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Structure and Applications of a Rhamnolipid Surfactant Produced in Soybean Oil Waste

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Abstract Soybean oil soapstock was utilized as an alternative carbon source for the production of rhamnolipids by *Pseudomonas aeruginosa* LBI strain. The chemical composition and properties of the rhamnolipid mixture obtained were determined to define its potential applications. The chemical characterization of the rhamnolipid has revealed the presence of ten different homologues. The monorhamnolipid $RhaC_{10}C_{10}$ and the dirhamnolipid $Rha_2C_{10}C_{10}$ were the main components of the mixture that showed predominance of 44% and 29%, respectively, after 144-h of cultivation. The biosurfactant was able to form stable emulsions with several hydrocarbons and showed excellent emulsification for soybean oil and chicken fat (100%). The rhamnolipid removed 67% of crude oil present in sand samples and presented antimicrobial activity against *Bacillus cereus* and *Mucor miehei* at 64 μg/mL and inhibition of *Neurospora crassa*, *Staphylococcus aureus*, and *Micrococcus luteus* at 256 μg/mL. The results demonstrated that the rhamnolipid produced in soybean oil soapstock can be useful in environmental and food industry applications.

Keywords Biosurfactants · Rhamnolipids · Soybean oil waste · Soapstock · *Pseudomonas aeruginosa*

Introduction

Biosurfactants are defined as a class of surface-active molecules synthesized by microorganisms. Some advantages of biosurfactants over synthetic surfactants such as

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mild production condition, lower toxicity, higher biodegradability, and environmental compatibility have prompted their applications in environmental protection as well as in food, cosmetic, and pharmaceutical industries [1–3]. However, the production of biosurfactants can be 50 times more expensive than the production of chemically derived surfactants [4]; thus, the economics is the main factor against their widespread use.

It is well known that the disposal of wastes is a growing problem, and new alternatives for their use should be studied as the treatment and disposal costs for these wastes are a vast financial burden to various industries [1]. The use of alternative low-cost substrates is an important strategy to facilitate industrial development of biosurfactant production. To this end, good components seem to be agroindustrial by-products or wastes, once these residues generally contain high levels of carbohydrates or lipids to support growth and surfactant synthesis [5]. Olive oil mill effluent [6], oil refinery wastes [7], waste fry oil [8], distillery and whey wastes [9], potato process effluent [10], and cassava wastewater [11] are some examples on the application industrial wastes as feedstock for biosurfactant production.

The world production of vegetable oils was about 134 million tons in 2008 and soybean oil production represented about 37 million tons. Brazil produced 6 million tons of soybean oil in 2008 and is considered one of the largest soybean and soybean oil producer (USDA annual report). Great quantities of waste are generated by the oil and fat industries: residual oils, tallow, soapstock, and frying oils. The production of soapstock, one of the wastes of the oil neutralization process, corresponds to 2–3% of the total oil production [12].

The rhamnolipids from *Pseudomonas aeruginosa*, which are among the most effective biosurfactants, are essentially constituted by a mixture of the homologues species $Rha_2C_{10}C_{10}$ and $RhaC_{10}C_{10}$ [13]. The surfactant properties of rhamnolipids depend on its composition and distribution of homologues that vary according to the bacterial strain, culture conditions, and medium composition [14]. *P. aeruginosa* LBI strain was isolated from a soil contaminated with hydrocarbons [15]. In previous work, we reported the production of rhamnolipid surfactant by this strain using different edible oil refinery wastes as carbon sources and soybean soapstock was the most promising substrate [16]. However, to improve commercial exploitation of the process, it is important to know the properties and potential applications of the product. The present work investigates the structure and applications of the rhamnolipid surfactant obtained when soybean oil soapstock is used as an alternative substrate.

Materials and Methods

Microorganism and Culture Conditions *P. aeruginosa* LBI bacterial suspension, obtained from a nutrient agar slant incubated for 24 h at 30 °C, was adjusted to DO_{610} 0.65 (approx. 10^8 cfu/mL), and 1 mL of this culture was inoculated on a 250-mL Erlenmeyer flask containing 50 mL of mineral salts medium [17] and 2% (*w*/*v*) of soybean oil as a carbon source. The inoculum was incubated for 24 h, 30 °C, 200 rpm on a rotary shaker (New Brunswick, USA), and an aliquot of 1 mL was added to the production medium. Biosurfactant production was performed on 125-mL flasks containing 25 mL of salts medium added of 2% (*w*/*v* total lipid) of soybean oil soapstock and incubated as described above for 144 h. Initial pH of broth was adjusted to 6.8–6.9. Experiments were conducted in three independent replicates. Error bars (when shown) represent standard deviation.

Rhamnolipid Quantification Rhamnolipids were quantified from the cell-free broth as rhamnose [18]. Rhamnolipid content was determined by multiplying rhamnose values by 3 [19].

Biomass Estimation Cell growth was estimated by the protein content of the culture using Lowry method [20].

Nitrate Concentration Nitrate concentration was estimated using the spectrophotometric UV method [21].

Surface Activity Measurement Culture samples were centrifuged at 8,000 *g* for 20 min to cell removal, and the supernatant was submitted to surface activity measurements. Surface tension was determined with a Krüss Tensiometer (Krüss, Germany) using the ring method. The critical micelle dilution (CMD^{-2}) was measured as the surface tension of 100 times diluted broth in distilled water.

Rhamnolipid Extraction Cells were removed from the culture broth by centrifugation at 10,000×*g* for 20 min. The biosurfactant was isolated from cell-free broth by acid precipitation after adjusting the broth pH to 2.0 using 6 N H_2SO_4 and keeping it at 4 °C overnight. The precipitate thus obtained was pelleted at 8,000×*g* for 20 min, redissolved in distilled water, adjusted to pH 6.1, and applied to an adsorption chromatography column filled with polystyrene resin, Amberlite XAD2 (Supelco, USA) previously equilibrated with 0.1 M potassium phosphate buffer (pH 6.1). The adsorption of the active compound was assayed by measuring the surface tension at the column outlet. Adsorption chromatography was terminated when the surface tension dropped below 35 mN/m [22]. The column was then washed with distilled water until a surface tension around 72 mN/m was attained. Rhamnolipids were eluted with methanol, and the solvent was evaporated to dryness under vacuum.

Structural Characterization of Rhamnolipid The analyses were performed with a triple quadrupole mass spectrometer Quattro II (Micromass, UK) equipped with a Z-spray interface using electrospray ionization in negative mode. The instrument was interfaced to a HP 1100 HPLC (Agilent Technologies, USA) equipped with a 150×4 mm Zorbax C_8 reverse phase column [23].

Emulsifying Activity Hydrocarbon source (6 mL) was added to 4 mL of surfactant solution (0.1% *w*/*v*) and vortexed at high speed for 2 min. After 24 h, the emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100.

Crude Oil Removal A biosurfactant solution (0.1% *w*/*v*) was utilized to wash sand samples contaminated with crude oil. The sand was added of 10% (*w*/*w*) of low viscosity crude oil and maintained at room temperature for 3 days. Then, 5 g of sand (50–80 mesh) samples were washed with rhamnolipid solution for 18 h at 200 rpm 30 °C. Aqueous solution was removed, and the sand was dried at 50 °C for 24 h. The sand sample was washed two times with dichloromethane (Tedia, USA), and the solvent was evaporated at 50 °C. The remaining oil was determined gravimetrically, and the percentage of oil removal was calculated using the following equation: $\text{Crudeoilremoved}\,(\%) = (\text{Oi} - \text{Or})/\text{Oi} \times 100\%$, where Oi is the initial oil in the soil (grams) before washing, and Or is the oil remaining in the soil (grams) after washing [24].

Antimicrobial Activity Antimicrobial activity of biosurfactant against bacteria, yeast, and fungi was determined by minimal inhibitory concentration (MIC) using the microbroth dilution method [25]. The bacterial strains were grown on Muller Hinton agar at 37 °C and the yeasts and fungi on Sabouraud Dextrose agar at 30 °C.

Results and Discussion

The biosurfactant was obtained after growth of *P. aeruginosa* LBI using soybean oil soapstock as the carbon source. The kinetics of rhamnolipid production using the alternative substrate is shown in Fig. 1. The nitrate was consumed during first 24 h of cultivation generating a biomass of 1.5 g/L. The bulk of rhamnolipid synthesis occurred after cell reached the stationary phase as a typical secondary metabolite. After 144 h, the rhamnolipid concentration was 11.7 g/L, and the conversion yield of carbon substrate to biosurfactant was around 75%. Similar results were obtained by Benincasa et al. [15] using sunflower oil soapstock as the carbon source. Bednarski et al. [7] also showed that the production of glycolipid surfactants from *Candida antarctica* and *Candida apicola* was increased from 7.3 to 13.4 g/L when soapstock was supplemented to culture medium.

The chemical characterization of the rhamnolipid revealed the presence of ten different homologues: $RhaC_{10}C_8$, $RhaC_{10}C_{10}$, $RhaC_{10}C_{12}$, $RhaC_{12}C_{12}$, $Rha_2C_{10}C_8$, $Rha_2C_{10}C_{10}$, $RhaC_8C_{10:1}$, $RhaC_{10}C_{10:1}$, $Rha_2C_{10}C_{10:1}$, and $Rha_2C_{10}C_{12:1}$. The product extracted from different cultivation times was analyzed, and the predominance of the homologues $RhaC_{10}C_{10}$ and $Rha_2C_{10}C_{10}$ as the main components of the mixture was found (Table 1). The concentration of both homologues varied during time; however, the monorhamnolipid

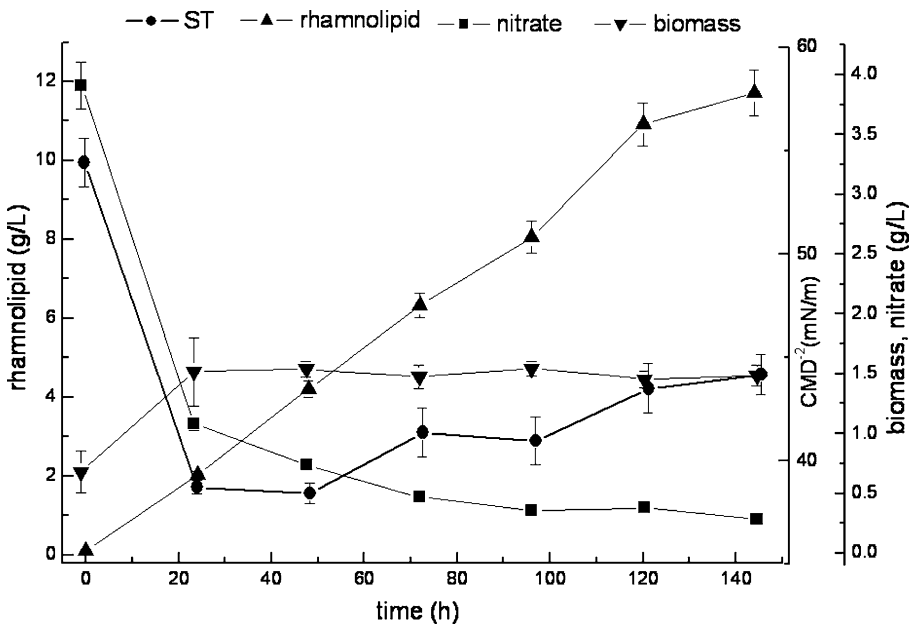

Fig. 1 Time course of rhamnolipid production by *P. aeruginosa* LBI using soybean oil soapstock. *Error bars* represent standard deviation

Table 1 Distribution of homologues (percent) present on rhamnolipid mixture produced in soybean oil soapstock at different cultivation times by *P. aeruginosa* LBI.

Rhamnolipid homologue	Cultivation time (h)					
	24	48	72	96	120	144
Concentration (%)						
$RhaC_{10}C_8$	1.56	1.9	2.06	4.44	4.25	4.41
$RhaC_{10}C_{10}$	42.18	59.02	66.86	38.44	40.85	43.97
$RhaC_{10}C_{12}$	ND	ND	ND	4.88	3.61	3.8
$RhaC_{12}C_{12}$	9.37	9.54	3.76	2.66	ND	ND
$Rha_2C_{10}C_8$	1.56	1.56	1.21	4.44	5.53	5.01
$Rha_2C_{10}C_{10}$	45.31	24.3	23.05	42.66	42.76	29.55
$RhaC_8C_{10:1}$	ND	0.52	0.36	ND	ND	ND
$RhaC_{10}C_{10:1}$	ND	0.69	0.72	ND	ND	ND
$Rha_2C_{10}C_{10:1}$	ND	ND	0.12	0.66	1.06	0.73
$Rha_2C_{10}C_{12:1}$	ND	1.56	1.82	1.77	1.91	1.76

ND not detected

($RhaC_{10}C_{10}$) was the major component of the final product extracted after 144 h of cultivation. The accepted pathway for rhamnolipid synthesis postulates that the monorhamnolipids are the precursors of the dirhamnolipids once the inactivation of *rhl* genes required for the synthesis of $RhaC_{10}C_{10}$ abolish rhamnolipid production [26]. Then, the changes in rhamnolipid distribution may account for the stages of their biosynthesis.

The level of rhamnolipid production by *P. aeruginosa* UG2 was affected by culture conditions but the distribution of homologues did not vary between cultures [27]. The rhamnolipid mixture obtained by these authors using corn oil as substrate had 60% of the dirhamnolipid ($Rha_2C_{10}C_{10}$) form. In our case, the monorhamnolipid showed an average predominance of 48.5% in contrast with 34.5% for the dirhamnolipid form. These results confirm the previous observations that the monorhamnolipid form predominates in culture when *P. aeruginosa* LBI was grown in soybean oil soapstock [16]. Nevertheless, Benincasa et al. [28] showed that after 84 h of cultivation in sunflower oil soapstock, this strain produced a rhamnolipid mixture where the dirhamnolipids isoforms predominate. These differences can be attributed to the carbon source composition, culture conditions as well as to the loss of some components during the purification steps. A recent report suggested that the amount of unsaturated fatty acids in the carbon source reflects in the unsaturation of rhamnolipid carbon chains. The authors reported that the amount of unsaturated fatty acids in substrate was 50%, and the unsaturated carbon chains in rhamnolipid mixtures were 30.9% [12], whereas in our work the unsaturated fatty acids concentration in soybean oil soapstock was 77% but the proportion of unsaturated rhamnolipid molecules was very low (Table 1). Figure 2 shows the high-performance liquid chromatography of the rhamnolipid extracted after 144 h of cultivation. The main components of the mixture showed retention times of 10.05 and 10.49 min corresponding to $RhaC_{10}C_{10}$ and $Rha_2C_{10}C_{10}$, respectively. The mass spectrum shows the presence of the pseudomolecular ions of *m*/*z* 503 and 649, which correspond to the $[M–H]^-$ forms of $RhaC_{10}C_{10}$ and $Rha_2C_{10}C_{10}$. As can be seen, the relative intensity also demonstrates the predominance of the monorhamnolipid homologue (Fig. 3). The identity of the pseudomolecular ions present in rhamnolipid mixtures were confirmed by their fragmentation profiles.

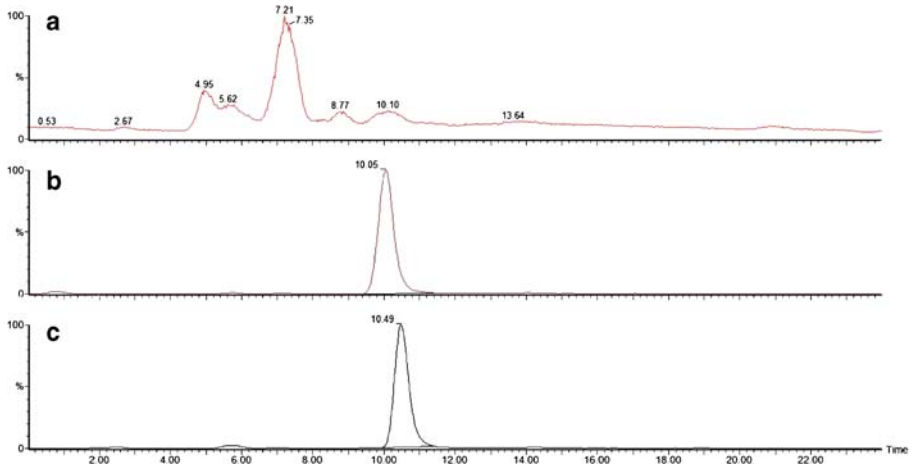

Fig. 2 High-performance liquid chromatography of rhamnolipid isolated after 144 h of cultivation. **a** Peak separation profile, **b** $RhaC_{10}C_{10}$ peak, **c** $Rha_2C_{10}C_{10}$ peak

The properties of the product obtained after 144 h of cultivation using soybean oil soapstock as carbon source were investigated. The surface tension of 0.1% (*w*/*v*) rhamnolipid solution was 26.9 mN/m, an interfacial tension against hexadecane 1.25 mN/m and a CMC of 51.5 mg/L [16]. The properties of rhamnolipids are dependent on bacterial strain, medium composition, and culture conditions that determine the composition and distribution of homologue molecules present on final product [14]. Values of CMC ranging from 10 to 234 mg/L and surface tension from 25 to 31 mN/m have been reported to different rhamnolipid mixtures [1]. However, as in this case, the mono- and dirhamnolipid were also the major molecules present in biosurfactant mixtures differing primarily on their predominance and concentration. To illustrate, the commercial rhamnolipid JBR 599 (Jeneil

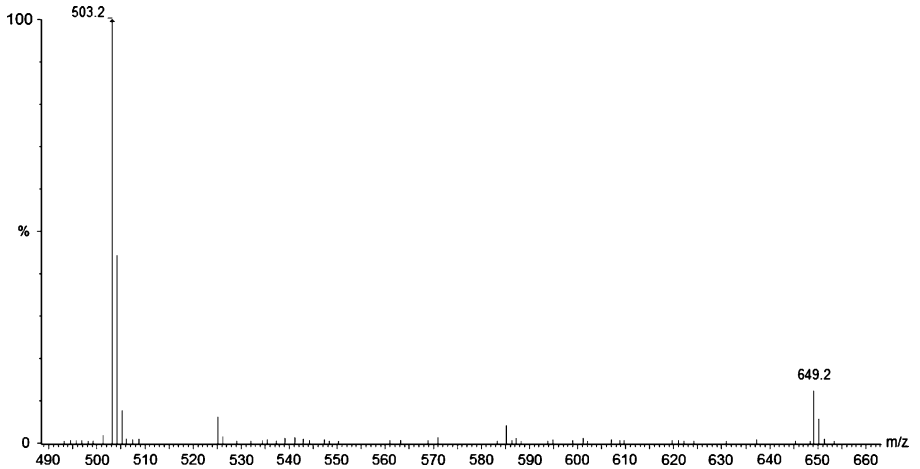

Fig. 3 Mass spectrometry (ESI-MS) showing the predominance of *m*/*z* 503 ($RhaC_{10}C_{10}$) and *m*/*z* 649 ($Rha_2C_{10}C_{10}$) ions

Biosurfactant Co., USA) has a purity of 99.9% and is composed of 51% of monorhamnolipids (37.7% $RhaC_{10}C_{10}$) and 44.9% of dirhamnolipids (33.2% $Rha_2C_{10}C_{10}$). This product shows a CMC from 100 to 125 mg/L, a surface tension of 29.0 mN/m, and an interfacial tension of 0.3 mN/m against paraffin oil.

The potential applications of the biosurfactant obtained in soybean soapstock were also investigated. The ability to form and stabilize emulsions is one of the most important features to be considered for the practical application of a surfactant. The emulsifying activity of rhamnolipid against different hydrophobic substrates is shown in Fig. 4. The biosurfactant was able to form emulsion with all hydrocarbons tested, which were stable up to 30 days. The rhamnolipid product was able to emulsify efficiently the aromatic hydrocarbons toluene and benzene as well as hexane, heptane, and kerosene showing potential to environmental applications such as bioremediation of pollutants and oil clean up. The excellent levels of emulsification observed for soybean oil (100%), chicken fat (100%), and mineral oil (71%) suggest that it can be useful for food industry. The results observed also suggest that the rhamnolipid mixture obtained from soybean oil soapstock forms emulsions more efficiently with long-chain fatty acids and triglycerides that predominate in oil and fats.

The capacity of aqueous rhamnolipid solution in removing crude oil from contaminated sand was also investigated. The washing with rhamnolipid was able to remove 67% of crude oil present in sand samples showing the potential of the product in oils spill accidents. Urum and Pekdemir [29] showed that a 0.1% of commercial rhamnolipid solution removed about 75% of crude oil from soil samples.

Antimicrobial activity of the rhamnolipid revealed activity against *Bacillus cereus* (64 μg/mL) and *Mucor miehei* (64 μg/mL) and some inhibition of *Neurospora crassa*, *Staphylococcus aureus*, and *Micrococcus luteus* at 256 μg/mL (Table 2). A rhamnolipid mixture obtained from *P. aeruginosa* AT10 growing on soybean waste fatty acids as carbon

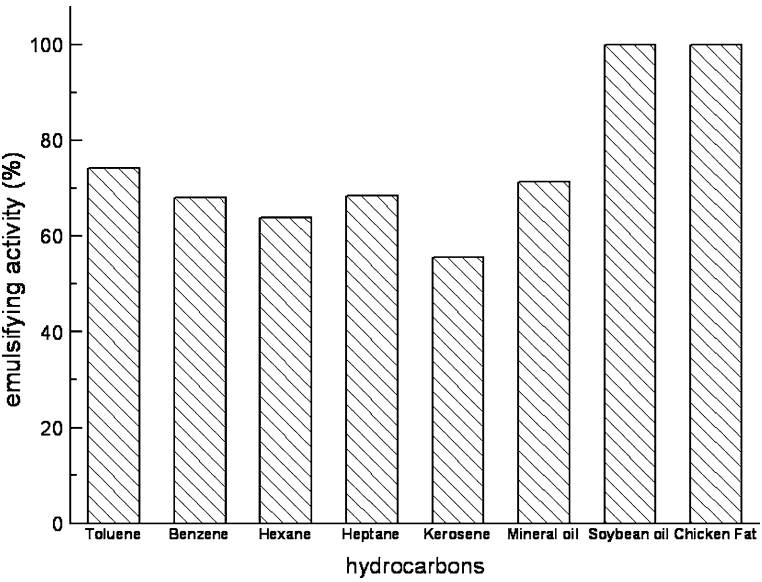

Fig. 4 Emulsifying activity of the rhamnolipid obtained in soybean oil soapstock

Table 2 Antimicrobial activity of the rhamnolipid produced in soybean oil soapstock.

Microorganisms	MIC (μg/mL)
Escherichia coli ATCC 11229	>500
Bacillus cereus ATCC 10876	64
Micrococcus luteus ATCC 4698	256
Pseudomonas aeruginosa ATCC 15442	>500
Rhodococcus equi ATCC 6939	>500
Salmonella cholerasius ATCC 10708	>500
Serratia marcescens ATCC 1953	>500
Staphylococcus aureus ATCC 25923	256
Candida glabrata ATCC 15126	>500
Kluyveromyces marxianus ATCC 16045	>500
Aspergillus niger ATCC 16404	>500
Mucor miehei ATCC 26282	64
Neurospora crassa ATCC 9277	256
Rhizopus microsporus ATCC 22959	>500

source also found a MIC of 64 μg/mL against *B. cereus*; however, MIC for *S. aureus* was 128 μg/mL and for *M. luteus* 32 μg/mL [30]. Benincasa et al. [28] reported that the rhamnolipid mixture containing 28.9% of $Rha_2C_{10}C_{10}$, 23% $Rha_2C_{10}C_{12:1}$, 23.4% $RhaC_{10}C_{10}$, 11.3% $RhaC_{10}C_{12}$, 7.9% $RhaC_{10}C_{12:1}$, and 5.5% $RhaC_{10}C_{12}$ showed a MIC of 4 μg/mL for *B. cereus*, 8 μg/mL for *S. aureus*, 128 μg/mL for *M. luteus*, and excellent levels of inhibition against fungi. The variation observed in results can be due to the differences in homologues composition of the rhamnolipid mixtures evaluated. When comparing the antimicrobial activity of two rhamnolipid mixtures obtained from different *P. aeruginosa* strains, Haba et al. [31] observed that *P. aeruginosa* AT10 rhamnolipids were most effective against fungi, and *P. aeruginosa* RL47T2 was more efficient against bacteria. The effects observed were attributed to the differences in homologue composition of both rhamnolipid mixtures. Owing to their intrinsic properties, surface-active compounds interfere with cell surfaces and disrupt microbial membranes. There is still little information available about antimicrobial action of rhamnolipid surfactants and the contribution of each homologous species on the final activity of the product.

We have demonstrated that the use of soybean oil soapstock as an alternative low-cost substrate for rhamnolipid production generates a product with potential industrial applications and, moreover, a process that can contribute to decrease the disposal of wastes in the environment.

Conclusions

The rhamnolipid produced by *P. aeruginosa* LBI using soybean oil soapstock as carbon source was characterized in terms of chemical structure. The product obtained showed the predominance of $RhaC_{10}C_{10}$ and $Rha_2C_{10}C_{10}$ homologues at a final concentration of 44% and 29%, respectively, after 144 h of cultivation. The results demonstrated that the rhamnolipid can be useful in environmental and food industry applications.

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