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Application of *Bacillus cereus* UCP 1615 Biosurfactant for Increase Dispersion and Removal of Motor Oil from Contaminated Sea Water

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Contaminations caused by hydrophobic compounds represent a permanent hazard to the environment and global development. Methods of cleaning and remediation are essential to avoid intoxication and death of living organisms after disasters with these compounds. The application of biosurfactants is one of the methods used to remove to remove oils. These amphipathic molecules reduce surface and interfacial tensions of two immiscible phases to improve mixing. Such compounds are produced spontaneously by various organisms, such as bacteria with the advantage of lower toxic activity, more biodegradability and stability under adverse environmental conditions when compared to chemical surfactants. Thus, this study investigated the potential application of a biosurfactant for enhanced removal capability of motor oil from contaminated water, under laboratory conditions. The biosurfactant was produced by *Bacillus cereus* UCP 1615 isolated from seawater, grown in mineral medium supplemented with 2% soybean waste frying oil and 0.12% peptone, in a bioreactor of 3 L, on a stirring of 250 rpm, for 48 h, at 28°C. After producing, were evaluated the surface tension, production and critical micelle concentration (CMC) of the biosurfactant, the obtained results were 27.5 mN/m, 4.6 g/L and 500 mg/L, respectively. Tests were conducted to examine the effectiveness of the biosurfactant. Firstly, it was evaluated the oil dispersion capacity in the crude biosurfactant using at the CMC concentration was enough to achieve favorable results (above 85%) in ratio 1:1 (biosurfactant/motor oil). Similar result was obtained with isolated biosurfactant in ratio 1:10. Thereafter, was measured the biodegradation using the biosurfactant and its microbial producing specie in the removal of oil contaminated seawater. The results showed that the presence of the biosurfactant in association with microorganism increased the degradation of the motor oil up to 96% in 27 days of incubation in seawater in relation to the control. Thus, the biosurfactant produced by *Bacillus cereus* UCP 1615, a promising strain that possibly had the metabolism stimulated to enhance the production of biosurfactant, has application potential for remediation processes in marine environments contaminated with hydrophobic compounds.

1. Introduction

Global development is tied to energy sources. Among these sources oil has its demand directly related to growing world industrialization. Although petrochemical plants and petroleum refineries are beneficial to society, they produce a large amount of hazardous waste (Geetha et al., 2018).

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However, accidents may occur during the petroleum exploitation and processing, bringing severe harmful and cumulative environmental impacts. Petroleum hydrocarbons are, in fact, the uppermost environmental pollutants to all biological systems (Ghazala et al., 2018). When in contact with water, the oil spreads forming a thin layer on the surface, which changes the exchange of gases between air and water and blocks the passage of sunlight. This blockage prevents the process of respiration and photosynthesis, impacting phytoplankton communities and causing a collapse in the food chain. Moreover, the development of marine animals are also affected, causing developmental anomalies, being harmful to human health, when in contact with the skin (Philibert et al., 2019; Laffon et al., 2016; Bachmann et al., 2014).

Methods of cleaning and remediation are essential to avoid intoxication and death of living organisms after disasters with hydrophobic compounds. There are physico-chemical methods for the removal of spilled oil, but these are not completely effective as well as being able to produce toxic byproducts. As an alternative to these methods, the most important are biosurfactants which are compounds formed by molecular structure containing hydrophilic and hydrophobic portions. They tend to distribute in fluid interfaces between layers with different degrees of polarity (oil / water), promoting a reduction of surface and interfacial tension, giving detergency, emulsification, lubrication, solubilization and phase dispersion (Santos et al., 2016). This biomolecule when compared to chemically synthesized counterparts, have many advantages because they are ecologically correct, biodegradable, less toxic, and produced by renewable sources (Henkel et al., 2017).

In this sense, the objective of the present study is to use a biosurfactant produced by *Bacillus cereus* UCP 1615 to enhance capability of dispersion, removal and biodegradation of motor oil from contaminated water.

2. Methods

2.1 Micro-organism

New strain of *Bacillus cereus* UCP 1615 was isolated from environmental water samples contaminated with petroleum derivates in the Suape Port, Ipojuca, Pernambuco, Brazil. The microorganism was maintained at 5 ºC on Nutrient Agar (NA) slants containing (w/v): yeast extract (0.3%), peptone (0.5%), NaCl (0.5%) and agar (1.5%).

2.2 Production of biosurfactant

The production of biosurfactant was performed in distilled water based medium with 2% soybean waste frying oil and 0.12 % peptone. The bioreactor (Marconi LTDA, Brazil) of 3 L, were kept under 250 rpm orbital agitation for 48 h at 28 ºC, with aeration (1.0 vvm). The culture medium was inoculated with a 24 h inoculum.

2.3 Surface tension and CMC determination

Surface tension and interfacial tension (against hexadecane) changes were carried out on the cell-free broth obtained by centrifuging the cultures at 5000 × g for 20 min by the ring method using a Sigma 700 Tensiometer (KSV Instruments Ltd., Finland) at room temperature. 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 could 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.

2.4 Biosurfactant isolation

The biosurfactant was extracted from culture medium after cell removal by centrifugation at 5000 × g for 30 min. The supernatant pH was adjusted to 2.0 with HCl 6.0 M, 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 procedure was repeated twice again. The product was concentrated from the pooled organic phases using a rotary evaporator (Resende et al., 2017).

2.5 Oil displacement test

The oil displacement test is a method used to measure the diameter of the clear zone, which occurs after dropping a surfactant-containing solution on an oil–water interface. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant. This assay was performed by adding 50 mL of distilled water to a petri dish with a diameter of 15 cm. After that, 100 mL of motor oil was dropped onto the surface of the water, followed by the addition of 10 or 100 mL of the cell-free broth (crude biosurfactant) over the oil layer. The biosurfactant at its CMC and 10 x CMC concentration was also tested. The results were considered positive when the drop of surface-active metabolites present in supernatants

repelled the oil formed clear halos. The diameters of the clear zones of triplicate experiments from the surfactant sample were determined after 30 seconds for an averaged value of the clear zone diameter and compared to a negative control using seawater (Resende et al., 2017).

2.6 Oil displacement test

Motor oil biodegradation experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL of seawater and 1% motor oil. The sterilized seawater plus motor oil medium was inoculated with 5% of 107 CFU/ml of the selected strain. The flasks were incubated at 28°C on a rotary shaker at 150 rpm. The experiments were conducted in three different sets as follows:
Set 1, seawater + motor oil + bacterial cells
Set 2, seawater + motor oil + bacterial cells + biosurfactant (at the CMC)
Set 3, seawater + motor oil + bacterial cells + biosurfactant (at twice the CMC)
Set 4, seawater + crude oil (control).
The samples were drawn on 9th and 27th day for estimation of motor oil degradation by gravimetric analysis. The residual motor oil was extracted in a pre-weighed beaker with hexane in a separating funnel. Extraction was repeated twice to ensure complete extraction. After extraction, hexane was evaporated in a hot air oven at 68–70°C the becker was cooled down and weighed. Cell-free control was incubated under similar conditions. The percentage of degradation was calculated as follows (Oleke and Glick, 2005):
% degradation = [amount of motor oil degraded / amount of motor oil added in the media] x 100.

3. Results and Discussion

3.1 Production of biosurfactant

The *Bacillus cereus* UCP 1615 was isolated from a marine and port region, a promising strain that possibly had the metabolism stimulated to enhance the production of biosurfactant. The produced biosurfactant decreased the surface tension of water from 72 to 27.5 mN/m. When it was produced in the 3 L bioreactor, it was observed a production of 4.6 g/L.
The results of surface tension are similar to those described in a study using *B. licheniformis* R2 in a 5 L fermentor. The authors have achieved a reduction to 28 mN/m in surface tension, although the yield was only 1.1 g/L (Joshi et al. 2015). In another study, using a biosurfactant by *B. subtilis* B20 grown with molasses in a 5 L fermentor, the production obtained was 2.29 g/L (Al-Bahry et al., 2013). Santos et al. (2016) consider the use of bioreactors becomes an even more attractive and promising alternative in comparison to the limitations bench top technique, such as shaker table, from the point of technical and economic views. In another study, the highest biosurfactant produced by *Bacillus pumilus* 2IR was 1.06 g/L using the 5 L bioreactor (Fooladi et al., 2018). Considering the production of others Bacillus genuses it was observed, the biossurfactant production distinctiveness of the strain used in this work.
The critical micelle concentration (CMC) from the biosurfactant produced by *Bacillus cereus* UCP 1615 was determined after plotting surface tension in function of biosurfactant concentration demonstrating a gradual decrease from 70 to 27 mN/m with the increase in biosurfactant until a concentration of 500 mg/L and remaining constant thereafter, as seen in Figure 1.

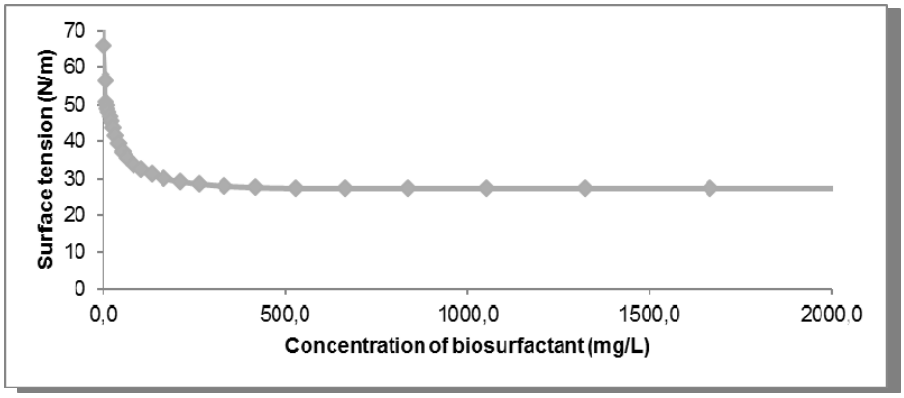

Figure 1: Graph of surface tension x concentration of isolated biosurfactant produced by Bacillus cereus UCP 1615 cultivated in mineral medium supplemented with 2% frying residual oil and 0.12% peptone for 48h

3.2 Motor oil dispersion

As it can be seen in Table 1, the crude and isolated biosurfactant from *B. cereus* UCP 1615 exhibited considerable oil dispersion capacity, indicating high surface activity. Oil spreading capacity depends on the decrease in water-oil interfacial tension due to the biosurfactant.

For environmental applications, the use of the biosurfactant in its crude form is a more viable alternative from the economic standpoint, since large volumes are required for such applications. This finding corroborates those reported for biosurfactants from *Bacillus mojavensis* I4 which achieved an oil displacement rate of 78%, in the ratio of 1:1.5 (Ghazala et al., 2018). Previous studies performed with crude biosurfactant from *Bacillus sp*. indicate an efficiency of 74% (Resende et al., 2017). Regarding the isolated biosurfactant, its use at the CMC was enough to achieve favourable results.

Table 1: Motor oil dispersion capacity of biosurfactant from Bacillus cereus UCP 1615 cultivated in mineral medium supplemented with 2% waste frying oil and 0.12% peptone for 48 hours (data expressed as mean ± standard deviation)

	Oil dispersion (%)		
Biosurfactant/oil ratio	Crude biosurfactant	Biosurfactant t(CMC)	Biosurfactant (10xCMC)
1:1	86 ± 0.3	76 ± 0.3	81 ± 0.6
1:10	55 ± 0.5	80 ± 0.5	88 ± 0.5

3.3 Biodegradation of motor oil

Four different sets were used to evaluate the biodegradation of motor oil, the results were recorded on the 9th and 27th day for each set (Table 2). The addition of the biosurfactant significantly increased the biodegradation of the oil (around 90%), as demonstrated in the second and third sets of the experiment. Moreover, the addition of the biosurfactant at the CMC was enough to achieve maximum degradation after 27 days. At the end of the experiment it was possible to observe the presence of biomass, which also shows the growth of the microorganism used. The results demonstrate that the biosurfactant produced is an efficient enhancer for the degradation of hydrophobic compounds. In addition to these results, studies indicate that biosurfactants maintain efficiency under extreme conditions of pH, salinity and temperature (Rufino et al., 2014).

Table 2: Motor oil biodegradation experiments conducted in seawater with and without biosurfactant from isolate B1 cultivated in mineral medium supplemented with 2% waste frying oil and 0.12% peptone for 27 days (data expressed as mean ± standard deviation)

Sets	Days/Oil removal (%)	
	9th	27th
Control: seawater + motor oil	12 ± 0.2	21 ± 0.4
Set 1: seawater + motor oil + bacterial cells	22 ± 1.5	32 ± 2.4
Set 2: seawater + motor oil + bacterial cells + biosurfactant at CMC	68 ± 1.3	96 ± 0.9
Set 3: seawater + motor oil + bacterial cells + biosurfactant at 2 x CMC	74 ± 1.6	97 ± 0.6

According to Santos et al. (2016), two degradation mechanisms may occur. The first involves the increase in the bioavailability of the hydrophobic substrate and the second includes the interaction between the biosurfactant and cell surface, which increases the hydrophobicity of the surface, enabling hydrophobic substrates to combine with bacterial cells more easily. According to Zheng et al. (2012), the variation in the biodegradation rate of a specific surfactant depends on its solubilisation capacity, which is determined by the properties of its micelles.

Several studies have described the capacity of bacterial biosurfactants to enhance the degradation of hydrocarbons. Studying *Oceanobacillus* sp. BRI 10, Jadhav et al. (2013) report similar results to those of the present investigation. A biosurfactant from *Candida tropicalis*, at the concentrations of 1xCMC, had already stimulated almost 60% biodegradation, with maximum above 70 % at 5xCMC (Almeida et al., 2018).

In another study, the diesel degradation capacity of a biosurfactant from *Bacillus amyloliquefaciens* An6 in a liquid medium was evaluated over a 15-day period and led to a 15 to 30% increase in degradation in a concentration-dependent manner (Faria et al., 2011). A biosurfactant from *Bacillus subtilis* CN2 increased the degradation of polycyclic aromatic hydrocarbons in motor oil more than twofold over an 18-day period (Bezza et al., 2015). The presence of a biosurfactant from *Bacillus* sp. have increased the degradation of the pollutant by 10% in sand contaminated with motor oil (Chaprão et al., 2015). A biosurfactant from *Pseudomonas*

cepacia CCT6659 increased the degradation of crude oil by bacterial cells to 83% in a 10-day period (Silva et al., 2014).

4. Conclusions

The biosurfactant produced by *Bacillus cereus* UCP 1615 presents satisfactory properties regarding the reduction of surface tension and oil dispersion capacity. The biomolecule has effectiveness and may aid in the biodegradation or removal of hydrocarbons in association with microorganisms. Production in bioreactors may be advantageous, enabling this biossurfactant production and industrial application. Therefore, this biomolecule has potential in the application of remediation processes in environments contaminated with hydrophobic compounds.

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