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Application of *Candida lipolytica* Biosurfactant for Bioremediation of Motor Oil from Contaminated Environment

Júlio César V. dos Santos[a], Emília Mendes da S. Santos[a], Yali A. da Silva[a], Isabela Regina Alvares da S. Lira[a], Renata Raianny Silva[a], Ítalo José B. Durval[a,b], Leonie A. Sarubbo[a, b], Juliana M. de Luna[a,b]

[a] Catholic University of Pernambuco, Príncipe Street, n. 526, Boa Vista, Code: 50050-900, Recife, PE, Brazil
[b] Advanced Institute of Technology and Innovation (IATI), Joaquim de Brito Street, 216, Code: 50070-280, Boa vista, Recife, PE, Brazil
*juliovasconcelos05@gmail.com

Contamination caused by hydrocarbons can produce permanent and irreparable damage to the environment. Biosurfactants' production has been intensively studied in the last years, for these agents are biodegradable and have applications in several industrial sectors. Moreover, they have, as well, managed to be ecologically compatible and to carry out the biodegradation of hydrophobic pollutants without damaging the environment. Bioremediation involves using microorganisms or their microbial metabolites for the degradation of contaminants, avoiding the use of chemicals. Biosurfactants are amphipathic biomolecules produced by microorganisms capable of allowing water-oil interaction and also providing biostimulation in the degradation process. In this work, it was used a medium formulated with distilled water supplemented with 2.5% corn steep liquor, 4.0% molasses, and 2.5% frying oil as substrates used to produce a biosurfactant *Candida lipolytica* (UCP 0988), at 28 ºC during 144 hours under 200 rpm. The biosurfactant's tensoactive properties were determined, and its isolation, toxicity, and bioremediation application were described. The surface tension values indicated a reduction in water tension from 72 to 28 mN/m and a yield of 21g/L in biosurfactant and Critical Micellar Concentration (CMC) of 0.5%. The biosurfactant produced demonstrated stability concerning emulsification and surface tension in extreme pH values (2 to 12), concentrations of NaCl (2 to 10%), and different temperatures (5 to 120°C), and it did not present toxicity to the vegetables. The crude or isolated biosurfactant showed efficiency in removing 57% - 70% of the motor oil in contaminated soil under static conditions. The results obtained demonstrated biosurfactant produced by *C. lipolytica* showed promising properties for use in bioremediation of hydrophobic compounds.

1. Introduction

Accidents that occurred with oil spills and its derivatives in Brazil, from 1975 to 2012, reached millions of liters that contaminated soils, rivers, and seas. Given this reality, the possibility of environmental contamination becomes real and imminent, with an urgent need to develop new technologies that may contain possible contamination (Karlapudi et al., 2018).

Bioremediation played an important role in cleaning up the spill of 41 million liters of oil caused by the ship Exxon Valdez, in the Gulf of Alaska, in 1989, initiating the development of this technology, demonstrating that there are good reasons to believe in the effective application of this method in the treatment of future oil spills in appropriate circumstances (Souza et al., 2014; Santos et al., 2020).

In the aforementioned accident with Exxon Valdez, the first step was physical washing with high-pressure water jets. Subsequently, chemical surfactants were applied to the polluted areas to accelerate petroleum-degrading microorganisms' growth and activity. Two or three weeks later, the regions treated with the surfactants were significantly cleaner than the control areas.

However, it was difficult to assess the treatment effects due to the heterogeneity of the contamination. Nonetheless, other studies have demonstrated the importance of using surfactants to increase oil biodegradation (Satpute et al., 2010; Durval et al., 2020).

In recent years, studies on biosurfactants' production have intensified due to these compounds' characteristics such as biodegradability, low toxicity, specificity, and stability under extreme environmental conditions of temperature, pH, and salinity (Perfumo et al., 2018; Santos et al., 2021).
The literature describes bacteria of the genera *Pseudomonas* and *Bacillus* as great biosurfactant producers. However, most biosurfactants of a bacterial origin are inadequate for use in the food industry due to their possible pathogenic nature. *Candida bombicola* and *Candida lipolytica* are among the most commonly studied yeasts for the production of biosurfactants (Santos et al., 2020).
Therefore, the aim of the present study the production of a new biosurfactant by the yeast *Candida lipolytica* UCP 0988 cultivated in a low-cost medium. We also describe the properties of the biosurfactant, toxicity, and application in the removal of a petroleum-based oil from terrestrial environment.

2. Materials and methods

2.1 Microorganism

Candida lipolytica (UCP 0988) was obtained from the culture collection of the Catholic University of Pernambuco, Brazil. The microorganism was maintained at 5ºC on Yeast Mold Agar (YMA) slants containing (w/v): yeast extract (0.3 %), malt extract (0.3 %), tryptone (0.5 %), D-glucose (1.0 %), and agar (5.0 %).

2.2 Preparation of inoculum

Inoculum were transferred to a tube containing YMA medium to obtain a young culture. Then the sample was transferred to flasks containing 50 ml of Yeast Mold Broth (YMB) medium, followed by incubation with constant stirring at 200 rpm and 28°C for 24 hours, obtaining the desired final concentration of cells (10^8 cells/ml).

2.3 Biosurfactant production media

The production of biosurfactant was performed in distilled water-based medium with 4.0% molasses, 2.5% corn steep liquor and 2.5 % of waste frying oil. The shake flasks were kept under 150 rpm orbital agitation for 144 h at 28 ºC.

2.4 Production of biosurfactant

Fermentations for the production of biosurfactant were performed in 1000-ml Erlenmeyer flasks containing 500 ml of production medium and incubated with the suspension of 10^8 cells/ml. The inoculum was added and the media were kept under orbital stirring at 200 rpm for 144 hours at a temperature of 28°C. Aliquots were withdrawn after fermentation for the determination of surface tension, emulsification activity, and yield of biosurfactant.

2.5 Surface tension and CMC determination

The measurement of the surface tension 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.

2.6 Isolation of biosurfactant

The biosurfactant was extracted from the fermented broth after removing the cells by centrifugation (5000 x g, 15 minutes, 4 °C). The same volume of ethyl acetate (1:1, v/v) was added to the cell-free broth. The mixture was vigorously stirred for 15 minutes and allowed to stand to separate the phases. The samples were extracted twice. The organic phase was evaporated at 40°C to remove the solvent (Silva et al., 2020).

2.7 Determination of emulsification activity

Emulsification index (EI) was measured using the method described by Cooper and Goldenberg (1987), whereby 2 ml of motor oil was added to 2 ml of the biosurfactant in a graduated screwcap test tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and the emulsification index was calculated.

2.8 Effect of environmental factors on biosurfactant activity

The effect of the addition of different concentrations of NaCl on the biosurfactant activity was investigated in the cell-free broth. A specific concentration of NaCl (2-10 %, w/v) was added, and surface tension and emulsification activity were determined as previously stated. The cell-free broth was also maintained at a constant temperature (5, 70, 100, and 120°C) used for surface tension and emulsification measurements. The effect of pH on surface

tension and emulsification was evaluated after adjusting the broth pH to 2, 4, 6, 8, and 10 with 6.0 M NaOH or HCl.

2.9 Phytotoxicity test

The biosurfactant's phytotoxicity was evaluated in a static assay involving seed germination and root growth of the vegetable cabbage (*Brassica oleracea*) based on (Tiquia et al., 1996). Test solutions were prepared in distilled water with biosurfactant concentrations of 1/2 x CMC, 1 x CMC and 2 x CMC (0.25, 0.5 and 1.0 g/l, respectively). After five days of incubation in the dark, seed germination, root growth (≥ 5 mm), and the germination index were calculated using the following formulas:

$$\text{Relative seed germination (\%)} = \frac{(\text{nº of seeds germinated in extract}}{\text{nº of seeds germinated in control)} \times 100} \quad (1)$$

$$\text{Relative root length (\%)} = \frac{(\text{mean root length in extract}}{\text{mean root length in control)} \times 100} \quad (2)$$

$$\text{Germination index} = \frac{[(\text{\% of seed germination}) \times (\text{\% of root growth})]}{100\%} \quad (3)$$

2.10 Removal of petroleum product adsorbed on sand by biosurfactant in packed columns – static assay

Glass columns (55 x 6 cm) were filled with approximately 200 g of sand contaminated with a 10% (v/w) solution of the hydrophobic contaminant. The surface was then flooded with 200 mL of a biosurfactant solution at a concentration corresponding to ½ CMC, CMC or 2 x CMC. The same volume of cell-free broth containing crude biosurfactant was also tested, while a column containing soil in 200 mL of water (without surfactant) was used a control. Samples were withdrawn to assess oil removal gravimetrically (Rufino et al., 2013).

3. Results and Discussion

3.1 Determination of surface tension and CMC of the biosurfactant

The biosurfactant produced by *Candida lipolytica* exhibited excellent capacities for reducing surface tension since the water surface tension was reduced from 70 mN/m to 29 mN/m, having a yield of 24 g/L, and an increase of concentration of biosurfactant up to 0.5% (Figure1). The biosurfactant from *C. lipolytica* demonstrated a greater capacity to reduce the tension than the surfactants of *C. lipolytica* (32 mN/m) (Rufino et al., 2007), of *C. glabrata* (31 mN/m) (Sarubbo et al., 2006), of *C. antarctica* (35 mN/m) (Adamczac; Bednarski, 2000), and of *Yarrowia lipolytica* (50 mN/m) (Gallert; Winter, 2002).

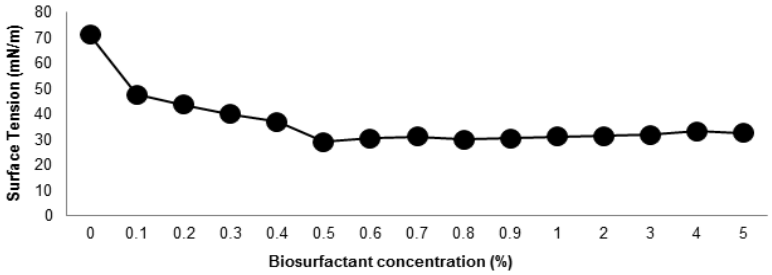

Figure 1: Critical Micellar Concentration (CMC) of the biosurfactant produced by C. lipolytica grown in distilled water supplemented with 4.0% molasses, 2.0% frying oil, and 2.0% corn steep liquor.

3.2 Emulsification index

Table 1 shows the emulsification index for the biosurfactant obtained from *C. lipolytica*, where 60% emulsification was observed for motor oil, followed by corn oil. When comparing with the values presented in the literature (Campos et al., 2014), obtained emulsification rates with the biosurfactant produced by *C. utilis,* using mineral medium supplemented with 5% canola frying oil and 6% glucose, of 43%, 73%, 73%, 33% and 30 % for soybean, sunflower, corn, rice, and engine oils, respectively.

Table 1: Emulsification index of the biosurfactant produced by C. lipolytica.

Biosurfactant	Emulsification (%)			
	Motor oil	Corn oil	Soy oil	Kerosene
	60%	58%	40%	30%

3.3 Surface tension stability of the biosurfactant

The stability of the biosurfactant produced by *C. lipolytica* was evaluated in the cell-free metabolic liquid after 144 hours of culture for different values of pH, temperature, and the presence of NaCl as a function of the surface tension of the biomolecule. The tests performed on the metabolic liquid free of cells concerning the pH variation demonstrated that the biosurfactant obtained did not present significant surface tension value changes. The stability of the biosurfactant produced by *C. lipolytica* has been tested over a wide temperature range. The biosurfactant was stable at the temperatures tested, as can be seen in (Figure 2A) variation of pH, (Figure 2B) variation of temperature, and resistance of the biosurfactant to NaCl addition (Figure 2C). The surface tension in the cell-free metabolic liquid containing the biosurfactant was stable, regardless of the added salt concentration. Freitas et al. (2016), with biosurfactant produced by *C. bombicola* shown similar results in relation to stability, where the biotensioactive obtained little variation in surface tension. The biosurfactant also obtained results similar to those of Santos et al. (2021), with stability in the surface tension when exposed at different pH values, temperatures and concentrations of NaCl values tested during the entire storage time, when compared to the control.

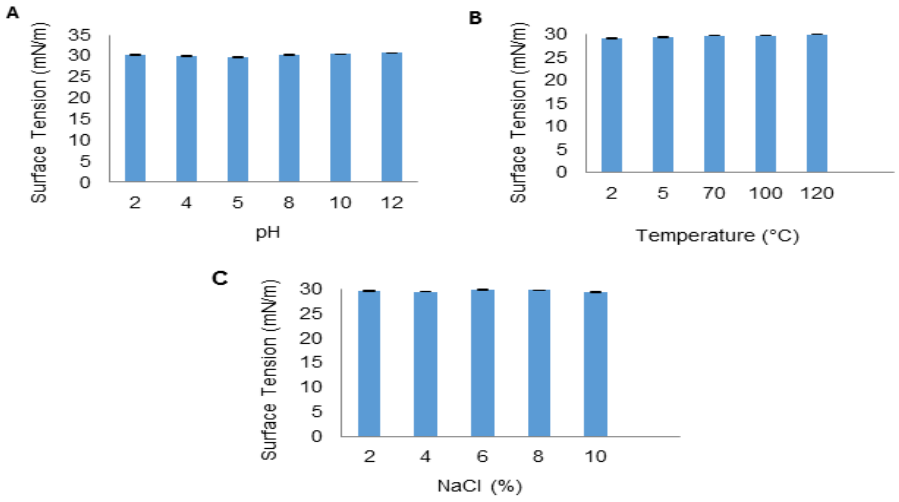

Figure 2: Stability of the surface tension of the C. lipolytica biosurfactant grown in distilled water supplemented with 4.0% molasses, 2.5% residual frying oil, and 2.5% corn steep liquor as substrates against variations in pH (A), temperature (B), and the addition of NaCl (C).

3.4 Phytotoxicity test with cabbage seed (*Brassica oleracea*)

Toxicity can be defined as a substance's ability to cause a harmful effect on a living organism, which depends on the concentration and properties of the chemical to which the organism is exposed and the time of exposure (Santos et al., 2017). The toxicity of the biosurfactant in cabbage (*Brassica oleracea*) is shown in Table 3. The literature considers a Germination Index (GI) of 80% to indicate the absence of phytotoxicity. The results obtained in this work and expressed show that the biosurfactants of vegetal and microbiological origin in the tested dilutions (1 x CMC, ½ CMC, and 2x CMC) did not present any inhibitory effect on the germination for the gherkin, tomato, and cabbage seeds, with IG higher than 80% for microbiological and plant biosurfactant, respectively, in the proposed concentrations, carried out in triplicate (Yerushalmi et al., 2003).

Table 2: Phytotoxicity of isolated biosurfactant from C. lipolytica grown in medium formulated with 4.0% molasses, 2.5% corn steep liquor, and 2.5% waste frying oil the seeds vegetable specie.

Vegetable seeds	Germination index (%)		
	Biosurfactant isolated by 0.25%	Biosurfactant isolated by 0.5%	Biosurfactant isolated by 1.0
Cabbage (*Brassica oleracea*)	100%	98%	90%

3.5 Removal of hydrophobic contaminant in the sand by the biosurfactant

Biosurfactants can emulsify hydrocarbons, increase solubility in water, and reduce surface tension, facilitating the release of these oily substances from soil particles (Banat et al., 2000). The removal potential presented by the biosurfactant produced by *C. lipolytica* is expressed in Figure 3.

The results obtained for the biosurfactant isolated from *C. lipolytica* demonstrated that the solution containing the metabolic liquid was able to remove 57% of the motor oil adsorbed on the tested sand sample. In the solution with the CMC, a 38% removal was observed. The solution containing 2xCMC was able to remove 48%, and the solution with ½ CMC removed 34% of the oil adsorbed on the sample. The control, formulated with distilled water, removed a 10% portion of the adsorbed oil. The biosurfactant produced by *C. Antarctica* presented the removal capacity of 50% of the oil adsorbed on sand (Adamczac; Bednarski, 2000). In comparison to the work done by Rufino et al. (2013), using packed columns, observed removal of 26% for biosurfactant crude (cell-free broth), and 33 and 37% for the isolated biosurfactant from *C. lipolytica* at CMC and 3 × CMC, respectively. The results obtained of the current study, with the together with the literature reports on the bioremediation of ground contaminated by petroleum derivatives, confirm the beneficial role of biosurfactants in the biodegradation of hydrocarbons.

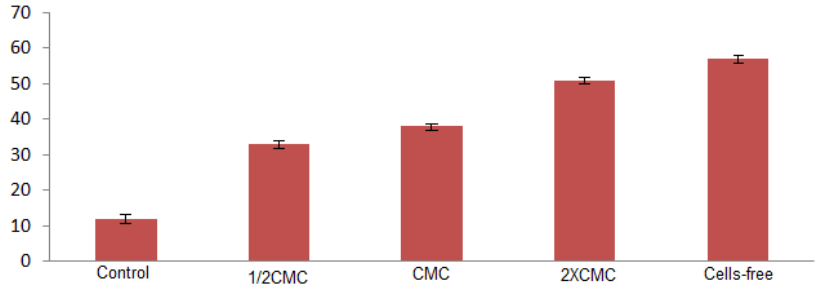

Figure 3: Removal of oil adsorbed on the sand by the remediation process using the biosurfactant produced by C. lipolytica in packaging columns through static testing.

4. Conclusions

The results obtained for the production of a biosurfactant by *C. lipolytica* using the medium formulated with agroindustrial demonstrate that the biomolecule presents promising properties with regard to surface tension and emulsification index. The biosurfactant maintained its stability in the extreme conditions of pH, salinity, and temperature, as well as low toxicity to plant seeds, showing potential for industrial application.

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