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ISOLATION AND MOLECULAR IDENTIFICATION OF HALOPHILIC ARCHAEA (Part –I) FROM SALINE SOIL

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ABSTRACT

Prokaryotics taxonomy is consider as a dynamic science**.** A handful of soil may contain many prokaryotic species. Due to molecular systematic, which led to the splitting of prokaryotes into bacteria and archaea. As molecular systematists continue to work on the phylogeny of prokaryotes. The use of polymerase chain reaction (PCR) has allowed for more rapid sequencing of prokaryote genomes. Archaea are highly diverse with respect to morphology, physiology, reproduction and ecology. As they are best known for growth in anaerobic, hypersaline, pH extremes, and high temperature habitats. Also found in marine arctic temperature and tropical waters. The research work in this is divided in two stages 1) Isolation and characterization of halophilic archaea 2) Phylogenetic identification of the haloarchaeal isolates. Section I is the introduction part Section II describes materials and methods for isolation, morphological and cultural characterization, cultivation and phylogenetic analysis of isolates. In Section III the experimental results showing cultural characteristics, growth requirements, phylogenetic trees and discussion with conclusion is included.

KEYWORDS: Extremophiles, Archaea, Osmolytes, Phylogenetic Analysis.

1. INTRODUCTION

All organisms in this biosphere are either directly or indirectly depend on microbial activities. So microorganisms are consider as key players in many important ecological processes like decomposition of organic matter, soil structure formation and recycling of essential elements (e.g., carbon, nitrogen, phosphorous, and sulfur) and nutrients and in xenobiotics. Hence, microbes play a critical role in modulating global biogeochemical cycles and influence all lives on Earth. In soil ecosystems, microorganisms are pivotal in suppressing soil-borne plant

diseases, promoting plant growth and in promoting changes in vegetation. An understanding of microbial dynamics and their interactions with biotic and abiotic factors is indispensable in bioremediation techniques, energy generation processes, and in biotechnological industries such as pharmaceuticals, food, chemical, and mining. The three fundamental questions that exist while discovering and characterizing any natural or artificial ecosystem are the following: (1) what type of microorganisms are present? (2) What do these microorganisms do? And (3) how do the activities of these microorganisms relate to ecosystem functions (e.g. Energy flow, biogeochemical cycling, ecological resilience)? Microbial ecology aims to answer these central questions and deals with the study of microorganisms and their interactions with each other and with their environment.[1] Moderate environments are important to sustain life. Moderate means environments with pH near neutral, temperature between 20°C and 40°C, air pressure 1 atm and adequate levels of available water, nutrients and salts. Many extreme environments, such as acidic or hot springs, saline and/or alkaline lakes, deserts and the ocean bedsore, also some extreme conditions like temperature, pressure, light intensity, oxygen and nutrient conditions is found in nature, which are too harsh for normal life to exist. Any environmental condition that can be perceived as beyond the normal acceptable range is an extreme condition. A variety of microbes, however, survives and grows in such environments over a wide range. These organisms, known as extremophiles, not only to tolerate specific extreme condition(s), but usually they require these for survival and growth also[2] Bacteria and Archaea are the most widely distributed organisms in these environments. Hypersaline environments constitute typical examples of environments with extreme conditions due to their high salinity, exposure to high and low temperatures, low oxygen conditions and in some cases, high pH values.[3,4] Archaebacteria were not recognized as a distinct form of life from bacteria until *1977*, when Carl Woese and George Fox determined this through RNA studies.[4,5] The haloarchaean and some extremely halophilic bacteria accumulate inorganic ions in the cytoplasm like K+, Na+, Cl- to balance the osmotic pressure of the medium, and also they have developed specific proteins which are stable and active in the presence of salts. While, moderate halophiles in their cytoplasm accumulate high amounts of specific organic osmolytes, which is functioning as osmoprotectants, providing osmotic balance without interfering with their normal metabolism of the cell.[4,6] Many bacterial products, which are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production.[7] As Carbon source is the key element for the growth of any microorganism.[8]

Table. 1: Classification and examples of extremophiles.

Environmental parameter	Type	Definition	Examples
Temperature	Hyperthermophile	growth >80°C	*Pyrolobusfumarii*, 113°C
	Thermophile	Growth 60-80°C	*Synechococcuslividis*
	Mesophile	15-60°C	*Homo sapiens*
	Psychrophile	<15°C	*Psychrobacter*, some insects
Pressure	Barophile	Weight loving	Unknown
	Piezophile	Pressure loving	For microbe, 130 MPa
Desiccation	Xerophiles	Anhydrobiotic	*Artemiasalina*; nematodes, microbes, fungi, lichens
Salinity	Halophile	Salt loving (2-5 M NaCl)	*Halobacteriacea, Dunaliellasalina*
pH	Alkaliphile	pH >9	*Natronobacterium, Bacillus firmus*OF4, *Spirulina spp.*(all pH 10.5)
	Acidophile	low pH loving	*Cyanidiumcaldarium,Ferroplasma sp.*(both pH 3)

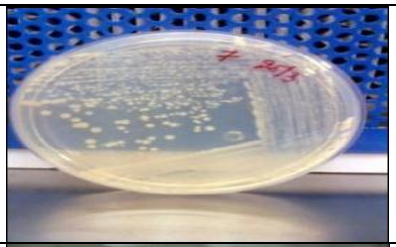
Gomes *et.al*. *2004*[9]

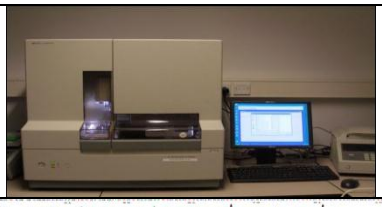
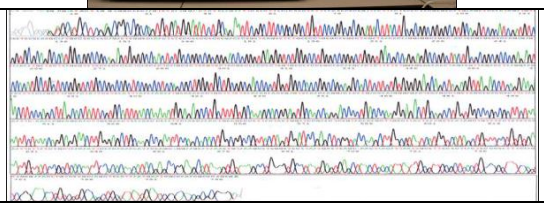
2. MATERIALS AND METHOD

2.1 Sampling

Sample collection site: Soil and Water samples were provided by Gujarat Biodiversity Gene Bank, GSBTM. The samples were: 363, 422, S-2 and S-5. pH of the soil samples was 7.5-8.0. Soil sample for viable bacterial counts were taken in sterile containers from Rann of Kutch.

2.2 Procedural workflow

1. Bacterial Colony		
2. DNA Extraction		
3. PCR		

4. Cycle Sequencing	
5. Sequencing and Sequence Analysis	

2.3 Materials and reagents

2.3.1 Media

- Halophilic Broth
- Agar Agar.

2.3.2 Reagents

- 1X TE buffer
- 10 % SDS
- 20 mg/ml Proteinase K
- Phenol: chloroform (1:1)
- Chloroform:Isoamylalcohol (24:1)
- Isopropanol
- 70 % ethanol
- 3 M sodium acetate (pH 5.2)
- Crystal violet (primary stain)
- Iodine solution/Gram's Iodine
- Decolourizer (e.g. ethanol)
- Safranin (secondary stain)
- PCR master mix
- PCR purification buffer
- Cycle sequencing mix
- Mili-Q-water
- Formamide

2.4 Enrichment of Archaeal Media: Halophiles were enriched in haloarchaeal broth containing Sodium chloride 250.0g, Potassium chloride 2.0g Magnesium Sulphate anhydrous

20.0g Yeast Extract 13.0g Agar 30.0g, D/w 1000ml, pH 7.2 for 10 days. From enriched 15%NaCl (w/v)halophilic broth, in 100ml flask, were incubated at 37C and kept on shaker at 100 rpm or 7days[10].

2.5 Isolation and characterization of Archaea

The various cultures were isolated from mixed cultures by streaking method and spreading method on above mentioned soild medium agar. These plates were incubated in incubator at 37 for 7 to 10 days. Individual colonies were sub cultured into mentioned agar plates containing different colonies until pure culture was isolated. Once all the isolates obtained were purified. Then After incubation time, colony characteristics and morphological properties were noted. Then DNA isolation is been done using Isolated colonies from culture were picked with the help of sterilized wire loop and added in the different reagent. Agarose gel Electrophoresis and PCR is performed to amplified the product, cycle sequencing and resulted in linear amplification of extension products. The extension products were then loaded into a genetic analyzer (DNA Sequencer) to determine the extension products.

2.6 Identification of microbes

The purified archaeal strains were identified based on its morphological, biochemical and 16S rRNA sequence. The 16S rRNA sequencing from isolated strains was accomplished by BioGene-Gujarat Biodiversity Gene Bank, India. Similarity analysis of the 16S rRNA sequences was conducted using the blast function of NCBI Gene Bank.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Archaeal isolates

Archaea were isolated and enumerated using the standard plate method. 0.1 ml of the proper dilution was used to surface inoculate nutrient agar media. From each dilution, five plates were inoculated at 37°C. Colonies were observed every day until optimal growth was observed. Colonies obtained on nutrient agar, were isolated and further purified. The isolates were stored at 4°C. For Identification of bacterial strain to carry out by 16S rRNA sequence analysis from the different isolates which have been submitted in the NCBI Gene Bank database under the different Bank ID. On the basis of morphological, biochemical characteristics and 16S rRNA sequences, isolated strains were identified as is showed in Table 2.

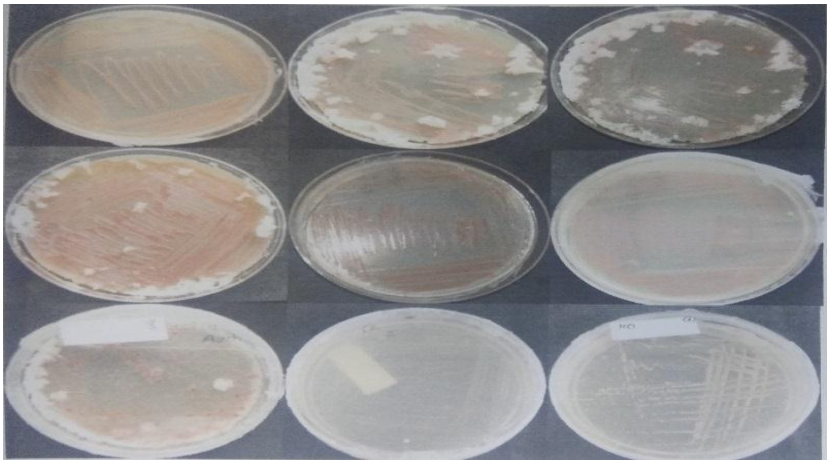


Fig. 1: Images of archaeal cultures grown in media.

3.2 DNA isolation and gel electrophoresis

DNA isolation from halophiles was carried out with two different protocols named as protocol 1 and 2. Protocol 1 was manual and protocol 2 was perpman protocol. 1% agarose gel electrophoresis was performed for visualization of DNA band. Concentration of DNA was checked by taking the absorbance at 260 nm and 280 nm on nanophotometer. Purity of DNA was checked by taking ratio of 260/280 nm.

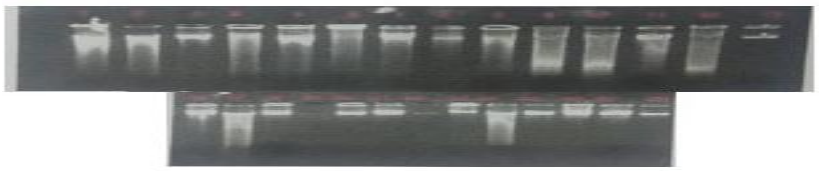


Fig. 2: Concentration and purity of DNA.

3.3 PCR amplification of DNA

Isolated DNA was amplified by using PCR amplification which was carried out in Thermo cycler. Amplified product was visualized on 2% agarose gel by electrophoresis. PCR product was further purified by Exo/Sap method.

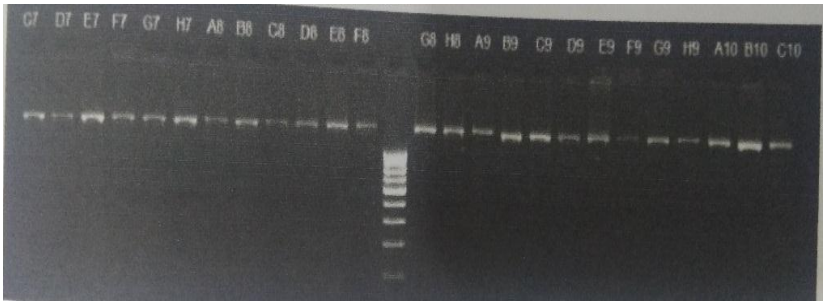


Fig. 3: Purified PCR product.

3.4 BLAST results

The sequences were interpreted and online local alignment tool BLAST was used to search and identify organism with same or similar sequences on NCBI database. The result of BLAST is given in table below.

Table. 2: Bacterial Identification by BLAST.

No.	Sample ID	Identified Organism	Query Coverage	Identity (%)	Bankit ID
1.	BAB 4199	*Halobacterium noricense*	98%	99%	1721995
2.	BAB 4204	*Halobacterium noricense*	99%	99%	1721995
3.	BAB 4201	*Haloferax alexandrines*	98%	98%	1721995
4.	BAB 4202	*Haloterrigena thermotolerans*	99%	99%	1721995
5.	BAB 4203	*Natrinema gari*	99%	98%	1721995
6.	BAB 4200	*Halobacterium noricense*	99%	99%	1721995
7.	BAB 4205	*Halobacterium noricense*	100%	99%	1721995

3.5 Colony characteristics and Gram reaction

Colonies of different strain on above mentioned agar plates appeared with irregular clusters, arranged in pairs, and tetrads. It grew in circular, entire, smooth, opaque, convex and creamy yellow, non-diffusible pigmented colonies. The strain was a Gram-positive, Gram negative, spherical, aerobic arranged.

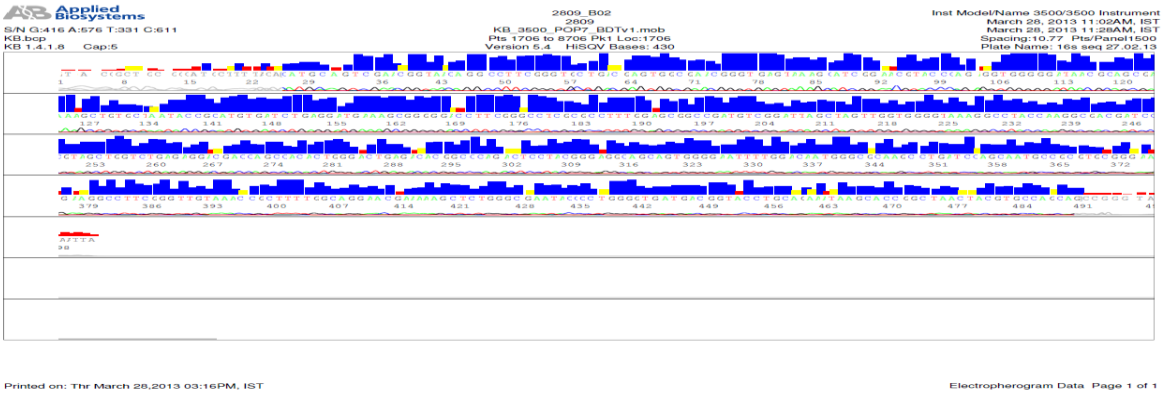
Table. 3: Colony characteristics.

Colony Morphology	BAB No.		
	4199	4200	4203
Size	Large	Small	Medium
Shape	Circular	Circular	Irregular
Elevation	Convex	Umbonate	Flat
Margin	Entire	Entire	Undulate
Appearance	Shiny	Shiny	Shiny
Optical Property	Opaque	Opaque	Translucent
Pigmentation	Red	Creamish	Yellow
Texture	Smooth	Smooth	Smooth
Gram Reaction	Gram positive	Gram negative	Gram positive

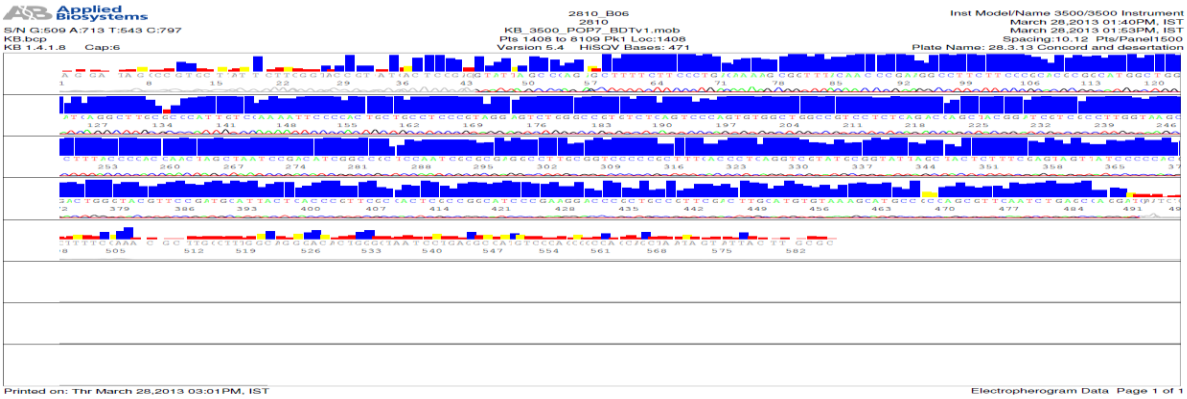
3.6 Sample run on DNA sequencer

Purified product from cycle sequencing was run on 3500xl Genetic analyser to obtain 16S rRNA gene sequence. Specific sequence was obtained for each of bacteria and archaea. After completion, sequences were presented as electropherogram by the CCD camera.

3.6.1 BAB 4199



3.6.2 BAB 4200



3.6.3 BAB 4203

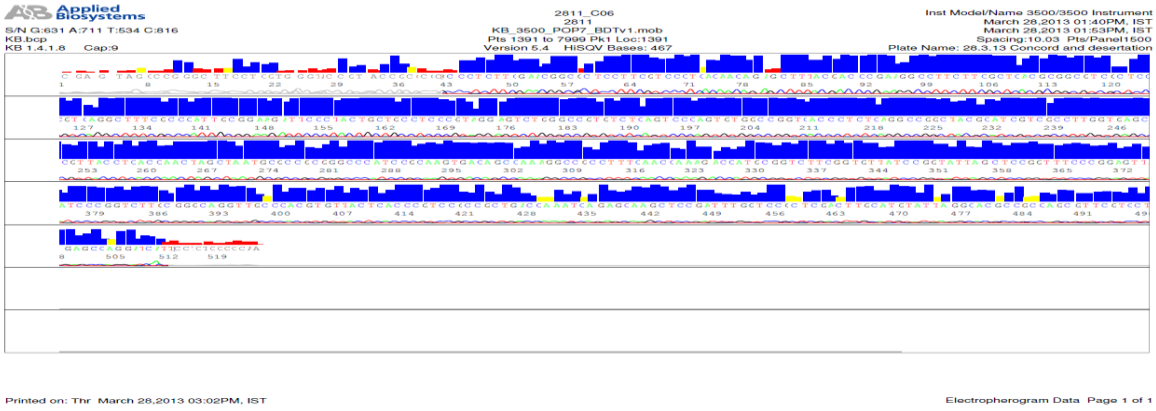


Fig. 4: Electrophorogram of Haloarchaea.

3.7 Phylogenetic Analysis

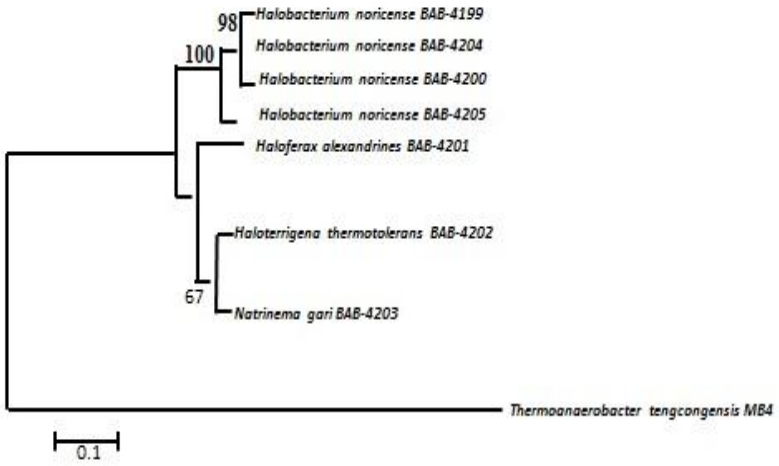


Fig. 4: Phylogenetic tree of Haloarchaea.

4. CONCLUSION

Haloarchaeal were isolated from Rann of Kutch in Gujarat, India. DNA isolation was carried from the for molecular identification of archaea. Further identification of haloarchaeal was performed by 16s rRNA gene sequencing.16s rRNA gene is universal gene for the identification of archaea. This gene is further amplifying by PCR and then sequenced using genetic analyser. Nucleotide BLAST was carried out to identify the archaea. Results from nucleotide BLAST showed that 4199 archaea have 99% similarity with *Halobacterium noricense* strain. From 99% similarity it was confirmed that 4203 was *Natrinema gari*. Further work can be carried out on the production and isolation of the enzymes from these many halophiles archaea and application of them in various industries can be find out.

5. ACKNOWLEDGEMENTS

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IMPORTANT

This publication is based on the review work and primary research work related to my Ph.D study.

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