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Diversity of Cultivable Halophilic/Halotolerant Eubacteria Isolated from Shache, Xinjiang, China and Characteristics of their Hydrolase

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For the past few years, bacteria that are grown in high-salt environment have been a focus of extremophiles research. They are distributed widely in different habitats and possess unique physiological properties. In this study, we obtained 97 halophilic or halotolerant eubacteria from saline-alkali soil in Shache County, Xinjiang Province, China, by selective media. Phylogenetic analysis of the partial sequences indicated that the isolates were affiliated with 19 genera of three orders. All 97 strains were screened for amylase, lipase, gelatinase and cellulase. Twenty-five strains were lipase producers, 27 gelatinase producers, 31 amylase producers, and none were cellulase producers. Combined hydrolytic activities were detected in some strains. Twenty-one isolates presented with two or more activities, but only five isolates presented with three hydrolytic activities. For the tolerability tests of amylase and lipase, we found that most of the isolates presented the highest amylase activity at 10% NaCl, pH 7.0 and 40°C, and the highest lipase activity at 5-10% NaCl, pH 8.0 and 30°C.

Key words: Halophiles, Halotolerant Bacteria, Amylase, Lipase, Gelatinase.

Saline environments contain thriving microbial ecosystems, and many studies have investigated the inhabitants of these systems in the past decade[1, 2, 3]. Microorganisms in saline environments are halotolerant or halophilic, and they are found in all three domains of life: Archaea, Bacteria and Eukarya. They contain representatives of many different physiological types, and are adapted to a wide range of salt concentrations up to saturation.

Previous reports revealed that archaea of family Halobacteriaceae growed at salt concentrations at or near NaCl saturation, but moderately halophilic bacteria preferred to grow below 10-15% NaCl.

It was reported that moderately halophilic bacteria strains are able to degrade organic pollutants, and diversity of their metabolites to adapt to extreme salinity, they were considered suitable candidates for potential application in industry[4].

Some of these halotolerant and halophilic species can be cultured to produce extracellular enzymes under conditions of high salt concentrations and high temperature[5]. Extracellular hydrolytic enzymes (such as amylases, proteases and lipases) have diverse potential applications in

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different areas such as the food industry, feed additives, biomedical sciences and the chemical industry[6]. The discovery of the industrial application of halotolerant enzymes that can withstand harsh conditions has greatly increased in recent years. However, their biotechnological possibilities have not been extensively exploited[7].

Hypersaline water and soil are distributed widely in Xinjiang Province in northwestern China, and halophilic microorganisms have been studied for many years in this area. Many halophilic/ halotolerant species have been found by different culture methods[8, 9]. However, there has been no report about extracellular hydrolases of halophilic/ halotolerant species in this specific area. In this study, we investigated the diversity of culturable bacteria from the saline-alkali soil in Shache, Xinjiang, and screened the extracellular hydrolytic enzymes of these isolates, and we found some potential salt-tolerant hydrolase producers in harsh conditions.

MATERIALS AND METHODS

Site description and Soil sample collection

Soil samples were collected from Shache (77°14.908'E, 38°25.049'N), which is in the west of Tarim Basin in Xinjiang Province, northwest of China. Saline soil in Shache is the typical type of saline soil in Xinjiang, where the main ions are chlorine and sulphate. The typical inland arid climate and special geographical conditions make salt accumulation on the surface of the soil profile. pH value of saline soil at sampling sites varied from 7.41-7.97. Soil samples were collected at a depth of 10-30 cm at each sampling site and stored in 50-ml sterile Falcon centrifuge tubes (Shanghai Sangon, China).

Enrichment and isolation of microorganisms

To isolate moderately halophilic and/or halotolerant bacteria, an enrichment strategy was established with five different media, which were named PT, HVA, HBCM, HNA and YCSS respectively (Table 1), and finally inoculated on Tryptone Soy Broth agar (TSB agar). One-gram soil samples were inoculated into 50 ml medium with 10% (w/v) NaCl in a 250-ml flask, and incubated at 37°C for 3 days, with shaking at 200 rev min^{-1}. Aliquots (0.2 ml) were spread onto Petri dishes using six different solid media (Table 1) and allowed to grow for 1-3 weeks at 37°C. Based on color and size, colonies were selected and further purified on TSB agar supplemented with 10% NaCl.

PCR amplification of 16s rDNA and phylogenetic analysis

Total genomic DNA of isolated microorganisms was extracted as previously described[10] and then used as a PCR template for identification of bacteria. PCR amplification was performed with primers SRR181F (5 -GTT TGA TCC TGG CTC AGG AC-3), SRR182R (5 -GGT GTT CCT CMH GAT ATC TG-3), SRR178F (5 -GAA CGC TGG CGG CGT GCT-3), and SRR179R (5 -GCG CAT TYC ACC GCT ACA CC-3) designed by conservative sequences of 16S rRNA gene. PCR was carried out in a total volume of 50 µl containing PCR buffer with 7.5 µM $MgCl_2$, 200 µM dNTPs, Taq DNA polymerase (1.0 U), 0.5 µM each primer, and DNA template (~80 ng). The amplification procedure was as follows: initial denaturation at 95°C for 4 min, and 30 cycles of denaturation at 95°C for 45 s, annealing at 70°C for 45 s, and extension at 72°C for 1 min, with final extension at 72°C for 10 min. The PCR products were examined on regular 1% agarose gel. For sequencing, the amplified PCR products were purified using a Sangon DNA Fragment Purification Kit and sequenced using an ABI 3730 automated sequencer at Shanghai Sangon Biotech (Shanghai, China). The sequences were aligned with ClustalW[11]. Phylogenetic tree (neighbor-joining) was constructed using MEGA 4.0 from dissimilar distances and pairwise comparisons with the Kimura 2-parameter model[12].

Screening of strains for extracellular enzyme activities

Extracellular amylolytic activity on plates was determined as described previously[6], using modified TSB medium supplemented with 0.5% starch. After incubation at 37°C for 7 days, the plates were flooded with I_2-KI reagent, and a clear zone around the growth indicated hydrolysis of starch.

Extracellular lipolytic activity of the isolates was detected by screening for halos around colonies after 10 days incubation at 37°C on TSB plates containing 1% Tween 80. Extracellular gelatinase activity was detected by gelatin liquefaction. Gelatin (1.5%) was used instead of agar as the curing agent. Liquefaction indicated

hydrolysis of gelatin. Extracellular cellulase production was determined by replica plating of halophilic/halotolerant isolates onto TSB plates containing $5gL^{-1}$ carboxymethylcellulose (Shanghai Sangon, China). The plates were incubated for 5 days at 37°C. Staining the plates with 0.03% Congo Red, a clear zone around a colony indicated carboxymethylcellulase production[13].

Effect of NaCl, pH and temperature on production of extracellular hydrolases

The production of salt-tolerant extracellular α-amylase and lipase was determined by incubating cultures on TSB agar supplemented with 0.5% soluble starch and 1% Tween 80, and production of gelatinase was detected by 1.5% gelatin instead of agar, and 0-20% NaCl was added to the medium. The effect of pH on hydrolase production was monitored in buffered TSB agar with variation of pH from 6.0 to 10.0. The NaCl concentration was maintained at 10% (w/v). The effect of temperature on hydrolase production was assayed by incubating hydrolase-producing strains on TSB agar with their optimal NaCl concentration and pH, and temperature of 10-60°C. Cultures were inoculated and their hydrolase activity was assayed as described above.

Effect of diverse ester on production of lipase

The production of salt-tolerant lipase was determined by incubating cultures on TSB agar supplemented with 1% Tween 20, Tween 40, Tween 60 and Tween 80. The lipolytic activity of the isolates was detected by screening for halos around colonies after 5 days incubation at 37°C on TSB plates.

Effect of cation and anion on activity of extracellular α-amylase of HVA-1(*Gracilibacillus* sp. H3)

In order to investigate the effect of different cations and anion on the activity of α-amylase, the activity of extracellular α-amylase of HVA-1 (*Gracilibacillus* sp. H3) was determined by mixing 500µl crude α-amylase liquid extracted from supernatant of fermentation liquid of HVA-1 (*Gracilibacillus* sp. H3) with 500µl 5% soluble starch, 1 mol/L Na_2MoO_4, Na_2SO_4, CH_3COONa, $NaNO_3$, $C_6H_5O_7Na_3$, NaCl and $MnCl_2$, $MgCl_2$, KCl, NaCl, LiCl had been added into the mixture, incubating at 37°C for 25min. Then mixed 750µl reaction liquid with 1ml Dinitrosalicylic acid reagent (DNS), the mixtures were heat for 8 min in boiling water bath, and then cooled to ambient temperature. The color intensities were measured by spectrophotometer at 575nm.

Nucleotide sequence accession numbers

The sequences reported in this study have been deposited in the GeneBank database (Accession nos. HQ620621-HQ620717)

RESULTS

Phylogenetic analysis of halophilic/halotolerant bacteria from Shache. A total of 97 eubacterial isolates were obtained from the saline-alkali soil samples. The strains were characterized phylogenetically by sequencing part of the 16S rRNA gene of all isolates. Fig. 1 shows the phylogenetic relationship of representative sequences. BLAST analysis of the partial sequences indicated that the isolates were affiliated with 19 genera of three orders (Table 2). Bacillales, the major group in this study, contained 71 isolates of 10 genera. Genus *Bacillus* was the most abundant, representing 17.5% of the isolates. The second was Halobacillus genus, representing 14.4% of the isolates. By contrast, the collection contained only one member from the genus *Salinicoccus* (HBCM-16).

The group of the Actinomycetales was represented by the genera Dietzia, Kocuria,

Table 1. Components of different media used in this study

Medium	Ingredients/L					
PT	casein peptone 7.5g	yeast extract 10g	NaCl 150g	phenol 690ml	Agar 18g	pH8.2-8.5
HVA	Humic acid 1g		NaCl 150g		Agar 18g	pH8.2-8.5
HBCM	casein peptone 7.5g	yeast extract 10g	NaCl 150g		Agar 18g	pH8.2-8.5
HNA	casein peptone 7.5g	yeast extract 10g	$NaMoSO_4$	150g	Agar 18g	pH8.2-8.5
YCSS	casein peptone 7.5g	yeast extract 10g	NaCl 250g		Agar 18g	pH8.2-8.5
TSB	casein peptone 15g	Soy peptone 5g	NaCl 100g		Agar 18g	pH 8.0-8.2

Micrococcus and *Nesterenkonia*. *Kocuria* contained 17.5% of the isolates. All of the isolates of the Actinomycetales were yellow or red pigmented and easy to identify on the plate. Finally, the group of the Oceanospirillales was represented by two genera, *Chromohalobacter* and *Halomonas*. Six suspected new species were discovered by their 16S rRNA gene sequence analysis (Table 3).

Selectivity of enrichment media

The distribution of isolates on the different enrichment media is HNA (34 isolates), HBCM (16 isolates), HVA (14 isolates), PT (28 isolates), and YCSS (5 isolates). According to the medium, 78.6% *Kocuria* and 100% *Planococcus* were isolated from HNA medium, 76.5% *Bacillus* were isolated from PT medium, and 50% *Halobacillus* were isolated from HVA medium. Only five isolates were found from YCSS medium, which contained 25% NaCl, and all of the isolates were affiliated with Bacillales (Table 2).

Screening for hydrolases activity

All of the 97 colonies were screened for amylase, lipase and gelatinase; 25 strains were lipase producers; 27 strains were gelatinase producers; and 31 isolates were amylase producers (Table 4).

Strains of *Halobacillus* were the most efficient salt-tolerant hydrolase producers. Among them, six strains had lipase activity, eight had gelatinase activity, and nine produced amylase. Strains affiliated to *Bacillus* were the second group to produce salt-tolerant hydrolase, and 18 isolates produced salt-tolerant hydrolase. Several

Table 2. Genera of halophilic/halotolerant strains isolated in five enrichment media

Genus	Species	HNA	PT	HBCM	HVA	YCSS
Bacillus	17	3	13	-	-	1
Chromohalobacter	1	-	1	-	-	-
Dietzia	1	1	-	-	-	-
Gracilibacillus	4	-	3	-	1	-
Halobacillus	14	1	-	5	7	1
Halomonas	4	1	-	1	2	-
Kocuria	14	11	2	1	-	-
Marinococcus	7	1	-	4	1	1
Micrococcus	2	2	-	-	-	-
Nesterenkonia	4	2	-	2	-	-
Oceanobacillus	6	2	2	-	2	-
Ornithinibacillus	4	-	4	-	-	-
Planomicrobium	4	4	-	-	-	-
Planococcus	2	2	-	-	-	-
Salimicrobium	2	-	-	-	-	2
Salinicoccus	1	-	-	1	-	-
Staphylococcus	2	-	-	1	1	-
Thalassobacillus	4	4	-	-	-	-
Virgibacillus	4	-	3	1	-	-

Table 3. Six suspected new species by blast 16s rRNA gene sequence in Genebank database

Strain (Accession No.)	Hit	Similarity
HNA-14 (HQ620634)	*Bacillus clausii* KSM-K16(AP006627)	96.348
HVA-10 (HQ620680)	*Halobacillus campisalis* ASL-17T(EF486356)	98.377
PT-5 (HQ620689)	*Ornithinibacillus bavariensis* WSBC 24001T(Y13066)	96.947
PT-11 (HQ620695)	*Oceanobacillus profundus* CL-MP28T(DQ386635)	97.267
PT-20(HQ620704)	*Ornithinibacillus bavariensis* WSBC 24001T(Y13066)	97.470
PT-28(HQ620712)	*Virgibacillus byunsanensis* ISL-24T(FJ357159)	97.935

combined activities were shown by some of these isolates. Combined hydrolytic activities of three substances were detected in five strains: PT-5 (*Ornithinibacillus* sp.), HVA-10 (*Halobacillus* sp.), HVA-3 (*Halobacillus* sp.), PT-9 (*Bacillus* sp.) and HNA-16 (*Bacillus* sp.). Besides, 16 strains were able to produce two extracellular enzymes.

Effect of NaCl, pH and temperature on hydrolases production

All the amylase producers had amylase activity at 5-10% NaCl, pH 7.0 and 30°C. They were divided into two groups: NaCl-dependent and NaCl-independent. Twenty-two isolates produced amylase activity in the absence of NaCl, but nine isolates could not produce it without NaCl. Each amylase-producing strain could tolerate changing NaCl concentration and pH within certain ranges. Nine isolates produced amylase activity between 0 and 15% NaCl, five isolates produced activity between 0 and 25% NaCl, and four isolates had

Table 4. Hydrolytic activity of Halophilic/Halotolerant isolates from saline soils in Shache

Genus	Strains	Tween 80 hydrolysis	Gelatin hydrolysis	Starch hydrolysis
Bacillus	17	3	9	6
Chromohalobacter	1	-	-	-
Dietzia	1	1	-	-
Gracilibacillus	4	2	1	4
Halobacillus	14	6	8	9
Halomonas	4	1	-	-
Kocuria	14	3	-	4
Marinococcus	7	1	1	-
Micrococcus	2	-	-	-
Nesterenkonia	4	-	-	-
Oceanobacillus	6	-	4	1
Ornithinibacillus	4	2	2	2
Planomicrobium	6	-	-	-
Salimicrobium	2	1	-	-
Salinicoccus	1	-	-	-
Staphylococcus	2	1	1	-
Thalassobacillus	4	4	-	4
Virgibacillus	4	1	1	1
Total numbers	97	26	27	31

Table 5. Hydrolytic Enzymes that are active in Polyextremic Environments

Enzyme	Producing Strain	30% NaCl	pH10	40°C	50°C	60°C
Amylase	*Gracilibacillus* sp. HVA-1	+	+	+	-	-
Amylase	*Halobacillus* sp. HBCM-8	+	+	+	-	-
Amylase	*Halobacillus* sp. HVA-10	+	+	+	+	-
Amylase	*Halobacillus* sp. HVA-12	+	+	+	-	-
Lipase	*Dietzia* sp. HNA-3	+	+	+	-	-
Lipase	*Thalassobacillus* sp. HNA-9	+	+	+	+	-
Lipase	*Thalassobacillus* sp. HNA-27	+	+	+	+	-
Lipase	*Thalassobacillus* sp. HNA-21	+	+	+	+	-
Lipase	*Halobacillus* sp. HVA-6	+	+	+	-	-
Lipase	*Ornithinibacillus* sp. PT-11	+	+	+	-	-
Lipase	*Salimicrobium* sp. YCSS-3	+	+	+	-	-
Gelatinase	*Virgibacillus* sp. HBCM-15	-	+	+	+	+

Table 6. Effect of different Ester on Production of Lipase

Strain	Gram reaction	Tween 20	Tween 40	Tween 60	Tween 80
HNA-5	+	+	+++	++	+++
HNA-9	+	++	+	+	++
HNA-13	+	-	+	+	+
HNA-16	+	++	-	++++	+
HNA-20	+	-	++	+	-
HNA-21	+	-	+	++	+++
HNA-27	+	++	+	++	+++
HBCM-4	+	++	+	++	+
HBCM-8	+	-	++	+	-
HBCM-13	+	+	+	+	+
HBCM-14	+	+	-	++	-
HBCM-16	+	++	-	-	+
HVA-5	+	+	+++	+	-
HVA-6	+	-	+	+	-
HVA-7	+	+	+++	+	-
HVA-8	+	-	-	+	+
HVA-10	+	+	+	-	-
PT-4	+	-	+	++	-
PT-5	+	-	+	+	-
PT-9	+	++	+++	+++	+
PT-11	+	-	+++	++	+
PT-20	+	++++	-	+	+
PT-22	+	++++	+	+	-
PT-26	+	++++	+	-	-
PT-28	+	+	-	+	-

amylase activity at 30% NaCl. Eighteen isolates showed amylase activity at pH 7.0-10.0, four at pH 6.0, but only one, HVA-5 (*Halobacillus*), at pH 6.0-10.0.

In all of the lipase producers, hydrolysis of Tween 80 depended on the presence of NaCl. Six isolates had lipase activity at 5-25% NaCl, and seven at 30% NaCl. Twelve isolates showed lipase activity only at pH 8.0.

Gelatinase activity was seen below 20% NaCl, and most strains produced gelatinase between 0 and 10% NaCl. None of the gelatinase producers hydrolyzed gelatin without NaCl. Fifteen isolates produced gelatinase activity at pH 7.0-10.0, and three at pH 6.0. HBCM-15 (*Virgibacillus* sp.) showed gelatinase activity at 60°C.

Effect of different esters on production of lipase producing

The strains hydrolyzing two substrates were shown in table 6. Tween 60 and Tween 40 were more preferred substrates(88% of strains were screened for hydrolyzing tween 60 and 76% of stains were screened for hydrolyzing tween 40), while 64% of strains screened for hydrolyzing tween 20, 52% of strains were screened for hydrolyzing tween 80. For the 25 strains, only 24% of the strains hydrolyzed all four substrates, 32% of the strains hydrolyzed three substrates and the remaining strains hydrolyzed two substrates. Among the 25 strains, only three strains, HNA-5, HNA-27 and PT-9, hydrolyzing four substrates, had high relative activities.

Active strains which can present enzyme activity at extreme environment

Active strains which can present enzyme activity at extreme environment were shown in the Table 7. Four strains, HVA-1 (*Gracilibacillus*), HBCM-8 (*Halobacillus*), HVA-10(*Halobacillus*) and HVA-12(*Halobacillus*), showed amylase activity, especially HVA-10 which could present the amylase activity at 50°C. The other four strains presented lipase activity at extreme environment. And among them, two strains, HNA-5(*Thalassobacillus*) and HNA-9

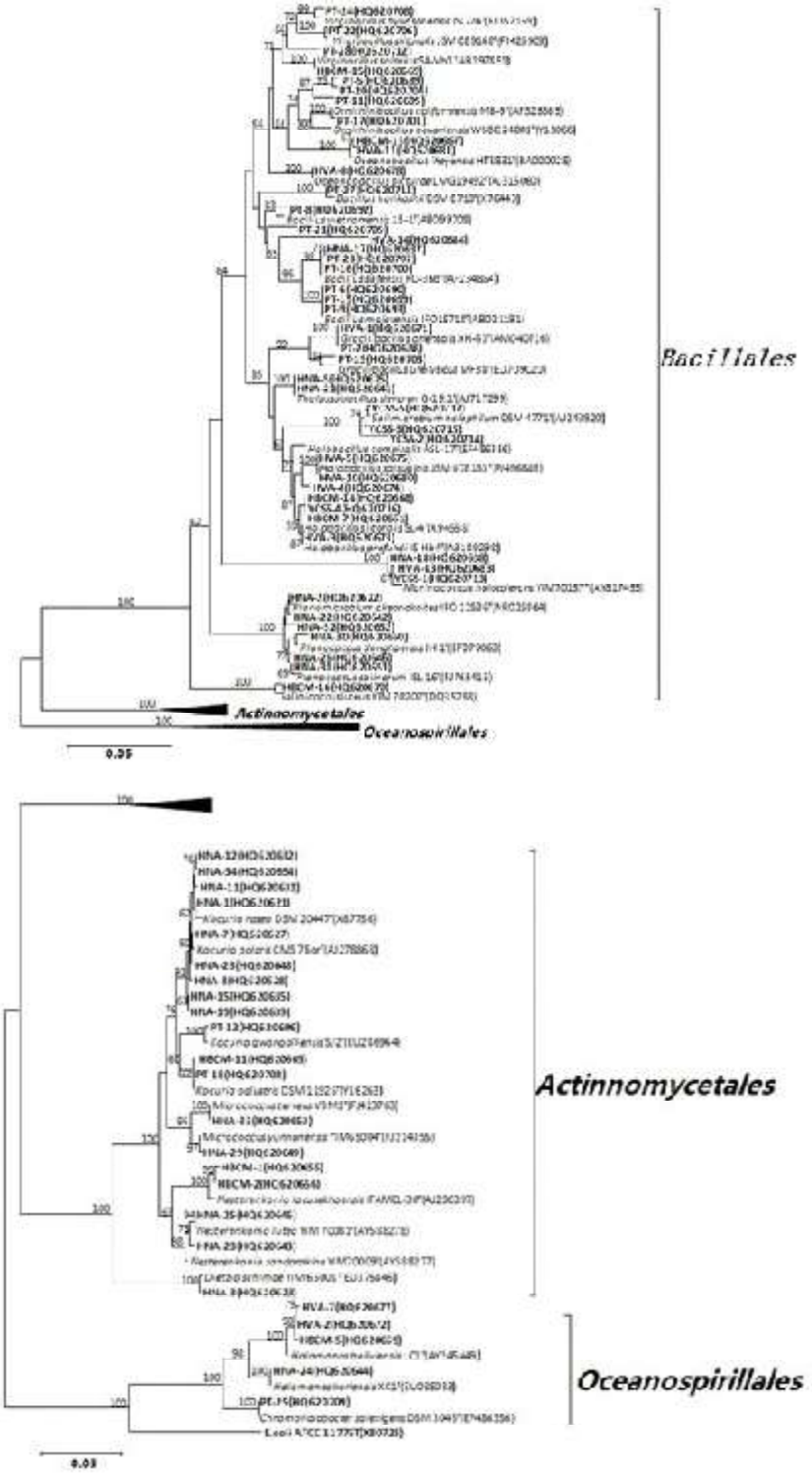

Fig. 1. Neighbor-joining phylogenetic tree of the partial isolates from saline soils of Shache. The nearest neighbors were revealed through BLAST search of non-redundant nucleotide sequences in the public databases as well as the nearest type strains (indicated with T). Bootstrap values (1000 replications) >50% are shown

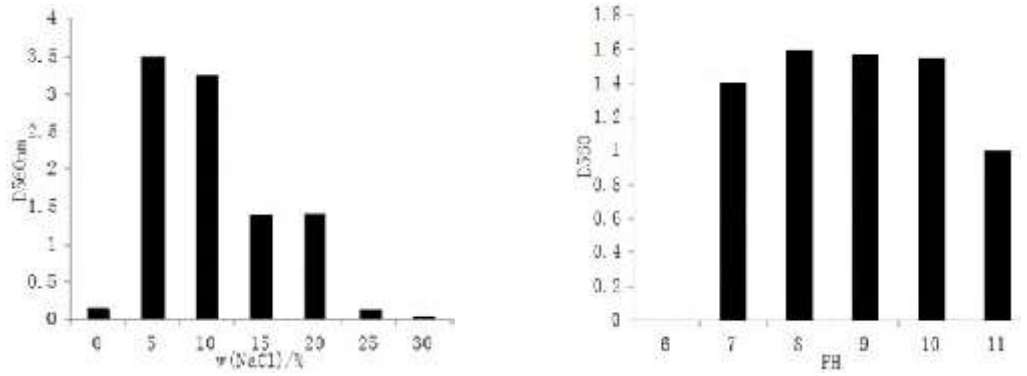

Fig. 2. Effects of NaCl concentration and pH on growth of *Gracilibacillus* sp H3

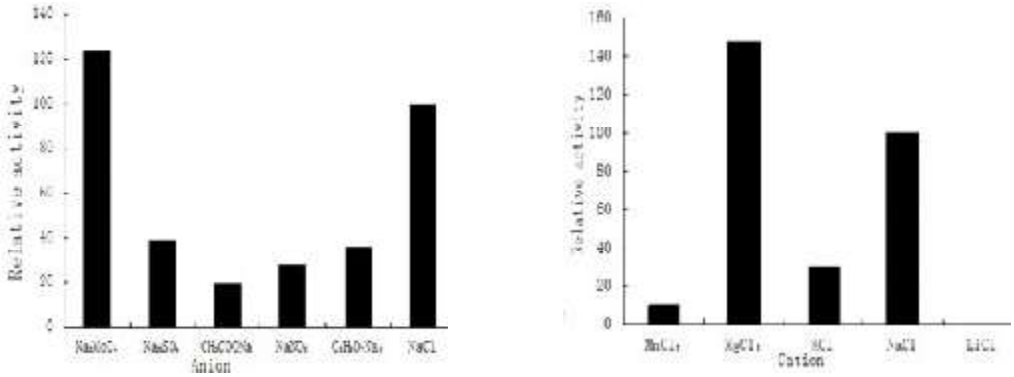

Fig. 3. Effect of cation and anion on the activity of extracellular α-amylase from *Gracilibacillus* sp. H3

(*Thalassobacillus*), showed the activity at 50°C.

Optimal NaCl concentration and pH on growth of HVA-1 (*Gracilibacillus* sp. H3) and the effect of cation and anion on the activity of extracellular α-amylase from HVA-1 HVA-1 (*Gracilibacillus* sp. H3)

HVA-1 (*Gracilibacillus* sp. H3) was a producer of extracellular μ-amylase. Further tests for its growth under different NaCl concentration and pH value display it was NaCl-dependent strain. Growth of HVA-1 (*Gracilibacillus* sp. H3) have be found under 5-30% NaCl and pH7-11, the optimal NaCl concentration was 5 %-10%, and the optimal pH value for growth was pH8-9 respectively(Fig.2), It was determined a moderately halophilic strain.

The effect of different ions on the activity of extracellular α-amylase from HVA-1 (*Gracilibacillus* sp. H_3) is shown in Fig.3. The anions SO_4^{2-}, CH_3COO^-, NO_3^- and $C_8H_5O_7^{3-}$ showed some inhibition to the activity of extracellular α-amylase, while the maximum activity was enhanced by $Mo0_4$. Among metal ions, the activity was enhanced by Mg^{2+}. While, K^+ and Mn^{2+} inhibited the activity of extracellular α-amylase and the maximum inhibition was caused by Li+ (100%).

DISCUSSION

Hypersaline environments such as solar salterns and salt lakes are inhabited by a broad spectrum of halophilic and halotolerant bacteria, including Gram-negative species (e.g. Halomonas, Chromohalobacter and Salinivibrio) and Gram-positive species (e.g. *Bacillus*, *Halobacillus*, *Marinococcus* and *Salinicoccus*)[6, 14]. Depending on the NaCl concentration required for optimal growth, they are classified into halotolerant organisms (<0.2 M NaCl), slight halophiles (0.2-0.5 M NaCl), moderate halophiles (0.5-2.5 M NaCl), and extreme halophiles (2.5-5.2 M NaCl)[14].

The results of the present study demonstrated a larger population of halophilic and halotolerant bacteria in saline-alkali soil in Xinjiang Province. The 97 isolates represent 18 different

genera: Bacillus, Chromohalobacter, Dietzia, *Gracilibacillus, Halobacillus, Halomonas, Kocuria, Marinococcus, Micrococcus, Nesterenkonia, Oceanobacillus, Ornithinibacillus, Planomicrobium, Salimicrobium, Salinicoccus, Staphylococcus, Thalassobacillus* and *Virgibacillus*. Most of the isolates were Gram-positive bacteria, and only three genera were Gram-negative. Among the isolates, *Bacillus* and *Halobacillus* were predominant, as reported previously[15, 16].

There were various nutritional requirements of these halotolerant/halophilic bacteria. Most strains that were affiliated to *Kocuria, Micrococcus, Planomicrobium, Planococcus* and *Thalassobacillus* preferred Na_2MoO_4, and not NaCl, and strains affiliated to *Halobacillus, Marinococcus* and *Salinicoccus* were grown mostly on media containing NaCl. Phenol-tolerant strains were isolated from saline soil, most of which were affiliated to the genera *Gracilibacillus, Ornithinibacilllus* and *Virgibacillus,* which were selected by PT medium containing 0.06% phenol and 10% NaCl (Table 3).

Only a few studies have reported that halophilic and halotolerant bacteria possess a wide spectrum of hydrolytic enzymes including α-amylases[17], proteases and lipase[6]. Some of these hydrolases have increased activity in the presence of NaCl and increased stability within a broad pH and temperature range[18]. In the study of Sanchez-Porro et al, only 23% of the 892 strains produced extracellular lipolytic activity when screened with Tween 80[6]. Among our study, most of the strains were negative for the production of hydrolytic enzymes, and only 25.7% of isolates were lipase producers, which is similar to the result of the Sanchez-Porro study. Strains affiliated to *Bacillus* and *Halobacillus* were efficient producers of salt-tolerant hydrolases, with 18 and 23 isolates, respectively, producing extracellular salt-tolerant hydrolases (Table 4). Five isolates were detected with combined hydrolytic activities.

Several studies have shown that enzymes derived from halophiles and halotolerant eubacteria are not only halostable, but also may be thermostable and alkali-stable[19]. According to the present study, 11 isolates showed hydrolase activity at 30% NaCl, four strains were amylase producers (HVA-1, HBCM-8, HVA-10 and HVA-12), and all of them were affiliated with *Halobacillus* except for HVA-1 (*Gracilibacillus* sp.). The other eight strains were lipase producers: three were affiliated to the genus *Thalassobacillus* (HNA-5, HNA-9, HNA-21 and HNA-27), and the others to Dietzia (HNA-3), *Halobacillus* (HVA-6), *Ornithinibacillus* (PT-5) and *Salimicrobium* (YCSS-3). HBCM-15 (*Virgibacillus* sp.) showed gelatinase activity at 60°C (Table 5). Two lipase and six amylase producers had hydrolase activities under extreme conditions of 25% NaCl, pH10.0 and 50°C (data not shown). It has been proven that many halophilic enzymes are polyextremophilic.

Further study showed that HNA-5 (*Thalassobacillus*) present high activity (Table 6) at extreme enviorment with 30% NaCl and 50°C(Table 7). For the above results, HNA-5 provide the possibility to use it for industrial application, especially for hydrolysis and esterification in lipid modification under extreme situation. Simultaneously, the deep further research of extracellular α-amylase of the HVA-1 (*Gracilibacillus* sp H3) is very meaningful to the promising industrial future because HVA-1 showed high activity at extreme environment.

Although halophilic and halotolerent bacteria produce extracellular salt-tolerant enzymes with great potential for use in industrial processes, only a few studies have reported them[14, 20]. The present study revealed a wide diversity of moderately halophilic and halotolerant bacteria in Shache, China, and their potential to produce three extracellular salt-tolerant hydrolases. Hydrolytic enzymes from these isolates could tolerant a wild range of NaCl concentrations, and some salt-tolerant hydrolases showed alkaliphilic and thermotolerant characteristics (pH10.0 and 50°C) at the same time. These results suggested potential resources of salt-tolerant hydrolases for different industrial processes.

REFERENCES

1. Ghozlan H., Deif H., Kandil RA., Sabry S., Biodiversity of moderately halophilic bacteria in hypersaline habitats in Egypt, *J. Gen. Appl. Microbiol.,* 2006; **52**: 63-72.
2. Sabet S., Diallo L., Hays L., Jung W., Dillon JG., Characterization of halophiles isolated from solar salterns in Baja California, Mexico, *Extremophiles*., 2009; **13**: 643-656.

3. Baati H., Amdouni R., Gharsallah N., Sghir A., Ammar E., Isolation and Characterization of Moderately Halophilic Bacteria from Tunisian Solar Saltern, *Curr. Microbiol.,* 2010; **60**: 157-161.
4. Oren A., Industrial and environmental applications of halophilic microorganisms, *Environ. Technol.*, 2010; **31**: 825-834.
5. Rohban R., Amoozegar MA., Ventosa A., Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran, Ind. *Microbiol. Biotechnol.*, 2009; **36**: 333-340.
6. Sanchez-Porro C., Martin S., Mellado E., Ventosa A., Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes, *J. Appl. Microbiol.,* 2003; **94**: 295-300.
7. Demirjian DC., Morís-Varas F., Cassidy CS., Enzymes from extremophiles, *Curr. Opin. Chem. Biol.*, 2001; **5**: 144-151.
8. Tang SK., Wang Y., Cai M., Luo K., Mao PH., Jin X., Jiang CL., Xu LH., Li WJ., *Microbulbifer halophilus* sp. nov., a moderately halophilic bacterium from north-west China, *Int. J. Syst. Evol. Microbiol.,* 2008; **58**: 2036-2040.
9. Wang Y., Tang SK., Luo K., Mao PH., Jin X., Jiang CL., Xu LH., Li WJ., Halomonas lutea sp. nov., a moderately halophilic bacterium isolated from a salt lake, *Int. J. Syst. Evol. Microbiol.*, 2008; **58**: 2065-2069.
10. Cui XL., Mao PH., Zeng M., Li WJ., Zhang LP., Xu LH., Jiang CL., *Streptimonospora salina* gen. nov., sp. nov., a new member of the family Nocardiopsaceae, *Int. J. Syst. Evol. Microbiol.*, 2001; **51**: 357-363.
11. Thompson JD., Higgins DG., Gibson TJ., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice, *Nucleic. Acids. Res.,* 1994; **22**: 4673-4680.
12. Tamura K., Dudley J., Nei M., Kumar S., MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 2007; **24**: 1596-1599.
13. Teather RM., Wood PJ., Use of Congo red polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, *Appl. Environ. Microbiol.,* 1982; **43**: 777-780.
14. Ventosa A., Nieto JJ., Oren A., Biology of moderately halophilic aerobic bacteria, *Microbiol. Mol. Biol. Rev*., 1998; **62**: 504-544.
15. Tiquia SM., Davis D., Hadid H., Kasparian S., Ismail M., Ahly S., Shim J., Singh S., Murray KS., Halophilic and halotolerant bacteria from river waters and shallow groundwater along the rouge river of southeastern Michigan, *Environ. Technol*., 2007; **28**: 297-307.
16. Romano I., Nicolaus B., Lama L., Manca MC., Gambacorta A., Characterization of a haloalkalophilic strictly aerobic bacterium, isolated from Pantelleria island, Syst. *Appl. Microbiol.*, 1996; **19**: 326-333.
17. Coronado MJ., Vargas C., Hofemeister J., Ventosa A., Nieto J., Production and biochemical characterization of an α-amylase from the moderate halophile Halomonas meridiana, *FEMS. Microbiol. Lett*., 2000; **183**: 67-71.
18. Wejse P., Ingvorsen K., Mortensen K., Purification and characterisation of two extremely halotolerant xylanases from a novel halophilic bacterium, *Extremophiles*., 2003; **7**: 423-431.
19. Setati ME., Diversity and industrial potential of hydrolaseproducing halophilic/halotolerant eubacteria, *Afr. J. Biotechnol.*, 2010; **9**: 555-1560.
20. Ventosa A., Nieto JJ., Biotechnological applications and potentialities of halophilic microorganisms, *World. J. Microbiol. Biotechnol.,* 1995; **11**: 85-94.