

## BIOSURFACTANT PRODUCTION BY *RHODOCOCCUS ERYTHROPOLIS* AND ITS APPLICATION TO OIL REMOVAL

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### ABSTRACT

The influence of different nutrients on biosurfactant production by *Rhodococcus erythropolis* was investigated. Increasing the concentration of phosphate buffer from 30 up through 150 mmol/L stimulated an increase in biosurfactant production, which reached a maximum concentration of 285 mg/L in shaken flasks. Statistical analysis showed that glycerol, NaNO<sub>3</sub>, MgSO<sub>4</sub> and yeast extract had significant effects on production. The results were confirmed in a batchwise bioreactor, and semi-growth-associated production was detected. Reduction in the surface tension, which indicates the presence of biosurfactant, reached a value of 38 mN/m at the end of 35 hours. Use of the produced biosurfactant for washing crude oil-contaminated soil showed that 2 and 4 times the critical micellar concentration (CMC) were able to remove 97 and 99% of the oil, respectively, after 1 month of impregnation.

**Key words:** Biosurfactant production, oil removal, *Rhodococcus erythropolis*.

### INTRODUCTION

The term surfactant is an abbreviation of the expression “surface active agents” (8). These compounds have been exhaustively studied, and they are fundamentally distinguished by their amphiphilic and amphipathic characteristics and by their ability to decrease surface and interfacial tensions of liquids. Biosurfactants are surfactants produced by microorganisms, either directly in microbial cell surfaces or by extracellular secretion. As amphiphilic molecules, biosurfactants contain hydrophilic and hydrophobic portions, and their structures are typically composed of one or more classes of compounds, including mycolic acids, glycolipids, polysaccharide-lipid complexes, lipoproteins, lipopeptides, phospholipids, and/or the microbial cell surface itself (12, 23).

The main biosurfactant market is the petroleum industry, where they have been incorporated into oil formulations and utilized in petroleum production and bioremediation. The advantages ascribed to biosurfactants include: their biodegradability, their reduced toxicity and the possibility to be produced by renewable raw material (16). These first two advantages make biosurfactants preferable to chemical surfactants, particularly for environmental applications. Consequently, enhanced oil recovery as well as soil bioremediation represents a large market (2, 18, 19). Due to their hydrophobicity, oil hydrocarbons bind consistently to soil particles. They also form holes in soil that may exclude water and nutrients. The surface area of oil can be increased by adding synthetic or biological surfactants, which increases the mobility and solubility of hydrocarbons and favors microbial

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degradation (15).

It has been reported that biosurfactants produced by the bacterial genus *Rhodococcus* are glycolipids with tensoactive properties (10). The bacterial cell wall of *Rhodococcus* has a highly organized and complex cell envelope, formed by a 30 to 54 carbon atom-containing peptidoglycan-arabinoglycan skeleton bonded to mycolic acids that represent more than 40% of the total cell wall. These acids can be found partially free as trehalose dimycolates and monomycolic lipids. Mycolic acids are 2-alkyl-3-hydroxy fatty acids of high molecular mass that are found exclusively in the cell envelope of bacteria belonging to the mycolata taxa, in which *Rhodococcus* spp. are included (20).

Studies on the kinetics of biosurfactant production and the nutritional requirements of microorganisms can generate important information about microbial metabolism that allows these parameters to be adjusted to meet the needs of biotechnology. Therefore, we investigated the influence of nutritional parameters and culture conditions on biosurfactant production by a strain of *Rhodococcus erythropolis*, and then we applied this knowledge to the treatment of crude oil-contaminated soil.

## MATERIALS AND METHODS

### Microorganism and growth conditions

*Rhodococcus erythropolis* ATCC 4277 was obtained from the bacteria collection of the Department of Microbiology, University of São Paulo, Brazil. The inoculum was grown in sterilized medium as described in Ciapina *et al.*, 2006, (4) and the cells were centrifuged (9000 x g) and used as fresh inoculum for shaken flask and bioreactor experiments.

### Shaken flask experiments

The basal medium used for all experiments contained NaNO<sub>3</sub> 3.4 g; NaCl 1.0 g; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.2 g; CaCl<sub>2</sub>.2H<sub>2</sub>O 0.02 g; FeCl<sub>3</sub>.7H<sub>2</sub>O 0.01 g per liter distilled water, pH 7.0.

Potassium phosphate buffer concentration varied from 30 up to 150 mmol/L for specific experiments. Glycerol was added as a carbon source to a final concentration of 2% (w/v). The medium was sterilized for 15 min at 121°C. The experiments were performed at 200 rpm, 37°C for 5 days.

### Batch Experiment in Bioreactor

A bioreactor Biostat<sup>®</sup> B 2.0 L model (B. Braun Biotech International, Germany) was run at 37°C with a constant dissolved oxygen level at 20% of saturation in the fermentation medium (maintained by the intensity of the agitation). The pH was maintained at 7.0 by automatic addition of hydrochloric acid and sodium hydroxide solutions.

### Experimental Design

A 2<sup>8-4</sup> fractional factorial design was used to evaluate biosurfactant production as a function of glycerol, NaNO<sub>3</sub>, NaCl, MgSO<sub>4</sub>.7H<sub>2</sub>O, FeCl<sub>3</sub>.7H<sub>2</sub>O, CaCl<sub>2</sub>.2H<sub>2</sub>O, yeast extract and a trace element solution, which composition is as follows (in g/L): ZnSO<sub>4</sub>.7H<sub>2</sub>O, 11.0; MnSO<sub>4</sub>.H<sub>2</sub>O, 6.0; FeSO<sub>4</sub>.7H<sub>2</sub>O, 1.0; CuSO<sub>4</sub>.5H<sub>2</sub>O, 0.04; CoSO<sub>4</sub>.7H<sub>2</sub>O, 0.04; H<sub>3</sub>BO<sub>3</sub>, 0.06; KI, 0.01; EDTA, 5.0. These independent variables with their real and encoded values are presented in Table 1. Statistical analysis was performed using *Statistica Statsoft* Version 6.0 (Tulsa, Oklahoma, USA).

**Table 1.** Variables studied and their levels in the 2<sup>8-4</sup> fractional factorial experimental design.

Variables	Low level	Center point	High level
	-1	0	+1
Glycerol (g/L)	10	20	30
Sodium Nitrate (g/L)	1	3.4	5.8
Sodium Chloride (g/L)	0	1	2
Magnesium Sulfate (g/L)	0	0.1	0.2
Ferric Chloride (g/L)	0	0.01	0.02
Calcium Chloride (g/L)	0	0.02	0.04
Yeast Extract (g/L)	0	0.1	0.2
Trace Elements (mL/L)	0	0.5	1

### Analytical methods

Microbial growth was monitored by biomass measurements by means of optical density observations of the cell suspension at 600 nm, followed by normalizing to the dry cell weight from a calibration plot.

Biosurfactant production was estimated by the concentration of polysaccharide. The choice of this method for surfactant quantitative determination was due to the ability of *R. erythropolis* to produce predominantly polysaccharide-class surfactant when glycerol is used as the carbon source. Polysaccharides were obtained by precipitation of the cell-free spent medium with 95% ethanol (12). After extraction, total sugars were estimated by the phenol-sulfuric method (6).

The emulsifying activity of the polysaccharide previously isolated was estimated by the method of Cooper and Goldenger, 1987 (5). Samples of 2 mL of cell-free spent medium were precipitated as described previously, and the precipitate was added to 2 mL of *n*-hexadecane and vortexed for 2 minutes and left to stand for 24 h. The Emulsification Index at 24 h (EI<sub>24</sub>) was given as a percentage consisting of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). The surface tension of the cell-free spent medium was measured by Du Nouy type tensiometer (Krüss Tensiometer, K11 model - Germany) utilizing the ring method as recommended by ASTM (1).

Glycerol concentration was analyzed by enzymatic-colorimetric assay using a triglycerides kit (GPO/POD – CELM/Brazil). The nitrate concentration was determined colorimetrically by the brucine sulfate method (4).

### Washing experiments

The method for washing crude oil-contaminated soil was from Urum *et al.*, 2003 (21). Four levels of biosurfactant

concentration were utilized: equal to the CMC, twice below the CMC, twice above the CMC and four times above the CMC (0.45 g/L, 0.23 g/L, 0.9 g/L and 1.8 g/L, respectively). Contaminated soil samples were divided into three groups: immediately contaminated, one-month contaminated and two-month contaminated. Five grams of the contaminated soil was introduced into flasks containing 25 mL of biosurfactant (precipitated polysaccharide) solution. Flasks were incubated at 30°C, 300 rpm, 120 min (4). After 3 min of decantation, 1 mL of the supernatant was washed with *n*-hexane to remove the crude oil extract (21). Absorbance of the *n*-hexane/crude oil solution was measured at 294 nm using DU70 Spectrophotometer (Beckman, Germany).

## RESULTS AND DISCUSSION

### Effect of phosphate concentration

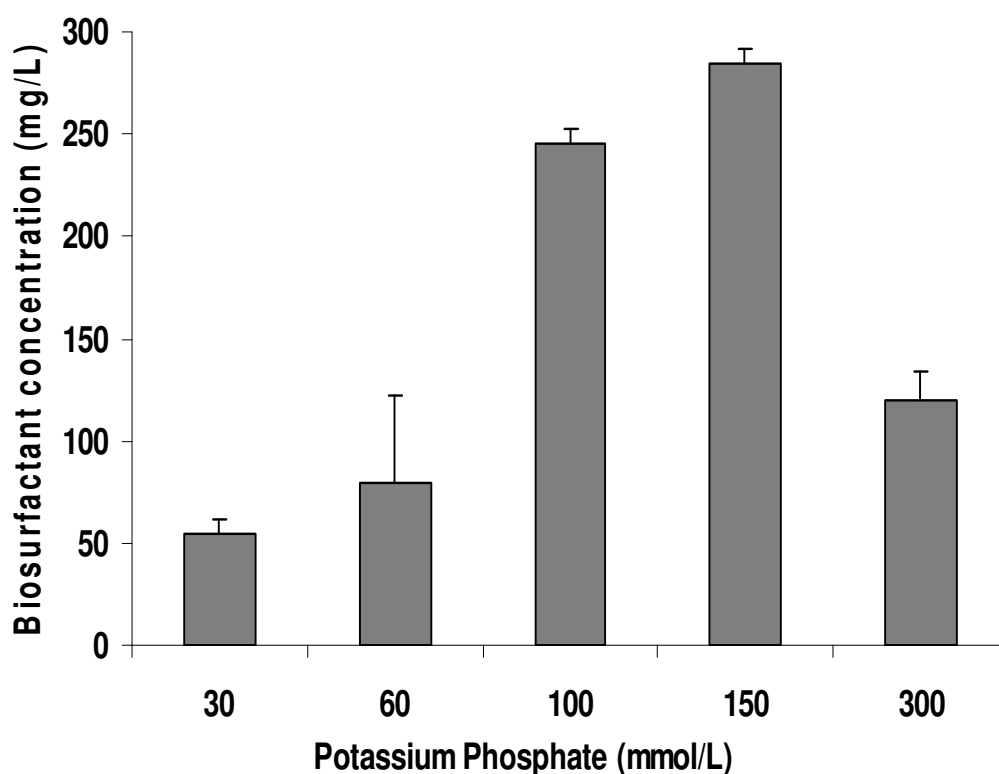
The phosphate buffer used to prevent pH decline had a strong influence on biosurfactant production and cell growth. In this study we varied the concentration of the phosphate buffer (K<sub>2</sub>HPO<sub>4</sub>:KH<sub>2</sub>PO<sub>4</sub> 1:1) from 30 to 300 mmol/L and evaluated its effect on fermentation. As shown in Table 2, concentrations equal to or higher than 60 mmol/L were sufficient to maintain the pH of the culture medium throughout the experiment. Although higher concentrations yielded more biomass, our results suggest that a phosphate concentration of 300 mmol/L modifies the surfactant properties of the product, as indicated by reductions in EI<sub>24</sub> and increases in surface tension. Higher values of EI<sub>24</sub> were obtained when 100 and 150 mmol/L phosphate buffer was added to the culture. At these concentrations, surface tension was reduced to 44.8 and 34.3 mN/m, respectively, comparable with values reported in the literature (7, 17).

**Table 2.** Observed values of pH, cell concentration, EI<sub>24</sub> and surface tension (ST) after 5 d of fermentation of *R. erythropolis* using potassium phosphate varying from 30 to 300 mmol/L

Response	Phosphate Concentration (mmol/L)				
	30	60	100	150	300
pH	5.3 ± 0.14	6.6 ± 0.03	6.6 ± 0.28	6.8 ± 0.14	6.8 ± 0.03
Cell dw (g/L)	1.2 ± 0.35	1.9 ± 0.43	2.4 ± 0.45	2.6 ± 0.53	3.4 ± 0.28
EI <sub>24</sub> (%)	25 ± 5.7	31 ± 4.2	36 ± 8.5	36 ± 5.6	10 ± 7.1
ST (mN/m)	52.0 ± 4.13	46.5 ± 5.08	44.8 ± 3.62	34.3 ± 3.25	49.6 ± 5.71

Figure 1, shows the amount of biosurfactant produced by *R. erythropolis* after five days of fermentation in different concentrations of phosphate buffer. The results demonstrated that increasing the concentration from 30 up through 150 mmol/L stimulates biosurfactant production. The maximum productivity was observed at 150 mmol/L, in which 285 mg/L of biosurfactant was obtained. This was the concentration used in further experiments. This behavior is consistent with studies

performed by Kim *et al.*, 1997 (11), who reported a positive influence of phosphate on biosurfactant production by *Bacillus subtilis*. On the other hand, Bazire *et al.*, 2005 (3) observed that a concentration of 75  $\mu\text{mol/L}$  of phosphate in LPM63 medium inhibits the cell-to-cell signal molecule N-butyryl-L-homoserine lactone ( $\text{C}_4\text{-HSL}$ ), which is involved in the *quorum sensing* system indirectly responsible for biosurfactant production in *Pseudomonas aeruginosa*.



**Figure 1.** Biosurfactant production by *R. erythropolis* grown in different concentrations of potassium phosphate. Values are expressed as means  $\pm$  S.D. ( $n = 3$ )

#### Effect of different nutrients on biosurfactant production

The results of the fractional factorial design with four center points are depicted in Table 3. The highest yield factor of product on substrate consumed ( $Y_{P/S}$ ) was obtained in essay 5 ( $Y_{P/S} = 19 \text{ mg/g}$ ), followed by essay 13 ( $Y_{P/S} = 14.5 \text{ mg/g}$ ). It is worth noting that in both essays the lowest level of glycerol and sodium nitrate and the highest level of yeast extract were used. Additionally, the influence of glycerol, sodium nitrate,

and yeast extract on the bioprocess was all statistically significant with values of  $p\text{-level} < 0.1$ , resulted from the *Statistica* version 6.0 (Microsoft  $\text{\textcircled{R}}$ ). Therefore, these variables were selected as for identifying the optimum region in the concentration range evaluated (Figure 2). Figure 2 (a) illustrates the combined effect of sodium nitrate and glycerol on the production yield factor ( $Y_{P/S}$ ). It is deducible that the influence of C:N ratio plays an important role on the

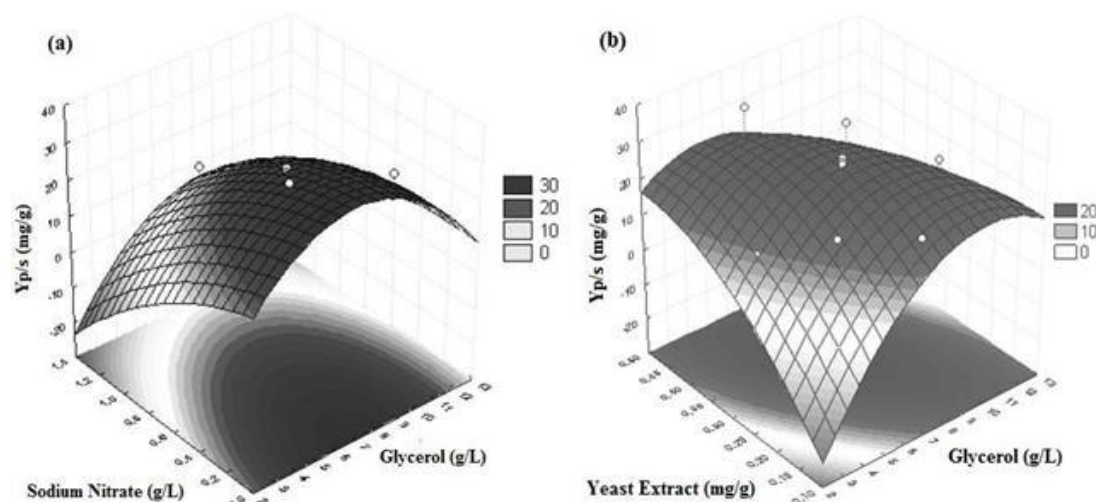
bioprocess performance. In this case, a C:N ratio of 21:1 was observed, also considering the presence of 10.5% total nitrogen in the yeast extract (data not shown). These results suggest that high C:N ratios are favorable for the bioprocess, corroborating other reports in the literature, which indicate that the stimulation of biosurfactant synthesis by *Rhodococcus* sp takes

place under nitrogen-limited conditions (11). Concerning the yeast extract, even low concentrations of this complex source of nutrients (0.1-0.2 g/L) enhanced considerably the bioprocess performance (Table 3 and Figure 2 (b)), pointing out that this complex source of nutrients is necessary since it induces highest production of biosurfactant by *R. erythropolis*.

**Table 3.** Variables studied (encoded values) and respective response in terms of  $Y_{p/s}$  in the  $2^{8-4}$  Statistical Fractional Factorial Design

Run	Glycerol	NaNO <sub>3</sub>	NaCl	MgSO <sub>4</sub>	FeCl <sub>3</sub>	CaCl <sub>2</sub>	Yeast Extract	Trace Elements	Yp/S mg/g
1	-	-	-	-	-	-	-	-	9,7
2	+	-	-	-	-	+	+	+	11,5
3	-	+	-	-	+	-	+	+	11,1
4	+	+	-	-	+	+	-	-	5,1
5	-	-	+	-	+	+	+	-	19,0
6	+	-	+	-	+	-	-	+	9,0
7	-	+	+	-	-	+	-	+	8,5
8	+	+	+	-	-	-	+	-	4,3
9	-	-	-	+	+	+	-	+	5,3
10	+	-	-	+	+	-	+	-	3,9
11	-	+	-	+	-	+	+	-	5,1
12	+	+	-	+	-	-	-	+	4,3
13	-	-	+	+	-	-	+	+	14,5
14	+	-	+	+	-	+	-	-	0,8
15	-	+	+	+	+	-	-	-	5,3
16	+	+	+	+	+	+	+	+	2,1
Center point	0	0	0	0	0	0	0	0	4,6 ± 1,3

Encoded values (-), (0) and (+) represents respectively, low level, center point (intermediate level) and high level for each variable studied. For glycerol, (-), (0) and (+) correspond to 10.0, 20.0 and 30.0 g/L; for NaNO<sub>3</sub> correspond to 1.0, 3.4 and 5.8 g/L; for NaCl correspond to 0.0, 1.0 and 2.0 g/L; for MgSO<sub>4</sub> correspond to 0.0, 0.1 and 0.2 g/L; for FeCl<sub>3</sub> correspond to 0.00, 0.01 and 0.02 g/L; for CaCl<sub>2</sub> correspond to 0.00, 0.02 and 0.04 g/L; for yeast extract correspond to 0.0, 0.1 and 0.2 g/L; and for trace elements (aqueous solution) correspond to 0.0, 0.5 and 1.0 mL/L, respectively.



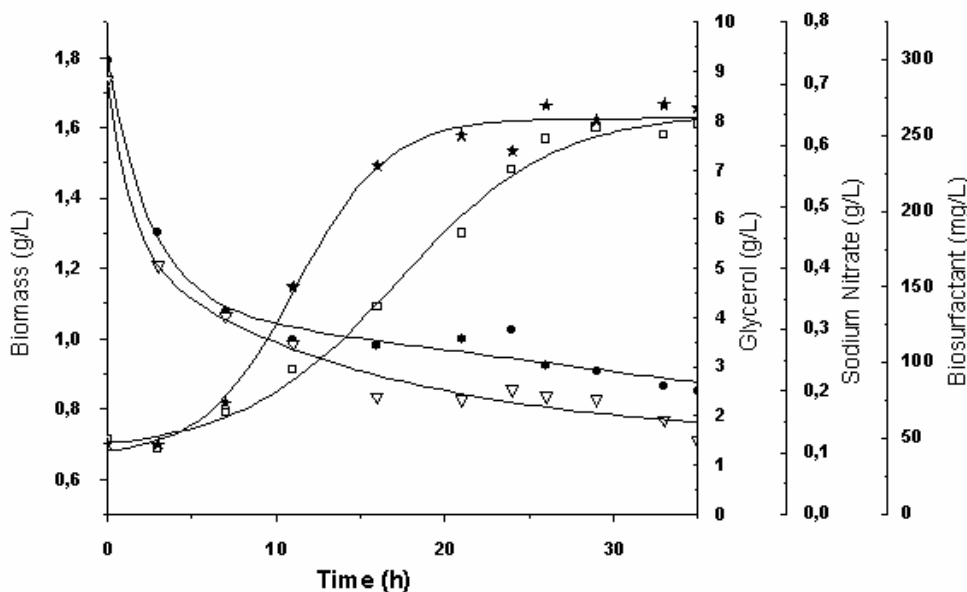
**Figure 2.** Surface-response graphs generated from the  $2^{8-4}$  Statistical Fractional Factorial Design, utilizing  $Y_{p/s}$  as a response factor: (a) glycerol and sodium nitrate and (b) glycerol and yeast extract influence on the response factor.

**Bioreactor batch experiment**

In order to validate the results obtained in the factorial experiment, the bioprocess was carried out batchwise in an instrumented bioreactor. This experiment employed concentrations of nutrients considerably more favorable for biosurfactant production.

Figure 3 shows the production of biosurfactant and cell growth in glycerol (10 g/L), sodium nitrate (1 g/L), yeast extract (0.2 g/L) and potassium phosphate (150 mmol/L). The exponential growth of *R. erythropolis* started after about 7 hours with a specific growth rate ( $\mu_x$ ) of 0.035 h<sup>-1</sup>; which remained constant for approximately 27 hours. Afterwards, the culture entered stationary phase and the glycerol and sodium

nitrate ratios in the culture were reduced to 2.9 g/L and 0.18 g/L, respectively. Biosurfactant production started in the early exponential growth phase and continued even after cell growth had ceased, indicating a typical semi-growth-associated mode of production. At the end of the bioprocess, the biosurfactant concentration reached 271 mg/L. The yield factors of biosurfactant per substrate consumed ( $Y_{P/S}$ ) and per cells grown ( $Y_{P/X}$ ) were 40.2 mg/g and 301 mg/g, respectively. These obtained parameters surpassed those found in shaken flasks, through experimental design. At the end of the experiment (after 35 hours), the minimum value of surface tension was 38 mN/m (data not shown), and an EI<sub>24</sub> of 40% for an n-hexadecane/water binary system was obtained.



**Figure 3.** Time-course of growth, biosurfactant production, glycerol and sodium nitrate consumption during cultivation of *R. erythropolis* in a bioreactor (□ biomass [g/L]; ▽ sodium nitrate [g/L]; ● glycerol [g/L]; \* biosurfactant [g/L]).

**Evaluation of the efficiency of the biosurfactant for washing crude oil-contaminated soil**

The emulsifying activity of each concentration used in this experiment is shown in Figure 4. Biosurfactant concentrations of 0.23 g/L, 0.45 g/L and 0.9 g/L resulted in EI<sub>24</sub> values of 27%, 40% and 60%, respectively.

Figure 5 shows the percentage of oil removed from the

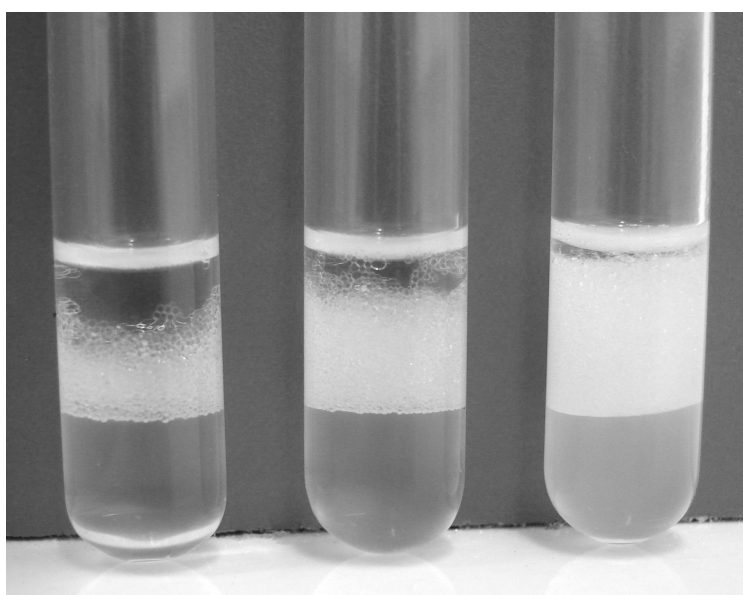
crude oil-contaminated soil. It is notable that, immediately after soil contamination, the oil-removal efficiency of the biosurfactant was nearly 100% at all concentrations used. Kuyukina *et al.*, 2005 (13), using biosurfactant produced by *Rhodococcus ruber* (utilizing n-hexadecane as the carbon source) at twice the CMC observed removal of 80% of the oil from immediately contaminated soil at 28°C.

At 0% biosurfactant concentration (washing solely with water), the reduction was about 76% for immediately contaminated soil. However, after a one-month contamination period, the oil-removal ability was considerably reduced, with only 7% efficiency in the treatment without biosurfactant.

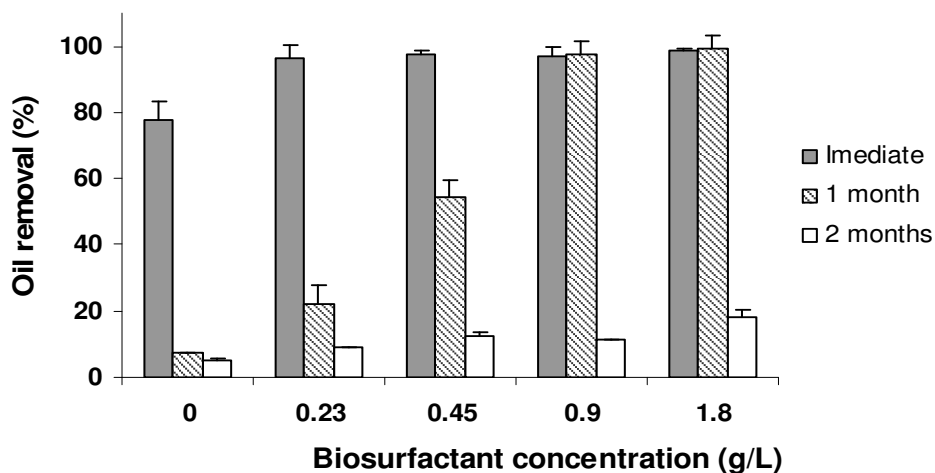
Biosurfactant oil-removal reached maximum efficiency for immediately and 1-month contaminated soils only when 0.9 and 1.8 g/L biosurfactant concentrations were applied. These

results corroborate those reported by Ivshna *et al.*, 1998 (9), who found that a concentration 2 g/L of biosurfactant produced by either *Rhodococcus erythropolis* or *Rhodococcus ruber* had the best effect on oil desorption from oil shale.

After a two-month contamination period, the efficiency of oil removal lowers dramatically, with a maximum value of 18% at the highest concentration of biosurfactant.



**Figure 4.**  $EI_{24}$  values obtained from biosurfactant solutions with concentrations twice below the CMC, equal to the CMC and twice above the CMC, from the left to right respectively.



**Figure 5.** Percentage of oil removed from sandy sediments by different concentrations of biosurfactants, immediately after the contamination and after 1 and 2 months.

This result suggests that time is a limiting factor for the performance of the biosurfactant, possibly due to greater adsorption of oil to the soil hindering the interaction between the biosurfactant solution and the oil. As the oil remains for longer times, the physical and chemical nature of the soil becomes modified, as noted by Urum *et al.*, 2005 (22). This occurs due to the loss of volatile components and oil of lower molecular weight, resulting in oil with higher density and viscosity. This phenomenon, known as *aging* (because it is time-dependent), is responsible for the persistence of hydrocarbons in soils because it makes the hydrocarbons less available to microbial and surfactant action. Additionally, as reported by Lee *et al.*, 2002 (14), clay and humus adsorption can reduce the effectiveness of (bio)surfactants for soil remediation, due to the negative charge of clay minerals interacting with the surfactants (positively or negatively charged) and decreasing micelle concentration and the extent of oil solubilization. Conversely, nonionic surfactants are less likely to be adsorbed to the soil.

### CONCLUSIONS

These studies demonstrate that the production of biosurfactant by *R. erythropolis* strain ATCC 4277 can be enhanced by the addition of experimentally defined concentrations of glycerol, sodium nitrate and yeast extract. Phosphate buffer varying from 60 to 150 mmol/L increases biosurfactant production and also maintain the proper pH for the fermentation process. Moreover, the results obtained in oil-removal experiments indicate the applicability of biosurfactants to soil bioremediation at low concentrations and at intermediate contamination periods, such as one month.

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