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Production of Biosurfactant by *Pseudomonas aeruginosa* Grown on Cashew Apple Juice

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Abstract

In this work, the ability of biosurfactant production by *Pseudomonas aeruginosa* in batch cultivation using cashew apple juice (CAJ) and mineral media was evaluated. *P. aeruginosa* was cultivated in CAJ, which was supplemented with peptone (5.0 g/L) and nutritive broth. All fermentation assays were performed in Erlenmeyer flasks containing 300 mL, incubated at 30°C and 150 rpm. Cell growth (biomass and cell density), pH, and superficial tension were monitored vs time. Surface tension was reduced by 10.58 and 41% when *P. aeruginosa* was cultivated in nutrient broth and CAJ supplemented with peptone, respectively. These results indicated that CAJ is an adequate medium for growth and biosurfactant production. Best results of biosurfactant production were obtained when CAJ was supplemented with peptone.

Index Entries: Biosurfactant; cashew apple juice; *Pseudomonas aeruginosa*; raw materials; rhamnolipid; fermentation.

Introduction

Biosurfactants are surfactants produced extracellularly or as part of the cell membrane by bacteria, yeasts, and fungi from various substrates including sugars, oils, alkanes, and organic sludges per solids *(1)*. Most microbial surfactants are complex molecules, consisting of different structures that include lipopeptides, glycolipids, polysaccharideprotein complex, fatty acids, and phospholipids *(2)*. They are amphipathic molecules with both hydrophobic and hydrophilic moieties *(3)* and are capable of

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reducing surface and interfacial tension *(4)*. Moreover, biosurfactants create micelles, enhancing the solubility of hydrocarbons (such as oil) in water or water in hydrocarbons *(4)*. Because of their surface-active properties and being environmentally friendly, biosurfactants are of great industrial and commercial interest. Potential industrial applications include enhanced oil recovery, crude oil drilling lubricants, surfactant-aided bioremediation of water-insoluble pollutants, and uses in the health care and food processing industries *(3,5)*. Furthermore, biosurfactants may be useful in agriculture, especially in formulation of herbicides and pesticides. In this application, the emulsifier is used to disperse the active compounds and very hydrophobic molecules in the aqueous solution *(6)*. Biosurfactant applications in the environmental industries have received more attention recently owing to their biodegradability, low toxicity, and effectiveness in enhancing biodegradation and solubility of hydrophobic compounds *(1)*. These biological compounds also have potential applications in agriculture, cosmetic, pharmaceuticals, detergents, food processing, laundry supplies, paint industries, and others *(7)*.

Bacteria of the genus *Pseudomonas* are known to produce a glycolipid surfactant containing rhamnose and 3-hydroxy fatty acids. The properties showed by rhamnolipids depend on their homologous composition and distribution that are determined by the bacterial strain, culture conditions, and medium composition *(8)*. Rhamnolipids are isolated from culture broth and can be produced using hydrophobic and hydrophilic substrates *(9)*. Although biosurfactants exhibit such important advantages, they have not been yet used extensively in industry because of relatively high production costs. One possible strategy for reducing costs is the utilization of alternative substrates such as agroindustrial wastes *(10)*. The main problem related to use of alternative substrates as culture medium is to find a waste with the right balance of nutrients that allows cell growth and product accumulation *(11)*. Natural cashew apple juice (CAJ) is an example of an inexpensive substrate in Brazil, as it is a byproduct of the cashew nut industry. In the north coast of Brazil, especially in the state of Ceará, the cashew agroindustry has an outstanding role in the local economy. The cashew apple, a pseudo fruit or peduncle, is the part of the tree that connects it to the cashew nut, the real fruit and a well-known product around the world. The cashew apple is a hard, pear-shaped, small, and nonclimacteric fruit, and is found in three colors: yellow, orange, and red. The most commonly commercialized ones are the yellow and red fruits. The edible portion, representing 90% of the fruit, is a pseudo fruit rich in vitamin C, flavor, and aroma. Internal and external market consumption of cashew nut, in the year of 2004, was about 232,000 t. However, only 12% of the total peduncle is processed *(12–14)* and it does not play an important role to the economy of the state. Furthermore, the majority of the cashew apple production spoils in the soil. These facts together with its rich composition (*see* Table 1), turns CAJ into an interesting and inexpensive (R$1.00/Kg) culture medium.

Table 1
CAJ Composition

Parameter	CAJ	References
Vitamin C (mg/100.0 g)	135.0–372.0	*12,13*
Brix	7.4	*13*
pH	3.8–4.2	*12,13*
Malic acid (g/100.0 g)	0.4	*13*
Total tannins (mg/100.0 g)	0.6	*13*
Condensed tannins (mg/100.0 g)	0.2	*13*
Calcium (mg/100.0 g)	0.9–5.4	*12*
Phosphorous (mg/100.0 g)	6.1–21.4	*12*
Iron (mg/100.0 g)	0.2–0.7	*12*
Carotene (mg/100.0 g)	0.03–0.74	*12*
Carbohydrates (g/100.0 g)	9.0–9.7	*12*
Reducing sugars (%)	10.7	*15*
Nonreducing sugars (%)	0.4	*15*
Starch (%)	8.5–2.7	*15*
Alanine (μmol/100 mL)	336.0	*16*
Serine (μmol/100 mL)	273.0	*16*
Phenylalanine (μmol/100 mL)	175.6	*16*
Leucine (μmol/100 mL)	178.0	*16*
Glutamic acid (μmol/100 mL)	148.4	*16*
Aspartic acid (μmol/100 mL)	87.6	*16*
Proline (μmol/100 mL)	158.7	*16*
Tirosine (μmol/100 mL)	115.5	*16*

Therefore, the aim of this work was to investigate the potential use of this alternative substrate (CAJ) as carbon source to rhamnolipid production by *P. aeruginosa* ATCC 10145. Surface-active properties and preliminary characterization of the biosurfactants obtained were also presented.

Materials and Methods

Microorganism

P. aeruginosa ATCC 10145, kindly donated by Dr. Fátima Borges from Empresa Brasileira de Pesquisa Agropecuaria (Embrapa)—Ceará, Brazil, was maintained on nutrient agar (Biolife) slants at 4°C.

Substrate Preparation

CAJ was withdrawn by compressing the cashew apple (*Anarcardium occidentale* L.). After compressing, the substrate was centrifuged at 3500 rpm for 20 min (BIO ENG, BE—6000, São Paolo, Brazil), filtered using a 25 μm filter paper, and diluted with water (1% [v/v]). CAJ supplemented with peptone, here named CAJP, was prepared by adding 5.0 g/L of peptone to diluted CAJ. Afterwards, pH was adjusted to 7.0 and it was sterilized by

filtering through a 0.45 μm Millipore membrane. Nutritive broth (NB) (5.0 g/L of peptone and 3.0 g/L of yeast extract) was distributed in flasks and sterilized in autoclave (Tecnal-AV-75, São Paolo, Brazil) at 1 atm, 121°C for 15 min.

Media and Growth Conditions

The bacterial strains were streaked in a nutrient agar slant and incubated for 24 h at 30°C. Three loops of culture were inoculated in 50 mL of NB (Biolife) in a 250-mL Erlenmeyer flask and incubated in a rotary shaker (Tecnal—TE240, BR) at 30°C and 150 rpm for 18–24 h. Afterwards, optical density (600 nm) of bacterial suspension was adjusted to 0.1 and an aliquot of 6 mL of inoculum (2%) was transferred to a 500-mL Erlenmeyer flask, containing 300 mL of medium, and incubated at 30°C, 150 rpm in a rotary shaker (Tecnal—TE240, BR). Samples were collected at time-defined intervals and submitted to analysis.

Biomass Content

Cell growth was determined by measuring the optical density of samples, using a UV-visible spectrophotometer (20 Genesis, BR) at 540 nm. Cell concentration was determined by dry weight by filtering through a 0.45 μm previously weighted Millipore membrane *(17)*.

Analytical Methods

Carbohydrates Concentration

Substrate concentration (glucose and fructose), present on CAJ, were measured by high-performance liquid chromatography using a Waters high-performance liquid chromatography equipped with a refractive index detector and a Shodex Sugar SC1011 (Karagawa, Japan) column (8.0×300 mm^2). Ultrapure water (MiliQ, Millipore, São Paolo, Brazil) was used as mobile phase under the following conditions: flow rate of 0.6 mL/min at 80°C.

Emulsification Activity

Emulsifying activity was determined according to Cooper and Goldenberg *(18)* with slight modifications: 2 mL of cell free supernatant was added to 2 mL of hydrocarbons (*n*-hexane, *n*-heptane, gasoline, kerosene, or soy oil), containing 0.2 mL of pink dye and the mixture was vortexed for 2 min. After 24 h, the height of emulsion layer was measured. The emulsifying activity (E_{24}) was calculated using Eq. 1 *(19)*.

$$E_{24}(\%) = \frac{H_{EL}}{H_S} \times 100 \quad (1)$$

where H_{EL} is the height of the emulsion layer and H_S is the height of total solution.

Surface Tension Determination

Surface tension was determined with a tensiometer (Torsion Balance of White Electrical Instrument, UK) at 30°C, according to the De Nöuy ring method. The surface tension measurements were performed using cell free supernatants obtained after centrifugation.

Rhamnolipid Extraction

Rhamnolipids mixtures were extracted from culture media after cell removal by filtering through a 0.45 μm Millipore membrane. The pH of supernatant was adjusted to 2.0 with H_2SO_4 (6 *N*) and an equal volume of $CHCl_3/CH_3OH$ (2 : 1) was added. The mixture was vigorously shaken for 5 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated again. The rhamnolipid product was concentrated from the pooled organic phase using a rotary evaporator. For further purification the viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45°C *(8)*.

Carbohydrate and Protein Analysis of Extracted Biosurfactant

The carbohydrate content of extracted biosurfactant was determined by Dubois method *(20)*. Protein concentration was assayed by the Bradford method *(21)*.

Statistical Analysis

All surface tensions and emulsification activities determinations were performed at least three times. Means and standard errors were calculated using the Microsoft Office Excel 2003 (Version 7).

Results and Discussion

Two complex media (CAJ and CAJP) and a defined media (NB) were used to grow *P. aeruginosa*. The effect of these media on cell growth and rhamnolipid production was investigated and results are pictured on Figs. 1–3. It can be observed that the variation in biomass concentration (optical density), in all media studied, is a typical curve of microbial growth. The same behavior was observed by several authors with different microorganisms *(22–24)*.

Table 2 presents results of growth and production of biosurfactant by *P. aeruginosa* in the different studied media. Maximum biomass concentrations were reached when NB and CAJP were used, 1.08 and 1.00 g/L, respectively. All media tested have favored extracellular production of active surface agent by *P. aeruginosa*. The maximum reduction in the surface tension (41%) was obtained for CAJP after 24 h of culture (Table 2).

A comparison between cell growth, surface tension, and substrate uptake allows observing that the biosurfactant production coincides with the consumption of substrate and the formation of a stationary phase. Several biosurfactants were recognized as secondary metabolites, whereas

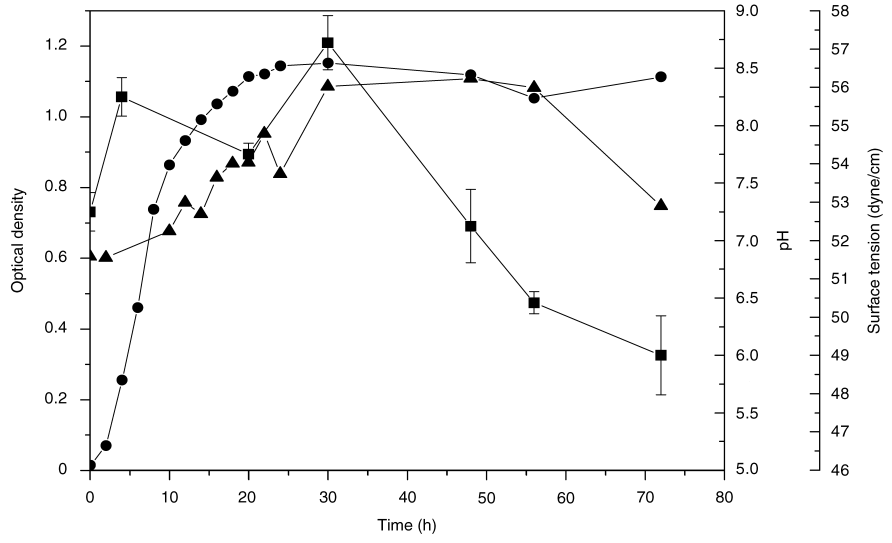

Fig. 1. Kinetics of growth and production of biosurfactants at 30°C and 150 rpm in NB by *P. aeruginosa*: (●) optical density (A_{600} nm), (■) surface tension, and (▲) pH.

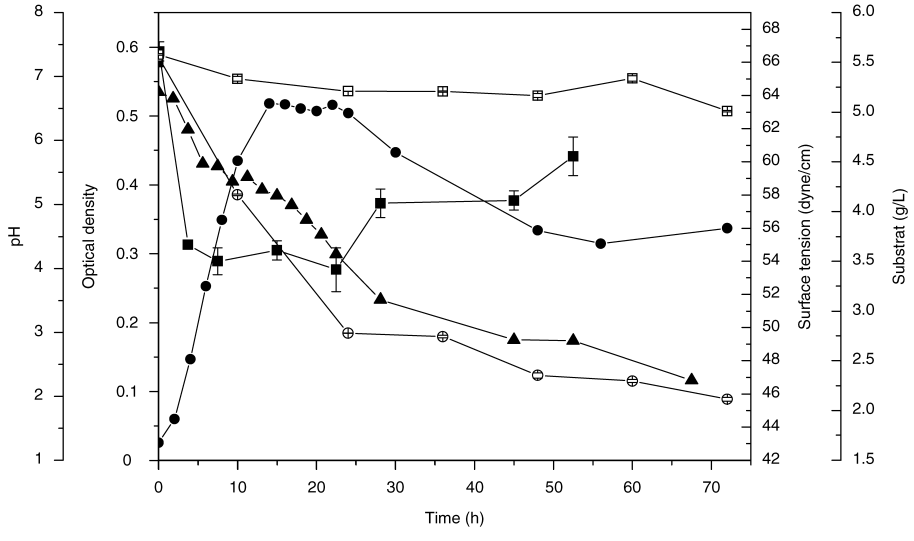

Fig. 2. Kinetics of growth and production of biosurfactants at 30°C and 150 rpm in CAJ 1 : 10 by *P. aeruginosa*: (●) optical density (A_{600} nm), (■) surface tension, (▲) pH, (○) glucose concentration (g/L), and (□) fructose concentration (g/L).

others were considered growth associated *(4)*. In this study, the observed behavior is typical of a secondary metabolite.

Other studies evidenced that *P. aeruginosa* was able to reduce the surface tension of soapstock media from 57.5 to 32.9 dyne/cm *(9)*. Brazilian native oils: buriti (*Mauritia flexuosa*), cupuaçu (*Theobroma grandiflora*), passion fruit

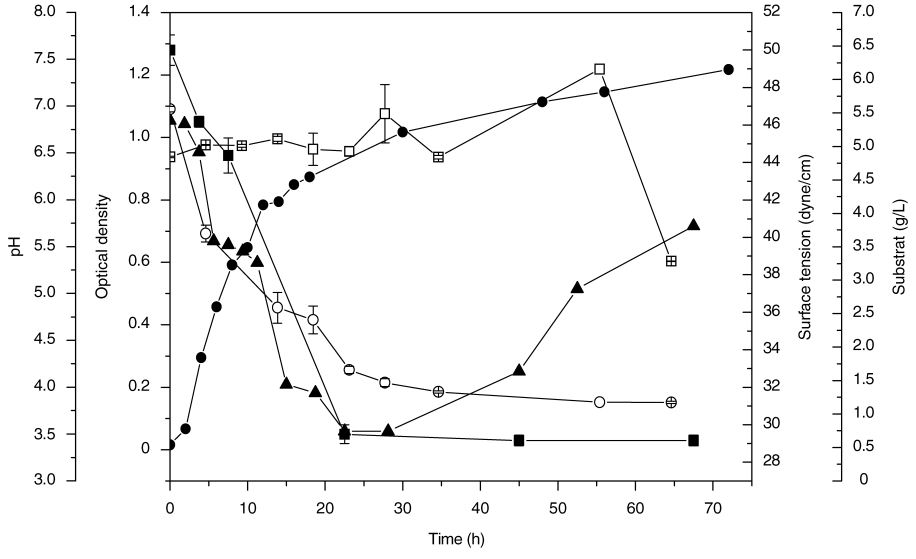

Fig. 3. Kinetics of growth and production of biosurfactants at 30°C and 150 rpm in CAJ supplemented with peptone by *P. aeruginosa*: (●) optical density (A_{600} nm), (■) surface tension, (▲) pH, (○) glucose concentration (g/L), and (□) fructose concentration (g/L).

Table 2
Effect of Different Media on Growth and Production of Biosurfactants by *P. aeruginosa*: CAJ 1 : 10, CAJ 1 : 10 Supplemented With Peptone (CAJP), and NB

Culture media	Biomass (g/L)	Final pH	Surface tension of media (dyne/cm)	Surface tension after cultivation (dyne/cm)	Reduction of surface tension (%)
CAJ	0.98	2.25	66.00 ± 0	44.37 ± 0.48[a]	32.77
CAJP	1.00	5.72	50.00 ± 0.81	29.50 ± 0.50[b]	41.00
NB	1.08	8.60	52.75 ± 0.5	47.17 ± 0.29[c]	10.58

[a]After 72 h of cultive.
[b]After 24 h of cultive.
[c]After 12 h of cultive.

(*Passiflora alata*), andiroba (*Carapa guianensis*), brazilian nut (*Bertholletia excelsa*), and babassu (*Orbignya* sp.) had also been evaluated as carbon sources to produce rhamnolipids for *P. aeruginosa* LB1 *(8)*. The highest rhamnolipids concentrations were obtained from brazilian nut and passion fruit, and surface tension varied from 29.8 and 31.5 dyne/cm, respectively *(8)*. In this article, when CAJP was used, the surface tension of the culture broth fell from 50 to 29.5 dyne/cm, and remained constant up to 70 h. Some authors *(23,25)* report the same behavior and explain that even in the

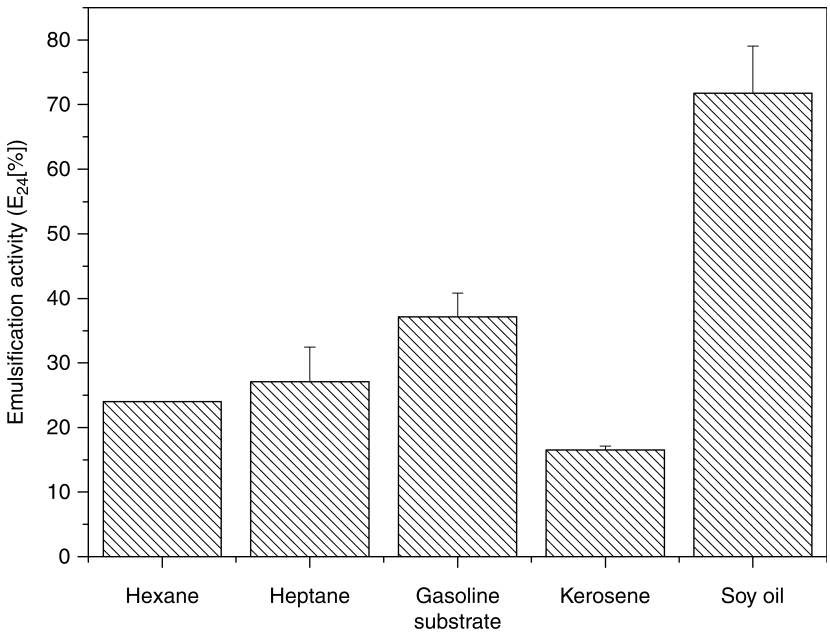

Fig. 4. Emulsifying activity (E_{24}%) of biosurfactant (produced from medium CAJ supplemented with peptone [CAJP]) obtained for different hydrocarbons. Error bars represent standard deviations.

presence of a small concentration of biosurfactant, the critical micellar concentration may be achieved from which no variation on the surface tension can be observed.

In Figs. 2 and 3, it can be observed that the microorganism consumed only glucose, as fructose concentration remained constant during the fermentation. The pH of the medium increased when using NB and reached values over 8.0, whereas CAJ and CAJP showed acidic conditions. Based on the obtained results, CAJP is a suitable substrate for growth and production of biosurfactant, because it has reduced the surface tension of the media to values below 30 dyne/cm *(24,26)*. Moreover, the exploitation of cashew apple will not only reduce the costs associated with biosurfactant production but will also utilize a surplus raw material that usually spoils in soil and contributes to ambient pollution.

The highest biosurfactant production occurred after 48 h of cultivation using CAJP (3.86 g by biosurfactant for 1000 mL de medium) and the poorest, when NB was used. An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase *(4)*. As shown in Fig. 4, all the hydrocarbons and soil oil tested served as substrate for emulsification by the biosurfactant. Soy oil (71.79% emulsified) was the best substrate, whereas kerosene (16.50% emulsified) was the poorest. Most microbial surfactants are substrate specific, solubilizing or emulsifying different hydrocarbons at different rate *(27)*.

No protein was detected in the extracted biosurfactant; however, it did contain carbohydrate (data not shown). The biosurfactant produced by the *P. aeruginosa* may be classified as a rhamnolipid. Rhamnolipids, trelalolipids, and sophorolipids are among the best known glycolipids produced by *P. aeruginosa (28)*.

Conclusion

In this article, three media were studied for the production of biosurfactant by *P. aeruginosa* ATCC 10145. Results indicate that the microorganism was able to grow and produce rhamnolipids when cultivated in all the media studied. However, best results of surface tension reduction were obtained when CAJP was used. Furthermore, *P. aeruginosa* grown on CAJP reduced the surface tension of the media to values below 30 dyne/cm, showing that CAJ, supplemented with peptone, is a suitable substrate for production of biosurfactant. The emulsifying activity of the produced biosurfactant was determined and it was able to emulsify hexane, heptane, gasoline, kerosene, and soy oil. Soy oil (71.79%) was the best substrate, whereas kerosene (16.50%) was the poorest. This initial study indicates that traditional carbon sources for biosurfactant production may be replaced by CAJ. Moreover, the use of CAJ as a culture medium would provide an alternative of waste management for the productive chain of cashew nut, an important industrial segment on the Northeast of Brazil.

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