Microbial Diversity of Halophilic Archaea and Bacteria in Solar Salterns and Studies on their Production of Antiarchaeal Substances

THESIS

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By

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Under the Supervision of

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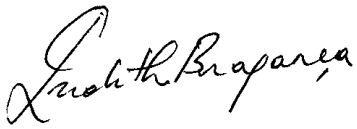
CERTIFICATE

This is to certify that the thesis entitled "**Microbial Diversity of Halophilic Archaea and Bacteria in Solar Salterns and Studies on their Production of Antiarchaeal Substances"** submitted by **Mr. Kabilan M.,** ID No **2008PH29009G** for award of Ph.D. of the Institute embodies original work done by him under my supervision.



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Dedicated to the saltern workers

white salt and dark lives…

To See a World in a Grain of Sand

And Heaven in a wild flower

Hold Infinity in the Palm of Your Hand

And Eternity in an Hour

-Auguries of Innocence

By

William Blake (1803)

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Abstract

Solar salterns are man-made ecosystems for the manufacture of salt in coastal regions. There is a gradual increase in the salinity from 3.5% to 37% at which salt is precipitated. Halophilic microorganisms ranging from bacteria to eukaryotes are known to inhabit salterns at various salinities. Solar salterns are the excellent systems for studying the microbial succession. India is the third largest producer of salt and it has area of 145308 hectares under salt production. In this study, representative salterns from the west coast (Ribandar, Siridao and Sinquetim in Goa) and from the east coast (Marakkanam and Vedaranyam in Tamil Nadu) of India were selected. Community composition and metabarcoding of halophilic microorganisms of solar salterns at different salinities and salt producing seasons was studied through culture dependent and culture independent techniques like denaturing gradient gel electrophoresis (DGGE) and Illumina MiSeq sequencing technology. The halophilic isolates were further screened for the production of the antiarchaeal substances.

Culturable diversity studies indicated that the solar salterns of Goa were dominated by the genus *Halococcus* during the preparatory phase. During the initial salt harvesting (ISH) and peak salt harvesting (PSH) phase, more diverse groups of halophilic archaea belonging to *Haloarcula, Haloferax, Halorubrum* and *Natrinema* were observed. *Halomonas* and *Bacillus* were the dominating halophilic bacteria of salterns of Goa. The salterns of Tamil Nadu showed the presence of *Haloarcula, Halorubrum, Halogeometricum* and *Halostagnicola* genera during the prolonged salt harvesting phase while *Halomonas* was the predominant bacterial member. Culture independent techniques involving DGGE analysis indicated that the sediments were equally colonized by Gram-positive and Gram–negative bacteria while the brines were dominated by Gram-negative bacteria. Metabarcoding studies using Illumina sequencing showed that *Haloarcula* and *Halorubrum* were the dominating haloarchaeal genera of the solar salterns of India while bacteria belonging to *Gammaproteobacteria* and *Bacteriodetes* were found to be dominant. *Halococcus* was the dominant haloarchaeal genus in the salt crystals.

Three potentially novel isolates (<99% 16S rRNA similarity values), *Haloarcula valismortis* BS3 (JCM30956), *Halorubrum chaoviator* BS17 (MCC2603) and *Halorubrum chaoviator* BS19 (JCM30957) were further characterized by phenotypic, chemotaxonomic

and genomic tools. The chemotaxonomic characterization of the isolates showed signature phospholipids of halophilic archaea, phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (Me-PGP) and phosphatidylglycerol sulfate (PGS). The major glycolipids of *Har. valismortis* BS3 were non-sulphated triglycosyl glycolipid (TGD-2) and diglycosyl diether (*DGD-2). The major glycolipid of the isolates Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 was sulfated mannosyl glucosyl diether (S-DGD-3). Whole genome sequencing indicated the genome size of *Har. valismortis* BS3 was 3.2 Mb and the G + C content was 64.54 and. The genome size of *Hrr. chaoviator* BS19 was 3.8 Mb and the G + C content was 67.61%. *Hrr. chaoviator* BS19 contained 42 tRNAs and 4 rRNAs and *Har. valismortis* BS3 contained 50 tRNAs and 1 rRNA.

Haloferax volcanii strain BBK2 and *Haloarcula valismortis* strain BS2 produced extracellular antiarchaeal substances. The cell free supernatant (CFS) of *Haloarcula vallismortis* strain BS2 containing the antiarchaeal substance was salt independent, withstood boiling (100°C) for 30 minutes and resisted the action of proteolytic enzymes. Partial purification indicated that the antiarchaeal substance might be a low molecular weight peptide of less than 3.5 kDa. This study demonstrates the adaptation and fluctuation of halophilic microorganisms through varying salinity and different salt producing phases.

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List of Abbreviations and Symbols

%	Percentage	µg	Micro gram
ACE	Abundance-Based Coverage	AHL	Acyl-homoserine lactones
AO	Acridin Orange	ATCC	American Type Culture Collection
AU	Arbitrary Units	AW	After Wash
Bé	Baumé	BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool	bp	base pair(s)
BSS	Basal Salt Solution	BW	Before Wash
C	Celsius	CCM	Closest Culturable Match
CDS	Coding DNA Sequences	Cdv-ABC	Cell Division ATP-binding cassette
CEM	Closest Environmental Match	CFS	Cell Free Supernatant
CFU	Colony forming unit	cg/g	Centi Gram Per Gram
cm	Centimeter	CP	Crystalliser Pan
CTAB	Cetyl Trimethyl Ammonium Bromide	DAPI	Diamidino Phenyl Indole
DCA	Detrended Correspondence Analysis	DDBJ	DNA Data Bank of Japan
DDW	Double Distilled Water	DEAE	Diethylaminoethanol
DGGE	Denaturing Gradient Gel Electrophoresis	DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid	dNTP	Deoxynucleotides
dsDNA	Double Stranded DNA	DTT	Dithiothreitol
E	East	ED	Entner Doudoroff
EDTA	Ethylene Diamine Tetra Acetic acid	EHM	Extremely Halophilic Medium
EP	Evaporator Pan	EPS	Exopoly Saccharide
ESCRT	Endosomal Sorting Complex Required for Transport	ESI MS	Electro Spray Ionisation Mass Spectroscopy
EtBr	Ethidium bromide	FISH	Fluorescence in situ hybridization
g	Gram	g/l	Gram Per Litre
G+C	Guanine Plus Cytocine	GC	Guanine Cytocine
GO	Gene Ontology	ISH	Initial Salt Harvesting
HMM	Hidden Markov Model	JCM	Japan Collection of Microorganisms

KAAS	KEGG Automatic Annotation Server	KAc	Potassium Acetate
kDa	Kilo Dalton	Kg	Kilo Gram
Km	Kilo Meter	Kton	Kilo Tonne
m	Meter	MALDI -TOF	Matrix-Assisted Laser Desorption / Ionization Time-of-Flight
MEGA	Molecular Evolutionary Genetics Analysis	Me-PGP	Methyl ester of Phosphatidyl Glycero Phosphate
MHM	Moderate Halophilic Medium	Ml	Milli Litre
mM	Milli Molar	MTCC	Microbial Type Culture Collection
MUSCLE	MUltiple Sequence Comparison by Log-Expectation	MWCO	Molecular Weight Cut Off
N	North	NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information	NCIM	National Collection of Industrial Microorganisms
NGS	Next Generation Sequencing	NGSM	NaCl Glucose Synthetic Medium
NHE	Na^+ H^+ Exchanger	NJ	Neighbour Joining
nm	Nano meter	NSM	NaCl Synthetic Medium
NT	NaCl Tri-Sodium Citrate	nt/s	Nucleotide/s
NTYE	NaCl Tryptone Yeast Extract	o	Degree
OD	Optical Density	ORF	Open Reading Frame
OTU	Operational Taxonomic Units	PAGE	Poly Acryl Amide Gel Electrophoresis
PANAM	Phylogenetic Analysis of Next generation Amplicons	PAST	Paleontological Statistics Software
PBHV	Poly Hydroxybutyrate-*co*-Hydroxyvalerate	PCI	Phenol-Chloroform-Isoamyl alcohol
PCO	Principal Coordinate Analysis	PCR	Polymerase Chain Reaction
PE	Polyethylene	PEG	Polyethylene glycol
PG	Phosphatidyl Glycerol	PSH	Peak Salt Harvesting
PGS	Phosphatidyl Glycero Sulfate	PHB	Polyhydroxybutyrate
PSH	Pre Salt Harvesting	PUFA	Producin of Polyunsaturated Fatty Acid
PVP	Polyvinylpyrrolidone	PVP	Poly Vinyl Pyrrolidinone
QS	Quorum Sensing	RDP	Ribosomal Database Project

Rf	Retention Factor	RNA	Ribo Nucleic Acid
RP	Reservoir Pan	rpm	Revolutions Per Minute
rRNA	ribosomal RNA	SC	Scheduled Castes
SCB	Sugar Cane Bagasse	S-DGD	Sulfated Diglycosyl Diether
SDS	Sodium dodecyl sulphate	sec	second
SEM	Scanning Electron Microscopy	SH	Salt Harvesting
sp.	Species	SS	Sediment Sample
SSU	Small Subunit	ST	Scheduled Tribes
S-TeGD	Sulfated Tetraglycosyl Diglycosyl Diether	S-TGD	Sulfated Triglycosyl Diglycosyl Diether
TACK	*Thaumarchaeota Aigarchaeota Crenarchaeota Korarchaeota*	TAE	Tris acetate EDTA
TAE	Tris base Acetic acid EDTA	TE	Tris-EDTA
TFA	Tri Fluoro Acetic acid	TGD	Triglycosyl Diglycosyl Diether
TLC	Thin Layer Chromatography	TN-M	Tamil Nadu Marakkanam
TNTC	Too Numerous to Count	TN-V	Tamil Nadu Vedaranyam
Tris HCL	*Tris hydrochloride*	µg	Micro Gram
µg/g	Micro Gram Per Gram	µm	Micro Meter
UPGMA	Unweighted Pair Group Method with Arithmetic Mean	USDA	United State Department of Agriculture
v/v	Volume by Volume	w/v	Weight by Volume
WGS	Whole Genome Sequencing	WS	Water Sample

Chapter I

Introduction and Review of Literature

1.1 General Introduction

Hypersaline environments are extreme habitats where the salt concentrations are much higher than the salinity of seawater (3.5%, w/vol). Apart from salinity, the organisms in these environments face extremes of temperatures, low oxygen and alkaline conditions. Yet organisms have adapted to survive in the extreme conditions (Ventosa, 2006). Hypersaline environments are inhabited by organisms belonging to the three Domains of life Archaea, Bacteria, and Eukarya (Oren, 2002). Solar salterns are man-made hypersaline ecosystems, where the salt (NaCl) is produced by sequential crystallisation of salts present in seawater. However, the microbiology of solar salterns is poorly understood.

The broad goal of this thesis was to characterise the microbial community composition of solar salterns through culture-dependent and culture-independent techniques. The solar salt production process in the salterns of Goa and Tamil Nadu was documented in detail (Chapter II). Solar salterns were studied for their culturable halophilic archaeal and bacterial diversity (Chapter III).Selected haloarchaeal isolates were further characterised through phenotypic and genotypic tools (Chapter IV). The non-culturable diversity of solar salterns was studied using DGGE and the metabarcoding studies were carried out using Illumina sequencing (Chapter V). The haloarchaeal isolates were screened for the production of the antiarchaeal substances and the active compounds were characterised and partially purified (Chapter VI).

1.2 Review of Literature

1.2.1 Systematics and phylogenetics of organisms

Taxonomic classification systems in the 15th and 16th centuries were based on the basis of phenotypic characteristics, behavioural traits, habitats and modes of locomotion. In the 18th century, the classification system had undergone major improvements with the adoption of binomial system of nomenclature. Major classifications were confined to plants and animals (Whittaker et al., 1959; Birch & Ehrlich, 1967). Since, the discoveries of Bacteria by Anton von Leeuwenhoek in 1676, many groups of bacteria were subsequently described which forced the taxonomists to reconsider the existing classification scheme to include them as well (Manktelow, 2010; Porter, 1976).

As most of the biologists were interested in studying the pathogenic bacteria, the earliest bacterial classification was dependent on the basis of morphology, growth and pathogenic potential. Meanwhile, other attempts were being made in parallel to classify

the microscopic and macroscopic life forms into prokaryotes (without nucleus) and eukaryotes (with nucleus) (Sneath, 1957; Kluyver& Van Neil, 1936). At the beginning of the 20th century, the data obtained from the studies on the physiological and biochemical properties of the bacteria further contributed to the confusion in bacterial taxonomy. Till the 7th edition of Bergey's Manual of Determinative Bacteriology, Bacteria were classified under plants (*Protophyta*) (Schleifer, 2009). In 1969, Robert Whittaker proposed the five kingdom classification which is still followed with minor tweaks. The classification system contains *Monera*, *Fungi*, *Protista*, *Plantae* and *Animalia* as separate Kingdoms and includes all unicellular life forms under *Monera* and *Protista* (Margulis, 1974).

In 1977, Carl Woese described the differences in the genetics and cellular composition between Bacteria and Archaeabacteria (now known as Archaea), till then considered to be the members of Bacteria. He further proposed the three-domain classification system with Bacteria, Archaea and Eukaryotes as separate domains, the highest level in taxonomy, on the basis of the difference in their ribosomal RNA gene sequences (Woese & Fox, 1977) (Fig. 1.1). Even before the three-domain classification, the presence of archaeal members (then recognised as Bacteria) were reported from the unusual locations like hypersaline regions, intestinal gut and hot springs (Nottingham &Hungate, 1968; Sehgal & Gibbons, 1960; Donk, 1920). But with the description of Archaea as a separate domain, many studies focussing on the microbial composition of the considered to be devoid of life locations, found a well-established and sophisticated community of microbes surviving. Following the common occurrences of archaea in extreme locations, the obvious conception developed was 'Archaea were thought to survive only in the extreme habitats'. However, later studies revealed that not only Archaea but also Bacteria and Eukaryotes can inhabit the extreme environments (Chao et al., 2005).

The phylogenetic tree suggested by Woese on the basis of 16SrRNA genes had suggested that Eukaryotes and Archaea are sister domains with a common ancestor. However, the phylogenomics analysis considering multiple genes has placed the Phylum *Eukaryotes* as a branch from *Crenarcahaeota*, indicating that eukaryotes evolved from archaea (Koonin, 2015). The discovery of TACK (*Thaumarchaeota, Aigarcheota, Crenarchaeota, Korarchaeota*) or proteoarchaeota superphyla and their genomic analysis revealed the presence of eukaryotic like proteins (Forterre, 2015).

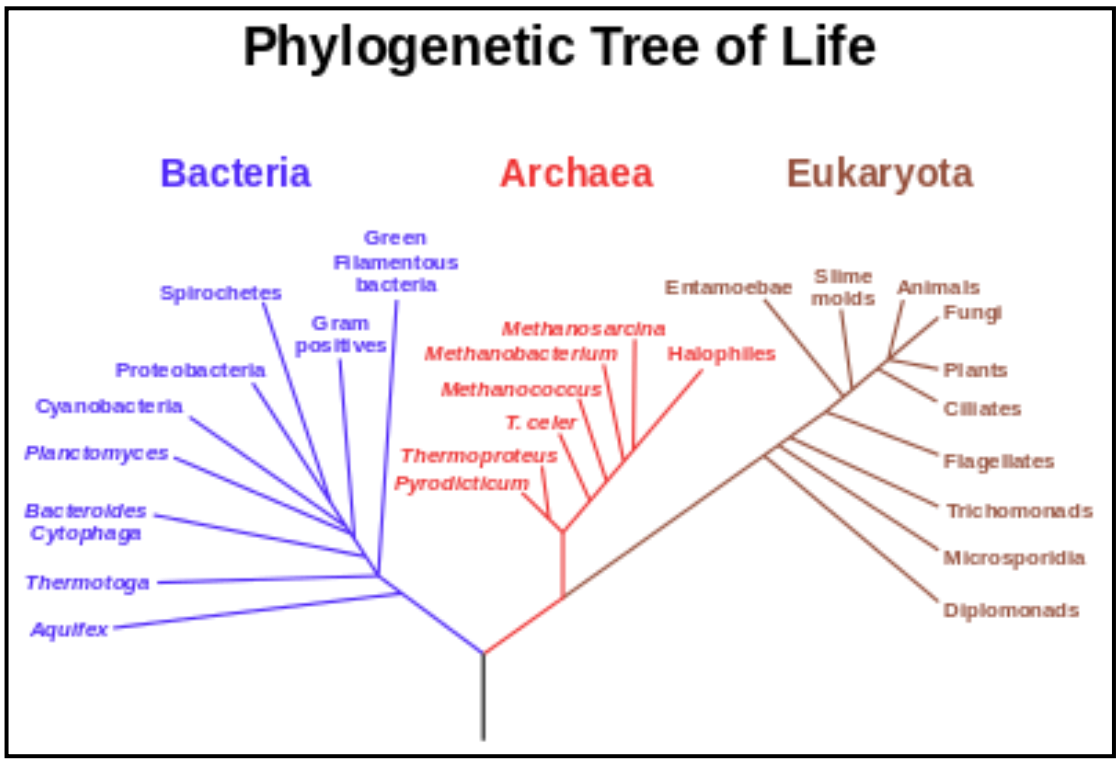


Fig. 1.1: The tree of life depicted by Carl Woese, based on the phylogenetic analysis of 16S rRNA gene of organisms (Woese et al., 1990).

For example, TACK contains FtsZ protein homologous to eukaryotic tubulin and CdvABC system similar to the eukaryotic ESCRT-III and Vsp4 proteins (Guy &Ettema, 2011). Recent discovery of *Lokiarcahaeota* adds more momentum to the endo-symbiotic theory of eukaryotic evolution. Phylogenetic analysis of the genomes revealed the monophyletic branching of *Lokiarchaeota* and Eukaryotes. Similarly, protein analysis revealed the presence of crenactins, gelsolins, ESCRT-III complex, Ras-like GTPases and ubiquitin system correlating with the ancestral eukaryotic protein candidates (Spang et al., 2015). Another group of archaeal clade called DPANN (*Parvarchaeota, Aenigmarchaeota, Diapherotrites, Nanohaloarchaeota, Pacearchaeota, Woesearchaeota,* and *Micrarchaeota*) representing a lineage of small sized archaea, is considered as archaeal ancestors, possibly due to their less complex genomes (Rinke et al., 2013). On considering these recent advancements in evolutionary biology, it is necessary to modify the tree of life proposed by Woese, by incorporating the novel archaeal lineages (Fig. 1.2) (Hug et al., 2016).

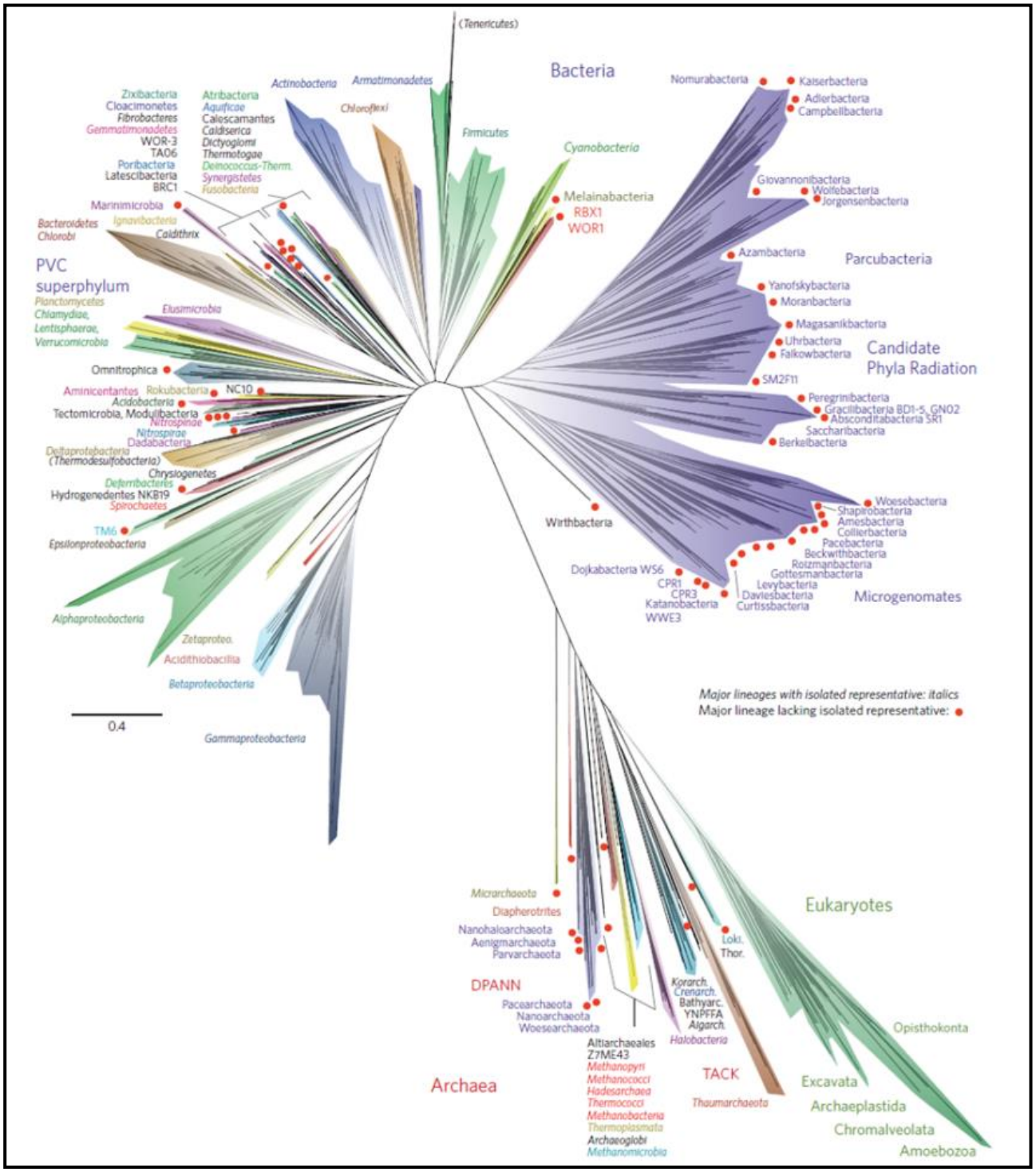


Fig. 1.2: Phylogenetic tree depicting the total diversity of organisms (adapted from Hug et al., 2016).

1.2.2 Extremophiles and their Habitats

Extremophiles (*extremus* meaning extreme and *philia* meaning love) are a group of organisms that survive in the most deleterious geochemical and physical conditions, often difficult for the otherwise mesophilic organisms to cope (MacElroy, 1978). Extremophiles are further divided into two categories. They are, i) organisms that are not native to the extreme conditions but can adapt and tolerate the extremeness and ii) organisms that are meant to thrive only in the extreme conditions as their cells are designed to do so. These extreme environments range from arid desert regions, polar regions to hypersaline environments (Bell & Callaghan, 2012). The various extremophilic environments and conditions experienced by the organisms in there are listed in table 1.1. Studying the diversity of extremophilic organisms can help us in understanding the boundaries of life and leads to an understanding the evolution of life. In the field of biotechnology, extremophilic organisms offer attractive solutions especially for the process industries (Herbert, 1992).

1.2.3 Halophiles

Halophiles (*hal*- sea or salt and *philos* – love) are one of the earliest groups of extremophilic organisms recorded (DasSarma&DasSarma, 2012). Records of the red colouration of salt producing areas, dating as back as 2700 B.C., indicates the halophilic bloom of salterns. There are several records indicating the red colouration of the salt lakes like Wadi Natrun lakes in Egypt and Dead Sea. One of the most well documented instances was by Charles Darwin in 1836 at the salt lake in Argentina and he had guessed that the red colouration might be due to the presence of microorganisms. Eventually it was noticed that the frequent spoilage of stored meat and fish was because of the usage of red salt contaminated with halophilic microbes. The first reported isolation of halophilic bacteria was from the discolorations of salted fish in 1919 (Lochhead, 1934). Studies related to the algal growth in the salt producing regions were carried out by Dunal as early as 1838 wherein he reported the isolation of a red unicellular alga and proposed that the red colouration of the brine could be due to the growth this alga (Dunal, 1838). In 1905, Hamburger gave a detailed description of this unicellular alga, now known as *Dunaliella salina* and the location of the β - carotene pigments, responsible for red colouration, in between the thylakoids of the chloroplast (Heidelberg, 1905).

Table 1.1: List of extremophilic habitats, the conditions experienced, and the adaptive strategies with an example organism.

Extremophile	Conditions	Adaptive strategies	Example organism
Temperature			
Hyperthermophiles	>80°C	Heat stable proteins with large hydrophobic core, increased surface charges, increased ionic interactions. Production of long chain, saturated, branched fatty acids and cyclic lipids	*Methanopyrus kandleri* (Slesarev et al., 2002)
Thermophiles	60 to 80°C		*Thermus aquaticus* (Brock & Freeze, 1969)
Psychrophile	0 to 20°C	Weak protein interactions with lower thermal stability and increased specific activity. High unsaturated fatty acids with increased ordered cellular water and reduced cell size	*Sychromonasantarticus* (Mountfort et al., 1998)
pH			
Acidophile	< 2	Proteins with high negative charge. Proton exclusion with efficient exclusion system and proton driven secondary metabolites	*Thiobacillus caldus* (Hallberg&Lindström, 1994)
Alkaliphile	>9	Membrane impermeability for OH ions. Efficient proton uptake and negatively charged cell wall polymers	*Desulfonatronovibrio hydrogenovorans* (Zilina et al., 1997)
Salinity			
Halophiles	2 to 5M NaCl	Salt-in strategy. Accumulation of compatible solutes. Efficient in transport system and protein modifications like increased acidic amino acids and decreased hydrophobic residues.	*Halobacterium salinarum* (Ventosa& Oren, 1996)
Radiation			
Radiation resistant	High energy gamma radiation and UV-B tolerant	High content of pigments and efficient DNA repair mechanisms	*Deinococcus radiodurans* (White et al., 1999)
Pressure			
Barophiles/ Piezophiles	> 400 to 500 atm	Increased binding capacity for enzymes. Poly unsaturated fatty acids in membranes. Pressure controlled gene expression.	*Thermococcus barophilus* (Marteinsson et al., 1999)
Dehydration			
Xerophiles	-4 to -70 MPa	Accumulation of osmolytes. Production of exopolysaccharides. Formation of spores.	*Xeromyces bisporus* (Dallyn& Everton, 1969)

atm - atmospheres; MPa -megapascal; UV - ultra violet

Simultaneously, studies were carried out on the biology of Great Salt Lake, Dead Sea and Wadi Naturn and many more eukaryotic organisms like protozoa and algae with few data on prokaryotes (Oren, 2012). Modern halophilic research started in the late 1950s, mostly in Canada and Norway, where the export of salted fish and cod was a major commodity. Scientists isolated numerous halophilic archaea from the salted fish and gave detailed descriptions of the isolates (Larsen, 1973 and 1968).

Based on the mineral composition, the water bodies are divided into thalasohaline and athalasohaline. Thalasohaline environments have mineral composition similar to marine environments with sodium and chloride being dominant element and the pH may vary from neutral to slightly alkaline (Camargo et al., 2004;Wais, 1988). In contrast, athalasohaline environments have magnesium and potassium as dominant ions with trace amounts of carbonate, chloride and sulfate. Their ionic strength is in complete contrast with the sea water and they contain high amount of organic carbon, nitrogen and phosphate compounds (Demergasso et al., 2004). Examples of athalasohaline environments include Dead Sea, soda lakes and alkaline soil (Oren, 2002).

Halophiles survive in a range of salinity as low as 2% and to as high as 37%. Halophiles are categorised based on the salinity requirements into four broad categories, namely (i) extreme halophiles, growing best in media containing 2.5–5.2 M (15 – 25%, w/vol) salt, (ii) borderline extreme halophiles, growing in media containing 1.5–4.0 M (7 – 20%, w/vol) salt, (iii) moderate halophiles, growing in media containing 0.5–2.5 M (3 – 15%, w/vol) salt and (iv) halotolerant microorganisms that do not show an absolute requirement for salt for growth but grow well up to often very high salt concentrations (considered extremely halotolerant if the growth range extends above 2.5 M (15%, w/vol) salt) (Kushner &Kamekura, 1988). Halophilic and halotolerant microorganisms are found in all three domains of life: Archaea, Bacteria and Eukarya. Almost all haloarchaeal genera are classified as extreme halophiles while bacteria are classified as moderate halophiles or halotolerant.

1.2.3.1 Phylogeny and Taxonomy

1.2.3.1.1 Halophilic Prokaryotes

The domain Archaea has five accepted phyla namely *Crenarchaeota, Euryarchaeota, Korarchaeota, Nanoarchaeota* and *Thaumarchaeota* followed by eleven proposed phyla. They are *Aenigmarchaeota, Aigarchaeota, Bathyarchaeota, Diapherotrites, Geoarchaeota, Lokiarchaeota, Micrarchaeota, Nanohaloarchaeota,*

Pacearchaeota, *Parvarchaeota*and *Woesearchaeota* (Eme& Doolittle, 2015b). All reported halophilic archaea fall under the phylum*Euryarchaeota*, class *Halobacteria*, order *Halobacteriales* and family *Halobacteriaceae* (Oren, 2002). At the time of writing, the family *Halobacteriaceae* contained 50 genera and 202 described species, making it one of the largest families containing cultivable species in the domain Archaea(http://www.ezbiocloud.net/). Apart from *Halobacteriacecae*, methanogenic halophilic archaea have also been found in the family *Methanocalculus* under order *Methanomicrobiales* and family *Methanosarcinaceae* under the order *Methanosarcinales* (Oren, 2002). At the time of writing, methanogenic haloarchaea included 4 genera and 11 species (http://www.ezbiocloud.net/).

The Domain Bacteria has 33 recognised phyla and halophilic bacteria are found in eight phyla. They are *Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes, Bacteroidetes, Thermotogae, Cyanobacteria* and *Tenericutes.* Unlike halophilic archaea, halophilic bacteria are highly diverse and distributed among various families. In some cases, there may be only a species of halophilic bacteria present in particular genera with rest of the members being non-halophilic. Though they are diverse, most of them are moderately halophilic with optimal salt concentration around 2 – 3 M. Large proportions of them are aerobic, motile and can degrade a wide range of substances for deriving energy (Rafael et al., 2011).

1.2.3.1.2 Halophilic Eukaryotes

Hypersaline environment were once thought to harbour only limited eukaryotic diversity. However, studies conducted in the past two decades, have revealed the vastness of eukaryotic diversity in hypersaline environments. Fungi are known to inhabit a variety of eco-niches and they are present in hypersaline environments too. Though many fungi have been isolated from the hypersaline environment, most of them like *Aspergillus*, *Penicillium* and *Eurotium* are halotolerant and are thus not true halophiles. The true halophiles are grouped under genus *Wallemia* under the class *Wallemiomycetes*, genus *Emericella*, genus *Trimmatostroma*, genus *Cladosporium*, genus *Aureobasidium*, genus *Candida* and genus *Hortaea*. Currently, there are 22 recognised halophilic fungal isolates (Gunde-Cimerman et al., 2009). Green algae *Dunaliella* has been isolated from various hypersaline environments and they act as the primary producers in the hypersaline environments. Other algal genera found in the hypersaline environments are *Asteromonas* and *Picocystis* (Seckbach, 2015; DasSarma&DasSarma, 2012). Apart from algae, diatoms

are also found in saline environments and they too can act as producers. They include members belonging to genus *Amphora*, *Nitzschia* and *Navicula*. Among the other unicellular eukaryotes, protozoans like flagellates and ciliates are frequently observed in hypersaline environments. Members of genus *Pleurostornurn*, *Halocafetaria*, *Trachelocerca, Metacystis, Chilophrya, Rhodopalophrya, Uronema, Condlylostoma, Palmarella, Nassula, Fabrea, Blepharisma, Cladotrichia, Euplotes, Podophrya, Trematosoma, Monosiga, Rhynchomonas*, *Phyllomitus*, *Tetramitus* and*Bodo*(Park & Simpson, 2015). Multicellular eukaryotes consists brine fly *Ephydra* and brine shrimp *Artemia*. They survive by feeding on the algal members of the hypersaline environments (VanStappen, 1996).

1.2.3.2 Physiology of Halophiles

Halophilism is determined by the ability of the organism to cope the stress posed by the salinity. Since biological membranes are permeable to water, normal cells would shrink in medium containing high salinity, resulting in cell lysis. Therefore, organisms living in the saline environments should have specialised adapted mechanisms for combating the high salinity (Joo& Kim, 2005). Though the mechanisms may vary between the organisms, the primary goal of the organism is to maintain the osmotic pressure in coherence with the external concentrations. In halophilic archaea the turgor pressure is higher than the external environment. Majority of Bacteria are moderately halophilic and Archaea are extremely halophilic organisms (Whatmore & Reed, 1990). The organisms usually employ two different mechanisms for combating salinity. They are,

1.2.3.2.1 Salt-in strategy

Organisms accumulate high intracellular KCl in response to the extracellular ionic concentration. Halophilic archaeal members belonging to the family *Halobacteriaceae*, the bacterial members belonging to order *Halanaerobiales* and genus *Salinibacter*, have been shown to adapt the salt-in strategy (Oren et al., 2002). The evidence for this strategy was first found in a study conducted on *Halobacterium salinarum*, which contained a high intracellular salt concentration. It was found that the cells contained Na^+, Cl^- and K^+ with predominant K^+ concentration (Litchfield, 1998).

1.2.3.2.2 Accumulation of compatible solutes

Halophilic archaea maintain low intracellular ionic concentration. Yet they survive in hypersaline environments by the accumulation of compatible solutes (Oren, 2008;Gunde-Cimerman et al., 2006; Oren, 1999).These compatible solutes are small organics compounds that do not interfere with the normal metabolism. They are also called as 'osmolytes' because of their regulation of osmotic pressure. Apart from combating salinity, these compatible solutes are stress protectants because of their protection against heat, desiccation, pressure and free radicals. Some of the accumulated compatible solutes are Glycine-Betaine, trehalose, ectoine, hydroxyl ectoine, sucrose, glucosyl glycerol, mannosylglyceramide, N-carbomyl glutamine amide and N-acetylglutaminylglutamine amide(Roberts, 2005).

1.2.4 Approaches to Study Biodiversity of Hypersaline Environments

1.2.4.1 Culture-Dependent Techniques

Initial attempts to culture halophilic microbes were carried out with fish broth, fish pieces soaked in brine, cod broth and powdered cod with gelatin or rice flour as the gelling agent. However, these cultivation attempts resulted in the growth of non-halophilic counterparts rather than halophilic organisms. In 1930s, it was recognized that, salinity of the medium is more important than the substrate on which they are grown. Media were then formulated with complex substrates like yeast extract, peptone with salinity around 10–15%, w/vol (Schneegurt, 2012).

Phenotypic techniques are usually carried out in the initial stages of the polyphasic approach in the identification of the culturable organisms (Mata et al., 2002). The techniques can give an insight on the biochemical and physiological profiles. The phenotypic characterisation involves analysis of the morphological traits like cell shape, size, motility, pigmentation and cell wall characteristics, biochemical traits like utilisation of various carbohydrates and metabolites, response to antibiotics and production of enzymes, physiological traits like optimal growth conditions. Cell membrane lipids, fatty acids and glycoproteins are also used as markers for characterising microorganisms. Since only a small fraction of organisms are culturable, phenotypic characterisation falls short in the taxonomic resolution to the species level of novel and closely related organisms (Oren, 1997).

1.2.4.2 Culture-Independent Techniques

Genomics has helped in unraveling the hidden treasures of hypersaline biodiversity. For example, *Salinibacter ruber*, a halophilic bacterium with archaeal life style was first identified in crystallizer pans of salterns through fluorescent *in-situ* hybridization (FISH) studies. Subsequently, medium was formulated and the bacterium was cultivated in the lab successfully (Anton et al., 2002). Similarly, in 1980 square shaped cells were first observed under microscopy in the coastal saline lakes in Egypt. And after much difficulty, this halophilic archaeon *Haloquadratum walsbyi* was cultured under laboratory conditions in 2004 (Burns et al., 2007). Genotypic techniques involving biomarkers and gene targeted sequencing has overcome the shortcomings of the phenotypic methods. These techniques have contributed a wealth of information in our understanding of the biodiversity of hypersaline regions.

1.2.4.1 Biomarkers

The most common non culturable technique employed is the direct enumeration of microbes directly under microscope and further quantification by staining the cells with 4',6-Diamidino-2-phenylindole (DAPI) and acridine orange (Pedros-Alio et al., 2000). Differentiation between live and dead cells can be achieved with LIVE/DEAD™ *Bac*Light® kit with some minor modifications in the protocol suited for hypersaline environments (Stan-Lotter et al., 2006). Cyanobacterial and algal density in the hypersaline environments can be measured with the estimation of the chlorophyll content (Estrada et al., 2004). Since haloarchaeal cell walls contain high-molecular-weight acidic glycoprotein, the bile salts such as desoxycholate and taurocholate can be used in the lysis of the cells, differentiating between archaeal and bacterial members and also between halococcal and non-halococcal members. Polar lipids are the excellent biomarkers in studying the community compositions. Archaeal cell walls contain diphytanyldiether lipids and bacteria contain ester linked lipids, the differentiation can be achieved through thin layer chromatography (TLC) and electro spray ionisation mass spectroscopy (ESI-MS). Certain glycolipids and phospholipids are present only in certain genera of halophilic archaea, making it a biomarker in further differentiating between the species. Fatty acids can serve as an important biomarker in differentiating between bacterial and eukaryotic halophilic members. They were also used in the differentiation of various members of cyanobacteria of Dead Sea (Litchfield et al., 2000; Oren &Guverich, 1993). Another important biomarker in characterising the halophilic communities is the

characterisation of their pigments. Halophilic archaea possess α-bacterioruberin and its derivatives, bacteria like *Salinibacter* contain C_{40} carotenoid salinixanthin and eukaryotes like *Dunaliella* contain β-carotenoids, the community compositions between these organisms can be monitored using the spectrophotometric analysis of the pigments. Osmotic solutes like glycine betaine accumulated by the bacterial members can help us in understanding the approximate composition of bacteria against archaea in a particular ecosystem (Oren et al., 2009;Oren & Rodriguez-Valera, 2001; Javor, 1983).

1.2.4.2 Genomic methods

Genotypic techniques can be applied to organisms irrespective of their physiological state and these techniques usually establish the taxonomic identity of the organisms in short durations. These techniques rely on the nucleic acids or a specific sequence in the DNA or RNA, termed as molecular markers for the identification of the taxonomic identity of the organisms. The information or sequences obtained from the gene fragments are then compared against the databases like SILVA, RDP and Greengenes (Fig. 1.3) (Das et al., 2014).

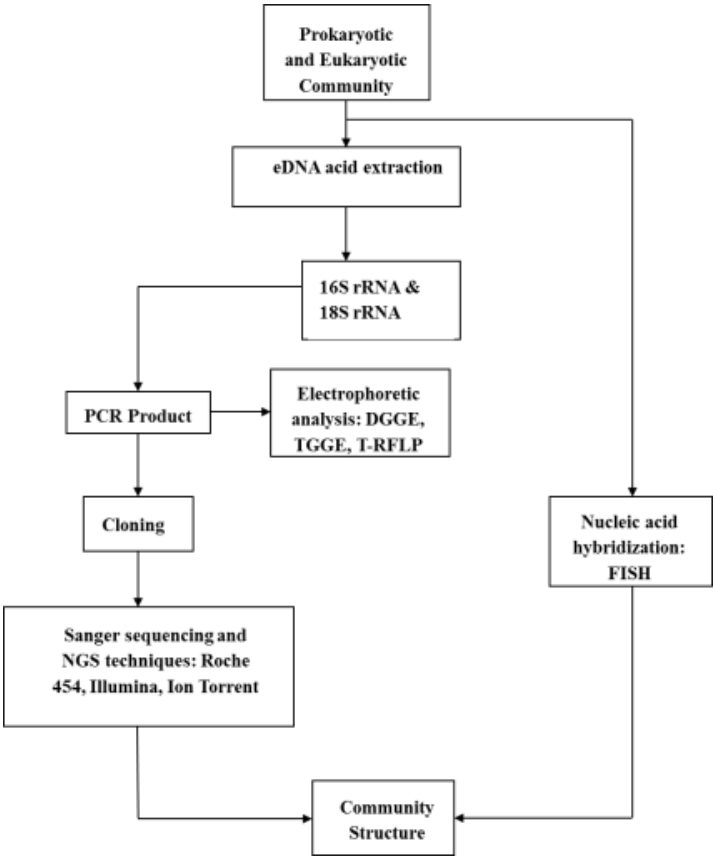


Fig 1.3: Nucleic acid-based methods in identifying prokaryotic diversity

The house-keeping genes are the usual targets as molecular markers in identifying the taxonomic identity of the cultured microorganisms and the community compositions of the hypersaline ecosystems through culture-independent techniques. Gene sequence analyses of the marker sequences can discriminate several strains of microorganisms in a more precise manner because of their identification in micro heterogeneity at species and strain level. There are several genetic markers for rapid cataloguing of gene families falling within definite taxonomic ranks. Different genetic targets for microbial identification are *16S rDNA, gyrB, rpoA, rpoB, rpoC, rpoD* genes, etc. Among all the molecular markers, the most widely used gene fragment for the identification of prokaryotes is 16S rRNA gene (Ramasamy et al., 2014).

The 16S small ribosomal RNA gene (16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes. The 16S rRNA genes comprise nine hypervariable regions (V1-V9) that demonstrate considerable sequence diversity among different bacteria, and nine conserved regions (C1-C9) that remain consistent (Fig. 1.4). 16S rRNA genes have been widely used for documentation of the evolutionary history and taxonomic assignment of individual organisms, as well as in characterization of microbial communities (Heuer et al., 1999). The advantages of using 16S rRNA as a molecular marker are, (i) the gene sequence is conserved sufficiently containing conserved, variable and hyper-variable regions (ii) around 1500 bp of sequence size which is relatively easy to sequence and large enough to contain sufficient information for identification and analysis of phylogeny (iii) Lateral transfer of this gene between taxa are rare and (iv) Since this gene has been widely sequenced in microbial diversity research, there are many reference databases including a large amount of sequences with taxonomic information, which is convenient to assign query sequences to known taxonomic groups and compare community composition across studies (Kim et al., 2011).However, despite these advantages of 16S rRNA as molecular marker, there are few disadvantages as well. For instance, if sequence variation of the 16S rRNA between two microorganisms is very small, distance measurements of this gene may not be able to provide accurate information, such as the presence of similar species in the same genus. And also multiple copy numbers of the 16S rRNA gene can overestimate the prokaryotic biodiversity (Case et al., 2007). Therefore, use of other house-keeping genes like *rpoB* (encoding the β-subunit of the bacterial RNA Polymerase), *rpoA* (encoding the α-subunit of RNA Polymerase), *rpoC* (encoding beta subunit of DNA dependent RNA polymerase), *gyrB* and *gyrA* (encoding DNA gyrase), *recA* (DNA damage repair, induction of SOS repair

and homologous DNA recombination), *ppk1*(encoding for polyphosphate kinase gene 1) (Das et al., 2014).

The phylogenomic identification of certain genera is difficult with the employment of single marker gene. The Multilocus sequence typing (MLST) improves the accuracy of the microbial systematics and the principle involves the measurement of DNA sequence variations using a set of house-keeping genes to characterise the strains (Maiden, 2009). A similar approach in archaeal taxonomy is the Multilocus Sequence Approach (MLSA), involving the sequencing of the five housekeeping genes (*atpB, EF-2, radA, rpoB* and *secY*) (Papke et al., 2011).

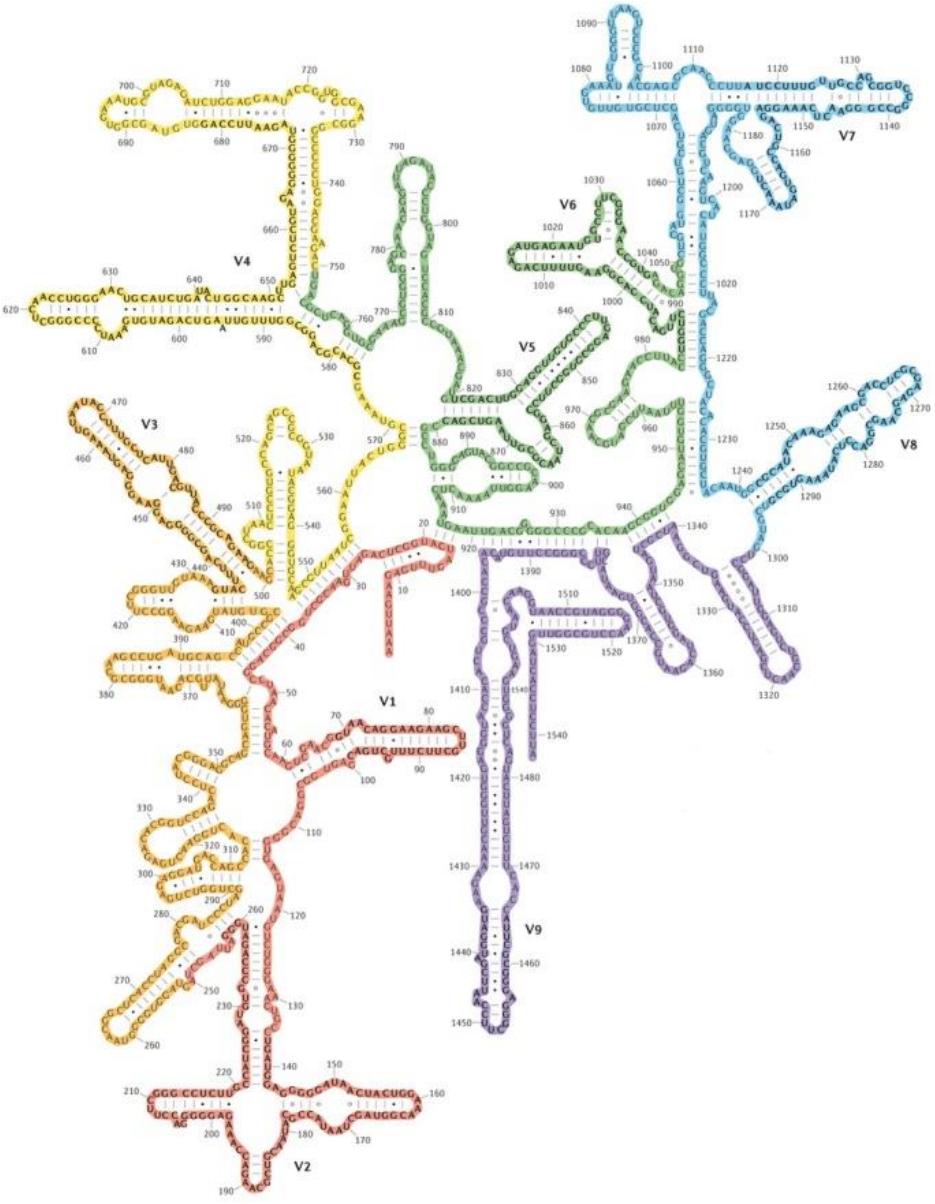


Fig 1.4: 16S rRNA secondary structure of *E. coli* and the distribution of variable regions(adapted from Y. Pablo (2014))

1.2.4.2.1 Denaturing Gradient Gel Electrophoresis

Similar length double stranded PCR products of different sequence composition can be separated using the Denaturing Gradient Gel Electrophoresis (DGGE) technique (Fig. 1.5). The technique exploits two principles of DNA composition and structure: the guanine-cytosine, with three hydrogen bonds is stronger than adenine-thymine and the reduced mobility of partially denatured double stranded DNA in a polyacrylamide gel. In this method, an amplicon of the 16S rRNA region is applied to a polyacrylamide gel with an increasing gradient of denaturants, urea and formamide. During the mobility of the DNA amplicons, the dsDNA will denature on reaching a particular denaturant composition, based on the GC content of the sequence. The amplicons with higher GC rich region will migrate further in the gel when compared with the lesser GC rich fragments. Since single stranded DNAs have greater mobility to prevent their complete run off from gel; a GC rich 40 base pair clamp is added at one end of the amplicon. Thus amplicons will have strands of denatured DNA with a bridge of GC rich double stranded sequence holding together. The amplicons have no mobility and hence are trapped in the gel. Since the GC content of every organism has a considerable variation the position of the band would be characteristic of particular taxa. Sequencing the amplicons eluted from the gel, the identity of the organisms can be confirmed (Muyzer et al., 2004; Muyzer, 1999; Muyzer & Smalla, 1998).

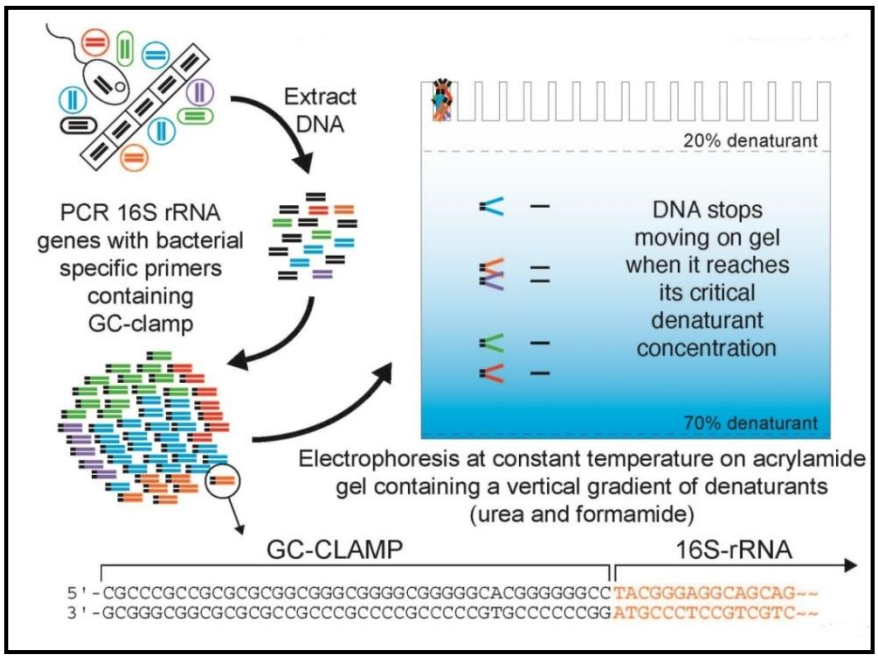


Fig. 1.5: Schematic representation of the principle of Denaturing Gradient Gel Electrophoresis (DGGE).

1.2.4.3 Metagenomics and the Next Generation Sequencing techniques

Quantifying the degree of microbial diversity was hampered by the inconsistency between the degree of the microbial community that could be measured and the actual community size. Though much advancement was made in sequencing technologies since Sanger demonstrated the DNA sequencing in 1977, the most important innovation occurred in 2005 with the demonstration of massively parallel sequencing or Next Generation Sequencing (NGS) (Schuster, 2007). High throughput by producing millions of sequences concurrently at much lower cost compared to Sanger sequencing is the salient feature of NGS. The advent of high throughput massively parallel sequencing technologies allowed for more environmental samples to be sampled at a higher level of phylogenetic diversity, generating more robust methods of inferences between environments (Morozova & Marra, 2008). The goal of a metagenomic sequencing study is to sequence an entire microbial community without isolating or cultivating individual organisms. This is accomplished by sequencing the mixture of bacterial genomes comprising the community of DNA (Shokralla et al., 2012).

1.2.4.3.1 Roche 454 Genome Sequencer

Prior to the sequencing reaction, DNA fragments are bound to beads in a 1:1 ratio. The bound DNA molecules are colonially amplified in an oil-water emulsion. The beads are then transferred into wells of picotiter plate containing enzymes and sequencing reagents. The 4 enzymes included in the pyrosequencing system are the Klenow fragment of DNA Polymerase I, ATP sulfurylase, Luciferase and Apyrase. The reaction mixture also contains the enzyme substrates adenosine phosphosulfate (APS), d-luciferin and the sequencing template with an annealed primer to be used as starting material for the DNA polymerase (Ahmadian et al., 2006).The Pyrosequencing is accomplished through incorporations of a deoxynucleotide triphosphates (dNTPs) base into a synthesized DNA chain which releases a pyrophosphate (Fig. 1.6). This is followed by the enzymatic production of ATP through the released pyrophosphate. The synthesised ATP is used in the production of a quantifiable light signal with the help of luciferase and the light signal is detected by a camera. This reaction is carried out on beads that contain millions of copies of a single DNA molecule. The sequential collections of images are analysed to measure the intensity of light. The amount of light generated determines a specific dNTP in that flow was incorporated and then translated to DNA sequence for each bead (Mardis, 2008).

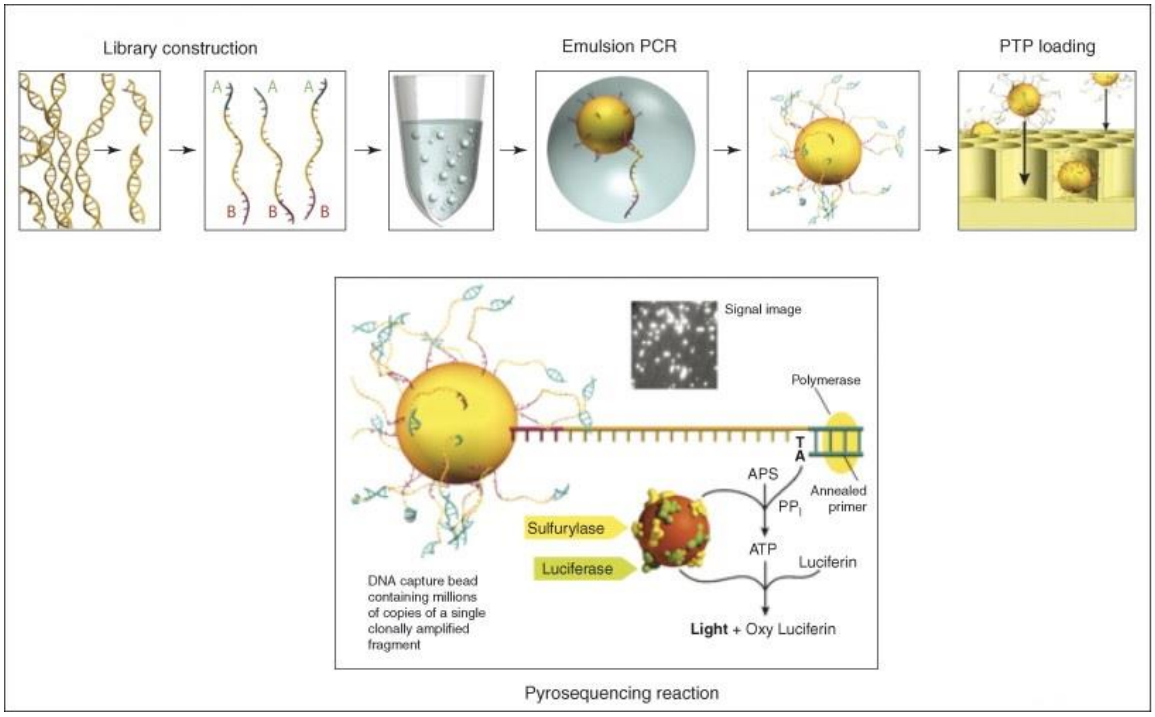


Fig 1.6: Workflow of Roche 454 pyrosequencing technology (adapted from Mardis, 2008)

1.2.4.3.2 Illumina Sequencing

Though much advancement was made in sequencing technologies since Sanger demonstrated the DNA sequencing in 1977, the most important innovation occurred in 2005 with the demonstration of massively parallel sequencing or Next Generation Sequencing (NGS). High throughput by producing millions of sequences concurrently at much lower cost compared to Sanger sequencing is the salient feature of NGS. Illumina sequencing is one of the NGS technologies working with the sequencing by synthesis strategy (Fig. 1.7). In this method, the DNA is fragmented into short sequences and the adapters, indices and terminal sequences are attached to the ends of the sequence through reduced cycle amplification. The DNA sample is then introduced onto an acrylamide coated glass slide with oligos attached on the surface. The sequence of the oligo will be complementary to the one of the terminal sequence adapters attached to the end of the DNA strands. Once the DNA strands are attached, bridge amplification is performed with a cluster generator. In this process, a single copy of DNA strand will be replicated to millions of identical strands through clonal amplification. A reversible terminator containing fluorescent labelled nucleotides is added in a sequential manner to the single stranded DNA molecule. Since each nucleotide has a characteristic output signal, after addition of each base, the signal is recorded and the unused nucleotides are washed away. Following this, the terminator dye, blocking the addition of next nucleotide, would be

removed and the cycle would be repeated again. Once the sequencing of a single strand is completed, the strand would be reversed and the sequencing would be carried out in the opposite direction (Shokralla et al., 2012; Caporaso et al., 2011; Mardis, 2008).

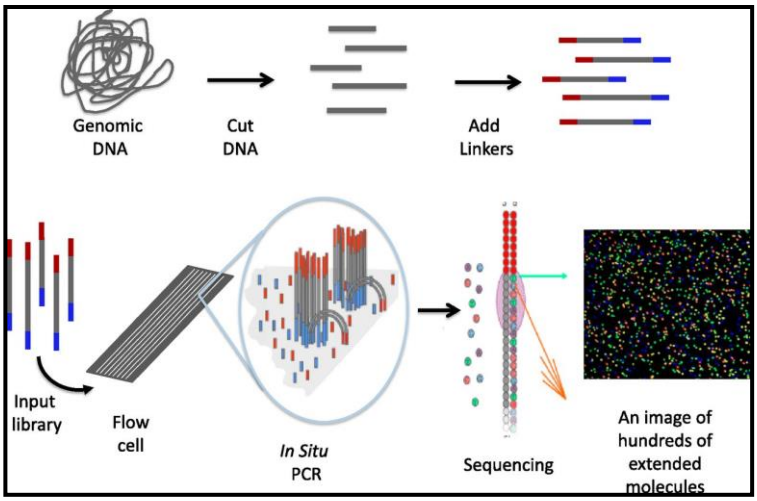


Fig. 1.7: Schematic representation of the work flow during Illumina sequencing (Johnsen et al., 2013).

Since millions of bases are generated per run, it is a difficult process to analyse the sequences. The general downstream processing also involves removal of noises generated, aligning the paired reads, performing sequence alignments and cluster analysis, phylogenetic analysis and statistical measures. Environmental DNA can be sequenced through two different approaches depending on the requirements of the study (Caporaso et al., 2012). Amplicon sequencing involves amplifying the 16S rRNA genes from the environmental DNA and sequencing will give a picture of the community structure. Other approach called metagenome shotgun sequencing involves sequencing the entire environmental DNA. Through this approach apart from the community structure, the functioning of the ecosystem as a whole can be analysed through the sequences of the metabolic genes. Whole genome of the organisms, the community structure of the ecosystems and the metabolic potential and functioning of the ecosystems can be studied in depth using Illumina technology. (Caporaso et al., 2012; Degnan & Ochman, 2012).

1.2.5 Microbiology of Solar Salterns

Multi-pond solar salterns present a gradient of salinities, from seawater salinity to halite saturation. The salt concentration in each pond is kept relatively constant, and microbial community densities are generally high (Oren, 2002). Few salterns that have been studied for their community composition are represented in Fig. 1.8. Preliminary

studies on determining the biodiversity of solar salterns employed culture-dependent techniques and culture-Independent techniques like cloning of 16S rRNA genes, DGGE and T-RFLP. Some of the haloarchaeal genera isolated from solar salterns are, *Haloferax*, *Natrinema*, *Halogeometricum*, *Halococcus*, *Haloterrigena*, *Halorubrum*, *Haloarcula*, *Halobacterium*, *Haloquadratum*and *Natronomonas*. Bacteria belonging to the phyla *Bacteriodetes*, *Proteobacteria* and *Firmicutes* dominate the salterns (Ventosa et al., 2014; Baati et al., 2010; Pasic et al., 2007; Sánchez-Porro et al., 2003; Benlloch et al., 2002).

NGS studies on the community analysis of hypersaline ecosystems are at its infant stages. Metagenomic sequencing of multiple hypersaline systems (Santa Polasaltern in Spain — 13–37% salinity), the hypersaline lake Tyrell in Australia (29% salinity) and crystallizer ponds in the USA (18–38% salinity) show equally high phylotypic diversity and the general dominance of Archaea, in particular the square-shaped halophilic archaeon *Haloquadratum*. Similar metagenomic study at Santa Pola saltern in Spain has led to the discovery of a novel uncultivated class, the 'Nanohaloarchaea' (Ventosa et al., 2014; Cowan et al., 2015).

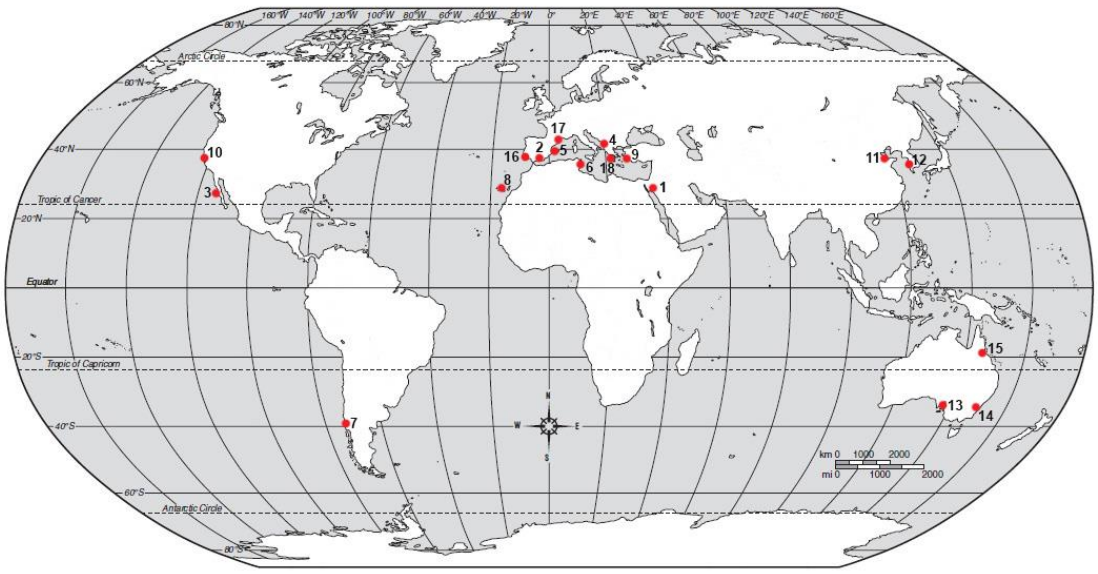


Fig 1.8: Major salterns around the world studied for their community compositions. 1 – Eliat, Israel; 2 – Santa Pola – Spain; 3 –Baja California, Mexico; 4 –Secovlje, Slovenia; 5 – Mallorca, Spain; 6 – Sfax, Tunisia; 7 – Maras, Chile; 8 – Grand Canary, Canary Islands; 9 – Eastern Anatolia, Turkey; 10 – San Francisco, USA; 11 – Tianjin, China; 12 – Daechon, Korea; 13 – Dry Creek, Australia; 14 – Bajool, Australia; 15 – Corio, Australia; 16 – Algarve, Portugal; 17 - Salin-de-Giraud, France; Missolonghi, Greece.

1.2.6 Halocins

Organisms of all three domains of life are known to produce proteinaceous antagonists, which can inhibit the growth of closely related strains, when competing for space and nutrients (Riley & Wertz, 2002). Inhibitory substances produced by archaea, "archaeocins", were first detected among extreme halophiles in 1982 and were given the name halocins (Rodríguez-Valera et al., 1982). Till date, all reported archaeocins are produced by halophilic archaea from the family *Halobacteriaceae* as secreted compounds and are termed as halocins (Shand & Leyva, 2008). Halocins are divided into protein halocins and microhalocins based on their molecular mass. Protein halocins include H1 and H4, with a size range of approximately 30-40 kDa (Shand & Leyva, 2007; Meseguer & Rodríguez-Valera, 1985). Microhalocins, such as H6/H7, R1, C8, S8, U1 and Sech7a are smaller than 10 kDa (Fig. 1.9). Microhalocins are characterized by the ability to withstand low salt concentrations, heat, and long-term storage while protein halocins are generally more sensitive to the above said conditions and lose their activity (Pašić et al., 2008; Shand & Leyva, 2007; Meseguer & Rodríguez-Valera, 1985).

1.2.6.1 Characteristics of Halocins

In 1994, Torreblanca reported that "Production of halocin is a practically universal feature of archaeal halophilic rods" after screening 147 isolates (Torreblanca et al., 1994). However, even after 21 years we have only 13 halocins characterized and purified (Table 1.2). Halocins share the following common features,

(i) Halocin genes are located on megaplasmids (mini-chromosomes)

(ii) Halocin genes have typical haloarchaeal TATA boxes and transcription factor B or B recognition elements (TFB/BRE)

(iii) Halocin transcripts are "leaderless", where the transcriptional start site is either coincident with or only a few basepairs (bps) upstream of the translational start codon ATG

(iv) Halocin preproteins appear to be exported by the twin-arginine translocation (Tat) pathway, as all have a Tat signal motif at their amino terminus

(v) Microhalocins are hydrophobic and are robust, as they can be desalted without losing activity, are insensitive to organic solvents such as acetonitrile and acetone, are relatively insensitive to heat.

(vi) Protein halocins (halocins H1 and H4) are heat-labile and lose activity when desalted below 5% (w/v) NaCl.

1.2.6.2 Mode of Action of Halocin

Halocins have been reported to generally kill the indicator / sensitive organisms by cell swelling followed by cell lysis (Pašicet al., 2008; Sun et al., 2005; O'Connor &Shand, 2002). The mechanism of action of halocin may involve modification of cell permeability or inhibition of Na^+/H^+ antiporter and Proton flux. Few halocins are said to be salt dependent since the protein loses its activity when the concentration of salts decreases beyond a minimum level (Price &Shand, 2000; Rodriguez-Valera etal., 1982). Halocin H6 produced by haloarchaea *Haloferax gibbonsii* was reported to inhibit Na^+/H^+ exchanger (NHE) in mammalian cells (Meseguer et al., 1995).

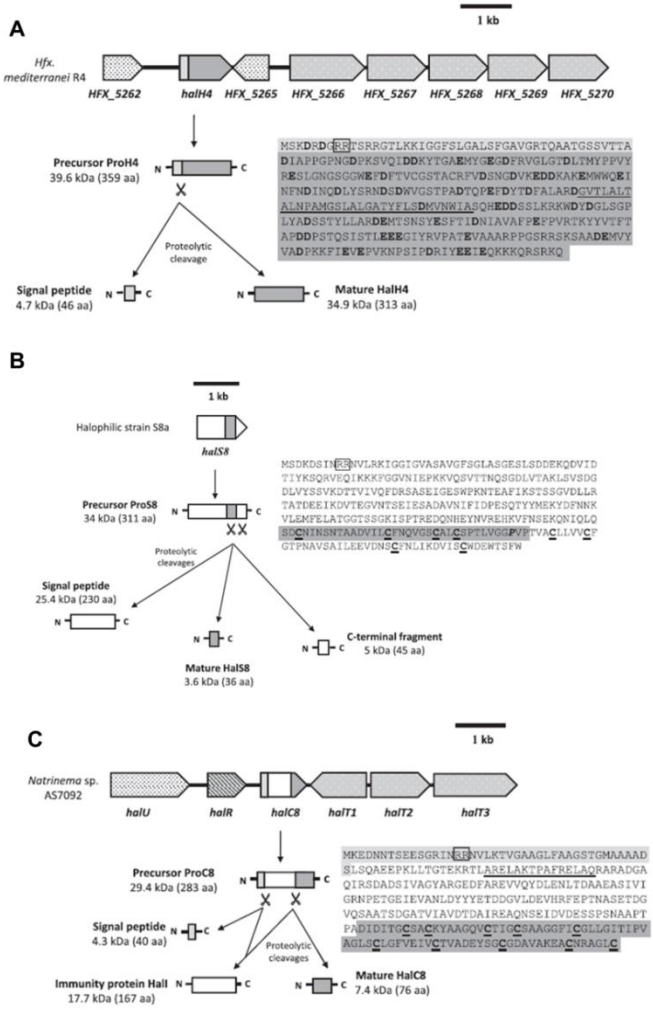


Fig. 1.9: A) Genetic locus around the halH4 gene involved in the biosynthesis of HalH4, maturation pathway and amino acid sequence of the precursor protein; (B) Genetic locus showing the halS8 gene involved in the biosynthesis of HalS8, maturation pathway and amino acid sequence of the precursor protein; (C) Genetic locus around the halC8 gene involved in the biosynthesis of HalC8, maturation pathway and amino acid sequence of the precursor protein.

1.2.6.3 Applications of Halocins

Haloarchaeal growth on hides could be prevented with natural antiarchaeal compound such as halocins (Birbir & Eryilmaz, 2005). Halocin H6/H7 from haloarchaea *Hfx. gibbonsii* was reported to inhibit the Na^+/H^+ exchanger (NHE) in mammalian primary cell culture from human skeletal muscle (myocytes and fibroblasts), macrophage, HEK293, NIH3T3, Jurkat and HL-1cells (Lequericaet al., 2006). Its cardio-protective efficacy on the ischemic and reperfused myocardium has also been evaluated.

Table 1.2: Halocins producing haloarchael strains and their characteristics.

Halocin	Producer Strain	Size kDa	Thermal Stability	Salt Dependence
A4	*Halobacterium* TuA4	< 5	> 1 week	No
C8	*Halobacterium* AS7092	7.4	> 1h at 100°C	No
G1	*Halobacterium* GRB	ND	ND	ND
H1	*Hfx. mediterranei* M2a	31	<50°C	Yes
H2	Haloarchaeaon GLA22	ND	ND	ND
H3	Haloarchaeaon Gaa 12	ND	ND	ND
H4	*Hfx. Mediterranei* R4	34.6	<60°C	Partial
H5	Haloarchaeaon Ma2	ND	ND	ND
H6/H7	*Hfx. gibbonsii* Ma2	3.8	> 90°C	No
R1	Haloarchaeaon GN101	3.8	60°C	No
S8	Haloarchaeaon S8a	3.58	100°C	No
SechA	*Hfx. mediterranei* SechA	10.7	80°C	Partial
SH10	*Natirnema* sp., BTSH10	20	< 40°C	Partial

ND - not determined; kDa- kilo Dalton

1.3 Gaps in Existing Research

It is evident from the literature that halophiles are still largely unexplored and with advancements in phylogenetics, the taxonomy of the halophiles is continuously changing (Oren, 2014). Solar salterns are excellent environments for exploring halophilic microorganisms when compared with other hypersaline environments (Javor, 2002). India is the third largest producer of solar salt and has 145308 ha under salt production (Salt Department, 2013 – 2014). In spite of huge area under salt production, halophilic biodiversity of Indian salterns is not documented. It is need of the hour to study the

biodiversity of Indian solar salterns which may help us in better understanding of the halophilic distribution, physiology and their ecological roles. Studying unexplored econiches like solar salterns can help us in isolating potential novel organisms harbouring diverse biotechnological applications.

Techniques so far employed have been culturable employing single or two media and non-culturable employing cloning of marker genes. With the advancements in molecular and computational phylogenetics, it is necessary to employ advanced techniques like next generation sequencing (NGS) for studying the community composition of solar salterns at various salinities. This will give us a deep insight of the interactions occurring between bacterial, archaeal and eukaryotic microbes in salterns at different salinities. Halocins are bacteriocin like antimicrobial proteins produced by halophilic archaea (Shand, 2006). Studies on halocin are still in its infancy. Therefore, it is proposed to study the biodiversity of solar salterns of India employing culturable and non-culturable techniques and screening the isolates for the production of potential antimicrobial substances.

1.4 Objectives of the Research Work

The following objectives were undertaken for study,

(i) Studying the halophilic archaeal and bacterial diversity of solar salterns during the preparatory phase, initial salt harvesting (ISH) phase and peak salt harvesting (PSH) phase through culture-dependent and culture-independent techniques.

(ii) Morphological, biochemical, chemotaxonomic and phylogenetic characterization of the culturable halophilic archaeal and bacterial isolates.

(iii) Screening and characterization of antimicrobial/antiarchaeal substances produced by the isolates.

Chapter II

Case studies on Solar Salt Production in Goa and Tamil Nadu

2.1 Introduction

Salt has been produced in India since ancient times. India's long stretch of coast lines and the favourable climatic conditions have made salt making a conducive activity. For several years, salt was produced at Rann of Kutch and along the coasts of Bombay, Bengal and Madras. Similar to European countries, salt production was monopolised by the government and rulers since ancient times. References linking to the taxes of salt have been mentioned in ancient texts dating back to 300 B. C. (Cirillo et al., 1994). During the colonial British rule, the salt was heavily taxed, creating a downfall in local labour force involved with the production of solar salt. This had created a major grievance among the public giving a flare to the independence movement (Jhala, 2009). India is the third largest salt producer only after China and USA. The main contribution comes from the states of Gujarat, Rajasthan and Tamil Nadu, which is about 90% of total production (Fig. 2.1). Salt production methods vary widely among different salt producing states. Sea water, sub-soil brine, lake brine and rock salt deposits are being used for salt production. Private sector contributes a major portion in salt production, contributing 90.3% of total production (Salt Department, 2014). To study the biodiversity of halophiles, a thorough knowledge of the climatic conditions experienced by the salterns and operation of salterns is essential. In this chapter, salt manufacturing process is compared between two states of Goa and Tamil Nadu.

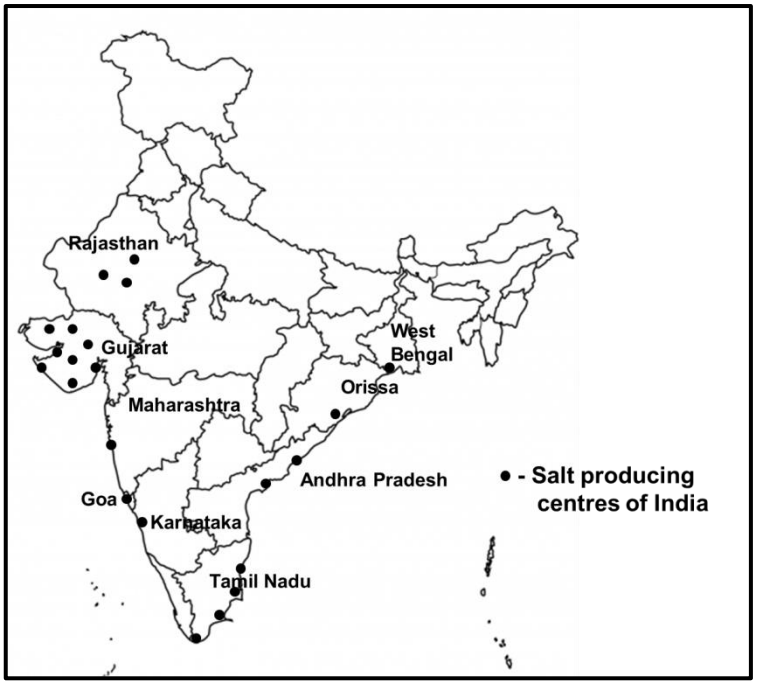


Fig. 2.1: Important salt producing states and their centres of India (Salt Department, 2014).

2.2 Case Study I: Salt Production in Goa

Goa, together with Daman and Diu, was a province under the Portuguese rule from 1510 and referred to as *Estado da India*. Goa was annexed by India on 19th December 1961 and liberated from the Portuguese rule (Pinho, 2007). Solar salt production in Goa had been an important industry until recently. Goa experiences tropical monsoon climate with hot summers followed by long monsoons from June to October. Goa has 9 rivers, the major being river Mandovi and Zuari and most of them form estuaries. These rivers experience high tidal influx during summers and therefore the salinity varies during monsoon (2-3%) and non-monsoon times (4-5%). Various factors such as favourable climatic conditions and easy accessibility to sea water have aided salt production through solar evaporation in Goa for hundreds of years (Mani et al., 2012a). Today, solar salt production has been declining due to low income generated competition from salt produced by other states, yearly damage of the saltern embankments and pollution. Currently there are 9 villages producing salt each having only a few operational salt pans (Mani et al., 2012b).

2.2.1 Historical Background

Solar salt production, described as a traditional village industry, in Goa has been practiced for the past 1500 years by various communities (Furtado & Fernandes, 2009; Sequeira, 2009). Since most of the rivers form estuaries and experience tidal influxes, salt production was started primarily in the coastal villages. Salt served as an important trade commodity too, playing a pivotal role in the economy of Goa. Salt produced in the salterns of Goa was considered to be of superior quality and was exported to Burma, Thailand and other Asian countries. With the Portuguese colonization of Goa in 1510, the salt production gained a huge momentum because of the increased demand for consumption. Portuguese cuisine required surplus salt and it was also used in balancing the hull of ships for sailing. With the maritime dominance of Portuguese, salt produced in Goa was exported even to the Middle East countries. Salt was thus the major export commodity of the '*Estado da India*' through the Mormugao port (Pinto, 1989; Scammell, 1982; Monteiro, 2001; Souza, 2009).

In 1880, a brief British control over the salterns of Goa even had health related consequences due to salt deficiency. After the British had taken over the salterns, the average quota of salt for an individual was reduced from 14.5 kg to 6.5 kg. This had forced many people to reduce the intake of salt which in turn resulted in hyponatremia

(Pinto, 1990). With the annexation of Goa by India in 1961, the salt production through natural evaporation faced severe decline and continues till date. Competition from iodized salt, availability of salt at less cost from other states and lack of skilled labour forced the saltern operators to abandon and look for other opportunities (Almeida, 1985).

2.2.2 Salt Production Areas

Goa has an area of 3,702 sq. km and lies between 14°54' to 15°48' North and 73°41' to 74°26' East, with a coastline of about 110 km. The Arabian Sea borders Goa on the west, Maharashtra to the north and Karnataka to the east and south. Goa consists of 443 villages at a population of 12 lakhs. A former union territory, Goa was added as the 25th state of Indian union on 30th May, 1987. It consists of 2 districts, North Goa and South Goa and made up of 12 talukas (Xavier, 1993). Goa receives an annual rainfall of about 280 to 480 cm, most of it during the months of June to October. The salterns receive high intensity sunlight and strong winds, making salt production a successful activity in Goa only during summers. Salt production was concentrated around thirty six villages mainly in the four talukas Pernem, Bardez, Tiswadi and Salcete (Fig. 2.2). These villages lie on the estuaries of rivers of Terekhol, Chapora, Baga, Mandovi, Zuari and Sal.

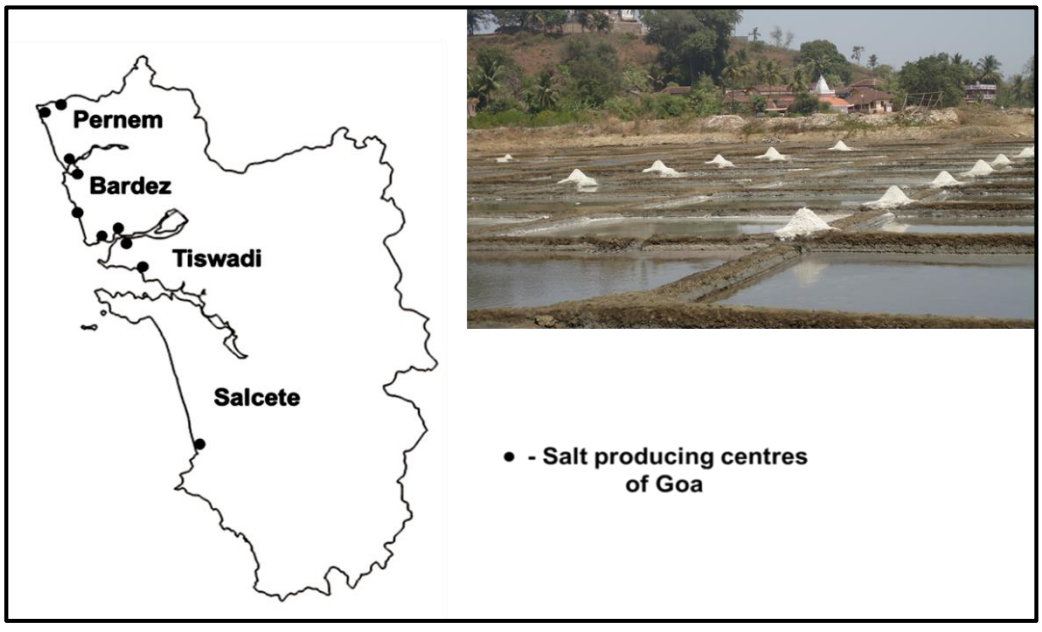


Fig. 2.2: Important salt producing talukas in Goa.

Currently, the number of salt producing villages has drastically reduced to 9 and the total area under current salt production is about 88 ha (Panigraphy et al., 2009) (Fig.

2.3). Because of the landscape and ownership of lands, all the salt pans in Goa come under the category of a small scale production (area of a single saltern less than 4.04 ha) and owned by the private sector. In 1876, Goa's salt production was about 44 kton and in 1961, reduced to 31 kton. In 2013-2014, Goa's total salt production was a mere 1.8 kton. This is very low when compared with India's total salt production in 2013-2014, which was about 23019 kton (Salt Department, 2014).

2.2.3 Communities Involved in Salt Production

In Goa, a unique organizational structure called *comunidade*, headed by a hereditary descendant, involves in governing villages and regulating the agricultural activities. Each village constitutes a *comunidade* and has its own rules depending on the local customs. This is one of the oldest administrative setup, which is in existence for the past thousand years and has been recognized by the constitution. In the past, *comunidade* was responsible for reclaiming the waterlogged lands (*Khazans*) along the coasts and making them suitable for agricultural activities, aquaculture, pisciculture and salt production. Therefore all the activities carried out in these *khazan* lands were regulated by *comunidade* including salt production. The income obtained from these *khazan* lands were utilized for the community development activities (DeSouza, 1990).

In Goa, five communities are involved in the salt production. They are *Mithgaudas, Gauddos, Bhandaris, Agris* and *Agers* (Sequeira, 2009; Singh et al., 2004). They either own the salterns or are employed in the salterns by one of these communities. The salt-making art was pioneered by the ancestors of the *mithgauda* community known as '*shannons*'. The *mithgauda* is a subdivision of the *gauda/govada* community mainly settled in Corgaon and Agarwada region of Pernem taluka. They are believed to have migrated from the Konkan belt of Maharashtra (Singh et al., 2004). Even though the procedure for producing salt followed by all the communities is same; there are some minor variations in the collection and heaping of salt crystals from crystalliser pans (CP). This indicates the evolution of salt production process within a community over a period of time. In the past salt production alone was a source of income for the people belonging to these communities. With the increase in profits and the importance emphasised on salt production by Portuguese, many people who had lands accessible to seawater started producing salt. After this, salt production process, dwelling only inside the communities, was getting popular among the general public. Salterns further provided employment opportunities for the migrant workers from the neighbouring states. In the past, many

people inherited the salt production as it was a community based activity. However with the advent of other job opportunities with better income, yearly damage of embankments protecting the salterns, people gradually abandoned this activity. In recent times many *comunidades* have stopped producing salt because of the lack of skilled labour thus leading to a reduction in number of operational salterns (Sequeira, 2009)

Fig. 2.3: A) Location of salterns at Velha, Goa in close vicinity to urban dwellings; B) Salterns of Agarvaddo, Goa on the banks of Chapora river; C) Operational saltern at Sinquetim, Goa during 2003 and D) Ceasing of salt production at Sinquetim salterns in Goa during 2015.

2.2.4 Process of Salt Production in Goa

Khazan lands of Goa are reclaimed mesohaline agricultural lands in the estuarine regions. At most of the places these *Khazan* lands are surrounded by a thick lush of mangrove vegetation. The salinity and tidal influx is regulated by embankments (dykes/bunds/*mero*) and sluice gates (*manos*) (Sonak et al., 2005). These sluice gates are symbols of rich cultural heritage and engineering skills. *Khazans* are described as contour controlled, topo-hydro engineering agro economic and agro-ecological sustainable productive systems. These *Khazan* lands are utilized for agriculture, horticulture, pisciculture and salt production (Kamat, 2004).

The salterns *(Mithache Agor or Mithagar)* in Goa experience two phases; namely the preparatory phase and the salt harvesting phase (Fig. 2.4). These salt pans are located in close proximity to the sea or may be located on the estuaries of a river. During monsoons, salterns lie submerged in rain water and therefore abandoned or utilized for aquaculture for breeding fish, shrimps and prawns (Rubinoff, 2001).

2.2.4.1 Preparative Phase (December to January)

The preparation of salterns is carried out from December to January. Before preparation, the previous embankments (dykes/bunds) that were damaged due to the monsoons are repaired. Rain water / sea water from the salterns are drained using motor pumps. Once the water has been completely drained, the preparation of saltern beds begin. The beds are ploughed, levelled by stamping and using a device called '*saalon*'. The extra clay is raked onto the walls of the bunds. *Saalon* has a long stick, approximately of 4 m in length, attached to a circular base. During this process the borders of different ponds are also constructed.

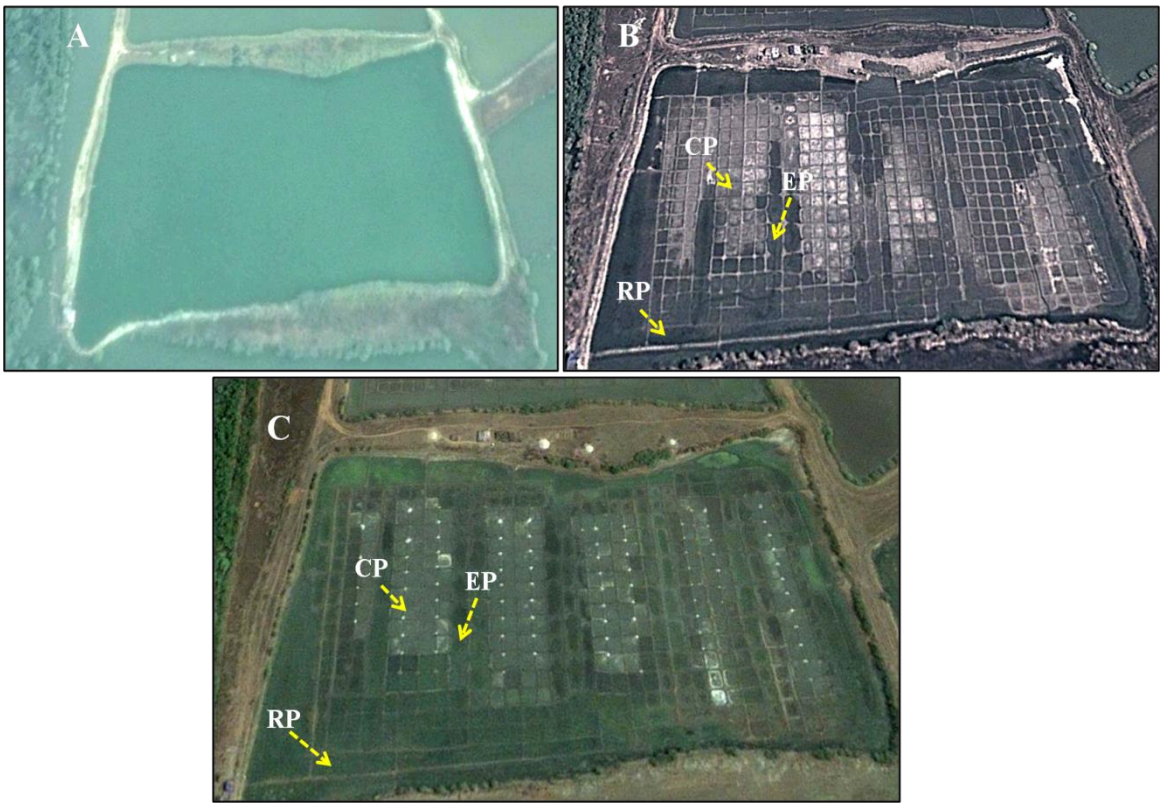


Fig. 2.4: Solar salterns at Siridao, Goa A) Flooded with rain water during monsoon; B) Salt pans during the preparatory phase and C) Salt harvesting phase. The salt crystals collected at the interjection between two crystalliser tanks are observed as white dots. Reservoir pan (RP), evaporator pan (EP) and crystalliser pan (CP) indicate the location of sampling sites.

The salterns consists of three distinct pans namely; reservoir pan (RP) (*tapovanim*/*tapounni*), evaporator pan (EP) (*podshing*) and crystalliser pan (CP) (*pikechim agor*) (Kamat, 1997) (Fig. 2.5). The entire pans are inter-connected to each other through an opening at the corners. Reservoir pan (RP) is used for storing the sea water during the time of tidal influxes and is connected to many evaporator pans (EP). Crystalliser pan (CP) in turn is fed by the evaporator pans (EP). The dimension of reservoir pan (RP) is 18-20 x 10-12 m, while that of the evaporator pan (EP) is of 18-20 x 6-8 m. In some salterns the reservoir pan (RP) may be of the same size as evaporator pan (EP); however the main difference is the depth of these two pans. Reservoir pans (RP) are around 20 inches deep, maintaining a water level of up to 15-18 inches deep while the evaporator pans (EP) are 10 inches deep in which water is filled for up to 5 inches. The reservoir pan (RP) is twice or thrice the size of the crystalliser pan (CP). The dimension of crystalliser pan (CP) is 6.5-8 x 4-5 m and the depth is of same as that of the evaporator pan (EP). The brine level in the crystalliser pan (CP) is maintained at a maximum level of 3 inches. The size of evaporator pans (EP) plays a critical role in the production of salt, bigger the size of evaporator pans (EP), better the production of salt.

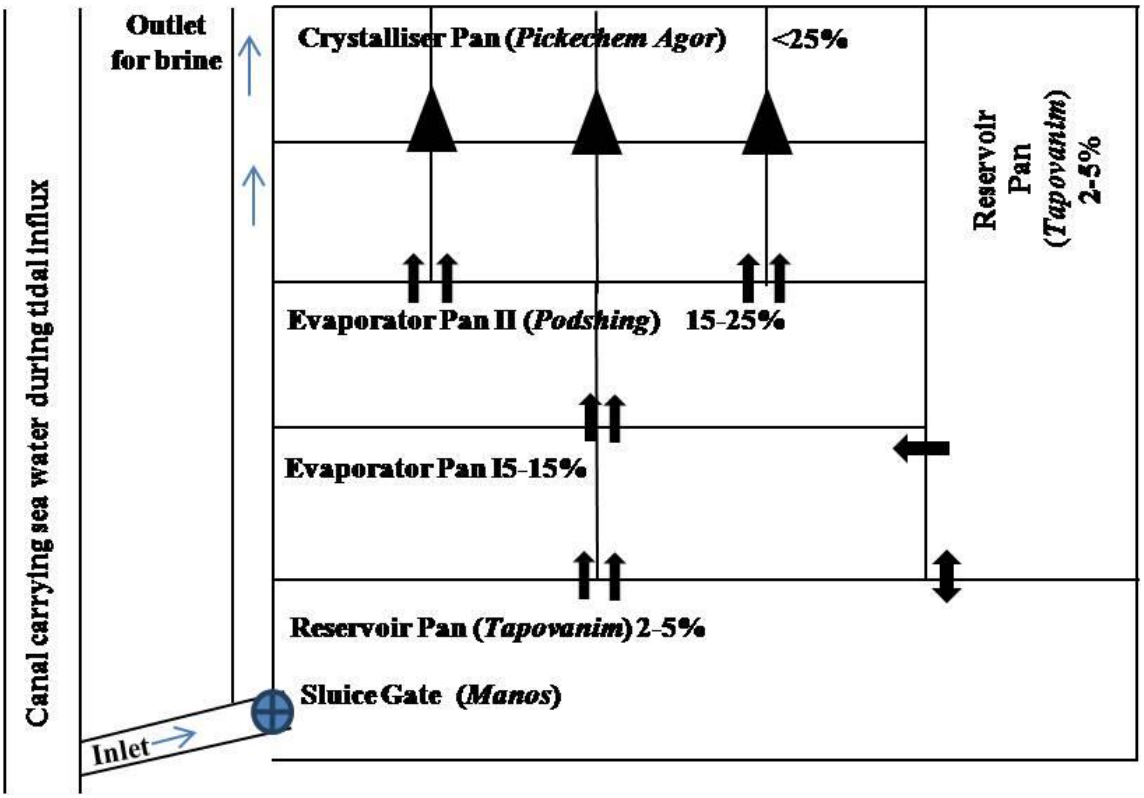


Fig. 2.5: Layout of a typical solar saltern of Goa.

The reservoir pan (RP) is connected with the creek or canals, supplying seawater, during tidal influxes, through a sluice gate (*Manos*). Sluice is made of wood and the gates are made up of clay mixed with hay. It helps in the controlled release of seawater into the salterns during high tide and prevents the backflow of water during the low tide, thereby maintaining the level of water in the reservoir pan (RP). Algal growth occurring in the pans are harvested regularly and used as fertilizers for coconut and cashew plantations. The reservoir pans (RP) are also used for pisciculture especially for the breeding of salt water fishes, from the months of October to December (Fernandes, 2006).

Once the salinity of seawater in the reservoir pan (RP) is around 5%, it is released to the first evaporator pan (EP). Calcium carbonate ($CaCO_3$) starts precipitating at salinity around 5% in the reservoir pan (RP) and completely precipitates in the first evaporator pan (EP). Once the brine attains salinity around 13-15%, it is released from the first evaporator pan to the second evaporator pan (EP). In the second evaporator pan (EP), calcium sulphate ($CaSO_4$) crystallises in the form of gypsum. These precipitates form a hard crust at the bed of the evaporator pans (EP). The brine, now having salinity around 23-25%, is released from the second evaporator pan (EP) to the crystalliser pan (CP). Sodium chloride (NaCl) crystallizes around 30%, first as flakes which float on the surface and latter settle at the bottom. The brine in the crystalliser pan (CP) appeared frothing due to the crystallisation of salt (Korovessis & Lekkas., 2009).

During the preparatory phase, seawater in the evaporator and crystalliser pan is allowed to stand and stirred time and again using a teeth shaped tool called '*danto*', for about 20 -25 days (Fig. 2.6). *Danto* has a long stick (approximately of 4 m in length), attached to a wooden block of 50-70 x 15-20 cm. The fed water is allowed to evaporate completely and the pans are fed again. This process is carried out for removing the extra clay, which in turn will be raked onto the walls of the pan, thus setting the salt pan beds. Fresh brine is released from the reservoir to the evaporator pan (EP) and finally to the crystalliser pan (CP) for salt crystallization. It takes ten days for the salt to crystallize during the first harvesting. Some crude salt (approximately 50 – 60 kg) is sprinkled over the crystalliser pans (CP) for aiding the salt formation and is repeated twice or thrice. Salt formed during the initial operations of salterns is not harvested (initial salt harvesting (ISH) phase). This hardens the beds and thus making them uniform for further harvesting. The salt harvested initially, contains lot of impurities due to the suspended clay particles and is brownish to grey in colour. This salt is not fit for consumption and is used as fertilizers or for curing of fish.

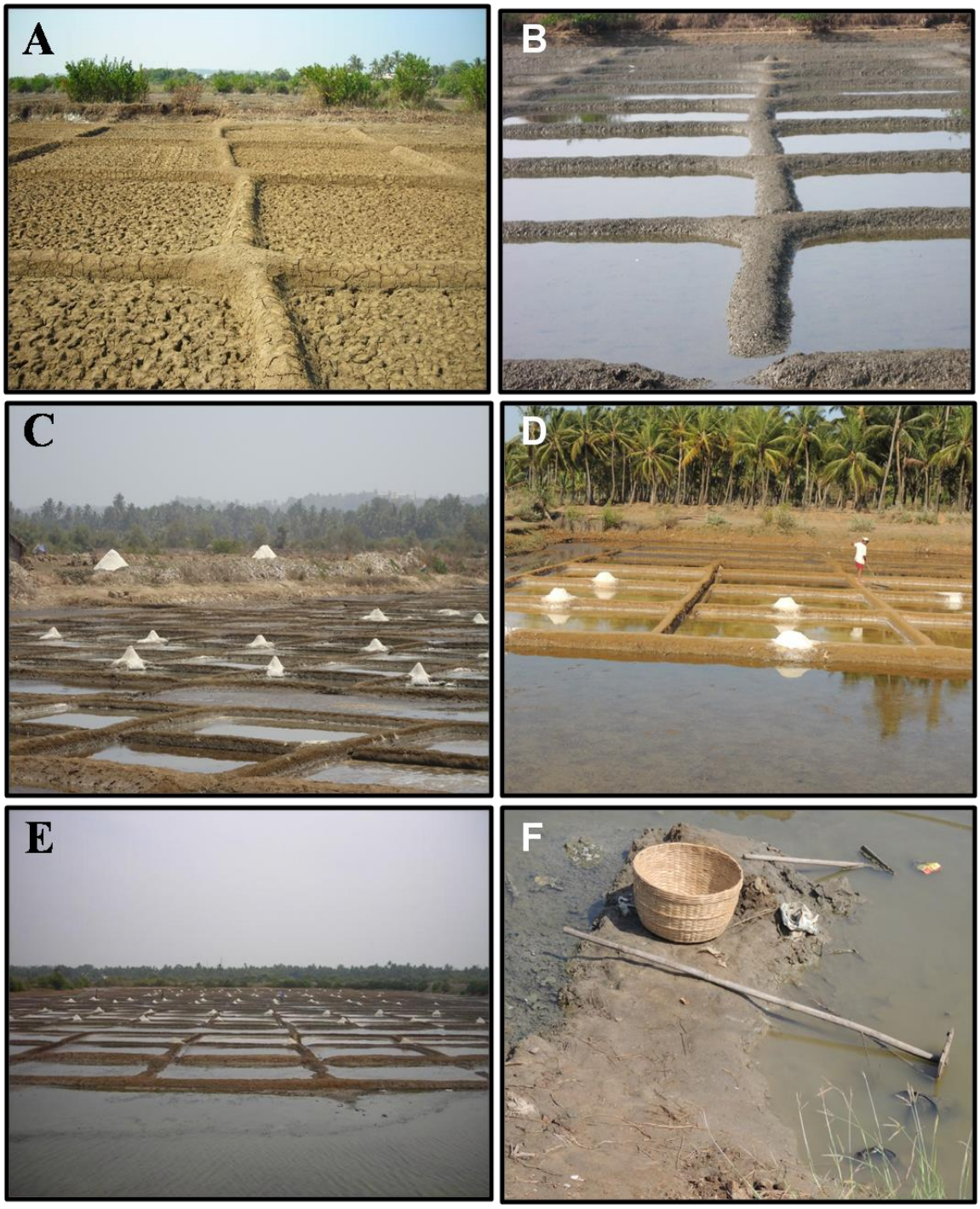


Fig. 2.6: A) Preparation of saltern bed after monsoon; B) Salterns fed with brine for salt production; C) Salt harvested and piled at the saltern junction; D) Saltern worker harvesting salt; E) Salterns of Siridao, Goa; F) Tools used for harvesting salt.

2.2.4.2 Salt Harvesting Phase (February to May)

Once the salterns are completely prepared, the peak salt harvesting (PSH) season begins, and it lasts from mid-February to May or early June, depending on the monsoon. The brine solution in the evaporator pan (EP) having a salinity of 23-25% is released to the crystalliser pan (CP) and salt will be harvested on a daily basis. The brine in the crystalliser pan (CP) is kept for not longer than a day or two, till the sodium chloride crystallises out. The salt crystals are harvested with the wooden rake, *'foyem'* (a long stick of approximately 4 m in length, attached to a wooden block of 50-70 x 15-20 cm) and piled as small heaps at the intersection of the pans. In the Pernem salterns, the salt crystals are heaped at the centre of the bunds. If the brine is kept for long time, salts of magnesium and potassium co-precipitates, making the salt unfit for consumption. In such situations, the entire mother liquor is let off in the drain and then restarted. Once sodium chloride precipitates out, the remaining brine rich in magnesium and potassium (bittern/mother liquor) is drained out in the canals.

The harvested salt is further purified and washed again with the concentrated brine solution for purifiction. It is then transferred through bamboo baskets and stored in a store house. The salt produced is transported to the local market through pickup trucks. The salt produced in Goa is of two grades: (i) fertilizer grade / preservative grade and (ii) consumption grade. Initial salt harvested (for a month) will be of fertilizer grade because of the mud and clay impurities. This salt is also used for the salting or preserving dry fish. The salt produced after the fine setting of the bed will be of consumption grade, which is used as a brine solution for pickling raw mangoes and in cuisine. The salt produced in different areas may render unique taste owing to the soil texture (Kamat, 1997; Fernandes, 2006; Parikkar, 2011; Khedekar, 2011).

2.3 Case study II: Salt Production in Tamil Nadu

Tamil Nadu is the second largest salt producer in the country next only to Gujarat. Given the fact that the state has about 1000 km of coast line, second longest in the country, salt production is carried since time immemorial (Ramesh et al., 2008). In contrast to salterns of Goa, the water used for the salt production is obtained from sub-surface brine as well as from the sea, their salinity varying from 4-5% (Palanichamy et al., 2006). Solar salt production in Tamil Nadu faces more problems related to the consequences of salt production industry such as poor attention towards workers health

and wage related problems, discharge from salterns affecting fishing and potable water quality in the vicinity of the salterns rather than the sustaining of industry .

2.3.1 Historical Background

The salt industry in Tamil Nadu dates back further than Goa with the role of salt as an important trade commodity, being mentioned in the inscriptions from 3 B.C.E. According to ancient Tamil landscape classification, the coastal and adjoining lands were called as '*Neithal*' and the main occupation of the inhabitants was salt making and fishing (Iniyan, 2015). The people involved in salt production were known as '*Umanar*' and their wives were called as '*Umathiyars*'. Usually, men were involved in salt production and the women were trading the salt produced. Salt and paddy, was often bartered for other goods like cotton and food grains. Evidences obtained from the *Chola* dynasty indicate that salt industry was under direct supervision of the government, for quality management and often it was taxed. Roads were constructed connecting the coastal towns to the interior cities and villages for the easy transportation of salt (Burrow, 1947).

Taxation of salt continued even during the British rule. British government monopolised and taxed the salt produced in India for encouraging the import from England. In 1900s the production and possession of salt was deemed illegal. However, in 1920s, because of doubling of the salt tax, there was a huge moment of unrest among people which led to the salt march, an important event in country's freedom struggle (Tomlinson, 1996; Wolpert, 2006). After independence, several private and government industrial firms were encouraged to produce salt, for meeting the country's salt demands and for exports. Currently, Tamil Nadu is a major exporter of industrial and edible salt to other Indian states and foreign countries (Salt Department, 2014).

2.3.2 Salt Production Areas

Tamil Nadu is the eleventh largest state with an area of about 130,058 sq. km and lies within coordinates 13^{o}09'N and 80^{o}27'E. Tamil Nadu is bordered by Bay of Bengal to the east, Indian Ocean to the south, state of Kerala to the west and the state of Andhra Pradesh to the north. Salt is currently produced in 10 districts out of 32 districts. They are Tuticorin, Nagapattinam, Ramnathapuram, Villupuram, Pudukkottai, Cuddalore, Thanjavur, Thiruvallur, Kanchipuram and Kanyakumari (Fig. 2.7). In contrast to Goa, Tamil Nadu receives rainfall from both north-east and south-west monsoon. The average annual rainfall is 94 cm with a share of 48% during north-east monsoon and 32% during south-west monsoon. Salt production is briefly halted from the months of November to

January due to the arrival of north-east monsoon. The total area under the salt production is 20000 ha and 50% the salterns come under Category I (saltern area exceeding 40 ha) according to the classification by the Salt Department of India. Tamil Nadu's salt production accounted about 2586 kton for the year 2013-2014 (Salt Department, 2014).

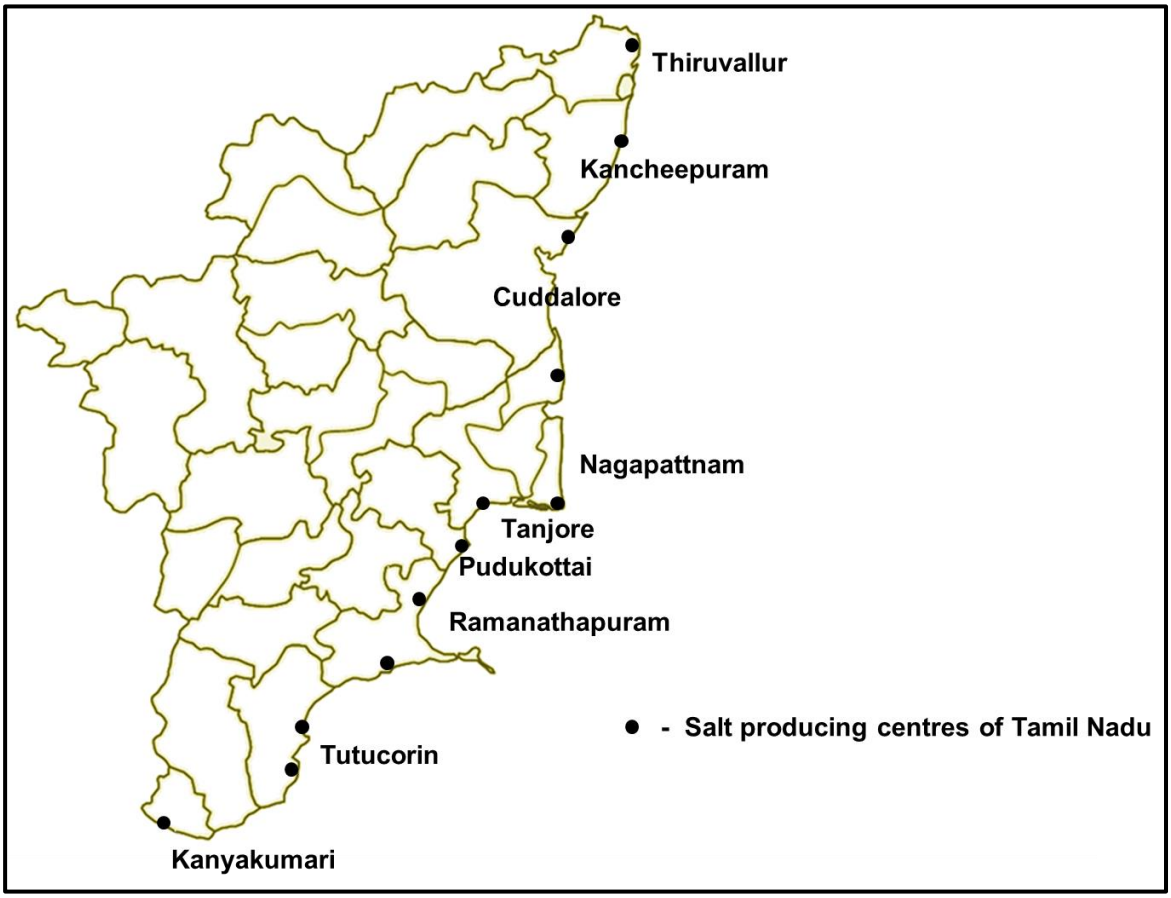


Fig. 2.7: Important salt producing districts and their centres of Tamil Nadu.

2.3.3 Communities Involved in Salt Production

Communities involved in the salt production are *Paratavar*, *Valayar* and *Meenavar*. Though fishing and salt making are their primary occupations, fishing has gained importance in recent times due to the high income generated while salt making has become an auxiliary occupation (Kanthimathi et al., 2008). In the medieval times, the salterns were under direct control of the rulers and later acquired by the British through The East India Company. Currently, the salterns are either owned by government or by industrial firms. The saltern workers of Tamil Nadu are recognised as farmers by the government and are eligible for various socio-economic and welfare schemes.

2.3.4 Process of Salt Production

The salt production in Tamil Nadu can be divided into two phases namely, preparatory phase (January - February) and salt harvesting phase (March – October). When compared to salterns of Goa, since the monsoon phase is shorter, Tamil Nadu salterns (*uppalam*) enjoy an extended period of salt harvesting. Apart from monsoons, since the coast of Tamil Nadu is located along Bay of Bengal, it is prone to unseasonal downpours, halting the salt production intermittently.

2.3.4.1 Preparative Phase (January to February)

After the monsoons, primary work of the saltern workers is to remove the silt and dirt washed into the salterns. This work is carried out manually irrespective of the scale of salterns. This is followed by the removal of vegetation bordering the salterns. The borders of the reservoir pan (RP) (*Theppam*), evaporator pan (EP) and crystalliser pan (CP) are reconstructed. In case of large salterns, the reconstruction task is carried through mechanized processes and while in small salterns the same task is carried out manually. Similar to the salterns of Goa, the reservoir pans (RP) are larger and deeper compared to the evaporator and crystalliser pans (CP). The dimensions of the reservoir pans (RP) vary from 20-50 x 20-50 m depending on the scale of salterns. The evaporator and crystalliser pans can vary from 14-25 x 14-25 m (Leema, 2007; Femitha & Vaithyanathan, 2012) (Fig. 2.8).

Clay soil is best suited for the salt production and is used in the construction of saltern beds. The clay soil will be procured commercially from the markets and transported to the salterns. Clay soil is then mixed in equal proportion with sand from sea shores. This is further supplemented with 3% lime and is made a homogenous mixture by the addition of 25% water. This mixture is then applied to the salt pan beds. The purpose of mixing sand is to give strength and hardness to the beds. In large salterns, a plastic sheet or tarpaulin is laid out and the salt pan beds are prepared over the sheets, to prevent the water seepage through the beds. In some places like Tutucorin, the natural soil is sandy in texture. Therefore, the clay is mixed directly with the sandy soil and the salt pan beds are prepared. The mixture is laid out on the salterns and flattened by the workers manually. In case of large salterns, rollers are used for flattening. The pans are constructed with a natural slope for the easy flow of water (Vaithyanathan, 2004; Shiba, 2008).

Fig. 2.8: A) Preparation of saltern bed at Marakkanam; B) Harvesting of salt at Marakkanam; C) Collection of salt from salt pans; (Pictures A-C adapted from https://www.behance.net) D) Salterns of Vedaranyam; E) Bore-well located in Marakkanam salterns; F) Salt crystals ready for harvest at Marakkanam salterns.

2.3.4.2 Salt harvesting phase (March to October)

Saltern beds are dried followed by the release of water from reservoir pans (RP) to other pans. Reservoir pans (RP) are periodically checked for algal and other weed growth. Mechanical pumps are employed for drawing water from sub-soil surface for the density and consistency of brine. The brine is filled in the reservoir pan and allowed to concentrate till the salinity reaches 5%. The brine is then transferred to the evaporator pans (EP). Once the salinity reaches 24%, the brine is transferred to the crystalliser pan (CP) and the salt crystallises around 35%. Entire process takes a week to two months, depending on the scale of saltern. The remaining bittern, rich in magnesium and potassium is drained through the outlet.

The salt formed is harvested after 4 days with a wooden tool which has a long stick of 3 meters in height, attached to a wooden block of 1.5 feet in length. The harvesting of the salt crystals will begin as early as 5 AM, to avoid the eye irritation caused by the reflection of sunlight from the salt crystals. The salt crystals are harvested and collected at the intersection of the crystalliser pans (CP). The harvested crystals are collected and heaped at a common place for transportation to the refineries. Salt heaps are washed off the impurities by spraying bore-well water at high speed. In case of industrial salt production, the salt crystals are washed with calcium sulphate solution.

After drying, the salt is further processed according to requirements. In Villupuram salterns, the salt crystals are directly packed and supplied to the local ration shops. However, in Tutucorin and Nagapattinam salterns, mobile pulverisers are used to grind salt crystals of desired size followed by packing and transportation. The salt crystals that are intended to be powdered and iodised are transported to the refineries usually located in the vicinity of salterns (Leema, 2007; Vaithyanathan 2004; Shiba, 2008).

2.3.5 Refining and iodisation of salt crystals

The harvested salt is transferred to a hopper located outside the refinery through tractors. The salt crystals are then transferred to the washing system through belt conveyers. Belt conveyers are fitted with magnets for removing metal impurities. Washed salt crystals enter a wet mill, where a saturated brine solution is added and slurry obtained is then transferred to slurry tank and mixed with more brine. The slurry is then pumped to the wash tank or elutriation tank to wash off impurities. The washed slurry is thickened by a thickener in the hydro cyclone and the clear brine is transferred to a brine tank.

Salt crystals are passed through a blender cum screw dryer, where potassium iodide solution is added. The mixing of iodine and salt crystals becomes uniform as they are passed through the dryer. The dry iodised salt coming out of the dryer is checked for their crystal size with a vibro screen, and transferred to a blender where it is mixed with free flowing agent (tri-calcium phosphate or calcium carbonate). The over-sized salt crystals are transferred to a pin mill, ground again and transferred back to the blender. The powdered salt coming out of the blender is transferred to the packaging section through a belt conveyer and packaged in desired packs and sizes (Aruna, 2011) (Fig. 2.9).

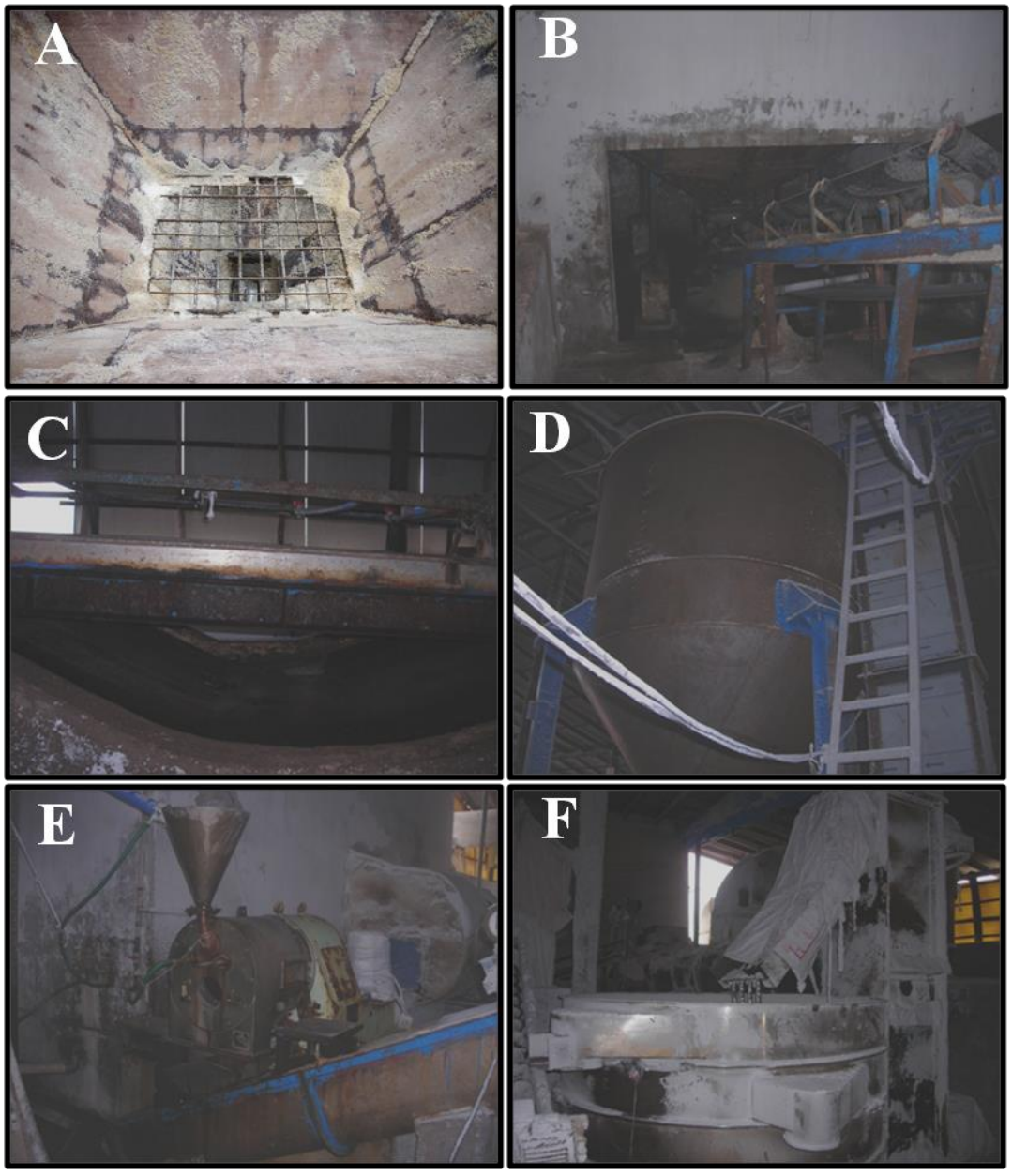


Fig. 2.9: Some equipment in salt refinery A) Hopper; B) Conveyer Belt; C) Washer; D) Settling Tank; E) Centrifuge; F) Grinder.

2.4 Conclusion

The salterns of Goa and Tamil Nadu are operated since ancient times. Goa has an area of about 88 ha under salt production with an annual production of 1.8 kton (2013-2014). Tamil Nadu has an area of about 2000 ha under salt production with an annual production of 2586 kton. Salterns in Goa experience two phases namely, preparatory phase from December to January, and salt harvesting phase, from February to June. Similarly, salterns in Tamil Nadu also experience two phases namely, preparatory phase from January to February, and salt harvesting phase, from March to October. The salt produced in Goa is of edible grade while the salt produced in Tamil Nadu salterns is of both edible and industrial grade. While the salt production in Tamil Nadu is a well-developed and growing industry, the salterns of Goa are declining due to the low income generated combined with the lack of skilled labour and competition from industrially produced salt.

Chapter III

Culturable Diversity of Halophilic Archaea and Bacteria in Solar Salterns of India

3.1 Introduction

Solar salterns are man-made systems providing a salinity gradient for the manufacturing of salt (NaCl). NaCl is obtained by the pre-crystallisation of calcium salts leaving behind magnesium and potassium salts (Javor, 2002). Organisms belonging to domain Bacteria, Archaea and Eukaryotes colonise these salterns. Organisms within salterns may thrive symbiotically. For instance, available organic nitrogen is converted to ammonia by microorganisms which in turn are utilized by *Dunaliella* for supporting the growth of brine shrimps (*Artemia*sp.). Biodiversity of salterns is in a constant flux (Oren, 2002). Although culture-independent methods have drastically improved in the past decade, culture-dependent methods are usually the first step in understanding the overall community composition and semi-quantitative diversity of the ecosystem (Hill et al., 2000).

India has 145308 hectares of coastal and inland regions under solar salt production (Salt Department, 2014). Since solar salterns located in the west and east coast of India experience differential climatic patterns, it was of interest to determine environmental impact on community structure. This chapter deals with the studies on the spatio-temporal variation of culturable halophilic prokaryotic diversity in solar salterns located on the east and west coast of India.

3.2 Materials and Methods

3.2.1 Sampling sites and sample collection

Five litres of brine/water and 2 kg of surface sediment/soil samples (at a depth of 0 – 7 cm) were collected aseptically from the following operational multipond solar salterns located on the west coast of India: Ribandar (15°30´N, 73°51’E) and Siridao(15°26´N, 73°52’E) in the state of Goa and on the east coast of India: Marakkanam (12°14´N, 79°55’E) and Vedaranyam (10°23´N, 79°36’E) in the state of Tamil Nadu. Sampling was carried out at solar salterns of Ribandar and Siridao during three different phases of salt production. Initial sampling was conducted in January 2012 (the preparatory phase) when the salterns were inundated with seawater. Subsequent samplings were conducted during the initial saltharvesting (ISH) phase in February 2012 and peak salt harvesting (PSH) phase in April 2012. Sampling in the salterns of Tamil Nadu was carried out in June 2012 during the peak salt harvesting phase only. Apart from the operational salterns, a discontinued solar saltern at Sinquetim (15°14´N, 73°57’E),

Goa was sampled in April 2012. Last reported salt production at Sinquetim was in the year 2003.The samples were stored at 4^{o}C if not processed immediately.

Salt crystals were also collected from Ribandar (Goa), Marakkanam (Tamil Nadu), Vedaranyam (Tamil Nadu) and commercial salt crystals from a grocery store.

3.2.2 Physicochemical analysis of the salterns

Temperature and pH of the sampling sites was measured using a thermometer and pH meter (Equip-tronics model EQ-632, India) respectively. Soil texture analysis was conducted based on the soil texture triangle (Appendix I) as recommended by USDA (U.S. Department of Agriculture). The salinity of the brine was measured using a brine hydrometer. Elemental analysis of brine/water and salt crystals were carried out as described in detail in Appendix II. Sodium and potassium content was measured through flame emission photometry (Systronics model 128, India). Calcium and magnesium concentration was determined by complexometric titration of Mg^{2+} and Ca^{2+} against ethylenediaminetetraacetic acid (EDTA).Chloride ion content was determined by argentometric titration against silver nitrate solution in the presence of potassium chromate as an indicator. Manganese ion composition was determined at SGS laboratories, India according to 21st edition of Standard Methods forthe Examination of Water and Wastewater (APHA, 2005).

3.2.3 Estimation of chlorophyll*a* concentration

3.2.3.1 Brine

Estimation of chlorophyll *a* was carried out using spectrophotometric method (Moss, 1967). Hundred ml of brine was filtered through magnesium carbonate coated Whatman GF/F filter paper. The filter paper was then minced and extracted with 90% cold ethanol using a mortar and pestle. The extract was incubated in dark for 10 minutes followed by centrifugation, before proceeding with spectrophotometric analysis.

3.2.3.2 Sediment

Chlorophyll *a* concentration in sediments was estimated by grinding 1 g of sediment in 90% cold ethanol for 10 minutes. Extract was then incubated overnight in dark and centrifuged. Clear extracts obtained were measured for their absorbance in a UV-Visible spectrophotometer (UV-2450, Shimadzu, Japan) at 664 and 647 nm.

3.2.3.3 Calculation

The amount of chlorophyll *a* present was calculated using the following formula (Lorenzen, 1967),

$$\text{Chl a (µg/ml or g)} = \frac{(11.93\ E_{664} - 1.93\ E_{647}) \times \text{(Volume of sample in ml)}}{\text{(Cuvette width in cm)}}$$

3.2.4 Enumeration of microorganisms in saltern sample

3.2.4.1 Sediments

Sediment samples (0.3 g) were vortexed for 5 minutes in 10 ml of 15% NaCl solution containing 4% formalin. Hundred microliters of this slurry was diluted in 10 ml of 15% saline containing 0.02% Triton X-100, followed by incubation at Room Temperature (30°C) for 15 minutes and sonication for 10 minutes. To this mixture, 0.1% of acridine orange solution was added and incubated in dark for 30 minutes. One ml of this solution was filtered through 0.22 µm-pore-size black polycarbonate filter paper (Millipore, USA). Microorganisms filtered on the filter membrane were enumerated under epifluorescence microscope (Olympus BX41, Japan). The microbial count was estimated from a count of at least 10 randomly chosen fields.

3.2.4.2 Brine/Water

For the enumeration of microorganisms present in brine/water, 90 ml of brine was incubated with 10 ml of 25% glutaraldehyde (Sigma, India) at 4°C for 10 minutes. About 10 ml of this glutaraldehyde-fixed sample was incubated in dark with 0.1% of acridine orange at Room Temperature (30°C) for 30 minutes. One ml of acridine orange stained brine sample was filtered through 0.22 µm-pore-size black polycarbonate filter paper (Millipore, USA). The filter paper was observed for microorganisms similar to the method described for sediment samples.

3.2.4.3 Calculation

1 ml or 1 g = (Average no. of microorganisms)x (Area of filter) x (dilution factor)/Area of filed view

3.2.5 Isolation, purification and maintenance of halophilic microorganisms

For the isolation of moderately halophilic bacteria, a loopful of sediment (~ 0.1 g) or brine (100 µl) was directly surface spread on agar plates of Moderate Halophilic Medium (MHM) (Table 3.1). For isolation of microorganisms from salt crystals two

different approaches were employed. First approach, involved dissolving the salt crystals in sterile distilled water to a concentration of 10% followed by surface spreading 100 µl on MHM agar plates. Second approach, involved adding 0.1 g salt crystals in liquid MHM medium followed by incubation at 37^{o}C with continuous shaking at 110 rpm. After 7 days of incubation, 100 µl of the enriched broth was surface spread on MHM agar plates. The plates were incubated at room temperature (30^{o}C) up to 30 days and analysed for the total viable count after 7 and 30 days.

Table 3.1: Composition of media employed in this study

Ingredients (gl^{-1})	**NTYE[a]**	**NT[b]**	**EHM[c]**	**JCM168[d]**	**MHM[e]**
NaCl	250.0	250.0	250.0	200.0	178.0
$MgSO_4.7H_2O$	20.0	20.0	20.0	20.0	1.0
KCl	5.0	2.0	2.0	2.0	2.0
Tryptone	5.0	-	-	-	-
Yeast extract	3.0	10.0	10.0	5.0	10.0
Beef extract	-	-	-	-	-
Trisodium citrate	-	3.0	-	3.0	-
$CaCl_2.2H_2O$	-	-	0.36	2.0	0.36
NaBr			0.23	-	0.23
$NaHCO_3$	-	-	0.06	-	0.06
Peptone	-	-	5.0	-	5.0
$FeCl_3.6H_2O$	-	-	Trace	0.036	Trace
Casamino acids	-	-	-	5.0	-
Sodium glutamate	-	-	-	1.0	-
$MnCl_2.2H_2O$	-	-	-	Trace	-

pH was adjusted to 7.0 – 7.4 with 1M NaOH. 1.8% agar was used as solidifying agent. a – Braganca and Furtado, 2009; b – Elevi et al., 2004; c - Enache et al., 2007; d – Minegishi et al., 2008; e – Ventosa et al., 1982)

Four different media were used for the isolation of extremely halophilic archaea. They were (a) NaCl Tryptone Yeast Extract (NTYE) medium (b) NaCl Trisodiumcitrate (NT) medium, (c)Japan Collection of Microorganisms 168 (JCM168) medium and (d) Extremely Halophilic Medium (EHM) (Table 3.1). For the isolation of extremely

halophilic archaea, a loopful of sediment (~ 0.1 g) or brine (100 µl) was directly surface spread on agar plates. Plates were incubated at room temperature (30°C) for 30 to 45 days in sterile plastic bags until colonies appeared. Distinct isolated colonies were streaked several times on the respective medium until a pure culture was obtained. Total viable microbial count was calculated after 7 and 30 days with the following formula,

Colony Forming Units (CFU) per ml or g = (No. of colonies x Dilution Factor)

Quantity of sample in ml or g

3.2.6 Molecular characterisation of halophilic isolates

3.2.6.1 Extraction of genomic DNA from isolates

Genomic DNA was extracted according to Pospiech and Numann (1995). Briefly, 2 ml of late-exponential phase culture grown in the respective medium was centrifuged at 12000 rpm for 3 minutes. The pellet was re-suspended in 400 µl SET buffer (50 mMTris-HCl, pH 8.0, 50 mM EDTA, 20% w/v Sucrose). Fifty µl lysozyme (10 mg/ml) and 20 µl proteinase K (15 mg/ml) was added and incubated at 37°C for 30 minutes. Then, 50 µl of 10% SDS was added and further incubated at 37°C for 30 minutes. To this mixture, 500 µl of Phenol: Chloroform: Isoamylalcohol (25:24:1, vol/vol/vol) was added and centrifuged at 13000 rpm for 10 minutes. To the supernatant, 300 µl of Chloroform: Isoamylalcohol (24:1, vol/vol) was added and centrifuged at 13000 rpm for 10 minutes. The aqueous phase was removed and the DNA was precipitated by adding one-third volume of isopropanol and incubating overnight at 4°C. The precipitated DNA was pelleted by centrifuging at 13000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol and re-suspended in TE (10: 0.1 mM) buffer.

3.2.6.2 Amplification of 16S rRNAgenes

3.2.6.2.1 Bacterial 16S rRNA

Bacterial 16S rRNAgenes were amplified using universal bacterial primers, 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′ (Weisburg et al., 1991). The PCR was performed in a thermal cycler (Applied Biosystems, USA) using following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min and 15 sec and final extension at 72°C for 10 min.

3.2.6.2.2 Archaeal 16S rRNA

Archaeal 16S rRNA gene fragments were amplified withuniversal archaeal primers, A109F AC(G/T)GCTCAGTAACACGT and 1510R GGTTACCTTGTTACGACTT (Mani et al., 2012a; Birbir et al., 2007). The PCR was performed in a thermal cycler (Applied Biosystems, USA) using following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 53.5°C for 30 sec and extension at 68°C for 1 min and final extension at 68°C for 10 min.

The bacterial and archaeal 16S rRNAPCR reaction mixtures contained 10X *Taq* buffer,2 mM $MgCl_2$, 10 mM of dNTPs (Sigma, India), 10 µM of eachprimer (IDT technologies, Singapore), 2 U *Taq* Polymerase and 10 ng template DNA. The PCR products were verified on a 1.5 % agarose gel through submerged DNA electrophoresis and was visualised over a UV illuminator (Bio-Rad, USA) after staining with ethidium bromide. Intact amplified PCR products were eluted from the gel and purified with gel elution kit (Chromous Biotech Pvt. Ltd., India), according to manufacturer's instruction.

3.2.6.3 Sequencing and phylogenetic analysis

The purified 16S rRNA gene products of around 1400 bp in size were sequenced using automated dideoxy chain termination method. Sequencing was carried out at Chromous Biotech Pvt. Ltd., India, Bio-Nano Electronics Research Centre, Toyo University, Japan, Iranian Biological Research Centre, Iran and Korea Basic Science Institute, Republic of Korea. Sequences were reviewed, corrected and assembled using Molecular Evolutionary Genetic Analysis (MEGA) software v5.0 (Tamura et al., 2011). Identification of phylogenetic neighbours and calculation of pair-wise 16S rRNA gene sequence similarity against the type strains with validly published names was achieved using EzTaxon-e BLAST analysis (Kim et al., 2012). The sequences of identified phylogenetic neighbours were retrieved from Ribosomal Database Project (RDP) release 11 and aligned with the sequences of the isolates using MUltiple Sequence Comparison by Log- Expectation (MUSCLE) (Edgar, 2004; Cole et al., 2009). Distances were calculated using the Kimura two-parameter (Kimura, 1980) correction in a pair-wise deletion procedure. The phylogenetic tree was reconstructed with Neighbour-Joining (NJ) algorithm using MEGA v5.0. Percentage support values were obtained using a bootstrap procedure with 1000 resampling.

3.3 Results

3.3.1 Sampling site description

Individual solar salterns were selected to represent different climatic and operational conditions and therefore salterns on the east and west coast were selected (Fig. 3.1). On the west coast of India, Ribandar and Siridao salterns located in the coastal state of Goa were selected. Sampling was carried out during three phases of salt production i.e., preparatory phase, initial salt harvesting (ISH) phase and peak salt harvesting (PSH) phase. Brines and sediments were collected from reservoir pan (RP), evaporator pan (EP) and crystalliser pan (CP) except for preparatory phase where a single brine and sediment sample was collected. In Sinquetim, sediment and water sample from the reservoir pan (RP) and a soil sample were collected.On the east coast, Marakkanam and Vedaranyam salterns located in the coastal state of Tamil Nadu were selected for this study. Sampling at Tamil Nadu salterns was carried out in reservoir pan (RP), evaporator pan (EP) and crystalliser pan (CP), during the peak salt harvesting (PSH) phase.

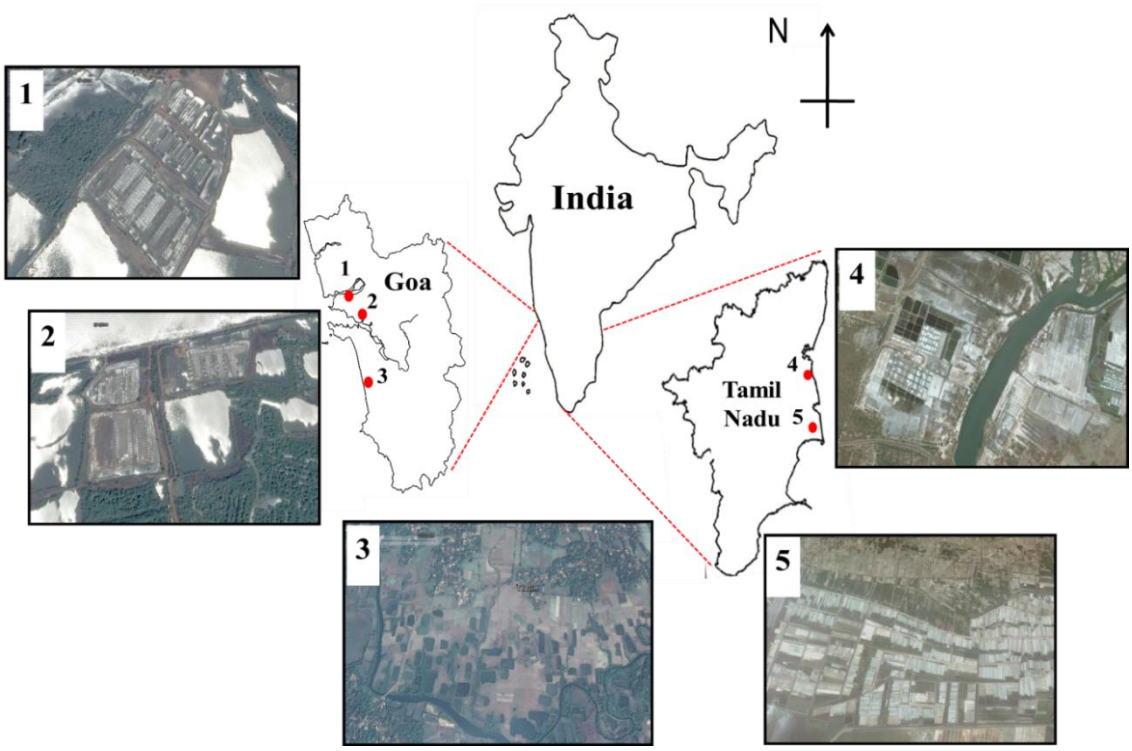


Fig. 3.1: Map of India showing the sampling sites along the west and east coast. 1. Siridao (Goa), 2. Ribandar (Goa), 3. Sinquetim (Goa), 4. Marakkanam (Tamil Nadu), 5. Vedaranyam (Tamil Nadu)

3.3.2 Physico-chemical analysis of solar salterns

3.3.2.1 Solar salterns of Goa

Soil texture analysis indicated that saltern soil of Goa is predominantly red clay. The temperature varied from 28 to 32°C and the pH varied from 6.5 to 7.8. Salinity of the solar salterns varied with the compartments of salt production. During the preparatory phase, the salinity of salterns was around 2%. However during the initial salt harvesting (ISH) phase, the salinity of reservoir pan (RP) varied between 2 – 2.4%, while the salinity of evaporator pan (EP) was about 14% and the salinity of crystalliser pan (CP) varied around 24%. Samples collected during the peak salt harvesting (PSH) phase indicated that the salinity of reservoir pan (RP) was 4%, while the salinity of evaporator pan (EP) was 17% and the salinity of crystalliser pan (CP) was about 28%.

The salterns were dominated by sodium and chloride ions. Sodium ion concentration was 8.6 g/l during the preparatory phase (Table 3.2). However during the initial salt harvesting (ISH) phase, the sodium ion concentration ranged from 9.9 (RP) to 68.2 g/l (CP). Similarly during peak salt harvesting (PSH) phase, concentration of sodium ion varied from 10.8 (RP) to 72.45 g/l (CP). Chloride ion concentration ranged from 18 to 19 g/l in the reservoir pan (RP) brine while in the evaporator pan (EP) brine the concentration ranged from 49.95 to 85g/l. Concentration of chloride ionranged from 119.28 to 141.8 g/l in the crystalliser pan (CP). Potassium ion concentration was 0.4 g/l in the reservoir pans (RP), while ranged from 1.1 to 2.2 g/l in the evaporator pan (EP) and between 4.2 and 4.5 g/l in the crystalliser pan (CP). Concentration of potassium ion in the preparatory phase brine was 0.2 g/l.Similar to potassium ion concentration, magnesium ion concentration increased with increasing salinity. Concentration of magnesium ionin the reservoir pan (RP) was 0.2 g/l with further increase to 0.4 g/l in the evaporator pan (EP) and to 8.6 g/l in the crystalliser pan (CP). The concentration of calcium ion remained high in the moderate salinity with values ranged from 1.1 to 1.3 g/l and with further increase in salinity; concentration of calcium was reduced to 0.3g/l. The concentration of manganese ions in Siridao saltern was 0.79 x 10^{-3} g/l and in Ribandar saltern was 7.9 x 10^{-3} g/l. Salinity of Sinquetim solar saltern water sample was 0.8%. Concentration ofsodium in the discontinued reservoir pan (RP) was 2.2 g/l and chloride was 5.6 g/l. Similarly, concentration of potassium, magnesium and calcium ion was 0.2 g/l (Table 3.2).

Table 3.2: Salinity and elemental composition of Siridao, Sinquetim and Vedaranyam saltern brine samples

Sampling site	Salinity (%)	Na^+ (g/l)	K^+ (g/l)	Mg^{2+} (g/l)	Ca^{2+} (g/l)	Cl^- (g/l)	Mn^+ (mg/l)
SPrep	2	8.6	0.2	0.1	0.3	19.6	ND
SISH – RP	2	9.9	0.4	0.2	0.5	18.4	ND
SISH – EP	13.6	35.19	1.1	0.4	1.3	65.51	ND
SISH – CP	25.2	68.2	4.2	8.6	0.3	119.28	ND
SPSH – RP	4	10.8	0.4	0.2	0.7	19	ND
SPSH – EP	17.4	45.02	2.2	0.3	1.1	85.44	0.79
SPSH – CP	28	72.45	4.5	8.6	0.3	141.8	ND
TN-V-RP	5	12.90	0.6	0.8	1.7	21.01	ND
TN-V-EP	18	46.57	2.5	2.7	0.3	73.7	ND
TN-V-CP	27	73.56	3.1	4.35	0.2	153.14	ND
SinqRP	0.8	2.2	0.2	0.1	0.2	5.6	ND

ND – Not Determined; S – Siridao; Prep – Preparatory phase; ISH- Initial salt harvesting phase;PSH – Peak salt harvesting phase; TN-V – Tamil Nadu Vedaranyam; RP - Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan;Sinq– Sinquetim.

3.3.2.2 Solar salterns of Tamil Nadu

Soil texture analysis indicated that the salterns of Tamil Nadu contain sandy black clay. The temperature of Tamil Nadu salterns at Vedaranyam and Marakkanamranged from 30 to 34°C and the pH ranged from 6.5 to 8.0. Salinity of the reservoir pan (RP) was 5% while the salinity of evaporator pan (EP) ranged around 18% and the salinity of crystalliser pan (CP) was about 28%.

Similar to salterns of Goa, salterns of Tamil Nadu were dominated by sodium and chloride ions. Concentration of sodium ion in reservoir pan (RP) was 12.90 g/l, in evaporator pan (EP) was 46.57 g/l and 73.36 g/l in crystalliser pan (CP) (Table 3.2). Concentration of chloride ion in reservoir pan (RP) was 21.01 g/l, in evaporator pan (EP) was 73.7 g/l and 153.14 g/l in crystalliser pan (CP). Potassium ion concentration increased from 0.6 g/l in reservoir pan (RP) to 3.1 g/l in crystalliser pan (CP). Magnesium ion concentration ranged from 0.8 g/l in reservoir pan (RP) to 4.35 g/l in crystalliser pan (CP). Concentration of calcium ion was highest in reservoir pan (RP) with 1.7 g/l and it decreased with increasing salinity to 0.2 g/l in the crystalliser pan (CP) (Table 3.2).

3.3.2.3 Salt Crystals

Salt crystal contained sodium and chloride as predominant ions with an average concentration of 35 cg/g (Na^+) and 55 cg/g (Cl^-) respectively (Table 3.3). Concentration of potassium and magnesium ions was 0.2 and 0.011 cg/g respectively, while the concentration of calcium ions was 0.23 cg/g (Table 3.3).

Table 3.3: Elemental composition of various salt crystals

Salt Sample	Na^+ (cg/g)	K^+ (cg/g)	Mg^{2+} (cg/g)	Ca^{2+} (cg/g)	Cl^-(cg/g)
Rib	33.58	0.24	0.011	0.25	55.78
TN-M	35.21	0.21	0.012	0.22	56.12
TN-V-BW	34.53	0.23	0.013	0.23	55.87
TN-V-AW	34.86	0.23	0.011	0.23	55.89
Com. Salt	36.19	0.26	0.012	0.22	55.13

Rib – Ribandar, TN –M - Tamil Nadu Marakkanam, TN – V - Tamil Nadu Vedaranyam, AW – After Wash, BW –Before Wash, Com. Salt – Commercial Salt, cg-centigram

3.3.3 Estimation of chlorophyll *a*

Spectrophotometric estimation revealed that solar salterns of Tamil Nadu contained higher concentration of chlorophyll *a* than salterns of Goa (Fig. 3.2). Concentration of chlorophyll *a* was found to be dominant in moderate salinity brines (~15%) and high salinity sediments (~28%). During the preparatory phase of salterns of Goa, chlorophyll*a* concentration of brine was higherthan sediment. Chlorophyll *a* concentration of Siridao saltern brine was 0.04 µg/ml and Ribandar saltern brine was 0.036 µg/ml. Chlorophyll *a* concentration of Siridao saltern sedimentwas0.024 and Ribandar saltern sediment was 0.025 µg/g. However during the initial salt harvesting (ISH) phase and peak salt harvesting (PSH) phase, sediments were found to contain higher chlorophyll *a* concentration than brine. Chlorophyll *a*concentration of Siridao saltern initial salt harvesting (ISH) phase brines ranged from 0.026 to 0.088 µg/ml and sediments ranged from 0.057 to 0.117 µg/g while peak salt harvesting (PSH) phase brines ranged from 0.038 to 0.084 µg/ml and sediments ranged from 0.057 to 0.191 µg/g.

Chlorophyll *a* concentration of Ribandar saltern brines obtained during initial salt harvesting (ISH) phase ranged from 0.03 to 0.083 µg/ml and sediments ranged from 0.045 to 0.134 µg/g while peak salt harvesting (PSH) phase brines ranged from 0.034 to 0.092 µg/ml and sediments ranged from 0.040 to 0.141 µg/g. Chlorophyll *a* concentration of the discontinued reservoir pan (RP) water obtained from Sinquetim saltern was 0.026µg/ml and sediment was 0.072 µg/g. The soil sample from Sinquetim saltern

contained chlorophyll *a* at a concentration of 0.006 µg/g.Chlorophyll *a* concentrations in Marakkanam saltern brines ranged from 0.004 to 0.081µg/ml while sediments ranged from 0.046 to 0.128 µg/g. Similarly, chlorophyll *a* concentrations in Vedaranyam saltern brines ranged from 0.061 to 0.12 µg/ml while sediments ranged from 0.071 to 0.24 µg/g (Fig. 3.3).

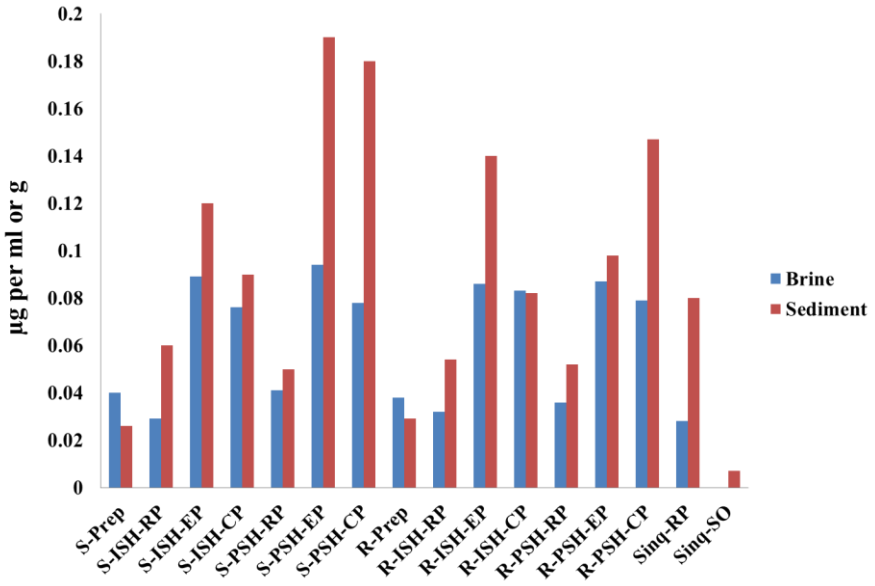


Fig.3.2: Chlorophyll *a* concentration of brine and sediment samples obtained from salterns of Goa.S – Siridao; R – Ribandar; Prep – Preparatory phase; ISH- Initial salt harvesting phase;PSH – Peak salt harvesting phase; TN-V – Tamil Nadu Vedaranyam; RP - Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan; Sinq– Sinquetim.

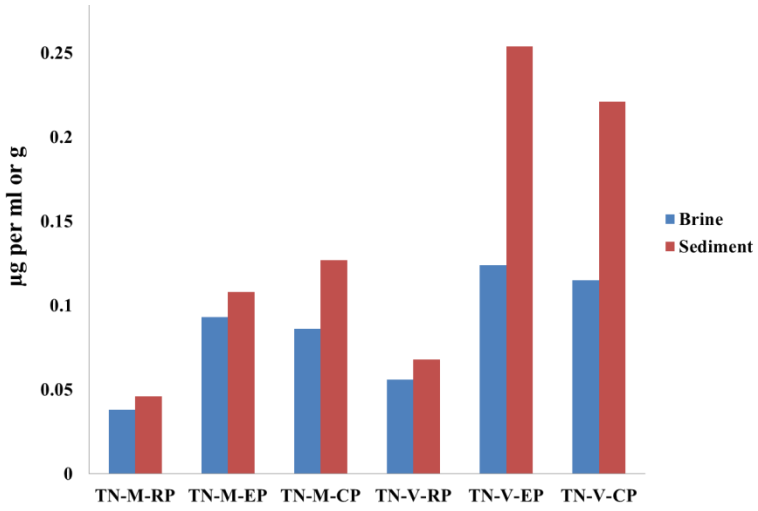


Fig. 3.3: Chlorophyll *a* concentration of brine and sediment samples obtained from salterns of Tamil Nadu. TN- Tamil Nadu; M – Marakkanam; V – Vedaranyam; RP - Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan.

3.3.4 Enumeration of microbial abundance

3.3.4.1 Microscopic enumeration of microorganisms

Acridine orange (AO) stained enumeration of saltern samples revealed that sediments contained higher microbial abundance compared to brines. The abundance of microorganisms in sediments obtained from Siridao salterns were 1.9 x 10^6 cells/g and Ribandar salterns were 2.32x 10^6cells/g (Fig. 3.4). Similarly, brine from Siridao showed an abundance of 1.74 x 10^6 cells/ml and Ribandar saltern showed an abundance of 1.94 x 10^6 cells/ml.When the microbial abundance was compared between the initial salt harvesting (ISH) phase and peak salt harvesting (PSH) phase, the latter was found to contain a high microbial abundance. Total count (TC) of AO stained Ribandar initial salt harvesting (ISH) phase brines showed abundance in the range of 2.38x 10^6 cells/ml to 3.78 x 10^6 cells/ml (mean 3.12x 10^6 cells/ml) and sediment showed abundance in the range of 3.51x 10^6 cells/g to 3.9x 10^6 cells/g (mean 3.74x 10^6 cells/g). The microbial abundance in Siridao brines during the initial salt harvesting(ISH) phase was enumerated in the range of 3x 10^6 cells/ml to 4.22 x 10^6 cells/ml (mean 3.67 x 10^6 cells/ml) and sediments raged from 3.41x 10^6 cells/g to 5.3 x 10^6 cells/g (mean 4.4x 10^6 cells/g).

Peak salt harvesting (PSH) phase brines obtained from Ribandar saltern showed abundance in the range of 3.21x 10^6 cells/ml to 3.82 x 10^6 cells/ml (mean 3.6x 10^6 cells/ml) and sediments showed abundance in the range of 3.9x 10^6 cells/g to 4.83 x 10^6 cells/g (mean 4.51x 10^6 cells/g).The microbial abundance in Siridao brines during the peak salt harvesting (PSH) phase was enumerated in the range of 3.6x 10^6 cells/ml to 3.91 x 10^6 cells/ml (mean 3.79 x 10^6 cells/ml) and abundance in sediments was in the range of 3.21x 10^6 cells/g to 5.38 x 10^6 cells/g (mean 4.34x 10^6 cells/g). Water sample obtained from the discontinued reservoir pan (RP) contained a microbial abundance of 2.63x 10^6 cells/ml and sediment sample contained a microbial abundance of 3.28x 10^6 cells/g while the soil sample contained 3.4 x 10^6 cells/g.

Salterns of Tamil Nadu showed a higher microbial abundance and in particular Vedaranyam salterns showed the highest total count (TC) of organism among all the salterns studied (Fig. 3.5). Total count (TC) of AO stained Marakkanam brines showed abundance in the range of 2.64 x 10^6 cells/ml to 3.71 x 10^6 cells/ml (mean 3.25x 10^6 cells/ml) and sediments showed abundance in the range of 3.32x 10^6 cells/g to 4.45x 10^6 cells/g (mean 3.98x 10^6 cells/g). Similarly, microbial abundance of Vedaranyam brines was in the range of 3x 10^6 cells/ml to 4.74 x 10^6 cells/ml (mean 4.11 x 10^6 cells/ml) and

in sediments microbial abundance was in the range of 3.82x 10^6 cells/g to 5.65 x 10^6 cells/g (mean 4.85 x 10^6 cells/g).

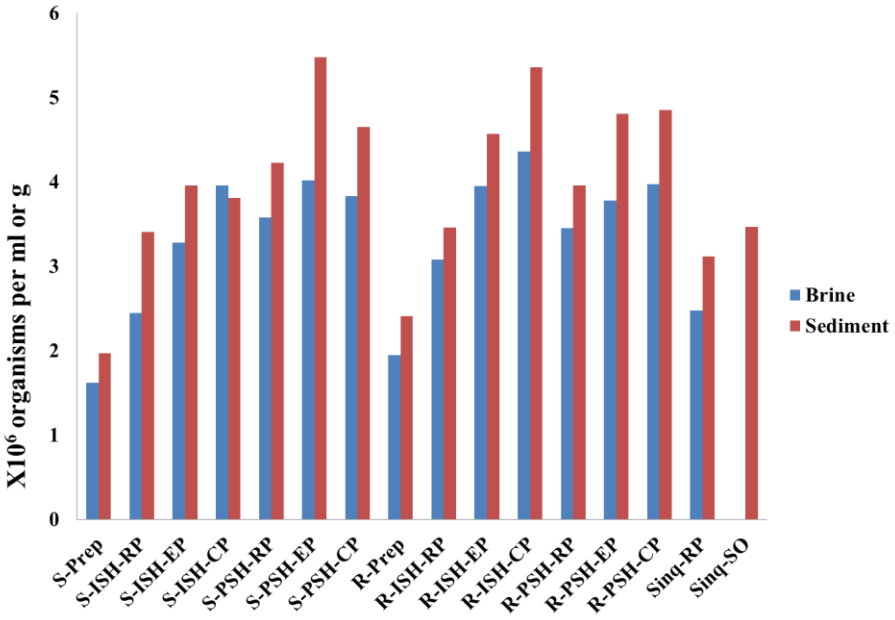


Fig.3.4: Total count (TO) of microorganism present in brine and sediment samples of salterns of Goa enumerated with acridine orange (AO) staining. S – Siridao; R – Ribandar; Prep – Preparatory phase; ISH- Initial salt harvesting phase;PSH – Peak salt harvesting phase; TN-V – Tamil Nadu Vedaranyam; RP - Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan;Sinq– Sinquetim.

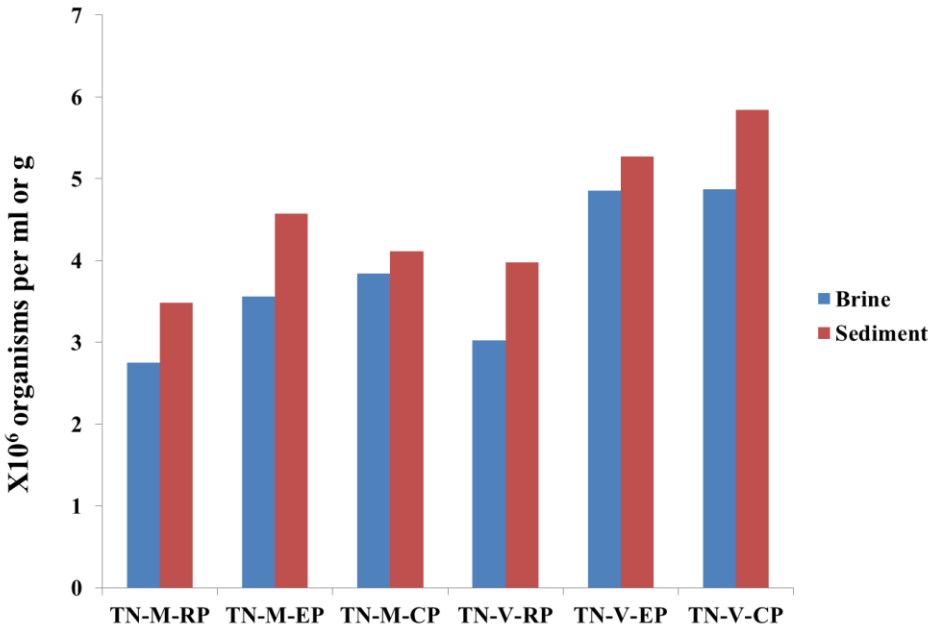


Fig.3.5: Total count (TO) of microorganism present in brine and sediment samples of salterns of Tamil Nadu enumerated with acridine orange (AO) staining. TN- Tamil Nadu; M – Marakkanam; V – Vedaranyam;RP - Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan.

3.3.4.2 Enumeration of viable halophilic microorganisms

Sediments and brine were spread-plated on NTYE, NT, EHM, JCM168 and MHM agar plates. Colonies appeared within 1-2 days on MHM agar medium, whereas 4-5 days were required to visualise white, cream and yellow pigmented colonies on other media. After 20 days, orange, pink and red pigmented colonies appeared on the extremely halophilic media plates (Fig. 3.6). Among the halophilic archaeal media employed, JCM168 showed higher viable counts of early days of incubation (within 3 days). The medium EHM was the slowest of all media to produce significant viable counts. After 25 – 30 days of incubation the viable counts obtained on all the media were comparable with the exception of MHM, which was deemed as too numerous to count.

The appearances of red and orange pigmented colonies were more prominent in the peak salt harvesting (PSH) phase samples obtained from salterns of Goa than initial salt harvesting (ISH) phase samples. Similarly,the appearance of pigment-less to yellow, orange and red pigmented colonies was a characteristic feature of both brine and sediment sample obtained from all three compartments of the saltern, reservoir pan (RP), evaporator pan (EP) and crystalliser pan (CP). Interestingly, Tamil Nadu saltern samples on EHM produced significantly higher viable counts at a lesser time when compared with the saltern samples of Goa. Similarly, the pink pigmented colonies appeared only in the media plates containing Marakkanam saltern sample.

The culturable microbial count (colony forming unit, CFU) was calculated after 7 and 30 days of incubation of media plates containing the saltern samples (Table 3.4). Similar to total counts obtained from AO stained enumeration, sediments produced higher viable counts than brines. Among the salterns on the east and west coast, salterns of Tamil Nadu produced higher viable counts when compared with the salterns of Goa. When the differences in viable microbial count were studied between the different phases of salt production in Goa, it was observed peak salt harvesting (PSH) phase produced more colonies than the other phases. Viable culture counts of the preparatory phase brine obtained from salterns of Goa ranged from 4.7×10^2 to 5.9×10^2CFU/ml and sediment samples ranged from$7.1 \times 10^2$to 8.9×10^2CFU/g (Table 3.4). The initial salt harvesting (ISH) phase brine produced viable counts ranged from 4.7×10^2 to 1.6×10^3CFU/ml and sediments produced viable counts ranged from$7.9 \times 10^2$to 4.8×10^3CFU/g respectively. Brines obtained from salterns of Tamil Nadu produced viable counts ranged from 6×10^2 to 3.1×10^3 CFU/ml and sediment produced viable counts ranged from $1.4 \times 10^3$to 7×10^3 CFU/g. Salt crystals produced a viable count of 3.8×10^3 CFU/g on average.

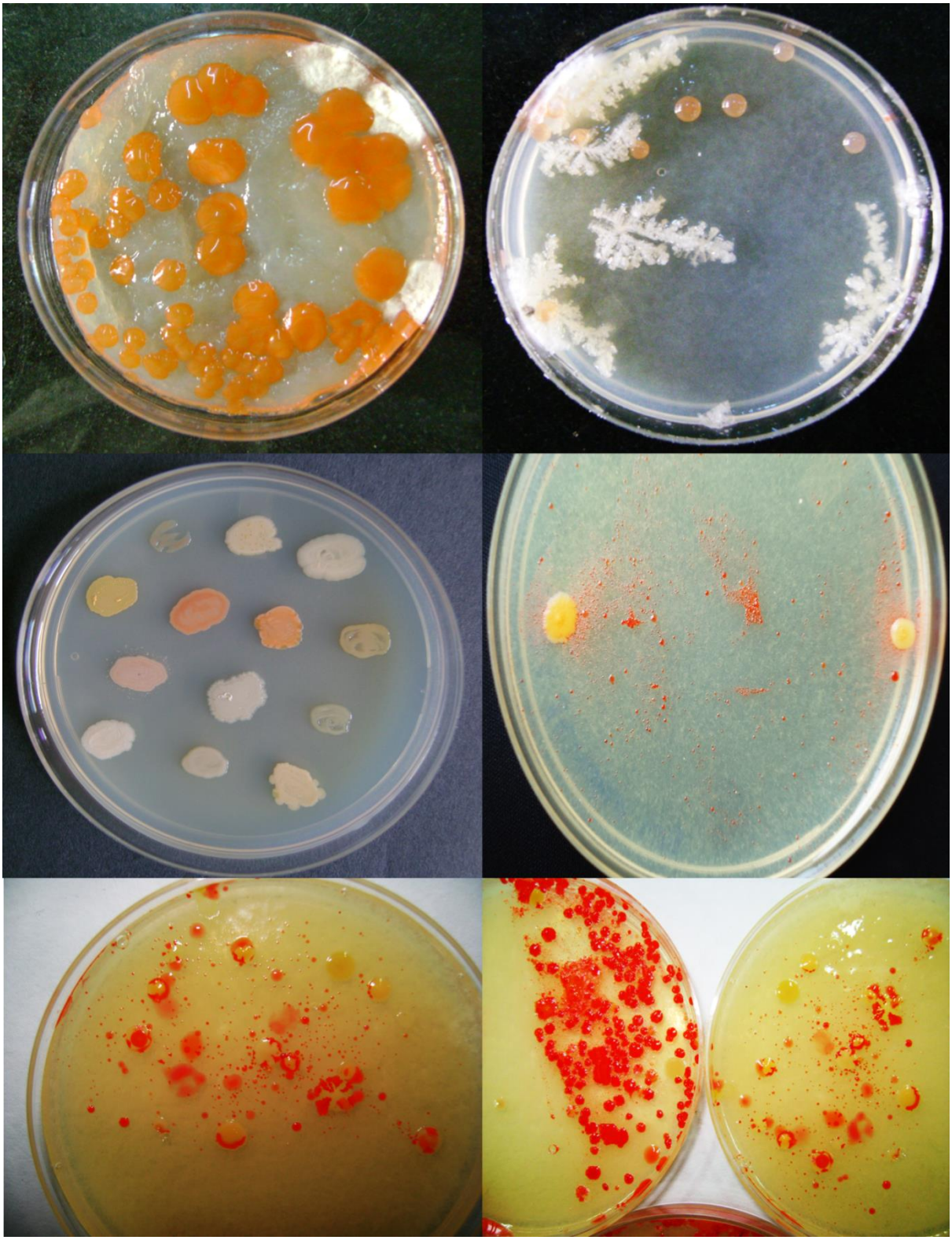


Fig. 3.6: Red pigmented haloarchaeal colonies on the extremely halophilic media plates after 30 days of incubation

Table 3.4: Total cultivable microorganisms (colony forming units) obtained from salterns of Goa and Tamil Nadu

Salterns	**Brine**		**Sediment**	
Goa	CFU/ml (7^{th} day)	CFU/ml (30^{th} day)	CFU/g (7^{th} day)	CFU/g (30^{th} day)
Preparatory Phase				
Siridao	5.2×10^2	5.9×10^2	8.1×10^2	8.9×10^2
Ribandar	4.7×10^2	5.3×10^2	7.1×10^2	8.3×10^2
Initial salt harvesting (ISH) phase				
Siridao	4.9×10^2	1.1×10^3	8.9×10^2	4.1×10^3
Ribandar	4.7×10^2	8×10^2	8.1×10^2	1.4×10^3
Peak salt harvesting (PSH) phase				
Siridao	6.1×10^2	1.6×10^3	9.7×10^2	4.8×10^3
Ribandar	5.3×10^2	1.2×10^3	7.9×10^2	2.9×10^3
Discontinued saltern				
Sinquetim	1.1×10^2	2.4×10^2	2×10^2	2.9×10^2
Tamil Nadu				
Marakkanam	6×10^2	2.1×10^3	1.4×10^3	5.2×10^3
Vedaranyam	7.1×10^2	3.1×10^3	2.7×10^3	7×10^3

3.3.4.3 Isolation, purification and designation of moderate and extreme halophilic microorganisms

White, cream and yellow colonies were selected from MHM medium for purification whereas pink, orange and red pigmented colonies were selected from NTYE, NT, JCM168 and EHM media. Cultures were purified through repeated streaking on respective agar medium to obtain pure culture (Fig. 3.7A& B). The isolates were labelled and maintained on respective media slopes/plates at Room Temperature (30^oC) or 4^oC. Pure isolates were designated as follows,

(i) BK (Bragança - Kabilan) series cultures (BK3, BK6, BK7, BK11, BK18, BK19 and BK20)

(ii) BBK (Bragança – Bhakti - Kabilan) series cultures (BBK1 and BBK2)

(iii) BS (Bragança - Salgaonkar) series cultures (BS1, BS2, BS3, BS4, BS5, BS6, BS7, BS8, BS11, BS13, BS15, BS16, BS17 and BS19)

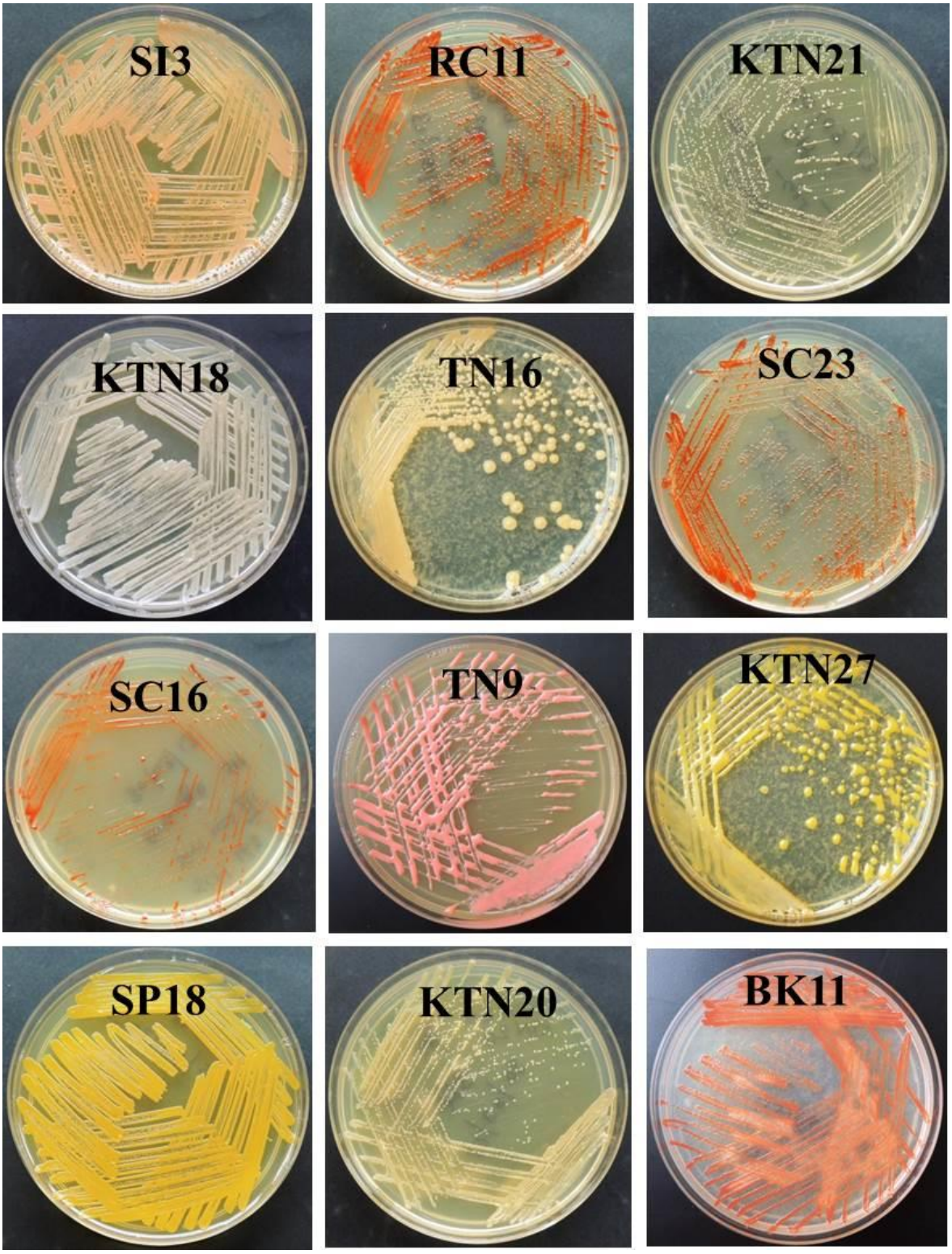


Fig. 3.7 (a): Some of the pure isolates obtained from solar salterns of Goa and Tamil Nadu.

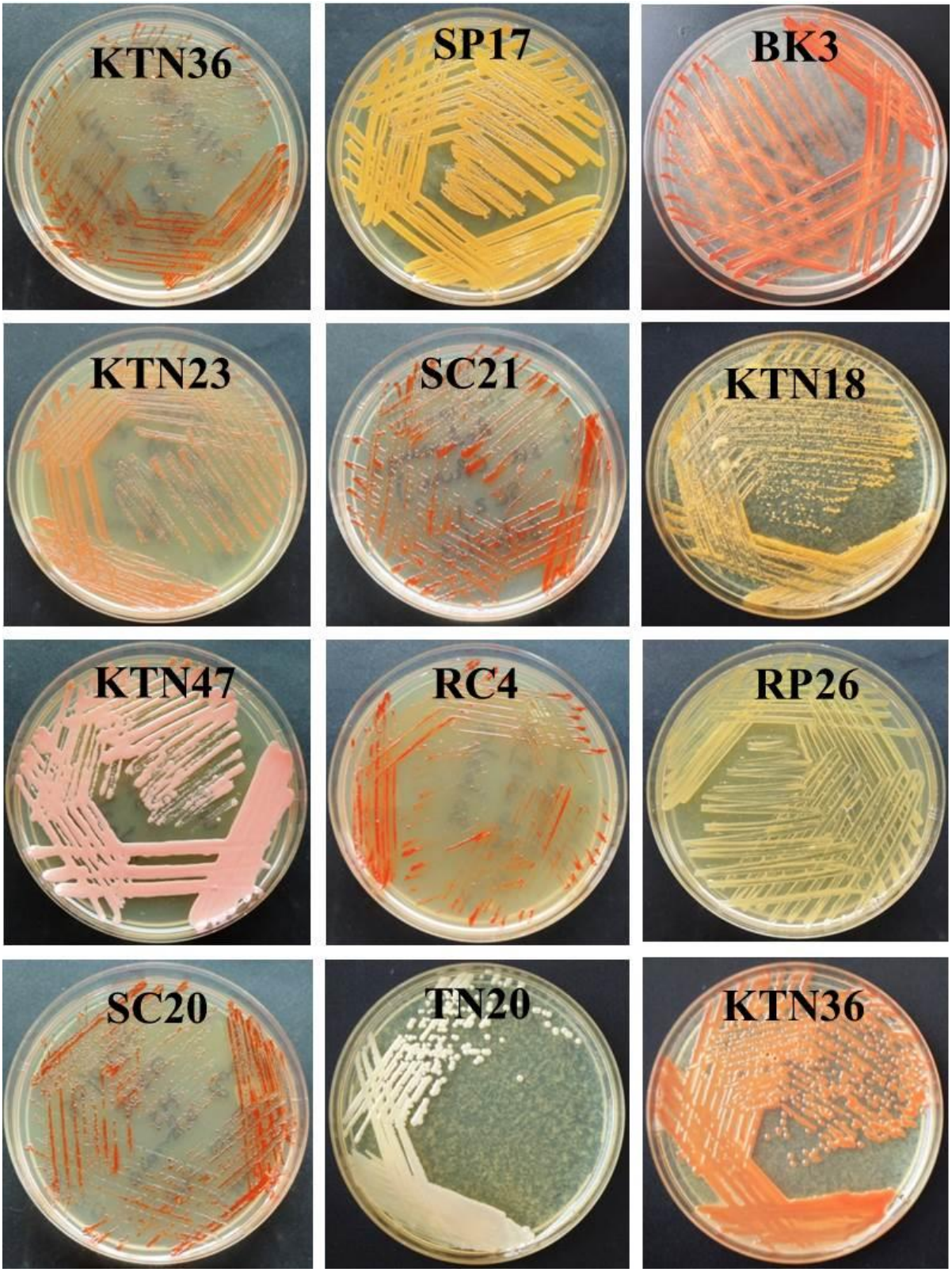


Fig. 3.7 (b): Some of the pure isolates obtained from solar salterns of Goa and Tamil Nadu.

(iv) TN (Tamil Nadu) series cultures (TN1, TN2, TN3, TN4, TN5, TN6, TN7, TN8, TN9, TN10, TN13, TN15, TN16, TN17, TN18, TN19, TN20,TN21).

(v) KTN (Kabilan Tamil Nadu) series cultures (KTN1 to KTN50)

(vi) RP (Ribandar Pre-salt harvesting) series cultures (RP1 to RP31)

(vii) RC (Ribandar Crystallisation) series cultures (RC1 to RC12)

(viii) SP (Siridao Pre-salt harvesting) series cultures (SP1 to SP25)

(ix) SC (Siridao Crystallisation) series cultures (SC1 to SC24)

(x) SI (Sinquetim) series cultures (SI1 to SI6)

(xi) S (Salt crystals) series cultures (S1 to S65)

3.3.4.4 Phylogenetic analysis of the isolates

A total of 270 isolates were obtained from all the salterns and salt crystals. Based on colony morphology and pigmentation, 68 distinct representative isolates were selected for further molecular characterisation. The 1400 bp 16S rRNA amplicon was sequenced and the phylogenetic identity of the isolates was established through pairwise sequence alignment against the type strains with validly published names using EzTaxon-e BLAST analysis (Kim et al., 2012). The phylogenetic analysis of the isolates obtained from salterns of Goa and Tamil Nadu revealed that 54 sequences belonged to the domain Archaea and 14 sequences belonged to the domain Bacteria (Table 3.5).

The 26 archaeal isolates obtained from the salterns of Goa were phylogenetically grouped into 5 genera: *Halococcus*, *Halorubrum*, *Haloarcula*, *Haloferax* and *Halostagnicola*. The most abundant culturable genera were *Haloarcula* (50%) and *Halococcus* (29%) followed by *Haloferax* (12%) (Fig. 3.8).The 6 bacterial isolates obtained from salterns were phylogenetically grouped into 5 genera: *Halomonas*, *Alkalibacillus*, *Halobacillus*, *Salinicoccus* and *Pontibacillus*. *Halomonas* (33%) was the dominant culturable bacterial genus in the salterns of Goa (Fig 3.8).

During the preparatory phase, the isolates obtained were phylogenetically related to genus *Halococcus* and the isolates obtained during initial salt harvesting (ISH) phase were related to genera *Halococcus* and *Haloferax*. However during the peak salt harvesting (PSH) phase, a high diversity of archaeal genera was observed with the recovery of 5 genera: *Halococcus*, *Halorubrum*, *Haloarcula*, *Haloferax* and *Halostagnicola*. No bacterial isolates were obtained during the peak salt harvesting (PSH) phase.

Table 3.5: Identified isolates with their sampling sites, respective media and their closest culturable match with similarity values.

Isolate Name	Isolation Source	Salinity	Media	Identification	% Similarity	GenBank/EMBL/DDBJ Accession No
BK3	Rib-WS-Prep	8%	NTYE	*Halococcus saccharolyticus*	99.52	HQ455793
BK6	Rib-WS-Prep	8%	NTYE	*Halococcus salifodinae*	99.39	AB588757
BK7	Rib-WS-Prep	8%	NTYE	*Halococcus saccharolyticus*	99.33	HQ455794
BK11	Rib-WS-Prep	8%	NTYE	*Halococcus saccharolyticus*	99.14	HQ455795
BK18	Rib-SS-Prep	8%	NTYE	*Halococcus saccharolyticus*	99.55	HQ455796
BK19	Rib-SS-Prep	8%	NTYE	*Halococcus salifodinae*	99.39	AB588758
BBK1	Rib-WS- ISH	27%	NTYE	*Halococcus salifodinae*	99.39	AB588755
BBK2	Rib-WS-ISH	27%	NTYE	*Haloferax volcanii*	99.79	AB588756
RP15	Rib-SS- ISH	15%	MHM	*Halomonas elongata*	99.45	AB904837
RP24	Rib-WS- ISH	15%	EHM	*Halomonas smyrnensis*	99.6	-
RP26	Rib-SS- ISH	3%	EHM	*Alkalibacillus salilacus*	99.8	-
RP31	Rib-WS-PSH	3%	EHM	*Haloferax prahovense*	99.54	AB904835
SP17	Siri-WS-PSH	3%	MHM	*Halobacillus alkaliphilus*	99.6	-
SP18	Siri-WS-PSH	3%	MHM	*Pontibacillus yanchengensis*	97.8	-
BS1	Rib-WS-PSH	15%	NT	*Haloarcula quadrata*	99.25	HQ455797
BS2	Rib-WS-PSH	15%	NT	*Haloarcula valismortis*	99.02	HQ455798
BS3	Rib-WS-PSH	27%	NT	*Haloarcula valismortis*	98.13	HQ455799
BS4	Rib-WS-PSH	27%	NT	*Haloarcula marismortui*	99.52	HQ455800
BS5	Rib-WS-PSH	15%	NT	*Haloarcula marismortui*	99.52	AB588759
BS6	Rib-WS-PSH	3%	NT	*Haloarcula salaria*	97.91	HQ455801
BS7	Rib-WS-PSH	15%	NT	*Haloarcula salaria*	99.47	HQ455802
BS8	Rib-SS-PSH	27%	NT	*Haloarcula marismortui*	99.66	-
BS11	Rib-SS-PSH	27%	NT	*Haloarcula japonica*	99.39	-
BS13	Rib-SS-PSH	15%	NT	*Haloarcula marismortui*	99.52	-
BS15	Rib-SS-PSH	3%	NT	*Haloarcula marismortui*	99.66	-
BS16	Rib-SS-PSH	15%	NT	*Haloferax prahovense*	99.72	-
BS17	Rib-SS-PSH	27%	NT	*Halorubrum chaoviator*	98.89	-
BS19	Rib-SS-PSH	3%	NT	*Halorubrum chaoviator*	98.90	-
BS20	Rib-SS-PSH	15%	NT	*Haloarcula marismortui*	99.46	-
TN4	Mar-WS-PSH	15%	NT	*Haloferax prahovense*	100	HF563070
TN5	Mar-WS-PSH	27%	NT	*Haloferax prahovense*	100	HF563071
TN6	Mar-WS-PSH	3%	NT	*Haloferax prahovense*	100	HF563072
TN8	Mar-WS-PSH	15%	NT	*Haloarcula hispanica*	99.56	HF563073
TN9	Mar-WS-PSH	15%	NT	*Halogeometricum boriquense*	100	HF547852
TN10	Mar-SS-PSH	27%	NT	*Haloferax prahovense*	100	HF563074
TN15	Mar-SS-PSH	3%	NT	*Haloferax prahovense*	100	HF563075

Isolate Name	Isolation Source	Salinity	Media	Identification	% Similarity	GenBank/EMBL/DDBJ Accession No
TN16	Mar-SS-PSH	15%	NT	*Salicolamarasensis*	99.75	HF563076
TN18	Mar-SS-PSH	27%	NT	*Halobacillus alkaliphilus*	99.42	HF563077
TN20	Mar-SS-PSH	3%	NT	*Salicolasalis*	99.53	HF563078
SC24	Siri-WS-PSH	27%	EHM	*Haloarcula marismortui*	99.38	AB904832
KTN50	Mar-SS-PSH	27%	EHM	*Haloarcula hispanica*	99.01	AB904831
KTN28	Veda-WS-PSH	3%	MHM	*Halorubrum xinjiangense*	98.25	AB904840
KTN49	Mar-WS-PSH	15%	EHM	*Halogeometricum boriquense*	99.52	AB904833
RC12	Rib-WS-PSH	15%	EHM	*Halococcus saccharolyticus*	99.14	AB904834
KTN23	Veda-WS-PSH	27%	JCM168	*Natrinema gari*	99.2	-
KTN47	Veda-WS-PSH	15%	EHM	*Halogeometricum boriquense*	100	-
SC21	Siri-WS-PSH	15%	EHM	*Haloarcula marismortui*	99.5	-
SC23	Siri-WS-PSH	3%	EHM	*Haloarcula marismortui*	99.6	-
KTN36	Veda-WS-PSH	27%	EHM	*Haloarcula marismortui*	99.7	-
RC11	Rib-WS-PSH	27%	EHM	*Haloarcula salaria*	98.7	-
SC20	Siri-WS-PSH	15%	EHM	*Haloarcula salaria*	99.5	-
KTN18	Veda-WS-PSH	15%	JCM168	*Halomonas elongata*	100	-
KTN20	Veda-WS-SH	3%	JCM168	*Halomonas denitrificans*	99.3	-
KTN21	Veda-WS-SH	27%	JCM168	*Halomonas elongata*	99.9	-
KTN27	Veda-WS-SH	27%	JCM168	*Staphylococcus arlettae*	99.8	-
RC9	Rib-SS-SH	27%	EHM	*Halostagnicolalarsenii*	99.86	-
SI3	Sinq-SS	-	MHM	*Salinicoccushispanicus*	98.9	-
SI5	Sinq-SS	-	MHM	*Haloferax prahovense*	100	AB904836
S56	TN-V-AW-SC	-	MHM	*Halococcus salifodinae*	98.52	AB904838
S46	TN-M-SC	-	MHM	*Halomonas elongata*	99.45	AB904839
S15	TN-V-AW-SC	-	JCM168	*Haloterrigenathermotolerans*	98.91	AB971350
S18	Comm. Salt-SC	-	NT	*Halococcus salifodinae*	98.46	-
S16	TN-V-AW-SC	-	JCM168	*Haloterrigenathermotolerans*	100	-
S8	Rib-SC	-	NTYE	*Halococcus salifodinae*	99.35	-
S5	TN-V-BW-SC	-	NTYE	*Halorubrum chaoviator*	99.74	-

S – Siridao; R – Ribandar; Prep – Preparatory phase; PSH – Pre-salt harvesting phase; SH – Salt harvesting phase; Sinq – Sinquetim; RP –Reservoir Pan; SO – Soil sample; TN- Tamil Nadu; M – Marakkanam; V – Vedaranyam; Rib – Ribandar; TN –M - Tamil Nadu Marakkanam; TN – V - Tamil Nadu Vedaranyam; AW – After Wash; BW –Before Wash; Com. Salt – Commercial Salt,

The 13 archaeal isolates obtained from the salterns of Tamil Nadu were phylogenetically grouped into 5 genera: *Halorubrum*, *Haloferax*, *Haloarcula*, *Halogeometricum*, and *Natrinema*(Fig. 3.8). *Haloferax* (38%) was the dominant cultivable archaeal genera followed by *Haloarcula* (23%) and *Halogeometricum* (23%). The 6 bacterial isolates recovered were phylogenetically related to 4 genera: *Halomonas*,

Staphylococcus, *Halobacillus* and *Salicola*. The most dominant bacterial genera was *Halomonas* (43%) followed by *Salicola* (29%) (Fig. 3.8).

The 7 archaeal isolates obtained from salt crystals were phylogenetically grouped into 3 genera: *Halococcus*, *Halorubrum* and *Haloterrigena*. *Halococcus* (50%) was the dominant culturable archaeal genera followed by *Haloterrigena* (33%). One bacterial isolate obtained was phylogenetically related to *Halomonas.*

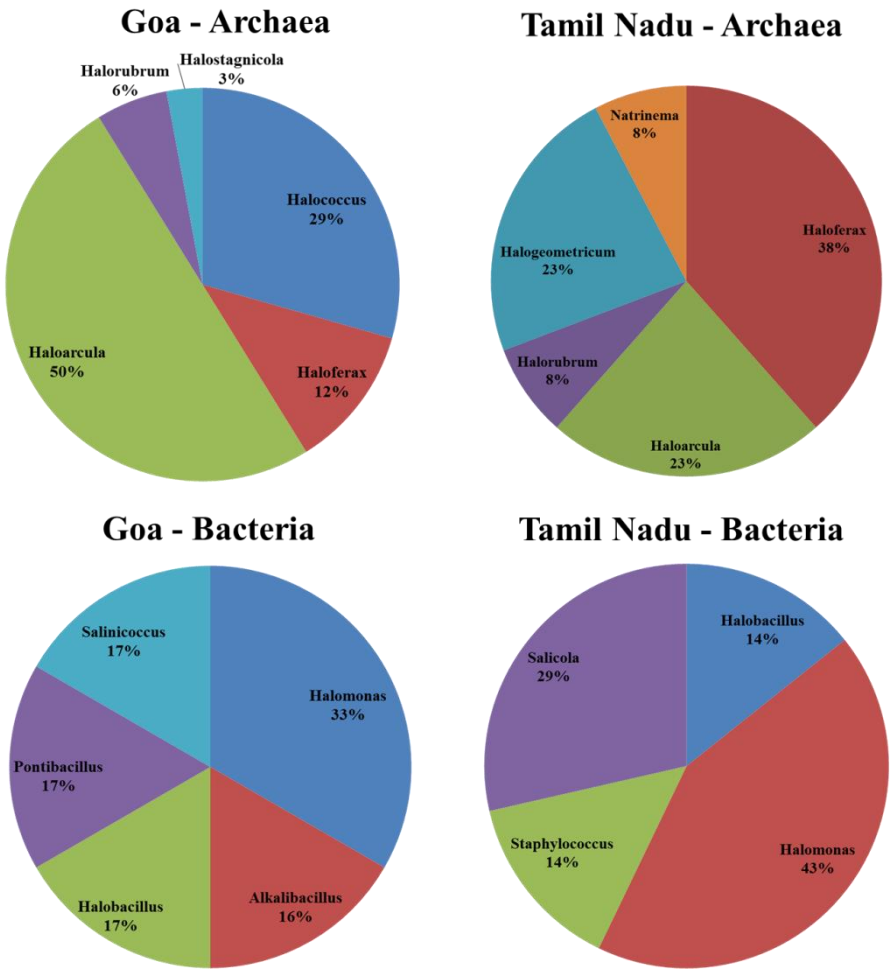


Fig 3.8: Relative abundances of different archaeal and bacterial genera isolated from the studied salterns of Goa and Tamil Nadu.

Phylogenetic tree was reconstructed using MEGA 5.0, based on the 16S rRNA gene sequences obtained from the isolates. Reference sequences were obtained from Ribosomal Database Project (RDP) version 11. The phylogenetic tree was reconstructed with Neighbour-Joining (NJ) algorithm using MEGA v5.0 and are elaborated below,

3.3.4.4.1 Solar salterns of Goa

Phylogenetic analysis of isolates obtained from the salterns of Goa during the preparatory phase indicated their closest relationship to (>99%) *Halococcus salifodinae* ATCC51437^T (AB004877) and *Halococcus saccharolyticus* ATCC49257^T (AB004876) (Fig. 3.10). Phylogenetic analysis of the isolates obtained during the initial salt harvesting (ISH) phase indicated the presence of 3 archaeal and 5 bacterial isolates.Among the isolates that have been phylogenetically assigned to domain Archaea, *Halococcus* sp. BBK1 displayed closest relationship (>99.0%) towards *Halococcus salifodinae* ATCC51437^T(AB004877) and *Halococcus saccharolyticus* ATCC49257^T (AB004876) while *Haloferax* sp. BBK2 and *Haloferax* sp. RP31 displayed closest relationship with (>99.0%) *Haloferax alexandrinus* JCM10717^T (AB037474), *Haloferax volcanii* ATCC29605^T (AY425724) and *Haloferax prahovense* DSM18310^T (AB258305) (Fig. 3.10).

Among the bacterial isolates, *Halomonas* sp. RP15 and *Halomonas* sp. RP24 displayed closest relationship (>99%) towards *Halomonas elongata* ATCC33173^T (X67023), *Halomonas maura* ATCC700995^T (AJ271864) and *Halomonas alkaliphila* DSM16354^T(AJ640133). *Alkalibacillus* sp. RP26 displayed closest relationship (>99.0%) towards *Alkalibacillus haloalkaliphilus* ATCC700606^T(AJ238041) while *Halobacillus* sp. TN18 showed closest relationship towards *Halobacillus alkaliphilus*DSM8525^T (AM295006), *Halobacillus salinus* JCM11546^T(AF500003)and *Halobacillus litoralis*ATCC700076^T(X94558). *Pontibacillus* sp. SP18 displayed 97.8% closest relationship towards *Pontibacillus yanchengensis*CCTCCAB209311 (EF533969) indicating the potential novelty of the isolate (Fig. 3.11).

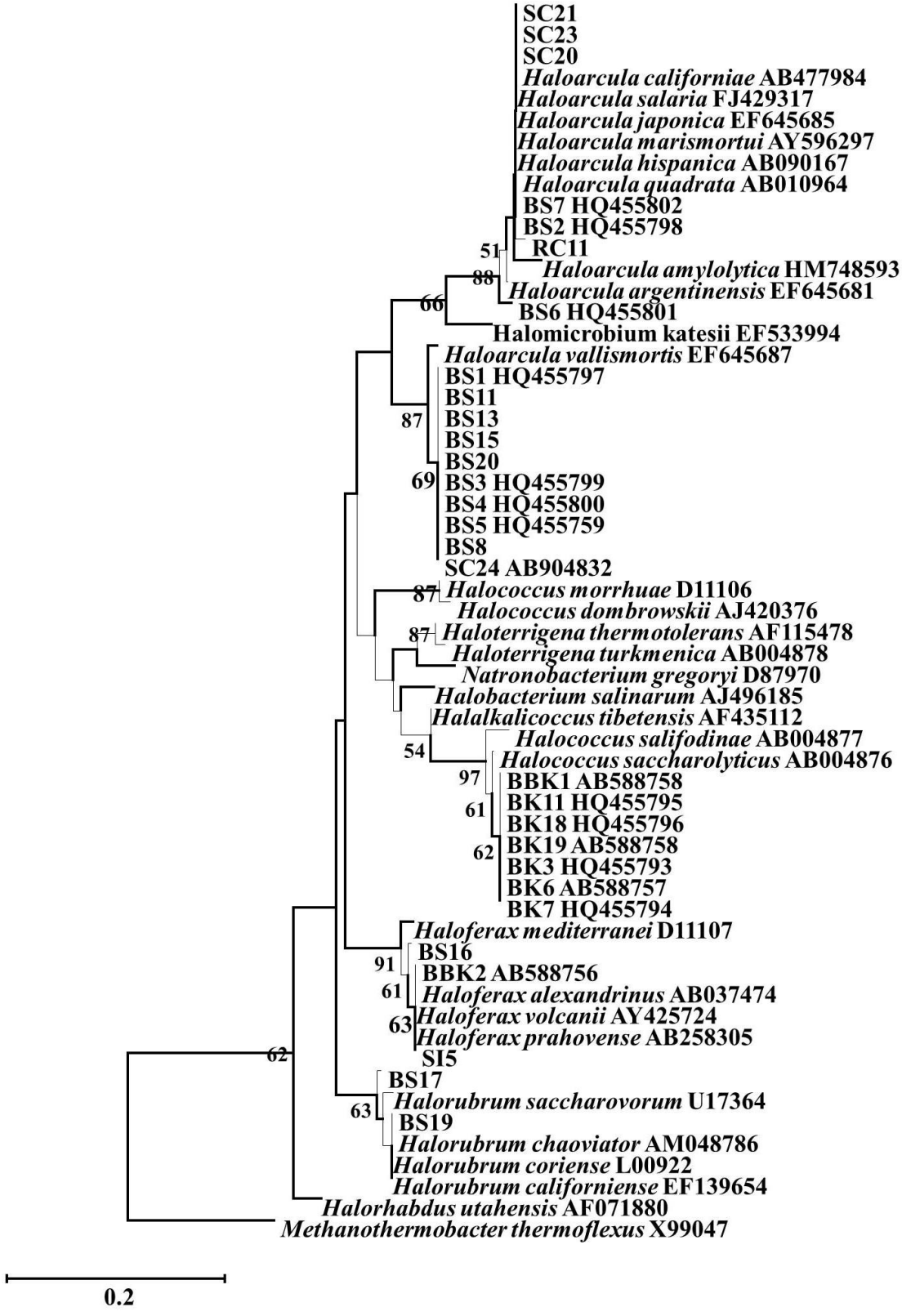


Fig. 3.10: Phylogenetic position of archaeal isolates recovered from salterns of Goa. The tree was constructed based on partial 16S rRNA gene sequences using the Neighbor-Joining method in MEGA 5.0.

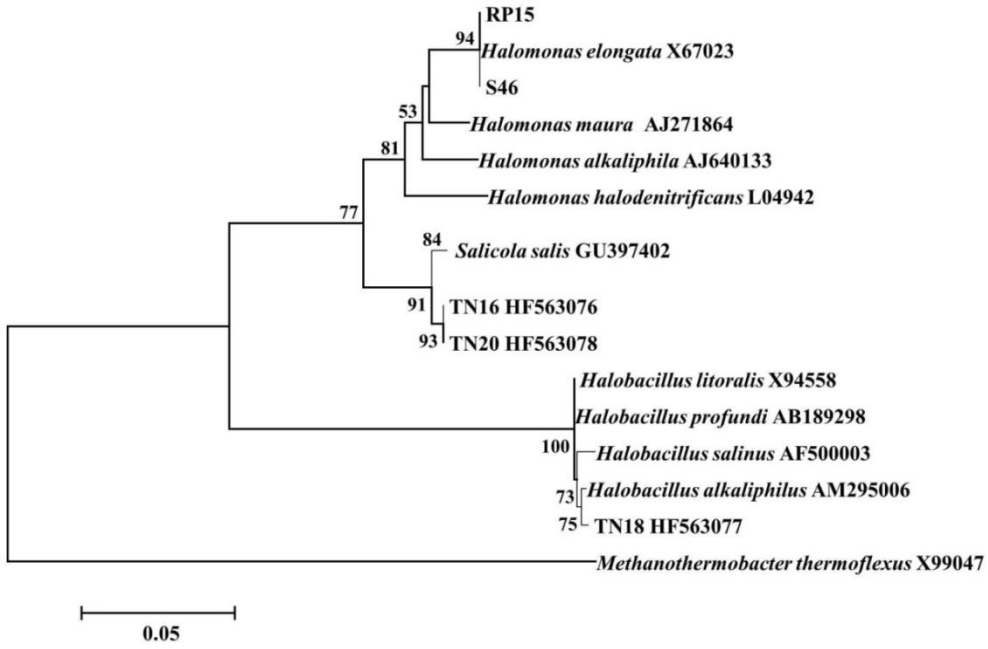


Fig. 3.11: Phylogenetic position of bacterial isolates recovered from salterns of Goa and Tamil Nadu. The tree was constructed based on partial 16S rRNA gene sequences using the Neighbor-Joining method in MEGA 5.0.

Isolates obtained in the peak salt harvesting (PSH) phase belonged to domain Archaea, *Haloarcula* sp. BS1, *Haloarcula* sp. BS2, *Haloarcula* sp. BS4, *Haloarcula* sp. BS5, *Haloarcula* sp. BS7, *Haloarcula* sp. BS8, *Haloarcula* sp. BS11, *Haloarcula* sp. BS13, *Haloarcula* sp. BS15, *Haloarcula* sp. BS20, *Haloarcula* sp. SC20, *Haloarcula* sp. SC21 and *Haloarcula* sp. SC23 were closely related(>99%) to *Haloarcula quadrata* ATCC700850^T(AB010964), *Haloarcula hispanica* ATCC33960^T (AB090167), *Haloarcula marismortui* ATCC43049^T (AB596297) and *Haloarcula salaria* JCM15759^T(FJ429317). *Haloarcula* sp. BS3 and *Haloarcula* sp. BS6 obtained from Ribandar brine sample displayed 98.13% and 97.91% relatedness to *Haloarcula marismortui* ATCC43049^T (AB596297) indicating the potential novelty of the isolates. Similarly, *Halorubrum* sp. BS17 and *Halorubrum* sp. BS19 obtained from Ribandar sediment sample displayed 98.89% and 98.90% relatedness towards *Halorubrum saccharovorum* ATCC29252^T (U17364) indicating the potential novelty of the isolates.

Haloarcula sp. RC11 obtained from Ribandar brine sample displayed 98.7% relatednesss towards *Haloarcula hispanica* ATCC33960^T (AB090167), again indicating the potential novelty of the isolate. *Haloferax* sp. BS16 displayed close relationship (>99.0%) with *Haloferax alexandrinus* JCM10717^T (AB037474), *Haloferax volcanii*ATCC29605^T (AY425724) and *Haloferax prahovense* DSM18310^T (AB258305).

Halococcus sp., RC12 was closely related (>99.0%) with *Halococcus salifodinae* ATCC51437^T(AB004877) and *Halococcus saccharolyticus* ATCC49257^T (AB004876). *Halostagnicola* sp. RC9 displayed close relationship (>99.0%) to *Halostagnicola alkaliphila* JCM16592^T(AB533255) and *Halostagnicola larsenii* DSM17691^T (AM117571)(Fig. 3.11). Among the 2 isolates obtained from Sinquetim saltern, one was phylogenetically affiliated with Bacteria and the other was affiliated to Archaea. *Haloferax* sp. SI5 displayed close relationship (>99.0%) to *Haloferax alexandrinus* JCM10717^T (AB037474), *Haloferax volcanii*ATCC29605^T (AY425724) and *Haloferax prahovense* DSM18310^T (AB258305). *Salinicoccus* sp. SI3 displayed 98.9% relatedness to *Salinicoccus hispanicus* ATCC49259^T (AY028927) indicating the potential novelty of the isolate.

3.3.4.4.2 Solar Salterns of Tamil Nadu

Haloferax sp. TN4, *Haloferax* sp. TN5, *Haloferax* sp. TN6, *Haloferax* sp. TN10 and *Haloferax* sp. TN15 were closely related (>99%)to *Haloferax alexandrinus* JCM10717^T (AB037474), *Haloferax volcanii*ATCC29605^T (AY425724) and *Haloferax prahovense* DSM18310^T (AB258305). *Haloarcula*sp. KTN36, *Haloarcula*sp. TN8 and *Haloarcula*sp. KTN50 were closely related (>99.0%) to *Haloarcula quadrata*ATCC700850^T(AB010964), *Haloarcula hispanica* ATCC33960^T (AB090167), *Haloarcula marismortui* ATCC43049^T (AB596297) and *Haloarcula salaria* JCM15759^T (FJ429317). Similarly, *Halogeometricum* sp. TN9, *Halogeometricum* sp. KTN49 and *Halogeometricum* sp. KTN47 were closely related (>99.0%) to *Halogeometricum borinquense* ATCC700274^T (AF002984) and *Halogeometricum rufum* JCM15570^T (EU887286). *Natrinema* sp. KTN23 was closely relatedtowards *Natrinemagari* JCM14663^T (AB289741) and *Natrinema pallidum* JCM8980^T (AJ002949). *Halorubrum* sp. KTN28 displayed 98.25% relatedness towards *Halorubrum xinjiangense* JCM12388^T(AY510707) indicating the potential novelty of the isolate (Figure 3.12).

Bacterial isolates, *Salicola* sp.TN16 and *Salicola* sp. TN20 was closely related (>99.0%) towards *Salicolasalis* LMG23122^T (DQ129689). *Halomonas* sp. KTN18, *Halomonas* sp. KTN20 and *Halomonas* sp. KTN21 was closely related (99.0%) similarity towards*Halomonas elongata* ATCC33173^T (X67023), *Halomonas maura* ATCC700995^T (AJ271864) and *Halomonas alkaliphila* DSM16354^T(AJ640133). *Halobacillus* sp. TN18 was closely related towards *Halobacillus alkaliphilus* DSM8525^T (AM295006),

Halobacillus salinus JCM11546^T (AF500003) and *Halobacillus litoralis* ATCC700076^T (X94558) (Figure 3.11).

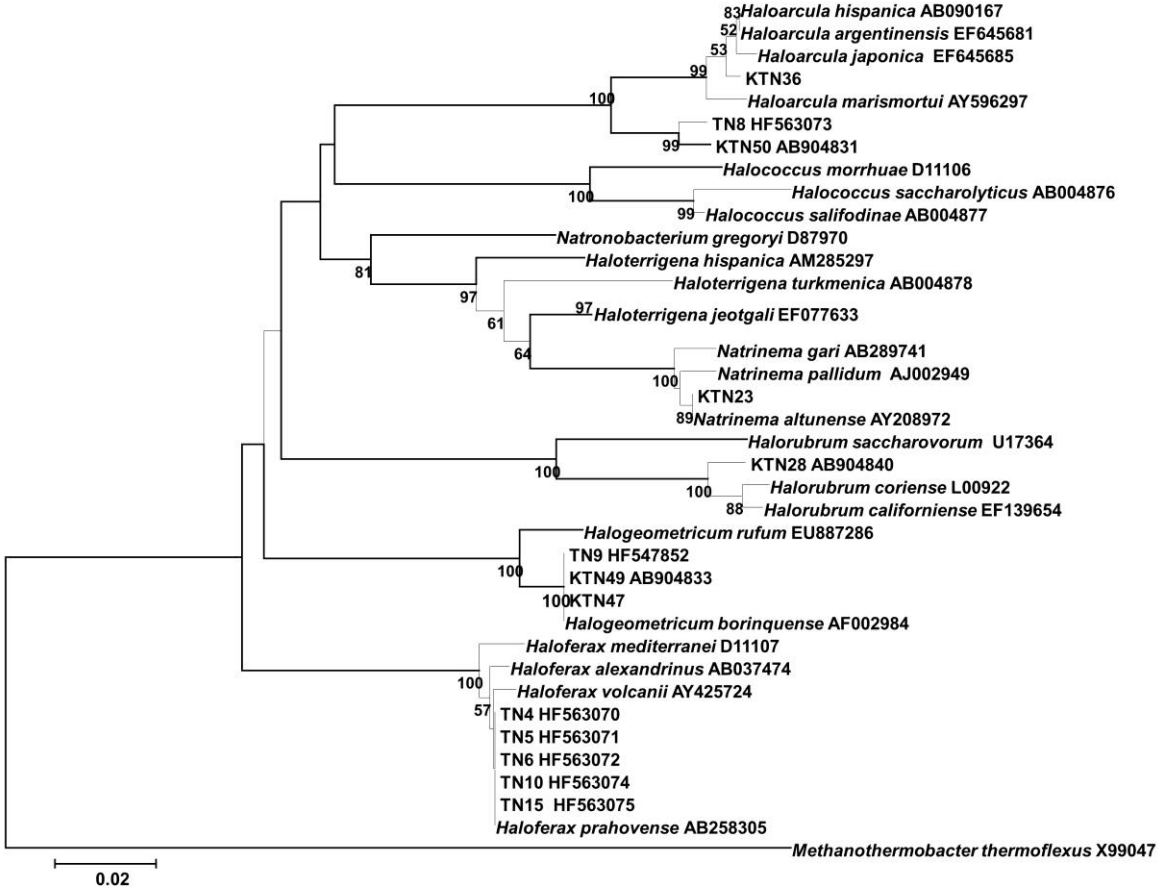


Fig. 3.12: Phylogenetic position of archaeal isolates recovered from salterns of Tamil Nadu. The tree was constructed based on partial 16S rRNA gene sequences using the Neighbor-Joining method in MEGA 5.0.

3.3.4.4.3Salt crystals

Among the 7 isolates obtained from salt crystals, 6 were phylogenetically affiliated to Archaea. *Halococcus* sp. S56 and *Halococcus* sp. S18 were 98.52% and 98.46%closely related with *Halococcus salifodinae* ATCC51437^T(AB004877) indicating the potential novelty of the isolates. *Halococcus* sp. S8 was closely related with *Halococcus salifodinae* ATCC51437^T(AB004877) and *Halococcus saccharolyticus* ATCC49257^T (AB004876). *Haloterrigena* sp. S15 was 98.91% related to *Haloterrigena turkmenica* ATCC51198^T(CP001860). *Haloterrigena* sp. S16 was closely related (>99.0%) with *Haloterrigena turkmenica* ATCC51198^T (CP001860) and *Haloterrigena thermotolerans* ATCC700275^T(AF115478). *Halomonas* sp. S46 was closely related (>99.0%) with *Halomonas elongata* ATCC33173^T (X67023), *Halomonas maura*

ATCC700995^T (AJ271864) and *Halomonas alkaliphila* DSM16354^T(AJ640133) (Fig. 3.13).

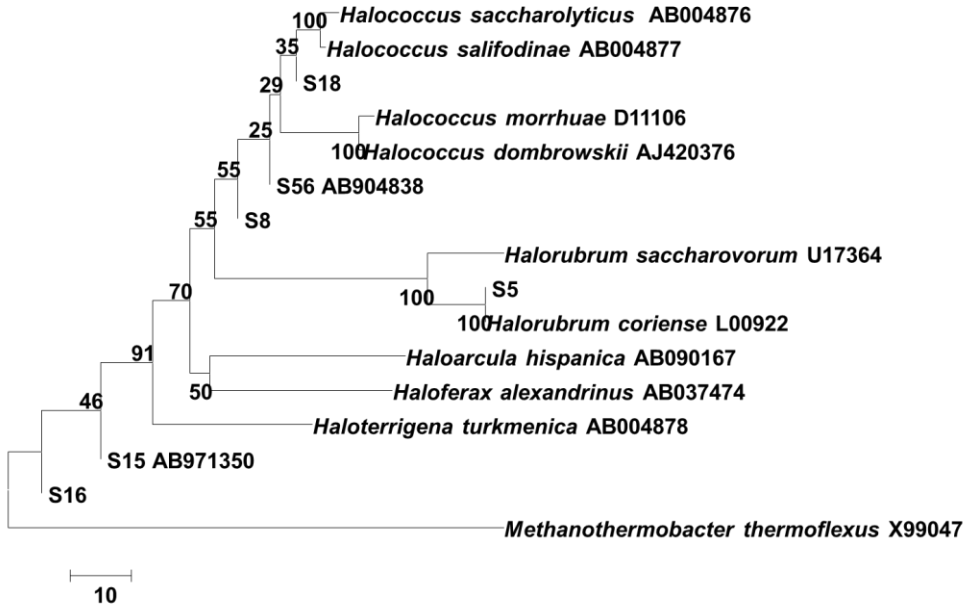


Fig. 3.13: Phylogenetic position of archaeal isolates recovered from salt crystals. The tree was constructed based on partial 16S rRNA gene sequences using the Neighbor-Joining method in MEGA 5.0.

3.4 Discussion

A detailed study on the physicochemical biological characteristics of the representative salterns along with microbial culturable diversity was carried out. Soil texture indicated the presence of clay soil during preparation of salterns. Clay soil is best suited for the manufacturing of solar salt since it has the maximum water retention capacity (Manikandan et al., 2009). Elemental analysis showed that thebrineswere dominated by sodium and chloride ions, indicating the thalasohaline nature of salterns (Oren et al., 2002). There was a gradual increase in the concentration of total ions with increasing salinity. However, the amount of calcium ions declined during the transition from intermediate salinity to high salinity. This may be due to the precipitation of calcium ions as calcium carbonate and calcium sulphate, during the intermediate salinity from 7 – 12%. Since the sampling was conducted before the complete precipitation of salt crystals, sodium and chloride ions were dominant along with the presence of potassium and magnesium salts. When the concentration of manganese was estimated between Ribandar and Siridao saltern, the former showed the presence of high (7.9 x 10^{-3} g/l) manganese content. This may attributed to the location of Ribandar salterns on the banks of Mandovi River. This river is an inland transport system for the transport of manganese ore, which

might have contaminated the salterns and its vicinity (Kerkar&LokBarathi, 2011). On analysing the ionic composition of salt crystals, chloride represented 50-55% and sodium represented 30 – 35%, indicating the halite nature of the salt crystals(Oren, 1994).

Studies have shown the presence of cyanobacteria and microscopic algae in the solar salterns and other hypersaline environments and their role as the producers in the ecosystem (Villaneuva et al., 1994; Elloumi et al., 2009). Thus, chlorophyll measurement of an ecosystem gives an insight of the distribution of phytoplankton community structure thereby productivity of the ecosystem (Riemann et al., 1989). Studies carried out in ESSA(Exportadora de Sal, SA)solar salterns, Mexico and Sfax salterns, Tunisia has shown that the concentration of chlorophyll *a* ranged from 0.004 to 0.085 µg/ml (Abid et al., 2008; Javor, 1983). Sediments from salterns of Goa and Tamil Nadu contained 10 fold higher concentrations than the reported values. However, Chlorophyll *a* concentrations in brines were comparable to other salterns round the world. When the chlorophyll *a*concentration of the brine samples remained relatively constant during the both phases of salt production in the salterns of Goa, the chlorophyll *a*concentration of sediment samples varied significantly between the salt producing phases. This may be attributed to the fact that during the initial stages of salt production, the development of benthic communities comprising of cyanobacteria and algae would be at their initial stages. However, with the continuous operation of salterns, a thick microbial mat would have developed contributing to the higher chlorophyll content. Similarly, the highest chlorophyll *a*concentration in the evaporator pans (EP) can be attributed to the presence of cyanobacteria like *Microcoleus chthonoplastes* and *Aphanothece halophytica* and with the increase in salinity, *Dunaliella salina*, the microalga takes over the role as sole producer in the crystalliser pans (CP) and contribute to the chlorophyll *a*concentration of crystalliser pans (CP) (Elloumi et al., 2009; Oren et al., 1992). Solar salterns of Tamil Nadu had higher chlorophyll *a* concentration than solar salterns of Goa, especially in the evaporator pans (EP) and crystalliser pans (CP). Since, Tamil Nadu solar salterns are operated for a longer time than the Goan salterns, the development of thick microbial mat with cyanobacteria and algae is expected, thereby contributing to the high chlorophyll *a* concentration. In Vedaranyam salterns, salt harvesting is done once in 7 -10 days thereby allowing more time for microbial mat development without any disturbances and this could have contributed to the highest chlorophyll *a* concentration among all the salterns investigated.

The brine and sediment samples were enumerated by staining with Acridine Orange (AO). Acridine orange stains both DNA and RNA at the excitation of 470 nm and the cells will appear orange to red if it contains more RNA than DNA while the cells will appear green if DNA outnumbers RNA. An active cell will contain more RNA while dormant or non-active cell will contain more DNA and hence former appear orange while latter appears green. The saltern samples contained more rods, curved and long than other shapes of cells. Square shaped cells typical of *Haloquadratum*, observed in the crystalliser pans around the world could not be found in the salterns of Goa and Tamil Nadu (Manikandan et al., 2009). The amount of cells were in the range of 10^6cells/ml, a value similar to the one found in other salterns around the world yet 10 fold less than some salterns(López-López et al., 2010; Anton et al., 1999; Elloumi et al., 2009). This may be due to the fact that, since most of the salterns in Europe and America harvest salts on a yearly basis against the Indian salterns, where the salt is harvested on a daily or weekly basis. The practice of frequent harvesting of salt prevents a stable microbial community to develop resulting in low cell numbers in solar salterns of India. Other reasons for slightly lower count could be attributed to the occurrence of less organic matter in the salterns for the microorganisms to feed upon and high predation rates (Maturrano et al., 2006).

Isolation of halophilic archaea from solar salterns is challenging. Since solar salterns are low diversity environments, serial dilution seldom results in consistent colony formation among different samples. Therefore, direct plating technique was used, without diluting the samples. The average CFU per ml or g found in salterns vary from 10^4 to 10^6 and in few nutritionally rich salterns the values can reach as high as 10^8organisms (Maturrano et al., 2006; Pasic et al., 2007; Birbir et al., 2007; Boujelben et al., 2014). However, in the other solar salterns of India the obtained viable counts were in the range of 10^3 and 10^4 CFU/ml (Manikandan et al., 2009; Jose &Jebakumar, 2012). The viable counts obtained in the Indian solar salterns under study are similar to the values obtained from salterns around the world. Since the Indian solar salterns are fed with oligotrophic waters, drawn from Arabian Sea and Bay of Bengal, the diversity of organisms in the salterns are low (Sreepada et al., 1996; Ram et al., 2007). Adding to the above fact, since solar salterns are themselves low nutrition environments, the diversity of organisms remains less throughout the salterns. The direct microscopic count yielded organisms in the order 10^6 and therefore the viable counts (0.5 – 0.8% of Acridine Orange (AO) count) shows the inability to capture the actual biodiversity of the salterns, in spite of employing

five different media. In the present study, among extremely halophilic media JCM168 and NT had highest viable count than EHM and NTYE. This may be due to the presence of tri-sodium citrate in NT and JCM168, acting as carbon source in enhancing fast growth of halophilic microbes. Apart from citrate, JCM168 contains casamino acid and glutamate, providing a source of amino acids, may have an advantage over other extremophilic media (Ceylan et al., 2012). MHM media was selected to capture halophilic bacteria and it supported the fastest growth of microbes among all the media. Though many bacteria can tolerate a wide range of NaCl concentration, they are intolerable to high magnesium concentration. So the low magnesium (2 g l^{-1}) content of MHM media would have allowed halophilic and halotolerant bacteria to colonise (Oren, 2012).

Majority of the organisms obtained were non-pigmented and they grew in a fastidious manner when compared with their pigmented counterparts. Since, most of the halophilic archaeal isolates possess the red coloured pigment, bacterioruberin, it is an important phenotypic trait to identify the halophilic archaea on media plates (Oren et al., 1992). Extremely halophilic organisms contain light driven proton pumps for maintaining their internal cellular ionic concentration. Therefore, their dependence on light exposes them to high UV radiation and hence pigmentation is one of the protective mechanisms against radiation induced DNA damage (Khanafari et al., 2010). Therefore, the appearance of orange/red coloured colonies indicated the presence of halophilic archaeal isolates.

Studies on the salterns around the world have identified *Haloarcula, Halorubrum* and *Haloferax* as the dominant cultivable archaeal genera (Luque et al., 2012; Pasic et al., 2007; Benlloch et al., 2001; Sabet et al., 2009; Gareeb&Setati, 2009). Similarly, these studied have identified bacteria belonging to *Proteobacteria, Firmicutes* and *Bacteriodetes* dominate the salterns. Some prevalent bacterial genera include *Salinibacter, Halomonas, Marinobacter* and *Bacillus* (Yeon et al., 2005; Sorokin et al., 2006; Baati et al., 2009; Chen et al., 2010; Ventosa et al., 1998). The salt pans of Goa are transient and operated only during the post monsoon season of January to May. During monsoons, the saltpans are inundated with seawater as well as rain water (Mani et al., 2012). Most of haloarchaeal strains are known to lyse in distilled water as they require at least 10% NaCl to maintain the integrity of their outer membrane. It is interesting to note that Halococcal microorganisms were most abundant, during the preparatory phase as they are resistant to lysis in lowering salinities than their counterparts (Oren, 2002). However, as salinity gradually increases, other members of haloarchaeal community start

to colonize the saltpans.To further confirm the survivability of Halococcal cultures obtained during preparatory phase, cell viability assay in distilled water was carried out. The isolates obtained from preparatory phase (BK series) were resistant to lysis in distilled water and 3.5% NaCl. However isolates obtained from initial salt harvesting (ISH) phase showed varied lysis on suspension in distilled water with the exception of BBK1 which was resistant to lysis. The isolate BBK2 lysed immediately, whereas BS4 and BS5 were observed to have delayed lysis. Viability assay was performed for BK6 obtained during preparatory phase and BBK2 obtained during initial salt harvesting (ISH)phase. On suspension of cells in distilled water, 3.5% NaCl and 30% NaCl, followed by plating revealed that isolate BK6 was viable even after 10 days on suspension in distilled water. Cells of BK6 also retained their coccoid shape, when observed microscopically. The isolate BBK2 survived for up to 24 h in 3.5% NaCl, but lysed immediately in distilled water showing no growth on plating.

It is interesting to note that Halococci were recovered almost as a pure culture in culturable form during the salt dilute phase (whereas other culturable genera could be recovered during the salt concentrated phase). A study by Fukushima et al. (2007), showed that the cells of *Halococcus* survived in seawater (salinity of which is 3.5%) upto 9 days without losing its cell rigidity. It is also possible that haloarchaea are trapped in the salt crystals and get deposited in the sediments. Therefore it is quite possible that even though the haloarchaeal members cannot flourish they can still retain their viability. Another possibility is presence of clay in these salterns. These clay particles have micropores on which the salt fluid gets filled along with the haloarchaeal members. These micropores could serve as a salt rich environment for the survival of haloarchaea (Benlloch et al., 2002; Jiang et al., 2007; Norton & Grant, 1988)

Though most of the halophilic archaeal isolates were obtained from intermediate (15%) and high salinity (27%) samples, few archaeal isolates like *Halorubrum*, *Haloarcula* and *Haloferax* were obtained from low salinity samples. Similarly, *Haloferax* isolate was obtained from the abandoned saltern reservoir pan (RP) sediment sample, where the salinity was 1%. Recent studies have shown that haloarchaea are being isolated from less hypersaline environments. Salt-marsh sediments, sulfur-rich spring and deteriorated ancient wall paintings has been investigated and found to have haloarchaeal members, predominantly belonging to *Haloferax* and *Halococcus* (Elshahed et al., 2004; Pinar et al., 2001). A study by Purdy et al., showed the presence of *Haloferax* and *Halogeometricum* in low salinity sediments. Similarly, isolation of *Haloferax* from low

salinity sulphur rich sediments has been reported. This shows the ability of halophilic archaea especially members like *Haloferax*, *to* grow in wide range of salinities (Oren &Hallsworth, 2014). It is also interesting to note the recovery of halophilic bacterial and halophilic archaeal isolates from the abandoned salterns. Both of the characterised isolates were recovered from the sediment sample of the reservoir pans. Though the salinity of the water samples was around 1%, the presence of micro sites of high salinity in the sediments could have helped the survival of halophilic isolates. Majority of the characterised halophilic archaeal isolates were obtained from extremely halophilic media and majority of characterised halophilic bacterial isolates were obtained from moderate halophilic medium. However, few isolates belonging to *Haloferax*, *Halorubrum* and *Halococcus* were isolated from the moderate halophilic medium. Though there is no difference in the concentration of NaCl, which is around 17.8%, the amount of magnesium is 0.2% as compared against 2% in other extremely halophilic media. Similarly, few bacterial isolates belonging to *Halomonas* and *Alkalibacillus* were isolated from extremely halophilic media. This shows the tolerance of halophilic bacteria in surviving wide range of ionic concentrations. In fact, *Halomonas* was isolated from three different media, namely JCM168, EHM and MHM. Therefore, this shows that both halophilic archaea and bacteria can survive in wide range of fluctuating environments.

Some isolates like *Halococcus* was isolated predominantly from NT and NTYE media while *Haloferax* and *Halomonas* was isolated from more than 3 media. This may because of the preferential selection of some isolates towards selective carbon sources while some isolates can have a potential of metabolizing multiple substrates. Some genera were found only in certain salterns whereas some genera were found in many locations. *Halococcus* were predominant in Goan salterns while *Halogeometricum* was isolated only from Tamil Nadu salterns. However, haloarchaeal genera like *Haloarcula, Haloferax, Halorubrum* and *Halomonas* were found in Ribandar and Siridao salterns. *Haloferax* was isolated repeatedly from Ribandar saltern during initial salt harvesting (ISH) and peak salt harvesting (PSH) phase. However, it was not present in any of the characterised isolates from Siridao salterns. This indicates the micro diversity of the individual salterns though these salterns are only 10 km apart. Salt crystals represent a unique ecosystem for isolating halophilic archaea. As the salt crystal is formed in crystalliser pans, several halophilic organisms are trapped inside the crystals providing an opportunity for isolating halophilic archaea. Several novel species of halophilic archaea have reported from the salt crystals in recent years (Kim et al., 2011; Mancinelli et al., 2009; Yimet al., 2014;

Cojocet al., 2009). *Halococcus* was the dominant species in the salt crystals after being isolated from salt crystals of Tamil Nadu and Goan salterns.

The present study revealed the presence of 9 (BS3, BS6, BS17, BS19, KTN28, RC11, S15, S18 and S56) and 2 (SP18, SI3) potentially novel isolates (similarity <99.0%) belonging to *Archaea* and *Bacteria*, subjected to DNA-DNA hybridisation experiments (Kim et al., 2014). This indicates that the solar salterns of India are yet to be completely explored and can be a good source of novel isolates producing industrially important biotechnological products.

3.5 Conclusion

The culturable microbial diversity of solar salterns of Goa on the west coast and Tamil Nadu on the east coast of India were studied for the culturable diversity of halophilic microorganisms. The detailed elemental analysis revealed that the salterns are dominated by sodium and chloride ions, indicating the thalasohaline nature. Chlorophyll*a* estimation indicated the salterns under investigation were moderate production environments. This study employed five different media for studying the culturable halophilic archaeal and bacterial diversity. Phylogenetic analysis of the purified isolates revealed that during the preparatory phase of salterns of Goa, *Halococcus* was the dominant genus. During the initial salt harvesting (ISH) phase, *Haloferax* and*Halococcus*were identified and during the peak salt harvesting (PSH) phase, archaeal organisms belonging to the genera *Haloarcula, Haloferax, Halorubrum* and *Halostagnicola* were identified. In Tamil Nadu salterns, this study revealed the presence of archaeal members belonging to genera *Haloferax, Halogeometricum, Halorubrum, Haloarcula* and *Natrinema*. Among the bacterial isolates, *Bacillus* and *Halomonas*were dominant in the solar salterns Tamil Nadu and Goa.Salt crystals harboured halophilic archaea belonging to *Halococcus* and *Halorubrum*, while the bacterial isolate belonged to *Halomonas*.

Chapter IV

Phenotypic, Chemotaxonomic and Genotypic Characterization of Halophilic Archaea

4.1 Introduction

The advancement in the field of genomics and proteomics is causing rapid shifts in the traditional techniques of bacterial identification, characterization and classification(Logue et al., 2008). The traditional bacterial classification system has difficulties owing to the microscopic size of the organisms and spotting visual morphological differences. However, bacterial taxonomy studiesthrough traditional techniques continue to be essential in understanding the biochemical and metabolic spectrum of the microorganism (Emerson et al., 2008). These traditional classification techniques are better aided by the in-depth understanding of their genetic make-up through whole-genome sequencing (WGS), achieved by high-throughput genome sequencing techniques.

In the past decade, more than 112 species belonging to the family *Halobacteriaceae* have been described (Oren, 2014). Halophilic archaea are considered to contain huge potential in the industrial biotechnological applications (Abrevaya, 2012). Screening the hypersaline environments for the isolation of novel isolates and studying their metabolic and biotechnological potential with traditional taxonomical tools coupled with advanced sequencing techniques, could help us in identifying novel value-added products. This chapter deals with the phenotypic, chemotaxonomic and genotypic characterisation of the selected halophilic archaeal isolates obtained from the culturable biodiversity studies of solar salterns described in the previous chapter.

4.2 Materials and Methods

4.2.1 Haloarchaeal isolates and media

Three halophilic archaeal isolates namely (i) *Haloarcula valismortis*BS3, (ii) *Halorubrum chaoviator*BS17, and (iii) *Halorubrum chaoviator* BS19 were selected for the phenotypic, chemotaxonomic and phylogenetic characterisation. These isolates were grown and maintained in NT medium. Whenever required, NaCl synthetic medium (NSM) consisting (gL^{-1}) NaCl- 200.0; $MgCl_2.6H_2O$-13.0; $CaCl_2.6H_2O$-1.0; KCl-4.0; $NaHCO_3$-0.2; NH_4Cl-2.0; $FeCl_3.6H_2O$-0.005; KH_2PO_4-0.5 was employed. pH was adjusted to 7.0 using 1M KOH and 0.2% sterile glucose solution was added as carbon source (Salgaonkar et al., 2011).

The phenotypic, chemotaxonomic and molecular characterization of the haloarchaeal strains wasperformed by following a polyphasic approach, according to the

guidelines included in the minimal standards recommended by Oren et al.,(1997& 2012) for description of new taxa of the order *Halobacteriales*.

4.2.2 Morphological characterization

Colony size, shape, margin, pigmentation, consistency, elevation and opacity of the three halophilic archaeal isolateswere examined on the 2-week old NT agar plates. The extent of cell lysiswas tested by suspending the cells in distilled water for a period of1week followed by examination under a microscope (Olympus BX41, Japan). Gram staining was carried out according to the modification of Dussault, 1955. Briefly, the extremely halophilic cell smears were prepared in a drop of 20% (w/vol) NaCl solution and air-dried. The cells were fixed and desalted in 2% acetic acid, air-dried and stained with primary stain, Crystal violet for 1min. The stain was discarded and the smear was covered with Gram's iodine for 30 sec. The smear was rinsed with water followed by decolourization with 70% ethanol for 30 sec and was counter-stained with safranin for 1 min. The slide was rinsed with water, dried and examined under oil immersion objective (100 X) of the phase-contrast microscope (Olympus BX41, Japan). Motility of the strains was observed under the phase-contrast microscope (Olympus BX41, Japan) using the hanging-drop technique.

Cell pellets of individual haloarchaeal isolates were dispensed in NSM medium to an absorbance of 0.8 at 600nm. Hundred µl of suspension was mounted onto circular glass cover slips, and air dried. The smears are then fixed with 2.0% glutaraldehyde fixative (prepared in NSM) and incubated at room temperature (30°C) overnight. The cover slips were then exposed, to a series of increasing gradient of acetone-water mixture corresponding to 30%, 50%, 70% and 90% for 10 min and followed by exposure to 100% acetone for 30 min. The cover slips containing the samples were placed on a stub, followed by coating with thin gold film using sputter coating device and then viewed under scanning electron microscope (SEM) (JEOL-5800 LV SEM, Japan).

4.2.3 Physiological characterization

Growth and optimum conditions of the isolates were determined in NT medium with different NaCl concentrations (0, 5, 10, 15, 20, 25 and 30% w/vol), incubation temperatures (15, 18, 20, 28, 35, 37, 40, 42 and 45 °C) and Mg^{2+} concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5% w/vol).The pH range for growth was determined at pH 5.0–10.0 (with intervals of 1 unit) using following buffers: 2-(N-

morpholino)ethanesulfonic acid (MES) (pH 5.5–6.7), piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.1–7.5), 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 6.5–7.9), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 6.8–8.2), Tricine (pH 7.4–8.8) and N-Cyclohexyl-2-aminoethanesulfonic acid (CHES) (pH 8.6–10.0) at a concentration of 25 mM. Cells were grown in 250 ml Erlenmeyer flasks with shaking (110 rpm) at 37 °C. Growth was monitored by measuring the turbidity at 600 nm at an interval of 24 hrs.

4.2.4 Biochemical characterisation

For determining the carbohydrate utilization pattern of the haloarchaeal isolates, stock of various sugars (10% w/vol) were made, sterilized separately at 121^{o}C for 15 minutes and added to the sterilized NSM medium (containing phenol red 0.018 g/l) to a final concentration of 1%. The sugars used were D-glucose, D-galactose, sucrose, D-fructose, ribose, D-mannitol, D-maltose, D-raffinose, L-arabinose, D-mannose, D-sorbitol and D-lactose.The tubes were incubated for 5 to 8 days at 37°C. Growth was observed by turbidity and production of acid and gas was observed by change in the colour of the medium from original red to yellow and gas bubble in Durham tube respectively.

Complex carbohydrate utilisation patterns of the haloarchaeal isolates were determined by adding 1% (w/vol) of yeast extract, tryptone, malt extract, peptone and casamino acids. The ability of the haloarchaeal isolates to utilise organic acids were determined by adding sodium acetate, sodium glutamate, sodium pyruvate, malic acid, ammonium oxalate, citric acid and oxalic acid to sterile NSM medium at concentration of 0.1% (w/vol). To test growth under anaerobic conditions, the strains were cultivated in NSM medium with KNO_3 (30 mM), L-arginine (5 g/l), and dimethyl sulfoxide (DMSO) (5 g/l) in screw-topped sealed vials. Indole production was determined by adding Kovac's reagent to the liquidNSM medium supplemented with 1% (w/vol) tryptone. H_2S formation was determined by the black sulfide precipitate in the soft agar medium containing 0.5% (w/vol) sodium thiosulfite. Nitrate reduction was tested in liquid NSM medium supplemented with 0.5% (w/vol) $NaNO_3$. Formation of gaseous products from nitrate was detected by the presence of gas bubbles in Durham tubes (Benson, 1965). All the tubes were incubated at 37^{o}C for 5 to 8 days.

Haloarchaeal isolates were screened for the production various hydrolytic enzymes. To determine extracellular amylase activity, isolates were streaked onto the NSM agar medium supplemented with 1% (w/vol) soluble starch (Hi-media, India) and

plates were flooded with iodine solution after growth was observed (15-20 days). Clearance around the growth against a dark blue background was taken as evidence of amylase activity. Similarly, protease and esterase activity was determined by observing the formation of clear/opaque zones around culture grown on NSM agar medium supplemented with 1% (w/vol) skimmed milk and 0.1% (vol/vol) Tween (Tween 20 and 80) (Gutierrez & Gonzalez, 1972; Oren et al., 2002). Gelatinase activity was determined by growing the isolates on NSM agar plates supplemented with 0.4% (w/vol) gelatin (Hi-media, India) and checking for clearance around the growth by flooding the plates with a solution of 15% (w/vol) $HgCl_2$ in 20% (w/vol) HCl. Catalase activity was determined by adding few drops of 3% H_2O_2 onto wet smear prepared from 1 week old culture. The smears were prepared on glass slides (Elevi et al., 2004). The presence of oxidase was determined with tetramethyl *p*-phenylendiamine-HCl. Appropriate positive and negative controls were run for all of the above tests and hydrolysis experiments were carried out in triplicates.

Antibiotic sensitivity of the strains were evaluated by spot-inoculating the haloarchaeal cultures on solid NSM medium containing 50 µg/ml of novobiocin, rifampicin, ampicillin, erythromycin, kanamycin, bacitracin, streptomycin and tetracycline. Plates were incubated at 37^{o}C for 5 to 8 days. Control plates without antibiotics were also inoculated with the respective cultures.

4.2.5 Screening of haloarchaeal isolates for PHA production

Haloarchaeal isolates were screened for the production of PHA. This was tested using NSM containing 1 and 2% (w/vol) glucose and starch as substrates. Fifty µl of Nile Red (Hi-media, India) was added to 100 ml of NSM agar medium such that the final concentration was 0.5µg/ml of medium [stock of 0.001% (w/vol) Nile Red in DMSO]. The isolates were streak/spot inoculated in triplicates on agar medium plates and incubated at 37^{o}C. Accumulation of PHA by haloarchaeal isolates was determined on exposure of the grown culture plates (5-8 days old) to UV light using Gel documentation system (BIO-RAD Laboratories CA, USA) (Spiekermann et al., 1999).

4.2.6 Screening for the production of antiarchaeal substances and growth factors

Haloarchaeal cultures were inoculated into a 100 ml Erlenmeyer flask containing 20 ml of NT medium and incubated at 37^{o}C with shaking (110 rpm) for up to 96 h. The culture broth was aseptically taken in a sterile Oakridge tube and centrifuged at 12,000

rpm for 30 min. The supernatant obtained was aseptically passed through 0.22 µm membrane filter (Millipore, India) for ensuring the complete removal of cells. The antiarchaeal activity of the cell free supernatant (CFS) tested against the indicator haloarchaeal strains. One ml of the indicator strain was aseptically transferred to a 100 ml of autoclaved medium containing 2% agar, which was precooled to 50^{o}C. The seeded agar medium was immediately poured into sterile Petri plates and allowed to solidify. Wells (7 mm in diameter) were punched aseptically with a sterile cork-borer and 100 µl of CFS was added to each well. The plates were incubated at room temperature (30^{o}C) and observed for zones of exhibition or inhibition. Sterile medium was used as control.

4.2.7 Chemotaxonomic characterisation

Cell pellets from 50 ml of 5 days old haloarchaeal isolates were suspended in 3.75 ml methanol:chloroform (2:1, vol/vol) and extracted for 4-6 h (Bligh and Dyer, 1959; Kates, 1995). The extract was collected by centrifugation at 10,000 rpm for 20 min, and the pellet was re-extracted with 4.75 ml methanol:chloroform:water (2:1:0.8, vol/vol). The extracts were combined and 2.5 ml of chloroform and 2.5 ml of water was added to it, for achieving phase separation. After centrifugation, the chloroform phase containing the lipids was collected in clean dry glass vial and dried by evaporation. Lipids were re-dissolved in 100 µl of chloroform and spotted on to silica gel plates (Silica gel 60 F254, Merck) using fine glass capillary tubes. The polar lipids were separated by single development using the solvent system, chloroform:methanol:aceticacid:water (85 : 22.5 : 10 : 4, vol/vol). Glycolipids were detected by spraying with 0.5% α – napthol in 50% methanol-water followed by 5% H_2SO_4 in ethanol and spots were visualized by heating the TLC plate at 100^{o}C. Phospholipids were detected by spraying separate set of plate with molybdenum blue spray reagent (Sigma-aldrich, India).

4.2.8 Growth kinetics

Growth pattern of the haloarchaeal isolates was studied in liquid NT medium. One percent mid-log culture was inoculated in 100 mL of NT mediumand the flasks were incubated at 37^{o}C and 110 rpm and the growth was monitored by recording the optical density at an interval of 24 h at 600 nm by UV–Vis spectrophotometer (UV-2450 Shimadzu, Japan).

4.2.9Genomic Characterisation of halophilic archaea through whole-genomic sequencing using Illimina NextSeq technology

4.2.9.1 Sequencing of genomic DNA

Har. valismortis BS3 and *Hrr. chaoviator* BS19 were grown aerobically in liquid NT medium (Table 3.1) at 37°C till the cells reached late exponential phase (after 7 days) of their growth phase. The cells were then harvested and the genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA), according to the manufacturer's instruction. The purity of DNA was checked by measuring the $A_{260/280}$ ratio on Nanodrop 8000 and it was quantified using Qubit® 2.0 Fluorometer. The paired-end sequencing library was prepared using Illumina TruSeq Nano DNA HT Library Preparation Kit. Two hundred ng of genomic DNA was fragmented by Covaris to generate a mean fragment distribution of 550 bp. Indexing adapters were ligated to the ends of the DNA fragments, preparing them for hybridization onto a flow cell. The ligated products were purified using SP beads supplied in the kit. The size-selected product was PCR amplified as described in the kit protocol.The amplified library was analysed in Bioanalyzer 2100 (Agilent Technologies, USA) using High Sensitivity (HS) DNA chip as per manufacturer's instructions. After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, library was loaded onto NextSeq for cluster generation and sequencing. The kit reagents were used in binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment. The entire library preparation and DNA sequencing was carried out at Xcelris Labs Ltd., India.

4.2.9.2 Bioinformatic analysis

The quality filtering of the raw data was carried out with Trimmomatic v0.30 (Bolger et al., 2014). The filtered data was assembled using CLC-Genomics workbench-6.0 with optimised parameters and Coding DNA Sequences (CDS) were predicated using Prodigal v2.60. The gene annotations of the predicted CDS were performed with BLASTX using NCBI blast+, against NR database. The significant E value of 10^{-5} was used as filter to retain significant Blast hits and remove short ambiguous alignments (Altschul et al., 1990). RNAmmer 1.2 Server and tRNAscan software was utilized to predict the tRNA and rRNA encoding genes (Lagesen et al., 2007; Lowe & Eddy, 1997).

The GO mapping was performed using Blast2GO program to classify the functions of the CDS based on Gene Ontology terms (Conesa et al., 2005). Ortholog assignment and mapping of the transcripts to the biological pathways were performed using KEGG automatic annotation server (KAAS). All the genes were compared against the KEGG database using BLASTX with threshold bit-score value of 60 (Moriya et al., 2007). The tools employed in the bioinformatic analysis of the reads are listed in Table 4.1.

Table 4.1: Softwares employed in the quality filtering and analysis of the raw sequences

Role	Software
Quality filtering of raw reads	Trimmomatic v0.30
De novo assembly of filtered reads	CLC Genomics Workbench – 6.0
CDS prediction	Prodigal v2.60
Gene annotations	BLASTX
rRNA gene prediction	RNAmmer 1.2
tRNA gene prediction	tRNAscan
Gene Ontology (GO) analysis	Blast2GO
Pathway predictions	KEGG Automated Annotation Server (KAAS)

4.3 Results

4.3.1 Morphological characterisation

Haloarchaeal isolates, *Har. valismortis* BS3, *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 were cocci in shape, non-motile and stained Gram-negative (Fig. 4.1). Colonies of *Har. valismortis* BS3, on NT agar medium were circular, convex, opaque with entire margins, about 0.5-1 mm in diameter (after 14 days of incubation at 37°C), exhibited light red pigmentation and had a butyrous consistency (Table 4.2). Colonies of *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19, on solidified NT agar medium were circular, convex, translucent with entire margins, about 1-2 mm in diameter (after 14 days of incubation at 37°C), exhibited brick red pigmentation and had a butyrous consistency. The isolates lysed when suspended in distilled water (Fig. 4.1 & Table 4.2).

4.3.2 Physiological characterisation

Har. valismortis BS3 required a minimum of 0.9 M (5% w/vol) NaCl while *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 required a minimum of 1.7 M (10% w/vol) NaCl for growth. Optimum growth of all the three isolates occurred at 4.3 M (25% w/vol) NaCl. The isolates were magnesium dependent and required at least 0.5% w/vol Mg^{2+} for growth and the optimum Mg^{2+}concentration was 3% w/vol. Growth of the isolates occurred between 28°C and 45°C with an optimum at 40°C. Growth of all the isolates was supported byNT medium at pH 5.0, 6.0, 7.0 and 8.0. No growth occurred at pH above 8.0 or below 5.0.

Table 4.2 Morphological characteristics of the halophilic archaeal isolates

Colony morphology	***Har. valismortis BS3***	***Hrr. chaoviator* BS17**	***Hrr. chaoviator* BS19**
Pigmentation	Light Red	Brick Red	Brick Red
Diameter	0.5-1 mm	1-2 mm	1-2 mm
Shape	Cocci	Cocci	Cocci
Elevation	Convex	Convex	Convex
Rim	Entire	Entire	Entire
Surface form	Smooth	Smooth	Smooth
Opacity	Opaque	Translucent	Translucent
Consistency	Butyrous	Butyrous	Butyrous

4.3.3 Phenotypic characterisation

Haloarchaeal isolates, *Har. valismortis*BS3, *Hrr.chaoviator*BS17 and *Hrr. chaoviator* BS19 were catalase- and oxidase-positive (Table 4.3). The isolates were able to utilise olive oil (1% w/vol) indicating the production of extracellular lipase, while starch (1% w/vol) was hydrolysed only by *Har.valismortis*BS3. The isolates could utilise D-glucose and D-maltose as the carbon source. Sucrose, D-Galactose and D-mannose was utilised by *Har. valismortis* BS3 and *Hrr. chaoviator* BS19 as carbon sources, while D-mannitol and D-sorbitol was utilised only by *Hal. valismortis*BS3. None of the isolates metabolised organic acids. All tested isolates reduced nitrate to nitrite aerobically and anaerobically. Similarly, all the isolates could grow anaerobically in the presence of DMSO andL-arginine. Among the complex carbon substrates tested, the isolates could utilise tryptone and yeast extract. However, casamino acid was utilised by *Har. valismortis* BS3 and peptone was utilised by *Hrr.chaoviator* BS19.

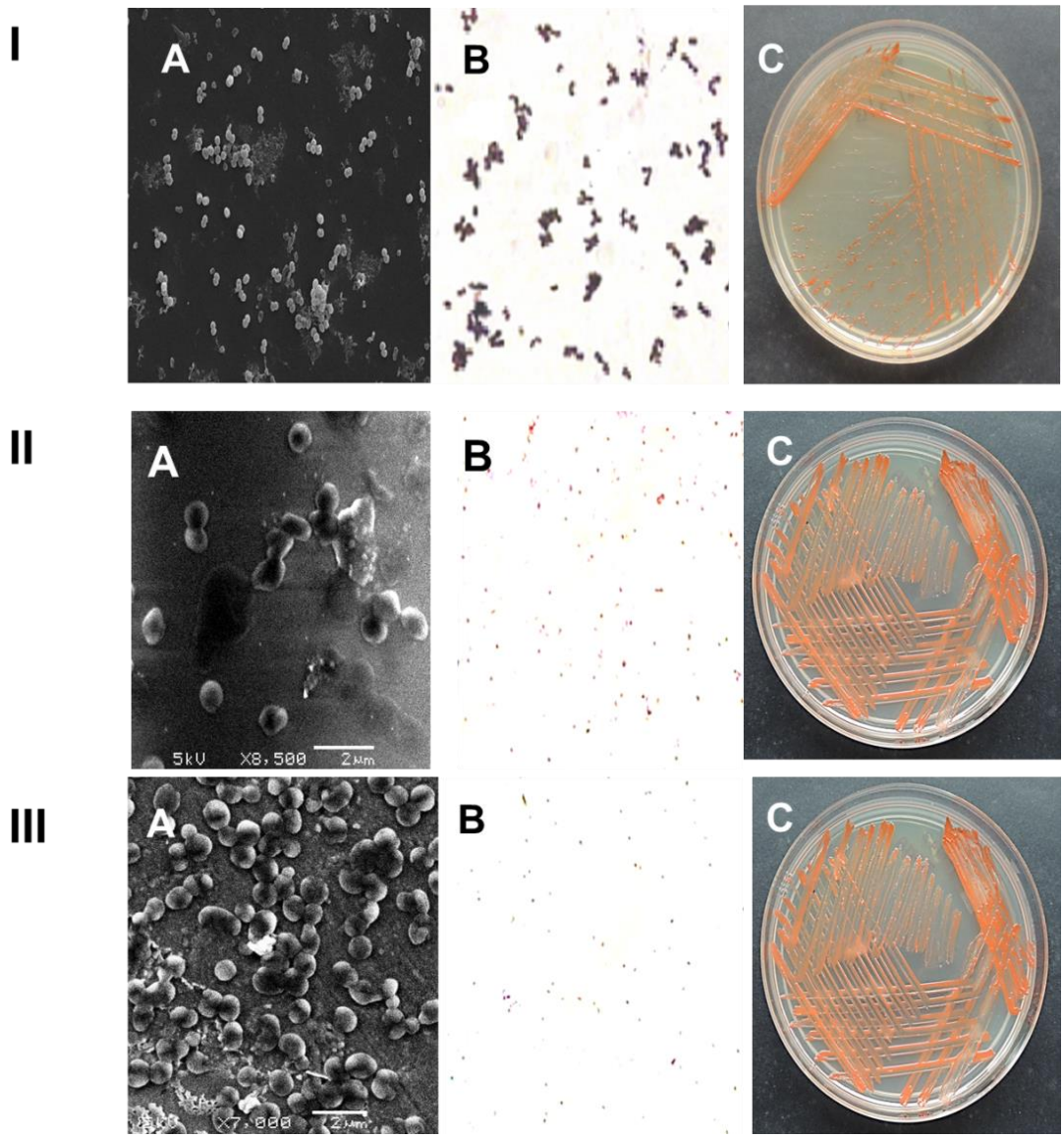


Fig. 4.1: I (A, B & C) - SEM photograph, Gram staining and the culture plate of *Har. valismortis*BS3; II (A, B & C) - SEM photograph, Gram staining and the culture plate of *Hrr. chaoviator*BS17; II (A, B & C) - SEM photograph, Gram staining and the culture plate of *Hrr. chaoviator*BS19

The 3 isolates were sensitive to novobiocin and rifampicin while isolate *Har. valismortis* BS3 alone was sensitive to bacitracin. The isolates did not accumulate intracellular PHA. Antiarchaeal substance was produced by the isolate *Halorubrum chaoviator* BS17.

Table 4.3: Phenotypic characteristics of the halophilic archaeal isolates

Characteristics	***Har. valismortis BS3***	***Hrr. chaoviator* BS17**	***Hrr. chaoviator* BS19**
Gram Stain	-	-	-
Motility	-	-	-
Cell lysis (in DW)	+	+	+
Catalase	+	+	+
Hydrolysis of:			
Starch (0.2%)	+	-	-
Tween 20 (0.1%)	-	-	-
Tween 80 (0.1%)	-	-	-
Skimmed milk (1.0%)	-	-	-
Olive oil (1.0%)	+	+	+
Urea (1.0%)	-	-	-
Acid production from: (0.5%)			
D-Glucose	+	+	+
Sucrose	+	-	+
D-Fructose	-	-	-
D-Mannitol	+	-	-
D-Maltose	+	+	+
D-Raffinose	-	-	-
L-Arabinose	-	-	-
D-Sorbitol	+	-	-
D-Lactose	-	-	-
D-Galactose	+	-	+
Glycerol	-	-	-
Ribose	-	-	-
D-Mannose	+	-	+
D-Xylose	-	+	+
Control (without sugar)	+	+	+
Organic acids (0.1%)			
Sodium acetate	-	-	-
Sodium pyruvate	-	-	-
Sodium Glutamate	-	-	-
DL-malic acid	-	-	-
di-ammonium oxalate	-	-	-
Lactic acid	-	-	-
Citric acid	-	-	-
Antibiotics (50µg/ml)			
Kanamycin	R	R	R
Rifampicin	S	S	S

Bacitracin	S	R	R
Streptomycin	R	R	R
Ampicillin	R	R	R
Erythromycin	R	R	R
Tetracyclin	R	R	R
Novobiocin	S	S	S
PHA Production from:			
Glucose (1% and 2%)	-	-	-
Starch (1% and 2%)	-	-	-
Antiarchaeal activity	-	+	-
Growth Factors	+	+	+

4.3.4 Growth kinetics of halophilic archaea

*Hrr.chaoviator*BS17 and *Hrr.chaoviator* BS19 grew slower as compared to *Har. valismortis* BS3. Growth curves of the isolates grown in NT medium at 37°C exhibited lag phase except *Har. valismortis* BS3. *Hrr. chaoviator* BS17 had a lag of 2.5 days while *Hrr.chaoviator* BS19 had a lag of 0.5 days in growth. *Har. valismortis* BS3 grew steadily in the NT medium and reached maximum OD_{600} of 2.25 on 5^{th} day. The maximum growth rate of the isolate was 0.451 and generation time was 1.53 $days^{-1}$. *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 reached maximum OD_{600} of 2.1 and 2 on 12^{th} and 11^{th} day of their growth respectively. *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 showed a growth rate of 0.245 and 0.289 and generation time of 2.82 $days^{-1}$ and 2.44 $days^{-1}$(Fig. 4.2).

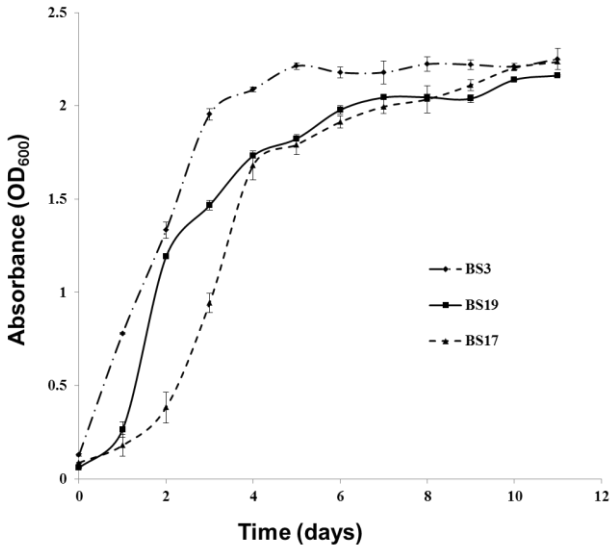


Fig 4.2: Growth profile of *Har. valismortis* BS3, *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 grown in NT medium

4.3.5 Polar lipid analysis of halophilic archaea

The major polar lipids of the three isolates were identified as phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (Me-PGP) and phosphatidylglycerol sulfate (PGS) (Fig. 4.3).The major glycolipids of *Har. valismortis* BS3 were non-sulphated triglycosyl glycolipid (TGD-2) and diglycosyldiether (DGD-2). The major glycolipid of the isolates *Hrr. chaoviator*BS17 and *Hrr. chaoviator* BS19 was sulfatedmannosylglucosyldiether (S-DGD-3). The polar lipid profiles of*Har. valismortis*BS3, *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 were chromatographically identical to the type strains, *Har. hispanica* JCM8911 and *Hrr. saccharovorum* JCM8865

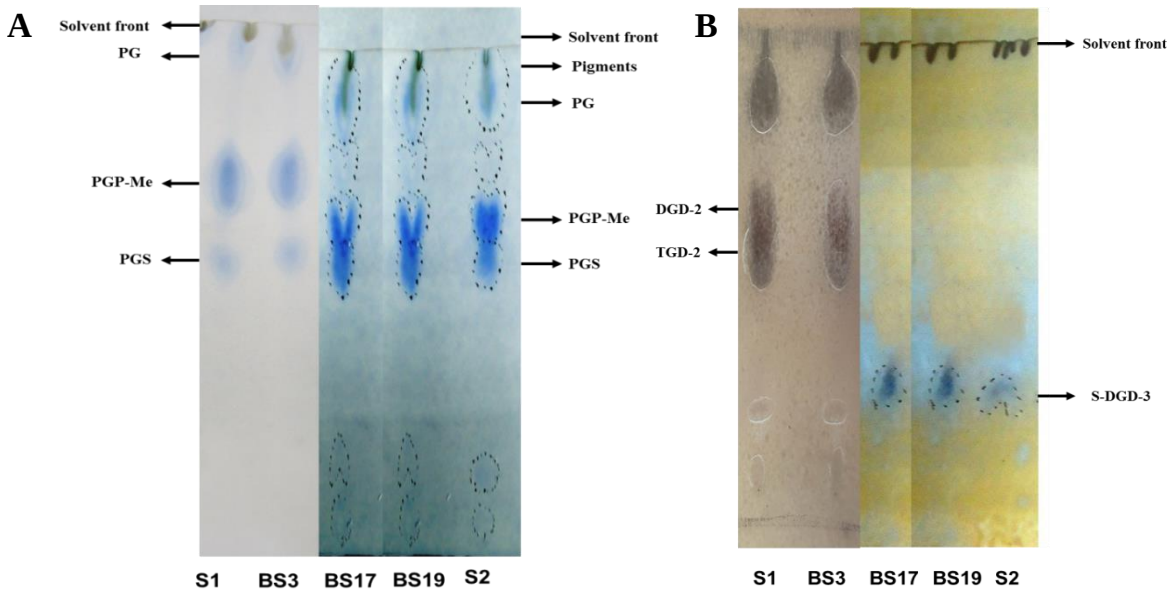


Figure 4.3: Polar lipid composition of the haloarchaeal cultures using thin layer chromatography. (A) Phospholipids and (B) Glycolipids. Lanes: S1 - *Har. hispanica* JCM8911^T; BS3 - *Har. valismortis* BS3; BS17 – *Hrr. chaoviator* BS17; BS19 – *Hrr. chaoviator* BS19; S2 - *Hrr. saccharovorum* JCM8865^T.PG - phosphatidylglycerol; PGP-Me - phosphatidylglycerol phosphate methyl ester; PGS - phosphatidylglycerol sulfate; S-DGD-3 - sulfatedmannosylglucosyldiether; DGD-2 - diglycosyldiether; TGD-2 - non-sulphated triglycosyl glycolipid.

4.3.6 Draft genomes of *Har. valismortis* BS3 and *Hrr. chaoviator* BS19

4.3.6.1 General features of the genomes

The genomic DNA libraries of *Har. valismortis* BS3 and *Hrr. chaoviator* BS19, prepared using TruSeq Nano DNA HT Library Sample Preparation Kit had a mean sizes of 618bp and 615bp, respectively. The libraries were sequenced on NextSeq 500 using 2

x150bp generating 4700027 reads from *Har. valismortis* BS3 and 7592570 reads from *Hrr.chaoviator* BS19. The reads obtained were filtered using Trimmomatic v0.30 with the following parameters, trimming of the adapters, sliding window trimming of 20 bp with a threshold value of 20, trimming of bases at the start and end of the reads with a threshold value of 20 and dropping the read length if the read is less than 40 bp. Assembled genome of *Har.valismortis* BS3 was 3.8 Mb size (Fig. 4.3) with a N50 value of 24946 bp while the assembled genome of *Hrr.chaoviator*BS19, the assembled genome was 3.2 Mb size (Fig. 4.4) with a N50 value 27667 bp. Both the organisms had their genome organised as a single chromosome (Table 4.4).

The G+C content of *Har.valismortis*BS3 was 67.61% and *Hrr.chaoviator*BS19 was 64.54%. The scaffolds were subjected to BlastN analysis against nt database of NCBI for detecting the presence of plasmids. The results indicated that *Hrr. chaoviator*BS19 harboured 5 plasmids and *Har. valismortis* BS3 harboured 8 plasmids. The presence of rRNAs was detected with RNAmmer software. This software is based on the Hidden Markov Model (HMM) and it predicts the rRNA sequences with high accuracy. It also predicted the presence of 4 rRNAs in *Hrr.chaoviator* BS19 and 1 rRNA in *Har.valismortis* BS3. The detection of tRNAs was carried out by tRNAscan-SE which revealed the presence of 42 tRNAs corresponding to *Hrr.chaoviator* BS19 and 50 tRNAs corresponding to *Har.valismortis* BS3 respectively.

Table 4.4: Genome attributes of *Hrr. chaoviator* BS19and *Har. valismortis*BS3

Attributes	BS19	BS3
Number of scaffolds	217	706
Number of contigs	345	1259
Genome size (bp)	3234604	3819804
Mean scaffold size	14906	3033
Scaffold N_{50}	27667	24946
Largest scaffold size	158262	112221
Smallest scaffold size	546	474
G + C content (%)	64.54	67.61
Protein-coding genes	3215	3974
tRNA	42	50
rRNA	4	1
Plasmids	5	8

Fig. 4.4: Circos plot explaining the genes in the reference genome (*Har.hispanica*ATCC33960^T) and *Har. valismortis*BS3 scaffolds. Outer circle with pink colour (Chromosome 1) and orange colour (chromosome 2) represents the reference genome (*Har.hispanica*ATCC33960^T); green colour circle represents the *Har. valismortis*BS3 scaffolds and the inner most circle with orange colour represents GC %).

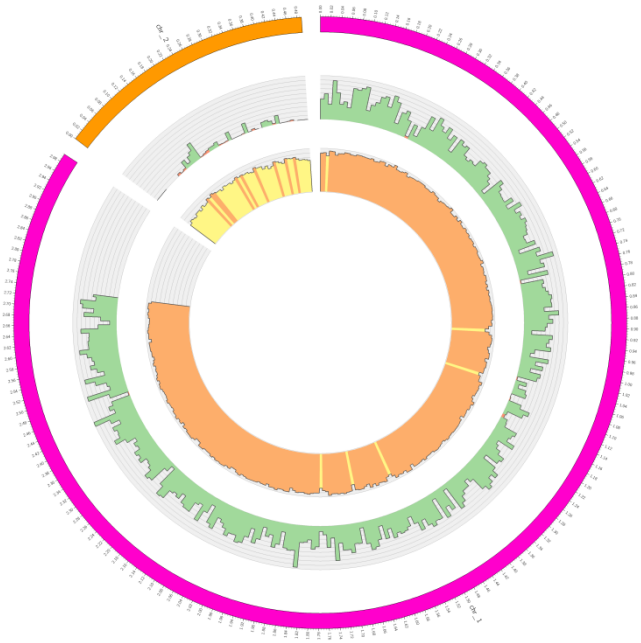


Fig. 4.5: Circos plot explaining the genes in the reference genome (*Hrr. lacusprofundi*ATCC43049^T)and *Hrr. chaoviator*BS19 scaffolds. Outer circle with pink colour(Chromosome 1) and orange colour (chromosome 2) represents the reference genome(*Hrr. lacusprofundi*ATCC43049^T) ; green colour circle represents the *Hrr. chaoviator* BS19scaffolds and the inner most circle with orange colour represents GC %).

4.3.6.2 Gene annotation and Gene Ontology (GO) analysis

The Coding Sequence (CDS) were predicted using Prodigal (PROkaryoticDYnamic programming Gene-finding Algorithm) v2.60. A total of 3974 CDS were identified from *Har.valismortis*BS3 and 3215 CDS were identified in*Hrr. chaoviator* BS19. CDS were then subjected to gene annotation studies by subjecting the CDS to BLASTX (Basic Local Alignment Search Tool) analysis against NR (Non Redundant) protein database. In *Hrr.chaoviator* BS19 genome, out of 3215 identified CDS, 3093 CDS were annotated for a known function and in *Har.valismortis* BS3, all 3974 CDS were annotated to have function.

The Gene Ontology mapping and annotation was carried out by Blast2GO program. After mapping, Blast2GO program determined the function of the annotated gene based on the GO ID and categorised in to three broad functional categories described above. The results indicated that in *Hrr. chaoviator* BS19, 3575, 834 and 2183 CDS were involved in biological processes, cellular component and molecular functions respectively. Among biological processes, maximum number of CDS were attributed to metabolic process (1286) followed by cellular process (921) and single-organism process (741) (Fig. 4.). Among the CDS assigned to cellular components, maximum number of CDS were assigned to cell (354) followed by membrane (286) (Fig 4.7). When the CDS assigned to molecular processes were analysed, highest number CDS were assigned to catalytic activity (1099) and binding (784) (Fig. 4.8)

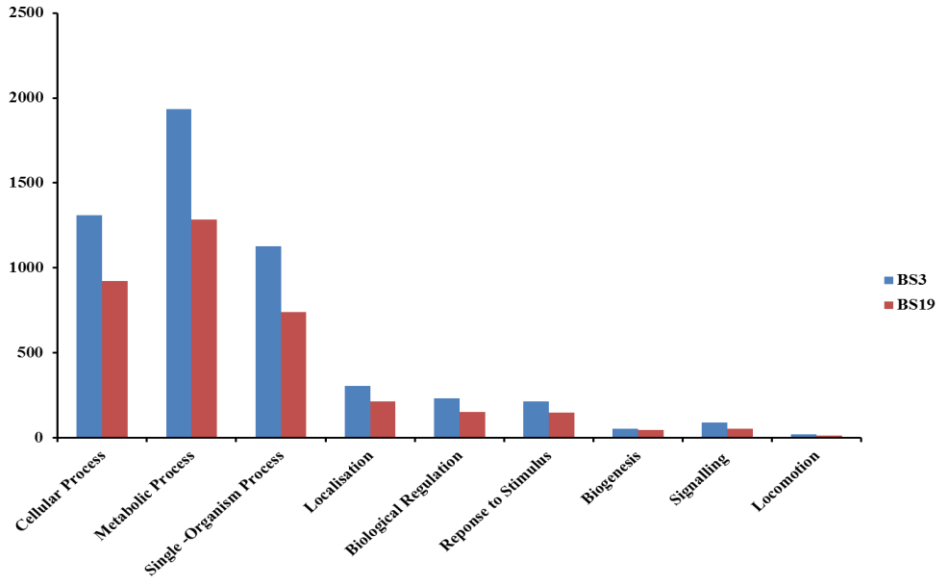


Fig. 4.6: CDS attributed to biological processes during GO analysis in the genomes of *Hrr. chaoviator* BS19 and *Har. valismortis* BS3

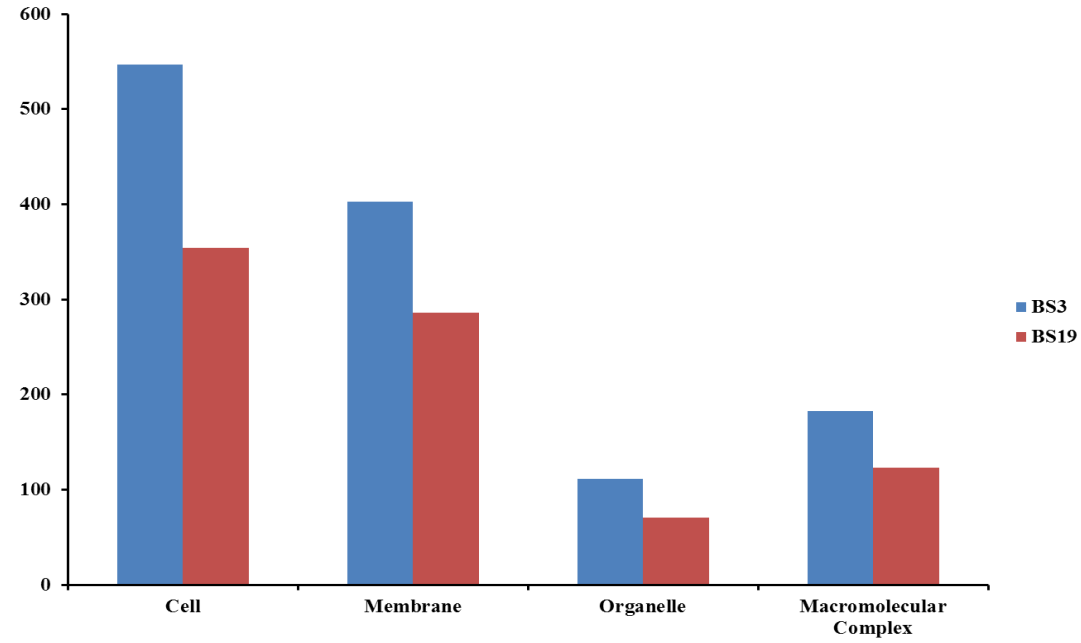


Fig. 4.7:CDS attributed to cellular component during GO analysis in the genomes of *Hrr. chaoviator* BS19 and *Har. valismortis* BS3

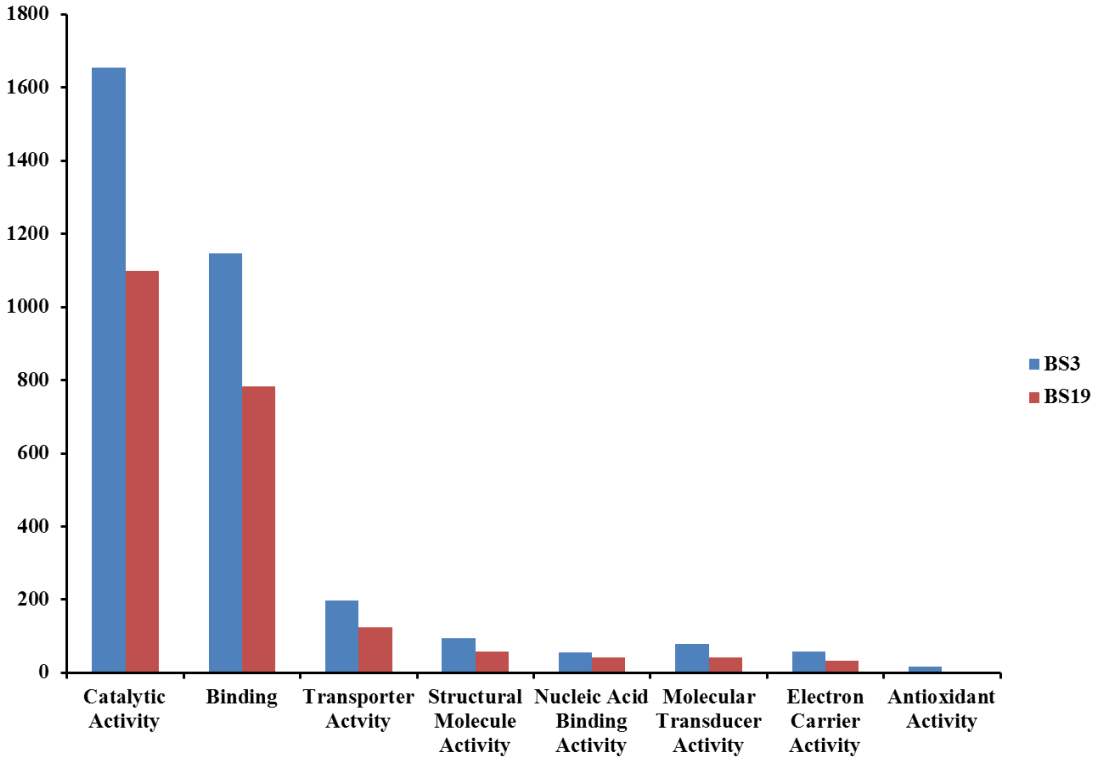


Fig. 4.8: CDS attributed to molecular function during GO analysis in the genomes of *Hrr. chaoviator* BS19 and *Har. valismortis* BS3

Similarly, in *Har. valismortis* BS3, 2234, 790 and 2183 CDS were involved in biological processes, cellular component and molecular functions respectively. Among biological processes, maximum number of CDS were attributed to metabolic process (1935) followed by cellular process (1311) and single-organism process (1126). Among the CDS assigned to cellular components, maximum number of CDS were assigned to cell (547) followed by membrane (403). When the CDS assigned to molecular processes were analysed, highest number CDS were assigned to catalytic activity (1655) and binding (1147).

4.3.6.3 Pathway analysis

The pathway analysis results can be classified into 4 categories. They are metabolic processes, genetic information processes, environmental information processes and cellular processes. A total of 1129 and 797 genes were assigned for metabolic processes in *Haloarcula sp.,* BS3 and *Halorubrum sp.,* BS19 genomes respectively. Similarly, 266, 138 and 23 genes were assigned under genetic information processes, environmental information processes and cellular processes in *Haloarcula sp.,* BS3 genome. In *Halorubrum sp.,* BS19 genome, 206 genes for genetic information processes, 95 genes for environmental information processes and 21 genes for cellular processes. Based on the annotated genes, metabolic pathways were predicted. The genetic makeover was similar in both organisms.

The *nuo*genes encoding for the subunits of NADH dehydrogenase was found in both the organisms. Other genes involved in oxidative phosphorylation like *sdh*, involved in succinate dehydrogenase were found to be present in a cluster and *cox*, involved in cytochrome c oxidase was also detected in *Har. vallismortis* BS3 and *Hrr. chaoviator* BS19. This study revealed the ability of *Har. vallismortis* BS3 and *Hrr. chaoviator* BS17 BS19 to synthesis histidine, tryptophan, leucine, serine, glycine, threonine, glutamine, aspartate and arginine. The presence of few genes involved in the biosynthesis of cofactors like riboflavin, coenzyme A, pyridoxal 5-phophate and nictotinamide were detected. One of the interesting features in the genomes of *Har.vallismortis* BS3 and *Hrr. chaoviator* BS19 was the presence of multiple pathways for the degradation of hydrocarbons and related compounds. Several genes related to ABC transporter like iron transporter, zinc transporter, phosphate transporter were identified in *Har. vallismortis* BS3 and *Hrr. chaoviator* BS19 genomes, may indicate that these strains are able to resist metal stress and can survive in heavy metal environment.

4.4Discussion

Taxonomy of halophilic archaea is continuously expanding with numerous novel halophilic archaeal strains being described in the past decade (Oren, 2014). Culturable diversity studies of the Indian solar salterns resulted in the identification of potential 9 novel haloarchaeal isolates (sequence similarity values less than 99.0%). Three strains were selected and studied in detail. Isolate BS3 belonged to *Haloarcula* and isolates BS17 and 19 belonged to *Halorubrum.*On comparing the biochemical characteristics of *Har.vallismortis*BS3 with the type strain *Har. vallismortis* DSM375, the ability of isolate BS3 to metabolise simple sugar substrates like glucose, sucrose and galactose and the inability to use sorbitol indicated that the isolate BS3 is different from the type strain of the genus *Haloarcula*. The optimal NaCl concentration of the genera *Halorubrum* and *Haloarcula* is between 15 – 25%. Similarly, the optimum pH is around 7.0 and the optimum temperature is around 40°C (Trigui et al., 2011; Camacho et al., 2014). The isolates employed in the study matched with the growth characteristics of their respective genera

Biochemical and chemotaxonomic characteristics of *Hrr. chaoviator* BS17 and *Hrr. chaoviator*BS19 were distinct among themselves indicating the two stains under consideration are different. Comparison with the type strain *Hrr. saccharovorum* JCM8865, the ability of the isolates to reduce nitrate indicated that the isolates are different from the type strain. When the growth kinetics of the isolates were compared to the type strains, the maximum growth rate of *Har. vallismortis*BS3 (0.451) was less than *Har.vallismortis*DSM375 (0.995). Similarly the growth rates of *Hrr. chaoviator*BS17 and BS19 (0.245 and 0.289) was also less compared to*Hrr.saccharavorum*JCM8865(0.984). This may be due to the different media employed in the growth of type strains and the physiological conditions of the environment from which the type strains were isolated (Robinson et al., 2005)

One of the characteristic features of the members of the family *Halobacteriaceae,*are the unique lipids with ether bonds and isoprenoid hydrophobic side chains (Kamekura, 1993, Kates, 1978; Oren, 2012; Oren, 2006). These polar lipids are used to discriminate between different genera and are used as chemotaxonomic signatures for certain genera (Oren et al., 2009). TLC chromatograms of polar lipids obtained from the haloarchaeal isolates, indicated the presence of PG, Me-PGP and PGS, typical of halophilic archaea (Oren and Rodriguez-Valera, 2001). The glycolipids, TGD-2 and

DGD-2 obtained from the chromatogram of *Har. vallismortis* BS3 correlated with the genus *Haloarcula.* Similarly the glycolipid profiles of *Hrr. chaoviator* BS17 and BS19 contained a single spot corresponding to S-DGD-3, correlating with the genus *Halorubrum* (Birbir et al., 2007).

The genome attributes of *Hrr. chaoviator* BS19 was compared with a well characterised haloarchaeon *Hrr.tropicale*V5 CECT9000. It was observed that *Hrr. chaoviator* BS19 contained 338230 bp, 31 protein-coding sequences and 10 RNAs less than *Hrr. tropicale*V5 CECT9000 (Sánchez-Nieves et al., 2016). When the genome features of *Har. vallismortis* BS3 was compared against the genome features of *Har. vallismortis* ATCC29715^T, it was observed that the type strain ATCC29715^T contained 110251 bp higher than the BS3 strain (Baliga et al., 2004). Similarly, *Har.vallismortis* BS3 was found to contain 110 genes and 33 ribosomes less than the type strain *Har. vallismortis* ATCC29715^T. Among the whole genomes of 5 *Haloarcula* isolates sequenced till now, strain *Har. vallismortis* BS3 has the highest G+C content, while the G+C content of *Har. vallismortis* ATCC29715^{T}is 61.79% (Yun et al., 2015). The high GC content of haloarchaeal genomes can be attributed to the selection pressure inserted by AT preferred IS (Insertion Elements). IS elements are segments of DNA that can transpose and integrate into the genome. This can cause physical disruption of the gene functions and reorganise genome sequences. In order to avoid the IS elements, the haloarchaeal genomes have moved towards the high GC content genome organisation (Ng et al., 2000; Baliga et al., 2004). Similarly, the presence of single rRNA gene in the isolate BS3 is surprising because all other sequenced *Haloarcula* members are known to contain more than one copy of rRNA. Presence of multiple copies of rRNA is essential for halophilic archaea because of their ability to speed up the translational process of proteins during multiple stresses (Klappenbach et al., 2000; Acinas et al., 2004).

The gene ontology (GO) analysis indicated that *Har. vallismortis* BS3, is more versatile in adapting to diverse environmental conditions when compared with *Hrr. chaoviator* BS19. This is due to greater amount of CDS attributed to metabolic process, cellular process and catalytic process. When the metabolic pathways were examined, a classical electron transport chain, which can be seen in aerobic halophilic archaea, consisting of four membrane-bound complexes was found in both *Har. vallismortis* BS3 and *Hrr. chaoviator* BS19 genomes. The four complexes are: NADH dehydrogenase (I), succinate dehydrogenase (II), ubiquinonecytochrom c reductase (III) and the cytochrome c oxidase (IV). Halophilic archaea do not possess the usual glycolytic pathway harboured

by bacteria and eukaryotes and instead it harbours a modified Entner-Doudoroff (ED) pathway. In the modified ED pathway, phosphorylation is postponed and Glucose is oxidized to gluconate via NADP+ -dependent glucose dehydrogenase.This modified pathway, typical of halophilic archaea was detected in both organisms. Genes involved in citric acid cycle and pentose phosphate pathway, typical of halophilic archaea was also observed (Sato &Atomi, 2011). WGS indicated the absence of majority of genes involved in the metabolism of galactose, fructose and mannose. This demonstrates the inability of the organisms in the metabolism of these sugars for energy.

4.5 Conclusion

Isolates *Har. vallismortis*BS3, *Hrr. chaoviator*BS17 and BS19 grew with an optimal requirement of 20% (w/vol) NaCl and a minimum of 0.5% (w/vol) magnesium for growth. Three isolates could reduce nitrate and were catalase positive. The isolates were pigmented from light red to brick red. The three isolates could utilise glucose and maltose. Whole genome sequencing indicated the genome size of *Har. vallismortis* BS3 was 3.2 Mb and *Hrr. chaoviator* BS19 was 3.8 Mb.The G + C content of *Har. vallismortis* BS3 was 64.54 and *Hrr. chaoviator* BS19 was 67.61. Isolate *Har. vallismortis* BS3 contained 42 tRNAs and 4 rRNAs while the isolate *Hrr. chaoviator*BS19 contained 50 tRNAs and 1 rRNAs respectively. Therefore, morphological, physiological, phenotypical, chemotaxonomic and genomic characterisation indicates that the isolates under study could be potentially novel.

Chapter V

Non-Culturable Diversity of Halophilic Prokaryotes and Eukaryotes in Solar Salterns of India

5.1 Introduction

Large numbers of microorganisms present in the environment remain uncultured due to the lack of ambient culturable conditions. It is estimated that only 1% of all microbes can be cultivated under laboratory conditions (Oliver, 2005). Biodiversity studies employing only culture-dependent techniques provide a limited insight into the community composition of the ecosystems (Amman et al. 1995). In spite of employing various media, still large numbers of halophilic isolates remains to be cultured. For a comprehensive picture, different culture-independent techniques are required for estimating the biodiversity of any ecosystem (Burns et al., 2004).

Culture-independent techniques like terminal-restriction fragment length polymorphism (T-RFLP),denaturing gradient gel electrophoresis (DGGE), high-throughput sequencing (HTS) techniques like pyrosequencing, Illumina, Ion Torrent, Pacific Biosciences employing 16S rRNA fingerprinting have provided us much valuable and diverse information in the past two decades (Schuster, 2007; Muyzer, 1999). Among the HTS techniques, Illumina has gained an edge in recent times particularly in the field of microbial ecology, because of the higher reads, great coverage and low cost per base, a prerequisite for metabarcoding studies (Quail et al., 2012).

HTS has contributed leaps and bounds in understanding the composition of microbial communities in various natural systems like marine environments, thermal springs and intestinal gut (Shokralla et al., 2012). However, very few studies have employed HTS for characterising the microbial diversity of hypersaline environments (Abed et al., 2015; Gomariz et al., 2015; Ventosa et al., 2014). Culture-independent studies on the community composition of the solar salterns of India are scarce and no study has been conducted employing high throughput sequencing technique. This chapter is aimed at studying and metabarcoding the spatial and seasonal biodiversity of solar salterns DGGE and Illumina sequencing respectively. This study is further aimed at understanding the microbial community fluctuations in the salterns across a geographical scale.

5.2 Materials and methods

5.2.1 Sampling sites and samples

Brine and sedimentswere collected fromreservoir pan (RP), evaporator pan (EP) and crystalliser pan (CP)from Siridao (Goa), during initial salt harvesting (ISH) and peak

salt harvesting (PSH) phase and fromVedaranyam (Tamil Nadu), during peak salt harvesting phase alone. An abandoned solar saltern at Sinquetim (Goa)was also sampled.Salt crystals from the salterns of Ribandar (Goa), Marakkanam (Tamil Nadu), Vedaranyam (Tamil Nadu) and solar salt obtained from a local grocery store were also used in this study.

5.2.2 Environmental DNA (eDNA) extraction from brine

Two hundred fifty ml of brine sample was vacuum filtered through 0.22 µm pore size polycarbonate membrane (Millipore, India). The filter membrane was cut into small pieces and proceeded as follows (Fig. 5.1):

5.2.2.1 Method A (Mani et al., 2015)

The membrane was suspended in 1 ml of cell suspension buffer (0.15M NaCl, 0.1M EDTA, pH 8.0) followed by 1 ml of lysis buffer (0.1M NaCl, 0.01M EDTA, 1% SDS, pH 8.0). To this suspension, 0.5 ml of 10% SDS was added and incubated at 55°C for 30 minutes for the extraction of eDNA.

5.2.2.2 Method B (Burgmann et al., 2001)

The membrane was suspended in 2 ml of lysis buffer (0.5M NaCl, 0.1M EDTA, 5% SDS, 1 M DTT) and incubated at 60°C for 30 minutes for the extraction of eDNA.

5.2.2.3 Method C (Mutlu et al., 2008)

The membrane was suspended in 1 ml of lysis buffer (0.1M Tris-HCl (pH 8.0), 0.1M EDTA, 0.75M sucrose) along with lysozyme at 1mg/ml concentration. This mixture was incubated at Room Temperature (30°C) for 60 minutes. Following this, proteinase K was added at a concentration of 1mg/ml followed by 10% SDS and the mixture was incubated at 55°C for 60 minutes. After incubation, 5M NaCl containing 10% CTAB was added to the eDNA extract and incubated at 55°C for 30 minutes.

5.2.2.4 Method D (Yeates et al., 1998)

The membrane was suspended in 2 ml of extraction buffer (0.1M Tris-HCl, 0.1M EDTA, 1.5M NaCl). 20% SDS was added to this suspension and incubated at 65°C for 60minutes. After incubation, half-volume of 30% polyethylene glycol and 1.6M NaCl was added and further incubated at Room Temperature (30°C) for 2 hours. The mixture was centrifuged at 6000 rpm for 10 minutes and to the pellet obtained, 7.5M potassium acetate was added to a final concentration of 0.5 M. The mixture was incubated on ice for

10 minutes and centrifuged at 6000 rpm for 10 minutes. The supernatant containing the crude eDNAwas removed to a fresh tube.

5.2.2.5 Method E (Benlloch et al., 1995)

The membrane was treated with 2 ml of sterile distilled water and 20% SDS. This mixture was incubated at 60°C for 60 minutes. Following incubation, proteinase K was added at a concentration of 1mg/ml and incubated further at 55°C for 60 minutes to obtain the crude eDNAextract.

Equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1,vol/vol/vol) was added to the crude eDNA extract obtained and centrifuged at 12000 rpm for 10 minutes. Chloroform: Isoamyl alcohol (24:1,vol/vol) was added to the supernatant obtained and centrifuged at 12000 rpm for 10 minutes. DNA was precipitated from the supernatant by adding 2 volumes of 95% ethanol and incubating overnight at room temperature. DNA was pelleted obtained by centrifuging the tubes at 12000 rpm for 30 minutes. DNA pellet obtained was washed with 70% ethanol, suspended in 100 µl TE (10:0.1 mM) buffer and stored at -20° C for further use.

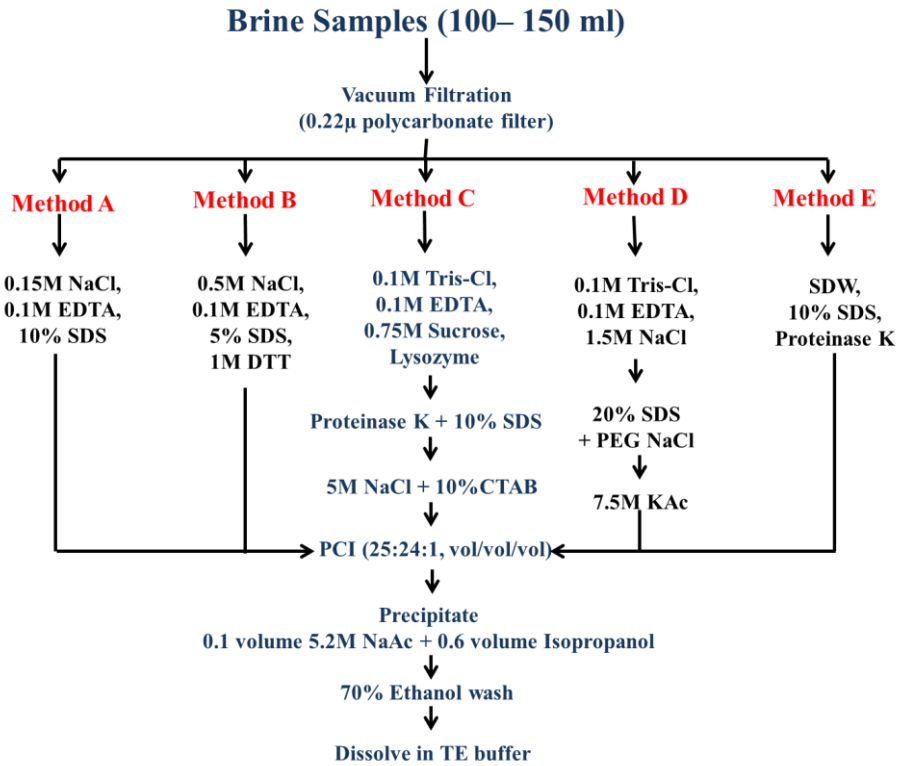


Fig. 5.1: DNA extraction methods employed for extracting eDNA from brine. Method C highlighted in blue resulted in successful extraction

5.2.3 eDNA Extraction fromsediment

5.2.3.1 Commercial DNA extraction kits

Macherey-Nagel Nucleospin DNA extraction kit, MoBio Power soil extraction kit and Qiagen faecal DNA extraction were used for extracting eDNA from saline sediments according to manufacturer's instructions.

5.2.3.2 Manual DNA extraction methods

Five grams of sediment was washed thrice with 100 mM sodium phosphate buffer (pH 8.0) and proceeded as follows (Fig. 5.2):

5.2.3.3 Method F (Bey et al., 2011)

The sediment sample was further washed four times with 1M NaCl. Following this step, 0.1M aluminium sulfate was added and vortexed for 10 minutes. The mixture was centrifuged at 6000 rpm for 10 minutes and the supernatant was decanted. Ten ml of extraction buffer(5M NaCl, 10% SDS, 0.1% β-mercaptoethanol) was addedto the sediment sampleand vortexed for 10 minutes and further incubated at 60°C for 60 minutes. The mixture was centrifuged at 6000 rpm for 10 minutes and the pellet obtained was treated with 7.5M potassium acetate to a final concentration of 0.5 M. The crude eDNA extract was incubated on ice for 10 minutes and centrifuged at 6000 rpm for 10 minutes.

5.2.3.4 Method G (Zhou et al., 1996)

The sediment sample was vortexed for 10 minutes with 10 ml of extraction buffer (100 mMTris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% CTAB). Proteinase K (1 mg/ml) was added and further incubated at 55°C for 60 minutes. Three ml of 10% SDS was added to the eDNA extract and incubated further for 60 minutes at 55°C with intermittent mixing.

5.2.3.5 Method H (modified from Zhou et al., 1996)

The sediment sample was vortexed for 10 minutes with 10 ml of extraction buffer (100 mMTris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 2.5% CTAB, 1% PVP) containing 0.1 and 0.5 mm glass beads. Lysozyme (1 mg/ml) and proteinase K (1 mg/ml) was added and incubated at Room Temperature (30°C) for 60 minutes. Three ml of 20% SDS was added to the crude eDNA extract and incubated at 55°C for 60 minutes.

To the crude eDNA extract obtained from the above methods, equal volume of Phenol: chloroform: Isoamyl alcohol (25:24:1,vol/vol/vol) was added and centrifuged at 12000 rpm for 10 minutes. The supernatant obtained was collected and an equal volume of chloroform: Isoamyl alcohol, (24:1, vol/vol) was added and centrifuged at 12000 rpm for 10 minutes. DNA was precipitated by adding precipitation reagent (0.1 volume of 3M sodium acetate (pH 5.2), 0.6 volume of isopropanol, 0.1% glycogen) and incubating the tubes overnight at Room Temperature (30°C). The DNA pellet was recovered by centrifuging the tubes at 12000rpm for 30 minutes. DNA pellet was washed with 70% ethanol and suspended in TE (10:0.1 mM) buffer and stored at -20°C for further use.

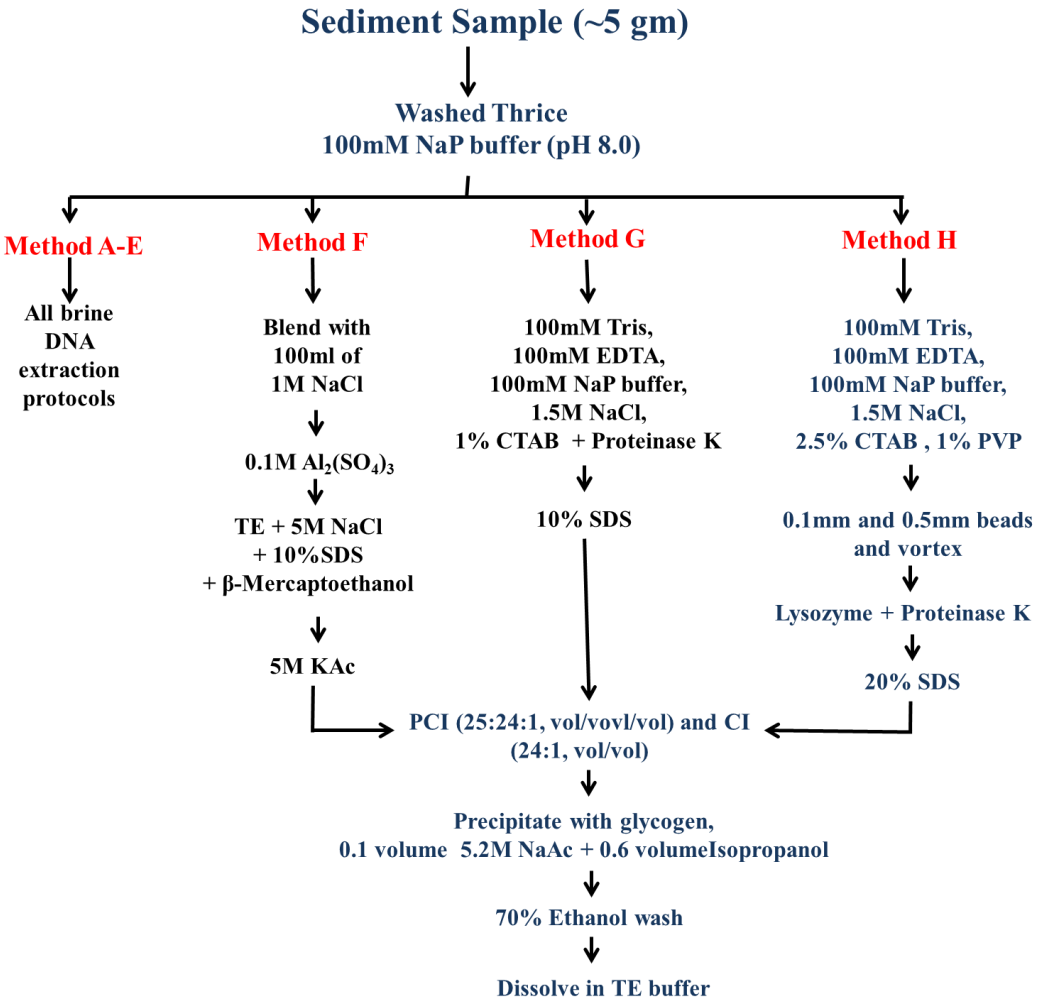


Fig. 5.2: DNA extraction methods employed for extracting eDNA from sediments. Method H highlighted in blue resulted in successful extraction

5.2.4 eDNA extraction from salt crystals

Ten grams of salt crystals were dissolved in 100 ml of sterile distilled water. The brine solution was then vacuum filtered through 0.22 µm pore size polycarbonate membrane (Millipore, India). DNA was extracted from the filter membrane using method H from the sediment eDNA extraction procedure.

5.2.5 Purification and quantification of DNA

The crude eDNAobtained from brine and sediment samples was subjected to 1% agarose gel electrophoresis in 1X TAE (Tris-acetate–EDTA) buffer. eDNAwaspurified using MoBioPowerClean DNA Clean-Up kit according to manufacturer's instructions. DNA was quantified and the purity was determined through A260/280 using a Nanodrop (Thermo Scientific, USA).

5.2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

5.2.6.1 Amplification of bacterial 16S rRNA genes

For amplifying the bacterial 16S rRNA gene fragment, three different approaches were utilised. First approach involved nested PCR with 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′ (Weisburg et al., 1991).PCR was performed in a thermal cycler (Applied Biosystems, USA) using following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min and 15 sec and final extension at 72°C for 10 min.The product obtained from the above PCR reaction was further amplified with theprimers 968F-GC 5'GCCCGCCGCGGCGGCGGGCGGGGGGGGGCACGGGGGGAACGCGAAGAACC TTAC -3' and 1401R 5'-CGGTGTGTACAAGACCC-3' (Watanabe et al., 2001)using the following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 1min and extension at 72°C for 1 min and 15 sec and final extension at 72°C for 10 min.

Second approach and third approach involved touchdown and direct PCR with the primers341FGC5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCCCT ACGGGAGGCAGCAG 3') (Muyzer et al., 1993) and 907R (5' CCGTCAATTCMTTTGAGTTT 3') (Haygood et al., 1992). The touch-down protocol was carried out with the following conditions,94°C for 5 min, 65°C for 1 min, 72°C for 3 min and 9 touchdown cycles of: 94°C for 1 min, 65°C (with a decreasing of 1°C in each cycle) for 1 min, 72°C for 3 min, followed by 20 cycles of: 94°C for 1 min, 55°C

for 1 min, 72°C for 3 min.The direct PCR program was out with the following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min and 15 sec and final extension at 72°C for 10 min.

Each PCR reaction mixture contained 10X *Taq* buffer,2 mM $MgCl_2$, 10 mM of dNTPs (Sigma, India), 10 µM of eachprimer (IDT technologies, Singapore), 2 U *Taq* Polymeraseand 10 ng template DNA. The PCR products were verified on a 1.5 % agarose gel through submerged DNA electrophoresis and was visualised over a UV illuminator (Bio-Rad, USA) after staining with ethidium bromide.

5.2.6.2 Amplification of archaeal 16S rRNA genes

Amplification of archaeal 16S rRNA was carried out with the nested pcrapproach using three different primer sets (Table 5.1).

Table 5.1: List of primers and annealing temperature employed for amplifying archaeal 16S rRNA gene.

Primer name	Sequence (5'-3')	Annealing Temperature	References
A109F	ACGGCTCAGTAACACGT		Großkopf et al., 1998
1510R	GGCTACCTTGTTACGA	53.5°C	Hiraishi, 1992
A109F	ACGGCTCAGTAACACGT		Großkopf et al., 1998
515R-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCGGCCAGCAGCCGCGGTAA	56°C	Reysenbach& Pace, 1995
21F	TCCGGTTGATCCYGCCGG		Massana et al., 1997
915R	GTGCTCCCCCGCCAATTCCT	58°C	Hoaki et al., 1994
344FGC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCGACGGGGTGCAGCAGGCGCG	60°C	Vetriani et al., 1999
915R	AGTGCTCCCCCGCCAATTCCT		Hoaki et al., 1994
340F	CCCTAYGGGGYGCASCAG		Gantner et al., 2011
1000R	GGCCATGCACYWCYTCTC	57°C	
340FGC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCCTACGGGG(C/T)GCA(G/C)CAG	57°C	Gantner et al., 2011
1000R	GGCCATGCACYWCYTCTC		

PCR was carried out with following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at (Table 5.1) for 1 min and extension at 72°C for 1 min and 15 sec and final extension at 72°C for 10 min.

Each PCR reaction mixture contained 10 X PCR buffer, 10 mM of each dNTPs, 2.5 mM $MgCl_2$, 2U of *Taq* Polymerase (Sigma), 10 mM primers (IDT Technologies) and 20 -50 ng of template DNA. The PCR products were checked by performing 1.5% agarose gel electrophoresis in 1X TAE buffer.

5.2.6.3 Denaturing gradient gel electrophoresis

Since archaeal 16S rRNA genes were not amplified by the primer sets, optimisation of PCR and DGGE (CBS Scientific Company, USA) (Appendix III) electrophoretic conditions for bacterial 16S rRNA gene amplicons were carried out. After optimisation, the non-culturable bacterial composition of brine, sediment and salt crystals was studied by loading 400 ng/µl of amplified bacterial 16S rRNA gene fragments onto 7.5% (w/vol) polyacrylamide gel (acrylamide:bis-acrylamide gel stock solution 37.5:1)with gradient of 40-75% (where 100% of denaturant consists of 7 M urea and 40% formamide). Electrophoresis was performed in 1X TAE buffer at 60°C with a constant voltage of 100 V for 10 h. DNA bands in the DGGE gels were visualised by silver staining (Appendix IV).

5.2.6.4 Phylogenetic analysis

Bands of interest were eluted and suspended in 100 µL distilled water at 4°C for overnight. Five µL of the eluent was used as template in the PCR reaction with same primers (without the GC clamp)and conditions as described above. PCR products were electrophoresed and eluted on a 2.0% agarose gel. The eluted PCR amplicons were purified and sequenced by Scigenom Labs (India) using an automated DNA sequencer (Applied Biosystems, India). Sequences were reviewed, corrected and assembled using Molecular Evolutionary Genetic Analysis (MEGA) software v5.0 (Tamura et al., 2011). Identification of phylogenetic neighbours and calculation of pair-wise 16S rRNA gene sequence similarity against the type strains with validly published names was achieved using EzTaxon-e BLAST analysis (Kim et al., 2012). The sequences of identified phylogenetic neighbours were retrieved from Ribosomal Database Project (RDP) release 11 and aligned with the sequences of the isolates using MUltiple Sequence Comparison by Log- Expectation (MUSCLE) (Edgar, 2004; Cole et al., 2009). Distances were calculated using the Kimura two-parameter (Kimura, 1980) correction in a pair-wise deletion procedure. The phylogenetic tree was reconstructed with Neighbour-Joining (NJ) algorithm using MEGA v5.0. Percentage support values were obtained using a bootstrap procedure with 1000 resampling.

5.2.6.4 Statistical analysis

The gel was scanned using TotalLab Quant software. Bands were identified automatically by the software, subtracting the background fluorescence and normalising the gel lanes. Each band was assumed to be an individual operational taxonomic unit(OTU) (Reche et al., 2005). Bands presence-absence and intensity data was further used for the statistical analysis. The similarity between the sampling sites was analysed using cluster analysis. A Dendrogram was constructed based on Bray-Curtis similarity distance measures with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) (Bray & Curtis, 1957; Michener &Sokal, 1957). Diversity indexes describing species richness and species evenness were calculated using the bacterial bands present in the DGGE gel. The Shannon–Weaver index of diversity (*H*) (Shannon and Weaver, 1963), Simpson index of diversity (*D*) (Simpson, 1949) and the equitability index (*E)* (Pielou, 1975) were calculated using the following formula,

$$H = -\Sigma (n_i/N) \log(n_i/N),$$

$$D = 1-\Sigma (n_i/N)^2$$

$$E = H/\log S$$

where 'n_i' is the relative surface intensity of each DGGE band, '*S'* is the number of DGGE bands (used to indicate the number of species) and '*N'* is the sum of all the surfaces for all bands in a given sample (used as estimates of species abundance). Sampling effort was validated by constructing rarefaction curves. Analytic Rarefaction 1.3 was used for calculating rarefaction curve by considering the number of OTUs observed against the number of sequences (Holland, 2003). To determine the habitability of the ecosystem, range-weighted richness (Rr) index was determined for each sample using the following equation,

$$Rr = (N^2 \times D_g)$$

where N represents the total number of bands in the gel, and D_g the denaturing gradient between the first and the last band of the gel (Marzorati et al., 2008). For determining the effect of salinity on the variation in bacterial diversity detrended correspondence analysis (DCA) was carried out using Paleontological statistics software (PAST) (http://folk.uio.no/ohammer/past/) (Hammer et al., 2001).

5.2.7 Illumina sequencing

The purified DNA obtained from the brine, sediment and salt crystals was subjected to Illumina sequencing. Amplification of the V3-V5 region of the 16S rRNA genes was performed using universal prokaryotic primers 515F 5'-GTGYCAGCMGCCGCGGTA-3'and 909R 5'-CCCCGYCAATTCMTTTRAGT-3' (Wang & Qian, 2009). Forward primer contained 8 base tag comprising of short nucleotide sequences linked with the primers for differentiating the sampling sites during bioinformatic analysis. PCR reactions were performed in duplicates with the following conditions:Initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min.

Amplification of the V3-V5 region of the 18S rRNA genes was performed using universal eukaryotic primers 515F (5'-GTGYCAGCMGCCGCGGTA-3') and 915R (5'-GTGCTCCCCCGCCAATTCCT-3'). Forward primer contained 8 base tag similar to prokaryotic primers. PCR reactions were performed in duplicates with conditions as following conditions: Initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min.

Each PCR reaction contained 10 X PCR buffer, 10 mM of each dNTPs, 2.5 mM $MgCl_2$, 2U of Taq Polymerase (Sigma), 10 mM primers (IDT Technologies) and 20 -50 ng of template DNA. The PCR products were verified on a 1.5 % agarose gel through submerged DNA electrophoresis and was visualised over a UV illuminator (Bio-Rad, USA) after staining with ethidium bromide.The PCR products were purified (QiagenMinElute kit) and quantified using Qant-iTPicogreends DNA Assay Kit before libraries preparation and sequencing on Illumina Miseq paired-end (2x300bp) technology (Genoscreen, France). The above processes were carried out according to manufacturer's instructions.

5.2.7.1 Bioinformatic analysis and statistics

The sequences were processed using PANAM (http://code.google.com/p/panam-phylogeneticannotation/downloads/list) (Taib et al., 2013; Hugoni et al., 2013) sequentially in the following manner: (i) Primary processing step involved stitching the paired-end reads using PANDAseq (Masella et al., 2012) with threshold value at 95%. Following this, the merged reads were checked for forward and reverse primer, barcodes

sequences and discarding the sequences with at most one mismatch. Finally, all the sequences with less than 200 bp were removed from the dataset. All these stringent quality filtering steps had eliminated about 15% of the total raw reads. (ii) Secondary processing step involved clustering of the quality checked sequences using USEARCH with a threshold value of 97%. (iii) Final step involved taxonomic assignment by the phylogenetic affiliation procedure of PANAM. Further, singletons were removed and samples were randomly normalised. Rarefaction curves, alpha diversity measures (Chao1, ACE and Shannon Indices) were calculated using R package (Oksanen, 2009).

5.3 Results

5.3.1 DNA extraction

Among the five methods employed for the extraction of DNA from brine only method C resulted in extraction of eDNA. Among the methods employed for saline sediments, method H resulted in the extraction of high molecular weight eDNA. All other commercial DNA extraction kits and manual DNA extraction methods failed to yield DNA from environmental samples. eDNA from the salt crystals was also extracted using method H (Fig. 5.3).

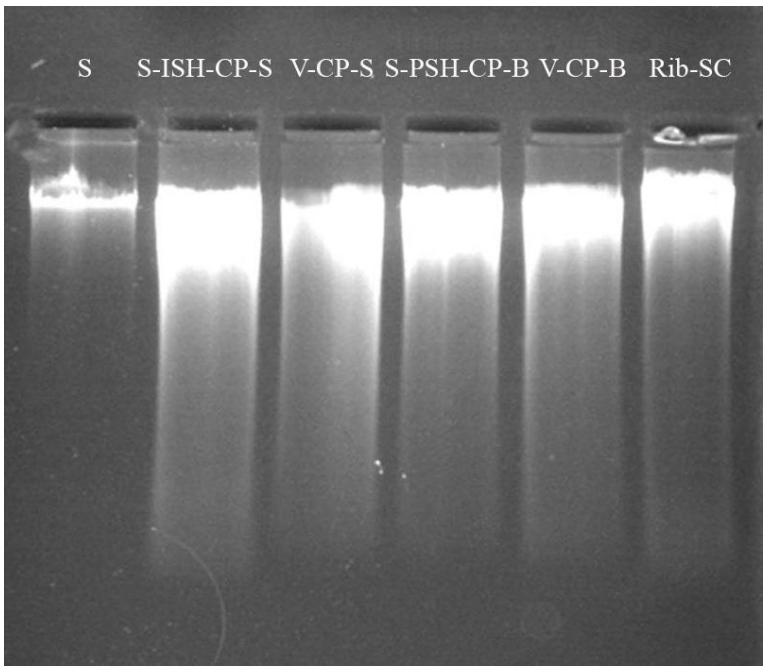


Fig. 5.3: eDNAobtained frombrine, sediments and salt crystals using the optimized protocols.Lambda DNA was used as the standard. S - Siridao; ISH – Initial salt harvesting; CP – Crystalliser pan; S – Sediment; B – Brine; V – Vedaranyam; PSH – Peak salt harvesting; Rib – Ribandar; SC – Salt Crystal

The preparatory phase brine obtained from Siridao salterncontained aneDNA concentration of 93.7 ng/µl and the eDNA concentration of sediment was 184.1 ng/µl. The eDNA concentration of initial salt harvesting (ISH) phase brines ranged from 141.3 to 89.1ng/µl and sediments ranged from 194.9 to 293.8 ng/µl. During the peak salt harvesting (PSH) phase, the concentration of eDNA in brine ranged from 76.2 to 124.1ng/µl and the concentration of eDNA in sedimentsranged from249.5 to 311 ng/µl. Brine obtained from Vedaranyam salternscontained eDNA in the range of91.4 to 128.1ng/µl and in the sediments eDNA concentrations ranged from 186.3 to 274.8 ng/µl. Salt crystals obtained from various salterns contained eDNA at an average concentration of 274.36 ng/µl. The purity ratio (A260/A280) of the eDNA varied from 1.77 to 1.84 (Table 5.2).

Table 5.2: Concentration and purity of eDNAobtained from various brine sediment and salt crystal samples.

Sample	DNA concentration in ng/µl		A260/280	
	Brine	**Sediment**	**Brine**	**Sediment**
Siridao				
Initialsalt harvesting (ISH) phase				
RP	94.6	284.5	1.81	1.83
EP	141.3	293.8	1.83	1.82
CP	89.1	194.9	1.79	1.80
Peak salt harvesting (PSH) phase				
RP	76.2	311	1.83	1.80
EP	124.1	284.6	1.78	1.82
CP	108.1	249.5	1.77	1.83
Preparatory Phase				
	93.7	184.1	1.82	1.81
Vedaranyam				
RP	128.1	259.1	1.83	1.84
EP	100.2	274.8	1.81	1.79
CP	91.4	186.3	1.79	1.78
Sinquetim				
RP	84.8	147	1.81	1.80
Soil	-	95.5	-	1.78
Salt Crystals				
Ribandar	240.3		1.81	
TN-M	271.2		1.80	
TN-V-BW	283.7		1.82	
TN-V-AW	314.8		1.80	
Comm. Salt	261.9		1.81	

S – Siridao; Prep – Preparatory phase; PSH – Pre-salt harvesting phase; SH – Salt harvesting phase; RP –Reservoir Pan; SO – Soil sample; V – Vedaranyam; Rib – Ribandar; TN –M - Tamil Nadu Marakkanam; TN – V - Tamil Nadu Vedaranyam; AW – After Wash; BW –Before Wash; Com. Salt – Commercial Salt,

5.3.2 DGGE profiling of bacterial 16S rRNA genes

DGGE was performed to study the difference in banding pattern between the three PCR approaches (Fig. 5.4). On visually inspecting the gel, it was observed that nested PCR approach resulted in optimal resolution, while in direct and touchdown PCR, fewer bands were obtained. Therefore, nested PCR approach employing primers 27F& 1401R followed by 968FGC & 1401R was used in amplifying the bacterial 16S rRNA genes. The concentration of PCR products loaded onto DGGE gel was optimised by loading 200 ng/µl, 400 ng/µl and 600 ng/µl of PCR product. On analysing the silver stained gel, it was observed that 400 ng/µl concentrations resulted in optimal resolution. Lanes with 200 ng/µl resulted in faint bands and 600 ng/µl resulted in band crowding (Fig. 5.4).Optimisation ofelectrophoretic conditions was followed by the bacterial diversity analysis of 16S rRNA amplicons obtained from brine and sediment eDNA (Figure 5.5&5.6).

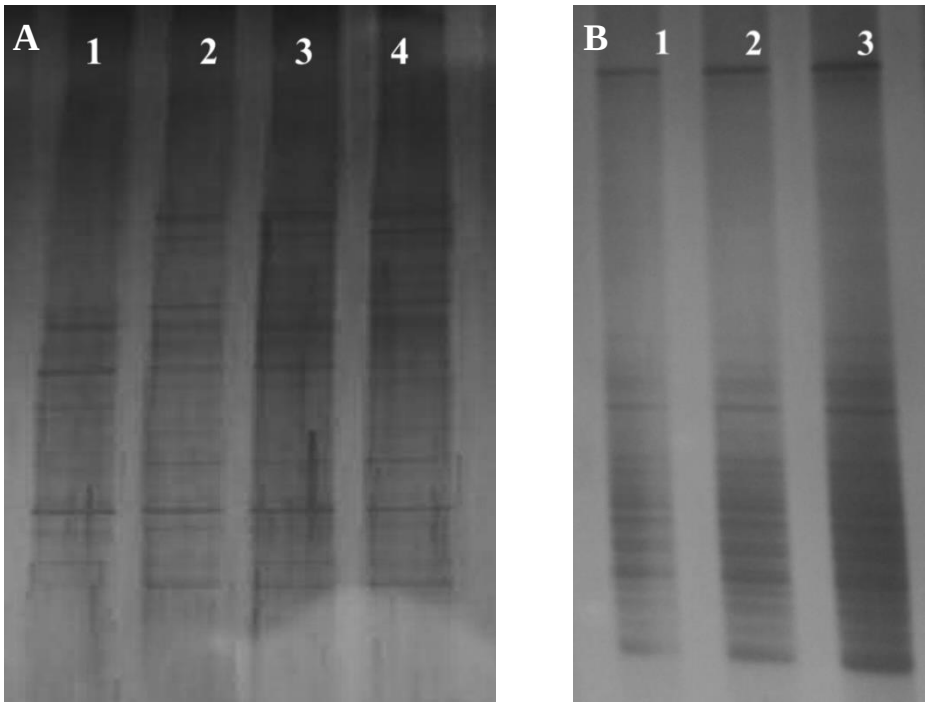


Fig. 5.4: A) DGGE profile of bacterial 16S rRNA sequences from Siridao ISH CP brine. 1 – Direct PCR (968FGC& 1401R), 2- Touch Down PCR (968FGC & 1401R), 3.Nested PCR (27F & 1401R and 968FGC & 1401R), 4. Nested and touch down PCR (27F & 1401R and 968FGC & 1401R). B) DGGE pattern of Vedaranyam CP sediment. 1- 200 ng per µl, 2- 400 ng per µl, 3- 600 ng per µl.

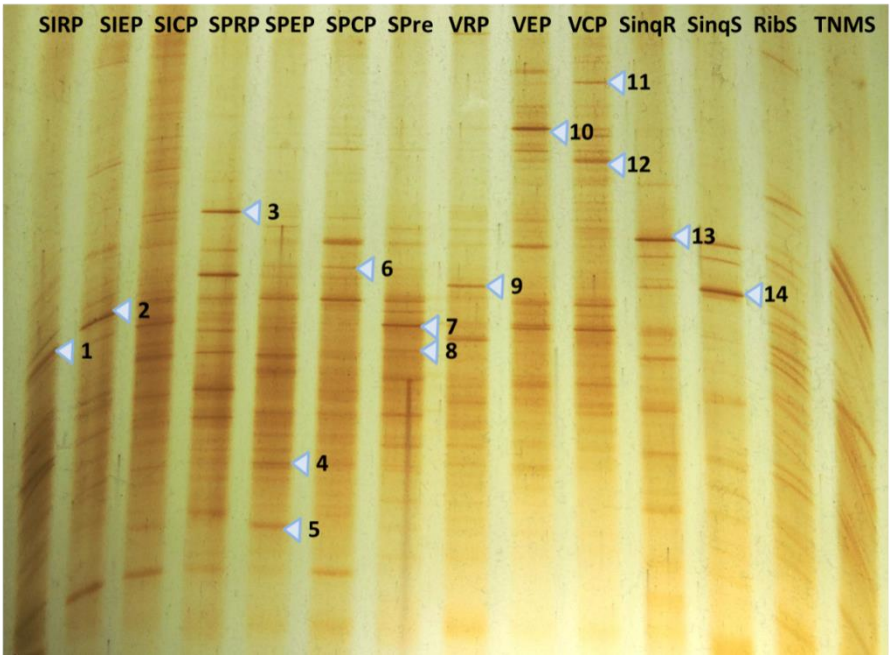


Fig. 5.5: DGGE profile of bacterial 16S rRNA obtained from sediments of solar salterns. S- Siridao; V – Vedaranyam; I – Initial salt harvesting phase; P - Peak salt harvesting phase; RP – Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan; Pre- Preparatory phase; Rib –Ribandar; TNM- Tamil Nadu Marakkanam; S – Salt crystals

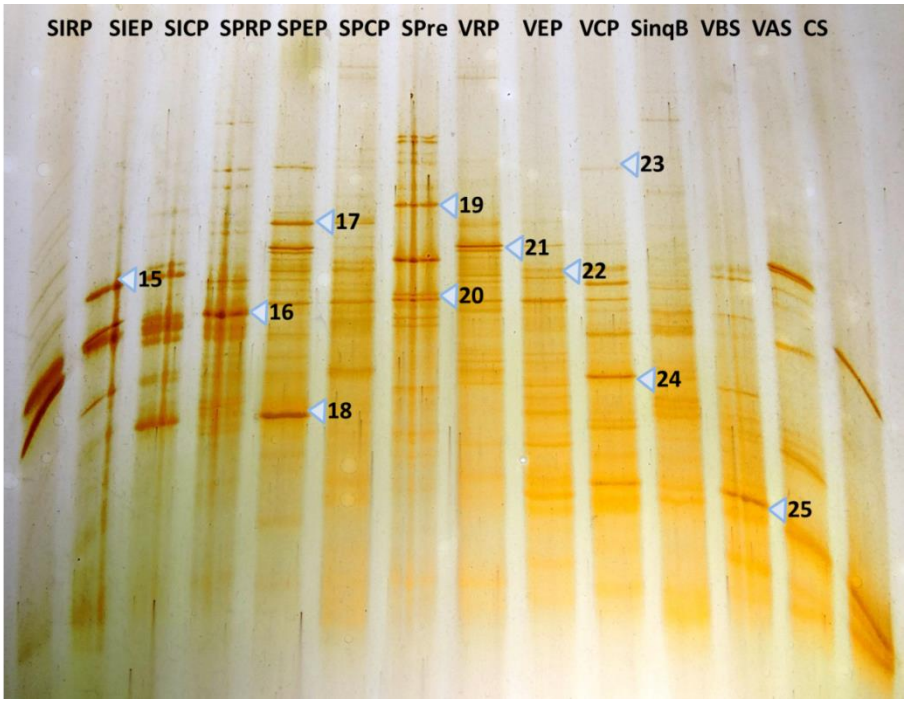


Fig. 5.6: DGGE profile of bacterial 16S rRNA genes obtained from brine samplesof solar salterns. S- Siridao; V – Vedaranyam; I – Initial salt harvesting phase; P - Peak salt harvesting phase; RP – Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan; Pre- Preparatory phase; V – Vedaranyam; B – Before wash; A – After wash; S – Salt crystals; CS – Commercial Salt

5.3.2.1 Phylogenetic Analysis

A total of 25 bands were eluted, purified and sequenced (Table 5.3). Culturable taxonomical identity of the sequences was established through EZ-taxon database and non-culturable taxonomical identity was established through pair-wise alignment of sequences using BLAST programme.

5.3.2.1.1 Closest culturable match (CCM) of sediment

As seen in Fig. 5.5, bands 1 and 2 corresponding to *Rhodococcus* with a similarity of 68% and 78% respectively were observed in sediment samples of both Siridao and Vedaranyam saltern at all salinities. Band 3 corresponding to *Anoxybacillus* with 49% similarity, was observed only in the reservoir pan (RP) sediment of Siridao saltern during peak salt harvesting (PSH) phase. Band 4 and 5 corresponded to *Exiguobacterium* and *Tepidibacillus* with a similarity of 65% and 51% respectively. Band 4 was observed in all salterns while band 5 was found only in evaporator pan (EP) sediment of Siridao saltern during PSH phase. Band 6 was observed only in the evaporator pan(EP) and crystalliser pan(CP) sediment of Siridao peak salt harvesting (PSH) phase and it corresponded to *Actinospica* with 70% similarity. Band 7 corresponded to *Dietzia* with 70% similarity and was observed at high intensity in the preparatory phase sediment of Siridao salterns though it was found during salt producing phases. Band 8 was observed only inthe preparatory phase sediment of Siridao saltern and corresponds to *Ornithinibacillus* with 51% similarity. Band 13 eluted from reservoir pan (RP) sediment of Sinquetim salterns corresponded to *Saccharopolyspora,* a soil bacterium with 84% similarity, while band 14 eluted from Sinquetim soil sample corresponded to *Nocardia* with 84% similarity (Figure 5.6 & Table 5.3). Band 9 corresponded to *Rhodococcus* with 77% similarity while band 10 corresponded to *Nocardia* with 74% similarity. Band 11 corresponded to *Oceanobacillus* with a similarity value of 51%. Band 12 corresponded to *Ornithinibacter* with 70% similarity, a marine bacterium. Bands 10, 11 and 12 were found only in the evaporator pan (EP) and crystalliser pan (CP) sediment of Vedaranyam salterns (Fig. 5.7 & Table 5.3).

5.3.2.1.2 Closest culturable match (CCM) of brine

Band 15 from evaporator pan (EP)brine from Siridao initial salt harvesting (ISH) phase corresponded to *Halomonas* with 73% similarity, a typical bacterium isolated from solar salterns. Similarly, Band 16 was observed in all the brine samples of Siridao saltern

and it corresponded to *Marinobacter* with 95% similarity. Band 17 obtained from Siridao peak salt harvesting (PSH) phase evaporator pan (EP) brine corresponded to *Rhodococcus* with 79% similarity and band 18 corresponded to *Pseudomonas* with 98% similarity. Both bands were eluted from evaporator pan(EP)brine belonging to peak salt harvesting (PSH) phase in Siridao salterns. Band 19 corresponded to *Marinobacter* with 81% similarity and band 20 corresponded to *Pseudomonas* 68% similarity. Band 19 appeared only in the reservoir pan (RP)brine of Siridao preparatory phase while band 20 was present in all brine samples of Siridao salterns (Fig. 5.7 & Table 5.3).

Band 21 obtained from reservoir pan(RP)brine from Vedaranyam saltern corresponded to *Rhodococcus* 76% similarity and band 22 corresponded to *Cobetia* with 62% similarity, a marine bacteria. Band 23 obtained from crystalliser pan (CP)brine from Vedaranyam saltern, corresponded to *Halomonas* with 50% similarity. Band 24 obtained from crystalliser pan (CP)brine of Vedaranyam saltern corresponded to *Nocardia* with 75% similarity. Band 25 from Vedaranyam saltern salt crystal sample corresponded to *Rhodococcus* with 78% similarity (Fig. 5.7 & Table 5.3).

5.3.2.1.3 Closest Environmental Match (CEM) of sediments

The BLAST analysis against non-culturable organisms indicated that the sequences displayed higher similarity against environmental sequences than cultured type strains. Bands 1, 2, 21 and 25 were related to uncultured *Rhodococcus* sp., with a similarity value of 82%, 89%, 89% and 92% while band 4 had a similarity value of 86% to uncultured bacterium isolate respectively. Band 3 had a similarity value of 75% to uncultured bacterium clone CUL2-s5. Band 5 corresponded to uncultured bacterium clone B12_1035 with a similarity value of 92%. Bands 6, 7, 10 and 24 corresponded to uncultured bacterium clone SH09-3-5 with similarity values of 79%, 70%, 81% and 82%. Band 8 corresponded to uncultured bacterium OM8 with a similarity value of 73%. Band 9 corresponded to uncultured bacterium clone SH09-3-8 with a similarity value of 79%. Band 12 corresponded to uncultured bacterium clone SH09-3-4 with a similarity value of 73%. Band 13 and 14 corresponded to uncultured bacterium clone 2_K08 and uncultured bacterium clone N5b with a similarity value of 89% and 86% respectively (Table 5.3 & Fig. 5.7).

Table 5.3: Closest culturable match (CCM) and closest environmental match (CEM) of the bacterial 16S rRNA sequences compared against EzTaon-e

Band Name	Sampling Site	Closest culturable match	% Similarity	Closest non-culturable match	% Similarity
1	Siri-PSH-RP-Sediment	*Rhodococcus soli*	68	Uncultured *Rhodococcus*sp.	82
2	Siri-PSH-EP-Sediment	*Rhodococcusfascians*	78	Uncultured *Rhodococcus*sp.	89
3	Siri-SH-RP-Sediment	*Anoxybacillus flavithermus*	49	Uncultured bacterium clone CUL2-s5	75
4	Siri-SH-EP-Sediment	*Exiguobacteriumencl ense*	65	Uncultured bacterium isolate	86
5	Siri-SH-EP-Sediment	*Tepidibacillusfermant ans*	51	Uncultured bacterium clone B12_1035	92
6	Siri- SH-CP-Sediment	*Actinospicadurhamen sis*	70	Uncultured bacterium clone SH09-3-5	79
7	Siri-Prep-Sediment	*Dietzia kunjamensis*	70	Uncultured bacterium clone SH09-3-5	70
8	Siri-Prep-Sediment	*Ornithinibacillushalo tolerans*	51	Uncultured bacterium OM8	73
9	Veda- RP - sediment	*Rhodococcus soli*	77	Uncultured bacterium clone SH09-3-8	79
10	Veda- EP - sediment	*Nocardia ninae*	74	Uncultured bacterium clone SH09-3-5	81
11	Veda- CP – sediment	*Oceanobacillus chungangensis*	51	Uncultured bacterium clone N5b	78
12	Veda- CP – sediment	*Ornithinibacter aureus*	70	Uncultured bacterium clone SH09-3-4	73
13	Sinq-RP-Sediment	*Saccharopolyspora spinosporotrichia*	84	Uncultured bacterium clone 2_K08	89
14	Sinq-Soil	*Nocardia acidotolerans*	84	Uncultured bacterium clone N5b	86
15	Siri-PSH-EP-Brine	*Halomonas pacifica*	73	Uncultured *Halomonas* sp.	78
16	Siri-SH-RP-Brine	*Marinobacter lipolyticus*	95	Uncultured *Marinobacter*sp.	97
17	Siri-SH-EP-Brine	*Rhodococcusyunnane nsis*	79	Uncultured *Rhodococcus*sp.	84
18	Siri-SH-EP-Brine	*Pseudomonas halophila*	98	*Pseudomonas* sp.BAB-4241	97
19	Siri-Prep-Brine	*Marinobacter nitratrireducens*	81	*Marinobacter*sp. AK64	83
20	Siri-Prep-Brine	*Pseudomonas halophila*	68	Uncultured bacterium clone ERB55	84
21	Veda- RP-	*Rhodococcusyunnane*	76	Uncultured	89

	brine	*nsis*		*Rhodococcus*sp.	
22	Veda- EP-brine	*Cobetia marina*	62	Uncultured bacterium KR	88
23	Veda- CP-brine	*Halomonas elongata*	50	Uncultured bacterium clone H77S1_82b10	75
24	Veda- CP-brine	*Nocardia ninae*	75	Uncultured bacterium clone SH09-3-5	82
25	Veda-SC-BW	*Rhodococcusyunnanensis*	78	Uncultured *Rhodococcus*sp.	92

5.3.2.1.4 Closest Environmental Match (CEM) of brines

Band 15 corresponded to uncultured *Halomonas* sp. with a similarity value of 78%. Bands 16 and 19 corresponded to uncultured *Marinobacter* sp with a similarity value of 97% and 83% respectively. Bands 17, 21 and 25 had similarity values of 84%, 89% and 92% to uncultured *Rhodococcus* sp. Band 18 was similar to *Pseudomonas* sp. BAB-4241 with a value of 97%. Band 20 had a similarity value of 84% to uncultured bacterium clone ERB55. Bands 22 and 23 had a similarity value of 88% and 75% to uncultured bacterium KR and uncultured bacterium clone H77S1_82b10 respectively (Table 5.3 & Fig. 5.7).

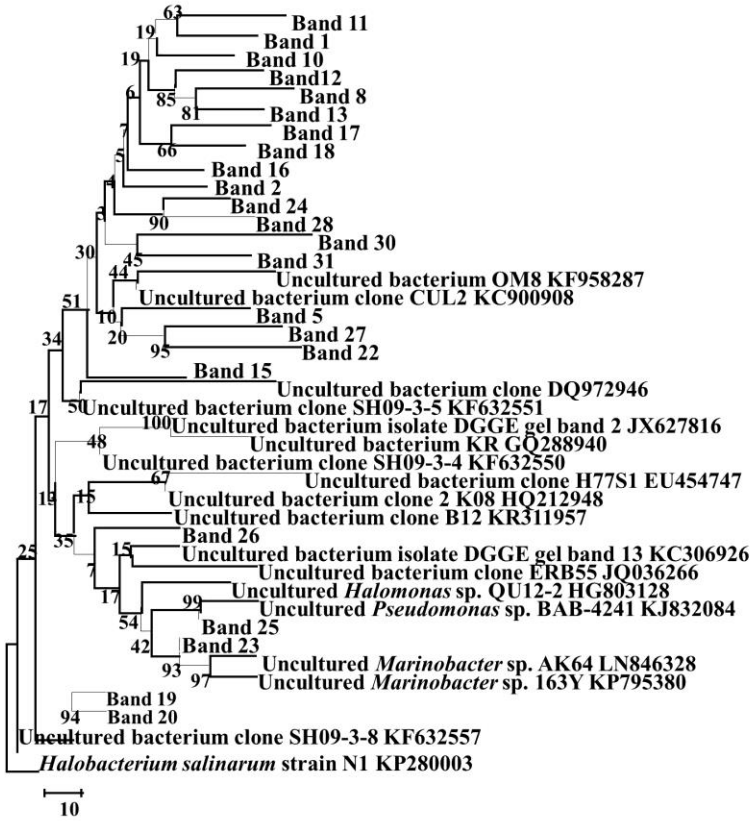


Fig. 5.7: Phylogenetic tree indicating the position of thesequence obtained from bands. Tree was constructed using Neighbour-Joining (NJ) method with MEGA 5.0.

5.3.2.2 Statistical analysis

Shannon index of that Siridao saltern preparatory phase brine was 2.552 and sediment was 3.111. Shannon indices of initial salt harvesting (ISH) phase brines ranged from 1.8 to 2.468 and the sediments ranged from 2.181 to 2.978. Peak salt harvesting (PSH) phase brines displayed Shannon indices ranged from 2.27 to 3.098 and Shannon indices of sediments was 3 on average. Brine and soil from Sinquetim saltern had a Shannon index of 2 on average while the sediment reservoir pan (RP) had a Shannon index of 3. Shannon indices ofbrines obtained from Vedaranyam saltern ranged from 2.611 to 3.118 and the sediments ranged from 2.773 to 3.481. Shannon index of salt crystals was 2 on average (Table 5.4).

Simpson index of Siridao saltern preparatory phase sediment was 0.9 and brinewas 0.8. Simpson index of initial salt harvesting (ISH) phase ranged from 0.8 to 0.9 and peak salt harvesting (PSH) phase ranged from 0.7 to 0.9. Sinquetim saltern contained Simpson index of 0.8 on average. Simpson index of Vedaranyam saltern brine was 0.8 and sediment was 0.9. Simpson index of the salt crystal was 0.8. Equitability index of Siridao saltern preparatory phase brine was 0.7 and sediment was 0.9.Equitability index of initial salt harvesting (ISH) and peak salt harvesting (PSH) phase ranged from 0.7 to 0.9. Sinquetim saltern displayed an equitability index of 0.8. Equitability index of Vedaranyam salterns ranged from 0.8 to 0.9. Salt crystal samples obtained from Vedaranyam saltern and commercial salt crystals had equitability index of 0.8 while the equitability index of Ribandar and Marakkanam salt crystal samples was 0.7 (Table 5.4).

The range-weighted richness of Siridao preparatory phase brine was 33 and sediment 49. Range-weighted richness of initial salt harvesting (ISH) ranged from 35 to 54 and peak salt harvesting (PSH) ranged from 38 to 59. Vedaranyam saltern brines had an average range-weighted richness of 43 and sediments had an average value of 53 (Table 5.5).The average range-weighted richness of salt crystals was 40. Rarefaction curves validated the sampling effort since the curves tended to plateau (Fig. 5.8). Detrended correspondence analysis (DCA) indicated that salt crystals had a similar biodiversity composition. Similarly, sediment and brine grouped together indicating no significant difference in the bacterial diversity composition. However, reservoir pan (RP) brine and sediment obtained from Siridao saltern had distinct composition which was evident by their distance from other samples (Fig. 5.9).

Table 5.4: Diversity indices [Shannon index (H), Simpson index (D) and equitability index (E)] followed by range-weighted richness (Rr)] of various sampling sites based on bands obtained in the DGGE gel.

Sample			Shannon index *H*	Simpson index *D*	Equitability index *E*	Range-weighted richness *Rr*
Siridao						
Initial salt harvesting (ISH) phase						
Brine						
RP			2.468	0.8791	0.7984	38.2
EP			1.8	0.766	0.7508	35.9
CP			2.212	0.7842	0.7385	37.1
Sediment						
RP			2.472	0.8671	0.8085	49.6
EP			2.181	0.8512	0.7699	46.3
CP			2.978	0.9437	0.9633	54.2
Peak salt harvesting (PSH) phase						
Brine						
RP			2.27	0.7571	0.6812	39.4
EP			3.098	0.942	0.92	40
CP			2.454	0.7957	0.7289	38.5
Sediment						
RP			3.187	0.9025	0.8577	51
EP			3.144	0.9487	0.937	59.7
CP			3.213	0.9332	0.9156	53.4
Preparatory Phase						
Brine			2.552	0.8142	0.7504	33.8
Sediment			3.111	0.947	0.9271	49.2
Vedaranyam						
Brine						
RP			3.118	0.9345	0.8843	45.8
EP			2.751	0.8979	0.8657	43.1
CP			2.611	0.8877	0.8714	41
Sediment						
RP			3.376	0.9466	0.9438	52.9
EP			3.481	0.9574	0.942	54.1
CP			2.773	0.9518	0.9314	55.6
Sinquetim						
Brine						
RP			2.621	0.8822	0.8479	47.3
Sediment						
RP			1.955	0.924	0.9141	53.5
Soil	2.147	0.815	0.8154		42	
Salt Crystals						
Rib	1.794	0.7133	0.6797		29.9	
TN-M	2.192	0.8433	0.7737		34.1	
TN-V-BW	2.357	0.8759	0.8931		51	
TN-V-AW	2.1	0.823	0.8186		46.4	
Comm Salt	2.224	0.8743	0.895		49.4	

S – Siridao; Prep – Preparatory phase; ISH – Initial salt harvesting phase; PSH – Peak salt harvesting phase; RP –Reservoir Pan; SO – Soil sample; V – Vedaranyam; Rib – Ribandar; TN –M - Tamil Nadu Marakkanam; TN – V - Tamil Nadu Vedaranyam; AW – After Wash; BW –Before Wash; Com. Salt – Commercial Salt; EP – Evaporator Pan; CP – Crystalliser Pan

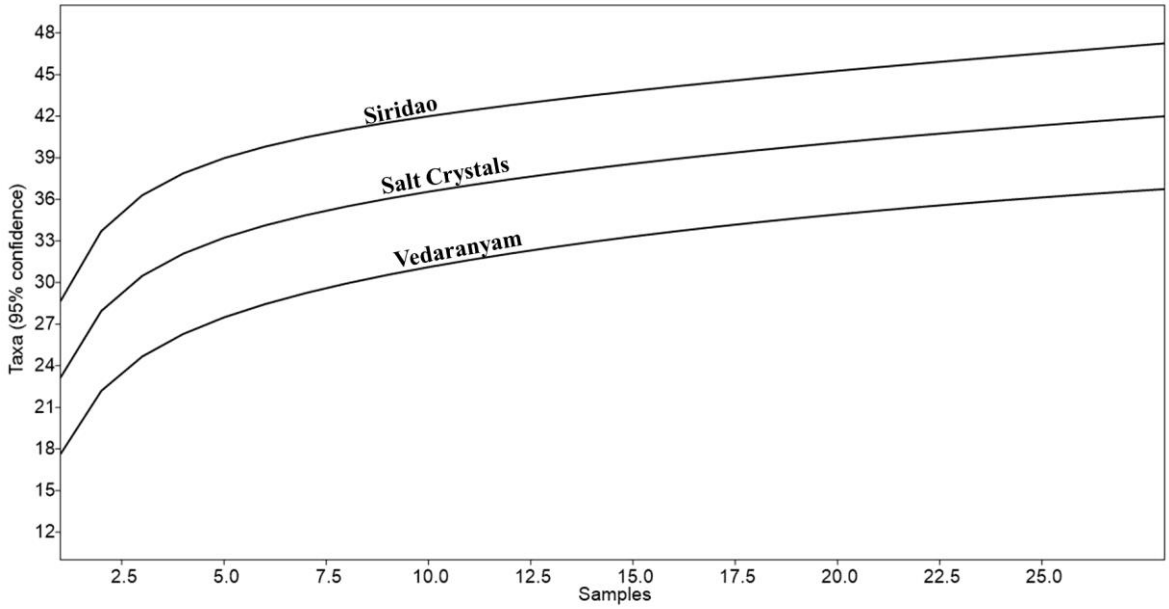


Fig. 5.8: Rarefaction curve of the samples validating the sampling effort, based on the DGGE profiles.

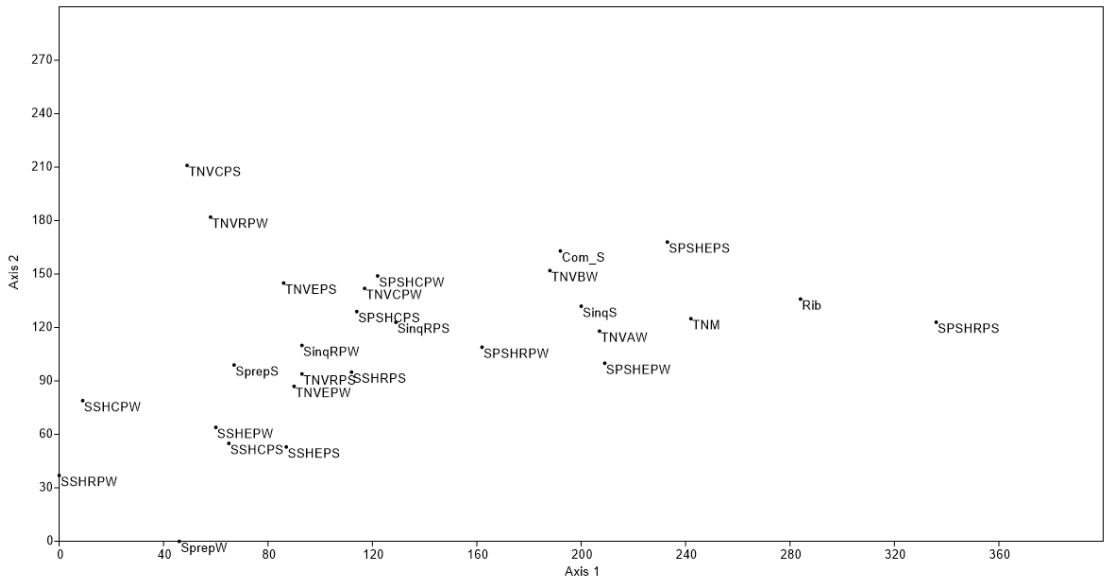


Fig. 5.9: Detrended correspondence analysis (DCA) of all the sampling sites based on the bacterial 16S rRNA genes

5.3.3 Illumina Sequencing

A total of 22 brine and sediment samples were studied for their prokaryotic community composition and 8 brine and sediment samples for eukaryotic community composition. Siridao brines obtained during initial salt harvesting (ISH) phase contained an average of 586023 prokaryotic reads and sediments contained an average of 6382 prokaryotic reads.Brines obtained during peak salt harvesting (PSH) phase showed an average prokaryotic reads of 789353 and the sediment sample showed an average prokaryotic reads of 10489. Brine sample obtained from Vedaranyam sample contained an average prokaryotic reads of 117460 and the sediments showed an average of 606535 reads. Sinquetim saltern contained an average of 71759 prokaryotic reads. Salt crystals showed an average of 40459 prokaryotic reads.

Siridao brines contained an average of 36283 eukaryotic reads and sediments contained an average of 1405 eukaryotic reads. Brines from Vedaranyam saltern showed an average of 12537 eukaryotic reads and sediments contained an average of 3182 reads. These reads were quality filtered were denoted as sequences. The sequences were assigned a taxonomic identity and normalised for further comparative and statistical analysis (Table 5.5).

Table 5.5: Sampling sites and the reads obtained by employing prokaryotic and eukaryotic primers

Sampling sites	Reads		
	Prokaryotes		Eukaryotes
Siridao	**ISH**	**PSH**	
	Brine		
RP	402348	589275	1639
EP	853366	916698	55680
CP	502356	862086	51530
	Sediment		
RP	5967	14691	1487
EP	8897	10320	1956
CP	4282	6458	773
Vedaranyam			
	Brine		
RP	167155		35336
EP	66134		507
CP	119093		1770
	Sediment		
RP	45887		5116
EP	51139		ND
CP	1722580		1249
Sinquetim			

RP sediment	9553	ND
Soil	81214	ND
Salt crystals		
Ribandar	41296	ND
Marakkanam	31269	ND
Vedaranyam-BW	47249	ND
Vedaranyam-AW	51598	ND
Commercial salt	30883	ND

ISH – Initial salt harvesting phase; PSH – Peak salt harvesting phase; RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.1 Prokaryotic metabarcoding of Siridao

Sequences obtained from Siridao salterns were normalised in PANAM at 4100 for each sampling site. A total of 5276 Operational Taxonomic Unit (OTUs) were obtained with a total read of 49200. The relative abundance of both Archaea and Bacteria across a salinity gradient through the relative abundance of 16S rRNA gene was investigated (Fig. 5.10). Sediments displayed an increase in the bacterial sequences and a decrease in archaeal sequences. However, an opposite trend was observed in brines. During the initial salt harvesting (ISH) phase, bacterial sequencesin sediments increased from 17% to 40% and from 21% to 51% during peak salt harvesting (PSH) phase. Similarly, archaeal sequences in sediments decreased from 83% to 60% (initial salt harvesting (ISH) phase) and 74% to 49% (peak salt harvesting (PSH) phase). However,during the initial salt harvesting (ISH) phase brine, there was an increase in the archaeal sequence from 34% to 91% while the bacterial sequences decreased from 66% to 9%. During the peak salt harvesting (PSH) phase, though the pattern was similar to initial salt harvesting (ISH) phase, there was an increase in bacterial sequences in reservoir pan (RP) and crystalliser pan (CP), when compared (Fig. 5.10).

5.3.3.1.1 Archaeal community composition

In sediments, sequences belonging to three phyla namely *Crenarchaeota*, *Euryarchaeota* and *Thaumarcheota* were detected, with *Euryarchaeota* being dominant, containing 99.5% sequences. Majority of the crenarchaeal and thaumarchaeal sequences were associated with the reservoir pan (RP) and evaporator pan (EP) samples. *Halobacteria* was the dominant class of *Euryarchaeaota* in sediments of Siridao salterns, contributing 99.5% sequences. The sequences could be assigned to 20 cultivable genera and 6 environmental groups (Fig. 5.11a).

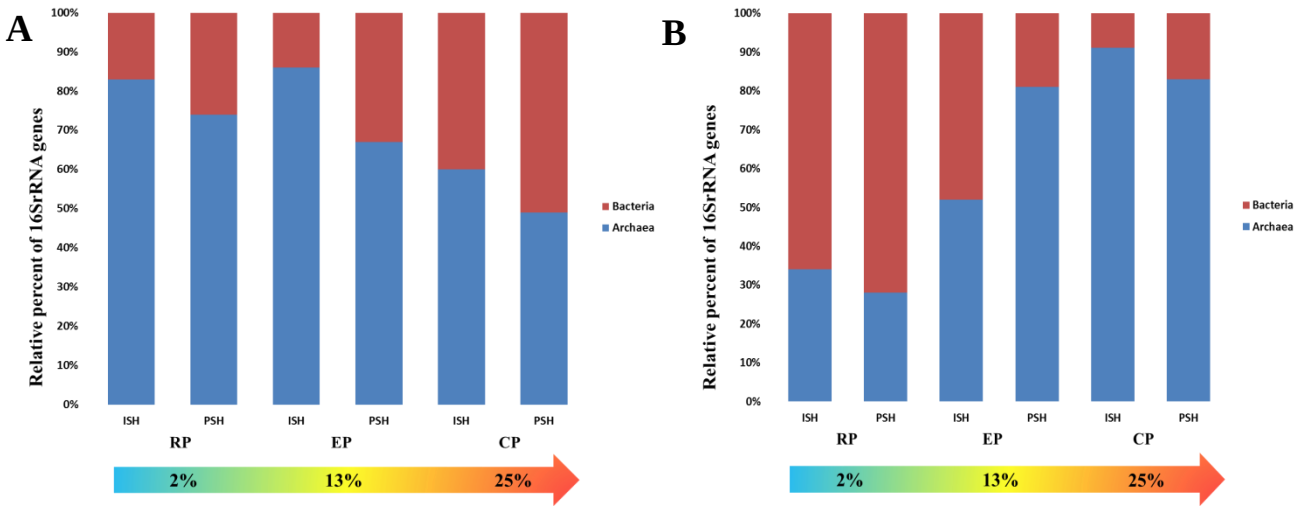
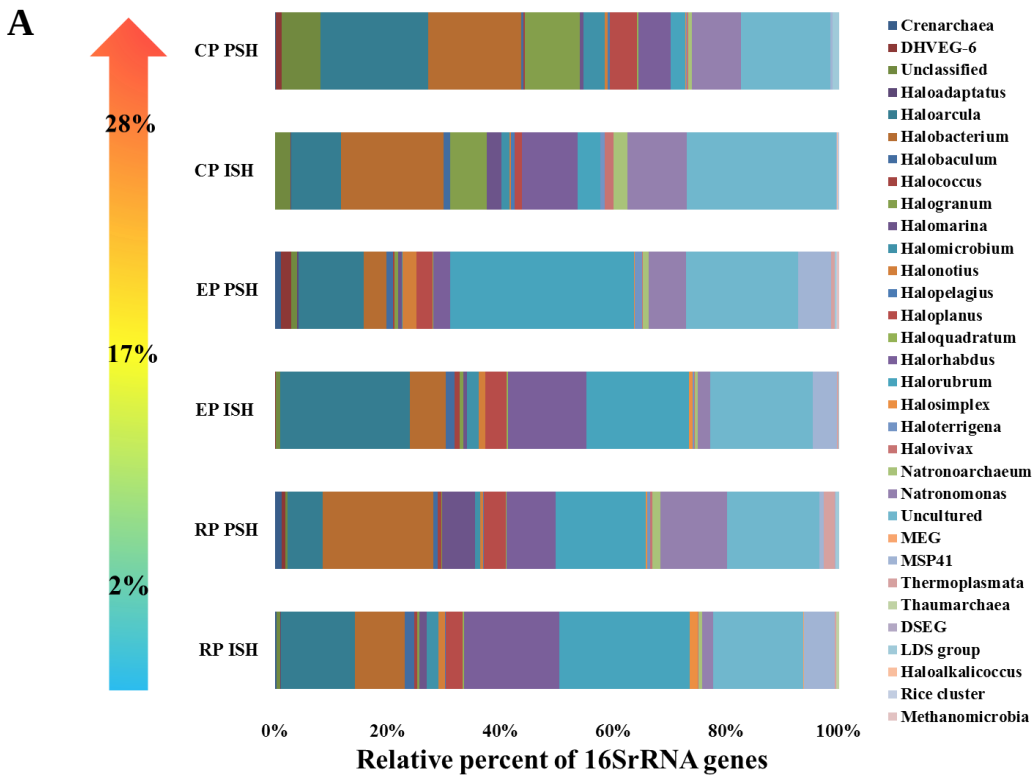


Fig. 5.10: Relative distribution of Archaea and Bacteria in (A) sediments and (B) brines of Siridao salterns.RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

The dominant genera (>5% of sequences) in the sediments were identified as uncultured haloarchaeon (18%), *Halorubrum* (16%), *Haloarcula* (13.6 %), *Halobacterium* (12.2%), *Halorhabdus* (9.6%) and *Natronomonas* (6.9%). However, the relative abundance of *Halorubrum* decreased to 2.5% in crystalliser pan (CP) when compared with reservoir pan (RP) and evaporator pan (EP) compartments, whereas, the percentage of *Halogranum* increased with salinity to 9.6% and was dominant in crystalliser pan (CP) sediment, during both initial salt harvesting (ISH) and peak salt harvesting (PSH) phase (Fig. 5.11a).

Sequences obtained in brines were affiliated with *Crenarchaeota* and *Euryarchaeota*, with the latter being dominant with 98.5% sequences. *Euryarchaeota* was dominated by *Halobacteria* (98.8%) and contained sequences assigned to 19 cultivable genera and 6 environmental bacteria. The dominant genera included *Halorubrum* (43%), *Haloarcula* (17%), uncultured haloarchaeon (11%), MSP41 (5.9%). The relative abundance of *Halorubrum*in initial salt harvesting (ISH) varied from 27% to 64% in reservoir pan (RP) and in peak salt harvesting (PSH) it varied from 20% to 48% in evaporator pan (EP) (Fig. 5.11b).



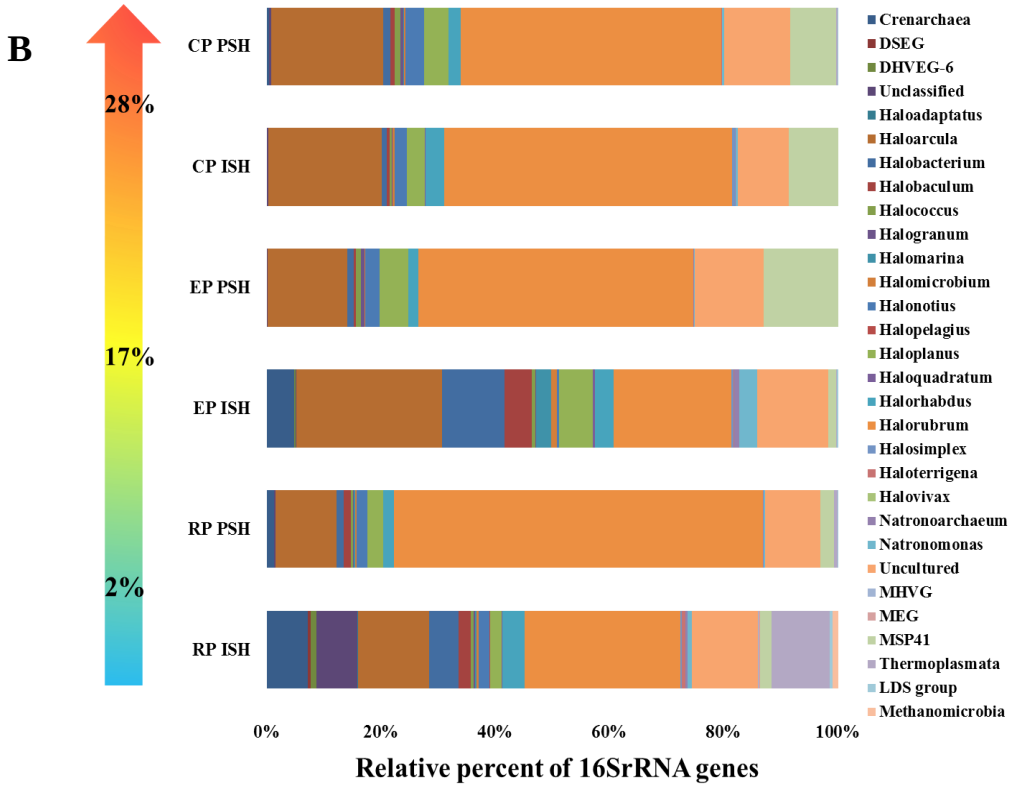


Fig. 5.11: Relative distribution of archaeal genera in (A) sediments and (B) brines of Siridao salterns. ISH – Initial salt harvesting phase; PSH – Peak salt harvesting phase; RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.1.2 Bacterial community composition

Taxonomic affiliation for Bacteria revealed the presence of 23 phyla in the sediments. The dominant phyla (>5% of sequences) were *Gammaproteobacteria* (33%), *Bacteriodetes* (28.2%), *Deltaproteobacteria* (10.3%), *Alphaproteobacteria* (7.2%), *Firmicutes* (5%). The dominant genera (>5%) were found to be clone ML602J-37 (8.1%), *Marinobacter* (5.1%). Reservoir pan (RP) compartments were highly diverse and richer than evaporator pan (EP) andcrystalliser pan (CP). *Bacteriodetes*, *Alphaproteobacteria* and *Gammaproteobacteria* were dominant in all the compartments and increased with salinity while relative percentage of all other phyla decreased with increasing salinity (Fig. 5.12a).

Bacteriodetes, *Firmicutes* and *Deltaproteobacteria* dominated the initial salt harvesting (ISH) phasewhile the latter two decreased with increasing salinity. However, *Gammaproteobacteria* dominated the salterns during thepeak salt harvesting (PSH) phase. At the genera level, bacterial fluctuations were observed to be higher than archaea. For instance, *Delsulfovermiculus*, *Rhodothermaceae*- like sequences and *Bacillus* decreased salinity gradient with the latter two found dominant only in initial salt harvesting (ISH) phase. Similarly, *Halomonas* was dominant in all the compartments,however only during initial salt harvesting (ISH) phase. Genera like *Idiomarina* and *Thioalkalispira* were dominant only during peak salt harvesting (PSH) phase.

The dominant phyla (>5% of sequences) in the brines were found to be *Bacteriodetes* (43%), *Gammaproteobacteria* (24%), *Firmicutes* (11%), *Alphaproteobacteria* (6.3%). The dominant genera were found to be sequences related to *Rhodothermaceae* (32%), *Halomonas* (6.3%), *Halovibrio* (5.2%). Similar to sediment samples, *Gammaproteobacteria* and *Bacteriodetes* were dominant in all the samples analysed, except for evaporator pan (EP) ininitial salt harvesting (ISH) phase, where *Firmicutes* were dominant. The concentration of *Chlorobi* and *Chloroflexi* increased with salinity predominantly during peak salt harvesting (PSH) phase. At genera level, *Rhodothermaceae* like sequences were dominant in the crystalliser pan (CP) while *Halomonas* dominated reservoir pan(RP) and evaporator pan (EP) compartments of solar saltern, during both phases (Fig. 5.12b).

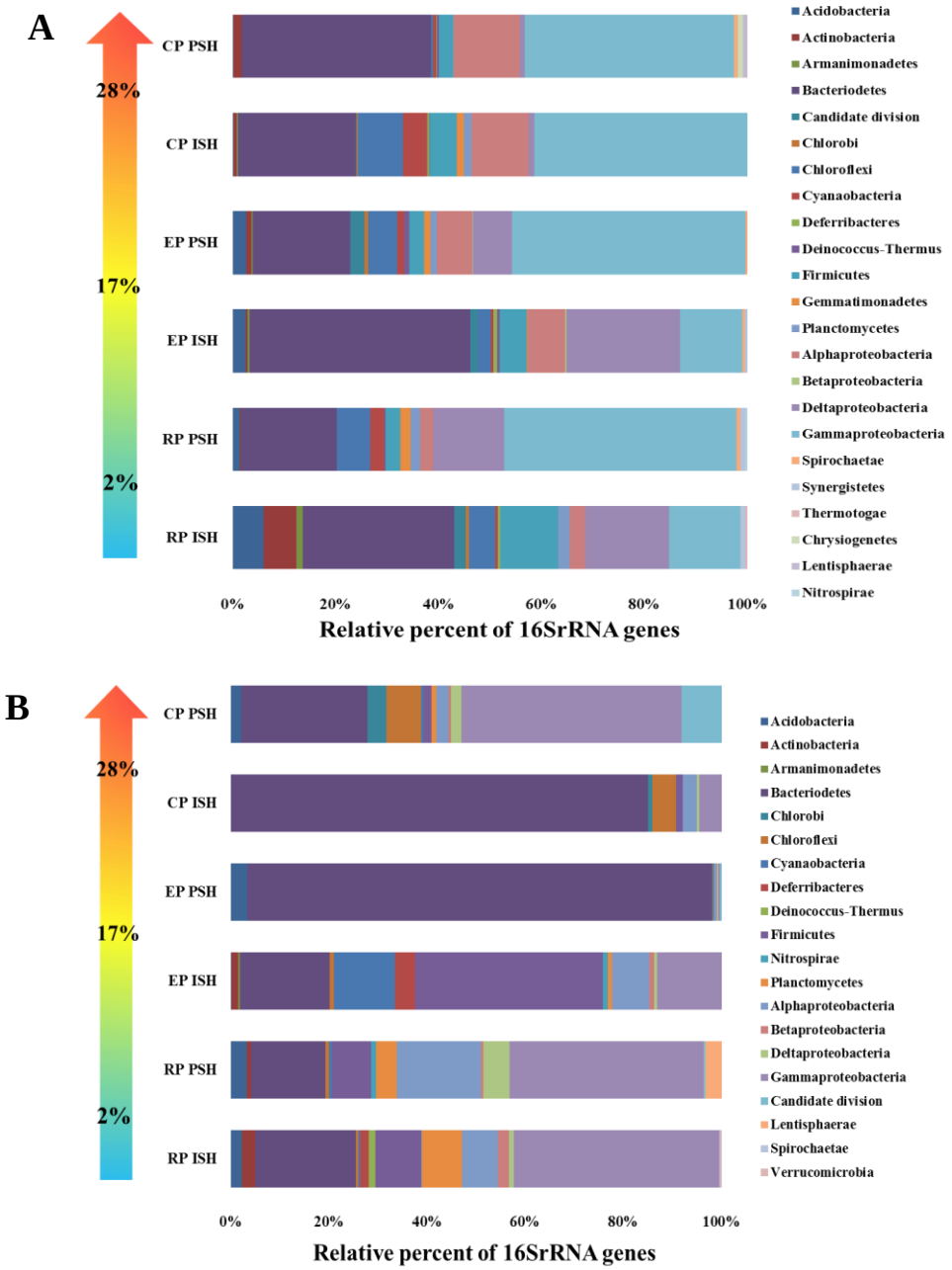


Fig. 5.12: Relative distribution of bacterial phyla in (A) sediments and (B) brines of Siridao salterns. ISH – Initial salt harvesting phase; PSH – Peak salt harvesting phase; RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.1.3 Statistical analysis

Rarefaction curves obtained from normalised data indicated the OTU richness and sampling effort. Sediments contained more OTUs than brines with the exception of crystalliser pan (CP) peak salt harvesting (PSH) phase. Among brines, crystalliser pan(CP)peak salt harvesting (PSH) phasecontained more OTUs when compared with other samples. When the rarefaction curves were compared between the two salt

producing seasons initial salt harvesting (ISH) and peak salt harvesting (PSH) phase, a clear pattern was not observed indicating the individuality of the sampling sites. Rarefaction curves belonging to brines tended to plateau than the curves from sediments (Figure 5.13a). Alpha diversity measures like Chao1, ACE and Shannon indices were calculated. Chao1 and ACE indicated the least species richness for crystalliser pan(CP) initial salt harvesting(ISH) phase brine and evaporator pan(EP) initial salt harvesting(ISH) sediment had relative maximum richness with other samples having an intermediary values. In general, sediments displayed high species richness when compared with brines. Shannon indices indicated the maximum diversity for the samples belonging to sediments from initial salt harvesting (ISH)phase and crystalliser pan(CP) initial salt harvesting(ISH) phase brine being the least diverse (Figure 5.13b).

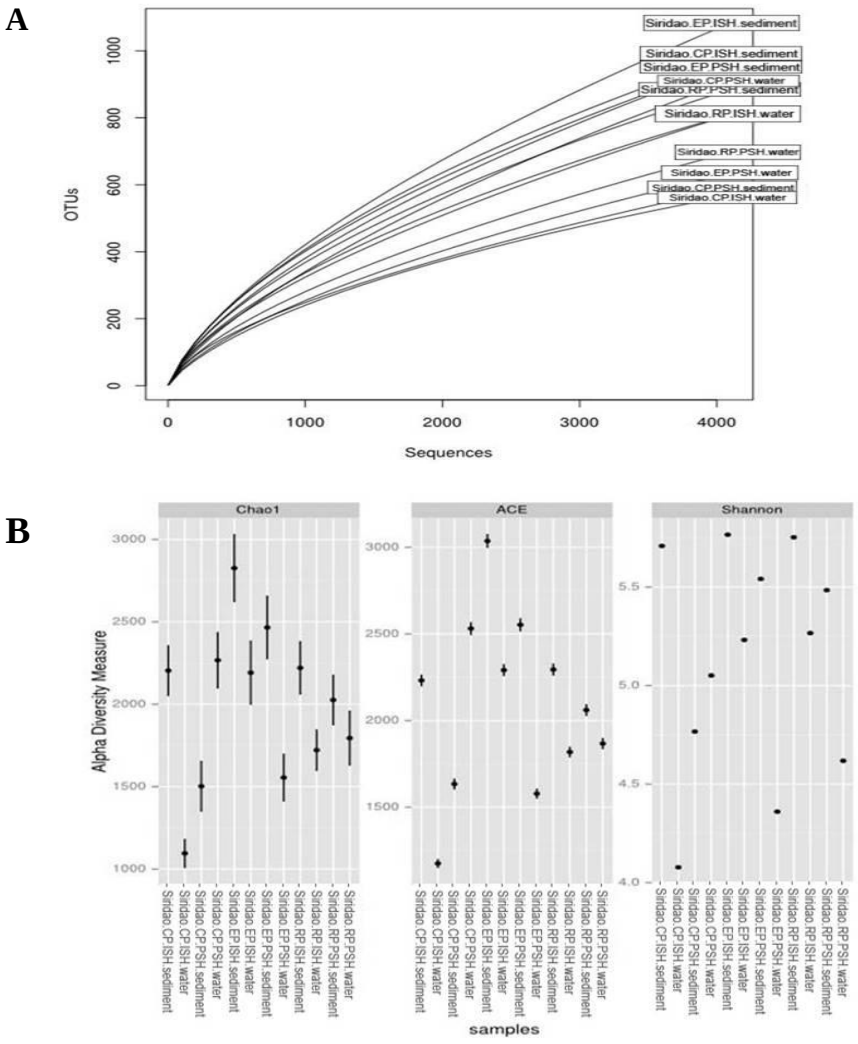


Fig. 5.13: (A) Rarefaction curves depicting the OTU richness among Siridao sample (B) Various diversity measures indicating the species richness (Chao1, ACE) and species eveness (Shannon indices) among Siridao samples.ISH – Initial salt harvesting phase; PSH – Peak salt harvesting phase; RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.2 Prokaryotic metabarcoding of Vedaranyam

Sequences obtained from Vedaranyam salterns were normalised at 45300 for each sampling site. A total of 2612 OTUs were obtained with a total read of 222683 sequences. In brines there was an increase in the archaeal composition from 9% reservoir pan (RP) to 51% evaporator pan (EP). However in crystalliser pan (CP), archaeal composition decreased to 14%. A decrease bacterial composition was observed through reservoir pan (RP) (90%) to evaporator pan(EP) (49%). However, crystalliser pan (CP) contained 89% of bacterial sequences. In sediments, the archaeal sequences decreased from 44% reservoir pan (RP) through 19% evaporator pan(EP) to 7% crystalliser pan(CP). The bacterial sequences increased from 56% reservoir pan (RP) through 81% evaporator pan(EP) to 93% crystalliser pan(CP) (Figure 5.14).

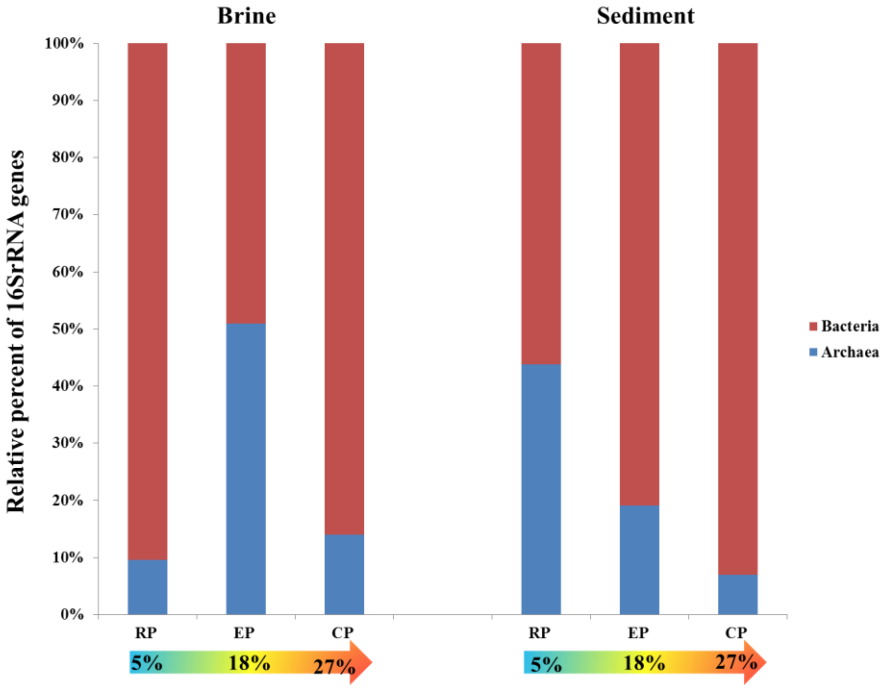


Fig. 5.14: Relative distribution of archaea and bacteria in sediment and brine samples of Vedaranyam salterns. RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.2.1 Archaeal Community Composition

In sediments, sequences belonging to three phyla namely *Crenarchaeota*, *Euryarchaeota* and *Thaumarcheota*were detected with *Euryarchaeota* being dominant. 6.5% sequences were attributed to *Crenachaeota*. *Halobacteria* was the dominant class of *Euryarchaeaota* in samples of Vedaranyam Salterns, contributing 95% sequences. *Thermoplasmata* another class of *Euryarchaeaota* contained 5% of sequences. The

sequences were assigned to 24 cultivable genera and 12 environmental groups. The dominant genera (>5% of sequences) in the sediments were identified as uncultured haloarchaeon (19%), *Halorhabdus* (11.3%), *Halobacterium* (10.8%), *Haloarcula* (9.5%), *Natronomonas* (7.6%), *Halogranum* (5.8%) and *Halorubrum* (5%). Among the brines, the dominant genera (>5% of sequences) were identified as uncultured haloarchaeon (22%), *Halobacterium* (15%), *Haloarcula* (11%), *Halorhabdus* (10%),*Natronomonas* (7.3%) and *Halorubrum* (6.6%). Maximum crenarchaeal and *Thermoplasmata* sequences belonged to reservoir pan (RP) sediment. *Halogranum* was highest in reservoir pan (RP) sediment and found to decrease with increasing salinity. *Halobacterium* was dominant in brines from reservoir pan (RP) and evaporator pan (EP). *Halorhabdus* was dominant in reservoir pan (RP) brine and in crystalliser pan (CP) sediment. *Halorubrum* was less dominant in reservoir pan (RP) sediment. However, with increasing salinity it was more dominant in evaporator pan (EP) and crystalliser pan (CP) brine. *Natronomonas* was dominant in reservoir pan (RP)and evaporator pan (EP) than crystalliser pan (CP) (Fig. 5.15).

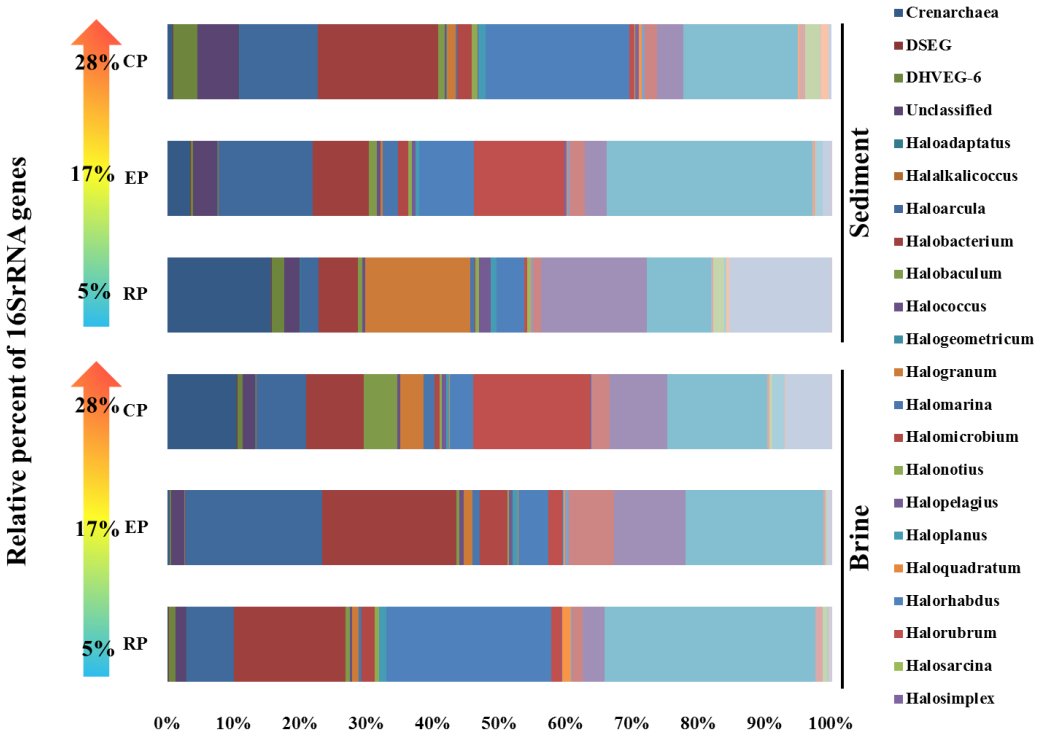


Fig. 5.15: Relative distribution of archaeal genera in sediment and brine of Vedaranyam salterns.RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.2.2 Bacterial Community Composition

Taxonomic affiliation for Bacteria revealed the presence of 26 phyla in the sediments. The dominant phyla (>5% of sequences) were *Firmicutes* (57%), *Chloroflexi* (15%) and *Gammaproteobacteria* (11%). The dominant bacterial genera (>5% of sequences) were *Halomonas* (20.6%) and *Bacillus* (14.8%). At phyla level, *Firmicutes*were dominant in reservoir pan (RP)brine. However with increase in salinity they were found to be dominant in evaporator pan (EP) and crystalliser pan (CP) sediment. *Gammaproteobacteria* was dominant in the brine at all salinities. *Alphaproteobacteria* and *Deltaproteobacteria* were dominant in reservoir pan (RP) sediment and evaporator pan (EP) brine and were less dominant at high salinities. *Bacteriodetes* was dominant in evaporator pan (EP) and crystalliser pan (CP) brines. At genera level, *Halomonas* was dominant in low salinity reservoir pan (RP) (14.5%) and high salinity crystalliser pan(CP) (54%) sediments. *Bacillus* was dominant in sediments at all salinities (7.9% in RP, 39% in EP and 40% in CP). Apart from this, some genera were dominant at only particular salinities. For an instance, *Brevibacillus* (14.5%) was dominant only in reservoir pan (RP)brine. *Aeromonas* was dominant in evaporator pan (EP)brine and *Idiomarina* was dominant in crystalliser pan(CP)brine. *Lysinibacillus* was dominant evaporator pan (EP) sediment (Figure 5.16).

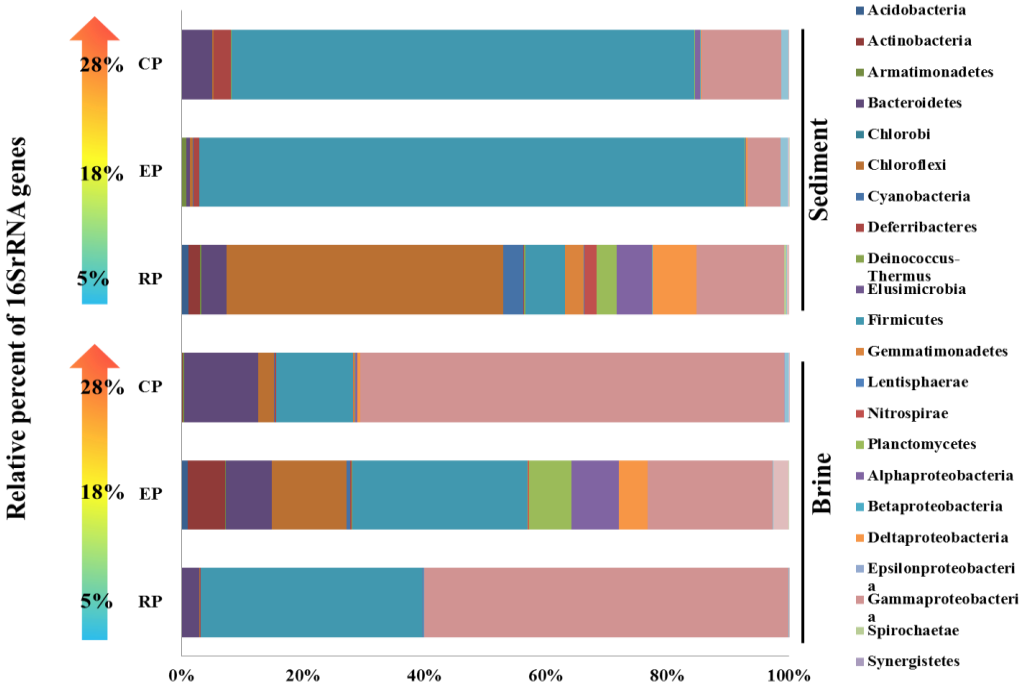


Fig. 5.16: Relative distribution of bacterial genera in sediment and brine of Vedaranyam salterns.RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.2.3 Statistical Analysis

Rarefaction curves indicated that curves tended to plateau indicating sufficient sampling. However, rarefaction curve of reservoir pan (RP) sediment and evaporator pan (EP) brine indicated insufficient sampling. Highest numbers of OTUs were observed for reservoir pan (RP) sediment and the lowest numbers of OTUs were observed for RP brine (Figure 5.17a). Alpha Diversity measures like Chao1 and ACE values indicated the highest species richness for reservoir pan (RP) sediment. Low species richness was observed for RP brine, evaporator pan (EP) sediment, crystalliser pan (CP) brine and CP sediment. Shannon diversity indicated highest diversity for reservoir pan (RP) sediment and evaporator pan (EP) brine. The lowest diversity pattern was observed for reservoir pan (RP) brine and crystalliser pan (CP) brine (Figure 5.17b).

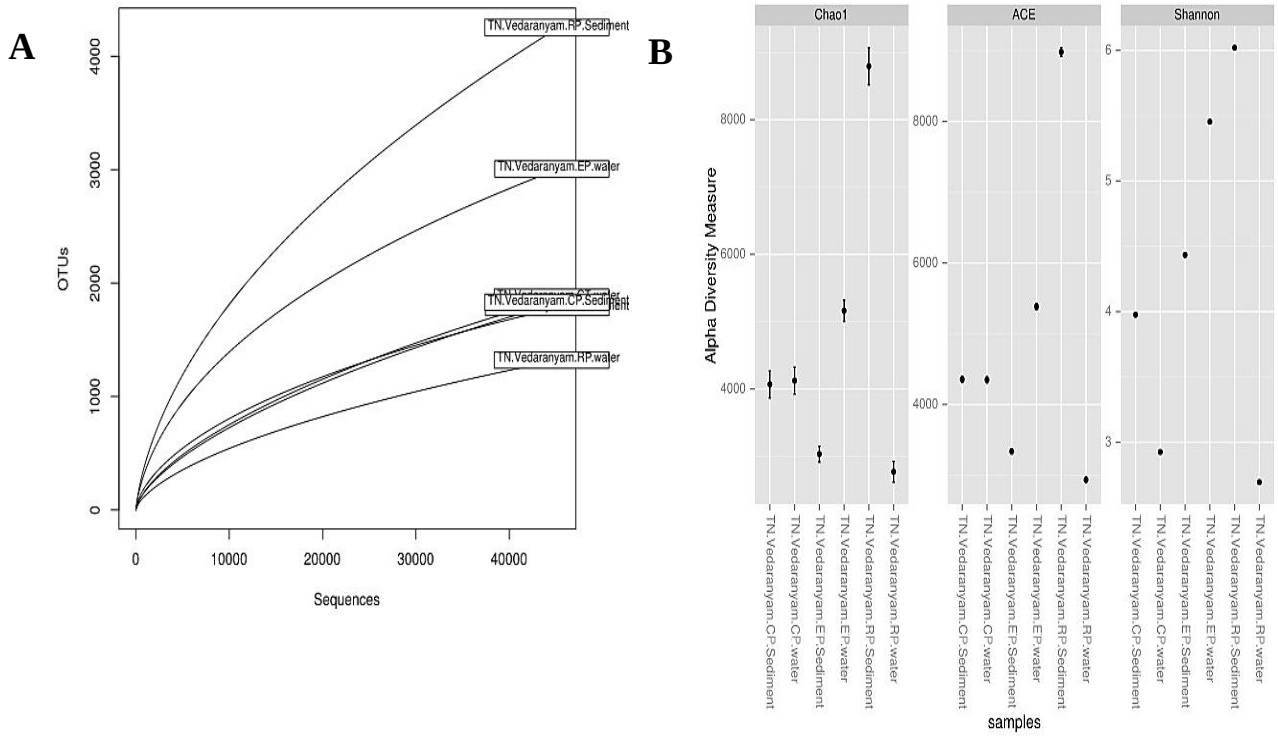


Fig. 5.17: (A) Rarefaction curves depicting the OTU richness among Vedaranyam samples. (B) Various diversity measures indicating the species richness (Chao1, ACE) and species eveness (Shannon indices) among Vedaranyam samples.RP –Reservoir Pan; EP – Evaporator Pan; CP – Crystalliser Pan

5.3.3.3 Prokaryotic metabarcoding of salt crystals

Sequences obtained from salt crystals were normalised at 30400. A total of 4231 OTUs were obtained with a total read of 152000 sequences. Among the salt crystals studied, highest archaeal community composition was observed for Marakkanam salt crystals (99%). Ribandar salt crystals had an archaeal composition of 90% and bacterial composition of 10%. When Vedaranyam salt crystals were compared, salt crystals obtained before wash had an archaeal composition of 88% and bacterial composition of 12%. However, in the salt crystals obtained after washing contained 83% archaeal sequences and 17% bacterial sequences. Commercial salt crystal sample contained an archaeal composition of 91% and bacterial composition of 9% (Figure 5.18).

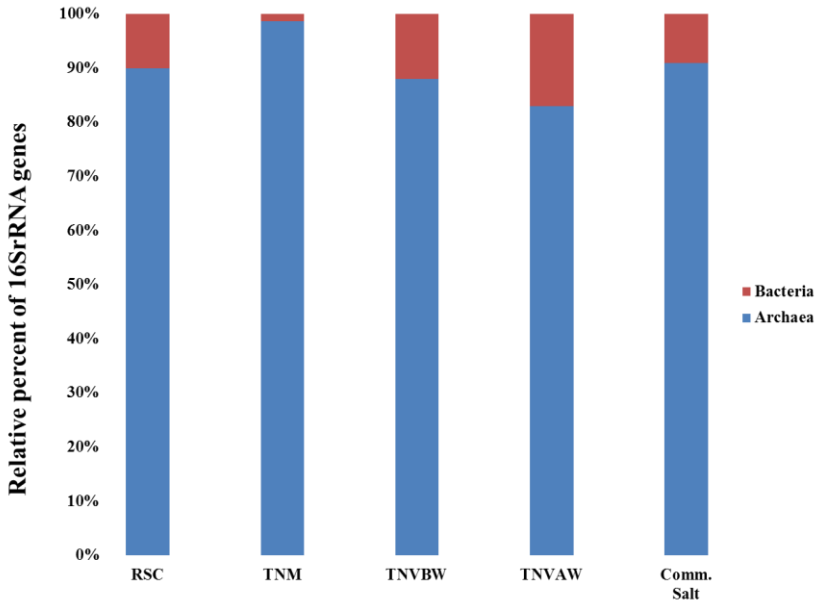


Fig. 5.18: Relative distribution of archaea and bacteria in various salt crystals. RSC – Ribandar salt crystals; TNM – Tamil Nadu Marakkanam; TNVBW – Tamil Nadu Vedaranyam Before Wash; TNVAW – Tamil Nadu Vedaranyam After Wash ; Comm. Salt – Commercial Salt crystals

5.3.3.3.1 Archaeal Community Composition

Among the salt crystals, sequences belonging to three phyla namely *Crenarchaeota*, *Euryarchaeota* and *Thaumarcheota*were detected with *Euryarchaeota* being dominant. *Halobacteria* was the dominant class of *Euryarchaeaota* contributing 99% sequences. Sequences were assigned to 22 cultivable genera and 9 environmental groups. The dominant genera (>5% of sequences) in the Ribandar salt crystals were identified as *Halorhabdus* (35%), *Halobacterium* (21%), Uncultured haloarchaeon (17%)

and *Halobaculum* (6%). The dominant genera (>5% of sequences) in the Marakkanam salt crystals were identified as *Natronoarchaeum* (24%), *Halobaculum* (21%), *Halobacterium* (15%), *Halorhabdus* (10%), Uncultured haloarchaeon (9%) and MSP41 (9%). The dominant genera (>5% of sequences) in the Vedaranyam salt crystals obtained before wash were identified as *Halorubrum* (39%), *Haloarcula* (13%), Uncultured haloarchaeon (13%), MSP41 (6%) and *Halobacterium* (5%). The dominant genera (>5% of sequences) in the Vedaranyam salt crystals obtained after wash were identified as *Haloarcula* (16%), Uncultured haloarchaeon (16%), *Halorhabdus* (13%), *Natronomonas* (12%), *Halorubrum* (11%) and *Halobacterium* (9%). The dominant genera (>5% of sequences) in the commercial salt crystals were identified as *Halobaculum* (26%), *Halorhabdus* (13%), Uncultured haloarchaeon (10%), *Halobacterium* (10%), *Haloarcula* (9%), *Natronomonas* (8%), *Halorubrum* (5%) and MSP41 (5%)(Fig. 5.19).

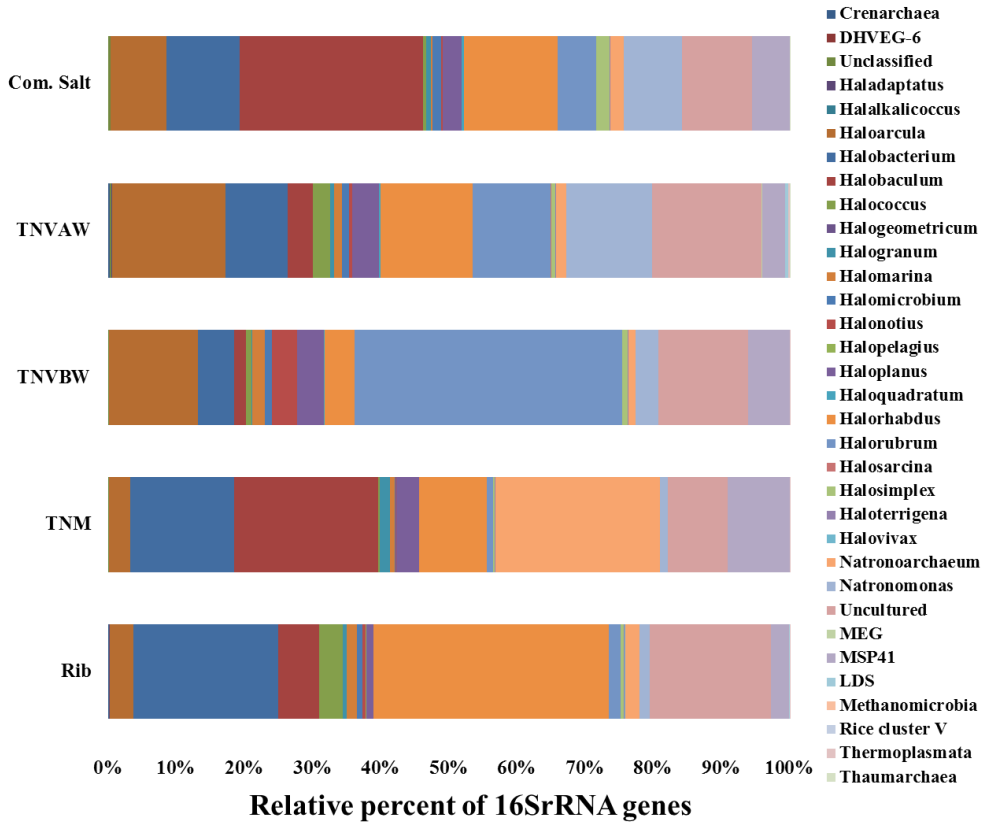


Fig. 5.19: Relative distribution of archaeal genera in various salt crystals. RSC – Ribandar salt crystals; TNM – Tamil Nadu Marakkanam; TNVBW – Tamil Nadu Vedaranyam Before Wash; TNVAW – Tamil Nadu Vedaranyam After Wash ; Comm. Salt – Commercial Salt crystals

5.3.3.3.2 Bacterial Community Composition

Taxonomic affiliation for *Bacteria* revealed the presence of 19 phyla in Ribandar salt crystal sample. The dominant phyla (>5% of sequences) were *Deltaproteobacteria* (37%) and *Bacteriodetes* (35%). Taxonomic affiliation for *Bacteria* revealed the presence of 15 phyla in Marakkanam salt crystal sample. The dominant phyla (>5% of sequences) were *Acidobacteria* (24%), *Cyanobacteria* (18%), *Gammaproteobacteria* (15%), *Bacteriodetes* (12%) and *Firmicutes* (10%). Taxonomic affiliation for *Bacteria* revealed the presence of 14 phyla in Vedaranyam salt crystal sample obtained before washing. The dominant phylum (>5% of sequences) was *Bacteriodetes* (92%). Taxonomic affiliation for *Bacteria* revealed the presence of 22 phyla in Vedaranyam salt crystal sample obtained after washing. The dominant phyla (>5% of sequences) were *Bacteriodetes* (59%), *Deltaproteobacteria* (13%), *Gammaproteobacteria* (10%) and *Alphaproteobacteria* (5%). Taxonomic affiliation for *Bacteria* revealed the presence of 15 phyla in commercial salt crystal sample. The dominant phyla (>5% of sequences) were *Bacteriodetes* (34%), *Deltaproteobacteria* (32%), *Armatimonadetes* (12%), *Acidobacteria* (10%) and *Firmicutes* (9%) (Fig. 5.20).

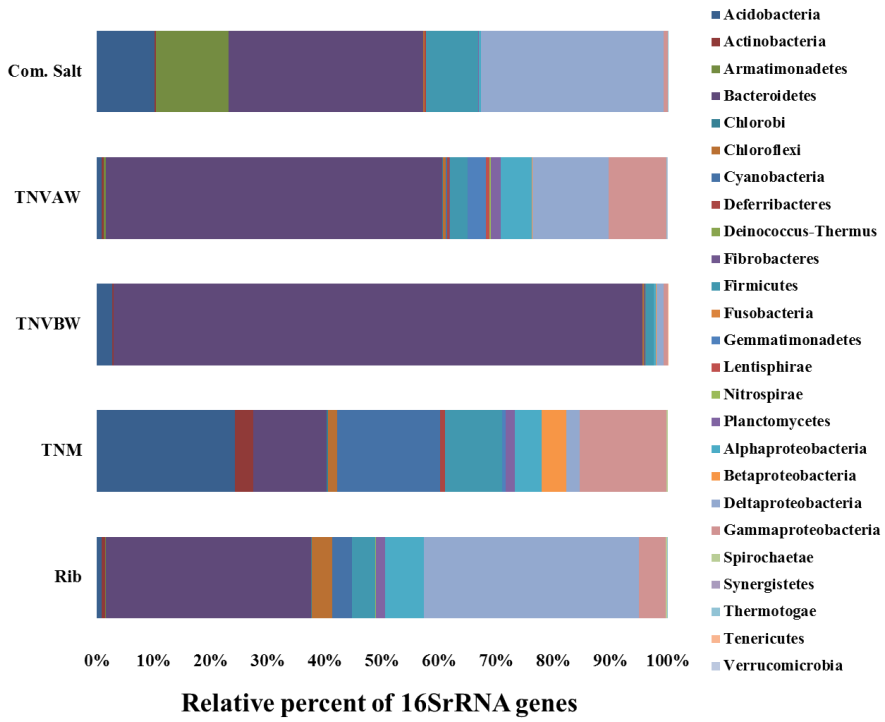


Fig. 5.20: Relative distribution of bacterial genera in various salt crystals. RSC – Ribandar salt crystals; TNM – Tamil Nadu Marakkanam; TNVBW – Tamil Nadu Vedaranyam Before Wash; TNVAW – Tamil Nadu Vedaranyam After Wash; Comm. Salt – Commercial Salt crystals

5.3.3.3.3 Statistical analysis

Rarefaction curve indicated insufficient sampling since the curves were not plateau except for Marakkanam salt crystals. Highest numbers of OTUs were observed for Ribandar salt crystal and the lowest numbers of OTUs were observed for Marakkanam salt crystal (Figure 5.21a). Alpha Diversity measures like Chao1 and ACE values indicated the highest species richness for Ribandar and Vedaranyam salt crystals. Moderate species richness was observed for commercial salt crystals. Lowest species richness was observed for Marakkanam salt crystals. Shannon diversity values indicated high diversity values for Vedaranyam salt crystal obtained after wash while the lowest diversity value was obtained for Marakkanam salt crystal. Other three samples contained moderate diversity values (Fig. 5.21b).

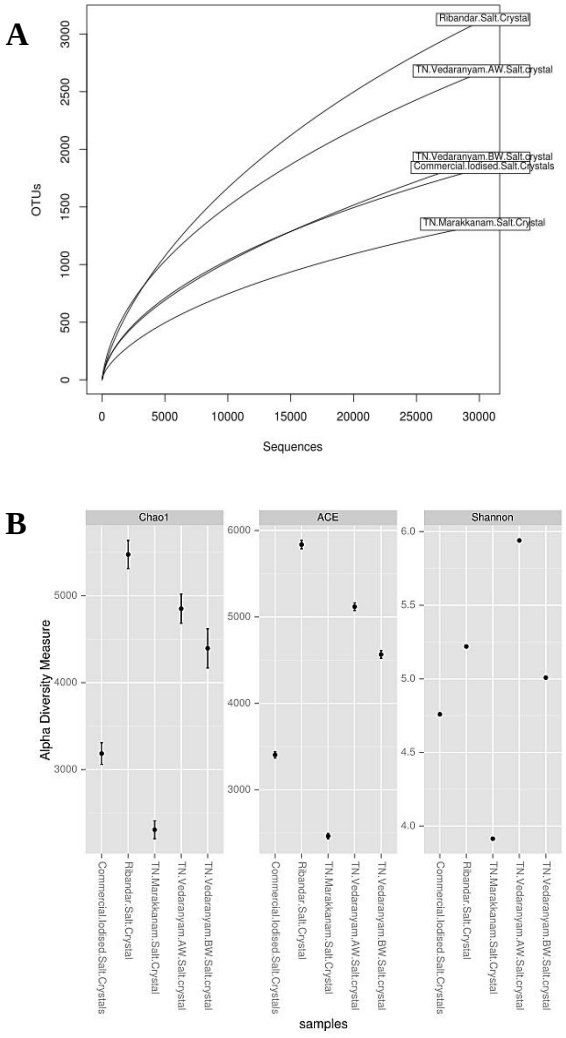


Fig 5.21: (A) Rarefaction curves depicting the OTU richness among various salt crystal samples. (B) Various diversity measures indicating the species richness (Chao1, ACE) and species eveness (Shannon indices) among various salt crystal samples.

5.3.3.4 Prokaryotic metabarcoding of Sinquetim

Sequences obtained from salt crystal samples were normalised at 9000. A total of 2868 OTUs were obtained with a total read of 18000 sequences. Sediment from RP had an archaeal composition of 23% and bacterial composition of 77%. Soil from abandoned salt producing areas contained sequences related to 14% archaea and 86% bacteria (Fig. 5.22).

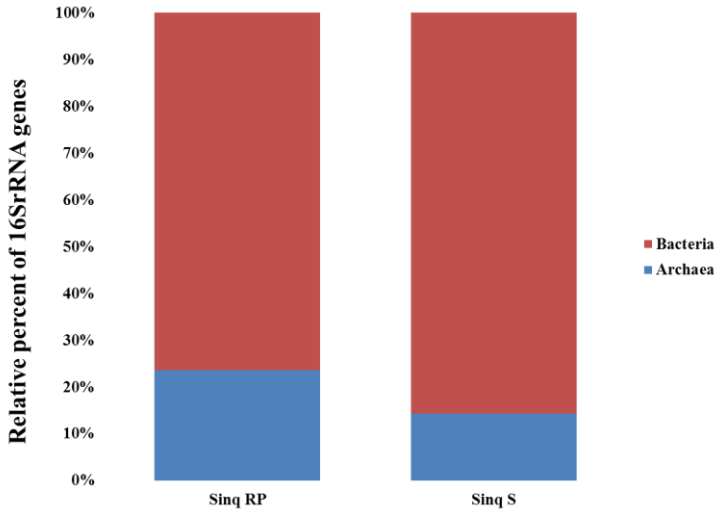


Fig. 5.22: Relative distribution of archaea and bacteria in reservoir pan (RP) and soil (S) samples of Sinquetim saltern.

5.3.3.4.1 Archaeal Community Composition

Sequences belonging to three phyla namely *Crenarchaeota*, *Euryarchaeota* and *Thaumarcheota* were detected in RP sediment, with *Crenarchaeota* being dominant with 60% sequences. *Halobacteria* was the dominant class of *Euryarchaeaota* contributing 24% sequences while 14% sequences were affiliated to *Thermoplasmata*. The sequences were attributed to 20 cultivable genera and 10 environmental groups. Uncultured haloarchaeonand *Halorubrum* was the dominant archaeal member with 2% sequences. Sequences belonging to three phyla namely *Crenarchaeota*, *Euryarchaeota* and *Thaumarcheota* were detected in soil sample with *Thaumarcheota* being dominant with 57% sequences. *Crenarchaeota* contained 6% sequences. *Halobacteria* was the dominant class of *Euryarchaeaota* contributing 34% sequences. The sequences of *Euryarchaeaota* were attributed to 15 cultivable genera and 5 environmental groups. The dominant phyla (>2% of sequences) were *Halorubrum* (5.8%), *Halorhabdus* (4.6%), *Haloarcula* (4.3%) and Uncultured haloarchaeon (3.7%)(Fig. 5.23).

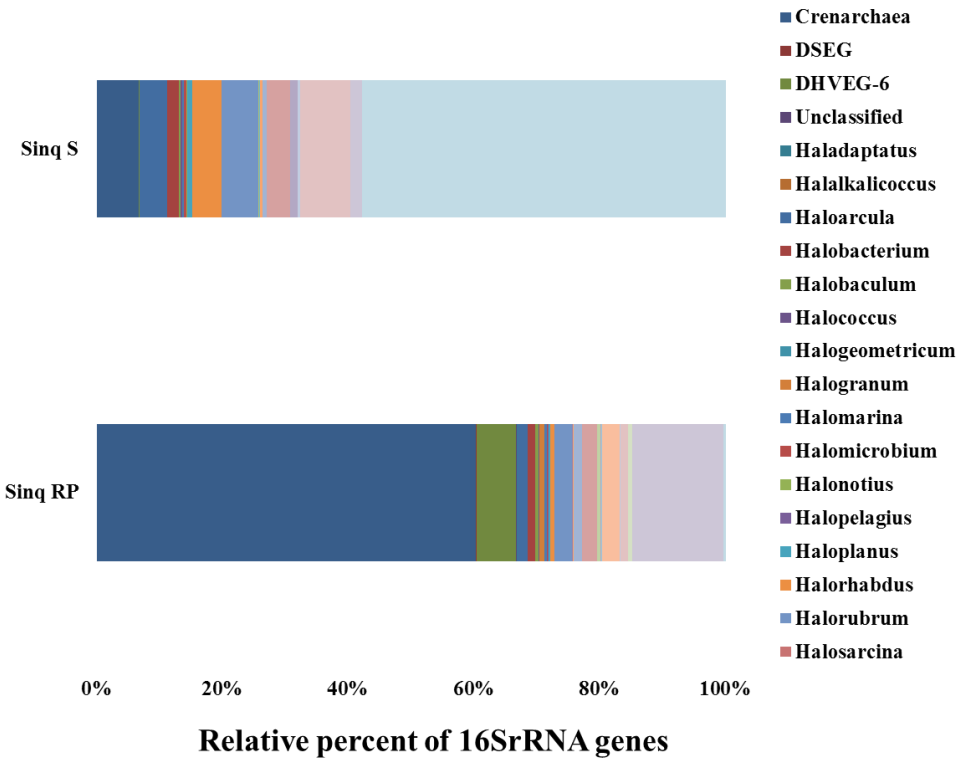


Fig. 5.23: Relative distribution of archaeal genera in reservoir pan (RP) and soil (S) samples of Sinquetim saltern

5.3.3.4.2 Bacterial Community Composition

Taxonomic affiliation for *Bacteria* revealed the presence of 26 phyla in RP sediment sample. The dominant phyla (>5% of sequences) were *Gammaproteobacteria* (16%), *Bacteriodetes* (14%), *Deltaproteobacteria* (13%), *Betaproteobacteria* (12%), *Choloroflexi* (12%) and *Firmicutes* (5%). The dominant bacterial genera (>5% of sequences) was *Halomonas* (8.6%). Taxonomic affiliation for *Bacteria* revealed the presence of 20 phyla in the soil sample. The dominant phyla (>5% of sequences) were *Actinobacteria* (32%), *Gammaproteobacteria*(19%), *Firmicutes* (17%), *Planctomycetes* (7%), *Acidobacteria* (6%), *Chloroflexi* (6%) and *Alphaproteobacteria* (5%). The dominant bacterial genera (>5% of sequences) were Uncultured bacterium belonging to order *Xanthomonadales*(11%) and *Dialister* (5%)(Fig. 5.24).

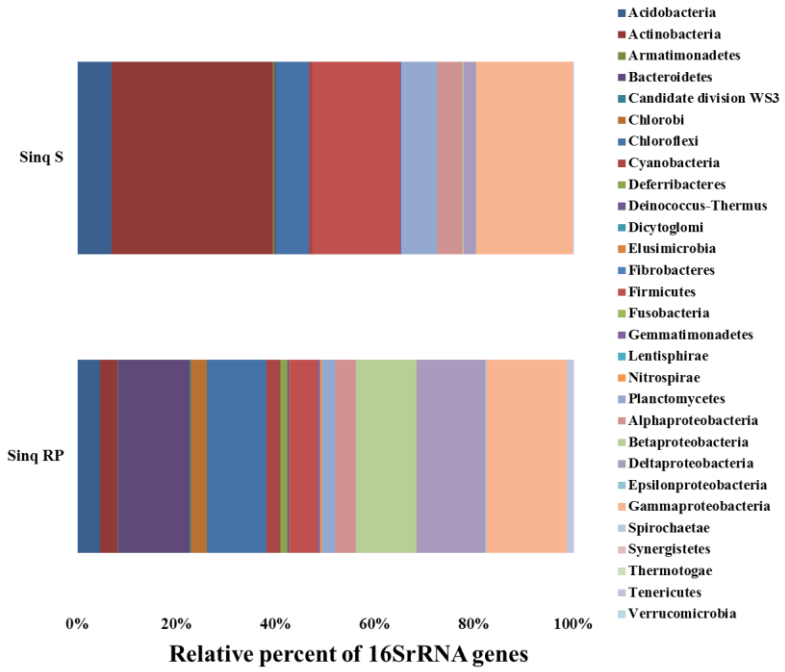


Fig. 5.24: Relative distribution of bacterial genera in reservoir pan (RP) and soil (S) samples of Sinquetim saltern.

5.3.3.5 Metabarcoding of Eukaryotes

Sequences obtained from Siridao and Vedaranyam brine and sediments were normalised at 409.

5.3.3.5.1 Siridao

Sequences belonging to *Euglenozoa* (Phylum), *Alveolata* (Clade), *Fungi* (Kingdom), *Rhizaria* (Super Group), *Haptophyceae* (division), *Stramenopiles* (Phylum) and *Viridiplantae* (Clade) belonging to eukaryotic domain in brine samples were detected. The dominant members (>5% of sequences) were *Viridiplantae* (37.3%), *Fungi* (32.1%) and *Alveolata* (30.8%). At the genera level, *Dunaliella* (32%) followed by *Cercozoa* (13%) was dominant. *Fungi* were dominant in reservoir pan (RP) with 64% sequences. *Alveolata* was dominant only in evaporator pan (EP) with 91% sequences. In crystalliser pan (CP), *Viridiplantae* was dominant with 93.7% sequences (Fig 5.25).

Sequences belonging to *Euglenozoa* (Phylum), *Alveolata* (Clade), *Fungi* (Kingdom), *Rhizaria* (Super Group), *Haptophyceae* (division), *Stramenopiles* (Phylum) and *Viridiplantae* (Clade) belonging to eukaryotic domain were detected in sediments. The dominant members (>5% of sequences) were *Viridiplantae* (44%), *Stramenopiles* (32%), *Fungi* (10%) and *Alveolata* (9%). At the genera level, *Dunaliella* (37%) was

dominant followed by *Aspergillus* (20%) and *Navicula* (9.6%). *Viridiplantae* was highest in reservoir pan (RP) with 74%. In evaporator pan (EP), their concentration was reduced to 49% and at crystalliser pan (CP), their concentration was further reduced to 10%. The number of sequences belonging to *Stramenopiles* increased from reservoir pan(RP) (8%) to crystalliser pan(CP) (68%) through evaporator pan (EP) (28%). Fungi followed similar pattern with highest percent of sequences (18%) in crystalliser pan (CP) (Fig 5.25).

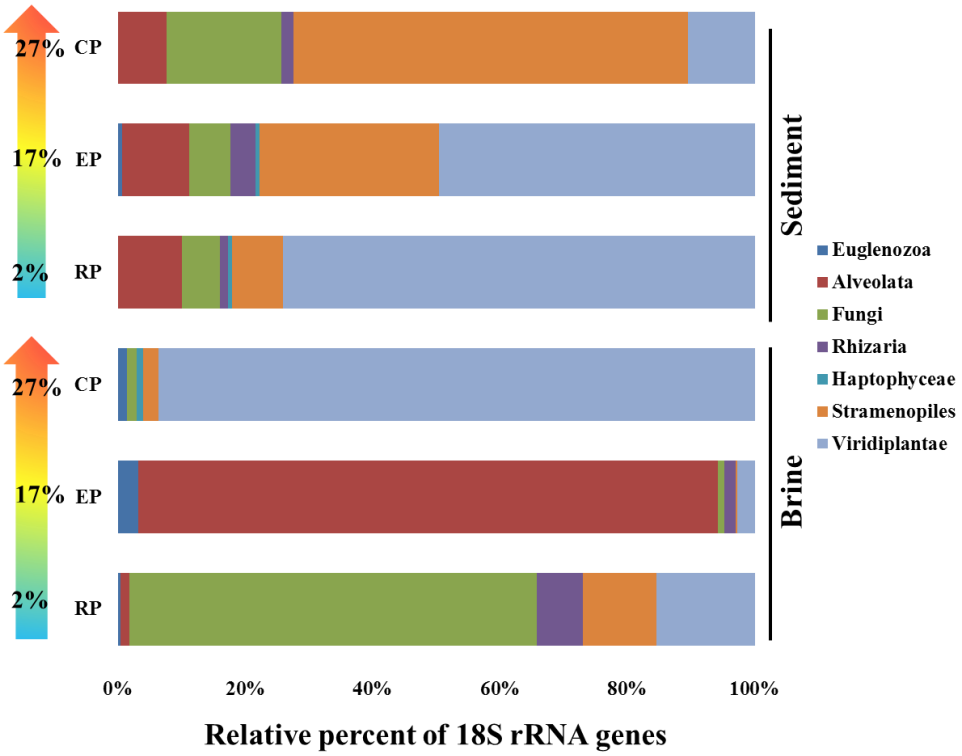


Fig. 5.25: Relative distribution of Eukaryotic genera in sediment samples of reservoir pan (RP), evaporator pan (EP) and crystallizer pan (CP) of Siridao saltern.

5.3.3.5.2 Vedaranyam

Sequences pertaining to *Euglenozoa* (Phylum), *Alveolata* (Clade), *Fungi* (Kingdom), *Rhizaria* (Super Group), *Diplomonadida* (Order), *Stramenopiles* (Phylum) and *Viridiplantae* (Clade) belonging to eukaryotic domain in brines were detected. The dominant eukaryotic members were *Viridiplantae* (63%), *Fungi* (20%), and *Alveolata* (10%). The dominant genera were *Blyxa* (75%) and *Condylostoma* (13.3%). *Viridiplantae* was dominant at low salinityreservoir pan (RP) (82%) and high salinitycrystalliser pan (CP) (70%). *Fungi* (50%) were dominant at evaporator pan (EP) (Figure 5.26). In the sediments, sequences belonging to *Euglenozoa* (Phylum), *Alveolata* (Clade), *Fungi* (Kingdom), *Rhizaria* (Super Group), *Diplomonadida* (Order), *Stramenopiles* (Phylum),

Viridiplantae (Clade) and *Parabasalia* (Class) were detected. The dominant eukaryotic members were *Fungi* (30%), *Viridiplantae* (27%), *Alveolata* (21%) and *Stramenopiles* (8%). The dominant genera were *Discocephalidae* (16%), *Dunaliella* (16%), *Leucosporidium* (12%) and *Aspergillus* (7%). Crystalliser pan (CP) sediments were dominated by *Viridiplantae* (33.3%) and *Fungi* (34%) while reservoir pan (RP)was dominated by *Alveolata* (26%)(Fig. 5.26).

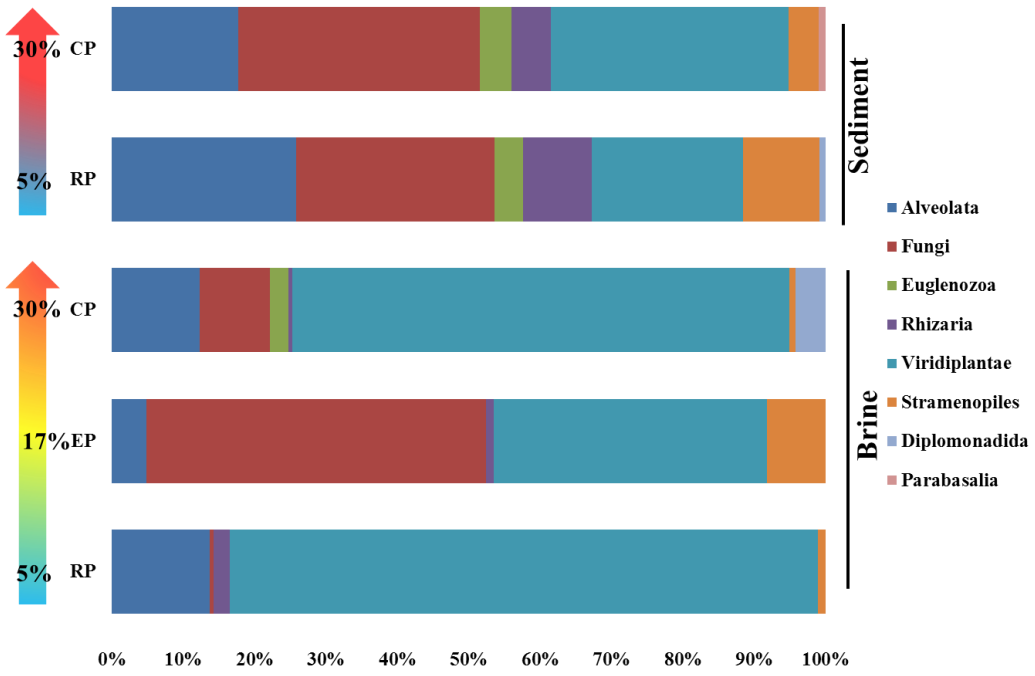


Fig 5.26: Relative distribution of Eukaryotic genera in brine samples of reservoir pan (RP), evaporator pan (EP) and crystallizer pan (CP) of Siridao saltern.

Statistical Analysis

Rarefaction curves indicated that the highest numbers of OTUs were observed for Marakkanam sediment samples and the lowest OTUs were observed for Siridao brine samples. The curves also indicated sufficient sampling for Siridao samples while insufficient sampling for Vedaranyam samples.

5.4 Discussion

Extraction of high molecular weight eDNA is essential for the microbial ecology studies for obtaining a complete profile of the microbial composition. For the extraction of eDNA from brine, five different approaches were used. All the methods contained SDS, known for denaturing the membrane proteins, as the cell lysis agent (Goldenberger et al., 1995). Method B contained DTT for reducing the disulfide bonds and thereby the contaminant proteins (Wang et al., 1993). All the methods contained appropriate incubation time for sufficient cell lysis to occur. However, after electrophoresis, it was observed that only method C resulted in the complete extraction of eDNA for all the brine samples. This may be due to the presence of sucrose in the lysis buffer which may provide better osmotic lysis when compared to sodium chloride present in buffers in other methods, considering the organisms under investigation are halophiles (Kieser, 1984). The use of lysozyme and proteinase K would have enhanced the cell lysis when compared to other methods which were devoid of enzymatic lysis (Wilson, 1987). DNA extraction from brine usually resulted in the co-precipitation of white polysaccharides. Marine algae are known for producing polysaccharides on cells lysis the polysaccharides dissolve in brine. These polysaccharides later precipitate during the DNA extraction procedure (Murray & Thompson, 1980). To circumvent this issue, CTAB was used in method C, which was successful in removing the polysaccharides. The addition of NaCl was necessary as CTAB binds with DNA when the NaCl concentration is less than 0.7M (Porebski et al., 1997).

Extraction of eDNA from hypersaline sediments is challenging due to the high salt content of the sample. Solar salterns contain clay particles that are permanently negatively charged. Since the bacterial and archaeal cells are negatively charged, the positively charged monovalent and divalent cations like sodium, calcium, potassium and magnesium sandwich the cells between the clay particles. Though the cells are lysed and DNA is released, it remains bound to the clay particles as well as bacterial cells because of high salt (Fukushima et al., 2007).

Commercial extraction kits did not successfully extract DNA from sediments and therefore manual extraction methods were used. Three different manual methods were selected for the extraction of eDNA. Among the three different approaches, method C employing a combination of chemical lysis, enzymatic lysis and mechanical lysis resulted in the successful extraction of high molecular weight eDNA. Soil is characterised by the

presence of humic acids, which get co-extracted along with the genomic DNA because of their similar properties. The humic acid content was reduced by treating the samples with PVP and the polysaccharide contamination was reduced with CTAB (Tsai & Olson, 1992). Method A involved chemical lysis with SDS and method B involved a combination of chemical and enzymatic lysis. However, mechanical disruption with glass beads helped in the lysis of cells attached to the clay particles.

During the amplification of 16S rRNA genes, it is generally recommended to adapt touch down or direct PCR approach. Touch down PCR program consists of gradual decrease in high to low annealing temperatures, thereby avoiding spurious PCR products (Korbie&Mattick, 2008). Nested PCR involves two rounds of amplifications, resulting in amplification of errors and in formation of duplexes. The erroneous PCR products can result in over estimating the diversity (Qiu et al., 2001). Phylogenetic analysis indicated the presence of 11 different bacterial genera in sediments. Occurrence of *Rhodococcus, Anoxybacillus, Exiguobacterium, Tepidibacillus, Actinospica, Dietzia, Saccharopolyspora,Ornithinibacillus, Ornithinibacter*and *Oceanobacillus* in salterns has been previously recorded (Barghini et al., 2014; Pikuta et al., 2000; Fruhling et al., 2002; Slobodkina et al., 2013; Cavaletti et al., 2006; Jin et al., 2012; Lu et al., 2014; Xiao et al., 2011&Lu et al., 2001). Bacteria belonging to 6 genera were observed in brine. *Halomonas, Marinobacter, Pseudomonas* and *Cobetia* have been previously reported from the solar saltern brines (Benlloch et al., 2002; Gauthier et al., 1992; Arahal et al., 2002).

It is interesting to note that bands in the DGGE corresponding to sediments were all of Gram-positive bacteria while bands belonging to brines were dominated by Gram negative bacteria. This corroborated with previous studies in salterns of Goa employing DGGE which indicated dominance of Gram-negative bacteria in brine (Mani et al., 2014). Previous studies from other salterns worldwide indicated that dominant bacterial phylotypes belonged to Gram negative bacteria (Baati et al. 2010; Demergasso et al. 2008; Tsiamis et al. 2008; Yeon et al. 2005). Given the cell membrane compositions, Gram-negative bacteria are more adapted to live in high ionic environments like solar salterns (Baati et al. 2010).

A few archaeal genera like *Halorubrum* and *Haloarcula* dominated the solar salterns and did not fluctuate significantly with the sudden onset of salinity or prolonged salinity. However significant difference in bacterial communities during the two different phases in Siridao salterns was observed. Distribution of Archaea and Bacteria were

different between brine and sediment samples and also between the various hypersaline environments studied. This shows the variation of community structure in individual compartments. Sediments support wide range of microbes than brine because of availability of organic nutrients and solar salterns were no exception to this pattern (Sander & Kalff, 1993). Sediments from Siridao ISH phase indicated high species evenness owing to a low salinity regime before the start of salt production process. This might have allowed diverse group of microbes to colonise the salterns. But a different trend was observed in samples collected in high salinity, with diversity measures indicating low species richness for CP ISH brine and CP PSH sediment with a corresponding high richness in CP ISH sediment and CP ISH brine respectively. It is hypothesized that with sudden onset of high salinity, most of the microbial groups would have lysed or would have become dormant and moved to sediment. In Vedaranyam salterns, brine contained low richness values compared to sediment samples.

It is commonly observed in the literature that the archaeal diversity increases with increase in salinity and simultaneously the bacterial diversity decreases (Rodriguez-Valera et al., 1985; Pedros-Alia et al., 2000; Oren, 1990). A similar pattern was observed in brine samples with increasing salinity except for Vedaranyam brine samples. However in sediments a different scenario was observed with a high percentage of haloarchaea in low salinity and decreasing further with increasing salinity. Probably as salinity increases, the archaea present in the RP sediment, slowly move to the brine column and dominate the brine samples of EP and CP. On the other hand, Bacteria dominating the brine at low salinities move towards the sediment with a corresponding increase of their numbers reaching their maximum in CP. Similar to the study carried out by Ghai et al (2011), in the moderate salinity compartments (19%), *Archaea* were dominant in brine samples. But it is not clear whether, the bacterial sequences recovered from sediment samples in CP compartment are from live bacterial cells or the exogenous DNA released after the lysis of bacterial cells. When observed between two salt producing seasons at Siridao, ISH and PSH, though the general pattern, discussed above remained same, the relative proportion of bacteria and archaea was different. During ISH phase, there was an increase in bacterial sequences, in all the compartments studied, except for EP. This indicates that bacteria could not with-stand the sudden flux in salinities during ISH and with a sustained salinity, more diverse bacterial group inhabit these ecosystems. This is emphasized by the fact that, archaeal community compositions (at least the dominant genera), were relatively stable over a gradient, while completely different bacterial genera were observed between

the two phases. Since the various compartments are interconnected, the microbial cells are transported from RP to CP through EP by a constant influx of brine. So the microbial cells that can withstand a wide range of salinity can survive and flourish on encountering optimal salinity. This is valid for Archaea since majority of the archaeal genera are dominant throughout the salinity gradient while bacterial genera fluctuate widely between the salt production phases and over the increasing salinity gradient.

In Vedaranyam brine, Bacteria remained high in EP and CP. The presence of high percent of *Bacteria* in high salinity brine sample can due to the abundance of halophilic bacteria. This was confirmed by the attribution of 65% percent of reads obtained from CP, to *Halomonas* a halophilic bacterium normally found in salterns. This can be due to available of particular metabolite or substrates that can preferable accelerate the growth of halophilic bacteria (Mata et al, 2002; Oren 1990). Dillon (2013) had reported the occurrence of high percentage of bacterial sequences in 38% salinity pond at Guerrero Negro salt pans in Mexico. The isolation of *Bacillu*s sp., from Marakkanam salterns (located at a distance of 229 km) tolerating 28% of NaCl indicates the common occurrence of bacterial members at high salinity ponds (Shanmugasundaram et al., 2015).

Salt crystals contained high archaeal content than bacteria as expected. The crystallisation point of NaCl crystal is 38%. Bacterial diversity at that salinity would be extremely low and therefore the salt crystals contained less bacterial members when compared to archaeal members (Vreeland et al., 2000 & 2007; Baati et al., 2010). When the community composition was examined between two different Vedaranyam salt crystals it was observed that salt crystals obtained after wash contained relatively high bacterial members than salt crystals before wash. Washing with water would have resulted in the lysis of archaeal members resulting in the high count for bacteria (Kushner &Kamekura, 1988). Sinquetim saltern was last operated in 2003 and therefore because of the monsoon rainfall every year the salinity would have gradually decreased. This makes the survivability of halophilic archaea difficult in these conditions. The community composition contained high bacterial sequences compared to archaeal sequences.

The solar salterns brine samples are characterised by low retention time because the brine is replenished on a daily basis as in Siridao or once in 4 days as in Vedaranyam. This gives the archaeal members a short time to grow in higher salinities. This is in contrast to the well-studied Bras del Port saltern in Spain, where the brine is replenished on a yearly basis (Guixa-Boixareu et al., 1996). So the short retention times, allows only the fast growing halophilic archaeal members to colonise the Siridao salterns. The

brineand sediment samples were dominated by the members of *Haloarcula* and *Halorubrum*, with latter dominating the brine samples. Other studies carried out in the salterns worldwide have also frequently isolated these members and sequences related to two haloarchaeal genera (Benlloch et al., 2001 & 2002; Anton et al., 1999; Pasic et al., 2005; Birbir et al., 2007). Haloarchaeal groups like *Halobacterium*, *Natraonomonas*, *Halomicrobium*, *Halomarina* and *Halorhabdus* were found predominantly in the sediment samples. This indicates the difference in the haloarchaeal community composition of brine and sediment samples, when the less dominant members are considered. *Halomicrobium* and *Halorhabdus* were found to dominate the solar salterns only during ISH phase and eventually decreased with time. This indicates that some members could tolerate and quickly adapt the sudden flux of increasing salinities and thrive but with the sustained salinity other members starts colonising (Yang & Cui, 2012; Dillon et al., 2015). On the other hand, *Haloarcula* was observed to be versatile in adapting to wide range of salinities in both brine and sediment samples. But *Halorubrum* was dominant only in RP and EP sediment samples but they were ubiquitous in brine samples at all salinities. We could also find on an average 18% and 10% of reads not affiliated to any cultured haloarchaeal species in sediment and brine, respectively. This indicates the possibility of novel species that is yet to be brought to culture. Study carried out in Ribandar salterns employing culturable techniques, located 10 km distance from the site under study, reported isolation of members belonging to *Halococcus* during PSH phase and *Halorubrum*, *Halococcus*, *Haloferax* and *Haloarcula* during SH phase (Mani et al., 2012). However, in Siridao salterns, *Halococcus* constituted only a minor fraction and we did not encounter *Haloferax* in any of the samples. This shows the variation in the haloarchaeal community structure between the closely located solar salterns inspite of similar operational procedure and geo-physical conditions.

In Vedaranyam saltern samples, *Halorhabdus* was dominant in RP brine and it decreased with increase in salinity with a corresponding increase in the sediment with highest in CP. This indicates the inability of *Halorhabdus* to thrive at high salinities. *Haloarcula* was found dominant in moderate salinity samples (EP) but not in RP and CP. *Halobacterium* were observed in at all salinities in both brine and sediment samples indicating their versatility. *Halogranum* was observed only RP sediment but absent from other samples. *Natronomonas* was predominant in RP sediment and with the increasing salinity it was dominant in EP and CP brine sample. When compared with Siridao salterns, Vedaranyam salterns exhibited diverse archaeal composition with more genera

equally dominant. In case of Siridao only two genera *Haloarcula* and *Haloferax* were dominant throughout the saltern. This could be attributed to the operational procedure of both salterns. Siridao saltern experiences sudden fluxes making it possible only for few genera to dominate. However, Vedaranyam salterns are operated for longer times providing an opportunity for diverse organisms to colonise.

In the salt crystals, *Halorhabdus* and *Halobacterium* were the dominant haloarchaeal genera in Ribandar samples. Surprisingly,in the culture dependent studies no isolates related to the above genera were obtained. This indicates the change in biodiversity pattern at high salinity especially during the salt crystallisation stages. The tolerance to high levels of magnesium and potassium may also play a key role in influencing community composition at salt crystallisation stages. The organisms tolerant to high levels of magnesium and potassium survive till crystallisation of salt and eventually get entrapped in salt crystals (Bolhuis et al., 2004). This can bring a change in the community composition between 30% and 37% salinity. When two salt crystal samples from Vedaranyam were examined, salt crystals obtained before wash were dominated with *Halorubrum* and *Haloarcula*. And the salt crystals obtained after washing contained an even distribution of haloarchaeal genera with *Haloarcula*, *Halobacterium*, *Halorhabdus*, *Halorubrum* and *Natronomonas*. Washing step is carried out to remove the impurities and traces of magnesium on the surface of salt crystals. Therefore, sudden exposure to brine may have caused lysis of susceptible halophilic archaeal members. *Haloquadratum*, a frequently isolated haloarchaeal member from saltern worldwide was less than 0.3% in any of the samples studied. This was similar to the salterns with short retention times similar to Indian salterns, where *Haloquadratum* contributed only a minor fraction (Manikandan et al., 2009).

Gram-negative bacteria are well adapted to live in highly fluctuating saline environments than Gram positive bacteria. This is validated by the presence of *Bacteriodetes* and *Gammaproteobacteria* being the dominant bacterial phyla followed by *Firmicutes* in Siridao. Other less dominant phyla like *Alphaproteobacteria* increased with increase in salinity and on the other hand *Deltaproteobacteria* displayed an opposite trend. We could find sequences related to *Rhodothermaceae* dominating the moderate and high salinity, EP SH and CP PSH brine samples respectively. This could be most likely be the sequences related to *Salinibacter* belonging to *Bacteriodetes* because of their frequent encounter in such salinities (Anton et al., 2008). However, during SH phase, CP was equally dominated by *Halovibrio*, *Rhodothermaceae* related sequences and *Salicola*.

This indicates the sustained salinity may favour diverse bacterial groups colonising salterns. Even though sequences related to *Rhodothermaceae* dominated high saline environments, they constituted only a minor fraction in sediments with majority being in brine. The preferential colonisation in brine may be because of the fact that *Salinibacter* is strictly aerobic and also because of the presence of xanthorhodopsin, light driven retinal proton pump, for harvesting energy from sunlight (Lopez-Lopez et al., 2010). *Halomonas*, one of the frequently isolated bacteria from moderately saline environments, was found in RP and EP compartment brine samples but in CP it formed a significant portion in the sediments. *Bacillus* was found to constitute significant portion in the EP but only during PSH and this may because of their halotolerance in resisting moderate salinities but lysing in high salinities. We could also find sequences related to *Desulfosalsimonas* and *Desulfovermiculus*, sulphate reducing bacteria dominating the moderate salinity sediments. Apart from these genera, we could affiliate good number of sequences to *Idiomarina* and *Marinobacter*, in high salinity sediments alone, indicating their possible dormancy or lysis. An even distribution of ML602J-37, an uncultured *Bacteriodetes* clone identified in a study conducted at Mono lake, was observed in sediment samples at all salinities and their role in solar salterns has to be studied (Baati et al., 2008).

In Vedaranyam salterns, brine samples were dominated by bacteria belonging to *Gammaproteobacteria*. However the sediment samples in EP and CP were dominated by *Firmicutes*. This pattern is similar to Siridao, where the brine samples were dominated by Gram negative bacteria. However, unlike Siridao, majority of bacterial sequences at high salinity belonged to *Halomonas*. The salt crystal samples were dominated by sequences belonging to *Bacteriodetes* and *Deltaproteobacteria*. *Bacteriodetes* sequences were related to *Salinibacter*, a bacterium typically found in high salinities (Bardavid et al., 2007). However, observing sequences related to *Deltaproteobateria* is surprising because the brine and sediment samples indicated that *Deltaproteobacteria* are found only in low salinity samples and they are absent at high salinity.

Eukaryotic diversity was low in both the salterns under investigation. This was validated by the failure in PCR amplification of 18S rRNA genes for majority of the samples. For the sequenced samples, the numbers of reads obtained were also low especially for the Siridao saltern samples indicating a very low eukaryotic diversity. *Dunaliella* dominated the brine samples at high salinity similar to other studies carried out at salterns in Portugal and Tunisia (Filker et al., 2015; Elloumi et al., 2009).

Correspondingly, there was increase in the sequences of diatoms *Naviculaceae* and *Bacillariaceae* in sediment samples. This can be attributed to the better osmotic adaption of *Dunaleilla*, which employs glycerol as an osmoprotectants (Bardavid et al., 2008). However, diatoms, those are dominant at low and intermediate salinities (RP and EP) do not contain specialised metabolic machinery for combating high salinity. At moderate salinity EP compartments, large proportion of sequences belonged to ciliates and in particular *Fabrea*. This organism has been previously reported from other environments with moderate salinity (8 – 12%) (Toumi et al., 2005). At low salinities in RP, many sequences belonging to Fungi were detected and they mainly belonged to *Aspergillus,* a known halotolerant member previously isolated from solar salterns (Cantrell et al., 2006). But with increasing salinity, Fungal sequences were less dominant and were observed in sediments. We could not detect any true halophilic fungi reported at high salinities indicating very low halophilic fungal diversity.

In Vedaranyam saltern sediment samples, the low salinity (RP) and high salinity (CP) samples contained a similar diversity pattern with members of *Aspergillus* and *Dunaliella*. Low salinity brine samples contained a huge proportion of sequences related to *Blyxa* an aquatic plant. This may be due to the location of Vedaranyam salterns close to the marshy lands that are common with *Blyxa* plant. At high salinities we could observe high proportion of *Dunaliella* and *Aspergillus*, to certain extent. At moderate salinities (EP), *Aspergillus* was the dominant member. Other studies had identified the moderate salinity regime supported highest eukaryotic diversity pattern (Filker et al., 2015; Elloumi et al., 2009). However, our studies revealed that Indian solar salterns contain low eukaryotic diversity when compared to other salterns. This may be due to the operational pattern of Indian salterns allowing less time for a eukaryotic community to develop.

5.5 Conclusion

This is the firstprokaryotic and eukaryotic metabarcoding study of the solar salterns of India. Various eDNA extraction methods were tried and the method C containing sucrose, lysozyme and CTAB was successful in extracting eDNA from brine. Similarly, method H containing CTAB, PVP, lysozyme combined with mechanical lysis resulted in successful eDNA extraction from sediments. DGGE studies identified that sediments wereinhabited by Gram-positive bacteria and samples by Gram-negative bacteria while brines were dominated by Gram-negative bacteria. Prokaryotic

metabarcoding through Illumina sequencing studies showed that Archaea were dominant in low salinity sediments while Bacteria were dominant in low salinity water. However, with increase in salinity, Archaea were dominant in brine while Bacteria were dominant in sediments. *Halorubrum*, *Haloarcula* and *Halobacterium*were the dominant halophilic archaeal members of the solar salterns. Bacteria belonging to *Bacteriodetes* and *Gammaproteobacteria* were identified as the dominant bacterial members. This study demonstrates the distinct microbial community structure of sediments and brine with enrichment of particular group of microorganisms along a salinity gradient.

Chapter VI

Screening and Partial Characterization of Antiarchaeal Substances Produced by Haloarchaea

6.1 Introduction

Production of peptide (<10 kDa) and protein (>10 kDa) antibiotics has been documented extensively in the domain Bacteria and Eukarya. However reports on the production of antimicrobial substances within the domain Archaea is scarce (Shand, 2006). The only known producers of antimicrobial peptides and proteins in the domain Archaea are halophilic archaea (phylum *Euryarchaeota*) and the genus *Sulfolobus* (phylum *Crenarchaeota*) (Besse et al., 2015). Antimicrobial peptides and proteins produced by the halophilic archaea are termed as halocins. Similar to bacterial antimicrobial substances, halocins are categorised into peptide or microhalocins (<10 kDa) and protein halocins (>10 kDa). Majority of halocins have a narrow spectrum action inhibiting organisms belonging to same phylum. However, some halocins like A4, R1 and S8 display cross-phyla interaction inhibiting crenarchaeal and other euryarcahaeal isolates belonging to methanogenic archaea, apart from inhibiting haloarchaeal isolates (Haseltine et al., 2001).

Though many haloarchaeal strains have been reported to produce halocins, only a handful of halocins have been characterised. Some of the characterised halocins include A4, G1, R1, H1, H2, H3, H4, H5, H6/H7, S8, C8 and Sech7a (Li et al., 2003; Price & Shand, 2000; Torreblanca et al., 1989; Torreblanca et al., 1994; Platas et al., 2002; O'Connor & Shand, 2002; Shand & Leyva, 2007). This chapter deals with the screening of the characterised isolates for the production of antimicrobial/antiarchaeal substances and its partial purification.

6.2 Materials and methods

6.2.1 Haloarchaeal isolates and media

Twenty four haloarchaeal isolates namely, (i) *Halococcus saccharolyticus* BK3, (ii) *Hcc. salifodinae* BK6, (iii) *Hcc. saccharolyticus* BK7, (iv) *Hcc. saccharolyticus* BK11, (v) *Hcc. saccharolyticus* BK18, (vi) *Hcc. salifodinae* BK19, (vii) *Hcc. saccharolyticus* BK20, (viii) *Hcc. salifodinae* BBK1, (ix) *Haloferax volcanii* BBK2, (x) *Haloarcula quadrata* BS1, (xi) *Har. vallismortis* BS2, (xii) *Har. vallismortis* BS3, (xiii) *Har. marismortui* BS4, (xiv) *Har. marismortui* BS5, (xv) *Har. salaria* BS6, (xvi) *Har. salaria* BS7, (xvii) *Har. marismortui* BS8, (xviii) *Har. japonica* BS11, (xix) *Har. marismortui* BS13, (xx) *Har. marismortui* BS15, (xxi) *Hfx. prahovense* BS16 (xxii) *Halorubrum chaoviator* BS17, (xxiii) *Hrr. chaoviator* BS19 and (xxiv) *Har. marismortui* BS20 were screened for the production of antiarchaeal substances. BK and BBK series

cultures were grown in NTYE (NaCl Tryptone Yeast Extract) medium while BS series cultures were grown in NT (NaCl Trisodium citrate) medium (Table 3.1).

6.2.2 Cell free supernatant (CFS)

Haloarchaeal cultures were inoculated into 100 ml Erlenmeyer flasks containing 20 ml of NTYE or NT medium and incubated at 37^{o}C with shaking (110 rpm) for up to 96 h. The culture broth was aseptically taken in a sterile Oak Ridge tube and centrifuged at 12,000 rpm for 30 min. The supernatant obtained was aseptically passed through a 0.22 µm membrane filter (Millipore, India) for ensuring the complete removal of cells.

6.2.3 Antiarchaeal activity assay by agar well diffusion assay

One ml of indicator haloarchaeal culture was aseptically transferred to a 100 ml autoclaved NTYE or NT medium containing 2% agar, which was previously pre-cooled to 50°C. The seeded agar medium was poured into sterile Petri plates and allowed to solidify. Wells (7 mm in diameter) were punched aseptically with a sterile cork-borer and 100 µl of CFS was added to each well. The plates were incubated at room temperature (30°C) for 10 days and observed for zones of inhibition if any. Sterile NTYE and NT medium were used as control.

6.2.4 Screening for extracellular hydrolytic enzymes

For determining extracellular amylase activity, isolates were streaked onto the NSM agar medium supplemented with 1% (w/v) soluble starch (Hi-media, India) and plates were flooded with iodine solution after growth was observed (15-20 days). Clearance zone around the culture against a dark blue background was considered as an evidence of amylase activity. Protease and esterase activity was determined by observing the formation of clear/opaque zones around culture, grown on NSM agar media supplemented with 1% (w/v) skimmed milk and 0.1% (v/v) Tween (Tween 20 and 80) respectively (Gutierrez & Gonzalez, 1972; Oren et al., 2002). Gelatinase activity was determined by growing the isolates on NSM agar plates supplemented with 0.4% gelatin (Hi-media, India) and checking for clearance around the culture by flooding the plates with 15% (w/v) $HgCl_2$ in 20% (w/v) HCl. Catalase activity was determined by adding drops of 3% H_2O_2 onto the smear prepared from ten day old culture. The smears were prepared on glass slides (Elevi et al., 2004). Appropriate positive and negative controls were employed for all the above tests and the experiments were carried out in triplicates.

6.2.5 Determination of physicochemical characteristics of CFS

The stability of CFS of *Hfx. volcanii* BBK2 and *Har. vallismortis* BS2 at different temperatures was determined by storing the CFS at Room Temperature (30^{o}C), 4^{o}C, -20^{o}C and -80^{o}C for 24 hours. The stability of CFS on heating was determined by exposing the CFS at 121^{o}C for 15 minutes and at 100^{o}C for different time periods (10 min, 30 min and 60 min). The stability of CFS in varying pH conditions (pH 2.0, 4.0, 6.0, 8.0 and 10.0) was determined by adjusting the pH of CFS with sterile 1N NaOH and IN HCl. The stability of CFS to proteolytic enzymes was determined by incubating the CFS with 1 mg/ml of Proteinase K (Thermo Fischer, India) and 1 mg/ml of Trypsin (Hi-media, India) followed by incubation at Room Temperature (30^{o}C) for 1 hour. Agar well diffusion assay was performed to determine the antiarchaeal activity of the treated CFS. Appropriate controls were used for all the assays. Further experiments were carried out with *Har. vallismortis* BS2 alone.

6.2.6 Quantification of antiarchaeal activity

The extent of antiarchaeal activity exhibited by *Har. vallismortis* BS2 CFS was quantified through serial two-fold dilution using sterile media as the diluent and expressed in arbitrary units per ml (AU/ml). One arbitrary unit is defined as the reciprocal of highest dilution showing inhibition of the indicator culture. Fifty µl aliquot of the dilution mixture was placed in wells on NT agar plate wells premixed with indicator strain and incubated at 37°C for the appearance of zones of inhibition. Arbitrary Units per ml (AU/ml) was calculated with the following formula,

$$\mathbf{AU/ml = 2^{n} \times 1000/V\ (\mu l)},$$

where, n is the highest dilution showing inhibitory activity and V is the volume of diluted CFS.

6.2.7 Time course experiment on production of antiarchaeal substances

Strain *Har. vallismortis* BS2 was grown at 37^{o}C and 110 rpm with an initial OD_{600} of 0.05. At regular intervals of 24 h, growth was determined by measuring the optical density (OD_{600}) of the liquid culture using UV-visible spectrophotometer (Shimadzu UV-2450, Japan) and the antiarchaeal activity was determined through agar well diffusion assay.

6.2.8 Salt Dependence Test

Ten ml of CFS was dialysed against 0%, 1%, 5%, 10%, 15%, 20% and 25% basal salt solution (BSS) in 3.5 kDa MWCO dialysis tubing (Spectrum Laboratories, USA) at room temperature for 48 hours with the change of BSS every 6 h. Basal salt solution was prepared by excluding yeast extract and tri-sodium citrate from the NT medium. Dialysate was concentrated using sucrose and checked for antiarchaeal activity through agar well diffusion assay.

6.2.9 Gel overlay assay

Har. vallismortis BS2 CFS was loaded on a 15% non-denaturing polyacrylamide gel (Native PAGE) (Appendix V) and Sodium Dodecyl Sulphate - polyacrylamide gel (SDS–PAGE) (Appendix V) (Lehrer et al., 1991). After electrophoresis, the gel was cut into two identical halves. In case of native PAGE, one half was neutralized using 0.01M phosphate buffer (pH 7.2) for 10 min while the SDS-PAGE, was washed overnight with 0.01% Triton X-100 after fixing the gel with fixing solution (50% methanol (v/v), 10% acetic acid (v/v) and 40 % water (v/v)). Following washing, the gels were placed in a sterile Petri dishes and overlaid with 25 ml of NT agar medium premixed with 1 ml of indicator strain *Hal. marismortui* BS4 . The plate was incubated at 37^{o}C for 5-10 days until growth of the indicator culture was visible. The other half of the gel was stained with silver nitrate (Appendix IV). The zone of inhibition obtained on the plates was correlated with the bands on the stained gel.

6.2.10 Antibacterial activity assay of CFS

The antibacterial activity exhibited by CFS was determined against two test organisms, Gram-negative *Escherichia coli* NCIM2345 and Gram-positive *Staphylococcus aureus* MTCC737 through agar well diffusion assay. Hundred microliters of the concentrated dialysate (dialysed against 0%, 5%, 10%, 15%, 20% and 25% BSS) was added to the wells in brain-heart infusion (BHI) agar plates, previously spread-plated with test organisms and were incubated overnight at 37°C.

6.2.11 Partial purification of antiarchaeal substances

6.2.11.1 Concentration of Cell Free Supernatant (CFS)

Initial concentration of the CFS was carried out using sucrose. Six hundred ml of CFS was transferred to 3.5 kDa MWCO dialysis tubing (Spectrum Laboratories, USA) and subjected to sucrose concentration for 4 h (Lee & Timasheff, 1981). The resulting

solution measured 100 ml and was designated as 6X concentrate. The 6X concentrate was analysed for antiarchaeal activity through agar well diffusion assay. About 50 ml of the 6X concentrate was further concentrated using 30 kDa NMWCO centrifugal concentrator (Spin-X UF, Corning, USA). Both permeate and retentate was checked for antiarchaeal activity through agar well diffusion assay. Retentate was designated as 30X.

6.2.11.2 Gel Filtration Chromatography

Gel filtration chromatography was carried out using Sephadex G-50 (Sigma-Aldrich, India).

6.2.11.3 Preparation of Column

Five grams of Sephadex G-50 (1.5-30 kDa range, Sigma-Aldrich, India) was suspended in 0.05 M Tris-Cl, pH 8.0 and hydrated for 15 min at 80°C. After removing the fine particles, the column was packed manually in a glass column (bed dimension of 1x55 cm). The packing was carried out at 4°C to avoid air bubble formation. Following packing, the column was equilibrated with 300 ml of 0.05 M Tris-Cl, pH 8.0. After equilibration, the bed volume (V_t) of the column was calculated with the following formula,

$$\textbf{Bed volume or volume of stationary phase } (V_t) = \pi r^2 h$$

where, r = radius of the column in cm; h = height of the column in cm. The void volume (V_o) is generally considered to be about 30% of bed volume (V_t).

6.2.11.4 Sample preparation and application on the column

One ml of 30X concentrate was applied onto the equilibrated column. The column was run at a flow rate of 0.150 ml/minute and 1 ml fractions were collected. The collected fractions were concentrated to 0.5 ml using a rotational vacuum concentrator (Martin Christ, Germany). The concentrated fractions were checked for protein concentration by measuring the absorbance at 280 nm (OD_{280}). The antiarchaeal activity of the concentrated fractions was determined through agar well diffusion assay. The fractions exhibiting antiarchaeal activity were pooled and further concentrated to 1 ml using the rotational vacuum concentrator.

6.2.12 Ion-Exchange Chromatography

The concentrated gel filtration fractions that contained high antiarchaeal activity were subjected to ion-exchange chromatography. The sample was loaded onto DEAE-Sepharose column (bed dimension of 1.5x15 cm) previously equilibrated with 0.05 M

Tris-Cl, pH 8.0. The column was first washed with one-column volume of 0.05 M Tris-Cl at a flow rate of 20 ml/h, followed by elution with a two-volume column of the same buffer containing a linear gradient of 0.1 to 0.5M NaCl. Several 2 ml fractions were collected and concentrated to 0.5 ml using a rotational vacuum concentrator (Martin Christ, Germany). The antiarchaeal activity of the concentrated fractions was determined through agar well diffusion assay. The active fractions were checked for their protein concentration by measuring the absorbance at 280 nm (OD_{280}). The fractions exhibiting antiarchaeal activity were pooled together and concentrated to 1 ml using rotational vacuum concentrator.

6.2.13 Mass spectrometry analysis of antiarchaeal substances

The fractions obtained after each concentration step along with the crude CFS and low range protein molecular weight marker ranging from 3.5 kDa to 43 kDa (Merck, India) were resolved on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was silver stained and the band corresponding to antiarchaeal activity, identified simultaneously with gel overlay assay, was excised. The partially purified protein bands from the gel fragments were eluted and subjected to peptide mass spectrometry analysis (CIF, UDSC, India) using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Applied Biosystems, USA). The obtained amino acid sequences were compared to deposited sequence NCBI and Swiss-Prot database using the Mascot search program.

6.3 Results

6.3.1 Screening for the production of antiarchaeal substances

The CFS obtained from 24 haloarchaeal isolates was screened against each other for determining the spectrum of antiarchaeal activity. A total of 28 antiarchaeal substance production-sensitivity interactions were documented. None of the strains were inhibited by their own CFS. Haloarchaeal isolates belonging to genera *Haloferax*, *Haloarcula* and *Halorubrum* displayed antiarchaeal activity against most of the isolates belonging to genera *Haloarcula* and *Halorubrum* (BS1, BS3, BS4, BS5, BS6, BS7, BS8, BS11, BS13, BS15 and BS19) (Fig. 6.1). Interestingly none of the isolates belonging to genera *Halococcus* and *Haloferax* were inhibited by the CFS of any of the isolates. None of the isolates belonging to *Halococcus* displayed any antiarchaeal activity.

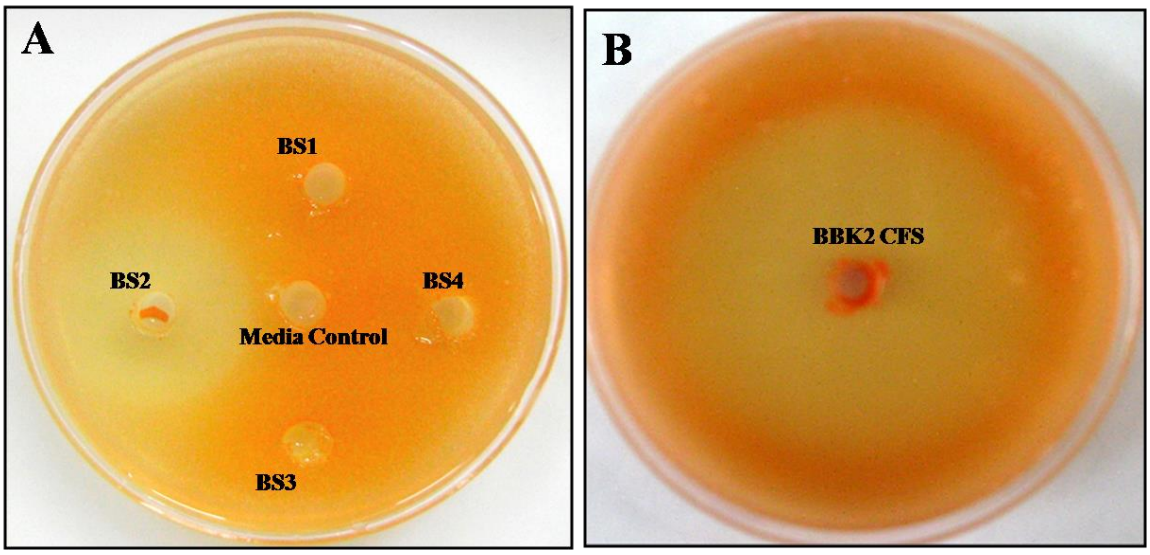


Fig. 6.1 Response of isolate (A) *Har. japonica* BS11 against *Har. argentinensis* BS1 CFS, *Har. japonica* BS2 CFS, *Har.valismortis* BS3 CFS and *Har. argentinensis* BS4 CFS. Sterile NT medium was used as control. (B) *Har. argentinensis* BS4 against *Hfx. volcanii* BBK2 CFS.

The CFS of *Har. vallismortis* BS2 and *Hfx. volcanii* BBK2 showed the best antiarchaeal activity by displaying largest zones of inhibition measuring 60 and 64 mm diameter respectively against the indicator strain *Har. marismortui* BS4. *Hfx. prahovense* BS16 displayed a broad-spectrum antiarchaeal activity against 9 isolates, highest among the studied isolates, however with a relatively smaller clearance zone of 20 mm on average. *Har. japonica* BS11 displayed antiarchaeal activity against 4 isolates with an average zone of inhibition of 14 mm. Isolates *Har. marismortui* BS15 displayed zones of inhibition of 24 mm against *Har. vallismortis* BS3, *Hrr. chaoviator* BS17 displayed zones of inhibition of 12 mm against *Har. marismortui* BS8 and *Har. marismortui* BS20 displayed zones of inhibition of 10 mm against *Har. vallismortis* BS3 (Table 6.1).

Table 6.1 Antiarchaeal activity exhibited by haloarchaeal cultures

	Zones of Inhibition										
	Indicators										
Producers	**BS1**	**BS3**	**BS4**	**BS5**	**BS6**	**BS7**	**BS8**	**BS11**	**BS13**	**BS15**	**BS19**
BS2	47	26	50	52	60	48	-	30	48	38	-
BS11	16	13	-	-	-	14	-	-	-	12	-
BS15	-	24	-	-	-	-	-	-	-	-	-
BS16	40	30	-	-	26	32	17	12	24	28	14
BS17	-	-	-	-	-	-	12	-	-	-	-

BS20	-	10	-	-	-	-	-	-	-	-	-
BBK2	28	38	64	ND	ND	ND	ND	ND	ND	ND	ND

"ND" Not determined; "-" No inhibition; Diameter of zone of inhibition in mm

Surprisingly growth enhancing activity was shown by the CFS of *Haloarcula*, *Halorubrum* and *Halococcus* (except BBK2) isolates against *Halococcus* and *Haloferax* isolates (Fig. 6.2). Among the genera studied, *Haloarcula* exhibited growth enhancing activity against highest number of isolates. The zones of growth enhancement on *Halococcus* varied from 12 to 40 mm with highest on *Hcc. salifodinae* BK6 with a zone of growth stimulation of 40 mm by the CFS of *Har. marismortui* BS15. *Halococcus* isolates BK3, BK6, BK7, BK19 and BBK1 displayed zones of growth stimulation against the CFS of *Haloarcula*, *Haloferax* and *Halorubrum* isolates. None of the *Halococcus* isolates exhibited growth enhancing activity against *Hfx. volcanii* BBK2, however only against *Hfx. prahovense* BS16. The zones of growth stimulation exhibited by CFS of *Halococcus* isolates on *Hfx. prahovense* BS16 varied from 18 to 27 mm. Interestingly, CFS from none of the other genera showed growth enhancing activity against *Hfx. prahovense* BS16 (Table 6.2).

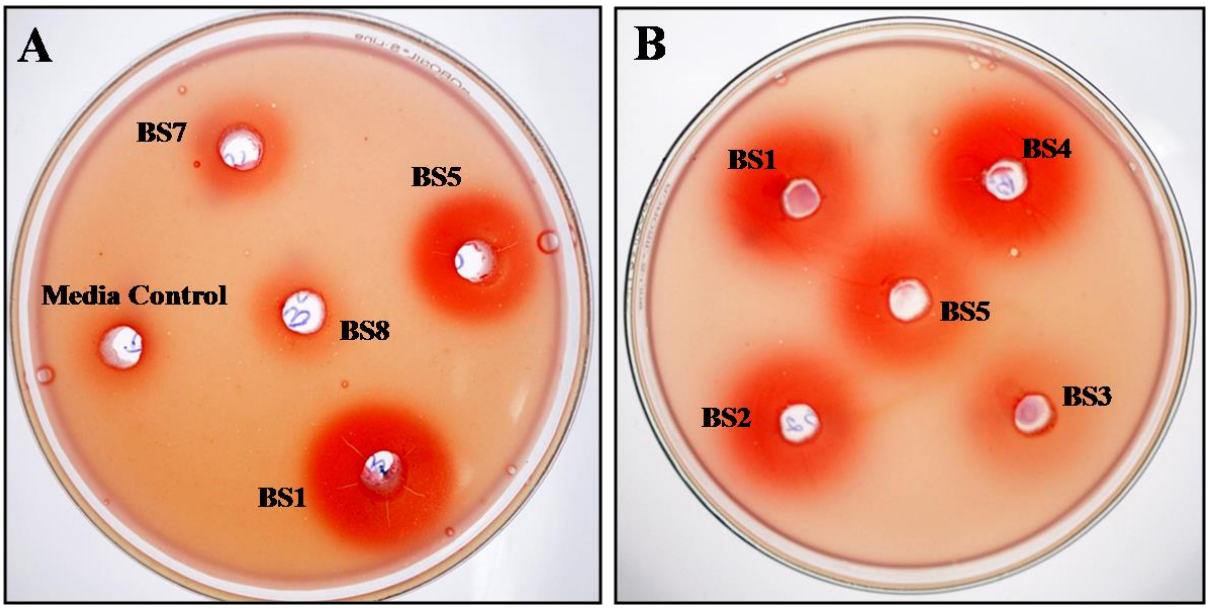


Fig. 6.2 Response of (A) *Hcc. salifodinae* BK19 against *Har. argentinensis* BS1 CFS, *Har. argentinensis* BS5 CFS, *Har. japonica* BS7 CFS and *Har. californiae* BS8 CFS. Sterile NT medium was used as control. (B) *Hcc. salifodinae* BK6 against *Har. argentinensis* BS1 CFS, *Har. japonica* BS2 CFS, *Har. vallismortis* BS3 CFS, *Har. argentinensis* BS4 CFS and *Har. argentinensis* BS5 CFS.

Table 6.2: Growth stimulation activity exhibited by the haloarchaeal isolates.

	Zones of growth stimulation									
	Indicators									
Producers	**BK3**	**BK6**	**BK7**	**BK11**	**BK18**	**BK19**	**BK20**	**BBK1**	**BBK2**	**BS16**
BS1	22	28	29	-	22	30	-	27	22	-
BS2	26	35	32	-	14	30	36	25	18	-
BS3	13	28	24	-	22	25	36	32	18	-
BS4	15	34	26	-	16	25	32	28	19	-
BS5	20	28	22	-	20	25	-	28	19	-
BS6	23	26	26	-	24	26	-	36	16	-
BS7	21	-	24	-	26	21	-	32	16	-
BS8	16	-	22	-	22	21	-	28	32	-
BS11	12	-	18	-	22	25	-	20	25	-
BS13	16	-	18	-	24	20	-	27	17	-
BS15	17	40	26	-	-	24	-	28	-	-
BS16	24	-	32	-	-	22	-	26	-	-
BS17	30	-	22	-	-	20	-	24	-	-
BS19	16	-	27	-	-	26	-	32	-	-
BS20	24	-	24	-	-	22	-	22	-	-
BK3	-	-	-	-	-	-	-	-	-	24
BK6	14	15	16	20	24	-	-	-	-	25
BK7	-	-	-	-	-	-	-	-	-	25
BK11	-	-	-	-	25	-	-	-	-	27
BK18	-	-	-	-	-	-	-	-	-	23
BK19	-	-	24	-	34	-	-	-	-	21
BK20	-	-	-	-	-	-	-	-	-	18
BBK1	-	-	-	-	-	-	-	-	-	20
BBK2	-	-	-	-	-	-	-	-	-	-

"-" No inhibition; Diameter of zone of growth stimulation in mm

6.3.2 Screening for the Production of Enzymes

Most of the isolates displayed amylase and gelatinase activity. All isolates belonging to *Halococcus* produced extracellular protease, which was seen as zone of clearance around the culture grown on skimmed milk supplemented media, whereas isolates belonging to other genera showed no protease activity. All isolates belonging to *Halococcus, Halorubrum, Haloferax* and *Haloarcula* tested catalase positive. All the isolates showed extracellular amylase production, with the exception of *Hfx. prahovense* BS16 and *Hrr. chaoviator* BS17. *Hfx. volcanii* BBK2 alone showed esterase activity,

indicated by the hydrolysis of Tween 80, while none of the isolates could hydrolyse Tween 20 (Table 6.3).

Table 6.3 Enzyme profiles of selected haloarchaeal isolates.

Isolate Name	Catalase (3%)	Hydrolysis of substrates				
		Starch (1 %)	Tween 20 (0.1%)	Tween 80 (0.1%)	Skimmed milk (1.0%)	Gelatin (0.4%)
BK3	+	+	-	-	+	+
BK6	+	+	-	-	+	+
BK7	+	+	-	-	+	+
BK11	+	+	-	-	+	+
BK18	+	+	-	-	+	+
BK19	+	+	-	-	+	+
BK20	+	+	-	-	+	+
BBK1	+	+	-	-	+	+
BBK2	+	+	-	+	-	+
BS1	+	+	-	-	-	ND
BS2	+	+	-	-	-	ND
BS3	+	+	-	-	-	ND
BS4	+	+	-	-	-	ND
BS5	+	+	-	-	-	ND
BS6	+	+	-	-	-	ND
BS7	+	+	-	-	-	ND
BS8	+	+	-	-	-	ND
BS11	+	+	-	-	-	ND
BS13	+	+	-	-	-	ND
BS15	+	+	-	-	-	ND
BS16	+	-	-	-	-	ND
BS17	+	-	-	-	-	+
BS19	+	+	-	-	-	+
BS20	+	+	-	-	-	+

ND not determined; + positive; - negative

6.3.3 Characterisation of antiarchaeal substance

Based on the diameters of zone of inhibition, CFS of *Hfx. volcanii* BBK2 and *Har. vallismortis* BS2 was further characterised. *Har. marismortui* BS4 was uesd as the indicator strain for all further experiments. The CFS of *Hfx. volcanii* BBK2 and *Har. vallismortis* BS2 was found to be stable over a pH range of 4.0-8.0 with a complete loss of activity in acidic (pH < 4.0) and alkaline pH (pH > 8.0) conditions. *Hfx. volcanii* BBK2 CFS was thermostable at 100^{o}C for 10 minutes whereas *Har. vallismortis* BS2 CFS retained its activity, even on heating at 100^{o}C for 30 minutes. However a complete loss in activity was observed on autoclaving (121^{o}C). The antiarchaeal activity of *Hfx.*

volcanii BBK2 CFS and *Har. vallismortis* BS2 CFS remained unaffected in a temperature range of -80 to 30ºC (Figure 6.1B). The antagonistic activity of *Hfx. volcanii* BBK2 CFS was completely lost upon treatment with proteinase K and trypsin whereas no loss in activity was observed for *Har. vallismortis* BS2 CFS (Table 6.4).

Table 6.4 Effect of heat, pH and enzyme on the activity of CFS of *Hfx. volcanii* BBK2 and *Har. japonica* BS2.

Treatment	BBK2 CFS Activity	BS2 CFS Activity
Heat		
100ºC for 10 min	+	+
100ºC for 30 min	-	+
100ºC for 60 min	-	-
121ºC for 15 min	-	-
Control (without heating)	+	+
pH		
2.0 and 10.0	-	-
4.0, 6.0 and 8.0	+	+
Enzymes (1mg/ml)		
Proteinase K	-	+
Trypsin	-	+
Temperature (24 hours)		
RT (30ºC), 4ºC, -20ºC and -80ºC	+	+

+ activity; - no activity; mg/ml - milligram per millilitre

6.3.4 Calculation of Arbitrary Units (AU/ml) for *Har. vallismortis* BS2 CFS

Antiarchaeal activity of *Har. vallismortis* BS2 CFS was quantified by performing a serial two-fold dilution against the indicator strain *Har. marismortui* BS4. The antiarchaeal activity gradually reduced with dilutions and completely disappeared at 14th dilution. The antiarchaeal activity of BS2 CFS was quantified as 81920 AU/ml (Fig. 6.3).

6.3.5 Time course experiment on antiarchaeal activity of BS2 CFS

Har. vallismortis BS2 exhibited a typical growth pattern when grown in NT medium with no lag phase. The strain remained in exponential phase for 4 days followed by stationary phase. Antiarchaeal activity of *Har. vallismortis* BS2 was first noticed at the transition from exponential to stationary phase on the 3rd day of growth.

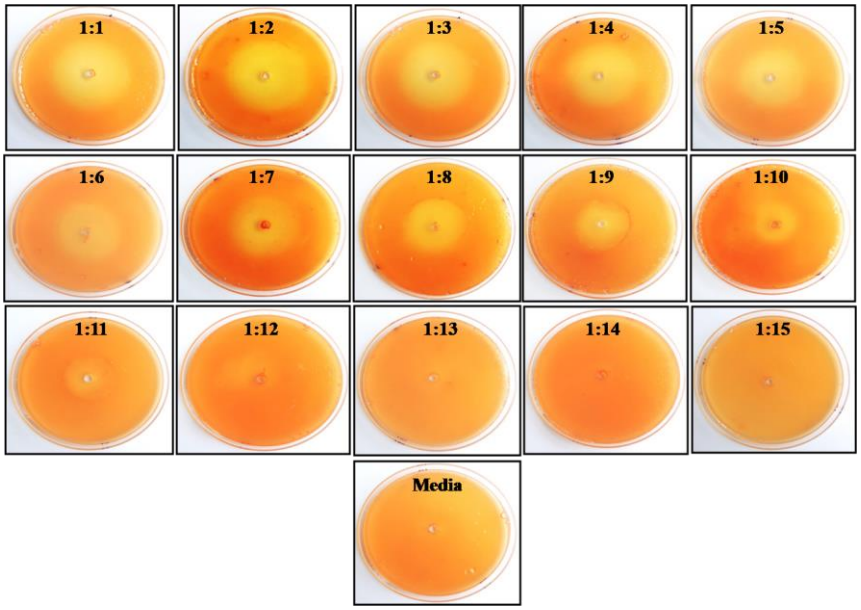


Fig. 6.3: Quantification of the antiarchaeal activity of *Har. vallismortis* BS2 CFS in arbitrary units (AU/ml)

The maximal production was attained on the 5th day of growth, when the isolate reached the stationary phase. The Antiarchaeal activity of BS2 remained constant throughout the stationary phase (Fig. 6.4).

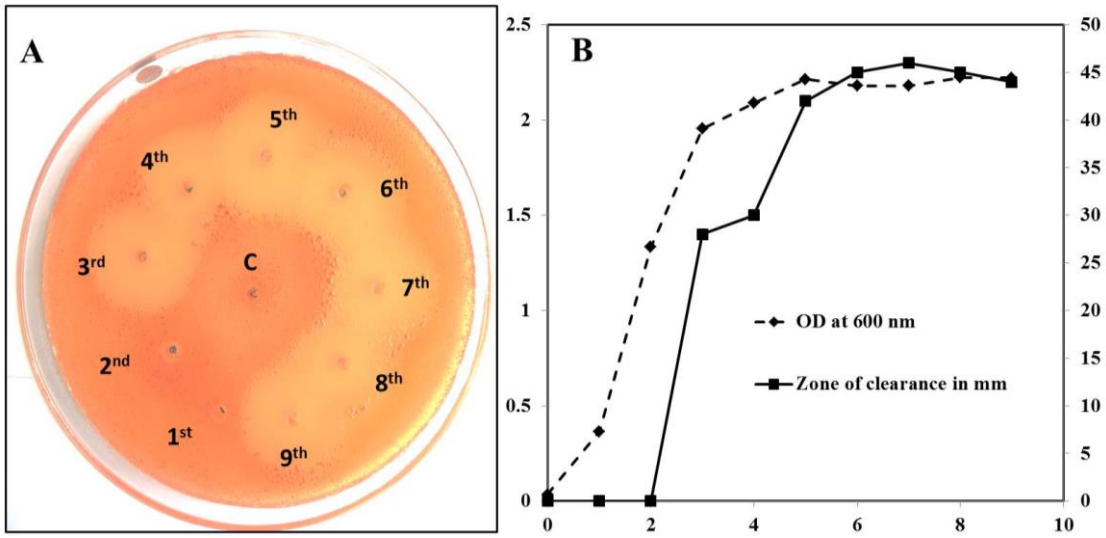


Fig. 6.4: (A) Response of *Har. marismortui* BS4 towards *Har. vallismortis* BS2 CFS obtained during various phases of growth. (B) Comparison of growth profile of *Har. vallismortis* BS2 against the antiarchaeal activity of the CFS

6.3.6 Salt dependence Assay of Antiarchaeal Activity of *Har. vallismortis* BS2 CFS

Dialysis of *Har. vallismortis* BS2 CFS against distilled water (0% BSS) resulted in 75% loss of activity. Similarly there was 40% and 20% loss in activity when the CFS was dialysed against 1% and 5% BSS respectively. However, no loss in activity was observed when the CFS was dialysed against 10%, 15%, 20% and 25% BSS (Fig. 6.5).

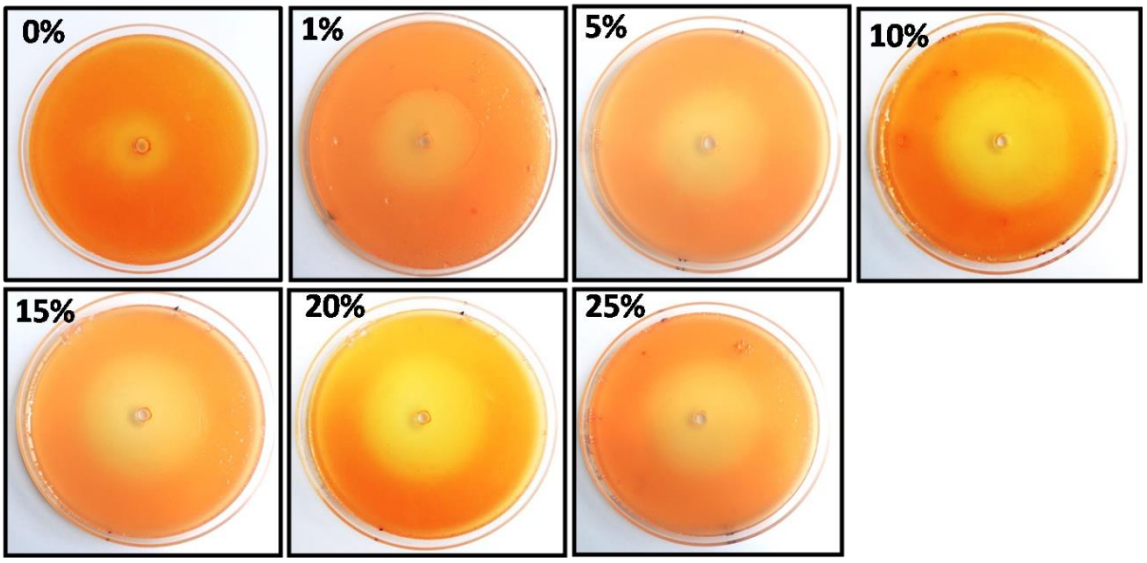


Fig. 6.5: Antiarchaeal activity of *Har. vallismortis* BS2 CFS dialysate obtained after dialysing against various salt concentrations of BSS

6.3.7 Gel overlay assay

The cell supernatant proteins were separated on a 15% Native PAGE and SDS-PAGE gel. Inhibition of the growth of indicator strain during gel overlay assay of Native PAGE and SDS-PAGE gel indicated the protein nature of the antiarchaeal substance. Zone of inhibition corresponded to the band present along the dye front in Native PAGE and SDS-PAGE gel indicating the low molecular weight (<3 kDa) of the antiarchaeal compound. The same position of the band in both the gels further indicated that the active antiarchaeal compound probably exists as a peptide (Fig. 6.6).

6.3.8 Activity of *Har. vallismortis* BS2 CFS against bacterial isolates

CFS of *Har. vallismortis* BS2 dialysates obtained after dialysis against 0%, 1%, 5%, 10%, 15%, 20% and 25% BSS, along with crude CFS, were checked for their antibacterial activity against Gram-negative *Escherichia coli* NCIM2345 and Gram-positive *Staphylococcus aureus* MTCC737. It was observed that the growth of *E. coli* NCIM2345 was not inhibited by crude and dialysed CFS of *Har. vallismortis* BS2.

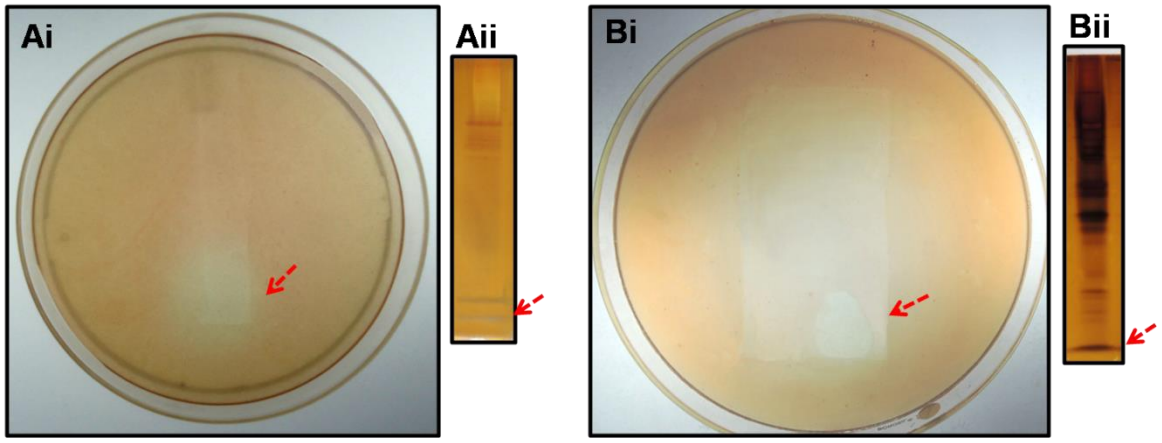


Fig. 6.6: (A) Non-denatured and (B) SDS-PAGE analysis of Har. vallismortis BS2 retentate (Aii and Bii) and its activity against Har. marismortui BS4 shown by gel overlay assay (Ai and Bi)

However, *S. aureus* MTCC377 displayed a mild bacteriostatic effect, indicated by a faint zone of clearance, while the dialysates did not inhibit the growth (Figure 6.7).

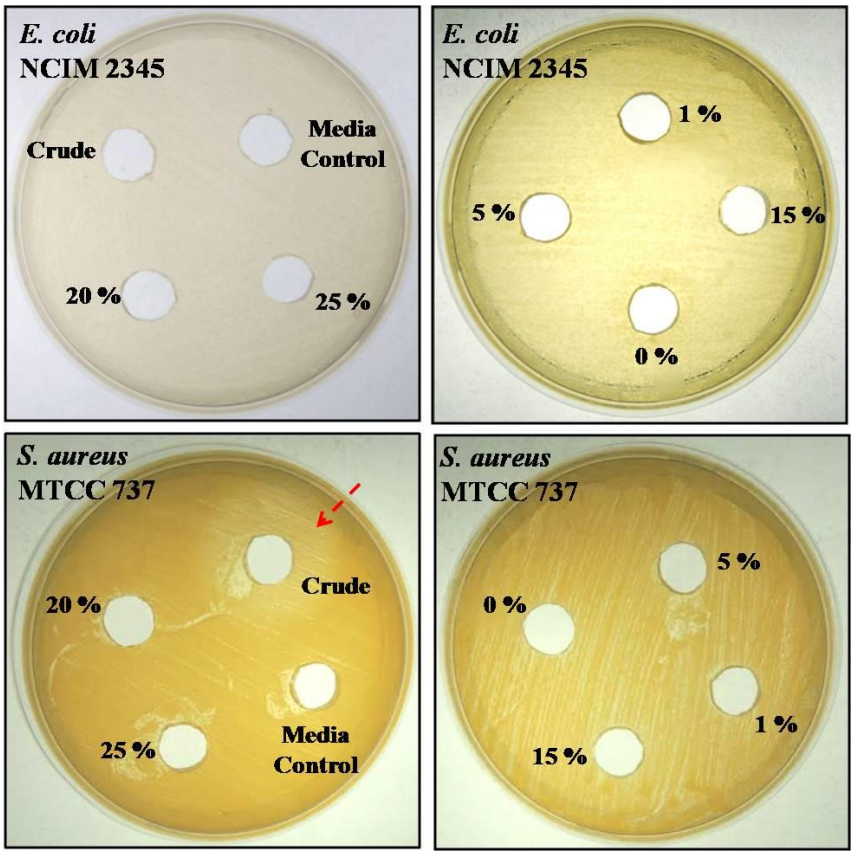


Fig. 6.7: Antibacterial activity of *Har. vallismortis* BS2 CFS and various dialysates obtained after dialysing against different salt concentrations of BSS on *Escherichia coli* NCIM2345 and *Staphylococcus aureus* MTCC737

6.3.9 Purification of *Har. vallismortis* BS2 CFS

6.3.9.1 Concentration of *Har. vallismortis* BS2 CFS

Har. vallismortis BS2 CFS was concentrated by sucrose. Agar well diffusion assay of the concentrate displayed 50% increase in the antiarchaeal activity, when compared to crude extract (Fig. 6.8). Sucrose concentrated CFS was further concentrated using centrifugal concentrator. On checking the obtained permeate and retentate for antiarchaeal activity through agar well diffusion assay, it was found that the retentate (designated 30X) showed the zone of inhibition against the indicator strain *Har. marismortui* BS4 while no antiarchaeal activity was detected in the permeate (Fig. 6.8).

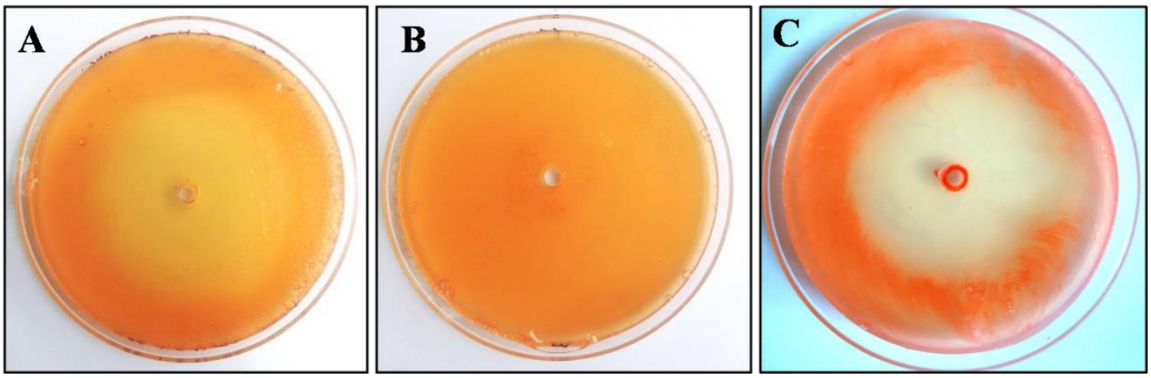


Fig. 6.8: Agar well diffusion assay of *Har. vallismortis* BS2 CFS (A) after sucrose concentration, (B) Permeate and (C) retentate obtained from 30 kDa centrifugal concentrator.

6.3.9.2 Gel filtration chromatography

The vacuum concentrated retentate fraction (30X) was applied on to a Sephadex G-50 gel filtration column. The bed volume (Vt) and void volume (Vo) of the column was calculated to be 27.5 ml and 8.25 ml respectively. On checking the fractions for antiarchaeal activity, it was found that the fractions numbered from 14 to 17 contained the active antiarchaeal compound, indicated by the zones of inhibition against the indicator strain *Har. marismortui* BS4 (Fig. 6.9).

6.3.9.3 Ion-exchange chromatography

Vacuum concentrated active fractions obtained from gel filtration chromatography were pooled and subjected to ion-exchange chromatography. Crude proteins were separated by DEAE-Sepharose into distinct peaks with active antiarchaeal compound eluting at 0.25M NaCl in fractions numbered from 40 to 43, determined through agar well diffusion assay (Fig. 6.10).

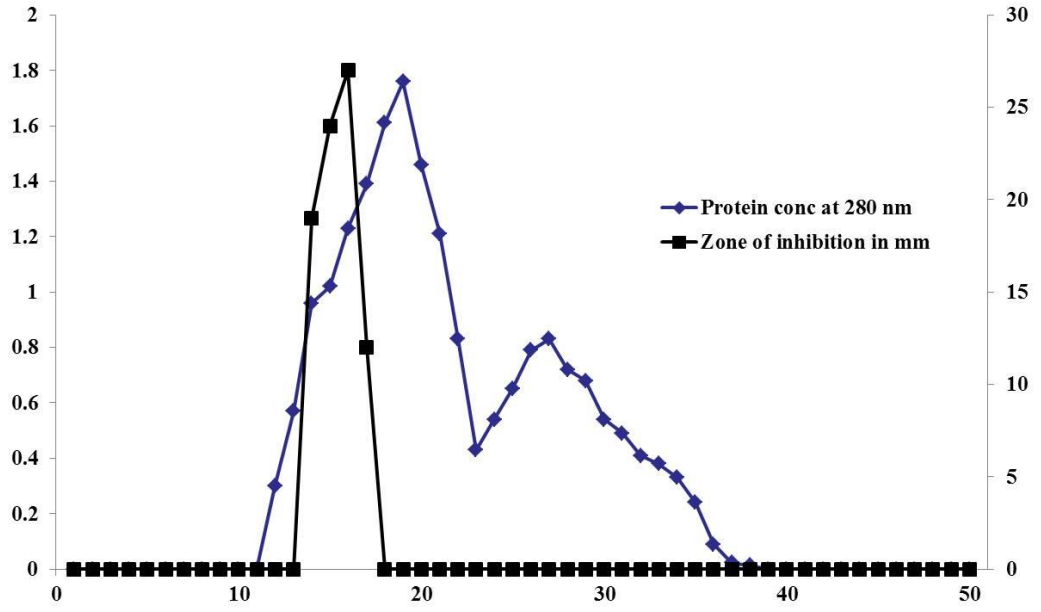


Fig. 6.9: Elution profile of *Har. vallismortis* BS2 CFS retentate obtained from 30 kDa centrifugal concentrator from a Sephadex G-50 gel filtration chromatography column.

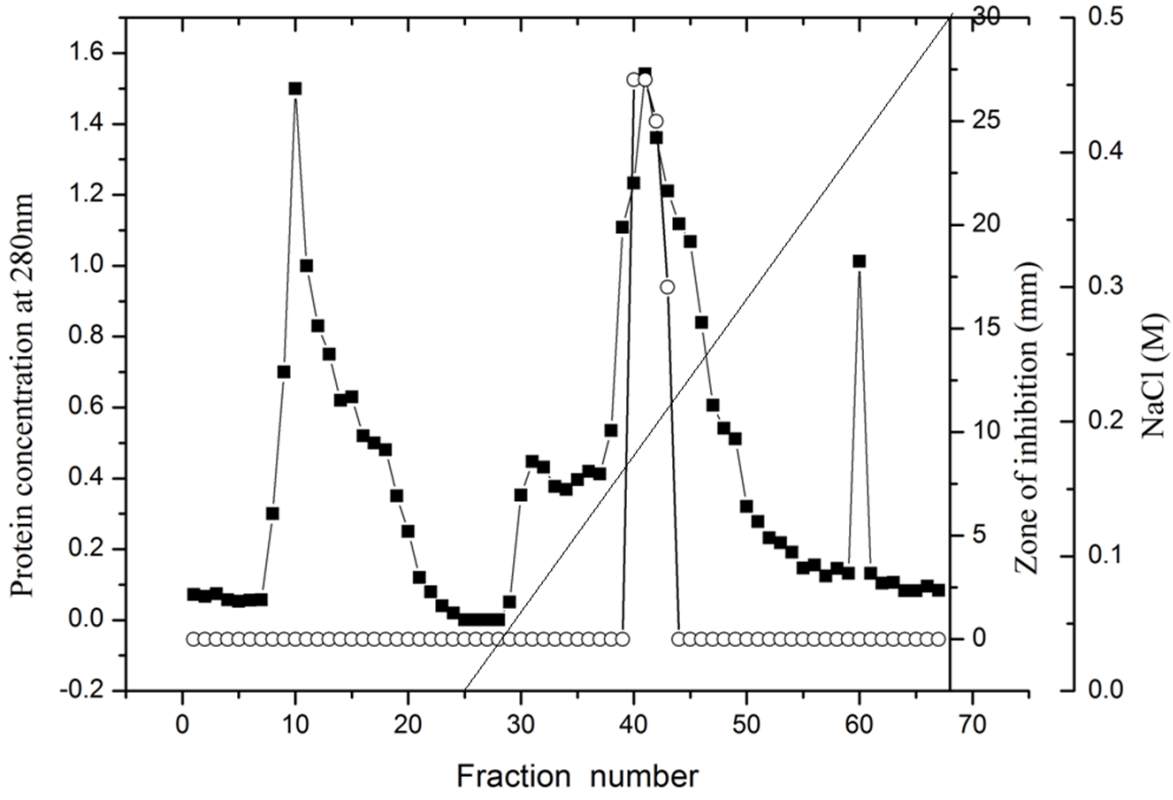


Fig. 6.10: Elution profile of *Har. vallismortis* BS2 CFS concentrate obtained from Sephadex G-50 gel filtration chromatography from DEAE-Sepharose chromatography column. Protein concentration at 280 nm (*squares*) and antiarchaeal activity as Zone of inhibition (*circles*) are shown. The NaCl gradient is represented as a *continuous line*.

6.3.9.4 Mass spectrometry analysis of antiarchaeal compound

The vacuum concentrated active fractions obtained from ion-exchange chromatography, along with the concentrates obtained from previous purification procedures and crude CFS were loaded onto 15% SDS-PAGE gel for electrophoresis (Fig. 6.11). The molecular weight of peptide band corresponding to the antiarchaeal activity was found to be less than 3.5 kDa and analysed by MALDI. The eluted band contained 20 different peptides and the Mascot search results indicated the obtained peptide sequences did not match with any of the known amino acid sequences in the protein databases.

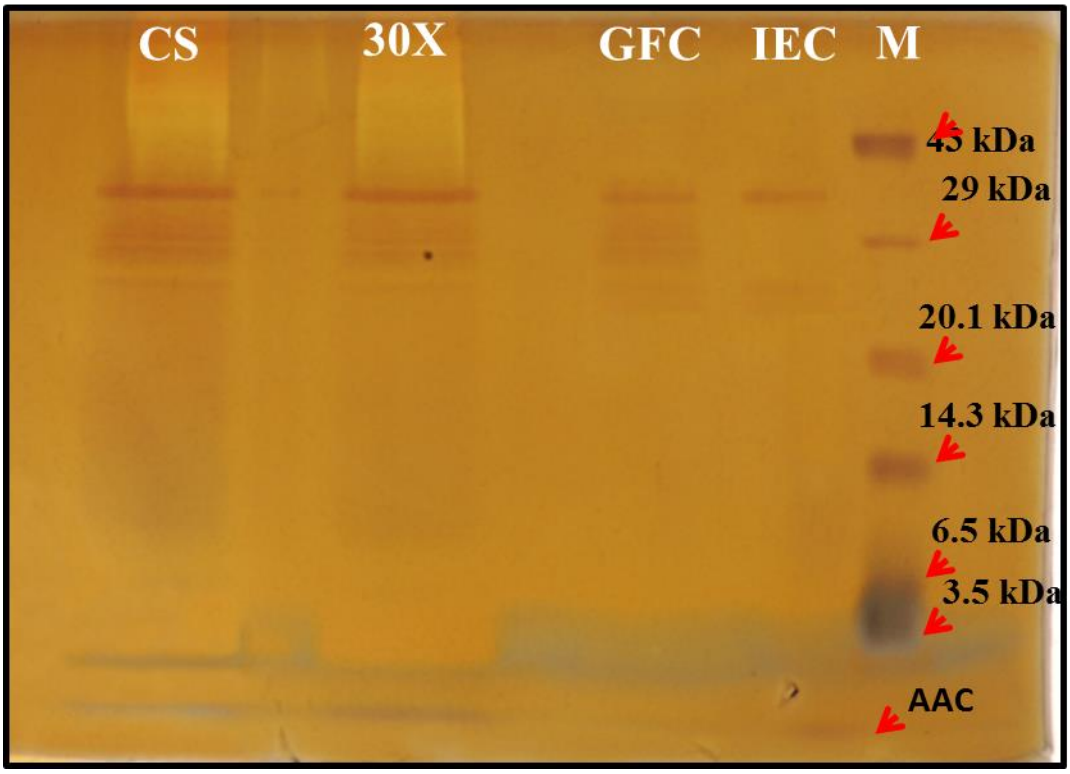


Fig. 6.11: SDS-PAGE analysis of *Har. vallismortis* BS2 CFS samples from each of the purification steps. CS - *Har. vallismortis* BS2 CFS; 30X - concentrated 30 kDa centrifugal filter retentate; GFC - concentrated active fractions obtained from Sephadex G-50 column; IEC – concentrated active fractions obtained from DEAE-Sepharose column; M – low range protein molecular weight marker (3.5 kDa – 43 kDa).

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
1794.7432	1793.7359	1792.9142	0.8218	1	46	4.9	1		IDTGEFGAILAMKGTNR
882.5273	881.5200	882.4923	-0.9723	0	30	2.6e+02	1		TVPSNPLR
1165.6942	1164.6869	1165.7183	-1.0314	0	27	6.1e+02	1		TNVIALVAPLR
1151.6812	1150.6739	1150.5731	0.1009	1	25	9.4e+02	1		TEAHKTEAHK
1334.8367	1333.8294	1334.6943	-0.8648	0	22	1.6e+03	1		TVALHEVDPAQR
842.4791	841.4719	841.4657	0.0061	0	22	2.1e+03	1		AEAVSIPR
1448.8604	1447.8531	1448.7484	-0.8953	1	22	1.6e+03	1		TPDALAVRHGEQR
951.6177	950.6105	951.5389	-0.9285	0	22	2.1e+03	1		TLAVGLLEH
1618.0007	1616.9934	1617.9202	-0.9268	1	21	1.8e+03	1		TPSPERSVPVLALPR
1434.8480	1433.8407	1434.8307	-0.9900	1	21	2.2e+03	1		TIGNVVALVERHK
982.5414	981.5341	981.5356	-0.0015	1	18	4.6e+03	1		TVPADPRAR
868.5164	867.5091	868.5130	-1.0039	1	17	4.7e+03	1		TAAKITHK
1901.1664	1900.1591	1901.0986	-0.9394	1	17	3e+03	1		TAIVGLLAEYLAGVLDKR
1007.6463	1006.6390	1007.6015	-0.9625	0	17	6.3e+03	1		TLAVPVSPPK
1051.6693	1050.6620	1051.6754	-1.0133	0	16	7.1e+03	1		TAIVIGIPIR
1033.6621	1032.6548	1032.5604	0.0944	0	15	1e+04	1		TSIFVTTHK
850.5067	849.4994	850.3821	-0.8826	0	14	1.2e+04	1		ANVEANSF
812.5259	811.5187	811.4188	0.0998	0	14	9.1e+03	1		GPGTPEVR
1302.8164	1301.8091	1302.6568	-0.8477	1	12	2e+04	1		AQAFVAESDPRI
1019.6472	1018.6399	1017.5859	1.0540	0	8	6.2e+04	1		TALVVSPFGK

Fig. 6.12: Amino acid sequences obtained from the eluted peptide band showing antiarchaeal activity.

6.4 Discussion

In this study, 24 haloarchaeal isolates belonging to 4 genera, *Halococcus*, *Haloferax*, *Halorubrum* and *Haloarcula* were screened for the production of antiarchaeal substances. Production of antihaloarchaeal substances/halocins have been reported from nine genera, *Halobacterium*, *Haloferax*, *Halorubrum*, *Haloarcula*, *Haloterrigena*, *Halogeometricum*, *Halogranum*, *Haloplanus* and *Natrinema* (Meknaci et al., 2014; Atanasova et al., 2013; Karthikeyan et al., 2013; Shand & Levya, 2007). In our study we found three genera namely, *Haloarcula*, *Haloferax* and *Halorubrum* producing antiarchaeal substances inhibiting other haloarchaeal strains. None of the isolates were inhibited by their own cell supernatants. A study conducted by Sun et al. (2005), demonstrated the cotranscription of gene encoding the halocin C8 and the immunity proteins ProC8 and HalI in *Halobacterium* sp. AS7092. The immunity conferring proteins were localised in the cellular membrane of haloarcheaon, protecting it against the action of its secreted halocin. Four genera, producing antiarchaeal substance, have been found as the dominant haloarchaeal genera in the salterns of Goa (Chapter 3). Solar salterns are nutrient limited environments with limited organic and inorganic nutrients in high salinity ponds while high zooplankton predation rates in the low and moderate salinity ponds. Therefore, halophilic archaea have adapted to live in these oligotrophic conditions by arresting their metabolism and the secretion of antimicrobial compounds (Gasol et al.,

2004). The secretion of antimicrobial or antiarchaeal compounds results in the lysis of susceptible strains thereby releasing the DNA and other intracellular materials. Since DNA is a reservoir of carbon (C), nitrogen (N) and phosphorous (P) and few halophilic archaeal genera like *Halobacterium* and *Haloferax* contain up to 30 genomic copies, genomic DNA released from the lysis of halophilic archaea can serve as a nutrient for the surviving halophilic archaea (Oren, 2014). It has been demonstrated in *Haloferax volcanii* that the extracellular DNA can serve as a source of phosphorous (Chimileski et al., 2014). Thus, the secretion of antiarchaeal substances can be an adaptive strategy employed by the halophilic archaea for survival during nutrient limiting conditions.

Interestingly, isolates belonging to the genus *Halococcus* neither synthesized nor were inhibited by the antiarchaeal substances produced by other genera. This could be attributed to the cell membrane of *Halococcus.* The cell membrane of *Halococcus* composed of highly sulfated heteropolysaccharide with a mixture of neutral and amino sugars, uronic acids, Glycine and gulosaminuronic acid (Kandler and Konig, 1998). This rigid cell wall could protect the Na^+/H^+ ion pumps located on the cell membrane from the action of the antiarchaeal substances (Meseguer et al., 1995). Another reason could be attributed to the production of protease enzymes that could inactivate the antiarchaeal substances.

While screening for the production of growth inhibitory substances, the production of growth enhancing substances by some of the haloarchaeal isolates was observed. The production of growth enhancing substances was confirmed by the presence of zones of exhibition around the wells. The zones of growth stimulation could be due to the secretion of some metabolites which would have promoted the growth of the indicator strains. The production of N-acyl-homoserine lactone (AHL), a quorum sensing molecule has been reported in *Natronocoocus occults* and *Haloterrigena hispanica* (Abed et al., 2013; Tommonaro et al., 2012; Paggi et al., 2003).

Thermal stability of growth inhibitory substance reveals the protein nature of the antiarchaeal substances/halocins. Protein halocins like H1 and H4 lose their activity on boiling at 100°C for more than 10 minutes while microhalocins like A4, C8 and S8 are resistant to boiling for prolonged times (Shand & Levya, 2007). *Har. vallismortis* BS2 CFS retained its antihaloarchaeal activity on boiling at 100°C for 30 minutes indicating, a possible presence of microhalocin. It was further confirmed by the fact that *Har. vallismortis* BS2 CFS was resistant to proteases - proteinase K and trypsin. the antiarchaeal nature of *Har. vallismortis* BS2 CFS was lost on adjusting its pH to 2.0 and

10.0. This character of *Har. vallismortis* BS2 CFS is different from characterised microhalocins, since the reported microhalocins are resistant over a broad pH conditions (pH 2.0 to 10.0). On the contrary *Hfx. volcanii* BBK2 CFS loss its activity on boiling at 100°C for 30 minutes and on treatment against protease enzymes, indicating the presence of protein halocins (>10 kDa). (Shand & Levya, 2007).

The production of halocin H1 by *Hfx.mediterranei* Xai3 occurs in the mid-exponential phase. However its activity is reported to have rapidly declined during the stationary phase. On the other hand, halocins like S8 by halophilic archaeon strain S8a, C8 by *Halobacterium* strain AS7092 and H4 by *Hfx.mediterranei* R4 have their halocin production in the late exponential phase, at the transition to stationary phase. While the activity of halocins S8 and C8 remains constant throughout the stationary phase, activity of halocin H4 declined rapidly with progression into the stationary phase (Shand & Levya, 2007). The antiarchaeal activity of *Har. vallismortis* BS2 CFS was evident during the late exponential phase and the activity remained at its maxima throughout the stationary phase. The decrease in the antiarchaeal activity of few halocins can be attributed to the production of proteolytic enzymes during the stationary phase that can render the halocins inactive. *Har. vallismortis* BS2 was protease negative and therefore the prolonged activity of its CFS in the stationary phase. The antiarchaeal activity of *Har. vallismortis* BS2 CFS remained high without any loss in its activity on dialysis against 10 to 25% BSS. Though *Har. vallismortis* BS2 CFS displayed a reduced activity on dialysis against 0% BSS, it can be considered as salt-independent halocin as salt dependent halocins display complete loss of activity when dialysed against water or 0% BSS (Shand, 2006). *Har. vallismortis* BS2 CFS did not show any antibacterial activity against *Escherichia coli* NCIM2345 or *Staphylococcus aureus* MTCC737. This is not surprising as microhalocins are known to contain a net neutral charge. As bacteria are negatively charged the interaction between halocins and bacteria is non-existent (O'connor & Shand, 2002).

Since *Har. vallismortis* BS2 CFS displayed microhalocins-like properties, corresponding purification procedures were adopted. Initial concentration steps involved sucrose and 30 kDa centrifugal concentrators. Employing centrifugal concentrator can serve two different purposes. While it the high molecular weight proteins can be separated from the low molecular weight proteins, the CFS was also desalted in the process for downstream purification procedures (Shand, 2006). The agar well diffusion assay indicated that entire antiarchaeal activity was retained in the retentate (30X) while

permeate showed no antiarchaeal activity. This is rather surprising because given the approximate molecular weight of antiarchaeal peptide (<3.5 kDa), it was expected to detect antiarchaeal substances in permeate. One possible reason could be due to the presence of hydrophobic amino acids in the protein leading to aggregation of peptides, preventing it from elution (Price and Shand, 2000). Antiarchaeal substance containing fraction eluted out immediately after void volume in the Sephadex G-50 column, indicating the aggregation of proteins similar to centrifugal concentration step. MALDI analysis indicated that the active antiarchaeal peptide is less than 3.5 kDa.

6.5 Conclusion

On screening 24 extremely halophilic archaeal isolates, *Hfx. volcanii* BBK2 and *Har. vallismortis* BS2, two potent producers were obtained. The CFS of *Har. vallismortis* BS2 withstood boiling (100°C) for 30 minutes and resisted the action of proteolytic enzymes. The antiarchaeal activity of *Hfx. volcanii* BBK2 CFS was lost on boiling for 30 minutes and on exposure to proteolytic enzymes. *Har. vallismortis* BS2 CFS was salt independent in total absence of NaCl. Partial purification indicated the antiarchaeal substance might be a potentially novel peptide with a molecular weight of less than 3.5 kDa.

Summary and Conclusions

Summary and Conclusions

Hypersaline environments constitute an important ecosystem with limited biodiversity, however contributing to nutrient recycling and acting as a reservoir of microorganisms with potential biotechnological applications. India has a large coast line (of about 7517 kms) with good sunlight and strong winds. Therefore solar salt production is an important economic activity of coastal people. Salt making is practiced in the states of Goa and Tamil Nadu since ancient times. Salterns of Goa experience three phases, preparative phase, initial salt harvesting (ISH) phase and peak salt harvesting (PSH) phase. Heavy rainfall from the months of June to October halts salt production completely. Salt pans are prepared from the month of November to January during the preparatory phase followed by the salt harvesting from February to May. Salterns of Tamil Nadu too experience similar phases of salt production. However, owing to the short monsoons, salt production is carried out for longer times from March to October.

Brine, sediments and salt crystals were collected from Ribandar, Siridao and Sinquetim salterns of Goa during three phases of salt production. Similarly, brines, sediments and salt crystals were collected from Marakkanam and Vedaranyam salterns of Tamil Nadu during the peak salt harvesting phase. Elemental analysis indicated that sodium and chloride ions dominate the salterns, defining the thalasohaline nature of the salterns. Salt crystals consisted of 90% sodium and chloride ions indicating the halite nature of the salt crystals. Salterns of Tamil Nadu contained higher chlorophyll content than salterns of Goa with high values observed for water and sediment samples. Chlorophyll *a* concentration of sediments was 0.075 µg/g on average and brines was 0.046 µg/ml on average. Total counts (TO) of acridine orange (AO) stained enumeration showed the microbial abundance in sediments was 3.74×10^6 cells/g and in brines was 3×10^6 cells/ml. Brines and sediments were plated out on 5 different media. On incubating the plates for 5-7 days, moderately halophilic isolates appeared and on further incubating the plates for up to 30 days, red – orange coloured colonies were observed. Viable counts indicated that MHM media contained 10^4 organisms/ml or g for sediments and brines, highest among all the media studied. Other extremely halophilic media contained viable counts in the range of 10^2 organisms/ml or g for sediments and brines.

A total of 270 isolates were obtained from various salterns and salt crystals. Based on the phenotypic characteristics like pigmentation, 65 isolates were selected for further molecular characterisation. Phylogenetic analysis indicated that 9 halophilic archaeal

isolates belonging to the genera *Halococcus, Haloterrigena, Halorubrum* and *Haloarcula* and 2 halophilic bacterial isolates belonging to genera *Salinicoccus* and *Pontibacillus* were novel (as the similarity of 16S rRNA is <99%). Preparatory phase of salterns of Goa was dominated by *Halococcus* isolates. During the initial salt harvesting (ISH) phase, *Haloferax*, *Halococcus* and *Halomonas* were detected and during the peak salt harvesting (PSH) phase, archaeal organisms belonging to the genera *Haloarcula, Haloferax, Halorubrum* and *Halostagnicola* were observed. In Tamil Nadu salterns, archaeal members belonging to *Haloferax, Halogeometricum, Halorubrum, Haloarcula* and *Natrinema* were obtained. Salt crystals harboured organisms belonging to the genera *Halococcus, Halomonas* and *Halorubrum*. Among the bacterial isolates, *Bacillus* and *Halomonas* were dominant in the solar salterns Tamil Nadu and Goa.

Three potential novel haloarchaeal isolates, *Haloarcula valismortis* BS3 (JCM30956), *Halorubrum chaoviator* BS17 (MCC2603) and *Halorubrum chaoviator* BS19 (JCM30957) were further characterised by phenotypic, chemotaxonomic and genomic tools. Biochemical characterisation of the isolates indicated a minimum NaCl requirement of 10% for the isolates *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 and 5% for the isolate *Har. valismortis* BS3. All the isolates were magnesium dependent requiring a minimum of 0.5% w/vol Mg^{2+} for growth and were pigmented light red to brick red. The isolates could reduce nitrate and could grow anaerobically in the presence of arginine and DMSO. The isolates utilised glucose and maltose. All the isolates were pigmented bright red. The chemotaxonomic characterisation of the isolates showed signature phospholipids of halophilic archaea, phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (Me-PGP) and phosphatidylglycerol sulfate (PGS). The major glycolipids of *Har. valismortis* BS3 were non-sulphated triglycosyl glycolipid (TGD-2) and diglycosyl diether (*DGD-2).* The major glycolipid of the isolates *Hrr. chaoviator* BS17 and *Hrr. chaoviator* BS19 was sulfated mannosyl glucosyl diether (S-DGD-3). Whole genome sequencing indicated the genome size of *Har. valismortis* BS3 was 3.2 Mb and the G + C content was 64.54 and. The genome size of *Hrr. chaoviator* BS19 was 3.8 Mb and the G + C content was 67.61%. *Hrr. chaoviator* BS19 contained 42 tRNAs and 4 rRNAs and *Har. valismortis* BS3 contained 50 tRNAs and 1 rRNA.

eDNA was extracted from brine, sediment and salt crystals using optimised protocols containing CTAB. Universal bacterial and archaeal primers were used for the

amplification of 16S rRNA genes. Three different PCR methods, direct, touch-down and nested approach were employed. DGGE analysis indicated that the sediments equally colonised by Gram-positive and Gram–negative bacteria and the brines were dominated by Gram-negative bacteria. Prokaryotic and eukaryotic metabarcoding studies on the solar salterns of India were carried out using Illumina MiSeq platform with a 2 x 300 bp paired-end run. The raw reads were quality filtered; paired-end merged and phylogenetically affiliated using PANAM (Phylogenetic Analysis of Next generation Amplicons).

Culture-independent studies through Illumina sequencing indicated that Archaea were dominant in low salinity sediments while Bacteria were dominant in low salinity water. However, with increase in salinity, Archaea were dominant in brine while Bacteria were dominant in sediments. Metabarcoding study identified that *Halorubrum*, *Haloarcula* and *Halobacterium* as the dominant halophilic archaeal members of the solar salterns. However, *Halobacterium* was never isolated through the culture dependent studies. Similarly, this study identified bacteria belonging to *Bacteriodetes* and *Gammaproteobacteria* as the dominant bacterial members. Though culture dependent studies resulted in the frequent isolation of *Halomonas*, bacterium belonging to phylum *Gammaproteobacteria* and *Bacillus* related genera belonging to phylum *Firmucutes*, the latter phyla represented only a minor fraction in Illumina metabarcoding studies.

On Screening 24 isolates for the production of antiarchaeal substances, it was found that *Haloferax volcanii* strain BBK2 and *Haloarcula valismortis* strain BS2 were the potent producers. CFS of *Har. vallismortis* BS2 withstood boiling (100°C) for 30 minutes and resisted the action of proteolytic enzymes while the antiarchaeal activity of *Hfx. volcanii* BBK2 CFS was lost on boiling for 30 minutes and on exposure to proteolytic enzymes. CFS of BS2 was salt independent with reduction in activity at low salinity. Partial purification indicated the antiarchaeal substance might be a potentially novel peptide with a molecular weight of less than 3.5 kDa.

This study is the first comprehensive report on the biodiversity patterns of solar salterns of India carried out using culture-dependent and culture-independent techniques like DGGE and Illumina MiSeq technology. This study can serve as model in understanding the fluctuation of microbes through varying salinity.

Future Prospects

Future Prospects

- Novelty of the cultures should be established by DNA-DNA hybridisation and MLSA analysis. They should also be deposited in the culture collections.

- Metabolic and biotechnological applications of the characterised halophilic archaeal and bacterial cultures should be studied.

- The sequence of the active antiarchaeal peptide should be obtained. Mechanism of action and biotechnological applications of the antiarchaeal compound could be explored

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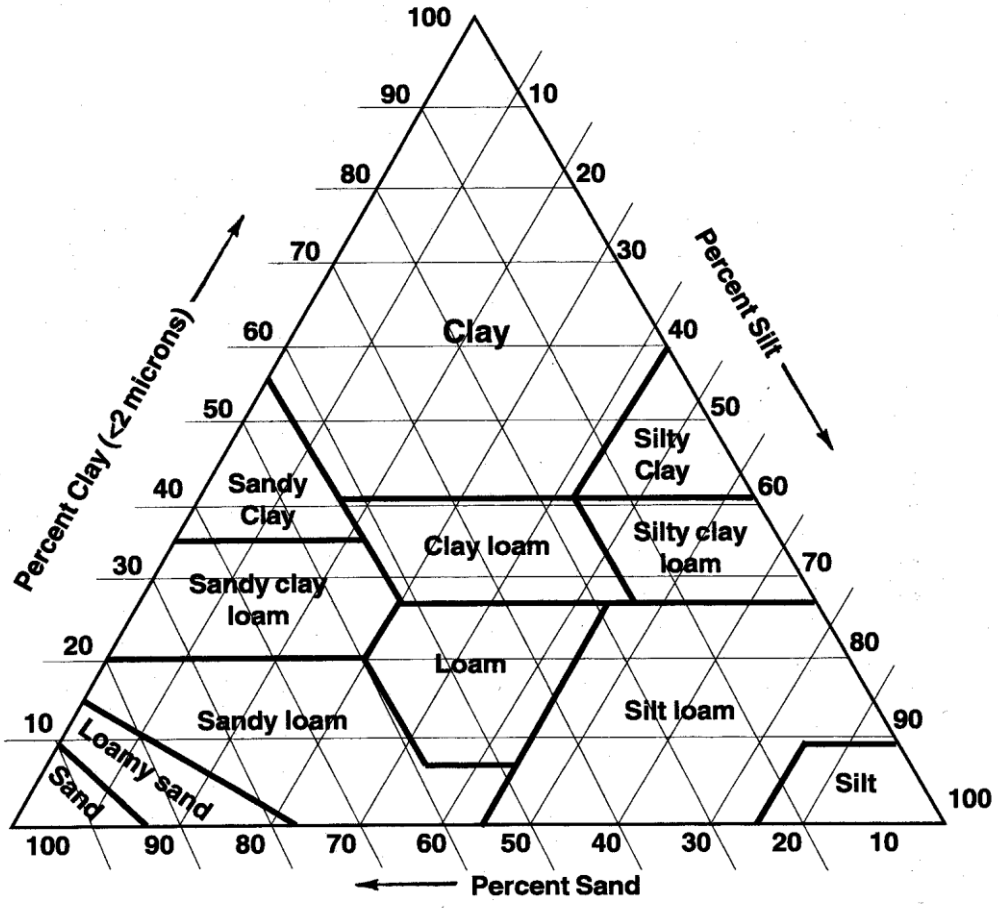
Appendices

Appendix I: Soil textural triangle describing the percent composition of clay, silt and sand in soil (adapted from http://www.nrcs.usda.gov/)



Appendix II: Elemental Estimation Methods

Preparation of sample for analysis

Water samples were filtered with a filter paper and the clear filtrate was used for the elemental analysis. For the elemental analysis of salt crystals, about 10 g of salt crystals were dissolved in de-ionised water, filtered and the clear filtrate was used for the estimation of elemental composition.

1) Estimation of Sodium and Potassium

Sodium and potassium content were measured using flame emission photometry (Grasshoff et al., 2009).

Principle: It involves the excitation of the atoms of alkaline and alkaline earth metals to the higher energy state with the help of thermal energy. When the atoms return to the ground state, the absorbed energy will be released in the form of radiation, at a particular wavelength characteristic of the element. Measurement of the emitted radiation in visible range can give quantitative and qualitative information on the element under investigation.

Procedure: Sodium stock solution of 1000 ppm was prepared by adding 2.5418 g of AR sodium chloride (NaCl) was dissolved in 1 litre of de-ionised water. Similarly, 1.909 g of AR Potassium chloride (KCl) was dissolved in 1 litre of distilled water to obtain a stock solution of 1000 ppm. Standard solution was prepared by diluting the stock solution with distilled water for obtaining the following concentrations, 25, 50, 75 and 100 ppm. In case of sediment samples, the stock and standard solutions were prepared in neutralised 1 N ammonium acetate (pH 7). Sodium and potassium concentrations were measured after selecting the appropriate filter and calibrating the instrument with standard solutions. Concentration of sodium and potassium in unknown samples were determined after appropriate dilutions.

11) Estimation of Calcium and Magnesium

The amount of calcium and magnesium in the samples was determined by complexometric titration of Mg^{2+} and Ca^{2+} against ethylene diamine tetra acetic acid (EDTA) (Harris, 1997).

Principle: This method involves determination of total hardness of the sample, followed by the determination of amount of calcium present. Since total hardness is contributed by Mg^{2+} and Ca^{2+} ions, the amount of magnesium ions can be determined by subtracting concentration of calcium from total hardness. In the total hardness determination, Mg^{2+} and Ca^{2+} forms stable colourless complex with EDTA at alkaline pH around 8. Eriochrome Black-T is used an indicator and the end point is the appearance of stable blue colour. For the determination of calcium ion concentration, the sample is titrated against EDTA but at a higher pH, around 12. Calconcarboxylic acid (Patton and Reeder's Reagent) is used as the indicator and colour change from red to blue indicates the end point.

Procedure: (i) Total Hardness: Ten ml of sample was taken and to this solution, 5 ml of ammonium chloride – ammonium hydroxide buffer and 2 drops of Erichrome black T indicator were added. The wine red solution was titrated against standardised EDTA till the colour changed to blue. The volume of EDTA (A) consumed equals the amount of calcium and magnesium and thereby the total hardness of the sample.

(i) Calcium Conc.: To determine the calcium concentration 10 ml of sample was mixed with 1 ml of 10% NaOH, and let it stand at room temperature for 5 minutes for the precipitation of magnesium ions as magnesium hydroxide. To this mixture, a pinch of Patton and Reeder's indicator was added and titrated against EDTA until it turned blue. The volume of EDTA (B) consumed denotes amount of calcium present. The titre value (A-B) gives the volume of EDTA consumed for magnesium alone.

Calculation

Ca (ppm) = Titre value (B) · (0.02N EDTA) · 0.02004 · (250/y) · 106 /x

Mg (ppm) = Titre value (A–B) · (0.02N EDTA) · 0.01216 · (250/y) · 106 /x

where y = Aliquote (10 ml), x = volume of sample taken (10 ml)

(Note: 1ml of normal solution of EDTA = 0.02004 g of Ca or 0.01216 g of Mg)

III) Estimation of chloride

Chloride ions present in the sample was determined by argentometric titration against silver nitrate solution in the presence of potassium chromate as an indicator (Skoog and West, 1969).

Principle: It involves the formation of white silver chloride as precipitates and the end point is the appearance of red colour due to the formation of silver chromate.

Procedure

About 10 ml of sample was titrated against standard $AgNO_3$(0.02 N) solution with few drops of potassium chromate solution as indicator. On titration, a curdy white precipitate of $AgCl_2$ was formed. The titration was continued till the formation of red coloured Ag_2CrO_4, indicating the end point of titration.

Calculation

Chloride (mg/l) = (T x 0.02 x 35.45 x 1000) / V

Where, T = Amount of silver nitrate solution utilised in ml

V = Volume of sample taken in ml

Appendix III: Denaturing Gradient Gel Electrophoresis (DGGE)

Gel Sandwich Preparation

One large and a smaller glass plate was cleaned with soap, dried, and cleaned with 96% ethanol. The gel bond was cut to the size of the larger glass plate. Some water was added to the surface of the large glass plate. The gel bond was placed hydrophobic side down on this glass plate (you can easily check this by adding a drop of water on the gelbond, it will roll off easily). The gel bond was fixed, without removing the paper sheet, with a roller, and then the paper sheet was removed. The gel bond was dried carefully with some tissues. The rubber gasket was placed in the large glass plate and ensured that there are no air gaps. A set of spacers was cleaned with 96% ethanol, and placed them on left and right site on the gel bond. The smaller glass plate was placed on top, clipped the larger and smaller glass plates with suitable clamps. Ensure that there is no leakage by filling the space with distilled water. The assembled set up was dried with blotting paper.

Preparing the Gel

The required gel solution was Prepared on ice, being high and low denaturant gel according to the mixing Table 1. The gradient maker and tubes were rinsed with distilled water. The screw was closed between the compartments of the gradient maker. The compartments were dried with a tissue. The high and low denaturant solutions were prepared in a 50 ml sterile Falcon tube. When the gel solutions are cooled, APS and TEMED was added to the high and low percentage denaturant solutions, as per Table 2. A magnetic bar was placed on the right compartment. The high solution was poured in the right compartment, and the low in the left. The gradient marker was placed in the magnetic stirrer rotating at minimal speed, opened the screw and the needle as placed between the glass plates. The needle was removed when the gel is poured, switched off the magnetic stirrer and transferred to the Erlenmeyer flask. The compartments were rinsed with distilled water and drain the system. The gel was left to polymerize for 1 h.

Polymerisation catalysts

- TEMED.
- Ammonium Per sulphate (10%): Always prepare fresh APS solution just before casting the Gel

Table 1: Denaturant solution (7.5% gel) (for 100 ml use)

	10 %	20 %	30 %	40 %	50 %	60 %	70 %	80 %	90 %	100 %
40%Acrylamide /Bis (ml)	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8
50X TAE Buffer (ml)	2	2	2	2	2	2	2	2	2	2
Formamide (ml)	4	8	12	16	20	24	28	32	36	40
Urea (g)	4.2	8.4	12.6	16.8	21	25.2	29.4	33.6	37.8	42

Added distilled water to 100 mL. Denaturant solutions were stored in amber bottles at 4°C.

Table 2: Denaturant gel Composition

	Low concentration	High Concentration
Denaturant solution	15 mL	15mL
APS (10%)	110µL	110µL
TEMED	8µL	8µL

Running a Gel

Fresh 1× TAE buffer was added to the buffer tank. Then the instrument was switched on for at least 90 min before electrophoresis, so that the buffer can equilibrate at 60oC. After 1 h of polymerization the comb was removed carefully. The clamps were removed and assembled the polymerized gel setup in the gel cassette. Switched off the instrument and the lid was removed. The sandwich gel cassette was placed into the buffer tank. The instrument was switched on until the upper buffer compartment is filled with buffer. All slots were rinsed with syringe and needle filled with buffer. Samples were loaded in the slots carefully. A mixture of DGGE-PCR products from different bacterial species and archaeal species was applied to DGGE gel as a marker to check the electrophoresis run and to compare fragment migration between gels. The power chord was plugged to the gel cassette. The instrument was switched on after returning the lid. The power supply was switched on and set to 85 V for 16 h.

Appendix IV – Silver Staining

Procedure:

1. Remove folia with gel and fix it in 10% acetic acid for 30 minutes.
2. Remove acetic acid and wash 3 times with distilled water for 5 minutes.
3. Meanwhile, prepare color solution. (should be freshly prepared)
4. Put the gel in color solution for 30 minutes. (should be closed)
5. Wash the gel for 20-30 seconds in distilled water.
6. Remove the distilled water and add 100ml of developing solution.
7. When the color turns yellow remove and add 500ml of developing solution.
8. Watch until band appears.
9. Remove developing solution.
10. Add 10% acetic acid and keep it for 10 minutes.
11. Remove acetic acid and 10% glycerol and keep it for 20 minutes.
12. Remove glycerol and wash it with distilled water.
13. Hang the gel for drying.

Reagents:

1. 300ml of 10% acetic acid.
2. Color solution – 0.3 g of $AgNO_3$
   0.45ml of formaldehyde (37%)
   300ml of distilled water
   Note: Always prepare freshly in an amber bottle and keep it in dark.
3. Developing solution - 12.5 g of Na_2CO_3 (or)
   33.75g of Na_2CO_3.10x hydrate (or)
   8.3g of Na_2CO_3 (free H_2O)
   600ml of distilled water
   1.2ml of formaldehyde (37%)
   0.6ml of Sodium thiosulfate (0.2%)

Appendix V: SDS-PAGE

The whole cell protein analysis was performed by SDS-PAGE (Laemmli, 1974).

Preparation of the gel

Glass plates and spacers were cleaned with acetone swab. Teflon spacers were placed on three sides except on top and clipped with clamps. Molten agar (1% W/V) was poured to seal the three sides from inside. Separating gel mixture was prepared. TEMED and APS were added last, solution swirled gently and poured into the glass plate sandwich to a level of about 4 cm from the top and overlaid with distilled water for uniform setting. After one hour, the water was drained. Stacking gel was poured on top of the separating gel. Teflon comb was inserted in the gel fluid immediately and allowed to set.

Electrophoresis

The comb was removed on setting the gel, 30 µl of protein samples were loaded into each well and the lower and upper tanks filled with running buffe. Power supply was set to a constant current of 30 m amps or 100 volts. When the tracking dye reached the bottom of the gel in about 4 h power supply was turned off. Plates were separated carefully and gel was stained.

Reagents for SDS-PAGE:

(a) Upper Tris (0.1 N)

Tris base: 3.03 g

SDS : 0.2 g

Distilled water: 40 ml

- pH adjusted to 6.8 with concentrated HCl

- Final volume to 50 ml

- Keep at 4°C.

(b) Lower Tris (1.5 M)

Tris base: 18.15 g

SDS : 0.4 g

Distilled water: 90 ml

- pH adjusted to 8.8 with concentrated HCl

- Final volume to 100 ml

(c) Acrylamide

Acrylamide: 29.2 g

Bisacrylamide: 0.8 g

Distilled water: 100 ml

- Filter through Whatman filter paper

(d) Sample buffer

Upper Tris (pH 6.8): 1.25 ml

10% SDS: 3.0 ml

Glycerol: 1.0 ml

β-mercaptoethanol : 0.50 ml

Distilled water: 4.75 ml

- Freeze

(e) Running buffer

Tris base: 3.03 g

SDS: 1.0 g

Glycine: 14.4 g

- pH adjusted to 8.3

- Final volume to 1 liter

(f) Tracking dye

Bomophenol blue: 0.1 g

50% sucrose: 100 ml

S.No.	Constituents	Separating Gel 10% T 0.27%C	Stacking Gel 4%T 0.21%C
1	30%T 8%C	8.4 ml	1.6 ml
2	Lower Tris	6.3 ml	-
3	Upper Tris	-	2.0 ml
4	Distilled water	10.4 ml	4.8 ml
5	APS (10)%	250 µl	100 µl
6	TEMED	20 µl	10 µl

APS – Ammonium per Sulphate

T – Acrylamide concentration expressed in terms, % T

C - Bisacrylamide concentration expressed in terms, %C

TEMED – Tetraethylenemethylethylenediamine

Appendix VI: List of Publications

1. **Kabilan M,** Bhakti Salgaonkar and Judith M.Bragança (2011) *Molecular Microbial Ecology Of Solar Salterns Of Goa And The Isolation Of Culturable Haloarchaea.* New India Publishers.

2. **Kabilan Mani**, Bhakti B. Salgaonkar**,** Deepthi Das and Judith M. Bragança (2012) **Community solar salt production in Goa, India.** *BMC Aquatic Biosystems*. 8:30. (doi:10.1186/2046-9063-8-30).

3. **Kabilan Mani**, Bhakti B. Salgaonkar and Judith M. Bragança (2012) **Culturable halophilic archaea at the initial and crystallization stages of salt production in a natural solar saltern of Goa, India.** *Aquatic Biosystems* 8:15. (doi: 10.1186/2046-9063-8-15).

4. Bhakti B. Salgaonkar**, Kabilan Mani,** Anjana Nair, Sowmya Gangadharan and Judith M. Bragança (2012) **Interspecific interactions among members of family *Halobacteriaceae* from natural solar salterns.** *Probiotics and Antimicrobial Proteins*. 4(2):98-107. (doi: 10.1007/s12602-012-9097-8).

5. **Kabilan Mani**, Sivaraman Chandrasekaran, Bhakti B. Salgaonkar**,** Srikanth Mutnuri, Judith M. Bragança (2014) **Comparison of bacterial diversity from solar salterns and a simulated laboratory study.** *Annals of Microbiology* **(**doi: 10.1007/s13213-014-0944-6).

List of other Publications

1. Bhakti B. Salgaonkar**, Kabilan Mani** and Judith M. Bragança (2011) **Sensitivity of Haloarchaea to eubacterial pigments produced by *Pseudomonas aeruginosa* SB1**. *World Journal Microbiology Biotechnology.* 27:799–804. (doi: 10.1007/s11274-010-0519-z).

2. Bhakti B. Salgaonkar, **Kabilan Mani** and Judith M. Bragança (2013**) Accumulation of polyhydroxyalkanoates by halophilic archaea isolated from traditional solar salterns of India.** *Extremophiles* 17: 787-795. (doi: 10.1007/s00792-013-0561-5).

3. Bhakti B. Salgaonkar, **Kabilan Mani** and Judith M. Bragança (2013) **Characterization of polyhydroxyalkanotes accumulated by a moderately Halophilic Salt pan isolate *Bacillus megaterium* strain H16.** *Journal of Applied Microbiology* 114:1347-1356. (doi:10.1111/jam.12135).

Appendix VII: List of Conferences

1. **Kabilan, M.** and Judith Bragança (2008) *"Isolation of Haloarchaea from solar salinity and low salinity coastal waters of Goa and Tamil Nadu, India"* in International Congress of Environmental Research (ICER-08) at BITS Pilani University on December 18 – 20, 2008.

2. **Kabilan, M.** and Judith Bragança (2008) "*Screening of Haloarchaeal Isolates for the Accumulation of Polyhydroxybutyrates*" in International conference on microbial diversity and metagenomics at Delhi University, Delhi on November 17 – 20, 2008.

3. Bhakti B. Salgaonkar, **Kabilan Mani** and Judith M. Bragança (2010) "*Biodiversity of Haloarchaea in Hypersaline Environments of Saltpans of Goa–India*". (Paper presented at the 9th International Conference of Halophilic Microorganisms HALOPHILES 2010), 29th June - 3rd July 2010, Beijing China.

4. **Kabilan M**, Bhakti Salgaonkar and Judith Bragança (2011) "*Molecular Microbial Ecology of solar salterns of Goa and the Isolation of culturable Haloarchaea*" in national conference on Microbial Diversity and its Application in Health, Agriculture and Industry at Indian Council for Agricultural and Scientific Research, Goa on March 4 – 5, 2011.

5. **Kabilan, M.** and Judith Bragança (2012) "*Culturable Haloarchaeal Diversity in Solar Salterns of Goa, India*" in 9th International Congress on Extremophiles, Sevilla, Spain on September 10 -13, 2012.

6. **Kabilan Mani**, B.B. Salgaonkar, Deepthi Das and J.M. Bragança (2013) "*Demonstrating the Evidence of Life in Extreme Conditions*" in 20th American Society for Microbiology Conference for Undergraduate Educators (ASMCUE), Englewood, Colorado, USA on May 16 -19, 2013.

7. Deepthi Das, Bhakti B. Salgaonkar, **Kabilan Mani** and Judith M. Bragança (2013) "*Resistance of Extremely Halophilic Archaea to Various Heavy Metals*". (Poster Presentation) HALOPHILES 2013 Conference, 23rd – 27th June 2013, University of Connecticut, Storrs, USA.

8. Bhakti B. Salgaonkar, **Kabilan Mani** and Judith M. Bragança (2012) "*Accumulation of Polyhydroxybutyrate by Halophiles from Solar Salterns of India*"**.** (Poster Presented) at *9th International Congress on Extremophiles 2012*, 10th-13thSeptember 2012, Sevilla, Spain).

Appendix VIII : Brief Biography of the Candidate

Personal Details

Name : Mr Kabilan Mani

Education : M.E. (Biotechnology), BITS Pilani University (2010)

: B.E. (Biotechnology), Anna University (2008)

E-mail : kabilan1987@gmail.com

Work experience

1) **Council of Scientific and Industrial Research,** Senior Research Fellowship (CSIR-SRF), 09/919(0017)/2012-EMR-I for the period of four years from 1st April 2012 to 31st March 2016.
2) **Raman Charpak fellowship 2014.** Worked at Microorganismes : Génome et Environnement, university of Blaise-Pascal, France under Prof. Didier Debroas.

Achievements

- **Department of Science and Technology (DST)** Government of India awarded travel grant for attending 9th International Congress on Extremophiles from 10th-13th September 2012, Seville Spain.

No. of publications : 4 (as first author) and 4 (as co-author)

No. of conferences / workshops attended : 8

Appendix IX: Brief Biography of the Supervisor

Prof. Judith M. Bragança completed her Ph.D. from Goa University in 2003 as a CSIR Fellow. Her Doctoral work elucidated the biodiversity of halophilic archaea and explored their mechanisms of resistance to arsenic and cadmium. Dr. Bragança joined the Department of Biological Sciences, BITS Pilani, K K Birla Goa Campus as a faculty in 2004 when BITS Pilani established its third campus in Goa. Since then she is involved in teaching a number of courses at the First Degree and Higher Degree Level. She has also been a Principal Investigator for in house as well as externally funded research projects. Prof. Bragança has over 20 publications in reputed peer reviewed journals and a number of conference proceedings to her credit.

Her research interests include Biodiversity of Halophiles, Biotechnological applications of halophilic archaea and bacteria and Metal tolerance in microorganisms. Under the BITS Pilani – University Immersion Scheme she visited Goethe-University Frankfurt, Germany during June – July 2013, and worked with Prof. Dr. Volker Muller, Dept. of Molecular Microbiology and Bioenergetics, Biozentrum. Prof. Bragança is a member of American Society of Microbiology (ASM), International Society for Salt Lake Research (ISSLR) and Association of Microbiologists of India (AMI). She has been actively involved as a reviewer of international journals from Elsevier, FEMS, Springer, etc. Presently she has three registered Ph.D. students under her tutelage, besides numerous thesis, dissertation and project students working with her.

Besides teaching, Prof. Bragança has been associated with BITS Pilani administration assuming multifarious responsibilities, mainly as Faculty-In-charge, Publications and Media Relations, Convener-Departmental Research Committee, besides being a member of various committees such as the Standing Committee for Students Discipline, Senate, Cross-campus Departmental Committee for Academics, Academic Counseling Board etc.