Production and characterization of biosurfactant by free and immobilized cells from Ochrobactrum intermedium isolated from the soil of southern Algeria with a view to environmental application

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\textbf{ABSTRACT}

In this paper, biosurfactant production by free and immobilized cells of Ochrobactrum intermedium has been studied. This bacterium strain was isolated from an Algerian crude oil-contaminated soil; hexadecane was used for the production as the sole carbon and energy source. The process was monitored by measuring the surface tension and emulsification index E24 for one week at 37 °C and neutral pH. For the production by immobilized cells, the concentrations of sodium alginate, calcium chloride and biomass were optimized. Results showed that O. intermedium entrapped in calcium alginate beads is able to preserve its viability and produce biosurfactants but with an effect on the production kinetics due to diffusional limitations of the alginate beads with greater stability with up to 75%. The product biosurfactant reduced the surface tension below 33 and emulsification index were 68%–93% after 48 and 72 h with free and immobilized cells, respectively. Also, the product belongs to the family of glycolipids and showed stability in a wide range of pH (2–12), temperature (25–120 °C), and to high salinity. Both products by strain O. intermedium, based on spectral features, have a chemical structure identical to that of glycolipids. The production yield of biosurfactant versus concentration of the hexadecane is 1292 g/g. Second, the surfactants are able to promote the solubility of polycyclic aromatic hydrocarbons (PAHs). Results show that the use of biosurfactant, produced by the isolated bacterial strain O. intermedium, obtained a better solubility of naphthalene and phenanthrene.

\textbf{Introduction}

The surfactants are amphipathic molecules which preferentially distributed at the interface between the fluid phases such as oil/water interfaces or air/water. These surfactant properties are capable of reducing the surface tension (ST) and interfacial tension and give excellent detergents, emulsifiers, foaming and dispersing products.

The surfactants are widely used for environmental purposes, industrial, agricultural, food, cosmetic and pharmaceutical applications. These compounds are chemically synthesized and can cause environmental problems, due to refractory and persistent nature of these substances [1]. The majority of commercially available surfactants are chemicals and petroleum products. They pose a risk to the environment because they are generally toxic and not biodegradable [2].

Therefore, in recent years, and thanks to the development of biotechnology, scientists have been interested in surfactants produced by microorganisms: biosurfactants or biological surfactants. They have the same surface-active properties as their chemical counterparts but have the advantage of being biodegradable, non-toxic and have a high ability for foam formation, greater selectivity, specific activity to extreme temperature, pH and salinity, the ability to synthesize from renewable substrates and ecological acceptability [3]. Although some best-known biosurfactants are of bacterial origin, such as that reported by different authors [4–7], the study biosurfactant production by bacteria has grown, production is reported mainly by Candida sp. genres, Pseudomonas sp. and Yarrowia sp. [8–10].

Optimizing the production of biosurfactant appears to be the main research area, due to low product yields. For this purpose, adequate culture medium, optimal conditions and the mode of culture are of major importance. Because of the small quantities produced, the recovery of biosurfactants from the culture medium is significant in the final production.
costs. Furthermore, the amphiphilic characteristic of these molecules increases the problem of separation, which is generally carried out by precipitation, organic extraction, adsorption or the ultrafiltration chromatography [11]. For this purpose, the immobilization of living cells in the porous support offers huge advantages in the continuous production of biosurfactants. It is an effective way to reduce the cost of product recovery, as growth and the phases of the product formation can be separated and substrate inhibition could be avoided due to diffusionless of the product formation can be separated and sub-

Cultivation of organism and production of the biosurfactants

For the preparation of inoculum, 50 mL of a preculture was used with a concentration of 8 g L⁻¹. The preculture contains (in g L⁻¹) came extract 5.0, peptone gelatin 10.0, and NaCl 10.

The cultures were grown in this medium for 24 h at 37 °C. The latter was used as inoculum to 8% (v/v) and then developed in an inorganic culture medium containing (in g L⁻¹) Na₂HPO₄ (2.2), MgSO₄·7H₂O (0.05), NaCl (0.05), KH₂PO₄ (1.4), FeSO₄·7H₂O (0.01), CaCl₂ (0.02), Extrait de levure (0.02), 1 mL of Oligo elements solution, which has the composition (in mg L⁻¹) of ZnSO₄·7H₂O (525), CuSO₄·5H₂O (705), CoCl₂·6H₂O (200), MnSO₄·4H₂O (200), NiSO₄·6H₂O (27), H₃BO₃ (15). The pH was adjusted to 7 and the medium was sterilized by autoclaving at 121 °C for 20 min. The nitrogen source used was NH₄NO₃ at a concentration of 1 g L⁻¹ containing hexadecane (2%, v/v) as the carbon source at 37 °C and at 200 rpm in shaker (KRÜSS KG, Germany) for 7 days. The culture broths were centrifuged for 15 min at 4500 rpm to remove the cells. Samples were withdrawn every 24 h to be analyzed.

Cells immobilization

The procedure of cell immobilization in calcium alginate was performed as follows. A preculture was launched for 48 h and was then centrifuged for 15 min at 4500 rpm. The biomass obtained was washed with sterile culture medium, centrifuged a second time for 15 min at 4500 rpm. Cells were mixed with a sterile solution of 4% (w/v) alginate, and then gradually added to the CaCl₂ solution by a sterile syringe; the separation of beads from the solution was carried out by filtration, the beads were washed with sterile distilled water and stored in the culture medium at 4 °C in firmly closed flasks or that used for immobilized cells experiments.

Optimizing concentrations

Under the same optimal conditions obtained for the free cell, cultivation was carried out for the cells immobilized in calcium alginate. The experiments were performed using an experimental methodology based on an experimental design used a particular modelling response surface RSM to optimize concentrations of alginate and calcium (CaCl₂). Responses were the ST and the index emulsification E24 (%).

Planning matrix, calculation of various parameters and statistical analysis were performed using the 6.0 MODDE software.
Biosurfactant functional characterization and application

The fermentation broth was centrifuged at 8000 rpm (D-78532 Tuttlingen, Hettich Zentrifugen, Germany) for 30 min to separate the biomass. The measurement of the ST was performed by the method of the ring broken away platinum with a ring tensiometer (Tensiometer K6, Krüss GmbH, Hamburg, Germany); the ST was measured for the obtained supernatant or the solution of biosurfactant using the du Nouy ring method [20]. pH was measured with a digital pH-meter (InoL, WTW, Germany). The emulsification is based on the ST. It was estimated by the emulsification index E24, that we put equal volumes of supernatant and hydrocarbon or oil (4 mL) in a test tube and mixed with a vortex (VTX 400, FRANCE) at maximum speed for 2 min. The emulsification index E24 [21] was determined after 24 h using the following Equation (1):

\[
E24\% = \frac{E}{E'} \times 100, \tag{1}
\]

where \( E \) is the length of the emulsified layer (mm), and \( E' \) the total length of the mixture (mm). The biosurfactants were recovered from the cell-free culture supernatants by cold acetone precipitation specific for glycolipid biosurfactant [22]. Three volumes of chilled acetone were added and allowed to stand for 24 h at 4 °C.

The presence of glycosidic groups in the molecule has been applied by rhamnose test using the method of Dubois et al. [23]. A volume of 1 mL of supernatant was added to 2 mL of 5% phenol reagent, and then 5 mL of pure sulphuric acid (98%) was added and incubated for 1 h at 65 °C [24]. Positive result was indicated by a reddish colour.

The foaming capacity was estimated by dissolving a part of the separated precipitate in distilled water using a test tube. The mixture was stirred manually for 30 s and left to stand for 5 min.

The CMC was the concentration beyond which the ST becomes constant. The CMC was determined by the dilution method by measuring the ST at each dilution, and then by tracing the surface of the tension curve as a function of the concentration of the solution of biosurfactant [25].

Using the rhamnose test described by Dubois et al. [23], it was found that the biosurfactant has carbohydrate moieties.

We have obtained infrared spectra using an infrared spectrophotometer Fourier transform (FTIR 8400 series, Shimadzu, Japan). Carbon tetrachloride (CCl4) was used to dissolve the biotensioactif (precipitate) and the analysis was performed at wavelengths between 500 and 4000 cm⁻¹.

Analysis by high-performance liquid chromatography was also carried out by dissolving the biosurfactant in acetonitrile (ACN), centrifuged and filtered, and then placed in a C18 column using a Shimadzu HPLC (LC-10ATyp pump with a UV detector SPD-10Avp, Japan). The surfactant was eluted under isocratic conditions at 1 mL min⁻¹ with a mixture of ACN and water (60:40, v/v) and detected at 280 nm [24]. The chromatographic analysis related to mass spectroscopy (LC-MS) was applied to better identify the biosurfactant product. The biosurfactant was dissolved in methanol. The LC-MS data were obtained using an Agilent 1100 series LC-MSD system coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. The chromatographic system was equipped with an automatic injector and a C18 reverse-phase column (2.1 × 150 mm). The HPLC analytical conditions were as follows: five microlitre of samples were injected at a split rate of 1:4. The column was eluted initially at a flow rate of 250 \( \mu \text{L min}^{-1} \) with a mixture of 25% ACN and 75% water, then by applying a linear gradient over 30 min to 50% ACN and 50% water and over 15 min to 90% ACN and 10% water. The column was then washed for 1 min, and ultimately re-equilibrated to 25% ACN and 75% water for 30 min. For electrospray ionization with positive ion polarity, the capillary voltage was set to 3.5 kV, the drying temperature to 350 °C, the nebulizer pressure to 40 psi, and the drying gas flow rate to 10 L min⁻¹. The maximum accumulation time was 50 ms, the scan speed was 26,000 mz⁻¹ s⁻¹ (ultrascan mode), and the fragmentation time was 30 ms.

Results and discussion

Preparation of calcium alginate beads and cells immobilization

The formation of beads was carried out with different concentrations of sodium alginate and calcium chloride. Optimal concentrations for obtaining a maximum yield of the biosurfactant are as follows: [alginate] equal to 5%, [CaCl₂] equal to 0.05 N and [biomass] equal to 0.148 g/100 mL alginate. With these concentrations we obtained very consistent beads and a good yield of biosurfactant.

Kinetics of biosurfactant production

We followed the kinetics of surfactant production for concentrations previously chosen. Figures 1 and 2
summarize the results obtained by measuring the surface tension and E24 for 120 h.

The ST reaches the minimum value (32.78 dyn/cm) with a maximum of emulsification index E24 equal to 93% after 72 h unlike the case of free cells or there was an increase after 48 h and decrease of E24, and after 48 h, the maximum biosurfactant quantity was produced for free cells (Figure 1), while to those immobilized, the maximum amount of the same biosurfactant was obtained after 72 h (Figure 2). It can be explained by the limitation in the case of the immobilized cells and there is a change in cell physiology following the fixing to the support in the optimal conditions [26] and the accumulated backlog for the production of a sufficient quantity of biosurfactant in comparison with the free cells is due to diffusional limitations of nutritious elements and oxygen inside the alginate beads and biosurfactant diffusing to the outside [27].

**Biosurfactant isolation, characterization and application**

The biosurfactant was precipitated by acetone, adding to the supernatant three equal volumes of acetone in an iced water bath, and allowed to stay for 24 h at 4 °C and centrifuged (200 r/min) (Centrifuge D-78 532 Tuttlingen). The biosurfactant obtained is dried in an oven (D-91126 Schwa Bach FR G) at 37 °C.

Production yield compared to the concentration of hexadecane biosurfactant was 1.616 g/g of hexadecane. This amount of surfactant is acceptable by comparing to other productions yields [28].

The precipitate obtained from the culture broth of isolate was a white crystalline powder, soluble in distilled water and/or methanol. The production yields using the biosurfactant production protocol described in the "Materials and methods" section was 2.0 g L⁻¹ on the basis of dry weight. These results are in accordance with those of Haba et al. [29]. Consistent with the data reported by Lotfabad et al. [30], both surfactants exhibited a high foaming power.

Rhamnose test based on the method of Dubois et al. [23] was positive, which indicates that the isolated biosurfactant can be glycolipids. Aqueous solutions of biosurfactant showed a good foaming stability. Total disappearance of the foam was detected after 2 h. The ST decreases with successive dilutions of biosurfactants (Figure 3), until the lowest value of ST which is equal to 31 dyn cm⁻¹ for a biosurfactant concentration greater than or equal to 1.5 g L⁻¹ for the isolated biosurfactant. These values define the CMC for the biosurfactant.

A positive result for rhamnose test. The deformation vibration at 1461 cm⁻¹ (Figure 4) confirmed the presence of alkyl groups. The valence carbonyl band was found at 1700 cm⁻¹ which is a characteristic of the ester compounds. The carbonyl group of ester has also
appeared at 1310 cm$^{-1}$, which corresponds to the deformation vibration of C–O, confirming that Desai and Banat [22] and Ron and Rosenberg [31] proved almost all biosurfactants have an ester group or carboxylic acid group in their structures. The adsorption bands located at 2723 and 1377–1458 cm$^{-1}$ (Figure 4) show that both products have a chemical structure identical to that of glycolipids. The peak of adsorption present at 2723 cm$^{-1}$ is that of the C–H stretching vibration of hydrocarbon chains, knowing that all classes of biosurfactants contain. Characteristic peak appears at 1774 cm$^{-1}$ that is relevant to the stretching vibration of C=O carbonyl group, while the bands from 1458 to 1377 cm$^{-1}$ confirms the presence of the bonds formed between the carbon atoms and the hydroxyl groups in the chemical structures of the glucosidic part.

From the obtained chromatogram (Figure 5), it is known that the biosurfactant produced by the bacterial strain O. intermedium is a mixture of two biosurfactants and based on the analysis by FTIR and rhamnose test, a high probability that they are rhamnolipids. One other work has shown that the product obtained is a mixture of rhamnolipids synthesized by Pseudomonas aeruginosa [32].

Analysis of the biosurfactant product by LC-MS (Figure 6) shows two peaks having the same molecular weight of 328 and fragmentation pattern at m/z 328.9, 312.8, 250.8, and 207.8.

The structural characterization has allowed us to identify preliminarily our biosurfactant. The biosurfactant produced by the strain is a glycolipid having as a mass 328 (fragmentation: 328/312/250/207)

(a) 328–312, the release of oxygen (16)
(b) 312–250, the release of a molecule of H$_2$O (18) with CH$_3$–CH$_2$–CH$_3$ bonds (44)
(c) 250–207, a release CH$_2$–CH$_2$–CH$_3$

The proposed structure is shown in Figure 7.

The ST of the biosurfactant was thermostable (average of 31 dyn cm$^{-1}$ at 20 and 100 °C) as shown in Figure 8(a). But emulsification index is slightly influenced by the change of temperature. These results show that our biosurfactant is stable comparing it with a synthetic surfactant giving a 70% of the emulsification index [33].

The ST of biosurfactant was stable from pH 4 (ST = 31.7 dyn cm$^{-1}$) to pH 11 (ST = 32.2 dyn cm$^{-1}$) (Figure 8(b)).
The effect of pH on the ST has been studied for biosurfactant produced by various microorganisms [34]. Similarly, the ST of liposan produced by *Candida lipolytica* remained stable between pH 2.0 and 5.0 [35], and that of emulsan produced by *Acinetobacter calcoaceticus* RAG-1 was stable between pH 5.0 and 6.0. With regard to E24, values were optimal from pH 7 to 11 (E24 average of 95), but they decreased at acid pH (70.58%). Similar results were obtained for biosurfactant produced by *P. aeruginosa* MR01 [30] comparison; the synthetic surfactant SDS maintains its emulsifying capacity for pH values ranging between 4.0 and 9.5. Emulsification capacity was very sensitive to the pH changes [36].

On the other hand, Figure 8(c) shows that the addition of NaCl in the range of concentrations tested (5–100 g L\(^{-1}\)) had only a weak effect on ST of the biosurfactant (average ST = 31 dyn cm\(^{-1}\)). This indicates that the biosurfactant is effective in the presence of monovalent ions (Na).

However, the E24 values decreased when the salt concentration was increased from 5 to 100 g L\(^{-1}\) (Figure 8(c)). There are reports that the presence of salts results in disruption of emulsions of oil and water, thus affecting the ST and emulsifying ability of surfactants [37]. This also indicates that biosurfactants produced by isolate *O. intermedium* is effective in the presence of monovalent ions (Na), while 20–30 g L\(^{-1}\) of salt is often sufficient to deactivate chemical surfactants [31,38].

From the results obtained, we noticed that the concentration of naphthalene and phenanthrene increased after the addition of biosurfactant. Then, biosurfactant products are able to solubilize the naphthalene and phenanthrene, with naphthalene solubilization rate greater than that of phenanthrene. This is due to the chemical structure of PAHs.

The solubility of naphthalene and phenanthrene in the solution increased more than 3 times and 1.5 times, respectively, compared to its value in the absence of biosurfactant.

Li and Chen [39] have shown that chemical surfactants (Triton X-100, Tween 20 and Tween 80) and a non-ionic biosurfactant (Tergitol) solubilized phenanthrene, also Ferradji et al. [40] proved the solubilisation of phenanthrene by biosurfactant.

Wong et al. [41] produced a biosurfactant by *A. calcoaceticus* BU03, which dissolved phenanthrene. Similarly, the solubilization of naphthalene using chemical surfactant (TX-100, CTAB) has been proven by Rao [42].

![Figure 5. Chromatogram of the biosurfactant produced by the strain *Ochrobactrum intermedium*.

![Figure 6. LC–MS analysis of the biosurfactant produced by the strain *Ochrobactrum intermedium*.

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and the biosurfactants produced by *Pseudomonas* spp. are able to solubilize naphthalene at neutral pH than the SDS and Triton. In general, the biosurfactant produced by *O. intermedium* showed potential application in petroleum sludge treatment, and environmental remediation [43].

The weight solubilisation ratio (WSR) value is defined as the amount of solubilized hydrocarbon per amount of surfactant, and hence corresponds to an increase in solubilize concentration per unit increase in micellar surfactant concentration. In the presence of an excess of hydrophobic organic compounds, the WSR is as in Equation (2) [44]:

\[
WSR = \frac{S_t - S_{cmc}}{C_s - CMC},
\]

where \(S_t\) is the apparent solubility of solute at a particular surfactant concentration greater than the CMC, \(C_s\) is the surfactant concentration at which \(S\) is evaluated, and \(S_{cmc}\) is the apparent solubility of PAH at the CMC. All concentrations are expressed in g L\(^{-1}\).

The highest solubility of naphthalene (0.175) and phenanthrene (0.054) was obtained for 2 g L\(^{-1}\) biosurfactant, and then decreased for increasing biosurfactant concentration. For lower values of the CMC, we noticed a decrease in the solubility of PAHs and we had obtained values equal to the solubility in water (32 mg L\(^{-1}\)). It's

![Proposed structure of the biosurfactant.](image)

**Figure 7.** Proposed structure of the biosurfactant.

![Influence of temperature on biosurfactant activity shown by E24 (◆) and surface tension (■).](image)

(a)

![Influence of pH on biosurfactant activity shown by E24 (◆) and surface tension (■).](image)

(b)

![Influence of salinity on biosurfactant activity shown by E24 (◆) and surface tension (■).](image)

(c)

**Figure 8.** (a) Influence of temperature on biosurfactant activity shown by E24 (◆) and surface tension (■). (b) Influence of pH on biosurfactant activity shown by E24 (◆) and surface tension (■). (c) Influence of salinity on biosurfactant activity shown by E24 (◆) and surface tension (■).
because of the existence of the monomers only, while from the CMC training micelles and then the aggregates allowed to have a better solubility. Thus, the excessive accumulation of biosurfactants at the interface facilitated interfacial tension reductions, resulting in higher solubility of the PAHs.

It is found that the production of biosurfactants is achieved by free and immobilized cells from the bacterial strain O. intermedium isolated from soil contaminated by hydrocarbons using hexadecane as carbon source and ammonium chloride as nitrogen source. The product exhibited a high level of thermal stability, a positive effect for increasing pH, and demonstrates a high level of tolerance to ionic strength. The procedure by immobilized cells showed production kinetics difference (72 h) caused by diffusional limitations.

This study showed that O. intermedium entrapped in calcium alginate beads is able to preserve its viability and produce biosurfactant with a sodium alginate concentration of 5%, a concentration of calcium chloride to 0.05 N, and 0.148 g of biomass in 100 mL of sodium alginate.

The solubility of naphthalene and phenanthrene was proven by calculating the WSR starting from the CMC biosurfactant. We can compare WSR values to those obtained with synthetic surfactants, such as SDBS (0.039), Triton X-100 (0.074) and RWS (0.016) at pH 7 [4]. Therefore, we suggest that biosurfactant can potentially and successfully be used for the remediation of soils contaminated with PAHs in the industrial sites.

Conclusions

O. intermedium strain was able to excrete biosurfactant with interesting surface-active properties for both free and alginate-entrapped cells in batch cultures. The effect of diffusional limitations of substrates and product observed with immobilized cells affected the production kinetics, since the required time to attain maximum yield was delayed. However, the emulsion stability was improved and less interfered by-products with the biosurfactant activity. The immobilization of cells was beneficial for biosurfactant recovery, if compared to previous work with free cells [5]. Any work on the optimization of biosurfactants production by cells immobilized in pilot scale could be of great interest before proceeding with large scale.

Purified biosurfactant had a CMC 1.5 g L⁻¹, which was much lower than that of chemical surfactants. Crude biosurfactants have also shown strong tolerance with a slight difference in heat, pH and salinity which shows clear perspectives for its use in extreme environmental conditions in bioremediation, pharmaceutical formulations and other industrial fields.

According to FTIR and LC-MS analyses and glycolipid test, the predicted probable chemical components of this biosurfactant were glycolipid compounds.

The findings in solubilization effect of biosurfactant demonstrated that this biosurfactant could enhance the water solubility of naphthalene and phenanthrene about 3 times and 1.5 times, respectively, compared to its value in the absence of biosurfactant. Therefore, we suggest that biosurfactant can potentially and successfully be used for the remediation of soils contaminated with PAHs in the industrial sites.

Disclosure statement

No potential conflict of interest was reported by the authors.

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