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Full Paper

Biosurfactant Production by *Pseudomonas aeruginosa* and *Burkholderia gladioli* Isolated from Mangrove Sediments Using Alternative Substrates

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Abstract: Biosurfactants are surface-active agents produced by a variety of microorganisms. To make biosurfactant production economically feasible, several alternative carbon sources have been proposed. This study describes biosurfactant production by strains of *Pseudomonas aeruginosa* and *Burkholderia gladioli* isolated from mangrove sediments in Northeastern Brazil and cultured in mineral media enriched with waste cooking oil. The biosurfactants were tested for drop collapse, emulsion formation and stability and surface tension. *P. aeruginosa* performed better both at lowering the surface tension (from 69 to 28 mN/m) and at forming stable emulsions (approximately 80%) at 48 hours of culture. The strains tested in this study were found to be efficient biosurfactant producers when cultured on substrates enriched with vegetable oil.

Keywords: biodegradation; waste cooking oil; vegetable oil; drop-collapse; emulsification index

1. INTRODUCTION

Biosurfactants or microbial surfactants are surface-active molecules produced by a variety of microorganisms [1]. Microemulsions formed by surfactants make it possible to mix otherwise immiscible solutions [2]. Surfactants are used in a range of industry sectors, such as crude oil, textiles, cosmetics, pharmaceutics, mineral oil and foods, as well as in the bioremediation of areas contaminated with heavy metals, including uranium, cadmium and lead [3,4]. Compared to chemical surfactants, biosurfactants are more tolerable to variations in pH, temperature and salinity, more selective and biodegradable and less toxic. In addition, biosurfactants may be produced in situ by microorganisms using organic contaminants as growth substrate [5, 6]. The use of biosurfactants has been limited so far due to the high cost of raw materials and processing. Alternative substrates have therefore been investigated, mostly derived from inexpensive agricultural waste or byproducts [7, 8].

Vegetable oils are natural products composed of a mixture of glycerol esters (triglycerides) and more or less unsaturated fatty acids containing 8–24 carbon atoms. They are widely employed in food preparation, Costa Neto and Rossi [9], have low melting points and are partly susceptible to oxidation [10]. It is difficult to estimate the amount of vegetable oil consumed in large urban centers due to its multiple uses.

Producing biosurfactants from renewable sources like vegetable oils or waste cooking oil and carbohydrates is a sound strategy for reducing industrial waste generation [11]. Waste cooking oil is an attractive alternative substrate in view of the abundance of both industrial and domestic supplies. However, it should be kept in mind that oil changes composition after being used, depending on the variety of food, the type of frying and the number of times it is

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used [12, 13]. Due to their versatility, biodegradability and low toxicity and to the development of new application technologies and improved production processes, biosurfactants will likely find ample industrial use in the near future. Much biotechnological research effort is therefore made prospecting for biosurfactant producers, including a number of microorganisms found in mangrove environments. Despite their ecological importance, mangroves are often heavily impacted by human activities, chemical pollution and oil spills.

Hydrocarbon contaminated sites can be considered as enrichment environments for selection of hydrocarbon degrading and/or biosurfactant producing microbial strains. Production of biosurfactants and bioemulsifiers by soil microorganisms provide them with an advantage in contaminated sited, since they can use water insoluble carbono sources for growth [14].

The present study describes the production of biosurfactants by bacteria isolated from mangrove sediments and cultured in mineral media enriched with vegetable oil used in deep frying. The process potentially mitigates pollution by providing a use for waste cooking oil and lowers the cost of biosurfactant production.

2. MATERIAL AND METHODS

Microorganisms

The strains were isolated from sediments and water from the mangrove swamp of the Cocó River in Sabiaguaba (outskirts of Fortaleza) and identified with the API 20E and 20NE test kits (BioMérieux) and complementary biochemical tests. The strains were kept in trypticase soy agar (TSA, Merck) in a B.O.D. incubator at 23ºC. Identified strains were included in the collection of bacteria in the laboratory by the Environment and Seafood Microbiology Laboratory of the Marine Sciences Institute (LABOMAR, Federal University of Ceará, Brazil).

Pre-inoculum

Pseudomonas aeruginosa and *Burkholderia gladioli* were selected and cultured in brain-heart infusion broth (BHI, Merck). Subsequently, 50-mL aliquots were placed in 125-mL Erlenmeyers and incubated overnight at 35ºC in an orbital lab shaker at 150 rpm (Tecnal TE- 420).

Adaptation

Pre-inoculum (1%) and cooking oil (5%) were added to 125-mL Erlenmeyers containing 50 mL BHI. The mixture was incubated for 24 hours at 35ºC in an orbital lab shaker at 150 rpm. Aliquots of the cultured microorganisms were seeded in TSA and incubated for 24 hours at 35ºC. The final inocula contained 10^8 CFU/mL [15].

Production of biosurfactants

Biosurfactants were produced in 250-mL Erlenmeyers containing 1% inoculum in 100 mL Bushneel Haas mineral medium (MM), supplemented with 2% glucose. A commercially available cooking oil was added at 2% and 5%, in duplicate. The mixture was incubated for 72 hours at 35ºC in an orbital lab shaker at 150 rpm and tested every 24 hours for drop collapse, emulsification index, surface tension and pH.

Qualitative drop-collapse test

The drop-collapse test was performed using a microtiter plate lid with 96 wells coated with 2 µL motor oil (10W-40, Castrol). At 24-hour intervals, 5-µL aliquots were drawn from the mixture in the shaker and dispensed in each well in triplicate, followed 1 minute later by evaluation of drop spreading. The negative control consisted of 5 µL deionized water dispensed in 3 wells. The test was considered positive when the drop spread on the oily surface. The test was also applied to 5 µL supernatant of the MM and cooking oil in which the bacteria were cultured, obtained by centrifugation for 10 minutes at 12,000 rpm.

Emulsification index

Subsequently, 2 mL sunflower oil, 2 mL corn oil or 2 mL hexadecane was added to the metabolic liquid in each experiment, in triplicate. Each mixture was then homogenized for 2 minutes in a test tube shaker (Phoenix AP 56) followed by 2 minutes' rest. The resulting layer of emulsion was measured with a caliper, as described by Das and coworkers [16]. The test tubes were stored at room temperature and stability was assessed at 24-hour intervals until 168 hours after starting the experiment. The emulsion volume (%EV) was calculated with the formula [16]:

%EV = emulsion height (mm) x cross-section area (mm^2)

total liquid volume (mm^3) (4000 mm^3)

Emulsion stability (%ES) over time was estimated with the formula [17]:

%ES = %EV, % at time *t*, h x 100

EV, % at 0h

Surface tension

The surface tension of the cell-free metabolic liquid was determined by the Du Noüy method using a Krüss K6 tensiometer with platinum ring calibrated with distilled water [18-19].

pH measurement

The pH of the cell-free metabolic liquid was measured at 24, 48 and 72 hours using a Hanna pH meter (model pH 211). Two standard buffer solutions, pH 4 and 7, were used to calibrate the equipment according to the manufacturer's instructions

3. RESULTS AND DISCUSSION

Both bacterial species produced biosurfactants. All strains of *Pseudomonas aeruginosa* in 5% of cooking oil produced biosurfactants. *Burkholderia gladioli* strain showed positive results in drop collapse test, except to 2% of metabolic liquid at 72 h and 5% of cell culture medium at 24 h. This was observed in media with cells and for supernatant only (Table 1). The table also shows when surfactant production occurred in the cell membrane (in nine situations). The drop-collapse test is based on the ability of biosurfactants to destabilize liquid droplets on an oily surface [20]. From the industrial point of view, biosurfactant-producing microorganisms are useful as they simplify the recovery process of bioproducts [21-22]. Using the drop-collapse test, Jain and coworkers [23] demonstrated that *P. aeruginosa* and *Bacillus subtilis* are capable of producing biosurfactants. Drop collapse is determined by the level of surface activity. In a study including 205 bacterial strains isolated from the environment, Youssef and coworkers [24] compared three methods of determining biosurfactant production: drop collapse, oil spreading and hemolytic activity. The first of these was found to be the most efficient at identifying biosurfactant-producing strains.

Burch and coworkers [25] compared biosurfactant production with an atomized oll assay for a large number of bacterial isolates and compared with a commonly used drop colapse assay. The atomized oll assay detected every strain that procedure a biosurfactant detectable by the drop colapse test, and also identified additional strains that were not detected with the drop colapse assay because they produced low levels of surfactans or hydrophobic surfactants such as pumilacidins.

According Abdel Mawgoud and coworkers [26] The development of more sensitive analytical techniques has lead to the further discovery of a wide diversity of rhamnolipid congeners and homologues (about 60) that are produced at different concentrations by various *Pseudomonas* species and by bacteria belonging to other families, classes, or even phyla. For example, various *Burkholderia* species have been shown to produce rhamnolipids that have longer alkyl chains than those produced by *P. aeruginosa.* For example, various *Burkholderia* species have been shown to produce rhamnolipids that have longer alkyl chains than those produced by *P. aeruginosa*.

Table 1. Detection of biosurfactant production by bacterial cells and cell-free culture supernatant from mangrove sediment isolates by qualitative drop-collapse test, using cooking oil as sole carbon source.

Carbon source concentration*	**Time**	**Microorganisms**			
		P. aeruginosa		*B. gladioli*	
		Cells	Metabolic liquid	Cells	Metabolic liquid
2%	24 h	-	+	+	+
	48 h	-	-	+	+
	72 h	+	-	+	-
5%	24 h	+	+	-	+
	48 h	+	+	+	+
	72 h	+	+	+	+

*= cooking oil
P. aeruginosa = Pseudomonas aeruginosa
B. gladioli = Burkholderia gladioli
+ = presence
- = absence

Emulsion volume (%EV) and Emulsion stability (%ES) were significant for *P. aeruginosa* culture enriched with corn oil at 2% and 5%. At 72 hours of experiment, %EV was approximately 70%, and %ES was 85% and 93%, respectively. Sunflower oil at 5% was also efficient: at 24, 48 and 72 hours, %EV was 73%, 65% and 73% and %ES was 78%, 88% and 97%, respectively.

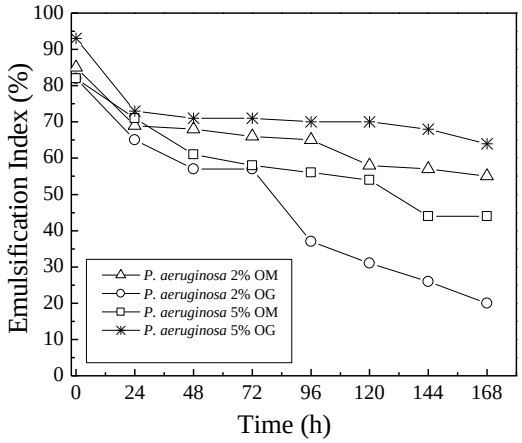
Bacillus subtilis and *P. aeruginosa* isolates from oil-contaminated soil were tested for emulsion stability using four carbon sources: vegetable oil, kerosene, petrol and diesel. Diesel yielded the highest level of emulsion stability with both bacterial species, followed by petroleum, kerosene and vegetable oil [27].

Iroha Okechukwu and coworkers [28] utilized cashew bagasse (*Anacardium occidentale*) as substrates for *Pseudomonas aeruginosa* to produce biosurfactants. The results reveal that all the culture supernatants formed emulsions above 50% when tested with olive oil, kerosene and Engine oil.

B. gladioli culture produced most biosurfactant when enriched with 5% corn oil. Production peaked at 72 hours when %EV was 69% and %ES reached 89%. The most promising results were observed for corn and sunflower oil. The emulsification index was insignificant when the culture was enriched with hexadecane (Figures 1 and 2).

Viramontes-Ramos [14] worked with a total of 324 bacterial strains, most were *Pseudomonas*, except for three strains (*Acinetobacter*, *Bacillus*, *Rhodococcus*) The highest emulsification index values of diesel, kerosene and motor oil were detected for *Pseudomonas strains* and seventenn (17) strains were positive for the drop-collapse.

According to Willumsen and Karlson [29] an emulsion may be considered stable if 50% of the original volume remains after 24 hours. In our study, emulsions remained stable for more than one week, indicating the biosurfactants were very stable. This is an important quality in a commercial product.



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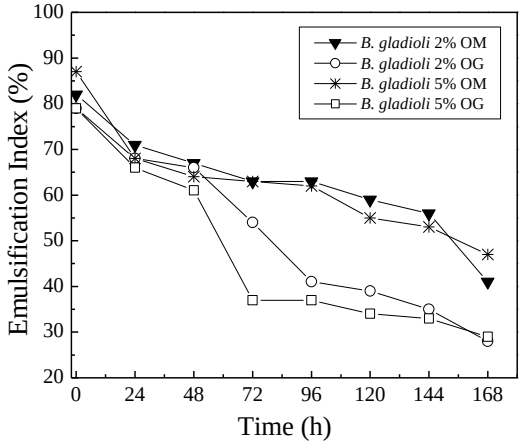

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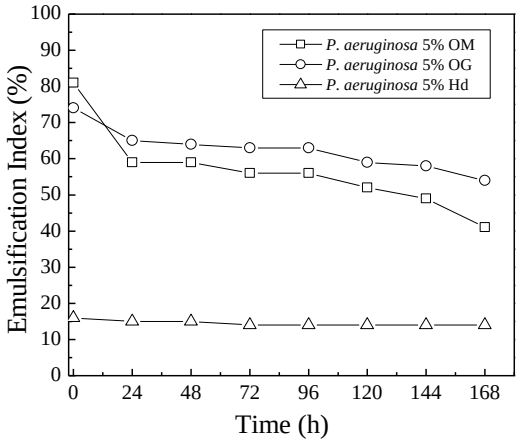

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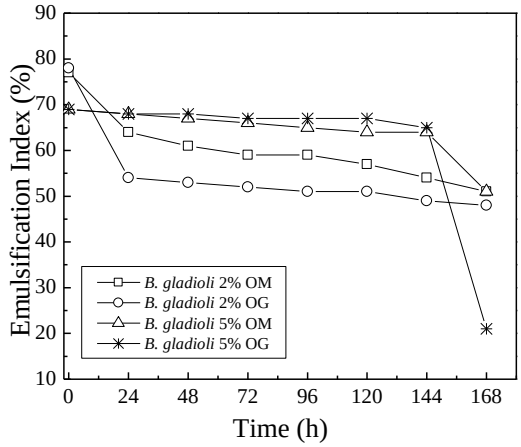

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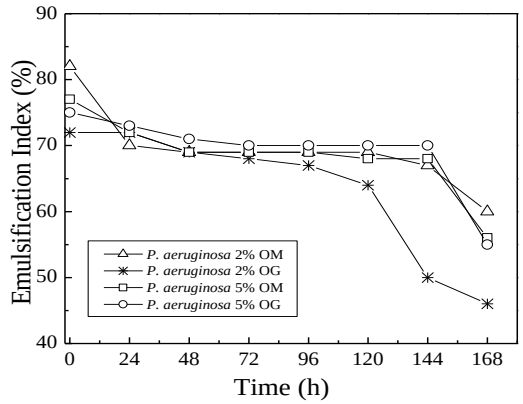

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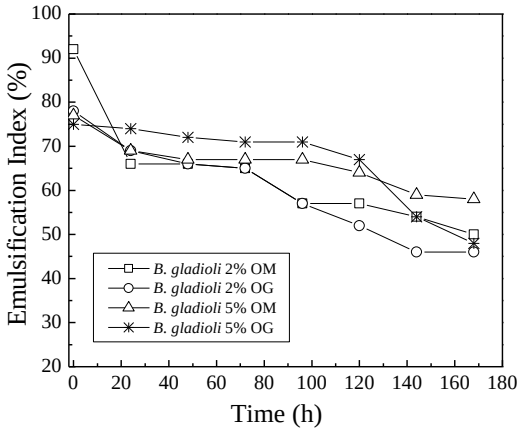
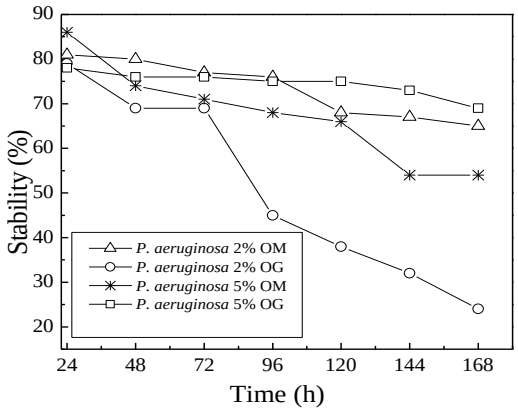


1f

Figure 1. Emulsion volume (%EV) of supernatants of *Pseudomonas aeruginosa* and *Burkholderia gladioli* cultured in mineral medium enriched with 2% and 5% cooking oil for 24 hours (1a and 1b), 48 hours (1c and 1d) and 72 hours (1e and 1f).



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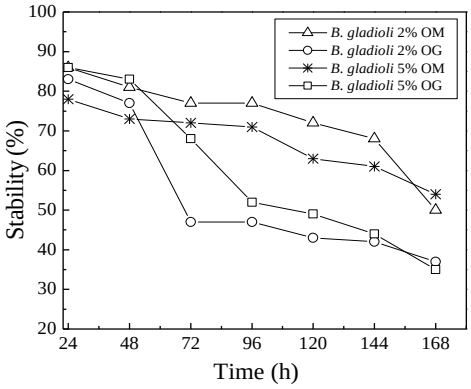

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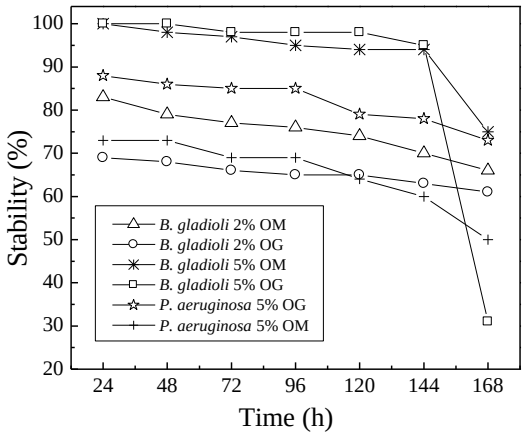

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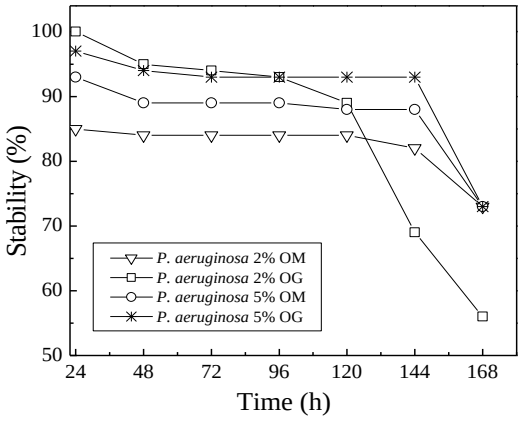

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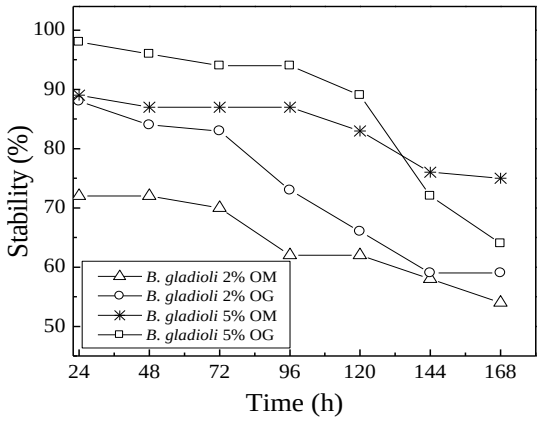

(2e)

Figure 2. Emulsion stability (%ES) of supernatants of *Pseudomonas aeruginosa* and *Burkholderia gladioli* cultured in mineral medium enriched with 2% and 5% cooking oil for 24 hours (2a and 2b), 48 hours (2c) and 72 hours (2d and 2e).

Surface tension decreased from 69 to 28 mN/m, with the greatest reduction (59%) observed for *P. aeruginosa* culture enriched with 2% cooking oil (Table 2). Similar results were reported by Lee and coworkers [30] using an isolate identified as *Klebsiella* sp. Y6-1. According to the authors, surface tension decreased by 45% during the first 24 hours.

The smallest reduction in surface tension (21.3%) was observed for *B. gladioli* culture enriched with 5% cooking oil (Table 2). A good biosurfactant producer should be able to reduce surface tension to less than 40 mN/m [9]

Thavasi and coworkers [31] measured the surface tension of cell free culture broth revealed that out of 105 strains screened, 79 (75.2%) strains showed reduction in surface tension and highest surface tension reduction was observed with 5 strains namely *Bacillus megaterium* (30.8±1.13mN/m), *B. subtilis* (38.75±0.3mN/m), *Corynebacterium kutscheri* (36.9±0.77mN/m), *C. xerosis* (37.8±0.42mN/m), *Lactobacillus delbrueckii* (32.5±0.70mN/m), *Pseudomonas aeruginosa* (28.7±0.98mN/m) and *P. fluorescens* (34.7±0.35mN/m).

According Uzoigwe and coworkers [32] It is important to note that the ability to reduce surface and interfacial tension stands as the distinctive contrast between biosurfactant and bioemulsifiers. Those molecules can both form stable emulsions but it is still unclear why bioemulsifiers do not show significant changes in surface/interfacial tension between diferent phases. This outstanding contrast between biosurfactants ans bioemulsifiers is especially important for accurate screening and identification procedures from microbial culture broths.

Table 2. Surface tension of metabolic liquid (cell-free medium) at 24, 48 and 72 hours in culture medium enriched with cooking oil at 2% and 5%.

Strain	Cooking oil concentration	Surface tension (mN/m)		
		24 h	48 h	72 h
Pseudomonas aeruginosa	2%	30.8 ± 0.5	28.9 ± 0.2	28.1 ± 0.2
	5%	43.9 ± 0.1	45.1 ± 0.1	41.8 ± 0.2
Burkholderia gladioli	2%	47.6 ± 0.4	40.1 ± 0.2	43.9 ± 0.1
	5%	50.2 ± 0.2	47.9 ± 0.1	45.4 ± 0.6

Values are averages of 6 repetitions ± standard deviation.

An important factor to be considered in the biodegradation of petroleum hydrocarbons is the pH value. The microorganisms tested in this study thrive at pH values between 6 and 8. However, pH values decreased during the first 24 hours, then remained stable at 3.0–4.0 until the end of the experiment. The observed decrease is probably due to the production of intermediate acids during the process [33]. According to Rufino and coworkers [34] the medium acidity is a parameter correlated with the efficiency of

biosurfactant synthesis, Bednarski and coworkers [35] based on experimental results, established that maintaining culture liquid acidity at the level of pH 5.5 produced the most desirable results in terms of glycolipid synthesis by yeast cultivated on oil refinery waste.

4. CONCLUSION

Cultures of *Pseudomonas aeruginosa* and *Burkholderia gladioli* isolated from mangrove sediments and water were efficient biosurfactant producers using inexpensive carbon sources. Waste corn and sunflower oil proved to be suitable substrates for biosurfactant biosynthesis, with potential for a variety of industrial applications. The identification of more readily available and less costly substrates is of great importance to industries engaged in biosurfactant production.

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