Solubilisation of naphtalene and phenanthrene by biosurfactant product by *brevibacterium lutescens*

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**ABSTRACT**

In this paper, biosurfactant production by free and immobilized cells of *brevibacterium lutescens* 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, emulsification index E24 during one week at 37°C and neutral pH. For the production by immobilised cells, the concentration of sodium alginate, calcium chloride and biomass were optimised. Results showed that *brevibacterium lutescens* entrapped in calcium alginate beads is able to preserve its viability and produce biosurfactant. An affection of the kinetics on the production due to diffusional limitations of the alginate beads with greater stability up to 75%. The product biosurfactant reduced the surface tension below 32-33 and emulsification index were 68-93% after 48 and 72h with free and immobilized cells, respectively. Also, the product showed stability in a wide range of pH (2-12), temperature (25-120°C), and to high salinity. Both products by strain *brevibacterium lutescens*, based on spectral features, have a chemical structure identical to that of glycolipids. The production yield of biosurfactant versus concentration of the hexadecane is 1.292g/g.

In second, the surfactants are able to promote the solubility of HAPs. Resulted show that the use of biosurfactant, produced by the isolated bacterial strain *Brevibacterium lutescens*, obtaining a better solubility of naphthalene and phenanthrene.

**Keywords:** biosurfactant , *brevibacterium lutescens*, immobilizad cells, surface tension, emulsification index, weight solubilization ratio.
1. 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 capable of reducing the surface 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 application of 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 chemical and are 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 to foam formation, greater selectivity, specific activity to extreme temperature, pH and salinity, the ability to synthesize from renewable substrates and ecological acceptability [3]. Although best known biosurfactants are of bacterial origin, such as reported by different authors [4-5-6-7], the study biosurfactant production by bacteria has grown, production is reported mainly by Candida sp, Pseudomonas sp. and Yarrowia sp [8-9-10].

Optimizing the production of biosurfactant appears to be main research areas, due to low product yields. For this purpose, the adequate culture medium, and the optimal conditions and the mode of culture, are of major importance. Because of the small quantities produced, the recovery of biosurfactant 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 diffusionelles limits [12-13]. Immobilized cells represent one of the current methods aim the delivery of organic products and alginate material is usually employed for this purpose and this method is known as nontoxic and economic method, which was that very used to produce biosurfactant [14-15].

Les hydrocarbures aromatiques polycycliques (HAP), qui sont distribués de façon ubiquitaire dans les sols et connu pour être cancérigène, tératogène et mutagène, sont toxiques pour les humains et peut être nocif pour les organismes du sol et plantes par transfert trophique [16]. Due to the low solubility in water and a high affinity to organic matter, PAHs are difficult to remove from the environment and the soil can a long-term source of groundwater contamination [17].

The presence of surfactants can increase the solubility of PAH and thus potentially increase their bioavailability [18] and can increase the apparent solubility of hydrophobic hydrocarbons by solubilizing them in the hydrophobic core of micelles. When the surfactant is above a certain concentration (critical micelle concentration, CMC) [19], (in this study we compared the production of a biosurfactant by free and immobilized cells by characterization of the product, in addition to the solubilistion naphthalene and phenantherene.

2. METHODOLOGY

2.1 Culture media

The bacterial strain used to produce the surfactant is brevibacterium lutescens isolated from soil contaminated by oil from the region hassi messaoud (Algeria) according to Ferhat et al., 2011 [5]. It was maintained on nutrient agar.

2.2 Cultivation of organism and production of the biosurfactant

For the preparation of the inoculum, 50 ml of a preculture was used with a concentration of 8g. 1^1. The preculture contains (in g. 1^1): carne extract 5.0, Peptone gelatin 10.0, and NaCl 10.

The cultures grow in this medium for 24h at 37^C. The latter was used as inoculum to 8% (v / v) and then developed in an inorganic culture medium containing (in g. 1^1):
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), 1ml of Oligo elements solution, the latter has the composition in mg.l⁻¹: 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.

1° containing hexadecane (2%, v/v) as the carbon source at 37°C and at 200 rpm in shaker (KRUSS KG, Germany) during 7 days. The culture broths were centrifuged for 15 min at 4500 rpm to remove the cells samples were withdrawn every 24h for analyze.

### 2.2. Cells immobilisation

The procedure of cell immobilization in calcium alginate follows the following steps:

A preculture was launched during 48 hours and is 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 was mixed with of a sterile solution of alginate 4% (w/v), then gradually added to the CaCl₂ solution by a sterile syringe, a separation of the beads from the solution is carried out by filtration, washed with sterile distilled water, and stored in the culture medium at 4 °C in flasks firmly closed or used for immobilized cells experiments.

### 2.3. Optimizing concentrations

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

Planning matrix and the calculation of the various parameters and the statistical analysis was performed using the 6.0 MODDE software.

### 2.4. biosurfactant functional characterization and application

The fermentation broth is centrifuged at 8000 rpm (D-78532 Tuttingen , Hettich Zentrifugen, Germany) for 30 minutes to separate the biomass. The measurement of the surface tension was performed by the method of the ring broken away platinum with a ring tensiometer (Tensiometer K6, Krüss GmbH, Hamburg, Germany), the surface tension is measured for the obtained supernatant or the solution of biosurfactant using the du Nouy ring method [20]. The pH was measured with a digital pH-meter (Inol., WTW, Germany). The emulsification is based on the surface tension. It is estimated by the emulsification index E24, we put equal volumes of supernatant and hydrocarbon or oil (4ml) 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 24h using the following equation (1):

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E24 = \frac{E}{E'} \times 100
\]

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. (1956) [23]. A volume of 1 ml of supernatant was added to 2 ml of 5% phenol reagent, and then 5 ml of pure sulfuric acid (98%). Incubated for 1 hour at 65 °C [24]. A positive result is indicated by a reddish color.

The foaming capacity was given by dissolved in distilled water in a test tube an amount of the separated precipitate. The mixture was stirred manually for 30 seconds and left to stand for 5 minutes.

The critical micelle concentration is the concentration, beyond which the surface tension is constant. The critical micelle concentration (CMC) was determined by the dilution method by measuring the surface tension 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].

The stability characterization was also studied, then how the activity of biosurfactant was affected by different temperature, pH and concentration of NaCl, so for 15 min at different temperatures: 20, 50, 70, 100 and 120 ° C, the surface tension and the index emulsification were measured for evaluated a thermal stability. Likewise the chemical stability was investigated as the influence of pH.
(2 to 11 for 15 minutes) and the influence of NaCl added to the supernatant at different concentrations (5, 15, 50 et 100 g/l) by measuring the surface tension and the index emulsification after 30 min.

The presence of carbohydrate moieties in the molecule has been applied by rhamnose test using the method of Dubois et al. (1956) [23]. A volume of 1 ml of supernatant was added to 2 ml of 5% phenol, followed by addition of 5 ml of pure sulfuric acid (98%). Incubated for 1 hour at 65 °C [24]. A positive result is indicated by a reddish color.

3. RESULTS AND DISCUSSION

3.1 Preparation of calcium alginate beads and cells immobilisation

The formation of beads was carried out with different concentrations of sodium alginate and calcium chloride. Optimal concentrations for obtaining a maximum yield of biosurfactant are: [alginate] equal to 5%, [CaCl$_2$] equal to 0.05N and [Biomass] equal to 0.148 gr / 100 ml alginate. This indicates to the beads have a good consistency for a good yield of biosurfactant.

3.2 Kinetics of biosurfactant production

We followed the kinetics of surfactant production for concentrations previously chosen. The figures 1 and 2 summarizes the results obtained by measuring the surface tension and E24 for 120 hours. Surface tension ST reaches the minimum value (32.78 dyn / cm) with a maximum of emulsification index E24 equal to 93% after 72h unlike the case of free cells or there was increased after 48 hours and decreased of E24 and after 48 hours the maximum biosurfactant quantity is produced for free cells (fig. 1) while to those immobilized the maximum amount of the same biosurfactant was obtained after 72h to (fig. 2). It can be explained by in the case of the immobilized cells is limited 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].

Fig.1. Biosurfactant production kinetics by free cells.
3.3 Biosurfactant isolation, characterization and application

The biosurfactant was precipitated by acetone, adding to the supernatant three equal volume of acetone in an iced water bath, allow 24 hours at 4 °C and centrifuged (200 tr / min) (Centrifuge D-78532 Tuttlingen). Le biosurfactant obtenu est séché dans une étuve (D-91126 Schwa Bach FR G) à 37 °C.

Production yield compared to the concentration of the hexadecane biosurfactant is 1.292 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. (2000) [29]. Consistent with the data reported by Lotfabad et al. (2009) [30] both surfactants exhibited a high foaming power.

Rhamnose test based on the method of Dubois et al. (1956) [23] was positive, which indicates that the isolated biosurfactant can be glycolipids. Aqueous solutions of biosurfactant showed good foaming stability. Total disappearance of the foam was detected after 2 h. The surface tension decreases with successive dilutions of biosurfactants (fig. 3), until the lowest value of surface tension which is equal to 32 dyne.cm-1 for a biosurfactant concentration greater than or equal to 2 g. l-1 for the isolated biosurfactant. These value define the CMC for the biosurfactant.

![Fig.2. Biosurfactant production kinetics by entrapped cells.](image)

![Fig.3. Critical micellar concentration of the biosurfactant.](image)
produced by various microorganisms [32]. Similarly, the surface tension of liposan produced by Candida lipolytica remained stable between pH 2.0 and 5.0 [33] (Cirigliano and Carman, 1985), and that of emulsan produced by Acinetobacter calcoaceticus RAG-1 was stable between pH 5.0 and 6.0. In regards to E24, values were optimal from pH 4 to pH 7 (E24 average of 95%) and they decreased at basic pH (pH = 11: E24 = 32.2%). These results are similar to those obtained by Pornsunthorntawee et al. (2008) [34]. Emulsification capacity was very sensitive to the pH changes [35]. On the other hand, the biosurfactant produced by strain Brevibacterium lutescens shows, the E24 values were optimal from pH 4 to pH 7 (E24 average of 95%) and they decreased at basic pH (pH = 11: E24 = 32.2%). Similar results were obtained for biosurfactant produced by P. aeruginosa MR01 [30]. In comparison, the synthetic surfactant SDS maintains its emulsifying capacity for pH values ranging between 4.0 and 9.5.

Figure 4c shows that the addition of NaCl in the range of concentrations tested (5 g. l\(^{-1}\) to 100 g. l\(^{-1}\)) had only a weak effect on surface tension of biosurfactant (average ST = 31 dyne.cm\(^{-1}\)). This indicates that biosurfactants is effective in the presence of monovalent ions (Na). While 20-30 g. l\(^{-1}\) of salt is often sufficient to deactivate chemical surfactants [32-36]. However, the E24 values decreased when the salt concentration was increased from 5 to 100 g. l\(^{-1}\) (Fig. 4c). There are reports that the presence of salts results in disruption of emulsions of oil and water, thus affecting the surface tension and emulsifying ability of surfactants [37]. This also indicates that biosurfactants produced by isolate Brevibacterium lutescens is effective in the presence of monovalent ions (Na). While 20-30 g l\(^{-1}\) of salt is often sufficient to deactivate chemical surfactants (Abu-Ruwaida et al., 1991; Banat, 1995),
Fig. 5a. Influence of salinity on biosurfactant activity.

The results obtained, we noticed that the concentration of naphthalene and phenanthrene increased after the addition of biosurfactant. Then biosurfactants products are able to solubilize the naphthalene and phenanthrene, with a naphthalene solubilization rate greater than 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 (2002) [38] have shown that chemical surfactants (Triton X-100, Tween 20 and Tween 80) and a nonionic biosurfactant (Tergitol) solubilized phenanthrene. WONG et al. (2010) [39] produced a biosurfactant by Acinetobacter calcoaceticus BU03, which dissolved phenanthrene. Similarly, the solubilization of naphthalene using chemical surfactant (TX-100, CTAB) has been proven by Rao (2009) [40] and the biosurfactants produced by Pseudomonas spp. are able to naphthalene solubility at neutral pH than the SDS and Triton. The 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 compound, the WSR is [41] in equation (2):

$$WSR = \frac{St - Scmc}{Cs - CMC}$$

where $St$ is the apparent solubility of solute at a particular surfactant concentration greater than the $CMC$, $Cs$ is the surfactant concentration at which $S$ is evaluated, and $Scmc$ 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 PAH and we had obtained equal to the solubility in water (32 mg. l$^{-1}$). this is due to the existence of 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 PAH.

4. CONCLUSIONS

The production of biosurfactants is achieved by free and immobilized cells from the bacterial strain Brevibacterium lutescens isolated from soil contaminated by hydrocarbons using hexadecane as carbon source and ammonium chloride, nitrogen source. Crude biosurfactants have also shown strong tolerance with a slight differences of heat, pH and salinity. The procedure by immobilized cells showed production kinetics difference (72h) caused by diffusional limitations. Immobilization of cells was useful for retrieving biosurfactant, when compared to earlier work with free cells (Ferhat et al. 2011). This study showed that Brevibacterium lutescens 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 0.05N, and 0.148g 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 compared 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.

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6. REFERENCES


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