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Enhancement of Rhamnolipid Production in Residual Soybean Oil by an Isolated Strain of *Pseudomonas aeruginosa*

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Abstract

In the present work, the production of rhamnolipid from residual soybean oil (RSO) from food frying facilities was studied using a strain of *Pseudomonas aeruginosa* of contaminated lagoon, isolated from a hydrocarbon contaminated soil. The optimization of RSO, ammonium nitrate, and brewery residual yeast concentrations was accomplished by a central composite experimental design and surface response analysis. The experiments were performed in 500-mL Erlenmeyer flasks containing 50 mL of mineral medium, at 170 rpm and 30 ± 1°C, for a 48-h fermentation period. Rhamnolipid production has been monitored by measurements of surface tension, rhamnose concentration, and emulsifying activity. The best-planned results, located on the central point, have corresponded to 22 g/L of RSO, 5.625 g/L of NH_4NO_3, and 11.5 g/L of brewery yeast. At the maximum point the values for rhamnose and emulsifying index were 2.2 g/L and 100%, respectively.

Index Entries: Biosurfactant; experimental design; *Pseudomonas aeruginosa*; rhamnolipids; surface tension; soybean oil.

Introduction

The surfactants constitute a very important class of chemical compounds widely used in a variety of industrial sectors because they act like dispersants and/or solubilizing agents of organic compounds. Most of the surfactants commercially used are synthesized from petroleum derivatives *(1)*. However, recently, the interest for microbial surfactants has significantly increased, especially because of its biodegradability *(2)*. Microbial compounds that have surfactant properties, i.e., reduce the surface tension (ST) and/or have high emulsifying capacity, are predominantly biosurfactants and consist of bacteria and fungi metabolic

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byproducts *(3)*. Microbial-produced surfactants offer several advantages over the equivalent chemical ones: low toxicity, temperature, pH, and ionic force tolerance, besides their possibility of being produced from renewable substrates *(4)*. The biosurfactants can be applied in agriculture for the pesticides and herbicides formulation, in the food industry as additives, and also in the pharmaceutics, textile, cosmetic, and petrochemical industries. In the latter, it is widely used for secondary oil recovery, oil residue removal, and mobilization and for bioremediation *(5)*.

When considering the biosurfactant potential, one must remember that these macromolecules are produced by a variety of microorganisms and that they have different chemical structures and surface properties. The kind and the quantity of the biosurfactant produced depend first on the type of microorganism that is producing it and on the carbon and nitrogen sources, trace elements, aeration, and other factors that can influence the microbial production of these compounds *(6)*. The glycolipids are the best-known microbial surfactants and, among them, the rhamnolipids are the most studied ones. These compounds contain 1 or 2 molecules of rhamnose bonded to 1 or 2 molecules of β-hydroxydecanoic acid *(1)*. Several species of *Pseudomonas* are able to produce large amounts of these compounds *(7)*. Some *Pseudomonas* sp. produce rhamnolipids that, when added to an oil/water system, are able to reduce the interfacial tension from 21 to 0.47 mN/m *(7,8)*. Because of the importance of substitution of chemical surfactants to lower- or nontoxic products, this work had as its main objectives the determination of the best experimental conditions for the rhamnolipid production by an isolated strain of *P. aeruginosa* in relation to the following variables: burnt oil concentration, ammonium nitrate (AN), and residual brewery yeast (RBY) using a central composite experimental design (CCD).

Materials and Methods

Microorganisms

The culture was isolated from the soil of a lagoon contaminated with diesel oil and gasoline located at the Rio das Pedras farm, Uberlândia, Minas Gerais, Brazil. The bacterial strain was identified as *P. aeruginosa* called strain PACL. The culture was maintained in bacto nutrient broth (BD, cod. 234000) supplied by BD (Becton Dickinson and Company) at 4°C.

Culture Isolation

The media proposed by Vecchioli et al. *(9)*, added with 0.5% (v/v) of residual soybean oil (RSO) as the single carbon source, was used for the bacterial cultures isolation, using the pour plate technique. Among the isolated microorganisms, the one that showed the best reduction of the culture medium ST after fermentation was selected and identified. The isolated microorganism was identified at Laboratório de Enterobactérias at Fundação Oswaldo Cruz through classical biochemistry methods.

Growth Medium and Conditions

The bacterial cultures were growth in the medium proposed by Santos et al. *(10)*, containing (g/L): NH_4NO_3 1.7, Na_2HPO_4 7.0, KH_2PO_4 3.0, $MgSO_4 \cdot 7H_2O$ 0.2, yeast extract 5.0, and glucose 10.0. The medium was autoclaved at 121°C for 15 min after adjusting the pH for 7.0. The production medium consisted of the same salts used in the growth medium with added burnt soybean oil (g/L): between 1.4 and 42.6, RBY (g/L): between 0.56 and 22.5, and AN (g/L): between 0.56 and 22.5.

Rotary Shaker Experiments

The experiments were performed in 500-mL Erlenmeyers flasks containing 50 mL of the medium. Typically, three loopfuls from the stock culture were cultivated in 100 mL of the medium proposed by Santos et al. *(10)* at 30 ± 1°C and 170 rpm for 24 h. After inoculating with 5 mL of the inoculum, the flasks were maintained in the rotary shaker at 170 rpm agitation rate for 48 h at 30 ± 1°C.

Central Composite Experimental Design

A central composite experimental design was used in order to optimize the process in relation to the following operational variables: RSO concentration, AN, and RBY. The levels of the studied variables were expressed in the codified form (nondimensional) using the following codifying equation:

$$X_n = \left[\left(X - X_0\right)\right] / \left(X_{+1} - X_{-1}\right) / 2$$

The statistical calculations were done through the Statistic 5.0 Software (Statsof Inc.).Table 1 shows the concentrations used in each of the 16 experiments of the central composite experimental design (CCD).

Analytical Methods

Rhamnose Concentration

The rhamnose concentration was determined according to the methodology described by Rahman *(11)*. The ST determination was performed using a Tensiometer (Fisher Scientific, model 21). The analyses were done at 25°C with the tensiometer previously calibrated. The emulsifying activity was determined for aviation kerosene as described by Cooper and Goldenberg *(12)*.

Results and Discussion

Table 2 shows the results obtained in the central composite design from the studied variables: RSO concentration (X_1), AN concentration (X_2), and RBY (X_3) using the isolated *P. aeruginosa* PATC strain.

Table 1
Concentrations Used for Each Variable in the 16 Experiments of the CCD

Experiment	Burnt oil (g/L)	AN (g/L)	RBY (g/L)
1	6.0	1.25	3.0
2	6.0	1.25	20.0
3	6.0	10.0	3.0
4	6.0	10.0	20.0
5	38.0	1.25	3.0
6	38.0	1.25	20.0
7	38.0	10.0	3.0
8	38.0	10.0	20.0
9	1.4	5.625	11.5
10	42.6	5.625	11.5
11	22.0	0.0	11.5
12	22.0	11.25	11.5
13	22.0	5.625	0.56
14	22.0	5.625	22.5
15	22.0	5.625	11.5
16	22.0	5.625	11.5

Table 2
Results of Rhamnose Production, EI (E24), and ST Obtained in the Experiments

Experiment	Rhamnose (g/L)	ST (mN/m)	E24 (%)
1	0.59	30.5	88
2	0.50	31	84
3	0.40	32	64
4	0.43	31.5	74
5	0.35	32.5	84
6	0.32	32.5	79
7	0.31	31.875	77
8	0.27	32.125	68
9	0.96	27.75	86
10	1.63	26.375	95
11	0.78	28.625	90
12	1.34	27	87
13	1.15	27.125	94
14	1.31	26.625	96
15	2.20	25.75	100
16	2.30	25.625	100

The results shown in Table 2 indicate that the maximum rhamnose syntheses were obtained in the fifteenth and sixteenth experiments in which the studied variables' concentrations were at the central point. This indicates that the variable maximum point in the optimization was near the central conditions of the design. The increase in the rhamnose

synthesis (RS) in the experiments promoted an increase in the emulsifying index (EI), demonstrating that both are probably directly related because the higher the biosurfactant concentration in the medium, the higher its capacity of being emulsified. Besides, the increase in the rhamnolipids concentration resulted in a decrease in the medium ST in all experiments performed.

In experiments one to eight, the alterations in the concentrations of RSO, AN, and RBY did not promote significant change in the medium ST. On the other hand, the lowest values of the ST were obtained with concentrations of RSO, AN, and RBY around 22, 5.625, and 11.5 g/L, respectively, which demonstrates that these concentrations are within the optimization region of the process. Besides that, all experiments were able to produce compounds that formed stable emulsions for 24 h. This analysis is a practical measurement of a biosurfactant utility because it gives the compound ability to emulsify nonmiscible liquids with stable emulsions formation. The determination of the significant parameters was performed through a hypothesis test (student's *t*-test) with 10% level of significance. The parameters that show a level of significance higher than this value were dismissed.

The empirically adjusted equations that represent the RS (R), ST (T_S), and the EI (I_E), are respectively, described in Eqs. 1–3.

$$R = 2.218 - 0.55\ X_1X_1 - 0.691\ X_2X_2 - 0.589\ X_3X_3 \quad (1)$$

$$T_S = 24.3769 + 2.209\ X_1X_1 + 2.662\ X_2X_2 + 2.096\ X_3X_3 \quad (2)$$

$$I_E = 102.86 - 4.937X_2 - \ 8.745X_1X_1 - 9.952\ X_2X_2 - 6.029\ X_3X_3 \quad (3)$$

In Eqs. 1 and 2, the isolated variables did not significantly influence the process, only the quadratic interactions did. But in Eq. 3, besides the quadratic variables, the AN also caused an increase in the EI response because when its concentration is lowered in the system, it promotes an increase in the response. The signs of these variable coefficients indicate a maximum point in the rhamnose production and in the EI, and a minimum point for the ST.

An algorithm done in the Maple V release 4 (Waterloo Maple, Inc., Canada) program was used to calculate the stationary point (P_0) for the ST and EI. These values are shown in Table 3. The λ's values that refer to the RS and to the EI indicate that these responses have a maximum point because they have equal and negative signs. However, the λs that refer to the ST indicate that this response has a minimum point as they present equal and positive signs.

The RS, ST, and EI, was 2.218 g/L, 25.372 mN/m, and 100%, respectively, in the optimization point from the codified variable values x_1, x_2, and x_3, as shown in Table 4. As expected, these values are very

Table 3
Stationary Point for the RS (R), ST, and EI (E24)

P_0	R	ST	E24
λ_1	–0.619	2.095	–10.812
λ_2	–0.587	2.141	–8.385
λ_3	–0.548	2.733	–5.530

Table 4
Codified Values of the Variables x_1, x_2, and x_3 in the Optimization Point

Coordinates	R	ST	E24
x_1	0.015	0.015	0.003
x_2	0.023	0.015	–0.249
x_3	0.007	0.008	–0.071

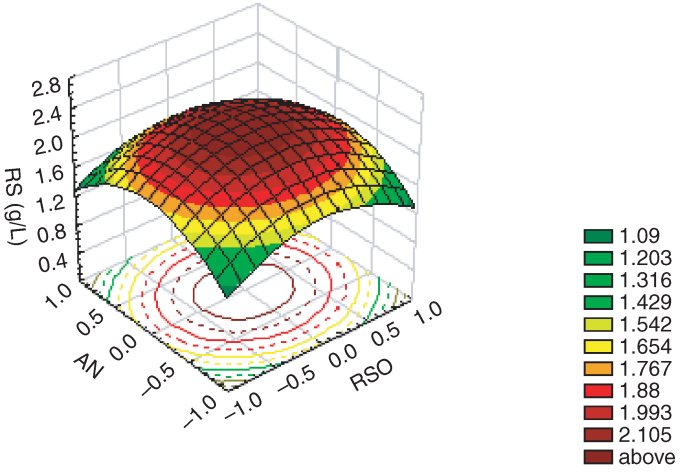

Fig. 1. Response surface for the RS in relation to the RSO and AN.

close to the ones obtained in experiments 15 and 16, as the variables of the maximization (rhamnose and EI) and minimization (ST) points are close to the central point.

Response surfaces were generated to facilitate the visualization of the effects of the independent variables: RSO, AN, and RBY on the RS, ST, and EI (Figs. 1–3). The maximum rhamnose production was obtained for values of RSO concentration and AN concentration in the central point region (Fig. 1). Figure 2 shows that the ST decreased for values of RSO a bit under the central point and for the values of residual brewery near the central point. The maximum EI was obtained for values of RBY a bit under the central point.

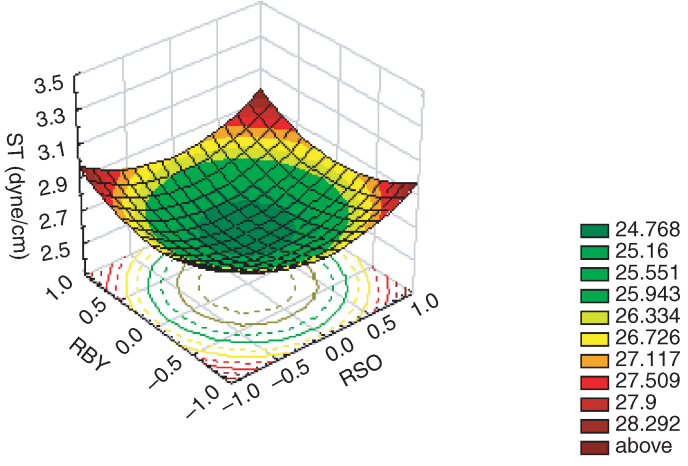

Fig. 2. Response surface for the ST in relation to the RSO and the RBY.

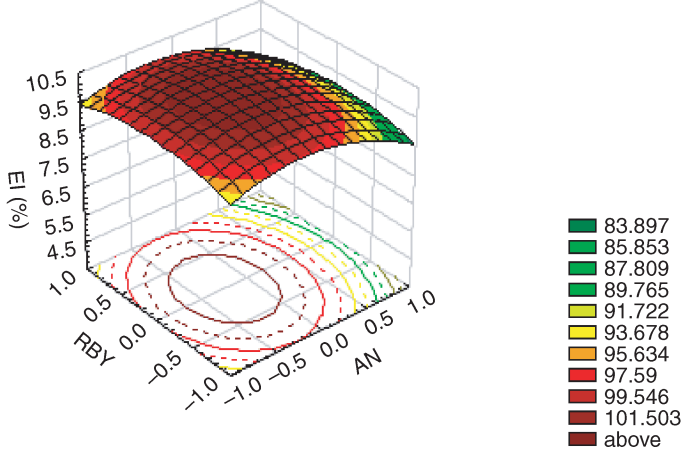

Fig. 3. Response surface for the emulsifying index (EI) in relation to the AN and the RBY.

Conclusions

The experimental design methodology was a very useful tool to determine the independent variables' behavior in rhamnose production, avoiding excessive analyses and showing general information about the influence of the independent parameters in the process. The results obtained in this work show that the isolated *P. aeruginosa* PACL presents good capacity of RSO degradation and great potential for biosurfactant production. Also, all experiments were able to produce compounds that formed stable emulsions for 24 h. The optimization of the analyzed responses showed that the best results found for the rhamnose concentration was 22.0 g/L of RSO, 5.7 g/L

of AN, and 11.0 g/L of residual brewery. All points were localized near the design central point.

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