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ORIGINAL ARTICLE

Extracellular hydrolytic enzymes produced by halophilic bacteria and archaea isolated from hypersaline lake

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Abstract

The screening of bacteria and archaea from Chott El Jerid, a hypersaline lake in the south of Tunisia, led to the isolation of 68 extremely halophilic prokaryotes growing in media with 15–25% of salt. Assessment of 68 partial 16S rRNA analyzed by amplified rDNA restriction analysis (ARDRA) revealed 15 different bacterial and archaeal taxonomic groups. Based on ARDRA results, phenotypic and hydrolytic activity tests, 20 archaeal and 6 bacterial isolates were selected for sequencing. The halophilic isolates were identified as members of the genera: *Salicola, Bacillus, Halorubrum, Natrinema* and *Haloterrigena*. Most of these isolates are able to produce hydrolytic enzymes such as amylase, protease, lipase, cellulase, xylanase, pectinase and some of them showed combined activities. *Natrinema* genus is an excellent candidate for lipase production. These results indicated that the extremely halophilic archaea and bacteria from Chott El Jerid are a potential source of hydrolytic enzymes and may possess commercial value.

Keywords Halophiles · Bacteria · Archaea · Hydrolytic enzymes · Hypersaline lake

Introduction

Hypersaline ecosystems, with salinity ranges at or near saturation are considered as extreme environments. Several studies of microbial diversity in these ecosystems have shown high microbial cell densities of halophilic microorganisms in each of the three taxonomic domains: Archaea, Bacteria and Eukarya. They are categorized as slight, moderate or extreme, based on the extent of their halotolerance. In highly saline conditions, halophilic microorganisms used various strategies to preserve their cell structure and function [38]. These microorganisms have several biotechnological applications like extracellular polysaccharides (EPS), polyhydroxyalkanoate used in biodegradable plastic production, pigments for food coloring, production of fermented foods, compatible solutes and hydrolytic enzymes [13]. The most studies on the microbial diversity and ecology of hypersaline environments have focused in the extracellular hydrolytic enzymes from moderate halophiles more than extreme halophiles [48]. Few reports described the diversity of extremely halophilic microorganisms producing hydrolytic enzymes in hypersaline habitats, such as solar salterns, salt lakes, saline deserts and saline deposits [16]. Thus, the detection and isolation of hydrolytic enzymes produced by extreme halophiles constitute an interesting research topic due to the great biotechnological potential exhibited by these enzymes. These enzymes including protease, lipase, amylase, cellulase, xylanase, and DNase, are considered as important candidates in a range of industries such as food processing, washing, biosynthetic processes and environmental bioremediation. Besides, many halophilic enzymes are also active

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and stable at high temperatures, providing a characteristic could be useful in some industrial processes [31, 37].

Chott El Jerid is the largest saline depression, located in the south-west of Tunisia (North Africa). It covers an area of approximately 5000 km^2. The chemical analyses of the brines showed that the waters are dominated by sodium, chloride followed by sulfate that classify Chott El Jerid as an thalassohaline environment despite its continental origin [24]. The study of the geological, sedimentological and hydrochemical aspects of Chott El Jerid have been investigated [24]. In addition, the microbial diversity inhabiting Chott El Jerid has been studied using cultivation approach [4, 17, 21, 32] and molecular tools [5, 35]. However, no studies describing the extracellular hydrolytic activities of extremely halophilic both bacteria and archaea from North Africa systems have been carried out. The purpose of this research was to isolate novel extremely halophilic aerobic bacteria and archaea from hypersaline lake, Chott El Jerid. It was also aimed to explore their potential for the production of hydrolytic enzymes.

Materials and methods

Site description, samples collection and physico-chemical analysis

Sampling was done from a continental salt lake: Chott El Jerid located in the south of Tunisia. Sample S1–10, a mixture of saturated salt waters and sediments, was collected in the dry season (October 2010) at 10 cm below the salt crust surface. Temperature, salinity, and pH of water sample were previously described [5]. Samples were collected into sterile flasks and kept aseptically at 4 °C until analyzed.

Enrichment and isolation of halophilic microorganisms

Enrichment cultures and isolation of aerobic halophilic microorganisms procedures were performed in medium containing (per liter): NaCl, 250 g; KCl, 4 g; $MgCl_2{\cdot}6H_2O$, 10 g; $MgSO_4{\cdot}7H_2O$, 15 g; $CaCl_2{\cdot}2H_2O$, 1 g; $NaHCO_3$, 0.5 g, yeast extract, 5 g; tryptone, 10 g; and glucose, 7 g. pH was adjusted to 6.8 before autoclaving. Solid media were prepared by adding 20 g l^{-1} agar. Strains were grown in 100 ml of medium in 250 ml Erlenmeyer flasks in a rotary shaker at under agitation at 100 rpm for 3 weeks. Aliquots (100 µl) of dilutions were plated onto agar medium. After 2 weeks of incubation at 37 °C, red, orange–red, pink, and yellowish colonies were obtained. Different colonies were picked and restreaked several times to obtain pure cultures. Microbial cultures were stored at − 80 °C in the isolation medium supplemented with 30% glycerol.

DNA extraction and PCR amplification

Genomic DNA of all isolates was extracted using a Wizard Genomic DNA Purification Kit as previously described [23]. Bacterial and archaeal 16S rRNA genes were amplified using primer sets FD1/1492R and 21F/1492R, respectively. PCR amplification was performed in a total volume of 50 µl mixtures containing 1× Taq buffer, 0.2 mmol each dNTP, 0.2 µmol of each primer, 50 ng DNA template and 1.25 U of Taq DNA polymerase (Fermentas). Archaeal PCR amplification conditions were as follows: 5 min at 94 °C, followed by 30 cycles (1 min of denaturing at 94 °C, 1 min of annealing at 55 °C, and 2 min of extension at 72 °C) and a final extension step of 10 min at 72 °C. Bacterial PCR amplification conditions were as follows: 5 min at 94 °C, followed by 30 cycles (45 s of denaturing at 94 °C, 45 s of annealing at 58 °C, and 1 min 45 s of extension at 72 °C) and a final extension step of 10 min at 72 °C. Amplification was carried out using a GeneAmp PCR 9700 System (Applied Biosystems).

Amplified ribosomal DNA restriction analysis (ARDRA) and statistical analysis

The 16S rRNA gene (1.5 kb) amplified by PCR was subjected to restriction digestion using *Hae*III (8 U), *Alu*I, *Mob*I, and *Bst*uI (10 U) restriction enzymes, according to the recommendation of enzyme producer (Life Technologies). The reaction mixture of 20 µl contained 12 µl of PCR product, 10 or 8 U of restriction enzyme and 2 µl of appropriate buffer. The amplified ribosomal DNA restriction analysis (ARDRA) was done using the same condition as previously described [23]. The ARDRA profiles were analysed using the PyElph software 1.4 [43]. Presence or absence of the bands in each lane of the DGGE gel was converted to a binary matrix. The Jaccard coefficient was used to estimate the similarity between patterns and a dendrogram was obtained by the method of UPGMA (unweighted pair group arithmetic average) using the software Past 3 [20]. ARDRA profiles were analyzed, and representative isolates were selected for nucleotide sequence determination.

Sequencing and phylogenetic analysis

PCR products were sequenced in the same manner as previously described [23]. In addition, phylogenetic analysis of all 16S rRNA genes sequences was carried out as previously reported [23]. The nucleotide sequences determined in this study have been submitted to the GenBank and assigned accession numbers KY129979 to KY129984 for

the bacterial sequences and KY129959 to KY129978 for the archaeal sequences.

Morphological characteristics of isolates

Morphological characteristics of isolated strains were examined on colony shape, cell morphology and motility. Cell morphology and motility of exponentially growing liquid cultures were examined on freshly prepared wet mounts by light microscopy.

Assays of hydrolytic enzymes in agar plate

A qualitative screening has been carried out to study the diversity of microorganisms producing hydrolytic enzymes using agar media supplemented with specific substrates for the enzymes of interest. Thirty microliters of lag-phase culture of each isolate was dropped onto 9 mm diameter of sterile disc. The inoculated discs were put onto the center of medium plates in which specific substrates have been incorporated. Plates were incubated at 37 °C for 3 weeks in duplicate. For protease detection, skimmed agar medium containing milk (50%) (v/v); 2% (w/v) agar (Bio Basic) supplemented with 0.5% (w/v) yeast extract and 1% tryptone (Bio Basic) was used as previously described [52]. Clear zones around the colonies were taken as evidence of proteolytic activity. To detect lipase production, the strains were cultured on rhodamine B agar plates. Sterilized medium was supplemented with 2.5% (v/v) olive oil and 0.001% (w/v) rhodamine B solution before pouring plates. The screening of lipolytic activity was monitored under UV light at 365 nm and the colonies with orange color zones were taken as evidence of lipase activity [7, 27]. Amylase screening was achieved based on the method of Amoozegar et al. [1] using 1% (w/v) starch (Sigma) and pectinase activity according to the method described by Soares et al. [49] using 1% (w/v) pectin. The plates were flooded with Lugol solution and the colonies with clear zones were identified as amylase and pectinase producing strains. For the screening of cellulolytic and xylanase activities, the isolates were cultured on agar plates containing carboxy methyl cellulose (CMC) 0.5% (w/v) and xylan 0.8% (w/v) (Sigma), respectively. After incubation, the plates were flooded with 0.1% congo red solution and then washed with 1 mol NaCl. The colorless halo zone surrounding colonies proved the presence of cellulolytic [50] or xylanase activity [55]. In order to detect the production of chitinase, the isolates were cultured on colloidal chitin agar medium plates (1%). Clear halos around the colonies indicated chitinase activity [41]. The ability of extreme halophilic strains to produce extracellular ABTS-oxidizing activity was determined using 0.35 g of 2,2′-azinobis3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma) supplemented by copper(II) sulfate as inducer [36].

Growth kinetics and enzymes production

Quantitative assays for 5 hydrolytic enzymes was performed using the production medium, as described above, supplemented with specific substrate for the enzyme of interest and inoculated with the corresponding strain. Flasks (in duplicate) were incubated in rotary shaker at 180 rpm and 37 °C. After every 24 h of interval, kinetics of archaeal growth and enzyme production were investigated. Optical density (OD) was measured by spectrometric method at 600 nm (UV/Vis Spectrometer T70+). For the enzyme assays, the content was centrifuged at 10,000 rpm for 20 min at 4 °C and the culture supernatant obtained was used as crude enzyme source.

Amylase, xylanase, pectinase and cellulase assays

The production medium was supplemented with 1% (w/v) starch (Sigma) for amylase activity, 1% (w/v) Birchwood xylan (Sigma) for xylanase activity and 1% (w/v) pectin for pectinase activity. These four enzyme activity were determined by measuring the amounts of formed reducing sugars using 3,5-dinitrosalicylic acid (DNS) reagent according to Miller [33]. The assays were performed by mixing 500 µl of culture supernatant (enzyme) with 500 µl of the substrate and incubating it in a water bath at 50 °C for 15 min (case of xylanase and amylase assays) or 30 min (case of cellulase and pectinase assays). The substrate specific for amylase activity consisted of 1% soluble starch in buffer A (Tris–HCl 20 mM, NaCl 2 M and $CaCl_2$ 10 mM; pH 7.4) [6]. Xylanase activity was measured using 1% of xylan; pectinase using 1% pectin and cellulase using 2% of carboxy methyl cellulose (CMC) as substrates dissolved in 50 mM sodium citrate buffer (pH 4.8). A volume of 3 ml DNS was then added into test tube to stop reaction and placed it in a boiling water bath for 10 min together with control. In the control, the enzyme was added after adding DNS reagent. Finally, the contents were cooled and diluted 5 times with distilled water and then vortexed. All assays were carried out in duplicates. The absorbance was measured using UV/Vis Spectrometer T70+ at 550 nm. One unit of xylanase or pectinase or amylase/cellulase activity was defined as the amount of enzyme produced 1 µmol of reducing sugars (as xylose or β-galacturonic acid or glucose, respectively) per minute per ml under the assay conditions.

Lipolytic activity assay

In 250 ml flasks containing 50 ml medium supplemented with 1% (V/V) Olive Oil. The lipase activity was determined by measuring the free fatty acids released using 0.1 N NaOH with a pH-stat (Metrohm, Swiss). The lipase activity was measured titrimetrically at pH 8 and 37 °C using olive oil emulsion obtained by mixing (3 × 30 s in a Waring blender),

10 ml of olive oil (Sfax-huile, Tunisia) in 90 ml of 10% GA (Gum Arabic). One unit of lipase activity corresponds to 1 µmol of fatty acid released per minute [11].

Results

Isolation of halophilic isolates from Chott El Jerid

The water of the sample (S1–10) was characterized by a salinity of 34.6%, a temperature at 23 °C, and pH at 6.61 [5]. A mixture of saturated salt waters and sediments from this sample was used to inoculate different enrichment cultures at various carbon sources in order to study the effect of carbon sources on composition of bacterial and archaeal communities. Four different enrichment cultures conditions were performed; (1) yeast extract (E); (2) tryptone (T); (3) yeast extract + tryptone + glucose (E + T + G); and (4) yeast extract + tryptone (E + T). A total of 68 extremely halophilic isolates, grew at 25% salt, were obtained from the four enrichment cultures tests. 36, 22, 3 and 7 isolates were obtained from T, E, G + T + E and T + E enrichment tests, respectively (Tables 1, 2).

Selection of halophilic isolates by ARDRA

Genomic DNA was extracted from all strains, and the 16S rRNA gene was amplified by PCR using specific primers for bacteria and archaea as described above. 21 and 47 isolates were confirmed to be bacteria and archaea, respectively. A representation of ARDRA patterns after full digestion with *Hae*III, *Alu*I, *Mob*I and *Bst*uI, restriction enzymes of the 16S rRNA gene from 68 isolates was compared. Dendrograms showed the relationships among ARDRA profiles from each enrichment culture (Figures S1 and S2). Clustering analysis of the archaeal ARDRA profiles indicated three, seven and one cluster groups from tryptone (Figure S1A), yeast-extract (Figure S1B) and tryptone + yeast-extract + glucose respectively. However, bacterial ARDRA profiles presented one cluster from each type of enrichment cultures (Figure S2A, B, C and D). Of the 47 archaeal clones analyzed, 11 different ARDRA patterns were identified. However, digestion of 21 bacterial sequences resulted in only four different patterns (Table 2).

Morphological characterization of halophilic isolates from Chott El Jerid

On the basis of the results of the enzymatic digestion profiles obtained and pigmentation colonial color, 35 isolates were selected to determine accurately the morphological characterization. In fact, the morphologies of all isolates were pleomorphic, short or long rod. Approximate cell dimensions were 0.1 × 4–7 µm (Table 1). Moreover, most of them were non-motile. Colonial pigmentation from all archaeal isolates ranged from blood-red to pale-pink. However, all bacterial colonies were yellow color. No growth was observed at NaCl concentrations less than 15% (w/v) for all isolates (Table 1). These results suggesting that all isolates should be considered as extremely halophilic according to the definition of Ventosa et al. [51].

Screening and preliminary characterization of the halophilic enzymes

To identify and characterize the enzymatic capabilities of the selected isolated strains (35 isolates), some biochemical tests were conducted (Table 1). Bacterial and archaeal isolates were tested qualitatively for their ability to produce eight different extracellular hydrolases (Fig. 1a; Table S1). According to the size of halos revealed in the specific plate assay, a total of 4, 7, 11, 11, 6, 6 halophilic isolates were able to produce amylase, lipase, cellulase, xylanase, pectinase, protease respectively (Table S1, Fig. 1a). None was able to produce chitinase and laccase activity. Within the domain *Archaea*, it was indicated that archaeal strains produced five types of enzymes including cellulase, xylanase, amylase, pectinase, and lipase (Fig. 1a). Within the domain *Bacteria*, our investigations showed four enzymatic activities including lipase, cellulase, xylanase and protease (Fig. 1a). There was no archaeal isolate which could produce the protease. Hydrolyze of olive oil was observed especially with archaeal isolates. Amylase and pectinase producers were not detected among our bacterial isolates.

Phylogenetic analysis

Based on the morphological characterization, the enzymatic digestion profiles obtained and the enzymatic activity tests, 26 representatives' bacteria and archaea were chosen for taxonomic and phylogenetic studies. The phylogenetic position of archaeal and bacterial strains were determined based on 16S rRNA gene sequencing. Trees depicting the phylogenetic relationship of the closet relatives were shown in Figs. 2 and 3. Archaeal isolates were belonged to the phylum *Euryacaryota* from the following genera: *Halorubrum, Natrinema* and *Haloterrigena*. This study presented *Halorubrum* as the predominant genus among isolates with considerably high similarities (> 99%) (Table 1). Archaea isolates are presented by 4 different species from the genus *Halorubrum*, one species from the genus *Natrinema* and one species from the genus *Haloterrigena*. 3 different groups are identified in our study. These groups showed high similarity (98–99%) with *Halorubrum chaoviator, Halorubrum lipolyticum, Halorubrum saccharovorum* and *Halorubrum terrestre*.

Table 1 Phenotypic and phylogenetic characteristics of selected isolates

Domain	Enrichment culture	Strains	Pigmentation	Cell morphology	Cell size; width and length (μm)	Mobility	0 g l^{-1}	150 g l^{-1}	Closest phylogenetic relative/accession number/similarity %
Bacteria	Tryptone	CEJTB24	Yellow-cream	Long rod	0.2×2–6	+	−	+	
		CEJTB26	Yellow-cream	Long rod	0.2×2–6	+	−	+	*Salicola salis* LMG 23122/(DQ129689)/99
		CEJTB35	Yellow-cream	Long rod	0.2×2–6	+	−	+	*Salicola marasensis* CECT 7107/(DQ019934)/99
		CEJTB44	Yellow-cream	Long rod	0.2×2–6	+	−	+	*Salicola marasensis* CECT 7107/(DQ019934)/98
	Yeast extract	CEJEB12	Yellow-cream	Short rod	0.4×4	+	−	+	*Bacillus qingdaonensis* JCM 14087/(AB571874)/98
	Tryptone + Yeast extract	CEJTEB26	Yellow-cream	Long rod	0.1×2–5	+	−	+	
		CEJTEB27	Yellow-cream	Long rod	0.1×2–5	+	−	+	*Salicola salis* LMG 23122/(DQ129689)/99
		CEJTEB29	Yellow-cream	Long rod	0.1×2–5	+	−	+	
		CEJTEB40	Yellow-cream	Long rod	0.1×2–5	+	−	+	
		CEJTEB42	Yellow-cream	Long rod	0.1×2–5	+	−	+	
		CEJTEB43	Yellow-cream	Long rod	0.1×2–5	+	−	+	
		CEJTEB44	Yellow-cream	Long rod	0.1×2–5	+	−	+	
	Tryptone + Yeast extract + Glucose	CEJGTEB102	Yellow-cream	Long rod	0.2×2–6	+	−	+	*Salicola marasensis* CECT 7107/(DQ019934)/97
Archaea	Tryptone	CEJTA9	Red	Long rod	0.2×2–5	−	−	+	*Halorubrum terrestre* JCM 10247/(AB090169)/99
		CEJTA11	Orange	Long rod	0.1×4–7	−	−	+	*Halorubrum saccharovorum* JCM 8865 (U17364)/99
		CEJTA17	Red	Long rod	0.2×2–5	−	−	+	*Halorubrum terrestre* JCM 10247/(AB090169)/99
		CEJTA19	Orange	Long rod	0.1×4–7	+	−	+	*Halorubrum saccharovorum* JCM 8865 (U17364)/99
		CEJTA20	Orange	Long rod	0.1×4–7	+	−	+	*Halorubrum saccharovorum* JCM 8865 (U17364)/99
		CEJTA22	Orange	Long rod	0.1×4–7	−	−	+	*Halorubrum saccharovorum* JCM 8865 (U17364)/99
		CEJTA30	Red	Long rod	0.2×2–5	−	−	+	*Halorubrum terrestre* JCM 10247/(AB090169)/99
		CEJTA34	Red	Short rod	0.1×2–4	+	−	+	*Halorubrum saccharovorum* JCM 8865 (U17364)/99
		CEJTA37	Red	Long rod	0.2×2–5	−	−	+	*Halorubrum chaoviator* DSM 19316 (AM048786)/99
		CEJTA38	Red	Long rod	0.2×2–5	−	−	+	*Halorubrum terrestre* JCM 10247/(AB090169)/99

Table 1 (continued)

Domain	Enrichment culture	Strains	Pigmentation	Cell morphology	Cell size; width and length (µm)	Mobility	0 g l^{-1}	150 g l^{-1}	Closest phylogenetic relative/accession number/similarity %
	Yeast extract	CEJEA2	Red	Long rod	0.1×2–5	+	–	+	*Halorubrum terrestre* JCM 10247/(AB090169)/99
		CEJEA5	Red	Long rod	0.1×2–5	–	–	+	
		CEJEA6	Red	Short rod	0.1×3	–	–	+	*Halorubrum lipolyticum* JCM 13559 (DQ355814)/98
		CEJEA7	Orange	Long rod	0.2×6	+	–	+	*Halorubrum saccharovorum* JCM 8865/(U17364)/99
		CEJEA8	Red	Long rod	0.1×2–5	–	–	+	
		CEJEA14	Red	Long rod	0.1×2–5	–	–	+	*Halorubrum chaoviator* DSM 19316 (AM048786)/99
		CEJEA16	Red	Short rod	0.2×2–4	–	–	+	*Halorubrum chaoviator* DSM 19316 (AM048786)/99
		CEJEA21	Red	Short rod	0.3×1–3	+	–	+	*Halorubrum saccharovorum* JCM 8865/(U17364)/99
		CEJEA22	Red	Short rod	0.2×3	+	–	+	*Halorubrum saccharovorum* JCM 8865/(U17364)/98
		CEJEA36	Pink	Pleomorphic	0.1–0.3×1–4	–	–	+	*Haloterrigena jeotgali* JCM 14585 (EF077633)/99
	Tryptone + Yeast extract + Glucose	CEJGTEA100	Orange	Long rod	0.2×2–7	–	–	+	*Natrinema altunense* JCM 12890 (AY208972)/99
		CEJGTEA101	Orange	Long rod	0.2×2–7	–	–	+	*Natrinema altunense* JCM 12890 (AY208972)/99

Two isolates, CEJGTEA100 and CEJGTEA101, present 99% similarity with *Natrinema altunense*, extremely halophilic archaeon isolated from a salt lake in Altun Mountain in Xinjiang, China [56]. CEJEA36 was related to *Haloterrigena jeotgali* isolated from traditional salt-fermented food from Korea [45].

Bacteria isolates were identified as members of the Class *Gammaproteobacteria* and *Bacilli* of the following genera *Salicola* and *Bacillus*, respectively with the dominance of *Salicola* genus (Fig. 3; Table 1). The isolate CEJB12 was related to *Bacillus qingdaonensis*, a moderately haloalkaliphilic bacterium isolated from a crude sea-salt sample collected near Qingdao in eastern China [54]. This result showed the presence of haloalkaliphilic bacteria in the Chott El Jerid which in accordance with studies of El Hidri et al. [17]. Five bacterial isolates CEJTB26, CEJTB35, CEJTB44, CEJTEB27 and CEJGTEB102 were related to *Salicola marasensis* and *Salicola salis*, extremely halophilic bacteria, isolated from the Maras solar salterns in Peru [30] and from Ezzemoul sabkha in Algeria [25], respectively.

Growth kinetics and enzymes productions by CEJTA37, CEJEA14 and CEJGTEA101

From 23 positive strains obtained from the primary screening method for measurement of enzymes productions based on the hydrolysis zones on agar plate assays, three archaeal isolates (CEJTA37; CEJEA14; CEJGTEA101) were selected for enzymes productions in liquid medium. *Halorubrum chaoviator* CEJEA14 was selected for amylase activity. The isolate *Halorubrum chaoviator* (CEJTA37) grew for xylanase and pectinase assays. *Natrinema altunense* (CEJGTEA101) was selected for pectinase, cellulase, xylanase and lipase activities. The growth curve observations and measurement of enzyme production results are given in Fig. 4. A pectinase activity was detected in CEJGTEA101 (0.067 U ml^{-1} at 144 h) and CEJTA37 (0.067 U ml^{-1} at

Table 2 Summary of the numbers of selected isolates

Domain	Enrichment culture	Tryptone (T)	Yeast-extract (E)	Tryptone + Yeast-extract (T + E)	Tryptone + Yeast-extract + Glucose (T + E + G)	Total
Bacteria	Numbers of isolates	12	1	7	1	21
	Selected isolates for phenotypic characterization and hydrolytic activity	4	1	7	1	13
	ARDRA profiles numbers	1	1	1	1	4
	Selected isolates for sequencing	3	1	1	1	6
Archaea	Numbers of isolates	24	21	–	2	47
	Selected isolates for phenotypic characterization and hydrolytic activity	10	10	–	2	22
	ARDRA profiles numbers	3	7	–	1	11
	Numbers of isolates for sequencing	10	8	–	2	20

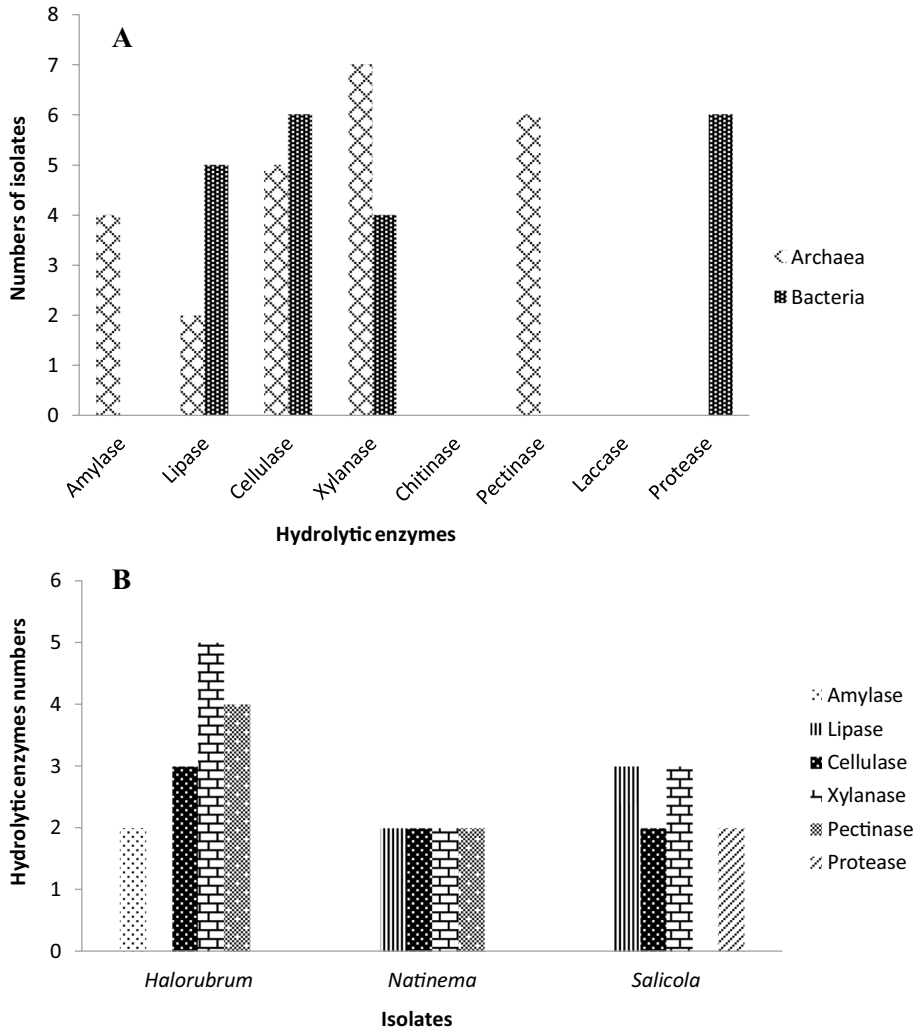


Fig. 1 **a** Hydrolytic enzymes production by haloarchaeal and bacterial isolates. **b** Hydrolytic activities among the representatives of the genera *Halorubrum, Natrinema* and *Salicola*

122 h) isolates. CEJEA14 isolate produce amylase (0.066 U ml^{-1} at 120 h). Xylanase production was in the isolate CEJTA37 (0.1 U ml^{-1} at 96 h) while the CEJGTEA101 isolate exhibiting the highest xylanase activity with 0.77 U ml^{-1} at 96 h. Maximum cellulase activity (0.5 U ml^{-1}) was produced by isolate CEJGTEA101 in accordance with

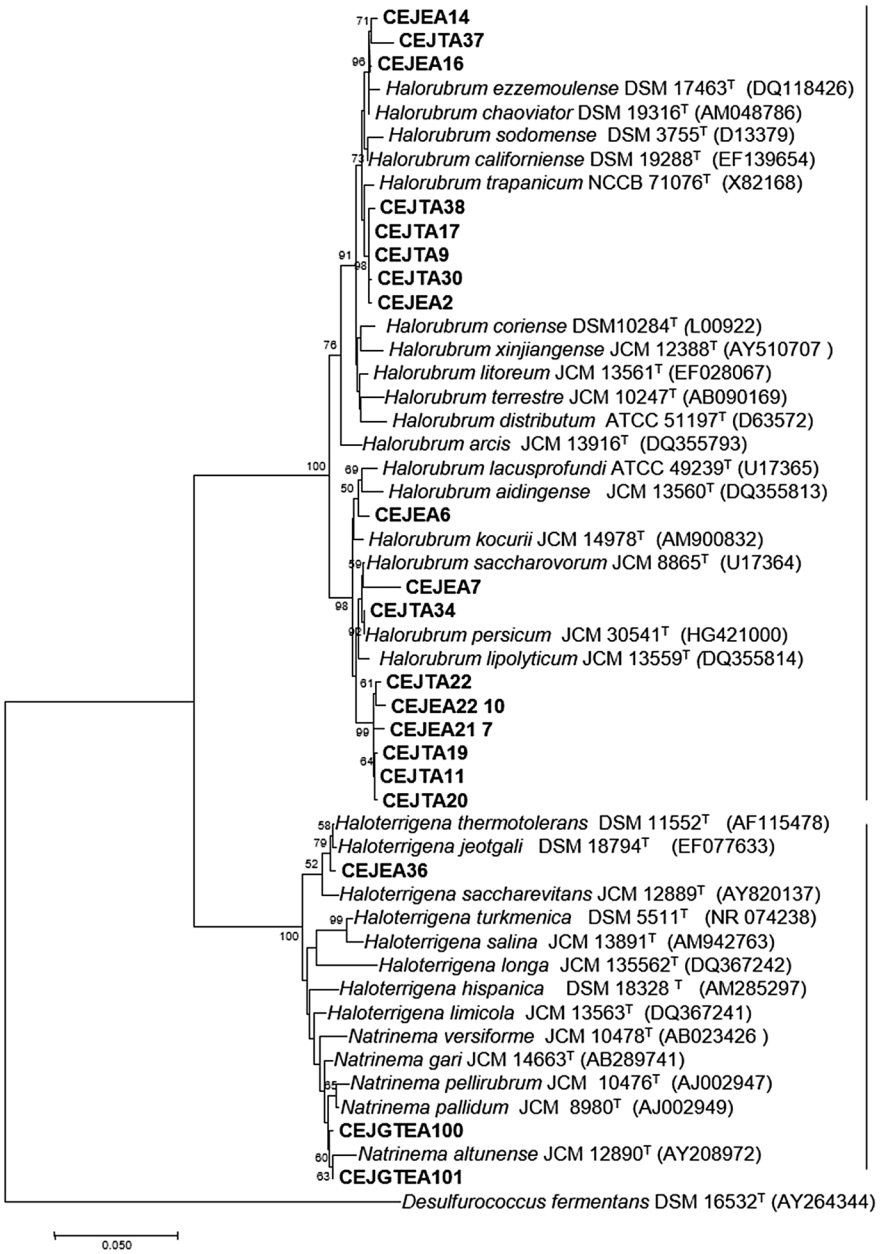


Fig. 2 Phylogenetic tree based on similarities of 16S rRNA sequences of archaeal isolates and its relatives. The tree is based on the Juke-Cantor model and the Neighbour-Joining method. The sequence of *Desulfurococcus fermentans* was used as the outgroup. Bootstrap values based on 100 replicates are shown

growth. This isolate CEJGTEA101 was obviously a producer of lipase with an activity of 432 U ml^{-1} after 96 h of incubation.

Discussion

Chott El Jerid is a hypersaline lake located in the south of Tunisia. The diversity of hydrolytic activity production of archaeal and bacterial microorganisms isolated from this ecosystem was investigated in this study. The combination of three different substrates in enrichment cultures led to the isolation of 68 extremely halophilic microorganisms from Chott El Jerid. 21 and 47 isolates are identified as bacteria and archaea with the amplification by PCR and specific primers, respectively. Based on the results of ARDRA, 35 isolates were chosen to determine their morphological characteristics and hydrolytic activities. The ability to produce 8 different hydrolases was tested among the isolates. A total of halophilic isolates were able to produce amylase, lipase, cellulase, xylanase, pectinase and protease. Neither chitinase nor laccase activity was detected among the 35 isolates. Figure 1 and Table S1 showed the hydrolytic activities of halophilic bacteria and archaea isolates.

Five hydrolytic activities including cellulase, xylanase, amylase, pectinase, and lipase were observed among

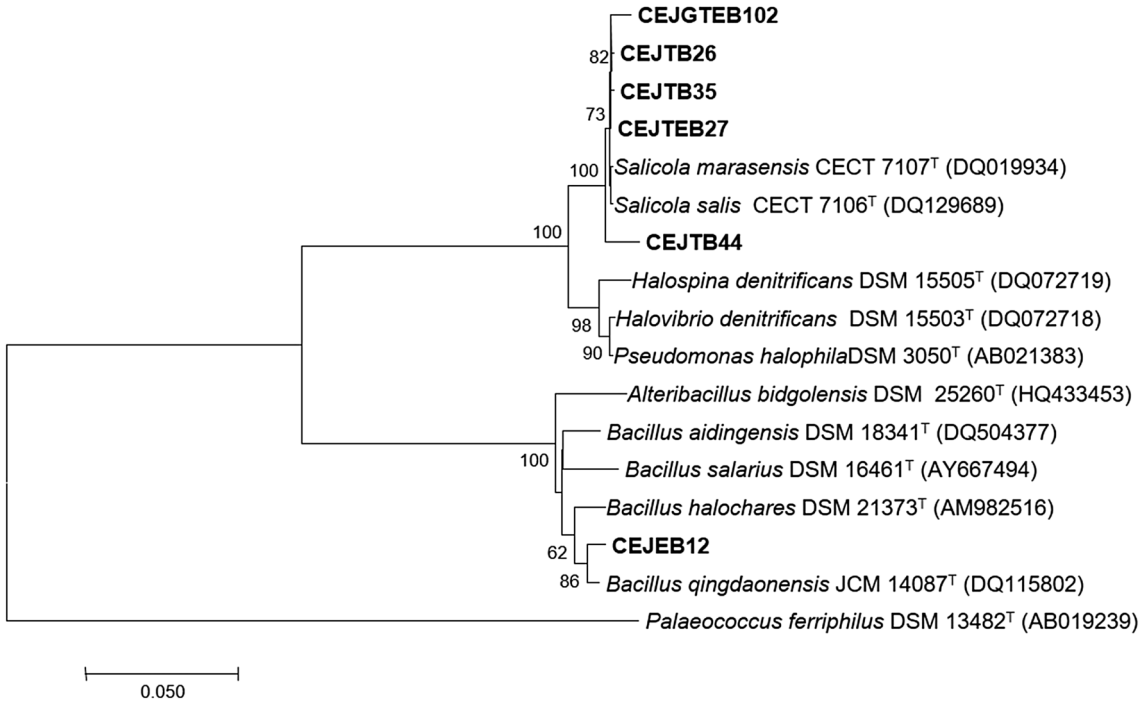


Fig. 3 Phylogenetic tree based on similarities of 16S rRNA sequences of bacterial isolates and its relatives. The tree is based on the Juke-Cantor model and the Neighbour-Joining method. The sequence of *Palaeococcus ferriphilus* was used as the outgroup. Bootstrap values based on 100 replicates are shown

extremely halophilic archaea. In similar studies, Makhdoumi Kakhki et al. showed the abundance of nine hydrolytic enzymes including DNase, amylase, lipase, inulinase, pullulanase, protease, cellulase, chitinase, xylanase by archaeal isolates from Aran-Bidgol Hypersaline Lake in Iran [29]. Birbir et al. determined the amylase, lipase, cellulase, β-galactosidase, gelatinase, caseinase, and DNase production among halophilic archaeal strains isolated from Tuzkoy salt mine in Turkey [8]. These results support previous studies on other hypersaline habitats except two hydrolytic activities including protease and chitinase which were not detected by archaeal isolates in our study.

Halophilic bacteria showed higher potential producing lipase, cellulase, xylanase and protease. These data are in agreement with other results previously reported. Rohban et al. elucidated the hydrolytic enzymes production by moderate and extremely bacterial strains isolated from different areas of Howz Soltan playa (Iran). They showed the secretion of lipase, amylase, protease, inulinase, xylanase, cellulase, pullulanase, DNase, and pectinase [46]. Moreover, Sánchez-Porro et al. investigated the diversity of moderately halophilic bacteria, producers of amylase, protease, lipase, DNase and pullulanase from salterns located in Almeria, Cadiz and Huelva (Spain) [47]. Cojoc et al. showed the ability of bacterial isolates collected from a subterranean rock salt crystal (Romania) to hydrolyze starch, gelatin, casein, tween 80, carboxymethyl cellulose and xylan [12]. De Lourdes Moreno et al. showed the abundance of four hydrolases including lipase, protease, amylase and nuclease produced by extreme halophiles in crystallizer ponds at two solar salterns in south Spain [15]. Moderately halophilic Bacteria, isolated from the largest hypersaline playa Aran-Bidgol in Iran, were able to produce a wide variety of enzymes including DNase, inulinase, amylase, lipase, pectinase, protease, chitinase, pullulanase, cellulose and xylanase [2]. From the same ecosystem, Chott El Jerid, extremely haloalkalitolerant bacteria were isolated and identified as *Halobacillus, Halomonas, Piscibacillus, Nesterenkonia* species producers of protease, lipase, DNase and amylase [17]. According to those studies above mentioned, the lipolytic, proteolytic, and amylolytic activities were predominant among isolates. However, our isolates showed high rate of cellulase, xylanase, lipase and protease activities. Amylase, pectinase and chitinase producers were not detected among our bacterial isolates.

Finally, among 35 isolates, 26 were genetically identified and phylogenetically analyzed. According to 16S rRNA gene analysis, the extremely halophilic archaeal isolates belonged to the genera *Natrinema, Halorubrum, Haloterrigena* and bacterial isolates to *Salicola* and *Bacillus. Halorubrum, Natrinema* and *Haloterrigena* were characterized as extremely halophilic archaeon. The genus *Halorubrum* is widely distributed in hypersaline environments [29]. 17 isolates were affiliated to *Halorubrum* spp.

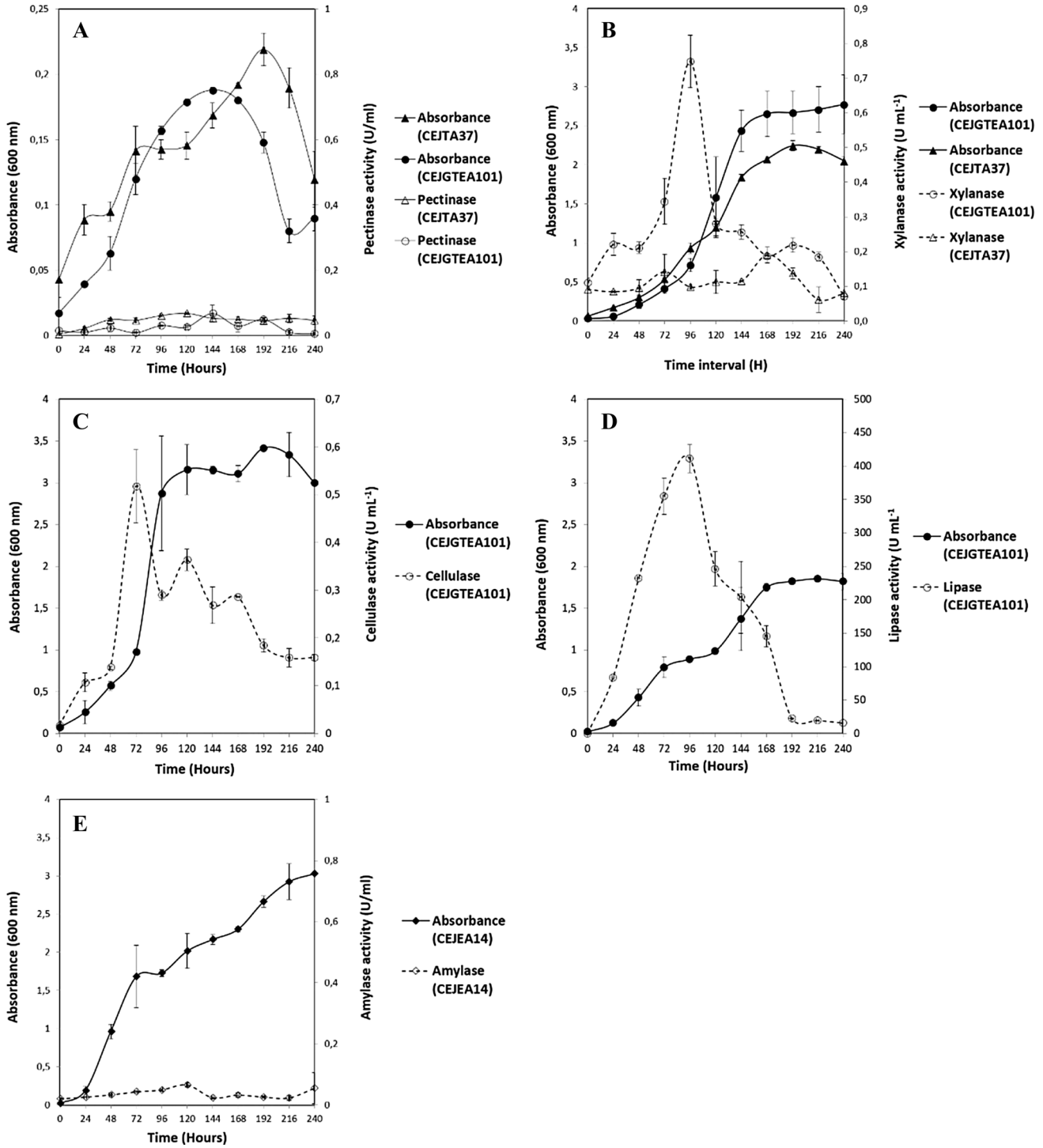


Fig. 4 Hydrolytic enzymes activities during the growth of archaeal isolates. Cell growth was determined at OD600 nm. Pectinase (**a**) and xylanase (**b**) activities of isolates CEJGTEA101 and CEJTA37. Cellulase (**c**) and lipase (**d**) activities of isolate CEJGTEA101. Amylase (**e**) activity of isolate CEJEA14. Values represent the means of two experiments ± confidence of intervals (error bars)

(Table 1). These isolates present one or two hydrolytic activities among amylase, cellulase, xylanase and pectinase enzymes (Fig. 1b). Makhdoumi Kakhki et al. [29] showed that the genus *Halorubrum* present the high rate of amylase, lipase, pullulanase, inulinase and DNase production. The most common hydrolytic enzymes in *Halorubrum* genus was amylase [15, 16, 29] which was in accord with our results. In this study, the CEJTA37 isolate, related to *Halorubrum*

chaoviator, produce pectinase (0.067 U ml^{-1}) and xylanase (0.1 U ml^{-1}). The biochemical properties of some haloarchaeal xylanases have been determined such as those produced by *Natrinema* sp. ssbjup-1 [42] and *Halorhabdus utahensis* [53]. The *Halorubrum chaoviator* CEJEA14 was able to produce amylase activities (0.066 U ml^{-1}). This activity was lower as compared with the other halophilic a-amylases produced from *Haloferax* sp. HA10 strain (4.2 U ml^{-1}) [3]. A number of an extracellular α-amylases were purified and characterized from haloarchaeal strains such as, *Halobacterium salinarum* [19], *Natronococcus* sp. strain Ah-36 [26], *Haloferax mediterranei* [44], *Haloarcula hispanica* [22], *Haloarcula* sp. strain S-1 [18] and *Halorubrum xinjiangense* [34].

Protease, lipase, pullulanase, CMCase, chitinase and inulinase activity were also observed in the *Natrinema* genus [29]. The *Natrinema* genus was the most potent isolate with six combinatorial enzymes production. The *Natrinema* genus present a high lipase activity [29]. In the present work, the CEJGTEA101 isolate, affiliated to *Natrinema altunense*, showed four hydrolytic activities including lipase, cellulase, xylanase and pectinase. This strain was able to produce lipase (432 U ml^{-1}), cellulase (0.5 U ml^{-1}), xylanase (0.77 U ml^{-1}) and pectinase (0.067 U ml^{-1}) under high salinity (25% NaCl). The characterization of xylanase and cellulase was investigated from extremely haloalkaliphilic archaeon *Natrinema* sp ssbjup-1, isolated from Lonar Lake [42]. In addition, the extremely halophilic archaeon *Halorhabdus utahensis*, isolated from the Great Salt Lake, showed an extracellular beta-xylanase activity [53].

It has been reported previously that lipolytic activity was screened in 35 haloarchaea from Algerian culture collection and the best strain producer TC6 was related to *Natronococcus* [7]. The extracellular lipase activities are quite common among halophilic Archaea (0.053–0.571 U ml^{-1}) [39, 40] including the genus *Natrinema* (0.062 U ml^{-1}) [39]. Lipase activity has been reported from *Natronococcus* sp. [9], *Haloarcula marismortui* [10] and *Haloarcula* sp. G41 [28]. *Haloarcula marismortui* synthesizes lipases intracellularly (490 U ml^{-1}) and extracellularly (260 U ml^{-1}) [10].

The CEJEA36 isolate related to the genus *Haloterrigena* is not able to produce qualitatively any hydrolytic activity.

On the other hand, bacterial isolates CEJTB26, CEJTB35, CEJTB44, CEJTEB27 and CEJGTEB102, related to the genus *Salicola*, were able to produce lipase, cellulase, xylanase, and/or protease activities except CEJTB26. Species of this genus have been reported to thrive abundantly and to compete with members of *Archaea* in crystallizer ponds of different solar salterns [30]. It is important to note that this bacterium *Salicola marasensis* IC10 produced an extracellular protease, (Salipro), and an intracellular lipase, (LipL) that showed interesting properties for use in different industries [15, 16]. Isolate CEJEB12, assigned to *Bacillus* genus, didn't show any hydrolytic activity. However, the moderate halophilic bacterium isolated from various [2, 16]. De Guzmán et al. [14] demonstrated the production of extracellular lipase by strain LV01 related to *Bacillus* genus with 0.079 U ml^{-1}.

As a conclusion, the diversity of hydrolytic activity production from archaeal and bacterial microorganisms isolated from Chott El Jerid was investigated in this study. 35 Halophilic isolates were able to produce amylase, lipase, cellulase, xylanase, pectinase and protease. None was able to produce chitinase and laccase activity. Pectinase and amylase enzymes were only detected in the archaeal species; however, in the bacterial species protease was the only activity. Extremely halophilic archaeal isolates were affiliated to *Natrinema, Halorubrum, Haloterrigena* and bacterial isolates to *Salicola* and *Bacillus* genera, of which several strains could produce hydrolytic enzymes. This study reported *Halorubrum* and *Salicola* as the predominant genera. The genera *Halorubrum, Natrinema* and *Salicola* showed the most enzymatic activity (Fig. 1b). Combined hydrolytic activity was observed in *Natrinema* and *Salicola* genera. According to the results, it is suggested that *Natrinema* and *Salicola* genera are excellent candidates for production the hydrolytic enzymes. Lipolytic activity produced by *Natrinema* under high salinity condition could made this strain an interesting candidate for future investigation. Those extremely halophilic isolates were selected for further studies for their great biotechnological applications with respect to their capacity to produce different hydrolases. It would be more constructive if these enzymes are purified from the isolates and then characterized, which is the next step in the current work.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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