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Biosurfactants Production by *Pseudomonas aeruginosa* FR Using Palm Oil

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Abstract

Biosurfactants production by a strain of *Pseudomonas aeruginosa* using palm oil as a sole carbon source was investigated. The experiments were carried out in 500-mL conical flasks containing 100 mL of mineral media supplemented with palm oil as the sole carbon source. The *P. aeruginosa* FR strain was able to reduce surface tension of three tested inorganic media. Rotation velocities from 100 to 150 rpm provided free-cell fermented media with the lowest surface tension of approx 33 mN/m. Emulsification index results of even 100% were achieved when diesel was used as oil phase. Eight surface-active compounds produced by the bacterium were identified by mass spectrometry.

Index Entries: Biosurfactants production; rhamnolipids; *Pseudomonas aeruginosa*; palm oil; *Ellaus guineensis* fruit; mass spectra.

Introduction

Surfactants are a wide class of amphiphilic molecules capable of reducing surface and/or interfacial tension between gasses, liquids, and solids. Therefore, these substances have been used in a great variety of products and processes in various chemical industries *(1,2)*.

The high toxicity, low biodegradability, and effectiveness in a narrow pH and temperature range of synthetic surface-active compounds raised the interest in surfactants produced by microorganisms, also called biosurfactants. These substances present ecological acceptance and effectiveness in a wide range of pH and temperature values *(**2,3**)*. Beside the surface and interfacial activities, some of the biosurfactants presented antifungal, antiviral, and soil clean-up processes, for example, metal sorption and enhancement of oil biodegradation *(3–5)*. Most biosurfactants are a complex mixture of molecules, comprised of various chemical structures, such as fatty acids, glycolipids, peptides, polysaccharides, and proteins *(1,6)*, that is, a function of the organism, raw matter, and process condition,

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increasing the interest in its composition, characterization, and production optimization (7,8).

Aiming at the final biosurfactant cost reduction, the development of economical alternatives for its production has been investigated. Thus, the use of low-cost raw matter appears as a natural choice to generate an overall economy. In this perspective, some substrates have been used for microbial surfactants production, such as agro-industrial wastes, hydrocarbon mixtures, and vegetable oil. Brazil is one of the world's largest producers of vegetable oil, for example, soybean oil, corn oil, babacu oil, and palm oil. Palm oil is extracted from *Ellaus guineensis* fruit, easily available in our country (Brazil). This oil is widely used in Brazilian foods because of its particular taste, odor, and thermal stability. Recently, various studies have been published on biosurfactant production by various bacterial genus. However, to our knowledge no reports have been published on the biosurfactants production from palm oil by *Pseudomonas aeruginosa* bacteria. The scope of this investigation was the production and characterization of biosurfactants synthesized by a *P. aeruginosa* FR strain growing on mineral media using palm oil as the sole carbon source.

Material and Methods

Microorganism

The bacterium strain employed in this study was isolated from soil contaminated with crude oil (Galeão Beach, Rio de Janeiro, Brazil). The microorganism was identified according to Palleronni (9). The strain was maintained on nutrient agar slant medium (Difco laboratories, 0001, Detroit, MI) at 4°C. The inocula were prepared using bacterial cells transferred from the storage culture to a test tube containing 10 mL of nutrient broth. After incubation at 29 ± 1°C for 24 h, the inoculum was propagated to a 500 mL flask containing 100 mL of the mineral medium with the following composition in (g/L): KH_2PO_4, 1.4; K_2HPO_4, 0.5; $CaCl_2 \cdot 2H_2O$, 0.02; $MnSO_4 \cdot 1H_2O$, 0.03; NH_4NO_3, 2.0. The pH of the medium was adjusted to 7.0 ± 0.2 using 1 *N* NaOH, supplemented with 0.5% (v/v) of palm oil and also incubated while agitating (150 rpm) for 24 h at 29 ± 1°C.

Growth on Mineral Media

Three inorganic media were used in this study on biosurfactants production containing distilled water, in g/L: (a) MM1: 1.0, $(NH_4)_3PO_4$; (b) MM2: 3.0, inorganic commercial fertilizer N:P:K (10:10:10), Ouro Verde Company (São Paulo, Brazil); and (c) MM3: 0.5, Na_2HPO_4; 4.5, KH_2PO_4, 2.0, NH_4Cl, 0.1, $MgSO_4 \cdot 7H_2O$. pH values of the media were adjusted to 7.0 ± 0.2 using 1 *N* NaOH, these media were sterilized autoclaving at 121°C for 15 min. Palm oil was sterilized separately at 111°C for 20 min and added aseptically (0.5% v/v) to the flasks containing the inorganic media, after cooling.

The bacterial concentration was monitored from 0 to 72 h of process. At programmed time intervals, colony forming unity (CFU) determinations were executed using the *pour plate* method, employing a nutrient agar medium. Petri dishes were incubated at 29 ± 1°C for 48 h, and the CFU enumeration was presented as a mean of three independent experiments.

Aliquots of 50 mL of fermented media were transferred to a conical separation flask for the removal of the palm oil. Aqueous phase was then centrifuged at 4000*g* for 15 min for cell removal. Supernatants were submitted to surface tension and the critical micelle dilution (CMD^{-1} and CMD^{-2}) was measured in a Sigma Tensiometer model 70 (Helsinki, Finland). The CMD^{-1} and CMD^{-2} were estimated by respective surface tension measurement of 10^{-1} and 10^{-2} diluted free-cell fermented medium in distilled water. All results were presented as a mean of three independent experiments.

Rotation Velocity Effect

The effect of rotation velocity on biosurfactant production was investigated using 500-mL conical flasks containing 100 mL of the MM3. The flasks were incubated using different rotary velocities: 50, 100, 150, and 200 rpm for 72 h at 29 ± 1°C. At programmed time intervals, aliquots of 80 mL of fermented media were transferred to conical separation flask for the removal of palm oil. Aqueous phase was then centrifuged at 4000*g* for 15 min for cell removal. Supernatants were submitted to surface tension, CMD^{-1}, CMD^{-2}, and emulsification index determinations. Emulsification capacities were determined by the addition, into crap tubes, of equal volumes of free-cell fermented media and selected hydrocarbon (hexane, jet fuel, and diesel). The tubes were mixed using a vortex for 2 min, and left to rest for 24, 48, and 72 h at 29 ± 1°C. The emulsification index was determined as the percentage of the height of the emulsion column.

Extraction and Characterization of the Biosurfactants

Rhamnolipids species were extracted from fermentation media using chloroform. The extract was dried under nitrogen atmosphere at 40°C and resuspended in methanol. This solution was submitted to high-performance liquid chromatography (HPLC) coupled to a quadrupole mass spectrometer in negative mode (Apploid 200 Varian San Francisco, CA). The chromatographic system was equipped with an automatic injector and a 250 × 4.6 mm^2 monocron C8 reverse phase column. Aliquots of 10 μL was injected into HPLC, and an acetronitrile–water gradient was used as mobile phase and the elution was started with 70% acetonitrile for 4 min, and subsequent acetonitrile concentration was raised to 70–100% for 40 min at 1.0 mL/min.

In a HPLC-electrospray mass spectrometer, postcolunm addition of acetone at 250 μL/min was executed using a syringe pump (Agilent 1100 thermo autosample, Paulo Alto, CA). Mobile phase and acetone were mixed in the system, and a split ratio of 1/10 was used to introduce the effluent

into electrospray. The injection was performed in full scan mode with mass range from 280 to 750 mass spectrometer in centroid mode using a frequency of 1/s and a interscan time of 0.1 s. Mass spectrometry was carried out using multiple reaction monitoring, dry nitrogen as carrier gas at 80°C and 400 L/h. The capillary was held at 3.5 kV potential and the extraction voltage was –34 V.

Results and Discussion

Mineral Media Screening

In Fig. 1A–C, the results of the cell count and the surface tension are presented as a function of the fermentation time when applied to the media MM1, MM2, and MM3, supplemented with palm oil. These test aim to investigate the ability of the *P. aeruginosa* strain to grow in media containing different kinds of nitrogen sources and the possibility to reduce costs by utilizing low-cost nutrient sources. For all the tested media, cell growth can be verified, which indicates that the *P. aeruginosa* strain was able to utilize nitrogen from various sources for cell multiplication. These results corroborate the ones presented by Bonilla *(8)* and Abalos et al. *(10)* who reported a growth of the *P. aeruginosa* strains in cultures containing different nitrogen sources. As presented in Fig. 1A–C, it can be observed that the microbial strain presented the lowest cell multiplication rate when applied to the MM1 medium. In this case, the highest cell concentration was 1.2×10^7 CFU/mL. When the MM2 and MM3 media were applied, similar maximum cell concentrations were obtained of approx 4.2×10^7 and 4.7×10^7 CFU/mL, respectively. These results indicate that the use of the medium containing commercial N:P:K fertilizer (MM2) or inorganic MM3 medium could promoted similar the bacterium growth. In studies related to the bioremediation of soil contaminated with crude-oil applying a mixed culture containing *P. aeruginosa*, Oliveira and de Franca *(11,12)* verified similar behavior, that is, the microbial growth occurred irrespective of the use of a complex mineral medium or commercial fertilizer.

In Fig. 1A–C it can also be verified that the biosynthesis of the emulsifiers was initiated in the exponential phase of the growth of the microorganism, continuing in the stationary phase, irrespective of the inorganic medium that was utilized. In the 24-h period of the process, a marked reduction of the surface tension of the medium occurred. It is key to emphasize that the lowest values for surface tension were achieved applying the MM2 and MM3 media, presenting values of approx 35 mN/m.

CMD is an indirect means of measuring the surfactant production related to the range of the critical micelle concentration *(13,14)*. The data presented in Fig. 1B and C demonstrate that the CMD^{-1} revealed a slight increase of the surface tension of the fermented medium, whereas the CMD^{-2} caused a remarkable increased of the surface tension, which is an indicator of the elevated quantity of surfactants in the respective media.

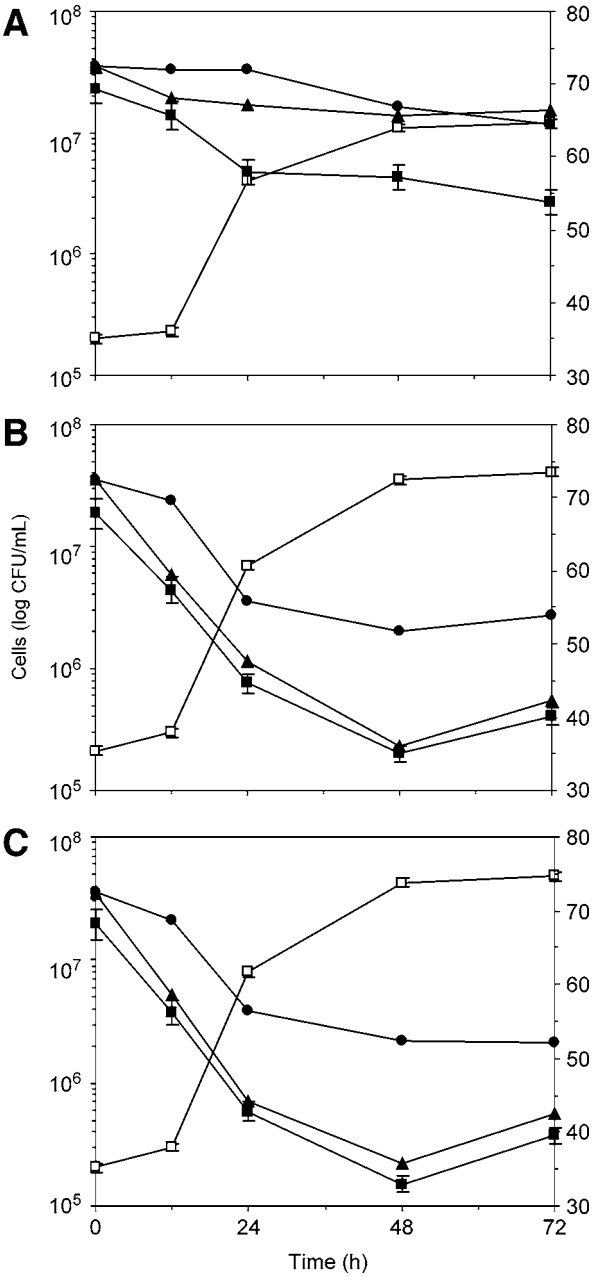

Fig. 1. Data of monitored parameters using three inorganic tested media: **(A)** MM1; **(B)** MM2; **(C)** MM3. (□), cells counting; (■), surface tension; (▲), CMD^{-1}; (●), CMD^{-2}.

When the MM1 medium was applied (*see* Fig. 1A), it was verified that both the CMD^{-1} and the CMD^{-2} presented high surface tension values, which was indicative of the low quantity of biosurfactants in the medium.

The selection of the mineral medium for the production of biosurfactants was based on the results of the surface tension of each of the three

Table 1
Surface Tension and CMD^{-1} Data Collected at 48 h of Bath Fermentations Using *P. aeruginosa* FR Growing on Inorganic Media MM2 Enriched With Palm Oil

Rotary velocity (rpm)	Surface tension (mN/m)	CMD^{-1} (mN/m)
50	55 ± 1.0	65 ± 0.7
100	39 ± 1.2	44 ± 0.9
150	35 ± 1.2	40 ± 1.0
200	51 ± 1.1	63 ± 0.8

studied media and of its CMD^{-1} and CMD^{-2}. The results indicate that the use of the media MM2 or MM3 promoted a higher biosurfactant production when compared with the mineral medium MM1. Furthermore, from the presented results it is verifiable that palm oil can be applied as a low-cost substratum, renewable and adequate for the surfactant production by *P. aeruginosa*. Because the commercial fertilizer is cheaper than the MM3 medium components, the use of the MM2 medium was favored in the subsequent tests for the bioemulsifier production by the strain that was studied.

Effect of Rotation Velocity

The variation in the agitation speed of the cultures of 50–200 rpm influenced the reduction of the surface tension of the medium. As can be observed in Table 1, the values for surface tension of the cell-free medium were higher for tests carried out employing 50 and 200 rpm. During the 48 h of the bioprocess, it was verified that the surface tension of the cultures agitated at 50 and 200 rpm was superior to 50 mN/m and presented a pronounced increase of the values for surface tension when submitted to the CMD^{-1} measuring. These results indicate a lower production of surface-active compounds under these process conditions. When the medium was agitated at 100 or 150 rpm, the lowest values of surface tension, CMD^{-1} and CMD^{-2} were achieved, pointing to greater surface-active compounds biosynthesis. The results now presented corroborate those reported by Cunha et al. *(15)* who found greater biosurfactants production by *Pseudomonas* spp. using ethanol-blended gasoline as the sole carbon source at 100 rpm. *P. aeruginosa* is a facultative microorganism that may present growth in environments with low oxygen concentrations *(9)*; the production of surface-active compounds, however, involves stages of oxidation of the substrate as described by Maier and Sobéron-Chávez *(16)*. The agitation speed of the medium is a determining factor in the mixture of the aqueous and oily phase as well as in the oxygen mass transfer into the cultures using agitated flasks *(17)*. Thus, the agitation speed may have influenced the surfactant production by the applied strain, promoting, in some cases, a phase mixture and/or an adequate oxygen transfer rate for bioemulsifier effective production.

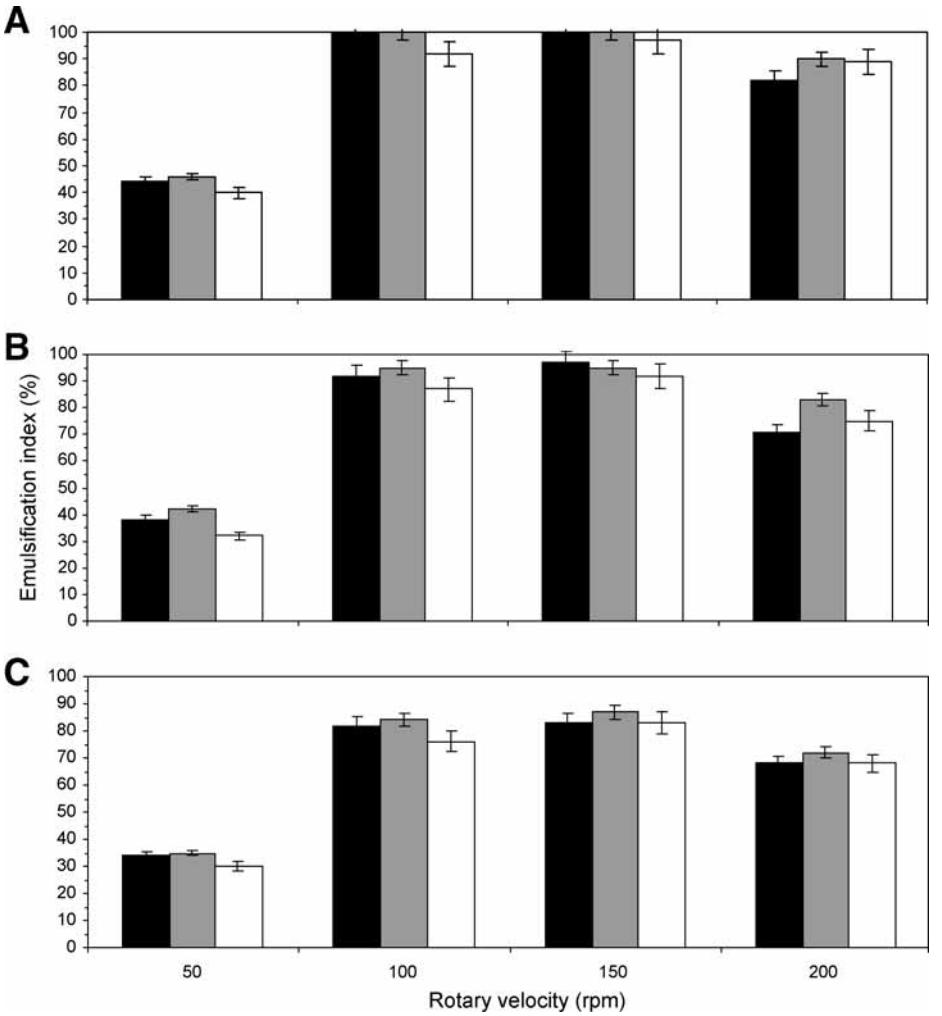

Fig. 2. Effect of rotary velocity on biosurfactant production at (■), 24 h; (■), 48 h; and (□), 72 h of the bioprocess, using different oil phases: **(A)** diesel; **(B)** jet fuel; **(C)** hexane.

Figure 2A–C represents the effect of the agitation on the emulsification index of the fermented cell-free MM2 medium obtained at 24, 48, and 72 h of process. It can be verified that rest time does not influenced emulsification index results, irrespective of agitation speeds of the cultures. It can be also verified that all the tested oily phases were emulsified. The use of 50 rpm furnished fermented media with lowest emulsification index, approx 30–45%. The cultures agitated at 200 rpm yielded free-cell fermented media with emulsification index from 55–80%. However, it is important to note that total emulsification was obtained with the fermented media from the cultures obtained at an agitation from 100 to 150 rpm. These results indicate that a higher concentration of surface-active compounds is obtained at rotation velocities from 100 to 150 rpm. Rahman et al. *(18)*

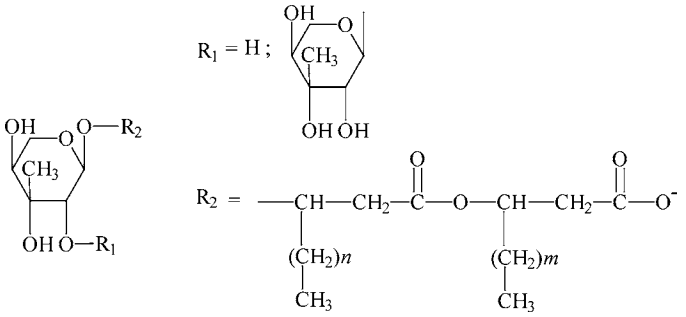

Fig. 3. Scheme of rhamnolipid general structure. $n = 4, 6, 8$ and $m = 4, 6, 8$ for length chain C_8, C_{10}, or C_{12} carbons atoms.

report on the surfactant production for two *P. aeruginosa* strains grown in a medium containing various carbon sources; also, they report an emulsification index from 25% to 90% for some oily phases such as hexane, diesel, and kerosene. In the present study, emulsification index of even 100% of these hydrocarbons was obtained with a stable time of up to 72 h, accounting for the good quality of the mixture of the surface-active compounds produced by the *P. aeruginosa* FR strain using palm oil as the sole carbon source.

Biosurfactant Characterization Using Mass Spectrometry

Some authors have been reporting on the characterization of surfactants produced by *P. aeruginosa* strains employing liquid chromatography coupled with mass spectrometry *(7,18–20)*. Muligan *(5)* and Santos et al. *(6)* report that *Pseudomonas* strains produce rhamnolipids from carbon sources that are either soluble or nonsoluble in water. The applied extraction method was based on the solubility of the lipids in chloroform and solvent strip under nitrogen stream at 40°C was performed aiming to avoid molecules oxidation. The extract was applied into the column of HPLC equipment and the elution with acetonitrile/water yielded the separation of eight fractions that were submitted to electronspray ionization. Analysis of mass spectra data verified a typical structure between the compounds, with the presence of rhamnose and lipidic group. Figure 3 presents the standard molecular structure of the rhamnolipids *(7)*, whereas Fig. 4 presents mass spectra of rhamnolipids mixture extracted from *P. aeruginosa* cultures on inorganic medium MM2 supplemented with palm oil as sole carbon source. Table 2 represents a synthesis of the data of the mass spectrometry of the each component of the rhamnolipids mixture.

The study of the mass spectrum of the rhamnolipids was initiated with the verification of the pseudomolecular ions and its fragments. The pseudomolecular ions with m/z 503 and 649 were most abundant in the samples, indicating a higher degree of biosynthesis, in agreement with the results reported by Déziel et al. *(20)*. The greater production of Rha-$C_{10}C_{10}$

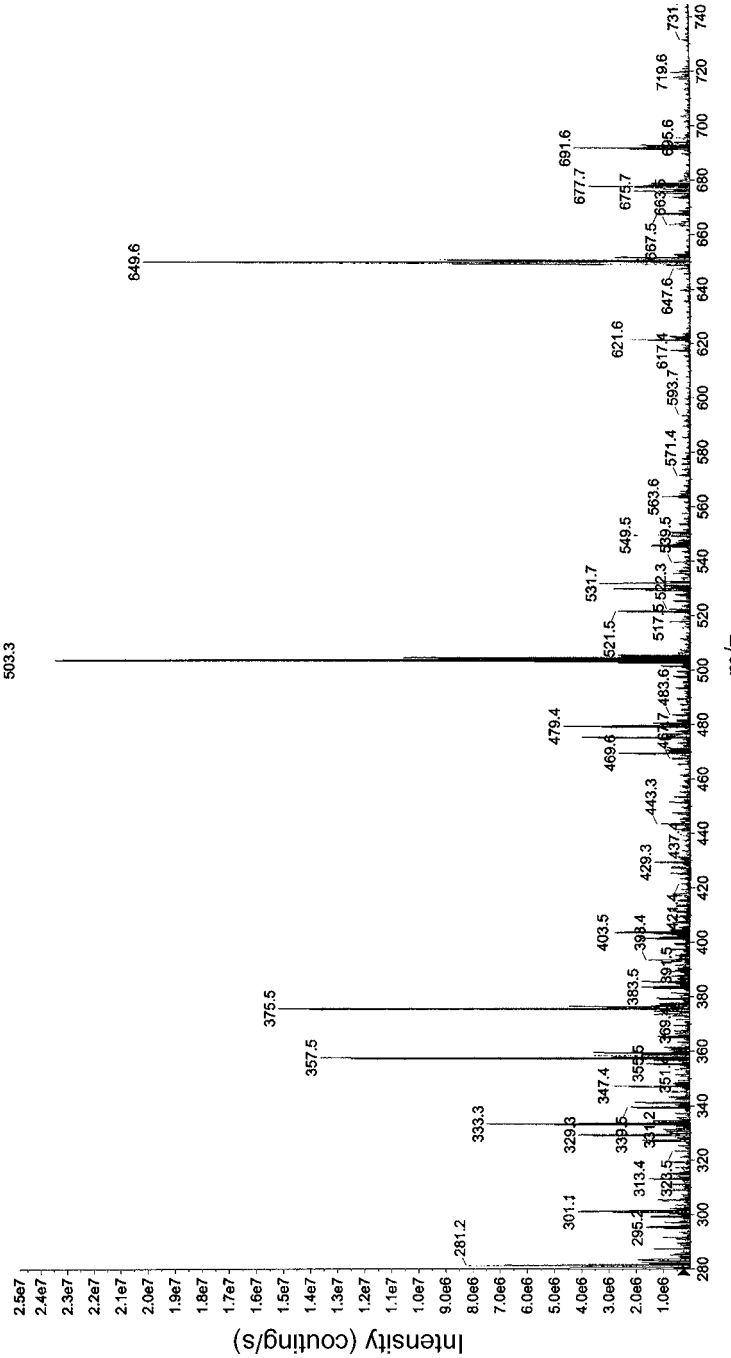

Fig. 4. Mass spectra of rhamnolipids.

Table 2
Mass Spectra Data of Isolated Rhamnolipds Produced by *P. aeruginosa* FR Growing on Inorganic Media MM2 Supplemented With Palm Oil

Rhamnolipids structure	Pseudomolecular ion (m/z)	Ion fragments (m/z)
R_1 = Rha; R_2= $C_{10}C_{12}$	677	163, 169, 187, 357, 503, 530, 650
R_1 = Rha; R_2 = $C_{10}C_{10}$	649	163, 169, 197, 311, 339, 479
R_1 = Rha; R_2 = C_8C_{10}	621	141, 163, 169, 311, 452
R_1 = H; R_2 = $C_{12}C_{10}$	531	161, 163, 169, 187, 333
R_1 = H; R_2 = $C_{10}C_{12:1}$	529	151, 163, 169, 187, 333
R_1 = H; R_2 = $C_{10}C_{10}$	503	103, 119, 163, 169, 333, 339
R_1 = H; R_2 = $C_{12:2}$	357	187, 333
R_1 = H; R_2 = $C_{8:2}$	301	131, 169, 187

and Rha-Rha-$C_{10}C_{10}$, m/z 503, 649, respectively, as compared with the other rhamnolipids species could be attributed to the culture conditions, carbon and nitrogen sources as well their ratio, as pointed by Santos et al. *(6)* and by Déziel *(20)*.

The pseudomolecular ions with 677, 621, and 649 m/z presented rhamnose group as R_1, and the others, pseudomolecular ion with m/z of 531, 529, 503, 357, and 301, has hydrogen as R_1. Some rhamnolipids, having unsaturated fatty acid group at side chain as also found, corresponding to the ions m/z 301, 357, 529 and corroborates with the rhamnolipid characterization results described in the literature *(19,20)*. The ion fragment at m/z 163 is related to the cleavage of the rhamnose group, and the fragments with the 119 and 103 m/z corresponding to its fragmentation *(18–20)*.

These data clearly show the synthesis of the rhamnolipids by *P. aeruginosa* FR growing in low-cost mineral media and carbon sources.

Conclusion

The synthesis of surfactants by *P. aeruginosa* FR strain was verified. The bacterium could grow in three inorganic media using of palm oil as sole carbon source, producing surface-active compounds. It was also verified that nitrogen source in the mineral medium and rotation velocity influenced biosurfactants production. The biosurfactants produced by bacterium growing in palm oil presents emulsification index of even 100%, when diesel was used as oily phase and temporal (72 h) stable emulsions were obtained. HPLC-mass spectrometry data clearly shows the presence of eight rhamnolipids species in the cultures. The results permitted us to conclude that rhamnolipids can be synthesized by *P. aeruginosa* FR using a low cost and renewable carbon source, the palm oil.

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