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ORIGINAL PAPER

Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran

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Abstract Screening of bacteria from different areas of Howz Soltan playa, a hypersaline lake in the central desert zone of Iran, led to the isolation of 231 moderately halophilic bacteria, which were able to grow optimally in media with 5–15% of salt, and 49 extremely halophilic microorganisms that required 20–25% of salt for optimal growth. These isolates produced a great variety of extracellular hydrolytic enzymes. A total of 195, 177, 100, 95, 92, 68, 65, 33, and 28 strains produced lipases, amylases, proteases, inulinases, xylanases, cellulases, pullulanases, DNases, and pectinases, respectively. In comparison with gram-negative bacteria, the gram-positive halophilic rods, showed more hydrolytic activities. Several combined activities were showed by some of these isolates. One strain presented 9 hydrolytic activities, 4 strains presented 8 hydrolytic activities, 10 strains presented 7 hydrolytic activities and 29 strains presented 6 hydrolytic activities. No halophilic isolate without hydrolytic activity has been found in this study. According to their phenotypic characteristics and comparative partial 16S rRNA sequence analysis, the halophilic strains were identified as members of the genera: *Salicola, Halovibrio, Halomonas, Oceanobacillus, Thalassobacillus, Halobacillus, Virgibacillus, Gracilibacillus, Salinicoccus,* and *Piscibacillus.* Most lipase and DNase producers were members of the genera *Gracilibacillus* and *Halomonas*, respectively, whereas most of the isolates able to produce hydrolytic enzymes such as amylase, protease, cellulose (CMCase) and inulinase, belonged to gram-positive genera, like *Gracilibacillus, Thalassobacillus, Virgibacillus,* and *Halobacillus.*

Keywords Biodiversity · Halophiles · Hydrolyses · Isolation · Screening

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Introduction

Hypersaline lakes, with salinity ranges at or near saturation are extreme environments; yet, they often maintain remarkably high microbial cell densities and are biologically very productive ecosystems [17, 18, 30]. To adapt to saline conditions, bacteria have developed various strategies to maintain cell structure and function. Studies of such bacteria are of great importance, as they may produce compounds of industrial interest, such as extracellular, hydrolytic enzymes that have diverse potential usage in biomedical science and chemical industries [11, 14, 29, 30]. Most industrial process are carried out under specific physicochemical conditions which may not be definitively adjusted to the optimal points required for the activity of the available enzymes; thus, it would be of great importance to have enzymes that exhibit optimal activities at various ranges of salt concentration, pH and temperature. Halophiles are an excellent source of such enzymes which are not only salt-tolerant, but also may be active at high temperature and pH values [7]. The isolation of moderate and extreme halophiles able to produce hydrolases will provide the

possibility to have optimal activities at different salt concentrations that could be useful in some industrial processes [22].

Moderately halophilic bacteria are a group of halophilic microorganisms able to grow optimally in media containing 3–15% NaCl [30]. They constitute a heterogeneous group of microorganisms including species belonging to various genera, such as *Halomonas* and *Salinivibrio,* which have been studied with respect to their ecology, physiology, biochemistry and genetics [30, 31].

Extremely halophilic bacteria grow optimally at salt concentrations from above 20% (w/v) to saturation [31]. They have the capability of producing different hydrolyses such as amylase and lipase. The potential importance of extremely halophiles in various industrial areas such as the leather industry [4] and food preservation [14, 29] is evident.

Iran consists of various saline environments including hypersaline lakes and playas, in which the microbial diversity has not been characterized; thus the potential of producing different hydrolytic enzymes among them remains unknown. Howz Soltan playa is located in central area of Iran, with an extension of about 240 and 280 km^2 in dry and wet seasons, respectively. The depth of salt layer which covers almost all the surface of the playa varies between 20 and 46 m and the pH of the water; saline soil and salt sediments differs between 6.5 and 8.2. Major chemical composition of the soil, brine, mud, and salt consists of NaCl, KCl, $MgSO_4$, $MgCl_2$, and Na_2SO_4 [24].

In the present study, we describe the screening for hydrolase-producing halophilic bacteria, isolated from Howz Soltan playa, a hypersaline environment located in eastern north of Qom province, in central area of Iran. So far, the microbial diversity of this ecosystem has not been studied. We have determined the capability of moderately and extremely halophilic bacteria for producing different extracellular hydrolyses, which will provide information about their potential utilization in industrial processes.

Materials and methods

Sample collection and growth conditions

The samples were collected during October and November (early wet season), May (early dry season) and August (dry season), of 2006 and 2007, respectively. Brine, multicolor solar salt, saline soil and saline mud samples were collected at different locations, at the surface and various depths with maximum distance of 4 km. Figure 1 shows the map of Howz Soltan Lake and the locations of sampling.

The temperature on the sampling sites varied between 11 and 17°C in wet seasons and 28–34°C in dry seasons. The pH of samples was 6.5–8.2. Samples were collected in

Fig. 1 Map of Howz Soltan Lake showing the sites (I, II, III, IV) used for sampling in this study

sterile plastic containers and were cultured not later than 18 h after collection. All samples were cultured in a saline nutrient broth with a final concentration of 10% sea salt consisting of (g/l): NaCl 81, $MgSO_4 \cdot 7H_2O$ 9.7, $MgCl_2 \cdot H_2O$ 7.0, $CaCl_2$ 3.6, KCl 2.0, $NaHCO_3$ 0.06, NaBr 0.026 for moderately halophilic bacteria [28] and 20% (w/v) for extremely halophilic microorganisms, supplemented with 5% (w/v) yeast extract. The pH of culture media was adjusted to 7.3 before autoclaving. Cultures were incubated at 34°C and 38°C in an orbital shaker, at 150 rev min^{-1}, during 3–7 days or more depending on the growth rate of isolates. When necessary, solid media were prepared by adding 12–15 g l^{-1} agar (Merck).

Screening of strains for extracellular hydrolytic activities

In order to detect the production of extracellular hydrolases, different enzymatic agar plate assays were performed with

the only exception of the pullulanase activity assay which was performed in liquid medium. The pH of all media was adjusted on 7.2–7.4, and 10% and 20% total salt were added for detecting hydrolytic activities for moderately and extremely halophilic bacteria, respectively. The different assay media used are described below.

Determination of extracellular amylase activity

The presence of amylolytic activity on plates was determined qualitatively following the method described by Amoozegar et al. [2], using starch agar medium (Merck) containing 10% or 20% (w/v) total salts. After incubation at 34–37°C for 1 week, the plates were flooded with 0.3% I_2–0.6% KI solution; a clear zone around the growth indicated the hydrolysis of starch.

Determination of extracellular protease activity

Proteolytic activity of the cultures was screened in skim milk agar containing 10% (w/v) skim milk, 2% (w/v) agar, supplemented with 10% and 20% (w/v) total salt for determining the hydrolytic activity of moderate and extreme halophiles, respectively. Clear zones around the growth after 7 days were taken as evidence of proteolytic activity [3].

Determination of extracellular lipase activity

To observe lipase production, the strains were cultured on nutrient agar plates containing olive oil (2.5%), victoria blue (0.4 mg l^{-1}) and appropriate salt concentration with an initial pH of 7.2–7.4. The plates were incubated at 37°C for 48 h and the colonies with blue color zones were identified as lipase producing strains [12, 21].

Determination of extracellular DNase activity

DNase activity of the strains was routinely determined using 42 g l^{-1} of DNase test agar medium (Merck), supplemented with 10% and 20% total salt for detecting DNase activity of moderately and extremely halophilic bacteria, respectively.

After incubation at 37°C for 7 days, the plates were flooded with 1 N HCl solution. Clear halos around the colonies showed DNase activity [16].

Determination of extracellular pectinolytic activity

The presence of pectinolytic activity on the plates was determined using a medium containing pectin 10 g l^{-1}, $(NH_4)_2SO_4$ 1.4 g l^{-1}, K_2HPO_4 2 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.02%, nutrient solution 1 g l^{-1} ($FeSO_4{\cdot}7H_2O$, 5 mg l^{-1}; $MnSO_4{\cdot}H_2O$, 1.6 mg l^{-1}; $ZnSO_4{\cdot}7H_2O$, 1.4 mg l^{-1}; $CaCl_2$, 2 mgl^{-1}), agar 20 g l^{-1}, 10% and 20% salts for moderately and extremely halophiles, respectively. After incubation at 37°C for 7 days, the plates were flooded with 0.3% I_2–0.6% KI solution. A clear zone around the growth showed pectinolytic activity [26].

Determination of extracellular inulinase production

The production of inulinase by halophilic strains was detected by preparing media containing inulin 2 g l^{-1}, $(NH_4)_2SO_4$ 0.5 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.2 g l^{-1}, KH_2PO_4 3 g l^{-1}, agar 20 g l^{-1}, supplemented with the appropriate concentration(10 or 20%) of salts for moderate and extreme halophiles, respectively. Inulin was used as the sole source of carbon in this medium; thus, bacterial growth after 48 h of incubation at 37°C, shows the presence of inulinase activity [1].

Determination of extracellular Cellulase (CMCase) activity

CMCase activity of the cultures was screened in a solid medium containing carboxy methyl cellulose (CMC) 5 g l^{-1}; $NaNO_3$ 1 g l^{-1}; K_2HPO_4 2 g l^{-1}; KCl 1 g l^{-1}; $MgSO_4$ 0.5 g l^{-1}; yeast extract 0.5 g l^{-1}; glucose 1 g l^{-1}; agar 17 g l^{-1}, and 10% and 20% salts for moderate and extreme halophiles, respectively. After incubation at 37°C for 7 days, the plates were flooded with 0.1% congo red solution. The clear zone around the colony indicated cellulase activity [34].

Determination of extracellular xylanase production

Xylanase activity was detected using a saline medium containing xylan 10 g l^{-1}; yeast extract 2 g l^{-1}; peptone 5 g l^{-1}; $MgSO_4$ 0.5 g l^{-1}; $CaCl_2$ 0.15 g l^{-1}; agar 20 g^{-1} and the appropriate concentration (10% or 20%) of salts. After incubation at 37°C for 48 h, the plates were flooded with 0.1% congo red solution. The clear zones around colonies indicated qualitative xylanase activity [32].

Determination of extracellular pullulanase activity

To detect pullulanase activity, the strains were cultured in saline liquid medium containing yeast extract 1 g l^{-1}; pullulan 5 g l^{-1}; appropriate concentration (10% or 20%) of salts and incubated for 48 h, at 37°C.

Clearness of medium after the addition of 97% ethanol indicated that the strains produced pullulanase because in presence of pullulan, the interaction between pullulan and ethanol leads to the formation of white precipitate of the non degraded pullulan [19].

Identification of the isolates

Morphological and physiological characteristics of the isolates were either studied on nutrient agar or in nutrient broth plus 10 or 20% (w/v) NaCl as recommended by Smibert and Krieg [25].

Growth at different salt concentrations (0, 2.5, 5, 7.5, 10, 15, 20, 25, and 30%, w/v) was tested on nutrient broth at pH 7.5. Growth was monitored by turbidity at OD_{600} using a spectroscopic method (model UV-160 A; Shimadzu).

Isolates which could grow at optimal growth 3-15% NaCl were considered as moderately halophilic strains [30], while isolates that could grow optimally at 15–25% NaCl concentrations, were considered as extreme halophiles [10].

Some strains were randomly selected with respect to their potential of producing extracellular hydrolytic enzymes and studied in detail. The genomic DNA of these strains was extracted by DNA extraction kit (Bioneer, South Korea) according to the manufacturer's recommended procedure and the 16S rRNA gene was amplified using the universal primers 8F (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1492R (5′-CACGGAT CCTACGGGTACCTTGTTACGACTT-3′). A PCR cycler (Biometra) was used for this amplification. Amplification reactions contained 1.25 µl of each primer, dNTP (10 mM) 0.5 µl, PCR buffer 2.5 µl, $MgCl_2$ (50 mM) 0.75 µl, template DNA 1 µl, DMSO 1.25 µl, smartaq DNA polymerase (Cinnagen, Iran) 0.5 µl, and dH_2O 16 µl, in a final volume of 25 µl.

The following conditions were used in the amplification of 16S rRNA gene: 95°C for 5 min., followed by 35 cycles of 95°C for 45 s, 55°C for 1 min and 72°C for 1.5 min, with final 10 min extension at 72°C. The PCR products were then checked on agarose gel with ethidium bromide staining. PCR product purification was conducted using PCR purification kit (Bioneer, South Korea). The purified PCR product was sequenced in both directions using an automated sequencer by Seq Lab laboratory (Germany). The phylogenic relationship of the isolates was determined by comparing the sequencing data with the related 16S rRNA gene sequences in the GenBank database of the National Center for Biotechnology Information, via BLAST search.

Phylogenetic analysis was performed using the software packages PHYLIP [5] and MEGA version 4 [9] after obtaining multiple alignment of data available from public databases by CLUSTAL_X [27]. Pairwise evolutionary distances were computed using the correction method [8] and clustering was performed using the neighbor-joining method [20]. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 resembling [6].

Results and discussion

Isolation and characterization of halophilic isolates from Howz Soltan Lake

Screening bacteria from saline soil, mud, brine and salt sediments of Howz Soltan lake led to the isolation of 231 moderately halophilic and 49 extremely halophilic bacteria among which there were 172 gram-positive rods, 56 gram-negative rods and 52 gram-positive cocci (Table 1). Most extremely and moderately halophilic isolates were found to be in multicolor salt sediments and saline soil/mud, respectively. Comparing with saline soil, mud and salt, the brine which was collected from playa consisted of low number of bacteria. The number of moderately halophilic isolates was remarkably higher than that of extremely halophilic bacteria. Non-halophilic bacteria were not found among these

Table 1 Sampling location, conditions, and distribution of halophilic isolates in Howz Soltan Lake

Location	Sample type	pH	Temperature (May 2007) (°C)	Total number of the isolates		
				Gram-positive rods	Gram-negative rods	Gram-positive cocci
Site I Thick salt layer, South	Salt sediments	6.5	29	11	7	14
	Brine	7.1	28	7	2	0
Site II Marshy margin, West	Saline soils	7.4	30	25	12	9
	Saline mud	6.5	31	20	0	2
Site III Marshy margin, East	Salt sediments	7.8	28	5	7	16
	Brine	6.5	28.5	9	5	0
	Saline soil	7.6	33	26	9	5
	Saline mud	7.8	34	23	4	0
Site IV Marshy margin, North	Saline soil	6.8	28	25	8	4
	Saline mud	6.6	30	21	2	2

isolates, probably due to the salt saturation in most of the areas in Howz Soltan habitat; their hypersaline conditions are not suitable for non-halophilic microorganisms.

Hydrolytic activity of halophilic isolates

The ability to produce nine different hydrolases was tested among the isolates. A total of 195, 177, 100, 95, 92, 68, 65, 33, 28 halophilic isolates were able to produce lipase, amylase, protease, inulinase, xylanase, cellulase, pullulanase, DNase and pectinase, respectively.

It is interesting to note that combined hydrolytic activity was also detected in many halophilic strains. One strain presented 9 hydrolytic activities, 4 strains presented 8 hydrolytic activities, 10 strains presented 7 hydrolytic activities, 29 strains presented 6 hydrolytic activities, 31 strains presented 5 hydrolytic activities, 14 strains presented 4 hydrolytic activities, 44 strains presented 3 hydrolytic activities, 69 strains presented 2 hydrolytic activities, and 32 strains presented one hydrolytic activity. These results support previous studies in other hypersaline habitats, but the number of strains showing combined hydrolase activity is higher in our study [15, 22].

Table 2 shows the hydrolytic activities of halophilic isolates from Howz Soltan playa. Greater hydrolytic activity was observed among gram-positive, moderately halophilic rods than gram-negative rods and gram-positive cocci. The gram-positive isolates showed higher amylolytic, proteolytic and inulinolytic activities, while gram-negative rods had mainly lipolytic, nucleolytic and pullulanolytic activities. The xylanolytic activity was shown to be similar by gram-positive and gram-negative rods. However, gram-negative rods showed mainly lipolytic activity, especially members of the genus *Salicola*. Among the gram-positive cocci, they produced mainly amylases, lipases and proteases, and interestingly, they presented more pectinolytic and cellulolytic activities than gram-positive and gram-negative rods. Most of the isolates with combined hydrolytic activities were moderately halophilic bacteria, which was a desirable result, as moderate halophiles have great biotechnological applications with respect to their ability to produce different hydrolyses.

Extremely halophilic bacteria which were fewer in number in comparison with moderate halophiles, showed higher potential producing amylase, lipase, cellulase and pectinase.

In similar studies, Sanchez-Porro and colleagues [23], showed the abundance of five hydrolytic enzymes including amylase, protease, lipase, DNase and pullulanase by

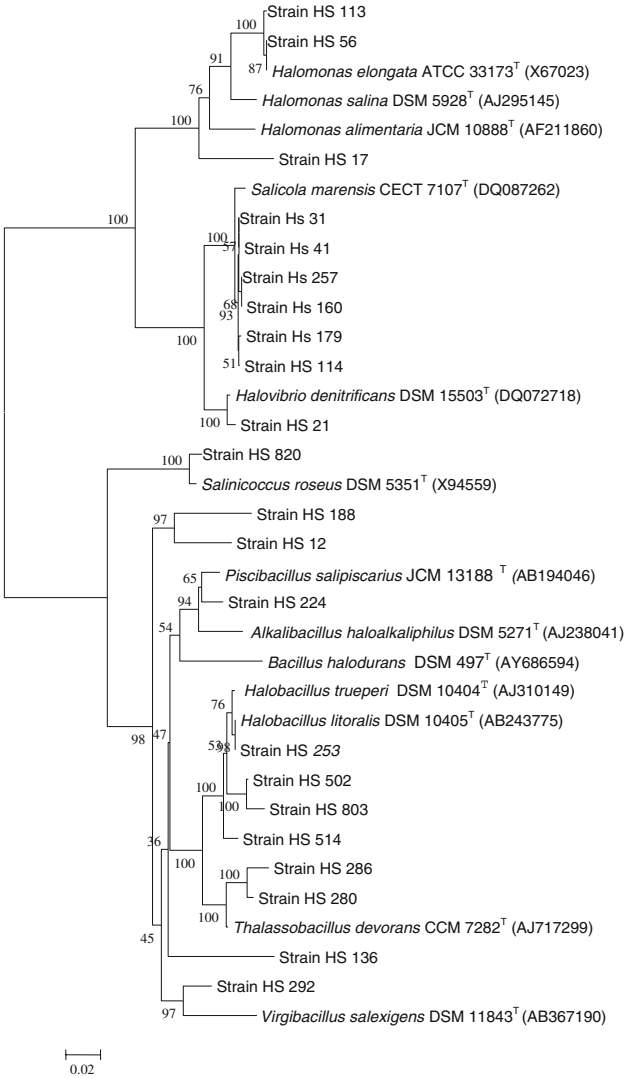

Fig. 2 Phylogenetic tree showing the position of the halophilic isolates, based on the partial 16S rRNA sequence comparison, obtained by the neighbor-joining method. The accession numbers for the reference strains are included in brackets. Bootstrap values are indicated on the branches

Table 2 Hydrolytic activity of halophilic isolates from Howz Soltan playa

Halophilic strains	Gram-positive rods	Gram-negative rods	Gram-positive cocci
Enzyme			
Amylase	123/172	38/56	26/52
Lipase	131/172	45/56	24/52
Protease	70/172	17/56	13/52
DNase	18/172	12/56	3/52
Xylanase	59/172	21/56	15/52
Pullulanase	33/172	27/56	5/52
Pectinase	13/172	7/56	8/52
CMCase	42/172	13/56	13/52
Inulinase	83/172	26/56	16/52

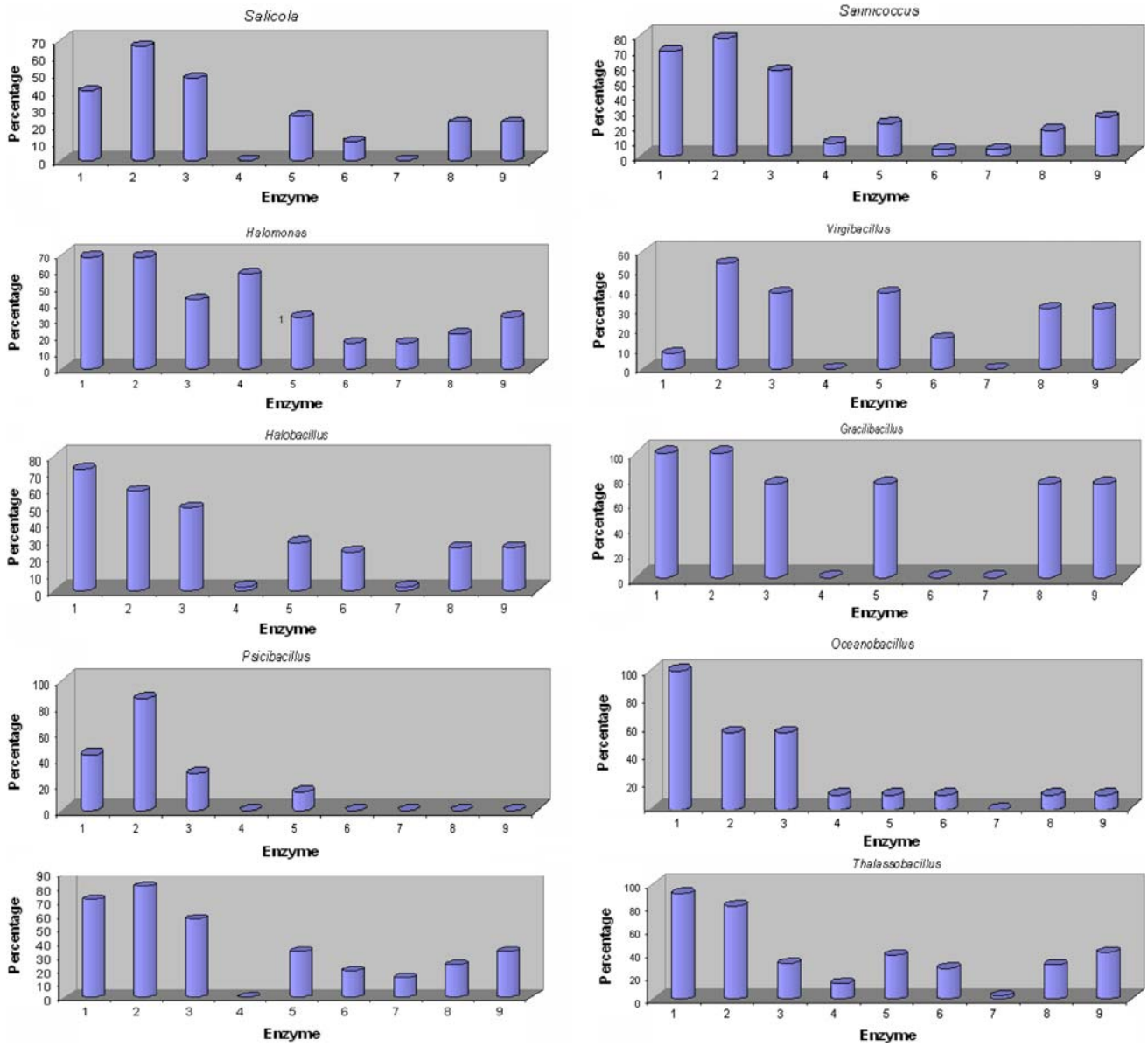

Fig. 3 Hydrolytic activities among the representatives of the genera *Salicola, Salinicoccus, Halomonas, Virgibacillus, Oceanobacillus, Thalassobacillus, Halovibrio, Halobacillus, Piscibacillus, Graciliba-cillus*. *1* Amylase, *2* lipase, *3* protease, *4* DNase, *5* xylanase, *6* pullulanase, *7* pectinase, *8* CMCase, *9* inulinase

moderately halophilic bacteria from salterns in Spain. Zavaleta and colleagues [33], determined the amylase, lipase and protease production among halophilic bacteria isolated from Pilluana brines, Peru. Furthermore, Moreno and colleagues [15] investigated the diversity of extreme halophiles, producers of lipase, protease, amylase and nuclease, in hypersaline ecosystems in South Spain. In the present study, the ability of halophilic isolates to produce nine different extracellular hydrolases has been investigated. We isolated strains with significant ability to produce inulinase, pectinase, cellulase and xylanase. These enzymes produced by halophilic bacteria are being reported here for the first time.

Identification of strains

On the basis of the phenotypic characteristics and the comparison of partial 16S rRNA gene sequences (950–1,100 nucleotides), the isolates were identified as members of the following genera: *Salicola, Halovibrio, Halomonas, Bacillus, Oceanobacillus, Thalasobacillus, Virgibacillus, Gracilibacillus, Halobacillus, Piscibacillus* and *Salinicoccus*. The tree showing the phylogenetic position of the isolates is shown in Fig. 2.

In contrast with the study of Sanchez-Porro et al. [22], according to which most of Gram-negative isolates belonged to the genus *Halomonas,* this study presented

Salicola as the predominant genus among gram-negative isolates. Among the gram-positive hydrolase-producing isolates, representatives of the genera *Virgibacillus* and *Thalassobacillus* were predominant. In addition, in this study, members of genera such as *Bacillus, Piscibacillus* and *Halobacillus* were isolated, while in the study by Sanchez-Porro et al. [22], no members of these genera were isolated.

On the other hand, in previous studies, strains of the genus *Salinivibrio* were found, but in the present study no strain of this genus was isolated. The reason might be related to the higher concentration of salt in Howz Soltan playa in comparison with those saline habitats studied in Spain. The optimal growth of members of *Salinivibrio* occurs in 2.5–10% NaCl [13], while the concentration of salt in Howz Soltan is saturated in dry seasons.

In contrast with the study of Sanchez-Porro et al. [22], in which some isolates were assigned to the genus *Chromohalobacter*, in this study we did not identify any member of this genus. Most amylase, DNase, and lipase producers were members of the genera *Oceanobacillus, Halomonas*, and *Gracilibacillus*, respectively (Fig. 3). Inulinase production was observed in isolates belonging to many genera, mainly members of the genera *Gracilibacillus, Halomonas*, *Virgibacillus, Halobacillus, Halovibrio,* and *Salinicoccus* (Fig. 3). Cellulolytic activity was detected in gram-positive rods, particularly members of the genera *Gracilibacillus, Virgibacillus,* and *Halobacillus* and xylanolytic activity was found in representatives of *Salinicoccus* as well as members of the *Bacillus*-related genera (Fig. 3).

None of isolates belonging to the genera *Virgibacillus, Oceanobacillus, Piscibacillus,* or *Gracilibacillus* were able to produce pectinase and the members of the genera *Virgibacillus, Piscibacillus, Gracilibacillus, Halovibrio* and *Salicola* failed to produce DNase (Fig. 3). It could be probable that the released activity of these two hydrolyses was not enough to cause visible clearing zone on the plates. Besides, they could be produced intracellularly; therefore, activities were not detected with the methods used.

Enzymes from halophiles are expected to show optimal activities in extreme conditions; thus, the possibility to have a wide variety of moderate halophiles producing extracellular hydrolytic enzymes will be of valuable importance for biotechnological applications. The halophilic isolates also showed the ability to tolerate a wide range of salinity, pH, and temperatures, and presented combined hydrolytic activity, which provides the advantage of uses in various industrial processes [11, 14, 29]. The discovery of biopolymer degrading enzymes offers a new horizon to remove oilfield waste where high temperature and salinity are typically found. Moreover, reaching information about nutritional requirements of these bacteria could help to plan strategies for bioremediation of oil and salt-contaminated drill cutting [22]; further studies are currently in progress in order to determine the diversity of halophilic bacteria and their ability to produce extracellular hydrolytic enzymes in other hypersaline environments and to select the best hydrolytic enzymes producers. Investigations should be directed towards the characterization of the hydrolases and the corresponding encoding genes to utilize their products in different industrial processes [22].

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