PRODUCTION OF BIOSURFACTANTS (RHAMNOLIPIDS) BY *Pseudomonas aeruginosa* ISOLATED FROM COLOMBIAN SLUDGES

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The biosurfactant production by strains of *Pseudomonas aeruginosa* isolated from Colombian hydrocarbon contaminated sludges has been determined. The methodology included the isolation of microorganisms, standardization of batch culture conditions for good surfactant production and characterization of the produced rhamnolipid. Several carbon sources were evaluated with regard to the growth and production curves. The stability of the rhamnolipid was also determined under variable conditions of pH, temperature and salt concentration. The strain *Pseudomonas aeruginosa* BS 3 showed biosurfactant production capabilities of rhamnolipid resulting in concentrations up to 2 $g \cdot dm^{-3}$ with surface tensions of 30 - 32 $mN \cdot m^{-1}$ in batch cultures with commercial nutrients.

Se determinó la capacidad de producción de biosurfactantes de cepas de *Pseudomonas aeruginosa* provenientes de lodos contaminados con hidrocarburo de diferentes regiones del país. La metodología utilizada comprendió el aislamiento de microorganismos, estandarización de condiciones de cultivo por lotes para una buena producción de tensoactivo y caracterización del ramnolípido producido. Se hizo énfasis en la evaluación de las fuentes de carbono más eficientes relacionada con las curvas de crecimiento y producción. También se determinó la estabilidad del ramnolípido frente a las variaciones de pH, temperatura y salinidad. La cepa *Pseudomonas aeruginosa* BS 3 permite producir biosurfactante del tipo ramnolípido con rendimientos hasta de 2 $g \cdot dm^{-3}$ con tensión superficial de 30-32 $mN \cdot m^{-1}$ en cultivos por lotes con nutrientes grado comercial.

Keywords: *biosurfactant, Pseudomonas, rhamnolipid, glycolipid, surface active compound.*

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INTRODUCTION

Surfactants are substances widely used in various industrial processes such as pharmaceutic, cosmetic, petroleum, food production and are frequently chemically synthesized. These chemical compounds are not biodegradable and can be toxic to the environment. Biosurfactants, however, have been shown in many cases to have equivalent emulsification properties and are biodegradable. Biosurfactants are surface active compounds mainly produced by microorganisms and synthesized in cells using diverse substrates, being totally biodegradable in aerobic and anaerobic conditions as well as non-toxic (Van Dyke *et al*., 1993; Kosaric *et al*., 1993; Banat, 1995; Ducret *et al*., 1995).

The emulsifying properties of these substances and/or their derived detergents are due to the existence of hydrophobic and hydrophilic moiety within the same molecule, which allows them to interact between two phases with different physicochemical characteristics. Biosurfactants can be glycolipids, lipopeptides/lipoproteins, lipopolysaccharides, substituted fatty acids and phospholipids according to their radicals (Duvnjak *et al*., 1982; Montes de Oca, 1992; Fiechter, 1992; Hommel, 1990).

The efficiency and effectiveness of these compounds are determined by their ability to reduce the surface tension of an aqueous solution and by the measurement of the critical micelle concentration (CMC) or concentration of biosurfactant necessary to achieve the lowest possible surface tension (Fiechter, 1992).

Rhamnolipids are normally synthesized by microorganisms to facilitate the uptaking of an insoluble substrate. For example, when oil is present in contaminated areas, the native microorganisms are selected for their assimilation capacity causing the availability and incorporation of the substrate to the cell for posterior metabolism (biodegradation) (Hommel, 1990; Zhang and Miller, 1994; Volkering *et al*., 1995)

There are many different microorganisms capable of synthesizing biosurfactants, *Pseudomonas fluorescens, P. aeruginosa,* are some of them (Kosaric *et al*., 1993; Hommel, 1990; Fiechter, 1992). Although extensively investigated and with a promising future, glycolipids have not been commercialized due to then high prize. Rhamnolipids, produced by *Pseudomonas* species are a kind of glycolipids (Hisatsuka *et al*., 1971; Hauser and Karnovsky, 1954).

Due to their biological origin and functional properties, biosurfactants can be used in various petroleum industrial processes, including emulsification and de-emulsification, separation, formation of low viscosity production of emulsions to transport heavy crudes, emulsion washing, formation of slurries, corrosion inhibition, enhance oil recovery, hydrocarbon biodegredation promotion (Kosaric *et al*., 1983; Díaz, 1991). If economically competitive, biosurfactants have the potential to replace synthetic surfactants, as they possess similar structural and physical properties, and are produced by renewable substrates, with the advantage of being degradable. When analyzing their economic viability, it is important to consider the nutritional requirements of the microorganism, the cost of the carbon sources employed (in bioremediation, a contaminating substance can be used as the substrate), the culture conditions and the design of a simple procedure to separate the product.

The aim of the present investigation was to select and evaluate the biosurfactant production capacity of native strains of *Pseudomonas aeruginosa* at laboratory level, with possible application in the petroleum industry.

EXPERIMENTAL METHODOLOGY

Microorganisms

Strains of *Pseudomonas aeruginosa* obtained by conventional methods (direct isolations on cetrimide and nutrient agar plates, and dilutions and growth in enrichment saline media), from sludges and waters taken from hydrocarbon contaminated areas in zones adjacent to petrochemical industries or crude oil spills.

The isolated bacteria were identified with the BIOLOG™ system for Gram negative bacteria; 95 tests were conducted to evaluate the carbon sources. The results were compared with the Microlog software database while the determination of the coefficient of similarity was used as the confidence criteria of the identification (Biolog, 1993).

The bacteria were frozen with glycerol at -193 K (fresh axenic culture in saline production medium: 20% V/V glycerol solution, in ratio 1:1) and fresh cultures on cetrimide agar plates (Merck, Darmstadt).

The selection and adaptation of the bacteria was

carried out by both quantitative tests of the production capacity of rhamnolipid in saline media, and qualitative tests for foam production and reproducibility of results.

Culture Media

The medium used for the growth and production of biosurfactants is a variation of the one reported by Hauser and Karnovsky (1954), using a 1% carbon source. It was identified as a Saline Production Medium (SPM) (all the components are per dm^{-3}): K_2HPO_4, 7 g; KH_2PO_4, 3 g; $MgSO_4$ $7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; glycerol, 10 cm^3 (Merck Darmstadt); pH 7,0. These chemicals were changed from analytical grades to commercial grades. Rhamnolipids are formed by *P. aeruginosa* grown on different carbon sources such as glycerol, glucose, vegetal oil, molasses and n-paraffin, etc., so as to evaluate and select an appropriate culture media (Guerra-Santos *et al*., 1984; Mercadé *et al*., 1993; Robert *et al*., 1991; Ramana and Karanth, 1989; Van Dyke *et al*., 1993; Zhang and Miller, 1994; Ghurye *et al*., 1994).

Nutrient and cetrimide agar plates were used to monitor and count the bacteria population.

Standarization of the culture conditions and saline composition

Different culture media were selected and evaluated with isolated strains in axenic cultures. The selection criteria was that more than 50% of the strains had grown, formed foam and produced a minimum of 500 $mgdm^{-3}$ of rhamnolipid. A 100 - 200 rpm range of agitation and 5,0 - 9,0 range pH were selected. (Van Dyke *et al*., 1993; Ramana and Karanth, 1989; Ghurye *et al*., 1994). Progressive microorganism adaptation processes by successive transferences to obtain strains that produce high level of rhamnolipid in less time were conducted. At the same time, the kinetic evaluations of the microorganisms were completed, which also favored the reduction of the process time.

The bacteria inocula was prepared by addition of cells from the solid medium to 5 cm^3 of SPM followed by incubation at 305 K (32 ºC) and rotatory agitation at 150 rpm. After 24 hours, this culture was transferred to another 50 cm^3 of sterile medium, which was incubated under the same conditions for approximately 7 to 10 days. A saline medium without inoculation using glycerol or the corresponding carbon source was employed as a negative control.

Evaluation of the carbon sources

Glycerol, sucrose, molasses, glucose and vegetal oil were evaluated in order to determine the most efficient carbon source. These sources were supplemented at 1% in SPM with analytical and commercial grade chemicals, and then evaluated following the general procedure.

Determination of rhamnolipid

The analytical evaluations for rhamnolipid were carried out by the phenol-sulfuric acid method, determining rhamnose in a glycerol and vegetable oil media and by the L-cystheine-sulfuric acid method for other sources of carbon containing glucose (Whistler and Wolfrom, 1962). Pretreatment of the samples was done by removing biomass by centrifugation at 6.000 rpm for 15 minutes. All measurements were carriet out in a UV-VIS Cary Varian 1E Spectrophotometer.

Equation 1 was used for the calculation of the concentration of rhamnose. These values were converted to rhamnolipid units.

$$[RL]_{Total} = [RL]_{480} - [Glucose]_{420\,-\,380} \qquad (1)$$

Measurement of surface tension and CMC determination

The surface tension was measured by the maximum bubble pressure method using a Sensadyne 6.000 tensiometer. The determination of the Critical Micelle Concentration (CMC) was conducted by conventional methods (Margaritis *et al*., 1979), using the rhamnolipid produced from glycerol as a carbon source in increasing concentrations of 50, 100, 200, 300, 500, 800, 1600 and 2.000 $mg \cdot dm^{-3}$, and the measurement of the associated surface tension.

Evaluation of the growth and production of rhamnolipid by *Pseudomonas aeruginosa*

These evaluations were achieved by performing growth and production experiments according to the culture conditions described above separately in 50 cm^3 flasks containing SPM with commercial grade chemicals and glycerol and vegetable oil as carbon sources.

Evaluating Biosurfactant Stability

For comparison purposes, the surfactants used were

Triton X-100 and LAS (linear alquilbenzene sulfonate) analytical and commercial grades. Triton X-100 is a nonionic surfactant and LAS analytical grade is linear anionic surfactant. The biosurfactant was produced according to the culture conditions described above. The biomass was removed by centrifugation and the level of rhamnolipid was determined. Stability was evaluated by measuring the surface tension. The sole indirect criteria applied to determine tenso-active activity was the measurement of the tension surface (Margaritis *et al*., 1979).

Assessment of the temperature resistance was carried out at 297, 313, 333 and 353 K. This involved exposing the product at each temperature for periods of 0,5 to 1 hour. The pH stability tests were performed by adding diluted sulfuric acid or sodium hydroxide so that solutions of pH 2 to 12 were obtained. Salt was added to the solutions in increasing sodium chloride concentrations (0, 1, 5, 10 and 20% (w/v)) in order to establish stability.

Evaluation of emulsifying properties

To determine the emulsifying properties of the biosurfactant, inverse O/W emulsions were prepared with heavy crude oil, varying the concentration of the surface-active ingredient.

RESULTS AND DISCUSSION

Evaluation of different native strains of *Pseudomonas*

Pseudomonas was selected for its nutritional and biochemical versatility as well as for the simplicity of the culture conditions; the latter being the main reason for their wide presence in different habitats (Krieg and Holt, 1984). Among the strains recovered from hydrocarbon contaminated sludges and waters, those that corresponded to the morphology of *Pseudomonas* were individually studied. The morphologic characteristics of these strains and the biosurfactant production results are shown in Table 1.

The time of appearance, the amount of foam generated, raising viscosity and darkering of the initial color through time were used as qualitative measures of the presence of the rhamnolipid. All the identified strains belong to the same species, *Pseudomonas aeruginosa*, but each microorganism had different morphological characteristics.

Taking into account the nutritional and activation characteristics for the kinetic study, a particular strain was considered rather than a group of microorganisms. Although the *Pseudomonas aeruginosa* BS 5 showed in some cases higher levels of production, the *Pseudomonas aeruginosa* BS 3 was selected for its production stability and reproducibility properties.

Influence of culture conditions on rhamnolipid production rates

Agitation. Repeated evaluation trials indicated that agitation did indeed influence production, and that the culture without agitation produced less rhamnolipid than with any of the agitation systems. The system without agitation does not present a good time-oxygen transfer which increases the process time for an efficient production. Additionally, the type of agitation employed was an important factor in the biosurfactant production. A higher concentration of rhamnolipid was achieved by rotatory shaking (orbital agitation) when compared to the thermoregulated bath (lateral agitation) and the control without agitation. The degree of homogenization of the culture achieved during agitation marked the difference between the two agitation systems (Table 2).

pH Value. The minimum surface tension and highest recovery of rhamnolipid was obtained with pH between 7,0 and 8,0 (Table 3). Besides, in all cases of the fermentation process, the pH decrease included alkaline pH. For this reason, it was decided to work at a neutral pH and thus eliminate the use of alkali and reduce process costs.

It was noticed that the pH value induced strong fluctuations in the production of rhamnolipid, probably due to the kinetic and metabolic behavior of the microorganism, which produces acids from carbohydrates as it grows thus decreasing the pH (Krieg and Holt, 1984). This condition affects the microorganism in distinct ways, one of which is the deactivation of its enzymes if the pH value is outside their normal range of operation. This effect is not drastic as alkaline pH values (Bohinski, 1991).

It is important to mention that the cultures were not maintained at a constant pH because it changed freely.

Table 1. Morphologic characteristics of native strains of *Pseudomonas aeruginosa* and rhamnolipid production capacity. Process time: 10 days; analytical grade medium; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH.

STRAIN	Origin	Microscopic morphology	Macroscopic morphology(+)	Surface tension ($mN \cdot m^{-1}$)	[RL]* ($mg \cdot dm^{-3}$)
BS 2	Sludge Ciénaga 1 y 2	CB long G(-)	Dark violaceous green colony, flat, sparkling, stuck to the medium.	33,8	1524,23
BS 3	Sludge Ciénaga 1 y 2	CB G(-)	Light brownish-green colony, undulating border, opaque, convex.	31,7	1600,03
BS 5	Sludge Ciénaga 1 y 2	CB small G(-)	Light green colony, smooth border, sparkling, convex.	33,3	1770,23
BS 6	Sludge Ciénaga 2	CB very small G(-)	Light green colony, sparkling, convex, smooth border.	32,0	1330,49
BS 7	Sludge Ciénaga 1	CB thin G(-)	Dark iridescent green colony, flat, smooth border, sparkling.	33,3	1392,32
BS 8	Sludge Ciénaga 1 y 2	CB small G(-)	Light green colony, smooth brownish border, sparkling, convex.	33,1	1492,35
BS 9	Sludge Ciénaga 1 y 2	CB thin G(-)	Dark iridescent green colony, flat, sparkling, smooth border.	32,7	1481,19
BS 11	Sludge Ciénaga 6	CB small G(-)	Light green colony, even border, sparkling, convex.	34,2	1424,52
BS 16	Sludge PTAR	CB large G(-)	Dark iridescent green colony, smooth border, sparkling, convex.	33,3	1116,95
BS 17	Water PTAR	CB G(-)	Large colony, uneven border, bright lime green, convex, sparkling.	31,6	1166,94
BS 18	Sludge Ciénaga 6	CB G(-)	Brownish-green colony, smooth border, convex, sparkling, large.	33,7	1601,09

CB : Cocci-rods morphology
[RL] : Rhamnolipid concentration(*Maximum values obtained)
PTAR : Planta de Tratamiento de Aguas Residuales (Waste-water treatment plant)
(+) Macroscopic morphology in Cetrimide agar.

Saline purity of chemicals. Initially, the cultures in analytical grade media exhibited higher production of surfactant than those performed in commercial grade media, suggesting that the purity of the components influences the output rate of the bacteria. However, as the bacteria progressively adapted to these components this performance criteria was inverted. This is possibly attributed to the presence of trace impurities or ions found in running water, which served as micronutrients for the growth and production of rhamnolipid.

Process time. The standardization of the culture conditions involved the progressive adaptation of the microorganisms to the culture medium. This was achieved by successive feeding steps with metabolically active seed cultures from previous experiments. As a result, the initial process time of 15 days was progressively reduced to 10 and 7 days.

Evaluation of carbon sources. The following carbon sources were evaluated: glycerol, molasses,

Table 2. Influence of agitation type on rhamnolipid production.
Process time: 10 days; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; analytical grade medium

STRAIN	Type of Agitation	Analytical		Commercial	
		Surface tension	[RL]	Surface tension	[RL]
		($mN \cdot m^{-1}$)	($mg \cdot dm^{-3}$)	($mN \cdot m^{-1}$)	($mg \cdot dm^{-3}$)
CONTROL	None	72,4	-	73,0	-
CONTROL	Rotatory	72,2	-	70,6	-
CONTROL	Lateral	71,2	-	69,8	-
BS 3	None	37,1	886,23	43,1	822,41
BS 3	Rotatory	33,8	1107,87	34,1	1402,73
BS 3	Lateral	38,2	824,75	34,1	1075,66

[RL] : Rhamnolipid Concentration

Table 3. Evaluation of the effect of pH on biosurfactant production.
Process time: 10 days; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; analytical grade medium

STRAIN	Inital pH	Final pH	Surface tension	[RL]
			($mN \cdot m^{-1}$)	($mg \cdot dm^{-3}$)
Control	7,0	7,0	72,2	-
BS 3	5,5	4,52	71,9	36,12
BS 3	5,5	4,55	71,4	264,23
BS 3	6,0	4,67	66,3	517,24
BS 3	6,0	4,64	67,6	894,04
BS 3	6,5	5,93	54,2	363,47
BS 3	6,5	6,02	50,9	782,80
BS 3	7,0	6,61	38,0	604,98
BS 3	7,0	6,60	37,4	564,48
BS 3	7,5	7,01	32,2	1422,82
BS 3	7,5	6,92	39,7	769,34
BS 3	8,0	7,24	35,7	1191,89
BS 3	8,0	7,24	33,8	1537,0

[RL] : Rhamnolipid Concentration

sucrose, glucose and vegetal oil.

The metabolic behavior of the BS 3 strain varied considerably with the different carbon sources. The general trend was an improvement in rhamnolipid production and a reduction in surface tension using commercial grade media. It is interesting to note, that although there were numerous interferences in the analytical method (other carbohidrates) to determine the rhamnolipid produced with sucrose and molasses, the surface tension results indicated that there was no biosurfactant production.

These interferences were due to the availability of more than one carbon substrate for the bacteria. As the analytical method included indirect determination of rhamnose concentrations (aldohexose), the non-degraded substrate caused interferences in the colorimetric method. This difficulty was overcame by carrying out evaluations at different wave lengths, which corrected the absorbance of the interferent.

Glycerol was observed to give the highest rates of production of the different soluble carbon sources studied. The production with vegetal oil was either similar or higher than the one obtained with the soluble sources. This implies that a selective pressure effect is acting on the microorganism, to produce the biosurfactant and thus makes the substrate available for metabolism (Mercadé *et al.,* 1993, Zhang and Miller, 1995). Table 4 shows the test results for the various carbon sources studied.

Determination of CMC of the rhamnolipid

The Critical Micelle Concentration of the rhamnolipid was determined in crude extracts (without purification), using glycerol as the carbon source. However, it should be noted that the carbon source is in fact irrelevant, since increasing concentrations of rhamnolipid are considered. The CMC of the rhamnolipid produced by *Pseudomonas aeruginosa* BS 3 was found to be in the 300 - 400 $mgdm^{-3}$ range. These results are given in Figure 1.

Table 4. Evaluation of soluble and insoluble carbon sources.
Process time: 7 days; Volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH

CARBON SOURCE	Saline medium	Surface tension ($mN \cdot m^{-1}$)	[RL] ($mg \cdot dm^{-3}$)
Glycerol	Analytical	35,5	1082,4
Glycerol	Analytical	35,4	1083,0
Glycerol	Commercial	32,1	1430,7
Glycerol	Commercial	32,0	1406,1
Control	Analytical	72,5	-
Glucose	Analytical	36,0	582,57
Glucose	Analytical	34,9	32,24
Glucose	Commercial	32.8	710,28
Glucose	Commercial	33,6	577,45
Control	Analytical	72,1	-
Sugar	Analytical	72,1	Interf.
Sugar	Analytical	72,2	641,85
Sugar	Commercial	72,4	315,1
Sugar	Commercial	71,6	660,6
Control	Analytical	72,2	-
Molasses	Analytical	70,6	Interf.
Molasses	Analytical	70,7	Interf.
Molasses	Commercial	70,4	Interf.
Molasses	Commercial	70,8	Interf.
Control	Analytical	71,5	-
Vegetal oil	Commercial	31,6	1455,93
Vegetal oil	Commercial	31,5	1352,8
Control	Commercial	64,2	N.D.
Control	Analytical	72,4	N.D.
Control	Commercial	72,3	N.D.

[RL] : Rhamnolipid concentration
Interf.: Interference in the method, overestimated value
N.D.: Not determined

Evaluation of the growth and production of the rhamnolipid by *Pseudomonas aeruginosa*

Growth and Production Curves. Growth and production curves using glycerol and vegetal oil in commercial grade media were used to compare the production of soluble and insoluble carbon sources.

During the first 30 hours of evaluation, the glycerol growth curve exhibited the latent and exponential phases (Figures 2 and 3). Discontinuity in the different parameters was observed in the following 18 hours (stationary phase) for all variables except for the absorbance (Figures 2, 3 and 4). Such phenomena can be associated with the formation of non determined

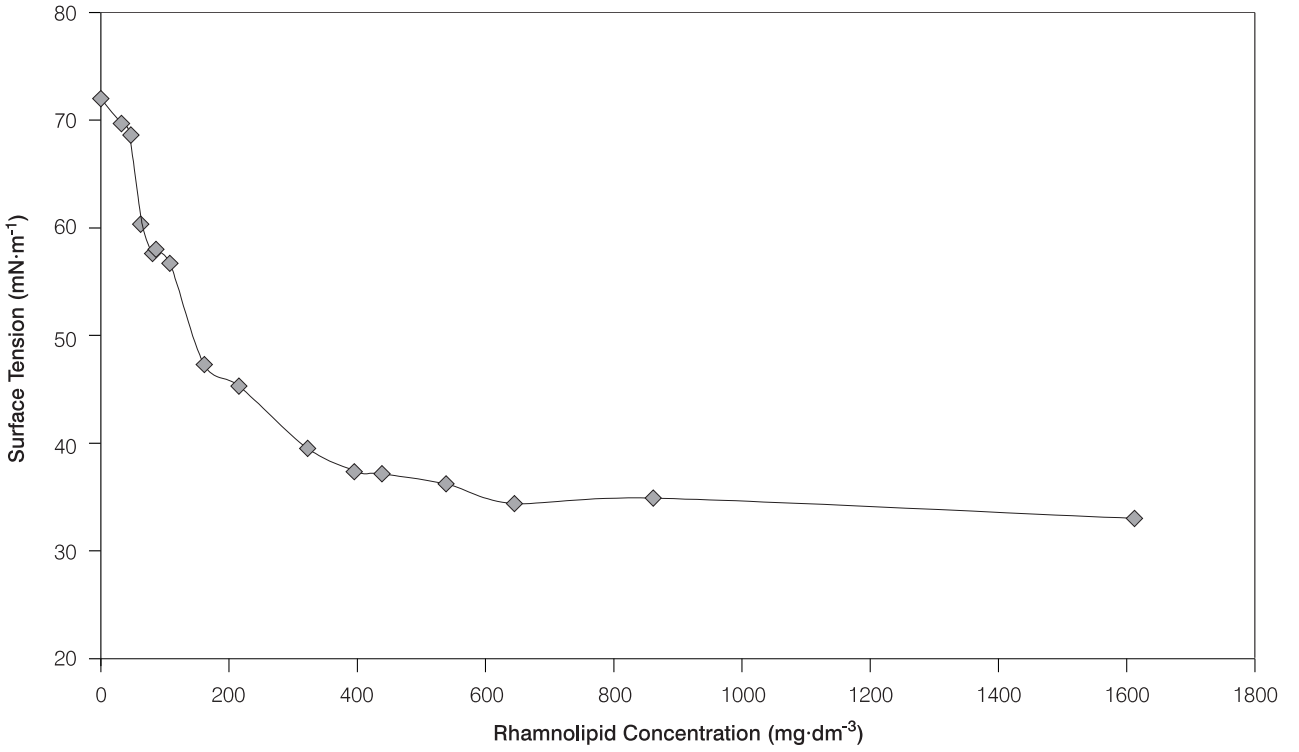

Figure 1. Critical Micelle Concentration (CMC) of the rhamnolipid from *Pseudomonas aeroginosa* BS 3.
Process time: 10 days; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH; analytical grade medium

byproducts of the bacterial metabolism which act as multiple carbon sources, producing a diauxic behavior. Moreover, during the stationary phase metabolic activity decreases, there is competition for the residual substrate and culture saturation occurs due to toxins and non metabolizable byproducts accumulation which can cause deviations in bacterial metabolism.

Surface tension was reduced by increasing the rhamnolipid concentration in the culture medium. The curve stabled off at a rhamnolipid concentration of 300 to 400 $mg \cdot dm^{-3}$ (CMC) after about 48 hours. The time-rhamnolipid concentration relationship indicates that biosurfactant production increased at around the 20 hours point (Figure 4). Although the rhamnolipid appears at the same time with the microbial growth, its accelerated production is present in the stationary phase. For this reason, the product formation appeared to be partly growth-associated as described by other authors (Persson *et al*., 1988; Guerra-Santos *et al.*, 1986). Moreover, the influence of limited concentration or of the source of inorganic nutrients such as nitrogen, iron, phosphorous on growth and biosurfactant production could be presented but not evaluated (Guerra-Santos *et al*., 1984; Guerra-Santos *et al.*, 1986; Persson *et al.*, 1988; Mulligan and Gibbs, 1989; Mulligan *et al*., 1989; Ochsner *et al*., 1995).

Figure 3 and 5 presents the microbial count, pH, surface tension and rhamnolipid concentration parameters produced using a commercial vegetal oil. The behavior was similar to the one observed with glycerol but more stable. The pH steadily decreased; rhamnolipid production started earlier (after 10 - 12 hours) which reflected in a reduction of surface tension and a slight increase of the microbial growth. This fact is characteristic of insoluble substrates which make a selective pressure in the first steps of the rhamnolipid production.

A drop was observed in the amount of rhamnolipid produced which is probably related to the reduction and stabilization of the microbial count in the stationary phase.

Comparing the two carbon sources used, higher

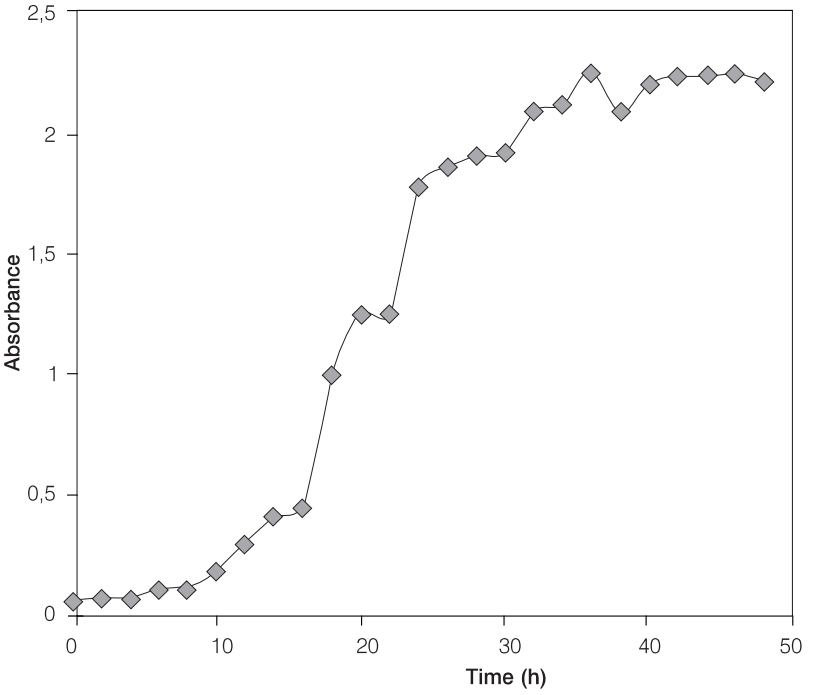

Figure 2. Growth and production kinetics of P*seudomonas aeruginosa* BS 3 with glycerol as carbon source. Absorbance vs time
Process time: 48 hours; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH; commercial grade medium

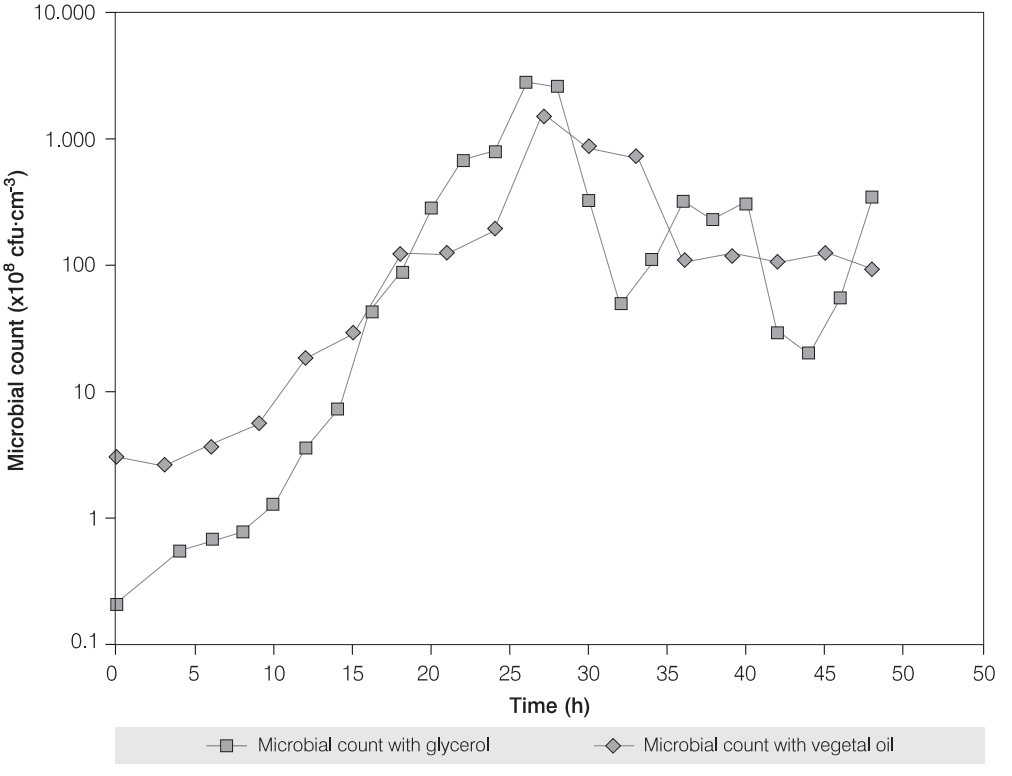

Figure 3. Growth kinetics of *Pseudomonas aeruginosa* BS 3 with glycerol and vegetal oil as carbon source.
Process time: 48 hours; volume: 50cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH; commercial grade medium

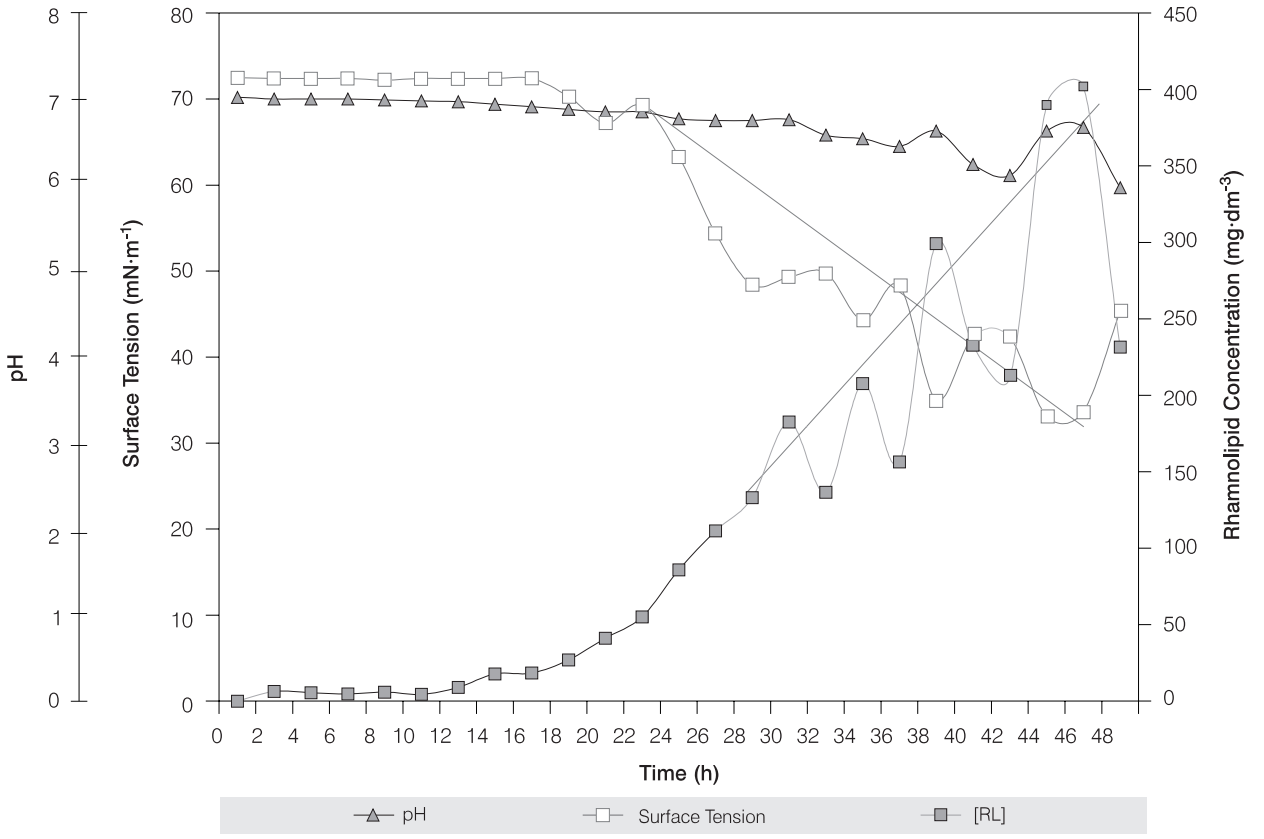

Figure 4. Growth and production kinetics of *Pseudomonas aeruginosa* BS 3 with glycerol as carbon source. Process time: 48 hours; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH; commercial grade medium

levels of rhamnolipid were produced using glycerol in stationary phase at 46 hours.

Evaluation of biosurfactant stability

The biosurfactant exhibited good tenso-active properties between a pH of 5 and 10 (Figure 6); resisted temperatures up to 353 K during one hour (Figure 7); and salt concentrations of up to 5% (Figure 8). A permanent surface tension of the aqueous solution in the 30 - 45 mNm^{-1} range was used as a qualitative measure of stability. The biosurfactant rapidly loses this property after this limit. At higher values of 45 mNm^{-1}, the surfactant losses its capacity to interact between two phases with different physicochemical characteristics (Kosaric, 1993).

The rhamnolipid showed greater temperature stability than the other surfactants studied, with tensions not exceeding 32 mNm^{-1}. The evaluation time was set taking into account variables such as costs, efficiency in real washing emulsion processes and operation speed in biodegradation systems, which must be completed in a similar period of time. With regards to salt concentration, only Triton X-100 was stable during the whole evaluation (34,5 - 39,3 $mN{\cdot}m^{-1}$), while LAS analytical and commercial grades were equivalent or less effective than the rhamnolipid. Triton X-100 showed the greatest stability to pH (surface tensions 34,1 - 34,8 $mN{\cdot}m^{-1}$), LAS gave tensions exceeding 38 $mN{\cdot}m^{-1}$, while the biosurfactant was more effective than the previously mentioned in the 5,5 - 7,5 pH range.

Evaluation of the biosurfactant emulsifying properties

Inverse low viscosity emulsions were prepared with the biosurfactant and Castilla heavy crude oil, using an active ingredient concentration of 500 $mg{\cdot}dm^{-3}$ or greater (with respect to the total emulsion volume). Inverse emulsions O/W 50/50 with 40 cP viscosity and 30 micron particle size were obtained. In addition, stable

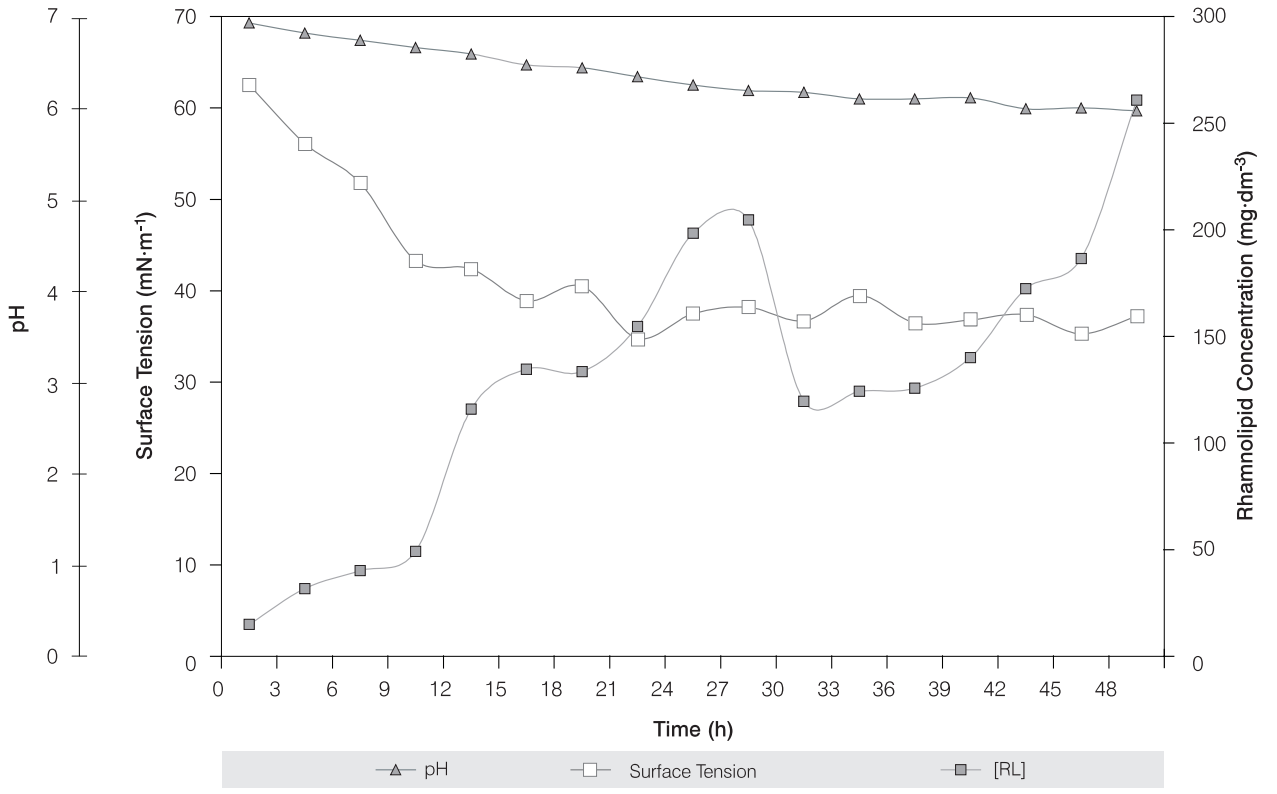

Figure 5. Growth and production kinetics of *Pseudomonas aeruginosa* BS 3 with vegetal oil as carbon source.
Process time: 48 hours; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH; commercial grade medium

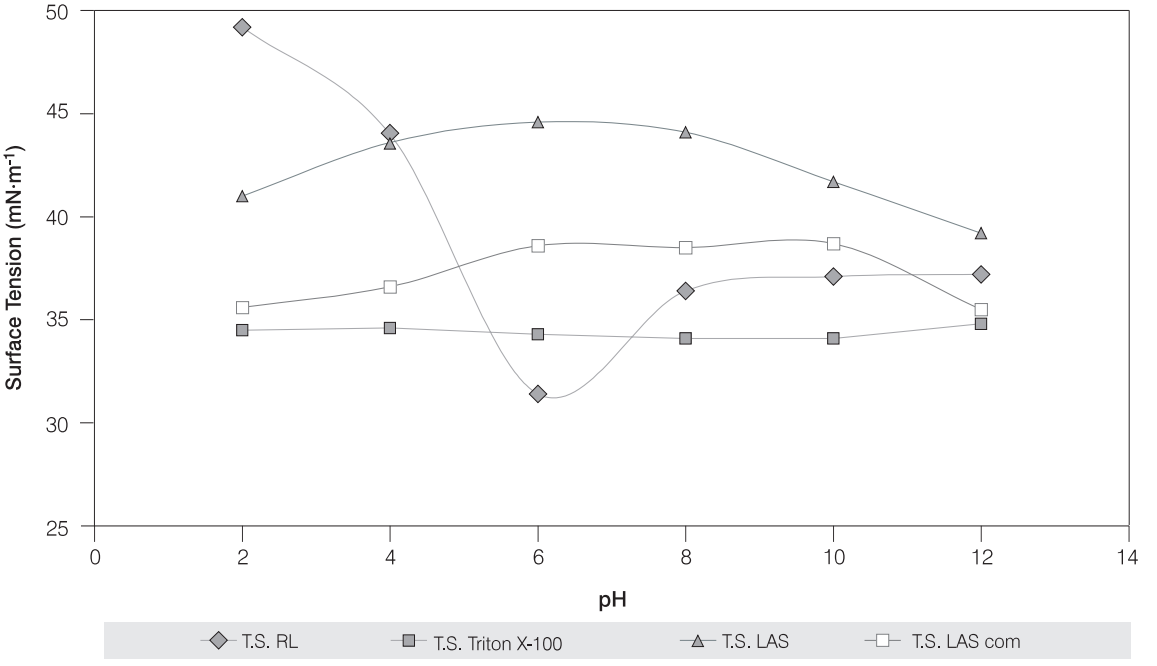

Figure 6. Variation of biosurfactant and synthetic surfactant surface tension with pH.
Process time: 7 days; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; commercial grade medium

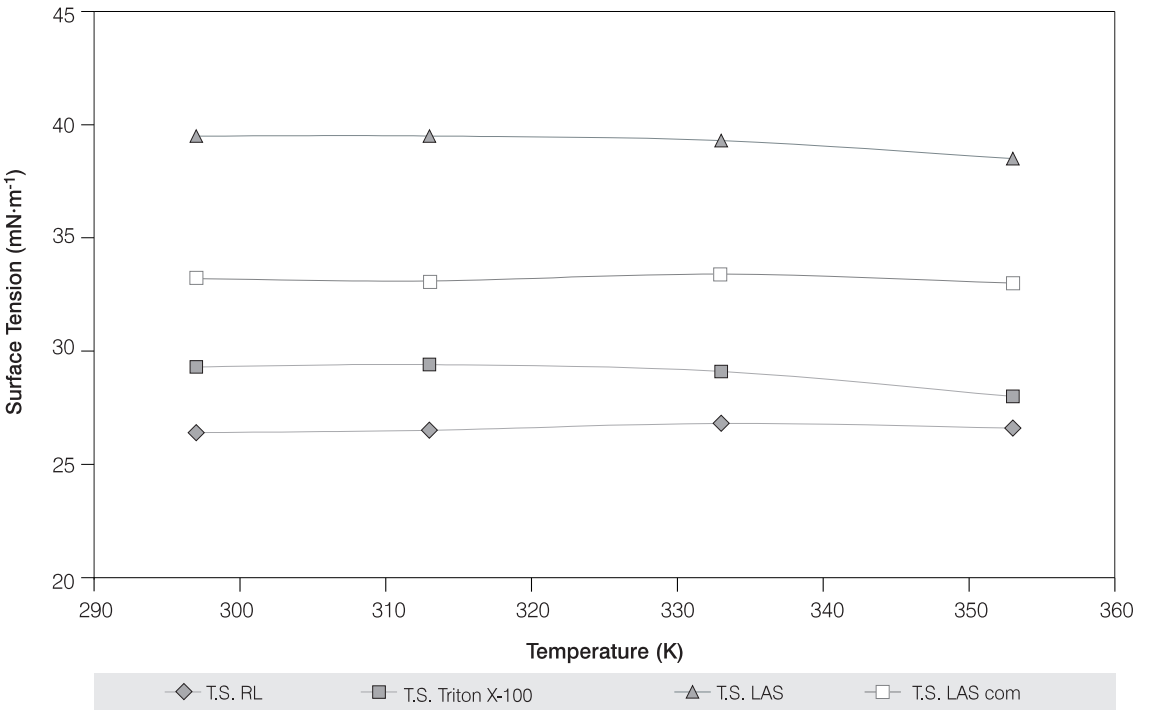

Figure 7. Comparison of thermostability of the biosurfactant with synthetic surfactants.
Process time: 7 days; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH; commercial grade medium.

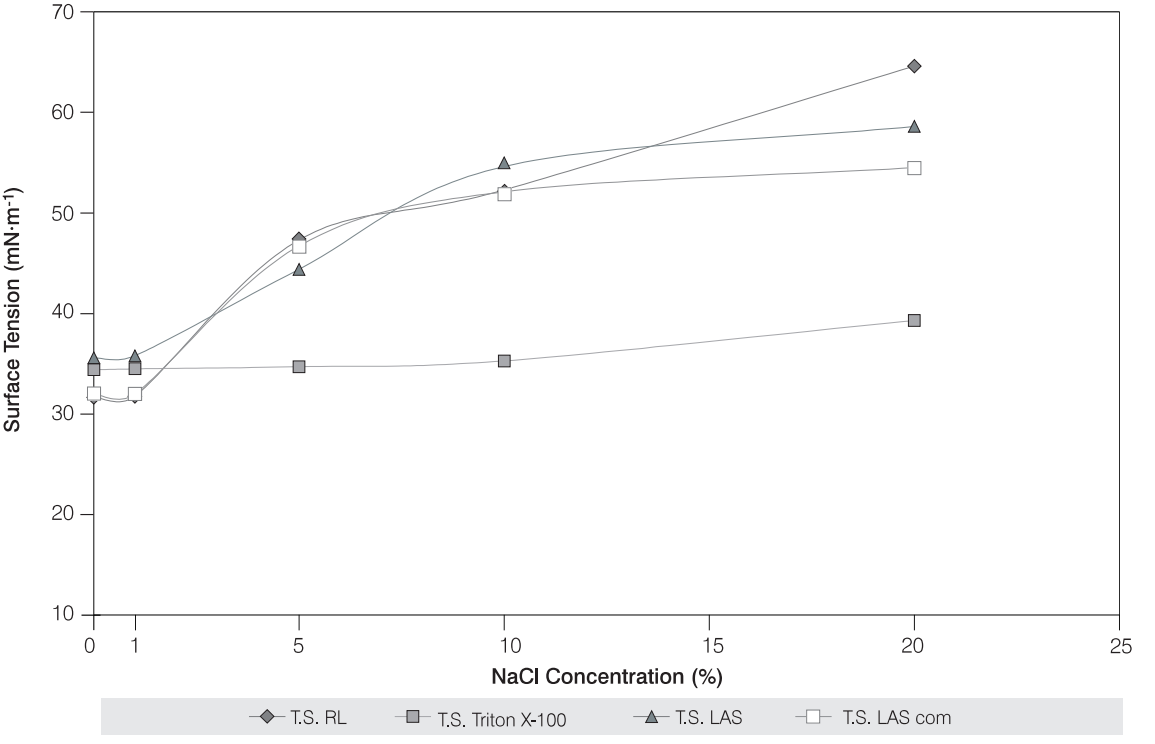

Figure 8. Comparison of salt concentraton stability of the biosurfactant with synthetic surfactants.
Process time: 7 days; volume: 50 cm^3; temperature: 305 K; agitation: 150 rpm; neutral pH; commercial grade medium.

inverse emulsions O/W 60/40 were formed using active ingredient concentrations of 800 $mg \cdot dm^{-3}$ or greater.

CONCLUSIONS

- Strains of *Pseudomonas aeruginosa* isolated from Colombian hydrocarbon contaminated sludges have the ability to produce surface active compounds. *Pseudomonas aeruginosa* BS 3 was selected as the most efficient strain in the evaluation.
- A methodology for producing high output levels of biosurfactant by batch cultures of *Pseudomonas aeruginosa* was determined. This involved controlling agitation, temperature, pH and salt concentration conditions. Media employing analytical grade chemicals were successfully replaced by commercial grade media. The emulsifying property of the biosurfactant was also verified with heavy crude oil.
- Soluble carbon sources were found to be the most effective ones for producing biosurfactants. However, insoluble carbon source acts as a limiting agent, such that the assimilation of the carbon source reduces biosurfactant production time.
- The rhamnolipid produced by *Pseudomonas aeruginosa* BS 3 is a partly growth-associated metabolite that starts its production in the first 24 hours of incubation. Production starts after aproximatelly 20 hours for soluble carbon sources and after 10 to 12 hours for the insoluble ones.
- The rhamnolipid produced by the bacteria is an efficient and effective tensoactive agent showing surface tension reductions up to 30 - 32 $mN \cdot m^{-1}$ for aqueous solutions.
- The Critical Micelle Concentration of the rhamnolipid was found to be in the 300 - 400 $mg \cdot dm^{-3}$ range (biosurfactant without purification). This non-purified condition makes it very economical for industrial production processes.
- Compared with other synthetic surfactants, the biosurfactant was found to be very stable under variable pH, temperature and salt concentration test conditions.
- More work remains to be done in order to evaluate and optimize the production and recovery of biosurfactants and their stability and emulsifying properties. It will also be important to conduct the chemical characterization of the molecules produced by *Pseudomonas aeruginosa* BS 3.

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