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MASS SPECTROMETRY ANALYSIS OF SURFACE TENSION REDUCING SUBSTANCES PRODUCED BY A PAH-DEGRADING *PSEUDOMONAS CITRONELLOLIS* STRAIN

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ABSTRACT

In this work we investigated the structure of the iron-stimulated surface tension reducing substances produced by *P. citronellolis* 222A isolated from a 17-years old landfarming used for sludge treatment in petrochemical industries and oil refinery. Its mass spectrum differs from *P. aeruginosa* spectrum, indicating that the surface tension reducing substances produced by *P. citronellolis* can be a new kind of biosurfactant.

Key words: *Pseudomonas aeruginosa*, rhamnolipid mixture, ESI mass spectrum, biosurfactant

We have recently reported a bacterial isolate *Pseudomonas citronellolis* 222A as a polycyclic aromatic hydrocarbon (PAH)-degrading bacteria and biosurfactant producer (6). This bacterium belongs to the *Pseudomonas aeruginosa* group, from the Pseudomonadaceae family and it was initially reported by Seubert (12) as an isoprenoid-degrading bacterium. More recently, *P. citronellolis* was isolated from soil as a hydrocarbon-degrading bacterial strain, which metabolizes citronellol (isoprenoid) and can also degrade the toxic hydrocarbon constituents present in oily sludge (3). In a further study we reported that *P. citronellolis* 222A showed the highest reduction of surface tension as compared with *P. aeruginosa* isolates and the addition of a soluble source of iron decreased surface tension (11). *P. citronellolis* 222A isolate showed a direct dependence on iron to stimulate surface tension reducing substances production that increased anthracene biodegradation (11). For *Bacillus subtilis,* Wei and Chu (13) reported that supplementation of iron enabled overproduction of a biosurfactant surfactin and increased biomass growth of this bacterium in a dose dependent manner. More recently, Wei *et al*. (14) verified addition of iron sulfate increased markedly surfactin production initially, but further iron addition led to a decrease in the concentration of surfactin, most likely due to acidification of the culture and consequent precipitation of surfactin. To our knowledge, the work of Santos *et al*., (11) showing the effect of iron on surfactant production by a *Pseudomonas citronellolis* was firstly reported on the literature. Despite that *P. citronellolis* belongs to the *Pseudomonas aeruginosa* group, we are considering the presence of a different surfactant structure as compared to the rhamnolipid produced by *P. aeruginosa* sp. In this work, we did the investigation on the structure of the iron-stimulated surface tension reducing substances produced by *P. citronellolis* 222A as compared to the structure of rhamolipid produced by *P. aeruginosa* LBI isolated from a petroleum-contaminated soil.

Pseudomonas citronellolis (isolate 222A) is a PAH-degrading bacteria and biosurfactant producer identified by 16S rRNA gene sequencing by Jacques *et al*. (6). It was isolated from 17-years old soil that was used for sludge treatment from petrochemical industries and oil refinery. *P. aeruginosa* LBI was isolated from petroleum-contaminated soil as previously characterized by Benincasa *et al.* (1) and its surfactant was used for structure comparison. For surfactant production, the isolate 222A was inoculated with an initial population of 2.0±0.2 Log CFU mL^{-1} in mineral medium (MM) (50 mL) as described by Jacques *et al.* (6), containing 250 mg L^{-1} of anthracene (three replicates) in the absence (control) and presence of 0.1 mM

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$Fe(NO_3)_3$ and incubated at 30ºC for 48 days with orbital shaking (150 rpm). Surfactant production was estimated by the emulsification index and by the reduction of the surface tension. Emulsification evaluation was performed by the presence and absence of cells into the MM (cells were removed by centrifugation at 10,000 rpm by 30 min, at 4ºC). Two mL of MM were mixed with diesel oil in a pirex glass tube (100 mm x 15 mm) using a vortex for 2 minutes. After that, the tubes were kept resting for 24 hours and then the volume and the stability of the emulsion were measured. The surface tension of the MM was measured in the absence of cells after samples equilibration (1h at 25ºC), using a model Lecont Du Nouy tensiometer. Sterile mineral medium was used as control (69.2 mM m^{-1}). Surface tension reducing substances were extracted from culture media after cell removal by centrifugation at 8000 rpm for 20 min. The supernatant pH was adjusted to 2.0 with 6N H_2SO_4, and an equal volume of $CHCl_3/CH_3OH$ (2:1) was added. The mixture was vigorously shaken for 5 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated. The extracted product was concentrated from the pooled organic phases using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45ºC, according to Costa *et al*. (4). Electrospray ionization (ESI) mass spectra were recorded on a high-resolution Q-Tof (Micromass, U.K.) mass spectrometer with a quadrupole (Qq) orthogonal time-of-flight configuration using operating conditions described in details elsewhere (4). The ESI mass spectrum in the negative ion mode was acquired using a capillary voltage of -3.5 kV, a cone voltage of 35 V and desolvatation gas (nitrogen) was heated to 100ºC. ESI tandem mass spectra were acquired by mass-selecting the target ion using the quadrupole mass analyzer followed by 25eV, collision induced dissociation using nitrogen in collision cell. The material was dissolved in methanol: water (1:1 v/v), filtered (0.22 mm) and introduced into the source at 15 ml min^{-1} with a syringe pump.

After 48 days of incubation into mineral medium containing 250 mg L^{-1} of anthracene *P. citronellolis* 222A isolate reduced surface tension from 69.2 to 36.2 mM m^{-1}. Addition of 0.1 mM of Fe-$Fe(NO_3)_3$ to the medium increased the reduction of surface tension to 26.0 mM m^{-1}, as reported by Santos *et al*. (11), being one of the smallest values reported in the literature, only compared to the surfactin produced by *B. subtilis* (14). This reduction was associated to the increase of the anthracene availability and consequently, increase of anthracene degradation (28%) estimated by gas chromatography (CG-MS), as compared with the result in the absence of iron. Beyond anthracene, iron addition at 0.1 mM $Fe(NO_3)_3$ increased the growth of the *P. citronellolis* 222A isolate living into a medium containing pyrene, phenantrene, gasoline and diesel oil. Emulsification was not detected in our work, indicating that the *P. citronellolis* 222A do not produce high molecular weight surfactant as a bioemulsificant.

Mass spectroscopy analysis of the rhamnolipid produced by *P. aeruginosa* LBI yielded an ESI mass spectrum with a predominant peak at *m/z* 649 and a second major component at *m/z* 503, which corresponds to the deprotonated molecules $[M-H]^-$ of the dirhamnolipid ($Rha_2C_{10}C_{10}$) and the monorhamnolipid ($RhaC_{10}C_{10}$), respectively (Figure 1A). These are biosurfactants frequently produced by *P. aeruginosa* and more studied and found in literature (1,4,5,8). In fact, ESI-MS detected a rhamnolipid mixture produced by this *Pseudomonas* with the presence of other components (*m/z* 621, *m/z* 677) (Fig. 1A). Pseudo-molecular ions above 621 probably represent fragments of the molecules of monorhamnolipids and dirhamnolipid (9). *P. aeruginosa* frequently produce a mixture of biosurfactants. Déziel *et al.* (5) identified the presence of the 21 types of rhamnolipids when *P. aeruginosa* 57RP grew using mannitol as unique source of carbon.

In our work, the same situation was observed with *P. citronellolis* 222A which also produced a mixture of substances with surface tension reducing proprieties (Fig. 1B). The substances extraction was not possible in the presence of iron, probably due to some chemical interference on extraction. In the absence of iron, the ESI-MS analysis of the reducing surface tension substances mixtures produced by *P. citronellolis* 222A revealed compounds of small *m/z*. The major component was pseudo-molecular ions at *m/z* 341 and two higher peaks detected as the anions of *m/z* 339 and 255; this indicates a production of components differents of *P. aeruginosa* pattern. Microorganisms synthesize a wide variety of low-molecular-mass biosurfactants (10). They are mainly rhamnolipids, trehaloselipids, sophorolipids, viscosin, surfactin, gramicidins, polymyxins, serrawettin, lipopeptides (10). However, bacterial cells can produce a large variety of surface tension reducing substances such as peptides, fatty acids, phospholipids and also antibiotics, that have low molecular mass than the named low-molecular-mass biosurfactants (7). In the ESI mass spectrum produced by *P. citronellolis* 222A it is possible to find structures similar to rhamnolipids produced by *P. aeruginosa*, as the anion of *m/z* 311 corresponds to a molecule with two rhamnoses (Rha_2) could indicate a production of dirhamnolipids by *P. citronellolis* (2).

Variations in the chemical structure of biosurfactants have been reported by several authors (1,7,10). These variations are obviously associated to species of bacteria that produce the biosurfactant, as well as variations in analytical methodologies used to characterize the molecules and growth conditions (5,9). Deziel *et al.* (5) show that the rhamnolipids produced by *P. aeruginosa* 57RP differs both in quantity and in structure depending on if the carbon source in mineral medium was mannitol or naphthalene. The most of the works in the literature use glycerol or vegetable oils (soybean, olive, sunflower, corn) as unique source of carbon to grow biosurfactant-producing microorganisms. To our knowledge, this is the first report about the structural characterization of surface tension reducing

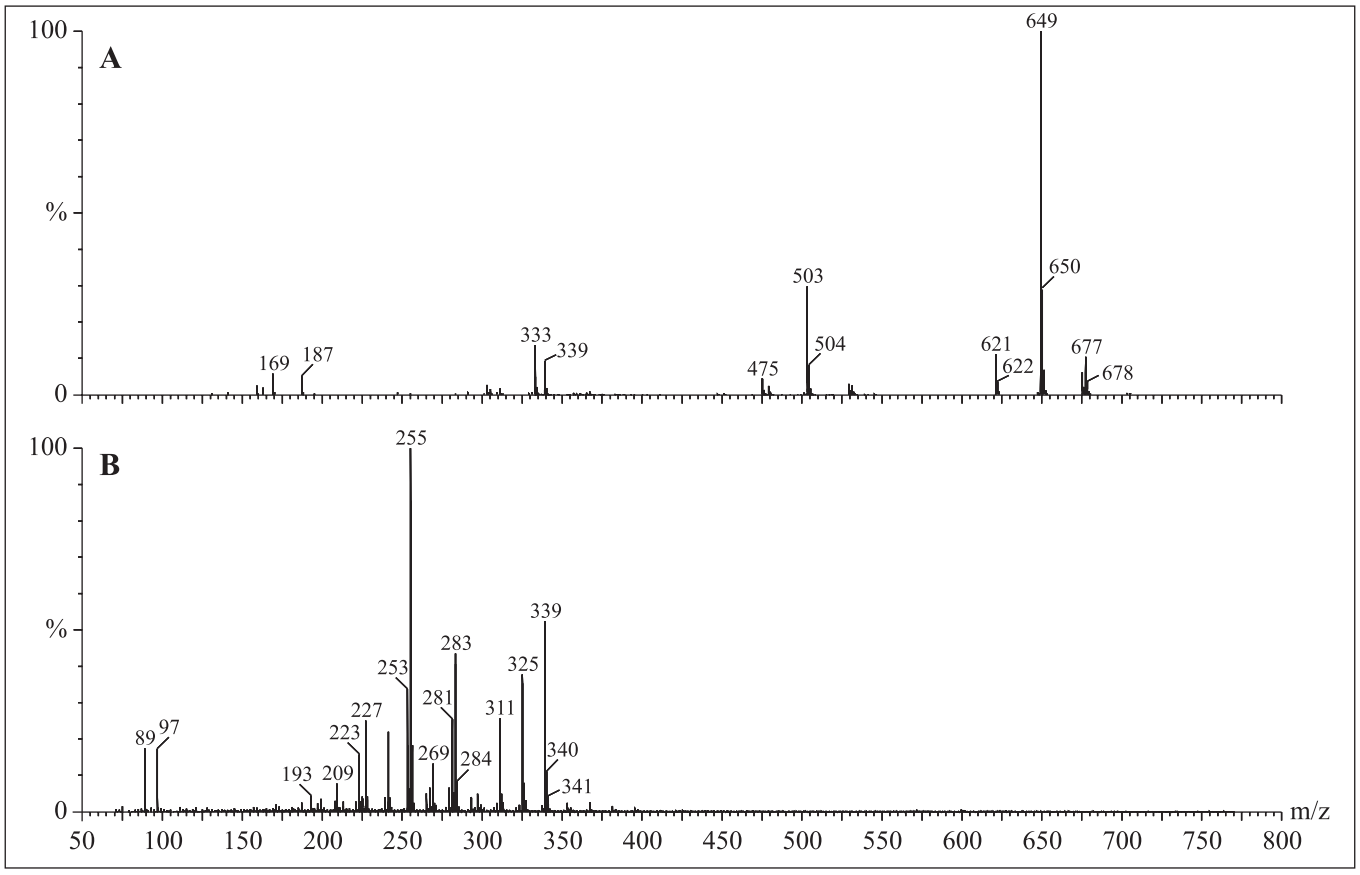

Figure 1. ESI mass spectrum for the rhamnolipids mixture produced by *P. aeruginosa* LBI (A) and the reducing surface tension substances produced by *P. citronellolis* 222A (B).

substances produced in anthracene. This in part could explain the differences between ESI mass spectrum of *P. aeruginosa* and *P. citronellolis*. It was observed that surface tension reducing substances produced by *P. citronellolis* structurally seem to be different of typical rhamnolipids produced by *P. aeruginosa*, which indicates the necessity of new studies (nuclear magnetic resonance, for example) aiming to identify completely the structures of these substances, which can be a new kind of biosurfactant.

RESUMO

Análise por espectrometria de massa de substâncias redutoras da tensão superficial produzidas por uma cepa de *Pseudomonas citronellolis* degradadora de hidrocarbonetos aromáticos policíclicos

Neste trabalho é apresentado um estudo a respeito da análise da estrutura de substâncias redutoras de tensão superficial produzidas por *Pseudomonas citronellolis* 222A estimulado pela presença de ferro. Esta bactéria foi isolada de um solo que há 17 anos vem sendo utilizado para o tratamento de borra oleosa proveniente da indústria petroquímica e de refinaria de petróleo. O espectro de massa difere do espectro de *P. aeruginosa*, indicando que as substâncias redutoras de tensão superficial produzidas por *P. citronellolis* podem ser um novo tipo de biosurfactante.

Palavras-Chaves: *Pseudomonas aeruginosa*, mistura ramnolipídica, espectro de massa ESI, biossurfactante

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