Strategies for improved rhamnolipid production by *Pseudomonas aeruginosa* PA1

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Rhamnolipids are biosurfactants with potential for diversified industrial and environmental uses. The present study evaluated three strategies to increase the production of rhamnolipid-type biosurfactants produced by *Pseudomonas aeruginosa* strain PA1. The influence of pH, the addition of endogenous autoinducers and the use of a fed batch process were examined. The culture medium adjusted to pH 7.0 was the most productive. Furthermore, the pH of the culture medium had a measurable effect on the ratio of mono- and dirhamnolipids synthesized. At pH values below 7.3, the proportion of monorhamnolipids decreased from 45 to 24%. Additionally, recycling 20% of the spent culture medium where *P. aeruginosa* was grown up to the later stationary phase was responsible for a 100% increase in rhamnolipid volumetric productivity in the new culture medium. Finally, the use of fed batch operation under conditions of limited nitrogen resulted in a 3.8-fold increase in the amount of rhamnolipids produced (1.29 g L^{-1} to 4.90 g L^{-1}, as rhamnose). These results offer promising paths to optimize processes for the production of rhamnolipids.

1 Strategies for improved rhamnolipid production by *Pseudomonas aeruginosa* PA1

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14

15   **Abstract**

16   Rhamnolipids are biosurfactants with potential for diversified industrial and environmental

17   uses. The present study evaluated three strategies to increase the production of rhamnolipid-type

18   biosurfactants produced by *Pseudomonas aeruginosa* strain PA1. The influence of pH, the

19   addition of endogenous autoinducers and the use of a fed batch process were examined. The

20   culture medium adjusted to pH 7.0 was the most productive. Furthermore, the pH of the culture

21   medium had a measurable effect on the ratio of mono- and dirhamnolipids synthesized. At pH

22   values below 7.3, the proportion of monorhamnolipids decreased from 45 to 24%. Additionally,

23   recycling 20% of the spent culture medium where *P. aeruginosa* was grown up to the later

24   stationary phase was responsible for a 100% increase in rhamnolipid volumetric productivity in

25   the new culture medium. Finally, the use of fed batch operation under conditions of limited

26   nitrogen resulted in a 3.8-fold increase in the amount of rhamnolipids produced (1.29 g L^{-1} to

27   4.90 g L^{-1}, as rhamnose). These results offer promising paths to optimize processes for the

28   production of rhamnolipids.

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30   **Keywords –** biosurfactant, rhamnolipid, homoserine lactones, *Pseudomonas aeruginosa*

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Introduction

Rhamnolipids are biodegradable biological surfactants. They present low toxicity and high resistance to extreme conditions of pH, salinity and temperature (Kesting *et al.*, 1996). Their surface properties, such as emulsification, dispersion, foaming, detergency, wetting and stabilization (Van Dyke *et al.,* 1993; Ishigami *et al.*, 1994; Desai and Banat, 1997; Bognolo, 1999), as well as their capacity for removing heavy metals (Torrens *et al.*, 1998; Lang and Wullbrandt, 1999; Kitamoto *et al.*, 2002) and their anticorrosive capacity (Araujo *et al.*, 2013), confer to these biosurfactants a variety of ecological (Rahman *et al.*, 2002) and commercial applications in the oil, pharmaceutical, food and chemical industries (Sinumvayo and Ishimwe, 2015).

Rhamnolipids are produced mainly by *Pseudomonas aeruginosa,* a Gram-negative bacterium that can be isolated from various habitats (water, soil or even plants). The control of rhamnolipid production in *P. aeruginosa* is performed by a regulatory system called quorum sensing that is controlled by acyl homoserine lactones. These lactones are signaling molecules that, paired with the regulators LasR and RhlR, initiate the expression of the enzymes involved in rhamnolipid synthesis (rhamnosyltransferases) (Ochsner *et al.*, 1994, 1995; Rahim *et al.*, 2001).

Efforts should be oriented toward the optimization of processes for the production of biosurfactants that result in high productivity on a commercial scale so that they can compete with synthetic surfactants in terms of cost. One of the strategies that has been suggested in the literature (Desai and Banat, 1997; Chayabutra *et al.*, 2000) is the limitation of nutrients, mainly nitrogen sources, as well as multivalent cations and some anions, as a condition for stimulating the synthesis of rhamnolipids by *P. aeruginosa*. In addition to nutritional modifications, changes

56 in physical factors such as temperature and pH can also influence the synthesis of rhamnolipids

57 by *P. aeruginosa*. Guerra-Santos and collaborators (1984) showed that rhamnolipid production

58 peaked at pH values ranging from 6.0 to 6.5 and decreased at values higher than 7.0. Another

59 approach to the induction of the rhamnolipid synthesis is the use of exogenous or endogenous

60 autoinducers (Ochsner and Reiser, 1995; Nakata *et al.*, 1998).

61   The aim of this work was to improve the cultivation conditions for the production of

62 rhamnolipids by a strain of *Pseudomonas aeruginosa* isolated from a Brazilian petroleum-

63 exploring environment. This study involved the investigation of the effect of a variation in pH

64 and the addition of endogenous homoserine lactones, as well as studies on fed batch operation.

65

66 **Material and Methods**

67 **Inoculum**

68   *Pseudomonas aeruginosa* PA1 (Santa Anna *et al.,* 2001) was maintained in a glycerol

69 solution (10% v/v) at −80°C. The thawed strain sample was inoculated onto YPDA plates (yeast

70 extract, 0.3%; peptone, 1.5%; dextrose, 0.1%; Agar, 1.2%) at 30°C for 48 h. The growth of the

71 inoculum was initiated by the addition of a loopful of cells from YPDA plates to a 1000 mL

72 Erlenmeyer flask containing 300 mL of medium with the following composition (per liter):

73 $NaNO_3$, 1.0 g; KH_2PO_4, 3.0 g; K_2HPO_4, 7.0 g; $MgSO_4.7H_2O$, 0.2 g; yeast extract, 5.0 g; peptone,

74 5.0 g and glycerol, 30 g. *P. aeruginosa* was grown at 30°C in a rotary shaker at 170 rpm for 24

75 hours. Cells were harvested by centrifugation (10,000g for 30 minutes) and used as the

76 inoculum.

77

78 **Influence of pH on rhamnolipid production**

79   The culture medium contained (per liter) 0.2 g of $MgSO_4$ $7H_2O$, 1.38 g of $NaNO_3$, and 30 g

80   of glycerol. The pH of the culture medium was adjusted from 6.0 to 8.0 with 0.062 M

81   KH_2PO_4/K_2HPO_4 buffer. Six 500 mL Erlenmeyer flasks containing a working volume of 100 mL

82   were prepared, inoculated with 0.12 g of cells (dry weight) and incubated at 30°C in a rotary

83   shaker at 170 rpm for 192 hours. At 24-hour intervals, samples were removed for the

84   measurement of cell growth and rhamnolipid concentration.

85

86   **Simple batch process supplemented with endogenous acylated homoserine lactones (AHL)**

87   The culture medium was prepared with the following composition (per liter): $NaNO_3$, 1.38

88   g; KH_2PO_4, 3.0 g; K_2HPO_4, 7.0 g; $MgSO_4.7H_2O$, 0.2 g and glycerol, 30 g. To each of five 500

89   mL Erlenmeyer flasks was added 100 mL of liquid medium supplemented with 1%, 5%, 10%,

90   15% or 20% (v/v) of cell-free spent culture supernatant from a 120 h-old *P. aeruginosa* PA1

91   culture. The control flask contained no endogenous AHL. The flasks were inoculated with 0.05 g

92   of cells (dry weight) and incubated at 30ºC and pH 7.0 in a rotary shaker at 170 rpm for 200

93   hours. Samples were removed at 24-hour intervals for the assessment of cell growth and

94   rhamnolipid production.

95

96   **Fed batch process: nitrogen and carbon feed**

97   The culture medium contained (per liter) 3.0 g of KH_2PO_4, 7.0 g of K_2HPO_4, 0.2 g of

98    $MgSO_4.7H_2O$, 0.46 g $NaNO_3$, and 10.0 g glycerol. Three 500 mL Erlenmeyer flasks (A, B and

99   C) were prepared with 100 mL of liquid medium. The flasks were inoculated with 0.05 g of cells

100   (dry weight) and incubated at 30ºC in a rotary shaker at 170 rpm for 240 hours. The frequency of

101   addition was a function of the amount of glycerol consumed in each flask. Flask A received 5

102   mL of a solution containing glycerol (200 g L^{-1}) and sodium nitrate (9 g L^{-1}) at each addition.

103   Flask B received 5 mL of a solution containing only glycerol (200 g L^{-1}), and flask C received 5

104   mL of sterile distilled water. Samples were removed at 24-hour intervals for the quantification of

105   cell growth, nitrate and glycerol consumption and rhamnolipid production.

106

107   **Thin-layer chromatography for rhamnolipid analysis**

108   The rhamnolipids were extracted with ethyl acetate from acidified (pH 2.0) cell-free

109   medium and analyzed by TLC on silica-gel-coated aluminum sheets (Macherey-Nagel®) using

110   CHCl$_3$:MeOH:AcOH (65:15:2) as the eluent (Schenk *et al*., 1995). The separated zones were

111   stained with orcinol-sulfuric acid reagent, followed by heating at 100ºC for 15 minutes. The

112   plates were scanned, and the relative quantities of the spots corresponding to the

113   monorhamnolipids and dirhamnolipids were determined by densitometry using Band Leader

114   (Ma'ayan Aharoni) and Micronal Origin (Micronal Software, Inc) software.

115

116   **Determination of cell, rhamnolipid, glycerol and nitrate concentrations**

117   Cell growth was assessed by measuring the absorbance at 500 nm, and the cell dry weight

118   (g/L) was determined using a standard calibration curve [ABS = 1.2595 x DW (g/L) – R^2=0.989],

119   considered to be valid for absorbance values up to 0.6 OD. Rhamnolipid quantification was

120   achieved indirectly by measurement of the rhamnose concentration using the method of Dubois

121   (1956). A 0.5 mL volume of cell-free supernatant was mixed with 0.5 mL of 5% phenol solution

122   and 2.5 mL of sulfuric acid and incubated for 15 minutes before measuring the absorbance at 490

123   nm. The results were compared with the analytical curve for rhamnose. Glycerol was quantified

124   by the GPO-POD enzymatic-colorimetric method using a kit for triglyceride determination from

125   LaborLab (Brazil). Nitrate was quantified through a colorimetric method using brucine sulfate.

126   Briefly, 2 mL of 0.6 g L^{-1} brucine sulfate in sulfuric acid solution (80%) was added to 0.5 mL of

127   sample, and the reaction mixture was heated in boiling water for 15 minutes. The reaction

128   mixture was immediately cooled in an ice bath, and the absorbance was measured at 410 nm.

129   The absorbance values were converted into concentration using an analytical curve for sodium

130   nitrate.

131

132   **Results and Discussion**

133   **Influence of pH on rhamnolipid synthesis**

134   The qualitative effect of pH on rhamnolipid synthesis was evaluated by thin layer

135   chromatography. Densitometric analysis of mono- and dirhamnolipids by TLC using (Figure 1)

136   furnished relative migration values close to 0.8 for the monorhamnolipids and 0.5 for the

137   dirhamnolipids. These values are in agreement with the migration rate of monorhamnolipids and

138   dirhamnolipids observed by Schenk and collaborators (1995), who employed TLC analysis under

139   the same conditions.
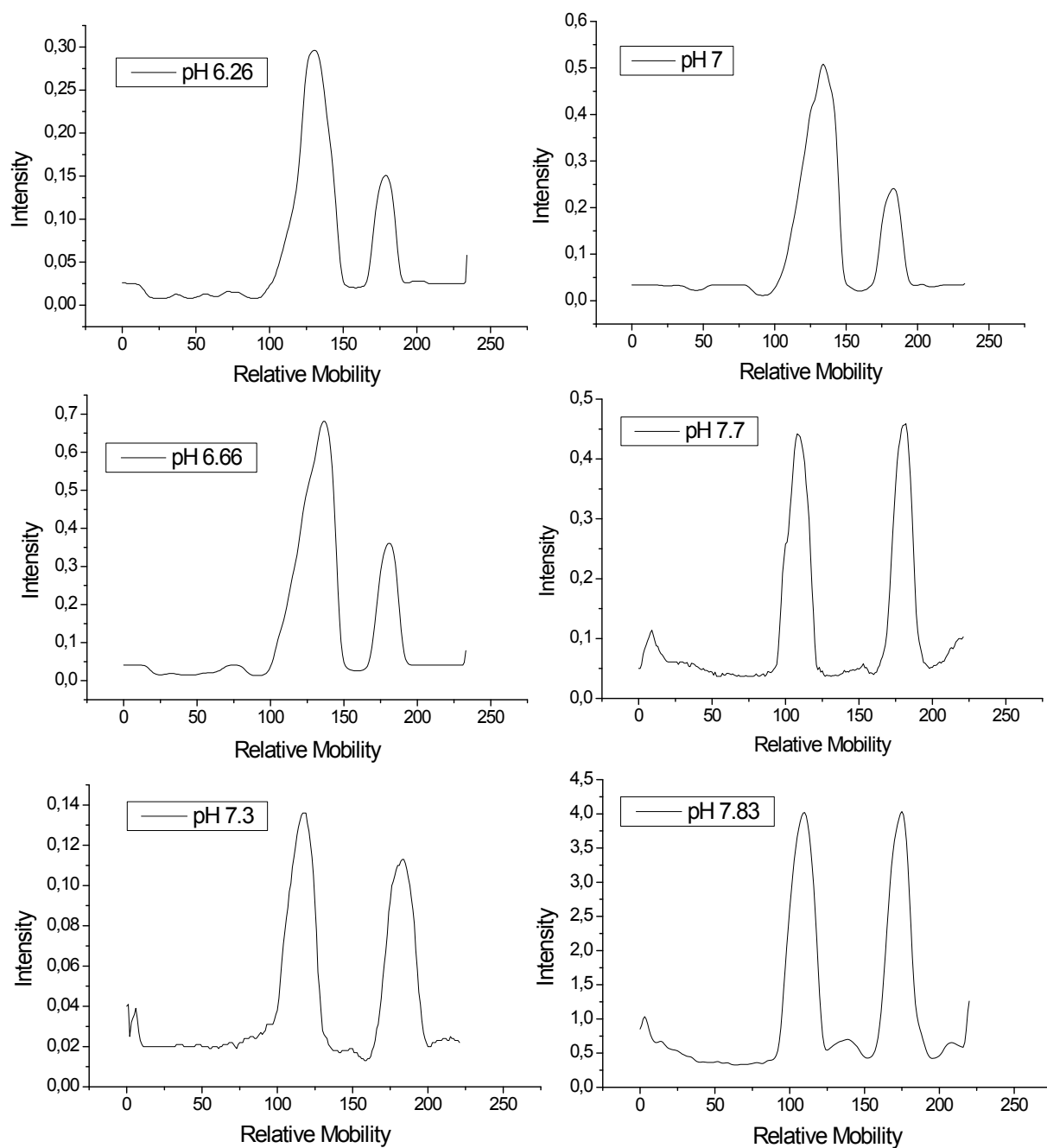
Figure 1: Densitometric measurements of the thin layer chromatographs of rhamnolipids at various pH levels.

144   As shown in the Table 1, the percentage of mono- and dirhamnolipids, determined by

145   densitometry of TLC, varied as a function of the pH of the culture medium. For pH ≤ 7.0, the

146   amount of monorhamnolipids produced was lower than the amount of dirhamnolipids. On the

147   other hand, at pH values higher than 7.0, the amount of mono- and dirhamnolipids was

148   approximately the same.

149

150   **Table 1**: Relative percentages of the rhamnolipid types synthesized as a function of the pH of the
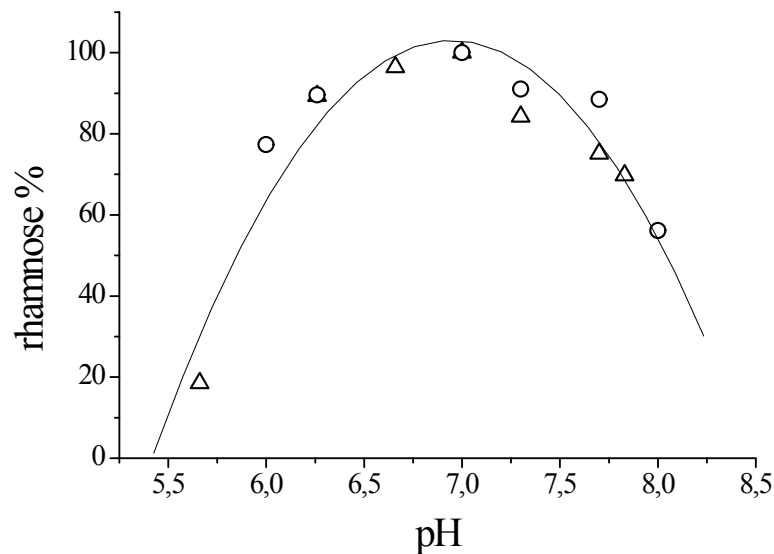
151   culture medium.

pH	Dirhamnolipids (%)	Monorhamnolipids (%)
6.26	74.35	25.65
6.66	75.35	24.65
7.00	76.41	23.59
7.30	55.25	44.75
7.70	51.66	48.34
7.83	52.75	47.25

152

153   It is possible that pH acts at a transcriptional level on the synthesis of one of the

154   rhamnosyltransferases or on the synthesis of a specific glycolipid transporter channel, or even on

155   the synthesis of L-rhamnose in *P. aeruginosa* (Olvera *et al*., 1999). *Escherichia coli* represents

156   an example in which various enzymes and periplasmic proteins are expressed in a pH-dependent

157   mechanism (Stancik *et al*. 2002). In any case, the possibility of obtaining a product with distinct

158   percentages of rhamnolipid types synthesized by the appropriate choice of pH allows for

159   different uses and applications for this natural formulation. Indeed, different rhamnolipid types

160   or its mixtures present emulsifying activity, critical micellar concentration, hydrophobicity or

161   bioavailability distinct from each other (Mata-Sandoval *et al*., 1999).

162   The quantitative effect of the pH of the culture medium on total rhamnolipid synthesis

163   demonstrated that the most productive pH was 7.0 (Figure 2).
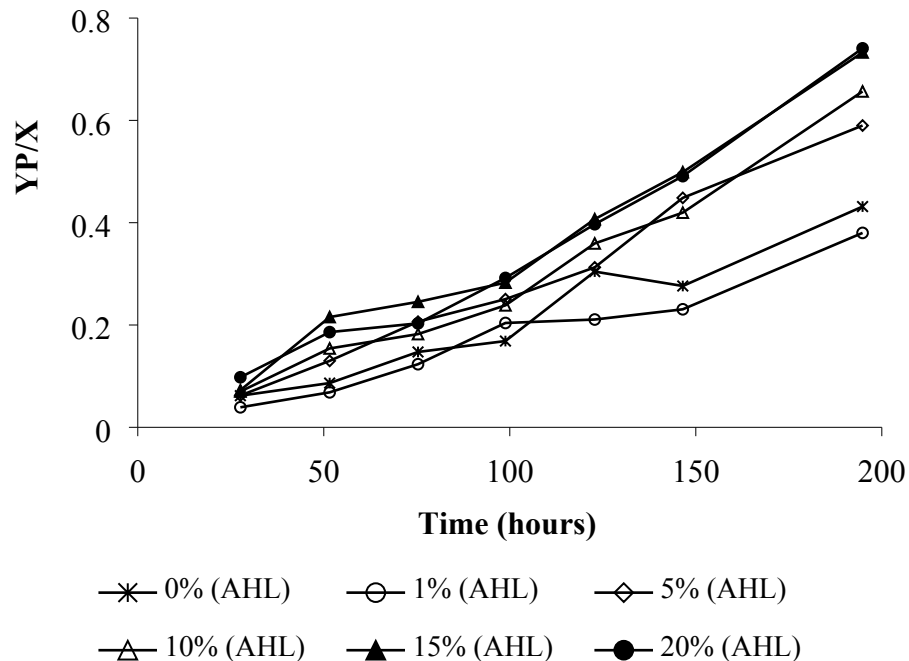
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165

166   **Figure 2**: Variation of rhamnolipid synthesis as a function of the pH of the culture medium. The

167   quantification of rhamnolipids by the Dubois method expresses the rhamnolipid content as the

168   rhamnose concentration. The triangles and circles correspond to independent experiments.

169

170   **Use of endogenous autoinducers of the *P. aeruginosa* quorum sensing system**

171   On the basis of an approach motivated by knowledge of the regulation of rhamnolipid

172   synthesis by the quorum sensing system in *P. aeruginosa* (Ochsner and Reiser, 1995), an

173   endogenous mixture of undetermined acylated homoserine lactones (AHL) present in the spent

174   cultivation medium where *P. aeruginosa* was grown for a 120-hour period (later stationary

175   phase) was used. The mass balance (carbon and nitrogen sources) present at the start of the

176   culture process was maintained with the same C/N ratio. The spent supernatant added to new

177   cultures operated in simple batch processes was shown to be extremely effective for the

178   induction of rhamnolipid synthesis. The addition of spent medium containing endogenous AHLs

179   at the start of new *P. aeruginosa* cultures promoted an increase in the specific yield of

180   rhamnolipids ($Y_{P/X}$) proportional to the volumetric fractions of supplemental spent culture

181   medium added (Figure 3).

Figure 3: Progress curve of specific yield ($Y_{P/X}$) in culture medium with different supplemental proportions of spent medium containing endogenous AHL.

186   The addition of endogenous AHLs to the *P. aeruginosa* PA1 culture increased the specific

187   rate of consumption of the carbon source (q_S) and the yield coefficient $Y_{P/S}$ (Table 2). This fact

188   suggests that the addition of AHLs would also alter the metabolism of nutrients that, in the final

189   analysis, would be coupled to the synthesis of the precursors for rhamnolipid and AHL

190   production. The volumetric productivity (Q_P) was also favored by the addition of endogenous

191   AHLs (Table 2).

192

193   **Table 2:** Process parameters of rhamnolipid production by *P. aeruginosa* PA1 in culture

194   medium supplemented with different percentages of spent medium containing endogenous AHL.

Condition	0%	1%	5%	10%	15%	20%
ΔRhamnose (g L^{-1})	2.15	2.02	3.52	3.63	3.96	4.21
ΔBiomass (g L^{-1})	4.98	5.31	5.96	5.53	5.40	5.68
$Y_{P/X}$ (g g^{-1})	0.431	0.380	0.590	0.657	0.733	0.741
$Y_{P/S}$ (g g^{-1})	0.145	0.149	0.142	0.145	0.164	0.171
Q_P (g L^{-1} h^{-1})	0.011	0.010	0.018	0.019	0.020	0.022
q_S (g L^{-1} h^{-1})	0.014	0.012	0.021	0.028	0.029	0.031

195   **ΔRhamnose:** increase in rhamnose concentration throughout the process; **ΔBiomass:** increase in cellular

196   concentration throughout the process; **$Y_{P/X}$:** yield factor of product synthesized based on biomass at the end of the

197   process; **$Y_{P/S}$:** yield factor of product synthesized based on consumed substrate; **Q_P:** volumetric productivity. **q_S:** rate

198   of glycerol consumption.

199

200   The calculated slopes of each curve shown in Figure 3 (ratio between $\Box Y_{P/X}$ and $\Box$Time)

201   were plotted against the log values of the spent culture medium and are presented in Figure 4.

202   This plot permits one to clearly observe that the increase in the production of rhamnolipids was

203   not due to an increase in cellular growth. These results suggest that the amount of AHLs added at

204   the beginning of cultivation probably contributed to the increase in the synthesis of

205   rhamnosiltransferases due to the natural unfolding of the quorum sensing system, which is

206   responsible for the transcriptional regulation of rhamnolipid synthesis (Ochsner and Reiser,

207   1995).

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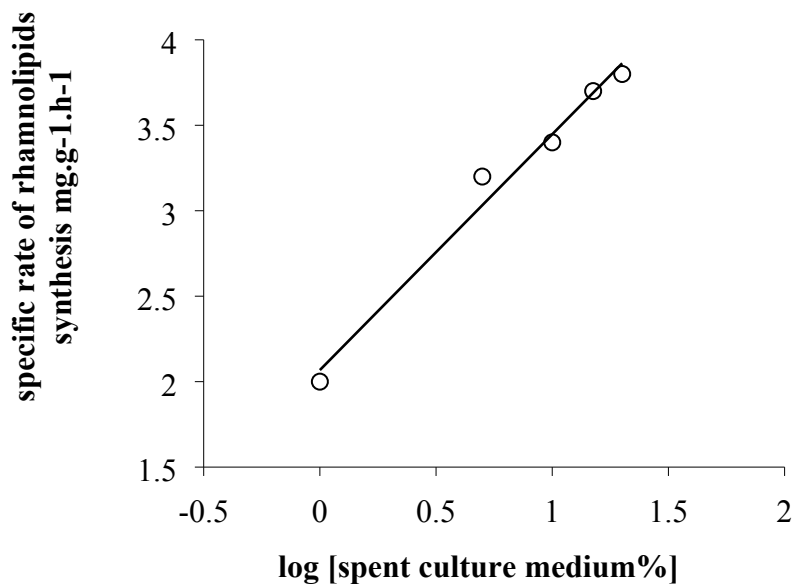
Figure 4: Relation between the specific rate of rhamnolipid synthesis and the addition of exogenous AHLs (expressed in the form of percent volume of culture medium containing endogenous AHL).
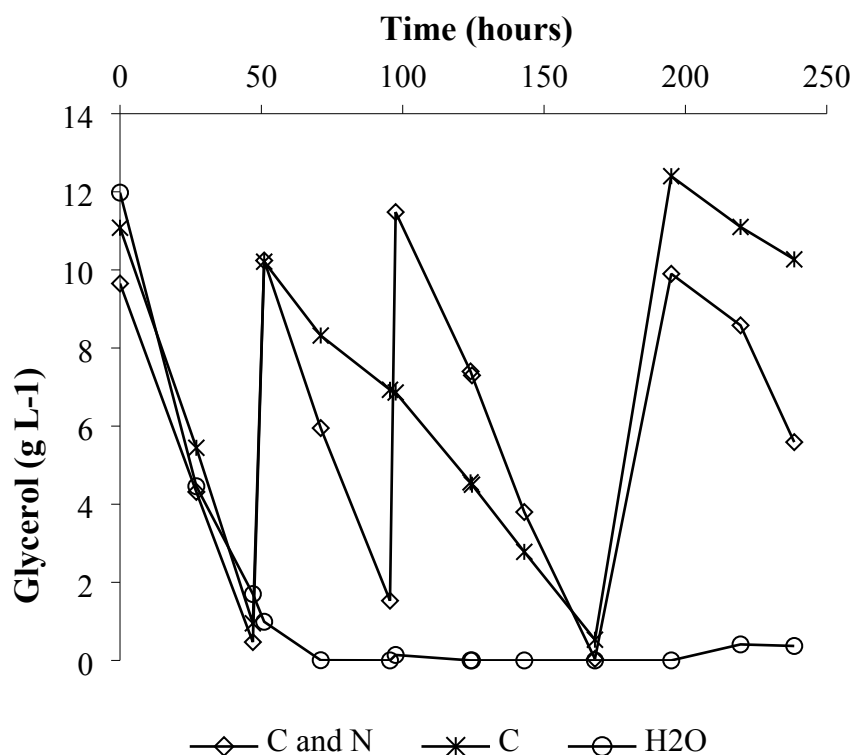
Fed batch strategy

Fed batch operation is another possible strategy to increase the production of rhamnolipids and was chosen to circumvent a possible negative effect, observed by Santa Anna *et al.* (2002), on the assimilation of nutrients when the culture medium contains glycerol concentrations higher than 3%, as well as the inhibition of the cellular growth in cultures of *P. aeruginosa* with nitrate concentrations higher than 0.1% (Chayabutra *et al.*, 2000). In addition, a process driven by fed batch can control and maintain the nutrient limits already established as being favorable for rhamnolipid synthesis (Desai and Banat, 1997; Chayabutra *et al.*, 2000).

The consumption of nutrients during the fed batch process was determined. In the control experiment, in which only water was fed to the medium, the carbon source (glycerol) and

224   nitrogen source ($NaNO_3$) present at the beginning were totally consumed after approximately 50

225   and 24 hours of cultivation, respectively (Figures 5 and 6).

226

227

228   **Figure 5:** Glycerol consumption during the fed batch process using different feeding strategies.

229

230   Although the rate of consumption of the nitrogen source ($NaNO_3$) was superior to that of

231   glycerol (Table 3), the addition of a solution containing glycerol and nitrate was accomplished

232   only as a function of the consumption of the carbon source (Figure 6). The rate of glycerol

233   consumption was dependent on the presence of a nitrogen source. This fact becomes evident

234   when the curves of glycerol consumption are compared amongst themselves, considering the

235   process in which glycerol and nitrate are added together and the process in which only glycerol

236   was added (Figure 5). It is reasonable to assume that the enzymatic machinery involved in

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237 glycerol metabolism, as well as in rhamnolipid biosynthesis, depends on the assimilation of

238 nitrogen and its conversion into catalytic proteins.

239

240 **Table 3:** Process parameters observed in fed batch processes under different feeding conditions.

Conditions	C+N	C	H$_2$O
ΔRhamnose (g L^{-1})	4.90	3.48	1.29
ΔBiomass (g L^{-1})	4.78	3.34	2.41
$Y_{P/X}$ (g g^{-1})	1.03	1.04	0.514
$Y_{P/S}$ (g g^{-1})	0.142	0.168	0.108
Q_P (g L^{-1} h^{-1})	0.021	0.015	0.013
$q_{S(Gly)}$ (g L^{-1} h^{-1})	0.168	0.073	0.197
$q_{S(NO3)}$ (g L^{-1} h^{-1})	0.027	0.027	0.027

241 **ΔRhamnose**: increase in rhamnose concentration throughout the process; **ΔBiomass**: increase in cellular

242 concentration throughout the process; **$Y_{P/X}$**: yield factor of product synthesized based on biomass at the end of the

243 process; **$Y_{P/S}$**: yield factor of product synthesized based on consumed substrate; **Q_P**: volumetric productivity; **$q_{S(gly)}$**:

244 rate of glycerol consumption; **$q_{S(NO3)}$**: rate of nitrate consumption. The parameters for the condition "H$_2$O" were

245 calculated at 100 h of culture.
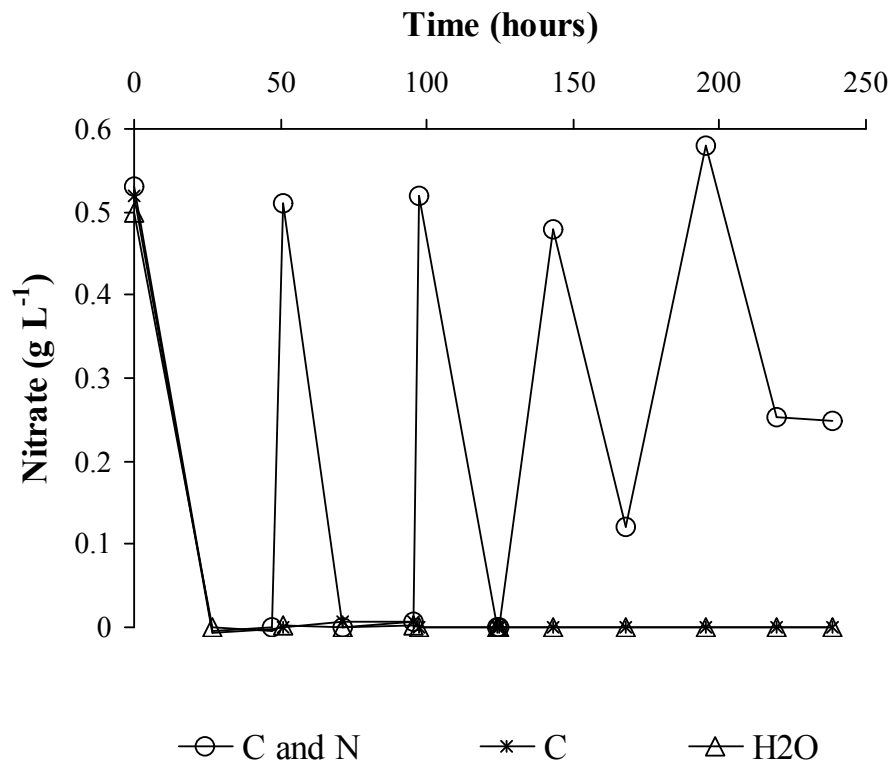
Figure 6: Nitrate assimilation during the fed batch process using different feeding strategies.

Ochsner *et al.* (1995) observed that the activity of the rhamnosyltransferase in *P. aeruginosa* during cultivation in a nitrogen-limiting medium containing glycerol as the carbon source showed maximum activity in the beginning of the stationary phase, which declined to zero in the late stationary phase. We suggest the hypothesis that exhaustion of the nitrogen source would not only limit cellular growth, but also the maintenance of the enzymatic machinery, mainly that involved in the metabolic pathways for rhamnolipid synthesis. When the fermentation system was fed with carbon and nitrogen sources, a higher cell yield and volumetric productivity of rhamnolipids was achieved (Table 3).

On the basis of the feeding conditions, together with the control experiment (without feeding), one can conclude that the exhaustion of carbon and nitrogen sources interrupted

259   rhamnolipid synthesis sooner (at 72 hours after initiating the cultivation) (Figure 7). On the other

260   hand, feeding the system with only glycerol led to an improvement when compared with the

261   simple batch (Figure 7) and resulted in higher $Y_{P/X}$ and $Y_{P/S}$ values (Table 3). Furthermore, the

262   feeding condition with both sources of nutrients (carbon and nitrogen) resulted in an even greater

263   increase in both the biomass produced and the volumetric productivity (Table 3).
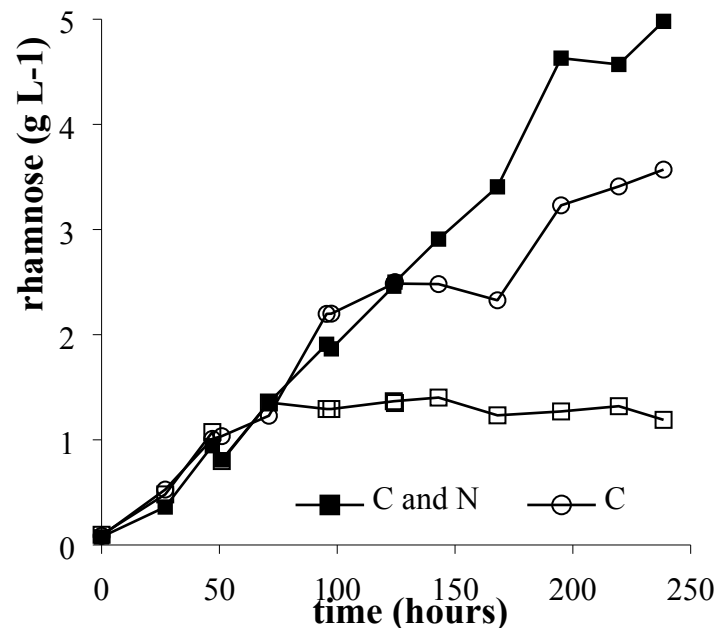
265   **Figure 7**: Time course of rhamnolipid production in the fed batch process using different feeding

266   strategies.

267

268   **Conclusion**

269   In addition to demonstrating the most appropriate pH for stimulating the production of

270   rhamnolipids, selective synthesis of different types of rhamnolipids caused by certain pH ranges

271   appeared as an unexpected and timely result. The reutilization or recycling of culture medium

272   containing endogenous homoserine lactones (autoinducers of the quorum sensing system)

273   produced by *P. aeruginosa* in new culture medium for the production of rhamnolipids was very

274 effective in the induction of rhamnolipid synthesis. A two-fold increase in volumetric

275 productivity was obtained using this strategy. The fed batch experiment using as strategy of

276 carbon and nitrogen source limitation was successful and could be employed along with an

277 appropriate formulation of the cultivation conditions (micronutrients, pH, supplementary AHLs).

278

279 **Acknowledgments**

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