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ORIGINAL ARTICLE

Comparison of Bacterial Biodiversity and Enzyme Production in Three Hypersaline Lakes; Urmia, Howz-Soltan and Aran-Bidgol

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Abstract This research is a comparative study on the diversity of halophilic bacteria with hydrolytic activities in three significant hypersaline lakes; Urmia in the northwest and Howz-Soltan and Aran-Bidgol in the central desert in Iran. Isolated strains from these saline lakes were found to be halotolerant, moderately and extremely halophilic bacteria. The bacteria in each saline lake were able to produce different hydrolytic enzymes including amylase, protease, lipase, DNase, inulinase, xylanase, carboxy methyl cellulase, pectinase and pullulanase. 188, 302, 91 halophilic strains were isolated from Urmia Lake, Howz-Soltan and Aran-Bidgol playa, respectively. The numbers of Gram-positive strains were more than Gram-negatives, and among Gram-positive bacteria; spore-forming bacilli were most abundant. Due to the unique physico-chemical conditions of the lake environments, the hydrolytic activities of isolated strains were significantly different. For instance, isolated strains from Howz-Soltan playa did not produce pectinase, DNase, amylase, lipase and inulinase, while the isolates from Aran-Bidgol playa had a great ability to produce pectinase and DNase. The strains from Urmia Lake were also good producers of DNase but failed to show any chitinase activity. The diversity of halophilic bacteria from the mentioned three saline lakes was also determined using PCR-amplified 16S rRNA followed by phylogenetic analysis of the partial 16S rRNA sequences.

Keywords Extremophiles · Halophile · Hydrolytic enzymes · Biodiversity

Introduction

Hypersaline lakes, with salinity ranges at or near saturation, are extreme environments that often maintain a remarkable cell density and are biologically very productive ecosystems. Halophilic bacteria have a great biotechnological potential to produce compatible solutes or hydrolytic enzymes. Hypersaline lakes are generally defined as those containing salinities in excess of seawater (3.5 % total dissolved salts). These lakes can be classified two types, based on origin and salt composition. Lakes with salt composition similar to seawater that named thalassohaline and athalassohaline lakes originated from evaporation of inland surface water which can differ in their ion composition compared with seawater derived lakes [1]. Iran possesses various hypersaline environments including permanent hypersaline lakes such as Urmia Lake and seasonal hypersaline lakes (playa) such as Aran-Bidgol and Howz-Soltan, which the microbial diversity of them has been little studied [2–4], that causes their potential usage in biotechnology remains unknown.

Urmia Lake is one of the largest permanent hypersaline lakes in the world. This lake is located in the northwestern

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of Iran and is an oligotrophic lake of thalassohaline origin with a total surface area between 4,750 and 6,100 km^2 and a maximum depth of 16 m at an altitude of 1,250 m. The lake is divided into north and south parts separated by a causeway. Due to the drought and increased demand for agricultural water in the lake's basin, the salinity of the lake has been raised to more than 300 g/L during recent years. The main cations of the lake water are Na^+ and K^+, Ca^{2+}, Li^+ and Mg^{2+}, and the main anions are Cl^-, SO_4^{2-}, HCO_3^-. The predominance of the Na^+ and Cl^- ions demonstrates the thalassohaline characteristic of Urmia Lake. Sodium ions are at slightly higher concentration in the south compared to the north of the lake which results in the shallower depth in the south and a higher evaporation rate [5].

Howz-Soltan playa is located in the central area of Iran, with an extension of about 240 and 280 km^2 in dry and wet seasons, respectively. The depth of the salt layer which covers almost all the surface of the playa varies between 20 and 46 m and the pH values of water, saline soil and salt sediments varies from 6.5 to 8.2. The major chemical composition of the soil, brine, mud and salt consist of NaCl, KCl, $MgSO_4$, $MgCl_2$ and Na_2SO_4 [2].

Aran-Bidgol playa is the largest hypersaline playa and is located in the northwest of Esfahan province, in the central area of Iran. This playa has been formed by deposition of halite sediments in different geological periods. It looks like a triangle which is headed toward the north direction, with an extension of about 647 km^2. The depth of salt layer which covers almost all the surface of the playa varies from 5 to 54 m, its pH is neutral (about 7.0–7.5) and its salinity reaches saturation. Most salts in this lake are NaCl, Na_2SO_4, $MgCl_2$ and $MgSO_4$ with traces of carbonate ions [3, 4].

Halophiles are salt loving microorganisms that inhabit hypersaline environments which can be classified as slightly, moderately and extremely halophilic, according to their requirement for NaCl [6]. These microorganisms produce compounds of industrial interest, such as extracellular hydrolytic enzymes, that are stable and efficient under conditions leading to precipitation or denaturation of most other proteins [7–13].

The isolation of moderate and extreme halophiles that are able to produce hydrolytic enzymes will provide the possibility to have optimal activities at different salt concentrations that could be useful in some industrial processes [2, 14, 15].

Based on difference between environmental conditions and physicochemical properties of three hypersaline lakes, in the present study, we aimed to compare biodiversity and enzymes production by halophilic bacteria isolated from these lakes. The study of these environments leads to the detection of microbial diversity and isolation of valuable microorganisms as a suitable source for extreme-enzymes production. So, we have determined the capability of moderately and extremely halophilic bacteria for producing different extracellular hydrolyses, which will provide information about their potential for utilization and processing in industrial level.

Materials and Methods

Sample Collection and Growth Conditions

Water and soil samples were collected from different saline environments during October and November (early wet season), April and May (early dry season) 2008. The samples were brine, multicolour solar salt, saline soil and saline mud. Figure S1 (all figures is shown as Online Resource in supplementary material) shows the location of these three hypersaline lakes on the map of Iran and the sampling location.

Samples were collected in 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 consisted of (g/L): NaCl 81, $MgSO_4 \cdot 7H_2O$ 9.7, $MgCl_2 \cdot 6H_2O$ 7.0, $CaCl_2$ 3.6, KCl 2.0, $NaHCO_3$ 0.06, NaBr 0.026 for moderately halophilic bacteria and 20 % sea salt for extremely halophilic microorganisms, supplemented with 5 % yeast extract. The pH of the culture media was adjusted to 7.3 before autoclaving. Cultures were incubated at 34 °C in an orbital shaker at 150 rev/min for 3–7 days or more depending on the growth rate of the isolates. Solid media were prepared by adding 12–15 g/L agar (Merck).

Screening of the Strains for Extracellular Hydrolytic Activities

To detect the production of extracellular hydrolyses; different enzymatic assays were carried out in agar plate assay, except pullulanase activity assay which was performed in liquid medium. The pH of all media could range from 7.2 to 7.4, and 10 and 20 % total salt was added for detecting hydrolytic activities in moderately and extremely halophilic bacteria, respectively. The different assay media used are described below.

Determination of Extracellular Amylase Activity

Amylolytic activity was determined qualitatively on plates by following the method described by Amoozegar et al. [8], using starch agar medium (Merck) containing 10 or

20 % total salts, followed by incubation at 34 °C for 1 week, the plates were flooded with 0.3 % I_2–0.6 % KI solution; a clear zone around the bacterial growth indicated the hydrolysis of starch.

Determination of Extracellular Protease Activity

Proteolytic activity of the cultures was screened in skim milk agar containing 10 % skim milk, 2 % agar, supplemented with 10 % and 20 % total salt for determining the hydrolytic activity of moderate and extreme halophiles, respectively. Proteolytic activity was proved by the clear zones appeared around the bacterial growth after 7 days [10].

Determination of Extracellular Lipase Activity

To observe lipase production, the strains were inoculated on nutrient agar plates containing olive oil (2.5 %), Victoria blue (4 mg/dL) and appropriate concentration of salt with an initial pH of 7.2–7.4. The plates were incubated at 34 °C for 48 h and the blue colour zones around the bacterial growth was used to identify the lipase producing strains [16, 17].

Determination of Extracellular DNase Activity

DNase activity of the strains was routinely determined using 42 g/L of DNase test agar medium (Merck), supplemented with 10 % and 20 % total salt for detecting DNase activity of moderately and extremely halophilic bacteria, respectively. The plates were incubated at 34 °C for 7 days, followed by flooding with 1 N HCl solution. Clear halos observed around the colonies were applied to express DNase activity [18].

Determination of Extracellular Pectinolytic Activity

The presence of pectinolytic activity on plates was determined using a medium containing (g/L): pectin 10, $(NH_4)_2SO_4$ 1.4, K_2HPO_4 2 and $MgSO_4{\cdot}7H_2O$ 0.02 %, nutrient solution 1 (g/L) ($FeSO_4{\cdot}7H_2O$ 5 mg/L, $MnSO_4{\cdot}H_2O$ 1.6 mg/L, $ZnSO_4{\cdot}7H_2O$ 1.4 mg/L, $CaCl_2$ 2 mg/L), agar 20 g/L, 10 and 20 % salts for moderately and extremely halophiles, respectively. After incubation at 34 °C for 7 days, the plates were flooded with 0.3 % I_2–0.6 % KI solution. A clear zone around the bacterial growth indicates pectinolytic activity [19].

Determination of Extracellular Inulinase Production

The inulinase production by halophilic strains was detected by preparing media containing (g/L): inulin 2, $(NH_4)_2SO_4$ 0.5, $MgSO_4{\cdot}7H_2O$ 0.2, KH_2PO_4 3, agar 20; supplemented with appropriate concentration of salt. Bacterial growth on plates containing inulin, as the sole source of carbon, after 48 h incubation at 34 °C was used to determine the inulinase activity [20].

Determination of Extracellular Carboxy Methyl Cellulase activity

Carboxy methyl cellulase activity of the cultures was screened in a solid medium containing (g/L): yeast extract 0.5, glucose 1, agar 17, carboxy methyl cellulose 5, $NaNO_3$ 1, K_2HPO_4 2, KCl 1, $MgSO_4$ 0.5. After incubation at 34 °C for 7 days, the plates were flooded with 0.1 % congo red solution. The clear zone around the colony indicated cellulase activity [21].

Determination of Extracellular Xylanase Production

Xylanase activity was detected using a saline medium containing (g/L): xylan 10, yeast extract 2, peptone 5, $MgSO_4$ 0.5, $CaCl_2$ 0.15, agar 20. After incubation at 34 °C for 7 days, the plates were flooded with 0.1 % congo red solution. The clear zones around colonies indicated qualitative xylanase activity [22].

Determination of Extracellular Pullulanase Activity

To detect pullulanase activity, the strains were cultured in saline liquid medium containing (g/L) yeast extract 1, pullulan 5, and appropriate concentration of salts and incubated for 72 h. The pullulanase activity was detected by clearness of medium observed followed by adding 97 % ethanol, since pullulan and ethanol interaction results in forming a white precipitate of non degraded pullulan [13]. Figure S2 shows hydrolytic activity of some strains qualitatively, using complicated appropriate culture media.

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 % NaCl. Gram reaction, motility, colony morphology and pigmentation, catalase, urease and oxidase activities, nitrate reduction, Tween 80 hydrolysis, Voges–Proskauer and methyl red reaction were checked as recommended by Sanchez-Porro et al. [23]. Acid production from carbohydrates, carbon and nitrogen sources utilization were performed according to Ventosa et al. [24]. Also, the susceptibility of the strains to antibiotics was determined on Muller–Hinton agar containing 10 and 20 % NaCl.

To determine the optimal growth temperature and pH, broth cultures were incubated at temperatures of 5–55 °C with 5 °C intervals and at pH values of 5–10 with 0.5 pH units' intervals. Growth at different salt concentrations (0, 2.5, 5, 7.5, 10, 15, 20, 25, and 30 %) was tested on nutrient broth at pH 7.5. Growth was monitored by optical density at OD_{600} using a spectroscopic method (model UV-160 A; Shimadzu).

Isolates with optimal growth at 3–15 % NaCl were considered as moderately halophilic strains [25], while isolates with optimal growth at 15–25 % NaCl concentrations were considered as extreme halophiles [1].

The production of extracellular hydrolytic enzymes was taken as an indicator to randomly select some strains. The genomic DNA 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′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CACGGATCCTACGGGTA-CCTTGTTACGACTT-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 (Bioneer, South Korea), template DNA 1 μL, DMSO 1.25 μL, smart Taq 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 products were analyzed on 1 % agarose gel with ethidium bromide staining and purified using PCR purification kit (Bioneer, South Korea). The purified PCR products were sequenced in both directions using an automated sequencer by Seq Lab (Germany). The phylogenetic relationships were analyzed by comparing the sequencing data with the related 16S rRNA gene sequences inferred from GenBank database of the National Center for Biotechnology Information, via BLAST search.

Phylogenetic analysis was performed using the software packages PHYLIP and MEGA version 5 [26] after multiple alignment of data available from public databases by CLUSTAL_X. The correction method was used to perform the pairwise evolutionary distances and the neighbour-joining method was used to accomplish the clustering. The evaluation of the tree topology of neighbor-joining data was performed using bootstrap analysis by performing 1,000 resembling [27–30].

Results and Discussion

In recent years, different screening programs have been performed in saline habitats in order to isolate and characterize the microbial sources with novel enzymatic activities or different properties compared to conventional enzymes. So, in this study we investigated microbial diversity, identification, classification and related hydrolytic enzyme patterns which is produced by identified strains from three hypersaline lakes in Iran.

Screening of bacteria from saline soil, brine, salt sludge and solar saltern of Urmia, Howz-Soltan and Aran-Bidgol Lakes led to the isolation 188, 302, 91 halophiles, respectively. The most extremely and moderately halophilic isolates from three hypersaline lakes were found to be in saline soil, solar saltern sediment, salt sludge and brine, in the order of frequency. As compared to these environments, the brine which was collected from three hypersaline lakes had low number of bacteria. The number of moderately halophilic isolates was remarkably higher than those of extremely halophilic bacteria (Fig. S3).

Among 153 moderately halophilic strains isolated from Urmia Lake, were 65 Gram-negative bacteria, 79 Gram-positive bacilli and 9 Gram-positive cocci. Of 231 moderately halophilic strains isolated from Howz-Soltan Lake, were 64 Gram-negative rods, 138 Gram-positive bacilli and 29 Gram-positive cocci and of 83 moderately halophilic strains isolated from Aran-Bidgol Lake were 24 Gram-negative bacteria, 44 Gram-positive bacilli and 17 Gram-positive cocci. Non-halophilic bacteria were not found among these strains, probably due to the salt saturation in most of the areas in three habitats.

Based on the ability to produce nine different hydrolysis enzymes by halophilic strains in Urmia Lake 57, 49, 64, 116, 97, 17, 51, 106 and 3 strains were able to produce amylase, protease, lipase, inulinase, xylanase, cellulase, pullulanase, DNase and pectinase, respectively. In Howz-Soltan playa 177, 100, 195, 95, 92, 68, 65, 33 and 28 strains were able to produce amylase, protease, lipase, inulinase, xylanase, cellulase, pullulanase, DNase and pectinase, respectively. In Aran-Bidgol playa 32, 20, 27, 40, 9, 11, 16, 40 and 24 strains were able to produce amylase, protease, lipase, inulinase, xylanase, cellulase, pullulanase, DNase and pectinase, respectively. Figure S4A–I shows comparative study of the hydrolytic activity of isolated halophilic strains from the three hypersaline lakes.

Among three hypersaline lakes, greater hydrolytic activity was observed in Gram-positive moderately halophilic rods than Gram-negative rods and Gram-positive cocci. In Urmia Lake, the Gram-positive strains showed higher amylolytic, proteolytic, nucleolytic, inulinolytic, xylanolytic and pectinolytic activities, while Gram-negative rods did not show any significant hydrolase production. The Gram-positive cocci isolates showed higher lipase and cellulase activities.

In Howz-Soltan playa 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 to Gram-positive and Gram-negative rods. However, Gram-negative rods showed mainly lipolytic activity. The Gram-positive cocci produced greatly amylases, lipases and proteases and interestingly, presented more pectinolytic and cellulolytic activities than Gram-positive and Gram-negative rods. In Aran-Bidgol playa, the Gram-positive strains showed higher amylolytic, proteolytic, inulinolytic, nucleolytic, pectinolytic, xylanolytic and pullulanolytic activities, while Gram-negative rods had mainly lipolytic, activity. Gram-positive cocci presented more cellulolytic activity than Gram-positive and Gram-negative rods.

According to the phenotypic characteristics and sequence comparison of amplified partial 16S rRNA gene of selected strains from the three hypersaline lakes, strains were belonged to the *Halobacillus*, *Marinobacter*, *Thalassobacillus*, *Halomonas* and *Halobacillus* genera in Urmia Lake; in Howz-Soltan playa the strains were *Virgibacillus*, *Bacillus*, *Oceanobacillus*, *Thalassobacillus*, *Piscibacillus*, *Gracilibacillus*, *Salicola*, *Halomonas* and *Halobacillus*; also *Thalassobacillus*, *Bacillus*, *Salinicoccus*, *Idiomarina*, *Salicola*, *Halomonas* were found in Aran-Bidgol playa. Figures S5, S6, and S7 show the phylogenetic positions of the isolates from Urmia, Howz-Soltan and Aran-Bidgol Lakes.

Generally, our studies showed that most isolated strains in three hypersaline lakes are belonging to Gram-positive and Gram-negative bacilli, Gram-positive cocci and extreme halophiles, respectively. However, extreme halophilic isolates in Howz-Soltan playa were more than Gram-positive cocci. Also, we found that the proximity of geography and similarity of physicochemical conditions in Aran-Bidgol and Howz-Soltan has also led to similarity in hydrolytic enzyme producing pattern of isolated strains in these lakes compared to Urmia Lake. Extremely halophilic bacteria which were lower in number in comparison with moderate halophiles, showed higher potential in producing amylase, lipase, pectinase, xylanase, amylase, lipase, inulinase, xylanase, cellulase, pectinase and protease, xylanase in Urmia, Howz-Soltan and Aran-Bidgol Lakes, respectively.

The diversity of hydrolytic enzymes activity in halophilic bacteria isolated from hypersaline environment was surveyed in this study. According to identification and classification of isolates and their enzyme activity patterns; in Urmia Lake, members of the genus *Halobacillus* produce cellulase, protease, amylase, pectinase, inulinase, members of the genus *Halomonas* produce inulinase, pullulanase, xylanase, members of the genus *Thalassobacillus* produce amylase, DNase, inulinase and members of the genus *Marinobacter* did not show any significant hydrolases production. In Howz-Soltan playa, genera *Halomonas*, *Thalassobacillus*, *Virgibacillus* are cellulose, inulinase, pullulanase, xylanase producers, genus *Piscibacillus* is a lipase producer, genera *Bacillus*, *Oceanobacillus*, *Gracilibacillus*, *Halobacillus*, *Halomonas* and *Salicola* are protease, amylase, pectinase, lipase producers and the genus *Halomonas* is DNase, protease and amylase producer.

In Aran-Bidgol playa, genera *Halobacillus* and *Thalassobacillus* produce DNase, lipase, inulinase, amylase, protease, cellulase, pectinase, pullulanase, genus *Halomonas* produced amylase, protease, lipase, DNase, inulinase, xylanase, pullulanase, pectinase, genus *Salicola* produced amylase, lipase, DNase, pullulanase and genus *Idiomarina* showed lipase and DNase activities. The halophilic isolates from the three hypersaline lakes were fine producers of extracellular, hydrolytic enzymes. Moderately halophilic spore-forming Gram-positive bacilli possess a great potential of hydrolyses production such as amylase, protease, inulinase, cellulose, pullulanase and xylanase. A few studies have been carried out concerning microbial diversity and extracellular enzymes production in other saline habitats in the world. For example, Sanchez-Porro et al. [14], showed the abundance of five hydrolytic enzymes including amylase, protease, lipase, DNase, pullulanase by moderately halophilic bacteria from saltern in Spain; Rohban et al. [2], investigated screening of halophilic bacteria producing nine extracellular hydrolyses from Howz-Soltan Lake, Iran, Makhdoumi et al. [15], investigated diversity of nine hydrolytic enzymes in haloarchaeal strains from Aran-Bidgol Lake, Iran and finally Kumar et al. [31] investigated screening and isolation of halophilic bacteria producing three industrially important enzymes. The aim of the present study was to analyze the ability of moderate and extreme halophilic bacteria to produce nine different extracellular hydrolases from Urmia Lake, Aran-Bidgol playa and Howz-Soltan playa. Strains were isolated with significant ability to produce inulinase, pectinase, cellulase and xylanase and finally biodiversity and enzyme production comparisons were performed in halophilic bacteria isolated from three hypersaline lakes in Iran.

Conclusion

This study provides the first publication on comparison of biodiversity and enzyme production of salt lakes for the first time in Iran. Further studies are currently in order to determine the diversity of halophilic bacteria and their ability to produce extracellular hydrolytic enzymes in the other hypersaline environments and to select the best hydrolytic enzymes producers. Since, halophilic bacteria

have the ability of quickly matched to changes in the salt concentrated environments and production of many metabolites and hydrolytic enzymes with high commercial value, so they are potential candidates for bioprocessing. Especially, many hydrolytic enzymes that produce by halophilic bacteria have tolerance to extreme conditions, so can be exploited wherever enzymatic transformations are required to function under these conditions such as in the presence of organic solvents, salt content and high temperature.

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