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# Effect of Medium Composition on Changes of Surface Tension During Cultivation of *Pseudomonas aeruginosa* LBM10 Growing on Glycerol

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**ABSTRACT**
This study evaluated the ability of biosurfactant production by *Pseudomonas aeruginosa* LBM10 using glycerol as the sole carbon source. Batch cultivations were performed at 30°C and 180 rpm. The effects of glycerol concentration, C/N ratio and C/P ratio on changes of surface tension were analyzed through a Central Composite Rotational Design. Nitrogen and phosphorus-limiting conditions favored the biosurfactant production. In order to maximize the surface tension reduction, contour plots constructed by predictive polynomial equations resulted in a glycerol concentration of 13.2 g/l, a C/N ratio of 80 and a C/P ratio of 147.2.
***Keywords*** – Biosurfactant, experimental design, nitrogen limitation, phosphorus limitation, rhamnolipids

## I. INTRODUCTION

Biosurfactants are microbial compounds able to reduce surface and interface tension in systems of different polarities. They contain a hydrophobic portion with little affinity for the bulk medium and a hydrophilic group that is attracted to the bulk medium. Biosurfactants can be produced by a wide range of microorganisms, distributed in various genera, mostly bacteria [1]. They are classified according to their chemical composition as glycolipids, lipopeptides and lipoproteins, phospholipids, fatty acids and neutral lipids [2].

Biosurfactants have been used industrially as adhesives, flocculating, wetting and foaming agents, de-emulsifiers and penetrants, based on their abilities to lower surface tensions, increase solubility, detergency power, wetting ability and foaming capacity. The petroleum industry has traditionally been the major user, for processes such as bioremediation schemes and enhanced oil recovery [3]. Biosurfactants increase the solubility of petroleum components in water to enhance bioavailability, leading to higher oil degradation rates [4].

Microbial surfactants can be synthesized from renewable substrates such as vegetable oils and sugars. They have a great chemical diversity, enabling more specific applications. They also have important features against synthetic surfactants, including high biodegradability, low toxicity, thermal and pH stability and resistance to high salt concentration [5].

The effectiveness of a biosurfactant is determined by its ability to reduce surface tension, which is a measure of the surface free energy per unit area, required to bring a molecule from the bulk phase to the surface [3]. Surface activity is verified through a substantial reduction of the culture medium tension or related phenomena [6].

*Pseudomonas* spp. bacteria are well known for producing glycolipid-type biosurfactants containing rhamnose and 3-hydroxy fatty acids, known as rhamnolipids [7]. Glycolipid production by *P. aeruginosa* was firstly reported by Jarvis & Johnson (1949) [8]. Rhamnolipid-type biosurfactants produced by *P. aeruginosa* are a mixture of homologous species composed of a hydrophilic part consisting of one or two rhamnose molecules, respectively termed mono-rhamnolipid and di-rhamnolipid, and by a hydrophobic part consisting of one or two fatty acids [9]. Rhamnolipids are the most promising biosurfactants in industry due to their physical-chemical and biological features and the high concentrations obtained relative to other biosurfactants [1].

Few biosurfactants have been used on an industrial scale because of the lack of cost effective production processes. A possible solution to enable large-scale production of biosurfactants is the use of low-cost renewable substrates. Furthermore, the search for new biosurfactant-producing microorganisms that can be grown economically on an industrial scale continues [10].

Biodiesel production from animal fats and vegetable oils generate approximately 10% (w/w) glycerol as the main by-product, and the excess glycerol may become an environmental problem, since it cannot be disposed of in the environment [11]. Thus, glycerol from biodiesel can be considered as a

new economically attractive renewable carbon source for the production of biosurfactants, considering their availability and costs, because the governments around the world are encouraging the production of biodiesel from vegetable oils in order to reduce the greenhouse effect [12].

In a previous study, *P. aeruginosa* LBM10, isolated from a southern coastal zone in Brazil, had satisfactory potential for producing rhamnolipid-type biosurfactant. The microorganism was found to produce biosurfactants from various cheap carbon sources such as soybean oil, soybean oil soapstock, fish oil and glycerol, all of which are available in the South of Brazil [12].

The aim of this work was to investigate the effects of glycerol concentration, C/N ratio and C/P ratio on surface tension reduction (STR) during *P. aeruginosa* LBM10 grown on glycerol to establish a medium composition that is more favorable for biosurfactant excretion.

## II. MATERIAL AND METHODS

### 2.1. Microorganism

The microorganism used in this study was *P. aeruginosa* LBM10, which was previously isolated from a southern coastal zone in Brazil and shown to produce rhamnolipid-type biosurfactant from glycerol [12].

### 2.2. Shaken flask cultivation

The strain was activated in Tryptic Soy Agar (TSA) tubes and cultivated at 30ºC for 48 h. Bacterial growth was scraped from each slant tube with the aid of 2 ml of 0.1% peptone diluent, and the suspensions from four tubes were inoculated into 500 ml flasks with cotton plugs containing 200 ml of the medium. They were then incubated in a rotary shaker at 30ºC and 180 rpm for an overall culture period of 144 h. Samples were taken at regular intervals and centrifuged at 3,000 rpm for 30 min. Supernatants were used for determining surface tension and glycerol concentration, and pellets were used to quantify the biomass.

The culture medium was made up of glycerol, $NaNO_3$, $KH_2PO_4$ (variable concentrations according to the experimental design), and $MgSO_4.7H_2O$ (0.2 g/l). The initial pH of the broth was adjusted to 6.5 prior to sterilization.

### 2.3. Experimental design

A Central Composite Rotational Design (CCRD), a total of 17 trials ($2^3$ plus axial points and three replicates at the central point), was used to determine the influence of the glycerol concentration, C/N ratio, and C/P ratio variables, with the STR as a response. The data were analyzed by Statistica 5.0 software (StatSoft, Inc., USA).

### 2.4. Analytical methods

The surface tension was determined by a tensiometer (Kruss Processor Tensiometer K-6, Germany), in accordance with the method recommended by Du Nouy.

The glycerol was assessed by the enzymatic-colorimetric method for triglyceride content evaluation. Supernatant samples (0.03 ml) were added to 3 ml of reagent (Liquiform®, Labtest, Brazil), and maintained at 37ºC for 10 min. The absorbance was measured at 500 nm, with glycerol as the standard [13].

The biomass was monitored by measuring the absorbance at 600 nm [14]. A calibration curve between $OD_{600}$ and the cell dry-weight concentration (g/l) was first established.

## III. RESULTS AND DISCUSSION

As shown in Table 1, 17 trials were proposed by CCRD to study rhamnolipid production evaluated by STR in the culture medium with the glycerol concentration, C/N ratio and C/P ratio variables. Glycerol was used as a carbon source for microbial growth, but it was not entirely consumed within 144 h of cultivation. The cell population did not achieve high biomass values, varying from 0.9 g/l (Trial 12) to 4.5 g/l (Trial 11). These values are in agreement with those obtained for *P. aeruginosa* growing on glycerol [4] and other carbon sources [1, 15].

Among the experiments, Trial 9 (13.2 g/l of glycerol, C/N and C/P ratio of 80) resulted in a high STR of the medium, from 61.1 mN/m to 31 mN/m, corresponding to a 53.1% reduction of surface energy. A potential biosurfactant producer should be able to reduce the surface tension of the medium to values below 40 mN/m [16]. Therefore, in this study, *P. aeruginosa* LBM10 demonstrated the ability to synthesize surface active compounds with glycerol as the sole carbon source in the different media under study.

Rhamnolipid production by *P. aeruginosa* PA1, isolated from the Northeast of Brazil, and cultivated in a medium containing commercial glycerol (1% v/v) as the sole carbon source at a C/N ratio of 20, showed a 48.2% reduction in surface tension. Moreover, using babassu oil, paraffinic oil and *n*-hexadecane as carbon sources at the same C/N ratio, the values of STR were 31, 4.4, and 47.4%, respectively [13].

*Pseudomonas* sp. 44T1 isolated from frying oil reduced the surface tension of the medium by 43.85 and 26.1% with olive oil and sunflower oil, respectively, as the sole carbon source [16]. *P. aeruginosa* ATCC 10145 synthesized surface active compounds and reduced surface tension by 32.8% for the cashew apple juice culture medium, and by 41% for the same medium added with 5 g/l peptone [15].

Table 1. Coded values, real values (in parentheses) and experimental data obtained from the assays in the CCRD.

| Trial | $X_1$ | $X_2$ | $X_3$ | $ST_i$ (mN/m) | $ST_f$* (mN/m) | STR (%) | Glycerol* (g/l) | Biomass* (g/l) |
|---|---|---|---|---|---|---|---|---|
| 1 | -1 (20) | -1 (40) | -1 (40) | 59.0 | 33.7 | 42.9 | 10.7 | 1.9 |
| 2 | +1 (40) | -1 (40) | -1 (40) | 62.5 | 32.5 | 48.0 | 35.5 | 1.4 |
| 3 | -1 (20) | +1 (120) | -1 (40) | 58.5 | 31.2 | 46.7 | 12.8 | 1.4 |
| 4 | +1 (40) | +1 (120) | -1 (40) | 60.5 | 31.7 | 47.6 | 33.2 | 1.3 |
| 5 | -1 (20) | -1 (40) | +1 (120) | 70.0 | 33.7 | 51.9 | 10.8 | 1.4 |
| 6 | +1 (40) | -1 (40) | +1 (120) | 57.6 | 34.5 | 40.1 | 34.7 | 1.6 |
| 7 | -1 (20) | +1 (120) | +1 (120) | 59.6 | 31.8 | 46.6 | 12.0 | 1.8 |
| 8 | +1 (40) | +1 (120) | +1 (120) | 61.4 | 39.6 | 35.5 | 31.3 | 1.7 |
| 9 | -1.68 (13.2) | 0 (80) | 0 (80) | 66.1 | 31.0 | 53.1 | 3.6 | 2.4 |
| 10 | +1.68 (46.8) | 0 (80) | 0 (80) | 70.2 | 33.4 | 52.4 | 30.4 | 2.6 |
| 11 | 0 (30) | -1.68 (12.8) | 0 (80) | 65.7 | 33.1 | 49.6 | 6.3 | 4.5 |
| 12 | 0 (30) | +1.68 (147.2) | 0 (80) | 64.7 | 40.6 | 37.3 | 21.7 | 0.9 |
| 13 | 0 (30) | 0 (80) | -1.68 (12.8) | 62.9 | 29.6 | 52.9 | 16.9 | 2.6 |
| 14 | 0 (30) | 0 (80) | +1.68 (147.2) | 66.8 | 37.5 | 43.9 | 18.5 | 2.2 |
| 15 | 0 (30) | 0 (80) | 0 (80) | 70.8 | 33.9 | 52.1 | 17.6 | 2.2 |
| 16 | 0 (30) | 0 (80) | 0 (80) | 68.5 | 33.4 | 51.2 | 16.2 | 2.4 |
| 17 | 0 (30) | 0 (80) | 0 (80) | 70.8 | 33.4 | 52.8 | 19.3 | 2.3 |

$X_1$: glycerol concentration (g/l); $X_2$: C/N ratio; $X_3$: C/P ratio; $ST_i$: initial surface tension; $ST_f$: final surface tension; STR: surface tension reduction.
* 144 h cultivation.

The rhamnolipid-type biosurfactant produced by *P. aeruginosa* LBI isolated from oil-contaminated soils furthered the STR of the mineral medium by 42.8% and by 40% with sunflower oil and olive oil, respectively [17]. Raza et al. [18] found a STR of 29.4% in the culture medium composed of 20 g/l refined canola oil using the mutant bacterium *P. aeruginosa* EBN-8.

A second order model was able to predict the STR (dependent variable) as a function of the glycerol concentration, C/N ratio and C/P ratio (independent variables). Analysis of Variance (ANOVA) was used to evaluate adequacy of fit. Based on the ANOVA, as shown in Table 2, a second order model was established for the STR.

Table 2. ANOVA for STR.

| Source of Variation | Sum of Squares | Degrees of Freedom | Mean Squares | $F_{calculated}$ |
|---|---|---|---|---|
| Regression | 429.25 | 7 | 61.32 | 6.69 |
| Residual | 82.55 | 9 | 9.17 | |
| Pure Error | 1.26 | 2 | | |
| Lack of Fit | 81.29 | 7 | | |
| Total | 511.80 | 16 | | |

$F_{tabulated}$:3.29; Correlation coefficient (R): 0.91.

Effects that were not statistically significant were ignored. The correlation coefficient was 0.91, and the F-value was 2 times higher than the listed value for a 95% confidence interval. Consequently, the model was found to be adequate to describe the response surface of STR (Fig. 1). The coded empirical model fitted by regression analysis is shown in Eq. 1.

$$STR\ (\%) = 51.71 - 1.32*(Glycerol) - 1.99*(C/N) - 3.61*(C/N)^2 - 1.93*(C/P) - 1.85*(C/P)^2 - 3.61*(Glycerol)*(C/P) - 1.65*(C/N)*(C/P) \quad (1)$$

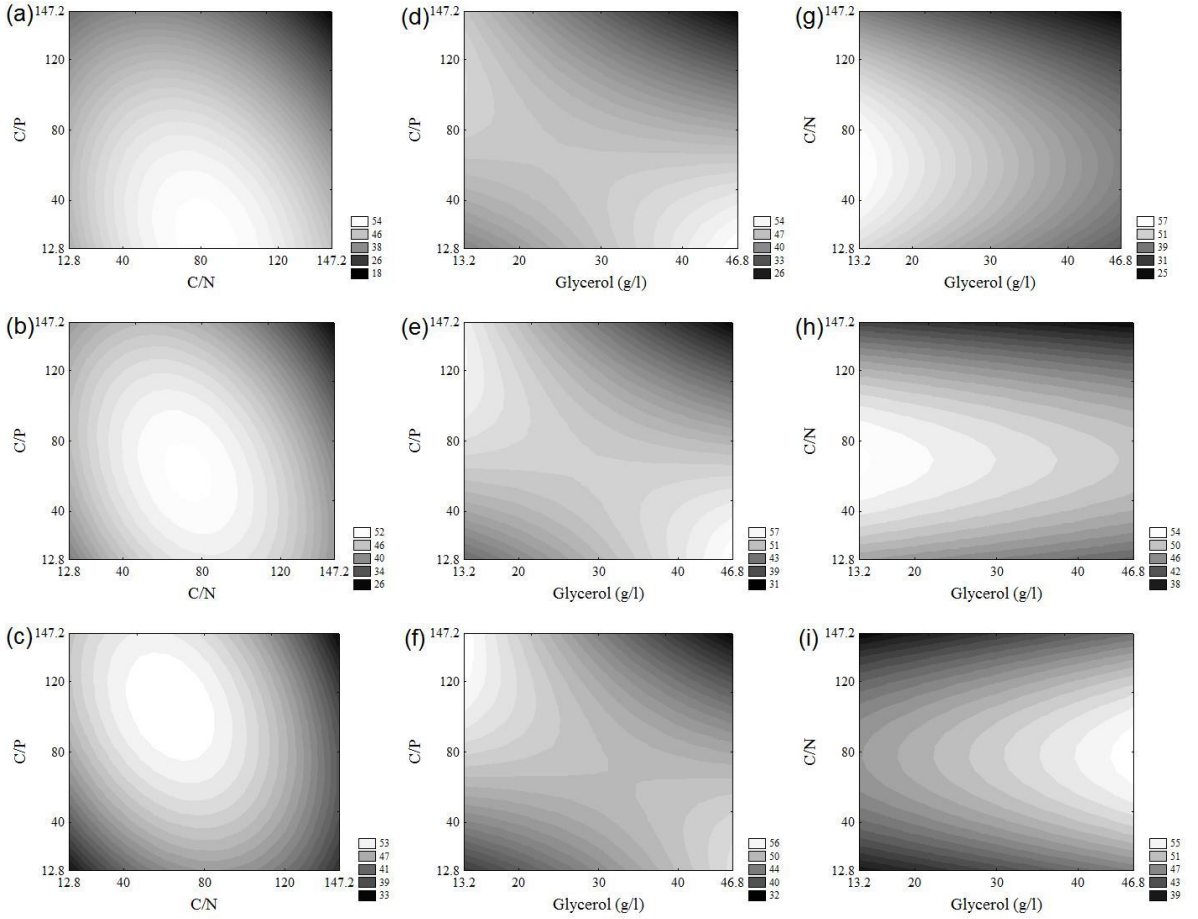


Figure 1. Contour plots for STR as a function of C/N ratio and C/P ratio with glycerol concentration at the levels 40 g/l (a), 30 g/l (b), and 20 g/l (c); as a function of glycerol concentration and the C/P ratio with the C/N ratio at the levels 120 (d), 80 (e), and 40 (f); as a function of glycerol concentration and the C/N ratio with the C/P ratio at the levels 120 (g), 80 (h), and 40 (i).

For a glycerol concentration of 40 g/l (Fig. 1a), the C/N and C/P ratios indicated for obtaining the highest STR (54%) were found to be approximately 80 and 12.8 to 40, respectively. When glycerol concentration was 30 g/l (Fig. 1b), the highest STR was obtained (52%) for the C/N ratio ranging from 50 to 100, and the C/P ratio from 25 to 100. For a glycerol concentration of 20 g/l (Fig. 1c), the highest STR (53%) was obtained for the C/N ratio ranging between 40 and 80, and the C/P ratio between 80 and 130.

Analysing Fig. 1d, two favorable points for STR were found when the C/N ratio was 120 when the C/P ratio was set between 100 and 147.2, at low glycerol concentration, or at low C/P ratio and high glycerol concentration (ranging from 12.8 to 40 g/l). Similar results were found for the C/N ratio between 80 and 40 (Fig. 1e and 1f). In these conditions, the STR values were found to be higher than 55%.

When the C/P ratio was set at 120 (Fig. 1g), the highest STR was obtained for the glycerol concentration of 13.2 g/l and the C/N ratio between 40 and 80. For an intermediate level (C/P of 80), the highest STR was obtained for the C/N ratio between 40 and 80, with glycerol concentration ranging from 13.2 to 20 g/l (Fig. 1h). For a C/P ratio of 40 (Fig. 1i), the highest STR was obtained for glycerol concentration between 40 and 46.8 g/l, and the C/N ratio approximately 80.

Therefore, the STR was found to be equal to or higher than 54% using glycerol at low concentration (13.2 g/l), the C/N ratio at approximately 80, and the C/P ratio at 147.2.

The most relevant STR values were found for nitrogen and phosphorus-limiting conditions, i.e. high C/N and C/P ratios, noting that the nutritional limitations direct the metabolism of *P. aeruginosa* LBM10 to the rhamnolipid-type biosurfactant synthesis. The rhamnolipid expression in *P. aeruginosa* is coordinately regulated at the transcriptional level by quorum-sensing systems depending on cell-density and regulators that respond presumably to a variety of signals. According to Bazire et al. [19], phosphate limitation is a signal that affects rhamnolipid production positively. This is in agreement with Guerra-Santos et al. [20] and Rashedi

et al. [21], who pointed out that the C/P ratio at 16 resulted in the highest rhamnolipid production. On the other hand, during the biosynthesis of rhamnolipid, lipid, not sugar, formation is the rate-determining factor, and nitrogen limitation may promote lipid accumulation [22]. Therefore, the C/N ratio is a vital factor influencing the performance of rhamnolipid production and some reports have mentioned that rhamnolipid production is more efficient under nitrogen-limiting conditions. Santa Anna et al. [13], Rashedi et al. [21] and Wu et al. [4] established C/N ratios of 60, 55 and 52, respectively, for the maximization of rhamnolipid production.

Moreover, the highest STR at the lowest glycerol concentration can be related to the inhibitory effect of high substrate concentration. According to Santa Anna et al. [13], high glycerol concentrations result in a possible inhibitory effect on the bacterium metabolism due to a likely nutrient transport deficiency.

## IV. CONCLUSION

*P. aeruginosa* LBM10 was able to assimilate glycerol as the sole carbon source, and is therefore a potential producer of rhamnolipid-type biosurfactant. The contour plots enabled us to find that a high STR (values equal to or higher than 50%) was obtained by glycerol at low concentration (13.2 g/l), with C/N and C/P ratios of approximately 80 and 147.2, respectively.

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