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Research Article

Isolation, Identification and Characterization of Microorganisms Isolated from Unexplored Saline Regions of Kutch, Gujarat, India

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Abstract

The present investigation reveals the diversity of bacteria, fungus and archaea along the different regions of Kutch area, Gujarat, India. The samples were collected from 3 selected site of Kutch area whose temperature was between 37 - 41°C, pH 8.5, EC 1480 µs, Salinity 9.01 and 1.85 ppm TDS. 52 bacterial, 17 extreme halophilic archaea and 2 halophilic fungus strains were isolated, among these all bacterial strains were selected for further study like enzyme productivity and anti-microbial activity because the main aim of research was to isolate organism which have multiple applications. Fifteen strains produced four or more than four enzymes which have industrial application (amylase, protease, cellulose, lipase, and chitinase) and one strain of Micrococcus showed antimicrobial activity against all test cultures. Eighteen archaea and two halophilic fungi that grew at 25% salt containing medium were also isolated.

Keywords: Bacteria; Fungus; Archaea; Kutch; Enzyme; Antimicrobial Activity; Salt Concentration

Introduction

Rann of Kutch is a salt marsh in the state of Gujarat, India. It can be divided into the Great and The Little Rann of Kutch. While the Great Rann of Kutch converts to a "White Desert" in the summer with thin layer of salt crust which becomes marshy island during rainy season, little Rann of Kutch represents salt crystallizers of India. On the whole the ecosystem of the Rann is largely undisturbed and would be the reservoir of myriad of microorganisms ranging from normal micro flora to extreme organisms which can thrive in the ever fluctuating harsh saline environments. Such places are always fascinating for understanding the evolution and survival of microbes in such harsh environments.

Lot of work has been done in terms of diversity studies in hyper saline systems. Ventosa [1] have worked on halophiles from unusual habitats, *Bacillus iranensis sp*. was isolated from saline mud of the hypersaline lake Aran-Bidgol in Iran [2]. In the Indian scenario hypersaline coastal regions of Gujarat, Maharashtra, Rajasthan and Tamil Nadu have been well studied and several Actinobacteria and Halophiles reported [3]. Halophilic Archaebacteria have been reported from desert of Rajasthan (Upasani 2007). Dave and Soni [4] have reported isolation of Halophilic and Alkalophilic bacteria from coastal regions of Gujarat. Thirteen bacterial strains were isolated from Kharaghoda area of Gujarat [5]. More than fifteen isolates were isolated from the different hyper saline soil of Little Rann of Kutch, Gujarat and most of them are used as biofertilizer [6].

Rann of Kutch has also been exploited for the isolation of Extremophiles. Notable studies include those involving isolation of extreme halophiles [7], Halophilic archaebacteria [8], halo tolerant alkaliphilic *Streptomyces* [9], moderately halophilic bacteria (Dey 2013). All these studies focus on the isolation of microbes using traditional methodology. A very interesting study of Pandit., *et al.* [10] revealed a diversity of 56 to 87% Bacteria and 8 to 40% Archaea using metagenomics which opened immense possibilities of further work.

In view of the work of Pandit., *et al.* [10] regarding the immense diversity and presence of genes of industrially important enzymes, this study was formulated to tap the diversity of Rann of Kutch for isolation of industrially important microbes specially focusing on those microbes which have multiple potential of bio-prospecting.

Thus, in this study undisturbed and unexplored soil samples were collected from three locations of Kutch (Sangur village near khavda, Mandvi beach and Great Rann of Kutchh). Microbial isolations were performed for isolation of bacteria, archaea and actinobacteria. These microbes were identified at the molecular level.

They were then tested for the antimicrobial potential and the ability to produce enzymes including Amylase, Protease, Cellulase, Lipase, and Chitinase [11-28].

Materials and Methods

Collection of soil samples

Soil samples were collected from different locations on the basis of differences in their physical parameters and to avoid recurrence of identical cultures. The collection sites includes Mandvi beach (N 22. 82513 E 69.33753), and Great Rann of Kutchh (N 23. 82763 E 69.52002) in Kutch area of Gujarat from the depth of 15 cm in sterile polythene bags and air tight container with the help of sterile spatula and were transported to laboratory for further processing.

Enrichment and isolation

At first, the soil slurry was made by suspending 1g of the collected dry soil in 10 ml distilled water. The slurry was mixed by vortexing for 1 minute, the coarse particles were allowed to settle and serial dilutions were made till 10^{-8}. 100 µl aliquots of dilutions 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} were spread on Starch casein Agar, Nutrient Agar and HV-YPC media in triplicates (A, B and C). All spread plates were incubated at 30°C for a period of 14 days. On day 14, colonies formed were observed and morphologically different colonies were subculture on Starch Casein Agar and HV-YPC media for further characterization and studies.

DNA amplification, sequencing and phylogenetic analysis

Genomic DNA was isolated from the isolates using Qiacube following the protocol given by the manufacturer. Lysis of cultures and chloroform wash were done manually and then the supernatant was subjected to column purification step, washing and elution in Qiacube. The eluted DNA was directly subjected to PCR amplification. Amplification of bacterial isolates was done using 16s universal primers 8F (5'3') and 1492R using touchdown PCR. Similarly, for amplification of Archaea, 21f and 958R were used and fungal DNA was amplified by ITS 1 and ITS 4. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence. PCR products were purified using EXOSAP. Cycle sequencing of purified PCR products was performed using BIGDYE employing same primers except for bacteria, where 704F and 907R primers were used. Cycle sequencing products were purified by using X-terminator and Sam solution. Sequencing was done using the 3500 genetic analyser from Applied Biosystems. Compilation of the individual sequences obtained was done using BIOEDIT software. Sequence homology was done using NCBI BLAST and the organisms were identified. Phylogenetic analysis was performed according to neighbour joining methods using MEGA version 7.0.

Enzymatic study

The isolates were screened for production of different enzymes using their respective media as mentioned in table 1. For this purpose, cultures were spot inoculated on assay plates and incubated for 4 - 7 days.

Name of Enzymes	Media	Method
Amylase	Minimal medium with starch	Clearing around the growth after flooding with iodine solution
Protease	Casein skim milk medium	Clearing around the growth
Cellulase	Carboxymethyl Cellulose medium	Clearing around the growth after flooding with iodine solution
Lipase	Tributyrin Agar media	Clearing around the growth
Chitinase	Chitin Agar media	Clearing around the growth

Table 1

Experiment was performed in triplicates. After incubation, plates were observed for zone of clearance due to enzyme activity.

Antibiogram analysis

This screening was done for 52 bacterial isolates. Analysis was done using crude extract obtained by spinning the culture broth at 5000 rpm for 2 minutes. The assay was conducted by agar well diffusion method (Perez., *et al.* 1990). Test cultures i.e. two gram positive and two gram negative were first spread on nutrient agar plates. Wells were punched using cup-borer and filled with 50 µl culture supernatant. Plates were incubated at 30°C for 24 hours. Sterile nutrient broth was taken as control. The plates were incubated over night at 30°C in incubator. The antimicrobial activity of microbes was analyzed by presence of inhibition zones.

Results

Physico-chemical properties of the samples

Both soil and water samples were collected from the hypersaline environment of Kutch. The color of water sample was light red to deep red indicating archaeal population. And the muddy soil with dark brown color was collected. The concentration of NaCl in majority of the water samples collected from hypersaline environments of the Kutch was near saturation, indicating the extremity of the environment. The pH of the samples varied from location to

location and ranged from 8.70 to 13.8. The total dissolved solids (TDS) of brine samples varied from 1.10 to 1.85 ppm, electrical conductivity (EC) of water sample varied from was 148 mS cm^{-1} and that of soil or mud sample was 118 mS cm^{-1}. The temperature of samples ranged from 20 to 26°C. The properties of the both samples is mentioned in the table 2.

Sample No.	Sample 1	Sample 2
Latitude	N 23. 82763	N 22.82513
Longitude	E 69.52002	E 69.33753
pH	13.8	8.70
Salinity	10.2	8.01
EC	148.0mS cm^{-1}	118.7 mS cm^{-1}
TDS	1.85ppm	1.14 ppm
Temperature	26.6°C	20.0°C

Table 2

Isolation of the microorganisms

There were numerous presences of microbes but the organisms with different cultural characteristics were selected. In case of extreme halophiles only colored colonies were counted. The prevalence of hard, sticky colonies was also observed which varied from light pink to cream. Two fungal cultures were also isolated which were found to be grown on the media.

A total of 71 isolates have been obtained in this study including bacteria, halophilic bacteria and archaea. Halotolerant Fungi have also been isolated which could grow on media containing 25% salt (Table 3).

Sr. No.	Microorganisms	Number of Isolates
1	Bacteria	48
2	Archea	13
3	Halophilic bacteria	08
4	Halotolerant fungi	02

Table 3

Identification of isolates based on 16S rRNA sequencing

Identification of isolates on the basis of 16S rRNA sequencing revealed the presence of many different groups of microbes ranging from Bacteria to Archea. Including some common generas like *Bacillus, Micrococcus, Staphylococcus, Providencia, Brachybacterium, Virgibacillus, Halobacillus, Haloarcula, Chromohalobacter, Heloferax, Halobacterium, Aspergillus*. Table 4 Shows the name of organisms along with their NCBI accession numbers.

Name of Organism	NCBI Accession number
Micrococcus luteus	KY681702
Virgibacillus chiguensis	KY681703
Kokuria palustris	KY681704
Bacillus sonorensis	KY681705
Bacillus infantis	KY681706
Bacillus cereus	KY681707
Bacillus anthracis	KY681708
Haloferax alexendrinus	KY681709
Pseudomonas putida	KY681710
Bacillus foraminis	KY681711
Micrococcus aloeverae	KY681712
Staphylococcus hominis	KY681713
Bacillus licheniformis	KY681714
Bacillus firmus	KY681715
Bacillus anthracis	KY681716
Staphylococcus epidermis	KY681717
Bacillus licheniformis	KY681718
Bacillus oceanisediminis	KY681719
Virgibacillus	KY681720
Bacillus firmus	KY681721
Bacillus siamensis	KY681722
Bacillus velenzensis	KY681723
Bacillus licheniformis	KY681724
Bacillus cereus	KY681725
Staphylococcus epidermis	KY681726
Bacillus endophyticus	KY681727
Bacillus endophyticus	KY681728
Bacillus anthracis	KY681729
Virgibacillus pantothenticus	KY681730
Providencia stuartii	KY681731
Bacillus subtilis	KY681732
Staphylococcus epidermis	KY681733
Bacillus licheniformis	KY681734
Bacillus licheniformis	KY681735
Bacillus licheniformis	KY681736
Bacillus flexus	KY681737
Bacillus thuringiensis	KY681738
Bacillus amyloliquifaciens	KY681739
Bacillus subtilis	KY681740
Brachybacterium paraconglom	KY681741
Micrococcus luteus	KY681742
Micrococcus endophyticus	KY681743

Bacillus subtilis	KY681744
Bacillus lichenifromis	KY681745
Bacillus licheniformis	KY681746
Staphylococcus sp.	KY681747
Haloferax sp.	KY681748
Haloferax sp.	KY681749
Haloferax volcanii	KY681750
Halobacterium salinarum	KY681751
Halomonas elongate	KY681752
Chromohalobacter salexigens	KY681753
Halobacterium salinarum	KY681754
Haloferax alexendrinus	KY681755
Halobacterium salinarum	KY681756
Haloferax lucentense	KY681757
Halomonas elongate	KY681758
Halomonas elongata	KY681759
Halobacterium salinarum	KY681760
Haloarcula amylolytica	KY681761
Paracoccus sp.	KY681762
Bacillus korlensis	KY681763
Bacillus sp.	KY681764
Bacillus amyloliquifaciens	KY681765
Arthrobacter sp.	KY681766
Halobacillus sp.	KY681767
Arthrobacter sp.	KY681768
Haloferax alexandrinus	KY681769
Halobacterium salinarum	KY681770
Aspergillus versicolor	KY681771
Aspergillus parasiticus	KY681772

Table 4

Enzymatic activity

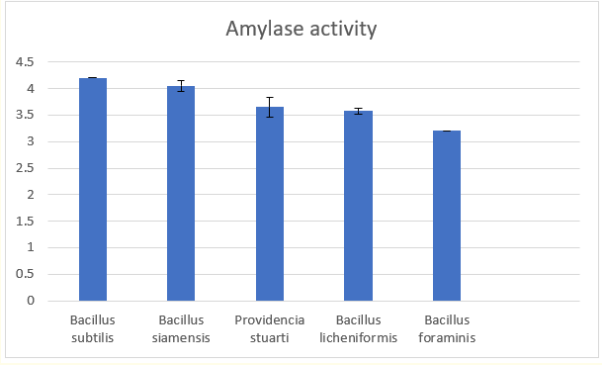
The bacterial isolates were screened for enzymatic and antimicrobial activities. Enzyme screening included industrially important enzymes like Amylase, Lipase, Protease, Cellulase and Chitinase. Maximum isolates were positive for protease activity and minimum number was for Chitinase enzyme (Table 5). The organisms showing maximum enzyme activity was analyzed (Graph 1 to 5).

Antimicrobial activity

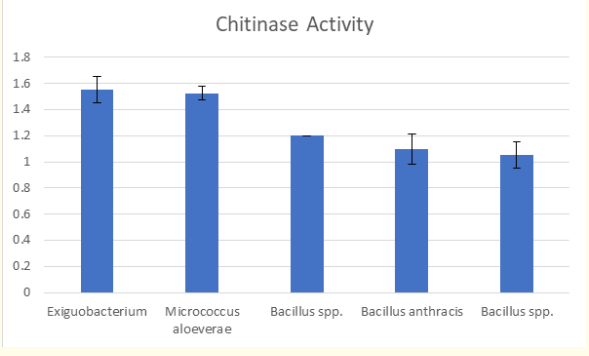
Total 48 bacterial cultures were screened for the antimicrobial activity. The activity was observed against two Gram negative and two Gram Positive test organisms. Maximum number of organisms

Sr. No.	Enzymes	Number of organisms showing Positive Result
1	Amylase	36
2	Protease	40
3	Lipase	33
4	Cellulase	38
5	Chitinase	19

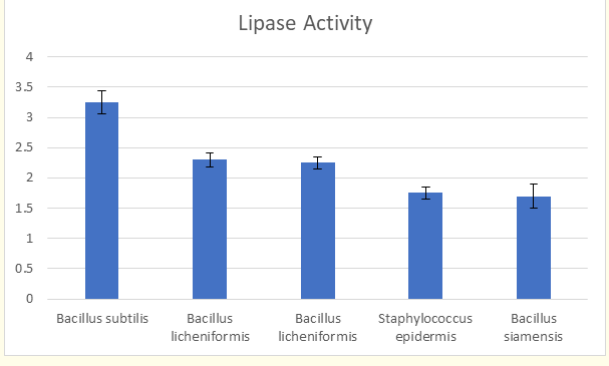
Table 5



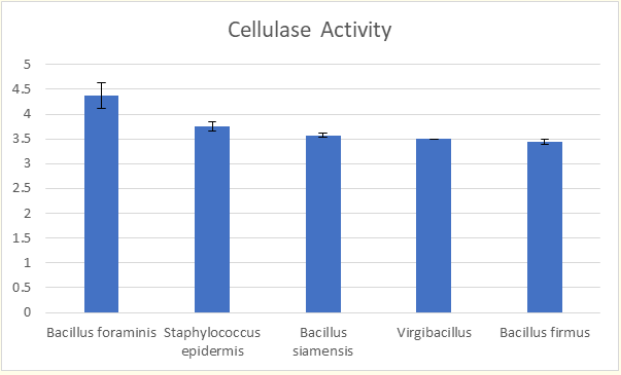
Graph 1: Shows organisms showing maximum amylase activity.



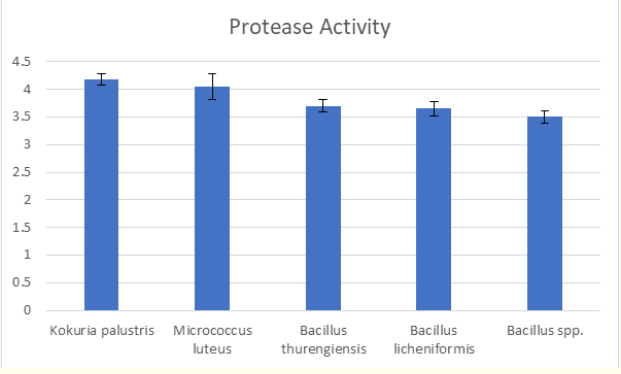
Graph 2: Shows organisms showing maximum chitinase activity.



Graph 3: Shows organisms showing maximum lipase activity.



Graph 4: Shows organisms showing maximum cellulase activity.



Graph 5: Shows organisms showing maximum protease activity.

were able to show positive activity against *Bacillus subtilis* and Minimum number of organisms were able to show Positive activity against *Enterobacter cloacae*.

Sr No.	Name of Test Organisms	No. of org. showing Activity
1	*Staphylococcus aureus*	10
2	*Bacillus subtilis*	47
3	*Pseudomonas aeruginosa*	06
4	*Enterobacter cloacae*	01

Table 6: Shows number of organisms showing antimicrobial activity against two gram positive and two gram negative pathogenic strains.

Discussion and Conclusion

In this study, we provide a preliminary description of a complex community of Microorganisms associated with saline soil of Kutch Gujarat. A total of 71 isolates have been obtained in this study including bacteria, halophilic bacteria and archaea. Halotolerant Fungi have also been isolated which could grow on media containing 25% salt.

The bacterial isolates were screened for enzymatic and antimicrobial activities. Enzyme screening included industrially important enzymes like Amylase, Lipase, Protease, Cellulase and Chitinase. Maximum isolates were positive for protease activity and minimum number was for Chitinase enzyme. The results of Amylase has wide range of industrial applications such as food, fermentation, and pharmaceutical industry due to its role in hydrolysis of starch molecules into glucose polymers. Protease is also one of the industrially important enzymes which are mainly used in Detergent, Tannery, Food, Metal recovery and wastewater treatment like industries. Lipase has industrial role in Pharmaceutical, Food, Detergent, Textile, Leather, and Cosmetic like industries. Cellulase is mainly used in pulp and paper, Textile, Bioethanol, Wine and Brewery, Food processing, Agricultural, and animal feed industries. Chitinase has mainly agricultural use chitinase producing organisms are used as bio-fertilizers. As the given organisms have been isolated from the hypersaline soil so they can be used as effective bio-fertilizers in saline regions.

Some of the isolates show the ability to produce multiple enzymes out of 52 bacterial isolates 10 organisms had shown the ability to produce all five enzymes. And 21 isolates had shown the ability to produce four enzymes.

Antimicrobial agents are widely used as pharmaceutical products and they have also been used in bio- pesticides. Many isolates have even shown the antimicrobial activity against both gram negative and gram positive test organisms. Out of 52 bacterial isolates 51 organisms have shown positive activity against *Bacillus subtilis*, 11 have shown positive activity against *Staphylococcus aureus*, 6 have shown positive activity against *Pseudomonas aeruginosa* and 1 have shown positive activity agent *Enterobacter cloacae*.

From these organisms one organism have also been reported which has ability to produce all 5 enzymes as well as it has shown antimicrobial activity against all sensitive test cultures. That organism has been identified as *Micrococcus aloeverae* (KY681712). With the use of this multiple ability organism many of the industrial queries can be resolved as many benefits can be obtained from a single organism having both economical and industrial benefits.

Kutch metagenomic studies have shown the presence of wide range of microbes from which few have been isolated and identified in the present study it also reveals the presence different enzymes so those organisms which are still uncultured can be cultured and many different industrially important enzymes can be obtained.

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