Physiochemical characterization of a biosurfactant produced by bacteria isolated from pharmaceutical process water

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ABSTRACT

The study was performed on samples from pharmaceutical process water. Only strains presumed to produce biosurfactants were selected depending on microbiological analyses. So, the main objective of this investigation was the isolation, identification and characterization of some microorganisms producing biosurfactants. These characteristics were compared with previous references. The capacity and conditions of biosurfactant production were determined via several tests such as the rate of biomass after incubation, pH, surface tension and emulsification index. Four strains have been identified, namely *Pseudomonas Spp1, Pseudomonas Spp2*, *Flavobacterium spp* and *Serratia liquefaciens*. The results obtained showed that the selected bacteria could produce biosurfactants with significant surface properties except for Pseudomonas Spp2. They were characterized in terms of structure, surface tension, emulsification capacity, thermal, and chemical stability. In addition, the emulsifying capacities were found to be very attractive; the best results were obtained for the products extracted from *Pseudomonas Spp1*, which were equivalent to those obtained from the reference *(Bacilllus subtilis).* Furthermore, all the biosurfactants have good thermal stability. However, their properties seem to be sensitive to pH; a basic pH leads to a lowering of the surface tension and an increase of the emulsifying capacity. Based on the spectral characteristics, the biosurfactants produced mainly by the isolated strains were found as glycolipid types.

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1. INTRODUCTION

Biosurfactants are amphiphilic molecules produced by some microorganisms. Their nature, as well as their surfactant capacity, are highly dependent on the type of microorganisms used (bacteria, yeasts, fungi), the strain tested and the nutrient substrate available for the cell development [1]. According to their emulsifying and solubilizing properties, these molecules have many potential applications covering a wide variety of fields such as environment, medicine, agriculture and pharmacy [2, 3, 4]. They are constituted of a polar hydrophilic part and a non-polar hydrophobic one. Generally, the hydrophilic group consists of amino acids, peptides or polysaccharides and the hydrophobic group consists of saturated or unsaturated fatty acids [5]. In contrary to synthetic surfactants, which are generally classified according to the nature of their polar group, biosurfactants are classified mainly according to their chemical composition and microbial origin [5, 6]. Therefore, various biosurfactants are listed, such as glycolipids, lipopeptids, phospholipids, neutral lipids, fatty acids or lipopolysaccharides [7, 8].

Microorganisms developing aerobically in an aqueous medium containing one or more carbon sources as carbohydrates, oils or hydrocarbons produce microbial biosurfactants. These organisms are usually bacteria, yeasts and fungi [9]. The physiological role of a biosurfactant is to allow microorganisms to develop on insoluble substrates by reducing the interfacial tension between water and the substrate, making the latter more easily accessible [10].

The bacteria used to produce biosurfactants are generally extracted from soils contaminated by hydrophobic molecules. They are isolated from their natural environment and cultivated in the laboratory [6]. This allows doing tests to choose the appropriate culture medium containing the best carbon source in order to obtain a maximum production rate. Although many species produce biosurfactants; the regulation of their synthesis is still poorly understood, except for the strains of *Pseudomonas aeruginosa* and *Bacillus subtilis* which are currently the most studied bacteria [11]. Biosurfactants are known to be non-toxic, biodegradable, and can be used under

extreme conditions [11, 12]. The success of their use and production requires a reduction in production costs. This goal can be achieved through the development of growth substrates of low-cost products [13].

These green-surfactants can be produced using agricultural resources and renewable wastes. This allows not only a significant reduction in production cost, but also a decrease in the quantities of biowastes to be treated. Furthermore, research on biosurfactants has increased significantly [14]. Recently, it has been reported that a large number of microorganisms producing biosurfactants can be isolated from polluted and unpolluted waters [11]. Thus, Carmen Rizzo et al. [15] showed that biosurfactants can be produced by microorganisms isolated from freshwater. Poonguzhali et al. [16] synthesized a biosurfactant with inexpensive rice water; they justified its use as a biological control agent. Nevertheless, it was not given to our knowledge a work on water collected from an industrial process network.

The main objective of this investigation was the isolation, identification, and characterization of some microorganisms producing biosurfactants from pharmaceutical process water.

2. MATERIAL AND METHODS

2.1. Bacterial strains

Pharmaceutical water samples were obtained from the water used in a process network of a pharmaceutical production unit (Medea, Algeria). They were collected aseptically using pre-sterilized vials, and filtered through a sterile Millipore filter (0.22 µm porosity). The filters having retained the microorganisms were placed in Petri dishes containing suitable solid culture media [17, 18] and then, they were incubated for 48 h at 37 °C for *Pseudomonas* and at 44 °C for *Enterobacteriaceae*. After incubation, individual colonies were randomly selected, picked and purified by repeated streaking under the same conditions. Subsequently, the selected isolates were identified by standard microbiological techniques [19].

For the comparison, two strains *Pseudomonas aeruginosa* ATCC 9027 and *Bacillus Subtilis* ATCC 6633 were used as reference strains. They were stored in test tubes containing nutrient agar at 4 °C and reactivated each month to constitute a working stock.

2.2. Microbial growth and strain characterization

In a sterile environment, and from a pure culture on nutrient agar, a surface of an isolated bacterium strain was introduced into a 250 mL Erlenmeyer flask. It contains a nutrient broth that has been autoclaved beforehand. The culture medium for *Bacilus subtilis* is composed of a solution containing 7 g/L of $NH_2HPO_4.2H_2O$, 3 g/L of KH_2PO_4, 1 g/L of NH_4HCl, 5 g/L of NaCl, 0.25 g/L of $MgSO_4.7H_2O$, 0.02 g/L of $CaCl_2$, and 4 g/L of Glucose; the pH was adjusted to 7. However, for the other strains, the culture medium is composed of 1 g/L of KH_2PO_4, 0.2 g/L of $MgSO_4.7H_2O$, 0.02 g/L of $CaCl_2$, 0.05 g/L of $FeCl_3.6H_2O$, 2 g/L of $NaNO_3$, and 30 g/L of glycerol; the pH was adjusted to 6.8 [20, 21].

For each liquid culture sample, biomass, pH, emulsification index (E_{24}) and surface tension (TS) were measured before and at the end of production. For the determination of biomass growth, the microbial suspension was separated and its absorbance was measured at 600 nm using a mini1240 UV spectrophotometer (Shimadzu, Japan). The pH of broth culture and supernatant were also controlled by a pH meter (Hanna Instruments PH 211). The TS was measured using a bidirectional tensiometer (CSC Du Nouy 70545). The E_{24} was calculated by measuring the layer of emulsion formed after 24 h. For this, equal volumes of collected supernatant and diesel (5 mL) were mixed using a vortex for 3 min and then left to stand for 24 h [22].

$$E_{24} = (E / E') \times 100 \quad (1)$$

where E is the volume of the emulsified layer and E', the total volume of the mixture.

2.3. Extraction and characterization of biosurfactants

After separating the biomass by centrifugation at 4000 rpm for 20 min at 4 °C, the supernatant was collected in tubes and used for biosurfactant extraction. The operation was carried out by pouring three volumes of icy acetone into a volume of supernatant and then left to stand for 24 h at 4 °C, so a white precipitate was formed. It was recovered by vacuum filtration and then by drying at room temperature [5].

The foaming capacity of the obtained biosurfactants was determined by manually shaking a test tube containing a solution of biosurfactant in distilled water for 30 s and then left to stand for 5 min. The emulsifying activity was evaluated by measuring E_{24} (Eq.1).

The effect of temperature on the thermal stability of biosurfactants was studied by measuring TS and E_{24} of the broth supernatant after incubation at different temperatures (20, 50, and 120 °C) for 15 min.

The chemical stability was evaluated by varying the pH of the supernatant from 2 to 11 and by measuring, after a rest period of 10 min, the TS and E_{24}.

On the other hand, the characterization of biosurfactants was performed using the Rhamnose test according to the method of Dubois [23] and, Fourier transform infrared (FT-IR) spectrometric analysis [24]. For this, an amount of 2 mg of the crude biosurfactant was ground with 200 mg of KBr powder to form a fine powder. The powder was then compressed to form translucent pellets that they were analyzed by FT-IR spectroscopy (Bruker, Germany) in the range of 400 to 4000 cm^{-1}.

3. RESULTS AND DISCUSSION

3.1. Strain isolation and identification

From the six samples collected, four strains were isolated and retained as strains presumed to produce biosurfactants. Based on Gram stain and oxidase test, the isolated strains were found to be gram negative. The API®20 E identification tests also made it possible to determine the genus of the bacteria [19]. The numerical profiles of this identification were 00000046, 00000045, 0042000 and 1306563, which correspond to the species *Pseudomonas Spp1, Pseudomonas Spp2, Flavobacterium spp* and *Serratia liquefaciens.*

The biomass rate was evaluated for different microorganisms to control the growth of bacterial strains. The optical density (OD) was measured before (at the inoculation of the culture media) and after a culture. The incubation time was determined according to strains types. The results of the measurements of OD and pH of the isolated strains as well as those of the reference are summarized in Table 1.

Table 1: OD, pH and TS of the isolated strains and reference

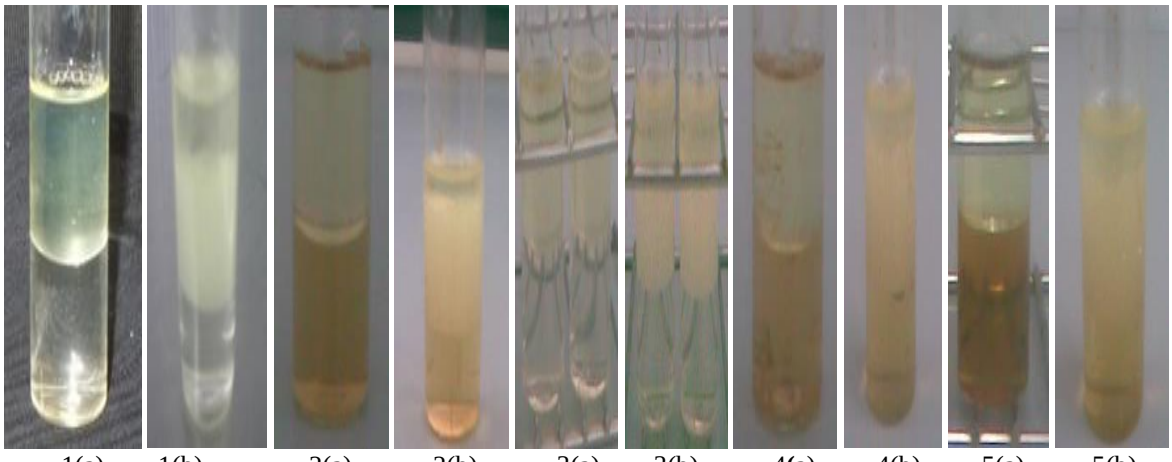
Microorganisms	At inoculation		After incubation		TS (mN/m)
	OD	pH	OD	pH	
Pseudomonas aeruginosa	0.792	6.80	1.052	6.51	45.6
Bacillus subtilis	0.736	7.01	2.185	4.50	39.0
Pseudomonas spp1	0.191	6.80	2.385	6.00	56.4
Pseudomonas spp2	0.721	6.84	0.800	6.80	61.0
Flavobacterium spp	0.190	6.82	1.653	5.70	36.1
Serratia liquefaciens	1.736	6.78	2.280	5.22	41.4

Analysis of the obtained results showed an increase in the biomass after the culture of the different strains, except *for Pseudomonas Spp2*, where the value of OD remained constant. In addition, a decrease was observed in the pH after the culture of different strains. This can be explained by the production and release of acids. In addition, the recorded TS values reflect good surface properties, except *Pseudomonas Spp2*, which had a high value of TS. This strain was not retained for further investigation.

3.2. Characterization of biosurfactants

3.2.1 Biosurfactant functional characterization

The precipitate obtained from the culture broth of the different isolates was a crystalline powder, soluble in water; it showed a high foaming power. The appearance of the emulsions formulated using the biosurfactants produced by the different isolated bacteria is shown in Figure 1. A good ability to emulsify diesel was observed for all samples.



1(a) 1(b) 2(a) 2(b) 3(a) 3(b) 4(a) 4(b) 5(a) 5(b)

Figure 1. Appearance of emulsions formulated using produced biosurfactants (1: *Pseudomonas aeruginosa*, 2: *Bacillus Subtilis*, 3: *Serratia liquefaciens*, 4: *Pseudomonas spp1*, 5: *Flavobacterium spp*; (a): before agitation, (b): emulsion after 24 h of rest; T = 25 °C)

The evolution of E_{24} of biosurfactants obtained from the selected strains is shown in Figure 2. It was observed that the diesel was emulsified by all strains used with E_{24} values varying between 43.4 and 59.09%. The highest emulsification activity was obtained by the biosurfactant produced by *Bacillus Subtilis* (59.09%), followed by that produced by *Pseudomonas spp1* (56.09%), *Pseudomonas aeruginosa* (55.42%), *Flavobacterium spp* (52.38%) and *Serratia liquefaciens* (43.4%).

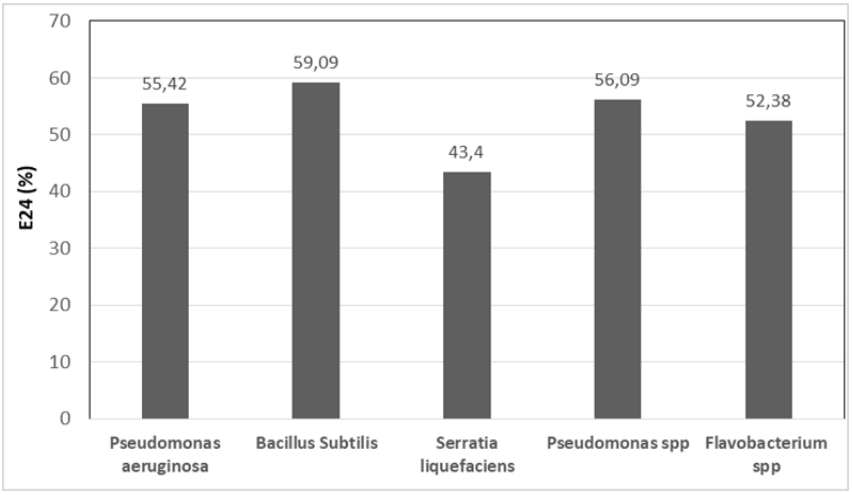
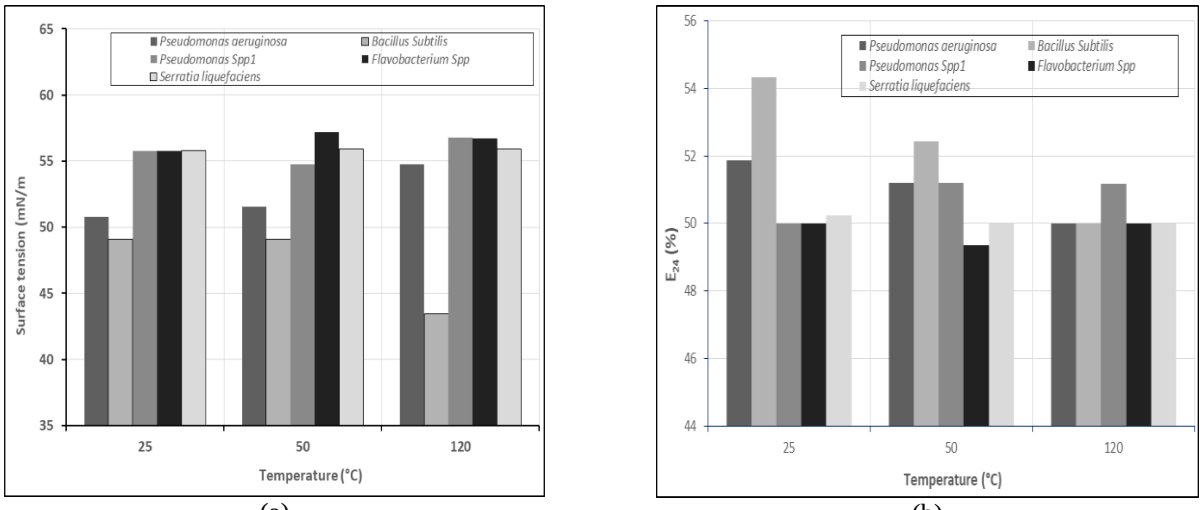


Figure 2. Emulsification activity (E_{24}) of biosurfactants against different microbial strains

In conclusion, the isolated strains allowed producing biosurfactants with a significant emulsification activity close to that obtained with the reference strains. These bacteria *(Bacillus, Pseudomonas, Flavobacterium and Serratia)* are already known for their production of biosurfactants [25].

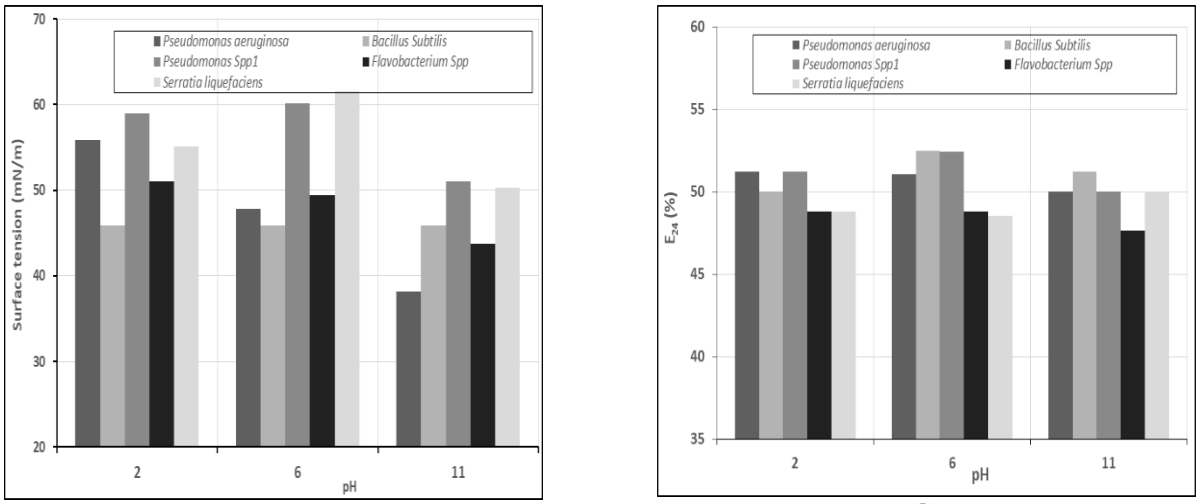
The effect of temperature on the stability of biosurfactants was evaluated by measuring TS and E_{24} as a function of three working temperatures (20, 50, and 120 °C). It was shown that the TS and E_{24} are influenced by the temperature except for the product obtained from *Serratia liquefaciens.* Between 25 and 50 °C, the TS is almost stable for biosurfactants derived from the two reference strains (Figure 3-a). However, above 50 °C, the TS of the biosurfactant from *Bacillus Subtilis* decreases, while of that obtained from *Pseudomonas aeruginosa* increases. The same results were observed for E_{24}; the emulsification activity is influenced by the temperature except for products obtained from *Flavobacterium Spp* and *Pseudomonas Spp1* where the effect of temperature is not important (Figure 3-b), their TS and E_{24} are constant. The biosurfactant produced from *Serratia liquefaciens* presented the best stability, its TS and E_{24} remained relatively constant with increasing temperature. These results demonstrated that the isolated strains made it possible to produce stable biosurfactants with significant surface properties.



(a) (b)

Figure 3. (a): Variations of surface tension (TS) as a function of temperature;
(b): Variations of E_{24} as a function of temperature

Furthermore, the variations of TS and E_{24} as a function of pH are illustrated in Figure 4. For the reference strains, an increase in pH from 2 to 11 leads to a decrease in the TS values from 55 to 45 mN/m for the product derived from *Pseudomonas aeruginosa* (Figure 4-a). For *Bacillus subtilus*, the TS remained nearly constant. However, the TS of biosurfactants from *Pseudomonas spp1* and *Serratia liquefaciens* increases for a pH between 2 and 6 then decreases considerably towards an alkaline pH (11). This proves that these bioproducts are stable at alkaline pH and their surface properties are much improved. This may be due to the change in the charge of the existing groups at the ends of the molecules [26]. For the product derived from *Flavobacterium spp,* the TS decreases with the increase in pH. Consequently, the basic pH reduces the TS and improves the surface properties.



(a) **(b)**

Figure 4. (a): Variations of surface tension as a function of pH; **(b)**: Variations of E_{24} as a function of pH

These results are in agreement with those obtained by other authors [27, 28]. In addition, E_{24} decreases slightly towards the basic pH (Figure 4-b). Since at a strongly basic pH, E_{24} still reaches higher values. However, the biosurfactants produced from *Pseudomonas spp1* and *Flavobacterium spp* have better activities at pH 6, while those produced from *Serratia lequefaciens* give better results at a basic pH. In this range of pH, a good emulsification is obtained, which is in agreement with the results of TS. In the case of biosurfactants obtained from *Serratia*, as those from *Pseudomonas* and *Flabobacterium*, high values of E_{24} and low TS are obtained at basic pH. Thus, it can be concluded that the biosurfactants produced by all the strains do not possess the same characteristics, whether reference strains or those isolated and identified.

3.2.2 Chemical structures

The carbohydrate test based on the Dubois assay was positive; this indicates that the isolated biosurfactants could be glycolipids. However, the type of glycolipids produced depends on the bacterial strain, the carbon source used, and the process strategy [29]. FTIR spectroscopy was used to identify their molecular structure. The characteristic bands of biosurfacatnts along with their assignment are listed in Table 2 in the FTIR spectra ranging from 350 to 4000 cm^{-1}. For all products obtained from the different isolated strains, O-H stretching vibrations at 3400−3500 cm^{-1} revealed alcohols. Similarly, O-H vibrations between 2800 and 3000 cm^{-1} confirmed the presence of carboxylic groups. The vibrations at 2921 cm^{-1}, 2851 cm^{-1}, and 1456 cm^{-1} are associated with the symmetrical vibrations of the (-CH) bond for the ($-CH_2$) and ($-CH_3$) groups for the saturated aliphatic chains (alkanes). The adsorption bands located between 2925 and 1650 with 1459 cm^{-1} indicate that all the substances have structures identical to those of glycolipids [30].

Table 2. Observed FTIR bands (cm^{-1}) in biosurfactants and their assignments

Assignment (cm^{-1})	Groupements	Intensity %	Strains
1654.07	C=C (acid)	53.0	*Pseudomonas aeruginosa*
1085.81	C-O (ester)	50.5	
983.16	C-H (alkene)	54.2	
1638.00	C=C (acid)	61.0	*Bacillus Subtilis*
1459.00	CH_2 ou CH_3 (ester)	63.0	
617.00	N-C=O (amide)	63.5	
1653.84	C=C (acid)	43.7	*Pseudomonas Spp*
1101.67	C-C (alkane)	45.9	
1654.00	C=C (acid)	42.0	*Flavobacterium spp*
1083.25	C-O (ester)	23.0	
864.99	C-H (alkene)	45.0	
598.00	C=O (amide)	30.0	
1655.89	C=C (acid)	26.0	*Serratia liquefaciens*
1085.99	C-O (ester)	21.0	
982.54	C-H (alkene)	25.0	

4. CONCLUSION

The results obtained showed that the strains *Pseudomonas Spp1, Flavobacterium Spp, and Serratia liquefaciens* could produce biosurfactants, based on their surface properties. The selection of the best strain was made according to its high emulsifying activity and minimum value of the surface tension. Thus, the Pseudomonas *Spp1* strain made it possible to obtain a biosurfactant with an emulsifying power equivalent to that of the reference *(Bacilllus subtilis).* Furthermore, it has been found that all biosurfactants are thermally stable. Nevertheless, it seems that their properties are sensitive to pH; a noticeable improvement was observed at basic pH. FT-IR analysis showed that biosurfactants obtained from different strains have almost the same functional groups, mainly acid and ester groups, which confirmed that their structure is of glycolipid type.

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