Applied Biotechnology: Isolation and Detection of an Efficient Biosurfactant from *Pseudomonas* sp. Comparative Studies against Chemical Surfactants

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***Abstract*—** *The use of biosurfactants became essential because of its multiple properties and applications. The high toxicity to the environment led to search for new alternatives such as the reduction or replacement by biological surfactants. Because of this, it is in our interest to produce biosurfactants from a non-pathogenic Pseudomonas. We obtained lower values of critical micelle concentration (CMC) from the culture broth than obtained from dodecyl sulfate sodium (SDS) and Pluronic F-68, used as pure surfactans. We found values of critical micellar concentration close to 0.15 mg/L in the purified fraction by adsorption chromatography. We determine by mass spectrometry this strain possibly produces two families of biosurfactants. Majority fraction might be formed by cyclic lipopeptides whose molecular weights could be located in the range of 1100-1200 Da. However, it is necessary perform confirmatory structural studies and to determine the specific structure of these analytes.*

***Keywords*— Biosurfactants, Critical Micellar Concentration, Mass Spectrometry, Pseudomonas, Surfactants.**

I. INTRODUCTION

Biosurfactants are a group of secondary metabolites synthesized by a great variety of micro-organisms. The properties of biosurfactants include the reduction of surface and interfacial tensions between liquids, solids and gases [1]. Due to their biodegradability and low critical micelle concentration (CMC) are ideal surfactants for environmental application [2]. These molecules have been studied extensively and now we have a good amount of information regarding their production, types and properties [3]. The principal action of these molecules will depend on its specific structure and production characteristics [4].

Biosurfactants possess a nonpolar region of long chain fatty acids and polar hydrophilic groups, such as carbohydrate, amino acid phosphate or cyclic peptides [5]. Because of this can be divided into two groups, low molecular mass this class includes phospholipids, glycolipids and lipopeptides. In general, show lower surface and interstitial tension. Another group is high molecular mass that used as emulsion stabilizing agents such as polymeric surfactants and lipoproteins [6], [3].

When are compared with synthetic surfactants, biosurfactants have several advantages, including high biodegradability, low toxicity, low irritancy, and compatibility with human skin [7], [8].

Biosurfactants not only act modifying the surface properties, but also alteration of compound bio-availability and interaction with membranes [9]. This explain their importance in the area of the therapeutic and biomedical [10], [8], [4].

Certain species of Pseudomonas are able to produce and excrete biosurfactants of great interest [11], [12]. The genomes of its species are very varied and flexible. This is reflected in their versatile secondary metabolism, which enables the production of a wide variety of organic compounds displaying a range of biological function including surfactants [13], [14]. The objective of this work is to produce biosurfactants with low values of CMC in culture supernatant and to identify the family of surfactant belong to which it belongs. It is important we to obtain these type of compounds from a non-pathogenic *Pseudomonas*. With this work, we started the studio of production of a surfactant of biological origin that can compete in the near future with synthetic surfactants.

II. MATERIALS AND METHODS

2.1 Microorganism

Bacterial strains were isolated from local soil (wild-type strain). It was identified as the genus *Pseudomonas* (strain B204). This strain was ceded for the Laboratory of Microbiology of the Faculty of Biochemistry and Biological Sciences, of the National University of the Litoral (UNL), Santa Fe, Argentina.

2.2 Production of biosurfactants from *Pseudomonas* sp.

Pseudomonas spp. was cultured, under controlled conditions of temperature and agitation, in an enriched medium similar to the published by Xia et al. (2004), pH 6.5-7.0 and using glycerol as source of carbon. Biosurfactant production was corroborated by measurements of surface tension of the culture supernatants, whose value should be between 31.5 and 37.5 mN/m. The pure cultures that comply this requirement were conditioned with a phosphate buffer and then were concentrated by ultra-filtration. The concentrated product (retentate) obtained was filtered until their sterility. Finally, it was lyophilized for storing to long term.

2.3 Extraction and quantification of biosurfactants

A technique of acidic precipitation was carried out, followed of extractions with solvents. At 3 mL of the concentrated lyophilized product was acidified to pH= 2 with 6 N hydrochloric acid and was extracted with an equal volume of chloroform/ethanol 3:1 (v/v) mixture and water as co-solvent of extraction, which was repeated three times. The phase obtained was dehydrated with anhydrous Sodium Sulfate, filtered and evaporated to dryness in rotary evaporator and finally weighed. With this data was calculated the % (w/w) and % (w/v) for estimating the yield of production for *Pseudomonas*.

Previously was performed a test of precipitation of biosurfactants and was isolated using acid precipitation. The precipitation of biosurfactants was confirmed for the increased in the tension surface of a known sample.

2.4 Thin layer chromatography (TLC)

The extraction was analyzed by thin layer chromatography (TLC), using silica gel 60 Fluka, for the biosurfactants compounds. As mobile phase was used chloroform/methanol/water 65:15:2 (v/v/v). The TLC plates were revealed with ultraviolet light and a solution of sulfuric acidic to 10% (v/v) in ethanol and were kept at 105 ºC for 5 min.

2.5 Critical micellar concentration (CMC)

A solution of lyophilized retentate of 2.4 mg/mL was prepared from which were made several dilutions in ultrapure water. The surface tension was measure at room temperature (25 °C) and it was compared with the measure of ultrapure water. The surface tension was determined by a Tensiometer Du Nouy (CSC Scientific Company, Fairfax Unites States), according to Du Nouy's ring method [15].

2.6 Analysis for mass spectrometry

The analyzes obtained by the technique of acidic precipitation were determined by electrospray ionization-mass spectrometry (ESI-MS). Negative and positive mass spectra were obtained on SQD2 single quadrupole mass spectrometer (Waters, Milford United States). The scan range used was 300 to 3000 m/z with the objective of detecting different families of biosurfactants. Methanol was used to dissolve the precipitate and was filtered with syringe filter of 0.22 μm Millipore. Were optimized the necessary parameters for to carry out then a chromatographic separation.

2.7 Isolation of majority biosurfactan by adsorption Chromatography

From of 1 g of lyophilized retentate at 45% (w/w) the biosurfactants were isolated by adsorption chromatography. A column of 30 cm long with 2 cm of internal diameter was used, to which was added 17 g of silica gel 60 to obtain a height of about 10 cm.

Chromatography was performed with a gradient of ethyl acetate: ethanol. The column wash was carried out with methanol. Chromatography was monitored by TLC in the same way as explained above. The thin layers were revealed with ultraviolet

light and a solution of sulfuric acidic to the 10% (v/v) in ethanol and were kept at 105 ºC for 5 min. For the detection of peptides, the dry plates were sprayed with a solution of 0.25% (v/v) ninhydrin in acetone.

The CMC of the fraction isolated by the column was determined in the same way as for the lyophilized retentate. In this case, it was started from an aqueous solution of 0.5 mg/mL of purified biosurfactant.

2.8 Ultra liquid performance chromatography (LC/MS) of the majority fraction

The analysis was performed in a SQD2 single quadrupole mass spectrometer (Waters) coupled to H-CLASS HPLC system (Waters, Milford United States). Ionization negative mode was used for chromatography experiments. The sample was dissolved in acetonitrile/water 1:1 (v/v) and was eluted with a water/acetonitrile gradient. The dimensions of column were 2.1 by 5 mm (ACQUITY BEH C18 1.7 µm, Waters, Milford United States), the injection volume was 3 µL and the flow rate was set to 0.3 mL/min. Spectra were taken in the m/z range of 300 to 3000 Da. The spray voltage of the mass spectrometer was 2 kV and the cone voltage 90 V. The desolvation temperature was 623 ºK and source temperature 423 ºK. The ion energy was set to 1 V. Mass Lynx (ver. 4.1) software was used for analysis and post processing.

2.9 Spectral scanning of fraction isolated of chromatography

The spectral scanning was performed of 200 to 800 nm in spectrophotometer UV-Visible (Perkin Ermer, Buenos Aires, Argentina) Lambda 20 software was used for analysis. The sample was dissolved in acetonitrile/water 4:1 (v/v).

III. RESULTS

3.1 Production, extraction and quantification of total biosurfactants

Through the values obtained from surface tension, we could ensure that we produce surfactants from nonpathogenic *Pseudomonas*. The values of surface tension (γ) measured in the culture broths were between 33 and 34 mN/m at a dilution 1/3000 (γ_{water} = 72.5 mN/m at 24 °C). The pH of broths was between 7.9 and 8.5 and their cellular concentration of 10^9-10^{10} CFU/ml. In the same dilution of retentate, the surface tension was 31 mN/m and lower values than 25 mN/m, showed the undiluted solution. The pH to the retentate was kept at 7.5. The tension superficial of undiluted permeate was around 25 mN/m, but increased to values close to water when was diluted to half.

The retentate was lyophilized and it was stored to -20 °C without losing its properties in the evaluated year. The yield of the process of lyophilized was 4.2 % (w/v).

We were able to estimate the amount of active principle released in the culture supernatant using the technique acidic precipitation. The yield of production was of 45 % (w/w) and 2% (w/v), possibly the rest consisting of proteins and the components of the culture medium. TLC plates were performed with isolated active principle and were observed in the plates two retention factor (R_f) values of 0.4 and 0.7, respectively. These showed positive reactions to sulfuric acidic reagent and in light ultraviolet.

It is important to clarify that was confirmed the precipitation of compounds of interest to pH 2 by surface tension measurements. A solution of γ = 28.5 mN/m at pH 7.5 increased the surface tension at 63 mN/m when the pH was decreased to 3.

3.2 Critical micelle concentration of the retentate (CMC)

The CMC is minimum surfactant concentration from which spontaneously begins the formation of micelles in the solution and for this reason the value of surface tension (γ) does not decrease more. The values of γ were plotted versus the values of concentration of different dilutions (Figure1) and the value of CMC of retentate was calculated from the turning point of the curve obtained. The value of CMC resulted to be 0.3 mg/mL and the surface tension was decreased to 29.6 mN/m.

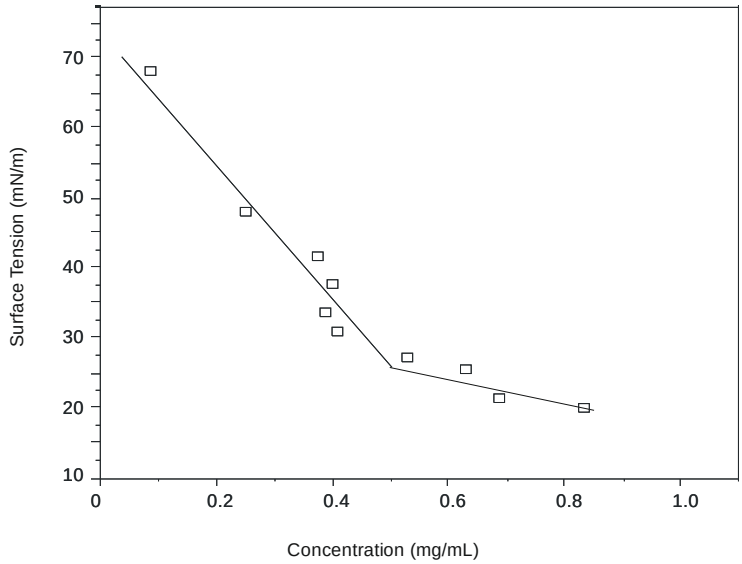

FIGURE 1. EFFECT OF RETENTATE CONCENTRATION ON SURFACE TENSION. THE INTERSECTION OF REGRESSION LINES DETERMINES VALUE OF CMC

3.3 Analysis of the product of precipitation by Mass Spectrometry

The negative-ion spectrum (Figure 2) showed a molecular ion of 1125.1 m/z of higher relative intensity. In the positive-ion spectrum this molecular ion peak appeared as 1148.8 m/z and 1170.8 indicating ions resulting from addition of one $[M+Na]^+$ and two sodium $[M+2Na]^+$ respectively. Lower molecular weight ions are also seen in the spectrum with lower relative intensity, such as masses of 502, 648 and 702 Da (Figure 2).

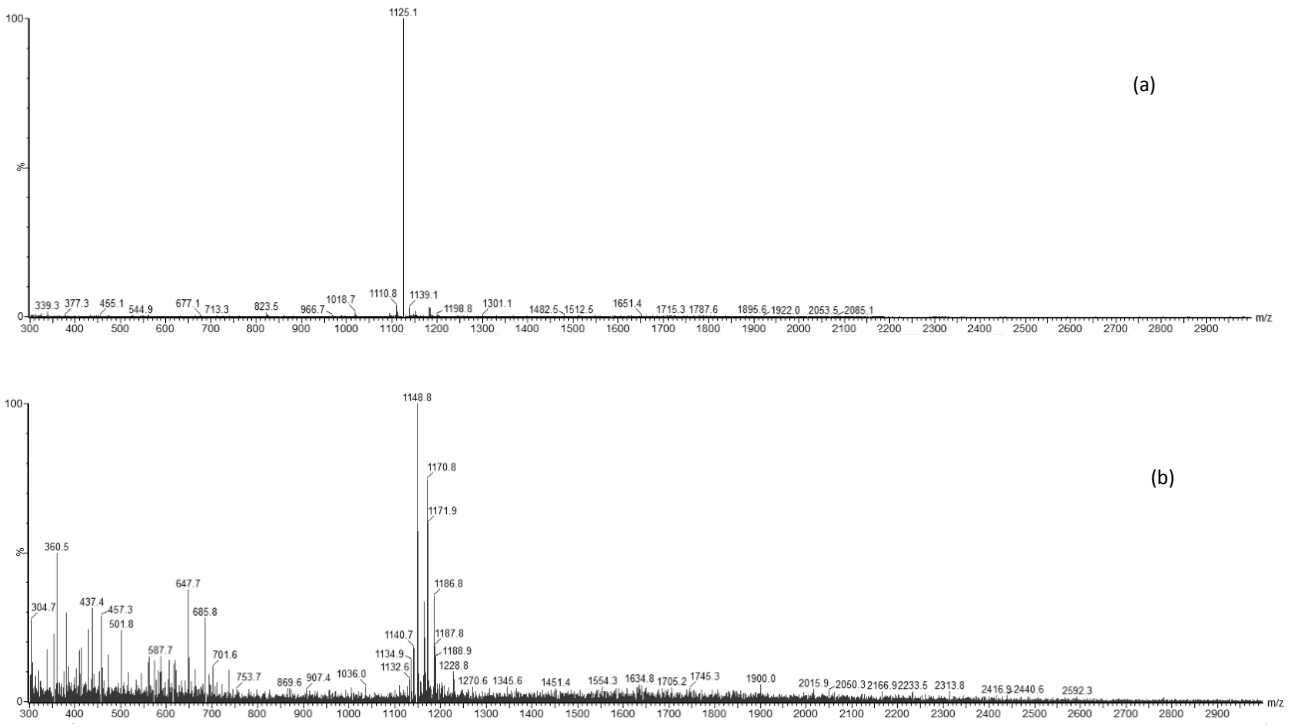

FIGURE 2. (A) ESI (-) MASS SPECTRA, AND (B) ESI (+) MASS PROFILE OF THE PRODUCT OBTAIN BY THE TECHNIQUE OF ACIDIC PRECIPITATION.

3.4 Adsorption Chromatography

The elution was achieved with a mixture of ethyl acetate/ethanol 1:1 (v/v). A fraction of 380 mg (38%) from 1 g of lyophilized retentate was obtained. The majority fraction showed a retention factor (R_f) of 0.4 in thin layer chromatography (TLC). This fraction showed positive reaction to sulfuric acidic reagent but negative reaction to ninhydrine.

CMC value was determined to confirm that isolated fraction from the column has surfactant activity. To calculate this was made a graph of surface tension versus concentration as shown in Figure 3. A value of CMC of 0.15 mg/mL was obtained. The value of surface tension at that concentration reached values of 29.6 mN/m.

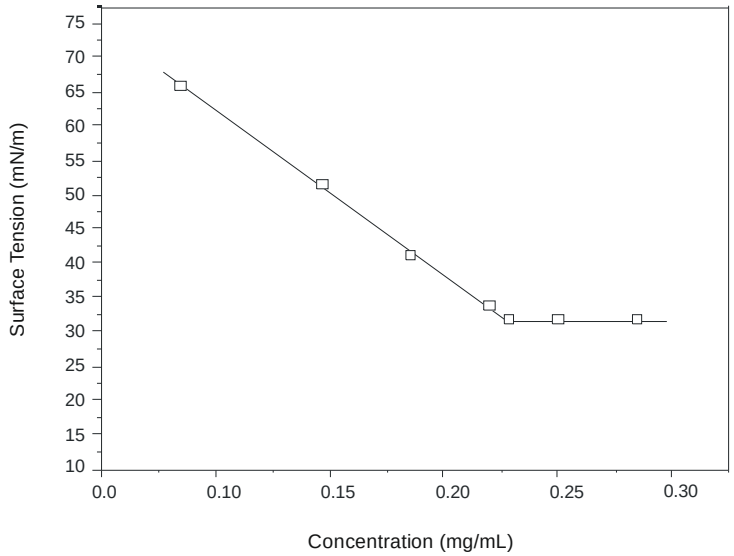

FIGURE 3. EFFECT OF ISOLATED BIOSURFACTANT CONCENTRATION ON SURFACE TENSION. THE INTERSECTION OF REGRESSION LINES DETERMINES VALUE OF CMC

3.5 Analysis by LC-MS

The majority fraction was analyzed by electrospray ionization-mass spectrometry (ESI-MS) coupled to a HPLC system. As shown in Figure 4 a series of negatively charged ions were observed in major faction isolated by adsorption chromatography. The analytes were eluted to a gradient of acetonitrile/water to 0.1% formic acid 65:35 (v/v) to 80:20 (v/v).

A retention time (RT) of 9.21 minutes was obtained by liquid chromatography-mass spectrometry LC-MS for the ion with the highest relative intensity in the form of negative ionization (Figure 4).This could be related to the deprotonated ion $[M-H]^-$ 1125.1 observed in electrospray ionization-negative ion mode (ESI-) mass spectrum, and with their corresponding sodiated ions m/z 1148.9 and 1171.8 found in electrospray ionization-positive ion mode (ESI+) mass spectrum (see Figure 2). The chromatogram showed also other less intense peaks m/z 1111; 1139 and 1154. These ions were not present in the (ESI-) mass spectrum of the product of precipitation in the Figure 2.

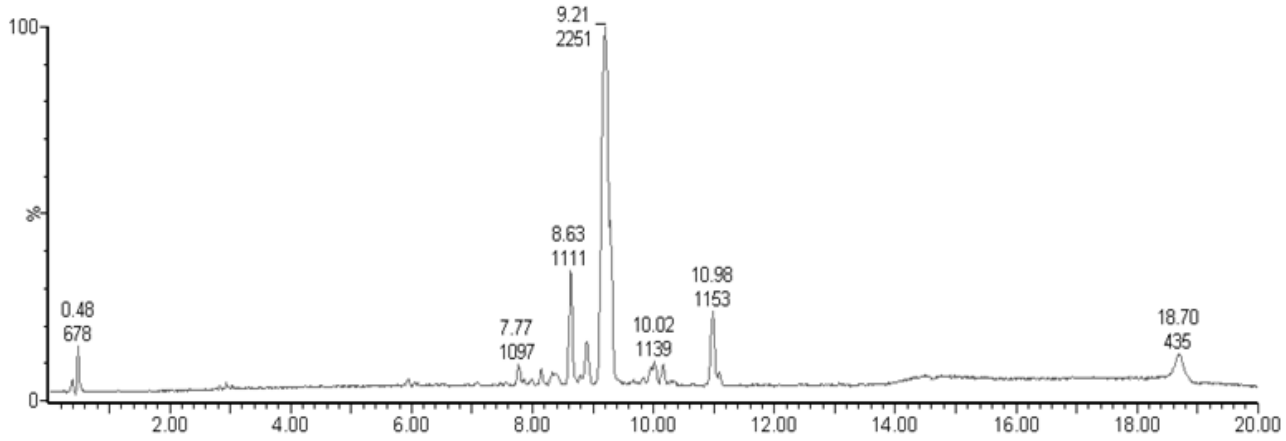

FIGURE 4. LC ESI-MS PROFILE IN NEGATIVE IONIZATION OF THE MAJOR FRACTION. THE SCAN RANGE WAS M/Z 300 TO 3000 DA.

3.6 Spectral scanning

The fraction obtained by chromatography showed a maximum of absorbance at 210 nm.

IV. DISCUSSION

The amount of surfactant needed to achieve the lowest possible surface tension is defined as the CMC, typically ranges from 1 to 200 mg/L for biological surfactants [16]. In the present study, the CMC of the crude biosurfactants and the purified majority fraction were investigated. It was possible to obtain a product with attractive surface tension values from unconcentrated culture broth. Values of CMC of dodecyl sulfate sodium (SDS) and Pluronic F-68, two chemical surfactants, were reported by O. Pornsunthorntawee et al. (2008) [17]. They found that Pluronic F-68 and SDS are able to reduce the surface tension of pure water in 42.8 and 28.6 mN/m, and the CMC values were approximately 350 and 1280 mg/L, respectively. In our case, were obtained lower values than pure chemical surfactants listed above. The retentate, reduced the surface tension of ultrapure water to 29.6 mN/m and the value of CMC was 300 mg/L. Even lower values were found for the fraction isolated by chromatography, which indicates that the compounds with greater surface activity were purified.

There are a wide variety of microorganisms have ability to produce biosurfactants, the genus *Pseudomonas* stands among them. It was reported that the strains of the genus *Pseudomonas* can produce cyclic lipopeptides and glycolipids such as rhamnolipids, these are two biosurfactants that differ in their structure and molecular weight [18]. The aim of our work is only identify the family of biosurfactants, since that in order to determine the specific structure of the compounds; it would necessary to perform Nuclear Magnetic Resonance studies and analysis of MS/MS.

Masses between 1000 and 1200 Da were observed by mass spectrometry and a maximum absorbance at 210 nm was found in the spectral scanning. This latter might indicate the presence of compounds in its structure containing peptide bonds, this could explain the high molecular weight found. The family of the compounds best known that contains hydrophobic and hydrophilic amino acids in their structure and surfactants activities are denominated cyclic lipopeptides [19]. There are two lipopeptides well described in the literature that belong to group of Viscosin, this are Viscosin and WLIP (white line-inducing principle). Its structure is very similar and both of them have a molecular weight of around of 1126 Da and 1149 Da when they form adducts with ion sodium [13], [20], [21]. The analytes observed by mass spectrometry could to form part of this family. The isolated compounds may not have free amino groups in their structure; this explains why the ninhydrin reaction was negative. This happens with Viscosin and WLIP. In contrast Viscosinamide has glutamine in its structure and the reaction of ninhydrin is positive [22].

Masses of 502, 648 and 702 Da were found, indicating that there possibly other family of compounds released to the culture broth for the strain used. These masses were described for mono and dirhamnolipids. Variations in rhamnolipid structures and their possible molecular weights are well known and are reported [23].

By acid precipitation and subsequent extraction we isolated and identified total biosurfactants. By acid precipitation technique we quantified total surfactant, and with extraction with solvents were eliminated proteins and other possible contaminants. However, in order to determine what proportion each produces, it is necessary to isolate each family of compounds. We isolate by adsorption chromatography a fraction of higher yield. The percentage of the major fraction corresponded to 84 % (w/w) of biosurfactants produced by *Pseudomonas*. In this fraction they were observed masses of 1100 to 1200 Da.

V. CONCLUSION

In the present work, we produce biosurfactants from non pathogenic *Pseudomonas*. We have obtained a possible product with surfactant activity through the addition of an ultrafiltration step in the production process, which is able to compete with a synthetic surfactant. This we demonstrate it with the CMC values achieved.

We detected two families of analytes secreted in the culture supernatant by mass spectrometry. We were able to isolate the majority fraction by adsorption chromatography; we believe that the strain used produced cyclic lipopeptides due to the molecular weights found. However, it is necessary to perform nuclear magnetic resonance studies and other structural studies that allow the detection of functional groups and confirm the above. We will continue to work on the characterization of the analytes found (biosurfactants), with possible applications as adjuvants in the agricultural sector.

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