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Assessment of four different methods for selecting biosurfactant producing extremely halophilic bacteria

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Biosurfactants are surface active agents with broad range commercial applications in various industries and have considerable advantages over their chemical counterparts. In this study, drop collapse assay, oil spreading assay, emulsification assay and surface tension measurement were compared for their reliability and ease of use to screen biosurfactant producing six extremely halophilic bacteria isolated from saline soil of Chott El Hodna-M'sila (Algeria), which is considered as a thalassohaline environment. Results from screening methods revealed that, CH2 and CH5 strains are potential candidates for biosurfactant production. Among these, there was a very strong negative linear correlation between the emulsification index and surface tension (r_s= - 0.987) and a strong negative linear correlation between the oil spreading technique and surface tension (r_s= - 0.971) and a weaker negative correlation between the drop collapse method and surface tension (r_s= - 0.807), suggesting that strains highly active in any one of these methods were active in other three methods. As every method has its advantages and disadvantages, a combination of different methods is appropriate for a successful screening.

Key words: Biosurfactant, drop collapse, emulsification stability, extremely halophilic bacteria, oil spreading, surface tension.

INTRODUCTION

Biosurfactants are natural surface active agents produced by bacteria, yeast, and fungi, having very different chemical structures and properties (Rone and Rosenberg, 2001; Chen et al., 2007). These biosurfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic domains that find application in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Rodrigues et al., 2006).

Nowadays, biosurfactants are used in industries as a cosmetic and special chemical substance, food, pharmaceutics, agriculture, cleansers, enhanced oil recovery

and bioremediation of oil-contaminated environments and can be effectively used in numerous processes, including enhanced oil recovery, transportation in pipelines, cleaning of oil storage tanks, refining and product formulation (Urum and Pekdemir, 2004; Mukherjee et al., 2006).

They are potential alternatives of chemically synthesized surfactant in a variety of applications because of their advantages such as lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelle concentration, each of production, ability to be synthesized from renewable resources, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Chen et al., 2007; Nitscheke and Costa, 2007).

For this reasons a variety of methods for the screening of new biosurfactant producing microbes has been developed and successfully applied. Since the 1970th there have been various trials in this field (Walter et al., 2010). Hence, for efficient detection of potential biosurfactant producers, combination of various screening methods are required which was evaluated during this study.

Thus, the main goal of this study was to introduce novel extremely halophilic bacteria with potential for biosurfactant/bioemulsifier production isolated from saline soils of Chott El Hodna-M'sila (Algeria). The potential of these isolates in producing biosurfactant was examined by drop collapse assay, oil spreading assay, emulsification assay and surface tension measurement.

Finally, this study is considered as a valorization of this environment which is a representative type of wetland in the Mediterranean by the extent of its size and its watershed, its location in the arid zone is another advantage to justify the rarity of this type of natural environment in one piece having undergone little or no significant changes by man.

Chott El Hodna is a model representative of the presence of several types of soil, bioclimates and biological species. For this reason, it was classified as humid zone of international importance by the RAMSAR convention in 2001. It presents a considerable ecological interest and accommodates a large number of animals and plants species diversifies of interest for biodiversity of the region El Hodna (Boumezbeur, 2002).

MATERIALS AND METHODS

Area study

Chott El Hodna is a shallow saline lake in Algeria; it is located within an endorheic basin in North-Central of Algeria (lat. 35°18'/35°32', long. 4°15'/5°05') (Figure 1). The peripheral zone of this Chott is characterized by wadis and oases which surrounds the central zone of the sebkha with a complete absence of vegetation. The basin of Hodna has an area of 8500 Km^2, but what is called the Chott has an area of 1100 Km^2 and is located about 400 m above sea level. It gets water only in winter and dry in summer, salt crusts

cover its full extent. Due to the high evaporation, it becomes regularly a desert of salt (Boumezbeur, 2002).

Sample collection and physicochemical analysis

Water samples (200 ml) were collected at 10 cm depth from the water surface with 250 ml sterile flasks. Samples were gathered during the winter season (January). A total of five water samples were collected from this lake, and dispatched on the day of collection in ice box to the laboratory. Physicochemical properties of the samples taken from Chott El Hodna were analysed according to the methods described by Rodier et al. (1996). Chemical properties included compositional estimation of Ca^{2+} and Mg^{2+}: by a complexometric method using ethylene-diamine-tetra-acetic acid (EDTA), HCO_3 and Cl^- by a titremitric method, SO_4^{-2} by UV/Vis spectrophotometry (Beckman/DU, 520), Na^+ and K^+ by spectrophotometry with flame ionisation (Jenwey PEP7) and nitrate by the sodium salicylate method. Physical parameters included pH and temperature measured *in situ* using a portable instrument. In addition, the colour and odour of the lake water were noted. The conductivity was monitored using a conductivity meter (Rodier et al. 1996).

Isolation of extremely halophilic bacteria

Five saline soil samples were collected in sterile plastic bags from Chott El Hodna-M'sila (Algeria). Each sample was inoculated for enrichment and was streaked on SG medium. This medium contained (g/L): NaCl, 250; $MgSO_4.7H_2O$, 20; KCl, 2; sodium citrate (trisodium salt), 3; casamino acids, 7.5; yeast extract, 1 and $FeSO_4.7H_2O$, 0.0023. The pH was adjusted to 7.3 with 1 M KOH. For solid medium, 20 g agar/L was added. Plates were incubated at 37°C. After two weeks incubation, representative colonies were transferred to fresh SG medium and isolated in pure culture (Ozkan et al., 2006).

Screening for haloarchaea

Haloarchaeal strains were selected among all isolates by their susceptibility to antibiotic and a bile acid. They were streaked on the solidified medium containing chloromphenicol at 20 mg/L. The cultures were incubated for ten days at 37°C. Strains that grew on the plates with chloromphenicol were regarded as Haloarchaea (Madalin et al., 2008).

Characterisation of isolates

Phenotypic characterisation was carried out in accordance with the recommended minimal standards for the description of new taxa in the order *Halobacteriales* (Oren et al., 1997). Cell motility and morphology of exponentially growing liquid cultures were examined using a microscope equipped with phase contrast optics. Colony morphology was observed on agar medium after incubation at 37°C for 10 days. Gram strains were carried out as described by Dussault (1995). Cytochrome oxidase, catalase, nitrate reduction, indol and H_2S production, hydrolysis of gelatin and starch were determined as explained before (Oren et al., 1997; Ozekan et al., 2007). The optimum salt concentrations for growth were determined in media containing 0-32% NaCl by turbidity measurement, pH from 2-11 and temperature from 0-60°C. Acid production was carried out in a medium with sugars as substrates. Antibiotic susceptibility was tested according to the methods described by Stan-Lotter et al. (2002).
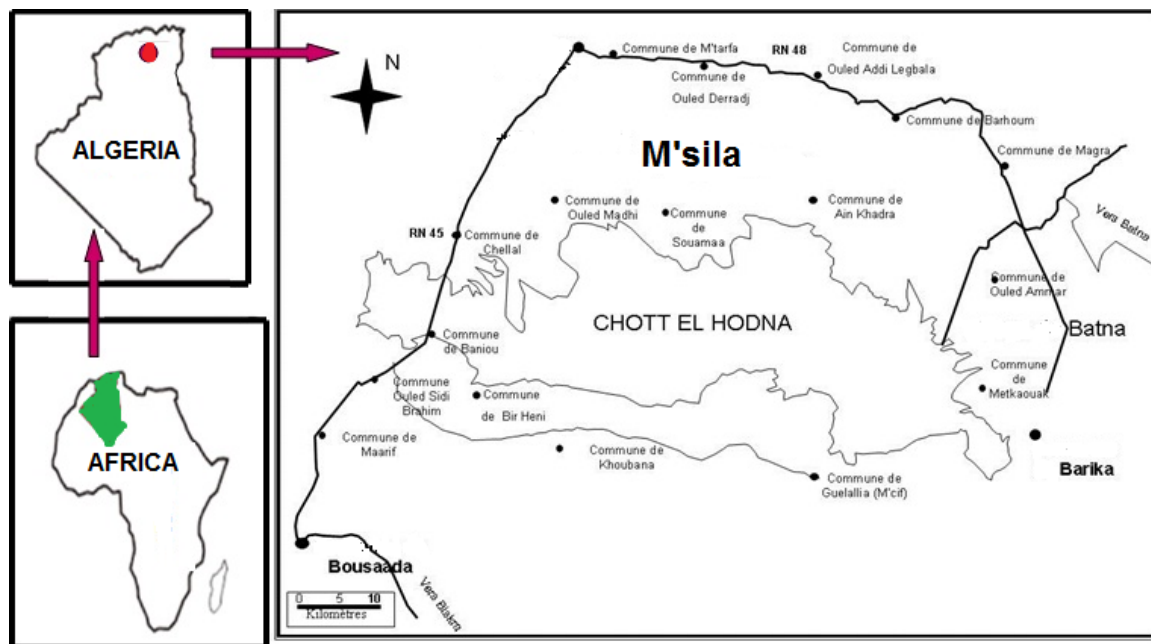
Figure 1. The area study is located in North-Central of Algeria.

Screening methods

Biosurfactant producing Haloarchaea were screened by using four methods. Experiments are done in three replicates.

Drop collapsing test

A modified oil collapse method was carried out using 96 well microtitre-plates containing 100 µl mineral oil, which was equilibrated for an hour at room temperature. 10 µl of supernatant of culture broth was added to the surface of a well and the picture captured after 1 min using × 10 objective lens of a microscope. Biosurfactant production was considered positive when the drop diameter was at least 0.5 mm larger than those produced by distilled water and also by culture medium as negative controls (Plaza et al., 2006; Youssef et al., 2004). The results were interpreted as follows « + » to « ++++ » corresponding to partial to complete spreading on the oil surface. Those cultures that gave rounded drops were scored as negative «-» indicative of the lack of biosurfactant production (Loganathan et al., 2010).

Oil spreading test

In oil spreading method, 50 ml of sea water synthetic was added to the large Petri plate (90 × 15 mm) followed by 20 µl of crude oil making a thin layer on the surface of the water. A 10 µl aliquot of supernatant was delivered onto the surface of oil (Morikawa et al., 2000). The triplicate assays from the same sample were determined (Rodrigues et al., 2006).

Emulsification index

After growing in standard for 7 days in an orbital shaker at 160 rpm and 40°C, cells were removed by centrifugation at 12,000 × g for 5 min at room temperature. 2 ml of the cell-free supernatant was mixed with 2 ml gazoil in a test tube (125 × 15 mm). This mixture was shaken for 2 min and then left to stand relative emulsion volume (EV,%) and emulsion stability (ES,%) were measured in intervals up to 24 h using the following Equations:

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

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

Emulsion formed by the isolates were compared to those formed by a 1% (w/v) solution of synthetic surfactant sodium dodecyl sulfate in deionised water as positive control and with sterile medium as negative control (Kebbouche-Gana et al., 2009). A criterion cited for emulsion stabilizing capacity is the ability to maintain at least 50% of original emulsion volume 24 h after formation (Nasr et al., 2009).

Surface tension measurement

The surface tension measurements of cell free supernatant were determined in a tensiometer (TD1C LAUDA). The values reported are the mean of three measurements. All measurements were made on cell-free broth (50 ml) collected at different time intervals after centrifugation (10 000×g for 25 min) at room temperature. The criterion used for selecting biosurfactant-producing isolates was the emulsification and the reduction of the surface tension of the medium to below 40 mN m[1-] (Ainon et al., 2013).

Statistical analysis of the correlation between different tests

A general rank correlation test according to Spearman, was conducted to determine the correlation between each of the four methods. The Spearman rank correlation coefficient, rs ranged

Table 1. Chemical and physical properties of Chott El Hodna Salt Lake compared to other hypersaline and marine ecosystems.

Ecosystem [b]	Chemical and physical properties [a]							
	pH	Na$^+$	K$^+$	Mg^{2+}	Ca^{2+}	Cl$^-$	SO$^{2-}_4$	HCO$^-_3$/CO$^{2-}_3$
Chott El Hodna- Algeria	**8.2**	**22.4**	**0.2**	**5.1**	**2.2**	**34.2**	**0.3**	**0.3**
Dead Sea	6.1	40	8	44	17	225	0.5	0.2
Wadi Natrun (Egypt)	11	142	2.3	< 0.1	< 0.1	155	22.6	67
Great salt Lake	7.7	105.4	6.7	11.1	0.3	181	27	0.72
Sea water	8.1	11	0.4	1.3	0.4	19	3	0.1

[a]Ions are represented as g per litre. For Chott El Hodna : Conductivity of the brine sample was 108300 µs/cm, ash content was 73.644 g/L and hardness was 725.60 °F. [b]Chemical and physical properties of some hypersaline and marine ecosystems (Gavrieli, 1997 ; Lmhoff et al., 1979; Post, 1981; Copin-Montégut, 1996).

between -1 (strong negative correlation) to +1 (strong positive correlation).

RESULTS AND DISCUSSION

Physicochemical properties of brine sample

Chemical and physical properties of brine sample taken from Chott El Hodna were compared to other hypersaline and marine ecosystems (Table 1). The results obtained indicate that the mineral content, pH and hardness of brine are suitable for the growth of haloarchaea.

As there is no previous description about a physical and chemical property of Chott El Hodna, these few tests was conducted to understand an outline of ecological condition where halophilic microbes were thriving. Chott El Hodna saline environment was an ideal habitat for haloarchaea and other halophiles, which were well adapted to gradual changes in Chott El Hodna ecosystem and was formed by ecological changes during last hundreds of years of evolution. The presence of all ions and a relatively high Mg^{2+} ion content (Table 1) enhances the natural enrichment of Haloarchaea (Bolhuis et al., 2006). But it contains about 73 and 74 g/L salts, approximately three times lower than the salinity of the Dead Sea, which is a source of extremely halophilic archaea. Typically, salt concentrations of 100 to 150 g/L are required for structural stability and viability of members of the Halobacteriaceae. Exposure to lower concentrations, even for short periods, leads to denaturation of the cells' proteins, including the glycoprotein cell wall present in most species, and the cells lyse. Still, there are reports of the isolation of Haobacteriaceae from low-salt environments, and some members have a surprising ability to survive exposure to low salt for prolonged periods. Isolates affiliated with the genera *Halococcus*, *Haladaptatus* and *Halogeometricum* obtained from a traditional Japanese salt field survived prolonged suspension at 50 g/L salts, and cells of an isolate related to *Haladaptatus paucihalophilus* even retained viability after nine days at 30 g/L (Fukushima et al., 2007).

To gain deeper understanding of Chott El Hodna salt lake, physicochemical properties of brine sample were determined, including pH, conductivity and ion composition (Table 1). The results indicate that the mineral content is dominated by chlorides (34.2 g/L) among the anions and sodium, magnesium and calcium among the cations (22.4 g/L, 5.1 g/L, and 2.3 g/L) respectively, which explains the slightly alkaline pH (8.2) of these lakes. As these environments result from evaporation of sea water (Oren, 2006), compared to the above, we can deduce that Chott El Hodna part of thalassohaline environment.

Extremely halophilc strains

In the current study six halophilic strains isolated from saline soil samples named CH1, CH2, CH3, CH4, CH5, CH6, were phenotypically characterized (Table 2), and compared with strains described previously by Oren, (2014). The strains studied belong to the family of *Halobacteriaceae*, order *Halobacteriales*. These strains were screened for biosurfactant production and emulsification activity.

Screening of biosurfactant production

The primary screening of biosurfactant producing halophilic archaea was carried out using, drop collapse and oil spreading techniques. Selecting of these methods was due to their strong advantages including simplicity, low cost, quick implantation and use of relatively common equipment that is accessible in almost every microbiological laboratory. Results obtained from various screening protocols are mentioned in Table 3 and Figure 2 (A, B and C.)

Drop collapse method

Jain et al. (1991) suggested the use of the drop collapse method as a sensible and easy to perform method which requires a small volume (5-10 µl) of culture broth or

Table 2. Features of the extremely halophilic strains.

Characteristics	CH1	CH2	CH3	CH4	CH5	CH6
Colony morphology (shape, relief, contour, size, and color)	Round Flat Regular Little Cream	Round Cambered Regular Big White	Round Flat Regular Little Orange	Round Cambered Regular Little Orange	Round Cambered Regular Little Translucent	Round Cambered Regular Big Translucent
Cell morphology	Rods	Pleomorphic	Rods	Rods	Cocci	Cocci
Gram reaction	-	-	-	-	-	-
Motility	+	+	-	-	-	-
Physiology						
NaCl tolerance (%)	8-25	10-30	20-25	20-25	10-30	15-25
pH tolerance	7-9	6-8	7-8	7-9	6-8	7-8
T° tolerance (°C)	10-45	35-45	25-45	35-45	25-45	35-45
Biochimical tests						
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Test (MR)	+	-	-	-	+	+
Test (VP)	-	+	-	-	-	-
Indol production	-	-	-	-	+	+
Nitrate reduction	+	+	+	+	-	+
Hydrogen sulfide test	-	-	-	-	+	+
Acid production from						
Mannose	-	-	-	-	-	-
Lactose	-	-	-	+	-	-
Fructose	-	+	+	+	+	+
Galactose	-	-	+	-	+	+
Xylose	+	-	+	+	+	-
Arabinose	-	-	-	-	-	-
mannitol	-	-	-	-	-	-
Glucose	+	+	+	+	+	+
Hydrolysis of						
Gelatin	-	-	-	-	+	-
Starch	-	-	ng	ng	-	ng
Sensivity to antimicrobial agents						
Penicilline	R	R	R	R	R	R
Ampicilline	R	R	R	R	R	R
Novobiocine	S	S	S	S	S	S
Bacitracine	S	S	S	S	S	S
Rifampicine	R	R	R	S	S	R
Anisomycine	S	S	S	S	S	S
Chloromphenicol	R	R	R	R	R	R

CH1, *Halomonas elongata*; CH2, *Natrialba asiatica*; CH3, *Ntrinema pellirubrum*; CH4, *Natrinema pallidum*; CH5, *Haladaptatus paucihalophilus*; CH6 *Haloplanus natans*; R, resistance; S , sensible; ng, no growth.

biosurfactant solution to test the surfactant property. In addition, it can be performed in Microplates (Tugrul and Cansunar, 2005). This assay has been applied several times for screening purposes (Batista et al., 2006; Bodour

Table 3. Drop-collapse test.

Strain	CH1	CH2	CH3	CH4	CH5	CH6
Motor oil	++	++++	+	+++	++++	++
Corn oil	++	++++	+	++	++++	+
Olive oil	+	++++	+	++	++++	+

Flat drops with a scoring system ranging from (+) to (++++) corresponding to partial to complete spreading on the oil surface, rounded drops were scored as negative (-) indicative of the lack of biosurfactant production. Sterilized standard medium used as negative control (-) and a solution of 1% SDS used as positive control (++++).
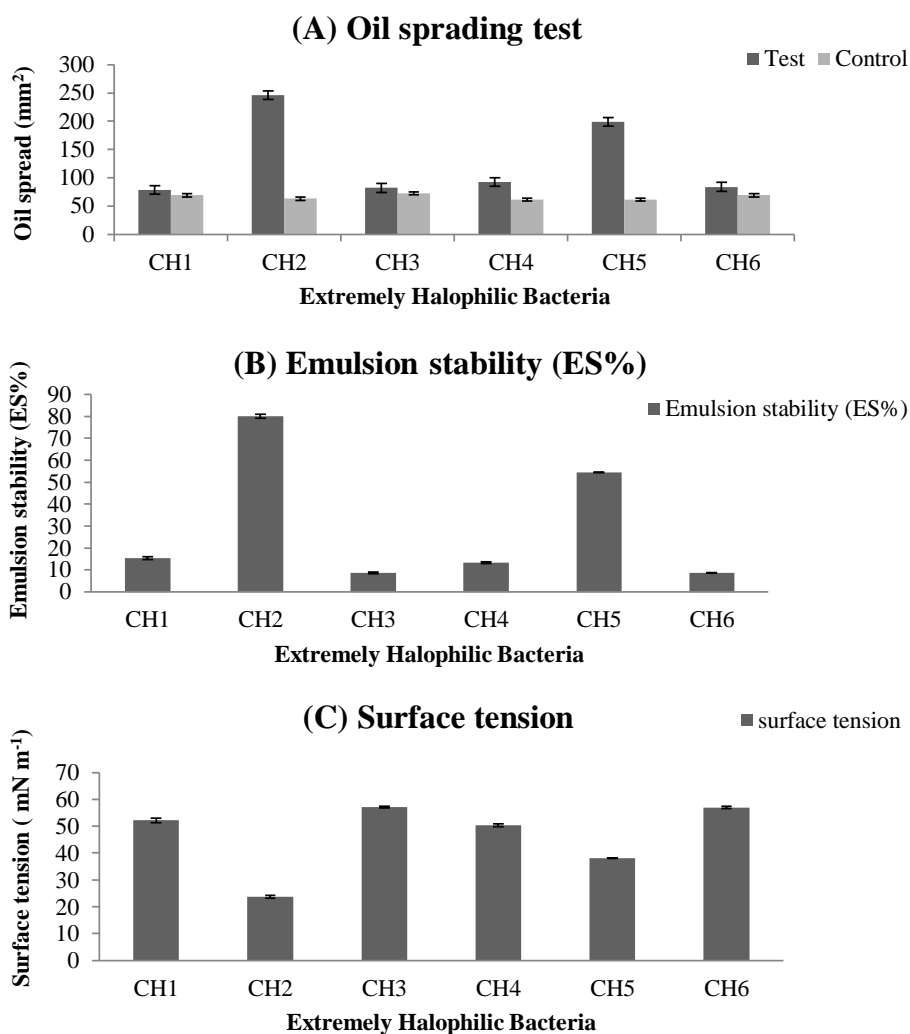

Figure 2. Oil spreading test, emulsion stability and surface tension of culture media without cells and relative volume of emulsions formed between cell culture media and diesel oil after growth of bacteria strains in standard medium for 7 days at 40°C and 200 rpm. Oil dispersion was expressed as mm^2 using the sterile standard medium as control (A); emulsion stability was as a percentage (B) and surface tension was expressed as mN m^{-1}(C). Values reported are average of three replicates.

et al., 2003; Plaza et al., 2006). The results of our experiments indicate that all strains tested positive for biosurfactant in the drop-collapse test reduced as shown as Table 3. Motor oil proved better to work with than olive oil and corn oil. Since, it has caused spreading of the sterilized 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.

In this experiment, cell free culture broth was used as the biosurfactant source. For strains which produce extracellular biosurfactant there was a drop collapse activity and four strains which do not produce biosurfactant the results were negative, which also inferred that to check the biosurfactant production of any microbial strain, cell free culture broth should be used instead of using culture broth with cells. This criterion will exclude microbial strains having high cell hydrophobicity but no biosurfactant production. Accuracy and reliability of results obtained in the drop collapse assay in this study were similar to the results reported by Kebbouche-Gana et al. (2009). The isolates with more than one positive response were exposed to comple-mentary screening. The latter screening stage included surface tension and emulsion activity measurements.

To further confirm the biosurfactant production of the above strains with positive and negative results, cell free culture broth from all six archaeal strains was subjected to oil spreading, emulsion stability and surface tension measurement experiments.

Oil spreading assay

Oil spreading assay results were in corroboration with drop collapse assay results. Strains found with positive drop collapse results were positive for oil spreading assay also (Table 3 and Figure 2A). These results confirmed the presence (for strains with positive results) and absence (for strains with negative results) of surface active compounds (biosurfactant) in the cell free culture broth. Morikawa et al. (2000), reported that the area of oil displacement in an oil spreading assay is directly proportional to the concentration of the biosurfactant in the solution. However, in this study there was no quanti-tative study conducted on biosurfactant concentration versus oil spreading activity, but a qualitative study to check the presence of biosurfactant in the cell free culture broth was in concurrence with the above mentioned earlier report. Similar results with drop collapse and oil spreading assay was reported by Youssef et al. (2004), while screening bacteria from biosurfactant production and also recommended that both drop collapse and oil spreading assay methods as reliable techniques for testing biosurfactant production. These results suggested that the oil-spreading technique is more sensitive than the other methods for biosurfactant detection in the supernatant from a culture medium.

Emulsification capacity assay

According to Willumsen and Karlson (1997), a criterion

used for selecting biosurfactant producers is the ability to maintain at least 50% of the original emulsion volume 24 h after formation. The results observed in this study (Figure 2B) reveal that from six strains screened, two (33.33%) strains showed positive emulsification activity. CH2 and CH5 strains showed that the relative emulsion stability formed: $80.00 \pm 1.0\%$ and 54.39 ± 0.2, respectively. Evaluating the emulsification capacity is a simple screening method suitable for a first screening of biosurfactant producing microbes. It is applied in many screenings (Chen et al., 2007), whereas, the Kerosene can be replaced with other hydrophobic compounds. In this study, gas oil was used as the hydrophobic substrate. Consequently, this method gives just an indication of the presence of biosurfactants.

Surface tension measurement

According to Bodour and Maier (1998), the criterion used for selecting biosurfactant producers is the ability to reduce the surface tension below 40 $mN.m^{-1}$. Surface tension measurement of cell free culture broth revealed that out of the six strains screened, two (33.33%) strains (CH2 and CH5) showed reduction in surface tension and the highest reduction was observed up to 23.7 ± 0.5 [1] and 38.1 ± 0.1 $mN.m^{-1}$, respectively (Figure 2C). There was a direct correlation found between drop collapse, oil spreading, emulsification stability and surface tension assays. Strains highly active in any one of these methods were active in other three methods. The direct measurement of the surface activity of the culture supernatant is the most straight forward screening methods. This gives a strong indication on biosurfactant production (Lin, 1996; Thavasi et al. 2011).

A comparison of the four methods using statistical analysis

Table 4 shows the coefficient of correlation between the four methods that were used to detect biosurfactant production. The Spearman rank correlation, ($r_s = -0.987$) showed a strong negative correlation between the emulsification index with measurement of emulsion stability in intervals up to 24 h and surface tension. A weaker negative correlation, ($r_s = -0.971$) was detected between the diameter of clear zone obtained with the oil spreading technique and surface tension. However, there was a weak negative correlation ($r_s = -0.807$) between drop collapse method and surface tension. Oil spreading technique and emulsification stability method were strongly correlated with Spearman rank correlation coefficient of $r_s=0.991$. However, a weak correlation was detected between drop collapse method and oil spreading method ($r_s=0.864$) and between drop collapse technique and emulsification stability method ($r_s=0.822$).

Table 4. Statistical correlation between different methods.

Spearman rank correlation coefficient (r_s)	Drop collapse	Oil spreading	Emulsification stability	Surface tension
Drop collapse	1			
Oil spreading	0.864	1		
Emulsification stability	0.822	0.991	1	
Surface tension	- 0.807	- 0.971	-0.987	1

Conclusion

Interest in biosurfactants has led to the development of a multitude of methods for the screening of biosurfactant producer strains. A combination of different methods is appropriate for a successful screening. In the present study two superior haloarchaea isolates CH2 and CH5 with biosurfactant-producing ability and the former with emulsion capacity were isolated from saline soil of Chott El Hodna-M'sila (Algeria). Their ability to reduce surface tension and emulsion capacity makes them new potential candidates for biosurfactant and bioemulsion production. Further studies have been initiated to identify their properties and consequently determine the potential of their different industrial applications in particular enhanced oil recovery application.

Conflict of interests

The authors did not declare any conflict of interest.

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