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Biosurfactant Formulation of *Pseudomonas cepacia* and Application in the Removal of Oil from Coral Reef

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The innovative biosurfactants arisen to replace chemical surfactants in bioremediation processes because of their effectiveness as dispersants, greater stability and biodegradability. Currently, the major market for biosurfactant is the petroleum industry, where these compounds can be used in the cleanup of oil spills, the removal of oil waste from storage tanks, enhanced oil recovery and bioremediation of soil and water. In this sense, this study investigated the stability of the biosurfactant produced by *Pseudomonas cepacia* CCT 6659 growing in culture medium containing 2 % residual canola oil, 3% corn steep liquor and 0.2 % $NaNO_3$ for 60 hours at stirring 250 rpm and 28 °C. In the cell-free metabolic liquid was added a salt of potassium sorbate (0.2 %) to the conservation of their surfactant properties. Heating, pH and salinity conditions were applied to evaluate the best application results of the biosurfactant for 120 days. The biosurfactant was also used in the engine lubricating oil removal impregnated in samples of coral reefs so as to evaluate its potential in removing petroleum compounds. The most significant results found for biosurfactant were surface tension of 25.92 mN/m, engine-oil emulsifying capacity of 99 % and dispersion 53.5 % oil in seawater. Moreover, the biosurfactant removed 84.5 % of the hydrophobic compound of the coral reefs samples. Based on the presented conservation economical method and in the proven resistance to extreme conditions, the biosurfactant demonstrated its potential for applications in the oil industry and in the environmental decontamination processes.

1. Introduction

Contaminants release, such as petroleum and its derived, into the environment is one of the main causes of global pollution. A large number of contaminants are toxic and carcinogenic, placing both human and animal health at risk. Through capillarity, hydrocarbons are adsorbed to surfaces and became trapped in a water immiscible phase, making difficult to remove these compounds from contaminated environments (Luna et al., 2013). Petroleum industry is the largest market for surfactants, where they can be used for removal/mobilization of oils encrusted in soil and storage tanks and bioremediation/dispersion of oily spots in the sea and oils removal from rocks and sand of the sea, increasing the recovery of the areas of environmental protection (Almeida et al., 2016; Silva et al., 2014b).
Surfactants are amphipathic molecules capable of reducing surface and interfacial tensions between liquids, Solids and gases. All surfactants have two ends, one of which is hydrophobic and the other hydrophilic. A hydrocarbon part usually comprises the hydrophobic end, which is less soluble in water, whereas the water-soluble hydrophilic end may be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Luna et al., 2016).
Currently, most compounds with surfactant properties on the market are mainly of synthetic origin. However, bacteria and archaebacteria are most responsible for the production of biological surfactant. Bacteria from the

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families *Pseudomonacea* and *Bacillacea* are able to produce efficient biosurfactants in the petroleum and polluting derivatives removal of water (Rocha e Silva et al., 2013). Ramnolipids produced by *Pseudomonas* are the best known glycolipid surfactants and their range of potential applications in the ceramic, food, cosmetic, pharmaceutical, metal, paper and environmental applications industries, such as bioremediation (Silva et al. 2013).

The main factor that restricts biosurfactants use in the market is their production cost when compared to their synthetic counterparts. The alternative use of low cost substrates, such as agroindustrial wastes, is an important strategy to reduce process costs and to provide industrial development of biosurfactant production (Rufino et al., 2014). Therefore, environmental and economic issues motivated this study, which presents biosurfactant production by a strain *Pseudomonas cepacia* CCT 6659 using a low cost optimized mineral medium supplemented with canola oil (residual frying) and corn step liquor substrates as substrates (Silva et al., 2013).

However, innovative biosurfactants replace chemical dispersants due to their numerous applications and their evaluated environmental benefits in relation to biodegradability and low toxicity. Stability is an essential factor in enabling the large-scale production, mainly of a biotechnology product that is slow to be produced in the face of the urgency of being applied in an oil disaster. All oil needs to be removed from the ocean within about 24 h after the spill. Therefore, the durability needs to be high for immediate use (Marchant; Banat, 2012).

The present study aimed to evaluate the biosurfactant behavior produced by *Pseudomonas cepacia* CCT 6659 under various properties presented and under specific conditions over time and in the biosurfactant maintenance by the addition of a chemical salt as an additive.

2. Materials and Methods

2.1 Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories, USA. Canola waste frying oil was received from a local restaurant in Recife-PE, Brazil and was stored according to supplier's recommendations and used without any further processing. Corn steep liquor was obtained from the factory Corn Products do Brasil, Cabo de Santo Agostinho-PE, Brazil.

2.2 Bacterial strain and preparation of seed culture

A strain of *P. cepacia* CCT 6659 was provided from the culture collection of the Fundação André Tosello de Pesquisa e Tecnologia, Campinas city, São Paulo, Brazil. The cultures were maintained in nutrient agar slants at 4 °C. For pre-culture, the strain from a 24 h culture on nutrient agar was transferred into 50 mL nutrient broth to prepare the seed culture. The cultivation condition for the seed culture was 28 °C, 150 rpm, and 10-14 h of incubation time.

2.3 Formulation

Pseudomonas cepacia (CCT 6659) strain was used for microbial biosurfactant production. Mineral medium used in the tests was supplemented with 2 mL of canola oil (frying residual), 3 mL of corn steep liquor and 0.2 g/L $NaNO_3$ in 500 mL Erlenmeyer flasks containing 250 mL of the medium and incubated with 1.5 % pre-inoculum and pH 7.0 with 250 rpm orbital shaking for 60 hours at 28 °C. After fermentation, the cell-free broth was submitted to a conservation method using 0.2 % of a preservative (potassium sorbate). After the treatment of the crude biosurfactant, broth was stored at room temperature (28–30 °C) for 120 days, with samples withdrawn at 15, 30, 45, 90, and 120 days (long term stability study). After each storage time, biosurfactant was subject to changes on pH (5.0, 7.0 and 9.0), addition of NaCl (1, 3 and 5 % w/v) and heating at 40 °C and 50 °C. Biosurfactant properties were checked by surface tension determination, emulsification activity and the dispersant capacity of motor oil in seawater to select the best conservation method (accelerated stability study).

2.4 Isolation of biosurfactant

Biosurfactant was extracted from the cell-free broth after cell removal by centrifugation at 5000×g for 20 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 v/v) 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. The pooled product from organic phase was dried in an oven until complete evaporation of the solvent at 80 °C to a constant weight (Silva et al. 2013).

2.5 Determination of surface tension and CMC determination

Surface tension of the isolated and formulated biosurfactant was determined with a Tensiometer (Sigma 700, KSV Instruments Ltd., Finland), using the Du Nouy ring method at room temperature (Silva et al., 2014a). The

critical micelle concentration (CMC) was determined using the same equipment, 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.

2.6 Emulsifying activity

Emulsification index (EI) was measured using the method described by Cooper and Goldenberg (1987), whereby 2 mL of a liquid hydrophobic compound (motor oil in this work) was added to 2 mL of the formulated 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 by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.7 Oil displacement test

The oil displacement test was carried out by slowly dropping 80 µL of motor oil onto the surface of 40 mL of seawater in a Petri dish (15 cm in diameter) until covering the entire surface area of the water. This was followed by the addition of 10 µL of the formulated biosurfactant onto the surface of the oil layer. The mean diameter of the clear zones of triplicate experiments was measured and calculated as the rate of the Petri dish diameter (Ohno et al., 1993).

2.8 Wash hydrophobic compound adsorbed on coral reef

The removal of motor oil adsorbed to the coral reef was evaluated 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 isolated biosurfactant at ½xCMC, 1xCMC, 2xCMC and 5xCMC and formulated biosurfactant, as illustrated in Figure 1. After the washing process, the percentage of removal was calculated. Following the washing of the porous surface, the samples were treated with 50 mL of hexane twice for the removal of residual oil. The solvent was evaporated at 50 ºC and the amount of oil removed was determined by gravimetry (Sarubbo et al. 2015).

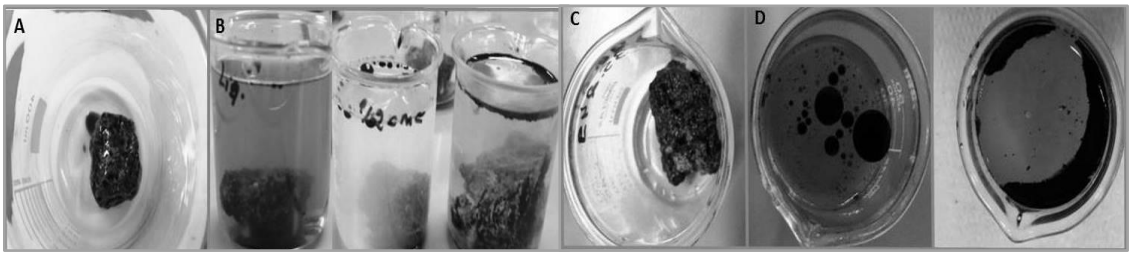

Figure 1: Illustration of porous surface before (A) and during (B) washing process with the biosurfactant from P. cepacia. (C) Oily solid surface washed and (D) Oily from Wash with the biosurfactant from P. cepacia CCT 6659.

3. Results and Discussion

3.1 Formulation

According Souza et al. (2014), one of the main requirements for biosurfactant formulation for industrial and biotechnological applications is that it should be stable over time and their properties should not significantly change with environmental variations and must support high autoclaving temperatures (121 ºC for 20 min), low temperatures (-18 °C for 6 months) and also extremes pH (5 and 11) found in the environment. Figure 2 shows surface tension values of the cell-free metabolic fluid produced by *Pseudomonas cepacia* CCT 6659 after being subjected to the storage process by the potassium sorbate salt addition as well as variations of pH (5, 7 and 9), temperature (40 and 50 °C) and NaCl concentrations (1, 3 and 5 %) to determine their stability at extreme environmental conditions. Through the 120 experiment days, the best results obtained for the surface tension of the metabolic liquid was found in the conditions of pH 7 (25.92 mN/m), at salinity of 3 % (26.63 mN/m) and heating at 50 °C (26.66 mN/m). With these results for the conservation method it was evidenced that the biosurfactant presented an excellent stability against the various adverse conditions during the period of 120 days.

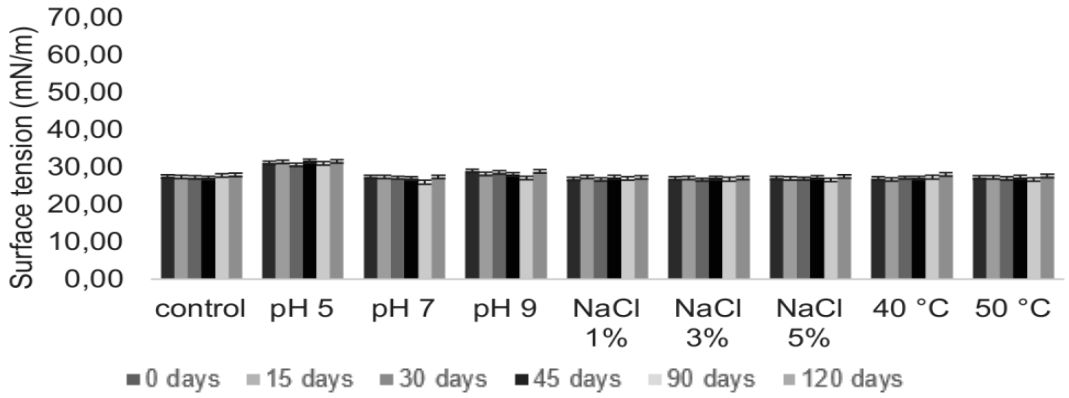

Figure 2: Surface tensions of biosurfactant added of 0.2 % potassium sorbate, over 120 days of conservation, under variation of pH, NaCl, heating and with the control of the conditions.

Emulsification index was evaluated in motor oil, according to Figure 3. Motor oil presented high emulsification rates above 90 % between 45 and 90 testing days, with percentages at 99 % after 90 days at pH 9. In addition, emulsification rates above 50 % were observed in almost all tested conditions (three pH ranges, heating at 40 °C and 50 °C and 3 % and 5 % salinity).

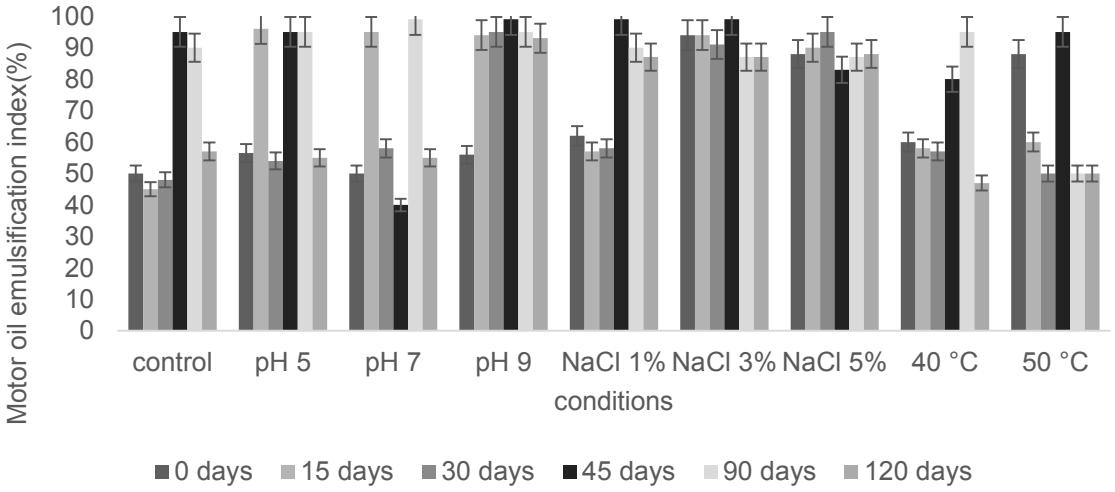

Figure 3: Motor oil emulsification capacity of biosurfactant conservaded with 0.2 % potassium sorbate addition over 120 days under pH, NaCl and heating.

The dispersant capacity of the formulated biosurfactant had behavior different in the conditions studied. Dispersion percentages related to the pH changes were higher at 120 days at pH 9, with a maximum value of 53.5 % (Figure 4). In general, the best dispersion results were observed under conditions of pH 9, 3 % salinity and heating at 50 °C. The dispersant capacity of a biosurfactant is of extreme importance when the intention is to treat marine environments contaminated with hydrocarbons, as this property helps accelerate the natural dispersion and degradation of the oil spill by breaking down the droplets, consequentially promoting a larger surface area for all degradation processes or photooxidation (Freitas et al., 2016).

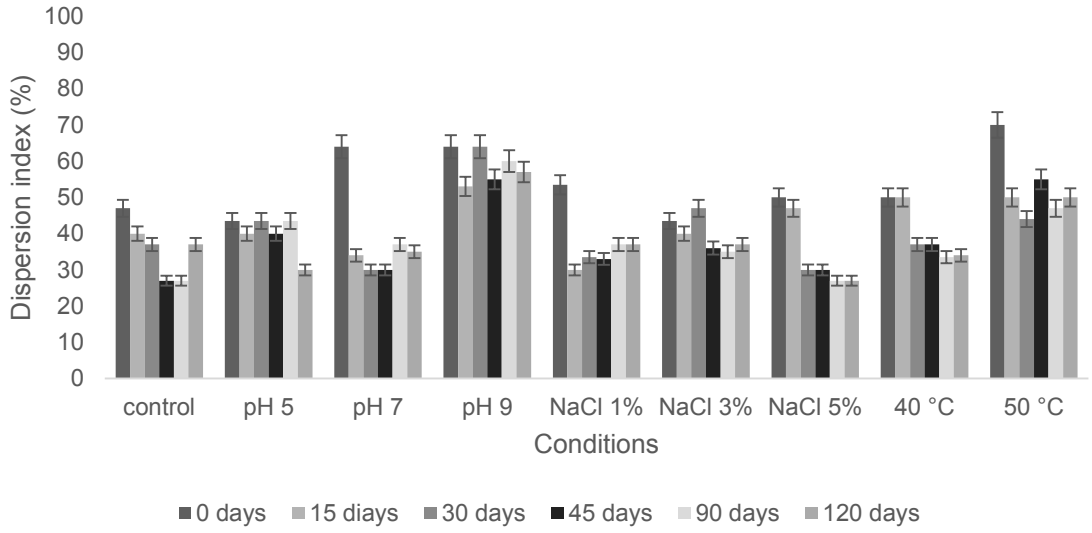

Figure 4: Motor oil dispersant capacity of biosurfactant over 120 days of conservation with 0.2 % potassium sorbate addition under pH, NaCl and heating.

3.2 Wash hydrophobic compound adsorbed on coral reef

The literature on the removal of oil from porous surface is scarce. However, Sarubbo et al. (2015) assessing the oil removal capacity of biosurfactant from *Pseudomonas* sp, found as a result, 46.1 %, 47.8 % and 70 % motor oil removal impregnated in marine stones using ½xCMC, 1xCMC and cell-free broth, respectively. Moreover, a 81,3 % and 68,62 % removal rate of motor oil adsorbed to a porous surface has been reported for the cell-free broth and the isolated biosurfactant at a concentration of 0.5 % produced by *P*. *cepacia*, respectively (Rocha e Silva et al., 2013). In the present study, a removal of 84.5 % was found as the best result (Table 1), demonstrating the application feasibility of *P. cepacia* biosurfactant (formulated and isolated) as dispersing agent adequate to cleanup of extremely sensitive ecosystem contaminated by hydrophobic pollutants, such as coral reefs that are delicate environments and of difficult access.

Table 1: Removal of hydrophobic contaminant adsorbed on marine stones by the cell free broth and the isolated biosurfactant produced by Pseudomonas cepacia CCT 6659.

Removal agent	Removal (%)
Formulated biosurfactant	84.5 ± 0.2
½ x CMC (300 mg/l)	68.4 ± 0.17
CMC (600 mg/l)	71.3 ± 0.19
2 x CMC (1200 mg/l)	68.3 ± 0.12
5 x CMC (3000 mg/l)	65.4 ± 0.11
Control (distilled water)	0.5 ± 0.14

Conclusion

The greatest impact of this work resided in the commercial formulation of a low cost biosurfactant using successfully industrial waste products. The present findings demonstrate that biosurfactant formulated maintained its tensioactive properties over a long storage period and removal results of oil adsorbed from coral reef clearly demonstrate the viability of application of this biomolecule as an agent for remediation processes.

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