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Evaluation of a co-product of biodiesel production as carbon source in the production of biosurfactant by *P. aeruginosa* MSIC02

Juliana Rabelo de Sousa[a], Jessyca Aline da Costa Correia[a], José Gustavo Lima de Almeida[b], Sueli Rodrigues[c], Otília Deusdênia Loiola Pessoa[b], Vânia Maria M. Melo[d], Luciana Rocha Barros Gonçalves[a,*]

[a] Department of Chemical Engineering, Federal University of Ceara, Fortaleza Brazil
[b] Department of Organic and Inorganic Chemistry, Federal University of Ceara, Fortaleza Brazil
[c] Department of Food Technology, Federal University of Ceara, Fortaleza, Brazil
[d] Department of Biology, Federal University of Ceara, Fortaleza, Brazil

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ABSTRACT

This work focuses on the use of a co-product of biodiesel production as carbon and energy source for the production of rhamnolipids by a new strain of *Pseudomonas aeruginosa* MSIC02. Different carbon sources were compared and the highest rhamnolipid concentration (1269.79 mg/L) was achieved when hydrolyzed glycerin was used. The study of nutritional and environmental conditions allowed an increase in the production of rhamnolipids. Maximum rhamnolipid concentration was attained at 18 g/L of glycerol, 4.0 g/L of $NaNO_3$, 62 mM of KH_2PO_4, pH 7.0 and 37 °C. Highest product yields on substrate and biomass and productivity were achieved at the same conditions. The biosurfactant showed good emulsifying properties (IE_{24} = 65%), being able to form emulsions with mineral and vegetable oils. The NMR spectrum of H^1 and C^{13} and mass spectra indicates that the purified product contained two types of rhamnolipids: L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL1) and L-rhamnosyl L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL2).

1. Introduction

The crescent interest worldwide in the use of fuels from renewable sources, for instance, sugarcane, lignocellulosic residues, oleaginous and animal fat, among others, has increased the demand for biofuels, such as ethanol and biodiesel. The use of those biofuels can reduce emissions of carbon monoxide, compared with fuels derived from oil [1].

Biodiesel is produced, primarily, by the transesterification of triglycerides with methanol or ethanol. After separation, two phases are obtained: an oil phase, consisting of alkyl esters (methyl or ethyl esters), and a phase rich in glycerin, consisting of glycerol, soap, alcohol and hydroxides. Glycerin is the main co-product from biodiesel production by transesterification and it is obtained in the raw form, which has a low commercial value due to the presence of several impurities [2]. However, this co-product may be used as a source of raw material for the production of high added value through chemical or biochemical conversion, such as polymers and additives for fuels, esters and ethers of glycerin [3]. Furthermore, the National Center for Agricultural Utilization Research has shown great interest in the use of glycerol as a substrate for the production of 3-hydroxypropionaldehyde (3-HPA), 3-hydroxypropionic acid, 1,3-propanediol, rhamnolipids (biosurfactants), among other products [4].

Surfactants are an important class of chemicals used in several sectors of industry. These compounds are currently available in the market but most of them are derived from petroleum, which gives them a non biodegradable character. Meanwhile, the growing increase in world production of these derivatives and the necessity of obtaining products compatible with the environment have given emphasis to the production of biological surfactants as an alternative to the existing products [5].

Biosurfactants constitute a class of surfactants that are produced from living organisms and can be found inside microbial cells or may be excreted extracellularly. Although presenting several advantages over the chemical surfactants, microbial surfactants are not yet widely used due to high production costs, associated with inefficient methods of recovery of the product and the use of expensive substrates. The problem of economic production of biosurfactants can be significantly reduced through the use of alternative sources of nutrients, readily available, low cost and enabling high concentrations of biosurfactant [6]. In addi-

* Corresponding author. Tel.: +55 85 33669611; fax: +55 85 33669610.
E-mail address: lrg@ufc.br (L.R.B. Gonçalves).

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tion, the optimization of culture conditions allows the increase in production levels. Data presented in the literature show that the production of rhamnolipids by bacteria of the genus *Pseudomonas* is strongly influenced by carbon and nitrogen sources, concentration of phosphorus, multivalent cations, carbon/nitrogen (C/N) and carbon/phosphorus (C/P) ratios [7–9]. The carbon sources used are very diverse, from simple sources, such as glucose [10] and glycerol [11], to more complex substrates, as lipids [12], alkanes [13] and esters of fatty acids [4], showing that *Pseudomonas* strains have a versatile metabolism.

Rhamnolipids produced by *Pseudomonas* strains are glycolipids formed by one or two molecules of rhamnose and a fatty acid chain. They are the most important classes of biosurfactants [14] and have applications in various industries and in bioremediation [15].

Therefore, the aim of this work was to analyze biosurfactant production by *Pseudomonas aeruginosa* MSIC02, and other related parameters, in a low cost medium, formulated using glycerin, a co-product of biodiesel production by transesterification.

2. Methods

2.1. Microorganism

P. aeruginosa strain MSIC02 was isolated from a crude oil contaminated soil. Its rRNA 16S sequence is deposited in the Genbank with the following access number: FJ876297. The strain was maintained on nutrient agar (beef extract 3 g/L, peptone 5 g/L and agar 15 g/L) slants at 4 °C and transferred monthly.

2.2. Raw material

Glycerin used in this work is the co-product of biodiesel production, resulting from the transesterification of castor bean oil by methanol in alkaline medium (NaOH) and was provided by Empresa Brasileira de Bioenergia Ind. Com Ltd. (EBB, Ceará, Brazil).

Crude glycerin was obtained after methanol removing by evaporation. Since heating at alkaline pH would lead to polymerization, due to the presence of NaOH used as catalyst in transesterification reaction (free alkalinity), the sample was previously neutralized (pH 7.0) with analytical grade H_2SO_4. After neutralization, it was kept under heating at 110 °C for 1 h under agitation to the evaporation of methanol.

Hydrolyzed glycerin was prepared by acid hydrolysis of crude glycerin, conducted at room temperature with concentrated H_2SO_4. The volume of H_2SO_4 needed was determined by the total alkalinity of glycerin (Eq. (1)). Then, water was added to the system in the ratio of 1:3 with respect to the mass of glycerin. The hydrolysis was conducted in a separation funnel for 24 h to allow phase settling to occur (glycerin and fatty acid). The resulting hydrolyzed glycerin was used as carbon source in batch experiments.

$$V_{H_2SO_4} = \frac{AT \times m}{N} \quad (1)$$

where $V_{H_2SO_4}$ is the volume of H_2SO_4 (L), AT is the total alkalinity, m is the mass of glycerin (mg) and N is the normality of H_2SO_4.

2.3. Characterization of raw material

Samples of crude and treated glycerin, by acid hydrolysis, were characterized for the presence of macro and micro elements. The micro elements were evaluated by atomic absorption in a Perkin Elmer Analyst 300 equipment [16]. Sulphur and phosphorus were analyzed by spectrophotometry using an UV-visible spectrometer at 600 nm [16]. All assays were conducted in triplicate. The total content of carbon was determined by high-temperature catalytic oxidation (HTCO) using a Shimadzu analyzer (TOC-V CPH/CPN).

2.4. Inoculum propagation

The strain of *P. aeruginosa* MSIC02 was transferred from a stock culture to a petri plate containing the same medium and incubated at 35.5 °C for 24 h. After this period, three loops of culture were transferred to 50 mL of nutrient broth (beef extract, 3 g/L, peptone 5 g/L) in a 250 mL Erlenmeyer flask. This was kept under agitation of 150 rpm at 30 °C for 18 h in a rotary shaker [17]. Then the optical density at 600 nm was determined by a spectrophotometer (Spectronic® 20 GENESYS) and adjusted, if necessary, to 1.25.

2.5. Fermentation media

Biosurfactant production was conducted using a mineral medium composed of (in g/L): KH_2PO_4 3.0, K_2HPO_4 7.0, $MgSO_4{\cdot}7H_2O$ 0.2, supplemented with different carbon (crude glycerin, hydrolyzed glycerin, soybean oil or castor oil) at 5% (w/v) and nitrogen ($NaNO_3$, $(NH_4)_2SO_4$ or peptone) sources at 2.4 g/L.

2.6. Culture conditions

Batch fermentations were conducted in 250 mL Erlenmeyer flasks containing 50 mL of medium. The culture flasks were incubated in rotary shaker at 150 rpm and 30 °C for 96 h. After this period, cells were separated by centrifugation at 10,000 × *g* for 15 min and the supernatant was used for analytical determinations. The experiments were performed in duplicate.

2.7. Optimization of media and cultivation conditions

The study of nutritional and environmental conditions on the production of biosurfactant was conducted in accordance with a 2^k full factorial design. The effects of phosphate and nitrogen concentrations, pH and temperature were evaluated through a 2^4 full factorial design with a triplicate in central point. In these experiments, the initial concentration of glycerol and inoculum were 18 g/L and 2% (v/v), respectively. The concentration of glycerol was kept constant in order to eliminate the effect of osmotic stress that it exerts on microorganisms in submerged fermentation process [1,8]. The levels were established in accordance with the literature [11,18]. Cell growth, biosurfactant concentration and surface tension reduction were the response variables. The pH of the medium was adjusted with a solution of KOH 3.0 M. Data analysis was performed by using Statistica 6.0 software.

2.8. Biosurfactant isolation and purification

Biosurfactants were extracted from the culture media after cell removal by centrifugation at 10 000 × *g* for 15 min. The pH of the medium was adjusted to 2.0 with H_2SO_4 6 N. Then, the recovery of the crude biosurfactant was performed by a liquid–liquid extraction, using a mixture of $CHCl_3$ and CH_3OH (2:1 v/v), according to Patel and Desai [19], then it was collected for further purification by column chromatography, according to Syldatk et al. [8], using silica gel 60 (70–230 mesh, VETEC). The purified biosurfactant was analyzed by thin layer chromatography (TLC), which was performed on precoated silica gel polyester sheets (Kieselgel 60 F_{254}, 0.20 mm, Merck) and developed by spraying a solution of vanillin/perchloric acid/EtOH followed by heating at 100 °C.

2.9. Analytical methods

2.9.1. Cell concentration

Cell growth was determined by measuring the optical density of samples, using a UV-visible spectrophotometer (20 Genesis, BR) at 600 nm. Cell concentration, in g/L, was determined by calibration curve of dry weight (g/L) versus optical density (600 nm).

2.9.2. Rhamnose concentration

Rhamnose concentration was determined by the orcinol–sulphuric method, described by Pham et al. [20]. The orcinol solution, 0.19% (w/v), was prepared by dissolving the reagent in a solution of sulphuric acid at 53% (v/v). Afterwards, a 0.1 mL-sample was added to 0.9 mL of this solution, followed by heating at 80 °C for 30 min. After 15 min of rest at room temperature, absorbance was determined at 421 nm using a spectrophotometer (Spectronic® 20 Genesys). Calibration curve was obtained by using standard solutions of rhamnose, whose concentrations ranged between 5 and 50 mg/L.

2.9.3. Glycerol concentration

Glycerol concentration was determined by High Efficiency Liquid Chromatography (HPLC) using a Waters high-performance-liquid chromatography equipped with a refractive index detector and a Shodex Sugar SC1011 column (8.0 mm × 300 mm). Ultra pure water (MiliQ) was used as mobile phase under the following conditions: flow rate of 0.6 mL/min at 80 °C and the volume of injection was 5 μL [17].

2.9.4. Raw material total alkalinity

Total alkalinity was determined by the following procedure. A sample of the raw material (Glycerin), around 2.0 g, was titrated with HCl 0.1 N using bromophenol blue as indicator. The total alkalinity was determined according to Eq. (2).

$$AT = \frac{(V_2 - V_b) \times N}{m} \quad (2)$$

where AT is the total alkalinity; m is the mass (g) of raw material; N is the normality of HCl; V_2 is the volume of HCl spent during the titration; V_b is the volume of HCl spent in blank

2.9.5. Emulsification index (E_{24})

Emulsification index was determined according to Cooper and Goldenberg [21], with slight modifications: 2 mL of cell free supernatant was added to 2 mL of

kerosene and the mixture was vortexed for 2 min. After 24 h, the height of emulsion layer was measured. The emulsifying activity (E_{24}) was calculated using Eq. (3):

$$E_{24}\ (\%) = \frac{H_{EL}}{H_S} \times 100 \quad (3)$$

where H_{EL} is the height of the emulsion layer and H_S is the height of total solution.

2.9.6. Surface tension

Surface tension, an indirect measure of the production of biosurfactants, was determined in the fermentation broth free of cells using a tensiometer (Krüss K6) at 25 °C, according to the De Nöuy ring method [22].

2.9.7. Emulsification activity

Emulsification activity was determined in accordance with the methodology described by Cirigliano and Carman [23], with slight modifications [24]: samples were filtered through a Millipore 0.45 μm membrane and the filtrate (1 mL) was placed in glass tubes (15 mm by 125 mm) and diluted with 1 mL of sodium acetate 0.1 M buffer (pH 3.6). Then, 0.5 mL of kerosene was added to the tube, which was vortexed. The resulting emulsion remained at rest for 10 min, and then the aqueous phase absorbance was measured in spectrophotometer (Spectronic® 20 Genesys) at 540 nm. A unit of emulsifying activity was defined as the amount of biosurfactant that changed the extinction of the aqueous phase 540 nm (A_{540}) by 1.0 under the conditions described above.

2.9.8. NMR and mass spectrometry analysis

Electrospray ionization-high resolution mass spectra were run on a quadrupole LCMS-IT-TOF (Shimadzu) spectrometer equipped with electrospray ionization source. ^{1}H (500 MHz), ^{13}C (125 MHz) and 2D NMR experiments were performed on a Bruker Avance DRX-500 spectrometer equipped with a 5 mm inverse detection *z*-gradient probe. ^{1}H and ^{13}C NMR spectra were measured at 27 °C using CD_3OD and $CDCl_3$ as solvent.

3. Results and discussion

3.1. Characterization of raw material

Glycerin, the main co-product of the biodiesel industry [2], has a very heterogeneous composition, being constituted of components from the original grease, as esters, triglycerides, fatty acids and soaps, and alcohol (ethanol or methanol) and hydroxides. The type of oleaginous, the transesterification process and the efficiency of the separation stage influence both composition and the proportion of glycerin components [25]. Therefore, in order to assess the presence of micro and macro nutrients in raw material used as a substrate, the physicochemical composition of crude and hydrolyzed glycerin was determined and the parameters evaluated are shown in Table 1.

The composition of crude and hydrolyzed glycerin differed in a few elements. The levels of iron, calcium and magnesium remained similar after acid hydrolysis. The sulphur content in hydrolyzed glycerin increased due to the large formation of sodium sulphate, as a result from the reaction between fatty acid salts (soap) and H_2SO_4. The concentrations of copper and manganese were below the detection limit of the equipment for all samples evaluated. The content of phosphorus was reduced from 12.7 ± 1.2 ppm to below the detection limit after acid treatment. The total carbon content was higher for crude glycerin when compared to hydrolyzed glycerin, probably, due to the presence of more complex carbon sources, such as methyl esters, fatty acids and esters of glycerol [1]. The characterization of the raw material provided important data that helped in the formulation of the fermentation medium.

Table 1
Physicochemical composition of crude and hydrolyzed glycerin, resulting from the transesterification of castor bean oil, utilized as substrate to biosurfactant production by *P. aeruginosa* MSIC02.

Elements	Crude glycerin	Hydrolyzed glycerin
Iron (ppm)	5.539 ± 0.127	5.163 ± 0.15
Zinc (ppm)	12.308 ± 0.60	23.628 ± 0.55
Copper (ppm)	ND	ND
Manganese (ppm)	ND	ND
Calcium (ppm)	6.148 ± 0.39	5.286 ± 0.48
Magnesium (ppm)	4.511 ± 0.067	5.894 ± 0.36
Sulphur (ppm)	ND	569.3 ± 25.5
Phosphoros (ppm)	12.7 ± 1.2	ND
Carbon total (mg/L)	306,800.0	288,350.0

ND: not detected by the analytical method used.

From the results pictured in Table 1, it can be observed that the crude and hydrolyzed glycerin contain important nutrients for cell growth and production of glycolipids, such as iron, zinc, calcium, magnesium and sulphur [8,13]. According to data from the literature [11,26], the amount of these micronutrients varies with the strain, however, the concentration follows, generally, an order of magnitude of ppm. It should be pointed out that media supplementation with phosphorus may be necessary, since the presence of this nutrient was not detected (Table 1) in crude nor hydrolyzed glycerin.

3.2. Effect of nutritional and environmental conditions on biosurfactant production by P. aeruginosa MSIC02

3.2.1. Effect of the carbon source

The use low cost carbon sources, such as vegetable oils and glycerol, are an interesting alternative to produce rhamnolipids by fermentation [11]. Therefore, the production of biosurfactants by *P. aeruginosa* MSIC02 using different carbon sources (crude and hydrolyzed glycerin, soybean oil and castor oil) was evaluated and the results of rhamnolipid concentration, surface activity and cell growth after 96 h of cultivation at 30 °C and 150 rpm are presented in Table 2. The crude and hydrolyzed glycerin differ on the content of glycerol, 60% and 95%, respectively (data not shown) and presence or absence of methyl esters, fatty acids and esters of glycerol [1]. It can be observed in Table 2 that the strain was able to use all carbon sources evaluated to grow and produce biosurfactants. The amount of rhaminolipid produced varied from almost 355 mg/L to more than 1200 mg/L, depending on the carbon source used. This result, when compared to other authors' [11,12,27,28], shows the potential of the *P. aeruginosa* MSIC02 in producing rhamnolipids. The most suitable substrate was hydrolyzed glycerin, allowing the production of 1269.79 mg/L of rhamnolipids, which is two to three times greater than those obtained for other sources. When crude glycerin, soybean oil and castor oil were supplied 354.63, 611.25 and 559.17 mg/L of rhamnolipids were produced, respectively. Our results are in agreement with the obtained by other authors: 690 mg/L [11], 620 mg/L [28], 1400–1500 mg/L [27].

The lower production of rhamnolipidis when using the crude glycerin as a substrate may be associated with the inhibitory effect of high salt concentrations derived from the transesterification

Table 2
Effect of carbon source and nitrogen source on rhamnolipid concentration, surface activity, cell growth and yield after 96 h of cultivation at 30 °C and 150 rpm.

Raw material	Nitrogen source	Final surface tension (mN/m)	IE_{24} (%)	Rhamnose (mg/L)	Biomass (g/L)	Ypx (g/g)
Soybean oil	$NaNO_3$	30.1 ± 0.1	62.2 ± 1.8	611.25 ± 19.2	0.531 ± 0.01	1.150
Castor oil	$NaNO_3$	32.0 ± 0.0	63.7 ± 0.9	559.17 ± 29.5	0.678 ± 0.06	0.824
Crude glycerin	$NaNO_3$	30.0 ± 0.0	68.8 ± 0.0	354.63 ± 12.9	0.623 ± 0.0	0.568
Hydrolyzed glycerin	$NaNO_3$	29.3 ± 0.1	63.0 ± 1.4	1269.79 ± 15.2	0.836 ± 0.02	1.519
Hydrolyzed glycerin	$(NH_4)_2SO_4$	29.1 ± 0.1	59.01 ± 1.2	898.26 ± 29.0	1.164 ± 0.04	0.771
Hydrolyzed glycerin	Peptone	30.1 ± 0.2	59.36 ± 0.5	503.13 ± 11.3	0.574 ± 0.0	0.876

process and pretreatment. According to the literature [29], the impurities in the raw material may affect microbial growth and production of metabolites. The same authors describe that crude glycerin, derived from biodiesel production, contains impurities, such as potassium and sodium salts [4–5% (wt/wt)]. This high salt concentration is not present in the hydrolyzed glycerin used in this work, since precipitation is observed during the stage of acid hydrolysis of the crude glycerin. Those facts may explain the obtained results.

In addition, different low cost substrates were investigated for biosurfactant production and the results are described in the literature [27,30,31]. Costa et al. [30] studied the use of alternative low-cost carbon substrates, such as glycerol, cassava wastewater (CW), waste cooking oil and CW with waste cooking oils, for the production of polyhydroxyalkanoates (PHAs) and rhamnolipids by different strains of *P. aeruginosa*. According to the authors, best rhamnolipid production (600 mg/L) was obtained with CW with cooking oil as the carbon source, which is much lower than the result achieved in this work when hydrolyzed glycerin was used as substrate (1269.79 mg/L). The same authors evaluated the use of glycerol as carbon source and rhamnolipid concentration varied between 175.3 and 264.2 mg/L. The production of rhamnolipids by *P. aeruginosa* LBI strain from oil wastes, as alternative low-cost substrates, was also evaluated [31]. The authors observed that soybean soapstock waste was the best substrate, generating 1170 mg/L of rhamnolipids, which is also lower than the concentration achieved in the present work. Additionally, the strain *P. aeruginosa* J4, isolated from the wastewater of a petrochemical plant, was able to produce up to 2000 mg/L rhamnolipids using sunflower oil as substrate [27]. Diesel and kerosene were also evaluated as a substrate, and the strain was able to produce 1300 mg/L and 710 mg/L, respectively.

As a result of the experiments, it was observed that the glycerol, the predominant carbon source that is present in hydrolyzed glycerin, showed higher potential for rhamnolipid production compared to other sources investigated, whose compositions have more complex carbon sources, such as triglycerides, fatty acids and esters of fatty acids. This result was similar to that achieved by Marsudi et al. [32] who assessed the simultaneous production of rhamnolipids and polyhydroxyalkanoates (PHA) by *P. aeruginosa* IFO3924 using palm oil as the sole carbon source. They observed that the strain secreted lipase, which hydrolyzed the oil into fatty acids and glycerol. Fatty acids became favorable carbon sources for cell growth and PHA production via β-oxidation and glycerol for rhamnolipid production via de novo fatty acid synthesis.

Although biosurfactant production was affected by the carbon source, emulsification index (above 60%) was similar for all assays, except the one achieved with crude glycerin that was a little higher (68%). All rhamnolipids displayed a good emulsification activity for kerosene, which clearly demonstrates its potential in a variety of commercial applications, such as bioremediation [27]. Furthermore, the surface tension of the cell-free fermented broth was reduced to 29–30 mN/m, except for the medium formulated with Castor oil (32 mN/m). According to the literature [12], the reduction of surface tension to those levels indicates the production of surface active compounds such as rhamnolipids.

Since the highest rhamnolipid production (1269.79 mg/L) with the best tensoative characteristics (decrease in surface tension to 29.3 mN/m and 63.0% of emulsification activity) was achieved with hydrolyzed glycerin, this carbon source was selected for further studies.

3.2.2. Effect of the nitrogen source

Medium constituents other than carbon sources also affect the production of biosurfactants. Among the inorganic salts evaluated in the literature, such as $(NH_4)_2SO_4$, NH_4NO_3 and $NaNO_3$ [7,11,33] nitrate supported maximum surfactant production by *P. aeruginosa* 44T [34]. Nevertheless, since the strain used in this work was not studied before for biosurfactant production, three nitrogen sources were evaluate. Table 2 shows that sodium nitrate ($Y_{P/X}$ = 1.5 g/g) is more effective than peptone ($Y_{P/X}$ = 0.9 g/g) and ammonium sulphate ($Y_{P/X}$ = 0.8 g/g). Table 2 also shows the results of cell growth, rhamnolipid concentration, emulsification index and surface tension for the different nitrogen sources evaluated. Although low surface tension of the culture broth was achieved for all nitrogen sources investigated, best biosurfactant production was noted in the medium containing nitrate as the nitrogen source. In this medium, rhamnolipid concentration at the end of essay was 1.4 and 2.5-fold higher when compared to the results achieved in the medium containing peptone and ammonium, respectively. Cell growth, on the other hand, was 1.4 and 2.0-fold higher when ammonium was used in the medium instead of nitrate and peptone, respectively.

The preference of nitrate as a nitrogen source for the production of rhamnolipids by bacterias of the genus *Pseudomonas* is quite studied in the literature [13,26,33]. Santa Anna et al. [11] attributed this preference to the ability of some microorganisms to undergo dissimilatory nitrate reduction to ammonium and then assimilation, which would simulate a condition of limiting nitrogen, encouraging rhamnolipids production.

Based on these results, $NaNO_3$ was used as a nitrogen source to study the effect of nutritional and environmental conditions on the production of rhamnolipids by *P. aeruginosa* MSIC02 according to a 2^4 full factorial design.

3.3. Effect of nutritional and environmental conditions on cell growth, rhamnolipid production and efficiency

Among the factors that, potentially, have influence on the biosynthesis of rhamnolipids by bacterias of the genus *Pseudomonas* are the carbon source, the effect of limiting nutrients, the C/N and C/P ratios [6,11]. The effect of limiting nutrients for the production of biosurfactants has been studied because of their importance on the microbial lipogenesis [6]. Generally, the accumulation of intracellular lipids is favored when there is some limitation of nutrients in the culture medium. Boulton and Ratledge [35] found that the limitation of nutrients (nitrogen) can stimulate the accumulation of lipids. During the biosynthesis of rhamnolipids, the formation of lipid, not sugar, is the limiting step [35]. The culture conditions and the availability of nutrients in the medium also influence the type and quantity of surfactant produced [8,12].

In this context, four factors that could influence biosurfactant production were considered: concentration of phosphate and $NaNO_3$, pH and temperature. Therefore, a 2^4 full factorial design was used to study the effect of nutritional and environmental conditions on the quantity of rhamnolipid produced, measured by the concentration of rhamnose. The experimental design and results are presented in Table 3.

A direct analysis of the results pictured at Table 3 shows that two conditions (runs 11 and 12) did not favor *P. aeruginosa* MSIC02 growth at 37 °C. Furthermore, at 30 °C, there was only a discrete cell growth (experiments 3 and 4) using the same fermentation media of runs 11 and 12. The temperature of 30 °C favored cell growth when compared to the tests conducted at 37 °C. There was an increase in the pH of the culture broth after 96 h in most of all experiments, except for those where growth was discrete or absent (experiments 3, 4, 11 and 12) and the central points, in which a small reduction in pH was observed (data not shown).

Fig. 1 shows the product yields on substrate ($Y_{P/S}$) and biomass ($Y_{P/X}$) as well as productivity (Qp) and glycerol conversion after 96 h of cultivation of *P. aeruginosa* MSIC02 at 150 rpm according to a full 2^4 factorial design. The consumption of glycerol was pro-

Table 3
Rhamnolipid production (rhamnose concentration), cell growth, surface tension and glycerol consumption after 96 h of cultivation of *P. aeruginosa* MSIC02 at 150 rpm according to a 2^4 full factorial design. Where x_1: concentration of $NaNO_3$ (g/L); x_2: concentration of KH_2PO_4 (M); x_3: pH; x_4: temperature (°C).

Run	x_1	x_2	x_3	x_4	Biomass (g/L)	Rhamnose (mg/L)	Initial surface tension (mN/m)	Final surface tension (mN/m)	Reduction of surface tension (%)
1	2.4	0.062	4.6	30.0	0.710	718.56	39.80	30.07	24.46
2	4.0	0.062	4.6	30.0	0.699	573.85	41.77	30.83	26.18
3	2.4	0.200	4.6	30.0	0.118	ND	41.00	46.00	0
4	4.0	0.200	4.6	30.0	0.132	ND	39.00	47.43	0
5	2.4	0.062	7.0	30.0	0.768	1138.22	44.00	33.00	25.00
6	4.0	0.062	7.0	30.0	0.762	1037.92	44.00	33.17	24.62
7	2.4	0.200	7.0	30.0	0.688	664.67	44.00	31.17	29.17
8	4.0	0.200	7.0	30.0	0.772	1009.98	44.00	31.07	29.39
9	2.4	0.062	4.6	37.0	0.417	128.24	44.93	34.97	22.18
10	4.0	0.062	4.6	37.0	0.483	589.82	44.00	36.87	16.21
11	2.4	0.200	4.6	37.0	0.001	ND	38.17	38.33	0
12	4.0	0.200	4.6	37.0	0.000	ND	38.00	37.63	0
13	2.4	0.062	7.0	37.0	0.676	1694.61	44.20	33.90	23.30
14	4.0	0.062	7.0	37.0	0.610	1908.18	44.00	34.00	22.73
15	2.4	0.200	7.0	37.0	0.638	698.60	41.93	31.67	24.48
16	4.0	0.200	7.0	37.0	0.580	1018.46	43.00	31.50	26.74
17	3.2	0.131	5.8	33.5	0.408	871.75	35.80	34.57	3.45
18	3.2	0.131	5.8	33.5	0.419	934.13	35.80	35.33	1.30
19	3.2	0.131	5.8	33.5	0.555	961.07	35.80	35.00	2.23

ND: not detected by the analytical method used.

portional to cell growth and production of rhamnolipids, since high values of $Y_{P/X}$ and $Y_{P/S}$ were achieved when glycerol conversion was also high. The highest values of $Y_{P/S}$ were obtained in runs 10 and 14. Productivity (Q_P) in experiment 14, however, was 3-fold higher than the obtained in experiment 10. $Y_{P/X}$ was also higher at experiment 14 compared to experiment 10 (2.5-fold). Therefore, best results of yield ($Y_{P/S}$ = 0.103 g/g and $Y_{P/X}$ = 3.129 g/g) and productivity (Q_P = 19.9 mg/L h) were achieved at the following conditions: 37 °C, pH 7.0, 62 mM of KH_2PO_4 and 4 g/L of $NaNO_3$ (experiment 14). Santos et al. [18], working with *P. aeruginosa* PA1 and glycerol as carbon source, achieved $Y_{P/X}$ = 0.488 g/g and Q_P = 18.0 mg/L h.

The experimental results pictured in Table 3 were used to estimate the variables main effects and the interactions among them. Fig. 2a pictures the Pareto chart for biomass production. According to Garrido-Lopez and Tena [36], the length of the bars is proportional to the absolute value of the estimated effects. In this work, the dashed line represents 95% of the confidence interval. Effects that cross this line are significant values with respect to the response. It can be observed that pH exerted a positive effect on biomass production. Temperature and phosphate concentration, on the other hand, exerted a negative effect. Nitrate concentration, at least in the range evaluated, exerted no effect on biomass production. The negative effect of phosphate concentration was, probably, due to increased osmotic pressure of the fermentation medium due to high concentration of salts. D'Souza-Ault et al. [37] studied the effect of osmotic stress by the addition of salts during cultivation of *P. aeruginosa* PAO1 and observed the reduction of specific rate of growth with increasing concentrations of NaCl.

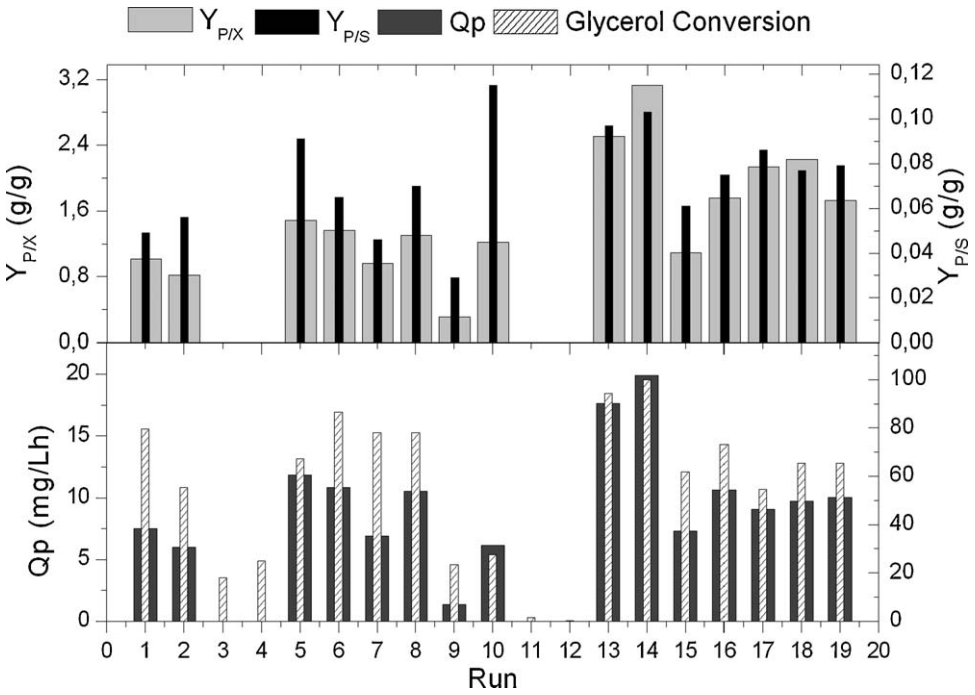

Fig. 1. Product yields on substrate ($Y_{P/S}$) and biomass ($Y_{P/X}$) and productivities (Q_P) for the experiments obtained from the factorial design 2^4 + 3 after 96 h of cultivation of *P. aeruginosa* MSIC02 to 150 rpm with glycerol as carbon source.

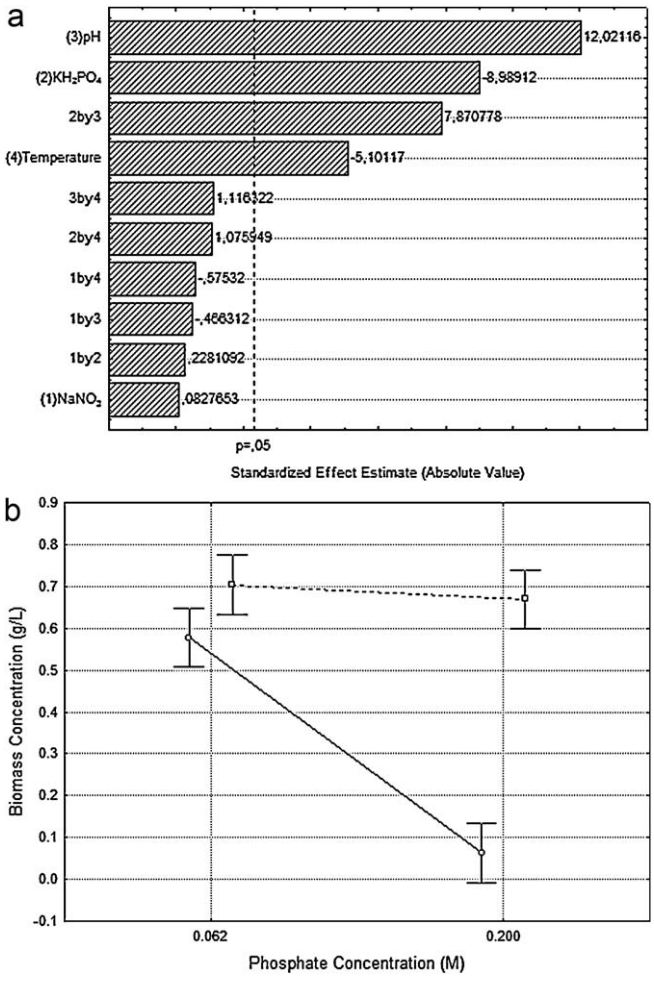

Fig. 2. Pareto charts of effects (a) and standard interaction plot (b) for biomass production by *P. aeruginosa* MSIC02. Factors: phosphate concentration and pH (pH 4.6 (○); pH 7.0 (□)).

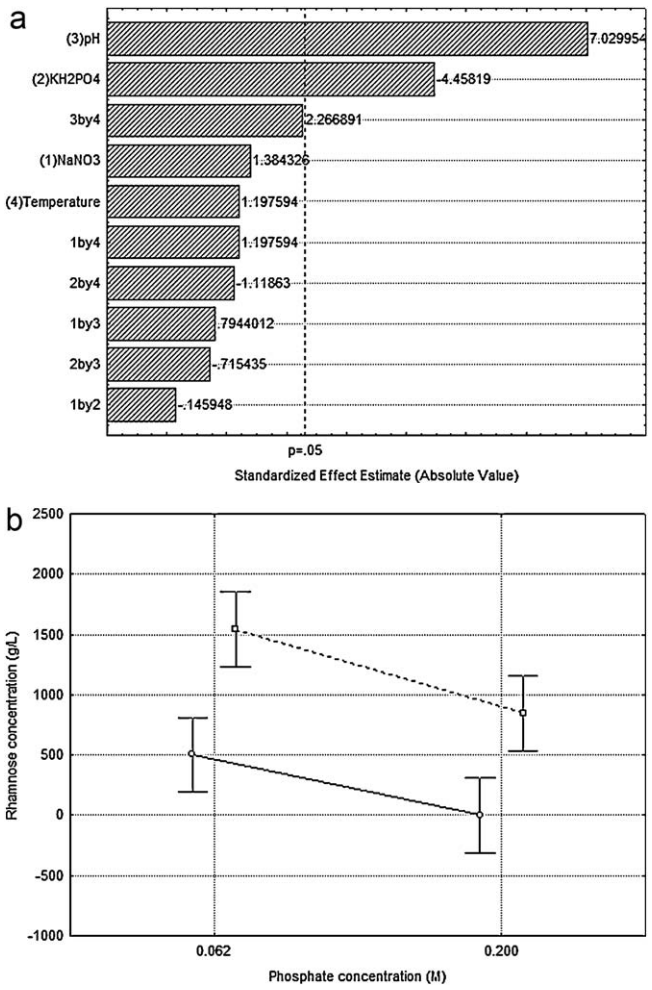

Fig. 3. Pareto chart (a) and standard interaction plot (b) for rhamnose production by *P. aeruginosa* MSIC02. Factors: phosphate concentration and pH (pH 4.6 (○); pH 7.0 (□)).

Fig. 2b presents the standard interaction plot for biomass concentration with phosphate concentration along the horizontal axis and pH levels (4.6 and 7.0) for each line, where means are indicated by points connected by lines. At pH 4.6, the effect of increasing phosphate concentration on biomass was more pronounced than that at pH 7.0. Based on this result, cell growth was not observed when 0.2 M of KH_2PO_4 and pH 4.6 were used. This is due, probably, to the effect of pH on the assimilation of phosphorus, which is an essential nutrient for growth of microorganisms.

Fig. 3a pictures the Pareto chart for rhamnolipid concentration. The factors pH and KH_2PO_4 showed significant main effects for the production of rhamnolipid. pH showed a positive effect, indicating that enhancing the pH value from 4.6 to 7.0 favored the production of biosurfactant. This result is similar to other authors [18] that achieved a higher concentration of rhamnolipid at this pH value. It can be observed that reducing phosphate concentration favored rhamnolipid production, since a negative effect is observed for phosphate concentration on the Pareto Chart pictured at Fig. 3a. Other authors [9] observed that increasing the C/P ratio, by reducing the concentration of phosphorus, favored the production of biosurfactant, obtaining a maximum accumulation of surfactant using a phosphate-limited complex medium (65 nmol/mL P_i).

Fig. 3b presents the standard interaction plot for rhamnolipid concentration with phosphate concentration along the horizontal axis and pH levels (4.6 and 7.0) for each line, where means are indicated by points connected by lines. It can be observed that, for both pH values evaluated, rhamnolipid production decreased with increasing concentrations of phosphate. Other authors [38] have observed the same results when evaluating the effect of phosphate limitation on the production of cell-to-cell signal molecules and rhamnolipid biosurfactants.

Another important indirect measure of biosurfactants production is the value of surface tension on the supernatant of a batch culture [27]. Furthermore, the ability to reduce the surface tension of a liquid is an indicative of the efficiency of a surfactant [39]. Therefore, the analysis of the effects of nutritional and environmental conditions on surface tension reduction (Table 3) was carried out by using a Pareto chart, pictured in Fig. 4. This Figure indicates that increasing pH and reducing temperature and phosphate concentration favored the reduction in surface tension (Fig. 4). The other factor (nitrate concentration) is a marginal statistically significant result for the studied response.

As illustrated in Table 3, surface tension decreased from 39.80 mN/m to a minimum value of 30.07 mN/m for experiment 1 and from 41.77 mN/m to 30.83 mN/m for experiment 2. These results showed the significant influence of the variables studied on the surface-active properties (Fig. 4) of the biosurfactant produced. Temperature and pH are reported in the literature [8] for interfering in the selective biosynthesis of rhamnolipids by *P. aeruginosa*, leading to a mixture of different proportions of these surfactant molecules, which may affect the properties of the supernatant. Sildatk et al. [8] found that the mixture of rhamnolipids synthe-

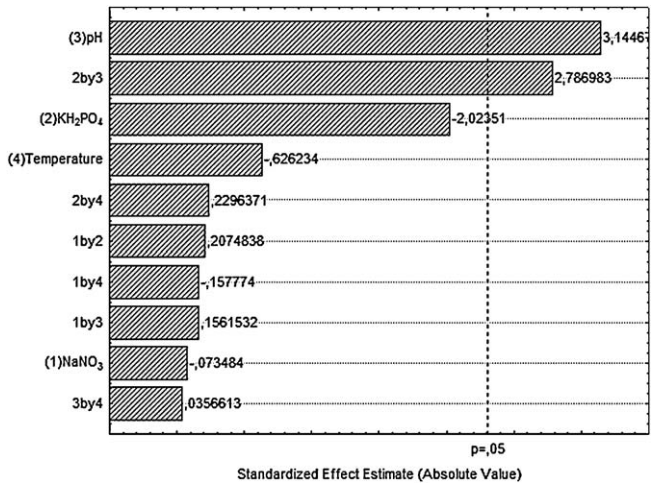

Fig. 4. Pareto chart for variable-response surface tension reduction obtained through the cultivation of *P. aeruginosa* MSIC02 to 150 rpm for 96 h according to a factorial design $2^4 + 3$.

sized by resting cells of *Pseudomonas* sp. DSM 2874, using glycerol and n-alkanes as carbon sources, was influenced by temperature. These authors observed that below 37 °C more hydrophilic rhamnolipids (R2 and R4) were synthesized. They also observed that the surface properties of the fermented broth varied according to the selective synthesis. Furthermore, the more hydrophilic molecules showed higher critical micelar concentration (CMC) and promoted a smaller reduction in surface tension of water, when compared to the more hydrophobic molecules of biosurfactant. Santos et al. [18] that studied the production of biosurfactants by *P. aeruginosa* PA1 from glycerol found that the changes in pH interfered in the synthesis of mono and di-ramnolipids. They also observed that di-rhamnolipids synthesis was favored in acid pH (5.66) while at neutral pH, the ratio between mono and di-rhamnolipids produced was approximately 1:1.

In summary, considering the results of rhamnolipid concentration, yields ($Y_{P/S}$ and $Y_{P/X}$) and productivity (Q_P), as well as the value of surface tension on the supernatant, the optimal culture conditions for biosurfactant production by *P. aeruginosa* MSIC02 were achieved when the assay was conducted at 37 °C and pH 7.0, using 18 g/L of hydrolyzed glycerin, 62 mM of KH_2PO_4 and 4 g/L $NaNO_3$.

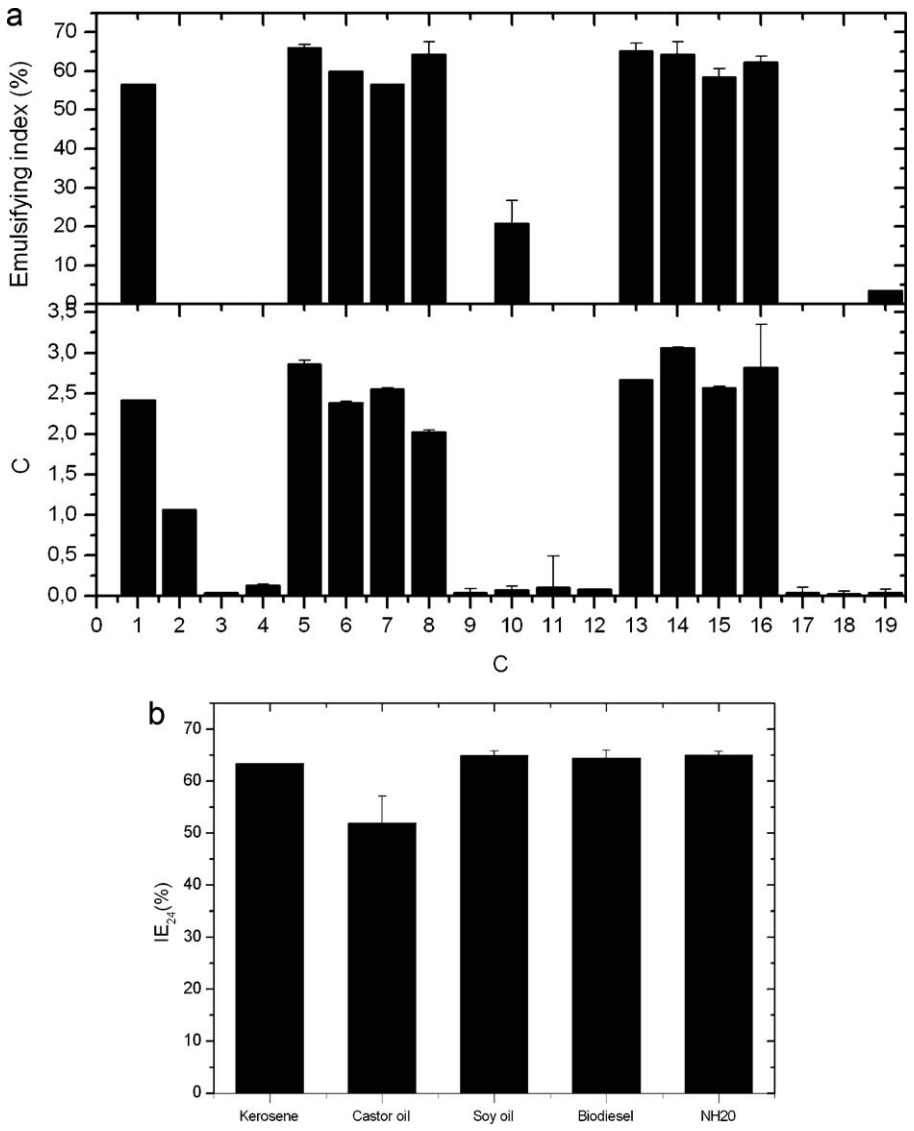

Fig. 5. Emulsifying index and emulsifying activity on kerosene of cell-free culture broth from the experiments carried out in accordance with the $2^4 + 3$ factorial design (a) and emulsifying index against different hydrophobic compounds of the rhamnolipid produced by *P. aeruginosa* MSIC02 in the media containing 4 g/L of $NaNO_3$ (g/L), 0.062 M of KH_2PO_4 at pH 7.0 and 37 °C (run 14) (b).

3.4. Evaluation of emulsifying properties of biosurfactants produced by P. aeruginosa MSIC02

Emulsifying properties of biosurfactants produced by *P. aeruginosa* MSIC02 under different nutritional and environmental conditions were determined by measuring the emulsifying activity, which determines the ability of biosurfactant in forming oil–water emulsion, and emulsifying index on kerosene, which determines the capacity of surfactant in forming emulsions, see Fig. 5.

It can be observed in Fig. 5a that higher and more stable emulsification activities, between 2 and 3 units, were detected for the biosurfactant produced in experiments 1, 5–8 and 13–16. Those results were superior to those described for the synthetic commercial surfactants tested by Amaral et al. [40]. They were also superior to those described to other biosurfactants from *Yarrowia lipolytica* [40], *C. lipolytica* IA [41] and *Nocardia* sp. L-417 [42]. An emulsification activity around 1 Unit was detected for the biosurfactant produced in experiment 2, however, no emulsification of kerosene was observed. The biosurfactant produced in experiment 10 was able to emulsify kerosene (E_{24} = 20%) without displaying emulsification activity. When cell-free culture broth from the experiments 3–4, 9–11 and 17–19 were tested, neither emulsification activity nor emulsifying index was detected.

Regarding emulsifying index, results showed a wide variation between the experiments (Fig. 5a). No measurable emulsifying index was obtained in experiments 2–4, 9, 11–12 and 17–19. For the other experimental conditions, emulsifying index ranged from 55% to 65%, except for experiment 10, which presented IE_{24} = 20.83%. Costa et al. [12] achieved an emulsification index of 70% and 65% for kerosene using, respectively, andiroba and buriti oil as carbon source for biosurfactant production by *P. aeruginosa* LBI.

A relationship between emulsifying activity and emulsifying index (Fig. 5a) can be noticed for most of the experimental conditions evaluated. Those experiments where a stable emulsion was formed, a high emulsifying activity, with a maximum of 3 units (run 14), was also achieved. That is to say that the biosurfactants produced in those experimental conditions show good emulsifying characteristics. Other authors [40] observed high values of emulsifying activity, about 2 units, after 50 h of cultivation of *Y. lipolytica* at 250 rpm. Liposan, a bioemulsifier produced by *Candida lipolytica*, presented emulsifying activity with n-Hexadecano of 0.98 U when grown in YNB medium supplemented with 5% of soybean oil [23].

The maximum emulsifying index (65%) and emulsifying activity (3 U m) were reached in the media containing 4 g/L of $NaNO_3$ (g/L), 0.062 M of KH_2PO_4 at pH 7.0 and 37 °C. The highest rhamnolipid concentration (1908.18 mg/L) was also obtained in these conditions (run 14), indicating a correlation between biosurfactant concentrations and emulsifying activity. Therefore, the surface activity of this biossurfactant to form emulsions was evaluated against different hydrophobic compounds (soybean oil, castor oil, biodiesel from castor oil and naftenic oil), see Fig. 5b. It can be observed that the biosurfactant displayed better emulsification for soybean oil, biodiesel from castor oil and naphthenic oil (from 64 to 65%) than for castor oil (52%). These results are in agreement with the literature. Ilori et al. [43] obtained up to IE_{24} = 65% by using the biosurfactant produced by the cultivation of *Aeromonas* spp. in crude oil against diesel oil. Other authors [12] observed that the biosurfactants obtained from the cultivation of *P. aeruginosa* LB1 in native oils provided a IE_{24} of 70–92% against kerosene.

3.5. Identification of rhamnolipids produced by P. aeruginosa MSIC02

According to the literature [12,15], the surfactant produced by genus *Pseudomonas* is a rhamnolipid. In order to confirm that information, the biosurfactant produced in this work was purified and analyzed by NMR and HR-ESI-MS. Purified rhamnolipid was obtained by isolation and purification in several steps, starting by the acidification of culture supernatant, free of cells, followed by a liquid–liquid extraction. The extracts obtained by liquid–liquid extraction were subjected to chromatographic separation with silica gel 60 as adsorbent. The fractions obtained from the column were analyzed by thin layer chromatography (TLC), which revealed the synthesis of two major compounds (data not shown). Compound **1** was isolated as a colorless resin, and its HR-ESI-MS spectrum revealed the sodium adduct ion at *m/z* 527.3213 $[M+Na]^+$ along with the potassium adduct ion at *m/z* 543.2889 $[M+K]^+$ indicating a molecular formula of $C_{26}H_{48}O_9$. The ^1H NMR spectrum of **1** showed signals related to a sugar moiety at δ 4.92 (1H), 3.86 (1H), 3.81 (1H), 3.72 (1H), 3.45 (1H) and 1.27 (3H) related a rhamnose moiety and, typical signals of oxymethine protons at 5.45 (1H) and 4.28 (1H). Additionally, signals for a methyl at δ 0.89 (6H) and methylene's groups at δ 2.55 and 2.42 were also observed. The ^{13}C NMR-CPD and DEPT spectra showed signals assigned to two carboxyl groups at δ 173.9 (C-6) and 171.6 (C-3), a unit of ramnose at 95.1, 73.2, 71.4, 71.3, 67.7 and 17.4. Signals for two extra oxymethine carbons at δ 71.9 and 70.6 and several methylene carbons were observed (Table 4). These findings were consistent with the structure of the L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate. Complete ^1H and ^{13}C NMR data (Table 4) were assigned through COSY, HMQC and HMBC spectra. Compound 2 was also isolated as a colorless resin. Its HR-ESI-MS spectrum afforded the sodium adduct ion at *m/z* 673.3832 $[M+Na]^+$ indicating a molecular formula of $C_{32}H_{58}O_{13}$. In accordance with the ^1H and ^{13}C NMR spectra the structural pattern of 2 was very similar to that of **1**, except for an extra rhamnosyl moiety. The ^1H NMR spectrum exhibited signal for two anomeric hydrogen at δ 4.91 (1H) and 4.88 (1H), which in the HMQC spectrum showed correlation with the carbons at δ 98.5 (C-1′) and 104.3 (C-1″), respectively. In the HMBC experiment the signal at δ 4.91 exhibited long-range correlation with the carbons at δ 74.7 (C-1) while the signal at δ 4.88 (H-1″) showed correlation with the carbon at δ 80.7 (C-2′) confirming the position of the two rhamnosyl units. Based on the above data, the structure of compound 2 was established as L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate.

Table 4
^{13}C (125 MHz) and ^1H NMR (500 MHz) spectral data for compounds 1 and 2, in CD_3Cl and CD_3OD, respectively.

Carbon	1		2	
	δ_C	δ_H	δ_C	δ_H
1′	95.10	4.92	98.49	4.91
2′	71.42	3.86	80.77	3.70
3′	67.77	3.72	70.38	3.65
4′	73.72	3.45	74.04	3.27
5′	71.34	3.81	72.12	3.96
6′	17.48	1.27	18.18	1.27
1″	–	–	104.34	4.88
2″	–	–	71.87	3.66
3″	–	–	70.27	3.62
4″	–	–	74.65	3.27
5″	–	–	72.41	3.65
6″	–	–	18.22	1.28
1	71.94	4.28	74.79	4.10
2	39.72	2.42	42.60	2.48
3	171.67	–	173.02	–
COOH	173.95	–	174.85	–
CH_3	14.30	0.89	14.57	0.95
4	70.65	5.45	73.82	5.30
5	39.19	2.55	41.39	2.55
CH_2	22.67/34.75	1.27/1.59	23.84/35.55	1.30/1.60

4. Conclusions

The production of rhamnolipids by *P. aeruginosa* MSIC02 varied depending on the raw material used, being hydrolyzed glycerin the best source of carbon and nutrients for growth and production of biosurfactants by *P. aeruginosa* MSIC02. The best productivity and yields were 19.9 mg/L h, $Y_{P/X}$ = 3.129 g/g and $Y_{P/S}$ = 0.103 g/g, respectively, achieved when the assay was conducted at 37 °C and pH 7.0, using 18 g/L of hydrolyzed glycerin, 62 mM of KH_2PO_4 and 4 g/L $NaNO_3$. The crude rhamnolipid gave high emulsifying activities against mineral and vegetable oils (IE_{24} = 65%). The NMR and mass spectra confirmed the presence of L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate. The properties of the biosurfactant in terms of its minimum surface tension, emulsification activity and the emulsion stability, revealed its potential for further environmental applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2011.06.016.

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