PRODUCTION OF RHAMNOLIPID BIOSURFACTANT BY *Pseudomonas aeruginosa* USING DIFFERENT BIOREACTOR OPERATING MODES AND CRUDE GLYCERIN

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ABSTRACT

Biosurfactants such as rhamnolipid (RML) are known for their wide range of applications, high biodegradability and production from renewable sources. Despite this, their low productivity and high cost compared to synthetic surfactants makes them a small part of the surfactant market. In this context, the aim of this work was to produce rhamnolipids (RMLs) using crude glycerin, a cheap and abundant raw material on the Brazilian market, using different modes of operation: single batch, pulse, continuous and sequential fed-batch The simple batch was tested with initial glycerol concentrations of 30, 39, 47 and 60g/L. The 47g/L initial glycerol simple batch strategy achieved final RML concentrations 8.7 and volumetric productivities (Pp) of 34.9 $mg.L^{-1}.h^{-1}$. Comparing these process with the pulse, sequential and continuous fed-batches, was achieved, respectively, concentrations of 8.4, 9.2, 5.7 and 11.2 g/L of RML; Pp of 34.9, 47.1, 10.5 and 47.2 $mg.L^{-1}.h^{-1}$; production of 22.3, 36.8, 59.4 and 44.9 g of RML; productivity (Q) of 139.5, 188.4, 104.6 and 188.6 mg/h of RML. Thus, under the conditions studied, the best strategies were the continuous fed-batch, which performed best in terms of concentration and productivity, and the sequential fed batch, which performed best in terms of RML mass production.

Keywords: Rhamnolipid, Bioreactor, Fed-batch, *Pseudomonas aeruginosa*, Glycerin.

1 INTRODUCTION

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic structures, which gives them the ability to reduce the surface or interfacial tension of the system in which they are applied. As a result, surfactants present good wettability, emulsification index, detergency, foaming and solubilization; therefore, they have possible applications in the cosmetics, agrochemical, food, oil, pharmaceutical, construction, textile and other sectors[1].The surfactants most commonly used on an industrial scale are produced by chemical routes and are usually synthesized from petrochemical sources. In addition to their low degradability, synthetic surfactants are known to be toxic to marine species and to cause harmful phenomena to the ecosystem, such as the "detergent swan" (formation of a layer of dense foam that carries pollutants over long distances, reducing the entry of sunlight and photosynthesis, causing mortality of local species) and eutrophication (since some detergents have phosphate groups).

In this context, the production of surfactants by biotechnological routes has emerged as an ecologically favorable alternative, given their better biodegradation time and production from renewable sources. Compared to synthetic surfactants, biosurfactants also generally have better critical micellar concentration; stability at different pH values, temperatures and ionic strengths; low toxicity to both wildlife and humans. Although these advantages, biosurfactants still represent a small part of surfactants market (USD 4.4B of USD 45.2B in 2023) mainly because their high production cost makes their large-scale production difficult[2,3].To minimize this barrier, several studies have been carried out using different strategies with the aim of making the process cheaper and increasing their productivity.

Thus, this work aimed to contribute to the feasibility of production and commercialization of rhamnolipid-type biosurfactants produced by the bacterium *Pseudomonas aeruginosa* in alternative bioreactor operating modes (simple batch, pulse fed, continuous fed, and sequential fed) using crude glycerin, which is a cheap and available agro industrial by-product in Brazilian market.

2 MATERIAL & METHODS

Rhamnolipids were produced using the LFM634 strain of the bacterium *Pseudomonas aeruginosa* obtained from the Bioproducts Laboratory Collection (tel: +55 11 3091-7352) (Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo). The microorganisms grew in rich medium with the following composition: $NaNO_3$ (1.0g/L), $MgSO_4.7H_2O$ (0.2 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (3.0 g/L), Peptone (5.0 g/L), Yeast Extract (5.0 g/L), Glycerol (30 g/L) [4]. The flasks were shaken at 200 rpm, at 30 ºC for 40 h and the contents were added into 2 mL slants with glycerol as cryoprotectant at final concentration of 20% v/v and stored at -20°C or -80°C. To prepare the pre-inoculum, the contents of the slant were added to a 1 L Erlenmeyer flask containing 300 mL of rich medium and cultivated for 40 h. The minimal medium was composed by: $NaNO_3$ (5.54 g/L), $MgSO_4.7H_2O$ (0.2 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (3.0 g/L), Yeast Extract (2 g/L), Glycerol (30 g/L)[4]. The bioreactor BioFlo® Celligen 310® (New Brunswick Science) with a nominal volume of 8 L, with 4 L of minimal medium, was inoculated with 10% v/v pre-inoculum. The temperature was maintained at 30 ºC, the mechanical agitation was performed by paddles stirred at

600 rpm, with surface aeration at a flow rate of 3 L/min, resulting in an initial oxygen transfer coefficient equivalent (kLa_{equiv}) equal to 1.7 h^{-1}. The fed medium contained glycerol, $NaNO_3$, $MgSO_4$, K_2HPO_4, KH_2PO_4; in the proportions 30: 5.5: 0.2: 7: 3. These conditions were maintained for all bioreactor cultivations. All the culture media were sterilized in an autoclave for at least 15 minutes at a pressure of 1 bar. The crude glycerin was obtained from the production of biodiesel and collected at the Montes Carlos Biodiesel Plant and provided by Petrobras.

During the cultivation of the LFM634 strain, cell growth curve was obtained by measuring the absorbance in a spectrophotometer at 600 nm, converting to cell concentration by the factor (f) of 0.8162; where [Biomass(g/L) = (f) . Abs]. Samples taken from the cultures were centrifuged for 10 minutes at 10^4 rpm. From the supernatant, the rhamnolipid concentration was determined by acidifying the sample to a pH close to 3 with concentrated sulfuric acid, followed by a water bath at 98ºC for 4 hours[5]. After neutralizing the sample, it is filtered and injected into the HPLC (HPLC Agilent Tecnologies) equipped with an HPX-87H column (BioRad - 300mm x 7.8 mm) to quantify rhamnose. Using a curve previously drawn up by mass spectrometry, it is possible to mathematically convert this value to rhamnolipid. Glycerol is determined directly in the HPLC at a temperature of 40°C, using 0.0032M sulfuric acid solution as the mobile phase with a flow rate of 0.6mL/min and a sample injection volume of 20 µL[6].

3 RESULTS & DISCUSSION

The first RML production strategy tested was the simple batch, which was carried out with initial glycerol concentrations of 29.8, 38.6, 47.4 and 60.5 g/L. (Figure 1) The processes reached concentrations of 4.2, 5.6, 8.4 and 8.7 g/L, respectively; and volumectric productivities (P_p) of 36.5, 36.5, 34.9 and 15.6 mg.L^{-1}.h^{-1}(Table 1). Thus, under the conditions studied, the process with an initial 47.4g/L of glycerol proved to be advantageous as it had both high concentration and productivity.

The second strategy studied was a pulse fed-batch, with initial glycerol concentration of 30g/L. When glycerol concentration was close to zero, the fed solution was added in suficient amount to reach 30 g/L. This process was repeated 3 times. At the end of the first cycle (simple batch), a RML concentration of 4.2 g/L was reached and in the following cycles, 9.2, 9.7 and 10.8 g/L were obtained, respectively; with P_p of 36.6, 47.1, 31.2, 25.2 mg.L^{-1}.h^{-1} (fig 1, Table 1) In this way, it is clear that the pulse-fed batch strategy is very interesting and should be limited to just one fed cycle, as the increase in RML concentration in the following cycles is not justified by the drop in productivity.

The third strategy studied was the sequential fed-batch. The process started with 30 g/L of glycerol and when the concentration droped to zero, half of the culture medium was removed and the same volume of fresh medium was added, in suficient amount to achieve the same initial glycerol concentration. After the first glycerol consumption cycle, 3 more feeding cycles were carried out. At the end of the first cycle, a RML concentration of 4.79 g/L was reached, and in the following cycles, 5.7, 5.6 and 6.8 g/L were obtained, respectively; with P_{Pcycle} of 28.8, 28.3, 23.8, 33 mg.L^{-1}.h^{-1} and P_P of 10.5 mg.L^{-1}.h^{-1} (fig 1, Table 1).

The fourth strategy was the continuous fed-batch, with initial glycerol concentration of 30 g/L. When the concentration approached zero, the feeding solution was initiated at a flow rate of 6.75 mL.L^{-1}.h^{-1}. This flow rate was adjusted throughout the process in order to maintain glycerol concentrations around 0.5 g/L - reaching 30.60 mL.L^{-1}.h^{-1}. At the end of 238 h, a concentration of 11.22 g/L of RML was reached with a P_p of 47.14 mg.L^{-1}.h^{-1} (fig. 1, Table 1)

Table 1 Comparison of RML production results by simple (47g/L), Pulse Fed, Countinuos Fed, Sequencial Fed-batch strategies.

Operating mode	Simple (30g/L)	Simple (39g/L)	Simple (47g/L)	Simple (60g/L)	Pulse Fed-Batch	Sequencial Fed-Batch	Continuous Fed-Batch
Glycerol(g)	119.3	154.6	189.4	242.8	226.0	451.8	460
Biomass(g)	22.1	19.1	22.6	22.5	31.4	80.1	36.5
RML(g)	16.9	22.3	33.3	34.8	36.8	59.4	44.9
RML(g/L)	4.2	5.6	8.4	8.7	9.2	5.7	11.2
Q (mg/h)	146.2	146.1	139.5	62.4	188.4	104.6	188.6
P_p (mg.L^{-1}.h^{-1})	36.5	36.5	34.9	15.6	47.1	10.5	47.2
$Y_{x/s}$ (g/g)	0.19	0.12	0.12	0.09	0.14	0.18	0.08
$Y_{p/s}$ (g/g)	0.14	0.14	0.18	0.16	0.16	0.13	0.10
$Y_{p/x}$ (g/g)	0.76	1.17	1.48	1.47	1.17	0.74	1.23

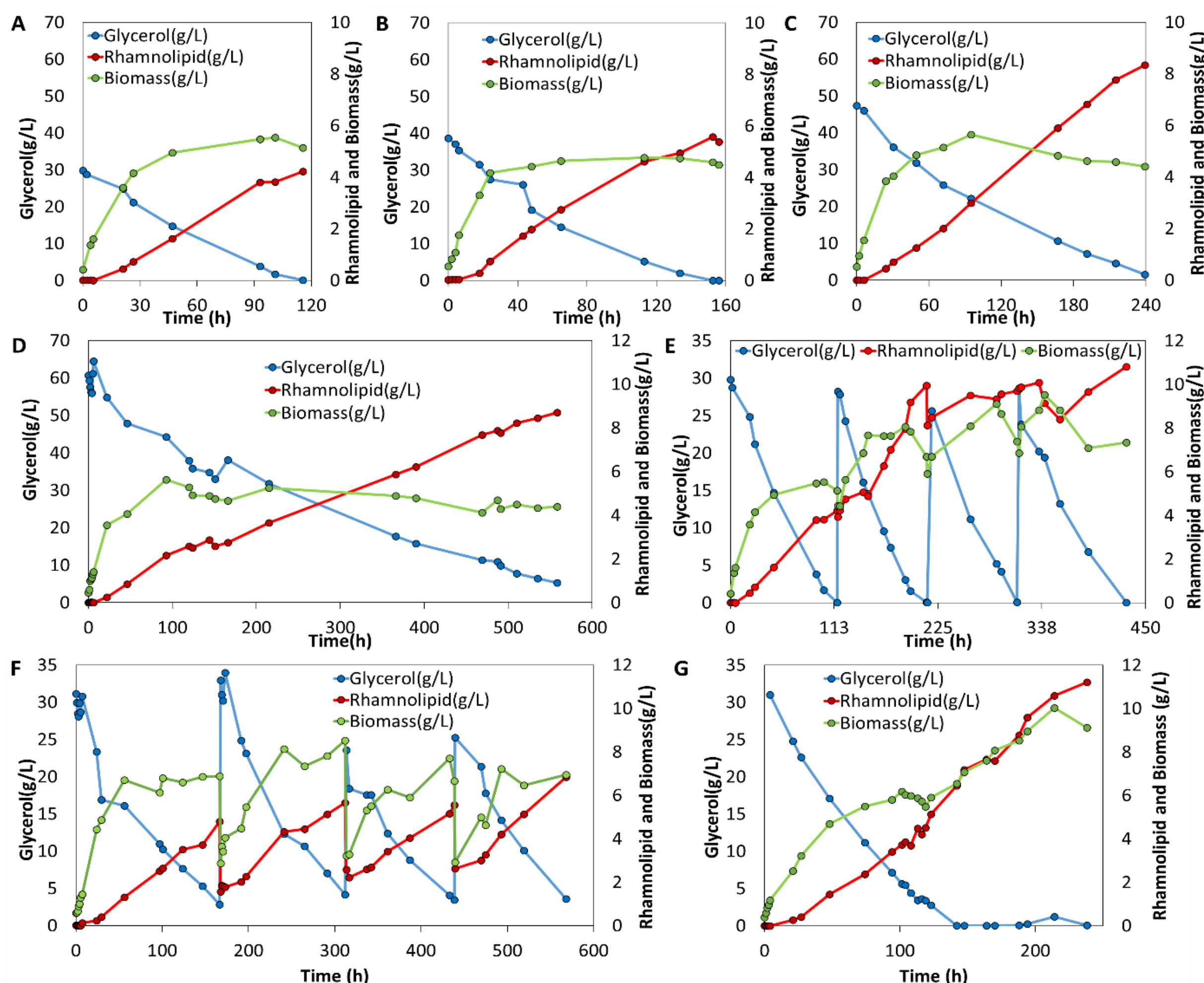

Figure 1 Rhamnolipid production kinetics of simple batch strategies with initial glycerol concentration of (**A**) 30g/L, (**B**) 39g/L, (**C**) 47g/L, (**D**) 60g/L, (**E**) pulse fed-batch, (**F**) sequential fed-batch and (**G**) continuous fed-batch.

4 CONCLUSION

At the scale and conditions studied, the continuous fed-batch was the best strategy, with the highest RML concentration (11 g/L) and volumetric productivity (47.2 $mg.L^{-1}.h^{-1}$), although the sequential fed-batch showed the highest absolute RML production (59.4g). The next stages of this work include a comparative economic analysis between the most interesting operating modes to see if the preference for continuous feeding is maintained.

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