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Full Length Research Paper

Evaluation of substrates from renewable-resources in biosurfactants production by *Pseudomonas* strains

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The aim of the present study is to evaluate biosurfactant production by two strains of *Pseudomonas* spp. using alternative carbon sources. The bacterial strains used were isolated from the Municipal Park of Mucugê, Bahia State, Brazil. Different vegetable oils and glycerol concentrations were evaluated and used individually, as the sole source of carbon and energy for the production of biosurfactant which was measured by the test of emulsification and surface tension. The best concentration of alternative substrates achieved was 0.5%, with emulsion formation rates ranging from 50 to 59% for both bacterial strains analyzed. The free-cell broth of *Pseudomonas* strains had the ability to reduce the surface tension of all culture media tested, although it was more effective when glycerol was used. The results confirm the potential of these alternative substrates, based on the availability and low cost of these raw materials, to reduce the final production cost and also the expenses with waste treatment.

Key words: *Pseudomonas*, biosurfactants, substrates, renewable-resources.

INTRODUCTION

Surfactants are a class of chemical compounds widely used in several industrial fields. The industrial demand for surfactants has increased to over 300% in the chemical industry for the last decades and the world production is estimated to exceed 3 million tons each year (Banat, 2000). However, there is a tendency in industrialized countries to replace synthetic surfactants for natural ones which are also called biosurfactants. This tendency is motivated by different advantages of biosurfactants such as, lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity, specific activity at extreme temperatures, pH and salinity and the ability to be synthesized from renewable feedstocks (Desai and Banat, 1997). Other important aspects are the growing environmental concern of consumers associated with new environmental laws and the fact that biosurfactants are not oil-derived, and then, not directly associated to oil price, which increases considerably on a day-to-day basis (Nitschke and Pastore, 2003).

Biosurfactants are a structurally diverse group of surface active molecules synthesized by microorganisms (Banat, 1995) and can be categorized as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids and neutral lipids(Benincasa et al.,

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Abbreviations: TSA, Triptic soyer agar; **MSM,** mineral saline medium; **TSB,** tryptic soy broth; **ST,** surface tension; **CMC,** critical micellar concentration; **SDS,** sodium dodecyl sulfate; **PAHs,** polycyclic aromatic hydrocarbons; **LCFAs,** long chain fatty acids.

2002). These amphiphilic compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce interfacial tension between different fluid phases. Their uses and potential commercial applications have been reported in several fields, including surfactant-assisted flooding for enhanced oil recovery in the oil industry, emulsifiers in the food industry and moisturizers in the cosmetic industry (Cameotra and Makkar, 2004).

However, biosurfactants are not yet widely used in industry due to high production costs associated to inefficient methods for product recovery and expensive substrates, the expected breakthrough in terms of applications of these biosurfactants remains to be achieved. The cost can be reduced by strain improvement, optimizing medium composition by statistical methods or by using alternative low-cost substrates. The choice of cheap raw materials is important to the overall economy of the process as they account for 50% of the final production cost and also reduce the expenses with waste treatment (Makkar and Cameotra, 1999).

Bacteria of the genus *Pseudomonas* has been highlighted by its ability to use mannitol, fructose, n-paraffins and vegetable oils as carbon sources to produce rhamnolipid-type biosurfactants, which are among the most effective surfactants known today (Raza et al., 2007). *Pseudomonas* strains are known to produce a glycolipid surfactant containing rhamnose and 3-hydroxy fatty acids (Lang and Wullbrandt, 1999). Although, there are several types of rhamnolipid species, all of them possess similar chemical structure and have an average molecular weight of 577 (Torrens et al., 1998). The crude biosurfactant extracted from the liquid culture of *Pseudomonas* strains were found to reduce the surface tension of water from 72 to 30mN/m, with an emulsification index of above 70% and a critical micelle concentration in the range of 5 - 200 mg/l, depending on the components of the mixture (Nitschke et al., 2005).

Large-scale biosurfactant production costs can be reduced through process optimization (Cunha and Leite, 1997; Lang, 2002). Optimizing factors that affect growth in biosurfactant producing organisms with potential for commercial exploitation is of paramount importance (Abouseoud et al., 2008). In this sense, the present work aimed to evaluate substrates from renewable resources as unique carbon and energy sources for biosurfactant production by *Pseudomonas* strains.

MATERIALS AND METHODS

Microorganisms

Pseudomonas spp. Slim03 and *Pseudomonas fluorescens* Slim15 strains colonies, isolated in the Municipal Park of Mucugê, Bahia State, Brazil, were seeded in triptic soyer agar (TSA) (Himedia) and kept in an incubator at 30°C, for 24 h. Afterwards, bacteria were suspended in saline solution (0.9%, w/v, NaCl) to a 10^8 ufc/ml concentration, based on an optical density scale (1.0) on a 600 nm filter, as standard inocula along the study.

Carbon sources

Alternative carbon sources such as corn and sunflower oils (crude commercial ones) and a standard source as glycerol (Merck) were tested individually as substrates for biosurfactant production.

Media and growth conditions

Three milliliter of each bacterial suspension were transferred to a 500 ml Erlenmeyer flask containing 100 ml of 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; pH 7.0) with different glycerol and vegetable oil concentrations (0.5, 1, 2 and 3%, w/v), as the sole carbon source. The samples were incubated on an orbital shaker at the speed of 180 rpm, for 5 days and 5 ml aliquots were taken from the bacterial cultures every 24 h and centrifuged (5.578 g for 20 min at 10°C) to obtain a cell-free broth.

Emulsification yield optimization

The MSM, as previously described, was used as base medium, changes were made in the glycerol and vegetable oil 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 was determined by the emulsification index (*E*24). The test was performed in 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 were determined using optimal culture conditions. Culture aliquots were removed every 24 h for absorbance readouts (600 nm) in the spectrophotometer (Cary, 50 Probe), according to the calibration curve of the dry weight versus absorbance.

Bacterial biomass concentration

The bacterial strains were seeded in TSA and kept in an incubator at 30°C, for 24 h. Afterwards, a colony was transferred to 500 ml Erlenmeyer flasks containing 100 ml 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 10°C) and the bacterial biomass was resuspended in distilled water to prepare the dry weight versus absorbance curve (600 nm).

Surface tension measurement

The cell-free broth surface tension (ST) of the bacterial strains tested under optima emulsification yield conditions was measured in a tensiometer (DataPhysics®, Oca15 plus), by the pendant drop method at the temperature of 28°C. The instrument was calibrated

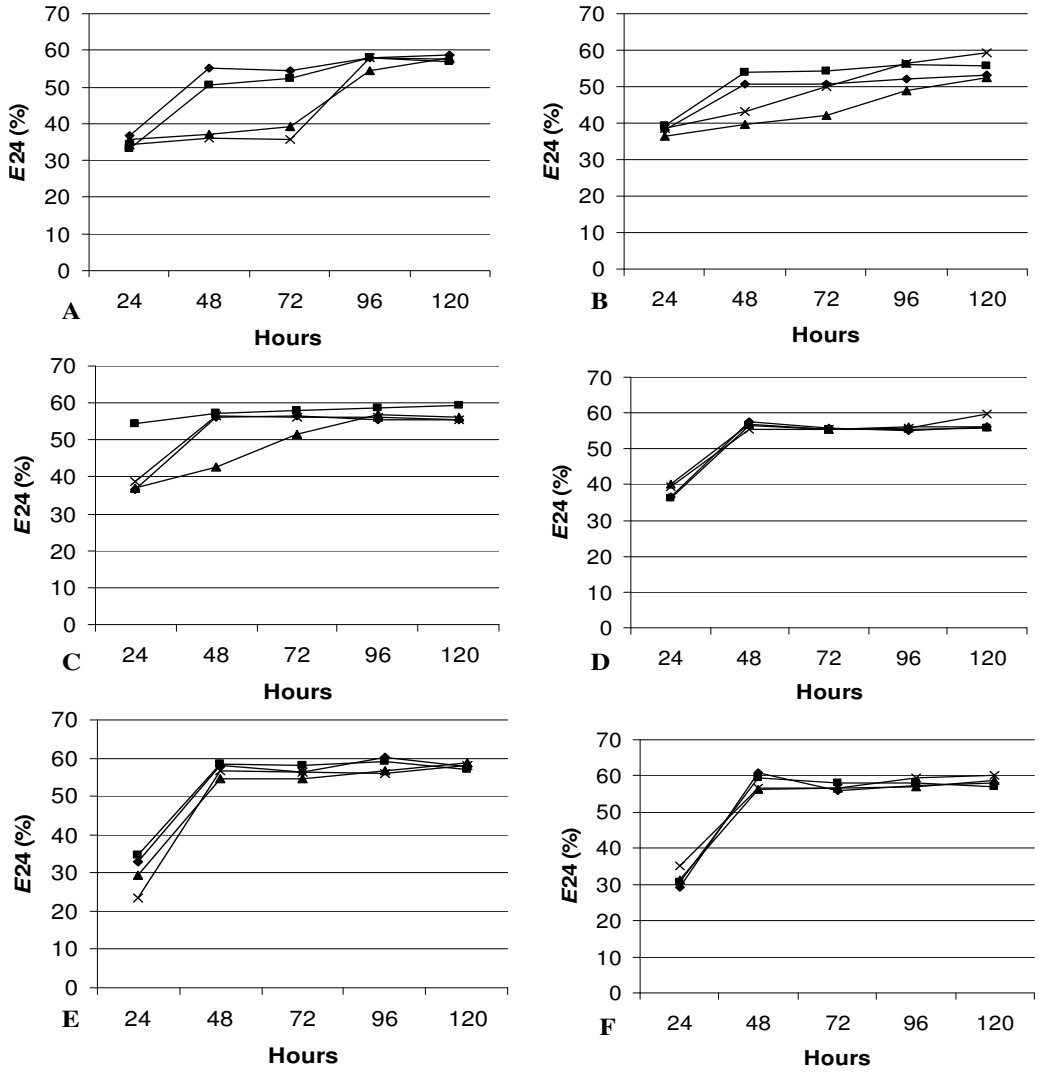

Figure 1. Carbon concentration effect (0.5%, ♦; 1%, ■; 2%, ▲; and 3%, **X**) on emulsification yield (*E*24, %). A, Slim03- corn oil substrate; B, slim15-corn oil substrate; C, slim03- sunflower oil substrate; D, slim15- sunflower oil substrate; E, slim03-glycerol substrate; F, slim15-glycerol substrate. Slim03 strain, *Pseudomonas* sp.; slim15 strain, *P. fluorescens*.

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 et al., 2006). The CMC was graphically estimated by the comparison of ST (mN/m) and cell-free broth concentration (%) (Fox and Bala, 2000). One percent sodium dodecyl sulfate (SDS) (J.T.BAKER) and non-inoculated MSM (addition of the respective substrates tested) were used as positive and negative controls, respectively, in all biosurfactant assays performed.

Data analysis

All biosurfactant production assays were performed in triplicate and the results of emulsification yield, cell growth and ST were evaluated by Kruskal-Wallis test, using GraphPad InsTat statistical package. Statistical significance was achieved by a $p < 0.05$.

RESULTS AND DISCUSSION

Effect of carbon concentration on emulsifier production

Pseudomonas spp. strains were able to produce biosurfactants by using the alternative substrates tested as the only source of carbon and energy (Figure 1). The vegetal oils proved to be promising substrates to be used for biosurfactants production, presenting emulsification indexes (E24) ranging from 33 to 59%. When Abouseoud et al. (2008) cultured a *P. fluorescens* strain using olive oil as the carbon source, they found an emulsifying activity of 49%. Based on our results, the use of other vegetal oils achieved even higher emulsifying activities,

indicating them to be better carbon sources for surfactant synthesis when compared to olive oil.

Cell-free broth obtained from vegetal oil-cultured bacteria presented a differentiated emulsifier production behavior (Figure 1). The corn oil emulsification index scored its highest result in concentrations of 0.5 and 1%, while all sunflower oil concentrations tested did not present differences. Nevertheless, 2% sunflower oil-cultured *Pseudomonas* spp. Slim03 strain presented a slower emulsifier production during 72 h (Figure 1). There were no statistically significant differences of E24 for sunflower oil at all nor in 0.5 and 1% corn oil concen-trations tested on 48 h-cultured bacteria. However, when comparing the cell-free broths obtained from cultures with 2 and 3% of corn oil, there was a statistically significant difference on emulsification related to concentration and culturing time. The E24 after 48 h of incubation shows that biosurfactant biosynthesis stopped and is probably due to the production of secondary metabolites which could interfere with emulsion formation and the adsorp-tion of surfactant molecules at the oil-water interface (Bonilla et al., 2005). The results show the ability of *Pseudomonas* spp. strains to grow and to produce, effi-ciently, biosurfactants with different concentrations of vegetal oils, emulsifying and stabilizing oil emulsions with different viscosities, for example, 5W-30 (ACDelco), 40 (SAE) and 25W-60 (Helix) mineral oils (results not shown). The use of vegetal oils as carbon sources to pro-duce biosurfactants seems to be an interesting and low cost alternative to the usual ones (Ferraz et al., 2002).

Bacteria culture supplemented with glycerol presented its best emulsification index (>54%) in 48 h. No bacteria growth or emulsification index was detected in control or non-inoculated flasks containing the respective analyzed carbon sources. The positive control flask, containing 1% SDS, presented average emulsification index of 65.13%. The results showed that substrate concentration did not interfere with biosurfactants production in the species studied. The values found did not present statistically significant differences when compared to tested concen-trations over time. However, Wei et al. (2005) observed that the biosurfactant level decreased sharply when glycerol concentration was over 2%. Moreover, the growth of *Pseudomonas aeruginosa* J4 completely terminated as the glycerol concentration exceeded 4%, resulting in negligible rhamnolipid production in the culture. However, the significant inhibitory effect of glyce-rol was not verified in this study and has not yet been revealed in the literature.

Results confirmed *Pseudomonas* spp. strains' ability to use water-soluble and insoluble substrates to produce biosurfactants, corroborating the results of Cooper et al. (1981). *Pseudomonas* strains studied release surfactants/ emulsifiers in order to facilitate assimilation of substrates (Bicca et al., 1999), being capable of emulsifying and solubilizing hydrophobic compounds in contaminated areas, bringing a distinct advantage for bioremediation processes (Cassidy and Hudak, 2001). Scheibenbogen et al. (1994) found that biosurfactant from *P. aeruginosa* UG2 was able to effectively remove a hydrocarbon mixture from a sand loam soil and that the degree of removal was dependent on the type of hydrocarbon removed and the concentration of the surfactant used. Moreover, Van Dyke et al. (1993) previously found that the same strain could remove, at a concentration of 5 g/l, approximately 10% more hydrocarbon from a sandy loam soil than from a silt loam soil and that SDS was less effective than the biosurfactant in removing hydrocarbon.

The ramnolipids from the same strain in a bioslurry could enhance the solubilization of four-ring polycyclic aromatic hydrocarbons (PAHs) more significantly than three-ring PAHs and that the biosurfactants were five times more effective than SDS (Deschênes et al., 1994). Synthetic surfactants used to increase contaminant solu-bility are often toxic, representing an additional source of contamination (Bognolo, 1999). However, biosurfactants have similar properties but are less toxic, biodegradable and can be produced *in situ*, at the contaminated site (Cha, 2000). These biosurfactants are not only of interest for bioremediation processes in the petroleum industry, but these compounds can be used also to enhance oil recovery from wells, reduce the heavy oil viscosity, clean oil storage tanks, increase flow through pipelines and stabilize water-oil emulsions (Bognolo, 1999).

Cell growth-associated emulsification yield

The 0.5% glycerol and vegetable oil concentrations were optimized to allow the cell growth-associated emul-sification yield and surface tension measurement, taking into account the lower cost of medium composition.

The *Pseudomonas* spp. strains showed a punctual difference on cellular growth when vegetal oils were used (Figure 2). The biggest bacterial biomass (6.6µg/ml) was obtained by *Pseudomonas* spp. Slim03 strain using corn oil as substrate. Maximum *E*24 value was reached after 48 h of vegetal oil culturing without significant differences on emulsifier production. The results showed differences of physiological behavior among the bacteria strains studied when cultured with corn oil which were not observed for sunflower oil (Figure 2). On the other hand, there was a parallel relationship between the substrate utilization, growth and biosurfactant production by the glycerol-cultured *Pseudomonas* strains studied (Desai and Desai, 1993), this may be explained by the extended growth phase (Das et al., 2009). These results indicate that biosurfactants production by microorganisms possibly occurs during exponential growth phase which suggests that biosurfactant is being produced as a primary meta-bolite, following the cellular biomass formation (Amiriyan et al., 2004). The growth-associated biosurfactant

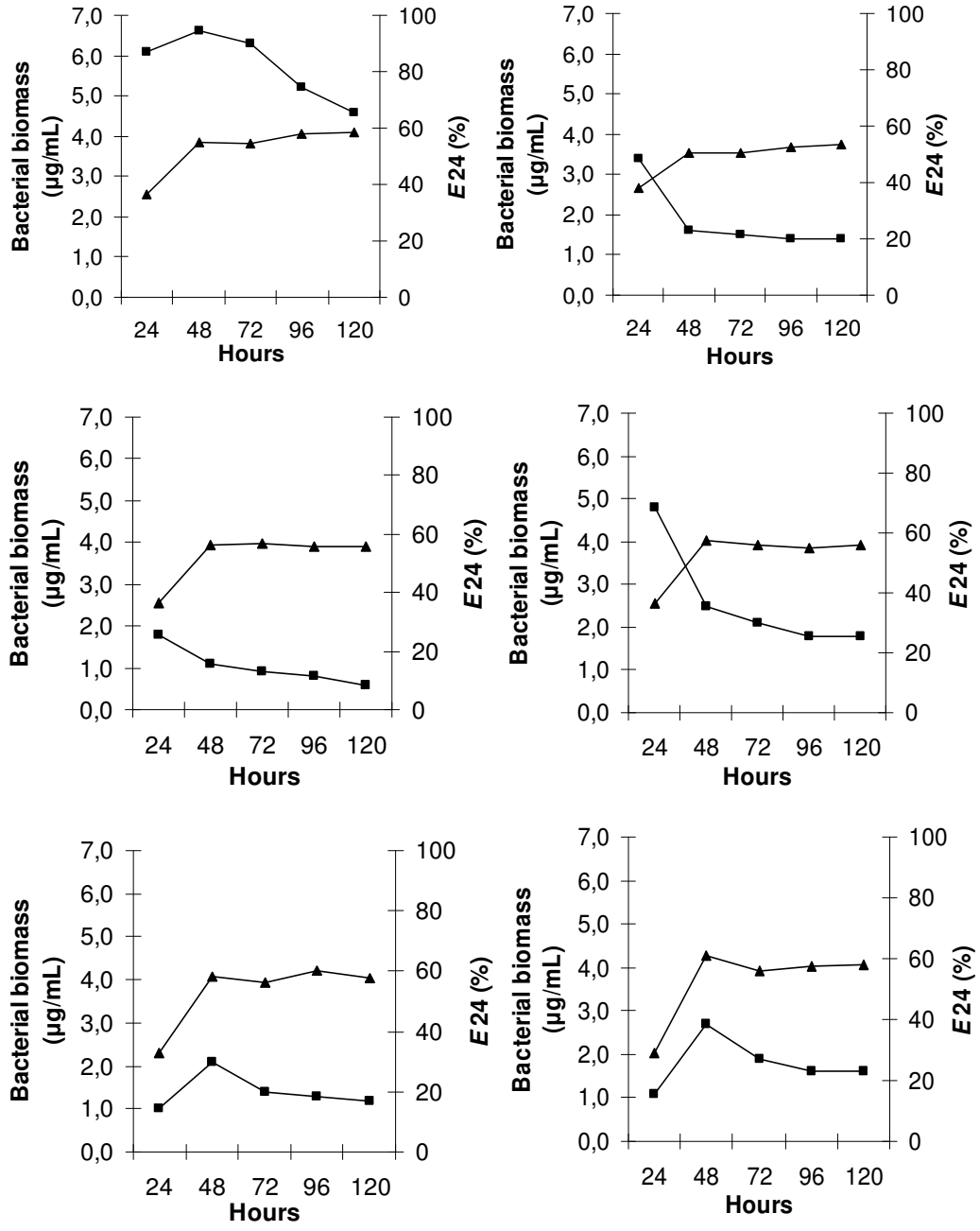

Figure 2. Variation in biomass concentration (µg/ml, ■) and emulsification index (%, ▲). A, Slim03-corn oil substrate; B, slim15-corn oil substrate; C, slim03- sunflower oil substrate; D, slim15-sunflower oil substrate; E, slim03-glycerol substrate; F, slim15-glycerol substrate. Slim03 strain, *Pseudomonas* sp.; slim15 strain, *P. fluorescens*.

production might facilitate cells' adherence to substrate molecules and metabolize them (Barathi and Vasudevan, 2001). Diversity exists among the biosurfactant producing microorganisms, suggesting that biosurfactant production is an important survival tool for producing microbes and appears to have evolved in an independent yet parallel fashion (Bodour et al., 2003).

Surface tension measurement

Results obtained in the biosurfactants production assays with different alternative substrates show their abilities to reduce ST of the tested media. The cell-free broths from *Pseudomonas* spp. Slim03 and *P. fluorescens* Slim15 strains presented excellent ST reduction in corn oil

supplemented medium from 76.35 to 35.26 and to 49.15 mN/m, respectively; in sunflower oil supplemented medium, from 78.06 to 38.12 and to 44.65 mN/m, respectively; and glycerol supplemented medium, from 78.24 to 31.75 and 30.85 mN/m, respectively (Figure 3). The analyzed vegetal oils presented great potential as substrates for biosurfactants production by *Pseudomonas* spp. strains, confirming the reports of Wei et al. (2005) analyzing *P. aeruginosa* ability to produce biosurfactants using different vegetal oils as substrates. The probable reason for this tendency of *Pseudomonas* strains may rely on the fact that it has a lipase activity which facilitates assimilation of fatty acids contained in vegetal oil fractions (Maier and Soberon-Chavez, 2000; Abouseoud et al., 2008), acting on these oils to form long chain fatty acids (LCFAs), composed of 12 to 18 atoms of carbon (Wei et al., 2005). The LCFAs can be β-oxidized to keep cellular growth or be transformed in a lipidic precursor in order to promote biosurfactant biosynthesis (Lang and Wullbrandt, 1999; Maier and Soberon-Chavez, 2000). Nonetheless, the best biosurfactants production rate associated with active surface property was observed with glycerol as the sole source of carbon.

Glycerol usage was indicated as the most efficacious substrate for surface tension reduction of the tested media which is easily observed by comparing with vegetal oils (Figure 3), confirming the results of Santa Anna et al. (2002). Das et al. (2009) considered the glycerol as a good carbon source for biosurfactant production. Bordoloi and Konwar (2007) showed that a *P. aeruginosa* (MTCC7815) bacterial isolate exhibited its maximum growth in glycerol containing medium and the biosurfactant produced by it lowered the surface tension of the culture medium from 70.2 to 29.7 mN/m. A number of microorganisms are able to grow on glycerol-containing media as the sole carbon and energy source (Seifert et al., 2001), such as *Klebsiella*, *Citrobacter, Clostridium* and *Enterobacter* in which glycerol is metabolized both oxidatively and reductively (Zhu et al., 2002). Like other small uncharged molecules, glycerol can cross the cytoplasmic membrane through passive diffusion. However, cells limited to passive uptake have a growth disadvantage at low concentrations of substrate (Voegele et al., 1993).

The cell-free broth from *Pseudomonas* spp. Slim03 and *P. fluorescens* Slim15 cultured on vegetal oils showed a critical micelle concentration (CMC) higher than those achieved with glycerol (control). However, cultures grown with glycerol as carbon source resulted in cell-free broths presenting CMC ranging from 30 to 40% for *P. fluorescens* Slim15 strain and 50 to 60% for *Pseudomonas* spp. Slim03 (Figure 3) and, as expected, there was a reduction on the surface tension of the drop related to increased cell-free broth concentration. In bioremediation process, the mechanisms of surfactant-enhanced soil washing occur when surfactant monomers increase the

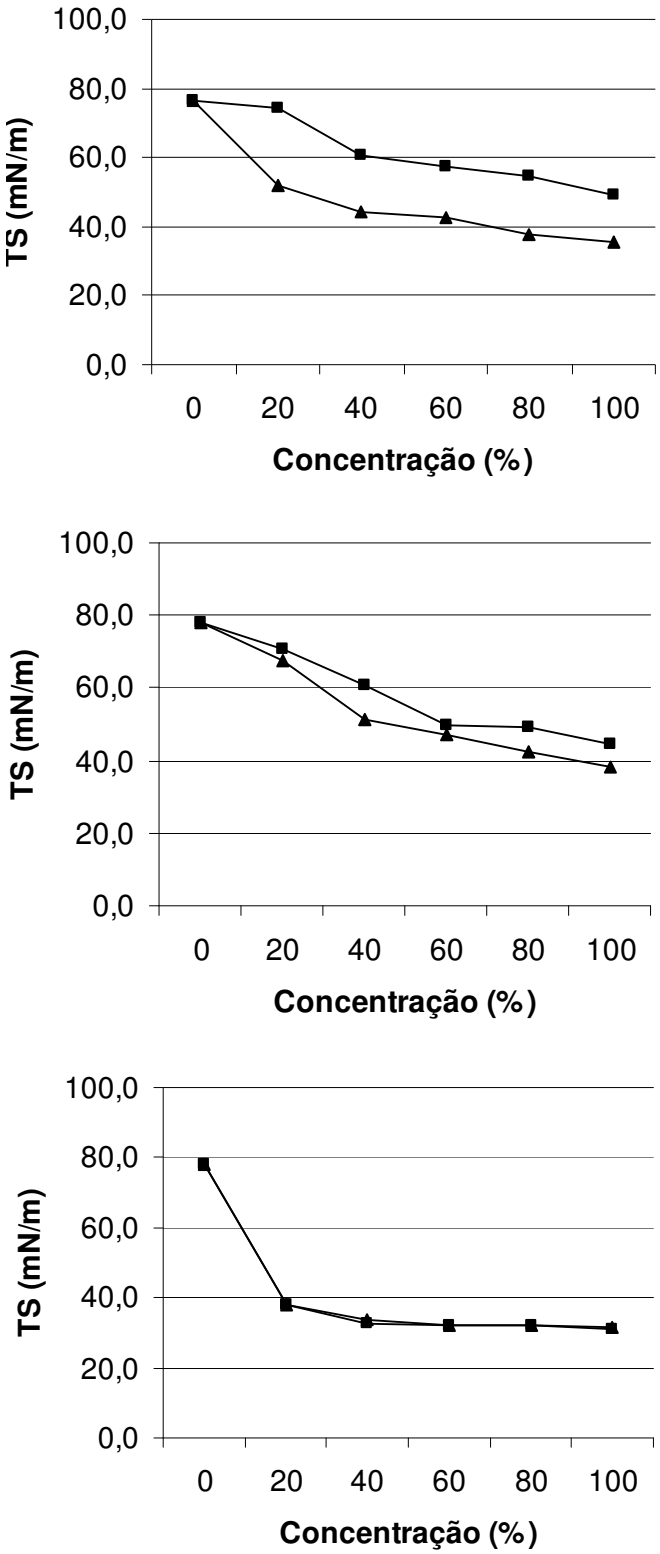

Figure 3. Surface tension (mN/m) versus cell-free broth concentration (%) of *Pseudomonas* spp. Slim03 (▲) and *P. fluorescens* Slim15 (■) strains cultivated with corn oil, sunflower oil and glycerol at 0.5% concentration. A, Corn oil substrate; B, sunflower oil substrate; D, glycerol substrate.

contact angle between the soil and hydrophobic contaminant, thereby promoting the separation of contaminant from soil particles and finally displacing the oil from the soil. After this stage, solubilization occurs when contaminants are partitioned from the soil into the hydrophobic core of surfactant micelles. Micellar phase bioavailability of hydrophobic organics means that contaminants partitioned into the micellar phase are biodegradable without having to transfer to the dissolved phase first (Deshpande et al., 1999).

The ST results presented great potential as the concentration of biosurfactants in cell-free broth may reach five to ten times the CMC values of an isolated biosurfactant (Batista et al., 2006). Vegetal oils and glycerol used as carbon source for biosurfactant production seem to be a good and economically possible alternative for usual ones (Boulton and Ratledge, 1987). Moreover, the biosurfactant released into the culture medium is interesting from an industrial point of view, because the product recovery process can be simplified (Cameotra and Makkar, 1998; Kuyukina et al., 2001).

Conclusion

The analyzed *Pseudomonas* spp. strains were able to produce biosurfactants efficiently using carbon renewable-resources. The optimum concentration of alternative substrata in the tests of production of biosurfactants was 0.5%. Glycerol was considered the best carbon source, which became a promising substratum for the mass production of biosurfactants and to make renewable energy available for sustainable biodiesel production. The potential of these alternative substrata can make possible the increase of biosurfactants production, in view of the availability and the low cost of the raw materials, reducing the final production cost and also waste treatment expenses.

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