

## Diversity, Distribution and Characterization of Halophilic and Halotolerant Bacteria Isolated from Marine Sediments for Producing Extracellular Hydrolytic Enzymes

Shiping Wei\*, Juan Yang, Rui Xiao, Jun Sun, Hao He and Hongpeng Cui

School of Marine Sciences, China University of Geosciences, 29 Xueyuan Rd.,  
Haidian District, Beijing 100083, China.

(Received: 10 July 2014; accepted: 06 September 2014)

A total of 114 halophilic and halotolerant bacteria were isolated from marine sediments. Phylogenetic analysis of the isolates showed that they belonged to 23 OTUs. 63, 52, 47, 57, 74, 15 and 4 isolates were able to produce protease, amylase, lipase, pectinase, pullanase, xylanase, cellulase, respectively. Combined hydrolytic enzyme activities showed that fifteen strains presented one hydrolytic activity, 32 strains presented 2 hydrolytic activities, 21 strains presented 3 hydrolytic activities, 26 strains presented 4 hydrolytic activities, 11 strains presented 5 hydrolytic activities and 2 strains presented 6 hydrolytic activities. The highest rates for production of protease, amylase, lipase, pectinase, pullanase, xylanase and cellulase were observed in members of *B. baekryungensis*, *Hallobacillus* sp., *B. pumilus*, *B. megaterium* or *P. chungwhensis*, *B. amyloliquefaciens*, *B. pumilus*, *B. baekryungensis*, respectively. However, the higher activities of protease, pectinase and pullanase were frequently produced by the members of *Halomonas* sp. *B. amyloliquefaciens* or *P. chungwhensis*, and *Vibrio* sp. respectively. This investigation showed that the diversity of halophilic and haotolerant bacteria from marine sediments may as a potential source of hydrolytic enzymes for industrial applications.

**Key words:** Marine sediments, Halophilic and halotolerant bacteria,  
Diversity and distribution, Hydrolytic enzymes.

Halophiles are organisms that live in high salt concentrations. According to the extent of their halotolerance, halophiles are categorized as slight halophiles that grow optimally at 2%-5%, moderate halophiles that grow optimally at 5%-20%, or extreme halophiles that grow optimally at 20%-30%. Moderately halophilic bacteria usually are defined as a group of halophiles able to grow optimally in media containing 3-15% NaCl<sup>1</sup>. Halophiles require the salinity to survive, while halotolerant organisms do not require salinity but can grow under saline conditions. Halophilic and halotolerant

microorganisms are widely distributed in the Earth ecosystems with a range of salt concentrations, such as the ocean, the Great Salt Lake, the Dead Sea, saline soils and evaporation ponds<sup>1</sup>.

Halophilic and halotolerant microorganisms have attracted considerable interest due to their considerable capability of producing compounds with great industrial potential<sup>2, 3</sup>. One of the most important biotechnological applications of these halophilic and halotolerant bacteria is focused on their production of diverse extracellular enzymes, such as amylase, lipase, caseinase, xylanase, inulinase, pectinase, cellulase, pullulanase, gelatinase, urease, glutaminase and asparaginase<sup>4</sup>, as the bacteria have the desirable physiological properties of stability and solubility at high salt concentrations, which

\* To whom all correspondence should be addressed.  
Tel: 86-10-82332449; Fax: 86-10-82320065;  
E-mail: weishiping@cugb.edu.cn

facilitate their use in the areas of food processing, feed additives, biomedical sciences and chemical industries<sup>2,5-7</sup>. Moreno *et al.*<sup>8</sup> summarized a number of halophilic bacteria showing hydrolytic activities have been isolated and characterized from different hypersaline habitats, such as solar salterns, salt lakes, saline deserts and saline deposits. Both Gram-positive and Gram-negative bacteria display a variety of hydrolytic enzyme activities<sup>9</sup>. The most diverse and abundant physiological group among hydrolytic producers are found in isolated environments, and multiple extracellular hydrolytic activities produced by one strain vary in different species. Even though considerable research into the hydrolytic activities produced by halophilic and halotolerant bacteria has been conducted, screening of the novel and higher activity produced halophilic and halotolerant bacteria is still ongoing.

In this study, we describe the diversity of halophilic and halotolerant bacteria isolated from different marine sediments and their capabilities for producing different extracellular hydrolytic enzymes associated with the environments and strain species. This data will provide valuable information about their potential utilization in industrial scale process.

## MATERIALS AND METHODS

### Sample sites and sampling

Marine sediments were collected from the tidal zones of Beidaihe (BDH, N119°31'21.893" and E39°50'21.903"), Qingdao (QD, N120°22'52.733" and E36°03'24.343"), Hainan (HNS, N20°00'29.183" and E110°32'33.343"; HNI, N19°25'27.073" and E109°59'05.863") at the coastal line of China in July, 2013. The marine sediments consist mainly of mud at BDH, sand at QD, mud at HNS, and silty sand at HNI. All samples were collected from the top layers (10 cm depth) of sediments and immediately transferred into the sterilized 50 ml centrifuge tubes, then stored at 4°C for subsequent analysis.

### Halophilic bacteria isolation and culture conditions

One gram of each sample was suspended in autoclaved saline water at 5% NaCl and diluted appropriately. One hundred milliliters of each diluted sample was plated on the isolation medium

containing (per liter): casamino acid 7.5 g, yeast extract 10 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 20 g, trisodium citrate 3 g, KCl 2 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 1.6 mg, NaCl 100 g, agar 15 g, pH 7.2-7.4. The plates were incubated at 30°C for 10 d. Based on the colony characteristics, different bacteria were selected and inoculated into liquid medium containing 5% NaCl. Pure cultures of each isolate were stored in 20% glycerol at -80°C for further identification and characterization.

### DNA extraction, PCR amplification and sequencing

Bacterial genomic DNA was extracted from pure culture with the fast spin kit (Invitrogen) following the manufacturer's instructions. Amplification of 16S rDNA gene was performed in 50 µl of reaction mixture containing 0.25 mM each primer of 27f (52 - GTTTGATCCTGGCTCAG-32) and 1492r (52 - TACCTTGTACGACTT-32), 0.2 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 5 µl of Taq buffer, and 5 U Taq DNA polymerase (Invitrogen, USA), and 10-20 ng template DNA. PCR was performed on a thermocycler with an initial denaturation at 95°C for 5 min. A total of 35 cycles, at 95°C for 50 s, 45°C for 50 s, 72°C for 1.5 min, followed by a final extension step at 72 °C for 10 min. The PCR products were visualized on an agarose gel, and the bands with the corrected size were excised and purified by using the Wizard SV gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's protocol. The partial 16S rDNA fragment was sequenced on an ABI 3730 automated DNA sequencer (Applied Biosystems).

### Phylogenetic analysis

Phylogenetic affiliation of each 16S rDNA sequence was initially queried by BLAST search to suggest the closest relatives against the GenBank database. The sequences were aligned with their relatives using Clustal W, and phylogenetic trees were constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm using the MEG 4 software. The partial sequences of 16S rDNA genes from the strains have been deposited in the GenBank database under the accession numbers from KF933605 to KF933718.

### Determination of extracellular hydrolytic enzyme activity

Activities of protease, amylase, lipase, pectinase, cellulase, xylanase and pullulanase were assayed using the media as the follows: medium

for determination of proteolytic activity (per liter) contains skim milk 15 g, yeast extract 2 g, NaCl 100 g, agar 20 g; medium for determination of amylolytic activity (per liter) contains starch 5 g, yeast extract 2 g,  $(\text{NH}_4)_2\text{SO}_4$  1.4 g,  $\text{K}_2\text{HPO}_4$  2 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g, NaCl 100 g, agar 20 g, pH 7.2; medium for determination of lipolytic activity (per liter) contains beef extract 3 g, peptone 10 g, tributyrin 6 ml, NaCl 100 g, agar 20 g; medium for determination of pectinolytic activity (per liter) contains  $(\text{NH}_4)_2\text{SO}_4$  1.4 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g,  $\text{K}_2\text{HPO}_4$  2 g, yeast extract 2 g, pectin 5 g, nutrient solution 1 ml ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  5 mg/l,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  1.6 mg/l,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  1.4 mg/l,  $\text{CaCl}_2$  2 mg/l), NaCl 100 g, agar 20 g; medium for determination of cellulase activity (per liter) contains carboxymethyl cellulose (CMC) 5 g,  $\text{NaNO}_3$  1 g,  $\text{K}_2\text{HPO}_4$  2 g, KCl 1 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g, yeast extract 2 g, glucose 1 g, NaCl 100 g, agar 20 g, pH 7.2; medium for determination of xylanase activity (per liter) contains yeast extract 2 g, peptone 5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{CaCl}_2$  0.15 g, xylan 10 g, NaCl 100 g, agar 20 g; medium for determination of pullulanase activity (per liter) contains yeast extract 2 g, pullulan 5 g, NaCl 100 g, agar 20 g.

To screen the hydrolytic enzyme activity produced by the bacteria, 0.5  $\mu\text{l}$  of pure culture of each strain was inoculated on the detection media described above and the plates were incubated at 30°C for 5 d. The presence of protease and lipolytic activities was directly identifiable from the plates as a clear zone developed around the colonies. For the assay of amylolytic and pectinolytic activities, the plates were flooded with 0.3%  $\text{I}_2$ -0.6% KI solution, and a clear zone around the growth colony identified an amylase or pectinase producing strain<sup>9, 10</sup>. The method of detection of pullulanase activity

was adopted by Rohban *et al.*<sup>10</sup> and Ruben *et al.*<sup>11</sup> with modifications. In the original report, the interaction between pullulan and ethanol leads to the formation of a white precipitate of non-degraded pullulan, thus allowing identification of pullulanase activity<sup>11</sup>. In this paper, a better result was obtained when the plates were flooded with 0.3%  $\text{I}_2$ -0.6% KI solution instead of 97% ethanol, with a clear zone around the colonies indicating the pullulanase activity produced by the strains. For detection of cellulose and xylanase activities, the plates were flooded with 10 ml 0.1% congo red solution and stained with the dye for 20 min. After draining off the dye, a clear zone surrounding the colony shows the cellulase and xylanase activities produced by the strains<sup>5, 9, 10</sup>.

All the plates were performed in triplicates and the diameters of each halo and each colony were measured.

#### Statistical analysis

Principal component analysis (PCA) was carried out to characterize distribution patterns of OTUs versus sampling locations using Canoco for Windows 4.5 program<sup>12</sup>. Ordination biplots include 4 variables of sampling locations and the total number of OTUs corresponding to each site.

## RESULTS

### Isolation and characterization of halophilic and halotolerant bacteria

After incubation at 30°C for 10 d, colonies were well-developed at salinity on the plates. Based on the colonial shapes (round, irregular and spreading or concentric), margin (entire, undulate lobate or filamentous), elevation (flat, convex, umbonate or umbilicate), pigment (pale yellow, pink-

**Table 1.** Isolation and characterization of halophilic and halotolerant bacteria

Location	Sediment property	Total number of isolates	Number of isolates classified by Gram staining		Number of isolates growing well on NaCl containing media <sup>a</sup>			
			Gram positive	Gram negative	5%	10%	15%	20%
BDH	mud	28	26	2	28	20	11	1
QD	sand	27	10	17	27	25	24	18
HNS	mud	30	30	0	30	21	12	7
HNI	silty sand	29	28	1	29	28	23	4

Note: <sup>a</sup> The growth of isolates on different salt concentration media was classified into four types, positive growth, moderate growth, weak growth and no growth. Only the strains showed positive and moderate growth were calculated as growing well on NaCl containing media.

red, creamy, white or off-white) and density (opaque or translucent), 28, 27, 30 and 29 strains were picked from the sediments of BDH, QD, HNS and HNI, respectively. Gram-staining shows that most of the strains isolated from BDH (92.9%), HNS (100%) and HNI (96.6%) belong to the group of Gram-positive bacteria, while a relatively higher ratio of Gram-negative bacteria (63.0%) was identified from QD (Table 1). A total of 114 strains were re-streaked on the media containing different concentrations of NaCl. All strains grew well on the media containing of 5% NaCl. When the concentration of NaCl was increased to 20%, most of them ceased growth or grew very weakly, however, 66.7% of the strains isolated from the sandy beach of QD grew well. When checking the strains on the alkaline pH media, all of them grew well in the range of pH 7-8.5.

#### Diversity and distribution of halophilic and halotolerant bacteria

A total of 114 bacterial 16S rDNA

sequences were analyzed and 23 operational taxonomic units were detected (OTUs, an OTU was defined as sequence sharing  $\leq 97\%$  identity). A phylogenetic tree of the 16S rDNA sequences revealed that 16 OTUs belonged to the class of *Bacillin* which included the four families *Bacillaceae*, *Planococcaceae*, *Bacillales Incertae Sedis* and *Paenibacillaceae*, 6 OTUs belonged to the class of *Gammaproteobacteria*, which included the three families *Halomonadaceae*, *Alteromonadaceae* and *Vibrionaceae*, and only one OTU was detected in the family of *Rhodobacteraceae*, lying within the class of *Alphaproteobacteria* (Fig 1). OTUs belonging to *Bacillaceae* showed the highest diversities, which comprised 56.5% of the total OTUs. 94 Gram-positive strains in Table 1 were belonged to *Bacillin*, and the other 20 Gram negative strains belonged to *Gammaproteobacteria* and *Alphaproteobacteria*, which indicated that the Gram-positive strains were the dominant strains

**Table 2.** Distribution and occurrence of OTUs and the number of isolates in the sampling locations

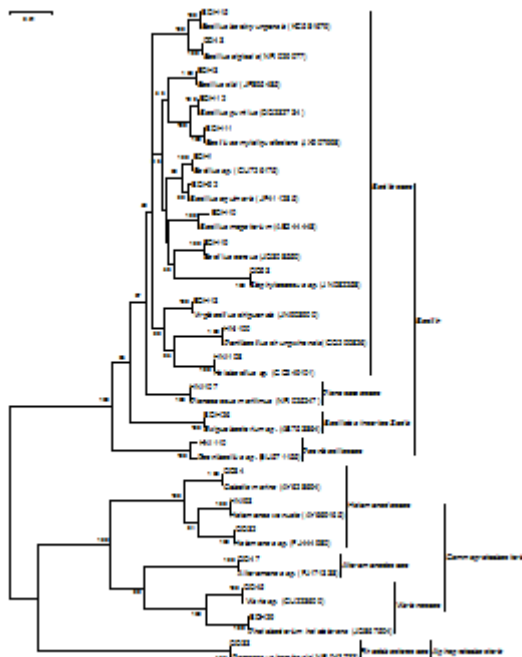
Class	Family	OTU of species <sup>a</sup>	Number of isolates <sup>b</sup>					
			BDH	QD	HNS	HNI		
<i>Bacillin</i>	<i>Bacillaceae</i>	<i>Bacillus baekryungensis</i> (Bba)	3	7	7	1		
		<i>Bacillus algicola</i> (Bal)	-	1	-	-		
		<i>Bacillus cibi</i> (Bci)	1	-	-	-		
		<i>Bacillus pumilus</i> (Bpu)	5	-	8	1		
		<i>Bacillus amyloliquefaciens</i> (Bam)	2	-	-	1		
		<i>Bacillus sp.</i> (Bsp)	2	-	2	-		
		<i>Bacillus aquimaris</i> (Baq)	4	-	3	-		
		<i>Bacillus megaterium</i> (Bme)	3	-	8	1		
		<i>Bacillus cereus</i> (Bce)	1	-	-	1		
		<i>Staphylococcus sp.</i> (Ssp)	-	1	-	-		
		<i>Virgibacillus chiguensis</i> (Vch)	2	-	-	-		
		<i>Pontibacillus chungwhensis</i> (Pch)	-	-	2	10		
		<i>Halobacillus sp</i> (Hbsp)	2	1	-	8		
		<i>Planococcaceae</i>	<i>Planococcus maritimus</i> (Pma)	-	-	-	1	
<i>Bacillales Incertae Sedis</i>	<i>Exiguobacterium sp</i> (Esp)		1	-	-	-		
	<i>Paenibacillus sp</i> (Psp)		-	-	-	4		
<i>Gammaproteo bacteria</i>	<i>Halomonadaceae</i>	<i>Cobetia marina</i> (Cma)	-	10	-	-		
		<i>Halomonas venusta</i> (Hve)	-	-	-	1		
		<i>Halomonas sp</i> (Hmsp)	-	3	-	-		
		<i>Alteromonadaceae</i>	<i>Alteromonas sp</i> (Asp)	-	1	-	-	
			<i>Vibrionaceae</i>	<i>Vibrio sp</i> (Vsp)	-	2	-	-
		<i>Alphaproteobacteria</i>	<i>Rhodobacteraceae</i>	<i>Photobacterium halotolerans</i> (Pha)	2	-	-	-
				<i>Paracoccus homiensis</i> (Pho)	-	1	-	-

Note: <sup>a</sup> An abbreviation for each bacterial species in the bracket was used for the Principal component analysis. <sup>b</sup> - no isolate.

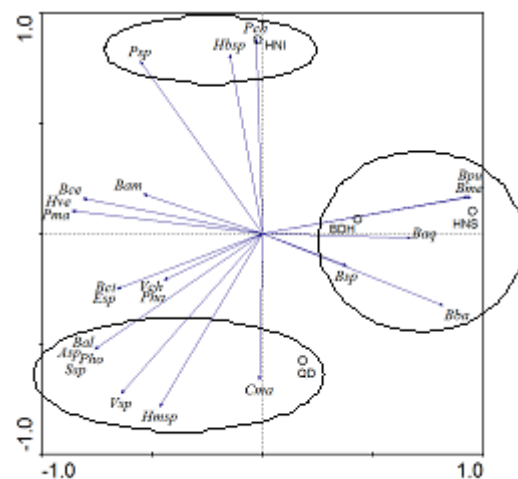
among the cultured moderately halophilic bacteria. Distribution of each OTU was evaluated across all the isolates associated with their sample locations. Out of total of 23 OTUs, 12, 9, 6 and 10 OTUs were detected in locations of BDH, QD, HNS and HNI, respectively. 91.7%, 44.4%, 100% and 90.0% of OTUs in BDH, QD, HNS and HNI were found in the taxonomic class of *Bacillia*, respectively. Corresponding to each location, 0.3%, 44.4%, 0% and 10.0% of the OTUs belonged to the class of *Gammaproteobacteria*. Only 11.2% of the OTUs detected in QD belonged to the class of *Alphaproteobacteria*.

From the occurrence of each OTU in the sample locations, it was shown that the total of 13 OTUs of the taxonomic family of *Bacillaceae* represented 89.3%, 37.0%, 100% and 79.3% of isolates in BDH, QD, HNS and HNI, respectively, which indicated that the isolates belonging to

*Bacillaceae* were the dominant strains in BDH, HNS and HNI. Then occurrence of OTUs in QD was remarkably different from that of in the other three locations. At QD, 2, 1 and 1 OTUs of the families *Halomonadaceae*, *Alteromonadaceae* and *Vibrionaceae* represented 48.1%, 0.4% and 7.4% of isolates, which indicated that the isolates belonging to *Halomonadaceae* were the dominant strains in QD (Fig. 1 and Table 2). Principal component analysis showed that community composition of bacteria between BDH and HNS was similar (Fig. 2). The isolates, which belonged to OTUs of *Bacillus baekryungensis* (*Bba*), *Bacillus pumilus* (*Bpu*), *Bacillus sp.* (*Bsp*), *Bacillus aquimaris* (*Baq*) and *Bacillus megaterium* (*Bme*), contributed significantly to abundance of community composition in both of the locations. The isolates belonging to OTUs of *Bacillus baekryungensis* (*Bba*), *Cobetia marina* (*Cma*), *Halomonas sp.* (*Hmsp*) and *Vibrio sp.* (*Vsp*) were clustered closely together in the location of QD. In contrast, the isolates belonging to the OTUs of *Pontibacillus chungwhensis* (*Pch*), *Paenibacillus sp.* (*Psp*) and *Halobacillus sp.* (*Hbsp*) were closely associated with the location of HNI.



**Fig. 1.** Phylogenetic relationship of 16S rRNA sequences of the isolates retrieved from marine sediments and related reference sequences. The phylogenetic tree was generated using approximately 1,500 bp of 16S rRNA genes by the neighbor-joining method. Bootstrap values are based on 1,000 replicates and are shown at the nodes with greater than 50% bootstrap support. The scale bar equals 1% nucleotide divergence.



**Fig. 2.** Correspondence analysis of biplots showing associations between bacterial OTUs and sampling locations. The biplots were created with 23 OTUs of halophilic and halotolerant bacteria and 4 sampling locations. The resulting ordination biplots approximated the weighted average of OTUs with respect to location variables, which are represented as arrows. The length of these arrows indicates the relative influence of OTU in defining the sampling locations based on bacterial community

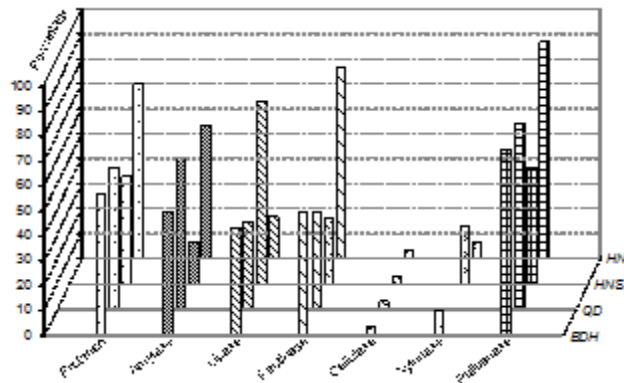
**Hydrolytic activity of isolates**

The ability to produce seven different hydrolytic enzymes was tested among the isolates. Of 114 isolates, 107 isolates were able to produce at least one of the hydrolytic activities, whereas 7 isolates were unable to produce any of the tested hydrolytic activities. A total of 63, 52, 47, 57, 74, 15, 4 isolates were able to produce protease, amylase, lipase, pectinase, pullanase, xylanase, and cellulase, respectively. Combined hydrolytic enzyme activities were detected and the results showed that the isolates have diversities for enzyme production. Fifteen strains presented one hydrolytic activity, 32 strains presented 2 hydrolytic activities, 21 strains presented 3 hydrolytic activities, 26 strains presented 4 hydrolytic activities, 11 strains presented 5 hydrolytic activities and 2 strains presented 6

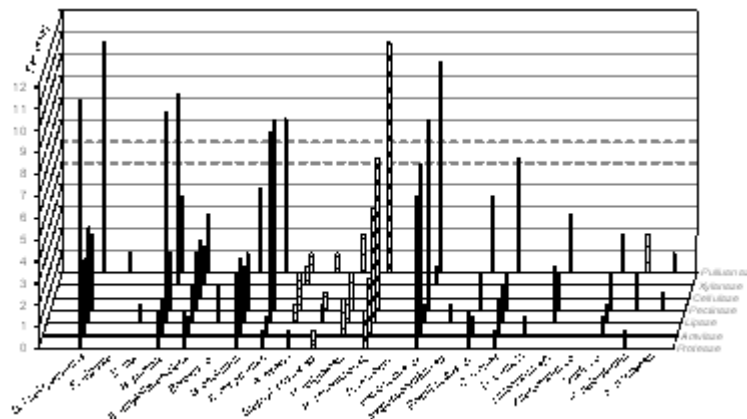
hydrolytic activities. Among the tested strains, *Halobacillus sp.* HNI111 and *Halomonas sp.* QD55 showed the highest protease activity, *B. amyloliquefaciens* BDH11 and BDH27 presented the highest amylase activities, *B. megaterium* HNS83 and *B. pumilus* BDH24, HNS77 and HNS83 presented the highest lipase activities, *B. amyloliquefaciens* BDH27, *B. cereus* HNI98 and *P. chungwhensis* HNI96 showed the higher pectinase activities, *Vibrio sp.* QD45 presented the highest pullanase activity, *B. pumilus* HNS74 presented the highest xylanase activity, and *B. amyloliquefaciens* BDH11 and HNI92 presented the highest cellulase activities.

**Characteristics of hydrolytic activity distribution associated with sample locations and strains**

Hydrolytic activities of the isolates with respect to the sample locations are shown in Fig. 3. The strains able to produce protease, amylase,



**Fig. 3.** Geographical distribution and occurrence of each hydrolytic activity produced by the isolates associated with the sampling locations



**Fig. 4.** Enzymatic profiles of the isolates and comparison of the rate for production of each enzymes among the strains

lipase, pectinase, pullulanase, xylanase and cellulase were widely distributed among the sample locations, whereas the strains able to produce xylanase and cellulase were not, with few strains able to produce xylanase and cellulase. The strains isolated from different locations showed the different frequencies of producing each kind of hydrolytic activity. These were as high as 70% of strains isolated from HNI, 60.7% of strains isolated from QD, 73.3% of strains isolated from HNS, 76.6% of strains isolated from HNI, 23.3% of strains isolated from HNS, and 86.7% of strains isolated from HNI were able to produce protease, amylase, lipase, pectinase, pullulanase and xylanase, respectively. The cellulase-producing strains were spread evenly among the four locations, reaching frequencies as low as 3.3%-3.6%.

Hydrolytic activities of the isolates with respect to the species are shown in Fig. 4. Although all of the species, except for *P. maritimus* and *Aleromonas sp.*, produced at least one of the hydrolytic activities, a higher ratio of hydrolytic activity producing strains were frequently observed within a number of limited species. Higher percentages of strains belonging to *B. baekryungensis* (11.4%) and *Halobacillus sp.* (7.0%) were able to produce protease than that of other species. 7.9% of strains belonging to *Halobacillus sp.* were able to produce amylase, which is greater than all other species. The highest percentages of strains producing lipase belonged to *B. pumilus* (9.7%) and *B. megaterium* (8.7%) and the highest percentages of strains producing pectinase belonged to *B. megaterium* (8.8%) and *Halobacillus sp.* (8.8%). It is interesting that only the strains belonging to the species of *Bacillus pumilus* were able to produce cellulase, and the percentage of producing cellulase (2.6%) was also lower than that producing other hydrolytic enzymes. Xylanase production was also found to be limited to species of *B. algicola*, *B. pumilus*, *B. amyliqueliefaciens*, *B. megaterium*, *P. chungwhensis* and *Halobacillus sp.*, and the highest percentage strains in producing xylanase was *B. pumilus* (8.8%). When compared with other species, the highest percentages of strains producing pullulanase belonged to *B. baekryungensis* (10.5%) and *Halobacillus sp.* (9.7%).

## DISCUSSION

Although halophilic and halotolerant bacteria have been extensively studied in various hypersaline environments<sup>1</sup>, only few studies have been carried out concerning their diversity and distribution in marine sediments. We isolated a total of 114 halophilic and halotolerant strains on agar plates from the marine sediments collected from BDH, QD, HNS and HNI. Phylogenetic analysis indicated that all isolates were members of 23 genera belonging to 8 families in 3 classes (Fig. 1 and Table 2). Community structure analysis showed that the species of *B. baekryungensis* (18 isolates), *B. pumilus* (14 isolates), *B. aquimaris* (7 isolates), *B. megaterium* (12 isolates), *Halobacillus sp.* (11 isolates), *P. chungwhensis* (12 isolates) and *C. marina* (10 isolates) were the dominant strains among the recovered isolates, where each of the other 16 species is represented by 4 or fewer isolates. In a study of marine sediment from Pichavaram, on the southeast coast of India, the dominant types of isolates developing on agar plates were assigned by taxonomy to the species of *Vibrio harveyi*, *Halomonas sp.*, *Vibrio fluvialis*, and *Halobacterium sp.*<sup>13</sup>. In another study, culturable moderately halophilic and halotolerant bacteria from the Moroccan marsh ecosystems of Lower Loukkos indicated that the isolates were dominated by *Bacillus aquimaris*, *Bacillus megaterium*, *Bacillus oceanisediminis*, *Bacillus hwajinpoensis*, *Bacillus safensis* and other unidentified *Bacillus sp.*<sup>14</sup>. The most concentrated brines, greater than 20% salt, are dominated by highly pigmented Archaea such as *Halobacterium*, *Haloferax* and *Haloarcula*<sup>7,15</sup>. In many cases, the bacteria from hypersaline aquatic systems are close relatives of marine organisms, but have increased salt tolerance<sup>16</sup>.

Distribution of the cultured halophilic and halotolerant bacteria showed that 16 out of 23 OTUs were members of 4 families in the class of *Bacillia* representing 94 isolates (82.5%). It was interesting that most of the strains isolated from BDH, HNS and HNI were Gram-positive bacteria, while most of the strains isolated from QD were Gram-negative. Principal component analysis showed that similar patterns of OTUs occurred between BDH and HNS, which mainly consisted of *B. baekryungensis*, *B. pumilus*, *Bacillus sp.*, *B.*

*aquimaris* and *B. megaterium*. We inferred that the two sites had similar environments as both of them were located in the high tidal zone and had sediments consisting mainly of mud. The sample site of HNI, located in an inter tidal zone, was a cross area of mud and sand. Only a few OTUs of HNI overlapped with those of HNS, even though they were located on the same coast. The occurrence of OTUs in QD was different from BDH, HNS and HNI, with most of the QD strains consisting of the Gram-negative bacteria, *Cobetia marina*, *Vibrio sp.* and *Halomonas sp.* These bacteria could tolerate salt concentration as high as 20% sodium chloride. We inferred that the sediment in the QD site consisted of sand, with the more erodible sand causing higher salinity in this area, leading to the development of a more salt-tolerant bacteria community. It was interesting that the community structure of the marine mud was mainly comprised of the genera of *Bacillus*, which was extensively represented in the hypersaline terrestrial ecosystems, such as saline soil<sup>17</sup>. The bacterial community of sand sediments collected from QD was very similar with those of hypersaline aquatic ecosystems, such as solar salterns<sup>7, 15</sup>.

Halophilic microorganisms have been investigated for their potential biotechnological applications in various fields, such as production of compatible solutes, biopolymers, enzymes, food biotechnology, and biological waste treatment<sup>2</sup>. Moreover, increasing concerns focus on the salt tolerant extracellular hydrolytic enzymes produced by moderately halophilic and halotolerant bacteria<sup>4, 8</sup>. Halophilic and halotolerant bacteria are an excellent source of enzymes exhibiting salt, pH and temperature tolerance<sup>18, 19</sup>. In this study, 114 halophilic and halotolerant bacteria strains were investigated and higher rates of production of protease, amylase, lipase, pectinase, and pullanase, but much lower rates of xylanase and cellulase, were observed among these strains. This was somewhat different from Rohban *et al.*<sup>10</sup>, who reported higher rates of production of amylase, lipase, protease, xylanase, and inulinase, but lower rates of pullulanase, pectinase, and cellulase production, among the halophilic strains isolated from Howz Soltan Lake. We inferred that this was due to the different strain species fostered by the different environments. Among the cultured

strains, most of them presented the combined hydrolytic activities and similar results to those reported by Rohban *et al.*<sup>10</sup> and Sánchez-Perro *et al.*<sup>19</sup>. We also noticed that most of the strains producing of hydrolytic were Gram-positive and of the class of *Bacillin* for the (Fig. 1 and Fig. 4). The genus *Bacillus* is well known as an enzyme producer and species belonging to this genus was frequently have been used in commercial production of enzymes<sup>20</sup>.

Geographical distribution of hydrolytic activities showed that activities of protease, amylase, lipase, pectinase, pullulanase and cellulase production by the isolates were widely distributed among the sampling locations. The strains isolated from HNI showed the higher rates for production of protease, pectinase and pullulanase, the strains isolated from QD presented the highest rates for production of amylase, and the strains isolated from HNS showed the highest rates for production of lipase, while lower rates for production of xylanase and cellulase were a common phenomenon distributed over all of the sampling locations. The each location presented different enzyme-producing activities by the isolates was clearly related to the different species community at each site. The highest rates for production of protease, amylase, lipase, pectinase, pullulanase, xylanase and cellulase were for members of *B. baekryungensis*, *Hallobacillus sp.*, *B. pumilus*, *B. megaterium* or *P. chungwhensis*, *B. amyloliquefaciens*, *B. pumilus*, and *B. baekryungensis*, respectively (Fig. 4). Rohban *et al.*<sup>10</sup> reported that most amylase, DNase, and lipase producers were members of the genera *Oceanobacillus*, *Halomonas*, and *Gracilibacillus* respectively. Cellulase producers were detected among members of *Gracilibacillus*, *Virgibacillus* and *Halobacillus*. Kakhki *et al.*<sup>5</sup> reported that under their conditions *Halorubrum* was the predominant genus showing the highest rates of amylase, lipase, pullulanase, inulinase and DNase production. Our data was much more similar to the results reported by Berrada *et al.*<sup>14</sup>, in which *Bacillus* was the predominant genus showing the highest production rates for amylase, lipase, DNase, protease and cellulase. It was interesting that highest average rates for production of the enzymes by a certain genus did not necessarily mean that the highest enzyme-producing activity



belonged to a strain from the same genus. For example, even though the highest average rate for production of protease was the species *B. baekryungensis*, the highest protease activity was observed in the strains of *Halobacillus sp.* HNI111 and *Halomonas sp.* QD55, which belong to the genera of *Halobacillus* and *Halomonas*, respectively. For pullulanase, although the strains belonged to the genera of *B. baekryungensis* and *Halobacillus sp.* showed highest average rates for production of pullulanase, a strain of *Vibrio sp.* QD45, belonging to the genus of *Vibrio*, presented the highest pullulanase activity (Table 2 and Fig. 4).

Finally, although a variety of halophilic and halotolerant bacteria was distributed in various environments and most of them presented enhanced extracellular hydrolytic enzyme activity, more work needs to be done with respect to industrial applications, such as characterization of the hydrolases, and isolation of the encoding genes for their use in genetic engineering<sup>10, 19</sup>.

#### ACKNOWLEDGEMENTS

The authors acknowledge James Hurley of Department of Plant Pathology and Microbiology, Texas A & M University, for making a critical reading and revision of this paper. This research was supported by the Fundamental Research Funds for the Central Universities (2652012138).

#### REFERENCES

- Ventosa, A., Nieto, J.J., Oren, A. Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.*, 1998; **62**: 504-544.
- Margesin, R. and Schinner, F. Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles*, 2001; **5**:73-83.
- Oren, A. Diversity of halophilic microorganisms: Environments, phylogeny, and applications. *J. Ind. Microbiol. Biotechnol.*, 2002; **28**:56-63.
- Mellado, E., Sanchez-Porro, C., Martin, S., Ventosa, A. Extracellular hydrolytic enzymes produced by moderately halophilic bacteria. In: Ventosa, A., (ed) *Halophilic Microorganisms*, Springer-Verlag, Berlin Heidelberg, 2004; pp 285-295.
- Kakhki, A.M., Amoozegar, M.A., Khaledi, E.M. Diversity of hydrolytic enzymes in haloarchaeal strains isolated from salt lake. *Int. J. Environ. Sci. Tech.*, 2011; **8**:705-714.
- Kulkarni, N., Shendye, A., Rao, M. Molecular and biotechnological aspects of xylanases. *FEMS Microbiol. Rev.*, 1999; **23**:411-456.
- Litchfield, C.D. and Gillevet, P.M. Microbial diversity and complexity in hypersaline environments: a preliminary assessment. *J. Ind. Microbiol. Biotechnol.*, 2002; **28**:48-55.
- Moreno, M.L., Pérez, D., García, M.T., Mellado, E. Halophilic bacteria as a source of novel hydrolytic enzymes. *Life*, 2013; **3**:38-51.
- Babavalian, H., Amoozegar, M.A., Pourbabae, A.A., Moghaddam, M.M., Shaker, F. Isolation and identification of moderately halophilic bacteria producing hydrolytic enzymes from the largest hypersaline Playa in Iran. *Microbiology*, 2013; **82**:466-474.
- Rohban, R., Amoozegar, M.A., Ventosa, A. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *J. Ind. Microbiol. Biotechnol.*, 2009; **36**:333-340.
- Ruben, D., Gonzale, E., Arenas, C., Vilches, E.B., De Billerbeck, M. Selective procedure for isolating microorganisms producing pullulanase and isoamylase. *Biotechnol. Tech.*, 1993; **7**:429-434.
- Ramette, A. Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl. Environ. Microbiol.*, 2009; **75**:2495-2505.
- Ganesan, S., Manoharan, N., Naveenkumar, S., Velsamy, G., Manivannan, S.P. Study on proteolytic treatment of textile fabric softness and smoothening using halophilic bacteria biopolymers. *Int. J. Environ. Sci.*, 2010; **1**:567-579.
- Berrada, I., Willems, A., Vos, P.D., EI-fahime, E., Swings, J., Bendaou, N., Melloul, M., Amar, M. Diversity of culturable moderately halophilic and halotolerant bacteria in marsh and two salterns a protected ecosystem of Lower Loukkos (Morocco). *Afr. J. Microbiol. Res.*, 2012; **6**:2419-2434.
- Rodriguez-Valera, F., Ventosa, A., Juez, G., Imhoff, J.F. Variation of environmental features and microbial populations with salt concentrations in a multi-pond saltern. *Microb. Ecol.*, 1985; **11**:107-111.
- Caton, T.M., Witte, L.R., Ngyuen, H., Buchheim, J.A., Buchheim, M.A., Schneegurt, M.A. Halotolerant aerobic heterotrophic bacteria from the Great Salt Plains of Oklahoma. *Microb. Ecol.*, 2004; **48**:449-462.

17. Ventosa, A., Mellado, E., Sánchez-Porro, C., Marquez, M.C. Halophilic and halotolerant micro-organisms from soils. In: Dion P, Nautiyal CS (eds) *Microbiology of Extreme Soils, Soil Biology* **13**, Springer-Verlag Berlin Heidelberg, 2008; pp 87-115.
18. Gomez, J. and Steiner, W. The biocatalytic potential of extremophiles and extremozymes, *Food Technol. Biotechnol.*, 2004; **2**: 223-235.
19. Sánchez-Porro, C., Martin, S., Mellado, E., Ventosa, A. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *J. Appl. Microbiol.*, 2003; **94**: 295-300.
20. McConnel, D.J., Cantwell, B.A., Devine, K.M., Forage, A.J., Laoide, B.M., O'Kane, C., Ollington, J.F., Sharp, P.M. Genetic engineering of extracellular enzymes systems of *Bacilli*. *Annual New York Academy of Science*, 1986; **469**: 1-17.