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Original Research Paper

Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates

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ABSTRACT

In the present study, four species of *Pseudomonas* (*P. cepacia, P. acidovorans, P. picketti* and *P. fluorescens*) were cultivated in different low-cost substrates and compared with regard to biosurfactant production. Surface tension was used as a preliminary screening standard for potential biosurfactant bacteria. The best result was obtained with *P. cepacia* grown in mineral medium supplemented with 2.0% corn steep liquor and 2.0% soybean waste frying oil for 144 h at 30 °C and 200 rpm. Kinematic studies on microorganism growth and biosurfactant production were performed. Surface tension of the medium was reduced to 27.57 mN/m at the end of the cultivation, yielding 5.2 g/L of isolated biosurfactant. Tests under extreme conditions of pH, temperature and NaCl indicated the stability of the biosurfactant for use in the treatment of oil-contaminated environments. The critical micelle dilution of the biosurfactant was determined and its use in the removal of motor oil from clay soil demonstrated rates greater than 80%. Washing experiments involving rocks and oily surfaces contaminated with motor oil demonstrated greater than 80% recovery rates. The crude biosurfactant was capable of dispersing approximately 80% of oil droplets in seawater and proved to be non-toxic to indigenous marine microbiota. The crude biosurfactant demonstrated no toxicity against seeds of *Brassica oleracea* or the microcrustacean *Artemia salina* employed as a bioindicator. The present findings indicate the application potential of the biosurfactant produced by *P. cepacia* in the oil industry as a complement to remediation processes involving contaminated soil and water.

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1. Introduction

Oil refineries and other large-scale industrial processes are potential sources of environmental pollution (Franzetti et al., 2010; Marchant and Banat, 2012). The largest oil spill in the world occurred in the Gulf of Mexico in 2010 following the explosion of an oil rig off the coast of the states of Louisiana and Mississippi (USA). After the sinking of the rig, the open ducts in the drilling area (depth of 1.5 km) continued spewing oil into the sea for a three-month period until finally capped. Official reports indicate the release of the equivalent of a thousand barrels of oil per day, with an estimated total of 3–4 million barrels of oil spilled, making it the largest environmental disaster in the history of the United States (British Petroleum, 2010).

The need to remediate contaminated areas has led to the development of novel technologies that enable the detoxification of contaminants in a non-conventional fashion beyond merely chemical or physical methods. The use of microorganisms and microbial products for the degradation of pollutants is one such technology, denominated bioremediation (Das and Mukherjee, 2007; Fracchia et al., 2012). However, the biodegradation of hydrophobic compounds, including petroleum hydrocarbons, is hampered by the bonding of these compounds to soil particles and their poor solubility in water, resulting in a low degree of bioavailability for microorganisms. The use of surfactants constitutes one of the most widely investigated methods for solving this problem (Calvo et al., 2009; Mulligan, 2009).

Most commercially available surfactants are derived from petroleum products. However, recent environmental control legislation has driven the development of natural surfactants as alternatives to existing products (Pacwa-Plociniczak et al., 2011). Compounds of a microbial origin that exhibit surfactant properties (biosurfactants) consist of metabolic by-products of bacteria, yeasts and filamentous fungi (Marchant and Banat, 2012). Although more attractive than their synthetic counterparts, biosurfactants are not yet competitive in the market due to functional reasons and high production costs. Thus, the success of biosurfactant production depends on the development of less costly

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processes, especially with regard to substrates, which account for 10–30% of the total production cost. Low-cost or underutilized substrates, such as industrial waste, can be used to address this problem (Sobrinho et al., 2008; Gusmão et al., 2010; Luna et al., 2013; Santos et al., in press).

Biosurfactants need to compete with petrochemical surfactants with regard to cost, function and production capacity linked to the needs of the intended application. The high cost of biosurfactant production can be absorbed in processes for which small amounts are needed, such as in cosmetics, medications and food products. However, in applications that require large amounts of surfactants, such as oil removal and recovery, the purification step remains a drawback to the use of compounds of a microbial origin (Banat et al., 2010).

The aims of the present study were to compare four species of *Pseudomonas* (*P. cepacia*, *P. acidovorans*, *P. picketti* and *P. fluorescens*) cultivated in different industrial wastes with regard to biosurfactant production and determine the best medium. A further aim was to investigate the isolation, tensoactive properties and toxicity of the biosurfactant selected as well as its application in the remediation of environments contaminated with hydrophobic pollutants.

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories (USA). Soybean waste frying oil and canola waste frying oil were obtained from a local restaurant in the city of Recife, state of Pernambuco, Brazil, stored according to the supplier's recommendations and used without any further processing. Corn steep liquor was obtained from “Corn Products do Brasil” in the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil. Sugarcane molasses were obtained from the São José sugar processing plant in the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil, and cheese whey waste was obtained from a local micro-producer of different types of cheese in the municipality of São Bento do Una, state of Pernambuco, Brazil. Vegetable fat waste and cottonseed refinery residue were obtained from a local food industry.

2.2. Microorganisms

Four species of *Pseudomonas (P. cepacia* CCT6659, *P. fluorenscens* ATCC13525, *P. picketti* CCT5031 and *P. acidovorans* CCT5040) from the culture collection of the André Tosello Research and Technology Foundation in the city of Campinas, state of São Paulo, Brazil, were tested for biosurfactant production. The cultures were 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 were 30 °C, 150 rpm and 10–14 h of incubation.

2.3. Fermentation media

The components of the production medium were dissolved in a mineral medium containing 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% KCl and 0.001% $FeSO_4 \cdot 7H_2O$ and the pH was adjusted to 7.0 by 1.0 M HCl. Different combinations of carbon and nitrogen sources were added to the medium: 2.0% canola waste frying oil, 2.0% cheese whey waste and 2.0% soybean waste frying oil plus 2.0% corn steep liquor. The other media were composed of distilled water supplemented with 2.0% soybean waste frying oil, 2.0% cottonseed refinery residue, 2.0% sugarcane molasses, 2.0% cheese whey waste and 2.0% vegetable fat residue. One 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 250 rpm for 144 h in a New Brunswick C-24 shaker (New Brunswick Scientific, NJ, USA). No adjustment of pH was performed during cultivation.

After selection of the best producer bacterium and medium composition, kinetics of microorganism growth and biosurfactant production were monitored for 144 h. For such, growth, pH, surface tension and biosurfactant concentration were evaluated at regular intervals.

2.4. Biomass determination

For biomass determination, 10-ml samples were centrifuged at 5000*g* for 30 min. The cell pellet was washed twice with distilled water to remove residue from the cultivation medium and centrifuged again. The biomass was dried in an oven at 105 °C for 24 h.

2.5. Surface tension and critical micelle dilution

Changes in surface tension were monitored in the cell-free broth obtained by centrifuging the cultures at 5000*g* for 30 min by the ring method using a Sigma 700 Tensiometer (KSV Instruments LTD - Finland) at room temperature. Tensiometers determine the surface tension with the aid of an optimally wettable ring suspended from a precision balance. The liquid is raised until contact with the surface is registered. The sample is then lowered again so that the film produced beneath the liquid is stretched. Maximum force is measured and used to calculate the surface tension. The instrument was calibrated against Mill-Q-4 ultrapure distilled water (Millipore, Illinois, USA). Prior to use, the platinum plate and all the glassware were sequentially washed with chromic acid, deionised water and acetone and flamed with a Bunsen burner.

The surface tension measurement was also employed to quantify the biosurfactant concentration using the critical micelle dilution (CMD) technique (Desai and Banat, 1997). When the surfactant concentration is greater than the critical micelle concentration, the surface tension remains constant at a minimum value. The surface tension increases only when the surfactant concentration drops below the critical micelle concentration upon dilution. In this present study, the minimum surface tension was in the range of 28–30 mN/m. The CMD was determined in the cell-free broth obtained by centrifuging the cultures at 5000*g* for 30 min. For the measurements, the cell-free broth was diluted several times with distilled water.

2.6. Effect of environmental factors on biosurfactant activity

The effect of the addition of different concentrations of NaCl on the activity of the biosurfactant was investigated in the cell-free broth. Specific concentrations of NaCl (2–10%, w/v) were added and surface tension was determined as described above. The cell-free broth was also maintained at a constant temperature (5, 70, 100 and 120 °C) for 60 min and used for surface tension and emulsification measurements. The effect of pH on surface tension was evaluated after adjustment of the broth pH to 2, 4, 6, 8, 10, 12 and 14 with 6.0 M NaOH or HCl.

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