Biosurfactant production from *Pseudomonas* strains isolated in rhizospheric soils from semi-arid area of Bahia, Brazil

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Abstract – The aim of this study was to select biosurfactant-producing bacterial strains from soils of plants in the semi-arid region of the state of Bahia, Brazil. The mineral salt medium (MSM) was used as base medium, changes being made in the glucose concentration, temperature, and pH during the optimization process. Two biosurfactant-producing bacterial strains were isolated from the soil of *Actinocephalus* sp. and belonged to the genus *Pseudomonas*. The emulsification indexes of these strains rose until 58%. They reduced surface and interfacial tensions of culture medium and crude oil to less than 33 mN/m and 3 mN/m, respectively. This study represented an unprecedented discovery of surfactant activity of the bacterial strains isolated from rhizospheric soils of plants from Northeast Brazilian semi-arid region.
Additional key words: bacterial, plants.

Resumo (Produção de biossurfactante por linhagens de P*seudomonas* isoladas do solo rizosférico no Semiárido da Bahia, Brasil) – O objetivo deste estudo foi selecionar linhagens bacterianas produtoras de biossurfactante do solo rizosférico de plantas na região semiárida do estado da Bahia, Brasil. O meio salino mineral (MSM) foi usado como meio base, sendo realizadas mudanças na concentração de glicose, temperatura e pH durante o processo de otimização. Duas linhagens produtoras de biossurfactante foram isoladas do solo rizosférico de *Actinocephalus* sp. e pertencem ao gênero *Pseudomonas*. Os índices de emulsificação destas linhagens aumentaram até 58%. Elas reduziram as tensões superficial e interfacial do meio de cultura e do óleo cru para menos de 33 mN/m e 3 mN/m, respectivamente. Este estudo representou uma descoberta inédita da atividade surfactante de linhagens bacterianas isoladas de solo rizosférico de plantas da região semiárida no Nordeste Brasileiro.
Palavras-chave adicionais: bactérias, plantas.

Plants and microorganisms exhibit complex interactions with the environment and produce small molecules, natural products useful for their survival. As a consequence of their biological role, these metabolites might exhibit a broad range of biological activities. In fact, natural products have played a fundamental role in useful bioactive substances for industrial applications (Pupo et al. 2006). Isolation of such microorganisms can lead novel bioactive natural products research for oil, pharmaceutical, and agrochemical industries (Strobel 2006).

The semi-arid region of the state of Bahia, especially the Chapada Diamantina, presents one of the most diverse ecosystems of the biosphere (Torres et al. 2003). The biodiversity of Chapada Diamantina presents microorganisms with biotechnological features applicable to oil industries due to the extreme conditions of its environment, such as highly saline, nutrient-poor and extremely acidic soils, in addition to its higher environmental temperatures (Gusmão & Maia 2006). Bioprospecting in such areas could be interesting for industrial applications, because organisms growing and producing bioactive substances under these conditions may be able to achieve better yields.

Furthermore, biosurfactants have many advantages when compared to synthetic surfactants. They may be more stable at the extreme conditions of oil reservoirs, less toxic, biodegradable, and show better efficiency for oil recovery. Another advantage of biosurfactants is that they can be modified by biotransformation to generate new products for specific requirements (Deleu & Paquot 2004). However, studies associated with its production in different culture media and physicochemical conditions are necessary to achieve the best competitive advantages (Mulligan 2005). Biosurfactant production depends primarily on microbial strain, carbon and nitrogen sources, pH, temperature, oxygen, and metal ion concentrations. Addition of water-immiscible substrates to media as long as nitrogen and iron limitations in the media results in an overproduction of some biosurfactants. The most promising applications are cleaning of oil-contaminated tankers, oil spill management, recovery of crude oil from sludge, enhanced oil recovery, and bioremediation of sites contaminated with

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hydrocarbons, heavy metals, and other pollutants (Desai & Banat 1997).

In this context, the aim of this study was to select biosurfactant-producing bacterial strains from soils (rhizospheric and adjacent) of plants in the semi-arid region of Bahia and to establish an optimal condition of emulsifier production in order to be useful for biotechnological applications in oil industry.

Materials and Methods

Sample collection. Samples were collected from rhizospheric (root-associated) and adjacent (root-dissociated by gentle shake) soils for each of the following groups of plants, endemic to the semi-arid region: *Actinocephalus* sp. (Koern.) Sano and *Syngonanthus mucugensis* Giul. (Eriocaulaceae) and *Stachytarpheta crassifolia* Schrad. (Verbenaceae). Two collections were made, one in the dry and the other in the rainy season, in two areas of the Mucugê Municipal Park (Parque Municipal de Mucugê – PMM), municipality of Mucugê, Bahia: Area 1 at 13°00'06"S and 41°20'54" W and Area 2 at 12°59'36"S and 41°20'33"W. The plant species were catalogued and identified in the Herbarium of the Universidade Estadual de Feira de Santana (HUEFS). Aliquots of soil samples corresponding to dry season were sent to the Physical Chemistry Laboratory (Embrapa Mandioca e Fruticultura) for physico-chemical analysis

Isolation of bacterial strains, media and growth conditions. Soil samples were collected in a depth of approximately 30 cm, sieved and homogenized. Ten grams of soil samples were placed in 500 ml Erlenmeyer flasks containing 90 ml saline solution (0.9% NaCl). Serial dilutions were made and aliquots of 0.1 ml transferred to the following culture media: King's B (composition, g/L: protease peptone n.03, 10.0; K_2HPO_4, 1.5; $MgSO_4.7H_2O$, 1.5; glycerol, 10; agar, 15.0; pH 7.0); Minimum Medium (MM) (composition, g/L: K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; NaCl, 0.1; $FeCl_3$, 0.01; NH_4NO_3, 0.5; agar, 15.0; pH 7.0); Mineral Saline Medium (MSM) (composition, g/L: K_2HPO_4, 4.0; Na_2HPO_4, 1.5; $NaNO_3$, 1.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.02; $FeCl_3.6H_2O$, 0.02; agar, 15.0; pH 7.0); and nutrient agar (Himedia). Selective culture media were used to isolate species of the genus *Pseudomonas* (agar Pseudomonas, agar Pseudomonas fluoresceine, agar Pseudomonas pyocyanin) (Himedia). The plates were incubated at 30°C, for 24 to 72 h. The different types of bacterial colonies obtained on the plates were then purified in tryptic soy agar (TSA).

Selection of biosurfactant-producing bacterial strains. The pure colonies were inoculated in 10 ml of MSM with 2% glucose (w/v) as the only source of carbon and energy. The incubation was kept for 48 h on an orbital shaker (180 rpm/30°C). The cells were pelleted by centrifugation (5.578 g for 20 min at 10ºC) (Universal 32R, Hettich) and the cell-free broth was used for selection by the drop-collapse method (Das et al. 1998). One per cent sodium dodecyl sulfate (SDS) (J.T. Baker) and non-inoculated MSM were used in all biosurfactant assays performed as positive and negative control, respectively.

Characterization of surfactant-producing bacterial strains. Morphological characterization of the biosurfactant-producing bacterial strains was done by Gram staining and conventional phenotypic tests. The bacterial strains were also identified preliminarily by a molecular technique, after genomic DNA extraction using Phenol-Chloroform-Isoamyl Alcohol (25:24:1, v/v), which detects the full length 16S rRNA region using the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG – 3') and rP2 (5'-ACGGCTACCTTGTTACGACTT – 3'), according to Weisburg et al. (1991). The 16S rRNA amplicons obtained were sequenced and analyzed by BLAST (NCBI, USA). Gas chromatographic analysis of whole cell fatty acid methyl ester (FAME) was also performed for further identification and grouping of isolates. A similarity index higher than 60% was used for clustering of isolates at species level (Sasser 1990). FAME profile is represented as a vector of the different relative FAME peak area percentages as calculated by the Sherlock MIS using the TSBA50 peak naming method (Slabbincka et al. 2009).

Emulsification yield optimization. The MSM, as previously described, was used as base medium, changes being made in the glucose concentration during the optimization process, aiming to achieve the optimum emulsification yield. The determined levels for each variable were used for later experiments.

The emulsification yield assay was performed in a 500 ml Erlenmeyer flask containing 100 ml of MSM (pH 7.0) with different glucose concentrations (0.25, 0.5, 1, 2, 3, and 4% w/v), as the sole source of carbon. To determine temperature and pH effects, MSM with the optimized glucose concentration (0.5%) was evaluated in a 5°C and 0.5 stepwise scales of temperature (25–40°C) and pH (5.0–8.0), respectively.

All tests were performed with a bacterial suspension of 10^8 c.f.u./ml, from the standard optical density scale (1.0) on a 600 nm filter. The samples were incubated on an orbital shaker at the speed of 180 rpm, for 5 days. Five milliliter aliquots were removed from the bacterial cultures at each following 24 h intervals and centrifuged (5.578 g for 20 min, at 10°C) to obtain a cell-free broth.

The emulsification yield was determined by the emulsification index (E24) which was performed in test tubes containing 2 ml of cell-free broth and 2 ml of mineral oil (Nujol). Emulsification activity was determined as a percentage of the height of emulsified layer (mm) divided by the total height of the liquid column (mm) (Das et al. 1998).

Cell growth-associated emulsification yield. The cell growth and emulsification yield ratio was determined using optimal culture conditions. Culture aliquots were removed from the flasks at each 24 h for absorbance readouts (600 nm) in the spectrophotometer (Cary, 50 Probe), aiming to determine bacterial biomass concentration, according to the calibration curve of the dry weight versus absorbance.

Bacterial biomass concentration. The bacterial strains were seeded in TSA and kept on an incubator at 30°C, for 24 h. Afterwards, a colony was transferred to 500 ml Erlenmeyer flasks containing 100 ml of tryptic soy broth (TSB), incubated at 30°C under agitation at 180 rpm for 48 h. The culture was centrifuged (5.578 g, for 20 min, at 10ºC) and the bacterial biomass was resuspended in distilled water to prepare the dry weight versus absorbance curve (600 nm).

Orcinol assay. Orcinol assay was used for direct assess of the amount of sugar in the sample (Chandrasekaran & Bemiller 1980). Sample was purified by first separating the cells from supernatant by centrifugation (10,000 g). The supernatant was then extracted with chloroform and ethanol. The 0.5 ml rhamnolipid sample was extracted with 1 ml chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.5 ml of H_2O was added. To 0.1 ml of each sample, 0.9 ml of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80ºC, the sample was cooled at room temperature and the OD 421 was measured. The sugar concentration was calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalent (RE) (mg/ml).

Surface tension measurement. The surface tension (ST) of the cell-free broth from the bacterial strains tested under optima emulsification yield conditions was measured in the tensiometer (DataPhysics®, Oca15 plus), by pendant drop method at 28ºC. The instrument was calibrated with distilled water with readout of 71.62 ± 1.0 mN/m (Chen et al. 2007). The biosurfactant concentration was expressed as critical micellar concentration (CMC) (Batista 2006). The value of CMC was given by point center from the curve of the graph surface tension versus concentration cell-free broth containing the biosurfactant (Fox & Bala 2000; Kim et al. 1997). The interfacial tension (IFT) between the cell-free broth and crude petroleum oil was also measured by the inverted needle technique (Joshi et al. 2008).

Statistical analysis. All the biosurfactant production tests were performed in triplicate. The results of emulsification yield optimization, surface and interfacial tension were evaluated by the Kruskal-Wallis test, using GraphPad InsTat statistical program, being considered significant with $p < 0.05$.

Results and Discussion

Isolation, selection, and characterization of bacterial strains. A total of 55 bacterial strains were obtained from both types of soil. Fifty-five per cent were isolated from *Syngonanthus mucugensis*, 29% from *Stachytarpheta crassifolia*, and 16% from *Actinocephalus* sp. Sixty-two percent of the bacterial strains were isolated from soils collected in the dry season.

Four bacterial strains showed tensoactive properties by the drop-collapse method, which is an indirect measure of surface activity of an oil-analyzed sample. The bead forms because the polar water molecules are repelled from the hydrophobic surface. In contrast, if the water droplet contains surfactant, the force or interfacial tension between the water drop and the hydrophobic surface is reduced, which results in the spreading of the water drop over the hydrophobic surface (Bodour & Miller-Maier 1998).

The Slim03, Slim04, Slim15, and Slim17 strains were characterized according to Table 1. Slim03 and Slim15 strains were isolated from soils associated to *Actinocephalus* sp. and the phenotypical test results presented characteristics of Pseudomonadaceae (Konemam et al. 2001). Hence, Pseudomonas selective media evaluation was carried out and presented positive results. Sequenced 16S rRNA gene analysis indicated that both isolates belonged to the genus *Pseudomonas*, as detected by 99% level of similarity presented by Slim03 strain for *Pseudomonas* sp. and 96% level by Slim15 strain for *Pseudomonas fluorescens* (Migula). The other two strains, Slim04 and Slim17, were collected from *Stachytarpheta crassifolia* and showed low similarity profiles (< 90%) for the genus *Bacillus* when 16S rRNA gene sequences were analyzed by BLAST. The TSBA50 identification library also presented low similarity index (58%) to one specie of the genus *Bacillus* (*Paenibacillus macerans*), according to FAME analysis.

Screening of biosurfactant-producing bacterial strains. Of the four bacterial strains analyzed, only *Pseudomonas* sp. Slim03 and *P. fluorescens* Slim15 were able to produce extracellular biosurfactant in MSM, indicating that the tensoactive action of the Slim04 e Slim17 strains are related to the property of the cell surface (Al-

Table 1. Characterization of surfactant-producing bacterial strains isolated from soil of plants in the semi-arid area of Bahia. + Positive test; - negative test.

Characteristics	Bacterial Strains			
	Slim03	Silm04	Slim15	Slim17
Cellular morphology	rod-shaped	rod-shaped	rod-shaped	rod-shaped
Gram staining	-	+	-	+
Oxygen reactivity	+	+	+	+
Oxydase	+		+	
Catalase		+		+
Motility	+	-	+	-
Lysine	+	-	+	-
Glucose	+	+	+	+

Tahhan et al. 2000). Some surfactants act bonded to the cell wall to regulate the properties of the cell adherence to and release from surfaces, what is one of their most important survival strategies for colonizing an ecological niche (Rosenberg & Ron 1999), such as the rhizosphere of plants. However, the *Pseudomonas* sp. Slim03 and *P. fluorescens* Slim15 strains probably emulsify and solubilize complex natural compounds present in the soil into simpler compounds, which are used by cell membrane as carbon and energy sources, through transportation and translocation of insoluble substrates (Bognolo 1999). This fact favors their survival in extreme environments (Batista 2006), in addition to promoting the plant growth (Costanza et al. 1997).

Effect of carbon concentration on emulsifier production. The cell-free broth from bacterial strains presented good emulsification index (E24) against mineral oil (Nujol), which ranged between 27 and 58%, according to the period of culture. Bacteria culture supplemented with 0.5 and 1% glucose presented its best emulsification index (> 55%) in the periods of 48 and 120 h. There was no significant difference between these two concentrations nor between the two tested strains. The 0.5% glucose concentration was optimized for the production of extracellular emulsifier, taking into account the lower cost of medium composition. Bacterial growth and emulsification index were not obtained in non-inoculated control flasks, with the respective concentrations of glucose analyzed.

Both soils of *Actinocephalus* sp. present a low organic potential, and this enables one to gain a better understanding of the physiological behavior of the tested microorganisms, bearing in mind that high concentrations of carbon source caused a significant suppression of the generation rate as well as biosurfactant level, what occurred in this study using the concentration of 4% glucose in MSM, for both bacterial strains analyzed (results not shown). In the study conducted by Wei et al. (2005), a reduction in the biosurfactant level (rhamnolipid) by *P. aeruginosa* J4 was also observed when glycerol was used as a carbon source at a concentration higher than 2% and complete inhibition of bacterial growth when concentration exceeded 4%.

Effect of pH and temperature on emulsifier production. The influence of pH on the culture medium had a direct effect on the synthesis of biosurfactant, in the tested scale of values. The biosurfactant activity of the bacterial strains was higher than 58% with initial pH 7.0. Similar pH (7.0) result was observed with species of *Aeromonas* isolated from a tropical environment (Ilori et al. 2005). There was a small reduction in the rate of emulsification when the pH was less than 6.0, but no significant difference. Nevertheless, the production of emulsifier was reduced when the pH was higher than 7.0, with significant difference of the emulsification yield ratio for the two tested strains (Figure 1).

The temperature had little or no effect on emulsifier production of the analyzed strains, confirming the reports of Batista et al. (2006). Wei et al. (2005) showed that surfactant production by *Pseudomonas aeruginosa* increased with temperature from 25 to 30ºC, remained nearly constant for 30 and 37ºC, and decreased slightly when temperature was further increased to 42ºC. Similar temperature (30–40ºC) results were also observed with *Aeromonas* spp. isolated from a tropical environment (Ilori et al. 2005).

The higher E24 was detected at temperatures of 30 to 40°C, being optimized temperature of 30ºC due to its proximity to the room temperature. There was no significant difference when compared to emulsified production in different tested temperatures. These culture conditions (30–40°C) may enable a greater enzymatic activity and thus accelerate cellular metabolism, resulting in a higher biosurfactant production. The E24 remained stable for longer than 30 days (results not shown). The stability of the emulsifier produced by the bacterial strains indicates the great economic potential of this biomolecule to the food industry, modifying consistency, texture and phase dispersion (Banat et al. 2000).

Cell growth-associated emulsification yield. The relationship between cellular growth and emulsifier production throughout time was determined for the bacterial strains after culture condition optimization (0.5% glucose, pH 7.0, 30°C), in which the measurement of cell growth was expressed as biomass concentration by biosurfactant activity, indirectly measured by the emulsification index. For both bacterial strains, the biosurfactant production possibly is associated with the cellular growth, as an increase in the biomass concentration leads to an increment in the production of emulsifier (Figure 2). In the case of a growth associated biosurfactant production, there is a parallel relationship between the substrate utilization, cell growth and biosurfactant production throughout time (Desai & Banat, 1997). The results suggest that production of biosurfactant by the bacterial strains analyzed occurs between the exponential growth phase (Amiriyan et al. 2004) and early stationary phase. Santos et al. (2010) also observed the association of cellular growth and biosurfactant production by *Pseudomonas* sp. Slim03 strain and *Pseudomonas fluorescens* Slim15 strains using glycerol and vegetable oils as substrate in the periods of 24 to 48 h.

Orcinol assay. The quantification of the sample extracted sugar was determined in optimal culture conditions. The results show that possibly the surfactant produced by *Pseudomonas* strains belong to group of glycolipids (rhamnolipid) or polymeric surfactants (carbohydrate-lipid-protein). The higher concentration (230.8 mg/L) of sugar was produced by *P. areuginosa* Slim03 strain when compared with the *P. fluorescens* Slim15 strain (190.1 mg/L). Wu et al. (2008) observed, with *P. aeruginosa* EM1 strain, that glucose (4%) was an effective carbon substrate for rhamnolipid production, producing 7.5 g/L of rhamnolipid. Wei et al. (2005) show that *P. aeruginosa* J4 strain was able to produce rhamnolipid efficiently with glucose (4%) medium at a concentration of 1.7 g/L.

Although vegetable oils have been frequently used as the carbon substrates for rhamnolipid production with *P. aeruginosa* strains (Maier & Chavez 2000; Rahman et al.

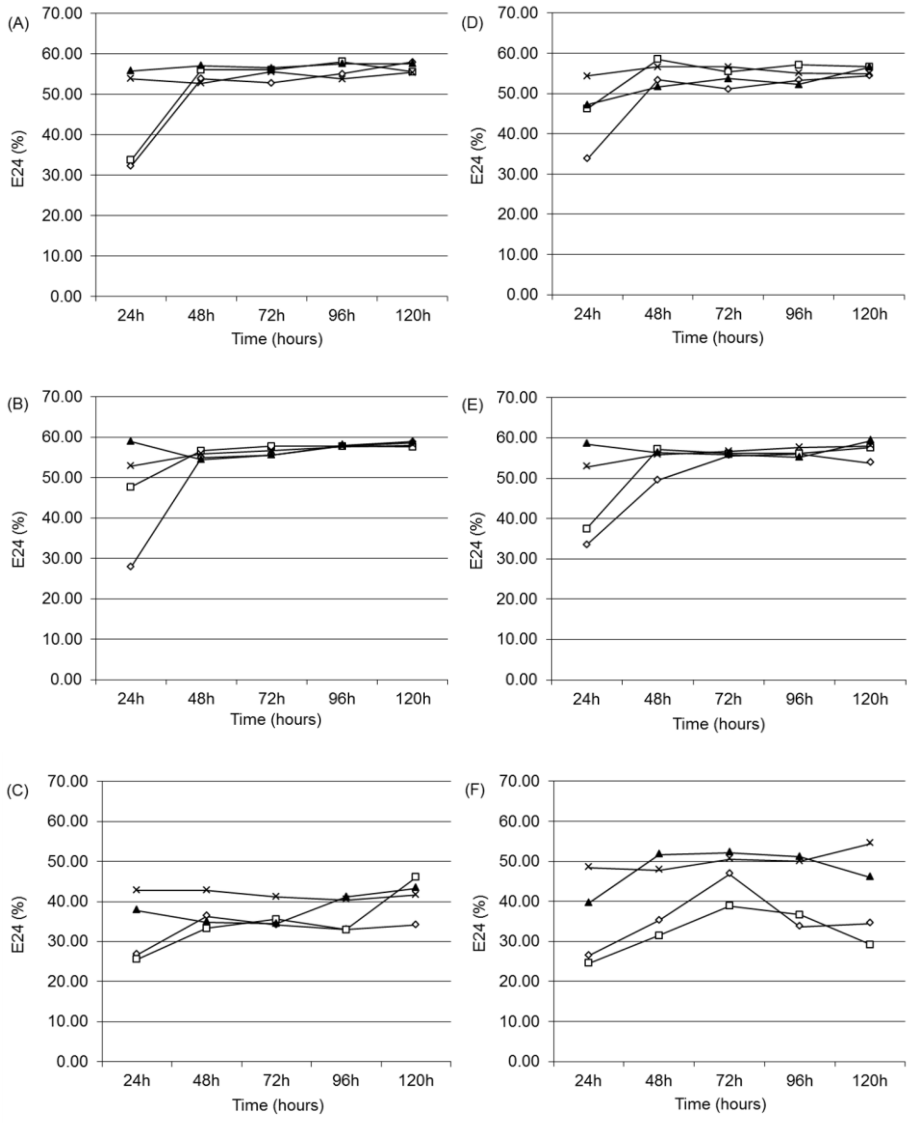

Figure 1. The effect of pH and temperature (25°C = ◊; 30°C = □; 35°C = ▲; and 40ºC = **X**) on emulsification yield: **A**, **B**, and **C** (at left)**-** *Pseudomonas* sp. Slim03 with pH 6.0, 7.0, and 8.0, respectively; **D**, **E**, and **F** (at right)**-** Slim15 *P. fluorescens* strains with pH 6.0, 7.0, and 8.0, respectively.

2002). Previous work reported a rhamnolipid concentration of 4.9, 5.4, and 4.8 g/L when sunflower, olive, and soybean oils (2%), respectively, were used as carbon sources by *P. aeruginosa* LBI (Benincasa et al. 2002). Wu et al. (2008) observed that *P. aeruginosa* EM1 strain attained a lower rhamnolipid yield from olive and soybean oils (8%) than that from glucose. The rhamnolipid production was 3.7 and 2.6 g/L for olive oil and soybean oil, respectively. Some reports show that vegetable oils were more efficient substrates in rhamnolipid production from *P. aeruginosa* strains when compared with glucose, glycerol, and hydrocarbons (Sandoval et al. 2001). However, the carbon source preference for rhamnolipid production seems to be strain dependent.

Surface tension measurement. The experiment was aimed at evaluating the tension-active properties of the cell-free fermented medium. Both *Pseudomonas* strains presented excellent surface tension reductions of the drop in the tested medium, *Pseudomonas* sp. Slim03 strain reduced from 73.52 mN/m to 31.14 mN/m and *P. fluorescens* Slim15 strain to 32.73 mN/m. These results were similar to the findings of Pornsunthorntawee et al. (2008) with *P. aeruginosa* SP4 strain, using soluble substrates as the only source of carbon and energy. The CMC of the cell-free broth containing the biosurfactant was approximately 40% to both bacterial strains tested. The results suggested that the crude biosurfactant provided excellent properties in terms of the reduction of surface tension and a low value of the CMC (Figure 3).

On this scale, one can note that increase in concentration reduced the ST until it attained a limiting ST value that corresponds to the CMC (Chen et al. 2007). In the study of

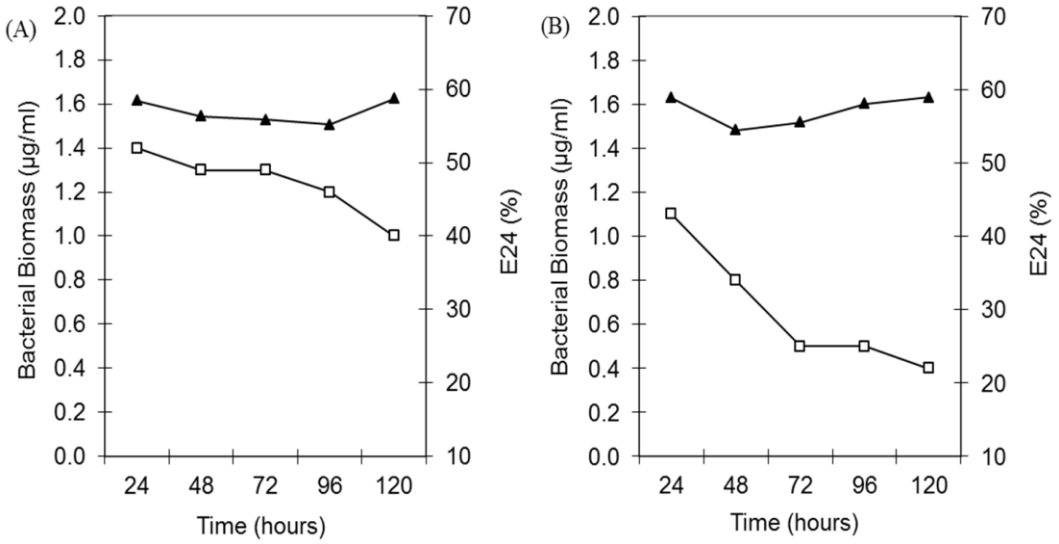

Figure 2. Variation in biomass concentration (µg/ml) (□) and emulsification index (E_{24}, %) (▲): **A-** *Pseudomonas* sp. Slim03 and **B-** Slim15 *P. fluorescens* strains, cultivated in MSM with 0.5 % glucose.

Batista et al. (2006), similar ST reduction behavior was observed as the concentration of the cell-free broth increased. Based on Kruskal-Wallis test, there was no significant difference ($p < 0.05$) in ST at the different concentrations of the analyzed samples. The result of ST is promising because, according to Batista et al. (2006), the concentration of the cell-free broth biosurfactant is equivalent to five to ten times above that of a CMC value of an isolated biosurfactant. The cell-free broth of *Pseudomonas* sp. Slim03 and *P. fluorescens* Slim15strains reduced the interfacial tension (IFT) of crude petroleum from 17.16 mN/m to 1.88 and 2.41 mN/m, respectively. The reduction in IFT indicates the ability of the surfactant to remove oil, reflected on the increase in the angle of contact and in the reduction of capillary force, which maintains the crude oil adsorbed in the soil (Urum & Pekdemir 2004). These biosurfactants shows potential biotechnological for bioremediation processes and also to enhance oil recovery in the petroleum industry, contributing positively to improve oil recovery by reducing IFT between oil and water and also by stabilizing water-oil emulsions (Bognolo 1999).

Conclusion

The biosurfactant-producing bacterial strains were isolated from soils of *Actinocephalus* sp., and belonged to the genus *Pseudomonas*. The results indicate that the culture optima conditions for the production of biosurfactant by the bacterial strains from semi-arid environments studied are MSM with 0.5 to 1 % glucose, pH 7.0, temperature of 30–40ºC, by 24 to 48 h of incubation. The *Pseudomonas* sp. Slim03 and *P. fluorescens* Slim15 strains have the ability to produce emulsifier and reduce the surface and interfacial tensions of water and crude petroleum, respectively. This study represented an unprecedented discovery of surfactant activity of the bacterial strains isolated from soils of plants from the semi-arid region, confirming the environmental influence on the metabolism of the tested microorganisms, and making them an interesting alternative for biotechnological applications.

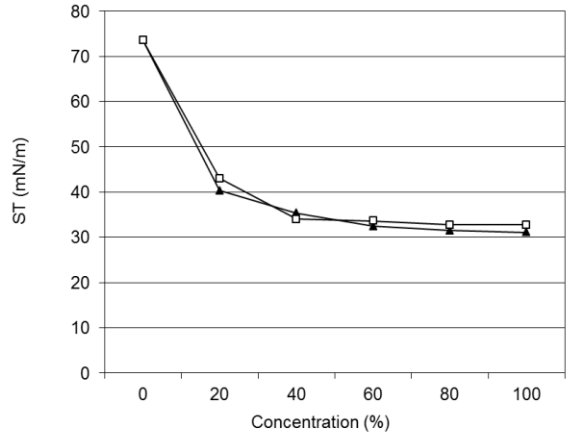

Figure 3. Surface Tension (mN/m) versus cell-free broth containing the biosurfactant (%) of *Pseudomonas* sp. Slim03 (▲) and *P. fluorescens* Slim15 (□) strains.

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