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PRODUCTION OF BIOSURFACTANT FROM A NOVEL ISOLATED STRAIN *BACILLUS AMYLOLEQUIFACIENS* K2E

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Abstract

Subject description: The functional and friendly-like ecological characteristics of biosurfactants allowing their exploitation in various fields. Biosurfactants show potential for application in the food industry, which they can be successfully used in food processing.

Objective: The purpose of this study is to carry out a study of a production of a biosurfactant using a locally new isolated bacterial strain.

Methods: The phylogenetic analysis, FTIR analysis, surface tension measurements, oil displacement test and the rheological test were the most important methods used in this study.

Results: The produced biosurfactant showed a good stability at extreme environmental conditions [temperatures (from 0 to 120 °C), pH (2–12) and salinity (up to 200 g/L)], over a time period up to three years. The phylogenetic analysis showed that strain K2E was very closely related to *Bacillus amylolequifaciens* (identity of 98.28%).

Conclusion: In the current study, in total, 77 hydrocarbon-degrading bacteria previously isolated from contaminated soil in the Algerian desert (Hassi-Messaoud, petroleum region), were individually screened for biosurfactant production. The strain K2E showed a particular performance in production of potent biosurfactant, able to reduce surface tension (ST) from (72 to 30.32 mN/m). This biosurfactant was produced by culturing producer strain in mineral salt medium (MSM) using olive oil (1%) as the only source of carbon and energy, yielded a maximum biosurfactant concentration (0.8 g/L) and having a critical micelle concentration (CMC) (0.75 g/L). The product obtained by dichloromethane extractor, is linked in lipopetide biosurfactant group as indicated by Fourier Transform Infrared (FTIR) spectroscopy. The obtained biosurfactant will find applications in the agri-food and environmental sectors.

Keywords: Biosurfactant, Production, Characterization, *Bacillus amylolequifaciens*.

PRODUCTION DE BIOSURFACTANT À PARTIR D'UNE NOUVELLE SOUCHE ISOLÉE *BACILLUS AMYLOLEQUIFACIENS* K2E

Résumé

Description du sujet : Les caractéristiques écologiques fonctionnelles des biosurfactants permettent leur exploitation dans divers domaines. Les biosurfactants présentent un potentiel d'application dans l'industrie alimentaire, où ils peuvent être utilisés avec succès dans la transformation des aliments.

Objectifs : Le but de cette étude est de réaliser une étude sur la production d'un biosurfactant en utilisant une nouvelle souche bactérienne isolée localement.

Méthodes : Dans la présente étude, au total, 77 bactéries dégradant les hydrocarbures précédemment isolées de sols contaminés dans le désert Algérien (Hassi-Messaoud, région pétrolière), ont été individuellement criblées pour la production d'un biosurfactant. Le produit obtenu par extraction au dichlorométhane, est lié au groupement lipopétide comme l'a indiqué la spectroscopie infrarouge à transformée de Fourier (FTIR)

Résultats : Une souche (appelée K2E) a montré une performance particulière dans la production d'un puissant biosurfactant, capable de réduire la tension superficielle (TS) de (72 à 30,32 mN/m). L'analyse phylogénétique a montré que la souche K2E était très proche de *Bacillus amylolequifaciens* (similitude de 98,28 %). Ce biosurfactant a été produit en cultivant la souche K2E dans un milieu de sels minéraux (MSM) en utilisant de l'huile d'olive (1%) comme seule source de carbone et d'énergie, a donné un rendement maximal de biosurfactant (0,8 g/L) et ayant une concentration micellaire critique (CMC) (0,75 g/L). Le biosurfactant produit a montré une bonne stabilité dans des conditions environnementales extrêmes [températures (de 0 à 121 °C), pH (2–12) et salinité (jusqu'à 200 g/L)], sur une période allant jusqu'à trois ans.

Conclusion :. Globalement, les résultats mettent en évidence les potentialités d'utilisation dans les préparations agro-alimentaire et dans le secteur environnemental.

Mots clés : Biosurfactant, Production, caractérisation, *Bacillus amylolequifaciens*

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INTRODUCTION

Environmental issues associated with biotechnological advancements create a notable market that can be an alternative to "greener" technologies. Over the years, more sustainable development involving the use of biological surfactants has been implemented [1-3].

The biosurfactants are produced by bacteria, fungi and yeasts [4-6]. Based on the biosurfactant-producing microbial species and the chemical structures, they can be divided into four groups: lipopeptides and lipoproteins, glycolipids, phospholipids, and polymeric surfactants [7-11]. These biomolecules own surface activities properties [12-14], and they are highly recommended for enhanced oil recovery [11,15]

The main drawbacks of the large-scale production of biosurfactants are low yields and high production costs [3]. Instead, the use of cheap substrates decreases for 10 to 30% of the total production cost [9, 16-18]. They possess several advantages over synthetic chemical-derived surfactants which include low toxicity, bioavailability, biodegradability, high foaming, environment-friendly, low cost in terms of feedstock availability in nature. The wide and varied potential applications of biosurfactants and their properties are very interesting which several authors described in detail their properties and their applications: In biotechnological industries (petroleum, food, as a biocontrol agent in agricultural applications, in downstream processing for bioprocessing applications, in health and beauty products for the cosmetic industries) and bioremediation [19]. Among these, lipopeptides are effective biosurfactants which are usually produced by *Bacillus*, *Pseudomonas*, and *Staphylococcus* strains [20-22]. The select criterion used of biosurfactants producers is the ability to reduce surface tension below 40 mN/m while the emulsifies producers are the the ability to maintain at least 50% of the emulsion original volume for 24 hours [2].

Hydrocarbon-contaminated sites are promising biotopes for isolating microorganisms, including bacteria, with high biosurfactant production capacity. For this purpose, a collection of bacterial strains, isolated from Algerian soil contaminated with crude oil and which have already shown the ability to degrade compound oil and olive oil, was set up to invest in applied research in the field of environmental and agro-food biotechnology [21,22].

The objective of this study was to characterize a biosurfactant produced by *Bacillus amylolequifaciens* K2E newly isolated in previous work in our laboratory. Accordingly, this study focused on the screening of hydrocarbonoclastic bacteria with biosurfactant production capacities

MATERIALS AND METHODS

1. Bacterial strains

Bacterial strains were isolated from a crude-oil-contaminated soil as described by Eddouaouda [2]. Roughly, 77 hydrocarbonoclastic, mesophilic and thermophilic bacteria with potential surfactant activity were obtained. Soil samples were collected from the desert at Hassi Messaoud-Ouargla region in Algeria (GPS coordinates: 6° 04′ 21" East, 31° 40' 57" North). The samples were transferred directly to sterile bottles and stored in the dark at +4 °C until analysis.

2. Media and conditions culture

- Luria Bertani (LB): 10 g peptone, 5 g yeast extract, 23 g NaCl in 1 liter of distilled water. The pH was adjusted to 7±0.2 with a solution of 1 M NaOH.
- Mineral salt medium (MSM) (in 1 L distilled water): 23 NaCl, 0.4 NH_4Cl, 0.3 KH_2PO_4, 0.3 K_2HPO_4, 0.33 $MgCl_2$, 0.05 $CaCl_2$ and 1 mL of trace metals containing (mg/L distilled water): 1500 $FeCl_2$ $4H_2O{\cdot}CoCl_2$ $6H_2O$ 190, 100 $MnCl_26H_2O$, 70 $ZnCl_2{\cdot}6H_3BO_3$, 36Ma $2MoO_4{\cdot}2H_2O$ and 6.7 mL HCl 35%. All media were autoclaved at 121°C for 20 minutes [15].

2.1. Screening of biosurfactant-producing bacteria

The strain K2E, among 100 strains tested, had shown a very interesting potential for biosurfactant production. The culture was carried out in 250 mL vials containing 100 mL of MSM added with 1% olive oil as the sole source of carbon and energy and consistently maintained at 45 °C and 150 $trmin^{-1}$ for 48 h. The culture medium was centrifuged at 6,000×*g* for 20 min and the detection of biosurfactant production by this microorganism was determined by measuring with a DuNouy Tensiometer model Sigma 700 (KSV Instruments LTD, Finland) and was used as a tool to select the carbon source in this experiment [23].

2.2. Selection of the optimal carbon source for biosurfactant production

Four carbon sources were used in this study to select the optimal one for maximum biosurfactants production: Glucose (1%, v/v), olive oil (1%, v/v), glycerol (1%, v/v), and olive oil mill (1%, v/v). All substrates were sterilized using a 0.22 µm filter. Surface tension reduction was measured as described above.

3. Identification of the strain K2E

Analytical profiling index (API) strip tests and 16S rRNA (rDNA) gene sequencing were carried out for the identification of the strain K2E. The nature of Gram staining, motility in hanging drop preparations, and physiological and biochemical characteristics of the strain K2E were investigated using API 50 CH strip in accordance with the manufacturer's instructions (bioMérieux, SA, Marcy-l'Etoile, France). The colony morphologies were done using cultures grown aerobically on nutrient agar. Cell morphology, motility, spore formation and presence of flagella were examined microscopically on fresh liquid cultures during exponential-phase after 18–24 h of incubation at 45 °C. Optimum temperature range for growth was determined by incubating the strain at temperatures ranging between 15 and 50 °C (in 5 °C increments) under aerobic conditions. The pH range for growth was examined in a range of pH values from 6 to 10. The effect of NaCl on bacterial growth was evaluated in presence of 0 to 25 % (w/v) NaCl. All the physiological tests were determined in nutrient agar medium. The polymerase chain reaction (PCR) amplification of the 16S rDNA gene was carried out with two universal primers, one forward and the other reverse, designed from the conserved zones within the rRNA operon of *E. coli* [24]. The forward primer (27F) was 5'-AGAGTTTGATCCTGGCTCAG-3' extended from base position 8 to 27; the reverse primer (1525R) was 5'-AAGGAGGTGATCCAAGCC-3' extended from base position 1,541 to 1,525. The genomic DNA of strain K2E was purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and then used as a template for PCR amplification (35 cycles, 94 °C for 35 s denaturation, 65 °C for 40 s primer annealing, and 72 °C for 60 s extension). The amplified ~1.5 kb PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), leading to pKL-16S plasmid (this study). The *E. coli* DH5α (F⁻*supE44 Φ80 δlacZ ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17* (r_k^-, m_k^+)*deoR thi-1* λ^- *gyrA96 relA1*) (Invitrogen, Carlsbad, CA, USA) was used as a host strain. All recombinant clones of *E. coli* were grown in LB broth media with the addition of ampicillin (100 µg/mL), isopropyl-thio-β-D-galactopyranoside (IPTG) (0.67 mM) and X-gal (360 µg/mL) for screening. DNA electrophoresis, DNA purification, restriction, ligation, and transformation were all performed according to the method previously described elsewhere [25].

3.1. Nucleotide sequence accession number

The nucleotide sequence data of *16S* rRNA (1522 bp) gene reported in this paper has been submitted to the DDBJ/EMA/GenBank databases under accession no. of **MW186767**.

3.2. Detection of biosurfactant production

To evaluate the production of biosurfactants by the strain K2E while using olive oil as substrate, emulsification activity, surface activities, , and hemolytique activity of the crude biosurfactant were determined as following.

3.2.1. Emulsification activity (E24)

A quantity of each biosurfactant was dissolved in distilled water in a test tube. The solution was manually agitated for 30s, and left to rest for 5 min. In order to assess the emulsifying power of the biosurfactant from strain K2E, it has been tested against four types of hydrophobic liquids, namely crude oil, gas oil, toluene, olive oil and vegetable oil. The mixture is stirred well at the vortex for 2 min (to obtain a homogeneous mixture), then let rest for 24 h before taking the measurements [2]. E24 (%) = Height of emulsion (cm) / (Height of the total mixture (cm) × 100

3.2.2. Surface activities

-Oil displacement test

Oil displacement experiment was performed as reported elsewhere [26]. To a Petri plate containing 20 mL of distilled water and 20 μL of crude petroleum oil were added, forming a thin film on the water surface. Then, 10 μL of culture supernatants was gently deposited on to the center of the oil film. If the biosurfactant is present, the oil will be displaced with an oil free clearing zone, the diameter of this clearing zone indicates the oil displacement activity of the biosurfactant. Distilled water was employed as a negative control.

-Surface tension measurement

To determine the surface activities of the obtained supernatants, ST measurements were carried out. The "Du-Nouy-Ring method" as described elsewhere [27-30]. was used to determine the surface tension of the supernatant. The ST was measured by means of a tensiometer with the ring method, using a platinum ring (De Nouy) with 3 cm in diameter at 25 °C. The ring was placed just below the surface of the supernatant solutions. The presence of biosurfactant in the solution was confirmed based on a decrease in ST of the supernatants against a control (distilled water). All measurements were repeated three times and their mean values were taken.

3.2.3. Hemolytic activity

Currently, many researchers use this technique to select new biosurfactant-producing isolates [15,31].

-Preparation of blood agar

Put a bottle of nutrient agar (200 mL) to liquefy in a water bath (90 °C), once liquefied it is allowed to cool a little to room temperature. Add 5 mL of sheep blood to the vial, mixing gently to prevent the cells from bursting. Pour into Petri dishes and let solidify at room temperature.

-Seeding

Using a sterile needle, do a central injection inoculation from the culture of the bacterial strain previously prepared, and reserve a Petri dish as a negative control. The dishes are incubated at room temperature. The reading is taken after 24 to 72 h in order to observe a positive hemolytic activity [32].

3.3. Biosurfactants extraction

The culture stock was centrifuged at 6,000 × *g* for 20 min to remove the cells. The clear supernatant is a source of raw biosurfactant. However, the biosurfactant was assumed to be recovered from the culture supernatant with pH adjustment at 2 with HCl 2N during the 24 h under refrigeration at +4 °C, but neither precipitation nor separation occurred, in this case, a production was carried out without adjustment of the pH to 2, and subsequently a hexane treatment is carried out for a total elimination of olive oil residues. The recovered supernatant has undergone three types of extraction with individual or mixed solvents used: ethyl acetate (v/v), chloroform/methanol (2/1), and dichloromethane (v/v), [33] before being concentrated in the rotary evaporator [1]. The crude biosurfactants were dried and weighed.

3.4. Partial characterization of biosurfactants

3.4.1. ST and CMC

The ST was measured by the ring method at 23±2 °C. The critical micelle concentration (CMC) of the biosurfactants was also determined according to the protocol cited by [2]. CMC is an important parameter during the evaluation of biosurfactant activity. The surface tension of surfactant reaches the lowest at its CMC. Above this concentration, no further effect can be observed on the surface activity. Measuring surface tension of serially diluted biosurfactant solution, the CMC was determined by plotting the surface tension versus concentration of biosurfactant in the solution.

3.4.2. Characterization of the crude biosurfactants by FT-IR

The infrared spectra were obtained using a Fourier transform infrared spectrophotometer. The biosurfactants were dissolved in one $mg.mL^{-1}$ (1%, w/v) methanol (CH_3OH) and then 20 µL of the solution is placed on the stainless steel plate of the FTIR device. The latter was left to rest for 10 min in the ambient air in order to evaporate the solvent. The optical absorption measurements were made in the medium-IR domains with wavelengths between 2×10^{-3} and 25×10^{-4} cm (500 and 4000 cm^{-1}) [2].

3.5. Biosurfactant stability studies

Stability studies were performed as cited elsewhere [34]. The samples, which contain the extracted biosurfactant, were used. To study the heat stability, samples were heated at 4, 25, 30, 40, and 70 °C for 24 h in a water bath. In addition, samples were also autoclaved (121 °C, 20 min). The effect of pH was studied by adjusting the pH of samples to 2, 4, 6, 8, 10, and 12 by using 1 N HCl/NaOH. The salinity stability was studied by adding different concentrations of NaCl (10, 20, 50, 100, 150, 200, and 250 g/L) to the samples. Following each treatment, the biosurfactant stability was evaluated using ST as mentioned earlier.

4. Statistical analysis

The statistical analysis of the data obtained was carried out using the STATISTICA software. All experimentations were performed in three independent replicates by referring at the control experiment. The results were expressed with standard deviation (mean ± SD).

RESULTS

1. Screening of bacterial strains growing on crude oil and producing biosurfactants

The screening methods can give qualitative and /or quantitative results. For a first isolates screening, qualitative methods are generally sufficient [35].

Table 1: Results after 72 h incubation of thermophilic strains in liquid LB test tubes and under 150-ppm agitation

Name of bacterial strain										
K2E is the performant strain productor of biosurfactant										
K2G	K1F	K7G	K1D	K1K	K7N	K7F	K2C	K1E	K7D	K7C
K2D	K2N	K2B	K7P	K1H	K1A	K7A	K2L	K7J	K7E	K2F
K1B	K7B	K1G	K2M	K1M	K7L	K7I	K7T	K2O	K2A	K7K
K1C	K7D	K2I	K1L	K1N	K1I	K7R	K7H	K2H	N1F	N1H
N1B	N1A	N1C	N1J	N1I	N1D	N1G	N1E	T7J	T1D	T7H
T2A	T7E	T7B	T2E	T2F	T1A	T2J	T2G	T1E	T7D	T1C
T7C	T1I	T7A	T1J	T1B	T2I	T7G	T7I	T1F	T1G	T1H

2. Carbon source selection

All the studies on the production of biosurfactants show, after the selection of the producing microorganism, the importance of the choice of the carbon source, be it a hydrocarbon, a carbohydrate, an oil, etc.

This source plays a determining role in the production rate as well as the nature of the biosurfactant synthesized. The results of the influence of carbon source on biosurfactant production by strain K2E, are shown in (figure 1)

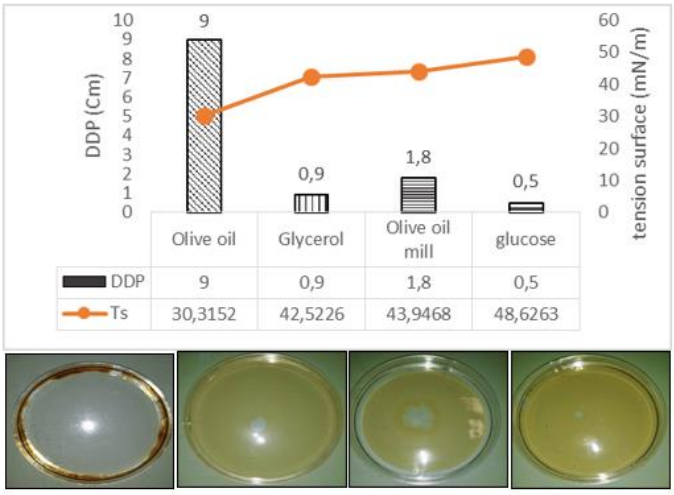

Figure 1: Influence of carbon source on biosurfactant production by strain K2E.

3. Phylogenetic affiliation and identification of the strain K2E

The strain K2E, already isolated and identified, can grow using olive oil as the sole source of carbon and energy.

4. Screening of biosurfactant activity

4.1. Surface Tension

Based on ST measurement, the cell-free supernatants of *Bacillus amylolequifaciens* strain K2E shows a remarkable reduction in surface tension minimal conditions (from 72 to 30.32 mN/m).

4.2. Oil displacement activity

The oil displacement method measures the surface activity of the cell-free supernatants tested against oil; the larger the diameter of displaced circle the higher surface activity of the surfactant. Biosurfactant produced by *Bacillus amylolequifaciens* strain K2E culture showed higher surface activity, the diameter of displaced circle was 9 cm. The strain presents a hemolytic activity (figure 2)

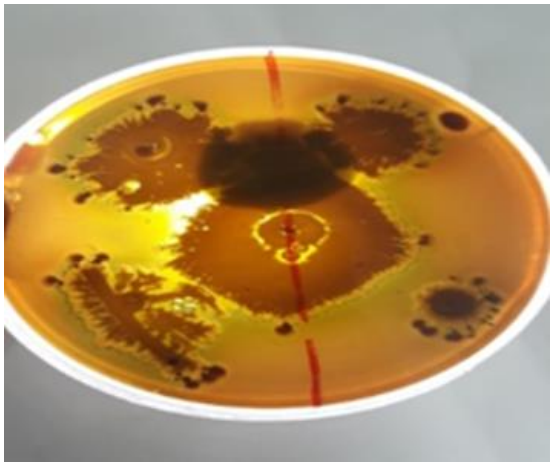

Figure 2: The hemolytic activity of the strain K2E

5. Partial characterization of biosurfactant and FTIR analysis

The biosurfactants from the *Bacillus amylolequifaciens* strain K2E was produced in MSM supplemented with 0.5% olive oil as the sole carbon. After fermentation for 1 day, the biosurfactant was extracted and subjected to characterization. Infrared absorption spectroscopy makes it possible to know the nature of the different chemical groups present in the biosurfactant of the strain The analysis of the FTIR absorption spectra (recorded in transmittance) made it possible to identify all the absorption bands present in an infrared spectrum and to predict the constituents concerned as shown in (figure 3).

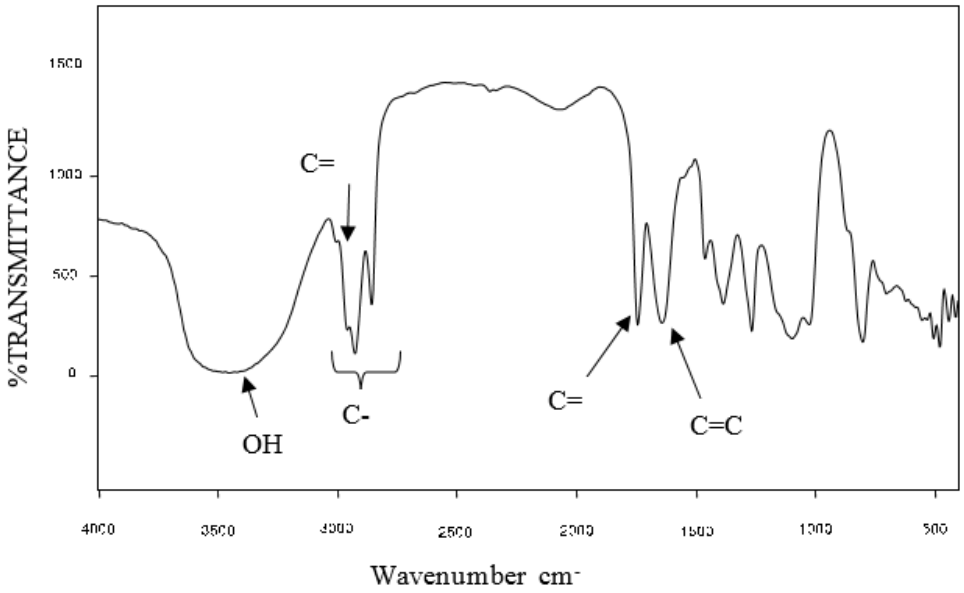

Figure 3: FT-IR absorption spectra of biosurfactant produced by *Bacillus amylolequifaciens*

5.1. Determination of critical micellar concentration

The surface tension is inversely proportional to the amount of biosurfactants, high concentrations of biosurfactants lower the surface tension to a minimum and stable value, called CMC. The experimental results show that the surface tension maintains a constant value (30 $mN.m^{-1}$) when the concentration of the biosurfactant produced using olive oil as a carbon source is equal to or greater than 750 mg/L.

5.2. Effect of temperature, pH and salinity on biosurfactant stability

The results obtained (figure 4) show that the biosurfactant is not affected by temperature variations. The product is stable for the increase in temperature. The thermal stability of the biosurfactant has been checked for the supernatant, the same surface tension was noted in the meantime temperature from 4 to 120 ° C (around 31 mN/m).

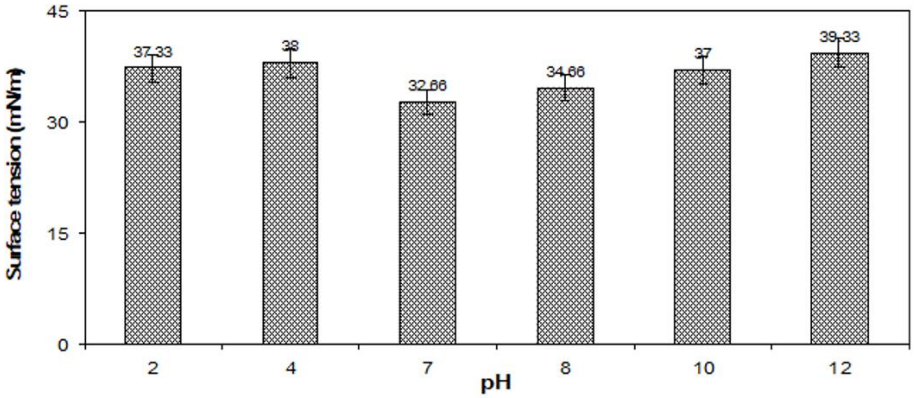

Figure 4: pH effect on the crude biosurfactant stability

The biosurfactant of our strain shows very good resistance in acidic and basic media over a very wide pH range varying from 2 to 12 (Fig. 5 and 6), and a salinity stability (up to 200 g/L)], over a time period up to 3 years.

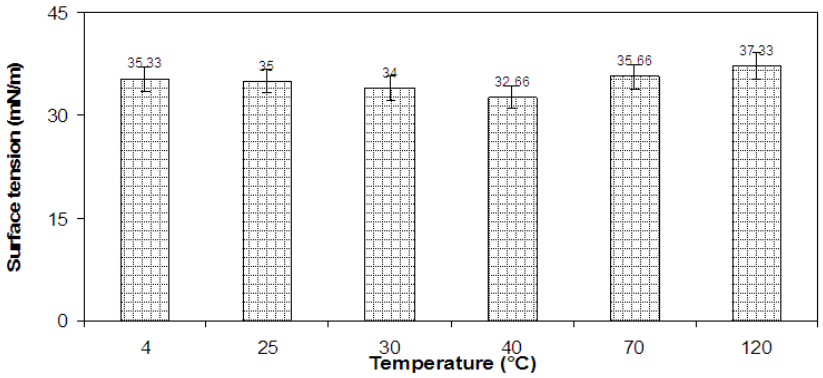

Figure 5: Effect of different temperature on the crude biosurfactant stability

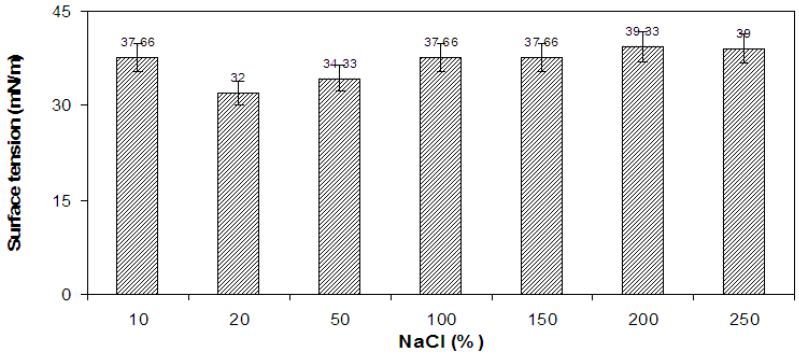

Figure 6: Salinity effect on the crude biosurfactant stability.

DISCUSSION

The most methods for a general screening of biosurfactant producing strains are based on the physical effects of surfactants. Alternatively, the ability of strains to interfere with hydrophobic interfaces can be explored. [35]. Surface tension, interfacial tension and stabilization of an oil-in-water emulsion are commonly used as surface activity indicators (table 1) [36]. The choice of the carbon source has a determining role in the rate of production as well as in the nature of the synthesized biosurfactant [37, 38].

All the studies on the production of biosurfactants show, after the selection of the microorganism producing, the importance of the choice of the source of carbon, whether it is hydrocarbon, carbohydrate, oil, etc. This source plays a decisive role in the rate of production as well as in the nature of the synthesized biosurfactant. For this purpose, four sources of 1% (v/v) concentration carbon were used: margine, glucose, olive oil and glycerol. The selection of the optimum source is carried out by the oil displacement test and the measurement of the surface tension. Figure1 shows the results regarding the effect of the carbon source on the production of the biosurfactant. They show that olive oil has a good production of biosurfactant which results in an interesting reduction in surface tension (30.32 $mN.m^{-1}$) with a large diameter of oil displacement (9cm). However, we note that the other used substrates do not produce biosurfactant (TS greater than 40). In addition, it was found that strain K2E gave the maximum surface activity after the first 48 h of incubation. Similar results were reported by Abousaoud et *al*. [39] where olive oil was the best source of carbon for the production of biosurfactants by *Pseudomonas aeruginosa* strain 44T1 [40]. The *Staphylococcus* sp. strain 1E uses olive oil for the production of biosurfactants as reported by Edouaouda et *al*. [41].

Edible vegetable oils are the most widely used in the production of biosurfactants, as in our study [42,43]; similar results showed that maximum production of biosurfactants was observed when using *Bacillus subtilis* strain SK320 grown at 37°C and 120 rpm in the base medium Bushnell Hass Broth (BHB) and 0.5% olive oil (Olio di Oliva, Sasso, Milan, Italy) as a source of carbon [44].

The regulation of biosurfactant production by *Bacillus stibtilis* strain SK320, grown on a base medium containing different substrates, followed by growth and production activity, was achieved at a concentration of 0.5%, v/v. This highlights our results using a concentration of 1% olive oil [44].

The K2E strain 16S rRNA gene sequence has perfect homology with those strains of *Bacillus amyloliquefaciens* and *Bcillus veleznsis* including the standard strain of *Bacillus amyloliquefaciens*. The K2E strain 16S rRNA gene sequence has a sequence homology of 98.55% with strains of the genus *Bacillus* and in particular with the strain of *Bacillus amyloliquefaciens* strain RB-25(CP054479) and *Bacillus amyloliquefaciens* strain 205(CP054415); the identification of the newly isolated bacterium (designated as K2E) was built in the basis of both catabolic and molecular methods. According to the methods described in the Bergey's Manual of Systematic Bacteriology, the morphological, biochemical, and physiological characteristics showed that the K2E isolate appeared in a bacilli form, and was an aerobic, spore-forming rods, Gram-positive, motile, and colonies are round, undulate, dull white and non-luminescent. In addition, it show positive reactions for catalase, oxidase, acetoin and phosphatase production, aesculin and starch hydrolysis, nitrate to nitrite reduction, and the Voges–Proskauer test and growth between pH 6 and pH 9 and between 20 and 37°C. The carbohydrate profile of the isolate was further investigated using API 50 CH gallery tests. The results showed that the strain metabolized d-glucose; d-cellobiose, d-fructose, ribose, d-xylose, galactose, mannose, inositol, mannitol, maltose, sucrose, trehalose, and d-lactose are readily utilized as energy sources in addition to other simple sugars. Glycogen, l-xylose, xylitol, d-arabinose, adonitol, sorbose, erythritol, inulin, d-arabitol, l-arabitol, capric acid, adipic acid, phenyl acetic acid, propionate, and glycine are not utilized as energy sources. The *16S* rRNA gene sequence from K2E was 98.28 and 97.48% similar to those of the *Bacillus amyloliquefaciens* strain F10-1^T (GenBank accession no. KY773617) and *Bacillus amyloliquefaciens* strain ATCC 23350^T (GenBank accession no. X60605), respectively. A phylogenetic tree based on the *16S* rRNA gene) showed that the novel isolates clustered with members of the genus *Bacillus*,

the nearest neighbour being *B. amyloliquefaciens* (98.28% sequence similarity). In brief, all the results obtained strongly suggested that this isolate should be assigned as *Bacillus amyloliquefaciens* strain K2E.

Surface and interfacial tension, stabilization of an oil and water emulsion is commonly used as a surface activity indicator [45]. K2E biosurfactant showed interesting surface tension reduction (30,31mN/m) and important emulsifying activity comparable to that found elsewhere [46]. K2E biosurfactant was stable even at high NaCl concentrations. In fact, there are reports that the presence of salts results in disruption of emulsions of oil and water, thus affecting the emulsification ability and surface tension of surfactants [47]. Therefore, the activity of biosurfactant preparation was evaluated in presence of different concentrations of NaCl to determine its field of application.

The ability to emulsify hydrocarbons was investigated using a wide range of pure and mixed substrates. The cell-free culture broth of *Bacillus amylolequifaciens* strain K2E successfully emulsified oil. The highest E24 value recorded by *Bacillus amylolequifaciens strin* K2E biosurfactant was 60% with oil.

Comparable results have been reported elsewhere, and they found that the emulsifier produced by *Ochrobactrum anthropi* had significant emulsification activity against various hydrocarbons such as octane, toluene, and crude oil [48]. In fact, the emulsification produced by microbes help to enhance the bioavailability of the oil leading to its utilization as a source of nutrient. Known emulsifying biosurfactants include glycolipids and lipopeptides. The presence of acetyl groups is linked with enhanced emulsification activities.

The results of hemolytic activity of K2E strain (figure 2) show the presence of a clear halo around the crop thing that confirms the production of a biosurfactant; biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay which was developed by Mulligan et *al*. [45]. Cultures are inoculated on sheep blood agar plates and incubated for 2 days at 25˚C. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies. Hemolysis can also be shown with purified biosurfactant [49]. The purified biosurfactant appeared as powdery and yellow residue. It was soluble in organic solvent such as ethyl acetate and water. Infrared absorption spectroscopy makes it possible to know the nature of the different chemical groups present in the biosurfactants of the strain K2E and thus to predict its nature.

The wide band of the vibration of the OH function of alcohols and in that of glycols is located at 3453 cm-1. In addition, vibrations of the C-H bonds of the methyl and methylene groups listed between 2958 and 2856 cm-1, the presence of peak is less intense but of great importance, implying the presence of an alkene (C=C) within the chemical structure located at 3004 cm^{-1}. The presence of the ester function is identified by the peak of the carbonyl function vibration (C=O) at 1742 cm-1. The presence of the structure of an alkene is confirmed by the presence of the peak obtained thanks to the vibration of the C=C link located at 1641 cm-1 (Fig. 3).

The CMC values confirm the minimum concentration of biosurfactant required for surface tension reduction. Thus, CMC is an essential method to detect the efficiency of a biosurfactant producer. In the present work, the extracted lipopeptide showed the lower CMC value of 75 mg/L while causing a significant surface tension reduction of water up to 32 mN/m.

The efficient surfactants have very low CMC values, i.e., less surfactant is required to decrease surface tension surface tensions versus [50, 52]. The surface tension of water decreased gradually with increasing biosurfactant concentration from 70 mN/m to 25 mN/m, with a biosurfactant concentration of 0.03%, and then remained constant. The CMC of the crude biosurfactant is within the range of CMC values reported for different types of biosurfactants produced by other *Candida* species [53, 56]. Such values are lower than those of synthetic surfactants in the partially purified and even in cell free broth preparations containing biosurfactants. The biosurfactant produced showed also a smaller CMC value than those of other biosurfactants from yeasts described in the literature, as values of 2.5% found for biosurfactants from *Candida lipolytica* and *Candida glabrata* [56], and of 0.6% for the biosurfactant from *Candida antarctica* [57].

Environmental conditions have an enormous influence on the metabolism of biosurfactants. This latter, exhibited better environmental compatibility than chemical surfactants, and possessed excellent stability with regards to pH, temperature, and salinity.

In fact, the applicability of produced biosurfactants in several fields depends on their stability at extreme environmental and operational conditions namely, pH, temperature, and salinity. The stability of biosurfactants at different salinity, pH and temperature values was measured and the results are illustrated in (Fig. 4, 5 and 6). The stability of the biosurfactant over various pHs is also an essential property that can affect its application. The biosurfactants activity was evaluated at the different pHs (2–14). At lower pH 4, the ST value was 38 mN/m. The highest activity was obtained at pH 7 where it was 33 mN/m. This reduction was mainly ascribed to the partial precipitation of biosurfactants [58]. In contrast, at higher pH values (10 and 12) the ST of biosurfactants increased, may be due to the occurrence of some alteration in the biosurfactant structure at these pH values which supports its use in industry. The work described elsewhere [59] showed that biosurfactant resulting from *Candida lipolytica* has a good resistance (ST=33.8 mN/m) in the acid media (pH=2), however a light increase in the ST (35.1 mN/m) was recorded in the alkaline media (pH=12).

On the other hand, the surface tension (average of 34 mN/m) of biosurfactant did not show any remarkable effect at a high temperature of 70 °C and remained without any significant increase on the surface tension capacity of the biosurfactant solution over different time intervals; therefore it was found that the biosurfactant mixture is thermally stable. As reported by the authors, the surface tension was slightly influenced by temperature for 1E biosurfactant [2].

The product was stable during the increase in temperature. The thermal stability of biosurfactant was demonstrated by retaining the same surface tension for a range of temperature from 4 to 55°C (average of 31 mN/m).

In addition, the salinity stability analysis was carried out, revealing that, the biosurfactant conserves its ability to decrease surface tension (30.32 mN/m) up to 200 g/L NaCl.

At 75 and 100 °C, the surface tension was increased (more than 35 mN/m) indicated the instability of the product at high–salinity. A slight increase in the surface tension is observed when the salt concentration is greater than 100 g/L.

Abouseoud and *al.*, reported that little changes were observed in the surface-active properties of *Pseudomonas fluorescens* biosurfactant with addition of NaCl up to 2.0 mol/L [27]. Ferhat and *al.*, showed that the surface tension of biosurfactant 1C or 7G remained stable in solution with increasing concentrations of NaCl from 5 g/L to 100 g/L (average = 31 mN/m) [21]. This indicates that biosurfactants produced by isolates *Ochrobactrum* sp. strain 1C and *Brevibacterium* sp. strain 7G, respectively are effective in the presence of monovalent ions (Na^+) [36].

CONCLUSION

The results of this study are of great interest, as it provides a low cost effective medium for the production of biosurfactants from a thermophilic strain newly isolated from an oilfield in Algeria, which identified as *Bacillus amylolequifaciens* strain K2E. The production of biosurfactants depend on the culture conditions, a maximum production is obtained with 1% of olive oil, a neutral pH and salinity of 1%. The biosurfactant produced by this strain has better resistance to extreme pH conditions (2–12) and salinity (up to 200 g/L). Indeed, it is capable of forming stable emulsions with a variety of hydrophobic compounds. The partial purified form of this agent might have a lipopeptide like structure as revealed by FTIR analysis. These results refer to the strong character of the biosurfactant its usefulness for industrial applications. Further surveys are needed to determine the structural identification of the biosurfactant nature using liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) and discovering its novel interesting applications (e.g. antibiofilm) for specific industrial applications.

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