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Aplication of Biosurfactants Produced by *Bacillus cereus* and *Candida sphaerica* in the Bioremediation of Petroleum Derivative in Soil and Water

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Oil spills, whether in an aquatic or terrestrial environment, cause the imbalance of ecosystems. It is essential to develop actions and strategies for the remediation of such accidents. Currently, chemical surfactants have been used in oil spills, although the use of these agents is increasingly restricted because of their toxic potential. One solution for the remediation of soils contaminated by oils is the use of biosurfactants, which are biodegradable and nontoxic. In this sense, the biosurfactants produced by *Candida sphaerica* (UCP0995) and *Bacillus cereus* using low cost substrates were used with their producer microorganisms in the remediation of motor oil contained in sand and sea water. Sand oil bioremediation experiments were carried out for 70 days, while in sea water the period was 30 days. The results showed that the addition of the biosurfactant increased the degradation of the oil in the sand to 90% during the first 15 days of the process using the biosurfactant produced by *C. sphaerica*, whereas using the *B. cereus* 90% oil removal was obtained from 60 days of experiment. With regard to the removal of oil in sea water, it was observed that the time in which the experiments were processed had a direct influence on the results, with removal percentages above 90% after 30 days of experiment for both biosurfactants. In this way, the biosurfactants produced, besides being obtained from low cost substrates, demonstrated efficiency in the removal of oils in sand and water, allowing the substitution of chemical treatment agents by environmental friendly agents.

1. Introduction

Oil is one of the most important resources of energy in the modern industrial world. As long as oil is explored, transported, stored and used there will be spillage risk. Oil spills impose a major problem on the environment (Silva et al., 2014). These disasters have devastating effects on marine and terrestrial environment. Due to this, the use of molecules with surfactant properties became an attractive alternative in the removal of these hydrophobic contaminants generated by the petroleum industry (Fracchia et al., 2012; Silva et al., 2014).
Most commercially available surfactants are derived from petroleum products; however, recent environmental control legislations have driven the development of natural surfactants as alternatives to the synthetic existing products (Silva et al., 2014).
These natural surfactants are called biosurfactants, amphipathic molecules with both hydrophobic and hydrophilic moieties. These biomolecules act in fluids with different polarities degrees (water/oil) allowing access to hydrophobic substrates and promoting surface tension decrease, an increase in contact area of insoluble compounds, an increase of mobility and thus facilitating the biodegradation of insoluble substrates (Freitas et al., 2016; Santos et al., 2017). This feature allows the reduction of surface and interfacial tensions

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and the formation of micro emulsions, where the hydrocarbons can be solubilized in water and vice-versa. Such properties enable a wide range of industrial applications involving detergency, emulsification, lubrication and phase dispersion (Almeida et al., 2017; Santos et al., 2017; Sarubbo et al., 2015).

The most important advantage of biosurfactants over chemical surfactants is probably their ecological acceptability. Biosurfactants are biodegradable and thus toxicity and accumulation problems in natural ecosystems are avoided. In the environmental sector, biosurfactants have potential applications in bioremediation and waste treatment because of their inherent degradability (Pacwa-Plociniczak et al., 2011; Santos et al., 2016).

Industries are currently attempting to replace some or all of chemical surfactants for sustainable biosurfactants (Marchant and Banat, 2012) although the high production of these biomolecules costs still remains a challenge. A key factor that regulates the success of biosurfactants production is the development of an economical process that uses low cost materials and offers a high yield. In fact, the use of low cost substrates is important for the global economy, as the substrate is responsible for up to 50% of the final production cost. Fortunately, biosurfactants can be economically produced from renewable resources such as vegetable oils, distillery residues and dairy residues (Rufino et al, 2014).

Thus, the objective of this study was to investigate the application of two surfactant agents from *Candida sphaerica* UCP0995 and *Bacillus cereus* UCP1615 as adjuncts in remediation processes of hydrophobic pollutants generated by the petroleum industry.

2. Materials and Methods

2.1 Microorganisms

The microorganisms *Candida sphaerica* UCP0995 and *Bacillus cereus* UCP1615 were catalogued in the culture collection of the Catholic University of Pernambuco, Brazil. The microorganisms were refrigerated at 5°C with YMA (Yeast Mold Agar) and Nutrient Agar, respectively.

2.2 Inoculum preparation

The inoculum of *C. sphaerica* was prepared by transferring cells grown on a slant to 50 mL of yeast mould broth (YMB). The seed culture was incubated for 24 h at 28ºC and agitated at 150 rpm. The inoculum (1%, v/v) was added to the medium at the rate of 10^4 cells/mL.

Bacillus cereus young culture was maintained after 24 hours of cultivation in nutrient agar medium. Cells were transferred to an Erlenmeyer flask containing 50 mL of nutrient broth with the following compositions: meat extract (0.5 %), peptone (1.5 %), NaCl (0.5 %), K_2HPO_4 (0.5 %) in pH 7,0. Cultivation conditions were carried out during 10-14 hours, 200 rpm and 28 °C to obtain an optical density of 0.7 (corresponding to an inoculum of 107 U.F.C./mL) at 600 nm. This inoculum was used at 2 % concentration in relation to the production medium final volume.

2.3 Biosurfactant production

For the production of the biosurfactant from *Candida sphaerica*, the medium was composed by 5 % oil soy residue and 2.5 % corn steep liquor dissolved in distilled water. The final pH of the medium was 5.5.

The biosurfactant production using *B. cereus* was carried out in mineral medium composed of 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.2 g/L $CaCl_2.H_2O$ and 0.05 g/L $FeCl_3.6H_2O$, supplemented with 2 % residual soybean oil and 0.12 % KNO_3. The final pH of the medium was 7.0. The media were autoclaved at 121 °C for 20 min.

Fermentations for biosurfactants production were carried out in 1 L Erlenmeyer flasks containing 500 mL of the production media and incubated with 1 % of *Candida sphaerica* and 5 % of *Bacillus cereus* from pre-inoculum. For biosurfactant production using *Candida sphaerica* was conducted at 28 ºC with shaking at 150 rpm for 144 h, while the biosurfactant production using *Bacillus cereus* was carried out at 28 °C with shaking at 150 rpm for 96 h. After fermentation, the metabolic broth obtained were centrifuged at 4400 rpm for 15 min.

2.4 Biosurfactant isolation

Isolation of the biosurfactant produced by *C. sphaerica*: Isolation of the biosurfactant was performed from cell-free metabolic broth obtained after centrifugation. Thereafter, the same volume of ethyl acetate (1:1 v/v) was added, the mixture being vigorously stirred for 15 minutes and allowed to stand to separate the layers. The samples were extracted twice. The organic phase was evaporated at 40 °C to solvent remove. The obtained residue was washed twice with hexane to remove the remaining oil and any hydrophobic substance, such as fatty acids and alcohols, which may have been formed during fermentation and the yield was obtained by gravimetry.

Isolation of the biosurfactant produced by *B. cereus*: Isolation of the biosurfactant was performed from cell-free metabolic broth obtained after centrifugation. The cell-free broth was extracted directly with ethyl acetate. The extraction was repeated three times using 1/4 of the volume of the solvent to metabolic broth and then vacuum filtered. The solvent was transferred to a separatory funnel where the aqueous phase was discarded. The solution was saturated with sodium chloride and stirred. The solvent portion was collected and added with desiccant (anhydrous magnesium sulfate, anhydrous sodium sulfate or anhydrous calcium chloride), filtered into a becker and placed in a hot plate. The product obtained was weighed after evaporation of the solvent to determine the yield of the biosurfactant produced.

2.5 Soil

Samples of normal sand for the cement test NBR 7214 (ABNT, 1982), whose organic matter is expressed in terms of tannic acid, at a level not exceeding 100 parts per million, being maintained between the sieves of nominal aperture of 0.3 mm was used for 0.15 mm (fine denomination) in the experiments. The sand samples used in the remediation experiments were autoclaved.

2.6 Experiment remediation of oil derivatives in soil

Samples of 10 g of sand contaminated with 5% motor oil were added to 100 mL of potable water and the mixture was enriched with 1 mL of cane molasses supplied from a local plant. This mixture was previously sterilized under flowing steam and constituted the control condition. Then, solutions of the biosurfactant isolated in the CMC (Critical Micellar Concentration - less amount of biosurfactant able of reducing to the maximum the surface tension of a liquid) and 2xCMC and 15 % of its microbial producer (15 % of the inoculum containing 10^7 CFU/mL, from an OD of 0.7 to 600 nm) previously cultured in its respective medium (YMB – Yeast Mold Broth for the *C. sphaerica* and NB - Nutrient Broth for the *B. cereus*) were added and the mixtures incubated at 28 °C at 150 rpm for 75 days, according to Table 1. Every 15 days of experiment 1 % of molasses was added to the mixtures. Samples of 5 mL were collected every 15 days (15, 30, 45, 60 and 75 days) to analyze the petroleum derivatives, totaling five samples. The percentage of oil degradation was calculated as the concentration of oil removed by gravimetry (JOO et al., 2008).

Table 1: Mixtures formulated for the experiments of biodegradation of engine oil in sand

Mixtures	Composition
Control	Contaminated soil + cane molasses
Condition 1	Contaminated soil + canes molasses + *C. sphaerica* or *B. cereus* cells
Condition 2	Contaminated soil + canes molasses + biosurfactant (in CMC) + *C. sphaerica* or *B. cereus* cells
Condition 3	Contaminated soil + cane molasses + biosurfactant (in 2xCMC) + *C. sphaerica* or *B. cereus* cells

2.7 Analysis of oil derivative removed from sand

The initial and final amounts of hydrophobic contaminant were determined in the aqueous phase and in the soil after extraction with n-hexane. 100 mL of n-hexane were added to aqueous phase and soil after washing in a separatory funnel and stirred for 10 min. This procedure was repeated as many times as necessary until the hexane phase was clear. The final extract of hexane and oil were rotoevaporated for hexane evaporation or the hexane was evaporated in an oven at 68-70 °C and the beaker containing the residual oil was weighed. The removal efficiency was evaluated by gravimetry after washing the liquid containing the contaminant removed and the soil containing the remaining contaminant with hexane.

2.8 Experiment remediation of oil derivatives in sea water

The experiments of biodegradation of motor oil were carried out in 250 mL Erlenmeyer flasks containing 50 mL of sea water collected in Suape Port (Pernambuco State, Brazil) and 1 % of motor oil. The medium was sterilized and then inoculated with 5 % inoculum (5 % of the inoculum containing 10^7 cells/mL and from a 0.7 to 600 nm O.D.) of each producing biosurfactant microorganism. The flasks were incubated in a rotary shaker at 150 rpm for 30 days, and samples were taken every 10 days of experiment, totaling 03 samples. The experiments were conducted under three different conditions, according to Table 2.

2.9 Analysis of oil derivative removed from sea water

The degraded oil was quantified in samples and in the post-extraction control medium with n-hexane. The residual oil was extracted in a separating funnel with the same volume of hexane. The extraction was

performed twice to ensure complete removal of the oil in the solvent. After extraction, the hexane was evaporated in an oven at 68-70 °C. The removal efficiency of the hydrocarbon compounds was calculated by the difference of the concentration between the control and the biodegraded samples, divided by the control concentration.

Table 2: Mixtures formulated for the experiments of biodegradation of engine oil in sea water

Mixtures	Composition
Control	Contaminated water
Condition 1	Contaminated water + *C. sphaerica* or *B. cereus* cells
Condition 2	Contaminated water + biosurfactant (in CMC) + *C. sphaerica* or *B. cereus* cells
Condition 3	Contaminated water + biosurfactante (2xCMC) + *C. sphaerica* or *B. cereus* cells

3. Results and Discussion

3.1 Remediation of oil derivatives in soil

The *C. sphaerica* UCP0995 and *B. cereus* UCP1615 potential for bioremediation was verified through a motor oil-contaminated sand with their biosurfactants. The solutions of the isolated surfactant under, at and above the CMC were tested, as well as in association with its producing microorganism, as shown in Figure 1.
As can be seen, the percentages of removal using the biosurfactant produced by *C. sphaerica* increases with its addition as compared to the condition without addition of biosurfactant. The highest removal percentage (98 %) was obtained for condition 3, at 75 days. However, from 15 days of experiment, percentages above 90 % were observed for conditions 2 and 3, which could reduce application costs.
On the other hand, the biosurfactant produced by *B. cereus* presented satisfactory results from 30 days of experiment, reaching 90 % with 60 days and 92 % with 75 days of experiment.
It was also possible to observe that the concentration of the biosurfactant alone influenced the percentage of removal, demonstrating the increase of the solubility capacity of the oil in the aqueous phase by the biosurfactants used.

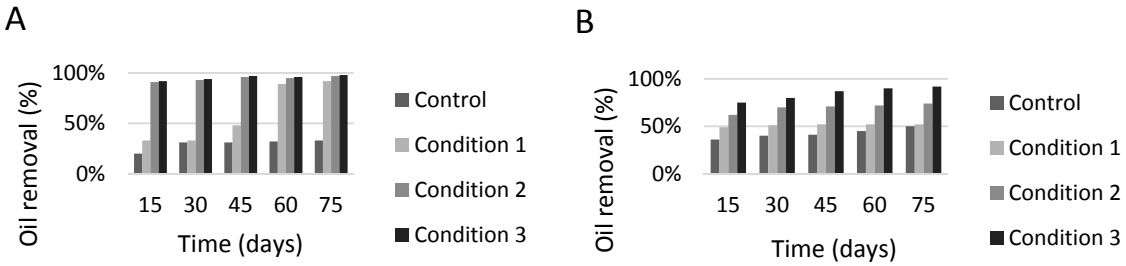

Figure 1 - Degradation of oil adsorbed in sand by bioremediation using the biosurfactant produced by C. sphaerica (A) and B. cereus (B)

The results obtained using the biosurfactant of *C. sphaerica* are excellent and corroborate the results obtained in the kinetic tests of oil removal, according to Luna el al. (2013).

3.2 Remediation of oil derivatives in sea water

The potential of the biosurfactants and their respective producers *C. sphaerica* UCP0995 and *B. cereus* UCP1615 for bioremediation in sea water was also verified. The results expressed in Figure 2 showed very satisfactory results.
The percentage of degradation by the microorganisms with the application of biosurfactants increased as the experiment was processed, showing the best results with 30 days of experiment. On the other hand, the results using the *B. cereus* biosurfactant showed good results from the first analysis (10 days). It was also observed that the increase of the biosurfactants concentration favoured the degradation for application with both biosurfactants.

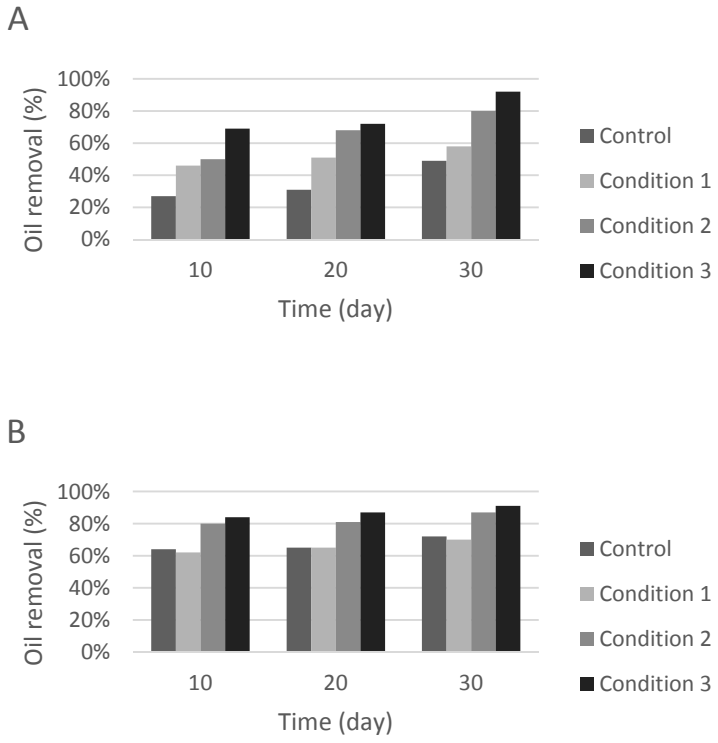

Figure 2 - Degradation of oil adsorbed in sea water by bioremediation using the biosurfactant produced by C. sphaerica (A) and B. cereus (B)

The bacterium *Pseudomonas cepacia* CCT6659 cultivated with 2% soybean waste frying oil and 2% corn steep liquor as substrates produced a biosurfactant with potential application in the bioremediation of soils. Four sets of biodegradation experiments were carried out with soil contaminated by hydrophobic organic compounds amended with molasses in the presence of an indigenous consortium. Significant oil biodegradation activity (83%) occurred in the first 10 days of the experiments when the biosurfactant and bacterial cells were used together, while maximum degradation of the organic compounds (above 95%) was found between 35 and 60 days. It is evident from the results that the biosurfactant and its producer species together are capable of promoting biodegradation to a large extent (Silva et al., 2014). In another study, the biosurfactant from *C. lipolytica* stimulated the degradation of motor oil by the seawater indigenous microorganisms (Santos et al., 2017). Chaprão et al. (2015) also described the application of two biosurfactants in the biodegradation of motor oil. Oil degradation reached almost 100% after 90 days in the presence of *Bacillus* sp. cells, while the percentage of oil degradation did not exceed 50% in the presence of *C. sphaerica*. The presence of the biosurfactants increased the degradation rate in 10–20%, especially during the first 45 days, indicating that biosurfactants acted as efficient enhancers for hydrocarbon biodegradation. In a similar study conducted in sea water, the effect of the biosurfactant from *P. cepacia* CCT6659 on the biodegradation of motor oil through the use of indigenous marine bacteria and fungi was evaluated over a 30-day period. The biosurfactant acted as a solubilizer of the motor oil, as demonstrated by the acceleration and growth of these microorganisms throughout the 30 days of cultivation in the presence of the biosurfactant (Rocha e Silva et al., 2014).

4. Conclusions

In the present study, the biosurfactants produced by *C. sphaerica* and *B. cereus* using industrial residues, showed efficiency in the degradation of oil in soil and sea water, reaching percentages above 90 % of contaminant removal. The results indicate the possibility of industrial application of these biomolecules in the remediation of impacted environments, reducing impacts on ecosystems.

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