.

Moreover, different incubations of soil contaminated with octane also were carried out in the absence of biosurfactants; these incubations (experiments 1 and 4) were employed as a control, and in addition, in some incubation, the soil was sterilized to evaluate the influence of the microbial activity in the bioremediation of hydrocarbon-contaminated soil (experiments 2 and 5). Table 1 shows the experiments carried out in this work.

Table 1. Experimental Conditions for the Different Assays

experiment	mg/kg octane	presence of BS	soil sterilization
1	700	no	no
2	700	no	yes
3	700	yes	no
4	70000	no	no
5	70000	no	yes
6	70000	yes	no

Table 2. Physicochemical Characterization of the Soil Employed in This Work

properties	units	value
pH_{H_2O}		5.1
pH_{KCl}		4.0
sand	%	69.7
coarse silt	%	3.0
fine silt	%	6.6
clay	%	20.7
texture		loam−clayey−sandy
TOC	g/kg	11.2
N	g/kg	0.9
C/N		12.4
OM	g/kg	19.3
octane	mg/kg	185
DHA	mg TPF/kg day	334

RESULTS AND DISCUSSION

Soil Characterization. The soil employed in this work presented pH = 5, and it was composed of 69.7% sand, with a 20.7% clay fraction. The OM content of the soil was 19.3 g/kg, and the total nitrogen concentration was 0.9 g/kg with a C/N ratio about 12.4. Table 2 shows the physicochemical characterization of soil employed in this work.

It is important to notice that assayed soil had 185 mg/kg of octane. This concentration of octane, found naturally in the soil, was due to the fact that the assayed soil was close to a road, where it was very easy for the soil to be contaminated by the traffic in the road. Consequently, it can be speculated that the soil employed in this work has microbial biomass and that it is not inhibited by the presence of octane. In fact, the DHA on soil was about 334 mg TPF/kg day, and when this soil was contaminated with higher doses of octane, the DHA on soil almost did not change in comparison with the soil with lower doses of octane. Table 3 shows the DHA of soil containing different concentrations of octane, in the presence or absence of biosurfactants from *L. pentosus*. For the same dose of octane, the DHA concentration in soil was higher in the presence of biosurfactant.

To carry out the bioremediation process successfully, it is important that soil contains microbial biomass and nutrients. Surfactants and biosurfactants can enhance the biodisponibility

Table 3. DHA of Soil, Contaminated with Different Doses of Octane, in the Presence and Absence of Biosurfactants

mg/kg octane	presence of BS	DHA mg TPF/day
189	no	334 ± 54
189	yes	354 ± 44
700	yes	378 ± 28
700	no	416 ± 47
70000	no	321 ± 30
70000	yes	335 ± 23

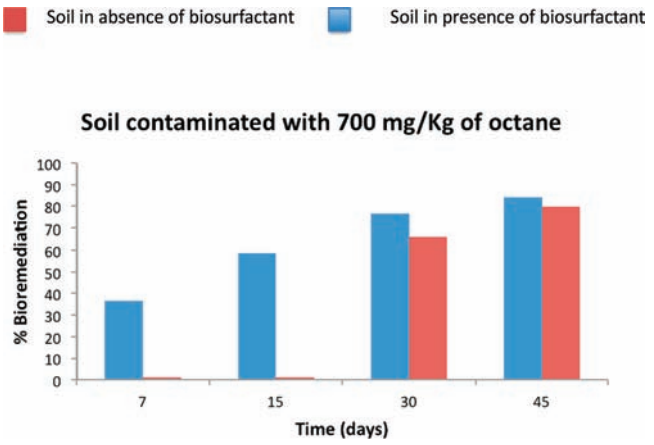

Figure 1. Percentage of bioremediation achieved for soil contaminated with 700 mg/kg of octane.

of the hydrophobic compounds in water, although it is the microbial biomass that removes the contaminants from the soil.

On the other hand, Thavasi et al.[13] evaluated the ability of biosurfactant from *L. delbrueckii* to remove crude oil in the absence or presence of fertilizer. These authors indicated that the main factor promoting oil biodegradation was the presence of biosurfactants rather the fertilizer. The utilization of fertilizer almost did not improve the biodegradation of crude oil in the presence of biosurfactant (only 7%). Moreover, in some studies, water-soluble fertilizers encountered problems such as washing away and rapid dilution in aquatic environment. Some authors[14] found that fertilizers only stimulate the early stage degradation rate of oil and that the final degradation efficiencies with fertilizers are not significantly different from those where no fertilizers are used. It is important to keep in mind that nutrients or fertilizer use may be essential in some environments with insufficient nutrient levels. The soil employed in this work has an important amount of OM and nitrogen; consequently, in a first attempt, any fertilizer was not employed to increase the biodegradability of octane in soil.

Bioremediation of Contaminated Soil by Using Biosurfactants from *L. pentosus*. The capability of biosurfactants and biosurfactant-producing bacterial strains to enhance organic contaminants availability and biodegradation rates was reported by many authors.[15−17] The advantage of biosurfactants produced by *L. pentosus* in comparison with other biosurfactants is that *L. pentosus* is a GRAS microorganism, generally regarded as safe, by the FDA (U.S. Food and Drug Administration). Moreover., the biosurfactants from *L. pentosus* are cell bound to the plasmatic membrane of lactobacillus and are easily extracted using PBS. This solution containing the biosurfactant can be employed

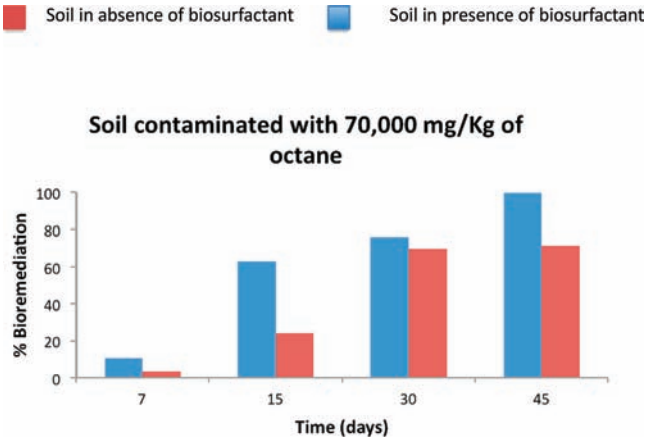

Figure 2. Percentage of bioremediation achieved for soil contaminated with 70000 mg/kg of octane.

directly in the bioremediation of contaminated soil with octane. The biosurfactant employed in this work was able to reduce the surface tension of water to 53 ± 2 mN/m, and also, it has bioemulsifier properties.[6,7]

Figure 1 shows the percentage of bioremediation of soil contaminated with 700 mg/kg of octane at different days of incubation in comparison with soil in the absence of biosurfactant from *L. pentosus*, whereas Figure 2 shows the same data for incubations carried out with soil contaminated with 70000 mg/kg of octane. Comparing Figures 1 and 2, it can be observed that after 7 days of incubation, using 700 mg/kg of octane, better percentages of bioremediation were achieved, and it is due to the fact that 70000 mg/kg of octane produces an important substrate inhibition. However, once microorganisms were adapted, the biodegradation occurred very fast. In both cases, higher differences in the percentage of bioremediation in comparison with the control at the first 15 days of incubation can be observed. At longer incubation times, the differences between soil in the absence and presence of biosurfactant were reduced. It can be speculated that a natural attenuation process can occur due to the adaptation of the biomass to the presence of octane in soil. However, the fact that biosurfactant accelerates the bioremediation of octane-contaminated soil is interesting, because the faster that the hydrocarbon is eliminated, there is more possibility to avoid washing away and rapid dilution in the aquatic environment of the hydrophobic contaminant. In the absence of biosurfactant, the first 15 days, octane exhibited limited bioavailability to microorganisms. Usually, hydrophobic contaminants tend to partition onto the soil matrix. This partitioning can account for as much as 95% or more of the total contaminant mass. Thus, this limits the concentration of hydrophobic contaminants available to the microbial population. Hence, certain hydrophobic contaminants can persist in the soil matrix for long periods of time.

Moreover, Figures 3 and 4 show the kinetic behavior of octane consumption during incubation experiments for soil contaminated with 700 and 70000 mg/kg of octane for 45 days. In both cases, when the soil was sterilized, the octane concentration was kept very high. This fact corroborates the importance of microbial biomass in the soil to metabolize hydrophobic contaminants. In Figures 3 and 4, it can be observed that the biosurfactant from *L. pentosus* clearly improved the solubilization of octane to be metabolized by the microbial biomass of soil.

Figure 3 shows, for soil contaminated with 700 mg/kg of octane, that biosurfactants improved the bioremediation of soil

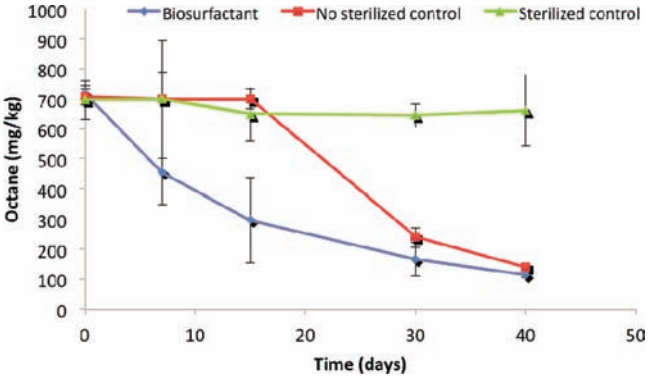

Figure 3. Kinetics of the octane biodegradation in soil containing 700 mg/kg of octane.

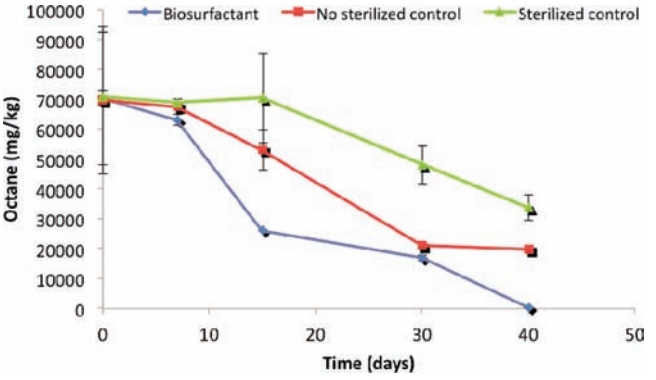

Figure 4. Kinetics of the octane biodegradation in soil containing 70000 mg/kg of octane.

in comparison with the controls, in the absence of biosurfactant. After 15 days of incubation, soil charged with biosurfactants reduced the octane concentration to 297.8 mg/kg, whereas soil in the absence of biosurfactant kept the octane concentration close to 700 mg/kg. It supposes that biosurfactants from *L. pentosus* were able to improve the octane elimination more than 57%, after 15 days of incubation, in comparison with the control. In the absence of biosurfactants, the soil needed about 30 days to reduce the octane concentration at values close to those achieved in the presence of biosurfactants after 15 days.

On the other hand, when soil was contaminated with higher doses of octane, the presence of biosurfactant also clearly improved the bioremediation of soil, and higher differences were achieved in comparison with soil contaminated with 700 mg/kg of octane. After 45 days of incubation, soil in the presence of biosurfactant reduced the octane concentration from 70000 to 293.8 mg/kg, whereas soil in the absence of biosurfactant kept the octane concentration at 19898 mg/kg, and in the sterile soil, the concentration of octane was 33745 mg/kg after 45 days of incubation. It can be speculated that the decrease of octane in the experiments carried out with sterile soil can be due to the loss of sterile conditions or at a partial evaporation of octane, although during incubations Erlenmeyer flasks were covered with cotton and aluminum foil.

The percentages of octane reduction in soil contaminated with 70000 mg/kg of octane was 62.8, 75.9, and 99.6 after 15, 30, and 45 days of incubation, respectively, whereas the biodegradation of octane in the control was 24, 69.8, and 71.5% after 15, 30, and 45 days of incubation, respectively (see Figures 1 and 2). Other authors achieved similar results during the bioremediation of contaminated sites with crude oil, diesel, or polyaromatic hydrocarbons. Obayori et al.[18] investigated the biodegradative properties of biosurfactant produced by *Pseudomonas* sp. LP1 strain on crude oil and diesel. The results obtained confirmed the ability of strain LP1 to metabolize the hydrocarbon components of crude and diesel oil. They achieved 92.34% degradation of crude oil and 95.29% removal of diesel oil. Biodegradative properties of biosurfactant producing *Brevibacterium* sp. PDM-3 strain were tested by Reddy et al.[19] They reported that this strain could degrade 93.92% of the phenanthrene and also had the ability to degrade other polyaromatic hydrocarbons such as anthracene and fluorene.

Moreover, Thavasi et al.[13] studied the effect of fertilizer and biosurfactants from *L. delbrueckii* on biodegradation of crude oil. After 7 days, maximum degradation of crude oil (75%) was observed by these authors, in experiments carried out in presence of bacterial cells, crude oil, biosurfactant, and fertilizer, and 68% of oil degradation was achieved by these authors in experiments carried out with bacteria cells, crude oil, and biosurfactants, in the absence of fertilizer, whereas in experiments carried out in the absence of fertilizer and biosurfactants, only 55% of crude oil was biodegraded. The presence of fertilizer only improved 7% the biodegradation of oil. In comparison with this work, we have obtained higher differences of octane biodegradation. After 15 days, soil charged with 700 mg/kg of octane in presence of biosurfactants reduced the concentration of octane to 58.6%, whereas the control, soil without, biosurfactant only reduced the octane concentration to 1.2%. The same behavior was observed in soil charged with 70000 mg/kg of octane. In this case, the presence of biosurfactant reduced the octane concentration to 62.8%, whereas the control, without biosurfactant, reduced the octane concentration to 24.0%.

In the assayed experiments, the hydrocarbon removal for a longer length of treatment became similar to the natural attenuation process. Other authors also achieved similar results working on the bioremediation of hydrocarbon-contaminated soil.[20,21] However, at initial times, biosurfactant from *L. pentosus* accelerated the bioremediation of contaminated soil in comparison with soil in absence of biosurfactant. This fact can be very interesting, mainly in the bioremediation processes carried out in situ, where it is very important to remove the contaminant from the soil as soon as possible.

The low water solubility of hydrophobic compounds like octane limits their availability to microorganisms, which is a potential problem for the bioremediation of contaminated sites. *L. pentosus* can produce biosurfactants that enhance the bioavailability of hydrophobic compounds, like octane, for bioremediation. Consequently, biosurfactant produced by *L. pentosus* could be employed for the bioremediation of contaminated soil.

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