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*Original Research*

# Isolation and identification of culturable halophilic bacteria with producing hydrolytic enzyme from Incheh Broun hypersaline wetland in Iran

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**Abstract:** Incheh Broun hypersaline wetland is located near the border of Turkmenistan in thenorth of Iran. This wetland is notable because of salinity (280g/l) and alteration in pH range (2.8 to 6.8). Eastern part of wetland is affected by wastewater of iodine extraction factory**.** Samples were taken from soil, water and salt. Totally, 400 bacterial strains were isolated of which 194 strains were Gram-positive bacilli, 184 strains were Gram-negative rod and 22 strains were Gram-positive cocci. According to phylogenetic analysis of 16S rRNA, selected strains were placed in three taxonomic phyla including *Firmicutes*, *Actinobacteria* and *Gammaproteobacteria*. Optimum growth was evaluated for salt and 22 strains were found to be moderate halophile and 33 strains were halotolerant. Production of lipase, amylase, gelatinase and protease was examined. Gram-positive bacilli were the main producers of hydrolytic enzymes. Gelatinase and protease were the most frequent enzymes. Gram–positive cocci were the main producers of lipase but they didn't produce amylase.

***Key words:*** Halophilic, halotolerant, hydrolytic enzymes, Incheh Broun wetland.

## Introduction

Attention to the diversity of microbial habitats, the functioning of complex microbial communities and the physiological features of community members is growing steadily. The biodiversity can be exploited for future sustainable biotechnological processes. Besides novel enzymes for specific purposes, novel microorganisms can also be exploited for a novel or improved biotechnological purposes. Operational techniques may help to screen microorganisms with interesting novel properties and to isolate and culture interesting novel strains. This work will lead to new views on ecosystems and biological function together with the biotechnology enabled by this science (1-5).

In the last few years, attention to hypersaline environments has been increased. In order to adapt to high saline conditions, halophilic microorganisms (grow in media containing between 0.5 M and 2.5 M NaCl) have developed various biochemical strategies to maintain cell structure and function such assynthesis compatible solute, bacteriorhodopsins, exopolysaccharides, hydrolases and biosurfactants (6). These compounds were clearly of industrial interest (7). Some Compounds, which are found in some halophiles are also made by many other microorganisms. However, sometimes there is a clear advantage to using halophiles for their production such as β-carotene production from *Dunaliella* (8). Halophilic enzymes have shown substantially different properties, including their requirements for the high salt concentrations in the range of 1–4 M for activity and stability. These enzymes have numerous applications in the industrial production of different products including detergents, foods, pharmaceuticals, leathers, diagnostic reagents and silver recovery (2, 9-12). In recent years, different screening programs have been performed in saline habitats in order to isolate and characterize novel enzymatic activities with different properties (13) such as extracellular lipase of *Salinivibrio* sp. which is active at 50 °C and nuclease H of *Micrococcus varians*sp. *halophilu*s which degrades RNA at 60 °C and 12 % salt (8).

The purpose of this study is the isolation and identification of culturable moderate halophilic and halotolerant bacteria and their extracellular enzymatic activity in InchehBroun hypersaline wetland located in the north of Iran near the Turkmenistan border. This wetland is approximately 100 acres and has a Mediterranean climate and alsothis wetland is remarkable because of salinity (280g/l) and variation of pH range (2.8 to 6.8).

## Materials and Methods

### Sample collection

Sediment and Water samples were collected from different depths in 4 different sites of Incheh Broun wetland in September 2014 (Figure 1). Water pH, salinity

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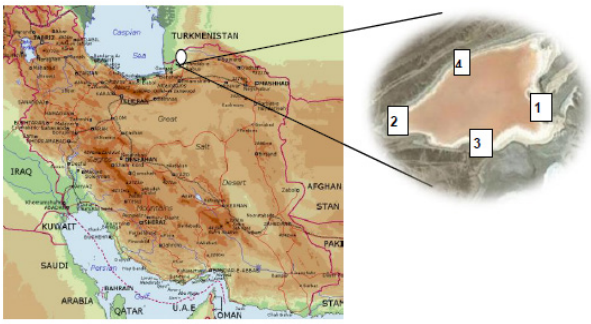


**Figure 1.** Location of InchehBoroun hypersaline wetland in Iranwhich was obtained from Google earth and sites of sampling.

and temperature were measured. Water was analyzed chemically in Behin Khakazma laboratory for measuring major elements and providing suitable medium.

**Isolation and purification**

Complex media used for isolation ofmoderately halophilic and halotolerant microorganisms contained 12% and 3% salt respectively, and we made each medium with two pH, 7 and 5.5. The modified HM medium used for isolation of moderate halophiles had the following composition; % (w/v): NaCl, 10.4; $MgSO_4 \cdot H_2O$, 0.83; $CaCl_2 \cdot 2H_2O$, 0.041; KCl, 0.16; $NaHCO_3$, 0.008; NaBr, 0.023; Bacto-peptone, 0.8; Yeast extract, 0.5; Glucose, 0.1; Agar, 1.5. SNA medium (Seawater Nutrient Agar) used for isolation of halotolerant bacteria contained % (w/v): NaCl, 2; $MgSO_4 \cdot H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.05; KCl, 0.05; Bacto-peptone, 0.5; Yeast extract, 0.1; Meat Extract, 0.2; Agar, 1.5. Samples were diluted in 3 % (w/v) sterile salt solution and were spread onto a culture medium mentioned above. Different colonies were picked after four weeks of incubation at 33˚C and were purified with several subcultures (14). Gram staining (Burke method) was performed and the result was confirmed by the KOH test. Motility was analyzed by the wet-mount method (15). Catalase, oxidase and nitrate reduction were checked as recommended by Smibert (16). The presence of endospore was investigated by Schaeffer–Fulton staining method (14). Colony morphologies were described by using standard microbiological criteria with special emphasis on pigmentation, diameter, colonial elevation, consistency and opacity. Optimal concentration of NaCl for growth was determined by inoculating the isolates into medium containing 0.5 % yeast extract with different concentrations of NaCl (0, 0.5, 3, 5, 7.5, 10, 12, 15 and 20 % (w/v) at pH 7.5. Absorbance was read at 600 nm, 24 h after inoculation. Growth at various temperatures (10, 15, 20, 25, 30,35,40,45 and 50 ºC) was determined. The pH range for growth was reduced and the final pH was adjusted between 4 and 10 (at intervals of 1 pH units) on liquid medium, which was used for isolation. For pH below 6, sodium acetate /acetic acid (17), for pH 6–9 HEPES and for pH above 9, $Na_2CO_3$/$NaHCO_3$ was used to adjust the pH of the medium.

**DNA extraction and PCR**

A total of 55 strains were selected randomly for 16S rRNA analysis. DNA extracted from pure cultures or cell pellets with the modified method and confirmed by electrophoresis agarose gel (18-20). The 16S rRNA gene was amplified by using universal primers, 16F27 and 16R1492 (21). Polymerase chain reaction (PCR) contained: PCR buffer (10X): 5 µl, $MgCl_2$ (5 mM):1.2 µl, Taq polymerase: 0.18 µl, each of the primers (1492R &27F) were used 1.2 µl, dNTP mix (10 mM): 1 µl and approximately: 4 µl (10–15 ng) template DNA in a 50 µl reaction volume. PCR conditions were as follows: denatured for 5 minutes at 95 ºC and subjected to 30 cycles of 60 seconds at 95 ºC, 45-60 seconds at 54-62 ºC and 45-60 seconds at 72 ºC. This was followed by a final elongation step for 10 minutes at 72 ºC. Temperature, annealing time and extension were separately optimized for each. PCR products were analyzed on 1% (w/v) agarose gels and sent for sequencing.

**Phylogenetic analysis**

Sequence data were analyzed with Chromas software and compared with registered sequences in GenBank database. Phylogenetic analysis was performed using ClustalX software (second edition) and phylogenetic trees were constructed using three different methods: maximum-likelihood (22, 23)maximum parsimony and neighbor-joining (24) algorithms integrated into the MEGA (fifth edition) software for phylogenetic inference.

**Hydrolytic enzyme production**

The presence of amylolytic activity on plates was determined qualitatively by following the method described by Amoozegar et al. (2003) (25). Starch agar medium (Merck) contained 3 % and 12 % (w/v) total salt. The plates were flooded after incubation at 33 °C for 2 weeks, with 0.3 % I2-0.6 % KI solution. A clear zone around the growth indicated the hydrolysis of starch. Proteolytic activity was determined by method described by Amoozegar et al. (2008) (26),. Skim milk agar containing 10 % (w/v) skim milk, 2 % (w/v) agar, supplemented with 3 % and 12 % (w/v) total salt was used for determining the hydrolytic activity of halotolerant and moderate halophiles, respectively. Clear zones around the growth were taken as evidence of proteolytic activity after 2 weeks. Lipolytic activity was detected by growth on a medium, which contained 1% Tween-80 (27). Gelatin-hydrolysis test was determined by a method described by Smibert & Krieg (1994) (16), depending on strains requirement, NaCl was added to the enzymatic medium and inoculated medium was incubated for 2 weeks at 33 ºC temperature.

**Results**

**Physicochemical properties of the site of studies**

Based on water chemical analysis (Table 1), this wetland is in thalasso haline hypersaline group because of the high rate of $Na^+$ and $Cl^-$. High concentration of $Cl^-$, $Ca^{2+}$, $Mg^{2+}$ and $K^+$ is considerable in comparison to other regions in site 2. Eastern part of wetland is affected by acidic waste water of iodine extraction factory, which resulted in change of water color to yellow or orange. As shown in Table 2, site 4 is closer to normal conditions than other sites.

**Microbial biodiversity analysis**

Totally 400 isolates were purified and classified

**Table 1.** Physico-chemical characteristics of the sampling sites.

| Specifications | Water of site 1 | Water of site 2 | Water of site 3 | Water of site 4 |
|---|---|---|---|---|
| **Color of Samples** | **yellow** | **Colorless** | **orange** | **black** |
| **Ion concentration %** | | | | |
| **$CO_3^{2-}$** | 0 | 0 | 0 | 0 |
| **$HCO_3^-$** | **0.92** | 0.14 | 0.023 | 0.053 |
| **CL -** | 7.8 | **23.7** | 20.9 | 5.67 |
| **$SO_4^{2-}$** | 0.42 | **0.96** | 0.86 | 0.88 |
| **$Ca^{2+}$** | 0.69 | **1.9** | 0.99 | 0.83 |
| **$Mg^{2+}$** | 0.3 | **1.6** | 0.45 | 0.4 |
| **$Na^+$** | 3 | 5.8 | **10.4** | 5 |
| **$Fe^{2+}$** | 0.001 | 0.000088 | **0.02** | 0.017 |
| **$Mn^{2+}$** | 0.000087 | 0.000087 | 0.0004 | 0.0004 |
| **$Cu^{2+}$** | 0.000001 | 0.000002 | 0.000016 | 0.000006 |
| **$Zn^{2+}$** | 0.000007 | 0.0000004 | 0.00003 | 0.000002 |
| **$K^+$** | 0.005 | **0.046** | 0.005 | 0.0056 |

**Table 2.** GPS data of sampling.

| Site of sampling | GPS | Average salinity % | Average pH |
|---|---|---|---|
| 1 | N: 37. 13.438E:54.30.224 | 26.8 | 4.2 |
| 2 | N: 37. 13.932 E:54.30.081 | 28.7 | 5.2 |
| 3 | N: 37. 13.829E:54.30.307 | 28.4 | 5.2 |
| 4 | N: 37. 13.517E:54.30.657 | 23.3 | 6.45 |

**Table 3.** Number of isolation in different sites.

| | | Number of isolation | | |
|---|---|---|---|---|
| | | HM medium | SNA medium | Total |
| Soil & water | Site 1 | 39 | 25 | 64 |
| | Site 2 | 41 | 45 | 86 |
| | Site 3 | 61 | 28 | 89 |
| | Site 4 | 72 | 37 | 109 |
| Salt | Sediment | 37 | 15 | 52 |

**Table 4.** The abundance of isolates on the basis of Gram staining.

| | Total | Site 1 | Site 2 | Site 3 | Site 4 |
|---|---|---|---|---|---|
| Gram-positive coccus | 20 | 6 | 5 | 2 | 7 |
| Gram-positive bacilli | 172 | 35 | 50 | 37 | 50 |
| Gram-negative bacilli | 167 | 24 | 40 | 39 | 64 |

based on Gram stain and KOH test. As shown in Table 3 and 4, 250 strains were obtained in HM medium and 150 strains obtained in SNA medium. Among 55 isolates which were selectedrandomly for sequencing, the lowest number were belonged to the HM medium at pH 5.5.

## Phylogenetic studies of the bacterial population

Based on 16S rRNA analysis, 55 strains were placed in three taxonomic phyla: *Firmicutes*, *Actinobacteria* and *Proteobacteria* which belonged to 15 genera and 33 species. 15 genera included: *Bacillus,* Marinobacter,*Halomonas*, *Kocuria, Oceanobacillus*, *Dietzia ,Virgibacillus, Chromohalobacter, Rhodococcus*, *Micrococcus, Paenibacillus, Halobacillus, Thalassobacillus, Arthrobacter* and *Salinithrix* . 16S rRNA sequencing showed that a total of 9 strains had 100 % similarity, 26 strains showed 98.5 to 99.8 % similarity.13 strains had 97- 98.4 % similarity which was showed significant differences in the level of species. 7 strains had less than 97 % similarity in 16S rRNA sequences (Figure 2, 3 and 4).One of the most important results of this study was to represents a novel species in a new genus within the family *Thermo actinomycetaceae,* with the name of *Salinithrix halophile.* The Frequency and variety of genera are shown in Figure 5. Selected strains were analyzed for halophilic and halotolerant behaviors. From the 55 strains analyzed, about 40 % of the strains (22 strains) were moderately halophilic bacteria and 60% of the strains (33 strains) were halotolerant. The most frequetly appearing moderate halophilic bacteria belonged to the genus *Marinobacter* and *Halomonas* and the major halotolerant bacteria belonged to the genus *Bacillus, Dietzia, Oceanobacillus* and *Kocuria.* The results are presented in charts in Figure 6.

## Identification of hydrolytic enzyme producing strains in the halophilic population

Production of amylase, protease, lipase and gelati-

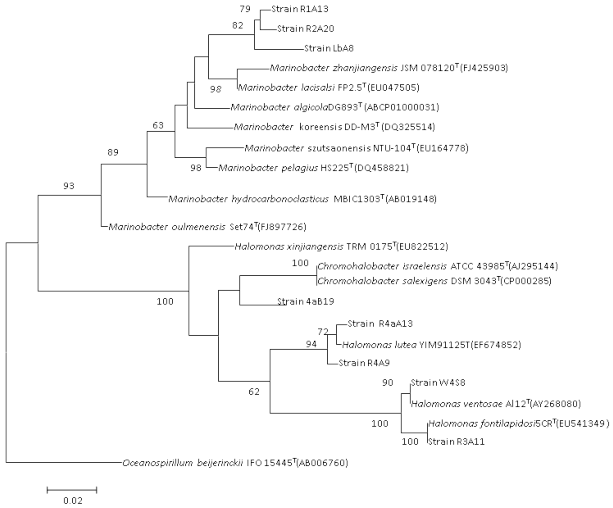


**Figure 2.** Maximum likelihood phylogenetic tree of 16S rRNA bacterial sequences for strains which related to phylum *Gammaprotobacteria*. The bar indicates a 2 % estimated sequence divergence.

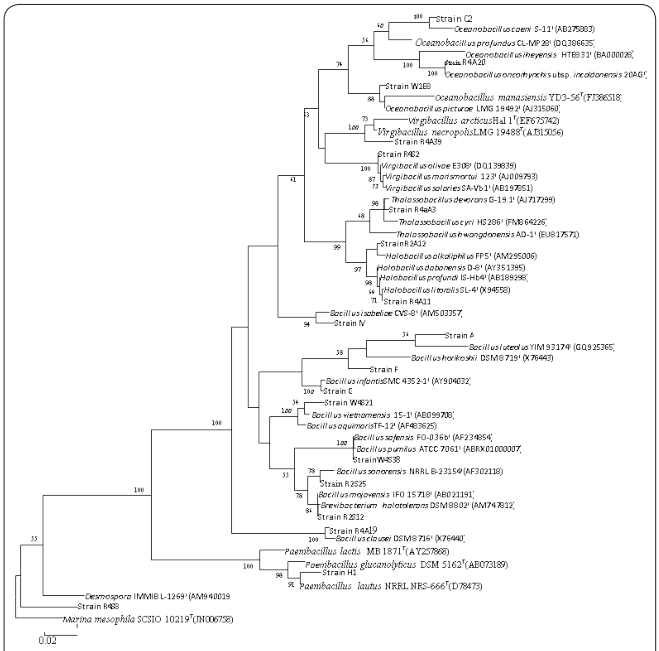

**Figure 3.** Maximum likelihood phylogenetic tree of 16S rRNA bacterial sequences for strains which related to class *Firmicutes*. The bar indicates a 2 % estimated sequence divergence.

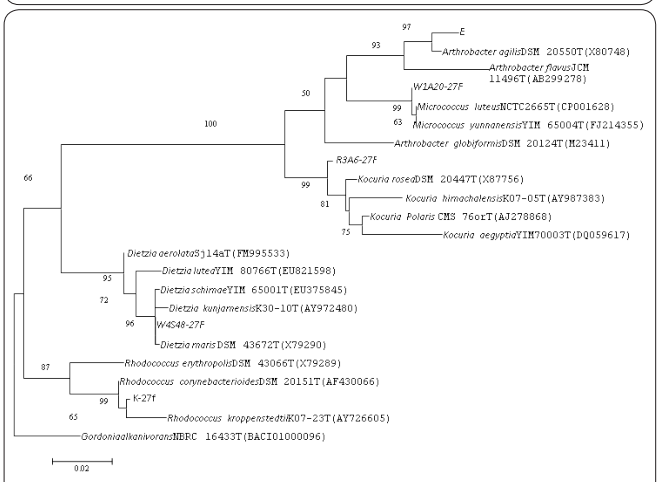

**Figure 4.** Maximum likelihood phylogenetic tree of 16S rRNA bacterial sequences for strains which related to class *Actinobacteria* The bar indicates a 2 % estimated sequence divergence.

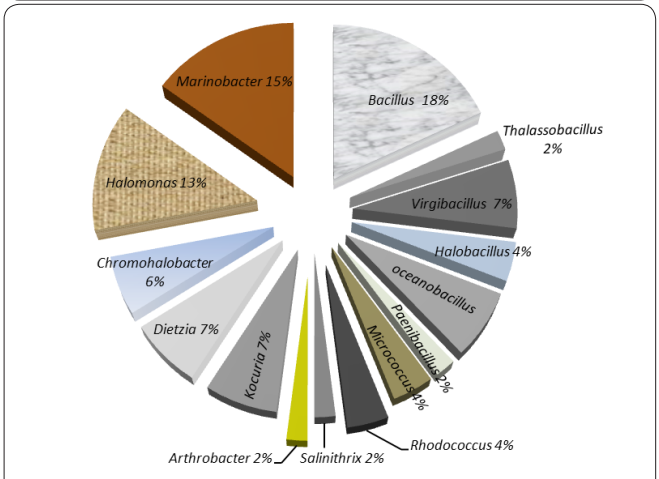


**Figure 5.** The abundance and diversity of genera which was isolated in each site.

nase were investigated in 55 strains. 25 strains showed remarkable ability in protease production. These belonged to genera *Bacillus*, *Virgibacillus*, *Oceanobacillus* and *Halobacillus* from Gram-positive bacilli, genera *Marinobacter* and *Halomonas* from Gram-negative bacilliand genera *Arthrobacter* and *Micrococcus* from Gram-positive coccus. Strains belonging to genera *Thalassobacillus*, *Bacillus*, *Marinobacter*, *Rhodococcus, Micrococcus, Dietzia* and *Kocuria* had a considerable role in lipase production. Among Gram-positive, the ability to produce amylase, protease, lipase and gelatinase was 22 %, 29 %, 10 % and 32 %, respectively. Gram positive cocci which were isolated such as*: Kocuria, Dietzia, Rhodococcus* and Micrococcus, we're not able to produce amylase. The ability to produce protease, lipase and gelatinase among Gram-positive cocci were 6%, 13% 20%, respectively. Among Gram-negative bacilli the ability to produce protease, lipase and gelatinase was 7%, 5% and 30 %, respectively. Total production of amylase, protease, lipase and gelatinase was 22, 43, 42 and 75 %, respectively. Comparison of enzyme production for different groups is presented in Figures 7 and 8.

## Discussion

Considerable point of this research is that culture

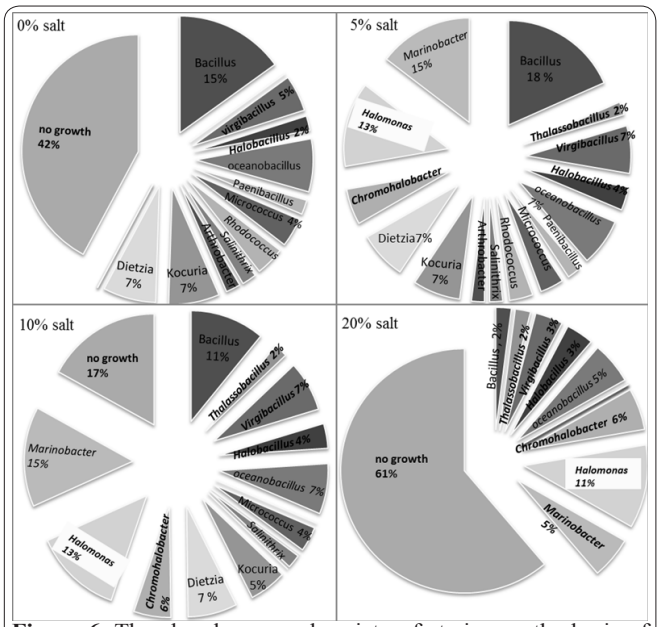


**Figure 6.** The abundance and variety of strains on the basis of response to salt concentrations.

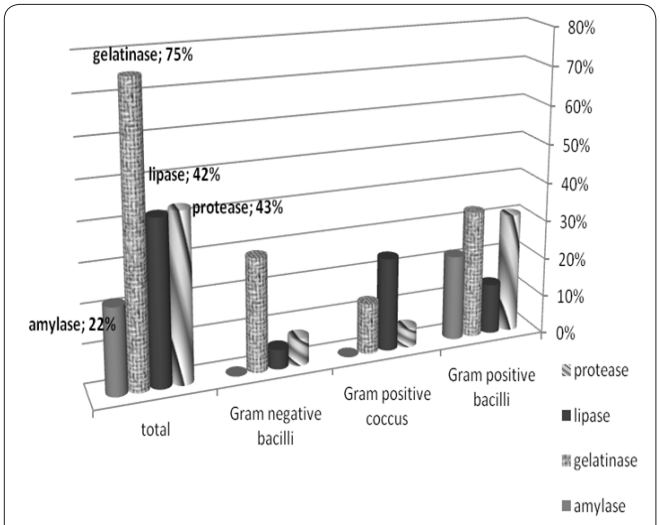


**Figure 7.** The percentage of hydrolytic enzymes by isolates (Total, Gram positive and negative bacilli and Gram-negative coccus).

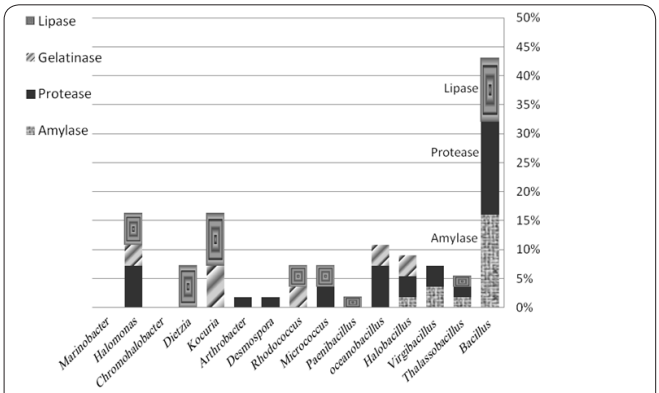


**Figure 8.** The percentage of hydrolytic enzymes by each of strains

method was used for isolating new strains. The results are only discussed about cultural groups. Comparing limited cultural groups with real microbial community and considering that diversity of unculturable microorganisms in this wetland has notbeen investigated yet, efficiency of methods employed inisolation was determined.

Frequency and variety of Gram-positive rods in this wetland, in comparison with studies on other hypersaline habitats were similar to the diversity of Great Salt Plainsin Oklahoma (14), hypersaline habitats in Egypt (28), and hypersaline playa in Iran.(22)

The most number of strains belonged to class *Firmicutes,* which were genetically close to thegenus*Bacillus*, *Oceanobacillus, Virgibacillus, Thalassobacillus*and*Halobacills*. The most frequent halotolerant genera in this research were *Oceanobacillus, Dietzia, Bacillus,* and *Kocuria*. In halophilic bacteria,*Marinobacter* and *Halomonas* were the most frequent genera. Gram-negative cocci were not isolated and Gram-positive cocci all belonged to the class *Actinobacteria.* Isolation of three genera *Dietzia, Salinithrix and Rhodococcus* of this hypersaline were considerable which was not mentioned in similar research. Frequency and variety of genera were shown in Figure 5 in different concentrations of NaCl. Biodiversity of each habitat was affected by climate and biological content. One of the important points was pH variation about this wetland. PH of this wetland was reported about 8 but an iodine extraction factory was built in 2006, which was drained acidic wastewater into the wetland. Just over 5 years, the pH of wetland was decreased at least by 3. Unexpectedly, acidophilic bacteria were not isolated from this wetland. On the one hand, two strains were isolated and were close to alkalophilic species *Halobacillus alkaliphiles* and *Bacillus horikoshii* and also optimum growth was observed for most isolates at pH 8.Salt extraction and returning water to the wetland was another stress for this ecosystem. These factors could be the reason for decreasing microbial diversity in this wetland. High microbial diversity was observed in site 4 of sampling, possibly because it is closer to the normal conditions. The main producers of hydrolytic enzymes were Gram-positive rods. These data are in agreement with other findings, which is consistent with the previously published data that showed Gram-positive bacteria as the dominant proteolytic isolates in the saline environments (9).The ability of 25 strains was remarkable in protease production. One of the most important hydrolytic enzymes produced by the strains was amylase. Main amylase producers were different species of the genus *Bacillus*. Gram-positive coccus and Gram-negative bacilli were identified in this study and did not produce amylase. Different strains ofgenera *Kocuria*, *Micrococcus*, *Halomonas, Oceanobacillus*and *Halobacillus* were gelatinase producers. Comparison of enzyme production was showed in different groups of isolates in Figures 6 and 7. This is the first study describing the culturable halophilic and halotolerant bacterial community of Incheh Broun hypersaline wetland in Iran. Our results confirmed that the presence of a considerable diversity of bacteria produced a variety of extracellular hydrolytic enzymes.

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