



Phylogenetic characterization and screening of halophilic bacteria from Algerian salt lake for the production of biosurfactant and enzymes

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ABSTRACT	

Environments containing significant concentration of NaCl such as salt lakes harbor extremophiles microorganisms which have a great biotechnology interest. To explore the diversity of *Bacteria* in Chott Tinsilt (Algeria), an isolation program was performed. Water samples were collected from the saltern during the pre-salt harvesting phase. This Chott is high in salt (22.47% (w/v)). Seven halophiles *Bacteria* were selected for further characterization. The isolated strains were able to grow optimally in media with 10–25% (w/v) total salts. Molecular identification of the isolates was performed by sequencing the 16S rRNA gene. It showed that these cultured isolates included members belonging to the *Halomonas*, *Staphylococcus*, *Salinivibrio*, *Planococcus* and *Halobacillus* genera with less than 98% of similarity with their closest phylogenetic relative. The halophilic bacterial isolates were also characterized for the production of biosurfactant and industrially important enzymes. Most isolates produced hydrolases and biosurfactants at high salt concentration. In fact, this is the first report on bacterial strains (A4 and B4) which were a good biosurfactant and coagulase producer at 20% and 25% ((w/v)) NaCl. In addition, the biosurfactant produced by the strain B4 at high salinity (25%) was also stable at high temperature (30-100°C) and high alkalinity (pH 11).

Key word: Salt Lake, *Bacteria*, biosurfactant, Chott, halophiles, hydrolases, 16S rRNA

INTRODUCTION: Saline lakes cover approximately 10% of the Earth's surface area. The microbial populations of many hypersaline environments have already been studied in different geographical regions such as Great Salt Lake (USA), Dead Sea (Israel), Wadi Natrun Lake (Egypt), Lake Magadi (Kenya), Soda Lake (Antarctica) and Big Soda Lake and Mono Lake (California). Hypersaline regions differ from each other in terms of geographical location, salt concentration and chemical composition, which determine the nature of inhabitant microorganisms (Gupta *et al.*, 2015). Then low taxonomic diversity is common to all these saline environments (Oren *et al.*, 1993). Halophiles are found in nearly all major microbial clades, including prokaryotic (*Bacteria* and *Archaea*) and eukaryotic forms (DasSarma and Arora, 2001). They are classified as slight halophiles when they grow optimally at 0.2–0.85 M (2–5%) NaCl, as moderate halophiles when they grow at 0.85–3.4 M (5–20%) NaCl, and as extreme halophiles when they grow at 3.4–5.1 M (20–30%) NaCl. Hyper saline environments are inhabited by extremely halophilic and halotolerant microorganisms such as *Halobacillus sp.*, *Halobacterium sp.*, *Haloarcula sp.*, *Salinibacter ruber*, *Haloferax sp.* and *Bacillus spp.* (Solomon and Viswalingam, 2013). There is a tremendous demand for halophilic bacteria due to their biotechnological importance as sources of halophilic enzymes. Enzymes derived from halophiles are endowed with unique structural features and catalytic power to sustain the metabolic and physiological processes under high salt conditions. Some of these enzymes have been reported to be active and stable under more than one extreme condition (Karan and Khare, 2010). Applications are

being considered in a range of industries such as food processing, washing, biosynthetic processes and environmental bioremediation. Halophilic proteases are widely used in the detergent and food industries (DasSarma and Arora, 2001). However, esterases and lipases have also been useful in laundry detergents for the removal of oil stains and are widely used as biocatalysts because of their ability to produce pure compounds. Likewise, amylases are used industrially in the first step of the production of high fructose corn syrup (hydrolysis of corn starch). They are also used in the textile industry in the de-sizing process and added to laundry detergents. Furthermore, for the environmental applications, the use of halophiles for bioremediation and biodegradation of various materials from industrial effluents to soil contaminants and accidental spills are being widely explored. In addition to enzymes, halophilic / halotolerant microorganisms living in saline environments, offer another potential applications in various fields of biotechnology like the production of biosurfactant. Biosurfactants are amphiphilic compounds synthesized from plants and microorganisms. They reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively (Akbari *et al.*, 2018). Comparing to the chemical surfactant, biosurfactant are promising alternative molecules due to their low toxicity, high biodegradability, environmental capability, mild production conditions, lower critical micelle concentration, higher selectivity, availability of resources and ability to function in wide ranges of pH, temperature and salinity (Rocha *et al.*, 1992). They are used in various industries which include pharmaceuticals, petroleum,

food, detergents, cosmetics, paints, paper products and water treatment (Akbari *et al.*, 2018). The search for biosurfactants in extremophiles is particularly promising since these biomolecules can adapt and be stable in the harsh environments in which they are to be applied in biotechnology.

OBJECTIVES: Eastern Algeria features numerous ecosystems including hypersaline environments, which are an important source of salt for food. The microbial diversity in Chott Tinsilt, a shallow Salt Lake with more than 200g/L salt concentration and a superficies of 2.154 Ha, has never yet been studied. The purpose of this research was to chemically analyse water samples collected from the Chott, isolate novel extremely or moderate halophilic *Bacteria*, and examine their phenotypic and phylogenetic characteristics with a view to screening for biosurfactants and enzymes of industrial interest.

MATERIALS AND METHODS: Study area: The area is at 5 km of the Commune of Souk-Naâmane and 17 km in the South of the town of Aïn-Melila. This area skirts the trunk road 3 serving Constantine and Batna and the railway Constantine-Biskra. It is part the administrative jurisdiction of the Wilaya of Oum El Bouaghi. The Chott belongs to the wetlands of the High Plains of Constantine with a depth varying rather regularly without never exceeding 0.5 meter. Its length extends on 4 km with a width of 2.5 km (Figure 1).



Figure 1: Location maps of Chott Tinsilt (google maps).

Water samples and physico-chemical analysis: In February 2013, water samples were collected from various places at the Chott Tinsilt using Global Positioning System (GPS) coordinates of 35°53'14" N lat. and 06°28'44" E long. Samples were collected randomly in sterile polythene bags and transported immediately to the laboratory for isolation of halophilic microorganisms. All samples were treated within 24 h after collection. Temperature, pH and salinity were measured *in situ* using a multi-parameter probe (Hanna Instruments, Smithfield, RI, USA). The analytical methods used in this study to measure ions concentration (Ca^{2+} , Mg^{2+} , Fe^{2+} , Na^+ , K^+ , Cl^- , HCO_3^- , SO_4^{2-}) were based on 4500-S-2 F standard methods described elsewhere (Association *et al.*, 1920).

Isolation of halophilic bacteria from water sample: The media (M1) used in the present study contain (g/L): 2.0 g of KCl, 100.0/200.0 g of NaCl, 1.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 g of Sodium Citrate, 0.36 g of MnCl_2 , 10.0 g of yeast extract and 15.0 g agar. The pH was adjusted to 8.0. Different dilutions of water samples were added to the above medium and incubated at 30°C during 2–7 days or more depending on growth. Appearance and growth of halophilic bacteria were monitored regularly. The growth was diluted 10 times and plated on complete medium agar (g/L): glucose 10.0; peptone 5.0; yeast extract 5.0; KH_2PO_4

5.0; agar 30.0; and NaCl 100.0/200.0. Resultant colonies were purified by repeated streaking on complete media agar. The pure cultures were preserved in 20% glycerol vials and stored at -80°C for long-term preservation.

Biochemical characterisation of halophilic bacterial isolates: Bacterial isolates were studied for Gram's reaction, cell morphology and pigmentation. Enzymatic assays (catalase, oxidase, nitrate reductase and urease), and assays for fermentation of lactose and mannitol were done as described by Smibert (1994).

Optimization of growth conditions: Temperature, pH, and salt concentration were optimized for the growth of halophilic bacterial isolates. These growth parameters were studied quantitatively by growing the bacterial isolates in M1 medium with shaking at 200 rpm and measuring the cell density at 600 nm after 8 days of incubation. To study the effect of NaCl on the growth, bacterial isolates were inoculated on M1 medium supplemented with different concentration of NaCl: 1%-35% (w/v). The effect of pH on the growth of halophilic bacterial strains was studied by inoculating isolates on above described growth media containing NaCl and adjusted to acidic pH of 5 and 6 by using 1N HCl and alkaline pH of 8, 9, 10, 11 and 12 using 5N NaOH. The effect of temperature was studied by culturing the bacterial isolates in M1 medium at different temperatures of incubation (4°C–55°C).

Screening of halophilic bacteria for hydrolytic enzymes: Hydrolase producing bacteria among the isolates were screened by plate assay on starch, tributyrin, gelatin and DNA agar plates respectively for amylase, lipase, protease and DNase activities. Amylolytic activity of the cultures was screened on starch nutrient agar plates containing g/L: starch 10.0; peptone 5.0; yeast extract 3.0; agar 30.0; NaCl 100.0/250.0. The pH was 7.0. After incubation at 30 °C for 7 days, the zone of clearance was determined by flooding the plates with iodine solution. The potential amylase producers were selected based on ratio of zone of clearance diameter to colony diameter. Lipase activity of the cultures was screened on tributyrin nutrient agar plates containing 1% (v/v) of tributyrin. Isolates that showed clear zones of tributyrin hydrolysis were identified as lipase producing bacteria. Proteolytic activity of the isolates was similarly screened on gelatin nutrient agar plates containing 10.0 g/L of gelatin. The isolates showing zones of gelatin clearance upon treatment with acidic mercuric chloride were selected and designated as protease producing bacteria. The presence of DNase activity on plates was determined on DNase test agar (BBL) containing 10%-25% (w/v) total salt. After incubation for 7days, the plates were flooded with 1N HCl solution. Clear halos around the colonies indicated DNase activity (Jeffries *et al.*, 1957).

Milk clotting activity (coagulase activity) of the isolates was also determined following the procedure described (Berridge, 1952). Skim milk powder was reconstituted in 10 mM aqueous CaCl_2 (pH 6.5) to a final concentration of 0.12 kg/L. Enzyme extracts were added at a rate of 0.1 mL per mL of milk. The coagulation point was determined by manual rotating of the test tube periodically, at short time intervals, and checking for visible clot formation.

Screening of halophilic bacteria for biosurfactant production.

Oil spread Assay: The Petridis base was filled with 50 mL of distilled water. On the water surface, 20µL of diesel and 10µL of

culture were added respectively. The culture was introduced at different spots on the diesel, which is coated on the water surface. The occurrence of a clear zone was an indicator of positive result (Morikawa *et al.*, 2000). The diameter of the oil expelling circles was measured by slide caliber (with a degree of accuracy of 0.02 mm).

Surface tension and emulsification index (E₂₄): Isolates were cultivated at 30 °C for 7 days on the enrichment medium containing 10-25% NaCl and diesel oil as the sole carbon source. The medium was centrifuged (7000 rpm for 20 min) and the surface tension of the cell-free culture broth was measured with a TS90000 surface tensiometer (Nima, Coventry, England) as a qualitative indicator of biosurfactant production. The culture broth was collected with a Pasteur pipette to remove the non-emulsified hydrocarbons. The emulsifying capacity was evaluated by an emulsification index (E₂₄). The E₂₄ of culture samples was determined by adding 2 mL of diesel oil to the same amount of culture, mixed for 2 min with a vortex, and allowed to stand for 24 h. E₂₄ index is defined as the percentage of height of emulsified layer (mm) divided by the total height of the liquid column (mm).

Biosurfactant stability studies : After growth on diesel oil as sole source of carbone, cultures supernatant obtained after centrifugation at 6,000 rpm for 15 min were considered as the source of crude biosurfactant. Its stability was determined by subjecting the culture supernatant to various temperature ranges (30, 40, 50, 60, 70, 80 and 100 °C) for 30 min then cooled to room temperature. Similarly, the effect of different pH (2–11) on the activity of the biosurfactant was tested. The activity of the biosurfactant was investigated by measuring the emulsification index (El-Sersy, 2012).

Molecular identification of potential strains. DNA extraction and PCR amplification of 16S rDNA: Total cellular DNA was extracted from strains and purified as described by Sambrook *et al.* (1989). DNA was purified using GeneClean® Turbo (Q-BIO gene, Carlsbad, CA, USA) before use as a template in polymerase chain reaction (PCR) amplification. For the 16S rDNA gene sequence, the purified DNA was amplified using a universal primer set, forward primer (27f; 5'-AGA GTT TGA TCM TGG CTC AG) and a reverse primer (1492r; 5'-TAC GGY TAC CTT GTT ACG ACT T) (Lane, 1991). Agarose gel electrophoresis confirmed the amplification product as a 1400-bp DNA fragment.

16S rDNA sequencing and Phylogenic analysis: Amplicons generated using primer pair 27f-1492r was sequenced using an automatic sequencer system at Macrogen Company (Seoul, Korea). The sequences were compared with those of the NCBI BLAST GenBank nucleotide sequence databases. Phylogenetic trees were constructed by the neighbor-joining method using MEGA version 5.05 software (Tamura *et al.*, 2011). Bootstrap resembling analysis for 1,000 replicates was performed to estimate the confidence of tree topologies.

Nucleotide sequence accession numbers: The nucleotide sequences reported in this work have been deposited in the EMBL Nucleotide Sequence Database. The accession numbers are represented in table 5.

Statistics: All experiments were conducted in triplicates. Results were evaluated for statistical significance using ANOVA.

RESULTS: Physico-chemical parameters of the collected water samples: The physicochemical properties of the

collected water samples are reported in table 1.

Temperature (°C)	10,6
pH	7,8,9
Salinity (g/L)	224,70
Conductivity (µs/cm)	18780
Turbidity (NTU)	1,13
Ca ⁺⁺ (mg/L)	701,40
Mg ⁺⁺ (mg/L)	6456,96
Fe ⁺⁺ (mg/L)	00
Na ⁺ (mg/L)	71425
K ⁺ (mg/L)	560,40
Cl ⁻ (mg/L)	124471,79
HCO ₃ ⁻ (mg/L)	195,20
SO ₄ ⁻ (mg/L)	16829,52

Table 1: Physicochemical properties of water from Chott Tinsilt.

At the time of sampling, the temperature was 10.6°C and pH 7.89. The salinity of the sample, as determined *in situ*, was 224.70 g/L (22,47% (w/v)). Chemical analysis of water sample indicated that Na⁺ and Cl⁻ were the most abundant ions (table 1). SO₄²⁻ and Mg²⁺ was present in much smaller amounts compared to Na⁺ and Cl⁻ concentration. Low levels of calcium, potassium and bicarbonate were also detected, often at less than 1 g/L.

Characterization of isolates. Morphological and biochemical characteristic feature of halophilic bacterial isolates: Among 52 strains isolated from water of Chott Tinsilt, seven distinct bacteria (A1, A2, A3, A4, B1, B4 and B5) were chosen for further characterization (table 2). The colour of the isolates varied from beige, pale yellow, yellowish and orange. The bacterial isolates A1, A2, A4, B1 and B5 were rod shaped and gram negative (except B5), whereas A3 and B4 were cocci and gram positive. All strains were oxidase and catalase positive except for B1. Nitrate reductase and urease activities were observed in all the bacterial isolates, except B4. All the bacterial isolates were negative for H₂S formation. B5 was the only strain positive for mannitol fermentation (table 2).

We isolated halophilic bacteria on growth medium with NaCl supplementation at pH 7 and temperature of 30°C. We studied the effect of NaCl, temperature and pH on the growth of bacterial isolates. All the isolates exhibited growth only in the presence of NaCl indicating that these strains are halophilic. The optimum growth of isolates A3 and B1 was observed in the presence of 10% NaCl, whereas it was 15% NaCl for A1, A2 and B5. A4 and B4 showed optimum growth in the presence of 20% and 25% NaCl respectively. A4, B4 and B5 strains can tolerate up to 35% NaCl.

The isolate B1 showed growth in medium supplemented with 10% NaCl and pH range of 7–10. The optimum pH for the growth B1 was 9 and they did not show any detectable growth at or below pH 6 (table 2), which indicates the alkaliphilic nature of B1 isolate. The bacterial isolates A1, A2 and A4 exhibited growth in the range of pH 6–10, while A3 and B4 did not show any growth at pH greater than 8. The optimum pH for growth of all strains (except B1) was pH 7.0 (table 2). These results indicate that A1, A2, A3, A4, B4 and B5 are neutrophilic in nature. All the bacterial isolates exhibited optimal growth at 30°C and no detectable growth at 55°C. Also, detectable growth of isolates A1, A2 and A4 was observed at 4°C. However, none of the bacterial strains could grow below 4°C and above 50°C (table 2).

Cellular/biochemical and growth parameter	<i>Halophilic bacterial isolates</i>							
	A1	A2	A3	A4	B1	B4	B5	
Cell morphology	Rods	Rods	Cocci	Rods	Rods	Cocci	Rods	
Motility	+	+	-	+	+	+	+	
Pigmentation	Pale beige	Yellowish	Pale yellow	Yellow	Beige	Orange	Orange	
Gram's reaction	-	-	+	-	-	+	+	
Catalase	+	+	+	+	-	+	+	
Oxidase	+	+	+	+	+	+	+	
Nitrate reductase	+	+	+	+	+	-	+	
Urease	+	+	+	+	+	-	+	
H ₂ S formation	-	-	-	-	-	-	-	
NaCl(%) optimum concentration	15 (5-25)	15 (5-25)	10 (2-25)	20 (8-35)	10 (2-25)	25 (8-35)	15 (8-35)	
pH for growth (optimum pH (range))	7.0 (6.0-10.0)	7.0 (6.0-10.0)	7.0 (6.0-8.0)	7.0 (6.0-10.0)	9.0 (7.0-10.0)	7.0 (7.0-8.0)	7.0 (6.0-9.0)	
Temperature optimum (range) (°C)	30 (4-48)	30 (4-48)	30 (10-40)	30 (4-48)	30 (10-40)	30 (20-37)	35 (10-50)	
Glucose	-	-	-	+	-	-	+	
Lactose	-	-	-	+	-	-	+	
Sucrose	-	-	-	+	-	-	+	
Mannitol	-	-	-	-	-	-	+	
Citrate	+	+	+	+	+	+	+	
Lysine	-	+	+	+	+	+	-	
Arginine	+	+	+	+	+	+	-	
Ornithine	+	+	+	+	+	-	+	
Tryptophan	+	+	+	-	-	+	+	
Indole	-	-	-	-	-	-	-	
Urea	+	+	+	+	+	-	+	

Table 2: Biochemical and growth characteristics of the halophilic bacterial isolates from Chott Tinsilt.

Plus (+) sign indicates the test as positive, whereas negative (-) sign indicates negative results. For biochemical test, Plus (+) sign indicates the growth of bacterial isolate and negative (-) sign indicates no detectable growth when streaked or spotted on M1 agar medium.

Screening of the halophilic enzymes: To characterize the diversity of halophiles able to produce hydrolytic enzymes among the population of microorganisms inhabiting the hypersaline habitats of East Algeria (Chott Tinsilt), a screening was performed. As described in Materials and Methods, samples were plated on solid media containing 10%-25% (w/v) of total salts and different substrates for the detection of amylase, protease, lipase and DNase activities. However, coagulase activity was determined in liquid medium using milk as substrate (figure 3). Distributions of hydrolytic activity among the isolates are summarized in table 4.

From the seven bacterial isolates, four strains A1, A2, A4 and B5 showed combined hydrolytic activities. They were positive for gelatinase, lipase and coagulase. A3 strain showed gelatinase and lipase activities. DNase activities were detected with A1, A4, B1 and B5 isolates. B4 presented lipase and coagulase activity. Surprisingly, no amylase activity was detected among all the isolates.

Screening for biosurfactant producing isolates: Oil spread assay: The results showed that all the strains could produce notable (>4 cm diameter) oil expelling circles (ranging from 4.11 cm to 4.67 cm). The average diameter for strain B5 was 4.67 cm, significantly (P < 0.05) higher than for the other

strains.

Surface tension and emulsification index (E24): The assimilation of hydrocarbons as the sole sources of carbon by the isolate strains led to the production of biosurfactants indicated by the emulsification index and the lowering of the surface tension of cell-free supernatant. Based on rapid growth on media containing diesel oil as sole carbon source, the seven isolates were tested for biosurfactant production and emulsification activity. The obtained values of the surface tension measurements as well as the emulsification index (E24) are shown in table 3. The highest reduction of surface tension was achieved with B5 and A3 isolates with values of 25.3 mN m⁻¹ and 28.1 mN m⁻¹ respectively. The emulsifying capacity evaluated by the E₂₄ emulsification index was highest in the culture of isolate B4 (78%), B5 (77%) and A3 (76%) as shown in table 3 and figure 2. These emulsions were stable even after 4 months. The bacteria with emulsification indices higher than 50 % and/or reduction in the surface tension (under 30 mN/m) have been defined as potential biosurfactant producers. Based on surface tension and the E24 index results, isolates B5, B4, A3 and A4 are the best candidates for biosurfactant production. It is important to note that, strains B4 and A4 produce biosurfactant in medium containing respectively 25% and 20%

(w/v) NaCl.

Stability of biosurfactant activities: The applicability of biosurfactants in several biotechnological fields depends on their stability at different environmental conditions (temperatures, pH and NaCl). For this study, the strain B4

appear very interesting (It can produce biosurfactant at 25 % NaCl) and was chosen for further analysis for biosurfactant stability. The effects of temperature and pH on the biosurfactant production by the strain B4 are shown in figure 4.

Isolates	Surface tension*	E ₂₄ **
A1	30.4 ±3.8	53 ±1.8
A2	31.7 ±1.9	56 ±2.3
A3	28.1 ±0.8	76 ±0.2
A4	30.2 ±2.7	65 ±0.3
B1	33.2 ±0.5	61 ±0.9
B4	29.8 ±1.6	78 ±0.7
B5	25.3 ±2.0	77 ±0.4

Table 3: Evaluation of the aqueous phase of suspensions of bacterial isolates from Chott Tinsilt after 7 days of growth in basal salt medium spiked with diesel oil. Errors bars are ± the standard deviation of the mean.

*Surface tension was expressed as mN m⁻¹ using basal medium as control (74.2 ±0.3 mN m⁻¹). **E₂₄ was expressed as percentage.

Strains Enzymes	Halophilic bacterial isolates						
	A1	A2	A3	A4	B1	B4	B5
Amylase	-	-	-	-	-	-	-
Gélatinase	+	+	+	+	-	-	+
Lipase	+	+	+	+	-	+	+
Coagulase	+	+	-	+	-	+	+
DNAase	+	-	-	+	+	-	+

Table 4: Effect of (A) temperature and (B) pH on biosurfactant stability produced by B4 strain.

Isolates closest match	Accession numbers	Closest relative	% Similarity	Origin of the
A1	HG939468	<i>Halomonas venusta</i> strain GSP24	96	Salt Plains
A2	HG939469	<i>Halomonas variabilis</i> strain GSP3	97	Salt Plains
A3	HG939470	<i>Staphylococcus arlettae</i>	95	Saline soil
A4	HG939471	<i>Halomonas</i> sp. M59	97	Solar salterns
B1	HG939472	<i>Salinivibrio costicola</i> subsp. <i>alcaliphilus</i> strain 18AG	96	Campania Region
B4	HG939475	<i>Planococcus citreus</i>	96	Saline Lake
B5	HG939476	<i>Halobacillus trueperi</i>	98	Human gut

Table 5: Bacteria 16S rRNA genes of isolates from Chott Tinsilt.

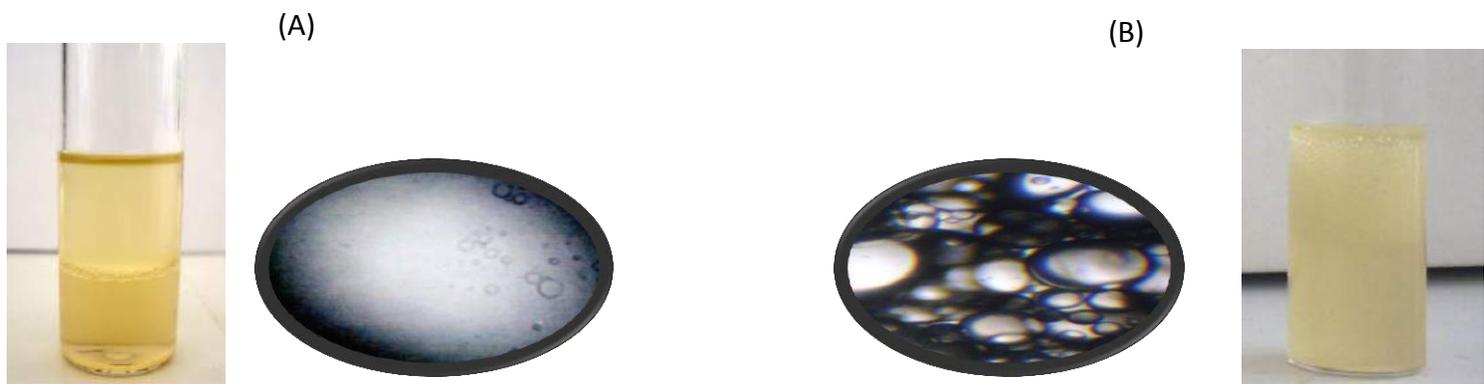


Figure 2: Emulsion aspect obtained by optical microscopy (GX40) for B4 strain, A) Negative emulsion, B) positive emulsion.

biosurfactant in medium containing respectively 25% and 20% (w/v) NaCl.

Stability of biosurfactant activities: The applicability of biosurfactants in several biotechnological fields depends on their stability at different environmental conditions (temperatures, pH and NaCl). For this study, the strain B4 appear very interesting (It can produce biosurfactant at 25 % NaCl) and was chosen for further analysis for biosurfactant stability. The effects of temperature and pH on the biosurfactant production by the strain B4 are shown in figure 4. The biosurfactant produced by this strain



Figure 3: Coagulase activity obtained for B4 strain. A) control; B) positive coagulase.

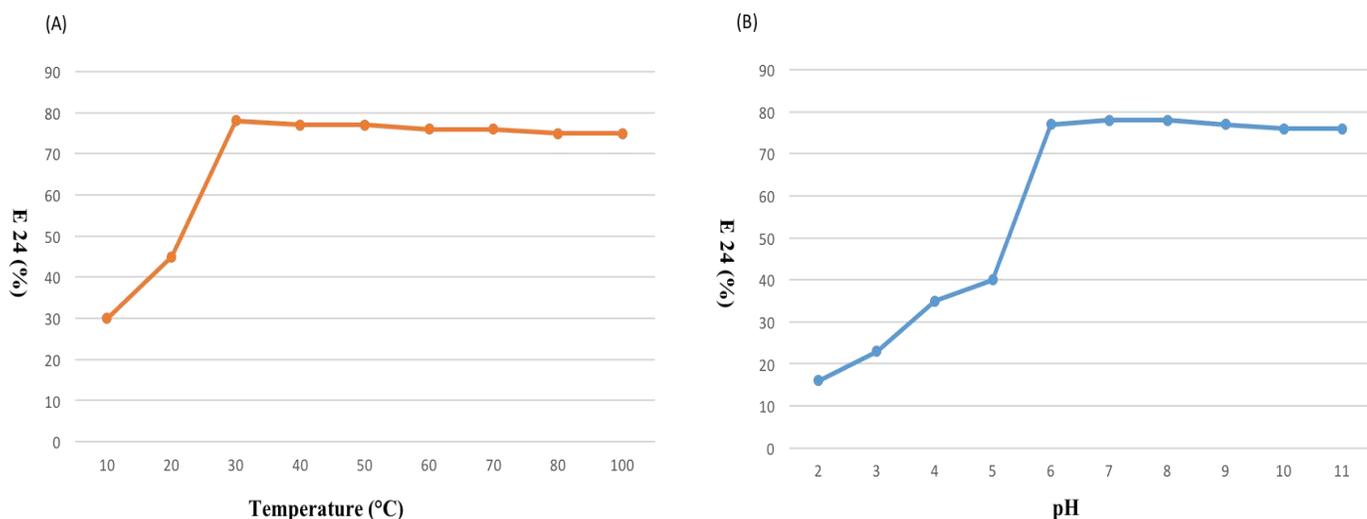


Figure 4: Effect of (A) temperature and (B) pH on biosurfactant stability produced by B4 strain.

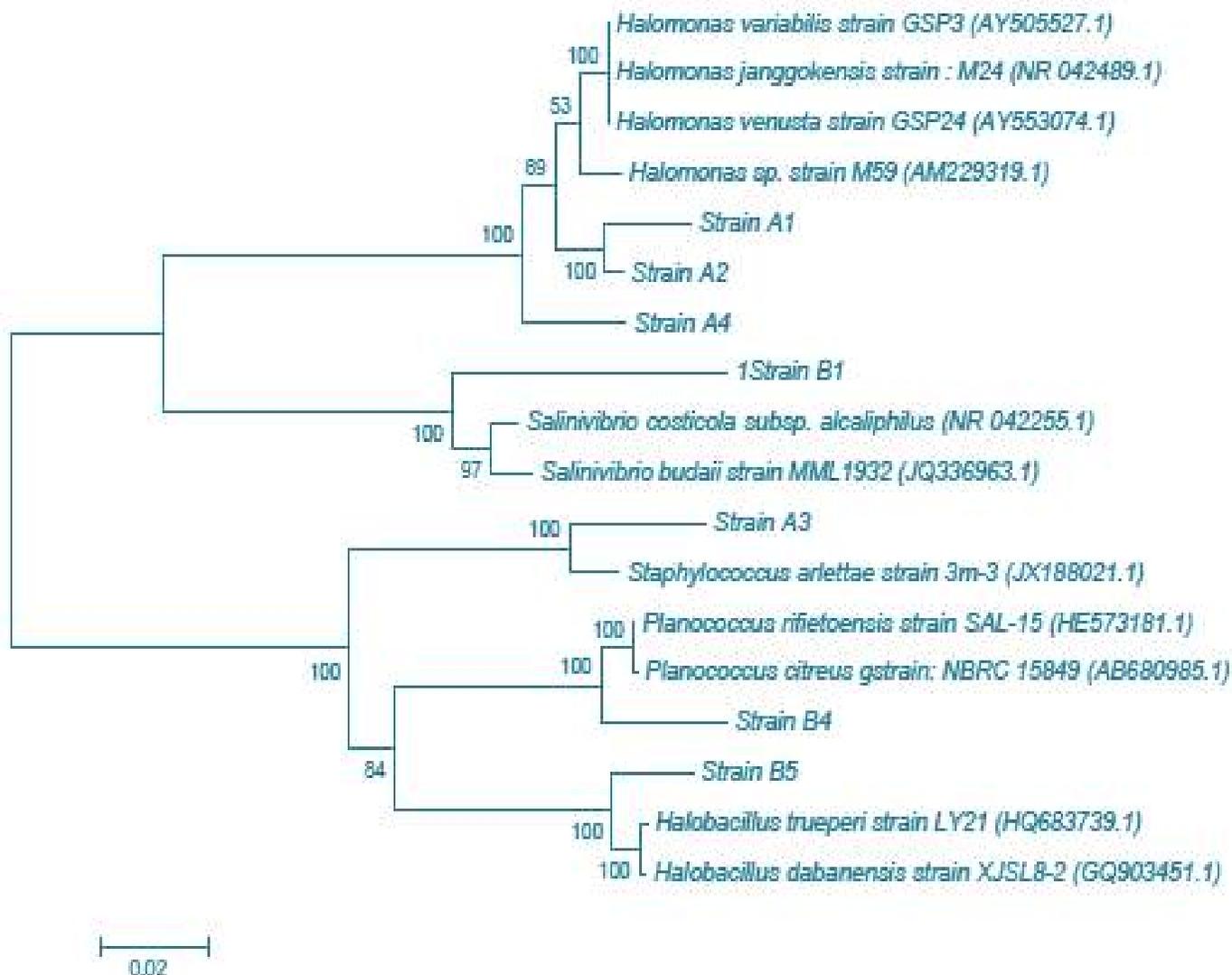


Figure 5: Phylogenetic analysis of bacterial isolates based on 16S rDNA sequences. Neighbor-joining phylogenetic trees depicting the interrelationships of 16S rDNA sequence of halophilic isolates A1, A2, A3, A4, B1, B4 and B5 with closely related halophilic isolates of their respective genera. The values at the tree nodes indicate percentages of recurring branches (1,000 bootstraps for resampling).

was shown to be thermostable giving an E-24 Index value greater than 78% (figure 4A). Heating of the biosurfactant to 100 °C caused no significant effect on the biosurfactant performance. Therefore, the surface activity of the crude biosurfactant supernatant remained relatively stable to pH changes between pH 6 and 11. At pH 11, the value of E24 showed almost 76% activity, whereas below pH 6 the activity was decreased up to 40% (figure 4A). The decreases of the emulsification activity by decreasing the pH value from basic to an acidic region; may be due to partial precipitation of the biosurfactant. This result indicated that biosurfactant produced by strain B4 show higher stability at alkaline than in acidic conditions.

Molecular identification and phylogenies of potential isolates: To identify halophilic bacterial isolates, the 16S rDNA gene was amplified using gene-specific primers. A PCR product of \approx 1.3 kb was detected in all the seven isolates. The 16S rDNA amplicons of each bacterial isolate was sequenced on both strands using 27F and 1492R primers. The complete nucleotide sequence of 1336,1374, 1377,1313, 1305,1308 and 1273 bp sequences were obtained from A1, A2, A3, A4, B1, B4 and B5 isolates respectively, and subjected to BLAST analysis. The 16S rDNA sequence analysis showed that the isolated strains belong to the genera *Halomonas*, *Staphylococcus*, *Salinivibrio*, *Planococcus* and *Halobacillus* as shown in table 5. The halophilic isolates A2 and A4 showed 97% similarity with the *Halomonas variabilis* strain GSP3 (accession no. AY505527) and the *Halomonas* sp. M59 (accession no. AM229319), respectively. As for A1, it showed 96% similarity with the *Halomonas venusta* strain GSP24 (accession no. AY553074). B1 and B4 showed for their part 96% similarity with the *Salinivibrio costicola* subsp. *alcaliphilus* strain 18AG DSM4743 (accession no. NR_042255) and the *Planococcus citreus* (accession no. JX122551), respectively. The bacterial isolate B5 showed 98% sequence similarity with the *Halobacillus trueperi* (accession no. HG931926). As for A3, it showed only 95% similarity with the *Staphylococcus arlettae* (accession no. KR047785). The 16S rDNA nucleotide sequences of all the seven halophilic bacterial strains have been submitted to the NCBI GenBank database under the accession number presented in table 5. The phylogenetic association of the isolates is shown in figure 5.

Discussion: The physicochemical properties of the collected water samples indicated that this water was relatively neutral (pH 7.89) similar to the Dead Sea and the Great Salt Lake (USA) and in contrast to the more basic lakes such as Lake Wadi Natrun (Egypt) (pH 11) and El Golea Salt Lake (Algeria) (pH 9). The salinity of the sample was 224.70 g/L (22,47% (w/v). This range of salinity (20-30%) for Chott Tinsilt is comparable to a number of well characterized hypersaline ecosystems including both natural and man-made habitats, such as the Great Salt Lake (USA) and solar salterns of Puerto Rico. Thus, Chott Tinsilt is a hypersaline environment, i.e. environments with salt concentrations well above that of seawater. Chemical analysis of water sample indicated that Na⁺ and Cl⁻ were the most abundant ions, as in most hypersaline ecosystems (with some exceptions such as the Dead Sea). These chemical water characteristics were consistent with the previously reported data in other hypersaline ecosystems (DasSarma and Arora, 2001; Oren, 2002; Hacène et al., 2004). Among 52 strains isolated from this Chott, seven distinct bacteria (A1, A2, A3, A4, B1, B4 and B5) were chosen for

phenotypique, genotypique and phylogenetique characterization.

The 16S rDNA sequence analysis showed that the isolated strains belong to the genera *Halomonas*, *Staphylococcus*, *Salinivibrio*, *Planococcus* and *Halobacillus*. Genera obtained in the present study are commonly occurring in various saline habitats across the globe. *Staphylococci* have the ability to grow in a wide range of salt concentrations (Graham and Wilkinson, 1992; Morikawa et al., 2009; Roohi et al., 2014). For example, in Pakistan, *Staphylococcus* strains were isolated from various salt samples during the study conducted by Roohi et al. (2014) and these results agreed with previous reports. *Halomonas*, halophilic and/or halotolerant Gram-negative bacteria are typically found in saline environments (Kim et al., 2013). The presence of *Planococcus* and *Halobacillus* has been reported in studies about hypersaline lakes; like La Sal del Rey (USA) (Phillips et al., 2012) and Great Salt Lake (Spring et al., 1996), respectively. The *Salinivibrio costicola* was a representative model for studies on osmoregulatory and other physiological mechanisms of moderately halophilic bacteria (Oren, 2006).

However, it is interesting to note that all strains shared less than 98.7% identity (the usual species cut-off proposed by Yarza et al. (2014) with their closest phylogenetic relative, suggesting that they could be considered as new species. Phenotypic, genetic and phylogenetic analyses have been suggested for the complete identification of these strains. These bacterial strains were tested for the production of industrially important enzymes (Amylase, protease, lipase, DNase and coagulase). These isolates are good candidates as sources of novel enzymes with biotechnological potential as they can be used in different industrial processes at high salt concentration (up to 25% NaCl for B4). Prominent amylase, lipase, protease and DNAase activities have been reported from different hypersaline environments across the globe; e.g., Spain (Sánchez-Porro et al., 2003), Iran (Rohban et al., 2009), Tunisia (Baati et al., 2010) and India (Gupta et al., 2016). However, to the best of our knowledge, the coagulase activity has never been detected in extreme halophilic bacteria. Isolation and characterization of crude enzymes (especially coagulase) to investigate their properties and stability are in progress.

The finding of novel enzymes with optimal activities at various ranges of salt concentrations is of great importance. Besides being intrinsically stable and active at high salt concentrations, halophilic and halotolerant enzymes offer great opportunities in biotechnological applications, such as environmental bioremediation (marine, oilfield) and food processing. The bacterial isolates were also characterized for production of biosurfactants by oil-spread assay, measurement of surface tension and emulsification index (E₂₄). There are few reports on biosurfactant producers in hypersaline environments and in recent years, there has been a greater increase in interest and importance in halophilic bacteria for biomolecules (Donio et al., 2013; Sarafin et al., 2014). Halophiles, which have a unique lipid composition, may have an important role to play as surface-active agents. The archae bacterial ether-linked phytanyl membrane lipid of the extremely halophilic bacteria has been shown to have surfactant properties (Post and Collins, 1982). Yakimov et al. (1995) reported the production of biosurfactant by a halotolerant *Bacillus licheniformis* strain BAS 50 which was able to produce a lipopeptide surfactant when cultured at

salinities up to 13% NaCl. From solar salt, *Halomonas sp. BS4* and *Kocuria marina BS-15* were found to be able to produce biosurfactant when cultured at salinities of 8% and 10% NaCl respectively (Donio *et al.*, 2013; Sarafin *et al.*, 2014). In the present work, strains B4 and A4 produce biosurfactant in medium containing respectively 25% and 20% NaCl. To our knowledge, this is the first report on biosurfactant production by bacteria under such salt concentration. Biosurfactants have a wide variety of industrial and environmental applications (Akbari *et al.*, 2018) but their applicability depends on their stability at different environmental conditions. The strain B4 which can produce biosurfactant at 25% NaCl showed good stability in alkaline pH and at a temperature range of 30°C–100°C. Due to the enormous utilization of biosurfactant in detergent manufacture the choice of alkaline biosurfactant is researched (Elazzazy *et al.*, 2015). On the other hand, the interesting finding was the thermostability of the produced biosurfactant even after heat treatment (100°C for 30 min) which suggests the use of this biosurfactant in industries where heating is of a paramount importance (Khopade *et al.*, 2012). To date, more attention has been focused on biosurfactant producing bacteria under extreme conditions for industrial and commercial usefulness. In fact, the biosurfactant produce by strain B4 have promising usefulness in pharmaceutical, cosmetics and food industries and for bioremediation in marine environment and Microbial enhanced oil recovery (MEOR) where the salinity, temperature and pH are high.

CONCLUSION: This is the first study on the culturable halophilic bacteria community inhabiting Chott Tinsilt in Eastern Algeria. Different genera of halotolerant bacteria with different phylogenetically characteristics have been isolated from this Chott. Culturing of bacteria and their molecular analysis provides an opportunity to have a wide range of cultured microorganisms from extreme habitats like hypersaline environments. Enzymes produced by halophilic bacteria show interesting properties like their ability to remain functional in extreme conditions, such as high temperatures, wide range of pH, and high salt concentrations. These enzymes have great economical potential in industrial, agricultural, chemical, pharmaceutical, and biotechnological applications. Thus, the halophiles isolated from Chott Tinsilt offer an important potential for application in microbial and enzyme biotechnology. In addition, these halo bacterial biosurfactants producers isolated from this Chott will help to develop more valuable eco-friendly products to the pharmacological and food industries and will be usefulness for bioremediation in marine environment and petroleum industry.

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