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Isolation of Halophilic Bacteria and Their Screening for Extracellular Enzyme Production

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Halophiles are those microorganisms which are found at high salt concentration. These microorganisms have the capability to form a wide array of bioactive substances that have different applications in various industries. This work focussed at the isolation and screening of various halophilic bacterial strains from different places in Rajasthan and Tamil Nadu. Using a complete medium with different salt concentrations, 70 halophilic bacteria were isolated from 4 different sites. These isolated bacteria were then examined and screened out for the synthesis of different extracellular enzymes, such as cellulase, lipase and amylase. Out of the total, 24 isolates were found positive for amylase, 9 for lipase and 16 for cellulase. These enzymes can perform the hydrolytic activity at high salt concentrations. The various halophilic bacterial strains isolated from various places show a prospective for use in a range of biotechnological and molecular biology experiments and the extremozymes obtained from these bacteria have great industrial importance.

Keywords: Extracellular-enzymes, Extremophiles, Halophiles, Halophilic-enzymes

Introduction

The microorganisms which have the capability to survive under extreme and harsh environments *i.e.* pH, temperature, salinity, pressure etc. are termed as extremophiles. There are many types of extremophiles each corresponding to different environmental conditions. Appropriately, there is a wide range of extremophiles, each comparing to various conditions in which microorganisms have inhabited. These microorganisms produce various compounds with bioactivity such as antibiotics, enzymes, pigments, etc.[1,2] In industries such as textile, pharmaceutical, food etc., these bioactive compounds have different applications.[3]

Extremophiles are classified into different categories on the basis of different conditions of their habitat. Among various extremophiles, halophiles are found in an environment with high salt concentration. Halophilic microorganisms are capable to withstand and survive under extreme salt concentrations by maintaining a balance between interior and exterior of cell allowing it to overcome osmotic stress.[4] This property of halophiles makes them more important in biotechnological applications. Halophiles are classified as extreme (20–25%), moderate (15–20%) and slight (5–10%) based on their salt tolerance.

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Halophilic microorganisms are the potential manufacturers of different biologically active compounds such as antibiotics, enzymes, pigments, etc. which have various applications in biotechnological industries.[5] Halophilic enzymes have developed particular characteristics that give them stability and solubility at high salt concentration. Extracellular enzyme from halophiles has not yet been widely studied.[6]

In recent years, much interest has been generated in a large variety of extracellular enzymes and other substances produced by halophilic microorganisms, such as DNases, proteases, lipases, pullulanases, amylases and cellulases because of their potential uses in industries and biotechnological application.[7,8] These halophilic enzymes are not only resistant to high salt concentration but can also withstand and work efficiently over a range of pH and they are also resistant to elevated temperatures. Enzymes isolated from halophiles have different properties from conventional enzymes. Therefore they offer important applications, like environmental bioremediation, food industry, and waste-water treatment.[9,10]

Salt Lake are the most suitable naturally occurring environment for halophilic bacteria. There are limited cases on the production of extracellular enzymes formed by bacteria isolated and cultured from Sambhar Lake[11] and Dairy waste.[12] However, the sites of Didwana salt

pan and Kaliveli Lake are still unexploired. So, it will be of great importance to isolate different extracellular enzyme producing microbes from these sites. This work focuses at isolation of halophilic bacteria from different sites and their screening for the different hydrolytic extracellular enzyme.

Materials and Methods

Sampling Sites

Sampling was done from different sites located in Rajasthan and Tamil Nadu for the isolation of halophilic bacteria. Samples were collected in sterile Petri-dishes and stored at low temperature in a refrigerator. The samples were collected from:

(a) Didwana salt pan – (soil sample) - Didwana (27.4°N 74.57°E) is a city in the Nagaur district of Rajasthan. The salt pans of Didwana also support commercial salt production.
(b) Sambhar lake – (soil sample)- Sambhar Salt Lake (26° 58′ 0″ N, 75° 5′ 0″ E) is India's biggest inshore salt lake that is located at a distance of 80 km south-west of Jaipur city and 64 km north-east of Ajmer, Rajasthan. It is the main source of Rajasthan salt production.
(c) Dairy – (waste-water sample)- It is Saras Dairy (Jaipur Dairy) located in Jaipur, Rajasthan. It has an effluent treatment plant from where the sample was collected.
(d) Kaliveli salt pan– (soil sample)- Kaliveli lake (12° 7′ 11.02″ N, 79° 51′ 27.66″ E) is located between Chennai and Pondicherry. It is the second-largest natural lake in southern India.

Isolation of Halophilic Bacteria

Isolation of halophiles was done on complete media[13] containing-Potassium dihydrogen phosphate (10 gm/l), glucose (10 gm/l), peptone (5 gm/l), yeast extract (5 gm/l) agar (15 gm/l) NaCl (10–30%), pH (7.0–7.4). Bacterial isolation was carried out by process of serial dilution (up to 10^{-7}) and also by direct sprinkling of soil samples on culture media. The petri-plates containing the sample were then incubated at 37°C. Bacterial colonies were observed after 24, 48 and 72 hours were streaked on medium with respective salt concentrations (10%, 15%, 20%, and 25%) for isolation of pure culture.

Screening of Halophilic Isolates for Extracellular Enzymes

Screening for Amylase

Screening of amylase producing halophilic bacteria was done by starch hydrolysis test on starch agar medium (gm/100 ml): yeast extract (0.5), starch (0.2), peptone (1.0), agar (3.0) and NaCl (10–25) (according to respective bacteria isolated). The bacterial isolates were streaked on petri-plates containing starch agar medium and these plates were incubated at 37°C for 48 hrs. After the 48 hours of incubation, the plates were flooded with Gram's iodine, presence of a clear zone around bacterial colonies indicated hydrolysis of starch.[14]

Screening for Lipase

Lipase producing strains from isolated halophilic bacteria were screened using medium described by Kumar *et al.*[15] (Agar 2.5 gm/100 ml, Tween 80 2%, Victoria blue 0.01%). The isolated bacterial cultures were inoculated on the medium and plates were incubated for 48 hours at 37°C in an inverted position. Bacteria that were producing lipase enzyme were showing a zone of hydrolysis around bacterial growth.

Screening for Cellulase

Cellulase activity of halophilic bacterial samples was assessed by screening for the formation of zone of hydrolysis around the bacterial isolates cultivated on CMC (Carboxymethyl cellulose) agar plates containing, KH_2PO_4 (0.5 gm/l), $MgSO_4$ (0.25 gm/l), CMC (2 gm/l), agar (15 gm/l), gelatin (2 gm/l) and NaCl (according to bacteria isolated on).[16] After incubation for 48 hours, 1% solution of congo red was flooded on plates.

Morphological Characterization

Isolated halophilic bacteria which were showing hydrolytic activity for enzymes were morphologically characterized by the colour of the colony, microscopic structure of the cell and Gram staining.[17]

Biochemical Characterization

Isolated halophilic bacteria showing enzymatic activity, the best activity showing bacteria were selected for biochemical characterization. Selected isolates were tested with various biochemical test viz. citrate utilization, MR-VP test, catalase, oxidase, indole, urease, nitrate reduction test and gelatin liquefaction, carbohydrate fermentation test, etc.[18]

Results and Discussion

In our study, the isolation of halophilic bacteria resulted in a total of 70 halophilic bacterial isolates from four different sites of India which includes hypersaline lakes, salt pans and industrial waste. Rohban *et al.*[19] reported halophilic/salt tolerant bacterial strains isolated and screened from various region of Howz Soltan playa (Iran). Cojoc *et al.*[20] isolated halophilic bacteria from a subterranean rock salt crystal (Romania). There are also

some reports on segregation of various bacteria capable to withstand high concentration of salt from different parts of India, Jayachandra *et al.*[21] from the west coast of Karnataka, Khunt *et al.* [13] wild ass excreta from Rann of Kutch (India). The number of halophilic bacteria isolated from different sites in our study is given in Table 1.

Screening for Extracellular Enzymes

These bacterial isolates were screened for different hydrolytic enzymes. The results of screening for extracellular enzymes and Gram's staining of the bacteria as given in Table 2 shows positive results in screening. Isolated bacteria are designated as "Dw" for Didwana salt pan (Dw10, Dw15, Dw20 and

Table 1 — Number of halophilic bacterial isolates from different sampling sites

Sampling site	No. of halophilic bacterial isolates
Didwana salt pan (Dw)	25
Sambhar lake (Sb)	20
Saras dairy (D)	10
Kaliveli salt pan (S)	15

Table 2 — Morphological characteristics, Gram reaction and extracellular enzymes produced by different halophilic bacterial isolates: Didwana Salt pan, Sambhar Lake, Saras Dairy, and Kaliveli Salt pan

Sampling site	NaCl Conc.	Morphology		Gram's staining reaction	Production of Amylase	Production of Lipase	Production of Cellulase
		Colony colour	Cell shape				
Didwana Salt pan							
Dw10-1	10%	Cream	Bacilli	+			++
Dw15-1	15%	White	Cocci	−	++	+	
Dw15-2	15%	White	Cocci	−	+		
Dw20-1	20%	Cream	Cocci	−			
Dw20-7	20%	Cream	Bacilli	+	+	++	
Dw20-8	20%	Cream	Cocci	−			
Dw20-11	20%	White	Bacilli	+	++		
Dw20-14	20%	Orange	Cocci	+	+		+
Dw20-15	20%	White	Cocci	+	++	++	
Dw25-1	25%	Pink	Cocci	−	++		+
Dw25-2	25%	Pink	Cocci	−	++		
Dw25-3	25%	Pink	Cocci	−	++		
Dw25-4	25%	Pink	Cocci	−	++		+
Sambhar Lake							
Sb10-1	10%	Cream	Bacilli	+			++
Sb10-4	10%	Cream	Cocci	+			++
Sb10-6	10%	Cream	Cocci	−			+
Sb15-1	15%	White	Cocci	−		++	
Sb15-3	15%	White	Cocci	+			+
Sb15-4	15%	White	Bacilli	+	+		
Sb15-8	15%	Cream	Cocci	+	+		
Sb15-10	15%	Cream	Cocci	−	+		
Sb15-11	15%	Pink	Bacilli	+	+		
Sb20-1	20%	White	Cocci	−		++	
Sb20-3	20%	Cream	Cocci	+	+		
Sb20-4	20%	White	Cocci	+		++	
Sb20-6	20%	White	Bacilli	+	+	++	
Sb20-8	20%	Cream	Cocci	+	++		
Saras Dairy							
D10-1	10%	White	Bacilli	+	++		
D10-5	10%	White	Bacilli	+	+++		++
D10-6	10%	White	Cocci	−			++
D10-7	10%	White	Bacilli	+	+++		++
Kaliveli Salt pan							
S10-1	10%	White	Cocci	−			++
S10-3	10%	White	Bacilli	+	++	++	+
S10-4	10%	Cream	Cocci	−	++		
S10-5	10%	White	Bacilli	+	+		
S10-6	10%	Cream	Bacilli	+	+++	++	+
S15-16	10%	White	Cocci	−			+
S15-18	15%	White	Cocci	+			+

Dw25), "Sb" for Sambhar lake (Sb10, Sb15 and Sb20), "D" for Saras dairy (D10 and D15) and "S" for Kaliveli salt pan (S10, S15 and S20). The "10", "15", "20" and "25" are labelled as the salt concentration at which they were isolated and the last number is the isolate number in that series. For example, Dw20-7 is the 7th isolate from 20% salt concentration (in the medium) of the Didwana salt pan sample.

The data from Table 2 confirms the extracellular hydrolytic activity of different isolated halophilic bacteria. Out of the total of 70 isolates, 24 showed amylase activity. The positive result of primary screening for amylase enzyme is shown in Fig. 1(a). Out of these 24-amylase positive bacterial strains, 10 were isolated from Didwana Salt Pan, 3 from Dairy waste, 7 from Sambhar Salt Lake and 4 from Kaliveli Lake. Out of these 24 amylase positive bacteria, 7 were isolated at 10%, 6 at 15%, 7 at 20% and 4 at 25% salt concentration. It was found that most of the isolated amylase positive bacteria showed Gram's positive reaction (16 Gram- positive and 8 Gram -negative); 13 were cocci in shape and eleven were bacilli in shape.

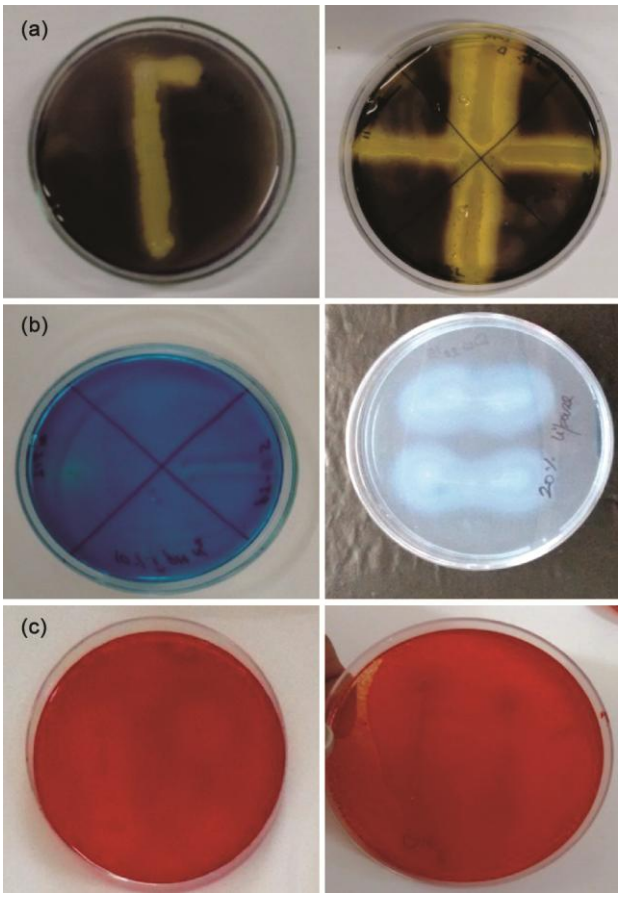

Fig. 1 — Petri plates showing positive results of screening for halophilic bacteria producing extracellular enzymes: (a) positive results of amylase, (b) positive results for lipase, (c) positive results for cellulase

Out of the total of 70 isolated halophilic bacteria, only 9 bacteria showed lipase activity. In Fig. 1(b) positive results of primary screening for lipase enzyme are shown. Of the 9 bacterial colonies which were lipase positive, 3 were isolated from Didwana Salt Pan, four from Sambhar Salt Lake and 2 from Kaliveli salt pan. None of the bacterial isolates from Dairy waste showed lipase activity. These lipase producers were isolated at different salt concentrations, out of nine 2 were isolated at 10%, 2 were at 15% and 5 were at 20% salt concentration. Bacteria which were showing lipase activity, 6 were Gram-positive and 3 were Gram-negative; 4 were found bacilli and 5 were cocci in shape.

Only 16 halophilic bacteria showed cellulase activity out of the total of 70 isolated halophilic bacteria. The positive result of cellulase enzyme primary screening is shown in Fig. 1(c). Among these cellulase positive bacteria, 4 were isolated from the Didwana salt pan, 4 were from Sambhar Salt Lake, 3 from Dairy waste and 5 from Kaliveli salt pan. These cellulase positive bacteria were isolated at the different salt concentrations, 10 were isolated at 10%, 3 at 15%, 1 at 20% and 2 at 25% salt concentration. Among these cellulase positive bacteria, 10 showed Gram's positive reaction and 6 showed Gram's negative reaction; 6 were bacilli in shape and 10 were cocci in shape.

A total of 49 bacteria were found positive for enzymes amylase lipase and cellulase. It was also found that among these bacteria, 11 were found to be positive for two or all the three screened enzymes. It was also observed that among the potential halophiles, the number of amylase producers were comparatively higher than for the other enzymes under study.

Similarly, Cojoc *et al.*[20] isolated enzyme-producing halophilic bacteria from subterranean rock salt crystal (Romania) and reported 3 amylases, 12 lipases and 2 cellulases. Their study also supports the multiple enzymatic activities of these bacteria. In a similar study, Rohban *et al.*[19] tested the different hydrolytic activity of isolated halophiles from Howz Soltan Lake, Iran and reported 177 amylase, 195 lipase and 65 cellulase producing bacteria. Their study also supports multiple hydrolytic activities as detected in our study. Khunt *et al.*[13]

Table 3 — Biochemical characterizations of 5 potential enzyme producers from halophilic bacterial isolates

Isolate / Test	S10-3	Sb10-7	D10-5	Dw20-15	Dw25-3
Motility	Non-motile	Motile	Non-Motile	Non-motile	Non-motile
Catalase test	+	+	+	+	+
Nitrate reductase test	+	+	+	−	−
H_2S production	−	−	−	−	−
Voges-Proskauer test	+	−	+	−	−
Oxidase test	−	+	−	−	−
Indole test	−	−	−	−	−
Gelatin hydrolysis	−	−	+	−	−
Casein hydrolysis	+	−	−	−	−
Urease test	+	−	+	+	−
Methyl red Test	−	−	−	−	−
Citrate utilization	+	−	+	−	−
Sugar utilization test					
Fructose	+	−	+	−	−
Dextrose	+	−	+	−	−
Glucose	+	+	+	−	−
Sucrose	+	−	+	−	−
Galactose	−	−	−	−	−

reported 8 amylases producing and 7 lipase producing halophilic bacteria from wild ass excreta, Rann of Kutch (India). Similarly, Karray *et al.*[22] isolated 4 amylases producing, 7 lipase and 11 cellulase producing halophilic bacteria from a Salt Lake in the south of Tunisia.

Biochemical characterization

Biochemical characterization of the 5 best potential enzyme producers from halophilic isolates was done. All the results of biochemical tests are given in Table 3.

The biochemical tests include various tests like catalase, citrate utilization, H_2S production, motility, urease, nitrate reduction, sugar fermentation etc. It was observed that all the tested halophilic bacteria have the capacity to produce catalase enzyme, suggesting their aerobic nature. The bacterial isolates, S10-3 and D10-5 showed positive results for nitrate reductase, citrate utilization, urease, Voges-Proskauer test and sugar utilization test. A similar type of result was also obtained by various investigators.[13,23,24] None of the tested bacterial isolates was found to be positive for H_2S production, indole test, Methyl red Test. These biochemical tests show different properties of bacteria and are also used for identifying bacteria according to Bergey's manual of systematic bacteriology.

Conclusions

In the present study a total of 70 bacterial isolates were found in different salt concentrations. Twenty-five halophilic bacterial isolates were derived from Didwana Salt pan; 20 from Sambhar Salt Lake, 10 from Saras Dairy and 15 from Kaliveli Salt pan. Out of these 70 halophilic isolates, 24 were showing amylase activity, 9 showed lipase activity and 16 showed cellulase activity at the specific salt concentrations. These bacterial isolates were showing hydrolytic activity at high salt concentrations. Therefore, the halophilic bacteria isolated from the above-mentioned sites are potential producers of different halophilic extracellular enzymes which can be used in future for industrial and biotechnological purpose.

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References

1 Gupta D G, Srivastava D S, Khare S, Prakash V, Extremophiles: an overview of microorganism from extreme environment, *Int J Agric Environ Biotechnol*, **7** (2014) 371–380.

2 Dumorné K, Camacho Córdova D, Astorga-Eló M & Renganathan P, Extremozymes: a potential source for industrial applications, *J Microbiol Biotechnol*, **27** (2017) 649–659

3 Espliego J M E, Saiz V B, Torregrosa-Crespo J, Luque A V, Carrasco M L C & Pire C, "Extremophile enzymes and

biotechnology," in *Extremophiles* (Boca Raton, FL: CRC Press), 2018, 227–248.

4 Ramírez D N, Serrano J A & Sandoval H, Microorganismos halófilos, Actinomicetos halófilos de México, Rev Mex Cienc Farm, **37(3)** (2006) 56–71.

5 Oren A, Industrial and environmental applications of halophilic microorganisms, *Environ Technol*, **31** (2010) 825–834.

6 Mellado E, Sánchez-Porro C, Martín S & Ventosa A, Extracellular hydrolytic enzymes produced by moderately halophilic bacteria, *Halophilic Microorganisms*, (2004) 285–295.

7 Jaeger K-E & Eggert T, Lipases for biotechnology, Curr Opin Biotechnol, **13** (2002) 390–397.

8 Pérez-Pomares F, Díaz S, Bautista V, Pire C, Bravo G, Esclapez J, Zafrilla B & Bonete M, Identification of several intracellular carbohydrate-degrading activities from the halophilic archaeon *Haloferax mediterranei*, *Extremophiles*, **13** (2009) 633–641.

9 DasSarma P, Coker J A, Huse V & DasSarma S, Halophiles, Industrial Applications, *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation and Cell Technology*, (2010).

10 Munawar N & Paul C Engel, Halophilic enzymes: characteristics, structural adaptation and potential applications for biocatalysis, *Curr Biotechnol*, **2** (2013) 334–344

11 Kumar S, Karan R, Kapoor S, Singh S P & Khare S K, Screening and isolation of halophilic bacteria producing industrially important enzymes, *Brazilian J Microbiol*, **43(4)** (2012) 1595–1603.

12 Garcha S, Verma N & Brar S K, Isolation, characterization and identification of microorganisms from unorganized dairy sector wastewater and sludge samples and evaluation of their biodegradability, *Water Resour Ind*, **16** (2016) 19–28.

13 Khunt M & Pandhi N, Moderate halophilic bacterial community in excreta of wild ass (*Equus hemionus* Khur). *Int J Biosci*, **1(5)** (2011) 31–37.

14 Gupta R, Gigras P, Mohapatra H, Goswami V K & Chauhan B, Microbial α-amylases: A biotechnological perspective, *Process Biochem*, **38** (2003) 1599–1616.

15 Kumar S, Arunasri R, Jayachandra Y & Sulochana, Screening of extracellular hydrolytic enzymes from *Marinobacter hydrocarbonoclasticus* Strain Ak5, The Bioscan, **5(1)** (2010) 97–99.

16 Sreedevi S, Sajith S & Benjamin S, Cellulase producing bacteria from the wood-yards on Kallai river bank, *Adv Microbiol*, **3(4)** (2013) 326–332.

17 Shirling E B & Gottlieb, Methods of characterization of Streptomyces species, *Int J Syst Bacteriol*, **61(3)** (1966) 313–340.

18 Cappuccino J G & Sherman N, *Microbiology- A Laboratory Manual*, 7th Edition California, (The Benjamin/Cummings Publishing Company Inc) 1992, 77.

19 Rohban R, Amoozegar M A & Ventosa A, Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran, *J Ind Microbiol Biotechnol*, **36(3)** (2009) 333–340.

20 Cojoc R, Merciu S, Popescu G, Dumitru L, Kamekura M & Enache M, Extracellular hydrolytic enzymes of halophilic bacteria isolated from a subterranean rock salt crystal, *Rom Biotechnol Lett*, **14** (2009) 4658–4664.

21 Jayachandra S, Kumar S A, Shouche Y & Sulochana M, Culturable diversity of extremely halophilic bacteria from west coast of Karnataka, India, *Int J Biol Pharm Allied Sci*, **2(2)** (2013) 391–405

22 Karray F, Abdallah M B, Kallel N, Hamza M, Fakhfakh N & Sayadi S, Extracellular hydrolytic enzymes produced by halophilic bacteria and archaea isolated from hypersaline lake, *Mol Biol Rep*, **45(5)** (2018) 1297–1309.

23 Gupta S, Sharma P, Dev K, Srivastava M & Sourirajan A, A diverse group of halophilic bacteria exist in Lunsu, a natural salt water body of Himachal Pradesh, India, *Springerplus*, **4** (2015) 274.