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Application of a Biosurfactant Produced in Low-cost Substrates in the Removal of Hydrophobic Contaminants

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This work describes the environmental application of a biosurfactant produced from *Pseudomonas* sp. cultivated in a mineral medium formulated with low-cost substrates. Fermentations were conducted in the mineral medium supplemented with 3 % corn steep liquor and 3 % molasses at 28 ºC during 144 h under 200 rpm. The medium surface tension was reduced to 29 mN/m. A Critical Micelle Concentration (CMC) of 0.5 % was obtained from the isolated biosurfactant. The application of the biosurfactant demonstrated an ability to remove 80.0 % of motor oil adsorbed on beaker surfaces. Stone washing tests showed 48.0 % removal at 0.5 % biosurfactant concentration and testing for oil removal in soil results showed around 63.0 % for the removal of motor oil adsorbed on fresh sand. The results obtained with the biosurfactant produced by Pseudomonas sp., under the conditions tested above show the promising properties of this biomolecule for use in bioremediation of hydrophobic compounds in soils and waters.

1. Introduction

Oil spill accidents result in significant contamination of the ocean and shoreline environments (Silva et al. 2014). It is estimated that 0.08 % - 0.40 % of the total worldwide production of petroleum eventually reaches the oceans (Sarubbo et al. 2012). Such incidents have intensified attempts to develop procedures and technologies for combating oil pollution in the environment. Soil that is accidentally contaminated with petroleum hydrocarbons can be remediated by physical, chemical, or biological methods (Rufino et al. 2013). However, new trends in soil and water restoration avoid introducing synthetic chemicals. Among the remediation techniques available for contaminated sites, bioremediation is regarded as environmentally friendly because it preserves the soil structure, requires little energy input, and involves the complete destruction or immobilization of the contaminants, although the efficiency of biodegradation of oil pollutants is often limited by their poor water solubility. One of the approaches to enhance biodegradation of oils is to use biosurfactants, which could increase solubility of oils in water to enhance the bioavailability of the hydrophobic substrates, leading to higher oil degradation rates (Benincasa, 2007).
Surface-active compounds of biological origin have attracted much attention and their popularity seems to steadily increase during recent years. This fact may be attributed to an evolved approach towards industrial production, which favors both environmental awareness and sustainability through use of renewable resources (Banat, 2010). The numerous advantages of biosurfactants compared to their synthetic counterparts are yet another reason why these compounds seem so promising. While biosurfactants are generally equally effective in terms of solubilization and emulsification, they are also considered to be biodegradable, less toxic, and thus by far, more environmentally friendly than synthetic surfactants. Since these molecules may be obtained from waste materials, their production also seems to be feasible in terms of economic justification (Banat, 2010). All these relevant traits contribute to a high applicability of biosurfactants, which currently stems to several branches of industry. The much extolled environmental friendliness combined with the ability to solubilize hydrophobic

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compounds may well explain why biosurfactants have also been recognized as excellent agents for improving bioremediation of contaminated environments (Sarubbo and Campos-Takaki, 2011). First and foremost, biosurfactants tend to interact with poorly soluble contaminants and improve their transfer into the aqueous phase. This allows for mobilization of recalcitrant pollutants which have been embedded in the soil matrix and their subsequent removal. The presence of biosurfactants may also lead to a potential enhancement of biodegradation efficiency. In this concept, the biosurfactant molecules act as mediators, which increase the mass transfer rate by making hydrophobic pollutants more bioavailable for microorganisms (Banat, 2010). This work describes the environmental application of a biosurfactant produced from *Pseudomonas* sp. cultivated in a mineral medium formulated with low-cost substrates.

2. Materials and methods

2.1 Microorganism

A strain of *Pseudomonas* sp., provided from the culture collection of the Fundação André Tosello de Pesquisa e Tecnologia, city of Campinas, state of São Paulo, Brazil, was tested as a biosurfactant producer. The culture war maintained on nutrient agar slants at 4 °C. For pre-culture, the strain from a 24 h culture on nutrient agar was transferred to 50 mL of nutrient broth to prepare the seed culture. The cultivation conditions for the seed culture.

2.2 Fermentation medium

The production medium was composed by 3 % sugar cane molasses and 3 % corn steep liquor as substrates (carbon and nitrogen sources) dissolved in the mineral medium, containing 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.02 % $MgSO_4.7H_2O$, 0.02 % $CaCl_2.H_2O$ and 0.005 % $FeCl_3.5H_2O$ and the pH was adjusted to 7.0 by 1.0 M HCl.

2.3 Biosurfactant production

Three percent aliquots (v/v) of the cell suspension (0.7 optical density at 600 nm), corresponding to an inoculum of 10^7 colony-forming units/ml, were used to inoculate 500 mL Erlenmeyer flasks containing 100 mL of sterile production medium. Cultivation was carried out at 30 °C with agitation at 200 rpm for 144 h in a New Brunswick C-24 shaker (New Brunswick Scientific, NJ, USA). No adjustment of pH was performed during cultivation.

2.4 Biosurfactant isolation

The biosurfactant was extracted from culture media after cell removal by centrifugation at 5,000 g for 30 min. The supernatant pH was adjusted to 2.0 with 6.0 M HCl, and an equal volume of $CHCl_3/CH_3OH$ (2:1) was added. The mixture was vigorously shaken for 15 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated twice again. The biosurfactant was concentrated from the pooled organic phases using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45 °C (Costa et al. 2009).

2.5 CMC and Surface tension determination

Surface tension changes were carried out on the cell-free broth obtained by centrifuging the cultures at 5,000 g for 20 min by the ring method using a Sigma 700 Tensiometer (KSV Instruments LTD - Finland) at room temperature. Tensiometers determine the surface tension with the help of an optimally wettable ring suspended from a precision balance. In the Ring method the liquid is raised until contact with the surface is registered. The sample is then lowered again so that the liquid film produced beneath the liquid is stretched. As the film is stretched a maximum force is experienced, the force is measured and used to calculate the surface tension. The instrument was calibrated against Mill-Q-4 ultrapure distilled water (Millipore, Ilhinois, USA). Prior to use the platinum plate and all the glassware were sequentially washed with chromic acid, deionised water, acetone and finally flamed with a Bunsen burner.
The critical micelle concentration (CMC) was determined by measuring the surface tensions of dilutions of isolated biosurfactant in distilled water up to a constant value of surface tension. Stabilization was allowed to occur until standard deviation of 10 successive measurements was less than 0.4 mN/m. Each result was the average of 10 determinations after stabilization. The value of CMC was obtained from the plot of surface tension against surfactant concentration. The CMC value was determined to be g/L of biosurfactant.

2.6 Washing of hydrophobic compound adsorbed to porous surface

The removal of motor oil adsorbed to rock was carried out by soaking the material in the contaminant until complete coverage and recording the volume spent. The material was then carefully placed in a 100 mL beaker

with the aid of a pincers and submitted to washing with the cell-free metabolic broth (crude biosurfactant) and with the isolated biosurfactant at ½ CMC and at the CMC concentration. After the culture process, the percentage of removal through washing was calculated. Following the washing of the porous surfaced, the samples were treated with 50 mL of hexane twice for the removal of residual oil. The solvent was rotoevaporated at 50 ºC and the amount of oil removed was determined by gravimetry (Silva et al. 2014).

2.7 Application of biosurfactant in hydrophobic contaminant cleaning test

To determine the cleaning ability of the biosurfactant, the inner walls of a beaker was coated with motor oil. To remove the adhered oil, 50 mL of the cell-free broth was added to the beaker, vortexed for 1.0 min and allowed to stand for 6 h. Following the washing, the residual oil was treated with 50 mL of hexane twice. The solvent was evaporated at 50 ºC and the amount of oil removed was determined by gravimetry (Pruthi and Cameotra, 2000).

2.8 Removal of motor oil through kinetic assay

The removal of motor oil from the contaminated soil was tested through the saturation of 50 g of soil with 5.0 g of motor oil. The following soils were tested: standard sand, NBR 7214 (ABNT, 1982) for which the organic matter is expressed in terms of tanic acid in level not superior to 100 ppm, (0.30 mm to 0.15 mm), beach sand, collected at Barra de Jangada beach, Jaboatão-PE, Brazil and clay soil (containing 50 % sand 48 % clay and 2.0 % de silt). The laboratory-contaminated soils were placed in 250 mL Erlenmeyer flasks, to which 50 mL of the cell-free metabolic broth (crude biosurfactant) were added. The Erlenmeyer flasks were shaken at 200 rpm for 24 h at 28 ºC. The entire content was then centrifuged at 5,000 rpm for 1,200 s. Following the washing of the soil, the samples were treated with 50 mL of hexane twice for the removal of residual oil. The solvent was rotoevaporated at 50 ºC and the amount of oil removed was determined by gravimetry (Luna et al. 2009).

3. Results and Discussion

3.1 Surface tension and CMC determination

The biosurfactant from *Pseudomonas* sp. exhibited the ability to reduce surface tension, since the water surface tension was reduced from 70 mN/m to 29 mN/m with increasing the concentration of the biosurfactant to 0.5 % (5,000 mg/L) (Figure 1). From this point, the concentration of the solution of biosurfactant caused no greater reductions in water surface tension, indicating that the CMC had been reached at this concentration.
This CMC value differs greatly from that of 53 mg/L reported for the rhamnolipids mixture produced by *P. aeruginosa* UG2 on corn oil (Zhang et al. 2005), or 230 mg/L found for a mixture of seven homologues (Abalos et al. 2001), or 120 mg/L found for a mixture of six homologues from *P. aeruginosa* LB1 cultivated in soapstock (Benincasa et al. 2004). The different CMC values may have resulted from differences in purity and composition of biosurfactants.

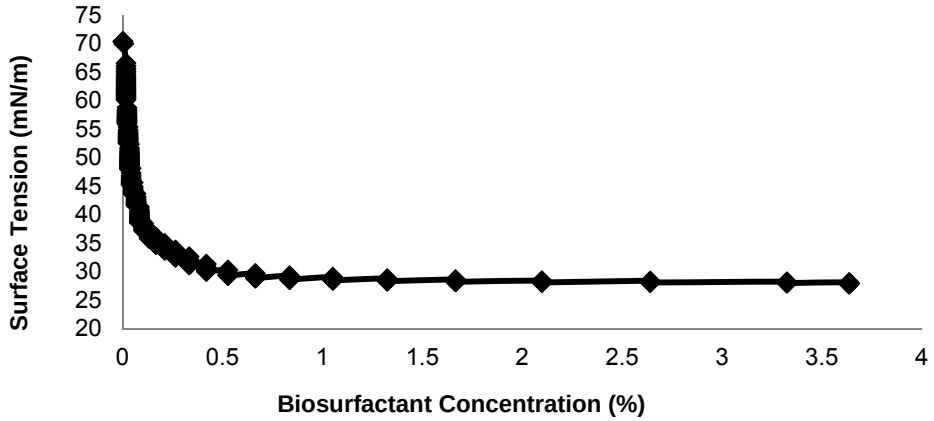

Figure 1: Critical Micelle Concentration of the biosurfactant from Pseudomonas sp. grown in mineral medium supplemented with 3% sugar cane molasses and 3 % corn steep liquor during 144 h at 200 rpm and 30 ºC

3.2 Washing of Hydrophobic Compound Adsorbed to Porous Surface

Experiments with the cell-free broth for removal of motor oil adsorbed on the porous surface showed a removal of 70.0 % of the oil after manual shaking for 5 minutes, demonstrating the potential of the crude biosurfactants a cleaning agent (Figure 2). Al ready at concentration of the CMC (0.5 %) the removal of oil reached 47.8 % while at ½CMC (0.25 %) the removal of motor oil reached 46.1 %.

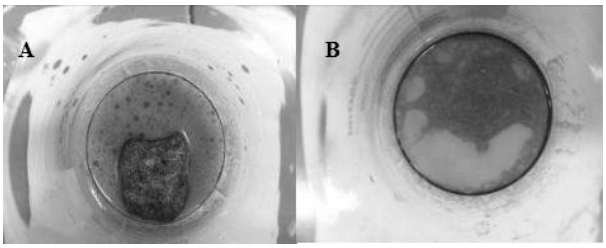

Figure 2: Illustration of porous surface before (A) and during (B) washing process with the biosurfactant from Pseudomonas sp.

Figure 3: Illustration of oily solid surface washed (A) and oily solid surface before non-washed (B) with the biosurfactant from Pseudomonas sp.

3.3 Application of Biosurfactant in Hydrophobic Contaminant Cleaning Test

The potential of the crude biosurfactant for cleaning a contaminated surface with a layer of oil was tested in a Becker contaminated with motor oil. After vortexing for 10 min and 24-h rest it was observed a complete removal of the oil layer of the walls of the beaker, which concentrated on the surface of the cell-free broth. On the other hand, with the isolated biosurfactant at concentrations of CMC and ½ CMC the results were lower, being 75 % for the biosurfactant at ½ CMC and 80 % with the biosurfactant at the CMC. The crude biosurfactant was more efficient and this is interesting for a promising application of the biosurfactant in the petroleum industry since.

3.4 Removal of motor oil through kinetic assay

Apart from the industrial applications of biosurfactants envisage, their application in the oil industry is one of the potential uses which requires lower purity specifications so that whole cell broth could be used, eliminating the purification steps that represent almost 60 % of the total production costs (Silva et al. 2014).

One of the main factors affecting the biodegradation efficiency of complex oily compounds is the low availability of contaminants for microbial attack (Abalos et al. 2004). An alternative to expand bioavailability and contaminant metabolism is increasing substrate solubilization by using biosurfactants. In the treatment of areas contaminated with complex compounds, which are difficult to degrade, it is both economically and environmentally interesting the use of the indigenous microorganisms that should present degradation capacity together with a biosurfactant (Cameotra and Singh, 2008).

Biosurfactant is a well-known surface active agent that generally used in improving the viability of contaminant to the microbial attack. The biosurfactant affect the biodegradation process by increasing the solubility and dispersion of the compound in two ways, i.e., increasing the surface area of hydrophobic water insoluble substrate or increasing the bioavailability of hydrophobic water insoluble substances (Franzetti et al. 2010).

The results demonstrated that the texture and size of the soils particles influenced the action of the biosurfactant, since the removal percentages were different when compared in the three sand samples. In this context, a higher removal was observed in the silt and in the sandy soils, as shown in Table 1. The clay soil has lower permeability due to formation of macro-pores between the grains of sand, through which water and air circulate more easily, as happens with the sandy soil.

Table 1: Removal of motor oil adsorbed in standard sand, beach sand and clay soil samples by the biosurfactant from Pseudomonas sp. and distilled water (as the control)

Sand type	Removal (%)	
	Cell-free broth (Crude biosurfactant)	Distilled water (control)
Standard sand	60.0%	13.9%
Beach sand	63.0%	15.0%
Clay sand	25.0%	10.0%

3.5 Removal of motor oil static assay

Laboratory studies on MEOR typically use sand-packed columns, which provide a suitable bench-scale approach to evaluate oil recovery for several reasons: it is an economic model; a battery of columns can be set up simultaneously; and they can simulate the oil recovery operations usually conducted in reservoirs (Suthar et al. 2008).

The crude and the isolated biosurfactant produced by *Pseudomonas* sp. were able to remove the motor oil from packed columns (Table 2 and Figure 4). The removal of the oil by the isolated biosurfactant varied depending on the concentration employed. The cell-free broth and the isolated biosurfactant at twice its CMC are almost equally effective in the removal of the motor oil pollutant (around 40 %). Thus, cell-free broth can be directly used without purification steps, which would further reduce 30 %-50 % of the production cost of biosurfactant.

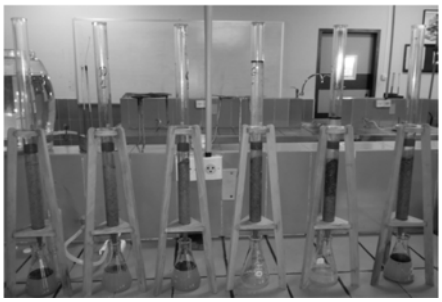

Figure 4: Illustration of packed columns

Table 2: Removal of motor oil adsorbed to sand in packed columns by the biosurfactant from Pseudomonas sp. and distilled water (as the control)

Solutions	Removal of motor oil (%)
Cell-free broth (Crude biosurfactant)	43±3.0
Biosurfactant (1/2 CMC)	15±2.1
Biosurfactant (CMC)	30±1.9
Biosurfactant (2xCMC)	40±2.5
Distilled water (control)	6±1.0

Studies carried out by Urum et al. (2003) demonstrated that the mobilization or solubilization of hydrophobic compounds by surfactants in san packed columns may or may not vary depending on the concentration employed. Cell-free broths containing *Pseudomonas aeruginosa* isolates cultivated in glycerol removed 49–54 % of crude oil contained in packed columns (Bordoloi and Konwar, 2008). The performance of water in the removal of motor oil was negligible as shown in Table 2. Khalladi et al. (2009), on the other hand, showed that the performance of water in the removal of diesel fuel was found to be non-negligible, while water contributed by 24.7 % in the global elimination of *n*-alkanes. High concentrations (2.5 and 5.0 g/L) of a biosurfactant isolated from *P.aeruginosa* 57SJ (CMC 400 mg/L) were needed to remove 70 % of pyrene adsorbed to soil (Bordas et al. 2007), while the biosurfactant produced by *Bacillus* species cultivated in residues of molasses and cheese whey removed about 30 % of the oil contained in a packed column (Joshi et al. 2008).

4. Conclusions

The biosurfactant produced by *Pseudomonas* sp. has potential for application in the remediation of soil and water contaminated with oil and oil products, considering the percentage of pollutant removal in different conditions. It is important to note that the best results were obtained with the crude biosurfactant, which represents a considerable reduction of the production costs of this compound, increasing the chances of an actual industrial application.

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