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Title: Isolation and characterization of halophilic archaea able to produce biosurfactants

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Abstract: Halotolerants microorganisms able to live in saline environments, offer a multitude of actual or potential applications in various fields of biotechnology. This is why some strains of Halobacteria from an Algerian culture collection were screened for biosurfactant production in a standard medium using the qualitative drop-collapse test and emulsification activity assay. Five of the Halobacteria strains reduced the growth medium surface tension below 40mNm-1 and two of them exhibited high emulsion-stabilising capacity. Diesel oil-in-water emulsions were stabilized over a broad range of conditions, from pH 2 to 11, with up to 35% sodium chloride or up to 25% ethanol in the aqueous phase. Emulsions were stable to three cycles of freezing and thawing. The components of the biosurfactant were determined; it contains sugar, protein and lipid. The two Halobacteria strains with enhanced biosurfactants producers designed strain A21 and strain D21 were selected to identify by phenotypic, biochemical characteristics and by partial 16S rRNA gene sequencing. The strains have Mg2+and salt growth requirements are always above 15% (w/v) salts with an optimal concentration of 15% to 20%. Analyses of partial 16S rRNA gene sequences of the two strains suggested that they were halophiles belonging to genera of the family Halobacteriaceae, Halovivax (strain A21) and Haloarcula (strain D21). To our knowledge, this a first report of biosurfactant production at such a high salt concentration.

Isolation and characterization of halophilic archaea able to produce biosurfactants

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Abstract

Halotolerants microorganisms able to live in saline environments, offer a multitude of actual or potential applications in various fields of biotechnology. This is why some strains of Halobacteria from an Algerian culture collection were screened for biosurfactant production in a standard medium using the qualitative drop-collapse test and emulsification activity assay. Five of the Halobacteria strains reduced the growth medium surface tension below $40mNm^{-1}$ and two of them exhibited high emulsion-stabilising capacity. Diesel oil-in-water emulsions were stabilized over a broad range of conditions, from pH 2 to 11, with up to 35% sodium chloride or up to 25% ethanol in the aqueous phase. Emulsions were stable to three cycles of freezing and thawing. The components of the biosurfactant were determined; it contains sugar, protein and lipid. The two Halobacteria strains with enhanced biosurfactants producers designed strain A21 and strain D21 were selected to identify by phenotypic, biochemical characteristics and by partial 16S rRNA gene sequencing. The strains have $Mg^{2+}$and salt growth requirements are always above 15% (w/v) salts with an optimal concentration of 15% to 20%. Analyses of partial 16S rRNA gene sequences of the two strains suggested that they were halophiles belonging to genera of the family *Halobacteriaceae*, *Halovivax* (strain A21) and *Haloarcula* (strain D21). To our knowledge, this a first report of biosurfactant production at such a high salt concentration.

Keywords: Halobacteria, Screening, Biosurfactant, surface tension.

Introduction

Biosurfactants are a diverse group of surface-active biomolecules produced by many living organisms [40, 6]. These amphiphilic compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce interfacial tension between different fluid phases. Their uses and potential commercial applications have been reported in several fields, including surfactant-assisted flooding for enhanced oil recovery in the oil industry, emulsifiers in the food industry, and moisturizers in the cosmetic industry [13, 22, 7]. Biosurfactants constitute a diverse group of surface-active molecules and are known to occur in a variety of chemical structures, such as glycolipids, lipopeptids and lipoproteins, fatty acids, neutral lipids, phospholipids, and polymeric and particulate structures [13].

The search for biosurfactants in extremophiles seems to be particularly promising since the biosurfactants of these organisms have particular adaptations to increase stability in adverse environment which can potentially increase their stability in the harsh environments in which they are to be applied in biotechnology [50].Some microorganisms can survive and grow over a wide range of salt concentration. In aquatic environments the conditions range from fresh waters (containing less than 0.05% w/v dissolved salts), through sea water with total salinities of 3.2% - 3.8% (w/v) to saturated salt solutions up to 30% (w/v) and above [37, 11].

There are very few reports on biosurfactant producer in hypersaline environments. Halophiles, which have a unique lipid composition (phytanylglycerol), may have an important role to play as surface active agents. The biopolymers secreted by halophiles are intrinsically highly stable and may have applications as mobility controllers and emulsifying agents in the oil industry [2, 13, 5]. The archae bacterial ether-linked phytanyl membrane lipid of the extremely halophilic bacteria has been shown to have surfactant properties [49]. Yamimov et *al* [57] reported the

production of biosurfactant by a halotolerant Bacillus species and its potential in enhanced oil recovery, *Bacillus licheniformis* strain BAS 50 was able to grow and produce a lipopeptide surfactant when cultured on variety of substrates at salinities up to 13% NaCl. The production of bioemulsiers from *Methanobacterium thermoautotrophicum* has been reported [53]. These bioemulsiers were active over a wide range of pH (5-10) and at very high salt concentrations (up to 200 g/l). Recently, interest in the mass cultivation of microorganisms from hypersaline environments has grown considerably, because this represents an innovative low technology approach to biotechnological exploitation [54]. In a screening program to obtain biosurfactant producer, we have isolated obligately halophilic microorganisms from a previously unexplored site in Algeria. Hypersaline environments where salinities exceed 1.5 M are usually dominated by prokaryotes. Two main groups are to be found: the moderately halophilic bacteria are more abundant at intermediate salinities (1.5 – 2.5 M), whereas the halophilic archaea (the halobacteria) dominate at salinities greater than 2.5 M, often imparting spectacular red pigmentation to the environment due to high levels of carotenoids [29]. Representatives of the majority of archaeal genera are characteristic of neutral saline environments (*Halobacterium*, *Halorubrum*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halobaculum*, and *Natrialba* spp) [29,35,36,39,43,47] whereas alkaline saline environments harbour haloalkaliphilic halobacteria such as *Natronomonas* and *Natronobacterium* spp [36].

The application of molecular and biochemical techniques has indicated that specific successions of halobacteria occur in hypersaline waters as the waters become concentrated.

Many neutral hypersaline environments at saturation point, harbour climax populations of halobacteria usually belonging to the genera *Halobacterium*, *Haloarcula* and *Halorubrum* [46]. Representatives of other genera are much less common [30,51], although detailed characterisation at the species level is seldom carried out. In this paper we report the

characterization of five halobacterial isolates from a sebkha near Ain Salah in Algeria, able to produce biosurfactants, whose production and partial characterization are described.

Materials and methods

Media and growth conditions

The bacterial isolates were routinely cultured in a standard medium containing (per liter) 125 g of NaCl, 160 g of $MgCl_2.6HO_2$, 5.0 g of K_2SO_4, 0.1 g of $CaCl_2$ $.2H_2O$, 1.0 g of yeast extract (Difco), 1.0 g of Casamino Acids (Difco), and 2.0 g of soluble starch (BDH). The pH of the medium was adjusted to pH 7.0 with NaOH. This medium was modified with respect to salt concentrations, nutrient and inhibitor contents as described below [47]. In most experiments, cells were grown in a horizontal shaking water bath (200 strokes per min) at 40°C in 100-ml Erlenmeyer flasks containing 50 ml of medium. To prepare agar plates, the media were solidified with 20 g of agar per liter. The media were sterilized by autoclaving. For further studies, the growth media was modified, it contained diesel oil (5% v/v) as the sole carbon source. Diesel used in the experiments was a standard Diesel fuel, without additives, obtained directly from Naftal Oil Refinery in Algiers.

Source of organisms

Halobacteria were enriched from 50 samples collected at 1-m intervals in the ponds located close to Ain Salah in Algeria. Enrichments were grown at 40°C, at different NaCl

concentrations (2.0–5.0 M) in the standard medium for 2 weeks. The cultures were purified by repeatedly streaking them on solid medium. Typically the isolates grew well after 7 days in the standard medium at 40°C, pH 7.0 with 3.5 M NaCl.

Morphological, biochemical and physiological characterization

Gram staining was performed by using acetic acid-fixed samples as described by Dussault [23]. Tests for catalase and oxidase activities, starch, gelatine, casein and Tween 80 hydrolysis, formation of indole from tryptophan, and nitrate reduction were performed by using standard procedures [28]. Growth response to NaCl was examined in liquid standard medium using serial NaCl concentrations ranging from 50 to 350 g l^{-1} and to pH by testing growth at pH 5 - 10. The growth response to temperature was examined by testing growth in liquid medium up to 60 °C. The requirement for Mg^{2+} for growth was tested qualitatively by growing the strains in standard liquid medium with and without $MgSO_4$, $7H_2O$. The utilization of sugars (glucose, fructose, galactose, arabinose, raffinose, xylose, cellobiose, sucrose and rhamnose) and the acid production from these compounds were determined in standard medium modified as follows: starch was omitted, and the yeast extract and Casamino Acids concentrations were reduced to 0.25 g/liter each or yeast extract and Casamino Acids were omitted, as described below. In the latter case, the media were amended with 0.1 g of NH_4Cl per liter and 0.01 g of KH_2PO_4 per liter [47]. Each potential carbon source was added to a final concentration of 5 g/liter from a concentrated sterile solution. Growth was monitored by determining the optical density of each culture at 600 nm, and the pH of each culture was compared with the pH of a control culture. A decrease in the pH to a value less than 6.0 was considered evidence of acid production. Starch hydrolysis was tested by flooding colonies grown on agar plates containing the standard growth

medium with an iodine solution. Susceptibility to antibiotic penicillin G (10U) was determined in liquid medium.

DNA extraction, polymerase chain reaction and sequencing 16S ribosomal DNA

Bacterial strains designated as A21 and D21 were selected for molecular identification. DNA was extracted from the polycarbonate filters as described by Minz et *al* [44], then the DNA was electrophoresed for 30 min at 100 V on 1% TAE agarose gel, excised from the gel and purified with a jet sorb gel extraction kit (Genomic DNA purification system-PROM, EGA). Purified DNA from the various strains was amplified using specific 16S rRNA archaeal primers, (21f 5'-TTCCGGTTGATCCYGCCGGA-3') and (958r 5'-YCCGGCGTTGAMTCCAATT-3') [20]. Each 50µl reaction mixture contained 5 µl of 10x PCR buffer , 5 µl of deoxynucleoside-triphosphate mix (2.5 nM each), 2.5 µl of bovine serum albumin, 0.5 µl of 21f primer (50 µM), 0.5 µl of 958r primer (50 µM), 0.5 µl of *Taq* polymerase (TaKaRa, Otsushiga, Japan), 1 µl of template DNA, and RNase/DNase-free water to a final volume of 50 µl. PCR was performed in 50µl glass capillaries using a Perkin-Elmer 480 thermal cycler. The following PCR program was used: 94°C for 30 s, followed by 30 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 45 s, followed by 72°C for 30 s. Phylogenetic analysis was performed using the software package BioNumerics (Applied Maths, Belgium) after including the sequence as received in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. This alignment was pairwise calculated using an open gap penalty of 100% and a unit gap penalty of 0%. Similarity matrix was created by homology calculation with a gap penalty of 0% and after discarding unknown bases. A resulting tree was constructed.

Screening for biosurfactant producing strains

The most important surface-active properties evaluated in screening microorganisms with potential industrial application are surface tension reduction, the emulsion forming and stabilizing capacity. The criterion used for selecting biosurfactant producers is the ability to reduce the surface tension below 40 mN m^{-1} [16, 9] while a criterion cited for emulsion-stabilizing capacity is the ability to maintain at least 50% of the original emulsion volume 24 h after formation [56]. Strains were cultivated on the standard medium and screening of biosurfactant producing colonies was performed using the qualitative drop-collapse test described by Jain et *al* [33] after being modified by Bodour and Maier [9]. Motor oil, corn oil and olive oil also were evaluated for use in this test. Two microliters of oil was applied to the well regions delimited on the covers of a 96 well microplates (Biolog, Hayward, CA) and left to equilibrate for 24 h. Five microliters of the seventh day strains cultures was transferred to the oil-coated well regions after centrifugation at 12,000g for 5 min to remove cells. Drop size was observed 1 min later with the aid of a magnifying glass, a result was considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by sterilized standard medium (negative control).

Emulsification activity assay and Surface tension measurement

Isolates testing positive in the drop-collapse test were also evaluated for emulsion forming and stabilizing capacity, according to the method proposed by Das et *al* [21], the surface tension of the cell-free supernatant was determined [48].

After growing in standard for seven days in an orbital shaker at 160 rpm and 40°C, cells were removed by centrifugation at 12,000g for 5 min and 2 mL of the cell-free supernatant were mixed with 2 mL kerosene in a test tube (100 mm x 15 mm). This mixture was shaken for 2

min and then left to stand. Relative emulsion volume (EV, %) and stability (ES, %) were measured in intervals up to 48 h using the following equations [21] :

$$EV, \% = \frac{EV; \%\ \text{emulsion height (mm)} \times \text{cross-section area (mm}^2\text{)}}{\text{total liquid volume (mm}^3\text{)}} \times 100$$

$$\% ES = \frac{\%\ EV; \%\ \text{at time } t; \text{ h}}{EV; \%\ \text{at 0 h}} \times 100$$

Emulsions formed by the isolates were compared to those formed by a 1% (w/v) solution of the synthetic surfactant sodium dodecyl sulphate (SDS) in deionised water, as proposed by Das et *al* [21]. During growing in standard medium, the surface tension of the cell-free supernatant (50 ml) collected at different time intervals after centrifugation (4500g) for 10 min, was determined using a KRUSS F6 tensiometer following the Wilhelmy plate measurement technique [48].

Properties of emulsions

Stabilization of emulsions from halophilic strain was evaluated over a range of chemical and physical conditions. The crude biosurfactant was dissolved in distilled water, and the pH was adjusted between 2 and 11 with HCI or KOH. After diesel oil was added, tubes were vortexed and the emulsions were measured after 1 h. The emulsifier was tested with 10, 15, 20, 25 and 35% (wt/vol) sodium chloride and 0, 10, 25, and 50% (vol/vol) ethanol in the aqueous phase. For the evaluation of stability, emulsions containing 0.14% (wt/vol) crude biosurfactant in distilled water and diesel oil were incubated at 4°C and room temperature for an extended

period. Emulsions were subjected to three cycles of heating (40°C, 16 h) and cooling (room temperature, 8 h) [13]. The stability of the formed emulsions (ES, %) were measured in intervals up to 48 h [21].

Surfactant isolation

A crude biosurfactant preparation was obtained by centrifuging (10,000 x g, 10 min, 4°C) the stationary-phase culture to remove the cells and adjusting the pH of the spent medium to 2 with 1 N HCl [17]. The acidified liquid was kept at 4°C overnight, and the precipitate that formed was collected by centrifugation (17,300 g, 30 min, 4°C). The precipitate was dissolved in distilled water, the pH was adjusted to 7.0 with 1 N NaOH, and the solution was lyophilized. The lyophilized material was designated the acid precipitate and analyzed by thin-layer chromatography [1]. All organic solvents used were commercially distilled and of the highest available purity (Sigma–Aldrich). Plates for thin layer chromatography (TLC) (Silica gel 60A), obtained from Merck, were washed twice with chloroform/methanol (1:1, v/v) and activated at 120°C before use. Glucids and proteins components were separated in solvent S1 (chloroform-methanol-acetic acid, 80:18:2 by volume). The solvent system S2 (chloroform-methanol-acetic acid, 97:2:1by volume) was used for lipid migration. The components were visualized by staining them with ninhydrin (5 mg of ninhydrin in a 50 ml butanol-50 ml acetone mixture) and heating them at 100°C for 5 min. The lipid components were detected as brown spots on the plate after spraying with chromosulfuric acid. Sugar compounds were located by charring at 110 °C for 5 min after spraying anthrone reagent as previously reported.

Results

Characterization of isolates

The enrichment procedure we used selects mainly halobacteria. Colonies from the enrichments that developed on solid media were about 1 mm in diameter, circular, entire and red-orange pigmented after 1 week of incubation at 40°C.

The strains had salt growth requirements that were always above 15% (w/v) salts with an optimal concentration of 15 to 20% and were considered extreme halophilics. These strains were presumptively identified as members of the family *Halobacteriaceae* on the basis of phenotypics characteristics (Table 2). The strains A21 and D21 were Gram negative, motile, catalase and oxidase positive. On the basis of the phenotypic features tested strains A21 and D21 showed phenotypics features resembling members of the genera *Halovivax* [15] and *Haloarcula* respectively. The phylogenetic position of strains A21 and D21 were determined (Fig.6), for the first strain designed A21, the partial gene sequence obtained was 400 nucleotides in length (GenBank, AM982815), for the second strain designed D21, the partial gene sequence obtained was 900 nucleotides in length (GenBank, AM982816). The sequences are comparable to 16S rRNA of other halophilic archaeon. We have demonstrated that strain A21 possessed similarities higher than 97% with those of the genus *Halovivax.* A similarity (based on a very small partial sequence) significant for possible species relatedness (>97%) is found with the two validly described *Halovivax* spp. Indicating that strain A21 may belong to one of these species of the genus *Halovivax*. Indeed, it was related to *Halovivax ruber* DSM 18193^T (98.6% similarity) and *Halovivax asiaticus* CECT 7098^T (98.4% similarity). Also, the partial 16S rRNA sequence of strain D21 was determined (900pb); the sequence was compared with the published 16S rRNA sequences of representative members of the Archaea. The

sequence showed a significant similarity (97%) for possible species relatedness is found with several validly described *Haloarcula* spp (Fig.6). To see distance matrix, it indicated that strain D21 may belong to one of these species. Following analyses for further species identification is suggested by the complete 16S rDNA sequence analysis and DNA: DNA hybridizations.

Screening and kinetic analysis of biosurfactant production

Five bacteria strains were selected for their ability to grow at neutral pH and at 3-5.2 M NaCl and were screened for biosurfactant production and emulsification activity. They are tested positive for biosurfactants in the drop-collapse test reduced, these organisms are A21, B21, C21, D21 and E21 (Table 1). In this study the drop collapse technique was only applied as a qualitative method to detect biosurfactant production. Motor oil proved better to work with than olive oil since, it did cause spreading of the sterilised standard medium used as negative control and produced plates in which drop diameter was most readily estimated, an important factor given this test is based on visual observation.

According to Bodour and Maier [9], the criterion used for selecting biosurfactant-producers is the ability to reduce the surface tension below 40 mNm $^{-1}$. Also, Willumsen and Karlson [56], a criterion cited for emulsion-stabilizing capacity is the ability to maintain at least 50% of the original emulsion volume 24h after formation. The 2 strains tested produced extracellular biosurfactant and produced a strong biosurfactant capable of generating a stable emulsion over several hours. However, cell-free supernatant from the five isolates (A21, B21, C21, D21 and E21) exhibited reduced surface tension (Table 1). Biosurfactants were either adhered to, or an integral part of, the cell surface of isolates that only reduced the surface tension in the presence of cells.

The relative emulsion volume (EV, %) was highest (75.2%) in the culture of the strain D21 followed by the strain A21 with the EV, % equal at 72.3%. In comparison, growth and surface tension (ST) decreased during eight 8 days of these five halophilics strains. The surface tension of the standard medium had a straight decreasing during the stationary growth phase (shown in Fig.1A and B). All of the strains tested show same kinetics of growth, we can see clearly that the production of biosurfactant takes place during the lag phase. The shapes of the curves are explained by a maximum production of the biosurfactants during this phase of the growth, they act as a primary metabolite. However, it is clear that strains D21 and A21 exhibited the highest surface activity with the lowest surface tension of 26.20 mN m^{-1} and 28.40 mNm m^{-1} respectively.

We saw the behaviour the strains A21 and D21 in the presence of a hydrocarbon in the culture medium. Thus for the later studies, the growth media was modified containing diesel oil (5% v/v) as the sole carbon source. The kinetics of growth was observed and shown in Fig. 2A and B. We note that the pH remains practically unchanged during the fermentation time of strains A21 and D21. The lower tension activity is marked for the strain D21. Surface tension is the force required to break the surface between two immiscible liquids. However, we note that the surface tension in the first days of fermentation is quite high but in the end of the third day, we see a decline in values until they reach 28mN/m and 34 mN / m for the two bacterial strains A21 and D21 respectively. As for the pH of the culture medium, there remains stable and stationary all along microbial growth. This result allows us to say that there is a production of biomolecules with tension-active properties as biosurfactants which cause the reduction of the surface tension of the medium. In addition and according to these results; it clear that these extremes halophilics bacteria grow on a medium containing hydrocarbons in a similar way that in the presence of the starch.

Chromatographic behaviour

Strain A21 presented lipids with a regular mobility in the solvent S2 (RF, 0.34) and the lipids gave a negative test on TLC plates for strain D21. Protein and sugar gave a positive test on TLC plates for both of strains A21 and D21, indicating that there is a production of the extracellular compounds by the strains A21 and D21. According these results, the biosurfactants produced by the halophilic bacteria D21 could be glycoproteins whereas for the halophilic strain A21, the components of the biosurfactant were found to contain sugar, protein and lipid. This could be a peptidoglycolipid thus, such as glycoproteins, glycolipids or lipopeptids.

Properties of the emulsions

Strains A21 and D21 were selected to study the stability of the emulsions formed under various conditions. The emulsions were tested for stabilization under a range of chemical and physical conditions which might be encountered in various applications. According to Cameron et *al* [13], it clear to facilitate the detection of the possible detrimental effects of pH, sodium chloride, or ethanol on emulsification. Emulsions from both strains, A21 and D21, were made with 0.05% (wt/vol) purified crude biosurfactant. The dilution by distilled water indicates that it does not have an effect on the emulsion produced. The emulsion stabilising capacity of the two bacterial strains A21 and D21 is kept constant with a value of 60.2 % and 58.82% respectively. The pH of the aqueous phase had little effect on the amount of diesel oil phase emulsified between pH 2 and 11 (Fig.3). In the basic environments, it is clear that the strain A21 has an emulsion stabilising capacity more important than those in the neutral and acidic environments, with an optimum at pH 9. Relative emulsion stability is equal to 100%. The

strain D21 shows the same the result except that its highest emulsion stabilising capacity appears with neutral pH. The effect of the change in pH does not appear to affect emulsions formed. These results demonstrate once again that it is possible to use these musts fermenting in sites polluted, for example, by oil and that whatever the pH values of the site to clean up. In the presence of 10 to 35% (wt/vol) sodium chloride in the aqueous phase, stable and strong emulsions were formed of both strains A21and D21 (Fig.4). According to the results obtained, strains A21 and D21 showed that the relative emulsion stability formed increases with increasing concentrations of ethanol until it reaches a maximum at a concentration of 25% (ES % = 93%). However, a higher concentration in ethanol (50%) caused a decrease in the relative emulsion stability (ES % = 88%). This demonstrates that the presence of high concentrations of ethanol has a positive effect on the stability of emulsion (Fig.5). The cycle of temperature (40 °C, 25 °C, 4 °C - 4 °C) has no effect on the stability of emulsions formed. But, the temperature cycle in reverse (-4 °C, 4 °C, 25 °C to 40 °C) causes the dispersion of the emulsion, so, this physical treatment provoke reduce of emulsion stability. Emulsions formed about the strains A21 and D21 were not disrupted by three cycles of heating to 40°C (16 h) and then cooling to 23°C (8 h). During storage at 4°C, the relative emulsion stability did not change over a 4 month period.

Discussion

This study broadens the field of biosurfactant producing microorganisms. Given the potential of biosurfactant for use as tools in different fields, our work suggests that it is interesting to search for biosurfactant in extremely halophilic archaeon. Certainly there is current interest in the production of other biosurfactants from halophiles; both archaeal and eubacterial, given the

possibility that biosurfactants adapted to high salt concentrations and temperatures will have improved stabilities when used in organic solvents. In the present study, the screening for biosurfactant production under hypersaline conditions is described for archaeal strains. Five Halobacteria strains were used throughout this work, two strains of them, strain designed A21 and strain designed D21 are identified as better biosurfactant producers using the qualitative drop-collapse test and the emulsification activity assay, they were selected for identification by phenotypic, biochemical characteristics and by partial 16S rRNA gene sequencing. We demonstrated that are able to grow in the presence of 20% NaCl. Analyses of partial 16S rRNA gene sequences of the 2 strains suggested that they were extremes halophiles belonging to genera of the family Halobacteriaceae, Halovivax (strain A21) and Haloarcula (strain D21). Also, halophiles have usually been overlooked in most screening programmes for exopolymers, recently, it has been found that *Haloferax mediterranei* produces a highly sulphated and acidic heteropolysaccharide (up to 3 g/l) which contains mannose as a major component [3, 4]. Such a polymer combines excellent rheological properties with a remarkable resistance to extremes of salinity, temperature, and pH [3]. Sulfated EPS is also notable for its role in inhibiting viral penetration into cells [32]. *Haloferax mediterranei* and *Haloarcula japonica* both produce sulfated EPS [6, 32]. The partial characterization of biosurfactants produced by strains A21 and D21 is the primary focus of the study. According to the results obtained, the biosurfactants produced by the halophilic bacteria D21 could be glycoproteins whereas for the halophilic strain A21, the components of the biosurfactants were found to contain sugar, protein and lipid. This could be a peptidoglycolipid thus, such as glycoproteins, glycolipids or lipopeptids. It is currently known that the type of biosurfactant made is dictated by the producing microorganism. One major class of biosurfactants is the glycolipids, which includes rhamnolipids, trehalose lipids, and sophorose lipids. Rhamnolipids are produced only by *P. aeruginosa* [10]; trehalose lipids are produced only by a number of closely related

genera, including *Rhodococcus*, *Nocardia*, *Corynebacterium*, *Tsukamurella*, *Gordonia*, *Mycobacterium*, and *Arthrobacter* [10] and other glycolipids produced by *Streptococcus thermophilus* [51]. Polymeric biosurfactant producers have been isolated from *Eubacteria*, *Eukaryotes*, and *Archaea* [10]. Moreover, the halobacterial membrane lipids exhibit many relevant properties; the ether-linked lipids possess very low melting points, are resistant to degradation by acids, alkalis, and heat and have an emulsifying ability, with an adequate hydrophile-lipophile balance, that produces good water-in oil emulsions [49]. An interesting potential application of the unique etherlinked lipids of the halobacteria is their use in novel types of liposomes, which have great value in the cosmetic industry. Such liposomes would be more resistant to biodegradation than those used at the moment and thus with a better shelf life, since halobacterial lipids are relatively resistant to the action of other bacteria [27]. Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10% (wt/vol), although slight reductions in the critical micelle concentration (CMC) were detected [45]. Furthermore, growth was not inhibited by the presence of crude oil. The biosurfactant produced was not affected by the temperatures, pHs, or NaCl. In this study, we observed that strains A21 and D21 were capable of reducing the tension surface of the medium up to 28 mNm^{-1} or more. Therefore, our own values are in agreement with values obtained by other researchers [41, 31] which are around 19-28 mNm^{-1}. The biosurfactant production is directly proportional to cell growth. When cell growth increases, surface tension decreases. The surface tension became constant and increased slightly during late stationary phase. Similar findings were also obtained for other surfactant-producing eubacteria [18, 5]. Cell-free supernatant from all halophilics strains tested in this study, exhibited reduced surface tension. Biosurfactants were either adhered to, or an integral part of, the cell surface of isolates that only reduced the surface tension in the presence of cells [16]. Isolates that liberate biosurfactants into the culture

medium are interesting from an industrial point of view, because the product recovery process can be simplified [26, 12, 38]. The biopolymers secreted by halophiles are intrinsically highly stable and may have applications as mobility controllers and emulsifying agents in the oil industry [3, 5]. There are very few reports on hydrocarbon biodegradation in hypersaline environments. Ward and Brock [55] have shown an inverse relationship between hydrocarbon biodegradation and salinity. Bertrand et *al* [8] reported the isolation of halophilic hydrocarbonoclastic bacteria, showing that hydrocarbon metabolism may occur in hypersaline conditions. In our studies, starch was a better carbon source than the other carbohydrates for screening biosurfactant-producing strains. Studies showed that few halophilic archaea are able to grow in aromatic compounds, but the production of biosurfactants was never demonstrated [19]. *Haloferax volcanii* D1227, a halophilic archaeon isolated from oil-brine-contaminated soil, was shown to degrade mono-aromatic compounds such as benzoate, cinnamate and 3-phenylpropionate [24]. More recently, *Haloarcula sp. D1* was shown to metabolize p-hydroxybenzoic acid (PHBA) [25]. Furthermore, the potential use application of Halobacteria (halophilic Archaea) for bioremediation of recalcitrant compounds in highly saline wastewaters for the accelerated remediation of hydrocarbon-polluted saline environments has been considered [7, 42]. The biological treatment of high saline effluents, such as the produced waters from the oil industry, has been studied [2]. Both selected strains are being identified and quantification and physicochemical characterization of their surface-active metabolites completed as the basis for optimization studies aimed at application in bioremediation.

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Fig. 1 Growth (A) and Surface Tension (B) decreases by cultures of five halophilic strains (A21, B21, C21, D21 and E21). Each culture of theses strains was grown at 40°C in standard medium. Values are averages for three cultures

Fig. 2 Kinetic of growth, emulsion stability, pH and ST decreases by cultures of Strain A21 (A) and Strain D21 (B). Each culture of theses strains was grown at 40°C in standard medium. Values are averages for three cultures

Fig.3. Influence of pH on the stability of the emulsion

Fig.4 Effect of different concentrations of salt (NaCl) on the stability of the emulsion

Fig.5 Effect of different concentrations of ethanol (%) on the stability of the emulsion

Fig.6 Phylogenetic dendrogram of halobacteria based on 16S rRNA gene sequence data, indicating the position of the strains A21 and D21. The tree was constructed using the neighbour joining method. The sequence data used were data for the following strains were obtained from the sequences collected from the international nucleotide sequence library EMBL, *Methanospirillum hungatei* was used as outgroup. Alignment was pairwise calculated

using an open gap penalty of 100% and a unit gap penalty of 0%. Similarity matrix was created by homology calculation with a gap penalty of 0% and after discarding unknown bases.

Table 1 Surface tension of culture media with and without cells and relative volume of emulsions formed between cell culture media and diesel oil after growth of bacteria strains in standard medium for seven days at 40°C and 200 rpm

Table 2 Characteristics that distinguish strain A21 and strain D21 from other related haloarchaeal genera. *Halovivax* and *Haloarcula*. Data from Castillo and *al* [15] and this study. +, Positive; -, negative; V, variable.

Figure

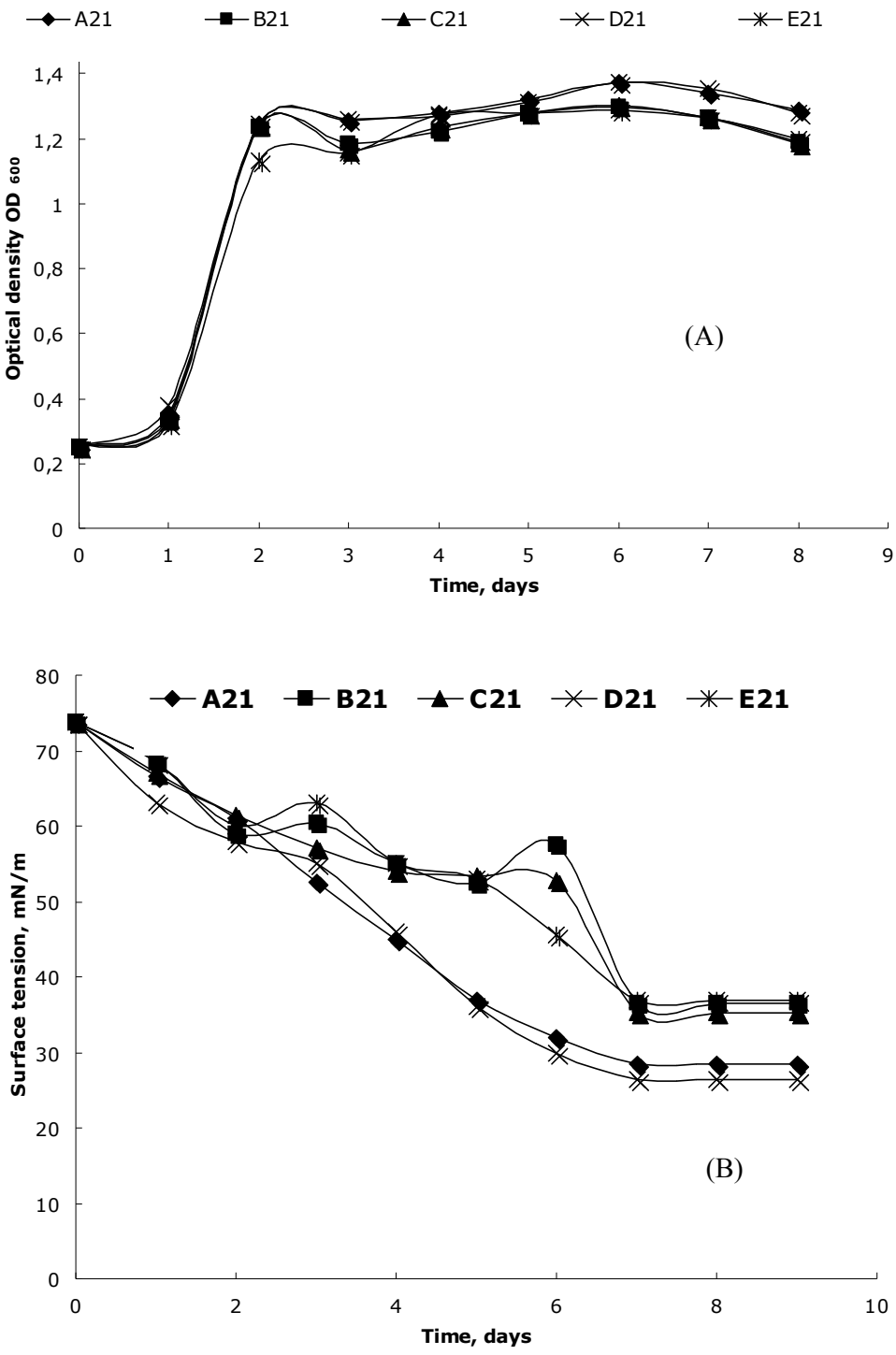
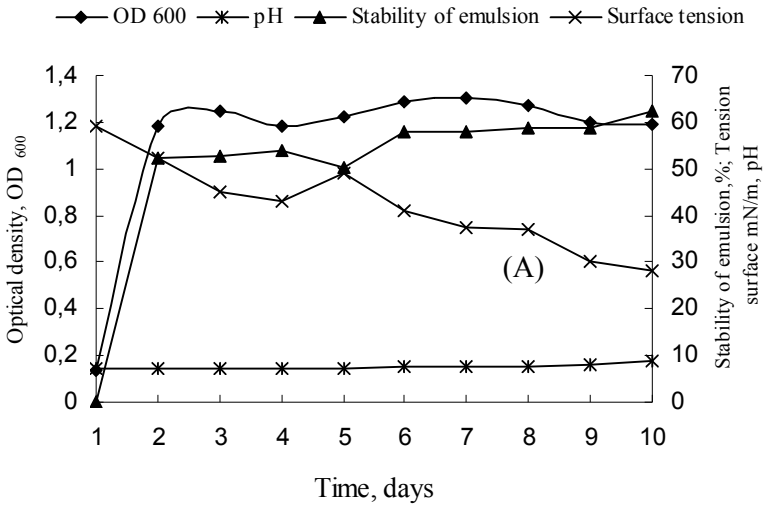

A21
B21
C21
D21
E21
Optical density OD 600
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
1
2
3
4
5
6
7
8
9
Time, days
(A)
80
70
60
50
40
30
20
10
0
Surface tension, mN/m
0
2
4
6
8
10
Time, days
(B)

Fig. 1


OD 600
pH
Stability of emulsion
Surface tension
Optical density, OD 600
Stability of emulsion,%; Tension surface mN/m, pH
(A)
Time, days

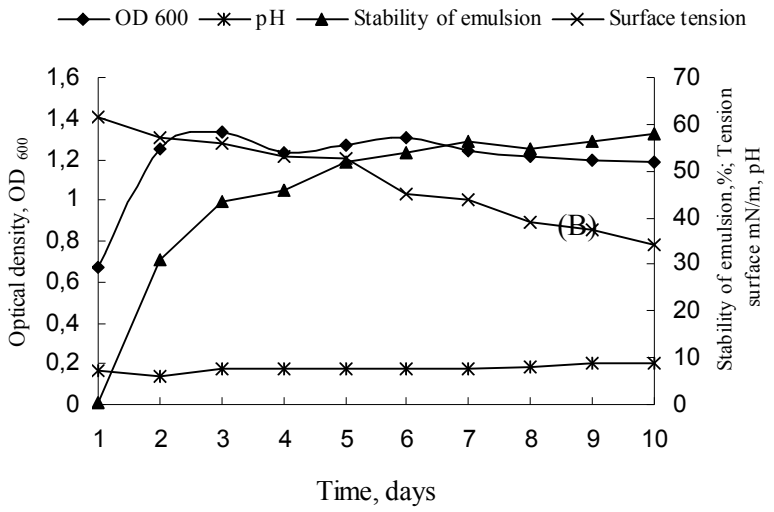

OD 600
pH
Stability of emulsion
Surface tension
Optical density, OD 600
Stability of emulsion,%; Tension surface mN/m, pH
(B)
Time, days

Fig. 2

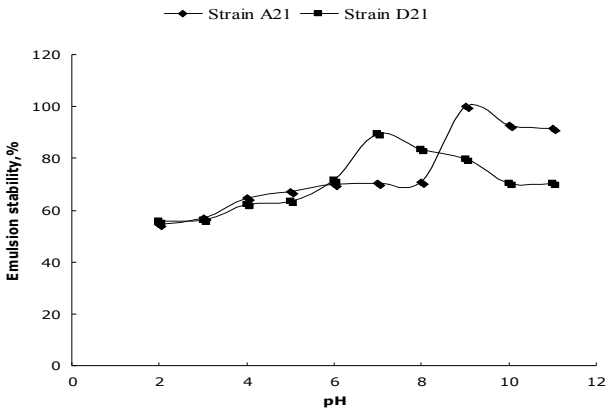

Strain A21
Strain D21
Emulsion stability,%
120
100
80
60
40
20
0
0
2
4
6
8
10
12
pH

Fig.3.

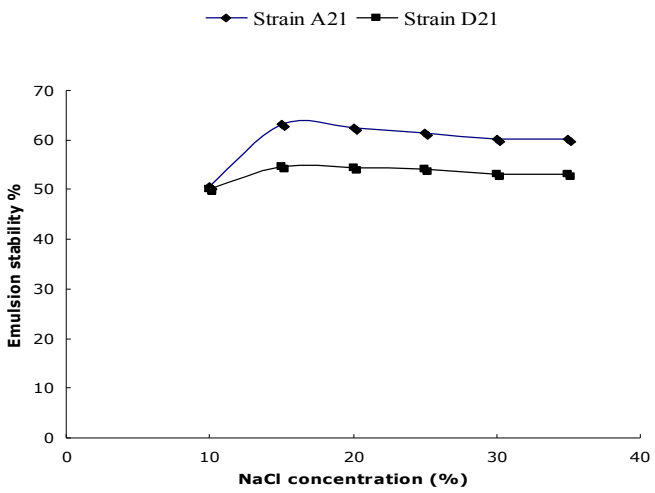

Strain A21
Strain D21
70
60
50
40
30
20
10
0
Emulsion stability %
0
10
20
30
40
NaCl concentration (%)

Fig.4

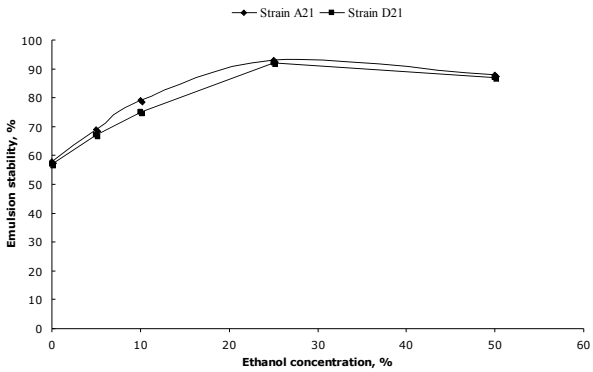
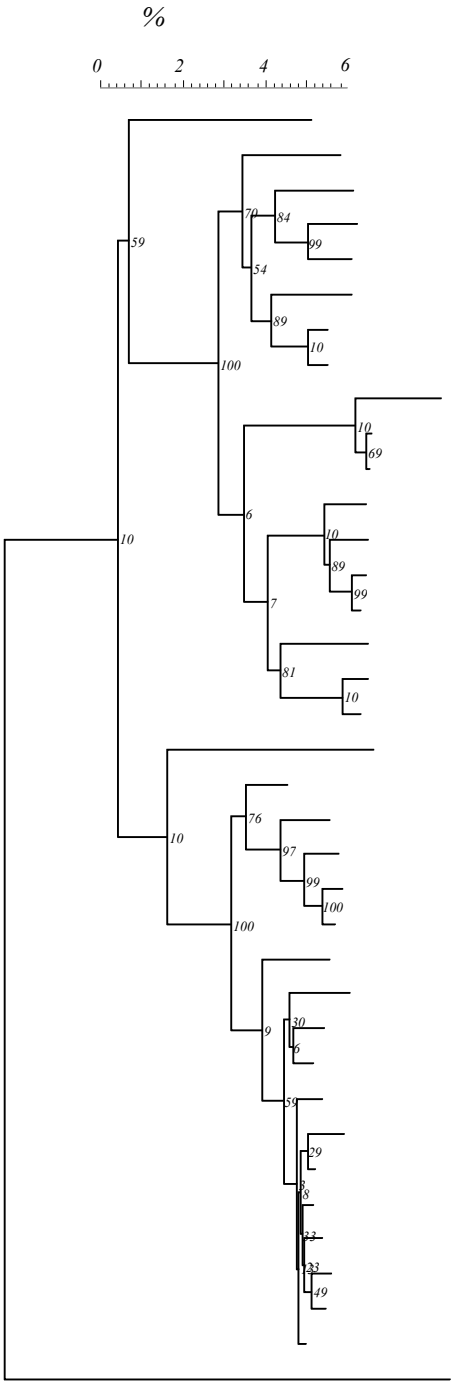

Strain A21
Strain D21
Emulsion stability, %
Ethanol concentration, %
0
10
20
30
40
50
60
70
80
90
100

Fig.5



JCM 8858T	*Natronomonas pharaonis*	*D87971*
NCIMB 2189T	*Natronobacterium gregoryi*	*D87970*
FERM P-11024T	*Natronococcus amylolyticus*	*D43628*
DSM 18795T	*Natronococcus jeotgali*	*EF077631*
NCMB 2192T	*Natronococcus occultus*	*Z28378*
C231T	*Halobiforma nitratireducens*	*AB045012*
JCM 11627T	*Halobiforma haloterrestris*	*AF333760*
JCM 12983T	*Halobiforma lacisalsi*	*AY277582*
Souche A21		
DSM 18193T	*Halovivax ruber*	*AM269467*
CECT 7098T	*Halovivax asiaticus*	*AM039978*
JCM 10478T	*Natrinema versiforme*	*AB023426*
JCM 12890T	*Natrinema altunense*	*AY208972*
NCIMB 786T	*Natrinema pellirubrum*	*AJ002947*
NCIMB 784	*Natrinema pallidum*	*AJ002948*
JCM 10989T	*Natrialba hulunbeirensis*	*AF262026*
ATCC 700177T	*Natrialba asiatica*	*D14123*
DSM 13077T	*Natrialba aegyptia*	*AF251941*
ATCC 700874T	*Halomicrobium mukohataei*	*D50850*
ATCC 33960T	*Haloarcula hispanica rrnB*	*AB090168*
ATCC 49778T	*Haloarcula japonica*	*D28872*
ATCC 700850T	*Haloarcula quadrata rrnB*	*AB010965*
JCM 13557T	*Haloarcula amylolytica rrnA*	*DQ826512*
ATCC 43049T	*Haloarcula marismortui rrnA*	*X61688*
ATCC 33800	*"Haloarcula sinaiiensis" minor gene*	*D14130*
NBRC 14741T	*Haloarcula vallismortis*	*D50851*
"B-2"	*"Haloarcula aidinensis"*	*AB000563*
JCM 13557T	*Haloarcula amylolytica rrnB*	*DQ826513*
JCM 13557T	*Haloarcula amylolytica rrnC*	*DQ854818*
Souche D21		
ATCC 33960T	*Haloarcula hispanica rrnA*	*AB090167*
ATCC 700850T	*Haloarcula quadrata rrnA*	*AB010964*
ATCC 43049T	*Haloarcula marismortui rrnB*	*X61689*
ATCC 33800	*"Haloarcula sinaiiensis" major gene*	*D14129*
ATCC 29715T	*Haloarcula vallismortis*	*U17593*
JCM 9737T	*Haloarcula argentinensis*	*D50849*
DSM 864T	*Methanospirillum hungatei*	*M60880*

Fig.6

Table

Table 1

Strains	Drops collapse	Surface Tension	Relative emulsion volume (%) after 48 h
			Cell-free Supernatant
A21	++++	28,4 ± 1,2	48.3 ± 0,6
B21	+++	36,4 ± 0,6	38.3 ± 0,7
C21	+++	35,5 ± 1,4	42.6 ± 0,5
D21	++++	26,2 ± 0,8	62.4 ± 0,4
E21	+++	36,9 ± 0,6	55.6 ± 0,6
1% SDS	++++	42.8 ± 0,6	23.5 ± 0,8

Surface tension was expressed as mN m^{-1} using standard medium as control (73.9 mN m^{-1}); Values reported are average of six to twelve replicates. %EV was as percentage.

Table 2

Characteristics	Strain A21	*Halovivax*	Strain D21	*Haloarcula*
Morphology	Coccus/pleomorphic	Rod/pleomorphic rod	Coccus/pleomorphic	Pleomorphic rod
Gram	-	-	-	-
Pigmentation	Pink-orange	Pale-pink	Pink-Red	Red
Catalase and Oxydase	+	+	+	+
NaCl optimum (M)	2.7M	3.4M	2.7M	2.5-4.3M
NaCl range (M)	2.7-4.5M	2.5-4.3M	2.7-4.5M	1.7-5.2
Mg++ optimum (M)	0.8M	0.05-0.1 M	0.5M	0.05-0.1 M
pH optimum	7-9, opt. 7	6-9, opt. 7-7.5	6-7.5, opt. 7	6.5-7.5
Temperature optimum (°C)	35-40°C	25-45°C, opt. 37°C	35-50°C	35-53°C
Nitrite from nitrate	-	-	-	+
Acid from carbohydrats	+	V	+	+
Growth on single carbon source	+	-	+	+
Indole from tryptophan	+	-	+	V
Hydrolysis of:				
Starch	+	-	+	V
Gelatin	+	+	+	V
Casein	+	+	+	-
Tween 80	+	+	+	V
Lysis in distilled water	Cells lyse	Cells lyse	Cells lyse	Cells lyse
Susceptibility to penicillin G (10U)	-	+	-	+