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 for increasing the production of rhamnolipid-type biosurfactants produced by *Pseudomonas aeruginosa* strain PA1. The influence of pH, the addition of *P. aeruginosa* spent culture medium 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 synthesized mono- and dirhamnolipids. At pH values below 7.3, the proportion of monorhamnolipids decreased from 45 to 24%. The recycling of 20% of the spent culture medium in 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 (2.9 g.L^{-1} to 10.9 g.L^{-1}). These results offer promising pathways for the optimization of processes for the production of rhamnolipids.

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

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15 **Abstract**

16   Rhamnolipids are biosurfactants with potential for diversified industrial and environmental

17 uses. The present study evaluated three strategies for increasing the production of rhamnolipid-

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

19 addition of *P. aeruginosa* spent culture medium and the use of a fed-batch process were

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

21 of the culture medium had a measurable effect on the ratio of synthesized mono- and

22 dirhamnolipids. At pH values below 7.3, the proportion of monorhamnolipids decreased from 45

23 to 24%. The recycling of 20% of the spent culture medium in where *P. aeruginosa* was grown up

24 to the later stationary phase was responsible for a 100% increase in rhamnolipid volumetric

25 productivity in the new culture medium. Finally, the use of fed-batch operation under conditions

26 of limited nitrogen resulted in a 3.8-fold increase in the amount of rhamnolipids produced (2.9 g

27 L^{-1} to 10.9 g L^{-1}). These results offer promising pathways for the optimization of processes for

28 the production of rhamnolipids.

29

30 **Keywords –**

31

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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 capacities (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 (Irfan-Maqsood and Seddiq-Shams, 2014; Randhawa *et al.*, 2014; Sinumvayo and Ishimwe, 2015).

Rhamnolipids are mainly produced 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 achieved by a regulatory system called quorum sensing that is controlled by autoinducers such as acyl homoserine lactones and Pseudomonas quinolone signal molecule. These signaling molecules, paired with the LasR and RhlR regulators, initiate the expression of the enzymes involved in rhamnolipid synthesis (rhamnosyltransferases) (Ochsner *et al.*, 1994, 1995; Rahim *et al.*, 2001; Reis *et al.*, 2011).

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 is the limitation of nutrients (Desai and Banat, 1997; Chayabutra *et al.*, 2000), mainly nitrogen sources, as well as multivalent cations (Syldatk *et al.*, 1985; Glick *et al.*, 2010) and some anions (Mulligan *et al.*, 1989; Clarck *et al.*, 2010), as a condition necessary for stimulating

56   the synthesis of rhamnolipids by *P. aeruginosa*. In addition to nutritional modifications, changes

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

58   by *P. aeruginosa* (Souza *et al*., 2011; Jamal *et al*., 2014). Another approach to the induction of

59   the rhamnolipid synthesis is the use of exogenous or endogenous autoinducers (Ochsner and

60   Reiser, 1995; Nakata *et al*., 1998; Galkin *et al*., 2014).

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 the

64   pH of the culture medium, the medium supplementation with recycled *P. aeruginosa* spent

65   culture medium and evaluation of process carried on fed-batch operation.

66

67   **Material and Methods**

68   **Inoculum**

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

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

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

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

73   Erlenmeyer flask containing 300 mL of medium with the following composition (per liter): 1.0 g

74   of NaNO$_3$, 3.0 g of KH$_2$PO$_4$, 7.0 g of K$_2$HPO$_4$, 0.2 g of MgSO$_4$.7H$_2$O, 5.0 g of yeast extract, 5.0

75   g of peptone, and 30 g of glycerol. *P. aeruginosa* was grown at 30°C and pH 7.0 in a rotary

76   shaker at 170 rpm for 24 hours. Cells were harvested by centrifugation (10,000*g* for 30 minutes)

77   and used as the inoculum.

78

Influence of pH on rhamnolipid production

The culture medium contained (per liter) 0.2 g of MgSO$_4$ 7H$_2$O, 1.38 g of NaNO$_3$, and 30 g of glycerol. The pH of the culture medium was adjusted from 5.7 to 8.0 with KH$_2$PO$_4$/K$_2$HPO$_4$ buffer by varying the mole fraction of salt species without changing the concentration of total phosphate ion, which was maintained at 0.062 moles per liter. Six 500-mL Erlenmeyer flasks containing a working volume of 100 mL were prepared, inoculated with 0.12 g of cells (dry weight) and incubated at 30 °C in a rotary shaker at 170 rpm for 192 hours. Samples were removed at 24-hour intervals for the measurement of cell growth and rhamnolipid concentration.

Simple batch process supplemented with recycled _P. aeruginosa_ spent culture medium

The culture medium was prepared with the following composition (per liter): 1.38 g of NaNO$_3$, 3.0 g of KH$_2$PO$_4$, 7.0 g of K$_2$HPO$_4$, 0.2 g of MgSO$_4$.7H$_2$O, and 30 g of glycerol. To each of five 500-mL Erlenmeyer flasks was added 100 mL of liquid medium supplemented with 1%, 5%, 10%, 15% or 20% (v/v) of cell-free spent culture supernatant from a 120 h-old _P. aeruginosa_ PA1 culture. The control flask contained no spent culture medium. The flasks were inoculated with 0.05 g of cells (dry weight) and incubated at 30ºC and pH 7.0 in a rotary shaker at 170 rpm for 200 hours. Aliquots (1.5 mL) were removed at 24-hour intervals for the assessment of cell growth and rhamnolipid production. The mass balance (carbon and nitrogen sources) present at the start of the culture process was adjusted, when necessary, to maintain the same C/N ratio and avoid undesirable side effect.

Fed-batch process: nitrogen and carbon feed

The culture medium contained (per liter) 3.0 g of KH$_2$PO$_4$, 7.0 g of K$_2$HPO$_4$, 0.2 g of MgSO$_4$.7H$_2$O, 0.46 g of NaNO$_3$, and 10.0 g of glycerol. The pH was adjusted to 7.0. Three 500-

103   mL Erlenmeyer flasks (A, B and C) were prepared containing 100 mL of the liquid medium. The

104   flasks were inoculated with 0.2 g of cells (dry weight) and incubated at 30ºC in a rotary shaker at

105   170 rpm for 240 hours. The frequency of addition of nutrients was a function of the amount of

106   glycerol consumed in each flask. Flask A received 5 mL of a solution containing glycerol (200

107   g.L^{-1}) and sodium nitrate (9 g.L^{-1}) at each addition. Flask B received 5 mL of a solution

108   containing only glycerol (200 g.L^{-1}), and flask C received 5 mL of sterile distilled water.

109   Aliquots (1.5 mL) were removed at 24-hour intervals for the quantification of cell growth, nitrate

110   and glycerol consumption and rhamnolipid production.

111

112   **Thin-layer chromatography for analysis of rhamnolipid types**

113   The rhamnolipids produced were extracted with ethyl acetate from acidified (pH 2.0) cell-

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

115   using $CHCl_3:CH_3OH:CH_3COOH$ (65:15:2) as the eluent (Schenk *et al.*, 1995). The separated

116   zones were stained with orcinol-sulfuric acid reagent, followed by heating at 100 ºC for 15

117   minutes. The stained plates were scanned, and the relative quantities of the spots corresponding

118   to the monorhamnolipids and dirhamnolipids were determined by densitometry using Band

119   Leader (Ma'ayan Aharoni) and Micronal Origin (Micronal Software, Inc) software to produce

120   two dimensional chromatograms.

121

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

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

124   (g.L^{-1}) was determined using a standard calibration curve [ABS = 1.2595 x DW (g.L^{-1}) –

125   R^2=0.989], considered to be valid for absorbance values up to 0.6 OD. Rhamnolipid

126   quantification was achieved indirectly by measurement of the rhamnose concentration using the

127   method of Dubois (1956) and was expressed as the rhamnolipid concentration using the factor

128   2.23 established by Kronemberger *et al.* (2008) by mass spectrometry measurements. A 0.5 mL

129   volume of cell-free supernatant was mixed with 0.5 mL of 5% phenol solution and 2.5 mL of

130   98% sulfuric acid and incubated for 15 minutes before measuring the absorbance at 490 nm. The

131   results were compared with the analytical curve for rhamnose. Glycerol was quantified by the

132   GPO-POD enzymatic-colorimetric method using a kit for triglyceride determination from

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

134   (ACS, 2006). Briefly, 2 mL of 0.6 g.L^{-1} brucine sulfate in sulfuric acid solution (80%) was added

135   to 0.5 mL of sample, and the reaction mixture was heated in boiling water for 15 minutes. The

136   reaction mixture was immediately cooled in an ice bath, and the absorbance was measured at 410

137   nm. The absorbance values were converted into concentration using an analytical curve for

138   sodium nitrate.

139

140   **Definition of process parameters utilized**

141   The process parameters utilized to evaluate the progress of improvement strategies for

142   rhamnolipid production are defined as follows. $\mathbf{t_f}$: final time of process (h); $\mathbf{t_i}$: initial time of

143   process (h); $\mathbf{\Delta t}$ (t_f-t_i); $\mathbf{P_f}$: final rhamnolipid concentration (g.L^{-1}); $\mathbf{P_i}$: initial rhamnolipid

144   concentration (g.L^{-1}); $\mathbf{X_i}$: initial cell mass concentration (g.L^{-1}); $\mathbf{X_f}$: final cell mass concentration

145   (g.L^{-1}); $\mathbf{S_f}$: final substrate concentration (g.L^{-1}); $\mathbf{S_i}$: initial substrate concentration (g.L^{-1});

146    $\mathbf{\Delta Rhamnolipids}$ (P_f - P_i); $\mathbf{\Delta Biomass}$ (X_f - X_i); $\mathbf{\Delta Substrate}$ (S_i - S_f); $\mathbf{Y_{P/X}}$ (ΔRhamnolipids ÷

147   ΔBiomass): yield of product synthesized per unit of cell mass produced (g.g^{-1}); $\mathbf{Y_{P/S}}$

148   (ΔRhamnolipids ÷ ΔSubstrate): yield of product synthesized per unit of substrate consumed (g.g^{-}

149 $^{-1}$); Q_P (ΔRhamnolipids $\div$ Δt): volumetric rhamnolipid production rate (g.L^{-1}.h^{-1}); $Q_{S(Gly)}$

150 (ΔSubstrate $\div$ Δt): volumetric glycerol consumption rate (g.L^{-1}.h^{-1}); $Q_{S(NO3)}$ (ΔSubstrate $\div$ Δt):

151 volumetric nitrate consumption rate (g.L^{-1}.h^{-1}); q_P ($Y_{P/X}$ $\times$ 1000 $\div$ Δt): specific rate of

152 rhamnolipids synthesis (mg.g^{-1}.h^{-1});

153

154 **Results and Discussion**

155   **Influence of pH on rhamnolipid synthesis**

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

157 chromatography. Densitometric analysis of mono- and dirhamnolipids performed by TLC

158 (Figure 1) furnished relative migration values close to 0.8 for the monorhamnolipids and 0.5 for

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

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

161 under the same conditions. Under the assay conditions, the more hydrophilic dirhamnolipids

162 interact more strongly with the TLC (silica gel) stationary phase because of the presence of two

163 rhamnose rings linked to lipid chain, whereas only one sugar ring exists in the

164 monorhamnolipids species.

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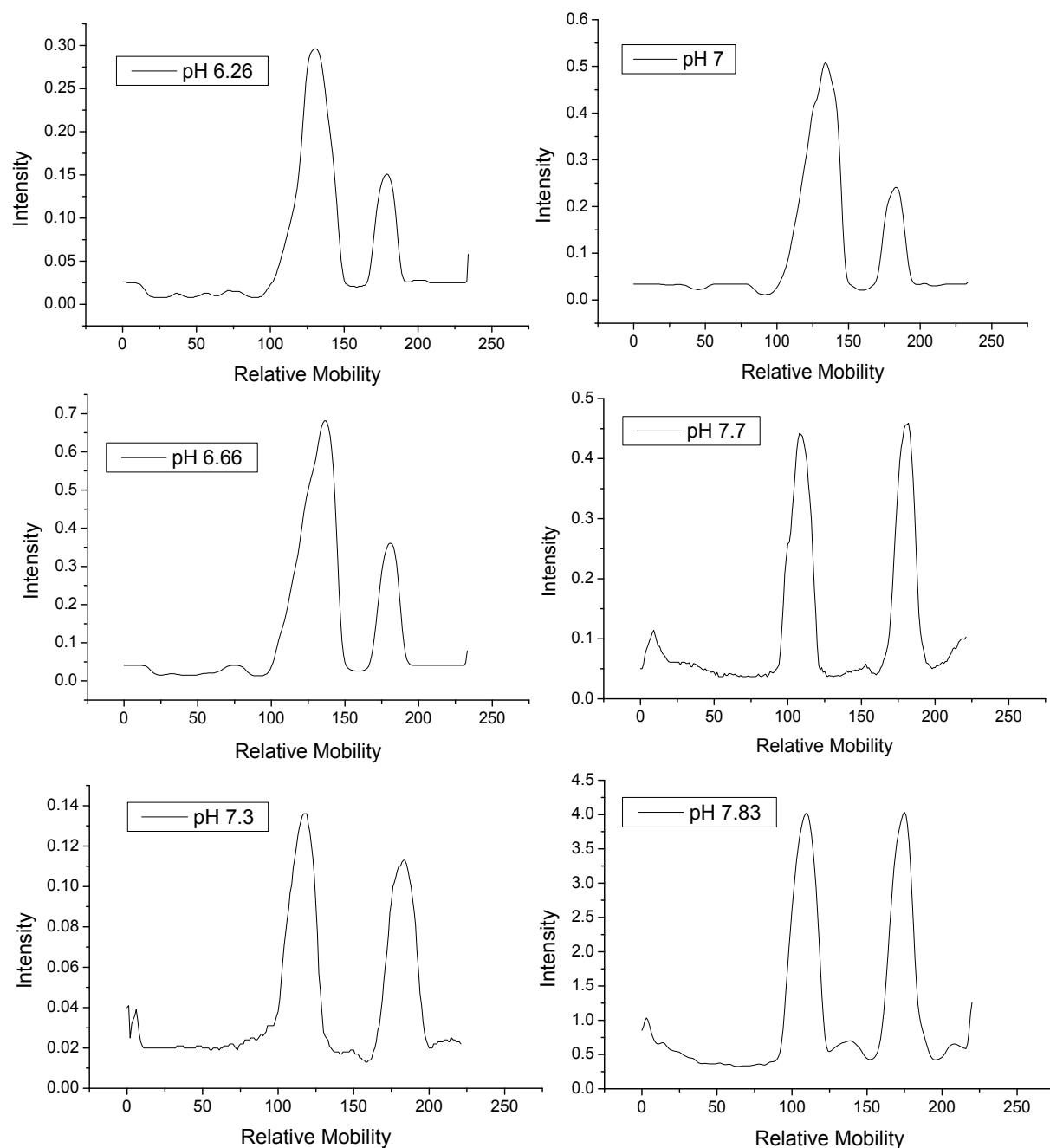
Figure 1: Densitometry of the thin layer chromatographs of rhamnolipids synthesized in culture

167   medium at different pH values.

168

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

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

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

172 other hand, at pH values higher than 7.0, the amounts of mono- and dirhamnolipids were

173 approximately the same. Mata-Sandoval, Karns and Torrens (2001) showed that, at pH 6.26 and

174 7.0, dirhamnolipids were the most abundant molecular species in the mixtures of rhamnolipids

175 synthesized by *P. aeruginosa* with corn oil as sole carbon source. However, they limited their

176 experiments up to pH 7.0.

177

178 **Table 1**: Relative percentages of the rhamnolipid types synthesized as a function of the pH of the

179 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

180

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

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

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

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

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

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

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

188   or their mixtures present emulsifying activity, critical micellar concentration, hydrophobicity or

189   bioavailabilities distinct from one other (Mata-Sandoval *et al.*, 1999). Costa *et al.* (2010), for

190   example, observed different CMC, emulsifying activity and interfacial tension values when they

191   compared two rhamnolipid preparations with distinct proportions of mono and dirhamnolipids

192   species.

193   From the viewpoint of quantitative analysis, the effect of the pH of the culture medium on

194   total rhamnolipid synthesis by *P. aeruginosa* PA1 demonstrated that the most productive pH

195   value was 7.0 (Figure 2). Guerra-Santos *et al.* (1984) found that rhamnolipid production by *P.*

196   *aeruginosa* cultivated in glucose peaked at pH values ranging from 6.0 to 6.5 and decreased at

197   values higher than 7.0. Jamal *et al.* (2014) achieved a maximum rhamnolipids yield (4.44 g.L^{-1})

198   at a pH level of 7.33 using glycerol as the carbon source and NaNO$_3$ as the nitrogen source.

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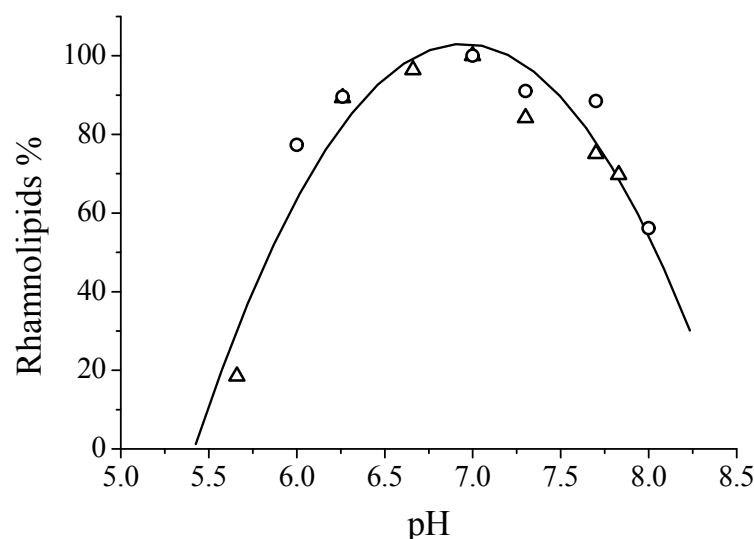
201   **Figure 2**: Variation of rhamnolipid synthesis as a function of the pH of the culture medium. The

202   triangles and circles correspond to independent experiments.

203

Use of recycled *P. aeruginosa* spent culture medium

204

In this approach, the free cell spent cultivation medium, where *P. aeruginosa* was grown

205

for a 120-hour period (later stationary phase), was used to induce rhamnolipid production. The

206

presence of a mixture of acylated homoserine lactones (AHLs) and Pseudomonas quinolone

207

signal (PQS) naturally secreted by *P. aeruginosa* in later stationary growth phase was reported

208

by several researchers (Ochsner and Reiser, 1995; Pesci *et al.*, 1997; Reis *et al.*, 2011). This

209

autoinducers are known to be responsible for the induction of the synthesis of rhamnolipids and

210

other virulence factors in *P. aeruginosa* (Fuqua and Greenberg, 1998; Williams *et al.*, 2000;

211

Diggle *et al.*, 2003).

212

The addition of spent medium containing endogenous autoinducers at the start of new *P.*

213

aeruginosa cultures resulted in an increase in the yield of rhamanolipids synthesized per unit of

214

substrate consumed ($Y_{P/X}$), as showed in Figure 3. The most significant differences among the

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$Y_{P/X}$ values were observed when the spent culture medium concentrations were changed from 1%

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to 5% and from 5 to 15% (Table 2).

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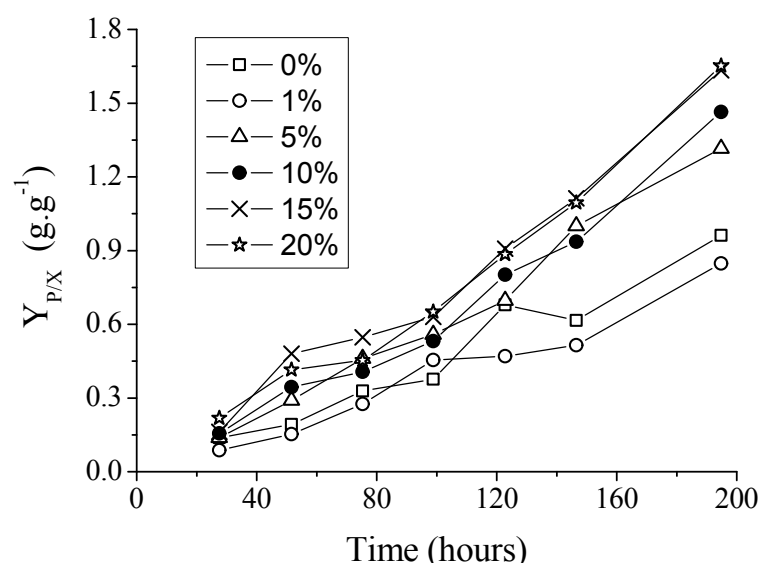
219 **Figure 3:** Progress curves of yield coefficient $Y_{P/X}$ in culture medium with different

220 supplemental proportions of 120 h-old *P. aeruginosa* spent culture medium.

221

222   The addition of spent culture medium to the new *P. aeruginosa* PA1 culture also increased

223 the volumetric glycerol consumption rate ($Q_{S(Gly)}$) (Table 2). This fact suggests that the addition

224 of endogenous autoinducers would also alter the metabolic rate of nutrients that, in the final

225 analysis, would be coupled to the synthesis of the precursors for rhamnolipid production. The

226 volumetric rhamnolipids production rate (Q_P) was also favored by the addition of endogenous

227 autoinducers (Table 2).

228

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

230 medium supplemented with different percentages of spent medium containing endogenous

231 autoinducers.

232

Parameters	0%	1%	5%	10%	15%	20%
ΔRhamnolipids (g L^{-1})	4.79	4.50	7.85	8.09	8.83	9.39
ΔBiomass (g L^{-1})	4.98	5.31	5.96	5.53	5.40	5.68
$Y_{P/X}$ (g g^{-1})	0.96	0.85	1.32	1.46	1.63	1.65
$Y_{P/S}$ (g g^{-1})	0.43	0.33	0.32	0.32	0.36	0.38
Q_P (g L^{-1} h^{-1})	0.027	0.026	0.044	0.047	0.051	0.054
Q_S (g L^{-1} h^{-1})	0.014	0.012	0.021	0.028	0.029	0.031

233 **ΔRhamnolipids:** difference between final and initial rhamnolipid concentration; **ΔBiomass:** difference between

234 final and initial cellular concentration; **$Y_{P/X}$:** yield of product synthesized per unit of cell mass produced; **$Y_{P/S}$:** yield

235 of product synthesized per unit of substrate consumed; **Q_P:** volumetric rhamnolipids production rate; **$Q_{S(Gly)}$:**

236 volumetric glycerol consumption rate.

237

238   The slopes calculated for each curve shown in Figure 3 using liner regression, called

239   specific rate of rhamnolipids synthesis, were plotted against the log values of the spent culture

240   medium concentration and are presented in Figure 4. This graph permits one to clearly observe

241   that the increase in the production of rhamnolipids was due to an increase in the capacity of the

242   microorganisms to synthesize rhamnolipids. These results suggest that the amount of spent

243   culture medium added at the beginning of cultivation probably contributed to the increase in the

244   synthesis of rhamnosiltransferases because the natural unfolding of the quorum sensing system,

245   which is responsible for the transcriptional regulation of rhamnolipid synthesis in presence of

246   endogenous autoinducers.

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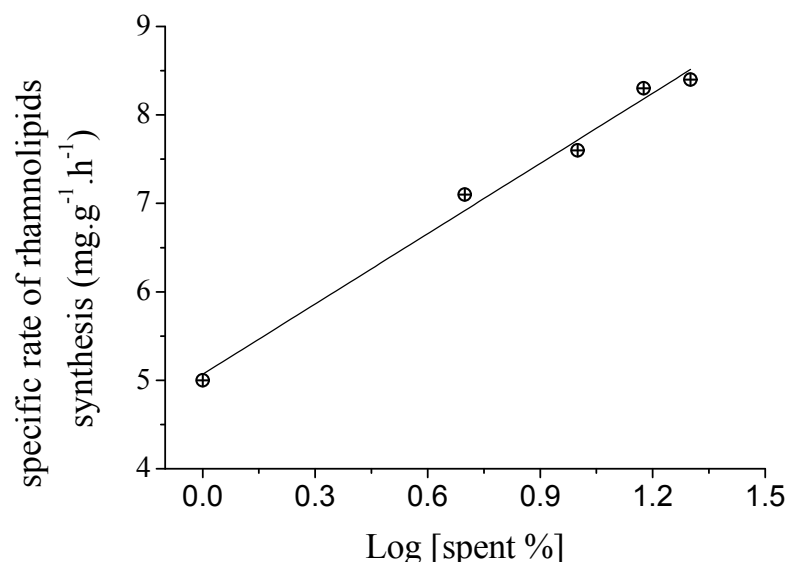
249   **Figure 4:** Relation between the specific rate of rhamnolipid synthesis and the addition of

250   different proportions of 120 h-old *P. aeruginosa* spent culture medium.

251

252   **Fed-batch strategy**

253   The fed-batch operation is another possible strategy for increasing the production of

254   rhamnolipids and was chosen to circumvent a possible negative effect, observed by Santa Anna

255   *et al.* (2002), on the assimilation of nutrients when the culture medium contains glycerol

256   concentrations higher than 3%. In addition, a process driven by fed-batch can control and

257   maintain the nutrient limits already established as being favorable for rhamnolipid synthesis

258   (Desai and Banat, 1997; Chayabutra *et al.*, 2000; Xavier *et al.*, 2011).

259   The consumption of nutrients during the fed-batch process was determined. In the control

260   experiment, in which only water was fed to the medium, the carbon source (glycerol) and

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

262   and 24 hours of cultivation, respectively (Figures 5A and 5B).

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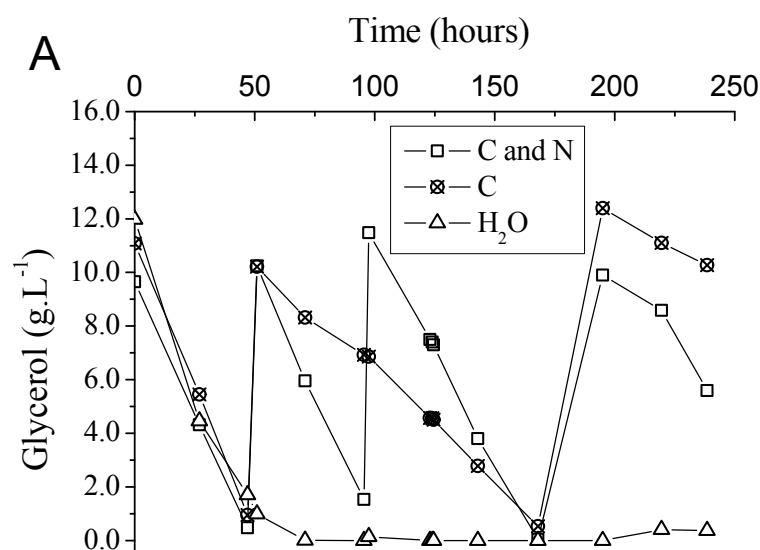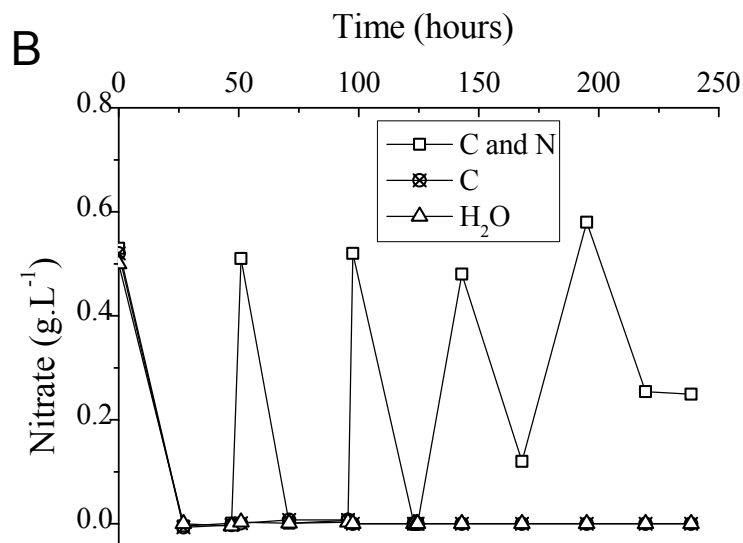
Figure 5: Progress curve of glycerol (A) and nitrate (B) consumption during the fed-batch

process using different feeding strategies. C and N – fed with carbon and nitrogen sources

together. C - fed only with the carbon source. H_2O – fed with water instead of nutrients.

269
270   Although the consumption of the nitrogen source ($NaNO_3$) occurred in shorter time

intervals than glycerol consumption (Figure 5A and 5B), the addition of a solution containing

glycerol and nitrate was performed as a function of the consumption of the carbon source (Figure

5A). At 143 hours after the beginning of the process, the volumetric glycerol consumption rate

decreased from 195 $mg.L^{-1}.h^{-1}$ to 162 $mg.L^{-1}.h^{-1}$. Because of this decay, only sodium nitrate was

added at this time to the flask originally fed with carbon and nitrogen sources with the objective

of maintaining the frequency of nitrate addition. The volumetric glycerol consumption rate

($Q_{S(Gly)}$) was higher in the presence of a nitrogen source (Table 3). This fact becomes apparent

when the curves of glycerol consumption are compared with one another, considering the

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

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

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

282   nitrogen and its conversion into catalytic proteins.

283

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

Parameters	C+N	C	H$_2$O
ΔRhamnolipids (g L^{-1})	10.93	7.76	2.88
ΔBiomass (g L^{-1})	4.78	3.34	2.41
$Y_{P/X}$ (g g^{-1})	2.29	2.32	1.19
$Y_{P/S}$ (g g^{-1})	0.33	0.35	0.21
Q_P (g L^{-1} h^{-1})	0.047	0.033	0.027
$Q_{S(Gly)}$ (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

285   **ΔRhamnolipids:** difference between final and initial rhamnolipid concentration; **ΔBiomass:** difference between

286   final and initial cellular concentration; $Y_{P/X}$: yield of product synthesized per unit of cell mass produced; $Y_{P/S}$: yield

287   of product synthesized per unit of substrate consumed; Q_P: volumetric rhamnolipids production rate; $Q_{S(gly)}$:

288   volumetric glycerol consumption rate; $Q_{S(NO3)}$: volumetric nitrate consumption rate. The parameters for the

289   condition "H$_2$O" were calculated at 100 h of culture.

290

291   Ochsner *et al.* (1995) observed that the activity of the rhamnosyltransferase in *P.*

292   *aeruginosa* during cultivation in a nitrogen-limiting medium containing glycerol as the carbon

293   source was the highest at the beginning of the stationary phase and declined to zero in the late

294   stationary phase. We suggest that exhaustion of the nitrogen source would limit not only cellular

295   growth, but also the maintenance of the enzymatic machinery, mainly that involved in the

296   metabolic pathways for rhamnolipid synthesis. In the present work, when the process of

297   rhamnolipids production was simultaneously fed with carbon and nitrogen sources, a higher cell

298   yield and volumetric productivity of rhamnolipids was achieved (Table 3).

299   On the basis of the feeding conditions, together with the control experiment (without

300   feeding), one can conclude that the exhaustion of carbon and nitrogen sources interrupted the *P.*

301   *aeruginosa* growth (Figure 6A) and the rhamnolipid synthesis (Figure 6B) sooner (at 72 hours

302   after initiating the cultivation). On the other hand, feeding the system with only glycerol led to an

303   improvement when compared with the simple batch (Figure 6A) and resulted in higher $Y_{P/X}$ and

304    $Y_{P/S}$ values (Table 3). Furthermore, the feeding condition with both sources of nutrients (carbon

305   and nitrogen) resulted in an even greater increase in both the biomass produced (Figure 6A) and

306   the volumetric productivity, reaching a rhamnolipid production value 40% higher (Table 3)

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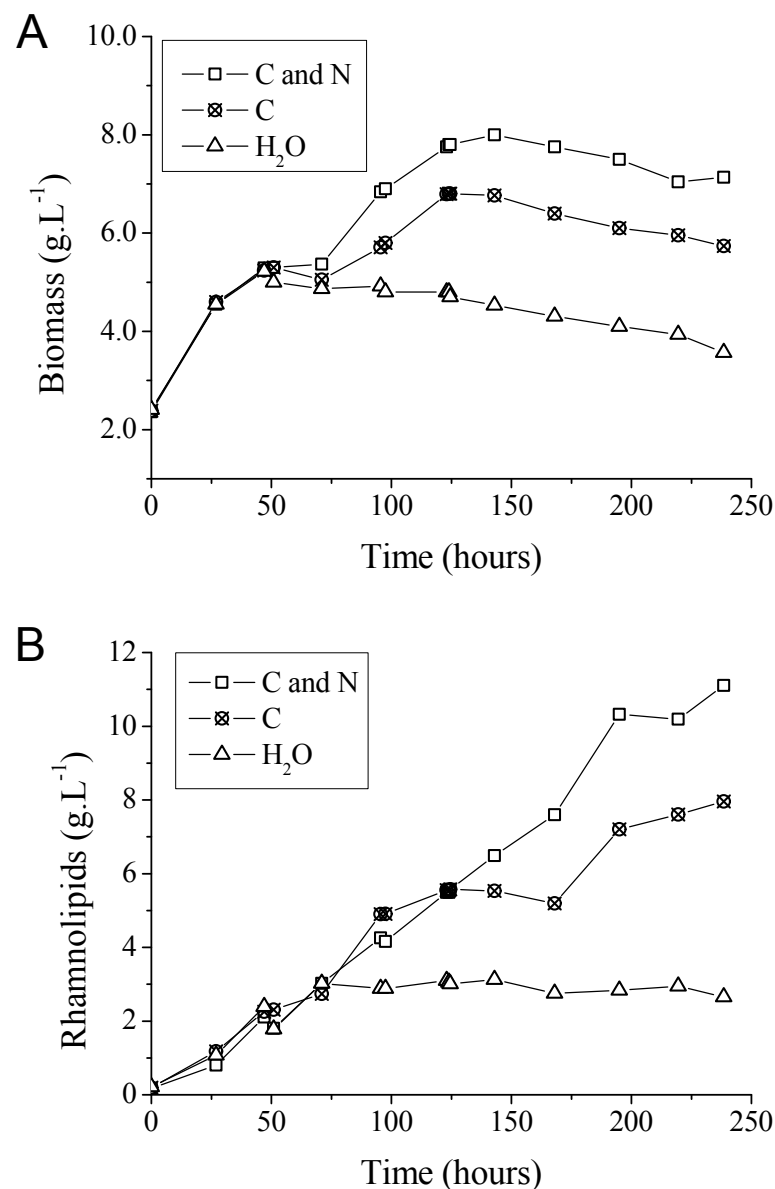
310 **Figure 6**: *P. aeruginosa* growth curves (A) and time course of rhamnolipid production (B) in the

311 fed-batch process using different feeding strategies. C and N – fed with carbon and nitrogen

312 sources together. C - fed only with the carbon source. H_2O – fed with water instead of nutrients.

313

314   After 125 hours from the start of the process, cell growth stopped in the fed-batch system

315 with both sources of nitrogen and carbon and in the system only fed with carbon source (Figures

316 6A and 6B). It is possible that the limitation of other nutrients has occurred at this point, such as

317 trace elements or oxygen. However, the production of rhamnolipids remained active mainly

318 when fed simultaneously with nitrate and glycerol. This behavior characterizes a semi-growth

319 associated profile.

320

321 **Conclusion**

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

323 rhamnolipids by *P. aeruginosa* PA1, selective synthesis of different types of rhamnolipids

324 caused by certain pH ranges appeared as an unexpected and timely result. The reutilization or

325 recycling of culture medium containing endogenous autoinducers of the quorum sensing system

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

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

328 productivity was obtained using this strategy. The fed-batch experiment using the limitation of

329 the carbon and nitrogen source was successful and could be employed along with an appropriate

330 formulation of the cultivation conditions (micronutrients, pH, supplementary autoinducers)

331

332   **Acknowledgments**

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