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Biosurfactant Production by *Rhodococcus erythropolis* Grown on Glycerol As Sole Carbon Source

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Abstract

The production of biosurfactant by *Rhodococcus erythropolis* during the growth on glycerol was investigated. The process was carried out at 28°C in a 1.5-L bioreactor using glycerol as carbon source. The bioprocess was monitored through measurements of biosurfactant concentration and glycerol consumption. After 51 h of cultivation, 1.7 g/L of biosurfactant, surface, and interfacial tensions values (with *n*-hexadecane) of 43 and 15 mN/m, respectively, 67% of Emulsifying Index (E_{24}), and 94% of oil removal were obtained. The use of glycerol rather than what happens with hydrophobic carbon source allowed the release of the biosurfactant, originally associated to the cell wall.

Index entries: Biosurfactant; *Rhodococcus*; oil removal.

Introduction

Biosurfactants are amphipathic molecules that can be divided in low-molecular-weight compounds such as glycolipids, phospholipids, and lipopeptides, which present lower interfacial tension and high-molecular-weight biosurfactants such as polysaccharides, proteins, lipoproteins, or complex of these biopolymers *(1)*. These high-molecular-weight compounds are associated with the production of stable emulsions, but the lowering of the surface tension or interfacial tension is not a usual trait of them and is called as bioemulsifiers *(2)*.

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The increasing interest in the potential applications of microbial surface-active compounds is based on their broad range of functional properties that include wetting, foaming, emulsification, viscosity reduction, phase separation, and solubilization. There are many areas of industrial application in which chemical surfactants could be substituted by biosurfactants in several fields as industrial cleaning, agriculture, construction, food, paper and metal industries, textiles, cosmetics, pharmaceutical, and petroleum and petrochemical industries, including applications in environmental bioremediation. Biosurfactants have gained attention because of their biodegradability, low toxicity, ecological acceptability, and ability to be produced from renewable and cheaper substrates *(3)*.

Surfactants are produced by a variety of microbes (bacteria and fungi), secreted either extracellularly or attached to parts of cells and are often only produced when growing on *n*-alkanes or other water-immiscible substrates to facilitate adhesion to hydrophobic substrates. However, some microbial surfactants can be produced on water-soluble growth substrates *(4)*.

The genus *Rhodococcus* is a group of Gram-positive bacteria exhibiting a diverse range of metabolic activities. Some of them have the ability to degrade a variety of organic compounds including polychlorinated biphenyls and aliphatic and aromatic hydrocarbons. This property is often accompanied by the ability to produce biosurfactants *(5)*. These molecules are predominantly glycolipids but other types have also been reported such as polysaccharides associated to the cell wall *(6,7)*.

Many of the potential applications that have been considered for biosurfactants depend on whether these can be produced economically in commercial quantities. The parameters that affected the economics of biosurfactant manufacture include the choice of nutrients and strain *(4)*. The purpose of this study was to investigate the production of biosurfactant by *Rhodococcus erythropolis* on glycerol and its application in hydrocarbon removal from oily sludge.

Materials and Methods

Bacteria

R. erythropolis ATCC 4277 was obtained from bacteria collection of the Department of Microbiology, University of São Paulo, Brazil.

Media and Growth Conditions

Inoculum

The inoculum was grown in sterilized medium containing 0.3% yeast extract; 1.5% peptone; and 0.1% glycerol, at 28°C in a rotary shaker at 200 rpm (throw = 5 cm), during 24 h of cultivation. The cells were centrifuged (10,000*g*, 10 min) and used as inoculum for shake flask and bioreactor experiments.

Shake Flask Experiments

The basal medium used for all culture experiments contained distilled water in the following amounts: 2 g/L KH_2PO_4, 1 g/L KNO_3, 2 g/L K_2HPO_4, 2 g/L $(NH_4)_2SO_4$, 1 g/L NaCl, 0.2 g/L $MgSO_4$, 0.02 g/L $CaCl_2$ $2H_2O$, 0.01 g/L $FeCl_3$ $7H_2O$, and 0.1 g/L yeast extract. The pH was adjusted to 7.0. The medium was sterilized for 15 min at 121°C. Glycerol and hexadecane were investigated as carbon sources to a final concentration of 1.5% (w/v). The experiments were performed at 28°C, at 200 rpm and on an orbital shaker for 1 wk.

Batch Experiment in Bioreactor

The basal medium containing glycerol (1.5%) was used. The bioreactor was run at 28°C, with a constant dissolved oxygen level of 30% of saturation in the fermentation broth (maintained by automatic stirred control). The pH was maintained at 7.0 by automatic addition of acid and base solutions. The fermenter used was a Biostat® B 2.0 L model (B. Braun Biotech International, Germany).

Biomass Content

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

Glycerol Concentration

The glycerol concentration was analyzed by enzymatic-colorimetric assay using a triglycerides kit (GPO/POD, CELM/Brazil).

Determination of Surfactant Production

The production of biosurfactant was estimated by the phenol-sulfuric method *(8)*. The choice of this method for surfactant quantitative determination was determined by the ability of *Rhodococcus* sp. to produce glycolipids and polysaccharides class surfactant (both with carbohydrates portion). The concentration of carbohydrate was reported as biosurfactant concentration.

Surface Characteristics

The whole broth of the bioreactor was sonicated (10 KHz, 10 min) before measurement for the release of the biosurfactant attached to the cell wall. The cells were removed by centrifugation at 10,000*g* for 15 min. The surface and interfacial tension of the cell-free spent medium was measured with a Du Nuoy ring tensiometer (Kruss Tensiometer, K9 model, Germany). Interfacial tension was measured with *n*-hexadecane as the oil phase. Emulsifying activity was estimated by the method of Cooper and

Table 1
Levels of Variables in Experimental Design to Hydrocarbons Removal Experiment

Variables	Low level (–)	Central point (0)	High level (+)
Oily sludge (g)	0.5	2	3.5
Agitation (rpm, throw = 5 cm)	100	200	300
Time (min)	30	75	120
Biosurfactant (g/L)	0.5	2.25	4

Goldenger *(9)*. Two milliliters of a cell-free spent medium were added to 2 mL of *n*-hexadecane and vortex for 2 min and left to stand for 24 h. The Emulsification Index (E_{24}) was given as percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm).

Evaluation of the Biosurfactant in Hydrocarbon Removal

The studies of submerged washing of oily sludge for hydrocarbon removal using the spent medium containing the biosurfactant were performed according to Urum *(10)*. The experiments were carried out following the experimental design as shown in Table 1, in which the variables investigated were oily sludge amount, agitation speed, process time, and biosurfactant concentration. The software *STATISTICA* (version 5.0, Statsoft Inc., Tulsa, OK) was used to generate the experimental design and the results were analyzed utilizing as response variable the removal of total hydrocarbons.

Results and Discussion

Shake Flask Experiments

Several works have reported the production of biosurfactant by *Rhodococcus* sp. using *n*-alkanes as the carbon source *(11–13)*, and observed the association of surfactants with the cell wall. According to Lang and Philp *(7)* only a minor portion of the produced surfactants is released.

In this study, a hydrophilic and hydrophobic carbon sources were evaluated in the production of biological active surface compounds. Biosurfactant concentrations of 0.4 and 0.1 g/L on glycerol and hexadecane, respectively, were obtained. In order to increase biosurfactant content, the effect of sonication was investigated. There was an increase of fourfold in surfactant release using sonication with the culture grown on hexadecane (0.4 g/L) and no significant difference (0.45 g/L) was observed with that culture grown on glycerol. Although production of surface-active lipids by *Rhodococcus* can be induced by the presence of *n*-akanes in culture medium *(7)*, these results showed that glycerol was able to promote the production of biosurfactant and allowed its release.

Physiologically, the production of biosurfactant is associated with the assimilatory mechanism to hydrophobic substrates. This mechanism

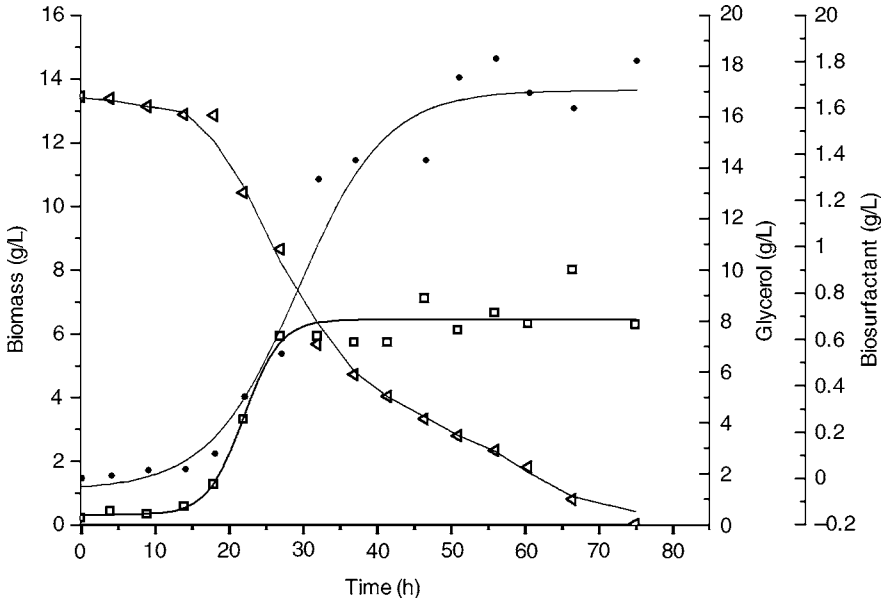

Fig. 1. Time-course of growth, biosurfactant production, and glycerol consumption during cultivation of *R. erythropolis* in bioreactor (□, biomass [g/L]; •, biosurfactant [g/L]; Δ, glycerol [g/L]).

would consist in direct contact of cells with large oil droplet with little or no emulsification, or the contact with fine oil droplet, culminating in emulsification. In the first, the biosurfactant is retained on the outer cell surface, facilitating the attachment and subsequent transport of hydrophobic compounds to the cell inside *(14)*. In the second case the free biosurfactant, released in the culture medium would form a hydrocarbon–surfactant complex that pseudosolubilize the substrates and hence increase availability to the cell *(15)*.

Batch Experiment in Bioreactor

Figure 1 shows the cell growth on glycerol and the synthesis of the biosurfactant. The exponential growth of *R. erythropolis* commenced at approx 14 h with a specific growth rate (μ_x) of 0.184/h, corresponding to a doubling time of 3.8 h. After 27 h, the culture achieved the stationary phase and the glycerol consumption rate was reduced from 0.64 to 0.15 g/L h. It is well known that the entrance in the stationary growth phase is associated with the depletion of any nutrient, even though carbon source is still available. The production of the biosurfactant started in early exponential growth phase and continued even when growth had ceased indicating a typical growth-semiassociated production. The biosurfactant concentration increased approximately threefold during stationary growth phase, accumulating 1.7 g/L after 51 h. In the end of the process, the volumetric productivity and the biosurfactant yield on cell growth were 0.033 g/L h

Table 2
Conditions and Results of the Experimental Design Method

Run	Oily sludge (g)	Agitation (rpm)	Time (min)	Biosurfactant (g/L)	Removal (%)
1	3.5	300	120	4	93.6 ± 3.1
2	3.5	300	120	0.5	57.7 ± 2.4
3	3.5	300	30	4	71.6 ± 2.2
4	3.5	300	30	0.5	22 ± 4.9
5	3.5	100	120	4	94.1 ± 2.9
6	3.5	100	120	0.5	24.9 ± 1.4
7	3.5	100	30	4	17.1 ± 1.3
8	3.5	100	30	0.5	88.9 ± 4.1
9	0.5	300	120	4	84 ± 3.2
10	0.5	300	120	0.5	87.8 ± 3.3
11	0.5	300	30	4	40 ± 2.4
12	0.5	300	30	0.5	65.1 ± 3.3
13	0.5	100	120	4	19.6 ± 0.9
14	0.5	100	120	0.5	32.6 ± 1.6
15	0.5	100	30	4	71.9 ± 1.9
16	0.5	100	30	0.5	63.8 ± 1.3
CP	2	200	75	2.25	64.3 ± 2.9

CP, central point.

and 0.28 g/g, respectively. The biosurfactant yield on glycerol consumed was 0.13 g/g.

The obtained production was equivalent to values reported for the production of biosurfactant by *Rhodococcus* sp. varying over a wide range (from 0.5 to 30 g/L) *(16)*. No excess of foam formation was observed. This is an advantage, when compared with other biosurfactant producing species, because the excessive foam formation in bioreactor is difficult to control *(17)*. In the end of the experiment, the minimum values of surface and interfacial tensions (with *n*-hexadecane) were 43 and 15 mN/m, respectively, comparable with values reported in the literature with glycerol as the carbon source *(18)*.

The E_{24} of 67% for a *n*-hexadecane-water binary system was obtained. It is a value comparable with results reported by other authors, working with *Rhodococcus* species *(12,16,19)*. This indicates that the biosurfactant produced in this study is a good emulsifier.

Evaluation of the Biosurfactant in Hydrocarbon Removal

In order to investigate the efficiency of the biosurfactant produced by this strain using glycerol as the carbon source, a preliminary experiment using the spent medium containing the surfactant was performed to verify the removal of hydrocarbons from oily sludge. The best condition pointed out by the experimental design is depicted in the experiment 5 (94.1%) (Table 2), confirming the good potential of this biosurfactant for oil

recuperation and environmental remediation. After oily sludge washings, the oil phase was maintained dispersed in the water phase, characterizing the removal phenomenon associated to dispersion and dislocation processes. This effect suggests the successfully oil recuperation in conventional separation system oil/water to industrial reprocessing.

These studies demonstrated that *R. erythropolis* ATCC 4277 strain grown on glycerol produces mainly free biological surfactant with surface active properties and good ability for emulsification. Furthermore, the excellent result obtained in the application of the produced biosurfactant in the hydrocarbon removal from oily sludge lead to a subsequent research for its chemical characterization, production optimization, and applicability evaluation.

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