Oil Wastes as Unconventional Substrates for Rhamnolipid Biosurfactant Production by *Pseudomonas aeruginosa* LBI

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Oil wastes were evaluated as alternative low-cost substrates for the production of rhamnolipids by *Pseudomonas aeruginosa* LBI strain. Wastes obtained from soybean, cottonseed, babassu, palm, and corn oil refinery were tested. The soybean soapstock waste was the best substrate, generating 11.7 g/L of rhamnolipids with a surface tension of 26.9 mN/m, a critical micelle concentration of 51.5 mg/L, and a production yield of 75%. The monorhamnolipid $RhaC_{10}C_{10}$ predominates when *P. aeruginosa* LBI was cultivated on hydrophobic substrates, whereas hydrophilic carbon sources form the dirhamnolipid $Rha_2C_{10}C_{10}$ predominantly.

Introduction

The interest in biosurfactants has increased considerably in recent years, owing to their potential for industrial applications in crude oil recovery, health care, food processing, and bioremediation of contaminated sites (*1*). Biosurfactants also show advantages over chemically manufactured surfactants because of their lower toxicity, biodegradable nature, and unique surface-active properties (*2*). Currently, the main drawback for the widespread use of biosurfactants is the disadvantageous economics of their production.

A possible strategy to reduce biosurfactants production costs is the use of inexpensive substrates such as agroindustrial wastes, once these residues generally contain high levels of carbohydrates or lipids to support growth and surfactant biosynthesis (*3*). Olive oil mill effluent (*3*), oil refinery wastes (*4*), distillery and whey wastes (*5*), potato process effluent (*6*), and cassava wastewater (*7*) are some examples of the application of food industry byproducts or wastes as feedstock for biosurfactant production.

The rhamnolipids from *Pseudomonas aeruginosa*, which are among the most effective biosurfactants, can be obtained from low-cost hydrophobic substrates, such as vegetable oils and wastes from the food industry (*8*). Rhamnolipids comprise a mixture of homologous species RL1 ($Rha_2C_{10}C_{10}$), RL2 ($RhaC_{10}C_{10}$), RL3 (Rha_2C_{10}), and RL4 ($RhaC_{10}$) (*9*). The biosurfactant properties of rhamnolipids depend on their composition and distribution of homologues that vary according to the bacterial strain, culture conditions, and medium composition (*10*).

Pseudomonas aeruginosa LBI has been isolated from a soil contaminated with hydrocarbons (*11*). Recently, the surfactant produced by this strain using sunflower oil soapstock was characterized as a new rhamnolipid mixture (RL_{LBI}) with unique surface-active and biological properties (*12*). Little is known, however, about how the biosynthesis of rhamnolipids by *P. aeruginosa* LBI is affected by the use of different hydrophilic and hydrophobic carbon sources. The present work investigates the production of rhamnolipid surfactants using conventional sugar substrates and alternative oil waste substrates. Surface-active properties and preliminary structural characterization of rhamnolipids obtained from wastes were also evaluated.

Materials and Methods

Microrganism and Culture Conditions. *Pseudomonas aeruginosa* LBI bacterial suspension, obtained from a nutrient agar slant incubated for 24 h at 30°C, was adjusted to DO_{610} 0.65 (ca. 10^8 cfu/mL), and 1 mL of this culture was inoculated in a 50 mL Erlenmeyer flask containing 50 mL of mineral salts medium (*13*) 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 in 125 mL flasks, containing 50 mL of salts medium with 2% (w/v) of each carbon source to be tested added 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.

Oily Wastes. Residues from soybean, corn, babassu, cottonseed, and palm oil refinery were added as carbon sources at approximately 2% (w/v) of total lipids.

Rhamnolipid Quantification. Rhamnolipids were quantified from the cell-free broth as rhamnose (*14*). Rhamnolipid content was determined by multiplying rhamnose values by 3 (*15*).

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Biomass Estimation. Cell growth was estimated by the protein content of the culture using the Lowry method (*16*).

Surface Activity Measurement. Culture samples were centrifuged at 8000*g* for 20 min for cell removal, and the supernatant was submitted to surface activity measurements. Surface (ST) and interfacial tension (IT) were determined with a Krüss Tensiometer (Krüss, Germany) using the ring method. Interfacial tension was performed against hexadecane. The critical micelle concentration (CMC) was determined by measuring the surface tension of serial dilutions of surfactant solution (0.1% w/v). 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 10000*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 8000*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 (*17*). 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.

Mass Spectrometry. Electrospray ionization mass spectra were recorded on a Q-Tof (Micromass, UK) mass spectrometer. The ESI mass spectrum in the negative ion mode was acquired using a cone voltage of ±35 V and desolvatation gas (nitrogen) temperature of 100 °C. ESI tandem mass spectra were acquired by mass-selecting the target ion using the quadrupole mass analyzer followed by 25 eV collision induced dissociation using nitrogen in the collision cell. The rhamnolipid material was dissolved in methanol/water (1:1 v/v), filtered (0.22 μm), and infused into the source at 15 μL/min with a syringe pump.

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

Oily Waste Characterization. Fatty acids composition of oil wastes was determined after methanolysis of sodium methoxide with a NaOH 0.5 N solution by standard techniques (*18*). The monomers were analyzed by gas chromatography using an Agilent 6850 Capillary Chromatograph (Agilent, USA). The GC operation conditions as described by Benincasa et al. (*11*).

Results and Discussion

First, we assayed for comparison different classes of substrates including two conventional (glucose and glycerol) and three unconventional substrates (used soybean oil, soybean oil soapstock, and chicken fat) for biosurfactant production by *P. aeruginosa* LBI. As Table 1 shows, although the amount of final biomass was similar for the five substrates tested, soybean soapstock waste is found to be the best substrate for rhamnolipid synthesis, showing also that final rhamnolipid concentration varies according to the carbon source utilized (Table 1).

Table 1. Biosurfactant Production by *P. aeruginosa* LBI Growing on Different Carbon Sources and Predominant Rhamnolipid as Determined by ESI(−)-MS Analysis

carbon source	ST[a] (mN/m)	biomass (mg/mL)	rhamnolipid (g/L)	predominant rhamnolipid homologue[b]
glucose	35.76	1.48	4.20	$Rha_2C_{10}C_{10}$
glycerol	34.80	1.73	8.05	$Rha_2C_{10}C_{10}$
used soybean oil	30.80	1.75	7.63	$RhaC_{10}C_{10}$
chicken fat	32.76	1.39	6.80	$RhaC_{10}C_{10}$
soybean oil soapstock	32.36	1.48	11.72	$RhaC_{10}C_{10}$

[a] Final surface tension of the broth. [b] For representative spectra see Figure 1.

Mata-Sandoval et al. (*19*) working with *P. aeruginosa* UG2 strain found that hydrophobic substrates generate greater amounts of rhamnolipids than hydrophilic carbon sources, whereas in our study, glycerol showed higher a rhamnolipid concentration than chicken fat and used soybean oil. This result suggests that glycerol is a potential low-cost substrate; moreover, its water solubility is advantageous because the analytical problems related to the presence of oil in culture broth can be eliminated.

Mass spectrometry using electrospray ionization in the negative ion mode (ESI-MS) has been used as a straightforward method to characterize rhamnolipids and their distributions of homologues (*20*, *21*). As Table 1 shows, the predominant rhamnolipid present in the biosurfactant mixture varies according to the carbon source used. That is, when *P. aeruginosa* LBI grows on hydrophilic substrates such as glycerol and glucose, the dirhamnolipid $Rha_2C_{10}C_{10}$ (RL1, *m/z* 649) predominates, whereas for the hydrophobic carbon sources the monorhamnolipid $RhaC_{10}C_{10}$ (RL2, *m/z* 503) is the predominant homologue present in the rhamnolipid mixture (Figure 1).

The ESI-MS/MS of the deprotonated molecules of both RL1 and RL2 (not shown) confirm their identity and the presence in the biosurfactant mixture, generating fragments of *m/z* 479, 311, and 169 for the dirhamnolipid (*m/z* 609) and fragments of *m/z* 333, 169, and 163 for the monorhamnolipid (*m/z* 503) as previously described in similar works (*12, 21*). Our results differ therefore from those of Mata-Sandoval et al. (*19*), who found that *P. aeruginosa* UG2 produce surfactant mixtures with similar rhamnolipid compositions regardless the carbon source. However, Deziel et al. (*20*) found that the rhamnolipids produced by *P. aeruginosa* 57RP differ both qualitatively and quantitatively depending on the carbon source. For mannitol they found that most rhamnolipids contains two fatty acid moieties, whereas for naphthalene about 80% of rhamnolipids contained just one fatty acid moiety. Syldatk et al. (*22*) found not only the carbon source but also the temperature and fermentation strategy affected the distribution of rhamnolipid homologues produced by *Pseudomonas* sp. DSM2874.

Rahman et al. (*23*) reported the production of rhamnolipid surfactants by the two oil-degrading *P. aeruginosa* strains DS10-129 and GS9-119 using soybean oil, safflower oil, and glycerol as substrates. Soybean oil increased the biomass and rhamnolipid production to severalfold that obtained with safflower oil and glycerol. The authors also observed that the dirhamnolipid $Rha_2C_{10}C_{10}$ (*m/z* 649) was the predominant homologue

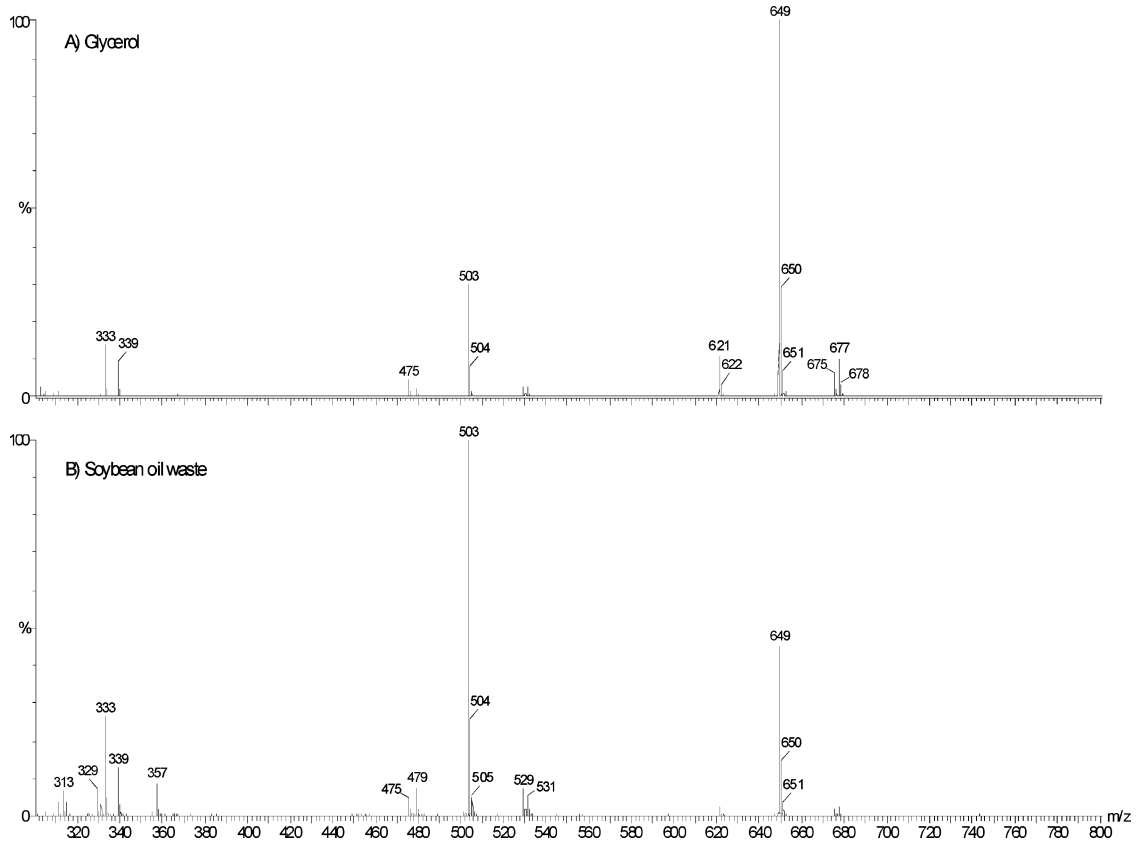

Figure 1. ESI mass spectra in the negative ion mode for the biosurfactant mixtures produced from (A) glycerol and (B) soybean oil soapstock. Note in A the predominance of RL1 (*m/z* 649), whereas RL2 (*m/z* 503) predominates in B.

synthesized, in contrast to the observations we made with *P. aeruginosa* LBI, where the monorhamnolipid was the predominant ion produced in oil substrates and the dirhamnolipid was the predominant in glycerol.

The oil refinery waste substrates evaluated herein are residues (soapstock and post-refinery fatty acids) produced in large quantities by the vegetable oil processing industry. These wastes represent 2−3% of the total oil processed and contain a considerable amount of oil that can be utilized as economic carbon sources for surfactant production.

On the basis of the results outlined in Table 1, which indicate that soybean oil soapstock is an attractive carbon source for biosurfactant production, we tested other oil refinery wastes for comparison. *Pseudomonas aeruginosa* LBI is able to grow and to produce surfactants using the oil waste substrates, but the fatty acids are consumed at different rates (Table 2). Linolenic acid is exhausted in soybean, but only 19% is utilized when cottonseed waste is used as the substrate. The decrease in total lipid content after incubation reached 77.5% for soybean, 57.5% for palm, 48.6% for corn, 47.4% for babassu and 8.5% for cottonseed waste. Note, however, that cottonseed generated 10.4 g/L of rhamnolipids (Table 3), likely owing to the use of glycerol (resulting from the hydrolysis of triglycerides) for surfactant synthesis, instead of the fatty acids. The variations observed in fatty acids consumption by *P. aeruginosa* LBI could be related to differences in triglycerides composition and the specificity of the bacterial lipase.

The surfactant from soybean soapstock shows the lowest surface and interfacial tensions (Table 3), whereas the product from cottonseed and babassu demonstrate poor surface-active properties. Biosurfactant produced from palm oil waste shows a good emulsification index against kerosene suggesting its potential use for bioremediation. Note that the final concentration of rhamnolipids is higher for the oil wastes as compared to the other types of substrates (Table 1). The variations observed in surface-active properties of biosurfactants obtained from the oil wastes are probably due to differences in individual homologue concentrations once the main molecules RL1 and RL2 are present in all samples and the monorhamnolipid is always the predominant molecule. Another possibility to explain the differences observed is the presence of impurities such as nonmetabolized co-

Table 2. Utilization of Fatty Acids from Oil Refinery Wastes by *P. aeruginosa* LBI after 144 h of Incubation

	% consumed				
fatty acid	cottonseed	babassu	corn	soybean	palm
C12 lauric		43.0			57.40
C14 miristic	47.22	57.66			70.0
C16 palmitic	10.64	57.44	68.44	75.97	66.47
C18 stearic	9.26	27.36	52.59	42.87	42.93
C18:1 oleic	7.71	46.09	47.98	67.25	60.23
C18:2 linoleic	15.55	36.20	42.34	82.20	52.47
C18:3 linolenic	19.15		55.22	100	74.20

Table 3. Surface-Active Properties of Rhamnolipids Obtained from Oil Refinery Wastes

	oil waste				
parameters	cottonseed	babassu	corn	soybean	palm
ST[a] (mN/m)	33.86	30.08	30.96	26.92	31.76
CMC (mg/L)	86.79	210.77	43.21	51.56	40.19
IT[a] (mN/m)	13.35	16.15	2.40	1.25	4.20
E_{24}(%)	10.1	20.0	20.0	55.7	71.4
RL (g/L)	10.55	8.65	13.46	11.72	8.62

[a] 0.1% aqueous solution.

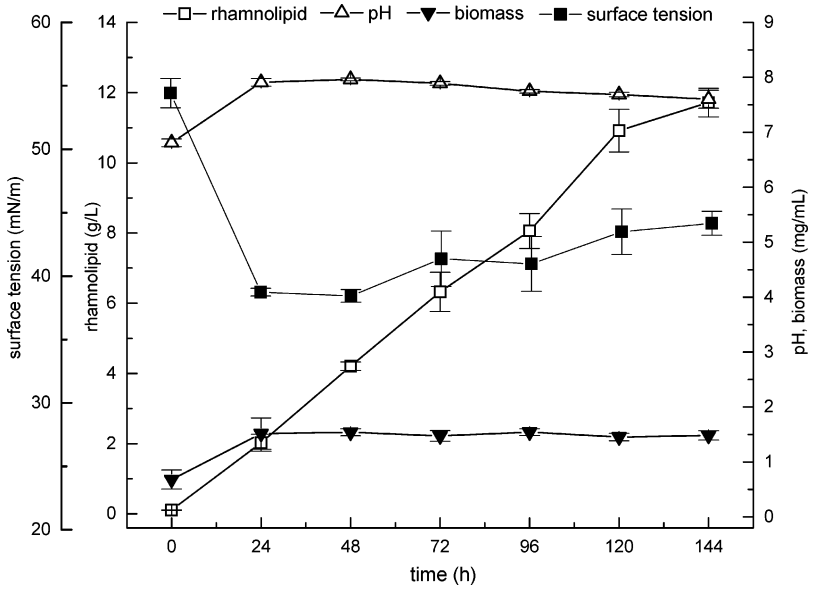

Figure 2. Time-course of rhamnolipid production by *P. aeruginosa* LBI using soybean oil waste. Surface tension is expressed as critical micelle dilution (CMD^{-2}).

extracted fatty acids from the culture broth that could influence the surface properties.

Preliminary structural characterization by ESI-MS reveals that the main rhamnolipids present when *P. aeruginosa* LBI is grown on the oil refinery wastes are RL1 and RL2, but the monorhamnolipid $RhaC_{10}C_{10}$ (detected as the deprotonated molecule of *m/z* 503) is the predominant in all waste samples evaluated. Other homologues such as $Rha_2C_{10}C_{12}$ (*m/z* 677), $RhaC_{10}C_{12:1}$ (*m/z* 529), $Rha_2C_{10}C_8$ (*m/z* 621), $RhaC_{10}C_8$ (*m/z* 475), and Rha_2C_{10} (*m/z* 479) are present as minor components (Figure 1). As Figure 2 shows, *P. aeruginosa* LBI biomass increases during the first 24 h and is subsequently maintained constant until the end of cultivation. The bulk of surfactant accumulation occurs during stationary growth as a typical secondary metabolite. Based on consumption of the carbon source, the production yield of rhamnolipid from soybean waste is around 75%.

It is known that the properties of rhamnolipids depend on the distribution of their homologues, but little is known about the contribution of each individual homologue in the surface properties of rhamnolipid mixtures. The dirhamnolipid $Rha_2C_{10}C_{10}$ shows lower CMC values (5 mg/L) than the $RhaC_{10}C_{10}$, which showed a CMC of 40 mg/L (*9*). The more hydrophilic rhamnolipids $RhaC_{10}$ and Rha_2C_{10} showed CMC values of 200 mg/L (*24*).

As we show here, the nature of the carbon source influenced the distribution of the rhamnolipid homologues synthesized by *P. aeruginosa* LBI suggesting that such synthesis can be directed to form preferentially a desirable rhamnolipid homologue by simply selecting the right substrate. This can be a useful tool when the influence of each homologue on rhamnolipid surfactant properties is better understood.

Soybean oil waste is found as the best substrate for surfactant production by *P. aeruginosa* LBI exhibiting therefore the greatest potential as an alternative economically viable carbon source.

Conclusions

The strain *P. aeruginosa* LBI was able to grow and to produce rhamnolipid surfactants using oil refinery wastes as carbon sources. Soybean soapstock generates the product with the best surface-active properties when compared with the other oil refinery wastes. The nature of the carbon source influences the distribution of rhamnolipid homologues synthesized by the bacteria.

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