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PRODUCTION OF BIOSURFACTANTS FROM *Pseudomonas aeruginosa* PA1 ISOLATED IN OIL ENVIRONMENTS

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Abstract - The potential production of rhamnolipid-type biosurfactants is assessed based on the development of a fermentative process with a strain of *Pseudomonas aeruginosa* PA1, which was isolated from oil production wastewater in the Northeast of Brazil. These production of molecules using different carbon (n-hexadecane, paraffinic oil, glycerol and babassu oil) and nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$ and CH_4N_2O) was studied. The best results were obtained when using glycerol as substrate. A C/N ratio of 60/1 and use of sodium nitrate as nitrogen source resulted in higher production of the rhamnolipid, expressed by rhamnose (3.16 g/L) and by the yield in relation to biomass (Yp/x = 0.70 g/g). Additionally, physical-chemical characteristics of the spent broth with and without cells were studied, providing a low critical micelle concentration of 19 mg/L and toxicity values of 13 and 13.8 mg/L using two test organisms, the micro crustacean *Daphnia similis* and the bacterium *Vibrio fisheri* (Microtox), respectively.
Keywords: production of biosurfactants, glycolipids, rhamnolipids, *Pseudomonas aeruginosa*, surface-active substances.

INTRODUCTION

Research in the area of biosurfactants has expanded quite a lot in recent years due to its potential use in different areas, such as the food industry, agriculture, pharmaceutics, the oil industry, petrochemistry and the paper and pulp industry amongst others. The development of this line of research is of paramount importance, mainly in view of the present concern with protection of the environment. Therefore, the most significant advantage of a microbial surfactant over chemical surfactants is its ecological acceptance because it is biodegradable and nontoxic to natural environments (Garcia, 1992).

In situ research has shown that biosurfactants can remove polluting agents from the environment. Biosurfactants produced by a strain of *Pseudomonas aeruginosa* SB30 were used to remove oil from gravel in the Exxon Valdez oil spill in Alaska. A 1% biosurfactants solution was enough to remove twice as much oil as water at temperatures ranging from 10^0 to 80^0C (Harvey et al. 1990). Nonetheless, in order to gain a significant share at the market, biosurfactants must be produced at low cost.

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Therefore, it is necessary to know more about the producing microrganism's physiology and the process engineering to develop the technology for these production of molecules, the use of cheap substrates being of utmost importance (Fiechter, 1992).

The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils, to produce rhamnolipid-type biosurfactants (Boulton and Ratledge, 1987). Several studies have been carried out to define the best ratio between carbon, nitrogen, phosphorus and iron needed to obtain high production yields.

Optimization of the carbon/nitrogen ratio in continuous cultures of *Pseudomonas aeruginosa* has been studied, indicating ratios between 15 and 23 as the optimum range for achieving high specific productivity of rhamnolipids, using glucose and vegetable oil as substrates, respectively (Ochener *e*t al. 1995). After nitrogen has been fully consumed, cell metabolism is directed to producing rhamnolipids, whose production increases after the exponential growth phase (Venkata and Karanth, 1989).

The purpose of this work was to study the production of a rhamnolipid-type biosurfactant by a strain isolated from oil environments, as well as to evaluate the tension-active proprieties and the toxicity of the spent broth.

MATERIALS AND METHODS

Isolation, Identification and Preservation of the Microorganism

The microorganism was isolated from wastewater samples collected from oil wells in the Northeastern of Brazil. The method of serial dilutions of the sample and plate count in selective medium Cetrimide agar (Merck, Darmstadt) was used for isolation purposes. The plates were incubated at 30^0C for 48 hours.

The bacterial culture was identified using the BIOLOG^{TM} automated identification system (Biolog, Inc., Hayward) for gram-negative bacteria. The results were compared with the Microlog software database in order to determine the coefficient of similarity of the genus established in the identification system (Biolog, 1993). The bacterium was maintained in liquid nitrogen in a glycerol solution (10% v/v) at -196^0C.

Inoculum

The strain was activated in a triptic soyer agar medium (TSA) (Merk-Datmstadt), cultivated at 30^0C for 48 hours and transferred to a 250 mL flask, containing 50mL of TSA. The flask was incubated at 30^0C and 250 rpm during 20 hours. Cells were havested by centrifugation at 7000 rpm during 20 minutes. The centrifuged microbial mass was suspended in a culture medium (medium salt production - MSP) with the following composition (g/L): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 3.0; $MgSO_4.7H_20$, 0.2. The pH was adjusted to 7.0 with a solution of KOH (1N) plus 1% v/v of glycerol P.A. (Merck) in order to obtain the initial inoculum concentration of 0.005, 0.075 and 0.1g/L, in accordance with a calibration curve of dry weight *versus* absorbance (Venkata and Karanth, 1989).

Fermentations

The production of rhamnolipids was studied during a seven-day fermentation period in flasks under agitation with the initial seeding material standardized in a culture medium, as mentioned previously, maintained at a temperature of 30^0C and stirred in a rotary shaker at 120 rpm. The carbon sources used were N-hexadecane (Merck, Darmstadt), paraffinic oil collected at flowing wells in the Buracica area of the state of Bahia, Brazil, consisting of 32% saturated hydrocarbons, 23% aromatics, 36% of resins and 9.1% asphaltenes), glycerol (PA - Merck, Darmstadt) and babassu oil (Du Reino). In addition to the carbon sources studied, the C/N ratio varied with the following concentrations of glycerol: 0.5, 1, 2, 3, 4, 5 and 6% v/v, corresponding to C/N ratios of 20, 40, 60, 80, 100 and 120. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, $NaNO_3$, $(NH_4)_2SO_4$ and CH_4N_2O were employed at the following concentrations: 1.45, 1.0, and 0.51 g/L and glycerol 3% v/v.

Biomass Concentration

Bacterial growth was monitored by measurement of absorbance (Micronal – mod. B442) at a wave length of 500 nm. Samples of 50 mL were removed from the flasks at regular intervals and centrifuged at 7000 rpm for 20 minutes. The centrifuged cells were suspended in 5 mL of distilled water and the biomass, expressed in dry weight (g/L), was obtained from a calibration curve.

Quantification of Rhamnose and Glycerol

The quantification of rhamnolipids expressed in rhamnose (g/L) was measured in the cell-free culture medium, using the phenol sulfuric acid method (Dubois et al. 1956). Glycerol was assessed by the enzymatic-colorimetric method for triglyceride content evaluation (CELM - Brazil).

Determination of the Critical Micelle Concentration (CMC)

The determination of CMC was performed by several dilutions of free-cell fermented medium containing rhamnolipids produced after seven days of fermentation. Superficial tension was measured using SIGMA 70 digital tensiometer (KSV Instruments LTD, Helsinki, Finland) at room temperature, as recommended by Du Nouy (ASTM D71, 1999).

Toxicity Assays

The toxicity assays were performed in the fermented medium with and without bacterial cells, using two organisms as toxicity indicators, a microcrustacean, *Daphnia similis*, and the bacterium *Vibrio fischeri*. Shortly thereafter, a culture suspension of the bacterium *Vibrio fischeri* was exposed to different concentrations of the sample of 0.281 to 45% v/v in water. Toxicity was measured in terms of luminescence reduction naturally emitted by the bacterium. The actual concentration of the sample that causes 50% of the lethal effect (CL50) was obtained by readings after 5 and 15 minutes of exposure, and the readings were done in piece of equipment known as the MICROTOX System.

The toxicity test using the microcrustacean *Daphnia similis* was carried out with organisms aged between 6 and 24 hours, exposed to different concentrations of samples (0.5; 1.0; 10; 50 and 100 v/v). Toxicity was measured in terms of the effects on mortality after 24 and 48 hours of exposure.

RESULTS AND DISCUSSIONS

Microbial Isolation, Identification and Preservation

The strain was identified as *Pseudomonas aeruginosa* by the BIOLOG^{TM} automated system and had a 99.7% similarity with the standardized strain of *Pseudomonas aeruginosa*, thus receiving code PA1. This strain showed an ability to use carbon sources, such as fructose, glucose, mannitol, mannose, glycerol and lactic acid (results not shown), which are known as good carbon sources for rhamnolipid production. (Venkata and Karanth, 1989).

Effect of the Carbon Source

The production of rhamnolipids by the *Pseudomonas aeruginosa* strain PA1, using substrates such as n-hexadecane, paraffinic oil, babassu oil and glycerol, is displayed in Table 1. The strain was able to use n-hexadecane, producing 130 mg/L of rhamnose, with a 47.7% drop in surface tension at the end of seven days of fermentation. The use of paraffinic oil, which is a very complex and heterogeneous carbon source, resulted in a considerable production of rhamnolipids (260 mg/L) however, practically no variation in surface tension was found at the end of fermentation (4.4%). This fact could probably be due to the formation of an emulsion during fermentation, which interfered in the quantification of the surface tension. The use of vegetable oil and glycerol as carbon sources to produce rhamnolipids seems to be an interesting and low cost alternative (Boulton and Ratledge, 1987). The bacterium produced 200 mg/L of rhamnolipids at the end of the fermentation with a drop of 31% in the surface tension of the spent medium when babassu oil was used as carbon source. As reported elsewhere, Table 1 shows a low initial superficial tension in the medium with babassu oil (40 D/cm) due to the tenso-active properties of the fatty acids in this vegetable oil. Pimienta et al. (1997) who carried out fermentation studies with strains of *Pseudomonas aeruginosa* grown in glucose, glycerol and commercial vegetable oil for a C/N ratio of 20/1, reported production of 700 mg/L, 1300 mg/L and 1400 mg/L of rhamnolipids, respectively, in seven days, showing the greatest potential for vegetable oil as substrate for rhamnolipid production. Nevertheless, it can be observed in Table 1 that the best rate of rhamnolipid production (690 mg/l) associated with the best surface-active characteristics (48.2% variation in surface tension drop) was achieved when glycerol was employed. This result was expected since this carbon source is taken up more easily than compared to the others. An abundant formation of foam was observed in the culture medium containing glycerol. Our results are in agreement with those obtained by Itoh et al. (1971), who worked with the strain *Pseudomonas aeruginosa* CFTR-6, which produced glycolipids

(620 mg/l) when glycerol (2% w/v) was used as carbon and energy source.

The microbial growth kinetics and rhamnolipid production in the fermentation with a 1% concentration of glycerol with a C/N ratio of 20/1 are represented in Figure 1 The stationary phase was reached after 40 hours of fermentation at the same time rhamnolipid production was increased. The rhamnolipid and biomass concentrations after 192 hours (eight days) were 1000 mg/L and 1470 mg/L, respectively. Glycerol was entirely consumed within 145 hours of fermentation and the rhamnolipid concentration peaked after another 100 hours. The production of this rhamnolipid is typical of a secondary metabolite and increased considerably in the stationary phase.

Table 1: Rhamnolipids and surface tension measurements at the end of seven days of fermentation by *Pseudomonas aeruginosa* PA1 using different carbon sources at a C/N ratio of 20/1.

Carbon Source	Rhamnose mg/L	Initial surface tension D/cm	Final surface tension D/cm	% Variation in surface tension
n-hexadecane	130	53.90	28.35	47.4
Paraffinic oil	260	54.00	51.60	4.4
Babassu oil	200	40.00	27.60	31
Glycerol	690	53.00	27.46	48.2

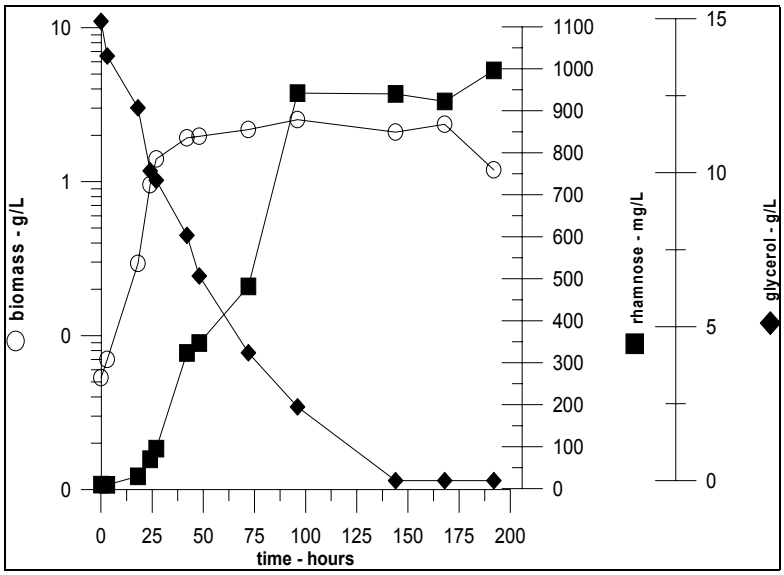

Figure 1: Microbial growth curve, rhamnolipid production and consumption of glycerol from the fermentation of *Pseudomonas aeruginosa* PA1 during 200 hours, using a 1% glycerol concentration.

Effect of Carbon/Nitrogen Ratio

Aiming at increasing the production of rhamnolipids by *Pseudomonas aeruginosa* PA1, a study with increasing glycerol concentrations (1; 2; 3; 4; 5 and 6% v/v) was conducted and a standardized inoculum of 0.1 g/L was employed. Figure 2 shows the yield factors relating substrate consumption to production ($Y_{P/S}$) and production to biomass ($Y_{P/X}$). The best results ($Y_{P/S}$ = 0.13g/g; $Y_{P/X}$ = 0.70 g/g) were obtained when glycerol was used in a concentration of 3% v/v, corresponding to a C/N ratio of 60/1. Additionally, it is possible to observe that the yield factor $Y_{P/S}$, decreased after this optimum glycerol concentration, reaching its lowest value ($Y_{P/S}$ = 0.086g/g) for the highest glycerol concentrations (6% v/v) thereby indicating a possible inhibitory effect on the bacterium metabolism due to a likely nutrient transport deficiency (Syldatk et al. 1985).

Effect of the Nitrogen Source

Figure 3 shows that sodium nitrate ($Y_{P/X}$ = 0.8 g/g) is more effective than ammonium sulfate ($Y_{P/X}$ =

0.4 g/g) and urea ($Y_{P/X}$ = 0.5 g/g). As shown in this figure, the use of nitrate at a C/N ratio of 60/1 implies better productivity than use of ammonium at the same C/N ratio, using 3% v/v of glycerol as carbon source. This result can be explained by the fact that nitrate first undergoes dissimilatory nitrate reduction to ammonium and then assimilation by glutamine-glutamate metabolism. This means that assimilation of nitrate as nitrogen source is so slow that it would simulate a condition of limiting nitrogen (Barber and Stuckey, 2000).

Pseudomonas aeruginosa is able to use nitrogen sources such as ammonia or nitrate. However, in order to obtain high concentrations of rhamnolipids it is necessary to have restrained conditions of this macro-nutrient. Our studies showed that nitrate is more effective in the production of rhamnolipids than ammonia and urea, which is in agreement with other studies reported in the literature (Syldatk et al. 1985, Ochsner et al. 1995, *Arino* et al. 1996).

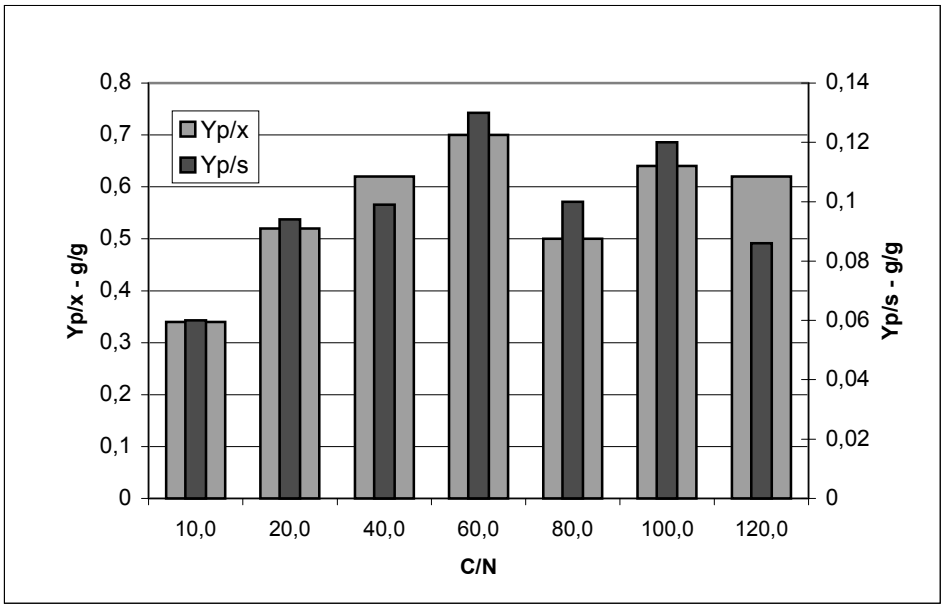

Figure 2: Yields of rhamnolipids related to biomass ($Y_{P/X}$) and to glycerol consumption ($Y_{P/S}$) for fermentations by *Pseudomonas aeruginosa* PA1 with different C/N ratios.

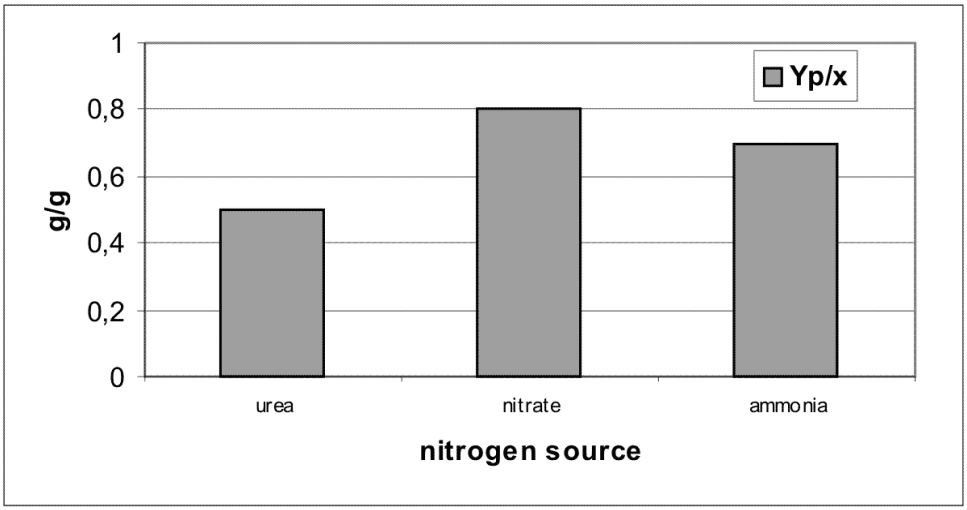

Figure 3: Effect of nitrogen sources on the production of rhamnolipids by *Pseudomonas aeruginosa* PA1.

Determination of the Critical Micelle Concentration

The experiment was aimed at evaluating the tension-active properties of the rhamnolipids accumulated in the fermented medium, using 3% v/v glycerol and 1.45 g/l sodium nitrate as the carbon and nitrogen sources, respectively. Figure 4 displays the results of superficial tension related to different concentrations of rhamnolipids present in free-cell fermented medium. The measurement for superficial tension of the medium at the end of fermentation was of 26.5 D/cm. At lower concentrations of rhamnolipids, high values of superficial tension were verified. It was also observed that the rhamnolipid concentration of 19 mg/L, corresponding to a superficial tension of 27 D/cm, was the point on the deflection curve; therefore it was assumed to be the critical micelle concentration of rhamnolipids that has satisfactory tension-active properties. Working with *Pseudomonas aeruginosa* 44T1, cultivated in 2% w/v of glycerol, Robert et al.(1989) observed a drop in the superficial tension of 30 D/cm in the free-cell fermented medium. The critical micelle concentration obtained by the authors was of 20 mg/L, very close to that obtained in our work..

Toxicity Assays

The purpose of this experiment was to evaluate the toxic effect of the fermented medium containing rhamnolipids produced by *Pseudomonas aeruginosa* PA1. Table 2 shows the results of toxicity, expressed in (CL50). It can be observed that the fermented medium both with and without cells had the same toxicity for both test organisms under standard conditions.

Lang and Wagner (1993) evaluated the toxicity of purified rhamnolipids produced by *Pseudomonas sp*. (ASPH - A1) using *Daphnia magna* as the test organism. They did not observe toxicity at a concentration of 200 ppm, considered for the critical micelle concentration the rhamnolipids found by the authors.

The results of 13.8 ppm, obtained using *Daphnia similis* as a toxicity indicator, was shown to be more toxic than the pure rhamnolipids described by Lang and Wagner (1993), which can be ascribed to the presence of other toxicity factors such as proteases, elastases, piocyanines and lipases. In despite of exhibiting a relative toxicity, biosurfactants have less impact the environment than chemical surfactants. Poremba et al. (1991) compared the toxicity of pure rhamnolipids with that of a synthetic chemical surfactant, using the *Photobacterium phosphoreum* bacterium. The chemical surfactant, Corexit, with an anionic characteristic identical to that of the rhamnolipids produced, displayed an inhibitory effect (CL50) of 5 ppm, which was ten times more toxic than the rhamnolipid-type surfactant.

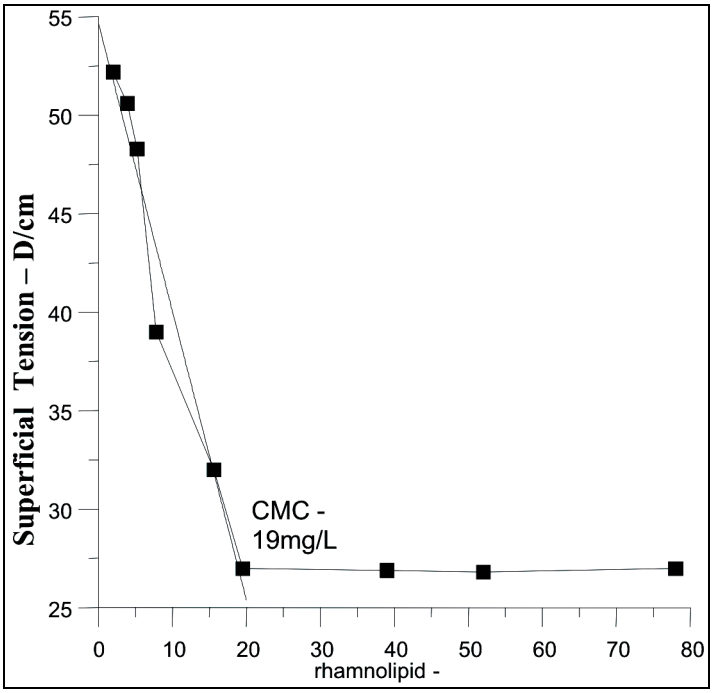

Figure 4: Superficial tension (D/cm) *versus* rhamnolipid concentration (mg/L) of fermented medium by *Pseudomonas aeruginosa* PA1.

Table 2: Estimate of toxicity the fermented medium with and without for *Pseudomonas aeruginosa* PA1 cells.

Sample	Microtox CL50 (ppm)	*Daphnia similis* CL50 (ppm)
Fermented broth with cells	13.0 +/- 0.4	13.8+/- 0.1
Fermented broth without cells	12.6+/- 0.5	13.0+/- 0.3

CONCLUSIONS

The strain isolated from oil environments was identified as *Pseudomonas aeruginosa.* It has the capacity to use carbon sources such as fructose, lactic acid, glucose, mannitol, mannose and glycerol.

This strain can produce rhamnolipid-type biosurfactants from substrates such as n-hexadecane, paraffinic oil, babassu oil and glycerol. However, the use of glycerol as carbon source showed the best results.

The variation in concentration of glycerol as carbon source from 1 to 6% v/v showed that with 3%v/v glycerol, the highest biomass concentration (4.05 g/L) and the greatest production of rhamnolipids (2.65 g/L) were obtained, and that when the concentration of glycerol rose above 3%v/v there was an inhibitory effect on microbial growth and the production of biosurfactants. This inhibitory effect was ascribed to problems linked to the solubility of glycerol and the difficulty of the bacterium to gain access to the nutrients in the culture medium.

The use of sodium nitrate (C/N = 60/1) caused an increase in the production of rhamnolipids of 3.16 g/L at the end of seven days of fermentation.

The critical micelle concentration of 19 mg/L was in agreement with other values reported in the literature, and the tension-active properties of these molecules indicate good prospects for application in industry, when compared to the values of the CMC of chemical anionic surfactants.

The fermented medium with or without cells showed a high level toxicity to the environment (13.0 and 13.8 mg/L), possibly due to the increase in production of the existing metabolites that have virulent characteristics. This toxicity becomes irrelevant, however when compared to that of commercial anionic surfactants such as Corexit (CL50 = 5 mg/L) which are used in the environment. These studies are of interest in order to optimize rhamnolipid production and therefore reduce production costs (Syldatk et al. 1985).

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