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Production of Biosurfactant from a New and Promising Strain of *Pseudomonas aeruginosa* PA1

L. M. Santa Anna,*[,1] G. V. Sebastian,[2] N. Pereira, Jr.,[3] T. L. M. Alves,[4] E. P. Menezes,[5] and D. M. G. Freire[6]

[1,2]Centro de Pesquisas da Petrobras (Petrobras Research Center-CENPES), [3]Escola de Química, UFRJ, [4]PEQ/COPPE/UFRJ, Rio de Janeiro, Brazil; [5]Fundação Tropical André Tosello, Campinas, São Paulo, Brazil; and [6]Faculdade de Farmácia, UFRJ, Rio de Janeiro, Brazil

Abstract

The *Pseudomonas aeruginosa* PA1 strain, isolated from the water of oil production in Sergipe, Northeast Brazil, was evaluated as a potential rhamnolipid type of biosurfactant producer. The production of biosurfactants was investigated using different carbon sources (*n*-hexadecane, paraffin oil, glycerol, and babassu oil) and inoculum concentrations (0.0016–0.008 g/L). The best results were obtained with glycerol as the substrate and an initial cell concentration of 0.004 g/L. A C:N ratio of 22.8 led to the greatest production of rhamnolipids (1700 mg/L) and efficiency (1.18 g of rhamnolipid/g of dry wt).

Index Entries: Production of biosurfactants; glycolipids; rhamnolipids; *Pseudomonas aeruginosa*; surface tension.

Introduction

The potential for the application of biosurfactant's has increased considerably in the last few years owing to their utilization in several areas. The most important advantage of a microbial surfactant in relation to a chemical one lies in its ecologic acceptance, because it is biodegradable and nontoxic in natural environments. Furthermore, biosurfactants can be applied in extreme temperature, pH, and salinity conditions, making them more versatile than industrial surfactants found in the market *(1)*.

*Author to whom all correspondence and reprint requests should be addressed.
Current address: Centro de Pesquisas e Desenvolvimento, Leopoldo A. Miguez de Mello, CENPES—PETROBRAS, Av. Hum, Quadra 7, Cidade Universitária, 1 Ilha do Fundão, Rio de Janeiro, Brazil, CEP 21949-900.

In situ investigations have shown the capacity of biosurfactants in removing pollutants from the marine environment. *Pseudomonas aeruginosa* SB30 biosurfactants were used to remove oil from gravel in the *Exxon Valdez* tanker oil spill in Alaska. A 1% solution of biosurfactants was enough to remove two times more oil than water in temperatures ranging from 10 to 80°C *(2)*. However, for biosurfactants to conquer a significant share in the market, they should be produced at low cost. Therefore, there is a need to better understand the metabolism, physiology, and industrial process parameters, in addition to using a cheaper substrate *(3)*.

Bacteria of the genus *Pseudomonas* can use different substrates such as glycerol, mannitol, fructose, glucose, *n*-paraffins, and vegetable oils to produce rhamnolipid-type biosurfactants *(4)*. Many studies have been conducted to determine the best carbon, nitrogen, phosphorus, and iron concentrations, to improve the bioprocess performance. The optimization of the ratio of carbon to nitrogen (C:N) has been researched in continuous cultures of *P. aeruginosa*, and ratios between 15 and 23 have been indicated as optimum values to yield a high rhamnolipid-specific productivity, using glucose and vegetable oil, respectively *(5)*. It has been determined that nitrogen depletion in the culture medium, cellular metabolism is directed toward rhamnolipid production, because several studies have detected their production after the exponential growth phase *(6)*.

The objective of the present study was to examine the production and molecular characterization of a rhamnolipid-type biosurfactant, produced by a bacteria isolated from petroleum environments.

Materials and Methods

Isolation, Identification, and Preservation of Microorganism

Isolation of the microorganism was carried out using samples from petroleum wells of the Northeast Brazilian region. Isolation was performed using the successive dilution method of the water sample and placed on Cetrimide agar plates (Merck, Darmstadt). Plates were incubated at 30°C for 48 h and the bacterial culture was isolated. Identification was done using the BIOLOG™ (Biolog, Hayward, CA) automated identification system for Gram-negative bacteria. Results were compared with the Microlog software database for determining the similarity coefficient for the type established in the identification system *(7)*. The isolated and identified bacteria was preserved by freezing with liquid nitrogen in a glycerol solution (10% [v/v]) at –196°C.

Preinoculum

The bacterium was reactivated in a trypticase soy agar (Merck) medium, cultivated at 30°C for 48 h, and then transferred to 250-mL conical flasks containing 50 mL of mineral medium with the following composition: 1.0 g/L of $(NH_4)_2SO_4$, 3.0 g/L of KH_2PO_4, 7.0 g/L of K_2HPO_4,

0.2 g/L of $MgSO_4 \cdot 7H_2O$, and 1% (v/v) glycerol at pH 7.0. The flasks were incubated at 30°C and 250 rpm for 20 h *(6)*.

Fermentation

Rhamnolipid production was studied during 7- to 10-d fermentation periods in 500-mL conical flasks containing 100 (medium volume:flask volume [MV:FV] of 0.2) or 200 mL (MV:FV of 0.4) of mineral medium as previously mentioned and 0.004 g/L of initial inoculum. The flasks were maintained at 30°C and agitated at 120 rpm in an incubator shaker (New Brunswick model G24; Edison, NJ). Carbon sources used, keeping a C:N ratio of 22.8, were *n*-hexadecane (Merck), paraffin oil (from production wells in the Buracica, BA, Brazil, containing 32% paraffins, 23% aromatics, 36% resins and 9.1% asphaltenes), glycerol (PA, Merck), and babassu oil (Du Reino).

Besides the effect of several carbon sources, the effect of inoculum concentration (0.0016, 0.004, and 0.008 g/L), the C:N ratio from the best carbon source, and the MV:FV ratio on rhamnolipid production were also investigated.

Analyses

Biomass Concentration

Bacterial growth was monitored by measuring the optical density (OD) of the prepared sample using a spectrophotometer (model B442; Micronal, Brazil) at 500 nm. A 50-mL sample was taken from the fermentation flask at the end of cultivation and OD was achieved by measuring after dilution. The sample was centrifuged at 7000 rpm for 20 min (model J2-21; Beckman). Centrifuged cells were suspended in 5 mL of distilled water. The dry weight of the biomass was found from a constructed calibration curve. The biomass was expressed as grams/liter.

Rhamnose and Glycerol Assay

Rhamnolipid quantification expressed as rhamnose (milligrams/liter) was evaluated in the cell-free spent medium using the phenol sulfuric acid method *(8)*. Glycerol was evaluated by an enzymatic-colorimetric method for the determination of triglycerides (CELM, São Paulo, Brazil) *(9)*.

Measurement of Surface Tension

Surface tension was measured in the cell-free spent medium using the SIGMA 70 digital tensiometer (KSV Instruments, Helsinki, Finland), at a temperature of 25°C, by the Du Nouy method *(10)*.

Extraction and Purification of Biosurfactant

After 7 d of fermentation, the culture medium was centrifuged at 4400 rpm for 15 min, and to the floating matter was added a solution of H_2SO_4 (5 *N*) until a final pH of 2.0 was obtained for the rhamnolipid precipitation. The precipitate was recovered in a 2:1 chloroform:ethanol solution.

After evaporation of the solvent, part of the residue was mixed with a KBr tablet to obtain its infrared absorption spectrum with the Fourier transform (Perkin-Elmer–2000). The remaining residue was used for the molecular characterization of rhamnolipid using the nuclear magnetic resonance (NMR) technique (Varian Inova 300 spectrophotometer).

Results and Discussion

Identification of Bacteria

The bacteria were identified as *P. aeruginosa* in the BIOLOG automated system with a 99% similarity to the standard strain *P. aeruginosa* and received the PA1 code. The bacteria used carbon sources such as fructose, D-glucose, mannitol, mannose, glycerol, and lactic acid, substances well known as good carbon sources for rhamnolipid production *(7)*.

Effect of Carbon Source

Experimental results for rhamnolipid production from *P. aeruginosa* PA1 using substrates such as hexadecane, paraffin oil, babassu oil, and glycerol are given in Table 1. *P. aeruginosa* PA1 was capable of using *n*-hexadecane as the sole carbon and energy source, producing 130 mg/L of rhamnolipid and a variation in the surface tension of 47.4% by the end of 7 d of fermentation. Suk et al. *(11)* used 3% hexadecane as the carbon and energy source, and after 3 d of fermentation with *P. aeruginosa* the surface tension of the medium was reduced from 72 to 30 D/cm with a reduction of 58%.

The utilization of paraffin oil, which has a complex and heterogeneous nature, resulted in reasonable rhamnolipid production expressed as rhamnose (260 mg/L); however, there was almost no change in surface tension by the end of the cultivation (4.4%). This fact is probably related to the formation of an emulsion during fermentation, which interfered with the determination of surface tension and made impossible the quantification of surface tension in a suitable manner. It was also shown from research by Hisatsuka et al. *(12)* that rhamnolipids in hydrocarbon fermentation contribute to stabilizing oil-in-water emulsions.

The utilization of vegetable oil and glycerol as carbon sources for rhamnolipid production seems to be a rather interesting and probably a low-cost alternative *(4)*. The bacteria produced 200 mg/L of rhamnolipid when babassu oil was used as the carbon source, and there was a decrease of 31% in surface tension by the end of cultivation.

However, Table 1 shows that the greatest rhamnolipid production (690 mg/L) with the best tensoactive characteristics (48% of decrease in surface tension) was achieved with glycerol, a carbon source easily assimilated. In addition to the quantity of rhamnolipid obtained in the medium (three to four times greater than those obtained for other sources), there was a quite large formation of foam in the cultivation floating material.

Table 1
Rhamnolipid Quantification and Surface Tension Measurement
After 7 d of Fermentation of *P. aeruginosa* PA1
Using *n*-Hexadecane, Paraffin Oil, Babassu Oil, and Glycerol
as Carbon Sources, for a C:N Ratio of 22.8

Carbon source	Rhamnose (mg/L)	Initial surface tension (D/cm)	Surface tension (D/cm)	Surface tension reduction (%)
n-Hexadecane	130	53.90	28.35	47.4
Paraffin oil	260	54.00	51.60	4.4
Babassu oil	200	40.00	27.60	31.0
Glycerol	690	53.00	27.46	48.2

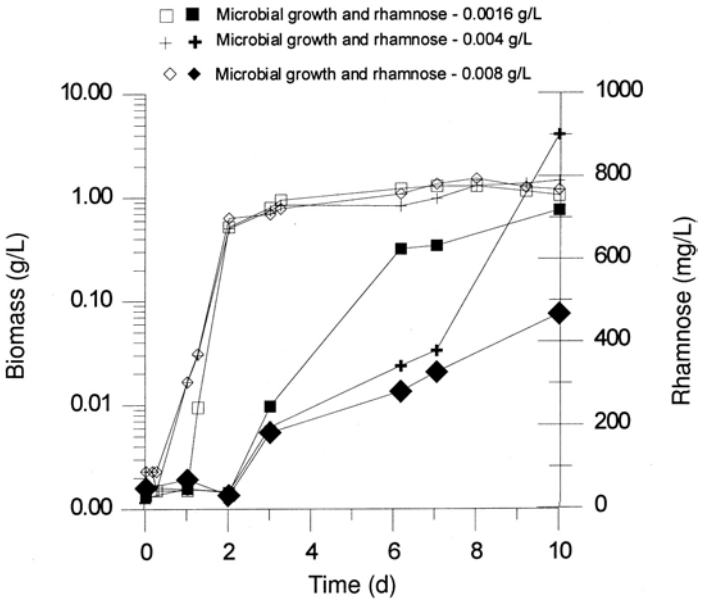

Fig. 1. Microbial growth curve and rhamnolipid production from the fermentation of *P. aeruginosa* PA1, over 10 d, using glycerol (C:N ratio of 22.8) with initial inocula of 0.0016, 0.004, and 0.008 g/L.

In agreement with our findings, studies conducted with a *P. aeruginosa* CFTR-6 have demonstrated a great capacity of the strain for glycolipid production (620 mg/L) when glycerol (2% [w/v]) is used as a source of carbon and energy *(6)*.

Effect of Inoculum Concentration

The effect of the initial inoculum concentration was studied using glycerol as the substrate. Figure 1 shows the microbial growth kinetics and rhamnolipid production in a 10-d fermentation with inoculum ratios of 0.0016, 0.004, and 0.008 g/L.

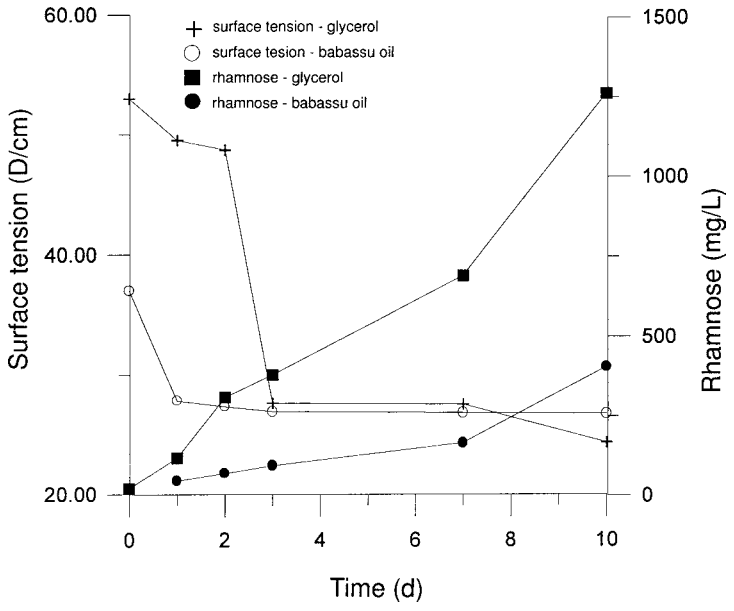

Fig. 2. Rhamnolipids production and surface tension measurements along *P. aeruginosa* PA1, fermentation using babassu oil and glycerol with a C:N ratio of 22.8.

The microbial growth profiles for the three different inocula were very similar with practically identical specific growth rates of 0.165 h^{-1} and the stationary phase beginning between 48 and 72 h. In the beginning of the stationary phase, we observed a substantial increase in the rhamnolipid concentration at the end of 10 d of fermentation, 720 and 900 mg/L, with 0.004 and 0.008 g/L of inoculum concentration, respectively. Similar to our study, other researchers have observed that the increase in rhamnolipid production occurs when microbial growth reaches the stationary phase, indicating its characteristic as a secondary metabolite *(5)*.

Effect of Ratio MV:FV

A ratio of MV:FV of 0.2, twice as great as in the previous experiments, was used, employing glycerol and babassu oil as substrates at a C:N ratio of 22.8. The fermentations were inoculated with a cell concentration corresponding to a inoculation of 0.004 g/L. Figure 2 shows the kinetic profiles of rhamnolipid production and surface tension over 10 d of fermentation.

Glycerol was still the best carbon source for rhamnolipid production; however, the use of a higher MV:FV ratio led to a twofold greater production of rhamnolipid (1400 mg/L), reaching a decrease in the surface tension of 24.6 D/cm at the end of 72 h of fermentation. When the MV:FV increased twofold, probability had increased oxygen supply in the medium just greater than the surface area in the flask.

Table 2
Rhamnolipid and Biomass Concentrations
After 7 d of Fermentation at Different Concentrations of Glycerol

Glycerol concentration (%)	C:N	Biomass (mg/L)	Rhamnose (mg/L)
0.5	11.4	1500	410
0.71	16.2	1950	710
1	22.8	2500	760

In the fermentation using babassu oil as the carbon source, production did not increase beyond 500 mg/L of rhamnolipid in 10 d of cultivation. Although surface tensions using either glycerol or babassu oil had reached similar values in 72 h of fermentation (24.6 D/cm), the rhamnolipid concentration was three times greater when glycerol was used as the substrate. Other researchers have obtained a similar reduction in the surface tension (25 D/cm) when glycerol was used in higher concentrations of approx 2% *(13)*.

At the beginning of fermentation with babassu oil, the culture medium already presented a low surface tension of 38 D/cm (Fig. 2). This fact can be ascribed to the presence of free constituent fatty acids in the oil with surfactant activity.

Effect of C:N Ratio

Table 2 gives the results for biomass production and rhamnolipid quantification at the end of 7 d of fermentation for various C:N ratios. The smallest C:N ratio used (0.5% [v/v] of glycerol) was limited in terms of carbon, since cell growth was about 60% lower compared with that using the highest C:N ratio. While the fermentation with the intermediate glycerol concentration (0.71% [v/v]) obtained good rhamnolipid production (710 mg/L), the fermentation using 1% (v/v) of glycerol yielded the greatest production of biomass (2500 mg/L) and rhamnolipid (760 mg/L).

The microbial growth kinetics and rhamnolipid production in the fermentation with a 1% (v/v) concentration of glycerol (C:N ratio = 22.8), MV:FV of 0.2, and inoculum of 0.004 g/L are represented in Fig. 3. The specific growth rate of the *P. aeruginosa* PA1 strain was 0.09 h^{-1}. The stationary phase was reached after 72 h of fermentation at the same time rhamnolipid production was increased. The rhamnolipid and biomass concentrations after 10 d were 1700 and 1470 mg/L, respectively. Glycerol was entirely consumed within 7 d of fermentation, and the rhamnolipid concentration peaked after another 3 d. The production of this rhamnolipid is typically of a secondary metabolite and increased considerably along the stationary phase.

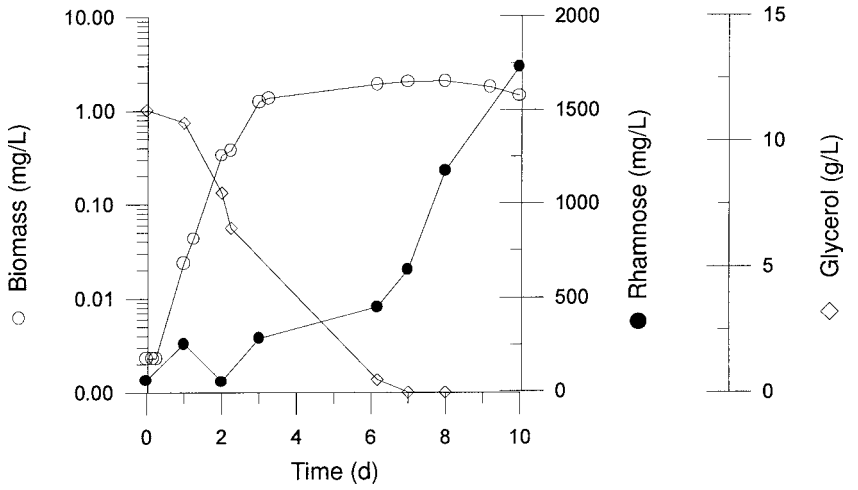

Fig. 3. Microbial growth curve, rhamnolipid production, and consumption of glycerol from the fermentation of *P. aeruginosa* PA1, over 10 d, using a 1% (v/v) glycerol concentration.

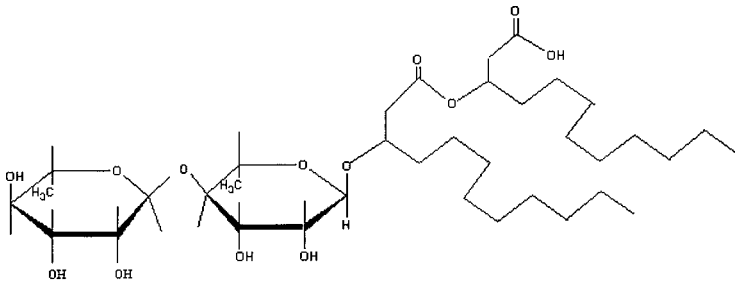

Fig. 4. Rhamnolipid structure of *P. aeruginosa* PA1 obtained from NMR analysis.

Molecular Characterization of Rhamnolipid

The structure of the rhamnolipid produced in the fermentation by the *P. aeruginosa* PA1 strain with 1% (v/v) glycerol (C:N ratio = 22.8), MV:FV of 0.2, and inoculum of 0.004 g/L is represented in Fig. 4.

The characterization of the molecular structure of the rhamnolipid was investigated by the combination of ^{13}C NMR and infrared spectroscopy *(14)*. Preliminarily compatible structures have been proposed. Two possible average structures were reached. One was a diester, and the other was an acid-ester molecule, which is shown in Fig. 4. Spectra of standard molecules in the literature could not allow a clear distinction among the carbonyls in the possible structures. The C=O deformation, which could be a definite difference in infrared spectra, was broad in the analysis of the samples and could not yield the necessary resolution to distinguish the possibilities. Therefore, the molecule has this undetermined structure. Another important aspect was the bond between the two rhamnose rings.

This could be compatible with a β 1.4 bond as in lactose and cellobiose, since the NMR spectrum showed resonance in the chemical shift of 103 ppm. However, that was not enough to ensure the bond position. Future studies will focus on the chromatographic purification of the rhamnolipids and on the definite proposition of the molecular conformation. Syldatk et al. *(15)* have proposed a very similar structure, with an α 1.2 bond between the cycles and with fewer carbons in the aliphatic chain of the esters of the rhamnolipid molecule.

Conclusion

The strain isolated from oil environments was identified as *P. aeruginosa*, showing a capacity of using carbon sources such as fructose, lactic acid, D-glucose, mannitol, mannose, and glycerol. This strain is capable of producing rhamnolipid-type biosurfactants from substrates such as *n*-hexadecane, paraffin oil, babassu oil, and glycerol. The rhamnolipid production kinetics by *P. aeruginosa* PA1 is typical of a secondary metabolite. The greatest rhamnolipid production (1700 mg/L) was obtained after 10 d of fermentation with 1% (v/v) glycerol (C:N ratio = 22.8) as the sole carbon source, MV:FV of 0.2, and initial inoculum of 0.004 g/L. The molecular characterization shows that the rhamnolipid is a disaccharide esterified in C-1, with acid having a chain size of 12 carbon atoms.

Acknowledgment

The molecular structure was determined by the group in the chemical sector at the Petrobras Research Center in Brazil for a future publication.

References

1. García, M. A. (1992), *Revista Instituto Mexicano Petroleo* **24,** 68.
2. Harvey, S., Elashvili, L., Valdes, J. J., Kamely, D., and Chakrabarty, M. (1990), *Biotechnology* **8,** 228–338.
3. Fiechter, A. (1992), *Tibtech* **1,** 208.
4. Boulton, C. and Ratledge, C. (1987), *Biosurfactants Biotechonol.* **25,** 47.
5. Ochener, U. A., Hembach, T., and Fiechter, A. (1995), *Adv. Biochem. Eng. Biotechnol.* **53,** 89.
6. Venkata Ramana, K. and Karanth, N. G. (1989), *J. Chem. Technol. Biotechnol.* **45,** 249.
7. Biolog (1993), MicroStation™ System Release, version 3.50.
8. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem.* **28,** 350–356.
9. Trinder, P. (1969), *Ann. Clin. Biochem.* **6,** 24–27.
10. American Society for Testing Materials. (1999), ASTM D971–99[a] Standard Test Method for Interfacial Tension of Oil Against Water by the Ring Method, American Society for Testing Materials.
11. Suk, W.-S., Son, H.-J., Lee, G., and Lee, S.-J. (1999), *J. Microbiol. Biotechonol.* **9(1),** 56–61.
12. Hisatsuka, K., Nakahara, T., Sano, N., and Yamanda, K. (1971), *Agric. Biol. Chem.* **35,** 686–692.
13. Banat, I. M. (1995), *Acta Biotechnol.* **15(3),** 251–267.
14. Ribeiro, A., Zhou, A., and Raetz, C. R. H. (1999), *Magnet. Reson. Chem.* **37,** 620–630.
15. Syldatk, C., Lang, S., and Matulovic, U. (1985), *Z. Naturforsch.* **40(1),** 61–67.