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Evaluation of Different Carbon and Nitrogen Sources in Production of Rhamnolipids by a Strain of *Pseudomonas aeruginosa*

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Abstract

Culture conditions involving variations in carbon and nitrogen sources and different C:N ratios were examined with the aim of increasing productivity in the process of rhamnolipid synthesis by *Pseudomonas aeruginosa*. In addition to the differences in productivity, the use of different carbon sources resulted in several proportions related to the types of rhamnolipids synthesized (monorhamnolipids and dirhamnolipids). Furthermore, the variation in nutrients, mainly the nitrogen source, resulted in different amounts of virulence factors, as phenazines and extracellular proteins. The data point out a new concern in the choice of substrate to be used for rhamnolipid production by *P. aeruginosa*: toxic byproducts.

Index Entries: Rhamnolipids; biosurfactant; virulence factors; *Pseudomonas aeruginosa*.

Introduction

Rhamnolipids produced by *Pseudomonas aeruginosa* are a most promising biosurfactant since they display a variety of commercial applications

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in the oil, pharmaceutical, and chemical industries *(1–3)*. Emulsification, demulsification, detergency, and wetting, among others, are important properties ascribed to such molecules. Major improvements in the productivity of bioprocesses are generally attributed to the development of superior strains via classic and recombinant genetic techniques; however, other parameters such as the nutritional and physical environment to which a microorganism is exposed are also known to significantly alter product yield. Thus, the evaluation of suitable substrates and the optimization of medium are essential for the development of this bioprocess, if it is to result in high yields and productivity values and diminish production costs. Among the substrates, the choice of carbon source exerts an important influence on the kinetics of uptake and its conversion in the desired product. In addition, there is a relation between the use of a specific carbon source and the proportion of rhamnolipids species synthesized (di- and monorhamnolipids) *(2–4)*. That difference in the composition of the rhamnolipid species affects global surfactant properties*(1)*. The nitrogen source is another component that exhibits an important role in the production of rhamnolipids. Nitrate salts, e.g., supported maximum surfactant production in *P. aeruginosa (2)*. The ratio of carbon and nitrogen nutrients is a fundamental parameter affecting the degree of productivity. The production of rhamnolipids can be carried out using diverse carbon sources, such as glycerol, mannitol, fructose, sucrose, glucose, ethanol, *n*-alkanes, *n*-paraffin, polycyclic aromatic hydrocarbons, vegetable oils *(5–10)*, and nitrogen sources, such as yeast extract, peptone, beef extract, soy flour, $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, and NH_4NO_3 *(1,2,8)*.

Another important aspect that should be taken into account in the choice of substrate is the extracellular product derived from secondary metabolism. In particular, in the rhamnolipid biosynthesis by *P. aeruginosa*, the synthesis of many virulence factors increases as a function of the substrate used. Several of the excreted proteins (e.g., elastase, Las A protease, phospholipase C [PLC], lipase, alkaline protease, Exoenzyme S, Toxin A) *(11,12)*, alginate, and phenazines affect the pathogenicity displayed by *P. aeruginosa*. Phenazine pigments, e.g., are secreted in copious amounts when *P. aeruginosa* grows aerobically in phosphate-poor medium, and exotoxin A and PLC are produced when iron and phosphate are limiting, respectively *(13)*. The major toxic phenazine produced by *P. aeruginosa* is pyocyanin *(14)*. Pyocyanin inhibits epidermal cell growth and lymphocyte proliferation, has antibiotic properties against other microorganisms, and inhibits mammalian cell respiration *(15,16)*.

The present work investigates and suggests the most appropriate carbon sources among glycerol, ethanol, soy oil, and olive oil, as well as between ammonium sulfate and sodium nitrate as sources of nitrogen for the production of rhamnolipids by *P. aeruginosa* PA1. The synthesis of undesirable byproducts such as pyocyanin and the variation in rhamnolipids species synthesized were also investigated.

Materials and Methods

Inoculum

P. aeruginosa PA1 *(17)* was obtained from a microorganism collection belonging to the Brazilian Oil Research Center (CENPES-Petrobrás/BR) and maintained in 10% glycerol at –80°C. The thawed strain sample was inoculated onto YPDA plates (0.3% yeast extract, 1.5% peptone, 0.1% d extrose, 1.2% agar) at 30°C for 48 h. The growth of inoculum was performed by the addition of a loopful of cells from YPDA plates to a 1000-mL Erlenmeyer flask containing 300 mL of medium of the following composition: 1.0 g/L of $NaNO_3$, 3.0 g/L of KH_2PO_4, 7.0 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 5% yeast extract, 5% peptone, and 3% glycerol. *P. aeruginosa* was grown at 30°C in a rotary shaker at 170 rpm. The cells were then harvested by centrifugation (10,000*g*, 20 min) and used to inoculate the bioreactors.

Media and Growth Conditions

Liquid media were prepared from a basal solution (MSP) having the following composition: 3.0 g/L of KH_2PO_4, 7.0 g/L of K_2HPO_4, and 0.2 g/L of $MgSO_4 \cdot 7H_2O$. This solution was supplemented with different carbon and nitrogen sources (different C:N ratios), and following sterilization, the media were inoculated with *P. aeruginosa* cells, and then cultivated in 500-mL Erlenmeyer flasks containing 100 mL of medium at 30°C in a rotary shaker at 170 rpm for 144 h. Glycerol substrate at 30 g/L and nitrogen sources ($NaNO_3$ and $[NH_4]_2SO_4$) with varying concentrations were used to obtain the desired C:N ratios (10, 20, 40, and 60). $NaNO_3$ (1.0 g/L) was used to determin C:N ratios with vegetable oils (soy oil and olive oil) and the C:N values of 27.5, 55.0, 110, and 165 were obtained. The production of rhamnolipids using ethanol at 2% (v/v) was performed varying the nitrogen sources: 5 g/L of yeast extract, 5 g/L of peptone, 1.85 g/L of $NaNO_3$, and 1.53 g/L of $(NH_4)_2SO_4$. For analysis of phenazines, medium MSP was supplemented with 3% glycerol and $NaNO_3$ or $(NH_4)_2SO_4$ to C:N ratios equal to 20.

Cell Biomass

The cell concentration was measured by absorbance at 500 nm, and the cell dry wt (g/L) was determined using a standard calibration curve (ABS = 1.2595 dry wt [g/L] – R^2 = 0.989) considered valid for absorbance values up to 0.6.

Quantification of Rhamnolipids

Indirect quantification of rhamnolipids was done by measuring rhamnose using the method of Dubois et al. *(18)*. A 0.5-mL vol of cell supernatant was mixed with 0.5 mL of 5% phenol solution and 2.5mL of sulfuric acid

and incubated for 15 min before measuring the absorbance at 490 nm. The results were compared with the standard curve for rhamnose.

Extraction of Rhamnolipid Mixture from Culture

The rhamnolipids were separated from the cells by centrifugation (10,000*g*, 4°C, 20 min), and the supernatant was acidified to pH 2.0 with 4.0 *N* HCl and kept at 4°C overnight. The resulting rhamnolipid precipitate was recovered by centrifugation (10,000*g*, 4°C, 30 min), resuspended with 0.04 *N* HCl, and extracted with the same volume of ethyl acetate. The organic phase was separated, dried with anhydrous $MgSO_4$, and evaporated under vacuum.

Analysis of Rhamnolipid Mixture by Thin-Layer Chromatography

The rhamnolipids extracted were analyzed by thin-layer chromatography (TLC) on silica 60 gel aluminum sheets (Merck, Darmstadt, Germany) using the solvent system $CHCl_3/CH_3OH/CH_3COOH$ (65:15:2) *(6,19)*. The separated zones were stained with vanillin–sulfuric acid (10% vanillin and 5%sulfuric acid in ethanol) reagent followed by heating to 100°C for 15 min.

Protein Quantification

Extracellular protein, obtained from the supernatant culture, was measured using the method of Lowry et al. *(20)*.

Measurement of Phenazines

The relative quantification of phenazines produced by *P. aeruginosa* was performed by measureing the absorbance spectrum between 200 and 500 nm (Beckman DU-70 spectrophotometer) after organic extraction in chloroform from cell-free culture medium (1:3) and analysis of principal wavelengths: 327, 309, 242 (pyocyanin); 368, 360, 350, 264 (1-hydroxyphenazine); 369, 251 (phenazine-1-carboxylic acid) *(15)*.

Results and Discussion

With the aim of improving the volumetric productivity (Q_P) in the rhamnolipid production, an experiment was carried out with different inoculum concentrations, and an increase of approx 62% in the volumetric productivity was achieved when the initial cell concentration rose from 0.32 g/L (Q_P = 0.0143 g/[L·h]) up to 3.0 g/L (Q_P = 0.0232 g/[L·h]), using glycerol as substrate (Fig. 1). Another aspect that was fundamental to improvement of productivity was the C:N ratio, since the best results were attained with higher values of this parameter (Figs. 2 and 3), corroborating the results reported in the literature indicating that the stimulation of rhamnolipid synthesis takes place under nitrogen-limited conditions *(1–3)*. Our results also seem to indicate that the influence of

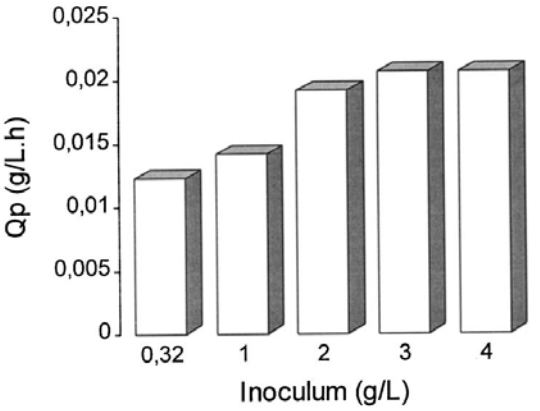

Fig. 1. Effect of inoculum size on volumetric productivity of rhamnolipids by *P. aeruginosa*.

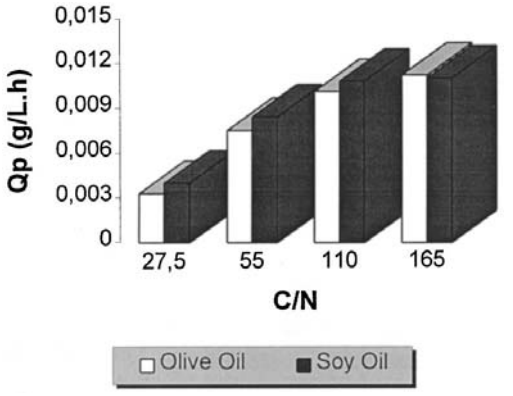

Fig. 2. Effect of variations in C:N ratio on volumetric productivity of rhamnolipids synthesized with vegetable oils and nitrate by *P. aeruginosa*.

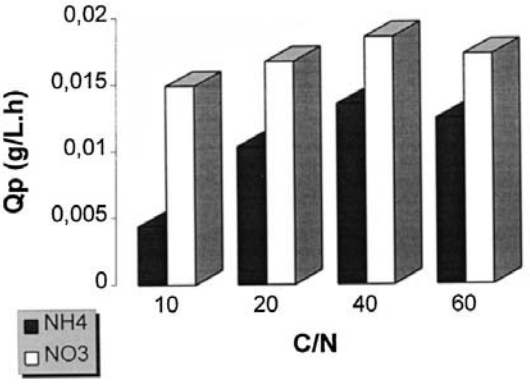

Fig. 3. Effect of variations in C:N ratio and nitrogen source on volumetric productivity of rhamnolipids synthesized from glycerol by *P. aeruginosa*.

C:N ratio is dependent on the metabolic route used for nitrogen source uptake. This is suggested by the response obtained following a comparison of nitrate and ammonium (Fig. 3).

The "door of entrance" of nitrogen in the metabolism of carbon is glutamate, which is formed by the reductive amination of α-ketoglutarate. There are two biochemical mechanisms through which this can occur. A one-stage reaction catalyzed by glutamate dehydrogenase is effective in environments where plenty of ammonium exists. The other mechanism, a process in two stages, in which glutamine is the intermediary, is used in conditions in which the ammonium concentration is low. This second mechanism allows the cells to use free energy through hydrolysis of adenosine triphosphate (ATP); thus, glutamine synthetase can assimilate ammonium from the environment. A correlation has already been noted between glutamine synthetase activity and nitrogen-limited conditions *(21)*. Additionally, since rhamnolipid by itself aids the uptake of carbon sources in conditions in which nitrogen is limiting, the pathway of enzyme glutamine synthetase is activated, consequently requiring a higher ATP production. Therefore, a higher consumption of the carbon source to support this new energy status is also necessary. This would lead to a cycle that would stimulate the synthesis of rhamnolipids. That hypothesis is supported by the well-known observation related to the increment in rhamnolipid productivity in nitrogen-limited conditions. As shown in Fig. 3, the use of nitrate in any C:N ratio results in better productivity compared with ammonium C:N ratios, using glycerol as carbon source. This can be interpreted as a simulation of nitrogen-limited conditions because nitrate first suffers dissimilatory nitrate reduction to ammonium *(22)*, and then assimilation by glutamine-glutamate metabolism. In other words, even with different C:N ratios, the assimilation of nitrate as nitrogen source is slower than assimilation of ammonium ions.

In our studies of the best choice of carbon source for improving productivity, glycerol, ethanol, soy oil, and olive oil were used as substrates. Although the literature shows the potential of ethanol *(8)* and vegetable oils *(3)* as good substrates for rhamnolipid production, the use of glycerol with a selected strain of *P. aeruginosa* resulted in better performance. The highest concentration of rhamnolipids (3.34 g/L expressed in rhamnose concentration) was obtained when glycerol was employed as substrate and nitrate as nitrogen source, while the use of ethanol (with yeast extract as nitrogen source), olive oil, and soy oil (with nitrate as nitrogen source) resulted in concentrations of these surface-active agents limited in 2.02, 1.61, and 1.59 g/L, respectively.

The partial characterization of the products obtained by *P. aeruginosa* PA1, using the TLC technique as described by Shenk et al. *(19)*, revealed the presence of two different areas with R_f (retardation factor) values equal to 0.74 ± 0.04 and 0.36 ± 0.04 (Fig. 4). According to Shenk et al. *(19)* and Arino et al. *(6)*, those R_f values (0.74 and 0.36) are consistent with the types monorhamnolipid and dirhamnolipid, respectively. The propor-

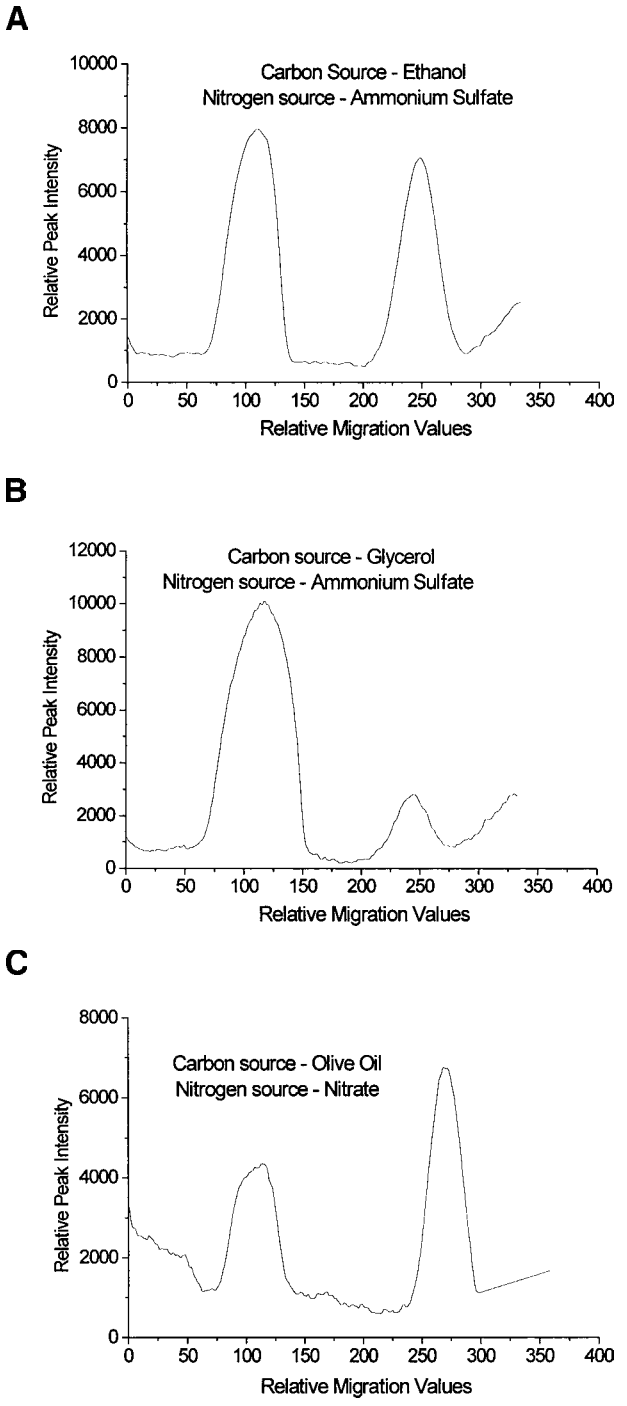

Fig. 4. Typical TLC densitometry of rhamnolipids. The first peak (R_f = 0.36) refers to the dirhamnolipids and the second peak (R_f = 0.74) to the monorhamnolipids. **(A)** Ethanol and ammonium sulfate as nutrients, **(B)** glycerol and ammonium sulfate as nutrients, and **(C)** olive oil and nitrate as nutrients.

Table 1
Proportion Between Species of Rhamnolipid Produced on Different Media Compositions

Carbon source	Nitrogen sources	Dirhamnolipids (%)	Monorhamnolipids (%)
Glycerol	$(NH_4)_2SO_4$	84.8	15.2
	$NaNO_3$	85.2	14.3
Ethanol	$(NH_4)_2SO_4$	58.8	41.2
	$NaNO_3$	73.5	26.5
Soy oil	$(NH_4)_2SO_4$	33.0	67.0
	$NaNO_3$	45.9	54.1
Olive oil	$(NH_4)_2SO_4$	38.7	61.3
	$NaNO_3$	43.7	56.3

tion between them was different based on the carbon source utilized (Table 1). There was a clear difference in the rhamnolipid proportion when glycerol was used compared with ethanol or vegetable oils. The nitrogen sources had little influence on the proportions of rhamnolipid species synthesized, with a slight increase in monorhamnolipid species when ammonium was used in place of nitrate, particularly when the carbon source was glycerol or vegetable oils. This different proportion among the types of rhamnolipids synthesized can represent an important change in the characteristics of the produced biosurfactant, affecting parameters such as the critical micelle concentration and interfacial tension *(4)*. Somehow, different carbon sources interfere either in the synthesis or, indirectly, in the reaction kinetics of the enzymes rhaminosyltransferase 1 or rhaminosyltransferase 2.

Other elements that varied with changes in the cultivation medium were virulence factors. The production of rhamnolipids by *P. aeruginosa* is associated with gene expression of virulence factors, such as pyocyanin, elastase, C-protease, alginate, and pyoverdin *(23–25)*, depending on the nature of the medium's constituents. Replacing ammonium with nitrate as a nitrogen source in the production of rhamnolipids from glycerol resulted in an increase in the level of phenazines synthesized (Table 2), and promoted a variation in the production and excretion of proteins (Fig. 5). In the presence of ammonium sulfate, the synthesis of extracellular proteins was increased at least 1.7 times using oil as carbon source in comparison to sodium nitrate (Table 3). This can result in liberation of significant amounts of toxic proteins, belonging to a pool of virulence factors. Figure 3 depicts the large variation in protein synthesis as a function of C:N utilized, when ammonium sulfate was used. The use of sodium nitrate did not result in variation in the synthesis of proteins, and unlike when ammonium was used, the level of proteins produced was low.

Table 2
Effect of Nitrogen Sources on Secretion of Phenazines in Production of Rhamnolipids Using Glycerol as Carbon Source

	Wavelength (nm)		
Nitrogen source	251	264	327
$NaNO_3$	0.198	0.130	0.139
$(NH_4)_2SO_4$	2.645	1.795	1.276

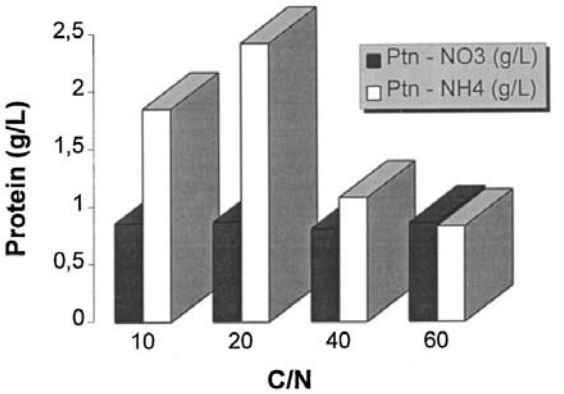

Fig. 5. Effect of variations in C:N ratios and nitrogen source in production of extracellular proteins. Ptnr, Proteins.

The ultraviolet-visible absorption spectra of a chloroform extract from cell-free supernatant of media production of rhamnolipids by *P. aeruginosa* revealed some maximum absorption peaks in characteristic wavelengths of phenazines (242, 251, 264, and 327 nm) (Fig. 6). These absorption spectra point to a mixture of phenazines that had a distribution variable with different nitrogen sources, when glycerol was used as carbon source (Table 2).

Conclusion

A new perspective is proposed suggesting that other factors should be considered in the choice of carbon, nitrogen, and other nutrient sources for the production of rhamnolipids from *P. aeruginosa* . Otherwise, one runs the risk of obtaining an unprofitable product owing to its high toxicity. The results of this preliminary study suggest that for the production of high levels of rhamnolipids with low incidence of toxic elements, it is necessary to employ glycerol and nitrate as carbon and nitrogen sources, respectively. Furthermore, this work showed the possibility of obtaining

Table 3
Effect of Nitrogen Source on Production
of Extracellular Protein from Several Carbon Sources

Carbon source	Nitrogen sources	Extracellular protein (%)	Protein (NH_4)/Protein (NO_3)
Glycerol	$(NH_4)_2SO_4$	72.5	2.63
	$NaNO_3$	27.5	
Ethanol	$(NH_4)_2SO_4$	75.3	3.05
	$NaNO_3$	24.7	
Soy oil	$(NH_4)_2SO_4$	64.2	1.79
	$NaNO_3$	35.8	
Olive oil	$(NH_4)_2SO_4$	63.0	1.70
	$NaNO_3$	37.0	

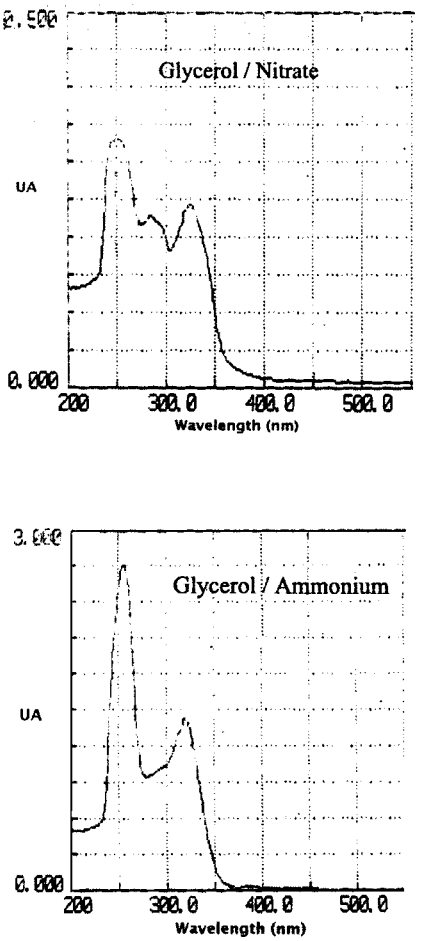

Fig. 6. Absorption spectra (200 at 500 nm) of phenazines derived from production of rhamnolipids by *P. aeruginosa*.

biosurfactant products for diverse applications by varying the proportion of the types of rhamnolipids as a function of carbon source utilized in the bioprocess.

Acknowledgments

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