International Journal of Molecular and Clinical Microbiology 10(2) (2020) 1369-1378

International Journal of
Molecular and Clinical Microbiology

Isolation and identification of halophilic and extreme halophilic bacteria from saline water wetland ecosystems and determination of their enzymatic profile in Golestan province, Iran

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ARTICLE INFO

Article history:
Received 23 Agust 2020
Accepted 28 November 2020
Available online 1 December 2020

Keywords:
Halophilic bacteria,
Extreme halophilic,
Enzymatic activity,
Saline water ecosystem

ABSTRACT

The aim of this study was to isolate halophilic and extreme halophilic bacteria from Incheh Borun saline water ecosystems including Alagol, Almagol and Ajigol wetlands, Shamushk and Shafi Abad dams, as well as Incheh and Qarniaraq volcanoes in Golestan province. The activity of amylase, protease and lipase enzymes was compared in them. The samples were isolated for 4 months from different areas with different EC and pH of the mentioned wetlands, dams and volcanoes. A total of 70 different water samples were collected from aquatic ecosystems that were purified by culturing on selected media. According to biochemical tests, 41 isolates were able to grow in saline environments, of which 63.4% were halophiles, which were observed only in wetlands and another volcanoes 36.6% were halotolerant and were excluded from the results. Alagol wetland had the highest abundance in halophilic species. The enzymatic activity of halophiles for amylase, protease and lipase hydrolase showed that 34.6% of the isolates contained all three enzymes, which include all three groups of gram-positive cocci, gram-positive and gram-negative bacilli. Comparison of enzymatic activity also showed that with the exception of two extreme species of Halophilus bacillus subtilis which were relatively slower in growth and had a longer period at the beginning and end of substrate application in culture medium, the rest of the isolates had almost the same range in growth time. They have the time of enzyme production and the time of completion of the substrate.

1. Introduction

Saline water ecosystems are extreme environments and often contain a wide range of salt-loving microbial agents. Therefore, the separation of medium and severe halophiles capable of producing extracellular hydrolase, which has the ability to work optimally at different salt concentrations, can be useful in industrial processes. Saline lakes with salinity ranges close to seawater are extreme environments but often have a wide range of microbial agents (Babavalian, 2007; Bagheri, 2009; Behrooz Rad, 2008). The microbes in these habitats include archaea, bacteria and eukaryotes. In some habitats, the presence of large numbers of these bacteria is known without the need for a microscope and through the formation of pink-red color by archaea (Haloquadratum and other Halobacteriales), bacteria (Salinibacter) and eucalyptus (Dunaliella salina). To adapt to saline conditions, bacteria have extensive strategies for maintaining their cellular structure and function. Studies of these bacteria are so important that they can produce and supply compounds that

can be used in industry; Such as hydrolytic enzymes that have wide potential for use in biomedical and chemical industries (Rad, 2008; Javid, 2004; Dideri Khamseh Motlagh, 2009; rahban, 2008).Most industrial processes are performed under special physicochemical conditions that regulate the optimal conditions for the activity of existing enzymes. Therefore, this issue is of great importance for enzymes that show optimal activity in a wide range of salt concentrations, pH and temperature. Halophiles are an excellent source of these enzymes, which are not only salt tolerant but also active at high temperatures and variable pH (Zahraei, 2007). Isolation of moderate and extreme halophiles capable of producing hydrolases that have optimum activity at different salt concentrations can be useful in some industrial processes (Mehrshad, 2011). Halophilic bacteria are the average group of halophilic microorganisms that can grow in environments containing 3-15% NaCl. They belong to a heterogeneous group of microorganisms including various genera such as Halomonas and Salinivibrio that have been studied in terms of ecology, physiology, biochemistry and genetics (3). Extreme halophilic bacteria grow optimally under saturated saline conditions of more than 20% (w / v). They can produce various hydrolase such as amylases and lipases. The potential importance of extreme halophiles has been observed and reported in various industries such as the leather industry and food preservation (Amoozegar, 2003; Dideri Khamseh Motlagh, 2009; rahban, 2008). Iran has a wide variety of saline environments, including salt lakes and beaches, the microbial diversity of which is unknown. Therefore, the potential for the production of various hydrolytic enzymes among them remains unknown (Arahal, 2002). Halophilic bacteria are potent organisms in production of novel bioactive antimicrobial compounds which might be considered in drug innovation and control of plant pathogens. The bacteria extracts were evaluated for their antifungal and antibacterial activities on human and Plant pathogenic strains.

This study highlights the therapeutic and prophylactic potential of B. subtilis extracts as antibacterial and antifungal agents (Elyasifar et al., 2019).

Halophilic bacteria grow over a wide range of salt concentrations. In this study we aimed to isolate and screen out the halophilic bacteria and to determine their activity for production of the bioactive compounds. In total, our finding illustrated that the maharlu salt lake might be considered a source of halophilic bacteria with potent activity for production of the bioactive compounds. In addition, isolation and characterization of these compounds culminate in the achievement of the new drugs (Hashemi et al., 2014).

Therefore, the aim of this study was to isolate halophilic and extreme halophilic bacteria from Incheh Borun, Almagol and Ajigol wetland ecosystems; Shamushk and Shafiabad dams of Incheh and Qarniagh flower springs in Golestan province and determination and comparison of amylase, protease and lipase enzymes in them were explained.

2. Materials and Methods

Sample and sampling: Samples were isolated for 4 months from June to September 2017 from Alagol, Almagol and Aji Gol wetlands around Incheh Borun city of Aq Qala functions in Gorgan, Shamushk dams in Gorgan and Shafi Abad in Fendersk Aliabad. Sampling of Qarniargh and Incheh volcanoes around Gomishan city of Gorgan was also considered in this research.

Alagol Wetland is located in the east of Aq Qala to Incheh Borun road, whose area reaches 2500 ha when it is flooded. Almagol Wetland, An international wetland with an area of 210 ha and a volume of water of 5 million m m^3 is located in the north of Aji Gol wetland. Aji Gol International Wetland with an area of 320 ha is located between Almagol and Alagol wetlands. This wetland is another wetland in Incheh Borun region, which is located in the west of Okhi Tappeh village and south of Tangli. The dam of Upper Shamushk village, with an area of about 6 haand a volume of water of about half a million mm^3, is located in the south of this village and the green forest area of this region. The dam of the historical village of Shafi Abad in the city of Ramyan was another example of the dams under study in this study and one of the spectacular attractions of the village of Shafi Abad. The Incheh Geyshan, also known as the Incheh Wetland, is located in the city of Aq Qala, on the way from Aq Qala to the border city of Incheh. The bed of this wetland is about 200 ha and is

about 26 km away from the center of Aq Qala city.

Qarniaragh is another volcanic eruption in Golestan province, which is located about 18 km northwest of Aq Qala city and near a village called Saqartpeh.

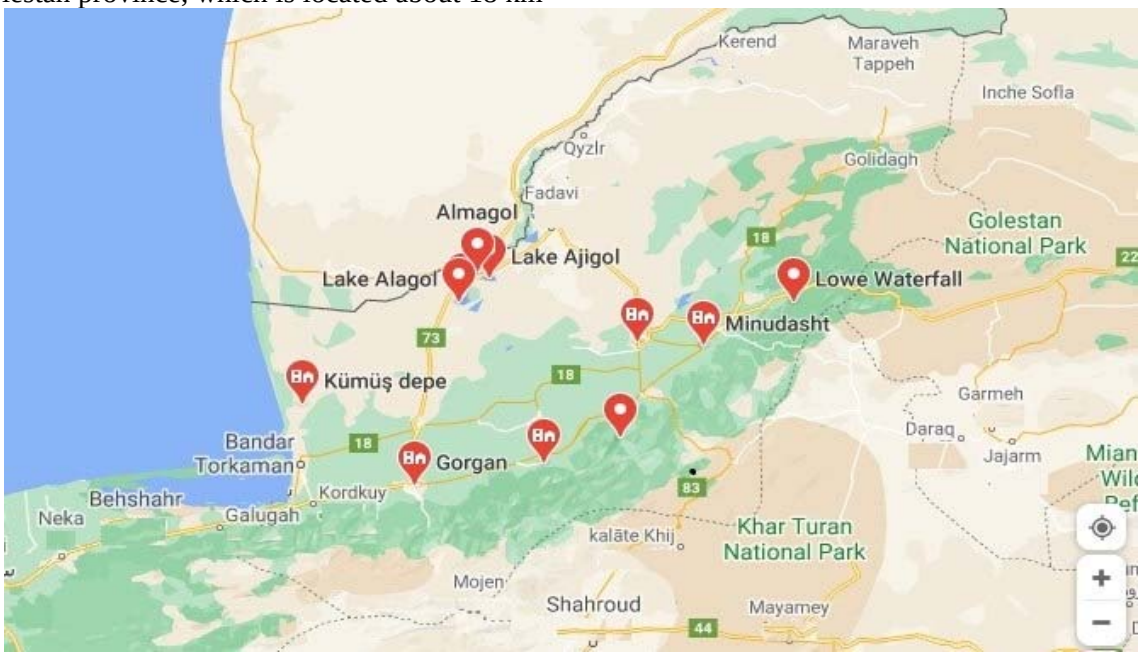

Figure 1. Geographical location of selected areas for sampling

2.1. Cultivation and isolation:

Samples were collected by sterile plastic containers, their pH and EC were measured and transferred to the laboratory and cultured in less than 8 hours. All samples in Saline nutrient broth with a final concentration of 10% sea salt containing NaCl in the amount of 81 g / l, MgSO4. 7H20 in the amount of 9.7 g / l, MgCl2.H2O in the amount of 0.7 g / l, CaCl2 in the amount of 3.6 g / l, KCl in the amount of 0.2 g / l, NaHCO3 in the amount of 0.06 g / l Liters and NaBr in the amount of 0.026 grams per liter; 10 and 20% (w / v) salt for medium halophilic microorganisms with 5% (w / v) of yeast extract and media containing 25% and 30% salt for extreme halophilic microorganisms with 5% yeast extract Was used. The pH of the culture medium was adjusted to 7.3 before autoclaving. Cultures were incubated at 37°C in orbital shaker at 150 rev / min for 7 days and after 7 days, 10% was cultured on nutrient agar medium.

A total of 70 different water samples were collected from aquatic ecosystems that were purified by culturing on selected media.

Then based on colony morphology with emphasis on pigment production, colony size and turbidity in salt concentrations and then performing various biochemical tests such as catalase, gelatinase, oxidase, citrate, urease, MRVP, fermentation / oxidation of sugars, identification of facial isolates accepted.

2.2. Enzymitic profile determination:

In order to determine the production of extracellular hydrolase, various enzymatic experiments were performed in agar medium. The pH of all media was adjusted to 2.7 to 7.4, and 20%, 25% and 30% salt were added to determine the hydrolytic activity of medium-sized and extreme halophilic bacteria, respectively. The ability to produce amylase, protease and lipase enzymes was also investigated by analyzing the substrate in the culture medium by the identified halophiles and also comparing the enzymatic activity in them. After PCR, the reaction products were electrophoresed on 1% agarose gel. The gel was

placed in an electrophoresis tank containing 1x TBE buffer with an initial voltage of 120 volts and after DNA was inserted into the gel, a secondary voltage of 80 volts was applied for 20 minutes. After the samples were more than two-thirds the length of the gel, the gel was placed in a uvidoc device and the position of the bands was examined by UV imaging. The band size was estimated by the gene ruler (Ladder) bp 10,000 - 250. (Azhar et al., 2014).

2.3. Molecular identification of selected isolates:

Isolates grown on selected culture media were selected for more accurate detection. Isolates were examined based on colony morphology. This parameter was determined by emphasizing appearance features, determining turbidity and color. Other characterizations were performed through culture growth at pH and optimum salt concentration. Also, preliminary biochemical tests of catalase, gelatinase, oxidase, citrate, urease, indole, methyl red, vozuose prosquer and fermentation / oxidation (lactose, sucrose, dextrose) to determine the genus of halophilic isolates along with 16 molecular confirmation method (rRNA) was performed after ensuring the enzymatic activity of the isolates and in order to definitively detect the species of salt-loving isolates of the aquatic ecosystems. (Azhar et al., 2014).

3. Results

The studies performed in saline media to differentiate between halophilic and non-halophilic bacteria showed that 41 microorganisms were able to grow in saline media based on the minimum amount of salt for initial enrichment, of which 15 species were not salt-loving and only species were salt tolerant (5%). Therefore, they were not included in the group of halophiles and after obtaining the data from the results.

Shamushk and Shafiabad seals had the highest frequency in salt tolerant species in their group and those that in general all isolated species. among were salt tolerant due to the low EC of these ecosystems and the amount of saline salts. The low content of them is comparable to other ecosystems. In the rest of the ecosystems, there were salt-loving isolates with a minimum growth potential of 10% salt.

According to the obtained data, it is clear that the highest number of species able to grow in saline environment has been isolated from Alagol wetland and the highest amount of salt-loving isolates among all ecosystems under study, belonged to this wetland. In total, the strains isolated from the aquatic ecosystems were 26 isolates (63.4%) salt-loving (halophilic) and 15 isolates (36.6%) were salt-tolerant, which were removed from the results. Therefore, in continuation of the reports, 26 salt-loving isolates identified from Alagol, Almagol and Aji Gol wetlands, as well as Qarniaraq and Incheh volcanoes have been studied, and the salt-tolerant isolates of Shamushk and Shafiabad dams have been Research objectives were set aside.

Then, by performing initial morphological and biochemical diagnostic tests, these 26 halophilic isolates obtained from saline water ecosystems were identified. The results showed that 14 halophilic isolates belonged to gram-positive cocci and their differentiation by biochemical tests determined their genus to some extent, all of which belonged to *staphylococci*. 10 halophilic isolates belonged to the group of gram-positive bacilli. According to the tests, they belonged to the genus *Bacillus* In addition, based on the experiments, two gram-negative halophilic isolates were detected whose genus was not detected in biochemical experiments. In general, the highest frequency of halophilic gram-positive cocci, which all belonged to the genus *Staphylococcus*, had a frequency of 53.8%, followed by gram-positive rod halophiles belonging to the genus *Bacillus* with a frequency of 38.5%. Salt-loving gram-negative bacilli also had the lowest frequency among halophiles with 7.7%.

After obtaining the data obtained from the preliminary identification of halophiles, their cultivation was performed in medium and severe saline environments to compare moderate and extreme halophil species. Isolates that were able to grow in only 10% salt were considered moderate halophiles and those that were grown in 20% or more were considered extreme halophiles (severe halophiles).

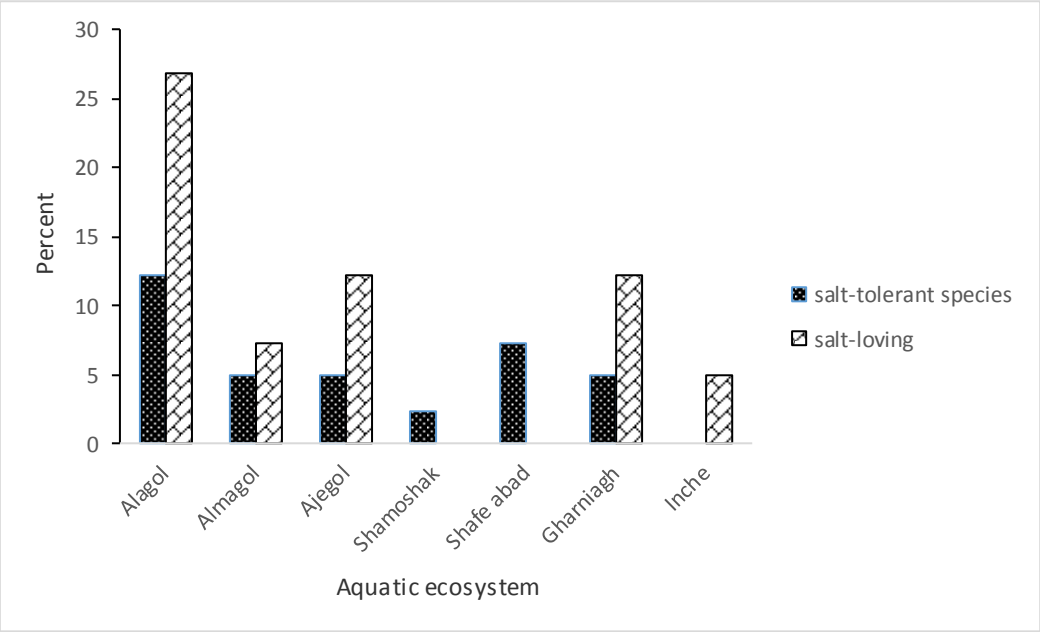

Figure 2. Abundance of salt-loving and salt-tolerant species in studied aquatic ecosystems

Table 1. Prevalence of moderate and extreme halophilic groups (severe) in bacteria isolated from saline water ecosystems

Type of ecosystem	Sampling location	Sampling depth water	number of samples	gram-positive cocci		gram-positive bacilli		gram-negative bacilli		Total	
				Average	Intense	Average	Intense	Average	Intense	Average	Intense
Wetlands	Alagol	10 to 20 cm	10	19.2	7.7	7.7	3.8	3.8	0	30.8	11.5
	Almagol	10 to 20 cm	10	7.7	0	3.8	0	0	0	11.5	0
	Aji Gol	10 to 20 cm	10	3.8	3.8	0	7.7	3.8	0	7.7	11.5
Mud volcanoes	Qarniarq	10 to 20 cm	10	3.8	7.7	7.7	0	0	0	11.5	7.7
	Incheh borun	10 to 20 cm	10	0	0	7.7	0	0	0	7.7	0
Total				34.6	19.2	56.9	11.5	7.7	0	69.2	30.8

Based on the data shown in Table 1, it is found that in all bacterial groups isolated from wetlands and geysers, the average halophilus has a higher percentage compared to the extreme halophilus and a total of 69.2 They include the percentage of total salt-loving isolates. Also, among the bacterial groups, gram-positive cocci had the highest frequency in both moderate and severe halophiles, which can be justified by the number of more isolates. After determining the class of halophilic isolates, both in terms of bacterial groups belonging to it and in terms of the intensity of salt-friendliness, the enzymatic performance of halophiles for three substrates of starch, protein and lipid, which are among the most consumed substances and their degrading enzymes. They are among the most widely used industrial cases, the results of which can be seen separately in the halophilic groups for the three enzymes amylase, protease and lipase in Table 2.

Table 2. enzymatic activity of isolated halophilic groups from saline water ecosystems

Halophilic group	Isolated number	Extracellular hydrolase		
		Amylase	Protease	Lipase
Gram-positive cocci	1	-*	+*	+
	2	-	-	+
	3	+	+	-
	4	+	-	+
	5	+	-	+
	6	+	+	-
	7	+	+	-
	8	+	+	-
	9	+	+	+
	10	-	+	-
	11	+	+	-
	12	+	-	-
	13	+	+	+
	14	+	+	+
Gram-positive bacilli	15	+	+	+
	16	+	+	-
	17	+	+	+
	18	+	-	-
	19	+	+	+
	20	-	+	-
	21	+	+	+
	22	-	+	+
	23	-	-	-
	24	+	-	-
Gram-negative bacilli	25	+	+	+
	26	-	+	+

*-: negative enzymatic activity +: positive enzymatic activity

Of the 26 halophiles isolated from saline water ecosystems, 9 isolates contained all three enzymes and were in all three groups of gram-positive cocci, gram-positive bacilli, and gram-negative bacilli. However, there was only one isolate among the halophiles that did not produce any of the three enzymes, which was a gram-positive bacillus. In other cases, the isolates produced one or two extracellular enzymes, which was determined by the presence of the substrates in the culture medium. The isolates producing two enzymes for amylase + protease, amylase + lipase and protease + lipase hydrolase were 6, 2 and 3, respectively. Single enzyme isolates for amylase, protease and lipase were evaluated in 3 cases, 2 cases and 1 case, respectively. In general, out of 8 extreme halophilic isolates, only 2 isolates had all three

enzymes and out of 18 average halophilic isolates, 7 isolates produced all three hydrolases in the environment. Extreme halophiles of two enzymes were 5 cases, of which 2 cases were related to amylase + protease enzymes, 2 cases were related to amylase + lipase and 1 case was related to protease and lipase. One case of single-enzyme halophilus had only the enzyme lipase, which did not produce any other extracellular hydrolase in the evaluations. Among the 18 average halophilic isolates, 7 bacteria produced all three enzymes, with the share of gram-positive cocci being three isolates and gram-positive and gram-negative bacilli each containing two isolates.

After determining the production status of extracellular hydrolase identified by halophilic isolates, the superior isolates producing all three enzymes in extreme or moderate groups underwent molecular confirmation tests to determine the type of genus and species Among the above isolates, there were 3 gram-positive cocci, 4 gram-positive bacilli and 2 gram-negative bacilli which were identified and recorded using 16S rRNA sequencing.

After determining the production status of extracellular hydrolase identified by halophilic isolates, the superior isolates producing all three enzymes in extreme or moderate groups underwent molecular confirmation tests to determine the type of genus and species. This process was performed by DNA extraction of isolates, PCR of 16S gene, electrophoresis of PCR products (Figure 3).

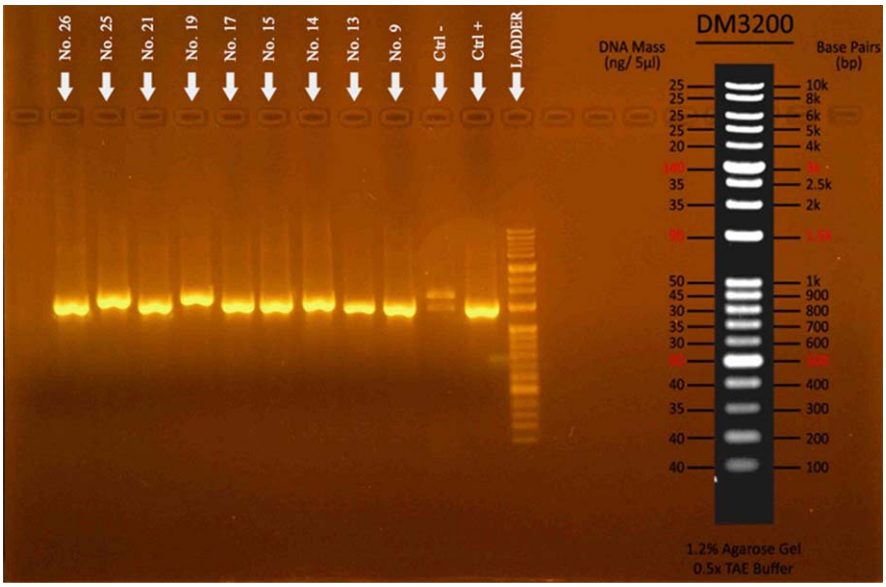

Figure 2. Electrophoresis of PCR products (16S gene) of isolates producing 3 enzymes

Table 3. Definitive diagnosis and characterization of extracellular hydrolase generating isolates

Bacterial group	Bacterial genus	Bacterial species	Intensity of halophilia	Separation place
gram-positive cocci	Planococcus	salinarum	medium	Alag Wetland
	Staphylococcus	capitis	medium	Aji Gol Wetland
	Staphylococcus	hominis	medium	Qarniarq volcano
Gram-positive bacilli	Bacillus	subtilis	Intense	Alag Wetland
	Bacillus	subtilis	Intense	Aji Gol Wetland
	Bacillus	licheniformis	medium	Alag Wetland
	Bacillus	thuringiensis	medium	Qarniarq volcano
Gram-negative bacilli	Citrobacter	murliniae	medium	Alag Wetland
	Pseudomonas	hibiscicola	medium	Aji Gol Wetland

The results of this study also showed that wetlands, as extreme environments in the amount of soluble salts, contain the largest groups of halophiles, followed by saline and semi-saline environments.

4. Discussion

Halophilic bacteria have high potential in biotechnological processes for at least two reasons. 1: Their activity in natural environments due to their participation in biochemical cycles C, N, S and P, formation and dissolution of carbonates, phosphate immobilization and the production of growth factors and nutrients is significant and 2: their nutritional needs are simple. Most of these microorganisms can use a wide range of compounds as a reservoir of energy and carbon. Most of them are able to grow in high concentrations of salt and also reduce the risk of contamination. In addition, several genetic tools have been developed for non-halophilic bacteria that can be applied to halophiles, making it possible for them to be genetically engineered. The results of studies showed that Halophilic bacteria also have the ability to produce compatible solutes, which are useful for the biotechnological production of smolites. The results of studies showed that examined the extracellular hydrolytic enzymes of halophilic bacteria isolated from subterranean salt rock crystals. Their study showed that the hydrolytic activity for Tween 80 and casein was predominant among isolated strains with a salt concentration of more than 2 M. The presence of combined hydrolytic activities in some isolated strains can be useful for use in some biotechnology activities in various fields of industry or agriculture. (Cojoc et al., 2009).

The results of studies showed that Some compatible salts, especially glycine, betaine, and actin, may be used as stress protectors against high salinity, thermal denaturation, enzyme stabilizers, nucleic acids, membranes, and the whole cell against drought and freezing. Also in research by Annapurna et al., (2012) Evaluated the ability to produce moderate halophilic proteases on Lake Sambhar and the shores of the Mumbai Sea. Casein substrates, blood stains on surgical instruments and hair were used to evaluate protease activity. Enzymes showed the ability to remove blood stains from surgical instruments. Bacillus subtilis isolates had optimum activity at pH 8 and 37°C and 10% NaCl with protease activity on casein and human hair These enzymes can be used in detergent production and leather processing technology (Annapurna et al., 2012). The results of studies showed that The industrial performance of these compounds in enzyme technology is promising. Also in research by Rahban., (2009) Isolated halophilic bacteria producing extracellular hydrolysis enzymes in Lake Hoz Sultan. These isolates produce a wide variety of extracellular hydrolytic enzymes. No halophilic isolates without hydrolytic activity were found in this study. Most lipase and DNase producers were members of the genus Gracilibacillus and Halomonas, respectively. The most isolates capable of producing hydrolytic enzymes such as amylase, protease, cellulase and inulinase belonged to gram-positive bacteria. The results of studies showed that Halophilic bacteria also produce a number of intracellular and extracellular enzymes and antimicrobial compounds that can also be used commercially. Also in research Studied the diversity of medium-sized halophilic bacteria producing extracellular hydrolytic enzymes. Bacterial screening of highly saline environments in southern Spain resulted in the isolation of 122 medium-sized halophilic bacteria. In this study, it was found that moderate halophilic bacteria contain enzymes such as amylase, DNase, lipase, protease and pollanase (Sanchez-Porro et al., 2003). Halophilic bacteria can produce enzymes that have optimum activity at high salinity and are useful for hard industrial processes; The subject of this study was 9 species of halophylls producing 3 enzymes of amylase, protease and lipase were isolated from saline water ecosystems in Golestan province.Golestan province is one of the relatively pristine regions of the country in the field of saline ecosystems that will have a high potential to isolate halophilic microorganisms and study the conditions of enzyme production in them. According to the results of this study, the isolation of halophilic and extreme halophilic bacteria from saline waters, wetlands, seals and geysers of the province and the study of enzymatic profile of amylase, protease, lipase hydrolase in them, can be a kind of process.

New applications in microbial biochemistry and enzymatic metabolites.

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