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Biodiesel byproduct bioconversion to rhamnolipids: Upstream aspects

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Abstract

This study focused on two important aspects of the upstream process: the appropriate use of crude glycerol as a low-cost carbon source, and strain selection. The effect of different crude glycerol concentrations on rhamnolipid biosynthesis by two *Pseudomonas aeruginosa* strains (wild type LBI and mutant LBI 2A1) was studied. Finally, the synthesized rhamnolipids were characterized by mass spectrometry. When both strains were compared, 50 g/L was the most favorable concentration for both, but *P. aeruginosa* LBI 2A1 showed an increase in rhamnolipid production (2.55 g/L) of 192% over wild type (1.3 g/L). The higher rhamnolipid production could be related to a possible mechanism developed after the mutation process at high antibiotic concentrations. Mass spectrometry confirmed the glycolipid nature of the produced biosurfactant, and the homologue composition showed a wide mixture of mono and di-rhamnolipids. These results show that high glycerol concentrations can inhibit microbial metabolism, due to osmotic stress, leading to a better understanding of glycerol metabolism towards its optimization in fermentation media. Since *P. aeruginosa* LBI 2A1 showed higher conversion yields than *P. aeruginosa* LBI, the use of a mutant strain associated with a low cost carbon source might improve biosurfactant biosynthesis, therefore yielding an important upstream improvement.

Keywords: Biotechnology, Microbiology

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1. Introduction

Biosurfactants are surface-active molecules that have emulsifying activity (Marchant and Banat, 2012), and the classification of these molecules depends on their chemical composition and structure. The best-studied biosurfactants are glycolipids, and within this group appear the rhamnolipids, which are mainly produced by bacteria of the genus *Pseudomonas* (Henkel et al., 2012).

Pseudomonas aeruginosa has been one of the most studied microorganisms for rhamnolipid production, since the properties of these molecules in solution enable a wide range of industrial and environmental applications, and their production yield is high with respect to cultivation time (Abdel-Mawgoud et al., 2011).

The effect of carbon source on rhamnolipid production in *Pseudomonas aeruginosa* has been studied. Hydrophobic carbon sources, such as vegetable oils and n-alkanes, have shown the best results for the production of rhamnolipid (Abdel-Mawgoud et al., 2011). Hydrophilic sources, such as glycerol, glucose, mannitol and ethanol, have also been used for production of these biosurfactants, but the production yield is inferior to that achieved with hydrophobic carbon sources (Nitschke et al., 2011). However, the use of hydrophilic carbon sources for rhamnolipid production can decrease the production cost because of improved downstream processing, which accounts for around ~60% of the total production cost (Mukherjee et al., 2006). Therefore, different strategies in the upstream and the downstream processes are being studied to increase efficiency (Abdel-Mawgoud et al., 2011; Díaz De Rienzo et al., 2016; Ma et al., 2016; Pereira et al., 2013).

A strategy for cost reduction and optimization of biosurfactant production is the use of raw materials for the fermentation medium, which can represent between 10–80% of the overall operating cost (Dhanaraja and Sen, 2014). The optimization of the fermentation process using a hydrophilic low-cost feedstock appears as a viable upstream strategy to reduce rhamnolipid production costs. The rapid progress of biodiesel production in Brazil and worldwide has resulted in an increase of crude glycerol as an industrial waste, which has a low market value and high disposal costs (Silva et al., 2009). A possible solution for the challenge of having this crude glycerol byproduct is to use it as a carbon source in biotechnological processes, such as in biosurfactant production (Khanna et al., 2012). This carbon source has shown promising results for rhamnolipid production, with maximum yields between 1 and 4 g/L (Bharali et al., 2014; Eraqui et al., 2016; Pereira et al., 2013), but there is still the challenge of optimizing the process to increase the production of the biosurfactant (Henkel et al., 2012).

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Another feasible strategy towards optimization of rhamnolipid production is the study of biosurfactant hyper-producer strains or mutants that can utilize raw materials, increase production yields and also, enhance biosurfactant characteristics (Dhanaraja and Sen, 2014). Hence, the aim of the present work was to evaluate the effect of varying the crude glycerol concentration on rhamnolipid production using *Pseudomonas aeruginosa* LBI strain in batch cultures, and to compare biosurfactant production between wild type *Pseudomonas aeruginosa* LBI and its mutant *Pseudomonas aeruginosa* LBI 2A1, using the most favorable glycerol concentration studied. Finally, the produced rhamnolipids were characterized.

2. Material and methods

2.1. Microorganisms

Pseudomonas aeruginosa LBI was isolated from petroleum-contaminated soil (Benicasa et al., 2002) and the mutant strain *Pseudomonas aeruginosa* LBI 2A1 was obtained by random transposon mutagenesis done a previous work from this lab, as a part of a doctoral thesis (Lovaglio, 2011). Each strain was maintained in nutrient broth plus 20% glycerol at −20 °C.

2.2. Raw material

The glycerol used in this work is the byproduct of biodiesel production, resulting from the transesterification of oil by methanol in alkaline medium (NaOH). The carbon source was provided by Biocapital Consultoria Empresarial e Participações S.A. (São Paulo, Brazil).

2.3. Culture medium and conditions

2.3.1. Culture medium

Lysogeny Broth (LB) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was used for the first pre-cultivation step. A Ca-free mineral salt solution (modified from Müller et al., 2011) with 1.5 g/L $NaNO_3$, 0.05 g/L $MgSO_4.7H_2O$, and 0.1 g/L KCl, containing a 0.1 M sodium phosphate buffer at pH 6.5 was used for the seed culture. A total of 1 mL/L of trace element solution (2.0 g/L sodium citrate $2H_2O$, 0.28 g/L $FeCl_3.6H_2O$, 0.87 g/L $ZnSO_4.7H_2O$, 1.2 g/L $CoCl_2.6H_2O$, 1.2 g/L $CuSO_4.5H_2O$, and 0.8 g/L $MnSO_4.H_2O$) was added to both seed and production media. Rhamnolipid production Ca-free medium consisted of 0.3 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L KCl, 15 g/L $NaNO_3$, with pH adjusted to 6.5. Crude glycerol was used as the carbon source and the concentration was half on the seed medium as was used for rhamnolipid production. This study was divided into three parts. First, the batch fermentation with *Pseudomonas aeruginosa* LBI was

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performed with different concentrations of crude glycerol (50, 70, 100, 150 g/L). Second, the rhamnolipid production of *Pseudomonas aeruginosa* LBI and *Pseudomonas aeruginosa* LBI 2A1 was compared using the lowest crude glycerol concentration tested initially. Finally, fermentation experiments were conducted with lower glycerol concentrations to confirm a limiting concentration (50, 30, 15, 5 g/L) for *Pseudomonas aeruginosa* LBI 2A1. Mineral salt solutions, phosphate sources, and crude glycerol were autoclaved separately for all experiments. All experiments were performed in triplicate.

2.3.2. Inoculum preparation

For the pre-culture each strain was re-activated in 25 mL of LB contained in a 125 mL Erlenmeyer flask with incubation at 37 °C and 200 rpm. After 24 h, 2.5 mL of the pre-inoculum were transferred to 500 mL Erlenmeyer flasks containing 20% volume of seed medium and they were incubated for 24–48 h at 37 °C, 200 rpm. For inoculation in the production medium, a theoretical initial OD_{580} of 0.08 was considered, and the appropriate inoculum volume of the seed culture needed to be calculated and transferred to the fermentation broth.

2.3.3. Rhamnolipid production in shaker

The rhamnolipid production was carried out in 1L Erlenmeyer flasks containing 30% production medium at 37 °C with agitation at 200 rpm for 120 h on rotatory shaker. Samples (10 mL) were collected every 24 h of fermentation, cells were separated by centrifugation at 6000 rpm for 30 min at 4 °C, and the cell pellet was used for dry biomass quantification, while the supernatant was submitted to analysis of rhamnolipid and substrate concentration.

2.4. Analytical methods

2.4.1. Rhamnolipid quantification

2.4.1.1. Rhamnolipid extraction

For rhamnolipid precipitation, 85% H_3PO_4 1:100 (v/v) was added to the cell-free broth. The biosurfactants were extracted using ethyl acetate 1:125 (v/v). The mixture was then shaken for 1 min and centrifuged at 10,000 rpm for 10 min and the upper phase was removed. The extraction process was repeated using the remaining lower phase.

2.4.1.2. Thin layer chromatography

The formation of rhamnolipid congeners was checked by thin layer chromatography on silica gel plates and chloroform/methanol/acetic acid (65:15:2 by vol) was

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used as the mobile phase. The spots were visualized with acetic acid/anisaldehyde/sulfuric acid (100:2:2 by vol) and heating at 100 °C.

2.4.1.3. High liquid performance chromatography

Rhamnolipid samples containing ethyl acetate were evaporated. Phenacyl esters of rhamnolipids were obtained as described before by Schenk et al. (1995). HPLC analysis was performed in a Shimadzu HPLC instrument coupled with a UV detector system. For calibration mono and di-rhamnolipid standard solutions (0.25; 0.50; 0.75; 1.0 g/L) were used. A reverse phase column (Shim-pack VP-ODS, Shimadzu − 150 mm × 0.46 mm, 5 μm silica gel) was used at 30 °C. The retention time for Rha-Rha-$C_{10}C_{10}$ was 25.2 ± 0.1 min and for Rha-$C_{10}C_{10}$ was 26.1± 0.1 min. The protocol for rhamnolipid separation and quantification was done as described by Müller et al. (2010).

2.4.2. Biomass quantification

Microbial growth was analyzed using a gravimetric method. The cell pellet was washed in 0.85% (w/v) NaCl solution (6000 rpm, 4 °C, 20 min), then was resuspended in 1 mL of 0.85% (w/v) NaCl solution and dried until constant weight.

2.4.3. Glycerol quantification

Substrate consumption was determined by high performance liquid chromatography, as described by McGinley and Mott (2008), with slight modifications. HPLC analyses were performed in a Shimadzu HPLC instrument coupled with a refractive index detector system. A Rezex ROA (Phenomenex, 300 17 × 7,8 mm) column was used, at 65 °C and 20 μL was injected with a flow rate of 0.5 mL/min. Five mM H_2SO_4 was used as the mobile phase and the retention time was 18 ± 0.1 min.

2.4.4. Characterization of rhamnolipids homologues

Electrospray ionization mass spectra were recorded on a 3200 QTRAP mass spectrometer, AB SCiex. A 3000 ppm stock solution of rhamnolipid was prepared with ultra-pure water as the solvent. The concentration of the sample was 20 ppm and a methanol/water (1:1) + 0.1% of ammonia hydroxide solution was used as the solvent. The sample was introduced into the source at a rate of 10 μl/min with a syringe pump. The spectra were acquired in the negative mode in a mass range 100–1000 m/z using the following conditions: −4500 V, Curtain Gas:10 psi, Gas 1:15 psi, DP (Declustering Potential): −20.0 V, EP (Entrance Potential): −7.0 V e CEP (Cell entrance potential): −12.0 V. The fragment ion experiment (MS2) was performed using a collision energy of 35.0 V +/− 15.0 V.

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2.4.5. Statistical analyses

The data normality was analyzed with a Shapiro-Wilk test ($\alpha = 0.05$). Data were compared by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test (key-Kr) when significant differences were found at $p = 0.05$. The software used was R (version 3.2.0).

3. Results

3.1. Effect of different crude glycerol concentrations on wild type *Pseudomonas aeruginosa* LBI rhamnolipids production

Pseudomonas aeruginosa LBI was able to grow in all crude glycerol concentrations tested, as shown in Fig. 1a. A significant preference for a lower concentration of glycerol (50 g/L) was observed ($p < 0.05$) (Table 1). The biomass production was 4.13 ± 0.03 g/L after 72 h of fermentation; the microorganism entered into a stationary phase at this point and presented a higher rhamnolipid production (1.3 ± 0.3 g/L), and completely depleted the carbon source (Fig. 1b–c). When 100 and 150 g/L of crude glycerol were tested, *P. aeruginosa* LBI entered a stationary phase at 24 h and 48 h, respectively, at 72 h of fermentation produced no significant rhamnolipids and only consumed 40% and 42% of the carbon source, respectively.

A peculiar behavior was observed with 70 g/L of crude glycerol, where by after 96 h, the biomass concentration increased significantly, nonetheless, the 72 h biomass value (3.53 ± 0.02 g/L) was taken into account, because the maximum rhamnolipid production (0.43 ± 0.01 g/L) occurred at that time with this glycerol concentration.

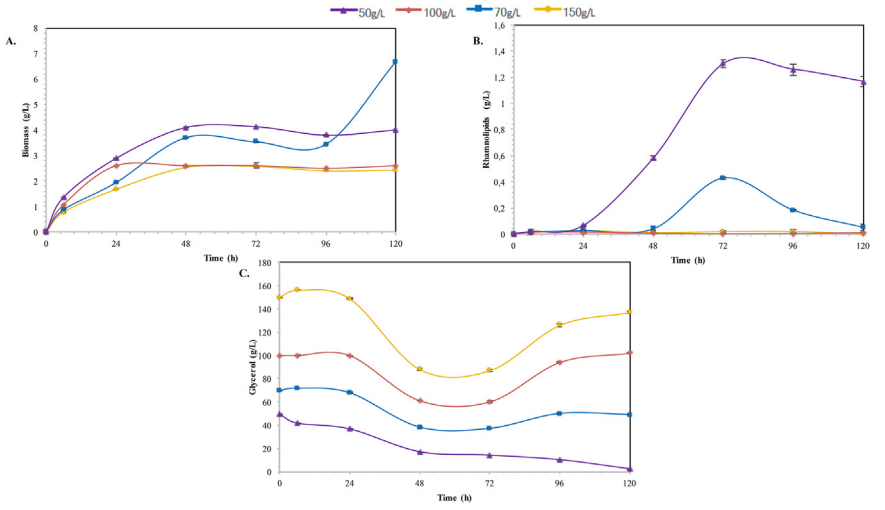

Fig. 1. Comparison on the effect of different crude glycerol concentrations (30 g/L – purple, 50 g/L – red; 100 g/L – blue; 150 g/L – yellow) on the fermentation with *P. aeruginosa* LBI. (A) Biomass production; (B) Rhamnolipids production; (C) Glycerol consumption.

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Table 1. Comparison of final glycerol concentration, rhamnolipids production and biomass at 72 h. a, b, c indicates significant differences between means within column, i.e. within rhamnolipids production at different glicerol concentrations; means followed by different letters differ significantly. Comparison of pair of means was conducted using Tukey Kramer HSD at $p < 0.05$.

Initial Glycerol (g/L)	*Glycerol consumption (%)*	Rhamnolipids Production (g/L)	Biomass (g/L)
50	72[a]	1.3 ± 0.3[a]	4.13 ± 0.03[a]
70	47[b]	0.43 ± 0.01[b]	3.53 ± 0.02 [b]
100	40[c]	0.002 ± 0.002[bc]	2.60 ± 0.1[a]
150	42[c]	0.017 ± 0.009[c]	2.57 ± 0.002[a]

With this concentration, the maximum consumption of the carbon source was 47% at 72 h.

3.2. Wild type *Pseudomonas aeruginosa* LBI vs its mutant *Pseudomonas aeruginosa* LBI 2A1

The lowest concentration of glycerol was used to compare rhamnolipid production between *Pseudomonas aeruginosa* LBI and its mutant *Pseudomonas aeruginosa* LBI 2A1 and the results are shown in Fig. 2a. A higher biomass production was observed with *Pseudomonas aeruginosa* LBI, which experienced rapid cell growth between 6 h and 48 h. In contrast, the mutant exhibited deceleration of microbial growth during the first 24 h of fermentation and the maximum biomass

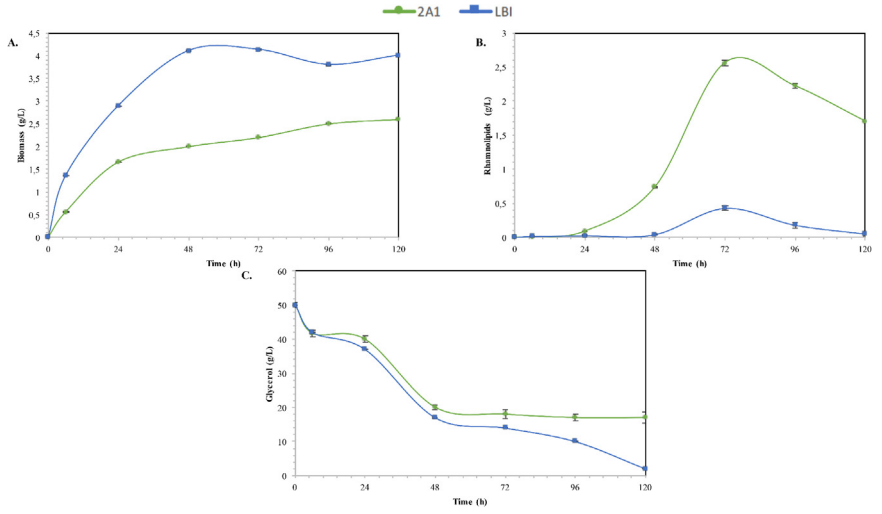

Fig. 2. Comparison of fermentation behavior between *Pseudomonas aeruginosa* LBI (blue) and *Pseudomonas aeruginosa* LBI 2A1 (green) (A) Biomass production; (B) Rhamnolipids production; (C) Glycerol consumption.

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concentration was 2.60 ± 0.01 g/L (Fig. 2b). Furthermore, *P. aeruginosa* LBI had managed to consume the entire carbon source after 120 h, while *P. aeruginosa* LBI 2A1 had consumed only 66% of the carbon source (Fig. 2c).

In spite of the non-significant difference in biomass production ($p > 0.05$), rhamnolipid synthesis by *Pseudomonas aeruginosa* LBI 2A1 was significantly higher ($p < 0.05$), with an increase of 192% (2.55 ± 0.01 g/L), over the wild type. In both cases, a decrease in rhamnolipid concentration was seen after 96 h, showing a possible use of the product as a carbon source (Fig. 2c).

3.3. Crude glycerol limiting concentrations on rhamnolipids production by mutant strain *Pseudomonas aeruginosa* LBI 2A1

Five different concentrations of the target carbon source were studied to determine a limiting concentration on rhamnolipid production for *Pseudomonas aeruginosa* LBI 2A1 (Fig. 3). As the glycerol concentration decreased, *P. aeruginosa* LBI 2A1 showed an increase in carbon consumption. The carbon source was completely depleted at 5, 15 and 30 g/L, and with 70 g/L of crude glycerol, the microorganism showed a similar pattern as *P. aeruginosa* LBI with an inhibition of the consumption of the carbon source (Fig. 3c). Furthermore, *P. aeruginosa* LBI 2A1 presented the higher rhamnolipid production with 50 g/L of crude glycerol. As the concentration of the carbon source decreased, the rhamnolipid production did as well, but the rhamnolipid production was the lowest obtained with the higher glycerol concentration (70 g/L).

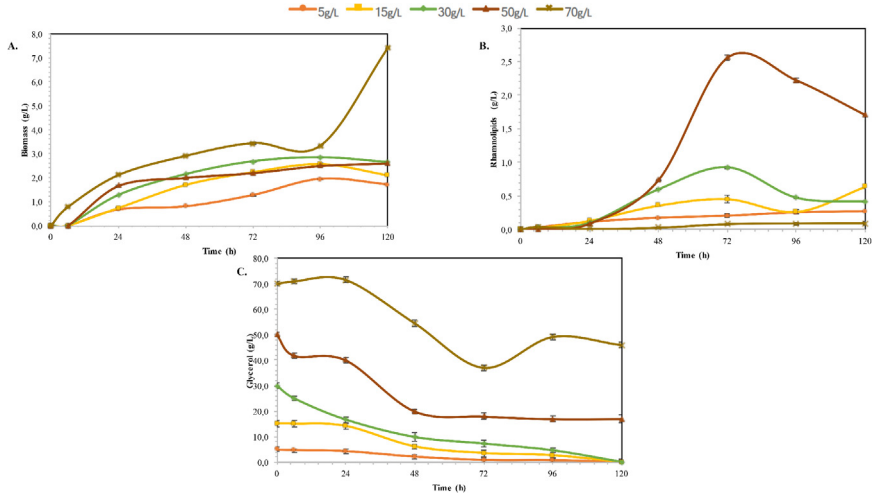

Fig. 3. Comparison on the effect of different crude glycerol concentrations (5 g/L – orange; 15 g/L – yellow; 30 g/L – green; 50 g/L – brown; 100 g/L – light brown) on the fermentation with *P. aeruginosa* LBI 2A1 (A) Biomass production; (B) Rhamnolipids production; (C) Glycerol consumption.

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No significant differences were observed when 50, 30, and 15 g/L of crude glycerol were used (Table 2). The peculiar behavior seen with *P. aeruginosa* LBI was also observed with the mutant at 120 h of fermentation which showed significant differences from all the other treatments ($p < 0.05$).

3.4. Homologues characterization

The mass spectra (ESI − MS) showed a mixture of mono and di-rhamnolipids, in total 9 rhamnolipids homologues with molecular weight between 475 and 677 were identified (Table 3).

The fragmented ion experiment showed the ions corresponding to the rhamnolipid homologues, and the existence of a lipid and a carbohydrate moiety for each homologue was confirmed. Fragmented ions m/z 475, 503, and 531 are shown in Fig. 4 (a, b, c, respectively). For $RhaC_8C_{10}$/$RhaC_{10}C_8$ (m/z 475), the two major ions resulting from the fragmentation were $[Rha]^-$ (m/z 163) and $[C_8C_{10}]^-$ (m/z 311). Mono-rhamnolipid $RhaC_{10}C_{10}$ (m/z 503) was fragmented into two major ions $[C_{10}]^-$ (m/z 169) and $[RhaC_{10}]^-$ (m/z 333). For $RhaC_{10}C_{12}$/$RhaC_{12}C_{10}$ (m/z 531), the mass spectra showed $[Rha]^-$ (m/z 163) and $[C_{10}C_{12}]^-$ (m/z 367).

Fig. 5 (a, b, c, respectively) shows fragmented ions m/z 621, 649 and 677, corresponding to the following di-rhamnolipid structures $RhaRhaC_8C_{10}$/$RhaRhaC_{10}C_8$; $RhaRhaC_{10}C_{10}$ and $RhaRhaC_{10}C_{12}$/$RhaRhaC_{12}C_{10}$ di-rhamnolipid. The fragmented íons for these congeners were: $[C_{10}]^-$ (m/z 187) and $[RhaRhaC_8]^-$ (m/z 457); $[C_{10}]^-$ (m/z 169) and $[RhaRhaC_{10}]^-$ (m/z 479); $[C_{12}]^-$ (m/z 197) and $[RhaRhaC_{10}]^-$ (m/z 479) or $[C_{10}]^-$ (m/z 169) and $[RhaRhaC_{12}]^-$ (m/z 508), respectively.

Table 2. Comparison of final glycerol concentration, rhamnolipids production and biomass at 72 h. a, b, c, d, e indicates significant differences between means within column, i.e. within rhamnolipids production at different glicerol concentrations; means followed by different letters differ significantly. Comparison of pair of means was conducted using Tukey Kramer HSD at $p < 0.05$.

Initial Glycerol (g/L)	Glycerol consumption (%)	Rhamnolipids Production (g/L)	Biomass (g/L)
5	84.5[a]	0.205 ± 0.03 [a]	1.267 ± 0.03
15	72.9[b]	0.453 ± 0.06 [a]	2.233 ± 0.01
30	60.5[c]	0.925 ± 0.02 [a]	2.667 ± 0.02
50	37[d]	2.558 ± 0.04 [b]	2.200 ± 0.01
70	7.5[e]	0.080 ± 0.02 [a]	3.433 ± 0.01

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Table 3. Relative abundance of rhamnolipid homologues produced by *Pseudomonas aeruginosa* LBI 2A1 determined using ESI–MS.

Homologues	$[M-H]^-$ m/z	Relative abundance (%)
$RhaC_8C_{10}/RhaC_{10}C_8$	475	40
$RhaC_{10}C_{10}$	503	45
$RhaC_{10}C_{12}/RhaC_{12}C_{10}$	531	37
$RhaRhaC_8C_{10}/RhaRhaC_8C_{10}$	621	63
$RhaRhaC_{10}C_{10}$	649	100
$RhaRhaC_{10}C_{12}/RhaRhaC_{12}C_{10}$	677	68

4. Discussion

Due to their high potential application in different fields, the optimization of rhamnolipid production using an industrial waste such as crude glycerol is an important issue on the upstream process. As a by-product from the biodiesel industry, crude glycerol has a complex composition (Xiao et al., 2013), and such impurities can interfere with microbial metabolism.

Upon studying the effect of different concentrations on *P. aeruginosa* LBI, crude glycerol was shown to influence the microorganisms since they presented an earlier stationary phase as the glycerol concentration increased. Similar results were observed by Souza et al. (2014) who obtained their best yield using 20 g/L of crude glycerol. On the other hand, crude glycerol impurities (salts, heavy metals) can be toxic in high concentrations, however, such impurities can be used as additional nutrients in the fermentation process described by Fu et al. (2015). These observations are in concordance with our results, which reflect a better adaptation to the carbon source at a lower concentration (50 g/L), and this is reflected in biomass production and significantly higher rhamnolipid production. Furthermore, the solubility of crude glycerol at elevated concentrations may be linked to the difficulty of the bacterium in metabolizing both the carbon source and other nutrients in the liquid culture medium, as described in other studies (Eraqui et al., 2016; Da Rosa et al., 2010; Santa-Anna et al., 2002; Silva et al., 2010).

Glycerol uptake has been reported to occur by facilitated diffusion in *Pseudomonas aeruginosa* through the porin OprB, the expression of which is controlled via catabolite repression (Hancock and Carey, 1980). With prolonged growth under glycerol limitation, *Pseudomonas aeruginosa* NM48 showed an over-production of the transport system that internalizes the glycerol that is subsequently phosphorylated and trapped into the cell, and this maintains the glycerol concentration gradient (Williams et al., 1994). Our results indicate that this transport system is occurring at low concentrations, and these concentrations were

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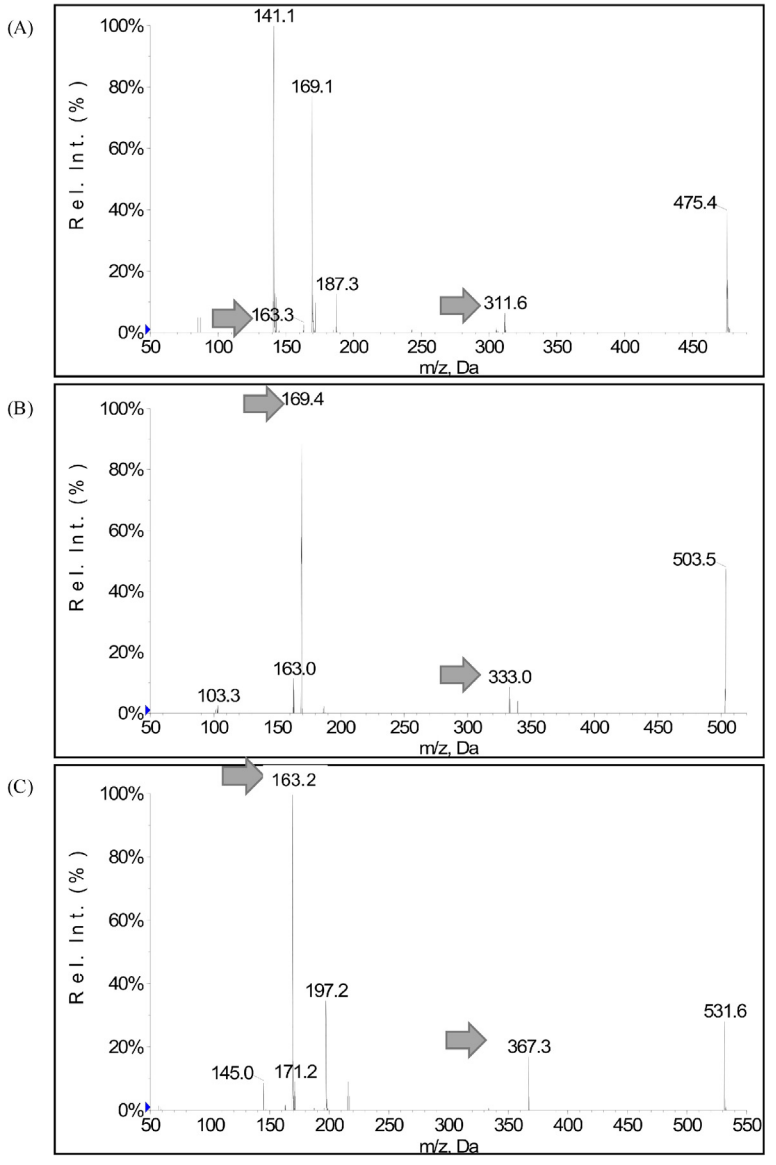

Fig. 4. ESI-MS/MS from pseudo-ions (*m/z*) (A) 457; (B) 503 (C) 631 from rhamnolipids produced by *Pseudomonas aeruginosa* LBI 2A1 with 50 g/L crude glycerol. Arrows indicate the fragmented ions of the homologues.

sufficient to promote cell growth. Glycerol was a limiting factor for rhamnolipid production below 30 g/L. The cell growth might not have been sufficient to stimulate the quorum-sensing cascade that leads to rhamnolipid gene-related transcription, mainly at the concentration of 5 g/L of crude glycerol.

High glycerol concentrations do not appear to stimulate the glycerol-uptake *glp* regulon, so in this limited condition (Williams et al., 1994; Schweizer and Po., 1994; Temple et al., 1998), glycerol passes passively but it might not be rapidly phosphorylated, which could lead to a release of the 'unphosphorylated' glycerol

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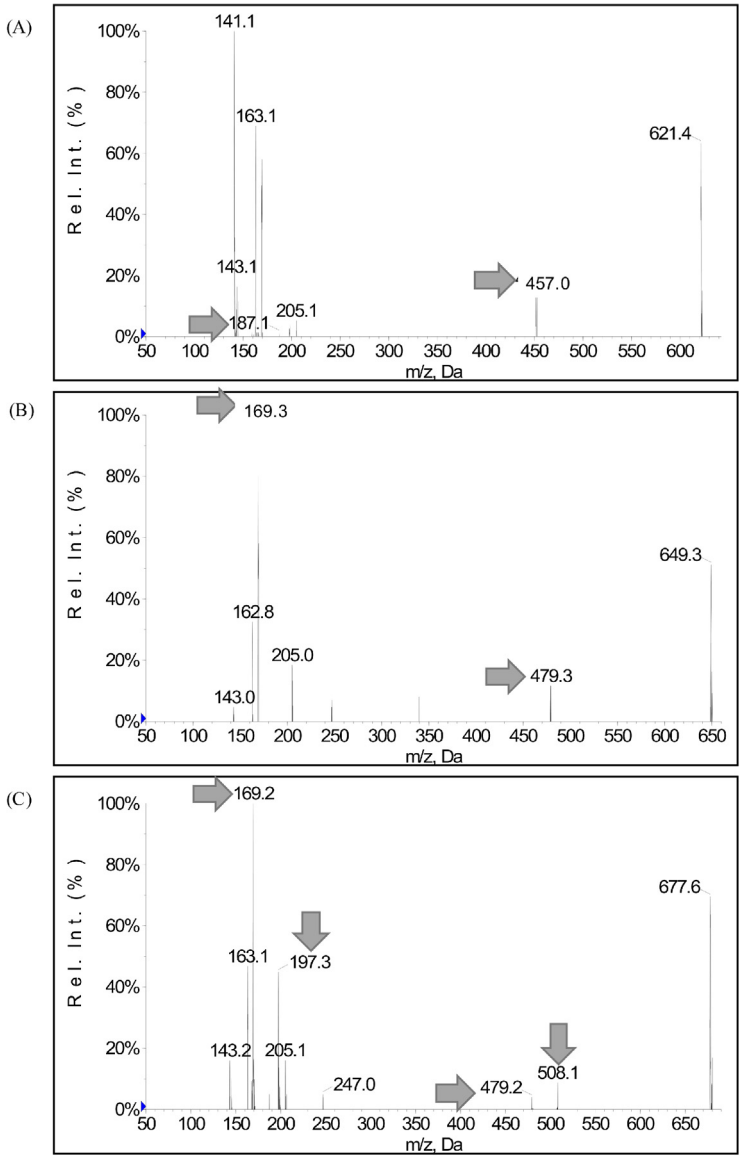

Fig. 5. ESI-MS/MS from pseudo-ions (*m/z*) (A) 621 (B) 649 (C) 677 from rhamnolipids produced by *Pseudomonas aeruginosa* LBI 2A1 with 50 g/L crude glycerol. Arrows indicate the fragmented ions of the homologues.

into the media, which may explain the increase of glycerol concentration in concentrations above 70 g/L.

The concentration of the carbon source was not a limiting factor for biomass production, but it could have been a limiting factor for rhamnolipid production, since crude glycerol contains high concentrations of Na^+, and the microorganism is under osmotic stress, which affects its metabolism (Fu et al., 2015). High Na^+ concentrations can influence the expression of genes related to rhamnolipid metabolism, which would affect the expression of the *rhl*I gene and, as a

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consequence, there would be a reduction in N-acylbutyryl-homoserine lactone (C4-HSL) production, which binds to the RhlR regulator to execute the transcriptional activation of the genes involved in rhamnolipid production (Bazire et al., 2005).

Since a higher production yield of rhamnolipid (g/L) was obtained using the lowest glycerol concentration (50 g/L), the yield with *P. aeruginosa* LBI was compared to the yield of its mutant: *P. aeruginosa* LBI 2A1. According to Lovaglio (2011), *P. aeruginosa* LBI 2A1 has a lower capacity to utilize hydrophilic carbon sources than *P. aeruginosa* LBI, thus, the difference in biomass production. Nonetheless, the mutant microorganism exhibited a greater rhamnolipid production, as described in other studies (Lovaglio, 2011). This could be related to the antibiotic selection pressure to which *P. aeruginosa* LBI 2A1 was subjected.

P. aeruginosa LBI 2A1 possibly developed a metabolic strategy related to enhanced rhamnolipid production. According to Pearson et al. (1999), the efflux pumps and the diffusion mechanisms are involved in N-(3-Oxododecanoyl)-L-homoserine lactone (3OC12-HSL), an auto inducer molecule that activates the cascade related to rhamnolipid synthesis. A mutation in the mechanism of efflux to resist the antibiotic could have influenced the rhamnolipid production of the mutant, since the process of antibiotic resistance is strongly related to virulence factor production via the Crc global regulator (Linares et al., 2010; Lovaglio, 2011). Also, it should be considered that *P. aeruginosa* LBI 2A1 may have mutations in genes not related to antibiotic resistance; instead, the mutations could be in genes responsible for repressor proteins of the rhamnolipid synthesis cascade.

The surface-active properties of rhamnolipids depend on the homologue composition, and this proportion depends on the carbon source used for the production, as well as the strain, the time, and culture conditions (Ji et al., 2016; Rikalovic et al., 2013). This variation on the composition of the homologues influences the physicochemical properties of rhamnolipids (Bharali and Konwar, 2011; Lovaglio et al., 2014). Nitschke et al. (2010) reported 10 rhamnolipid homologues produced by *Pseudomonas aeruginosa* LBI using soybean oil waste. Lovaglio et al. (2014), identified nine rhamnolipid congeners produced by *Pseudomonas aeruginosa* LBI 2A1 using sunflower oil, castor oil and corn soapstock.

P. aeruginosa LBI 2A1 grown on crude glycerol produced a mixture of rhamnolipids containing 6 homologues including mono and di-rhamnolipids, similar findings have been reported in the literature (Liu et al., 2014; Haba et al., 2014; Monteiro et al., 2007; Perfumo et al., 2006; Varjani and Upasani, 2016). Mono-rhamnolipid Rha-C_{10}-C_{10} is the precursor of Rha_2-C_{10}-C_{10}, according to the generally accepted pathway for rhamnolipids synthesis suggesting that for every di-rhamnolipid detected its mono-rhamnolipid congener should be detected (Déziel et al., 1999), in this study in deed were.

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Furthermore, Lovaglio et al. (2014) observed that the rhamnolipid profile was similar when produced using three different carbon sources, as hydrophobic carbon sources. The metabolism of the lipid portion probably occurred through ß-oxidation, the limiting factor might have been rhamnose production for constituting the hydrophilic moiety of rhamnolipids (Lovaglio et al., 2015). As the carbon source used in this study was hydrophilic, it could be that the limiting factor for rhamnolipid production might be the hydrophobic portion through the *de novo* synthesis pathway. Subsequently, the conversion of glycerol might be happening using the Entner-Doudoroff route, for dTDP-L-rhamnose precursor synthesis (Lovaglio et al., 2015).

The effective use of crude glycerol as the raw substrate with an overproducer strain can reduce rhamnolipid production costs. Here we confirmed that *Pseudomonas aeruginosa* LBI 2A1 is suitable for the optimization of rhamnolipid production using crude glycerol, and that the homologues produced by this strain with different carbon sources might go through different metabolic routes that could be investigated for optimization of the upstream bioprocess.

Declarations

Author contribution statement

Ana Maria Salazar-Bryam: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Roberta Barros Lovaglio: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jonas Contiero: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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