Bioprospecting of Halophilic Archaea from Indian Solar Salterns

THESIS

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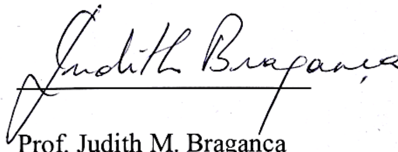
CERTIFICATE

This is to certify that the thesis entitled **"Bioprospecting of Halophilic Archaea from Indian Solar Salterns "** submitted by Deepthi Das, ID No. 2010PH290008G, for award of Ph.D. degree of the Institute embodies original work done by her under my supervision.

Signature of the Supervisor: 

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"It always seems impossible until it is done." ~ Nelson Mandela

<u>This work is dedicated to</u>

My dear ones who told me,

"You Can!"

ABSTRACT

This study aims at isolating halophilic archaea from the solar salterns of India and studying them for various biotechnological prospects. Salterns of India constitute a vast resource of archaeal organisms which have not been explored for their potential biotechnological applications.

Fifteen extremely halophilic isolates obtained from the solar salterns of Goa (west coast) and Tamil Nadu (east coast), India were isolated on five different nutrient rich halophilic media. Fourteen extremely halophilic archaea spanning 6 different genera namely, *Haloarcula* sp. (strains E1, BS2 and E2)*, Halococcus* sp. (strains M3, BK6 and E4)*., Halorubrum* sp. (strains M2, BS17 and M5)*, Halogeometricum* sp. (strain E3)*, Haloterrigena* sp. (strain J1)*, Haloferax* sp. (strains M1, BBK2 and E5.) and one extremely halophilic bacterium *Halomonas* sp. (strain M4) were obtained in the study. The main areas of study were halophilic enzymes, haloarchaeal pigments and the tolerance/resistance of halophilic archaea to metals/metalloids.

Halophilic archaeal isolates were screened for the production of various hydrolytic enzymes (protease, amylase, lipase, esterase, pectinase and cellulase). Seven amylase producers, 3 protease producers, 1 lipase producer, 6 esterase producers and 5 pectinase producers were obtained. Cellulase producers were not seen among the screened halophilic archaeal isolates.

Protease production by *Halococcus* sp. strain E4 was carried out in Norberg-Hofstein (NH) medium supplemented with 1% skimmed silk (SM). The optimum pH and temperature for production of protease was found to be pH 7.0 – 8.0 and 37 °C, respectively. The E4 protease was partially purified and a yield of 32% and a purification fold of 6.39 was obtained. The molecular weight of the active protease fractions was found to be around 67 kDa and 46 kDa. The protease was found to be a metalloprotease and the optimum conditions were pH 8.0 and temperature 60°C. Metal ions Ca^{2+}, Fe^{2+}, and Mg^{2+} contributed to an increase in activity. The E4 protease could tolerate 1% (v/v) non-ionic detergents such as Triton X-100, Tween 80 and was active in organic solvents such as methanol, ethanol and acetone. This is the first report on production and characterization of a protease from the genus *Halococcus*.

Optimum amylase production was observed in NH medium supplemented with 1% starch by *Halogeometricum* sp. E3 in the stationary phase. The optimum pH of the medium was pH 7.0 – 8.0 and temperature was 37 °C for production of the enzyme. The amylase was concentrated 1.64 folds by ultrafiltration with 10 kDa membrane. The E3 amylase was active from pH 5.0 to pH 10.0 and optimum pH required for activity was 8.0-10.0. The amylase was active from 25 °C to 80 °C while the optimum temperature required for activity was 60 °C. It was active in the presence of 1% non-ionic detergents Triton X-100 and Tween 80 and organic solvents such as ethanol, methanol, acetone and butanol. Metals Ca^{2+}, Mg^{2+}, Zn^{2+} increased the activity whereas metals Mn^{2+}, Ba^{2+}, Co^{2+} decreased the activity of the enzyme. This polyextremophilic amylase was found to be an α-amylase. This is the first report of an amylase from the genus *Halogeometricum*.

Bright orange pigments from extremely halophilic archaea, *Haloarcula* sp. (strain BS2 and strain E2) as well as *Halorubrum* sp. (strain M5) were characterized using various techniques and the main component was found to be bacterioruberin. Other derivatives of bacterioruberin: mono-anhydrobacterioruberin, bis-anhydrobacterioruberin, and 2-isopentenyl-3,4-dehydrorhodopin were also detected in the study. The minor carotenoids of lycopene and β carotene were identified in both *Haloarcula* and *Halorubrum* pigments.

Photoprotective activity of the haloarchaeal pigment was evaluated by challenging keratinocyte cell line (HaCaT) and carcinoma cell line (HeLa) with UV after exposure to the pigment. It was seen that in the conditions studied, the pigment extract did not offer photoprotection. However, the pigment extracts from both *Haloarcula* sp. strain E2 (HA) and *Halorubrum* sp. strain M5 (HR) exhibited a protective antioxidant activity, comparable to that of vitamin E, against oxidative stress induced by H_2O_2 and arachidonic acid.

Haloarchaeal strains belonging to four different genera (viz. *Haloferax* sp. strain BBK2, *Halorubrum* sp. strain BS17, *Haloarcula* sp. strain BS2, and *Halococcus* sp. strain BK6) were tested for resistance/ tolerance against metals/ metalloids (viz. Cd, Cu, Pt, Zn, Se and Te). The extremely halophilic archaeon *Haloferax* sp. strain BBK2 grew in the presence of Cd in both complex and minimal media with NaCl concentration varying from 15% to 25%. Cadmium accumulation of 21.08% in the presence of 0.5 mM Cd and 15.19% in presence of 1 mM Cd was observed. Interaction between polysaccharides, proteins, lipids and Cd was recorded. The SEM EDX and XRD analysis proved that the Cd was accumulated by intracellular cell components probably as CdS nanoparticles.

Halococcus sp. strain BK6, *Haloferax* sp. strain BBK2, *Halorubrum* sp. strain BS17 *and Haloarcula* sp. strain BS2 were able to tolerate and grow in complex as well as minimal media in presence of Zn and ZnO NPs. The $ZnCl_2$ resistance in complex/minimal media was seen as *Halococcus* sp. strain BK6 (2.0/1.0 mM) >*Haloferax* sp. strain BBK2 (2.0/1.0 mM) >*Halorubrum* sp. strain BS17 (0.5/0.5 mM) > *Haloarcula* sp. strain BS2 (0.5/0.1 mM) whereas for ZnO NPs resistance was BK6 (2.0/1.0 mM) >BBK2 (2.0/0.5 mM) >BS17 (0.5/ 0.5 mM) >BS2 (0.1/0.1 mM). Among the four genera, *Haloferax* sp. strain BBK2 showed a higher Zn accumulation. The amount (percent) of Zn sorbed on the surface of cells of *Haloferax* sp. strain BBK2 grown in the presence of $ZnCl_2$ was greater (21.77 %) than that of cells grown in presence of ZnO NPs (14.89 %).

Haloarcula sp. strain E2 tolerated and grew in 0.5mM/1mM platinum (Pt) in EHM broth/ agar. Pt was accumulated intracellularly upto 20.84% of wet weight of cells. Organic matter, especially protein interaction with platinum could be inferred from FTIR spectrum. TEM analysis showed irregular shaped and spherical nanoparticles of the size ranging from 5-20 nm. This is the first study showing Pt resistance and accumulation in haloarchaeal cells.

These findings suggest that halophilic archaea from Indian solar salterns can serve as sources for various high value low volume products such as poly extremophilic enzymes and potent antioxidants. High metal resistance of various haloarchaeal strains may be useful in bioremediation of various heavy metals such as cadmium and zinc. Green synthesis of platinum nanoparticles by halophilic archaea which could be used as electrocatalysts or catalytic converters etc., magnetic nanopowders, polymer membranes, coatings, nanofibers, etc., was demonstrated.

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List of Abbreviations and Symbols

AAS – Atomic Absorption Spectroscopy

ATCC - American type culture collection

ATP – Adenosine Tri Phosphate

ATR- Attenuated total reflectance

BABR - Bisanhydrobacterioruberin

BHT - Butylated hydroxytoluene

BLAST - Basic Local Alignment Search Tool

BR - Bacterioruberin

BSA - Bovine Serum Albumin

CBB – Coomassie Brilliant Blue

CM - Complete Media

CM Cellulose- Carboxy methyl cellulose

CPD – Critical Point Drier

Da/ kDa – Dalton/ kilodalton

DDBJ- DNA Databank of Japan

DGD - Diglycosyl Diether

DMEM- Dulbecco's Modified Eagle's Medium

DMSO- Di-methyl Sulphoxide

DNA - Deoxyribonucleic Acid

DNS - 3,5-Dinitrosalicylic acid

DPPH- 1,1-Diphenyl-2-picrylhydrazyl radical

DW- Distilled Water

EDAX- Energy Dispersive Analysis of X-rays

EDTA- Ethylene Diamine Tetra Acetic acid

EHM - Extremely Halophilic Medium

FBS - Fetal Bovine Serum

FTIR-Fourier Transform Infrared Spectroscopy

GPC - Gel Permeation Chromatography

HA – *Haloarcula* pigment

HPLC - High Performance/Pressure Liquid Chromatography

HR – *Halorubrum* pigment

JCM - Japan Collection of Microorganisms

LCMS - Liquid Chromatography-Mass Spectrometry

LPSN - List of Prokaryotic Names with Standing in Nomenclature

MABR – Monoanhydrobacterioruberin

MEOR - Microbially Enhanced Oil Recovery

MHM - Moderate halophilic medium

MIC - Minimal Inhibitory Concentration

MTCC - Microbial Type Culture Collection

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide

MUSCLE - MUltiple Sequence Comparison by Log Expectation

MWCO - Molecular Weight Cut Off

NADH - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

NCBI - National Centre for Biotechnology Information

NGSM - NaCl Glucose Synthetic Medium

NH – Norberg-Hofstein

NP/np - Nanoparticles

NSM - NaCl Synthetic Medium

NSMY- NaCl Synthetic Medium supplemented with 0.1% Yeast extract

OD- Optical Density

PCI- Phenyl Chloroform Isoamyl-alcohol

PCR - Polymerase Chain Reaction

PDA- Photodiode Array

PG - Phosphatidyl Glycerol

PGP Me- Monomethylated Phosphatidyl Glycerophosphate

PMSF - Phenyl Methane Sulphonyl Fluoride

PPM - Parts Per Million

RNA - Ribonucleic Acid

RO- Reverse Osmosis

ROS - Reactive Oxygen Species

rRNA - ribosomal RNA

RT - Room Temperature

S-DGD - Sulfated Diglycosyl Diether

SDS- Sodium Dodecyl Sulphate

SDW - Sterile Distilled Water

SEM- Scanning Electron Microscopy

SM – Skimmed Milk

S-TeGD- Sulfated Tetraglycosyl Diglycosyl Diether

S-TGD- Sulfated Triglycosyl Diglycosyl Diether

TE- Tris-EDTA

TEM -Transmission Electron Microscopy

TLC- Thin Layer Chromatography

TPP – Triple Phase Partitioning

UV – Ultra Violet

VE – Vitamin E

XRD - X-ray diffraction

µ - Growth rate

Chapter I

Introduction and Review of Literature

"Science is an endless search for truth. Any representation of reality we develop can be only partial. There is no finality, sometimes no single best representation. There is only deeper understanding, more revealing and enveloping representations."- Carl R. Woese (2004)

1.1 INTRODUCTION

Halophiles are salt-loving organisms (Halo - salt; phile - loving) (Oren, 2008). Various shades of red colour imparted to aquatic environments, cured fish and other animal products led to the discovery of halophilic archaea a century ago (Harrison and Kennedy, 1922; DasSarma et al., 2010; Oren, 2012). Although salts are required for growth of all organisms, halophiles are characterized by their tolerance/ requirement of hypersaline conditions for growth (DasSarma and DasSarma, 2017). Archetype of halophilic organisms are found in all three domains of life, for example, *Halobacterium* sp. NRC-1, an archaeon; *Halomonas* sp., a bacterium; *Dunaliella salina*, a unicellular eukaryote (Pavitra et al., 2017). Multicellular halophilic eukaryotes include brine shrimp and brine fly larvae (DasSarma and DasSarma, 2012).

Based on optimum salinity required for growth, they can be classified as Non-halophiles (<1% NaCl), Halotolerant (non-halophiles which can tolerate high salt concentrations), Slight halophiles (1-3% NaCl), Moderate halophiles (3-15% NaCl), Extreme halophiles (>15% salt) (Kushner and Kamekura, 1988; Kushner, 1993; Biswas and Paul, 2017). Though halophiles are now known to be present ubiquitously, they are generally found in higher numbers in several hypersaline econiches (DeLong, 1998; Moissl-Eichinger et al., 2017).

Hypersaline environments (salt concentrations higher than that of sea water i.e., 3.5% dissolved salts) are broadly classified as thalassohaline and athalassohaline. Thalassohaline environments are mostly formed by the evaporation of sea water and are characterized by an ionic composition that is predominantly like sea water with the major cation and anion being Na^+ and Cl^-, respectively. The pH is neutral or slightly alkaline. Athalassohaline environments have an ionic composition that varies greatly from that of sea water. They may contain very

high concentration of carbonates/bicarbonates and may have divalent ions such as Mg^{2+} or Ca^{2+} as the dominant cations and their pH may range from slightly acidic to highly alkaline. Solar salterns are an example of thalassohaline environments and is a reservoir for halophilic organisms with potential applications in biotechnology industry (Oren, 2002 a; Babavalian et al., 2013).

There are two main adaptation mechanisms used by halophiles to survive in hypersaline environments. Aerobic and extremely halophilic archaea as well as few anaerobic halophilic bacteria accumulates inorganic ions (generally KCl) to balance the osmotic pressure (Oren, 1999, 2002 a; Yin et al., 2015). The presence of high salt in the cytoplasm requires adaptation in the protein machinery of the haloarchaeal cells. Hence, they have developed acidic proteins to increase solvation and improve function in high salinity (DasSarma and DasSarma, 2012). Halophilic or halotolerant bacteria accumulate or produce high concentrations of various compatible solutes/ osmolytes (i.e., water soluble organic compounds of low molecular weight) to deal with the osmotic pressure in a salty environment (Oren, 2002 a; Oren, 2008; Quillaguamán et al., 2010; Roberts, 2005). Osmolytes have neutral charge at physiological pH and do not interfere with any metabolic processes (DasSarma and DasSarma, 2012). They stabilize the biological structures thereby imparting resistance to multiple stresses like salts, heat, desiccation or even freezing conditions (Delgado-García et al., 2012). Common osmolytes found in halophilic bacteria are ectoine/ hydroxyectoine. Amino acids, glycine, betaine and other osmotic solutes have also been reported to be accumulated in small amounts (Louis and Galinski, 1997; Vargas et al., 2008; Ventosa and Nieto, 1995; Yin et al., 2015)

The halophiles have developed resistance to various toxic substances including heavy metals to survive in their hypersaline econiche. They produce specialized biomolecules like pigments, biosurfactants, specialized proteins, extracellular polysaccharides (EPS), polyhydroxyalkanoates, etc., to adapt to the deleterious hypersaline environment. They also have some distinct advantages in comparison to other microorganisms like a high potential for genetic manipulation; ease of culturing and maintenance as well as a minimum necessity for maintaining aseptic conditions as the salt present in the growth medium acts as a deterrent for contaminants. These attributes of halophilic organisms, along with the fact that they hold

enormous untapped potential, command the attention of marine biotechnologists world-wide. (Oren, 2010; Biswas and Paul, 2017; Pavitra et. al., 2017).

1.2 REVIEW OF LITERATURE

1.2.1 Archaea:

Earth's microbiota is remarkably diverse with the presence of life being discovered even in econiches normally deemed uninhabitable (DeLong, 2003). Archaea forms the third domain of life, which thrives in but is not limited to extremes of pH, salinity, temperature, etc (Fig 1.1). The word 'archaea' comes from the Greek word 'arkhaios' meaning 'ancient/ primitive'.

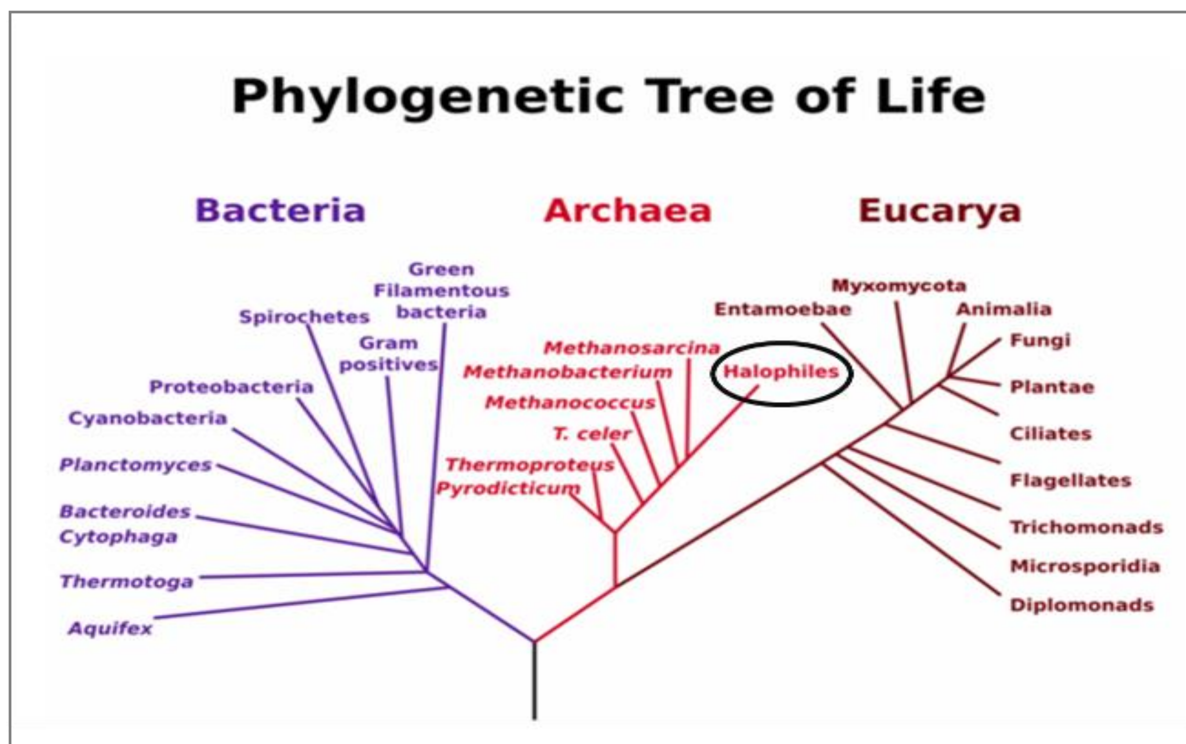


Fig 1.1: The phylogenetic rooted tree showing the three domains of life: Bacteria, Archaea and Eukarya. Reproduced with permission from Woese et al., 1990. Copyright (1990) National Academy of Sciences.

The third domain, apart from Eukarya and Bacteria, was proposed by Carl Woese and co- workers in the late 1970s through their ribosomal RNA studies (Woese and Fox, 1977). Though they were thought to reside only in extreme conditions, now, with the advent of

advanced techniques, more light has been shed on their cosmopolitan presence (Cavicchioli, 2011). Table 1.1 compares the characteristic features of the three domains of life.

Table1.1 A comparison between the characteristics of Bacteria, Archaea and Eukarya (Aves et al., 2012; Cavicchioli et al., 2011; Raymann et al., 2014).

Trait	Bacteria	Archaea	Eukarya
Nucleus	Absent	Absent	Present
Telomeres	Absent	Absent	Present
Organelles	Absent	Absent	Present
Histones	Absent	Present	Present
Spliceosomal introns	Absent	Absent	Present
Carbon linkage in lipids	Ester	Ether	Ester
Phosphate backbone of lipids	Glycerol-3-phosphate	Glycerol-1-phosphate	Glycerol-3-phosphate
First amino acid in protein synthesis	Formyl methionine	Methionine	Methionine
Ribosomes	70S	70S	80S
Operons	Present	Present	Absent

Carl Woese (1993) remarked that even after a decade of the acceptance of archaea as the third domain of life, not much effort was devoted to the study of archaea since it was viewed as bacteria-like organisms for a long time. He recommended a more phylogenetically proper perspective of seeing archaea as quite distinct from bacteria in genetic make-up and stressed on its relationship with various aspects of eukaryotes. He also speculated that a study of archaea might even shed light on the nature and evolution of eukaryotic cells (Woese, 1993).

1.2.2 Hypersaline econiche

Hypersaline environments are universal and are expanding because of natural as well as human activities. Many industrial processes use salts which, when finally released into the environment as effluent, causes an increase in salinity in the area. Also, some natural ecological occurrences such as petroleum reserves are generally associated with hypersaline brines. Halophiles are known to grow and thrive in such environments which can be detrimental to the growth of other organisms (DasSarma and DasSarma, 2012). Halophilic microorganisms have been isolated from sources as diverse as salted foods, solar salterns, Dead sea, salt mines, Permian rock salt and even the plumage of captive flamingoes (Roh et. al., 2009; Mani et. al., 2012; Oren et. al., 1990; Norton et. al., 1993; Stan-Lotter et. al., 2002; Yim et. al., 2015) Viable haloarchaea have been isolated from fluid inclusions from geographical formations that are millions of years old. Fig 1.2 shows orange coloured haloarchaea entrapped in salt crystals (Braganca and Furtado, 2009).

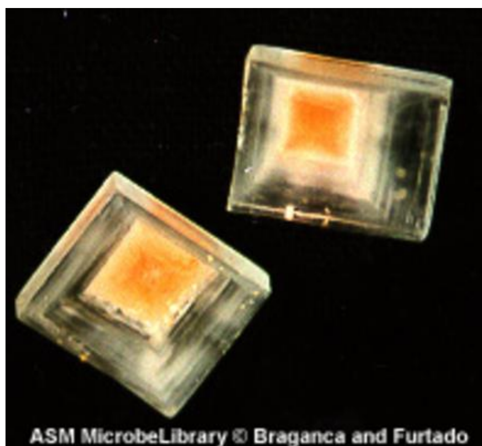


Fig 1.2 Haloarchaeal organisms entrapped in salt crystals obtained from solar salterns of Goa, India (Braganca and Furtado, 2009) Licensed by CC BY-NC-ND 4.0.

To appreciate the need for their adaptations in these econiches, there is a necessity to understand hypersaline environment. Whether thalassohaline or athalassohaline, the salt content of a hypersaline environment can range from 3.5% to 35%. As the salt content of a solution increases, the water content per unit volume decreases. Brock and co-workers defined low-water content or water activity('a') as the "ratio of the vapor pressure of the air in equilibrium with a substance or solution to the vapor pressure at the same temperature of pure water". Water activity is unitless and it ranges from 0 to 1 with pure water and sea water having

the 'a' value of 1 and 0.980, respectively. Growth is supported only up to a water activity of 0.72. Halophilic archaea grow at water activity approaching this lower limit (Brock et al., 1994). A decrease in water activity implies lesser water availability for cellular functions such as dissolution of nutrients, general metabolic processes, proteins and nucleic acids hydration, etc.

High total ionic composition; stable high temperatures, intense light and ultra violet rays (in solar salt pans), extreme pH, or presence of electron acceptors other than O_2 are some of the other distinctive attributes of hypersaline environments. Consequently, halophiles have acquired compensatory mechanisms and/or adapted their biochemistry survive in this hostile milieu (Litchfield, 1998).

1.2.3 Halophilic archaea

Halophilic archaea or Haloarchaea belongs to the class *Halobacteria*, which represents one of the largest groups within the domain Archaea (Gupta et al., 2015). Use of culture dependent and culture-independent methods have triggered a continuous increase in the number of known haloarchaeal genera and species (Galinski and Trüper, 1994). Haloarchaeal classification has been carried out using conserved signature proteins and conserved signature insertions/deletions as phylogenetic markers. According to this classification, the class *Halobacteria*, within the phylum *Euryarchaeota,* is divided into three orders: *Halobacteriales*, *Haloferacales* and *Natrialbales* (Gupta et al., 2015; Gupta et. al., 2016). The class *Halobacteria* currently includes 177 species with validly published names, placed in 48 genera (LPSN 2015, Antunes, 2017).

1.2.4 Characteristic features of haloarchaea

Most of the haloarchaea produce isoprenoid-derivative pigments which contributes to their red, orange or pink color (Oren and Rodríguez-Valera, 2001). Occasionally, haloarchaeal blooms cause the whole water body to turn pink or red (Fig 1.3). Though many species of this class are commonly isolated from high salt environments (Grant et al., 2001a; McGenity and Oren, 2012), the salinity range required for growth of different members vary considerably with some

species requiring only 0.8M NaCl for growth (McGenity and Oren, 2012). Low salt tolerant haloarchaea have been found in estuarine environments (Bochiwal, 2009).

Haloarchaea may be differentiated from halophilic bacteria on the basis of the absence of murein in their cell walls, the presence of pseudouridine or 1-methyl pseudouridine instead of ribothymidine in the 'common arm' of the tRNAs (Krieg, 2001; Grant et al., 2001b; Oren, 2006), their possession of ether linked lipids, as well as other attributes commonly shared with other members of the archaeal domain (Kates, 1996; Kamekura and Kates, 1999; Oren, 2006).

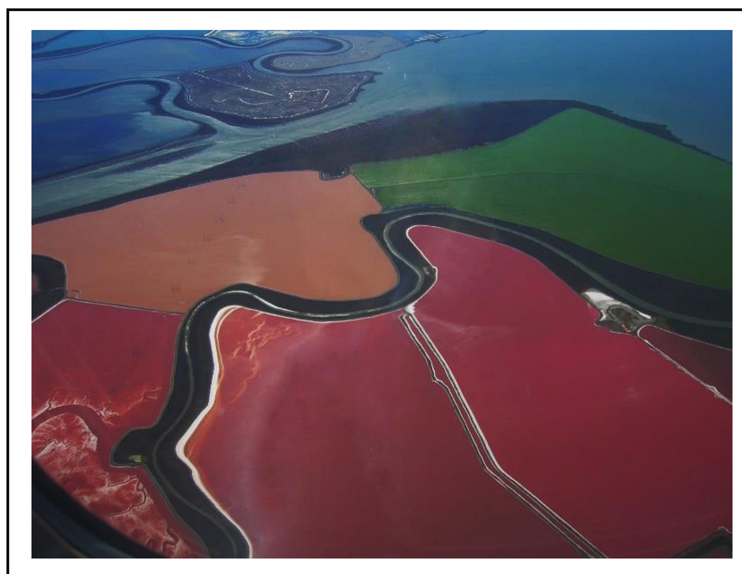


Fig 1.3: Aerial view of the salt evaporation ponds of San Fransisco Bay Area. The red colour being attributed to the haloarchaeal blooms. https://commons.wikimedia.org/wiki/File: SanFranciscoBaySaltPonds.jpg (Under CC-BY-SA 3.0 license).

The cell walls of haloarchaea, are commonly composed of glycoprotein subunits rather than peptidoglycan (typical of bacteria), (Guan et al., 2012); however, some members have cell walls that are made up of complex polysaccharides or repeating units of a poly(L-glutamine) glycoconjugate (Niemetz et al., 1997; Oren, 2012).

They are very diverse with respect to their morphology, physiology and other characteristics. Fig 1.4 shows a mixed culture of halophiles (A) and a pure culture of the unique square archaeon *Haloquadratum* sp. (B).

While most haloarchaea exhibit Gram-negative staining, staining is found to be variable in some (Grant et al., 2001b; Oren, 2006). They can be alkaliphilic, acidophilic and/or psychrotolerant; motile or non-motile; and their morphology can be coccoid, rod shaped and even triangular or square shaped; most of them being pleomorphic depending on variation in growth conditions like higher temperatures or the lower sodium chloride (NaCl) concentrations compared to the optimal growth conditions. (Grant et. al., 2001b; Oren, 2006; Burns et. al., 2007; Minegishi et. al., 2010b, Hezayen et. al., 2001).

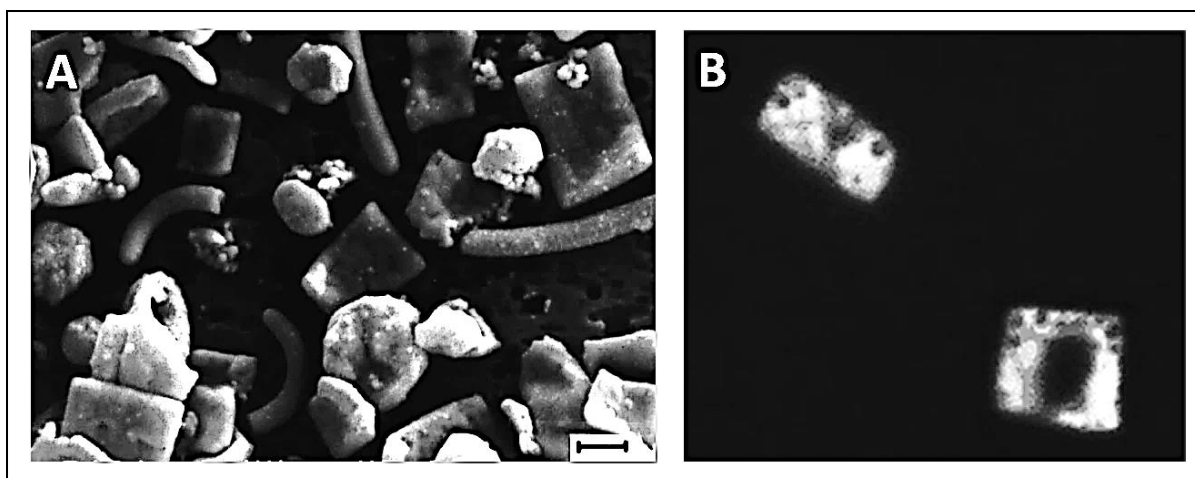


Fig 1.4 (A) Mixed community of halophilic microorganisms from a saltern crystallizer pond near Alicante, Spain. Reproduced with permission (CCC License No:4372450165036) from Oren, 2002 b (B) Unique square cells of *Haloquadratum* sp. Reproduced with permission from Burns et. al., 2007 (CCC Licence No:4353120367777).

Polar lipids in haloarchaea have been analyzed extensively by various groups (Kates, 1996; Kamekura et al., 1997; Kamekura and Kates, 1999; Oren, 2006, 2012). High G+C content, characteristic of haloarchaea, stabilizes the DNA in presence of the high cation concentrations in their cytoplasm (Litchfield, 1998). Polyploidy is a common trait in haloarchaea. This provides ecological and evolutionary advantages like protection against the DNA damage caused by irradiation, desiccation or mutagens (Zerulla and Soppa, 2014).

1.2.5 Salt adaptation in haloarchaea

An excess of salts causes osmotic imbalance in cells. Sodium is also known to have electro-chemical and osmotic interactions with proteins and nucleic acids thereby disrupting their function/ conformation. Aggregation of proteins and collapse of their 3D structure is a typical effect of salt in mesophilic proteins. Hence, for survival, halophiles encounter a constant energy stress to cope with the entry of excess sodium into the cell (Oren, 2002b; Valentine, 2007). Response to this continual energetic challenge is an important factor which differentiate archaea from bacteria with the key role played by membrane composition supported by several additional adaptations in every level of cell function. In general, bacteria concentrate more on utilizing new or variable resources and less on adapting to energy stress. The adaptations of archaea to chronic energy challenges provide a distinct benefit in a variety of adverse milieu (Valentine, 2007).

Glycosylation of haloarchaeal proteins of the S-layer in haloarchaea is an adaptation for growth in hypersaline conditions and it is also believed to offer protection against proteases. Salinity of the surrounding environment determines the type of sugars involved in N-glycosylation of the S-layer and the position in which these sugars are added to this glycoprotein (Guan et. al., 2012; Jarrell et. al., 2014).

Halophilic proteins are known to contain a higher ratio of acidic amino acids like glutamic acid/ aspartic acid in comparison to basic amino acids (Fig 1.5). This seems to be one of the requirements for protein activity at higher salinity. The surface negative charge imparted by the acidic amino acids increase the layer of hydration around the protein thereby increasing its solvation and preventing the aggregation and precipitation which is generally seen in the non- halophilic proteins under conditions of high salinity (Reed et al.,2013; Dym et al., 1995, Karan et al., 2012). These proteins are also characterized by decreased number of Lysine residues, a higher content of small non-polar hydrophobic residues like Glycine, Alanine and Valine (Madern et al., 1995).

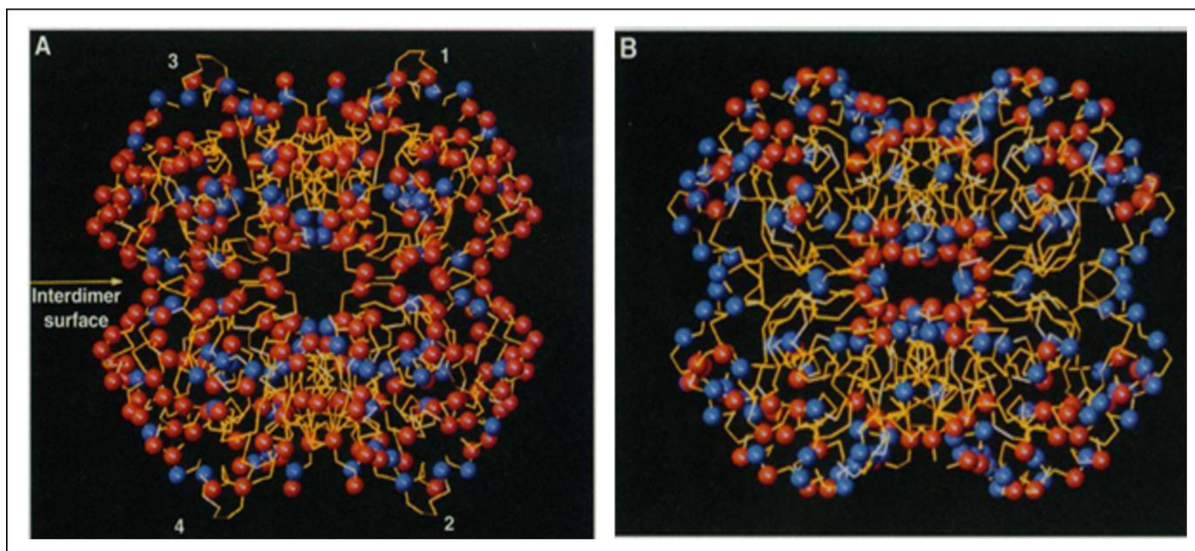


Fig 1.5 Comparison of the three-dimensional tetramer structures of halophilic malate dehydrogenase (A), and its non-halophilic counterpart (B). Red balls-acidic residues; blue balls- basic residues. The numbers 1 to 4 indicate four different monomers. (Dym et al., 1995). CCC Licence No: 4372930217205.

Ion-pair or salt-bridge is another major contributor of protein stability (Dill, 1990; Pace, 1990). Salt bridges are electrostatic bonds between oppositely charged groups. In halophiles, they are more abundant and stronger in comparison to mesophiles and even thermophilic proteins (Dym et al., 1995). 80% of salt-bridges in halophilic archaea are conserved and are involved in halostability. Halophiles employ almost twice the number of salt bridges compared to their non halophilic counterparts. Halophilic proteins have lower content of bulky hydrophobic residues. This deficit of hydrophobic force is balanced by the increased number of salt bridges. The extensive network salt-bridges in halophilic proteins which improve cooperative stabilization of protein structure indicates their extremophilic design (Nayek et al., 2014).

Many species of haloarchaea produce buoyant gas vesicles (DasSarma and Arora, 1997). Molecular oxygen has low solubility in concentrated salt solutions found in hypersaline environments particularly at higher temperatures. The gas vesicles in the cells enable them to float to the surface where more oxygen is present. This might also facilitate photophosphorylation due to increased availability of light. Another interesting fact is the left-

handed Z-DNA conformation seen in few haloarchaeal DNA; but whether this has any implication in extremophilicity is yet to be explored (DasSarma and DasSarma, 2012).

1.2.6 Bioprospecting of halophilic archaea

Mankind has been using halophiles knowingly or unknowingly for at least 5000 years. The reddening of the brines, due to an increase in the number of haloarchaea, contributes to the absorption of light energy, thereby increasing water evaporation and speeding up the process of salt crystallization (Oren, 2002; Lee, 2013). Other ancient applications include the production of fish sauce, soy sauce and other traditional fermented foods. Present day applications/ potential applications of haloarchaea and their metabolites may be summarized as follows:

1.2.6.1 Enzymes from halophilic archaea (Halophilic enzymes/ Halozymes)

Haloarchaeal enzymes are biocatalysts/ enzymes produced by halophilic archaea and are noted for their ability to function under one or more extreme conditions of salinity, temperature, pH, low water activity etc. (Bonete and Martínez-Espinosa, 2011). Research on the physiology and enzymology of haloarchaea is still in infancy but the interest on various aspects of haloarchaea is gaining ground in the recent times from their genomic as well as proteomic point of view (Joo and Kim, 2005; Karadzic and Maupin-Furlow, 2005; Kirkland et al., 2008). The discovery of extremophilic enzymes are revolutionizing the field of biotechnology. (Fore et al., 2006). Though the enzymes from halophilic archaea have the catalytic activity comparable to their bacterial counterparts, the ability to function in high salinities and conditions of low water activity makes them unique (Mevarech et al., 2000; Britton et al., 2006). The same properties such as the presence of high surface negative charge which increase their stability in high salt conditions could also cause the stability to decrease in the absence of salts, which in turn might limit its application ((Reed et al., 2013; Madern et al., 2000; van den Burg, 2003). However, few mutational studies have shown that it is possible to increase the salt dependence of a non-halophilic protein and also to decrease the salt dependence of a halophilic protein (Esclapez et al., 2007; Tadeo et al., 2009). Ongoing studies

on combining halophilic enzymes and reverse misclles will further expand the range of applications for these enzymes (van den Burg, 2003; Marhuenda-Egea and Bonete, 2002).

The industrial enzymes market is estimated to be valued at $4.61 Billion in 2016 and is projected to grow at a Compound Annual Growth Rate (CAGR) of 5.8% from 2017 to 2022 (Markets and markets, 2016). The global market for industrial enzymes is segmented into cleaning agents, animal feed, food and beverages and biofuel production, and was valued at $3.1 billion in 2010 (BCC research, 2012). The major enzymes involved in these applications can be classified broadly as carbohydrases (amylase, pectinase, cellulase, xylanase, etc.), proteases, lipases and others. Carbohydrases represent the fastest growing section in terms of growth rate. With newer applications such as egg processing, protein fortifying being developed, the search for novel and exotic enzymes from unexplored sources is gaining ground (Markets and markets, 2014). Most industrial processes are carried out in harsh physiological conditions such as extremes of pH, temperature, pressure, low water activity, etc. Extremozymes or enzymes from extremophilic microorganisms which have their optima at various extremes are the ideal candidates (Kakhi et al., 2011). Apart from being highly efficient, the increased use of enzymes has helped lower the use of hazardous / harmful chemicals hereby reducing the environmental impact. (Grant and Heaphy, 2010; Sinha and Khare, 2012a).

Halophiles form a relatively unexplored group of extremophiles compared to thermophiles, alkaliphiles etc. Though they have been used traditionally for solar salt production and even for processing of foodstuffs, few products from halophiles (for example, beta carotene from *Dunaliella* and ectoine from moderately halophilic bacteria) have been able to penetrate the global market (Oren, 2010). Halotek, a German company established in 2016, have started marketing products like bacteriorhodopsin, membrane lipids, carotenoids etc. from *Halobacterium salinarium* (http://halotek.de) but no haloarchaeal enzyme has reached the level of commercial production and distribution till date. Interest in hydrolases from extremely halophilic organisms began as early as 1956 when Baxter and Gibbons suggested that all enzymes from obligate extreme halophiles are halophilic (Baxter and Gibbons, 1956). In the last decade or two, many hydrolases from various halophilic archaeal

sources have been screened, isolated and purified and ongoing research for novel sources of extremozymes is ensuing across the world (Akmoussi-Toumi et.al., 2018; Menasria et. al., 2018; de Castro et al., 2006; Kakhki et al., 2011; Minegishi et al., 2013; Moshfegh et al., 2013; Pérez-Pomares et al., 2003; Siroosi et al., 2014). Table 1.2 shows few haloarchaeal enzyme producers.

Halophilic archaea cope with several stresses simultaneously viz., high salinity of upto 30%, high temperature, ultra violet radiations, pH variations, etc. Time and again, it has been proven that, the enzymes from these organisms display polyextremophilicity i.e., the ability to function optimally in more than one of these extreme conditions (Karan et al., 2012).

Types of stress encountered in industries like high temperature and high pH can be dealt with by specialized enzymes from thermophiles and alkaliphiles, respectively (Oren, 2010). However, the condition most frequently encountered in synthetic chemistry, and in various chemical purification methods is the ‘dry’ or low water activity condition.

The halophilic enzymes or halozymes function optimally under such conditions and it has been proved that the high salt required to maintain the function and structure of the halozymes may be partially replaced by organic solvents such as di-methyl sulphoxide (DMSO) (Karan et al., 2012; Alasafadi and Paradisi, 2013). Hence it is very important to identify new sources and study these unique enzymes as well as its producers in detail to fine tune the catalytic activity in order to make an entry in the industrial level. These enzymes have a huge market in detergent industry where chemical formulations have to be designed for high pH and varying ionic conditions. Many hydrolases (proteases, lipases, detergents, cellulases, etc.) are already in use in several modern laundry detergents (Grant and Heaphy, 2010; Sinha and Khare, 2012b).

The enzymatic potential of the various halophilic organisms isolated from hypersaline niches of the Indian subcontinent has hardly been explored (Kumar et al., 2012). Part of this study focuses mainly on the isolation of extremely halophilic archaea from various salt pans of India, their characterization by morphological, biochemical and molecular techniques as well as screening for the production of various hydrolytic enzymes.

Table 1.2 Extremely halophilic archaea producing few biocatalytically relevant enzymes (Adapted from Antunes et al., 2017 with permission. CCC License No: 4406990862018)

Enzyme	Organism	Stability/activity	Reference
β-Galactosidase	*Haloferax lucentense*	Optimal activity at 23 % NaCl	Holmes et al. (1997)
β-Xylanase	*Halorhabdus utahensis*	Optimal activity at 5–15 % NaCl	Wainø and Ingvorsen (2003)
β-Xylosidase	*Halorhabdus utahensis*	Optimal activity at 5 % NaCl	Wainø and Ingvorsen (2003)
Amylase	*Halobacterium salinarum*	Optimal activity at 1 % NaCl	Good and Hartman (1970)
Amylase	*Halorubrum xinjiangense*	Optimal activity at 23 % NaCl	Moshfegh et al. (2013)
Amylase	*Haloferax mediterranei*	Optimal activity at 17 % NaCl	Pérez-Pomares et al. (2003)
Amylase	*Natronococcus amylolyticus*	Optimal activity at 15 % NaCl	Kobayashi et al. (1994, 1992)
Amyloglucosidase	*Halorubrum sodomense*	Optimal activity at 7.5 % NaCl	Oren (1983); Chaga et al. (1993)
Class I fructose aldolase	*Haloarcula vallismortis*	Optimal activity at 2.5 M KCl	(Krishnan and Altekar 1991)
Lipase	*Natronococcus* sp.	Optimal activity at 23 % NaCl	Boutaiba et al. (2006)
Protease	*Natronobacterium* sp.	Optimal activity at 5.5 % NaCl	Yu (1991)
Protease	*Haloferax mediterranei*	–	Stepanov et al. (1992)
Protease	*Halobacterium salinarum*	Optimal activity at 23% NaCl	Ryu et al. (1994)
Serine protease	*Halobacterium salinarum*	–	Izotova et al. (1983)
Serine protease	*Natrialba asiatica*	Optimal activity at 10−15 % NaCl	Kamekura and Seno (1990); Kamekura et al., 1992
Serine protease	*Natrialba magadii*	Optimal activity at 6–9 %NaCl	Giménez et al. (2000)
Serine protease	*Natronococcus occultus*	Optimal activity at 6 % NaCl	Studdert et al. (1997)

1.2.6.2 Haloarchaeal pigments

Most haloarchaea have red to orange coloration imparted mostly by carotenoid pigments (Tomita,1983, Yatsunami et al., 2014). A molecule specific entity (chromophore) in the pigment captures photons and facilitates the excitation of an electron to a higher orbital; the visual effect or the colour observed being caused by the non-absorbed energy reflected or refracted into the eye (Delgado-Vargus et al., 2000). The initial reports of carotenoid production by members of *Halobacteriaceae* family were published in 1966-1967 (Schwieter et al., 1966; Kelly and Jensen, 1967).

The non-polar lipids of haloarchaea are largely C50 carotenoids, namely bacterioruberins (Fig 1.6) as well as its derivatives 2-isopentenyl-3,4-dehydrorhodopin (IDR), bisanhydrobacterioruberin (BABR), and monoanhydrobacterioruberin (MABR) as the major carotenoids (Kushwaha et al., 1974; Rønnekleiv and Liaaen-Jensen, 1995; Kushwaha et al., 1975; Kelly and Jensen, 1967) along with small fractions of C-40 carotenoids (such as lycopene and β-carotene) and C30 isoprenoids (squalenes), retinal, vitamin MK-8 and C40-carotenoids such as carotene and lycopene, as the minor carotenoids (Asker et al., 2002; Yatsunami et al., 2014). Some species may also produce the ketocarotenoid canthaxanthin in addition to other carotenoids (Naziri et al., 2014).

There is very little information in the literature about the carotenoids of halophilic archaea in comparison those from other organisms. Despite the vast number of reports on that subject, only 1.3% of them are related to haloarchaeal carotenoids (Rodrigo-Banos et al., 2015). The first report on biosynthesis of carotenoids in haloarchaea stated that the biosynthetic pathway for synthesis of C40 carotenes started from isopentenyl pyrophosphate which was converted to trans-phytoene, to trans-phytofluene, to ζ-carotene, to neurosporene, to lycopene, to gamma-carotene, and finally lead to the formation of β-carotene. This pathway is unique: the cis isomers of phytoene and phytofluene, components of the main pathway of carotene biosynthesis in higher plants, are not present in halophilic archaea (Kushwaha et al., 1976).

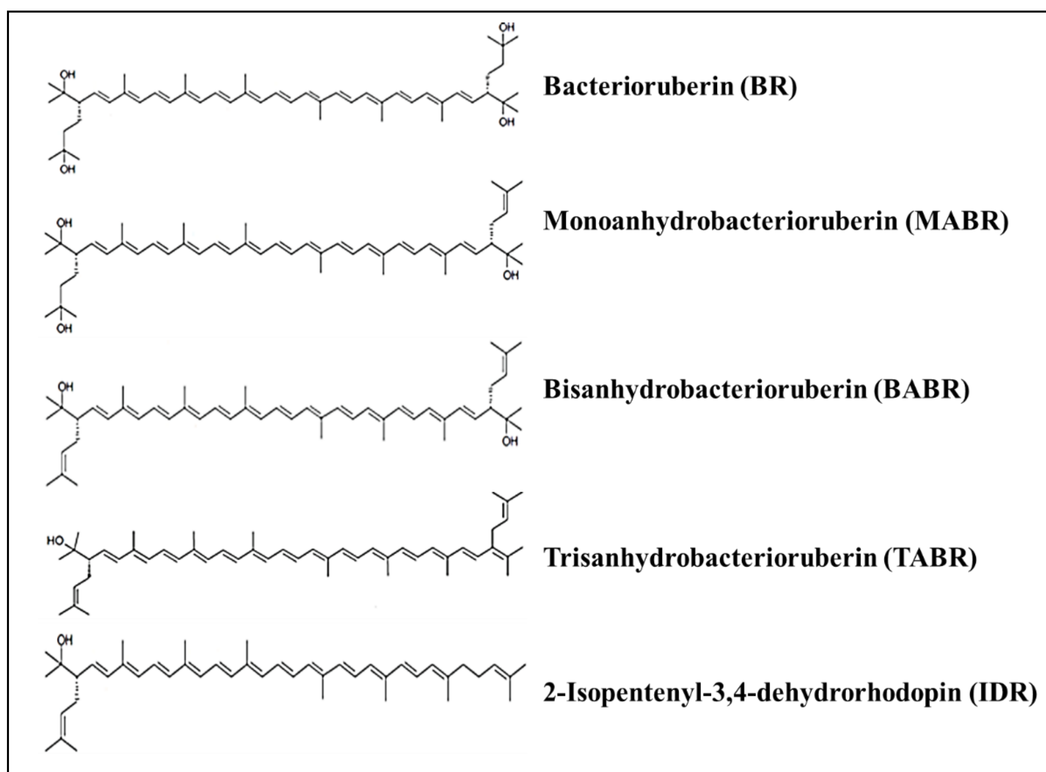


Fig 1.6 Chemical structures of bacterioruberin, the major pigment found in most haloarchaea, and its derivatives. Reproduced with permission from Rodrigo-Baños et al., 2015. (CC BY 4.0)

However, the studies showing the effect of nicotine and glycerol in bacterioruberin synthesis hypothesized that bacterioruberin is formed by addition of C5 isoprene units to each end of the lycopene chain, followed by the introduction of four hydroxyl groups (Kushwaha and Kates, 1976; Kushwaha and Kates, 1979).

Reports on bacterioruberin synthesis by *Halobacterium* NRC-1 shows the existence of multiple genes for several steps in carotenoid production indicating the possibility of more than one biosynthetic pathway (Peck et al., 2001; DasSarma et al., 2001). Genes named c0507, c0506, and c0505, identified in *Haloarcula japonica*, encoded a carotenoid 3,4-desaturase (CrtD), a bifunctional lycopene elongase and 1,2-hydratase (LyeJ), and a C50 carotenoid 2″,3″-

hydratase (CruF), respectively. These enzymes catalyze the reactions that convert lycopene to bacterioruberin in this organism (Fig 1.7) (Yang et al., 2015).

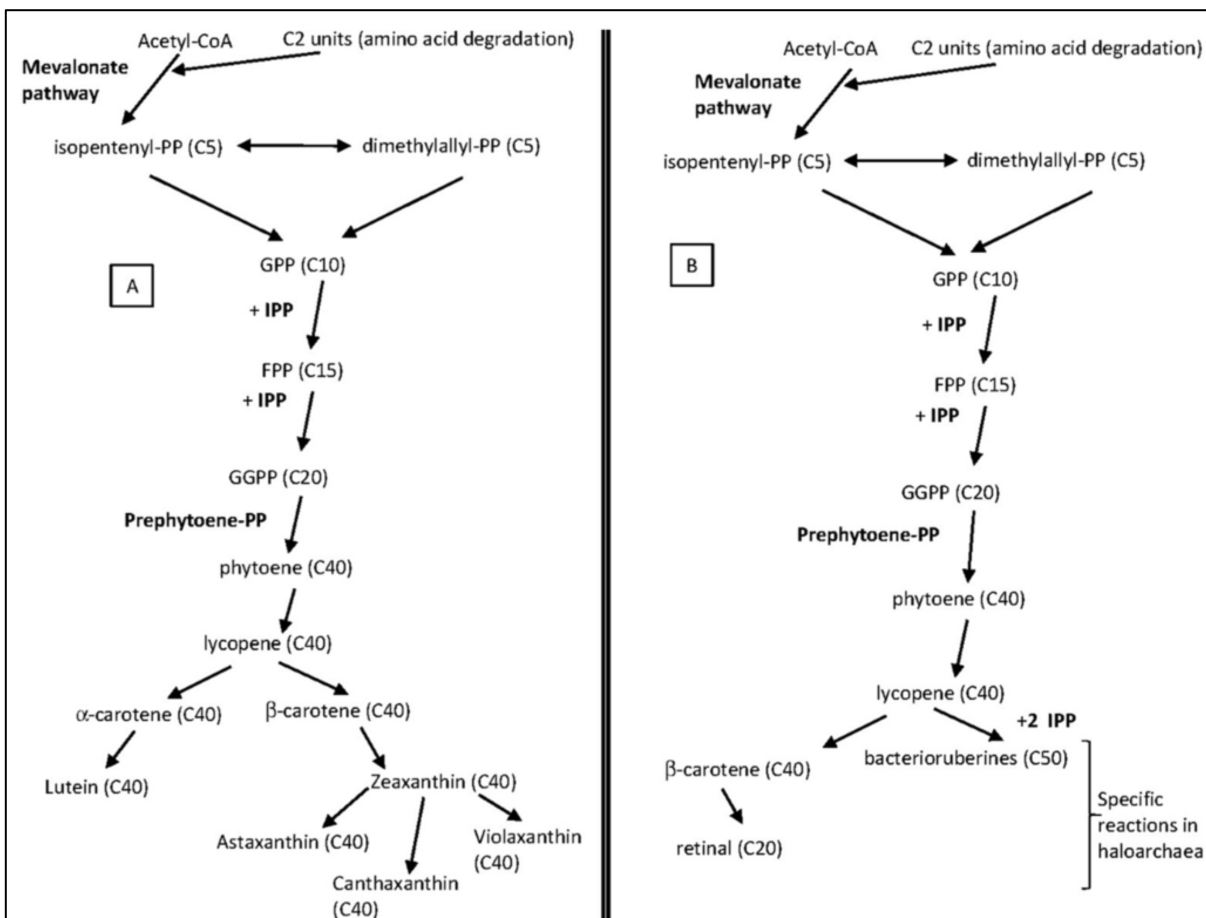


Fig 1.7 Comparison between the biosynthesis of isoprenoids in photosynthetic organisms (A) and the biosynthesis pathway proposed in haloarchaea (B) on the basis of the results reported from *Halobacterium*, *Haloarcula* and preliminary evidence from *Haloferax* genomic analysis. IPP: isopentenyl-diphosphate; GPP: geranyl diphosphate; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate. Reproduced with permission from Rodrigo-Baños et al., 2015. (CC BY 4.0)

Carotenoids are competent scavengers of hydroxyl free radicals and singlet oxygen species and reactive nitrogen species; their antioxidant properties being attributed to their chemical structure, mainly, the number of conjugated double bonds and the type of structural end-group (Yatsunami R et al., 2014, Shahmohammadi et al., 1998, Albrecht et al., 2000, Abbes et al., 2013, Chisté et al., 2011). One such carotenoid with well-established antioxidant property is β-carotene (Sies and Stahl, 2004a). β-carotene has been commercialized and is

currently in use as a natural food colorant, antioxidant, multivitamin preparations, health food products (nutraceuticals) and even as an additive in cosmetics (Margesin and Schinner 2001; Oren 2002, 2010). The hydroxyl free radical scavenging effect of bacterioruberin from *Rubrobacter radiotolerance* was much higher than commercially available β-carotene using thymine degradation studies (Saito et al., 1997).

The scavenging capacity of bacterioruberin extracted from *Haloarcula japonica* also exhibited higher 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging than β-carotene (Yatsunami et al., 2014). This is due to the presence of 13 conjugated double bonds in bacterioruberin as opposed to 11 in β-carotene. Ourisson and Nakatani (1989) reported the role of bacterioruberin in reinforcing the membrane structure of *Halobacterium* spp. The photoprotective effect of haloarchaeal pigment has been demonstrated using a colored wild type strain and a colorless mutant of *Halobacterium salinarium* (Dundas and Larsen, 1963). However, there have been very limited reports on the application of bacterioruberin as an antioxidant.

In the 1990s, several independent studies showed that the halophilic archaea can be used as a good source of natural carotenoids as they produce these pigments at high concentration. This increased the interest in large scale production by utilizing suitable bioprocess engineering tools, like customized bioreactors (Rodrigo-Baños et al., 2015).

1.2.6.3 Metal tolerance and accumulation in haloarchaea:

Rapid industrialization and other anthropogenic activities have resulted in drastic environmental pollution. Natural processes like surface runoffs, weathering and/or erosion and anthropogenic activities like mining, industrial effluents, agricultural runoffs and sewage have led to the accumulation of toxic metals and their derivatives like nanoparticles in the environment (Weber et al., 2013; Zhao et al., 2012). There have been world-wide reports on metal (Cd, Cr, Cu, Mn, Pb, Zn, Ni and Fe) contamination of soil (sediments) and water bodies like rivers, lakes, estuaries, etc. due to industrial processes (Jain et al., 2007; Amin et al., 2009; Kaushik et al., 2009; Rath et al., 2009; Uluturhan, 2010). These polluted environments set the stage for the transformation of native microflora into potential metal-resistant strains (Naik and Furtado, 2017). Few metal tolerant halophilic archaea and their MIC have been tabulated in

Table 1.3. The potential applications of metal tolerance and accumulation in microorganisms are:

(i) Bioremediation: High concentrations of heavy metals maybe found in econiches like solar salterns (Pereira et al., 2013) and estuaries (Ratheesh Kumar et al., 2010) because they form effective ecological sinks for water borne metals from river or sea water (Chapman and Wang, 2001). Natural and anthropogenic activities like erosion, mining, agriculture, and waste disposal further contribute towards metal pollution at these sites (Ross, 1994; Tabak et al., 2005). The use of microbial-based bioremediation attracts considerable interest, and research on the use of halophiles for metal bioremediation is flourishing (Bini, 2010). *Halobacterium* and *Haloferax* sp. has been used for treatment of industrial wastewaters containing high salt or hypersaline environments polluted with nitrates, nitrites (Kapdan and Erten, 2007; Martı´nez-Espinosa et al., 2007).

(ii) Green synthesis of metal nanoparticles: Metal(loid)s resistance observed in halophilic archaea made them likely candidates for the environmentally-sound green synthesis of metal nanoparticles (NPs). Silver nanoparticle production has been reported in *Halococcus salifodinae* BK3 (Srivastava et al., 2013), selenium nanoparticles (SeNPs) by *Halococcus salifodinae* BK18 (Srivastava et al., 2014). Since metal uptake and synthesis of NPs are intracellular, haloarchaea have an added advantage as they can be used simultaneously for metal(loid)s bioremediation and NPs synthesis (Antunes et al., 2017).

1.2.6.4 Bioplastics

Polyhydroxyalkanoates (PHAs) are a heterogenous family of polyesters, produced as intracellular carbon storage compounds by halophilic organisms. Some examples are PHB, P(HB-co-HV), etc. (Salgaonkar and Braganca, 2015). They are biodegradable, biocompatible and water impermeable- properties which make them a practical alternative to polyethylene and polypropylene which are non-biodegradable oil-derived thermoplastics. (Divya et al., 2013; Margesin and Schinner, 2001; Ventosa and Nieto, 1995).

Table 1.3 Heavy metal tolerance of halophilic archaea isolated from various econiches

Haloarchaeal sp.	Isolation site	Metal ion(s)	MIC (mM)	References
Halobacterium sp.	Solar salterns, Spain	Zn^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}, As^{3+}, Cd^{2+}	0.5, 1–2.5, 0.5, 0.01, 20, ≤2.5	Nieto et al. , 1987
Halocula sp.	Solar salterns, Spain	Zn^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}, As^{3+}, Cd^{2+}	0.05, 2.5, 0.05, 0.01, 10, 0.05	Nieto et al., 1987
Halobacterium sp.	Estuarine salterns, Goa, India	Li^{+}, As^{5+}, As^{3+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}/Cd^{2+}/Fe^{2+}/Fe^{3+}	200, 60, 3, 50, 5, 2.5, 2.0	Khandavilli et al., 1999
Halobacterium sp. NRC-1	Salt lake	Fe^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}	7.5, 2, 0.6, 0.05, 2, 1.2	Kaur et al., 2006
Haloferax mediterranei	Solar saltern	Zn^{2+}, Ni^{2+}, Pb^{2+}, Cr^{6+}	1, 2.5, 2.5, 5.0	Popescu and Dumitru, 2009
Haloferax sp. TL 5	Telega salt lake, Romania	Zn^{2+}, Ni^{2+}, Pb^{2+}, Cr^{6+}	0.5, 2.5, 2.5, 5.0	Popescu and Dumitru, 2009
Halobacterium saccharovorum, *H. salinarum*, *Natronobacterium gregoryi*	Solar salterns Tamil Nadu, India	Zn^{2+}, As^{3+}, Cd^{2+}	0.01, 0.001, 0.001	Williams et al., 2012
Haloarcula sp. IRU1	Hypersaline Urmia lake, Iran	As^{5+}	0.65	Taran et al., 2013
Haloferax sp. BBK2	Solar salterns , Goa, India	Cd^{2+}	4.0	Das et al., 2014
Haloferax volcanii BBK2, *Halorubrum* strain BS17, *Haloarcula japonica* BS2, *Halococcus* strain BK6	Estuarine salterns,	Zn^{2+}	1.0, 0.5, 0.5, 1.0	Salgaonkar et al., 2015

1.2.6.5 Bacteriorhodopsin

Bacteriorhodopsin, discovered in the early 1970s, is an interesting 25 kDa integral membrane protein molecule present in most members of the Class *Halobacteriaceae* (Ovchinnikov et al., 1979; Hampp, 2000; Oren, 2002). It is a key player in the haloarchaeal photosynthetic system where it acts as a light driven proton pump which when coupled with a membrane-bound ATPase generates energy (i.e. Adenosine triphosphate, ATP). Bacteriorhodopsin is known for its (a) photosensitivity and cyclicity to illumination and (b) remarkable stability to chemical as well as thermal degradation (Hampp, 2000) which promote its application in various aspects such as optical computing and new types of optical memories, artificial neural networks, spatial light modulators, holography, artificial retina, etc (Margesin and Schinner, 2001).

1.2.6.6 Other applications

Exo-polysaccharides produced by haloarchaea has a huge potential in paper, textile, paint and pharmaceutical industries as gelling agents, emulsifiers, thickeners and stabilisers (Litchfield, 2011; Ventosa and Nieto, 1995). Additional applications include use of gas vesicles for bioengineering, microbially enhanced oil recovery (MEOR) processes, saline soil recovery for agriculture, liposomes with increased resistance for cosmetic industry, etc. (Litchfield, 2011; Oren, 2002; Ventosa and Nieto, 1995).

An understanding of the full potential of haloarchaea will stem from further studies of their biodiversity and a detailed insight into their molecular functions. Remaining bottlenecks may be eliminated with the development of more efficient and reliable bioprospecting tools. (Antunes et. al., 2017; Yin et. al., 2015). Areas which can be termed as hypersaline environments are on the rise due to various anthropogenic and natural activities. Hypersaline environments may even be created as in case of saltpans. The occurrence of novel and unique biomolecules in halophiles and a possibility for their mass cultivation using artificial hypersaline environments suggests that their products would form a huge part of the market in future (DasSarma and DasSarma, 2012).

1.3 GAPS IN EXISTING RESEARCH

It is evident from the literature that halophiles are a reservoir of biotechnologically valuable products and are still largely unexplored as compared to Eubacteria. The objectives were framed by taking the following gaps in account:

1. Its known that the biodiversity differs with differing geographical locations. Enormous potential benefits of halophilic archaea from salt pans of India have not been well documented.
2. The search for novel enzymes which can catalyse reactions under harsh ambient conditions (extremozymes) have gained renewed interest because of two main reasons: (i) biocatalysts/ enzymes are known for their specificity (ii) they are less damaging to the environment than other inorganic/ chemical catalysts currently in use. Halophiles which are known to be a source of polyextremophilic enzymes have not been explored much in this aspect.
3. The haloarchaeal pigments have a well-established role in protection of haloarchaeal cells from the effect of UV stress encountered in the environment. These pigments, which are generally C50 carotenoids. However, there are hardly any reports regarding the application of these pigments with respect to their photoprotective or antioxidant activity.
4. Haloarchaea inhabit coastal and hypersaline econiches where the metal concentration is quite high due to the fact that they serve as sinks for runaway industrial waste etc. Hence, they are inherently resistant to metals, which, in the same amounts will be generally toxic to other organisms. There are only a few studies on metal accumulation in haloarchaeal cells. Their potential in bioremediation and their role in nanoparticle synthesis needs to be explored further.

1.4 AIMS OF THIS RESEARCH WORK

This study aims to shed light on the enzymatic potential of haloarchaea isolated from Indian solar salterns, to characterize pigments and study their photoprotective and antioxidant effects on keratinocytes (HaCaT) and cancerous (HeLa) cell lines and finally to gain an idea about the metal resistance and accumulation in halophilic archaea and to check nanoparticle synthesis.

1.5 OBJECTIVES OF THE RESEARCH WORK

- Identification and screening of halophilic archaea for their metal tolerance and capability to produce various hydrolytic enzymes and pigments
- Optimization of culture conditions for production of enzymes and partial characterization of amylase and protease
- Characterization of pigments and evaluation of its photoprotective and antioxidant properties
- Evaluation of metal resistance in halophilic archaea

Chapter II

Identification and characterization of extremely halophilic isolates obtained from various solar salterns of India

2.1 INTRODUCTION

Solar salterns are an example of thalassohaline environments and are reservoirs for halophilic organisms (Oren, 1998; Babavalian et al., 2013). Halophiles are relatively unexplored compared to other extremophiles such as acidophiles, thermophiles, etc. with reference to their biotechnological applications (Oren, 2010). They must cope with several stresses simultaneously such as high salinity up to saturation conditions, high temperature, ultra violet radiations, high pH, etc. Halophiles use two different mechanisms to combat the high salinity in their surroundings. The moderately halophilic as well as the halotolerant organisms accumulate small molecules called osmolytes which serve as osmo-protectants for the cell components. Halophilic archaea as well as a few extremely halophilic bacteria accumulate ions such as K^+, Na^+, Cl^- etc. in their cytoplasm to maintain osmotic balance with their surrounding environment (Karan et al., 2012; de Lourdes et al., 2013).

Haloarchaea exhibit various morphologies of rods, discs and cocci. Some also possess triangular, square shaped or pleomorphic morphologies. They have unique lipids with ether bonds and isoprenoid hydrophobic side chains (Kamekura, 1993; Kates, 1978; Oren, 2012). These polar lipids serve as chemotaxonomic signatures and are employed routinely to discriminate between different genera. They are mostly aerobic and are known to survive in acidic as well as basic pH. Several distinctive features such as purple membrane having bacteriorhodopsin, a photo-repair system of red- orange pigments, high tolerance to metal ions and various salts are worthy of interest. This chapter deals with the isolation of haloarchaeal cultures from various solar salterns of India in different halophilic nutrient media. A total of 15 isolates were obtained and identified using morphological, chemotaxonomic and molecular techniques and the culture sequences were deposited in DNA Data Bank of Japan (DDBJ)/ GenBank.

2.2 MATERIALS AND METHODS

2.2.1 Materials:

All the chemicals used in the study, procured from Hi Media, Mumbai, India, were of certified AR grade unless otherwise specified. Glassware was washed with water rinsed with distilled

water before each use. Media was sterilized at 121ºC and 15 psi pressure. Distilled water was used for all media preparation.

2.2.2 Sample collection, enrichment, growth and maintenance of halophilic cultures

Samples were collected from three different salt pans in Goa, i.e., Sinquetim (15º14'N; 73º57'E), Siridao (15º26'N, 73º52'E), Ribandar (15º3º′N, 73º51′E), as well as from two salterns viz. Marakkanam (12º12'N; 79º52'E) and Vedaranyam (10º35'N; 79º.78'E) in Tamil Nadu. Sediment samples (~10 grams) and water samples (~ 1litre) were procured separately in sterile bags and bottles respectively.

The samples were stored at 4ºC till further use. Five different media (i) Extremely Halophilic Medium (EHM) (Salgaonkar and Braganca, 2015; Salgaonkar, 2015), Moderately Halophilic Medium (MHM) (Ventosa et al,1982), NaCl Tryptone media (NTYE) (Braganca and Furtado, 2009), NaCl Tri-sodium citrate media (NT) (Elevi et al., 2004) and modified JCM 168 (Enache et al., 2007) with NaCl content varying from 17.8 to 25.0% were used for enriching the halophilic organisms (Table 2.1).

Hundred microliters of the liquid samples were directly spread on all five nutrient media agar plates. The sediment samples were suspended in 15% NaCl solution; vortexed and centrifuged. 100µl of the supernatant was spread on to all five nutrient media plates. The plates were incubated for 10-15 days at room temperature. The colonies obtained were purified by successive re-streaking on the respective nutrient media till isolated colonies were obtained. The pure cultures obtained were grown in the respective nutrient media in which they were isolated. The culture plates were stored at room temperature in sealed plastic covers and sub-cultured at 30-40 days interval.

To revive the microbial cultures after a long period of time, glycerol stocks preparation is important. Therefore, the culture was grown up to an OD_{600nm} of 1.0 and mixed with 40% sterile glycerol in 1:1 ratio by vortexing. These stocks were labelled and stored at -80ºC.

2.2.3 Identification of the isolates

2.2.3.1 Morphological characterization

(i) Gram Staining:

Cell suspensions were prepared on clean glass slides in a drop of 15% (w/v) NaCl solution and air-dried. The cells were desalted with 2% (v/v) acetic acid (Dussault, 1955) followed by standard Gram staining (Appendix I) and observed using phase contrast microscope (100X objective; Olympus BX41) (Mani et al., 2012b).

Table 2.1: Composition of the nutrient media* used for isolation and maintenance of halophiles used in the study.

Ingredients (g/L)	EHM	MHM	NTYE	NT	JCM168
NaCl	250	178	250	250	200
$MgSO_4$	20	1	20	20	20
KCl	2	2	5	2	2
Yeast extract	10	10	3	10	5
Tryptone	-	-	5	-	-
Trisodium citrate	-	-	-	3	3
$CaCl_2$	0.36	0.36	-	-	2
Peptone	5	5	-	-	-
Casamino acids	-	-	-	-	5
Sodium glutamate	-	-	-	-	1
NaBr	0.23	0.23	-	-	-
$NaHCO_3$	0.06	0.06	-	-	-
$FeCl_3$	trace	trace	-	-	0.036
$MnCl_2$	-	-	-	-	trace

*pH was adjusted to 7.0-7.4 using 1M NaOH. 1.5-1.8% agar-agar was used as solidifying agent

(ii) Scanning Electron Microscopy:

Fifty to hundred microliters of cell suspension in 15% (w/v) NaCl solution was mounted onto glass coverslips, air dried and desalted with 2% acetic acid (Dussault, 1955). The cells were fixed with 2% gluteraldehyde. The cells were dehydrated using a series of

increasing concentrations (10-100%) of acetone (Mani et al., 2012b). These dehydrated samples were dried further using Critical Point Dryer (CPD) (Leica EM CPD 300, Austria). The samples were then coated with Gold- Palladium using Leica EM ACE-200 sputter coater and mounted onto aluminum stubs for analysis using scanning electron microscope (Quanta FEG 250, Netherlands). Detailed protocol for sample preparation is given in Appendix II.

2.2.3.2 Biochemical characterization

(i) Polar lipid analysis: Halophilic isolates in their exponential phase of growth (5-6 days old culture) were centrifuged at 10,000 rpm for 10 min to separate the cells. The cell pellets obtained were suspended in a mixture of chloroform: methanol in the ratio 1:2 (v/v). The extraction process was carried out for 4-6 hrs. The suspension was centrifuged (10,000 rpm, 20 min) and the supernatent was stored in a dark bottle. The pellet was vortexed with methanol: chloroform: water in the ratio 2:1:0.8 (v/v). The supernatent obtained after centrifugation of this suspension was pooled with the stored supernatent and a mixture of chloroform: water (1:1 v/v) was added to achieve phase separation. The organic phase containing the lipids was separated completely by centrifugation, collected in a clean dry glass container, and allowed to dry by evaporation. Thin layer chromatography was carried out on silica gel plates (Silica gel 60 F254, Merck). The dried lipids were resuspended in chloroform and approximately10-100µl was spotted on the TLC plates. Chloroform: methanol: acetic acid: water (85: 22.5: 10: 4, v/v) was used to separate the lipids (Oren et al., 1996; Litchfield et al., 2000; Elevi et al., 2004). Glycolipids were visualized by spraying with 0.5% (w/v) α – napthol in 50% (v/v) methanol-water followed by 5% (v/v) H_2SO_4 in ethanol and spots were detected by heating the TLC plate at 100°C. Phospholipids were visualized by spraying molybdenum blue spray reagent (Sigma-Aldrich) (Bligh and Dyer, 1959; Kates, 1977).

(ii) Carbohydrate utilization: A stock (10% w/v) of various sugars (viz., glucose, ribose, sucrose, fructose, mannitol, maltose, sorbitol, and lactose) were prepared and sterilized separately at 121°C for 15 minutes. For qualitatively analyzing the carbohydrate utilization, each sugar solution was added to sterilized Norberg and Hofstein medium to obtain a final concentration of 1% (v/v). Growth was observed by visually checking for turbidity. The results were noted after 10-15 days of growth at 37°C (Salgaonkar, 2015).

(iii) Pigment analysis: The cells were separated by centrifugation at 10,000 rpm, 4°C for 15 minutes. Chloroform: methanol (1:2 v/v) was added to the wet cell pellet and vortexed for 5 minutes (Das et al., 2014). The suspension was centrifuged and the supernatent was taken for pigment analysis. The pigment was analyzed by spectrophotometric scan in the range of 200 to 800 nm using a UV- Visible Double Beam Spectrophotometer (Shimadzu, Japan, UV-2450) with chloroform: methanol (1:2 v/v) as the blank. The extraction procedure was carried out under dim light and the pigment extracts were collected in tubes covered with aluminum foil as the pigments tend to get photo-oxidized.

2.2.3.3 Molecular characterization

(i) Genomic DNA Extraction:

Archaeal isolates: One ml of culture broth (5–6 days old) was centrifuged at 5,000 rpm for 5 min. The pellet was resuspended in 200 µl of sterile distilled water. To this, 200 µL of buffer-saturated phenol was added and incubated at 60ºC for 60 min. This suspension was centrifuged at 8,000 rpm for 5 min. Four hundred microliters of cold ethanol were added to the aqueous phase for DNA precipitation. The precipitated DNA was washed with 70 % ethanol and resuspended in nuclease free water (Dyall-Smith, 2008).

Bacterial isolate: Two ml of 12 hours old bacterial culture in MHM medium was centrifuged at 10,000 rpm for 5 min. The pellet was re-suspended in a mixture containing 400 µL SET buffer (Appendix III), 50 µL lysozyme (10 mg/ml) and 20 µL proteinase K (15 mg/mL) and, incubated at 37°C for 30 min after which, 50 µL of 10% (w/v) SDS was added. The mixture was incubated for another 30 min at 37°C. After incubation, 500 µl of PCI (Appendix III) was added and mixture was centrifuged at 13,000 rpm for 10 min. Following this, 300 µL of CI (Appendix III) was added to the supernatant and centrifuged at 13,000 rpm for 10 min. The aqueous phase was removed, and the DNA was precipitated by adding 1/3rd volume of isopropanol and incubating at 4°C overnight. The precipitated DNA was pelleted by spinning at 13,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol and resuspended in TE buffer (Appendix III) (Pospiech and Numann, 1995).

(ii) Amplification of 16S rRNA gene using PCR:

Archaeal isolates: The extracted genomic DNA was used as template for the amplification of 16S rRNA gene fragment with primers A109(F) AC(G/T) GCTCAGTAACACGT and 1510 (R) GGTTACCTTGTTACGACTT (Mani et al., 2012 b ; Birbir et al., 2007). Each PCR reaction mixture contained 10X Taq buffer, 2 mM $MgCl_2$, 10 mM of dNTPs (Sigma), 10 µM of each primer (IDT technologies, Singapore), 2U Taq Polymerase and 1 µl of template DNA. The reaction was carried out as follows:

i. Initial denaturation for 5 min at 94ºC
ii. Denaturation for 30 s at 94ºC
iii. Annealing for 40 s at 53.5ºC
iv. Elongation for 60 s at 68ºC (35 cycles of ii, iii, iv)
v. Final elongation for 5 min at 68ºC.

Bacterial isolates: 16S rRNA gene fragment was amplified using 27(F) 5′-AGAGTTTGATCMTGGCTCAG3′ and 1492(R) 5′-GGTTACCTTGTTACGACTT-3′ (Frank et al., 2008) under the following conditions:

i. Initial denaturation for 5 min at 94°C
ii. Denaturation for 30 s at 94°C
iii. Annealing for 30 s at 52°C
iv. Elongation for 75 s at 72°C (30 cycles of ii, iii, iv)
v. Final elongation was for 10 min at 72°C.

Amplified product was subjected to electrophoresis on a 1.5% agarose gel and was found to be approximately 500-1400 bp in size. The purified product was sequenced bi-directionally, using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). 16S rRNA sequences were submitted to DDBJ (DNA Databank of Japan)/ NCBI (National Centre for Biotechnology Information). Multiple sequence alignment was done using MUltiple Sequence Comparison by Log- Expectation (MUSCLE). Distances were calculated using the Kimura two-parameter (Kimura, 1980) correction in a pair-wise deletion procedure. The phylogenetic tree was reconstructed with Neighbor-Joining (NJ) algorithm using Molecular Evolutionary

Genetic Analysis MEGA X. Percentage support values were obtained using a bootstrap procedure using 100 replicates (Tamura et al., 2011; Wright, 2006; Mani et al., 2014).

2.3 RESULTS AND DISCUSSION

2.3.1 Sampling sites

The sampling sites are from the west coast (Siridao, Ribandar and Sinquetim in Goa) and east coast (Vedaranyam and Marakkanam in Tamil Nadu) of India. Goa is lined by Arabian sea whereas Tamil Nadu has its coastal area facing Bay of Bengal in the east and Indian Ocean in the south. The rainfall patterns are considerably different in these two states and this plays an important part in salt production pattern and the resident microbial community (Litchfield et. al., 2000; Mani et al, 2012 a).

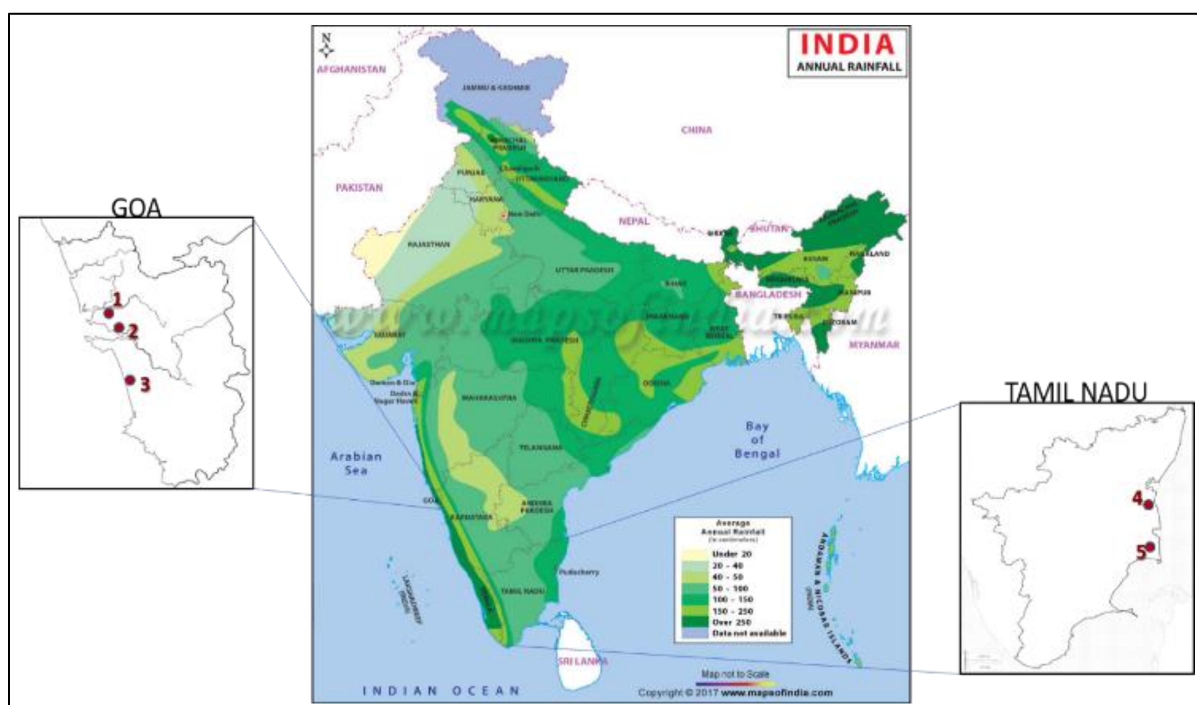


Fig 2.1: Sampling sites: Siridao (1), Ribandar (2), Sinquetim (3) in Goa and Marakkanam (4), Vedaranyam (5) in Tamil Nadu. Annual rainfall obtained in various parts of India is depicted in the central map (www.mapsofindia.com).

Goa experiences higher seasonal fluctuation with heavy rainfall in the monsoon season accounting for over 95% of the total annual rainfall. Hence, salt production cycle has three stages (i) monsoon period (ii) pre-salt harvesting phase (iii) salt harvesting phase. In this period (spanning the entire year), the salinity will increase from ~3% in the monsoon period to ~30%

in the salt harvesting phase. Total annual rainfall in Tamil Nadu is less than 25% of the rainfall received in Goa, and the rainfall follows a more distributed pattern with, the monsoon period contributing towards 30-40 percent of the annual rainfall. Therefore, salt production occurs throughout the year without a drastic variation in the salinity (Mani et al., 2012a; Kaur and Purohit, 2012). Sinquetim is a non-functional saltern whereas the other 4 are fully functional (Kabilan, 2016).

2.3.2 Halophiles: Enrichment, growth and maintenance

The samples obtained from various sites were plated on five different media and visually distinct colonies obtained were purified by repeated streaking. Fifteen of these culture isolates were used in various aspects in this study (Table 2.2).

Isolates E1, E2, E3, E4 and E5 were isolated and maintained in EHM media; M1, M2, M3, M4 and M5 isolated and maintained in MHM media; BS2 and BS17 in NT media; BBK2 and BK6 in NTYE media; J1 in JCM168 media. The pigmentation of most of the isolates was in different shades of orange ranging from pink, to orange and orangish red colour. Only strain M4 appeared cream.

Haloarchaea and extremely halophilic bacteria obtained from neutral thalassohaline environment generally have an optimum pH requirement of pH 6.0 to pH 8.0 (Grant, 2004). Na^+ and K^+ is form an obligate requirement as it helps the haloarchaea maintain osmolarity (Martin et al., 1999). Mg^{2+} is required for the activity of many haloarchaeal enzymes (Madern et al., 2000). The cream colonies (Isolate M4) started appearing after 1-2 days. The pigmented colonies appeared after 10-15 days. The orange colouration is associated with halophilic archaea due to the presence of bacterioruberin, a C50 carotenoid present in the membrane of the organism.

Visually different cultures were purified by repeated streaking and started appearing in 7-10 days after acclimatization to the growth medium. The growth was faster if the plates were incubated at 37 ^{o}C but this caused the salts in the media to crystallize out more rapidly Hence, room temperature was preferred for the long-term maintenance of cultures. Fig 2.2 shows some of the pure isolates used in the study.

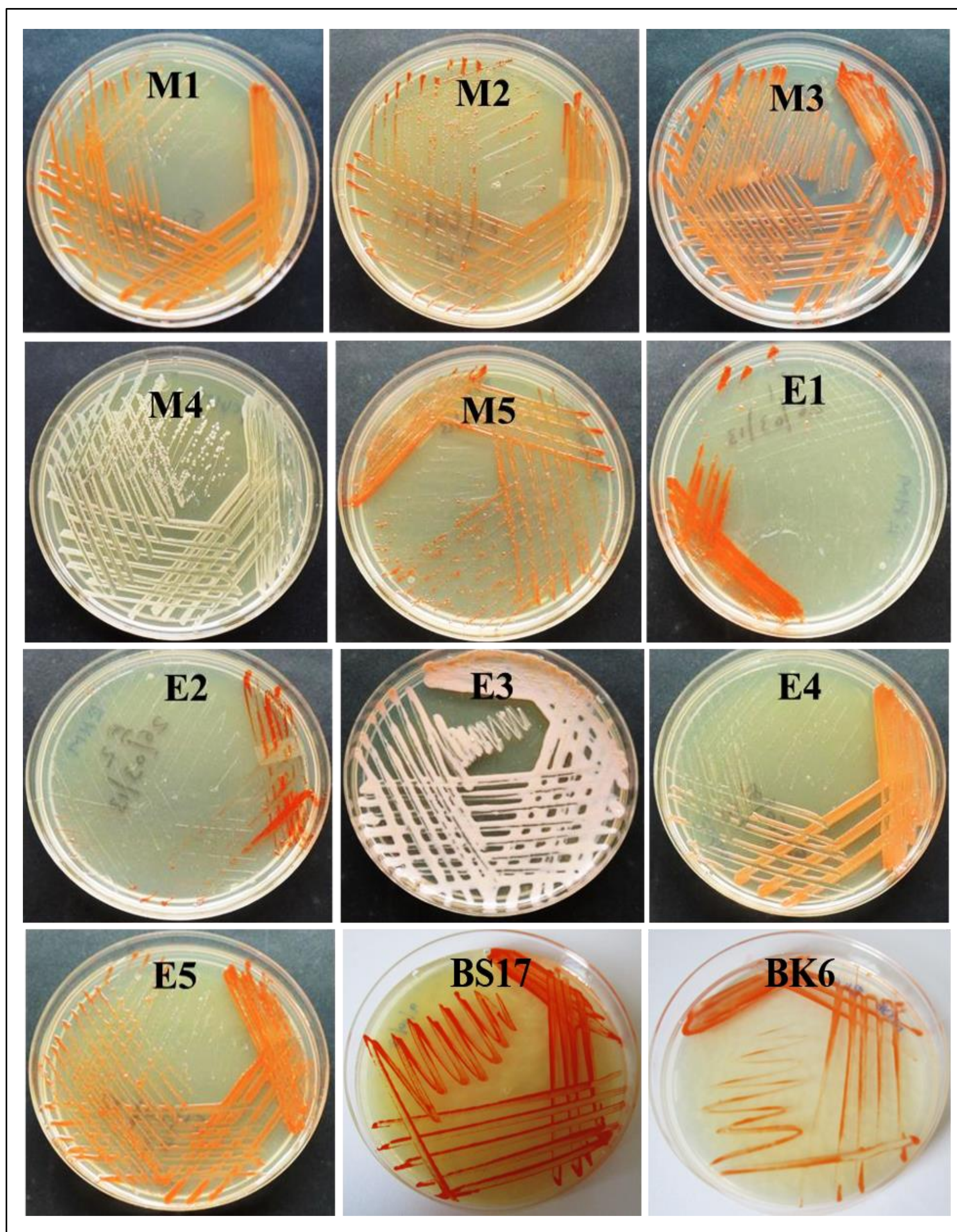


Fig 2.2: Pure cultures of the extremely halophilic archaeal isolates obtained from solar salterns of Tamil Nadu (Marakkanam, Vedaranyam) and Goa (Siridao, Ribandar, Sinquetim), India.

2.3.3 Identification of halophilic isolates

2.3.3.1 Morphological characterization

All halophilic isolates stained Gram negative and most appeared to be coccoid in morphology except for M4 and BS17 (rod and short rods respectively) when observed using a phase contrast microscope (1000X magnification). SEM imaging (Fig 2.3) revealed that the size of the haloarchaeal cells ranged from 0.7µm to 1.6µm arranged as single cells, double cells, in chains or in clusters, cocci, disk shaped or highly pleomorphic morphology (Table 2.3). Cocci morphology is characteristic of *Halococcus* sp. *Haloarcula* and *Halogeometricum* sp. cells are known to be triangular or even pleomorphic in shape (Montalvo-Rodriguez et al., 1998). Rod shaped and pleomorphic *Halorubrum* cells have also been reported (Cui et al., 2007; Trigui et al., 2011).

Table 2.2: The sampling site, growth media, pigmentation and colony morphology of the purified strains used in the study.

Strain	Sampling site	Salinity	Growth media	Pigmentation	Colony Morphology
M1	Sinquetim	0.8%	MHM	Light orange	Circular
M2	Ribandar	2-24%	MHM	Orange	Circular
M3	Vedaranyam	5-28%	MHM	Bright orange	Circular
M4	Marakkanam	5-28%	MHM	Cream	Circular
M5	Vedaranyam	5-28%	MHM	Orange-red	Circular
E1	Marakkanam	5-28%	EHM	Orange-red	Circular
E2	Siridao	2-24%	EHM	Orange-red	Circular
E3	Marakkanam	5-28%	EHM	Light pink	Circular
E4	Ribandar	2-24%	EHM	Orange	Circular
E5	Ribander	2-24%	EHM	Orange	Circular
BS2	Ribandar	15%	NT	Bright orange	Circular
BS17	Ribandar	27%	NT	Orange Red	Circular
BBK2	Ribandar	27%	NTYE	Orange	Circular
BK6	Ribandar	8%	NTYE	Orange	Circular
J1	Vedaranyam	5-28%	JCM168	Light orange	Circular

MHM- Moderately halophilic media, EHM- Extremely halophilic media, NT- NaCl Tri-sodium citrate media, NTYE- NaCl Tryptone media, JCM 168- Japan Collection of Microorganisms 168

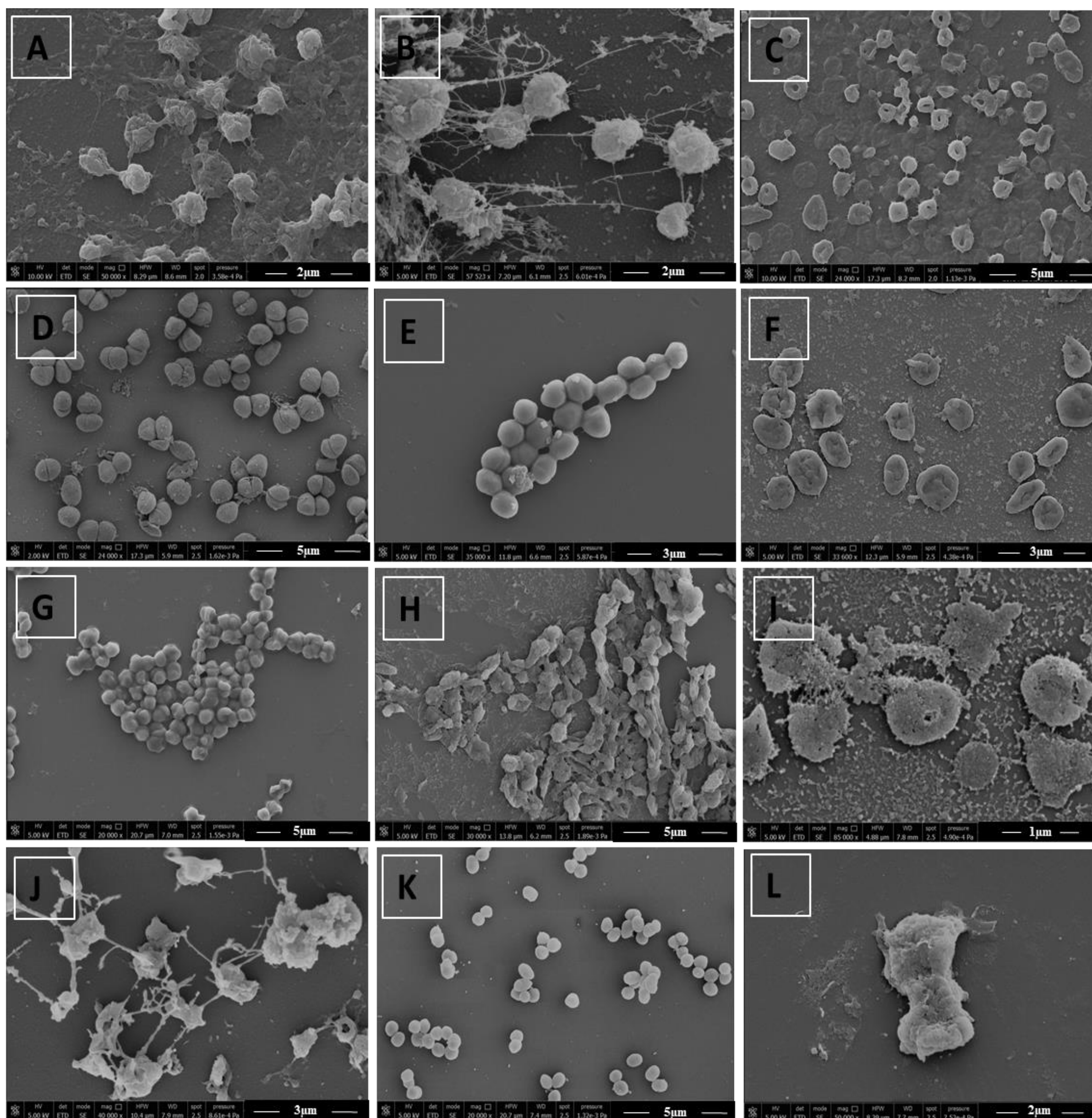


Fig 2.3: Scanning electron micrographs of haloarchaeal isolates showing the different morphologies: A- E1, B-E2, C-E3, D-E4, E-E5, F-M2, G-M3, H-M5, I-BS2, J-BBK2, K- BK6, L-BS17 (The size scale is indicated in each figure)

Table 2.3: Morphological characteristics of the extremely halophilic isolates ascertained by Gram staining, Phase contrast microscopy and SEM imaging.

Strain	Gram Character	Morphology (100X)	Morphology (SEM)	Size	Cell arrangement
M1	Negative	Cocci	ND	ND	-
M2	Negative	Cocci	Disk shaped	1.104μm	Singles
M3	Negative	Cocci	Cocci	816.5nm	Cluster
M4	Negative	Rods	ND	ND	Singles/chains
M5	Negative	Cocci	Pleomorphic	750nm	-
E1	Negative	Cocci	Cocci	720nm	Cluster
E2	Negative	Cocci	Cocci	948nm	Cluster
E3	Negative	Cocci	Pleomorphic	822.2nm	-
E4	Negative	Cocci	Cocci	1.004μm	Double
E5	Negative	Cocci	Cocci	996.3nm	Cluster
BS2	Negative	Cocci	Pleomorphic	907.3nm	-
BS17	Negative	Short rods	Pleomorphic	1.602μm	-
BBK2	Negative	Cocci	Cocci	770.2nm	
BK6	Negative	Cocci	Cocci	780nm	Double
J1	Negative	Cocci	ND	ND	-

2.3.3.2 Biochemical Characterization

(i) Polar lipid composition

Total lipids were extracted following the procedure described by Elevi et. al., 2004. The extracts thus obtained are presented in Fig 2.4. The varying colouration is due to the carotenoid pigments which get eluted along with the lipids.

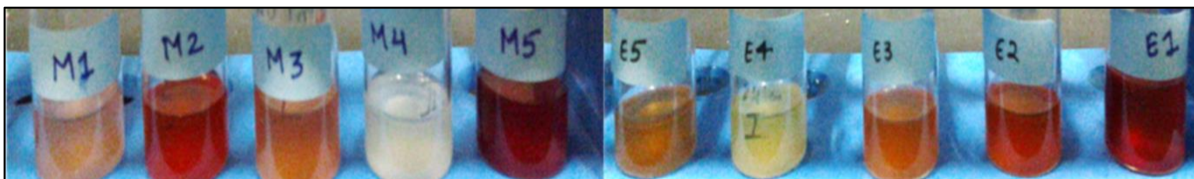


Fig 2.4: Vials showing polar lipids extracted from several extremely halophilic isolates.

Thin layer chromatography was performed to separate the polar lipids extracted from the halophilic isolates (Fig. 2.5). The polar lipids of haloarchaea consists of one major phospholipid and one major glycolipid accompanied by a few minor phospholipids and glycolipids. A combination of phospholipid and glycolipid structures are recognized as molecular markers for the taxonomic classification of family *Halobacteriaceae* (Kamekura and Kates, 1999). Strain M1, M2, E1 and E5 showed the presence of monomethylated derivative of phosphatidyl glycerophosphate (PGP-Me) as the major phospholipid and phospatidyl glycerol (PG) as a minor phospholipid accompanied by sulfated diglycosyl diether (S DGD) as the major glycolipid which suggests that they could belong to the genera *Haloarcula, Haloferax, Halorubrum* or *Halococcus* (Kamekura, 1999; Kamekura and Kates, 1999; Moldoveanu et. al., 1990).

Strain E4 and M3 exhibited nearly identical profiles and the presence of PGP-Me, PG, S DGD, sulfated triglycosyl / tetraglycosyl diether (S TGD/ S TeGD) and also a glycolipid X at an Rf of 0.01. A similar profile and an unnamed glycolipid, Glycolipid X, from *Halococcus hamelinensis* was described by Goh and coworkers in 2006 (Goh et al., 2006; Kamekura and Kates, 1999). In strain E3 a lack of glycolipids was noted which point towards the possibility of it belonging to the genus *Natronococcus* or *Halogeometricum.*

The lipid profile of E2 showed the presence of phospholipid PGP-Me and glycolipid S DGD. Since this is found in many genera, E2 could belong to *Haloarcula, Halorubrum, Haloferax, Halococcus* or *Haloterrigena* (Kamekura and Kates, 1999). Strain M5 showed the presence of only PGP- Me and glycolipid X; Very faint spots of PGP-Me and PG were detected in M4. Since these did not correspond to any known pattern, an inference regarding the identity could not be drawn for Strain M4 and M5 from the chromatogram.

The major phospholipid in several haloarchaeal genera (viz., *Halobacteria, Haloarcula, Haloferax*, *Halococci, Natronobacteria* and *Natronococci*) is identified to be PGP-Me which is found in all the isolates tested (Kates, 1993). Archaeal polar lipids or the phospholipid ether lipids, are composed of di- and tetraethers of glycerol (archaeols and caldarchaeols, respectively) or more complex polyols with side chains consisting of C15, C20, C25 or C40 isoprenoids. Extreme halophiles contain only archaeol derived lipids.

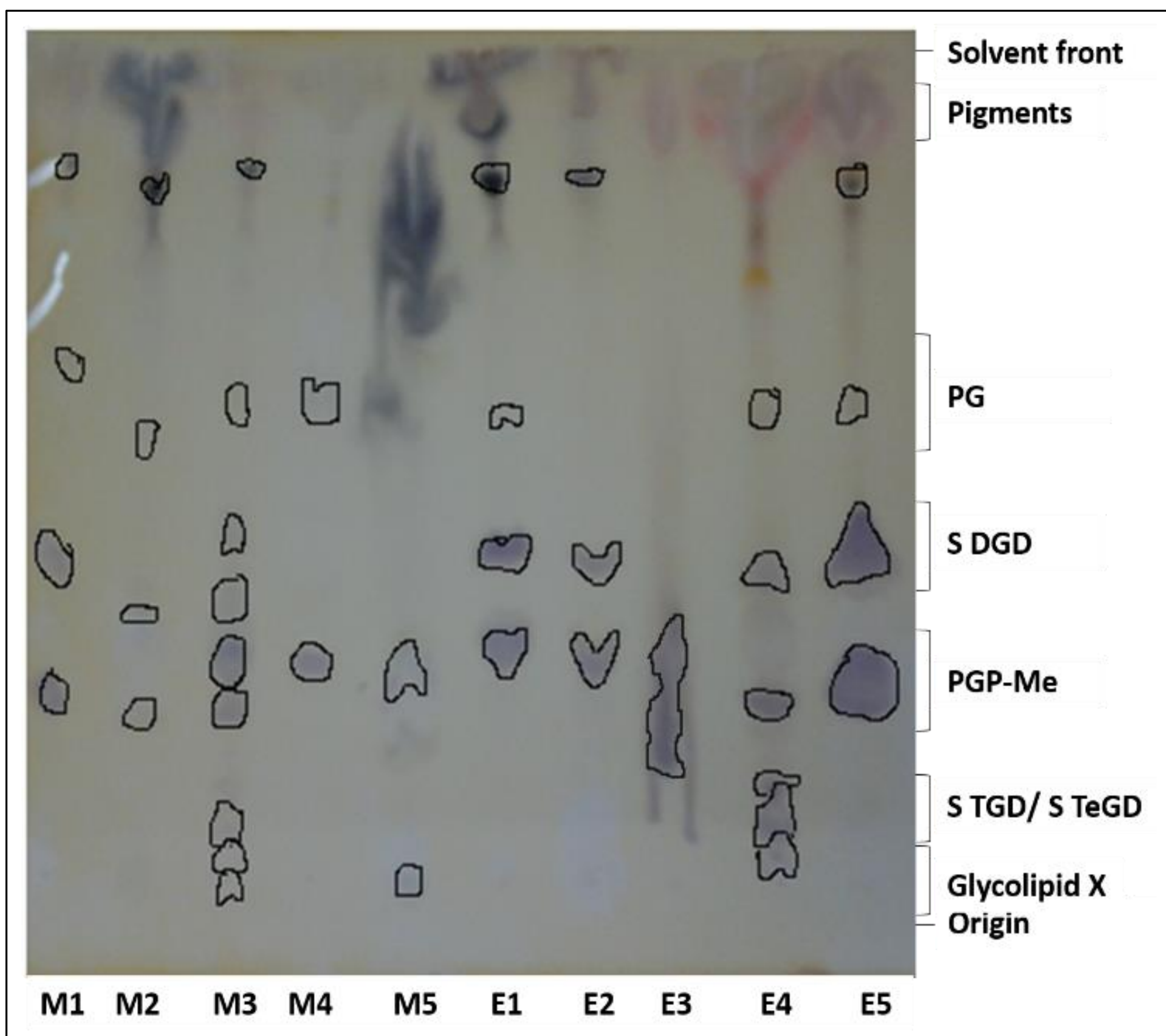


Fig 2.5 Thin layer chromatography of total polar lipids extracted from halophilic isolates strains M1, M2, M3, M4, M5, E1, E2, E3, E4 and E5. Phospholipids and glycolipids were visualized using molybdenum blue spray reagent and α-naphthol spray respectively.

The polar lipids of archaea are unique and readily distinguished from the acyl phospholipids of bacteria and eukaryotes. Archaeal lipids contain two phytanyl chains ether linked to glycerol or other alcohols (viz. nonitol). The acyl groups are normally fully saturated isoprenoids (De Rosa et al., 1991) containing two fatty acyl chains ester linked to a glycerol molecule with the third -OH of glycerol bound to phospho/ glycol containing moieties. (Kate,1993; Gattinger et al., 2003). The ether lipids of archaea are more stable and less permeable than ester linked lipids of other forms of life. This forms one more adaptation of archaeal cells to survive in extreme conditions (Elferink et al., 1992; Thompson et al., 1992; Konings et al., 2002)

(ii) Carbohydrate utilization:

The isolates were found to utilize various sugars. Ribose was utilized by all strains tested except for M4. Fructose was metabolized by 7 of the 10 strains tested whereas glucose was used up by only 5 strains. Lactose was utilized by 3 strains and sucrose by 2. Sorbitol and mannitol were utilized only by Strain E2 and maltose was not metabolized by any strain. Strain E2 was able to utilize ribose, glucose, sorbitol, mannitol, fructose, sucrose and lactose. Strain M5 could not utilize any of the sugars tested. each. The results have been tabulated in Table 2.4.

Table 2.4 Carbohydrate utilization by the extremely halophilic isolates

STRAIN	CARBOHYDRATE						
	GLUCOSE	SORBITOL	MANNITOL	FRUCTOSE	SUCROSE	LACTOSE	MALTOSE
M1	-	-	-	+	-	-	-
M2	+	-	-	-	-	+	-
M3	-	-	-	+	-	-	-
M4	+	-	-	-	+	+	-
M5	-	-	-	-	-	-	-
E1	+	-	-	+	-	-	-
E2	+	+	+	+	+	+	-
E3	-	-	-	+	-	-	-
E4	+	-	-	+	-	-	-
E5	-	-	-	+	-	-	-

'+' indicates growth/ utilization; '-' indicates ' no growth/ non-utilization'

Haloarchaea are either aerobes of facultatively anaerobic heterotrophs. Some are capable of growing in a wide range of energy sources whereas others such as *Haloquadratum walsbyi* have specific demands for growth (Burns et. al., 2007; Bardavid and Oren,2008). The Embden – Meyerhof pathway seems to be incomplete in halophiles as inferred from an absence of phosphofructokinase. The enzymes of semi phosholylated Entner-Doudonoff pathway for glycolysis is present and are highly conserved in most sequenced haloarchaea suggesting a

common ancestry. However, genome comparative analysis of several haloarchaeal genera reveal that haloarchaea prefer amino acids over sugar as energy and carbon source. One exception is seen in the case of *Halorhabdus utahensis* which seems to be well adapted to grow on carbohydrates (Anderson et. al., 2011).

(iii) Pigment characterization

Each purified halophilic isolate was unique and exhibited a characteristic pigmentation when grown in nutrient rich media. This is attributed to the presence of pigments in them. Strain M4 was devoid of any pigmentation and appeared as cream coloured colonies on MHM agar plate. The pigments from the extremely halophilic isolates were analyzed using UV Visible spectrophotometer. The pigment profiles of the cultures were seen to have their λ_{max} at around 395, 472, 506 and 539 (Fig. 2.6) which indicates the presence of bacterioruberin class of pigments, characteristic pigment present in halophilic archaea.

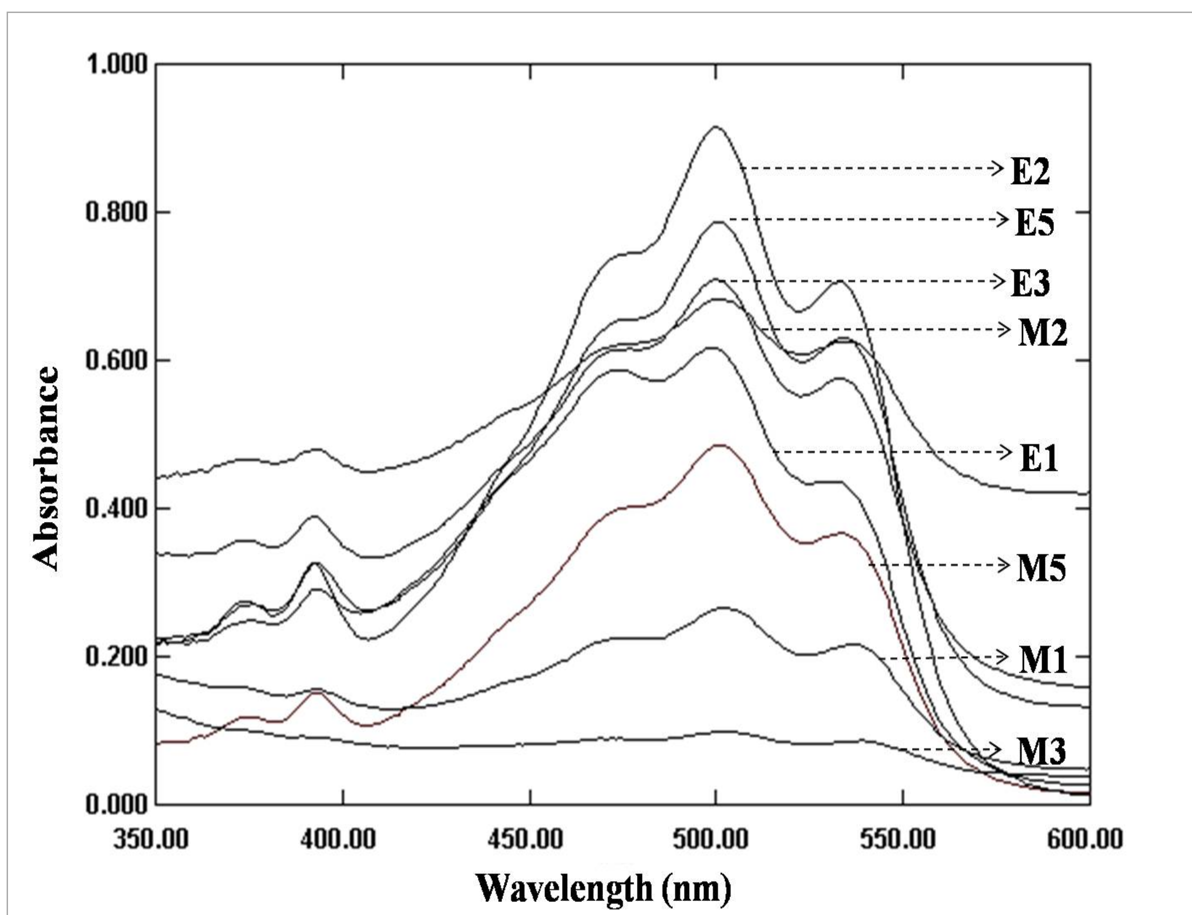


Fig. 2.6 Spectrophotometric scan of the pigments extracted from several halophilic isolates using chloroform: methanol (1:2)

The carotenoid pigments serve to protect the cells from the harmful effects of UV radiation and assists in photoreactivation (Rodrigo-Baños et al, 2015).

2.3.3.3 Molecular characterization of the halophilic isolates used in the study:

Phylogenic analysis of all the halophilic isolates were carried out using 16S rRNA gene sequencing. Among the isolates obtained from the solar salterns of India, 14 were phylogenetically affiliated to Archaea and 1 to Bacteria. These 15 cultures were grouped under 6 different genera. Phylogenetic tree analysis (Fig. 2.7) and sequence comparison with the GenBank database showed that strains M1 and E5 were most closely related to *Haloferax prahovense* strain TL6 (100 and 99.85% similarity), BBK2 was most closely related to *Haloferax denitrificans* strain ATCC 35960 (99.80%), whereas the strain BS17 was most closely related to *Halorubrum xinjiangense* strain CGMCC 1.3527 (98.84%), M2 and M5 belonged to the genus *Halorubrum*. The strains M3 and BK6 showed 99.18 and 99.86% similarity respectively to *Halococcus agarilyticus* strain 62E whereas E4 showed 99.86% similarity to *Halococcus saccharolyticus* strain DSM 5350.

Strains E1, E2 and BS2 belonged to the genus *Haloarcula* and strain J1 belonged to the genus *Haloterrigena*. Strain E3 was most closely related to *Halogeometricum borinquense* strain DSM 11551 (99.69%). The bacterial strain M4 was most closely related to *Halomonas elongata* DSM2581 (99.29%).

The obtained 16S rRNA sequences were submitted in DDBJ under the accession numbers (given in brackets): Strain E1 (AB904831), Strain E2 (AB904832), Strain E3 (AB904833), Strain E4 (AB904834), Strain E5 (AB904835), Strain M1 (AB904836), Strain M2 (MK810744), Strain M3 (AB904838), Strain M4 (AB904839), Strain M5 (AB904840), Strain J1 (AB971350), Strain BS17 (AB971753), Strain BBK2 (AB588756), Strain BK6 (AB599757). Strain BS2 (HQ455798) was submitted in GenBank (Table 2.5).

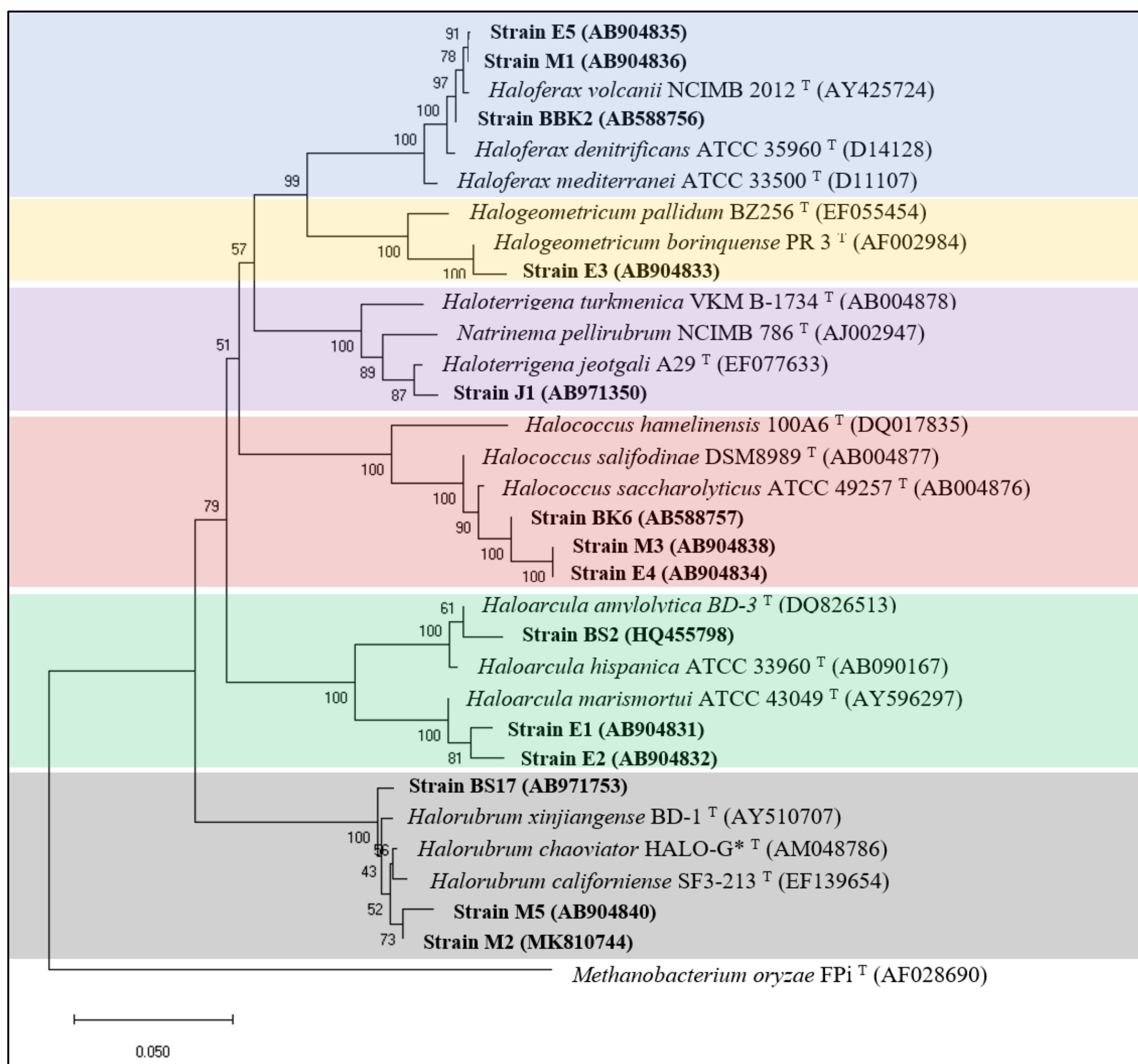


Fig. 2.7 Phylogenetic tree indicating the positions of isolated haloarchaeal strains. The evolutionary history was inferred using the Neighbour-Joining method with bootstrap values for 100 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X.

Table 2.5 Extremely halophilic isolates obtained from solar salterns of India.

Strain	Identification	Closest phylogenetic neighbour	Sequence similarity with closest phylogenetic neighbour	Length of sequence (bp)	Accession number
BS2	*Haloarcul*a sp.	*Haloarcula vallismortis* ATCC 29715(T)	98.70	923	HQ455798
BK6	*Halococcus* sp.	*Halococcus agarilyticus* 62E	99.79	1474	AB588757
BBK2	*Haloferax* sp.	*Haloferax denitrificans* ATCC 35960	99.80	1473	AB588756
BS17	*Halorubrum* sp.	*Halorubrum xinjiangense* CGMCC 1.3527	98.84	1470	AB971753
M1	*Haloferax* sp.	*Haloferax prahovense* TL6	100.00	644	AB904836
M2	*Halorubrum* sp.	*Halorubrum sodomense* DSM 3755	99.47	1321	MK810744
M3	*Halococcus* sp.	*Halococcus agarilyticus* 62E	99.18	611	AB904838
M4	*Halomonas* sp.	*Halomonas elongata* DSM 2581	99.29	722	AB904839
M5	*Halorubrum* sp.	*Halorubrum sodomense* DSM 3755	98.87	798	AB904840
E1	*Haloarcula* sp.	*Haloarcula hispanica* ATCC 33960	99.01	507	AB904831
E2	*Haloarcula* sp	*Haloarcula marismortui* ATCC 43049	98.92	646	AB904832
E3	*Halogeometricum* sp.	*Halogeometricum borinquense* DSM 11551	99.69	646	AB904833
E4	*Halococcus* sp.	*Halococcus saccharolyticus* DSM 5350	99.86	945	AB904834
E5	*Haloferax* sp.	*Haloferax prahovense* TL6	99.85	651	AB904835
J1	*Haloterrigena* sp.	*Natrinema pellirubrum* DSM 15624	98.54	549	AB971350

2.4 SUMMARY AND CONCLUSION

Extremely halophilic isolates obtained from the various solar salterns of Goa and Tamil Nadu, lining the west and east coast of India were isolated on five different nutrient rich halophilic media. Visually different colonies were purified using repeated streaking in the respective media in which they were isolated. They were characterized using various morphological, chemotaxonomic and molecular methods and their identity was established using sequence comparison by Mega X. Their sequences were submitted in DDBJ/ GenBank and accession numbers were obtained. The 15 halophilic isolates belonged to 6 different genera of extremely halophilic archaea (*Haloarcula, Halococcus, Halorubrum, Halogeometricum, Haloterrigena, Haloferax*) and one extremely halophilic bacterium belonging to genus *Halomonas*.

Chapter III

Screening of extremely halophilic archaeal isolates for the production of various hydrolytic enzymes and the partial characterization of protease and amylase

3.1 INTRODUCTION

Halophilic archaea, which are found in abundance in hypersaline econiches, accumulate K^+ ions intracellularly for osmoregulation (Marhuenda-Egea and Bonete, 2002). This requires the entire cell machinery to be adapted to a high salt environment. Halophilic archaeal enzymes typically require salt for maintaining their structural and functional stability (Mevarech, 2000). These enzymes are characterized by a high surface negative charge due to the presence of acidic residues which bind strongly to water molecules and help them maintain their structure even in low water conditions (Karan et. al., 2012). Other factors which contribute to their stability in high salt environment are the presence of salt bridges and a weakening in the hydrophobic interactions in the level of core hydrophobic contacts (Nayek et. al., 2014; Siglioccolo et. al., 2011).

After the discovery of thermostable DNA polymerase, there has been a renewed interest in the enzymes which can function in harsh conditions deemed extreme for the normal mesophilic organisms (Vieille and Zeikus, 2001). A great deal of research is aimed at finding novel enzymes from different sources which have potential application in various industries (Karan et. al., 2012). Effective enzymes able to function in non-aqueous environments may be applied in biofuel and bioenergy applications where large quantities of organic solvents are involved (Deng et. al., 2005; Fjerbaek et. al., 2009). Other industries where haloarchaeal enzymes can find an important place are detergent, laundry, food, textile, paper and pulp industries etc.

Great variation has been observed in the diversity of haloarchaeal community isolated from different geographical locations (Munson et. al., 1997; Oh et. al., 2010; Mani et. al., 2012). The bioprospecting of halophilic archaea from Indian salterns would be of great value to understand the biocatalytic activity in these environments and is expected to provide novel biological sources for hydrolytic enzymes with potential biotechnological applications (Thombre et. al., 2016).

In this study, we have screened halophilic isolates spanning 6 genera of halophilic archaea for the production of various extracellular hydrolytic enzymes such as protease,

amylase, lipase, esterase, pectinase and cellulase. Also, attempts on partial purification and characterization of two enzymes, amylase and protease, were carried out.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Chemicals for the preparation of growth medium and other substrates were purchased from Hi Media, Mumbai, India. Double distilled water was used for the preparation of media.

3.2.2 Growth of extremely halophilic archaea

Ten extremely halophilic archaeal isolates spanning 6 genera *Haloarcula* sp. (Strain E1, E2), *Halogeometricum* sp. (Strain E3), *Halococcus* sp. (Strain E4, M3), *Haloferax* sp. (Strain E5, M1), *Halorubrum* sp. (Strain M2, M5) and *Haloterrigena* sp. (Strain J1) was used in the study. Strains E1, E2, E3, E4 and E5 were maintained in EHM media; JCM 168 media was used to grow and maintain strain J1 and strains M1, M2, M3 and M5 were maintained in MHM media. The cultures were maintained on the respective nutrient media plates at room temperature in zip-lock covers and sub-cultured every 30-45 days.

3.2.3 Screening of halophiles for the production of various enzymes

The various strains were screened for the production of extracellular hydrolytic enzymes by plate assay method. Activity was checked in three different minimal media viz., Norberg Hofstein (NH) media, NaCl Synthetic Media (NSM) and NSM supplemented with 0.1% YE (NSMY). The media was supplemented with the substrate of the enzyme which is to be checked. 50 µl of the log phase culture was spotted on all plates except the ones for checking lipase and esterase activity where 50 µl of the culture was added to wells bored in the solid media (Kakhki et. al., 2011).

3.2.3.1 Determination of amylase activity

Plate assay was carried out in the three media described above (NH, NSM, NSMY), supplemented with 0.5%, 1% and 2% (w/v) soluble starch. The media was predigested at 100ºC to dissolve starch completely and then sterilized by autoclaving. The different cultures were spotted on the solid medium. The plates were incubated at room temperature for 15-20

days and then flooded with 0.3 % I_2–0.6 % KI (w/v) solution. Amylase production was indicated by the formation of a clear zone around the culture against a blue-black background.

3.2.3.2 Determination of protease activity

Different volumes of sterile skimmed milk (SM) were separately added to the three media (NH, NSM and NSMY) to get the final substrate concentrations of 0.5%, 1% and 2% (v/v). Cultures were spotted on the plates. The plates were incubated at room temperature for 10-15 days. Formation of a halo/ clearance around the culture indicated the production of protease.

3.2.3.3 Determination of lipase activity

Olive oil (0.5%, 1%, 2% v/v) was supplemented in the three media. Fifty microliters of culture was added into wells bored in the solid medium. The plates were observed for the presence of white sediment around the culture growth which indicates lipase production after 15 days incubation at room temperature. White sediment is formed because of the interaction between the free fatty acids (released after lipase activity) and the calcium present in the media (Gupta et. al., 2003).

3.2.3.4 Determination of cellulase activity

Cellulase activity was checked in the three media, supplemented with 0.5%, 1%, 2% (w/v) CM cellulose. Cultures were spotted on the plates. The plates were incubated at room temperature for 20 days and then flooded with 0.1% Congo Red solution. Cellulase production is indicated by the formation of a clear zone around the culture.

3.2.3.5 Determination of pectinase activity

Plate assay was carried out in the three media (NH, NSM, NSMY), supplemented with 0.5%, 1%, 2% (w/v) sterile pectin. Cultures were spotted on the plates. The plates were incubated at room temperature for 20 days and then flooded with 0.3% I_2–0.6% KI (w/v) solution. Pectinase production was indicated by the formation of a clear zone around the culture against a blue-black background.

3.2.3.6 Determination of esterase activity

Esterase activity was determined on plates supplemented with 0.5%, 1%, 2% (v/v) of Tween 20 and Tween 80 in NS, NSMY and NH medium. Fifty microliters of culture was added into the wells bored in the solid medium. Free fatty acids released due to esterase activity binds with calcium present in the medium to form insoluble white precipitates around the culture growth which indicates esterase production after 15 days incubation at room temperature (Gupta et. al., 2003).

3.2.4 Protease from *Halococcus* sp. Strain E4 -partial purification and characterization

3.2.4.1 *Halococcus* sp. strain E4: growth and protease production

Five hundred milliliters of sterile NH+1% skimmed milk (SM) medium (pH adjusted to 7.2 with NaOH) in 2 L Erlenmeyer flasks were inoculated with 1% E4 inoculum grown in EHM media. The culture was incubated at 37 ^{o}C and 110 rpm till it reached the stationary phase which is visually indicated by the colour change of the culture medium from pale yellow to orange. Growth was monitored by measuring the cell density (OD at 600nm) and the specific activity was calculated as described below (Section 3.2.4.2(ii)) at 24-hour intervals.

3.2.4.2 Assays for protease activity:

A qualitative (Gelatin well assay) and a quantitative (Azocasein) assay was carried out for the detection and estimation of protease present in a sample.

(i) Gelatin well assay:

Gelatin 1% (w/v) was dissolved in 50 mM Tris-Cl buffer supplemented with 4 M NaCl and 10 mM $CaCl_2.2H_2O$ (pH 8.5). 1.5% agar was added to it and the media was sterilized by autoclaving at 121 ^{o}C for 15 min. 15-20 ml of this sterile media was poured into a sterile Petri dish and left to solidify at room temperature. Wells of 0.5cm diameter were bored in the media using a sterile well borer. The plates were allowed to dry for 24 hours at 37 ^{o}C. The sample to be assayed (~50-100 µL) was added to the well and the plates were incubated overnight at 37 ^{o}C. A developing solution containing 15% (w/v) mercuric chloride

in 20% HCl(aq) was poured on the media and the clear zone obtained after precipitation of gelatin was used to detect the protease activity of the sample (Vidyasagar et. al., 2006a).

(ii) Azocasein assay:

Azocasein assay was carried out as described by Vidyasagar et. al., 2006 (b). 1mL of 0.4% azocasein in 0.1 M glycine- NaOH buffer pH 10.0 containing 20% NaCl were taken in 1.5 mL microcentrifuge tubes. This substrate solution is preincubated at 60 ^{o}C. The reaction was initiated by the addition of 50-100 µL of enzyme solution. The mix is incubated for 15' at 60 ^{o}C. Addition of 500 µL of 20% TCA(aq) terminated the reaction by precipitation of proteins. The tubes were incubated at room temperature for 30 minutes. The supernatent collected after centrifugation at 10,000 rpm for 5 min was analyzed by a UV Visible spectrophotometer. OD was taken at 540 nm.

One unit of azocaseinase activity was defined as the amount of enzyme that produced an absorbance change of 0.01 per min under standard assay conditions. Specific activity is the activity of the enzyme per milligram protein. Protein content was estimated using Bradford assay (Appendix IV)

3.2.4.3 Optimization of protease production

The condition for protease production was optimized by keeping all parameters constant except for the one which is being optimized. In each case, growth was monitored by OD at 600 nm and the specific activity was calculated as described in Section 3.2.4.2(ii). Readings were taken once the cultures reached stationary phase (Day 6).

(i) NaCl Concentration: Sterile NH +1% SM was prepared with varying NaCl concentrations (10%, 15%, 20%, 25% and 30%). The pH of the medium was adjusted to 7.0 with 2 M NaOH. 1% of E4 culture was inoculated and the culture was incubated at 37 ^{o}C and 110 rpm.

(ii) Initial pH of the medium: NH +1% SM was prepared, and the pH of the medium was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 with 6 N HCl/ 2 M NaOH. The medium was then sterilized and 1% of E4 culture was inoculated. The culture was incubated at 37 ^{o}C and 110 rpm.

(iii) Temperature: Sterile NH +1% SM was prepared. The pH of the medium was adjusted to 7.0 with NaOH. 1% of E4 culture was inoculated and the culture was incubated at RT, 37 ^{o}C and 45 ^{o}C. The shaker speed was set at 110 rpm.

(iv) Aeration: Sterile NH +1% SM was prepared. The pH of the medium was adjusted to 7.0 with NaOH. 1% of E4 culture was inoculated and the culture was incubated at 37 ^{o}C. One set was kept in an orbital shaker at 110 rpm. Another set was maintained at stationary conditions.

Sample analysis was carried out in triplicates and the statistical analysis was performed with EXCEL 5.0 (Microsoft Corp, Unterschleissheim, Germany); a two-tailed paired Student's *t*-test was used. All data are presented as mean $\pm$ SDs.

3.2.4.4 Crude enzyme extraction

After optimization of culture conditions, mass culture was carried out in multiple 2L Erlenmeyer flasks having 500 mL of sterile NH+1% SM (pH 7.5) containing 25% NaCl; inoculated with 1% E4 grown in EHM media in the log phase of growth. The flasks were incubated at 37 ^{o}C in an orbital shaker set at 110 rpm. Once the cultures reached late log phase, the cells were separated by centrifugation at 10,000 rpm and 4 ^{o}C for 20 min. The supernatant was decanted into sterile blue capped bottles under aseptic conditions. Filtration using 0.22 µm filter was carried out to ensure complete removal of cells. The filtrate served as the crude enzyme. The activity was checked by gelatin well assay as well as azocasein assay and the activity and specific activity was calculated as mentioned in Section 3.2.4.2(ii)

3.2.4.5 Concentration and purification of protease

3.2.4.5.1 Ultrafiltration

Ultrafiltration technique is basically a pressure induced separation of solutes from a solvent through a semi permeable membrane. The crude enzyme was concentrated 50 times using Amicon Stirred cell Ultrafiltration unit and a regenerated cellulose membrane having a molecular weight cut off (MWCO) of 10 kDa and 30 kDa. In both cases, the permeate and retentate was tested for activity using gelatin well assay as well as azocaseinase assay.

Retentate which had protease activity was used for further analysis. Total activity, Yield%, and purification fold was calculated as follows:

Total Activity	= Activity x Total volume of sample
Yield %	= (Total Activity of sample x 100) / Total activity of crude supernatent
Purification Fold	= Specific activity of sample / Specific activity of crude supernatent

3.2.4.5.2 Organic solvent precipitation

Addition of miscible organic solvents to a solution will decrease the dielectric constant of water and displaces water molecules from the solvation shell of the protein causing the protein molecules to precipitate. Crude Extract was stored at 4 ^oC. Acetone or ethanol (chilled to -20 ^oC) was added at the rate of 10 ml/min with vigorous stirring. A volume of acetone or ethanol corresponding to four times of the original crude enzyme volume was added. Mixture centrifuged at 4 ^oC for 15 minutes. Pellet dissolved in 50mM Tris-Cl buffer (pH 7.5) + 4 M NaCl + 10 mM $CaCl_2$ and checked for activity. Supernatent is returned to cold bath and extraction procedure repeated once more. Pellet after centrifugation dialyzed with 50mM Tris-Cl buffer (pH 7.5) + 4 M NaCl + 10 mM $CaCl_2$ to remove the traces of organic solvent from the sample.

3.2.4.5.3 Gel filtration chromatography

Gel filtration chromatography separates proteins, peptides, and oligonucleotides based on size. Molecules move through a bed of porous beads, diffusing into the beads to greater or lesser degrees. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more slowly, while larger molecules enter less or not at all and thus move through the bed more quickly. Both molecular weight and three-dimensional shape contribute to the degree of retention. Gel Filtration Chromatography/ Size exclusion chromatography is used for analysis of molecular size, for separations of components in a mixture, or for salt removal or buffer exchange from a preparation of macromolecules.

(i) Preparation of the column:

The specific instructions for the packing of each column was supplied along with the matrix. In general, Sephadex G50/ Sephadex G75/ Sephadex G100 was suspended in sterile distilled water and allowed to swell till the volume became constant. Sepharcryl S 200 HR was obtained in a presoaked condition. The matrix was washed three times with distilled water to remove any broken beads or impurities. After wash, the matrix was degassed and then packed on to a glass column (Borosil). Packing must be carried out such that there are no air bubbles in the column. Once the column was packed it was equilibrated with the column buffer 50 mM Tris Cl buffer containing 2 M NaCl and 10 mM $CaCl_2$. The experimental void volume was calculated as the volume required to elute Blue Dextran (prepared in the same buffer) from the column. The bed volume was calculated each time by using the equation:

$$V = \pi r^2 h$$

where V is the bed volume, r is the radius of the column and h is the height of the packed matrix. Theoretical void volume was calculated each time as 30% of the bed volume. If experimental void volume and theoretical void volume are approximately same, the packing is considered fit to be used for separation.

(ii) Sample preparation and loading:

The crude concentrated enzyme was centrifuged at 4 ºC and 10,000 rpm for 5 minutes to remove any suspended particles. The clarified enzyme sample was added along the sides of the column so that the matrix is not disturbed. A sample volume of 0.5- 2% of the bed volume gave best resolution, though, up to 5% gave acceptable separation while using Sephadex G50.

(iii) Separation by gel filtration:

Gel filtration was carried out in four stages using different matrices Sephadex G50 (1.5-30 kDa), Sephadex G75 (3-80 kDa), Sephadex G100 (4-150 kDa) and Sephacryl S 200 (5-250 kDa). First the concentrated crude enzyme sample was loaded on Sephadex G50 (1.5-

30 kDa). 1.5 ml fractions were collected separately and stored at 4 ^oC. Each fraction was checked for protease activity by gelatin well assay. The active fractions were pooled together and stored at 4 ^oC. This process was repeated till all concentrated crude enzyme was passed through the Sephadex G50 column. The total pooled active fractions were concentrated using ultrafiltration(10 kDa). At each stage the protease activity was quantified using azocasein assay and specific activity was calculated. The concentrated pooled fraction was loaded on all the other columns to check which one gave better resolution and the results have been documented.

3.2.4.5.4 Poly acrylamide gel electrophoresis (PAGE) and zymography:

Non-denaturing PAGE was carried out as described in Appendix VI to analyze the protein profile at various stages of purification. Zymography was carried out as described in Appendix VII. The substrate used, and the time of incubation was standardized to obtain the activity of the protease in the gel.

3.2.4.6 Characterization of partially purified E4 protease:

(i) pH: The activity of the semi-pure protease was tested in varying pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0) to find out the optimum pH for activity. The following systems were used 50 mM acetate buffer, pH 5.0-6.0; 50 mM Tris-HCl buffer, pH 7.0-8.0; 50 mM glycine-NaOH buffer, pH 9.0-11.0; each containing 4 M NaCl and 10 mM $CaCl_2$

(ii) Temperature: Proteolytic activity was tested at different temperature between (4 ^oC, 25 ^oC, 37 ^oC, 45 ^oC, 60 ^oC and 80 ^oC) at optimum pH. Aliquotes were withdrawn at desired time intervals and proteolytic activity was measured under standard assay condition.

(iii) NaCl: Effect on enzyme activity was determined by estimating the azocaseinase activity of purified enzyme in presence of varying NaCl concentrations. The substrate dilutions (1% casein) were prepared in 50 mM Tris-HCl buffer pH 8 containing 1.0, 2.0, 3.0, 4.0 and 5.0 M NaCl. The enzyme assay was carried out at 37°C using these substrates in the assay system and the values were expressed as relative activity.

(iv) EDTA and Metal ions: The reaction mixture (1 mL) was prepared by adding 500 µL of the *Halococcus* sp. E4 protease solution to 500 µL of EDTA and metal ion solution such that

the final concentration of EDTA and metal ions were 10 mM and 2 mM respectively. The effect of various metal ions like $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $BaCl_2$, $MgCl_2$, and $HgCl_2$ were studied. The enzyme-inhibitor mixture containing 25% (w/v) NaCl was pre-incubated for 30 min at room temperature. The residual activity was then determined under optimal conditions (25% NaCl, pH 8.0 and 60 °C). Activity of enzyme prepared, incubated under similar condition without any additives, was taken as 100% (Vidyasagar et. al., 2009; Pathak & Sardar, 2014).

(v) Detergents: The compatibility of the crude protease as a detergent additive was determined by testing its stability towards some surfactants. Enzyme preparation was incubated with SDS (1% w/v), Triton X-100 (1% v/v) and Tween 80 (1% v/v) for 1 hour at 40 °C and then the proteolytic activities were measured under standard assay condition. Activity of enzyme prepared, incubated under similar condition without any additives, was taken as 100%.

(vi) Organic solvents: To examine stability of the protease to different organic solvents, 1.0 volume of organic solvent was added to 4.0 volumes of the protease solution and incubated at 30 °C with constant shaking at 150 rpm for 7 days. After incubation for appropriate periods of time, aliquots were withdrawn, and remaining activity was measured under pre-determined pH and temperature (Akolkar et. al., 2008; Manikandan et. al., 2009)

Sample analysis was carried out in triplicates and the statistical analysis was performed with EXCEL 5.0 (Microsoft Corp, Unterschleissheim, Germany); a two-tailed paired Student's *t*-test was used. All data are presented as mean $\pm$ SDs.

3.2.5 Amylase from *Halogeometricum* sp. strain E3

3.2.5.1 *Halogeometricum* sp. strain E3: growth and amylase production

Five hundred milliliters of NH medium + 1% starch (w/v) (pH adjusted to 7.2 with NaOH) in 2 L Erlenmeyer flasks was predigested to dissolve the starch. It was sterilized by autoclaving and the sterile medium was inoculated with 1% *Halogeometricum* sp. E3 inoculum grown in EHM media. The culture was incubated at 37 ºC and 110 rpm till it reached the stationary phase which is visually indicated by the colour change of the culture

medium from pale yellow to pinkish orange (due to the production of pigments by the organism). Growth was monitored by measuring the cell density (OD at 600nm) and the specific activity was calculated as described below (Section 3.2.5.2(ii)) at 24-hour intervals.

3.2.5.2 Assays for amylase:

A qualitative (Starch well assay) and a quantitative (Dinitro-salicylic acid) assay was carried out for the detection and estimation of amylase present in the sample.

(i) Starch well assay:

Starch 0.25% (w/v) was dissolved in 50 mM Tris-Cl buffer supplemented with 4 M NaCl and 10 mM $CaCl_2.2H_2O$ (pH 8.5). 1.5% agar was added to it and the media was sterilized by autoclaving at 121 ^oC for 15 min. 15-20 ml of this sterile media was poured into a sterile Petri dish and left to solidify at room temperature. Wells of 0.5 cm diameter were bored in the media using a sterile well borer. The plates were allowed to dry for 24 hours at 37 ^oC. The sample to be assayed (~50-100 µL) was added to the well and the plates were incubated overnight at 37 ^oC. A developing solution containing 50 mM HCl, 2.5 mM KI and 5 mM Iodine was poured on the media and the clear zone obtained used to detect the amylase activity in the sample (Hutcheon et. al., 2005).

(ii) Dinitro salicylic (DNS) acid assay:

Five hundred microliters of crude enzyme was added to equal volume of substrate solution (1% soluble starch in 50 mM buffer containing 4 M NaCl and 10 mM $CaCl_2$). This mixture was incubated at 70^oC for 30 min. A volume of DNS reagent equal to that of the reaction mixture (1 mL) was added and the reaction was terminated by boiling the samples for 10 min. once the samples cooled to room temperature, OD was taken at 540 nm. Dilutions were performed wherever required (Miller, 1959; Moshfegh et. al., 2013). Standard curve for calculation of amylase activity is given in Appendix V.

One unit of amylase activity was defined as the amount of enzyme required to produce 1 mM maltose per minute under standard assay conditions. Specific activity is the activity of the enzyme per milligram protein. Protein content was estimated using Bradford assay (Appendix IV)

3.2.5.3 Optimization of amylase production

The condition for amylase production was optimized by keeping all parameters constant except for the one which is being optimized. In each case, growth was monitored by OD at 600nm and the specific activity was calculated as described in Section 3.2.5.2(ii). Readings were taken once the cultures reached stationary phase.

(i) NaCl concentration: Sterile NH +1% (w/v) starch was prepared with varying NaCl concentrations (10%, 15%, 20%, 25% and 30%). The pH of the medium was adjusted to 7.0 with NaOH. 1% of *Halogeometricum* sp. E3 culture was inoculated and the culture was incubated at 37 ºC and 110 rpm.

(ii) Initial pH of the medium: NH +1% (w/v) starch was prepared, and the pH of the medium was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 with 6N HCl/ 2M NaOH. The medium was then sterilized and 1% of E3 culture was inoculated. The culture was incubated at 37ºC and 110 rpm.

(iii) Temperature: Sterile NH +1% (w/v) starch was prepared. The pH of the medium was adjusted to 7.0 with NaOH. 1% of E3 culture was inoculated and the culture was incubated at RT, 37°C and 45°C. The shaker speed was set at 110 rpm.

(iv)Aeration: Sterile NH +1% (w/v) starch was prepared. The pH of the medium was adjusted to 7.0 with NaOH. 1% of E3 culture was inoculated and the culture was incubated at 37ºC. One set was kept in an orbital shaker at 110rpm. Another set was maintained in stationary condition.

Sample analysis was carried out in triplicates and the statistical analysis was performed with EXCEL 5.0 (Microsoft Corp, Unterschleissheim, Germany); a two-tailed paired Student's *t*-test was used. All data are presented as mean $\pm$ SDs.

3.2.5.4 Crude enzyme extraction

After optimization of culture conditions, mass culture was carried out in multiple 2L Erlenmeyer flasks having 500 mL of sterile NH+1% (w/v) starch (pH 7.5) containing 25% NaCl; inoculated with 1% E3 grown in EHM media in the log phase of growth. The flasks were incubated at 37 ^{o}C in an orbital shaker set at 110 rpm. Once the cultures reached late log phase, the cells were separated by centrifugation at 10,000 rpm and 4 °C for 20 min. The supernatant was decanted into sterile blue capped bottles under aseptic conditions. Filtration using 0.22 µm filter was carried out to ensure complete removal of cells. The filtrate served as the crude enzyme. The activity was checked by DNS assay and the activity and specific activity was calculated as mentioned in Section 3.2.5.2 (ii).

3.2.5.5 Concentration and purification of amylase

3.2.5.5.1 Ultrafiltration:

The crude amylase was concentrated 50 times using Amicon Stirred cell Ultrafiltration unit and a regenerated cellulose membrane having a molecular weight cut off (MWCO) of 10 kDa and 30 kDa. In both cases (viz., 10 kDa and 30 kDa), the permeate and retentate was tested for activity. Retentate of 10kDa membrane which had high amylase activity was used for further analysis.

3.2.5.5.2 Organic solvent precipitation:

Crude extract was stored at 4°C. Acetone/ ethanol extraction was carried out as mentioned in Section 3.2.4.5.2.

3.2.5.5.3 Purification of amylase:

Purification was attempted using various techniques like gel filtration chromatography, affinity chromatography and triple phase partitioning.

3.2.5.6 Poly acrylamide gel electrophoresis (PAGE) and zymography:

PAGE (non-denaturing) was carried out as described in Appendix V to analyze the protein profile at various stages of purification. Zymography was carried out as described in Appendix VI.

3.2.5.7 Characterization of amylase from *Halogeometricum* sp. strain E3:

(i) pH: The activity of E3 amylase was tested in varying pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0) to find out the optimum pH for activity. The following systems were used 50 mM acetate buffer, pH 5.0-6.0; 50 mM Tris-HCl buffer, pH 7.0-8.0; 50 mM glycine-NaOH buffer, pH 9.0-11.0; containing 4M NaCl and 10mM $CaCl_2$.

(ii) Temperature: Amylase activity was tested at different temperature between (4°, 25°, 37°, 45°, 60° and 80°) at optimum pH. Aliquotes were withdrawn at desired time intervals and the activity was measured under standard assay condition.

(iii) NaCl: Effect on enzyme activity was determined by estimating the amylase activity of purified enzyme in presence of varying NaCl concentrations. The substrate dilutions (1% starch) were prepared in 50 mM Tris-HCl buffer pH 8.0 containing 1.0, 2.0, 3.0, 4.0 and 5.0 M NaCl. The enzyme assay was carried out at 37 °C using these substrates in the assay system and the values were expressed as relative activity.

(iv) EDTA: The effect of the lack of metal ions was determined by testing its stability towards chelating agent, EDTA. The enzyme was incubated with EDTA for 1 hour at 40°C and then the activities were measured under standard assay condition. Activity of enzyme prepared, incubated under similar condition without any additives, was taken as 100%.

(v) Metal ions: The effect of various metal ions (at a final concentration of 2mM) on amylase activity was investigated by adding metal salts like $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $BaCl_2$, $MgCl_2$, and $HgCl_2$ to the reaction mixture (Pathak & Sardar, 2014).

(vi) Detergents: The compatibility of the crude amylase as detergent additive was determined by testing its stability towards some surfactants (SDS, Triton X-100, and Tween-80). Enzyme preparation was incubated with different additives (at a final concentration of 1%) for 1 hour at 40 °C and then the proteolytic activities were measured under standard assay condition. Activity of enzyme prepared, incubated under similar condition without any additives, was taken as 100%.

(vii) Organic solvents: To examine stability of the amylase to different organic solvents, 1.0 volume of organic solvent was added to 4.0 volumes of the protease solution and incubated at

30 °C with constant shaking at 150 rpm for 7 days. After incubation, remaining activity was measured under pre-determined pH and temperature (Akolkar et. al., 2008; Manikandan et. al., 2009).

Sample analysis was carried out in triplicates and the statistical analysis was performed with EXCEL 5.0 (Microsoft Corp, Unterschleissheim, Germany); a two-tailed paired Student's *t*-test was used. All data are presented as mean $\pm$ SDs.

3.3 RESULTS AND DISCUSSION

3.3.1 Screening of halophilic archaeal isolates for extracellular hydrolytic enzymes production

Ten haloarchaeal cultures viz., *Haloferax* sp. strains M1 and E5, *Halorubrum* sp. strains M2 and M5, *Halococcus* sp. strains M3 and E4, *Haloarcula* sp. strains E1 and E2, *Halogeometricum* sp. strainE3 and *Haloterrigena* sp. strain J1 were screened for the production of amylase, protease, lipase, esterase, pectinase and cellulase using plate assays (Fig 3.1).

The producers for amylase were *Halorubrum* sp. M2 and M5, *Halococcus* sp. M3 and E4, *Haloarcula* sp. E1 and E2, and *Halogeometricum* sp. E3. Out of these, E3, M5 and E1 were found to be the best producers. Amylase production was indicated by the zone of clearance around the culture after adding iodine solution (Fig 3.1A). In the three media used, NH and NSMY showed maximal activity compared to NSM without yeast extract. α-Amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) are a class of industrial enzymes that finds application in diverse areas such as food, laundry, brewing, textile and even in pharmaceutical industry. Many sources have been identified among halophilic and non-halophilic bacteria such as *Nesterenkonia* sp. and *Bacillus* sp., as well as eukarya such as *Trametes trogii* and *Aspergillus niger* but very few have been found among halophilic archaea. (Shafiei et. al. 2010; Liu and Xu 2008; Levin et. al.,2008; Mahadik et. al., 2002). The amylase produced by *Halorubrum* sp. (Moshfegh et. al., 2013) and *Haloarcula* sp. have been characterized but we report for the first time, the production of amylase from *Halococcus* sp. and *Halogeometricum* sp.

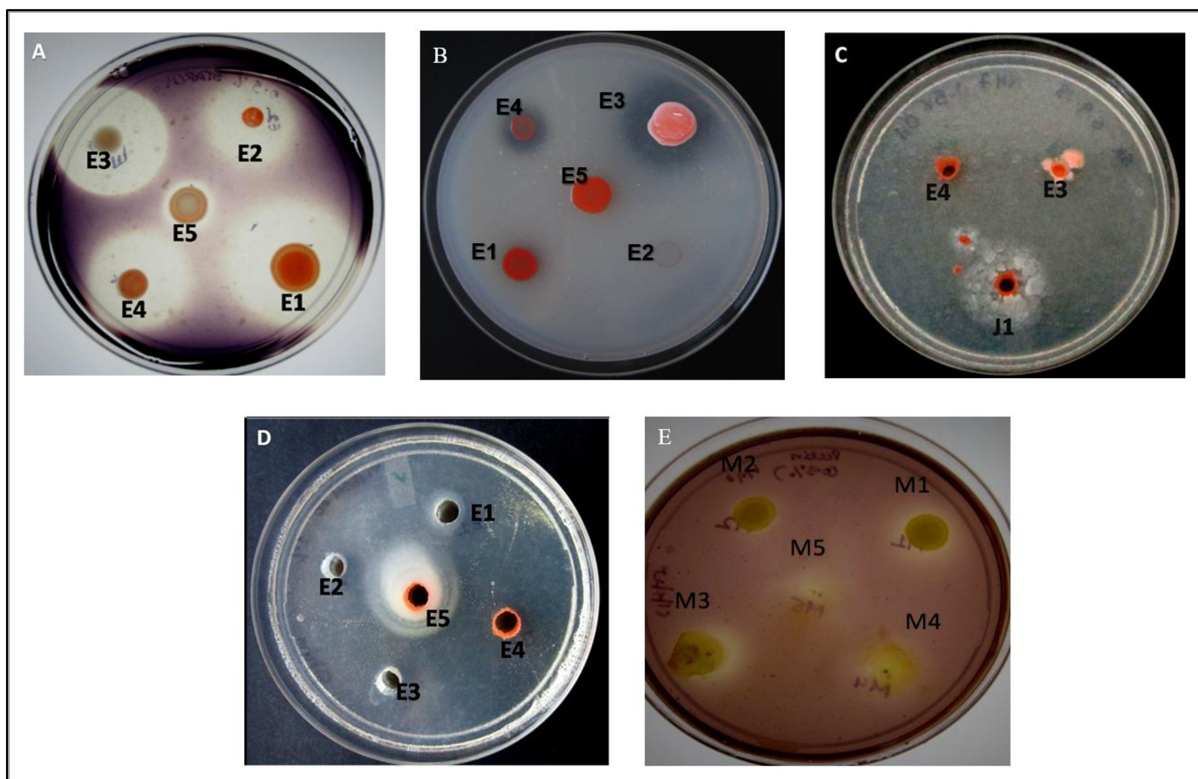


Fig 3.1 Screening for extracellular hydrolytic enzymes using plate assays exhibiting (A) amylase activity, (B) protease activity, (C) lipase activity, (D) esterase activity and (E) pectinase activity. NH/ NSMY/ NSM media supplemented with the substrates specific to each enzyme was used for the study. The zone of clearance indicates enzyme activity except in case of lipase and esterase where the activity is indicated by formation of a white precipitate.

Protease activity was observed in *Haloferax* sp. M1, *Halogeometricum* sp. E3, *Halococcus* sp. E4 and *Haloterrigena* sp. J1.The activity was indicated by the zone of clearance around the culture(Fig 3.1B). E3 showed the best activity. Yeast extract did not have much effect in the production of protease. NSM and NSMY showed comparable activities while in NH media, the activity was slightly less. Protease from *Halogeometricum* sp. has been studied by Vidyasagar (Vidyasagar et. al., 2006a; Vidyasagar et. al., 2006b). A thermostable protease has been characterized from *Haloferax* sp. (Manikandan et. al., 2009). Protease from *Halococcus* sp. and *Haloterrigena* sp. has not yet been studied in detail.

Lipase activity was seen only in one of the isolates, *Haloterrigena* sp. J1 (Fig 3.1C). It was observed that NH media having 0.5 % olive oil showed maximum activity seen as a white precipitate of calcium salts of the fatty acids released. Growth of the organism and subsequent production of lipase was profoundly affected by the absence of yeast extract as

seen in NS medium where lipase production was negligible. There are very few reports of true lipase activity from haloarchaea. Recently, organic solvent-tolerant lipase from *Haloarcula* sp. G41 and organic solvent-tolerant, detergent-stable lipase from *Haloferax* sp. was isolated and characterized (Li and Yu, 2014; Akmoussi-Toumi et. al., 2018). Though *Haloferax* sp. and *Haloarcula* sp. were screened in our study, lipase activity was not observed.

Halorubrum sp. M1, *Halococcus* sp. M3, *Haloarcula* sp. E1 and *Haloferax* sp. E5 showed positive result for esterase in Tween 20 while in Tween 80, *Halorubrum* sp. M1, *Halorubrum* sp. M2, *Haloarcula* sp. E1, *Haloferax* sp. E5 showed esterase activity observed as a white precipitate formed around the culture (Fig 3.1D). *Haloferax* sp. strain E5 was found to be the best producer as it produced the maximum amount of white precipitate around the culture. Esterase activity is common among halophilic archaea like *Haloarcula* sp. (Camacho et. al., 2009; Müller-Santos et. al., 2009), *Natronococcus* sp (Singh and Singh, 2017), *Haloferax* sp. (Rathod et.al., 2016), *Halococcus* sp. (Legat et. al., 2013), Halorubrum sp. (Cui et. al., 2006; Zhang and Cui, 2014). Active microbial lipolytic enzymes have been discovered from marine sediments (Li et. al., 2014) as well as surface and deep-sea seawater (Chu et. al., 2008; Fang et. al., 2014) implying their potential role in marine organic carbon degradation and recycling (Zhang et. al., 2017).

M1, M3, E1, E2, and E4 showed moderate pectinase activity in NSM medium having 0.5 % pectin as the sole carbon source (Fig 3.1E), while none of the haloarchaeal isolates screened in this study showed cellulase activity. Results are tabulated in Table 3.1.

All organisms were able to produce more than one enzyme. *Halorubrum* sp. (strain M2), *Halogeometricum* sp. (strain E3) and *Haloterrigena* sp. (strain J1) were able to produce 2 hydrolytic enzymes; *Halococcus* sp. (Strains E4 and M3), *Haloferax* sp. (strain M1) and *Haloarcula* sp. (strain E1) produced 3 enzymes each. These results have been summarized in Fig 3.2.

Table 3.1 Enzyme activity exhibited by various haloarchaeal isolates obtained from the solar salterns of Goa and Tamil Nadu, India.

Enzyme	Producer strains	Best producer(s)
Amylase	M2, M3, M5, E1, E2, E3, E4	E3, M5, E1
Protease	M1, E3, E4, J1	E3, E4
Esterase	M1, M2, M3, E1, E5	E5
Lipase	J1	J1
Pectinase	M1, M3, E1, E2, E4	M1,M3
Cellulase	-	-

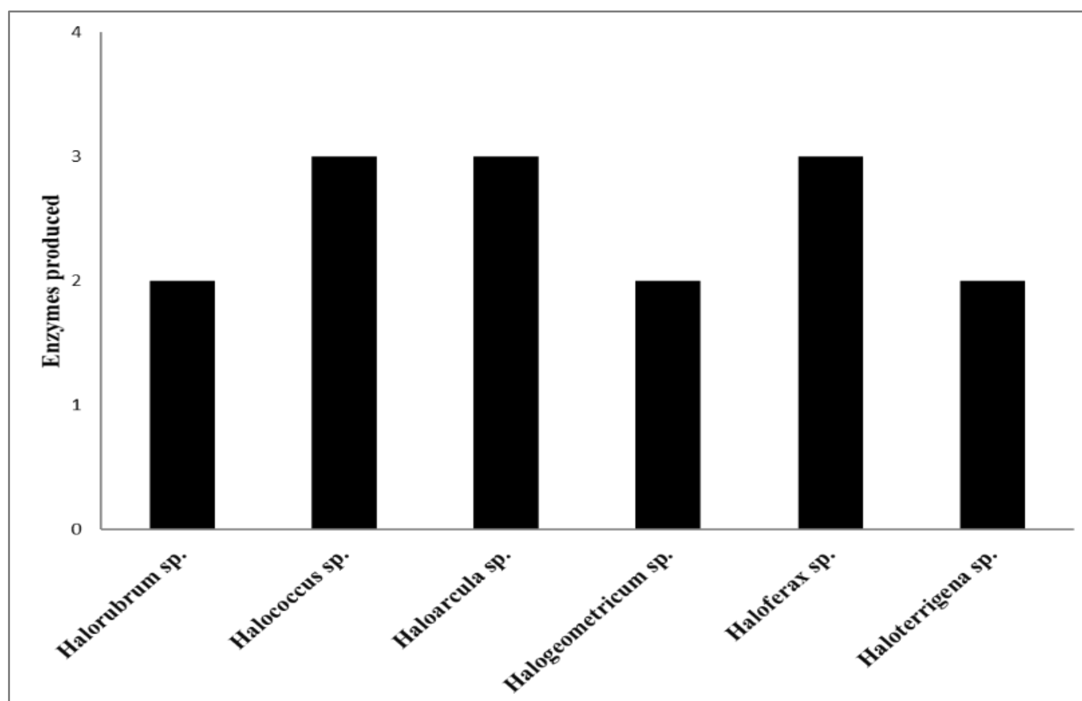


Fig 3.2: Halophilic archaea capable of producing multiple hydrolytic enzymes, obtained in the study

More than one type of hydrolytic activity was observed in many of the haloarchaeal isolates. Similar findings have been previously reported (Rohban et. al., 2009; Moreno et. al., 2007 and Sanchez-Porro et. al., 2003). Recent study on 68 haloarchaea isolates isolated from Algerian arid and semi-arid wetland ecosystems showed that 89.7% and 52.9% of the isolates produced at least two and three hydrolytic enzymes respectively (Menasria et. al., 2018). The drawback of using haloarchaea for the production of these enzymes is their slow growth and production rate (Ventosa et. al., 1998). But once the novel halozymes are identified and characterized, they can be overexpressed in non-halophilic organisms.

3.3.2 Protease production from *Halococcus* sp. strain E4

Halococcus sp. strain E4 was initially grown in NH +1% skimmed milk (SM) at pH 7.0, temperature 37ºC and 110 rpm in a shaking incubator. The inoculum used was 1% of E4 grown in EHM (approximately 0.6 OD at 600nm). The growth curve as well as protease production is shown in Fig 3.3.

There was lag period of two days for *Halococcus* sp. strain E4 (grown in NH + 1% SM), after which exponential growth phase started. The extracellular protease production also increased along with the growth and the protease activity peaked in the stationary phase of growth from Day 6, declining thereafter. In *Halobacterium* sp. and *Natronolimnobius innermongolicus* maximal protease production was reported at its stationary phase of growth (by Day 4 and Day 7 respectively) after which there was a fall in the protease production (Elbanna et. al., 2015, Selim et. al., 2014).

3.3.2.1 Optimization of protease production:

(i) NaCl concentration: The optimum NaCl concentration for protease production was found to be 20-25% NaCl. Considerable growth was seen till 30% NaCl (visual observation) but the production of protease dropped drastically after salinity was increased from 25% - 30% NaCl (Fig 3.4).

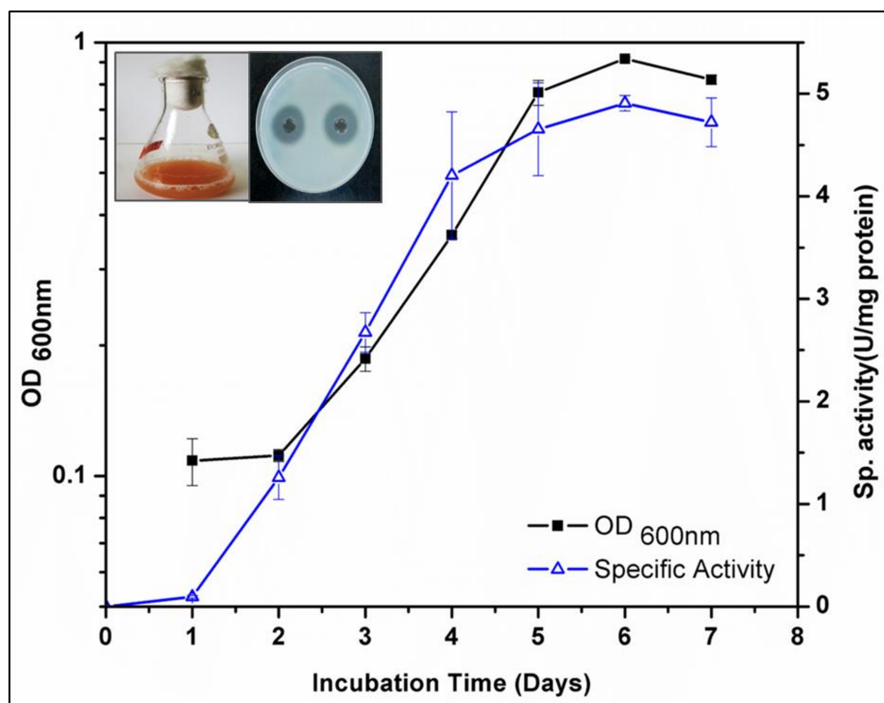


Fig 3.3 Growth profile and protease production by *Halococcus* sp. strain E4. Inset shows the liquid culture of E4 grown in NH+1%SM (left) and the gelatin well assay representing protease activity (right) on Day 6.

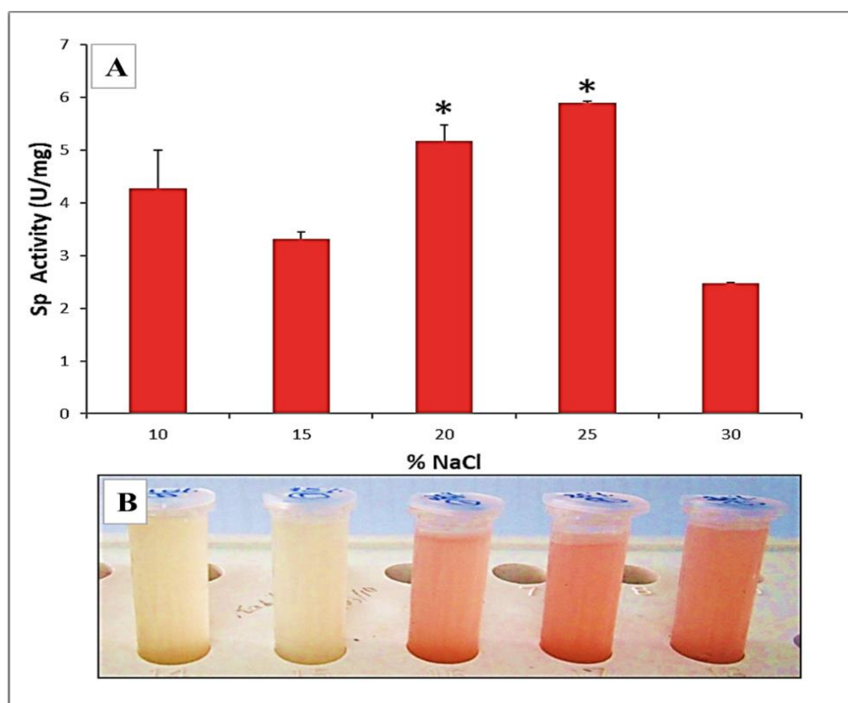


Fig 3.4 Effect of NaCl on protease production (A) and growth (B) of *Halococcus* sp. strain E4 on the 6th day of growth (* $p < 0.05$).

(ii) Initial pH of the medium: The optimum pH for protease production was found to be pH 7-8 though growth indicated by turbidity and development of orange colour was seen in a wider range of pH 5-9 (Fig 3.5). *Halobacterium* sp. strain HP25 also showed an optimum protease production at neutral pH (Elbanna et. al., 2015).

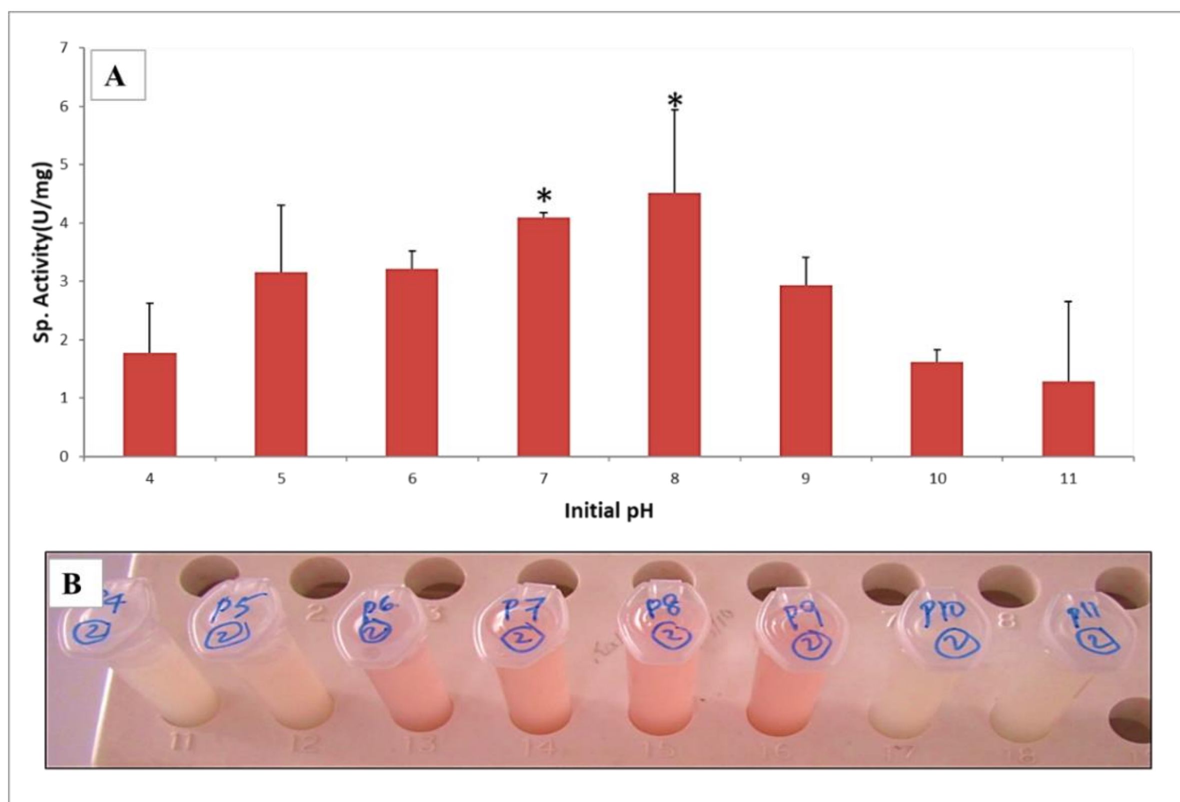


Fig 3.5 Effect of pH on protease production (A) and growth (B) of *Halococcus* sp. strain E4 on the 6th day of growth (* $p < 0.05$).

(iii) Temperature: Highest growth was observed at 45°C but maximum protease production was seen to be at 37°C. At room temperature, the production was significantly reduced (Fig 3.6A). Hence, for further experiments, the optimum temperature of 37°C was used. The drastic decrease in protease production with increase in temperature from 40-45°C was reported in *Halobacterium* sp. even though there was no significant difference in the growth (Elbanna et. al.,2015).

(iv) Aeration: The culture kept in stirring condition showed higher protease production than the one which was kept stationary. The latter showed decreased growth as well (Fig 3.6B).

Stirring causes an increased aeration as well as an increase in nutrient uptake which probably led to increased protease production (Beg et. al., 2003; Potumarthi et. al., 2007) Hence, aeration is an important parameter in the growth and production of protease from *Halococcus* sp. strain E4.

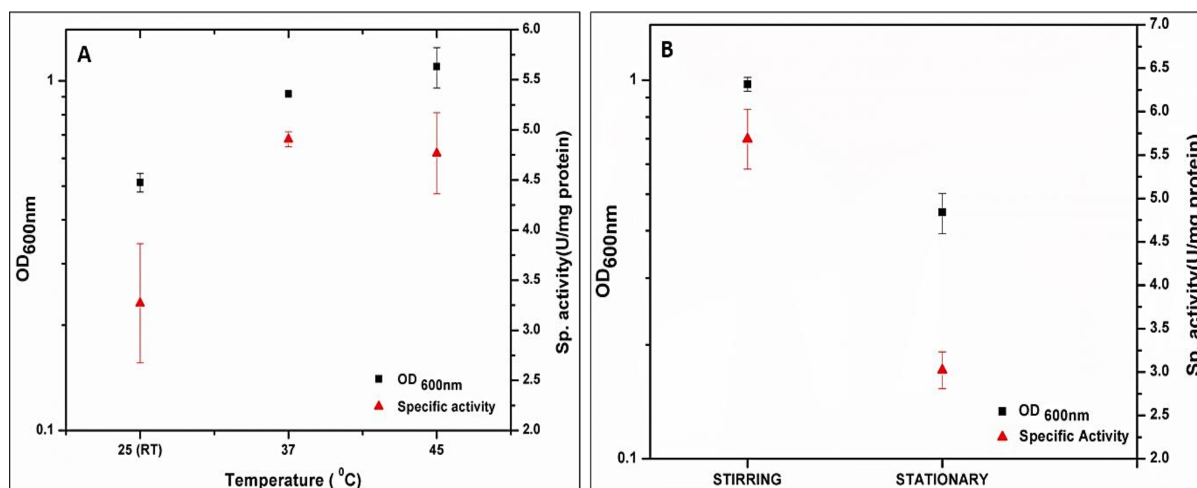


Fig 3.6 The effect of temperature (A) and aeration (shaking condition) (B) on the growth and protease production of *Halococcus* sp. strain E4

The optimum conditions for protease production from *Halococcus* sp. E4 were thus found to be a temperature of 37°C, pH 7-8, in NH +1% SM containing 25% NaCl in shaking condition. In these conditions, the culture reached stationary phase on Day 5 and the peak production of protease was observed on Day 6. These culture conditions were used in all further experiments.

3.3.2.2 Concentration of protease produced by *Halococcus* sp. strain E4

The E4 culture grown in optimum conditions for 6 days was centrifuged to obtain supernatent which served as the crude extracellular protease enzyme extract. This crude enzyme was concentrated using (i) ultrafiltration and (ii) organic solvent precipitation.

(i) Ultrafiltration: The crude protease extract was subjected to ultrafiltration using membranes of MWCO 10 kDa or 30 kDa (Fig 3.7). Results are tabulated in Table 3.2. The protein activity was found to be maximum in the retentate of 30kDa membrane. Hence, the retentate of 30kDa membrane was used for further purification. Ultrafiltration is a preferable

technique for the concentration of haloarchaeal proteins because it can be operated in high salt conditions.

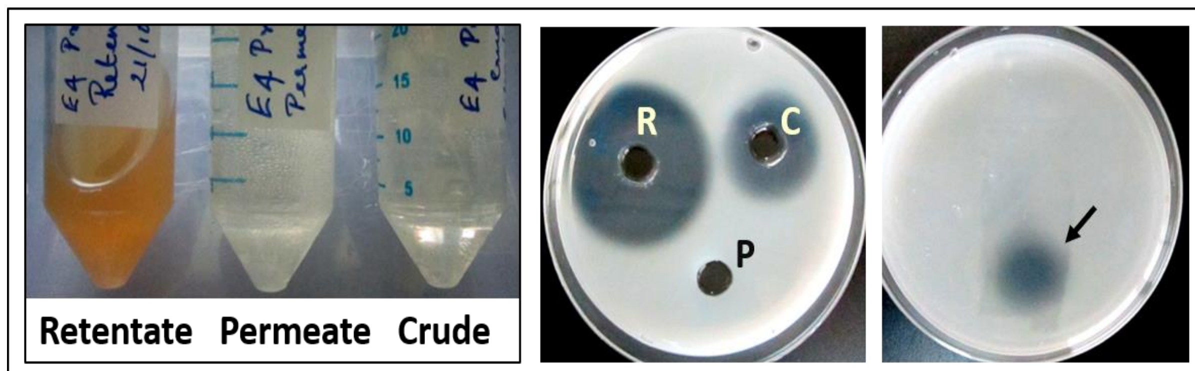


Fig 3.7 The retentate and permeate solution in comparison with the crude enzyme extract (Left panel); Gelatin well assay showing the zone of protease activity in 50µl each of retentate(R), Permeate(P) and Crude(C) (Center panel); Gel overlay assay of the retentate on a gelatin plate showing the zone of activity indicated by black arrow (Right panel).

(ii) Organic solvent precipitation: The precipitation of protease was carried out using acetone and ethanol. *Halococcus* sp. E4 protease after concentration showed a specific activity of 0.94 and 1.03 for acetone and ethanol respectively (Table 3.2). Ultrafiltration is more effective than organic solvent precipitation (Table 3.2). Hence for further purification, the crude protease extract was concentrated using ultrafiltration with 30 kDa MWCO regenerated cellulose membrane.

3.3.2.3 Purification by gel filtration chromatography

Different combinations of gel filtration columns were used to maximize both the purity and yield. Sequential purification with gel filtration columns of different bead sizes yielded a partially purified protease. The best combination was to run multiple rounds of gel filtration chromatography with Sephadex G50 and then pool all the active fractions (2ml each) from the different iterations, and concentrate it using ultrafiltration Fig. 3.8 shows the protein profile obtained after a single run of Sephadex G50 with the active fractions as Fraction 4-8.

Table 3.2 Comparison of different methods used to concentrate protease enzyme from *Halococcus* sp. E4.

	Sp. Activity (U/mg)	Yield (%)	Purification fold
Crude	5.4	100	1
Ultrafiltration			
10kDa Retentate	1.04	38.24	0.21
10kDa Permeate	0.11	7.93	0.02
30kDa Retentate	1.66	78.39	0.33
30kDa Permeate	0.13	3.77	0.03
Organic solvent precipitation			
Acetone	0.94	21.96	0.19
Ethanol	1.03	25.08	0.21

Multiple runs were carried out using the concentrated protease, the active fractions were pooled and concentrated using ultrafiltration. A non-denaturing PAGE (7%) was carried out with the crude, permeate, retentate and the active pooled fractions. A gel overlay assay was also carried out to check for protease activity (Fig 3.9). Only the retentate (protease concentrated by ultrafiltration using 30 kDa membrane) showed activity in the gel overlay assay though quantification with azocasein showed an increase in the specific activity which is substantiated by the protein profile obtained from the Sephadex G50 run.

The pooled active fractions from Sephadex G50 was pooled, concentrated using a 30 kDa membrane and loaded on a Sephadex G100 column. 10% non-denaturing silver stained PAGE showed the presence of multiple proteins and zymogram showed a zone of clearance corresponding to protease activity rather than bands corresponding to protease activity (Fig 3.10).

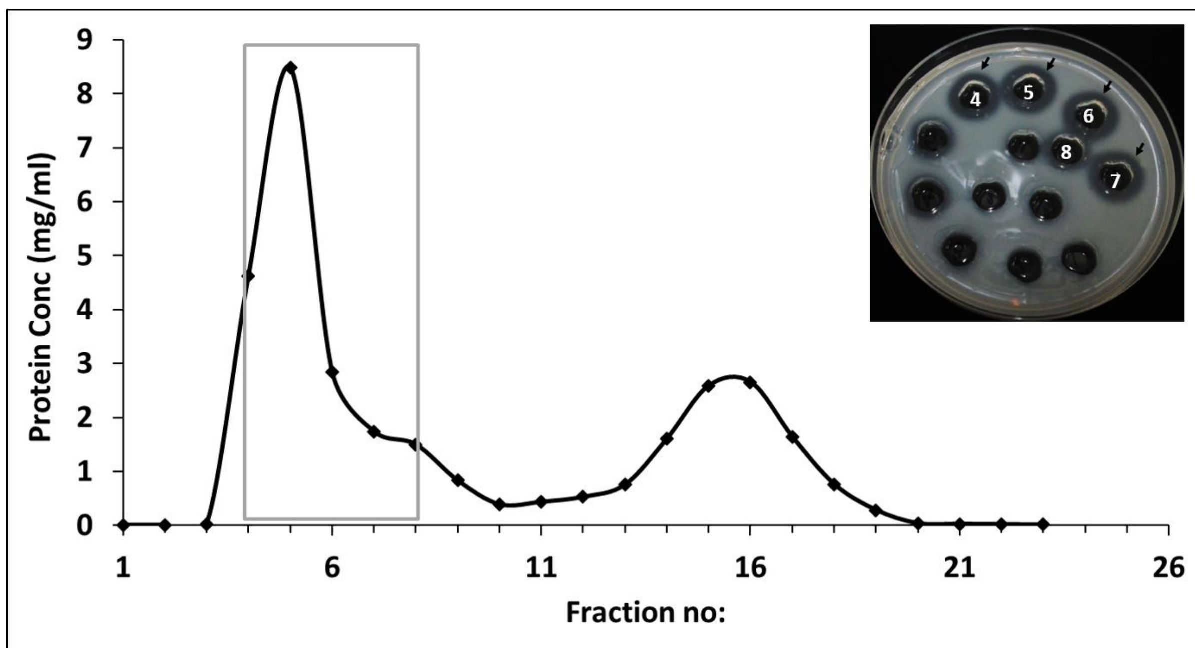


Fig 3.8 The protein profile obtained from Sephadex G50 column. Fractions 4-8 (indicated by a rectangle) showed protease activity. Inset shows the gelatinase well assay with the active fractions indicated by black arrows.

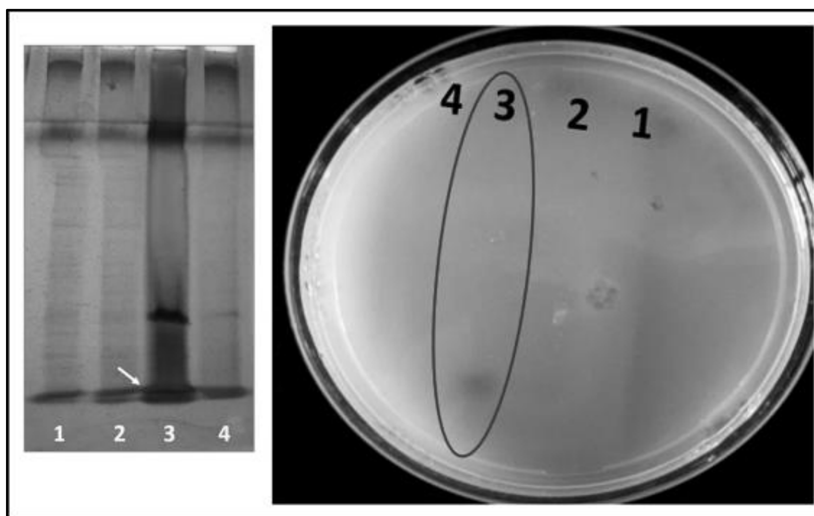


Fig 3.9 Non-denaturing (7%) polyacrylamide gel stained with silver stain (Left) showing the crude protease (Lane 1); 30 kDa Permeate (Lane 2); 30kDa Retentate (Lane 3). Equal volumes were loaded on to the gel and then a gel overlay assay was carried out with 0.25% gelatin as substrate. Retentate showed a clearance corresponding to protease activity.

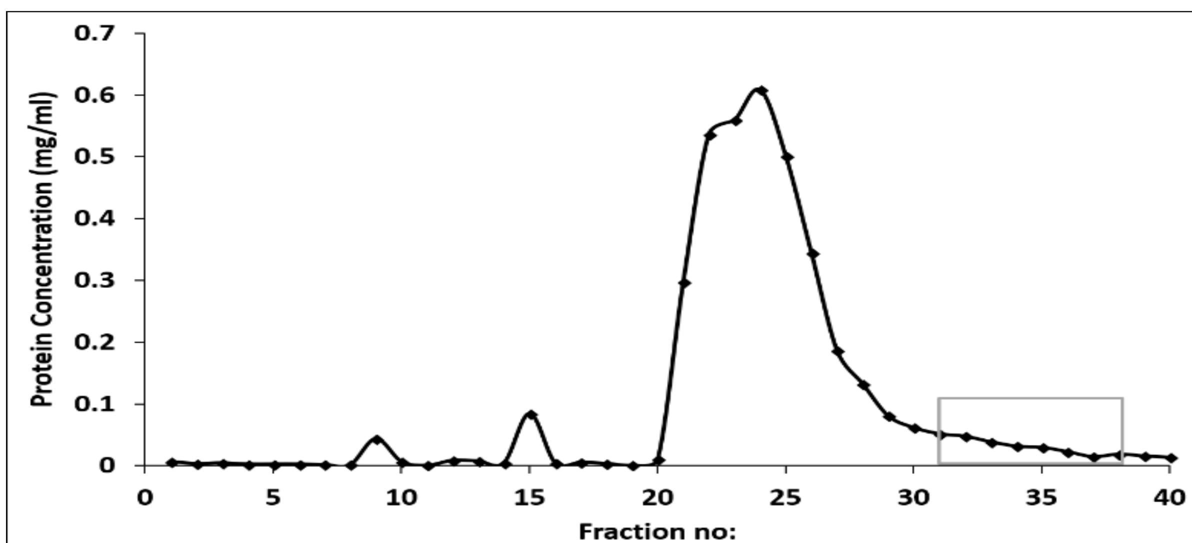


Fig 3.10 Protein profile obtained from Sephadex G100 column (Left). Fractions 9-13 (indicated by a rectangle) showed protease activity. Non-denaturing PAGE (10% gel) silver stained to reveal the proteins present in the pooled active fractions (Lane 1) and the corresponding zymogram showing a zone of clearance implying protease activity (Lane 2).

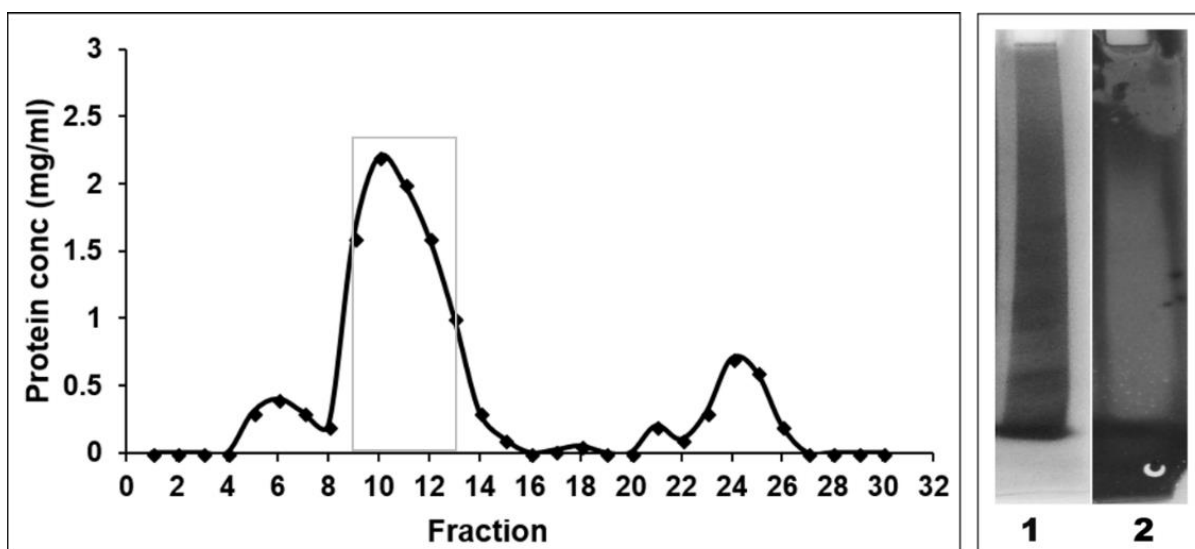


Fig 3.11 Protein profile obtained from Sephacryl S 200HR column. Fractions 31-38 (indicated by a rectangle) showed protease activity.

The pooled active fractions were further concentrated by ultrafiltration with a 30 kDa membrane and the retentate was loaded on to a Sephacryl S 200HR column. The protein

profile of the chromatographic run is shown in Fig 3.11. A higher level of purification with a 6.39 purification was obtained after this step (Table 3.3).

Table 3.3 Purification table of protease from *Halococcus* sp. strain E4

Purification step	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Sp. Activity (U/mg)	Purification Fold	Total Activity (U)	Yield (%)
Crude supernatent	2000	0.52	0.097	5.41	1.08	1046	100
Retentate	40	20.49	12.34	1.66	0.33	819.96	78.4
Sephadex G50	20	45	8.41	5.35	1.07	900	86.0
Sephadex G100	10	59	2.98	19.80	3.96	590	56.4
Sephacryl S 200HR	5	67	2.1	31.90	6.39	335	32.0

The pooled active fractions were run on a non-denaturing gel (10 %) which showed the presence of several proteins with protease activity (Fig 3.12). The CBB stain showed a major contaminant which does not show protease activity. The bands corresponding to the active proteases was not visible with CBB staining. The major active fractions of around 67 kDa and 46kDa can be purified further using size-based techniques. Another protein with approximately 38kDa molecular weight was also observed was too close to the major protein contamination present. Hence it will be impractical to separate it using any size-based technique. The clearance obtained below 20kDa might be due to the presence of salt as salt precipitates were observed in that area.

Further characterization has been carried out with the semi pure active concentrated fraction obtained from Sephacryl S 200 HR.

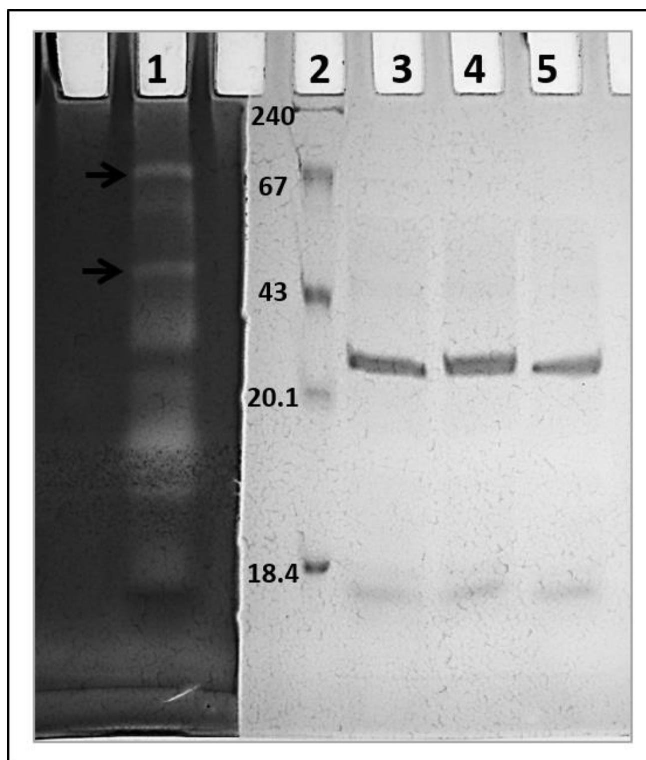


Fig 3.12: 10% non-denaturing gel showing *Halococcus* sp. E4 protease the zymogram and the CBB stain with native PAGE marker. Clear bands in dark background (Lane 1) corresponds to protease activity. Lane 2- Marker; Lane 3, 4, 5- partially purified protease (in triplicates)

A serine protease purified from *Halogranum rubrum* by using ultrafiltration followed by ion exchange chromatography with DEAE Sepharose and then Gel filtration by Sephadex G100 showed a molecular mass of 47 kDa which may be similar to the protein isolated from *Halococcus* sp. E4 (Gao et. al., 2017). The major protease from *Natrialba magadii* had an apparent molecular mass of 36 kDa which may be similar to the 38kDa protease seen in *Halococcus* sp. E4 (Giménez et. al., 2000). These similarities can be validated only after the proteases from E4 are purified to homogeneity.

3.3.2.4 Characterization of protease from *Halococcus* sp. E4

(i) pH: The optimum pH for protease activity was observed at pH 8.0 though comparable activity was seen in the pH range 8.0-10.0 (Fig 3.13A). This is a typical characteristic of haloarchaeal enzymes which usually prefer slightly alkaline to alkaline pH 10.0-11.0 for their activity. Examples are proteases from *Natrialba magadii* (pH 8.0–10.0), *Natronococcus*

occultus NCBM 2192 (pH 7.0-9.0) and *Halogeometricum borinquense* TSS101 (pH 10.0) (Giménez et. al., 2000; Studdert et. al., 2001; Vidyasagar et. al., 2006).

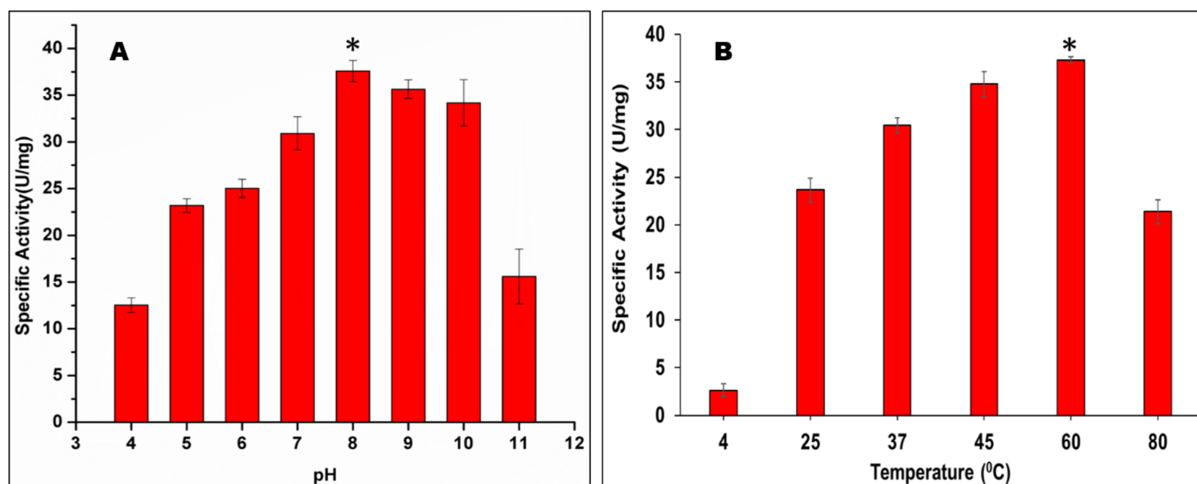


Fig 3.13 Effect of (A) pH and (B) Temperature on the activity of protease from *Halococcus* sp. strain E4 (* $p< 0.05$).

(ii) Temperature: Proteolytic activity was tested at different temperature between (4 ^oC, 25 ^oC, 37 ^oC, 45 ^oC, 60 ^oC and 80 ^oC) at the optimum pH 8.0. It was seen that protease activity increased with increase in temperature with maximum activity observed at 60 ^oC. A drastic decrease in activity was seen at 80 ^oC (Fig 3.13B) though a residual activity of around 70% was retained even at this high temperature. High temperature optima are not uncommon in halophilic enzymes owing to the fact that they generally reside in hypersaline econiches where they have to cope with varying temperatures which is more than what 'normal' growth conditions. *Natrialba magadii*, *Natronococcus occultus* NCBM 2192, *Natrinema* sp. J7, *Halogeometricum borinquense* TSS101, *Haloferax lucentensis* VKMM 007 are few halophilic archaea whose protease exhibits a pH optimum of 50-60 ^oC (Giménez et. al., 2000; Studdert et. al., 2001; Shi et. al., 2006; Vidyasagar et. al., 2006; Manikandan et. al., 2009).

(iii) NaCl: Highest proteolytic activity was seen at 4 M (23.4% w/v) NaCl though comparable activity was observed from 3 M (17.5% w/v) -5 M (29.2% w/v) NaCl (Fig. 3.14). As seen with most haloarchaeal proteins, the extracellular protease from *Halococcus* sp. E4 showed negligible activity in the absence of salt, underlining the role of salt in haloarchaeal enzyme activity and stability (Giménez et. al., 2000).

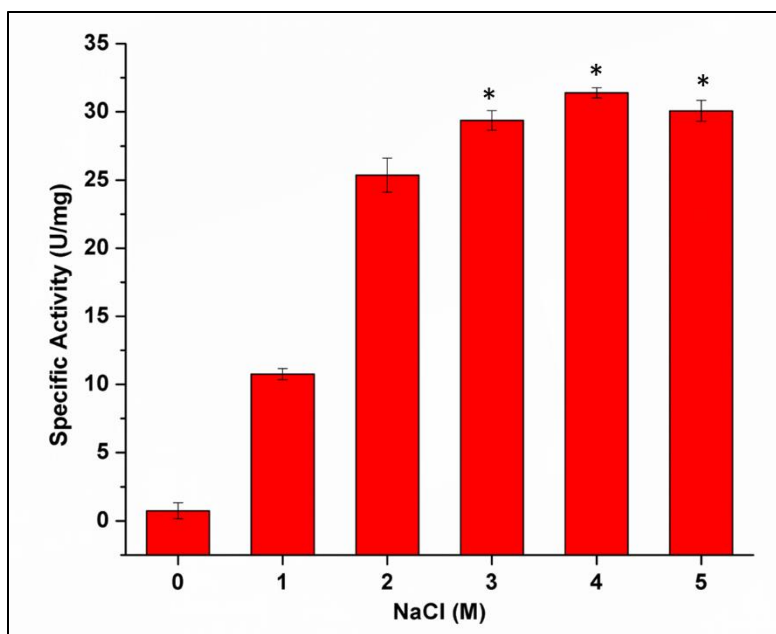


Fig 3.14 Effect of NaCl on the activity of protease from *Halococcus* sp. strain E4 (*p< 0.05).

The halophilic proteins are known to have highly negative surface charge due to the exposure of acidic residues on its surface (Reed et. al, 2013). This negative charge is neutralized by tightly bound water dipoles. This increases their solubility and flexibility in molar salt concentrations, thereby stabilizing the protein structure, preventing protein aggregation where their non-halophilic counterparts would have become rigid and non-functional (Dym et. al., 1995). The requirement of molar concentrations of salt for halophilic protein stabilization has been attributed to low affinity binding of salt to specific sites on the protein surface required to stabilize the active conformation of the protein (Nayek et. al., 2014; Mevarech et. al., 2000; DasSarma and DasSarma, 2015).

(iv) EDTA and Metal ions: EDTA had a significant effect on the protease activity of *Halococcus* sp. E4 protease activity with the residual activity reduced to around 40% in comparison with the control indicating that this enzyme may require metal ions as cofactor for its activity (Fig 3.15). Similar inhibition (33% residual activity with 10mM EDTA) was observed in case of halo-alkali-thermophilic protease from *Halobacterium* sp. strain HP25 (Elbanna et. al., 2015). Most haloarchaeal proteases characterized till date are serine proteases and Ca^{2+} is known to have an important role in maintaining the activity of serine

protease (Ward, 1983). Ca^{2+} also has the ability to alleviate enzyme denaturation and increase thermostability (Hutadilok-Towatana et. al., 1999; Vidyasagar et. al., 2006)

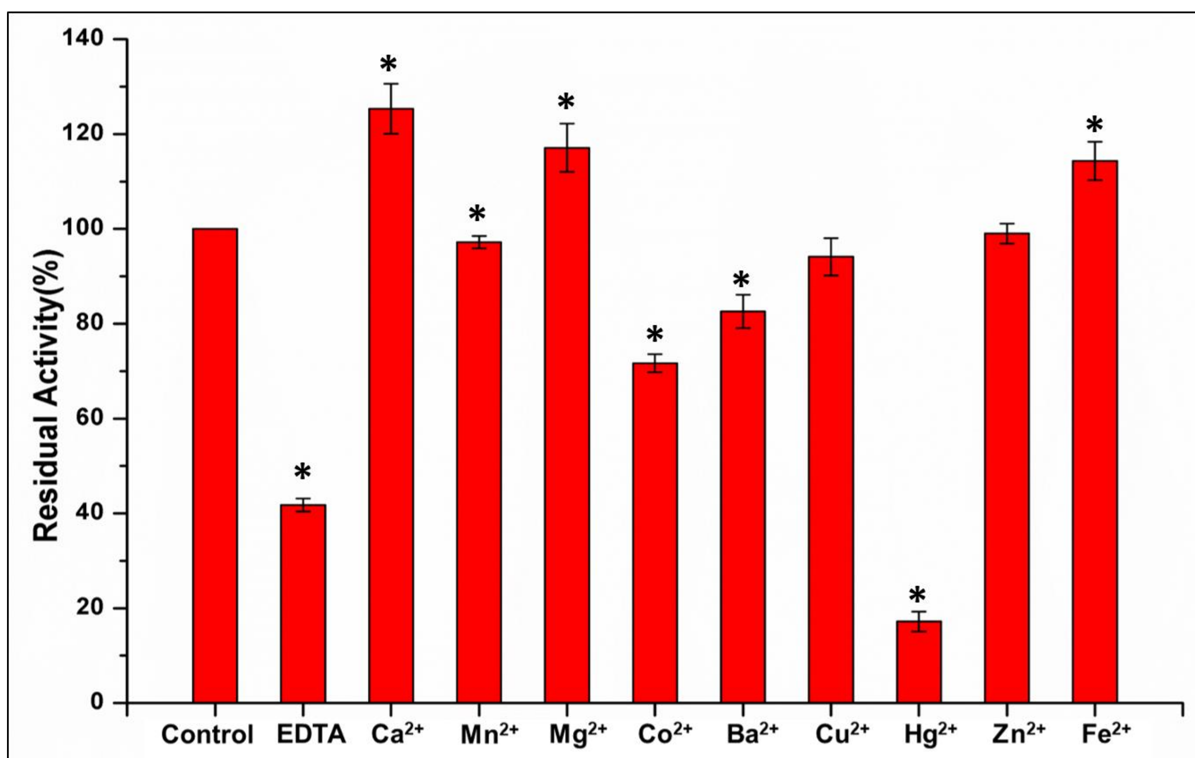


Fig 3.15 Effect of EDTA (10mM) and metal ions (2mM) on the activity of protease produced by *Halococcus* sp. E4 (* $p < 0.05$).

(v) Organic solvents: *Halococcus* sp. E4 protease was found to be stable in ethanol and acetone without much loss of activity even after 7 days of exposure (Fig 3.16 A). With methanol, more than 70% of enzyme activity was retained. Organic solvents create an environment with low water activity due to which most proteins lose their structure and consequently their function. However, haloarchaea which are adapted to hypersaline low water activity environments produces enzymes which are inherently stable in these conditions (Ruiz and De Castro, 2007; Manjula, 2014). An organic solvent tolerant halophilic protease was characterized from *Halobacterium halobium* (Kim and Dordick, 1997) but protease from another halophilic archaea *Natrinema* sp. BTSH10 was found to be unstable in organic solvents other than ethanol (Manjula, 2014).

(vi) Detergents: *Halococcus* sp. E4 protease exhibited a considerable stability in the presence of non-ionic detergents (Triton X-100 and Tween 80) but was considerably affected

by SDS, which is an anionic detergent (Fig 3.16 B). Detergent stable protases have been previously purified from few halophilic archaea such as *Halobacterium* sp. strain HP25, *Haloferax* sp. strain APP15. The unusually stable properties of these enzymes may allow it to find potential application as an additive in the laundry detergent industry. (Elbanna et. al., 2015; Pathak and Sardar, 2014).

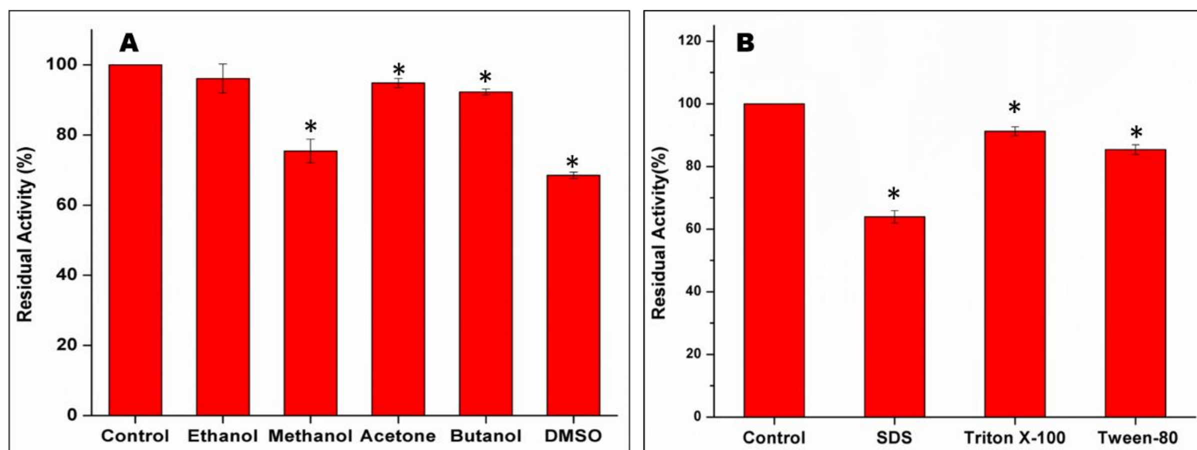


Fig 3.16 Effect of Organic solvents (A) and Detergents (B) on activity of protease produced by *Halococcus* sp. E4 (* $p < 0.05$).

3.3.3 Amylase production by *Halogeometricum* sp. strain E3

3.3.3.1 Growth and amylase production of *Halogeometricum* sp. strain E3

Halogeometricum sp. E3 grew well in NH +1% starch, pH 7 and temperature 37 ^{o}C in shaking conditions reached stationary phase in 2-3 days. The amylase production was increased as the culture approached stationary phase, with maximum amylase production on Day 4 (Fig 3.17). Hence, all further experiments were carried out with amylase extracted on Day 4, when OD at 600 reached 1.27.

3.3.3.2 Optimization of culture conditions for production of amylase from *Halogeometricum* sp. E3

(i) Aeration: There was a significant increase in the growth of *Halogeometricum* sp. E3 with agitation when compared to that grown in stationary condition (Fig 3.18). A 4-fold increase in amylase production was also observed when the culture was stirred. The effect of aeration has not been documented much in case of halophilic archaea but *Halobacillus* sp.

(Amoozegar et. al., 2003), *Marinobacter* sp. EMB8 (Kumar and Khare, 2015) showed an increase in the production of amylase with increased aeration.

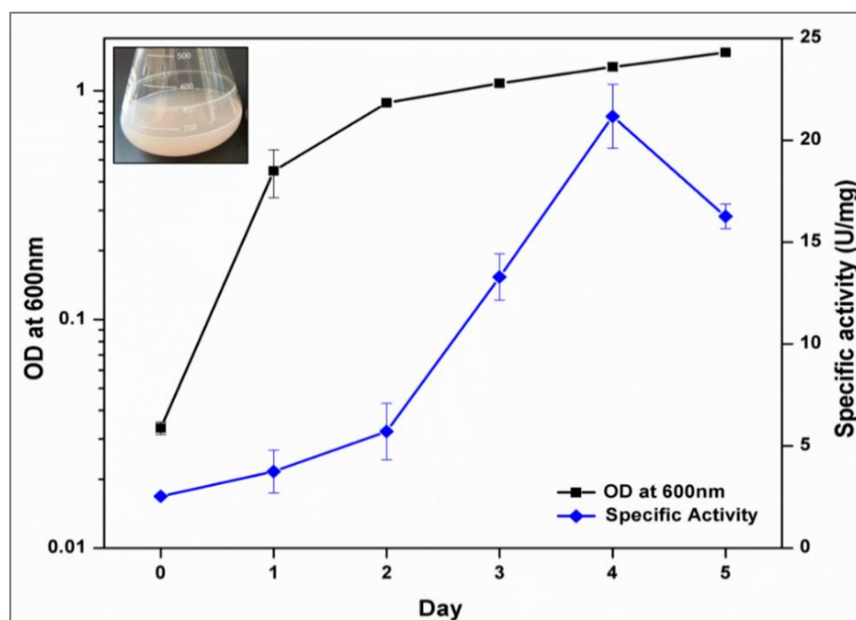


Fig 3.17 Growth of *Halogeometricum* sp. E3 in NH media supplemented with 1% starch, pH 7 and the corresponding amylase production. Inset shows the liquid culture of pink coloured *Halogeometricum* sp. strain E3.

(ii) Temperature: Growth of *Halogeometricum* sp. E3 increased with temperature but there was a drastic decrease in the production of amylase after 37 ^{o}C (Fig 3.18). Similar results were seen with other halophilic archaea. *Halorubrum xinjiangense* and *Halobacterium* MMD047 has an optimum temperature at 40 ^{o}C whereas *Haloferax* sp. has an optimum temperature of 37 ^{o}C respectively (Moshfegh et. al., 2013; Shanmughapriya et. al., 2009; Bajpai et. al., 2015).

(iii) pH: The optimum pH for growth of *Halogeometricum* sp. E3 was pH 7.0-8.0 (Fig 3.19). The protease activity was also optimum at the same pH (7.0-8.0) after which both the growth and protease declined with the increase in pH. pH has an important role in growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium affect the synthesis and secretion of amylase just like its stability (Fogarty and Kelly, 1990). In acidic medium, the growth and amylase production were negligible. *Halobacterium salinarum* MMD047 also showed similar pH preference with pH

optimum for amylase production at 7.0 and but it could grow in a wider range of pH 5.0-9.0 (Shanmughapriya et. al., 2009).

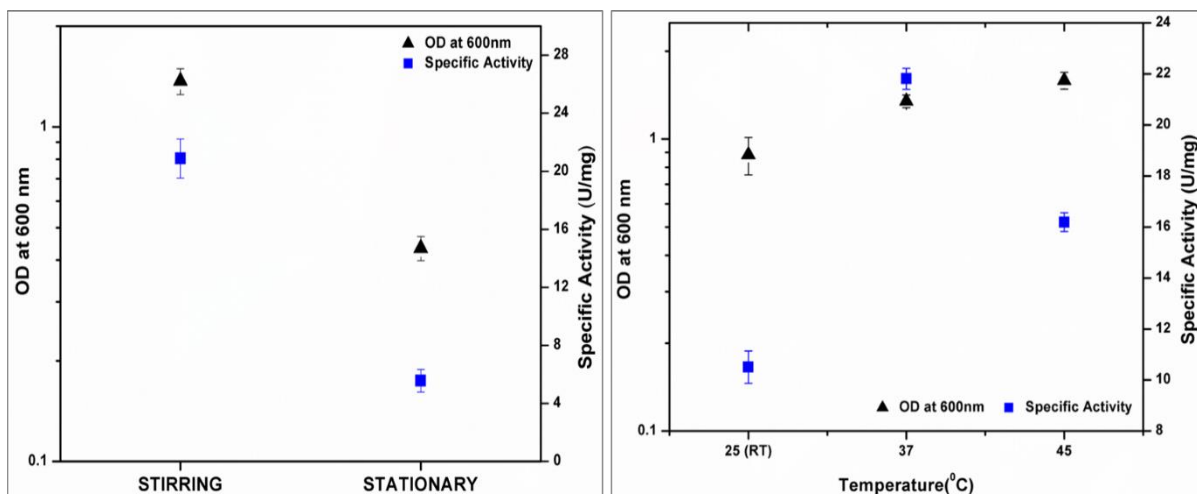


Fig 3.18 The effect of stirring/agitation (Left) and temperature (Right) on the growth of *Halogeometricum* sp. E3 and its amylase production. Data taken on Day 4 of growth.

(iv) NaCl: For *Halogeometricum* sp. E3, the optimum NaCl required for growth and amylase production was 3-4 M NaCl (Fig 3.19).

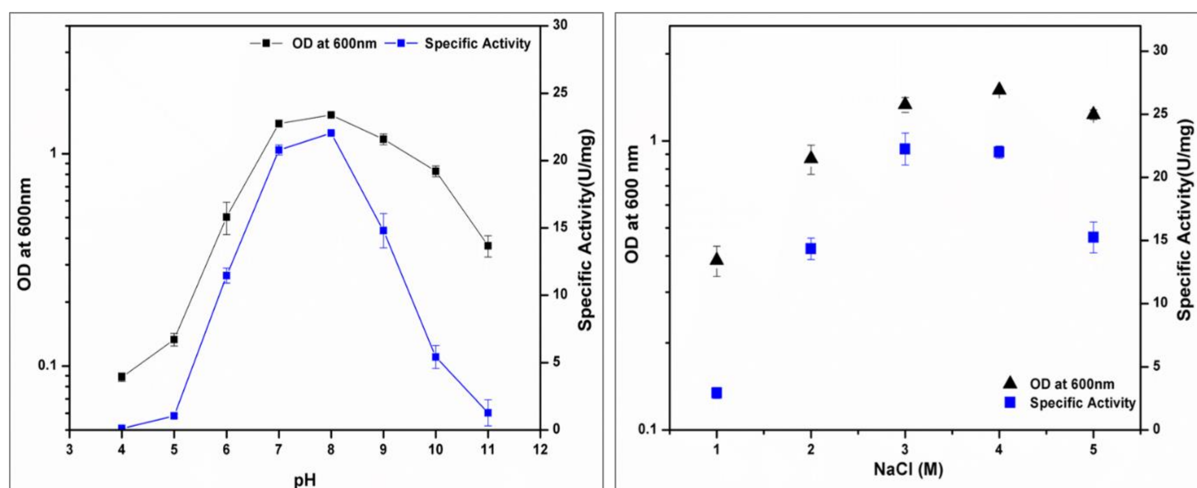


Fig 3.19 Effect of pH (left) and NaCl concentration (right) on the growth of *Halogeometricum* sp. E3 and its amylase production

Molar concentrations of NaCl is typically required for maintaining the stability and function of haloarchaeal enzymes (Karan et. al, 2012). The optimum salt requirement for amylase production in halophilic archaea is mostly found to be species dependent, ranging from 2.5 M for *Natronococcus* sp. strain Ah-36 (Kobayashi et. al., 1992), 3 M for *Haloferax* sp. HA10 (Bajpai et. al., 2015), 4 M for *Halorubrum xinjiangense* (Moshfegh et. al., 2013) to 4.3 M NaCl for *Halobacterium halobium* (Patel et. al., 1993).

(v) Starch (Substrate) concentration: Growth and amylase production increased with the increase in starch content till a concentration of 1.5% starch. Beyond that concentration, the media got saturated with starch and hence could not be tested. The optimum concentration for amylase production was seen to be 1-1.5% starch (Fig 3.20).

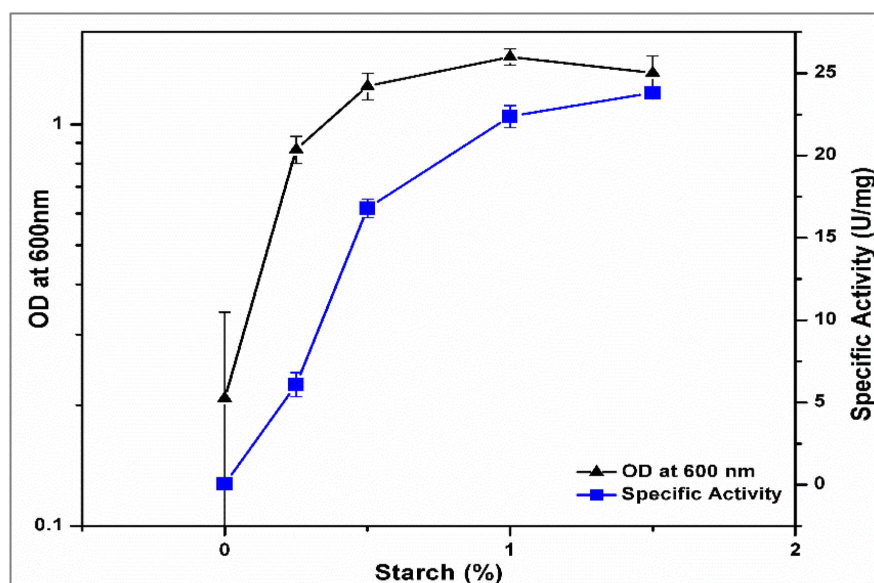


Fig 3.20 Effect of Starch (Substrate) concentration on the growth of *Halogeometricum* sp. E3 and its amylase production

Amylase production from *Halogeometricum s*p. E3 was observed to be inducible with starch as can be inferred from the increase in amylase production with the increase in starch concentration in the media. Amylase production is known to be inducible in most halophiles with the preferred inducers being starch, dextrin and maltose (Kumar et. al., 2016). In contrast, *Natrialba aegyptiaca* strain 40T could produce amylase constitutively though productivity was low. Its optimum requirement was 0.05% (w/v) starch whereas for *Haloferax mediterranei* and *Haloarcula* sp. S-1 the optimum requirement was 0.2 and 1%

(w/v) soluble starch respectively (Hagaggi et. al., 2013; Pérez-Pomares et. al., 2003; Fukushima et. al., 2005).

3.3.3.3 Concentration of crude amylase from *Halogeometricum* sp. E3

Amylase was concentrated by ultrafiltration and ethanol/ acetone precipitation (Table 3.4). Though purification was attempted by several techniques such as affinity chromatography, gel filtration chromatography and triple phase partitioning or by a combination of these techniques, the results were not conclusive. In each step, starch and salt was seen to interfere with the purification process.

Table 3.4 Purification table for amylase concentrated using various techniques

Concentration step	Vol (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Sp. Activity (U/mg)	Purification Fold
Crude supernatent	1000	120.340	6	20.06	1.00
Retentate 10 kDa	70	380.36	11.59	32.82	1.64
Retentate 30 kDa	60	210.50	10.34	20.36	1.01
Permeate 10 kDa	930	80.42	3.76	21.39	1.07
Permeate 30 kDa	940	105.36	4.75	22.18	1.11
Acetone	25	195.31	20.25	9.64	0.48
Ethanol	25	173.77	19.01	9.14	0.46

3.3.3.4 Non-denaturing PAGE and zymogram

Native PAGE and zymogram was carried out to check for the molecular weight of the extracellular amylase from *Halogeometricum* sp. E3 but since the zymogram corresponding to the protein bands exhibited the binding of starch to the proteins (rather than the clearance/ amylase activity), molecular weight could not be elucidated. Further characterization was carried out using the concentrated crude enzyme (Fig 3.21).

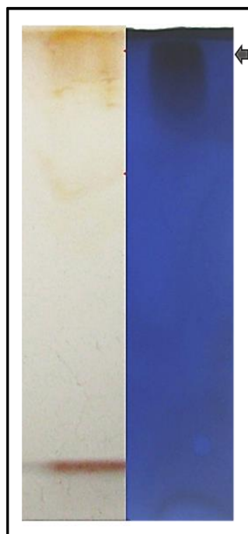


Fig 3.21 Non-denaturing PAGE (silver stained) and zymogram showing the binding of starch to the protein

3.3.3.5 Characterization of amylase from *Halogeometricum* sp. E3

(i) Thin layer chromatography: The major product obtained after reaction of starch with E3 amylase was maltose (Fig 3.22). Glucose was undetectable. Starch substrate, maltose and glucose were used as controls. There are three types of amylase – α, β and γ amylase. α-Amylase cleaves internal α-1, 4-glycosidic linkages in starch to yield glucose and maltose. β-Amylase is an exo-hydrolase enzyme that hydrolyses α-1, 4-glucan linkages to yield successive maltose units. γ-Amylase cleaves α (1-6) glycosidic linkages and α (1-4) glycosidic linkages to yield glucose. γ- amylase is most efficient in acidic environments (Sundarram and Murthy, 2014; Sivaramakrishnan et. al., 2006).

(ii) Optimum pH and temperature for E3 amylase activity: The optimum pH and temperature required for *Halogeometricum* sp. E3 amylase was 8.0 – 10.0 and 60°C respectively. The activity of E3 amylase increased with increasing pH till pH 10.0 after which there was a drastic decrease in amylase activity (Fig 3.23). This amylase was active in the temperature range 25.0 - 80.0 with around 50% residual activity at a high temperature of 80°C.

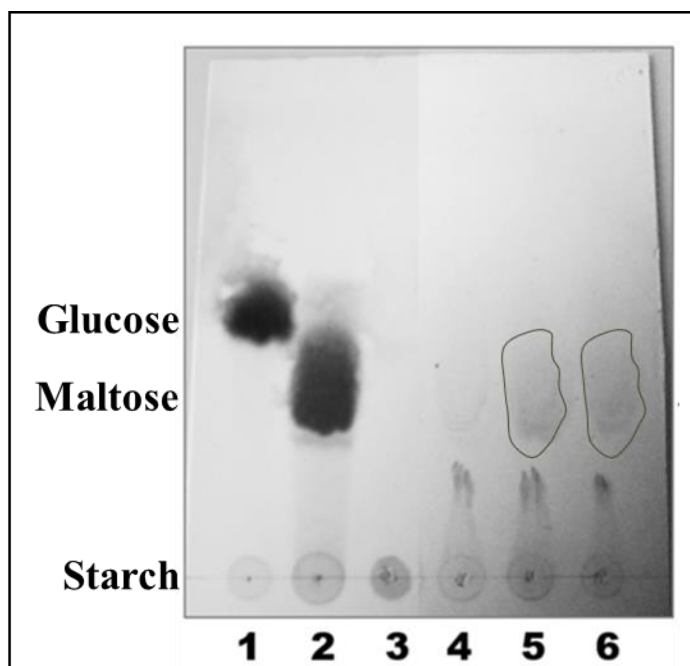


Fig 3.22 Thin layer chromatography showing the products of *Halogeometricum* E3 amylase. 1-Glucose, 2-Maltose, 3- Starch (Substrate), 4- 10kDa Permeate, 5- 10 kDa Retentate, Crude E3 amylase

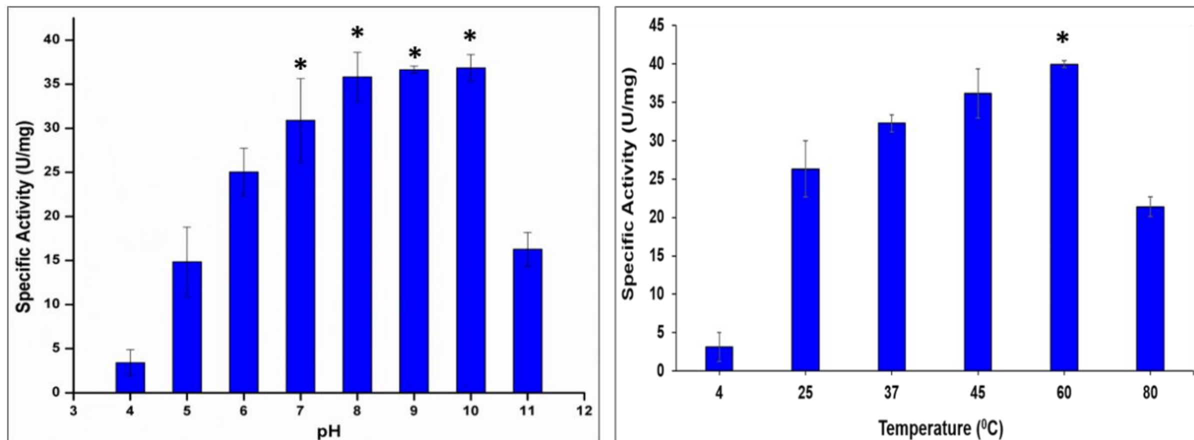


Fig 3.23 Effect of pH (Left) and Temperature (Right) on the activity of amylase produced by *Halogeometricum* sp. E3(* $p < 0.05$).

Though a temperature optimum of 60ºC is common among haloarchaeal amylases, pH optima from 8.0 – 10.0 is unusual. A 74 kDa amylase from *Natronococcus* sp. strain Ah-36 showed pH optimum 8.7 and temperature optimum at 55ºC (Kobayashi et.al., 1992). An organic solvent tolerant 70 kDa amylase from *Haloarcula* strain S-1 (Fukushima et. al.,

2005) and 60 kDa amylase from *Halorubrum xinjiangense* (Moshfegh et. al., 2013) showed a pH optimum 7.0 and 8.5, and Temperature optimum 50°C and 70 °C respectively.

(ii) Optimum NaCl concentration required for *Halogeometricum* sp. E3 amylase activity: The optimum NaCl concentration required for the optimal activity of E3 amylase was found to be 3.0 – 4.0M though high amylase activity was seen in the range of 2.0 – 5.0M NaCl. Around 50% reduction in activity was observed when the salt concentration was reduced from 2M to 1M (Fig 3.24).

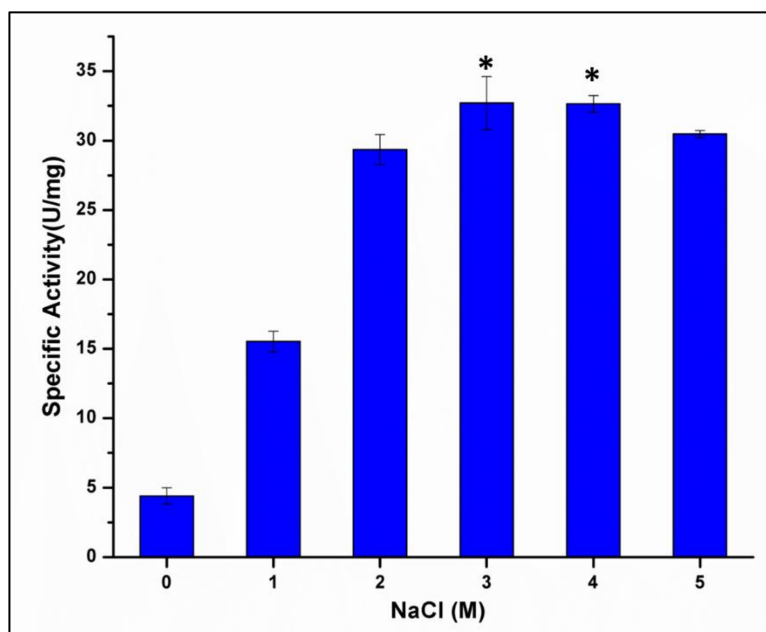


Fig 3.24 Effect of NaCl concentration on the activity of amylase produced by *Halogeometricum* sp. E3(* $p < 0.05$).

The importance of salt in maintaining the structure and function of haloarchaeal proteins have been described previously (Section 3.4.2.4 (iii)). Similar salt requirements are seen in other haloarchaeal strains such as *Natronococcus* sp. strain Ah-36, *Haloferax mediterranei*, *Haloarcula* strain S-1, *Haloarcula hispanica, Halorubrum xinjiangense* with optimum NaCl requirements of 2.5 M, 3.0 M, 4.3 M, 4-5 M and 4 M respectively (Kobayashi et. al., 1992; Pérez-Pomares et. al., 2003; Fukushima et. al., 2005; Hutcheon et. al., 2005; Moshfegh et. al., 2013).

(iii) Effect of EDTA and different metal ions on E3 amylase activity: In the presence of 10mM EDTA there was almost complete cessation of amylase activity underlining the importance of metal ions in the activity of amylase. Ca^{2+} ions showed the maximum positive influence on the activity of E3 amylase with Mg^{2+} and Zn^{2+} being the other two ions showing a positive influence on amylase activity. Not much change was observed with the addition of Cu^{2+} and Fe^{2+} ions. Mn^{2+}, Ba^{2+}, Co^{2+} and Hg^{2+} adversely affected the function of E3 amylase with Hg^{2+} being the most potent inhibitor among the ions tested (Fig 3.25).

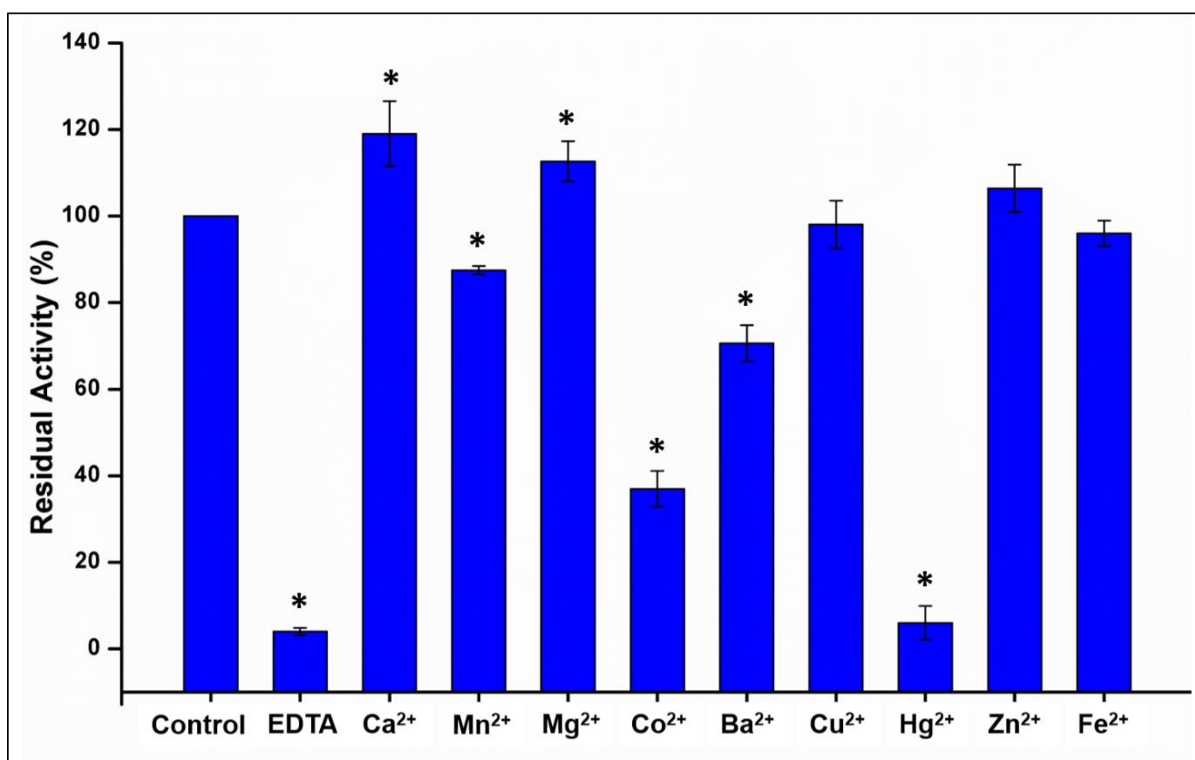


Fig 3.25 Effect of EDTA and different metal ions on the activity of amylase produced by *Halogeometricum* sp. E3 (* $p < 0.05$).

The complete inhibitory effect of EDTA and the positive effect of Ca^{2+} ions imply that the amylase from *Halogeometricum* sp. E3 is an α-amylase. α-amylases are metalloenzymes having calcium ion as a cofactor. Similar irreversible loss of α-amylase activity was also observed in *Haloferax mediterranei* upon addition of EDTA (Kumar et. al., 2016; Pérez-Pomares et. al., 2003).

(iv) Effect of detergents and organic solvents on E3 amylase activity: Non-ionic detergents Triton X-100 and Tween 80 did not have much effect on the activity of E3 amylase but anionic detergent SDS was highly inhibitory. Organic solvents such as ethanol, methanol and acetone did not affect the amylase activity. Butanol showed 80% residual activity while DMSO caused a drastic reduction in *Halogeometricum* sp. E3 amylase activity (Fig 3.26).

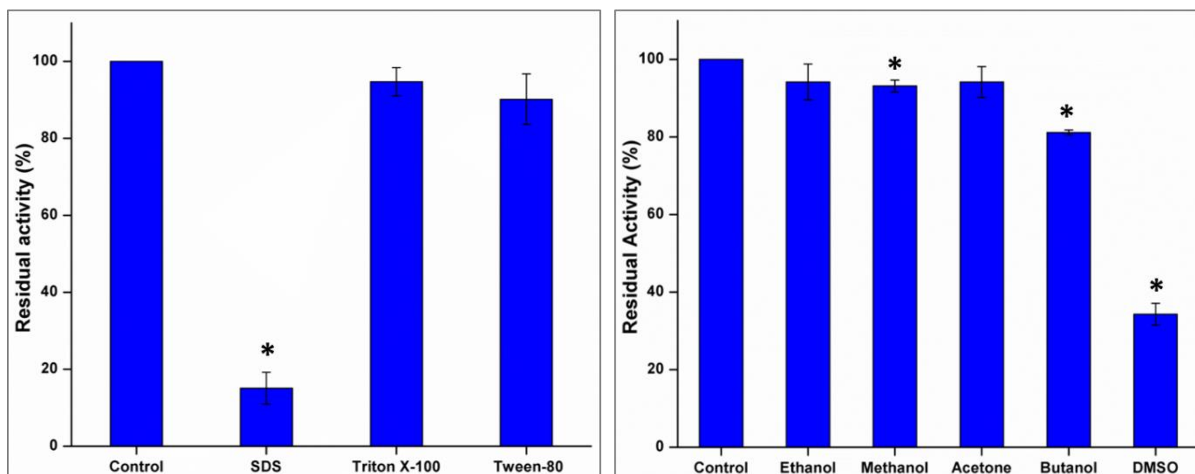


Fig 3.26 Effect of detergents and organic solvents on amylase activity from *Halogeometricum* sp. E3 (* $p < 0.05$).

The unique feature of organic solvent and detergent tolerance renders amylase from *Halogeometricum* sp. E3 to be a highly useful enzyme of biotechnological interest for enzymatic hydrolysis of starch, employing organic solvents as well as textile and laundry industries.

3.4 SUMMARY AND CONCLUSION

Halophilic archaea isolated from the solar salterns of Goa and Tamil Nadu, India namely, *Haloarcula* sp. strains E1 and E2, *Halogeometricum* sp. strain E3, *Halococcus* sp. strains E4 and M3, *Haloferax* sp. strains E5 and M1, *Halorubrum* sp. strains M2 and M5, were screened for the production of various hydrolytic enzymes. Of these, 7 amylase producers, 3 protease producers, 1 lipase producer, 6 esterase producers and 5 pectinase producers were obtained in the study. Cellulase producers were not seen in the screened halophilic archaeal isolates.

Protease produced by *Halococcus* sp. strain E4 was produced in NH medium supplemented with 1% skimmed milk (SM). Growth was observed till NaCl concentration of 30% , in a wide range of pH 5.0-9.0, and a temperature range of 25 - 45ºC. However, optimum NaCl, pH and temperature for production of protease was 25%, 7.0 - 8.0 and 37 ºC respectively. E4 protease was partially purified using ultrafiltration and gel chromatography and a yield of 32% and a purification fold of 6.39 was obtained, the molecular weight of the protein fractions was found by native PAGE to be around 67 kDa and 46 kDa. The protease was found to be metalloprotease. Significant protease activity was observed in the pH range of pH 5.0-10.0 and temperature range of 25-80 ºC. However, optimum conditions for protease activity were pH 8.0 and Temp 60 ºC. Metal ions Ca^{2+}, Fe^{2+}, and Mg^{2+} contributed to an increase in activity. The enzyme was able to tolerate 1% (v/v) non-ionic detergents such as Triton X-100, Tween 80 and was active in organic solvents such as methanol, ethanol and acetone. This is the first report on production and characterization of a protease from *Halococcus* sp.

Amylase from *Halogeometricum* sp. E3 was concentrated 1.64 folds by ultrafiltration using a 10 kDa membrane. Optimum amylase production was observed in NH medium supplemented with 1% starch in the stationary phase. The initial pH of the medium was 7.0-8.0 and temperature 37 ºC. Though purification was attempted using triple phase partitioning (TPP) and gel filtration chromatography, presence of starch and NaCl interfered with the purification techniques used. It was active from pH 5.0 to pH 10.0 and optimum pH was at 8.0 – 10.0. The amylase from *Halogeometricum* sp. E3 was active from 25 ºC to 80 ºC while its temperature optimum was at 60 ºC. It was active in the presence of 1% non-ionic detergents, Triton X-100 and Tween 80 and organic solvents such as ethanol, methanol, acetone and butanol. Ca^{2+}, Mg^{2+}, Zn^{2+} increased the activity whereas metals Mn^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+} decreased the activity of the enzyme. The polyextremophilic amylase was found to be α amylase and this is the first report of an amylase from *Halogeometricum* sp.

The ability of halophilic archaea to produce various hydrolytic enzymes were explored in this study and novel archaeal sources have been found. These polyextremophilic enzymes have high potential for use in harsh industrial conditions.

Chapter IV

Characterization of haloarchaeal pigments and their effect on human cell lines under various stress conditions

4.1 INTRODUCTION

The non-polar lipids of halophilic archaea are mainly C50 carotenoids, with bacterioruberins as the major carotenoids (Kushwaha et. al.,1974; Rønnekleiv and Liaaen-Jensen,1995) and C30 isoprenoids squalenes, retinal, vitamin MK-8 and C40-carotenoids such as carotene and lycopene, as the minor carotenoids (Asker et. al., 2002). Ourisson and Nakatani (1989) reported the role of bacterioruberin in reinforcing the membrane structure of *Halobacterium* spp.

Carotenoids are competent scavengers of hydroxyl free radicals and singlet oxygen species and reactive nitrogen species; their antioxidant properties being attributed to their chemical structure, mainly the number of conjugated double bonds and the type of structural end-group (Yatsunami et. al., 2014, Shahmohammadi et. al., 1998, Albrecht et. al., 2000, Abbes et. al., 2013, Chisté et. al., 2011). One such carotenoid with well-established antioxidant property is β-carotene (Sies and Stahl, 2003). Stahl and co-workers demonstrated that β-carotene and a combination of β-carotene and vitamin E administered orally over a period significantly reduced the incidence of ultra violet (UV) light induced erythema by filtering out blue light in humans (Stahl et. al., 2000).

The carotenoid pigments in halophilic archaea serve to protect the archaeal cells from the harmful effect of UV radiation and assists in photoreactivation (Rodrigo-Baños et. al., 2015). Till date, to the best of our knowledge, only two non-pigmented haloarchaea have been reported: *Halorhabdus utahensis* (Antunes et. al., 2008) and *Halarchaeum acidiphilum* (Minegishi et. al., 2010). Shahmohammadi and co-workers reported an increase in resistance to UV radiation and ionizing radiation (γ-radiation) in *Halobacterium salinarium* as compared to *Escherichia coli*. (Shahmohammadi et. al., 1998). The photoprotective effect of haloarchaeal pigment has also been demonstrated using a colored wild type strain and a colorless mutant of *Halobacterium salinarium* (Dundas and Larsen, 1963).

This chapter deals with an assessment of the stability and characterization of the pigment as well as an evaluation of its effect on UV exposed keratinocyte cells (HaCaT). The pigments from *Haloarcula* sp., strain BS2 and strain E2 as well as *Halorubrum* sp., M5 were characterized, identified and evaluated for their antioxidant properties and their effect on HaCaT (keratinocyte) and HeLa (cancerous) cells undergoing different types of stresses.

4.2 MATERIALS AND METHODS:

4.2.1 Materials

Chemicals for the preparation of growth medium, 2,2′-diphenyl-1-picrylhydrazyl free radical (DPPH), butylhydroxytoluene (BHT), ascorbic acid were purchased from Hi media (India). Methanol, acetone, and chloroform were from SD Fine (India); high-performance liquid chromatography (HPLC)-grade methanol were obtained from Merck (Darmstadt, Germany); HPLC-grade water (18.2 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA). All cell culture reagents were obtained from Hi-media.

4.2.2 Halophilic archaeal cultures

The *Haloarcula* sp. strain E2 and BS2 as well as *Halorubrum* sp. strain M5 was grown and maintained in EHM, NT and MHM media (Table 2.1) respectively at 37 ^{o}C in a shaking incubator at 110 rpm. The strains have been described in detail in Chapter 2. Extraction was carried out once the cultures attained bright orange colour (in 7-10 days). The stock cultures were maintained at room temperature and routinely sub-cultured every 30-45 days.

4.2.3 Pigment production and extraction

Pigment production was carried out in batches of 500 ml nutrient rich media in 2 L Erlenmeyer flasks and inoculated with 1% (v/v) log phase culture. The flasks were incubated in shaking condition at 110 rpm and 37 ℃. Pigment was recovered once the cultures developed bright orange/ reddish orange pigmentation (in 4-5 days). Cells were harvested from 500 ml culture by centrifugation (10,000 rpm, 4 ^{o}C, 20 min). The cell pellet was then suspended in minimal volume of 10% NaCl solution. Extraction was carried out in a dark room with chloroform: methanol mixture in the ratio 1:2. Each solvent containing 0.05% BHT was added separately to the cell pellet and vortexed. Then it was allowed to settle, and the supernatant was decanted into a brown bottle. The extraction process was carried out till the cell pellet became white in colour. To each 100 ml solvent extract collected, 20 ml of hexane: 25% NaCl (aq) in the ratio 1:1 was added and stirred vigorously. This was allowed to separate out into two layers. The hexane layer was decanted out and evaporated using rotary vacuum evaporator. The dried pigment extract was weighed and stored at -20 ^{o}C in labelled amber microfuge tubes

for further use (Abbes et. al., 2013). The extraction process as well as all experiments were carried out in dim light unless otherwise mentioned.

4.2.4 Characterization of the haloarchaeal pigments

4.2.4.1 UV Visible spectrophotometry

The pigment extract (Approx. 0.5 µg/mL) was dissolved separately in various solvents/ oils (viz., chloroform: methanol (1:2), acetone, water, soybean oil, coconut oil) and scanned in the wavelength range of 300 – 600 nm using UV-Visible Double Beam Spectrophotometer (Shimadzu, Japan, UV-2450).

4.2.4.2 Thin Layer Chromatography (TLC)

TLC was carried out as described in Strand et. al., 1997. The pigment extract was dissolved in acetone and 10-100µL was spotted on Silica Gel 60 F254 plate. Solvent system used was 50% acetone in heptane. The solvent vapors were first allowed to saturate the TLC chamber sealed with a glass lid. The inside of the lid was lined with petroleum jelly to prevent loss of solvent vapors. No special visualization technique was used as the compounds were colored (Fang et. al., 2010; Strand et. al., 1997).

4.2.4.3 Fourier Transform Infrared Spectroscopy (FTIR)

A drop of the pigment extract dissolved in methanol was placed on the Attenuated total reflection (ATR) unit and the sample was analyzed by FTIR spectroscopy. The atmospheric CO_2 and water were removed by comparison with background scan.

4.2.4.4 Raman spectroscopy

Raman spectroscopy is one of the vibrational spectroscopic techniques used to provide information on molecular vibrations and crystal structures. This technique uses a laser light source to irradiate a sample, and generates an infinitesimal amount of Raman scattered light, which is detected as a Raman spectrum using a CCD camera. The characteristic fingerprinting pattern in a Raman spectrum makes it possible to identify substances including polymorphs and evaluate local crystallinity, orientation and stress. A hand-held Raman device Tactic ID GP (B&W TEK) with excitation wavelength 785 nm was used to analyze the Raman spectra of the pigments. 1 ml of each pigment dissolved in methanol was transferred to a glass ampule

of appropriate size and placed in the sample holder. The spectrum obtained was then analyzed manually.

4.2.4.5 Reversed Phase High Performance Liquid Chromatography (RP HPLC)

The dried pigment extract was dissolved in HPLC grade methanol and 200 µL of this sample was injected into an HPLC system equipped with a C18 Zorbax 5 µm analytical column (4.6 × 250 mm) at a flow rate of 1 mL/min. Isocratic elution was carried out with 100% methanol. This was optimized after trying out various gradients of water and methanol. Eluted peaks were detected by a photodiode array detector (PDA). The retention times of the components were compared with standard literature.

4.2.4.6 Liquid Chromatography-Mass Spectrometry (LCMS)

Chromatographic separation was performed on an Agilent 1200 series HPLC system including a quaternary pump and a degasser equipped with a G1315B Diode Array Detector. The accompanying Agilent LC Chemstation was employed for instrument control, data acquisition and processing. Chromatographic separation was performed using C18 Zorbax 5 µm analytical column (4.6 × 250 mm) by isocratic elution with a flow rate of 0.8 ml/ min. The mobile phase was composed of 95% methanol and 5% of 0.1% formic acid in Sterile MilliQ water. Samples were kept on ice and wrapped with aluminum foil to reduce isomerization and oxidation of carotenoids by light irradiation. The temperature was maintained at 20 °C and UV detection was performed at 450 nm. The mass spectra from 6530 Q-TOF LC/MS were recorded in the positive ion mode in the mass range from 400 to 800 m/z. Ions were monitored in the scan mode. The identification of carotenoids was performed by comparing retention time, and characteristics of the mass spectra (protonated molecule ($[M+H]^+$) and its MS/MS fragments. All the carotenoids were monitored at 450 nm with a UV-visible detector.

4.2.5 Antioxidant activity

The antioxidant activity of the pigment extracts was quantified by the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay as described by Jiménez-Escrig et. al., 2000. Briefly, 500 µl of the sample or blank was mixed vigorously with 60 µM ethanolic DPPH 1:1 (v/v) ratio and incubated at room temperature for 30 min. Absorbance was measured at 580

nm. The radical scavenging activity (RSA) of the samples were calculated using the following equation in which A_0 is the OD of control and A is the OD of sample at 580 nm.

$$\textbf{Radical Scavenging Activity (\%)} = (1 - A/A_0) \times 100$$

Sample analysis was carried out in triplicates and the statistical analysis was performed with EXCEL 5.0 (Microsoft Corp, Unterschleissheim, Germany); a two-tailed paired Student's *t*-test was used. All data are presented as mean $\pm$ SDs.

4.2.6 Stability of the pigment extract

For potential application as a sunscreen agent, the stability of the pigment extract in direct sunlight was evaluated. Stability assessment was carried out by exposing each pigment solution (Pigment dissolved in one of the following solvent/ oils: chloroform: methanol (1:2), acetone, water, soybean oil, coconut oil) to bright sunlight (between 11 AM to 1 PM) for a period of 30 minutes. Spectral scans were carried out in the range 300 – 600 nm before and after sunlight exposure.

4.2.7 Cell culture experiments

Immortalized human keratinocyte (HaCaT) cell line and human cervical carcinoma cell line (HeLa) cells were cultured in complete media containing Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS)- hereafter referred to as 'complete media'- at 37 °C in a humidified atmosphere with 5% CO_2. Cells were sub-cultured when they reached 80-90% confluence and experiments on them performed when they were 50-60% confluent. The compatibility and effect of pigment extract on HaCaT (human keratinocyte cell line) and HeLa cells were studied under various stress conditions like UV exposure and oxidative stresses (H_2O_2 and Arachidonic acid). For all experiments, 96 well tissue culture plates treated with poly-D-Lysine with a cell seeding density of 2 $x10^4$ per well (counted using Neubauer Hemocytometer) was used. Cell viability was quantified using MTT assay (Section 4.2.7.1). Morphological changes were observed under Nikon Eclipse TS100 inverted phase contrast microscope and photographed using Nikon Coolpix L22 camera. All experiments with pigment extracts were performed under dim light and plates covered with aluminum foil to minimize any change in pigment due to external light.

4.2.7.1 MTT (3 - (4,5 dimethylthiazol – 2 - yl) - 2,5 - diphenyl tetrazolium bromide) assay

Cells were seeded at a density of 2 x 10^4 cells per well in a 96 well tissue culture plate (treated with poly - D- Lysine) in 100 µl of complete media and incubated at 37 ºC in a 5% CO_2 atmosphere for 24 h. After 24 h, treatment (UV exposure or with test substance) was carried out and the plates were incubated for another 24 h. Untreated cells were used as the control. After incubation, 20 µl of 5 mg/ml MTT was added to each well and further incubated for 4 h (Abdullah et al., 2014). The incubation was followed by removal of the contents of the wells and dissolution of the formazan crystals in 100 µL/well of dimethyl sulfoxide (DMSO) solution and absorbance taken at 570 nm and 630 nm (background) on Micro-ELISA plate reader. Cell viability was expressed as percentage of absorbance in comparison to control, untreated cells MTT assays are generally performed in the dark since MTT reagent is light sensitive (Mosmann, 1983).

4.2.7.2 Biocompatibility of pigment extracts by cytotoxicity measurement

Dried pigment extract dissolved in dimethylsulphoxide (DMSO)/ ethanol served as a stock which was diluted with DMEM so that the final concentration of DMSO was less than or equal to 0.1%. An equal number of HaCaT/ HeLa cells (2 x 10^4 cells - counted using Neubauer Hemocytometer) were seeded on to poly-D-lysine treated plates. After 24 hours of incubation, the media was replaced by serum free media containing varying amounts of haloarchaeal pigment (0, 0.5, 2.5, 5, 10, 25, 50, 75, 100, 125 and 200 µg/mL). The cells were observed, and viability was assessed by MTT assay after 24 hours incubation. The experiments were performed in triplicates for repeatability.

4.2.7.3 Effect of pigment on cells exposed to ultra violet (UV) rays

HaCaT/ HeLa cells (2 x 10^4 cells/ well) were grown in 96 well plates in complete media for 24 hrs. The pigment extracts were added separately into each well in the concentration 0, 10, 25, 50, 75, and 100 µg/mL and incubated for 24 hrs. The media in each well was then drained out and all wells except the controls were exposed to UV light (Philips 15W/G15 T8– 49 J/cm^2) for varying time periods (0.5-30 min). Fresh complete media was then added to all the wells and the cells were further incubated in normal growing conditions for 24 hrs after

which cell viability (MTT) assay was performed (Offord et. al., 2002). The cells treated with only UV, only pigments and the untreated cells served as appropriate controls.

4.2.7.4 Oxidative stress induction

The pigment extract's antioxidant effect was evaluated using cells treated with sub-lethal concentrations of pigment extracts and vitamin E (positive control) for 24 hours. Oxidative stress was induced by (a) arachidonic acid and (b) hydrogen-peroxide (H_2O_2). After 24 hours of reaction, the complete medium was replaced by 200 µl of serum-free DMEM containing arachidonic acid (60 µM) (Mosmann, 1983) or H_2O_2 (50 µM) as a pro-oxidant (Garcia-Alonso et al, 2007), and incubation was extended to 24 hours. The pigment's protective effect against cell viability reduction as induced by oxidative stress was assessed using the MTT assay (as described in Section 4.2.7.1).

4.2.7.5 Statistical analyses

Statistical analyses were performed with EXCEL 5.0 (Microsoft Corp, Unterschleissheim, Germany); a two-tailed paired Student's t-test was used. Comparisons were made within groups, between treatment and control (same subjects), and between groups. Differences were analyzed at each time point. All data are presented as means $\pm$ SDs.

4.3 RESULTS AND DISCUSSION

4.3.1 Pigments of *Haloarcula* sp. Strain BS2

4.3.1.1 Pigment production and characterization

Haloarcula sp. strain BS2 is a bright orange pigmented, Gram negative cocci, isolated from brine collected from the solar salterns of Ribandar, Goa (Salgaonkar et. al., 2012). This strain forms circular, butyrous colonies on NT agar plates. Pigment was extracted in the stationary phase (Day 5-6) when BS2 culture attained bright orange colour (the growth curve of BS2 is shown in Fig.4.1). The culture on NT agar and NT broth medium as well as the extracted pigment is shown in Fig. 4.2.

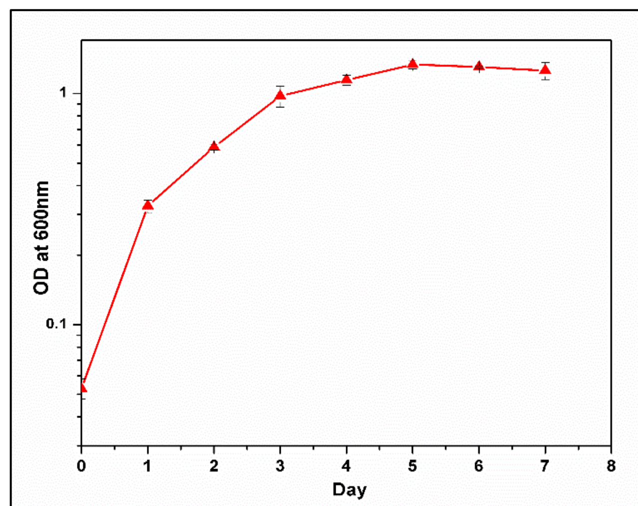
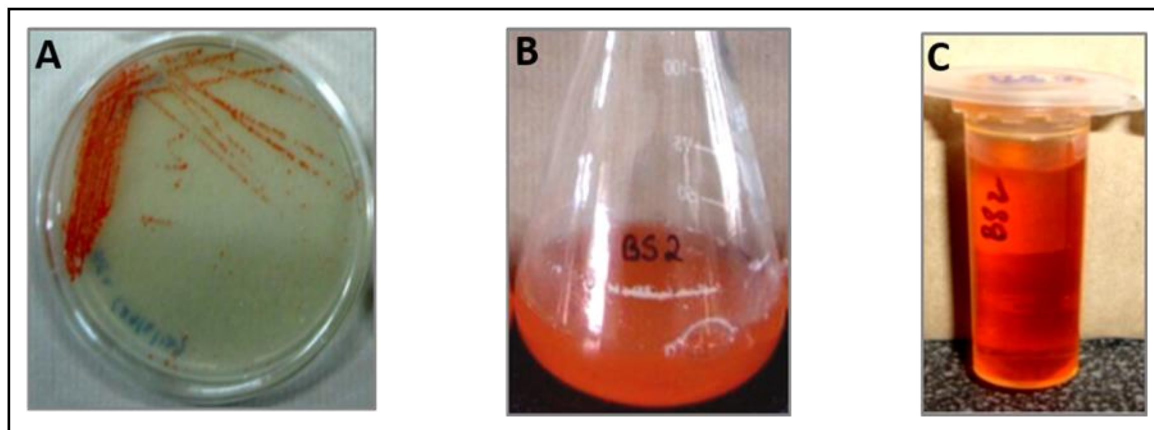


Fig 4.1: Growth of extremely halophilic archaea, *Haloarcula* sp. strain BS2 in NT medium.

Fig 4.2: *Haloarcula japonica s*train BS2 (A) on NT agar plates, (B) on NT broth, (C) pigment



extracted using chloroform: methanol (1:2)

The pigment when dissolved in organic solvents (1) acetone (2) chloroform-methanol (1:2) and analyzed spectrophotometrically showed characteristic three fingered peaks at 467, 493, and 527 nm and two cis maxima at 370 and 385 nm (Fig. 4.3A). The dried pigment dissolved in water showed a slight shift towards the red end of the spectrum. Relative decrease in the intensity of the three main peaks was also observed (Fig. 4.3B). When soybean oil and coconut oil was used as the solvent, the characteristic pattern remained identical to that in polar organic solvents.

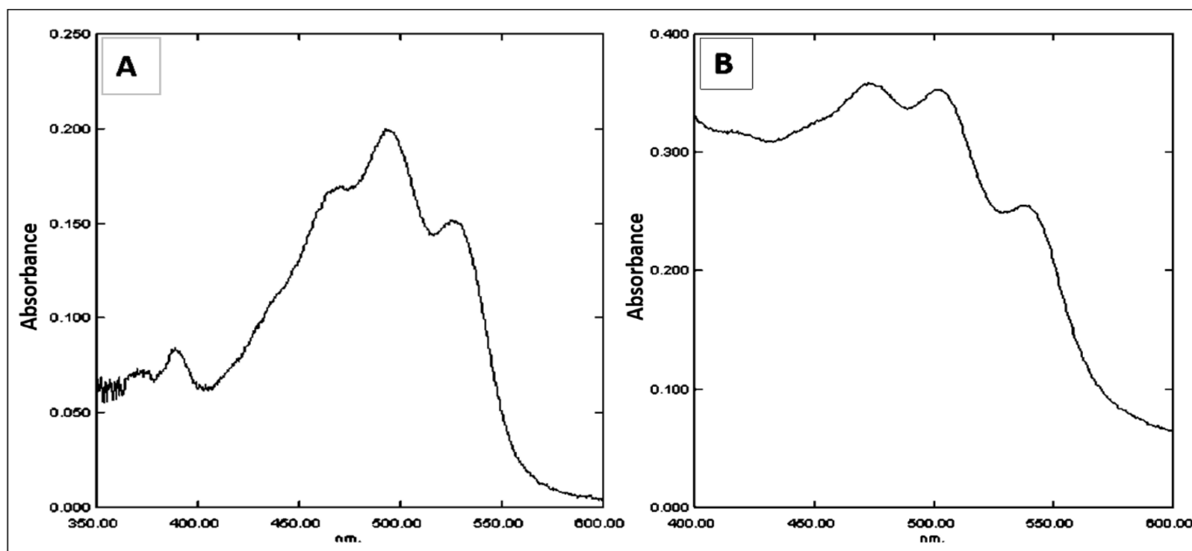


Fig 4.3 UV-Vis spectra of BS2 pigment dissolved in (A) chloroform-methanol(1:2) (B) water

Haloarchaea are known to produce C50 carotenoids which originate from the terpenoid biosynthetic pathway. The major ones being bacterioruberin and its precursors monoanhydrobacterioruberin, bisanhydrobacterioruberin, and trisanhydrobacterioruberin. They may be present in cis or trans isomeric forms. The structure of bacterioruberin has 13 conjugated double bonds which has strong absorption at around 450-550 nm range of the visible spectrum (Calegari-Santos et. al., 2016). Britton (1995) described that bacterioruberin and its derivatives exhibited the distinctive spectral peaks 370 and 385 nm for two cis peaks (Britton, 1995; Yachai, 2009). These characteristic peaks were observed in case of BS2 pigments indicating the presence of bacterioruberin in this organism.

The TLC profile of pigments extracted from BS2 (Fig. 4.4) showed three prominent dark orange spots of Rf 0.117, 0.294, 0.559. The Rfs match that of bacterioruberin (BR), mono-anhydrobacterioruberin (MABR) and bis- anhydrobacterioruberin (BABR) (Fang et. al., 2010). This confirms the presence of C50 carotenoids in *Haloarcula japonica* strain BS2. Other light orange and yellow colored bands were also visible indicating the presence of other minor pigments like lycopene, canthaxanthin, 3-hydroxy-echinenone, lycopersene, phytoene, phytofluene, and 2-isopentenyl-3,4-dehydrorhodopin, presumed to be precursors to the major C50 carotenoids (Calegari-Santos et. al., 2016).

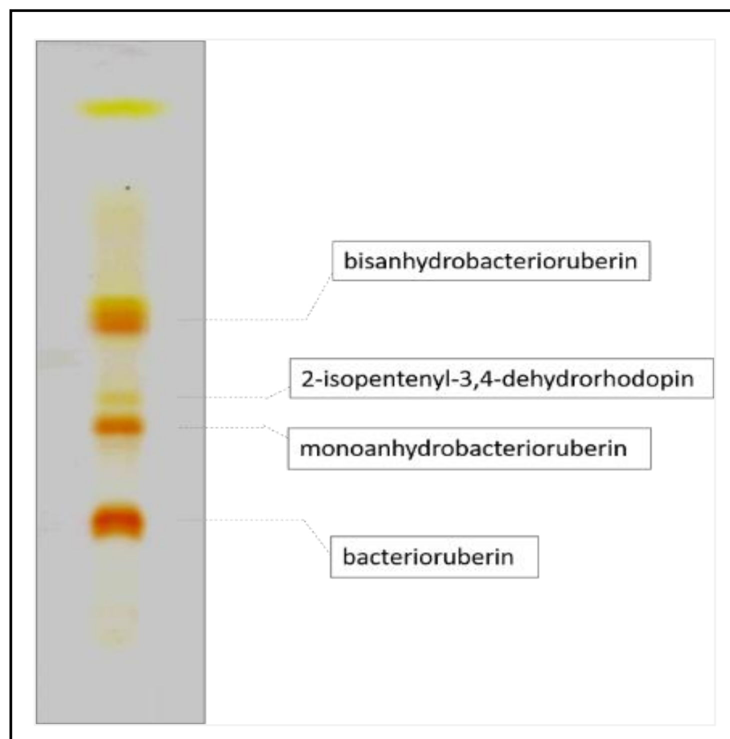


Fig. 4.4 Thin Layer Chromatogram (TLC) of the pigment extracted from *Haloarcula japonica* Strain BS2 with heptane-acetone (1:1) as the mobile phase

4.3.1.2 Stability of pigment in sunlight

Stability of the pigments extracted was analyzed in different solvents like chloroform-methanol (1:2), acetone, water, soybean oil and coconut oil.

i. Organic solvents and water: The pigment extract turned colorless after 30 minutes of exposure to bright sunlight in polar solvents used: a) chloroform-methanol (1:2), b) acetone and c) water. The pigment peaks were found to decrease in intensity as the time of exposure increased. Substantial lowering in intensity of the peaks was recorded every 5 minutes (Fig 4.5 A).

ii. Soybean oil and coconut oil: Soybean oil stabilized the haloarchaeal pigments and the color was retained even after 30 min of exposure to bright sunlight. An increase in the peak intensity was recorded after 30 min of exposure to sunlight (Fig 4.5 B). Coconut oil did not stabilize the pigment and it was decolorized in 30 min of exposure to sunlight.

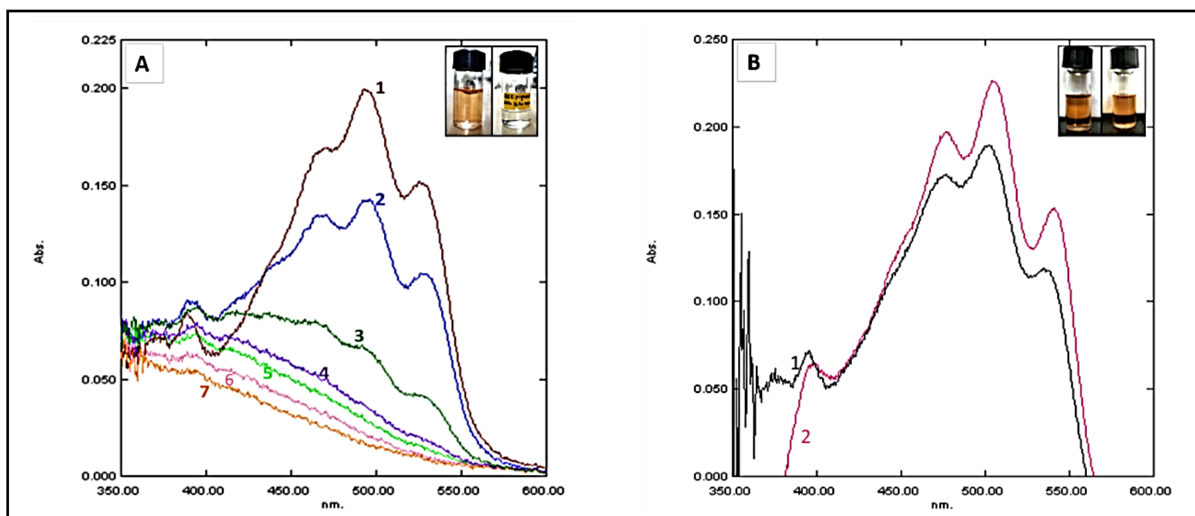


Fig. 4.5 UV-Vis spectra of BS2 pigment; (A) pigment dissolved in chloroform- methanol (1:2) and exposed to bright sunlight. Scanning done in every 5-minute interval. **1**- unexposed pigment, **2**- 5 min exposure, **3**- 10 min exposure, **4**- 15 min exposure, **5**- 20 min exposure, **6**- 25 min exposure, **7**- 30 min exposure (B) pigment dissolved in soybean oil and exposed to bright sunlight. Scanning done after 0 and 30 minutes of exposure. **1**- unexposed pigment, **2**- 30min exposure. Inset shows pigment before and after 30 minutes' exposure to sunlight.

Production of pigment is known to vary with incident light intensity, NaCl concentration in the medium, dissolved oxygen, etc. depending on the strain used (Fang et. al., 2010, Calegari-Santos et. al., 2016, Rodrigo-Baños et. al., 2015). The C50 carotenoids are sensitive to light and heat. The stability assessment of the pigment in various polar solvents showed that the haloarchaeal pigment decolorized on exposure to sunlight. Probably, they were converted to colorless derivatives in the presence of sunlight as theorized by Fang and co-workers (Fang et. al., 2010). This was seen by the decrease in intensity of the characteristic three shoulder peaks at 467, 493, and 527 nm in the BS2 pigment extracts.

It was observed that soybean oil could markedly increase the stability of the pigment as compared with solvents such as acetone, water, chloroform: methanol (1:2). Spectral scans showed a bathochromic shift of the absorption maxima towards the red end of the spectrum when soybean oil was used to dissolve the pigments. This agrees with the work done by Yachai where a 12 nm shift of absorption maxima was reported when the pigment dissolved in soybean oil as compared to that in ethanol (Yachai, 2009). Sachindra and Mahendrakar (2005) showed similar results with the carotenoid astaxanthin where the absorption maxima of astaxanthin in soybean oil was 487 nm and that in ethanol was 478nm. The polarities of a chromophore in

the ground and excited states are different. Hence, when they are dissolved in solvents having different polarities, there is differential stabilization of the ground and excited states depending on the solvent polarity. This creates a variation in the energy gap between these electronic states which is observed as the bathochromic shift (Yachai, 2009).

4.3.1.3 Effect of UV exposure to keratinocytes

In HaCaT cell lines, rapid cell death occurred upon UV irradiation. Partial loss of membrane integrity and adherence property was observed in the cells exposed to UV for a minimum of 5 minutes. As the duration of UV exposure increased, the number of attached cells decreased drastically. As massive cell death was seen to occur after 15 minutes, it was decided to restrict the time of exposure to 15 minutes (Fig. 4.6).

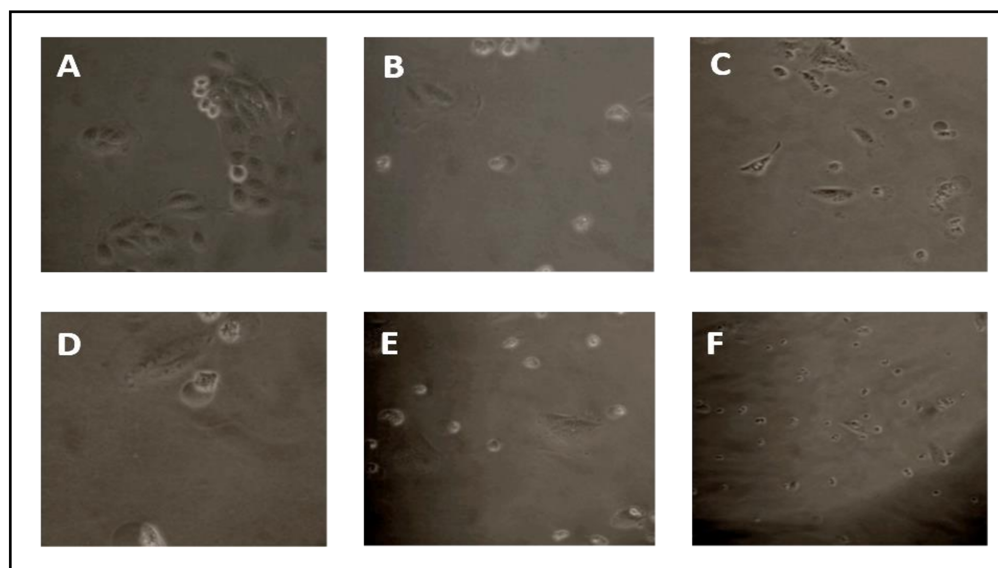


Fig. 4.6 Cell morphology by phase contrast microscope. Control cells, unexposed to UV(A); Cells exposed to UV for: 5 min (B), 10 min (C), 15 min (D), 20 min (E), 30 min (F). Photomicrographs taken after incubating cells for 24 hours in normal growing conditions following UV exposure.

4.3.1.4 Effect of haloarchaeal pigments on keratinocytes

On exposure of HaCaT cells to 5, 10, 25, 50 and 100 μg/mL concentration of pigment, it was seen that cells could attach and grow to form a confluent layer even in concentrations as high as 100 μg/mL. However, at 50 μg/mL and 100 μg/mL pigment extract there was the formation of stress granules (Fig. 4.7).

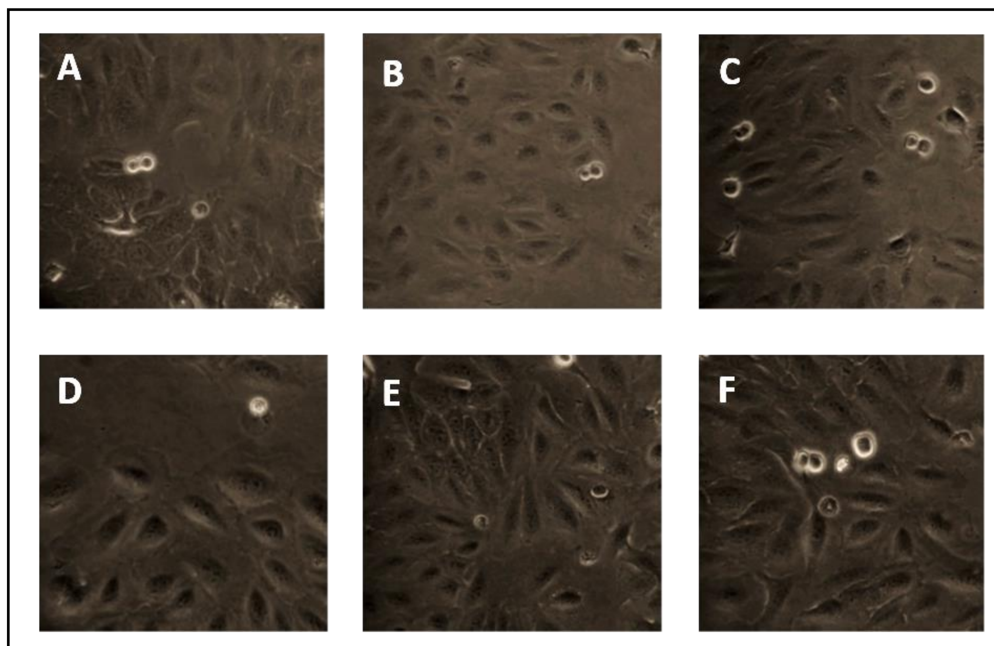


Fig. 4.7 Cell morphology by phase contrast microscope. Cells treated with varying concentrations of pigment: Control cells, untreated with pigment (A), 5 µg/mL (B), 10 µg/mL (C), 25 µg/mL (D), 50 µg/mL (E), 100 µg/mL (F). Photomicrographs taken after incubating cells for 24 hours in normal growing conditions following pigment treatment.

Cell viability was assessed using MTT assay. Till 100 µg/mL of pigment extract, there was no significant reduction in cell viability ($p < 0.05$). 86% of cell viability was retained even at the highest concentration of 100 µg/mL pigment used in this study (Fig 4.8).

Exposure to UV radiation has an established role in skin carcinogenesis. Erythema solare is a condition which refers to the reddening of skin along with tenderness of skin, blistering and sometimes second degree burns on the skin in response to UV exposure. Photooxidation is also linked with premature skin ageing. While there are reports evaluating the photoprotective effect of carotenoid pigments in the native haloarchaeal cells (Shahmohammadi et. al., 1998) and as oral supplement in humans (Sies and Stahl, 2004), there are hardly any studies showing the effect of external delivery of these carotenoid pigments on

keratinocytes. In our study, it was proved that bacterioruberin did not significantly affect the cell viability of HaCaT cells till a high concentration of 100 µg/ml was used ($p < 0.05$).

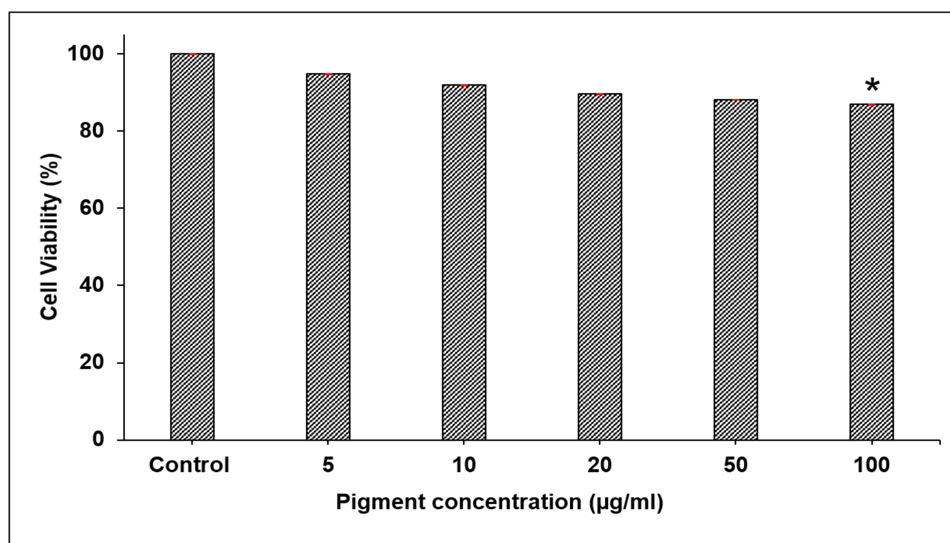


Fig. 4.8 Effect of different concentrations of pigment extract from *Haloarcula* sp. strain BS2 on the cell viability of keratinocyte (HaCaT) cell line (* $p<0.05$).

Carotenoids can act as a pro-oxidant in conditions of high oxygen tension, unbalanced redox reactions in the cell, or even high carotenoid content in the cell. This could explain the decrease in cell viability at higher carotenoid concentrations. The cell type also influences the activity of carotenoids mostly due to differences in uptake of carotenoids into the cells (Palozza et. al., 2003; Young and Lowe, 2001).

4.3.1.5 Study of photoprotective effect of haloarchaeal (BS2) pigment

To evaluate the photo protective action of BS2 pigment, the cells which were treated with varying concentration of pigments were exposed to UV radiation for 15 minutes and the cells were observed after a period of 24 hours. Morphologically, the cells treated only with UV had very diffused membrane structure and lost their adherence property almost entirely. With increasing concentration of pigment extract, it was observed that the number of attached cells kept on increasing and the membrane integrity had improved (Fig. 4.9). In contrast, the cell viability studies by MTT assay showed that there was no significant difference in the viability of cells after the addition of varying concentration of pigment extracts (Fig. 4.10).

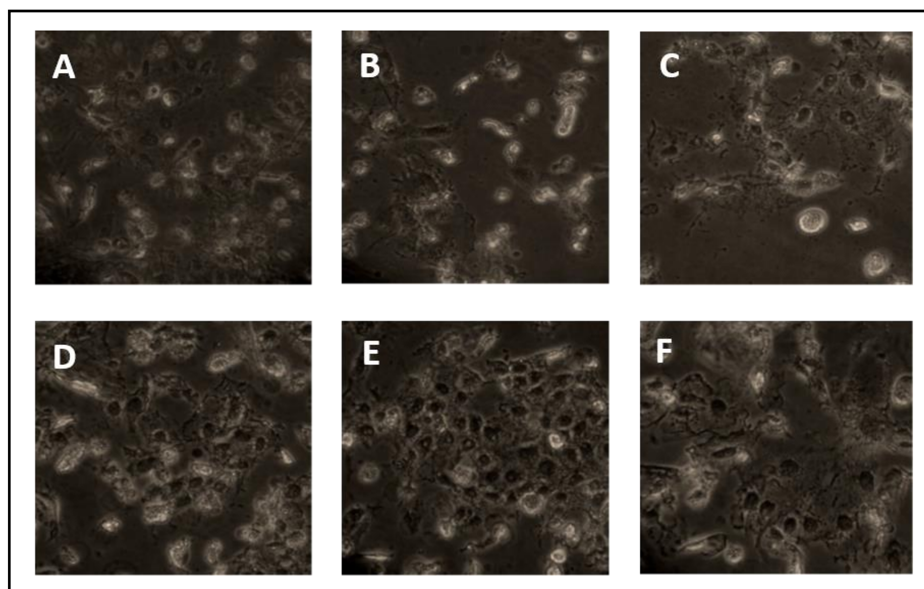


Fig. 4.9 Cell morphology by phase contrast microscope. All cells were exposed to 15 min of UV radiation, incubated in normal growing conditions for 24 hours and subsequently treated with varying concentrations of pigment: Control cells, untreated with pigment (A), 5 μg/ml (B), 10 μg/ml (C), 25 μg/ml (D), 50 μg/ml (E), 100 μg/ml (F). Photomicrographs taken after incubating cells for 24 hours in normal growing conditions following pigment treatment.

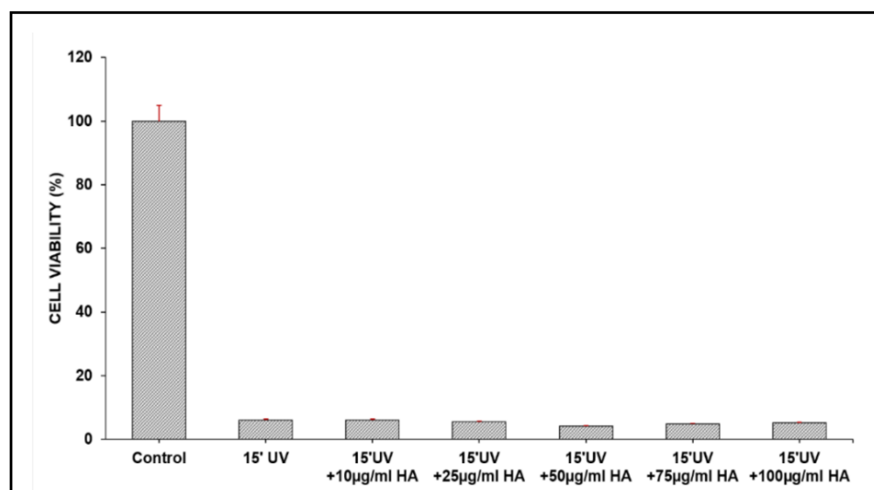


Fig. 4.10 Cell viability of HaCaT cells challenged with UV rays for 15 minutes after exposure to varying concentrations of pigment extract from *Haloarcula* sp. strain BS2

UV damage occurs at the DNA level, majorly as pyrimidine dimers and at the protein levels as the denaturation of proteins and as cellular stress by Reactive Oxygen Species (ROS) (Allegra et. al., 2003).

It was observed that the cell viability did not increase significantly in comparison with the cells exposed only to UV. Even with increasing concentration of pigment, there was no improvement in the cell viability. The antioxidant properties of bacterioruberin are well documented but the carotenoid itself might have got oxidized in the presence of UV light. Carotenoids from *Rhodotorula rubra* MTCC 1446 are known to get degraded on exposure to light (Kaur et. al., 2009). Offord et. al in 2002, in experiments conducted on human keratinocytes, reported that vitamin C, vitamin E and carnosic acid have photoprotective activity in vitro whereas lycopene and β carotene needed the presence of vitamin E for stabilization, suggesting the necessity of an antioxidant protection of the carotenoids for their biological responses.

4.3.2 Studies with pigment extracts from *Haloarcula* sp. strain E2 and *Halorubrum* sp. strain M5

4.3.2.1 Haloarchaeal culture growth and pigment extraction

The cultures *Haloarcula* sp. E2 and *Halorubrum* sp. M5 grown in liquid nutrient media were allowed to grow till the culture broth became bright orange in colour at around Day 5-6 at an OD_{600} of 1.3-1.5 (Fig 4.11). Pigment extraction was attempted with different solvents like acetone, methanol and chloroform: methanol (1:2 v/v).

In case of E2, acetone and methanol extraction yielded poor results whereas extraction of pigment was efficient in chloroform: methanol mixture. Cells of M5 lysed easily and pigments could be extracted using all three solvents. Acetone was the most efficient solvent among these for M5. Pigment extracts were subjected individually to two phase separation which resulting in clear bright orange pigment dissolved in hexane.

4.3.2.2 Pigment characterization

4.3.2.2.1 Thin layer chromatography (TLC)

Thin layer chromatography of the pigments extracted from E2 and M5 are shown in Fig. 4.12. E2 pigment (HA) showed an almost identical TLC profile to that of *Haloarcula* sp. BS2 (Fig. 4.3) which is as expected because they are two different strains of *Haloarcula* sp.

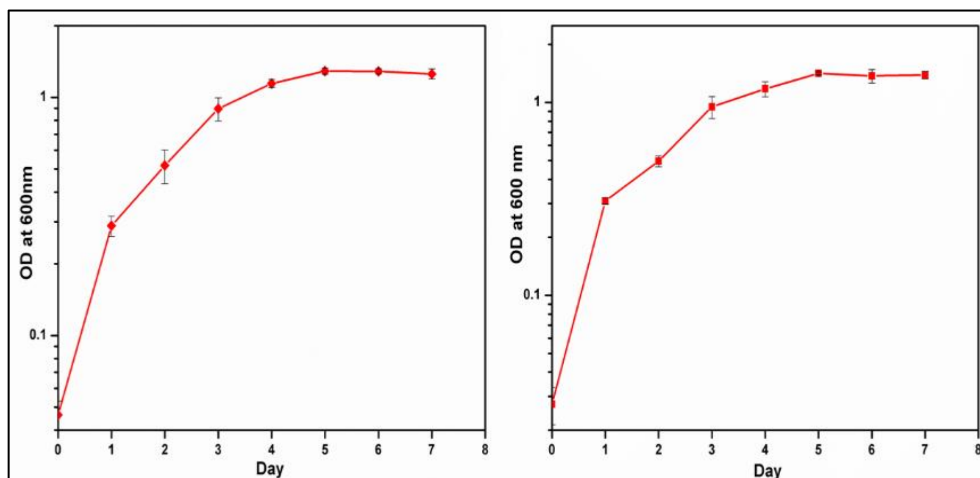


Fig 4.11: Growth of Halophilic archaea *Haloarcula* sp. E2(A) and *Halorubrum* sp. M5(B)

M5 pigment (HR) showed the presence of lesser number of components in comparison with HA. Both HA and HR showed the presence of bacterioruberin (BR), an intense orange coloured spot at Rf = 0.117 along with the derivatives of BR such as MABR of Rf = 0.294 and 2-isopentenyl-3,4- dehydrorhodopin (IDR) of Rf = 0.352. The Rf of bacterioruberin and its derivatives are in agreement with those reported in Strand et. al., 1997 and Fang et al, 2010. Interestingly, one major derivative, BABR (Rf = 0.559) was absent in HR.

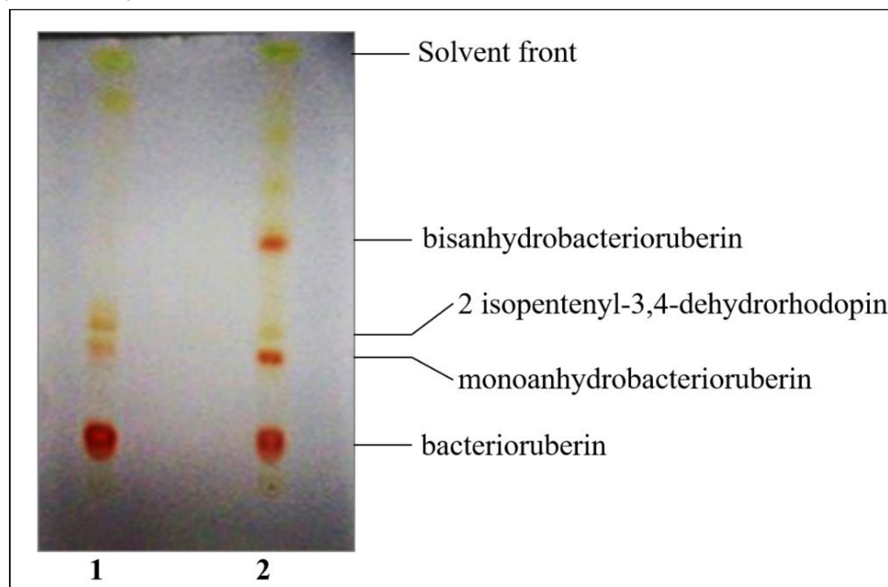


Fig: 4.12 Thin layer chromatogram of *Halorubrum* pigment (HR) – Lane 1 and *Haloarcula* pigment (HA) - Lane 2 obtained using the solvent system heptane: acetone (1:1 v/v)

4.3.2.2.2 Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR spectra obtained for *Haloarcula sp.* E2 pigment and *Halorubrum sp.* M5 pigment were almost identical with peaks at 1650 cm^{-1} (conjugated C-C stretching), 2850-2950 cm^{-1} (for C-H) and 3200-3400 cm^{-1} (for O-H) which are characteristic of bacterioruberin, a C-50 carotenoid and derivatives (Fig 4.13). Bacterioruberin (BR) has a distinct molecular structure having a primary conjugated isoprenoid chain with 13 C=C units with no subsidiary conjugation from any terminal groups, which contain four OH groups while its derivatives MABR, BABR and IDR have 3, 2 and 1 -OH group(s) respectively (Oren, 2011; Jehlička et. al., 2014).

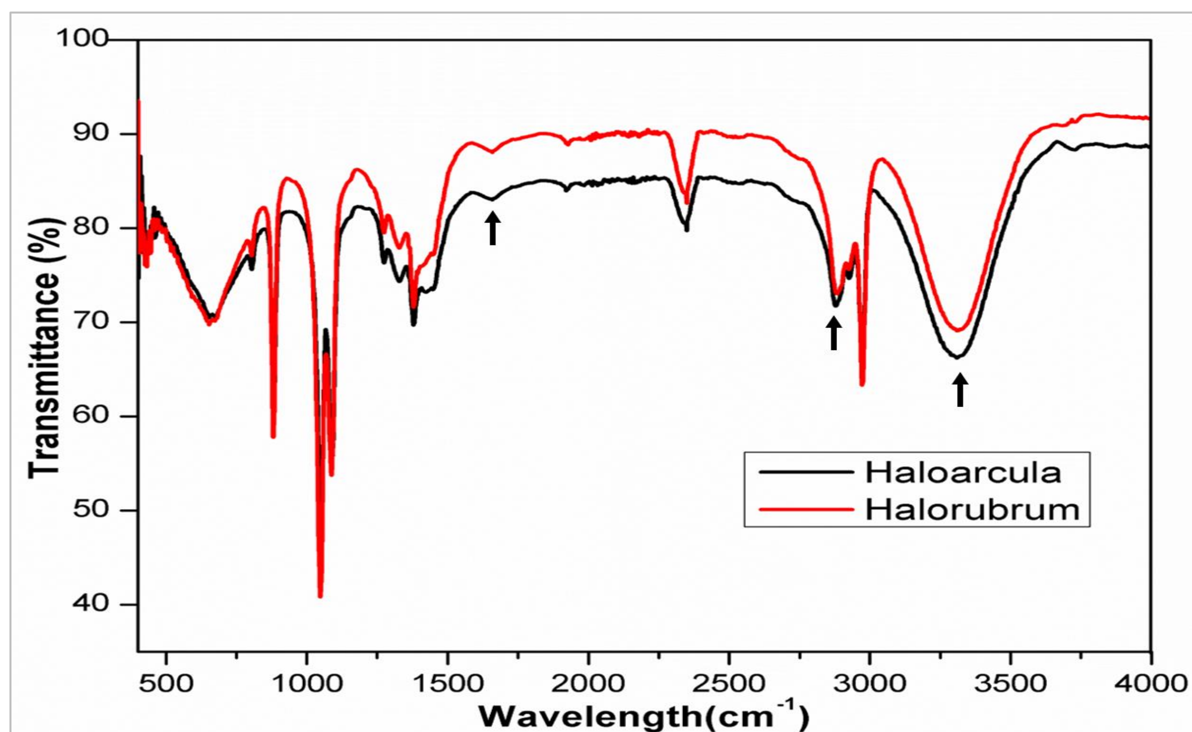


Fig 4.13: FTIR of *Haloarcula* sp. E2 pigment (HA) and *Halorubrum* sp. M5 pigment (HR): (1) conjugated C-C stretching at 1650 cm^{-1}, (2) C-H bond at 2850-2950 cm^{-1} (3) O-H bond at 3200-3400 cm^{-1}

4.3.2.2.3 Raman spectroscopy:

Consistent Raman spectra was obtained across replicates for both haloarchaeal pigment HA and HR, with only minor shifts between them (Fig 4.14). Raman spectroscopic studies show C=C stretching at 1505–1508 cm^{-1}; a C-C stretching at 1148–1152 cm^{-1}. A minor peak at 996-1010 cm^{-1} corresponding to C = CH bending was obtained in HA and HR pigments.

Similar results were reported for bacterioruberin and its derivatives from *Halobacterium salinarum*, *Halorubrum sodomense* and *Haloarcula marismortui* 43049 confirming its presence in HA and HR pigments. (Marshall et. al., 2007; Jehlička et. al., 2014; Camacho-Córdova et. al., 2014).

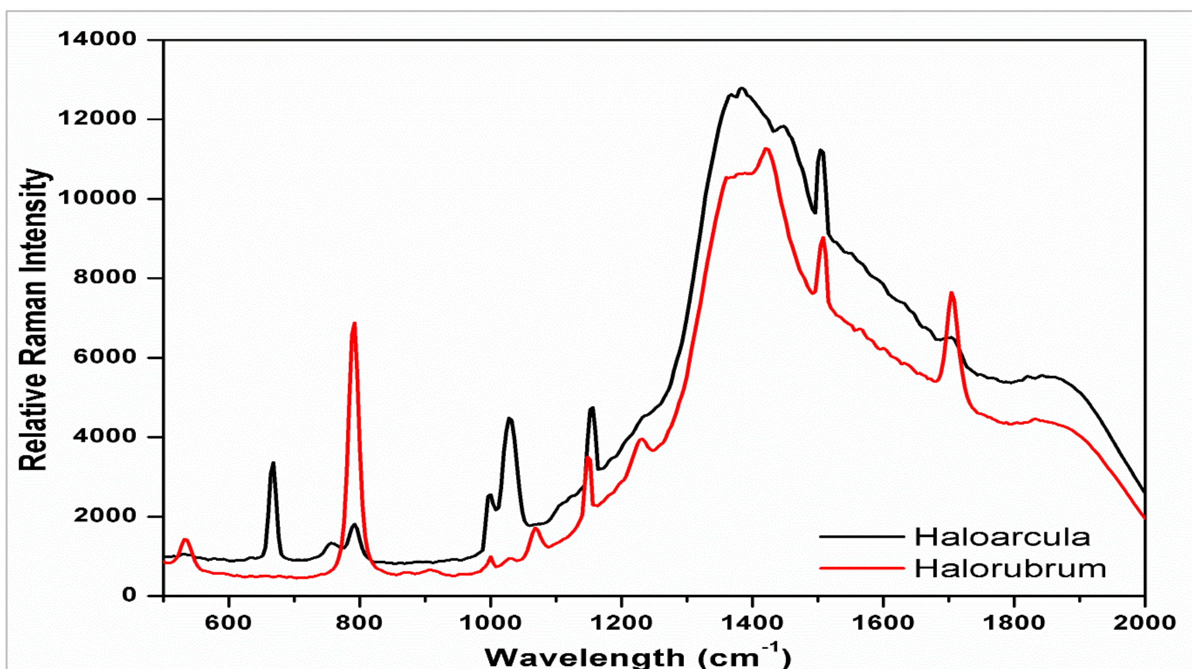


Fig 4.14: Raman Spectra of Haloarcula pigment and Halorubrum pigment showing peaks at 1505–1508 cm^{-1}, 1148–1152 cm^{-1} and 996-1001 cm^{-1} corresponding to C=C stretching, C-C stretching and C = CH bending respectively.

4.3.2.2.4 Reverse Phase High Performance Liquid Chromatography (RP HPLC):

The pigment extracts from E2 (HA) and from M5 (HR) was separated into its components by using RP HPLC (Fig 4.15). The chromatogram showed the presence of 13 peaks in HR and 19 peaks in HA. This corresponded well with the TLC profile also as more number of spots were seen in the HA profile in comparison to HR profile. Peaks 3-11 in both HA and HR were identical in terms of profile and retention time. 19 peaks were observed in case of *Haloarcula* (HA) pigment extract and 13 compounds were obtained in *Halorubrum* pigment (HR). Comparison with retention time in standard literature and TLC confirmed the presence of bacterioruberin and its derivatives.

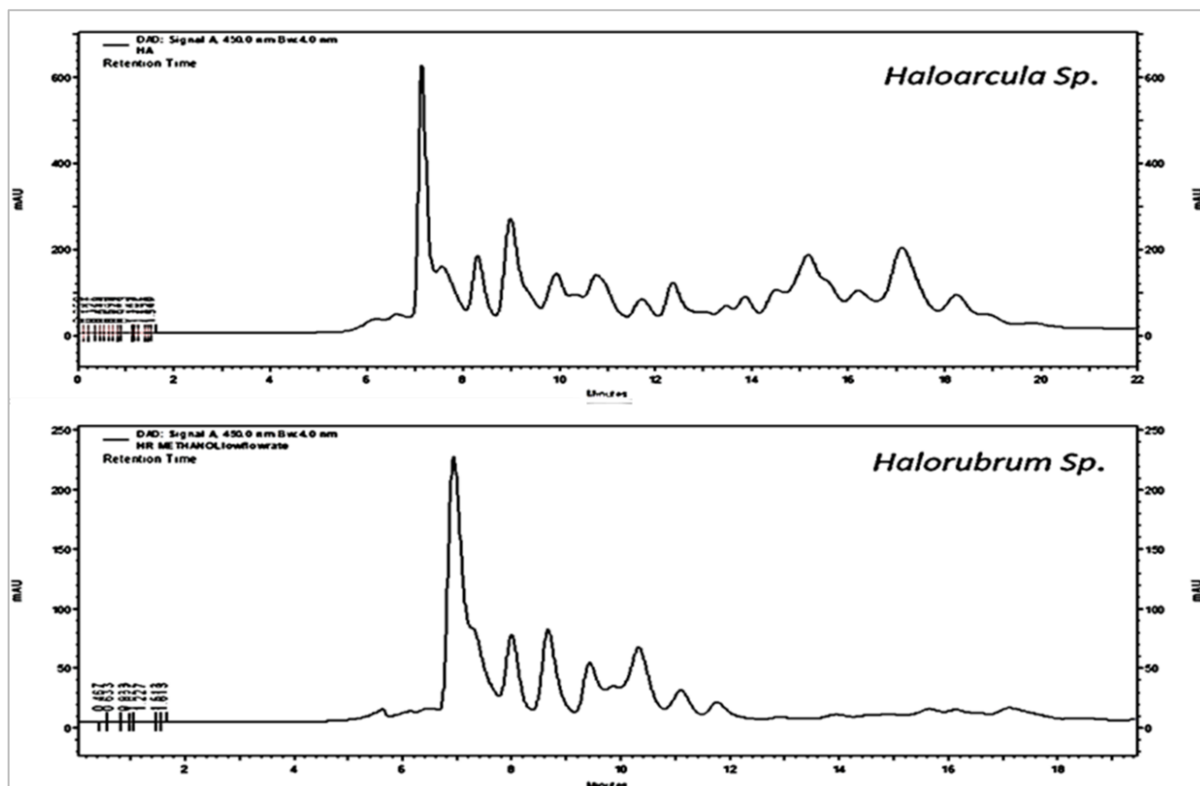


Fig 4.15: The reverse phase HPLC profiles of the pigment extract of *Haloarcula* sp. E2 and *Halorubrum* sp. M5.

4.3.2.2.5 Liquid Chromatography Mass Spectrophotometry (LCMS):

The M/z $[M+z]^+$ value in positive ion mode showed the M^+(m/z) value of 741.5, 723.5, 705.5, 620.5 corresponding to bacterioruberin, monoanhydrobacterioruberin, bisanhydrobacterioruberin and isopentenyl dehydrorhodopin respectively were obtained in case of *Halorubrum* pigment (HR) proving the presence of bacterioruberin and its derivatives (Abbes et. al., 2013; Yatsunami et al., 2014). Minor carotenoids (Lycopene and β-carotene) was also detected at M^+(m/z) value of 537.2. Similar results were obtained in *Halorubrum* sp. TBZ126 (Naziri et. al., 2014). In *Haloarcula* pigment (HA), fragments with M^+(m/z) value of 743.6 [741+H+H], 702.8 [705-H-H-H] and 537.2 were detected indicating the presence of bacterioruberin, bisanhydrobacterioruberin and lycopene/β-carotene (Yatsunami et. al., 2014).

4.3.2.3 Antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay:

The antioxidant activity of haloarchaeal pigments was evaluated by DPPH radical scavenging activity. This increased with the increase of the quantity of extract used in the assay. This dose dependent trend corresponds well with previous reports (Yachai, 2009; Squillaci et. al., 2017). The effective concentration for 50% scavenging (EC_{50}), determined by using a linear regression equation (fitted to % radical scavenging activity Vs concentration of the compound), increased in the order Vitamin E < HA < HR (Table 4.1). The %RSA of the haloarchaeal pigment extracts HA (from *Haloarcula* sp. strain E2) and HR (from *Halorubrum* sp. strain M5) were similar to that of vitamin E ($p > 0.05$). The results are shown in Table 4.2. Similar antioxidant studies with the pigment extracts from *Haloterrigena* sp. reported a higher antioxidant activity with an RSA of 66% by 7 µg/mL of that pigment extract (Squillaci et. al., 2017).

Table 4.1 DPPH radical scavenging activity of each compound with respect to the effective concentration (EC_{50}) required for scavenging 50% of the free radicals in solution.

Sample	EC_{50} (µg/mL)
HA	274.8 ± 6.8
HR	286.8 ± 3.7
Vitamin E	212.2 ± 2.0

Table 4.2 The % Radical scavenging Activity (RSA) of each sample in varying concentrations. Each % RSA value is the mean ± standard deviation of three replicate analyses

Sample	Concentration (µg/mL)	%RSA
HA	100	25.1± 1.5
	200	38.8±2.1
	300	55.7±2.1
	400	65.5±2.1
HR	100	21.1±1.9
	200	35.6±.09
	300	53.4±3.8
	400	64.4±0.9
Vitamin E	100	36.0±0.8
	200	51.3±1.0
	300	66.6±1.4
	400	80.0±0.8

4.3.2.4 Cell Culture studies

4.3.2.4.1 Biocompatibility of pigment extract by cytotoxicity measurement

The biocompatibility study was carried out on two cell lines: transformed keratinocyte cell line (HaCaT) and cervical carcinoma cell line (HeLa). Cell viability assay shows that HR (pigment from *Halorubrum* sp. strain M5) was more cytotoxic than HA (pigment from *Haloarcula* sp. strain E2) to HaCaT and HeLa cells. HaCaT and Hela cells treated with 200 µg/mL HA retained around 60% cell viability whereas the cells treated with 200 µg/mL HR retained only 10% cell viability.

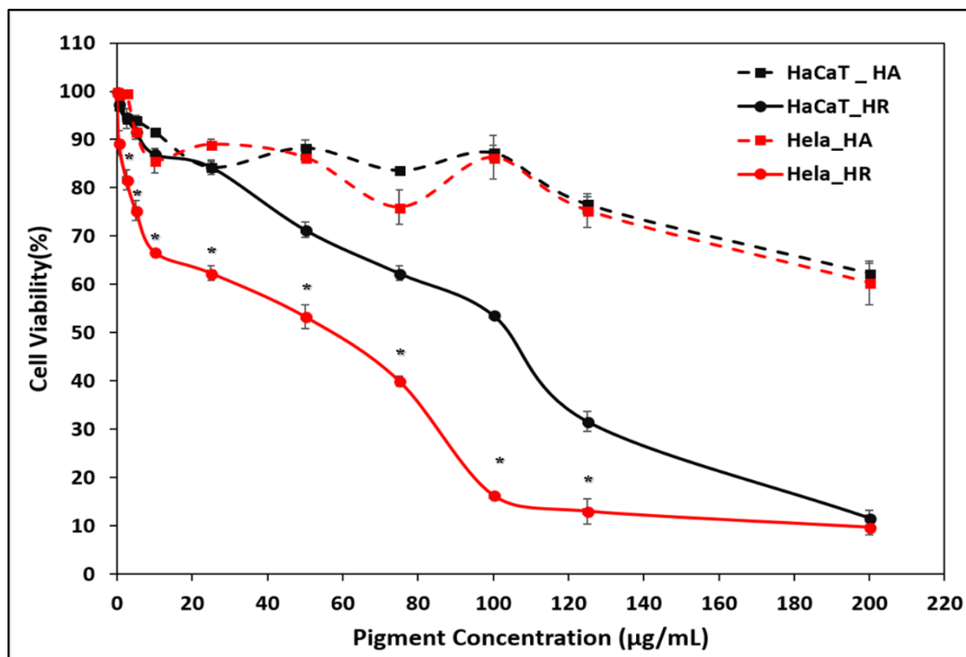


Fig 4.16 The change in cell viability of HaCaT cells (black) and HeLa cells (red) with increasing concentration of pigment extracts HA (dashed line) and HR (solid line). Untreated cells served as the control. Statistically significant difference between HeLa and HaCaT cells treated with HR pigment is indicated by* ($p<0.05$).

4.3.2.4.2 Effect of pigment on cells exposed to ultraviolet (UV) rays

HaCaT and HeLa cells were challenged with UV exposure after treatment with varying amount of HR and HA pigments (Fig 4.17 and Fig 4.18). Control cells which were neither exposed to UV nor pigments were considered as having a cell viability of 100%.

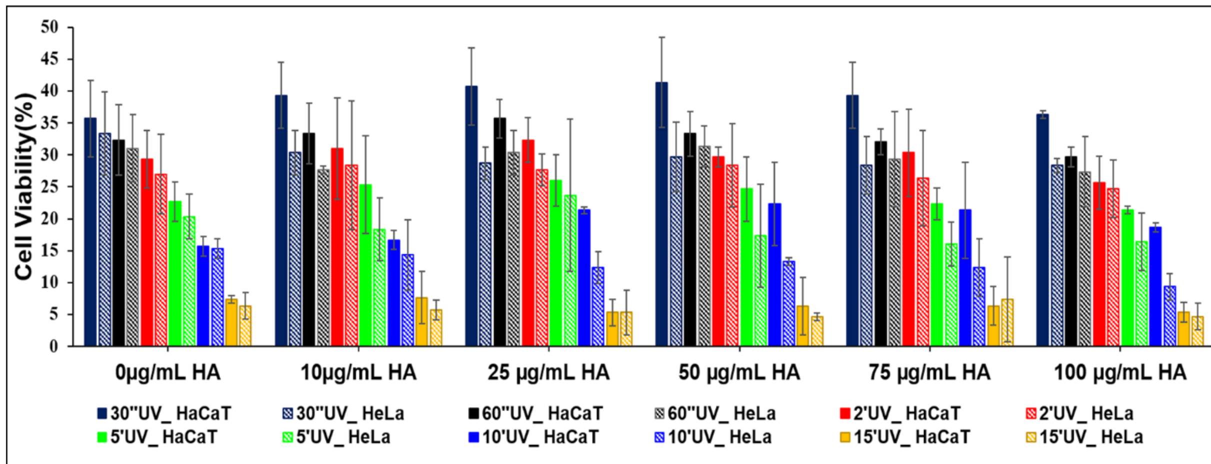


Fig 4.17 HaCaT and Hela cells were first exposed to *Haloarcula* pigment (HA) and then challenged with UV for different durations. The change in cell viability is shown in the graph.

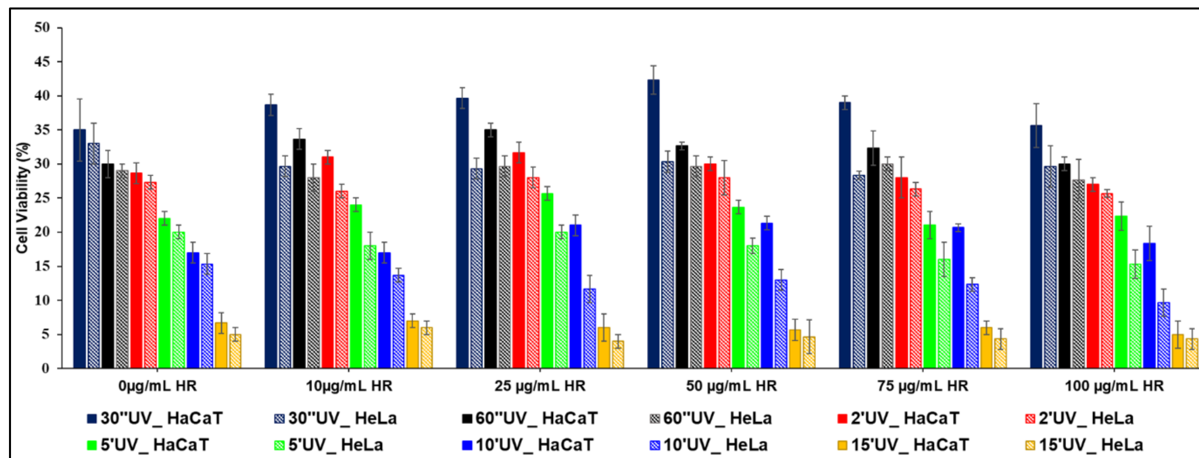


Fig 4.18 HaCaT and Hela cells were first exposed to *Halorubrum* pigment (HR) and then challenged with UV for different durations. The change in cell viability is shown in the graph.

When HaCaT/ HeLa cells were exposed to 30 sec of UV radiation, a cell viability of 35% / 33% was observed. With the addition of various concentrations of haloarchaeal pigments HA or HR, there was no significant change in the cell viability. As the time of exposure of cells to UV radiation increased from 30 sec to 15 min, the cell viability decreased in a dose dependent manner from 35% - 7% in HaCaT cells and from 33% - 6% in HeLa cells. The addition of varying amount (10-100µg/ mL) of pigment extracts HR or HA did not increase or decrease the HaCaT/ HeLa cell viability significantly.

UV irradiation is known to induce photooxidative damage due to the production of reactive oxygen species (ROS) like peroxyl radicals, superoxide radical anion and singlet molecular oxygen. Photooxidative stress affects cellular lipids, proteins, and deoxyribonucleic acid (DNA) and is hypothesized to contribute to erythema as well as premature aging of the skin, skin cancer, degenerative diseases etc. (Darr and Fridovich, 1994; Stahl, 2000, Kaur et. al., 2008). Scientific evidence suggests that carotenoids and tocopherols are efficient ROS scavengers and antioxidants (Tinkler et. al., 1994; Sies and Stahl, 1995). They also play an important role in the reduction of lipid peroxidation (Packer and Cadenas, 2002).

A significant photoprotective action of haloarchaeal pigment extracts, containing bacterioruberin and its derivatives, was therefore expected. However, photoprotection was not observed in the UV exposed cells. This could be because of the degradation of the pigment during UV exposure (Offord et. al., 2002). The administration of pigments in specialized formulations, micellar form, microemulsions, water-dispersible beadlets, enriched bovine serum, artificial liposomes, or nanoparticles, may increase its stability and facilitate absorption into cells (Xu et. al., 1999; Auge et. al., 1998; Williams et. al., 2000; Pfitzner et.al., 2001; Junghans et. al., 2001).

4.3.2.4.3 Effect of pigment on cells exposed to oxidative stress

Hydrogen peroxide and arachidonic acid were used to induce oxidative stress in HaCaT/ HeLa cells. Cells were treated with the haloarchaeal pigments, HA and HR, after which they were challenged with 50 µM hydrogen peroxide (H_2O_2) / 60 µM arachidonic acid (AA).

In the absence of pigments, 64% / 50% cell viability was retained after treatment with H_2O_2 in case of HaCaT/ HeLa cells (Fig 4.19). On treatment with increasing concentration of *Haloarcula* pigment HA, the cell viability increased in a dose dependent manner till 75 µg/mL. On addition of 75 µg/mL of HA, significant increase ($p < 0.05$) in cell viability was observed with 92% / 68% cell viability being retained for HaCaT/ HeLa cells. This was equivalent ($p > 0.05$) to the cell viability of HaCaT / HeLa cells (91% / 60%) treated with 75 µg/mL of vitamin E (positive control).

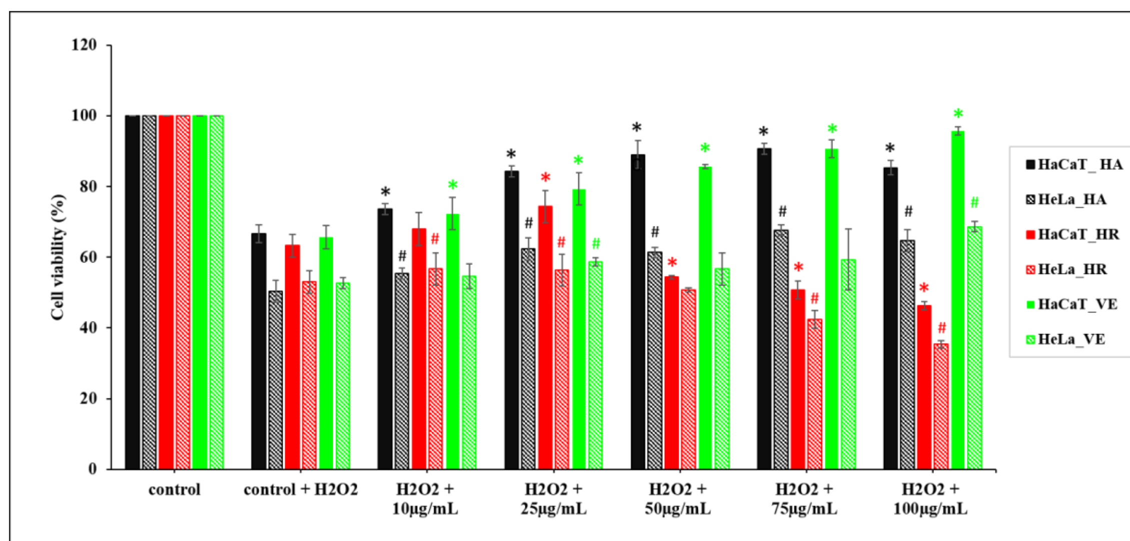


Fig 4.19 HaCaT and HeLa cells on exposure to oxidative stress (H_2O_2) after treatment with pigment extracts HA/HR or vitamin E (positive control). The significance (* $p < 0.05$) of each value was calculated with respect to the corresponding negative control, i.e., control + H_2O_2.

On treatment with increasing concentration of *Halorubrum* pigment HR, the cell viability increased in a dose dependent manner only till 25 µg/mL. On addition of 25 µg/mL of HR pigment, 75%/ 56% of HaCaT/ HeLa cell viability was retained. This was comparable ($p > 0.05$) to the cell viability (80%/ 60%) of HaCaT/ HeLa cells treated with 25 µg/mL of vitamin E (positive control).

When challenged with arachidonic acid also, the same pattern of stress response was seen in the cell lines with HR having its maximum effect at 25 µg/mL and decreasing after that and HA showing the maximum protection at 50-75 µg/mL (Fig 4.20). Halophilic pigment extracts showed a significant increase in cell viability after exposure to oxidative stresses such as hydrogen peroxide and arachidonic acid. These results corresponded well with the reports by Abbes et. al., 2003 which described the protective antioxidant activity shown by the carotenoid extracts from *Halobacterium halobium* on HepG2 cells under oxidative stress induced by H_2O_2 and arachidonic acid.

Carotenoids are involved in the scavenging of reactive oxygen species, peroxyl radical and singlet molecular oxygen which are created due to oxidative stress. Xu and co-workers reported that lycopene, a C40 carotenoid pigment (with 9 conjugated double bonds), diminishes the cytotoxicity induced by arachidonic acid in human liver cancer cell line, HepG2

cells (Xu et. al., 2003). The antioxidant protective effect of beta carotene, a C40 carotenoid pigment (with 11 conjugated double bonds), by suppression of upregulation of heam oxygenase-1 gene expression has been reported in UVA challenged human dermal fibroblasts (FEK 4) (Trekli et. al., 2003).

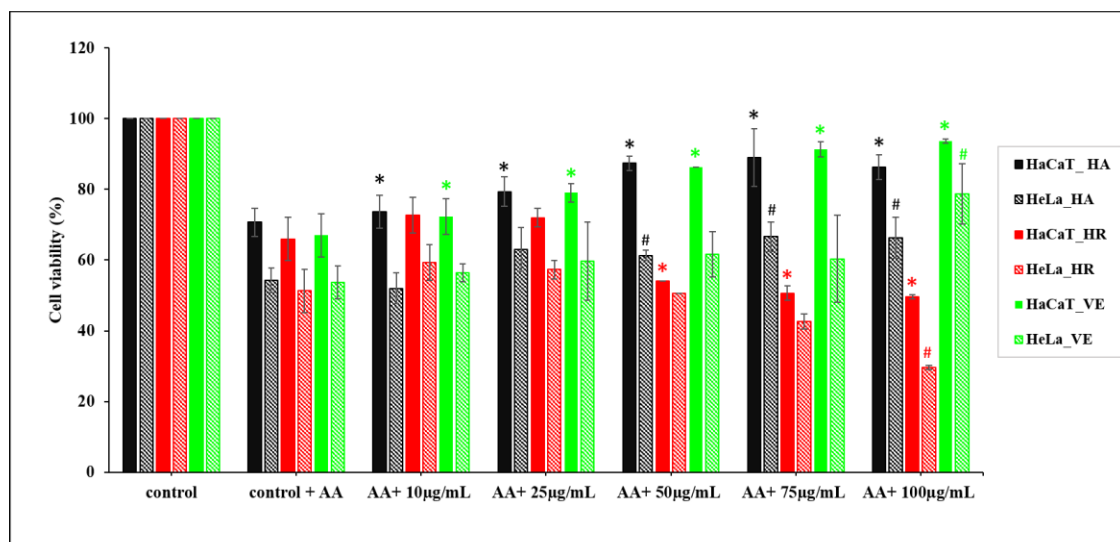


Fig 4.20 HaCaT and HeLa cells on exposure to oxidative stress arachidonic acid (AA) cell viability after treatment with pigment extracts HA/HR or vitamin E (positive control). The significance (* $p < 0.05$) of each value was calculated with respect to the corresponding negative control, i.e., control + AA.

HaCaT and HeLa cells on exposure to oxidative stress (H_2O_2) after treatment with pigment extracts HA/HR of each value was calculated with respect to the corresponding negative control, i.e., control + H_2O_2.

4.4 SUMMARY AND CONCLUSION

Pigments from orange coloured, extremely halophilic archaea, *Haloarcula* sp. (Strain BS2 and Strain E2) as well as *Halorubrum* sp. (Strain M5) were characterized using various techniques such as UV-Visible spectroscopy, thin layer chromatography, Raman spectroscopy, FTIR and LCMS. The main component was found to be bacterioruberin with a mass $[M/Z]^+$ of 741. Derivatives of bacterioruberin, like monoanhydrobacterioruberin, bisanhydrobacterioruberin, and 2-isopentenyl-3,4- dehydrorhodopin were also detected in the study. Minor carotenoids like lycopene and β carotene was identified in both *Haloarcula* and *Halorubrum* pigments.

Photoprotective activity of the haloarchaeal pigments was evaluated by challenging keratinocyte cell line (HaCaT) and carcinoma cell line (HeLa) with UV after exposure to the pigments. It was seen that in the conditions studied, the pigment extract did not offer photoprotection. However, it was observed that, the pigment extracts from both *Haloarcula* sp. (HA) and *Halorubrum* sp. (HR) showed antioxidant activity comparable to that of vitamin E. The pigments offered a protective effect against induced oxidative stresses such as H_2O_2 and arachidonic acid in HaCaT and HeLa cell lines. This study concluded that halophilic archaea isolated from the solar salterns of India have a potential to open new promising opportunities for the development of bioactive agents.

Chapter V

Study of metal tolerance in extremely halophilic archaea

5.1 INTRODUCTION

Metal pollution is continually rising with increasing urbanization (Ahmed and Malik, 2012). Industry effluents, vehicle exhaust, mining, electronic wastes, all contribute to an increase in metal content in soil and water bodies (Zhuang and Gao, 2014). This impacts the terrestrial as well as aquatic environment in drastic ways (Kumar et al., 2011). Bioaccumulation and bio-amplification occurs as the metals move up the food chain (Croteau et. al., 2005).

Natural water bodies are sometimes converted to hypersaline environments due to the dumping of industrial wastes or mining activities (Das et. al, 2014). Haloarchaea are organisms which thrive in hypersaline environments which very often also double up as sinks for the metals from various effluents. Hence, they generally develop a high resistance to various metals. In the recent past, there has been a lot of interest in the mechanism of haloarchaeal metal resistance, intracellular accumulation of various metals as well as their metal nanoparticle production (Naik and Furtado, 2017)

In this chapter, the resistance of four haloarchaeal genera *Halococcus* sp. (strain BK6), *Haloferax* sp. (strain BBK2), *Halorubrum* sp. (strain BS17), and *Haloarcula* sp. (strain BS2 and E2), were tested against common metals/ metalloids viz., Cadmium (Cd), Zinc (Zn), Copper (Cu), Platinum (Pt), Selenium (Se) and Tellurium (Te) released into the environment in higher quantities due to anthropogenic activities.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Soluble salts of Copper ($CuSO_4.5H_20$; Mol wt: 249.677 g mol^{-1}), Cadmium ($CdCl_2.2H_2O$; Mol wt: 201.32 g mol^{-1}), Selenium ($Na_2SeO_3.5H_2O$; Mol wt: 263.01 $gmol^{-1}$), Tellurium (K_2TeO_3; Mol wt: 230.69 $gmol^{-1}$) and Zinc ($ZnCl_2$; Mol wt: 136.28 $gmol^{-1}$) were purchased from SD fine Chem. Pvt Ltd Mumbai, India. Zinc oxide nanoparticles (ZnO NPs; Mol wt: 81.37 $gmol^{-1}$) with average particle size $<$ 100 nm and Pottassium hexachloroplatinic acid (K_2PtCl_6; Mol wt:485.99 g mol^{-1}) were obtained from Sigma Aldrich, USA. All other chemicals were procured from Hi-media. Deionized water was used for preparing media. Milli Q water was used for metal solution preparation, dialysis etc.

5.2.2 Halophiles, growth and maintenance:

Haloarchaea spanning 4 genera viz. *Haloferax* sp. Strain BBK2; *Haloarcula* sp. strain BS2 and strain E2; *Halorubrum* sp. strain BS17; *Halococcus* sp. strain BK6. BBK2 and BK6 were maintained in NTYE medium; BS2 and BS17 were maintained in NT medium and E2 was maintained in EHM medium. The culture identities and characteristics have been described in Chapter 2.

5.2.3 Screening for tolerance of halophiles to various metals/metalloids:

The strains *Haloarcula* sp. BS2, *Halorubrum* sp. BS17, *Haloferax* sp. BBK2, and *Halococcus* sp. BK6 were screened for their ability to tolerate various metals like Copper (Cu), Cadmium (Cd), Platinum (Pt) and Zinc (Zn) as well as to metalloids Tellurium (Te) and Selenium (Se). A loopful of each culture was streaked on their respective nutrient media plates containing 0, 0.5 and 1mM of the metal/ metalloid to be tested. Strain E2 was only tested for platinum resistance. Resistance of (i) *Haloferax* sp. strain BBK2 against Cd (ii) four genera (BS2, BS17, BBK2, BK6) against Zinc Salt (Bulk) and ZnO nanoparticles as well as (iii) E2 against Pt was explored in detail based on the screening results.

5.2.4 Cadmium Resistance in *Haloferax Sp.* Strain BBK2

5.2.4.1 Effect of Cd on the growth of BBK2

(i) In complex (NTYE) and minimal (NGSM) media:

BBK2 (log phase culture) was inoculated in flasks containing complex/ minimal media and varying concentrations (0.5mM,1mM, 2mM, 4mM) of Cd. Control flasks without Cd were maintained for all experiments. All the flasks were incubated (37 °C, 110 rpm) in a shaker incubator. The culture growth was recorded as optical density at 600 nm by UV–Vis Double Beam spectrophotometer (UV-2450 Shimadzu, Japan). The growth rate (μ) was calculated from plot of optical density (OD_{600nm}) against time using the curve-fitting DMFit programme (http://www.ifr.ac.uk/safety/ DMfit) (Baranyi and Roberts, 1994).

(ii) Growth kinetics:

Growth pattern of BBK2 in two sub-lethal concentrations of Cd was studied in NTYE and NGSM media. An inoculum of 1% log phase BBK2 culture was added to 100 mL of media containing 0, 0.5 and 1.0 mM Cd. The experiment was carried out in triplicates. The flasks devoid of Cd served as control. All flasks were incubated in a shaker incubator (37 °C, 110 rpm). 1ml aliquotes were removed at 24 hour-intervals. The growth was monitored by recording the optical density at 600 nm by UV–Vis Double Beam spectrophotometer (UV-2450 Shimadzu, Japan).

(iii) In varying NaCl concentrations:

Growth of BBK2 was assessed at various salt concentrations ranging from 5% NaCl to 30% NaCl in the presence of 1 mM Cd in NGSM medium. Appropriate controls without Cd were maintained under the same conditions. The experiment was repeated thrice to ensure reproducibility.

(iv) In varying pH

Growth of BBK2 was assessed at a pH range of 4.0–10.0 in the presence of 1 mM Cd in NGSM containing 20% NaCl. Appropriate controls without Cd were maintained in the same conditions.

5.2.4.2 Pigment analysis in presence of metals:

Pigments were extracted from 10 mL of 9–10 days old (stationary phase) culture. For this, the cells were harvested by centrifugation at 8000 rpm for 10 min. A mixture of chloroform: methanol (2:1 v/v) was added to the wet cell pellet and vortexed for 10 min. The orange coloured organic fraction containing the pigment was separated from the cell debris by centrifugation at 8000 rpm for 10 min. The extraction continued till the cell debris was colorless/ white. The pooled supernatant was then scanned between 190 and 800 nm using a UV–Vis Double beam spectrophotometer (UV-2450 Shimadzu, Japan).

5.2.4.3 Whole cell protein analysis

Approximately 80 mg of cells were incubated in sterile distilled water for 30 min at room temperature (27 °C). The cell suspension was lysed by freeze thaw method. It was frozen at -20 °C for 15 min and thawed at 80 °C for 5 min. This was repeated thrice to ensure complete lysis. The protein released was estimated by Lowry's method (Lowry et al., 1951) and equal amount of protein was then analyzed by standard SDS PAGE procedures (Laemmli, 1970).

5.2.4.4 Cd Accumulation studies

(i) Atomic absorption spectroscopy (AAS):

10 mL of culture, grown in the presence of Cd (0.5 mM and 1.0 mM), was centrifuged at 10000 rpm for 10 min. The cell pellets were digested with nitric acid: sulphuric acid in the ratio 3:1 (v/v). Further complete hydrolysis of the cells was ensured in a dry sand bath at 80–100 °C till clear solution was obtained. This solution was then analyzed by GBC Atomic Absorption Spectrophotometer. Standard curve was obtained using a stock of 10 mg L^{-1} Cd^{2+} solution.

(ii)Scanning electron microscopy – electron dispersive X-ray spectroscopy (SEM EDX)

(a) **Intact cells analysis:** Cells of BBK2 were pelleted at 10,000 rpm and 100µl of this suspension was mounted onto glass coverslips, air dried and desalted with 2% acetic acid. The cells were fixed with 2% glutaraldehyde, overnight (10–12 h). The slides were then exposed to a series of increasing acetone concentrations (10%, 30%, 50%, 70%, 90%), each for 10 min and finally with 100% acetone for 30 min. These dehydrated samples were air dried and mounted onto stubs. The fixed samples were coated with gold for SEM–EDX analysis (JEOL-5800 LV SEM). Cobalt stub was used as the standard for EDX analysis.

(b) Dialyzed cells analysis: Cells from 100 mL medium were pelleted and then dialyzed against distilled water for 12 h with regular changes of distilled water every hour for the removal of NaCl and other media components. Dialysis ensured that the cell membranes were disrupted. The dialysate was then dried completely at 80 °C and analyzed by SEM–EDX.

(iii) Fourier transform infrared spectroscopy (FTIR):

500 mL culture grown in the presence and absence of Cd was centrifuged at 10,000 rpm for 10 min. The cell pellet thus obtained was dried at 80°C in a hot air oven. The dry residue was ground using a mortar and pestle and the powder was analyzed by FTIR spectroscopy.

(iv) X ray diffraction (XRD) studies:

500 mL culture grown in the presence 0.5 mM Cd was harvested by centrifuging at 10000 rpm for 10 min. The cell pellet obtained was dialyzed against distilled water for 24 h with regular changes of water after every 1 h. The dialyzed samples were dried at 80 °C in a hot air oven for 48 h. The dried cell sample was pulverized using a mortar and pestle and the cell powder was X-rayed over 5–75° using the Rigaku Mini-Flex II powder X-ray diffractometer.

5.2.5 Resistance to Zinc metal salt (bulk) and Zinc nanoparticles:

Strains BS2, BS17, BK6 and BBK2 were evaluated for their resistance to Zinc in bulk metal form and in nanoparticle form.

5.2.5.1 Haloarchaeal culture and growth medium:

Haloarchaeal strains belonging to four different genera were used in this study. *Haloarcula* sp. BS2, *Halorubrum* sp. BS17, *Halococcus* sp. BK6, *Haloferax* sp. BBK2. BBK2 and BK6 were grown in NTYE medium, while BS2 and BS17 were grown in NT medium, both containing 25 % (w/v) NaCl. The metal studies were carried out in minimal NGSM media (Salgaonkar et al. 2012).

5.2.5.2 Minimum inhibitory concentration (MIC) studies:

MIC of the heavy metal and metal nanoparticles (NPs) was determined by growing the cultures in complex (NTYE/NT) and minimal media (NGSM) incorporated with $ZnCl_2$ and ZnO NPs. The MIC was noted by gradually increasing the concentration of metal and metal NPs in the media until the culture ceased to grow. The concentrations of $ZnCl_2$ and ZnO NPs

employed for MIC studies were 0.1, 0.5, 1.0, 2.0 and 4.0 mM. The growth was recorded after 5–15 days of incubation.

5.2.5.3 Growth kinetics in presence of Zn and ZnO NPs

The growth kinetics were studied by culturing the selected strains in NGSM with Zn and ZnO NPs, concentrations non-detrimental to their growth. They were BK6 (0.5 mM), BBK2 (0.5 mM), BS2 (0.1 mM) and BS17 (0.1 mM). Respective controls were maintained without the Zn and ZnO NPs for all experiments. All the flasks were incubated at 37^oC and 110 rpm. The culture growth was monitored after every 24-h interval at 600 nm by UV–Vis Double beam spectrophotometer (UV-2450 Shimadzu, Japan). The growth rate (µ) and lag phase time (k) were calculated from plot of optical density ($OD_{600\ nm}$) against time using the curve fitting DMFit programme (http://www.ifr.ac.uk/safety/ DMfit) (Baranyi and Roberts 1994).

5.2.5.4 Pigments analysis of haloarchaeal strains grown in presence of Zn and ZnO NPs

Pigments were extracted from stationary phase culture (8–10 days old). The cells were harvested by centrifugation at 8000 rpm for 10 min. To one set of the cell pellet, acetone was added and to the second set chloroform: methanol in the ratio of (2:1 v/v) was added. The mixture was vortexed for 5 min until entire pigment (orange–red) was extracted in the solvent. The solvent fraction containing the pigment was separated from the cell debris (colorless or white) by centrifugation at 8000 rpm for 10 min. The supernatant was then scanned between 190–800 nm using UV–Vis Double beam spectrophotometer (UV-2450 Shimadzu, Japan).

5.2.5.5 Zinc accumulation study

(i) Atomic absorption spectroscopy (AAS)

10 ml of respective haloarchaeal culture, grown in the presence of $ZnCl_2$ and ZnO NPs (0.1 and 0.5 mM), was centrifuged at 10,000 rpm for 15 min. The cell pellets were hydrolysed with concentrated nitric acid: sulphuric acid in the ratio of 3:1 (v/v). The mixture was further completely digested in a sand bath at 100^oC for 3 h till clear solution was obtained (Das et al., 2014). The solutions were analyzed for Zn content by GBC atomic absorbtion spectrophotomer

(AAS) after appropriate dilution. Standard curve was obtained using a stock of 10 mg/l Zn^{2+} solution. The cell dry weight (CDW) of the haloarchaeal cultures were determined by centrifuging the culture broth at 10,000 rpm for 15 min and drying overnight at 80 ^{o}C.

(ii) SEM–EDX analysis

Late log phase cells of all four haloarchaeal isolates *Haloferax* sp. strain BBK2, *Halococcus* sp. strain BK6, *Haloarcula* sp. strain BS2 and *Halorubrum* sp. strain BS17 grown in the presence of Zn/ZnO NPs were smeared onto glass slides/coverslips, air dried and desalted with 2 % acetic acid. The cells were fixed for 10 h with 2 % glutaraldehyde. Samples were dehydrated by exposing to a series of increasing acetone concentrations (10, 30, 50, 70, 90 %), each for 10 min and finally with 100 % acetone for 30 min. The samples were mounted onto stubs followed by gold coating for SEM–EDX analysis (JEOL-5800 LV SEM). Cobalt stub was used as the standard for EDX analysis.

(iii) X-ray diffraction studies (XRD)

100 ml of cells of *Haloferax* sp. strain BBK2 grown in the presence of 0.5 mM ZnCl2/0.5 mM ZnO NPs was harvested by centrifuging at 8,000 rpm for 20 min. The supernatant was discarded, and the cell pellet obtained was dialyzed against distilled water for 20 h with regular changes of water after every 3–4 h. The dialyzed samples were dried at 80°C in a hot air oven for 24 h. With the help of mortar and pestle, the dried cells were pulverized, and the X-ray diffraction of the powdered samples was carried at 5°–75° with scanning speed of 2° min^{-1} using the Rigaku Mini-Flex II powder X-ray diffractometer. Cells of *Haloferax* sp. strain BBK2 grown in absence of metal prepared in the same way were used as control.

5.2.6 Platinum resistance in *Haloarcula* sp. strain E2

5.2.6.1 Growth Kinetics:

The growth kinetics of E2 in NT medium in the presence of platinum (Pt) was assessed by the absorbance at 600nm in a UV Visible spectrophotometer. Readings were taken every 24 hours for a period of 7 days.

5.2.6.2 Pt accumulation studies:

The accumulation of platinum in E2 cells were studied using AAS, FTIR, XRD SEM EDX and TEM analysis.

(i) Atomic absorption spectrophotometry (AAS):

10 mL of culture, grown in the presence of Pt (0.5 mM) was centrifuged at 10000 rpm for 10 min. The cell pellets were processed as described in Section 5.3.3.4(i). This solution was then analyzed by GBC Atomic Absorption Spectrophotometer. Standard curve was obtained using a stock of 10 mg L^{-1} Pt from a standard solution (1000 mg/L Pt in 5% hydrochloric acid) obtained from Sigma.

(ii) Fourier Transform Infra-Red Spectroscopy (FTIR):

500 mL culture grown in the presence and absence of Pt was centrifuged at 10,000 rpm for 10 min. The cell pellet thus obtained was dried at 80 °C in a hot air oven. The dry residue was ground using a mortar and pestle along with KBr. This mixture was pelleted and analyzed by FTIR spectroscopy (Das et al., 2014).

(iii) X Ray Diffraction (XRD):

Hundred milliliters of cells of *Haloarcula* sp. strain E2 grown in the presence of 0.5 mM Pt was harvested by centrifuging at 10,000 rpm for 20 min. The supernatant was discarded, and the cell pellet obtained was dialyzed against distilled water for 24 h with regular changes of water after every 3-4 hrs after which these samples were treated with 5M NaOH (to remove organic matter) and then washed with Milli Q water. The samples were dried at 80ºC for 24 hrs and pulverized. The X-ray diffraction of the powdered samples was carried at 10–75º with scanning speed of 20 min^{-1} using Rigaku Mini-Flex II powder X-ray diffractometer. Cells of *Haloarcula strain* E2 grown in absence of metal prepared in the same way were used as control.

(iv) Scanning Electron Microscopy- Electron Dispersive Spectroscopy (SEM EDX)

Late log phase cells of *Haloarcula* sp. strain E2 grown in the presence of 0.5mM Pt was smeared onto glass slides/coverslips, air dried and desalted with 2 % acetic acid. The cells

were fixed for 10 h with 2 % glutaraldehyde. Samples were dehydrated by exposing to a series of increasing acetone concentrations (10, 30, 50, 70, 90 %), each for 10 min and finally with 100 % acetone for 30 min. The samples were mounted onto stubs followed by carbon coating for SEM–EDX analysis (JEOL-5800 LV SEM).

(v) Transmission electron microscopy (TEM):

100 ml of cells of *Haloarcula* strain E2 grown in the presence of 0.5 mM Pt was harvested by centrifuging at 10,000 rpm for 20 min. The supernatant was discarded, and the cell pellet obtained was dialyzed against distilled water for 24 h with regular changes of water after every 3-4 hrs after which these samples were treated with 5M NaOH (to remove organic matter) and then washed with Milli-Q water. The samples were dried at 80ºC for 24 hrs and pulverized. The powder sample was dispersed in Milli-Q water by sonication (MicrosonTM Sonicator) at 0 °C for 15 min at 3RPS (40 W). The colloidal solution of Pt thus obtained was drop-coated on carbon-coated copper TEM grids. The TEM images were obtained using Philips (Model- CM200) transmission electron microscope (resolution 2.4 A˚), which was operated at an accelerating voltage of 190 keV.

5.3 RESULTS AND DISCUSSION

5.3.1 Screening for tolerance of haloarchaea to various metals/elements:

Preliminary screening process showed the tolerance of halophilic archaea to the metals/metalloids Cd, Cu, Pt, Zn, Te and Se in varying degrees (Fig 5.1). Cadmium was seen to be tolerated only by BK6 and BBK2. Both could grow up to 1mM Cd. Haloarchaeal isolates BBK2, BK6, BS2, BS17 could tolerate 2mM copper and slight growth was seen in BK6, BS17 up to 4mM Cu. 1mM Pt was tolerated by BS2, BS17, BK6, E2 and BBK2 could tolerate 0.5mM. Luxuriant growth was observed in BS2 and BK6 in presence of 4mM Se. In BS17 growth was seen up to 2mM Se and slight growth was seen in 4mM Se. In BBK2, there was only slight growth from 0.5 to 4mM Se. BBK2 was not able to tolerate tellurium. In 0.5mM Te, BS2 and BS17 showed brown coloured culture while in 1.0mM Te, these two cultures turned black. BK6 could grow only till 0.5mM Te. Resistance was seen in BK6 and BBK2 for up to 2.0 mM $ZnCl_2$ while BS17 and BS2 could grow up to 0.5 mM $ZnCl_2$ whereas for ZnO

NPs BK6 and BBK2 showed a resistance of 2.0mM. BS17 and BS2 could grow till 0.5mM and 0.1mM ZnO NPs respectively. These results are summarized in Table 5.1.

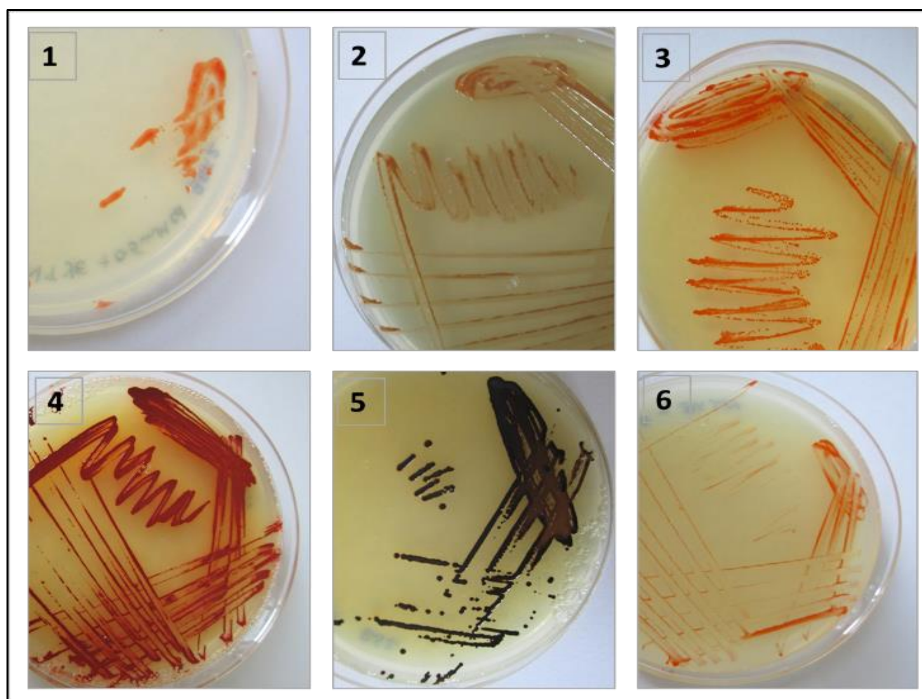


Fig 5.1: Growth of various haloarchaeal isolates in solid nutrient media containing various metals/metalloids. *Haloferax* sp. BBK2 grown in 0.5mM Cd (1), *Halococcus* sp. BK6 grown in 2mM Cu (2), *Haloarcula* sp. E2 grown in 1mM Pt (3), *Haloarcula* sp. BS2 grown in 4mM Se (4), *Haloarcula* sp. BS2 grown in 1mM Te (5) and *Halococcus* sp. BK6 in 0.5mM Zn (6).

Metal/ Metalloid	Tolerant Haloarchaeal Strains and MIC (in brackets)
Cadmium (Cd)	BK6(1.0mM); BBK2(1.0mM)
Copper (Cu)	BK6(4.0mM); BBK2(2.0mM); BS2(2.0mM); BS17(4.0mM)
Platinum (Pt)	BK6(1.0mM); BBK2(0.5mM); BS2(1.0mM); BS17(1.0mM); E2(1.0mM)
Zinc(Bulk) (Zn)	BK6(2.0mM); BBK2(2.0mM); BS2(0.5mM); BS17(0.5mM)
Zinc(Nanoparticle)(Zn)	BK6(2.0mM); BBK2(2.0mM); BS2(1.0mM); BS17(0.5mM)
Selenium (Se)	BK6(4.0mM); BBK2(4.0mM); BS2(4.0mM); BS17(4.0mM)
Tellurium (Te)	BK6(0.5mM); BS2(1.0mM); BS17(1.0mM)

Table 5.1: Tolerance/ resistance of the haloarchaeal strains *Halococcus* sp. BK6, *Haloferax* sp. BBK2, *Halorubrum* sp. BS17 as well as *Haloarcula* sp. BS2 and E2 to various metals/metalloids.

5.3.2 Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2[¶]

5.3.2.1 *Haloferax* strain BBK2

Morphologically the colonies of the haloarchaeal isolate BBK2 appeared as circular (2–3 mm in diameter), orange pigmented, mucoid/slimy with entire margin and convex elevation on NTYE medium. The cells stained Gram negative and appeared coccoid under phase contrast microscopy. SEM images depicted an involuted morphology of the cells characteristic of the genus *Haloferax*. The culture was identified as *Haloferax volcanii* by 16S rRNA gene sequencing (Mani et al., 2012). Among the 40 genera of the family *Halobacteriaceae* known till date (Oren and Ventosa, 2013), the genus *Haloferax* is the most widely explored for its various biotechnological potential (Zhao et al., 2013). However, its tolerance to metals such as Cd has not been explored.

5.3.2.2 Effect of cadmium on the growth of *Haloferax* strain BBK2

(i) In complex NTYE and minimal NGSM media

Haloferax strain BBK2 was able to grow best at 0.5 and 1.0 mM Cd. Though it tolerated up to 4 mM of Cd, the growth was considerably less in comparison with the control (Fig. 5.2).

In NTYE medium, the specific growth rate and the doubling time was relatively unaffected in the presence of Cd (1 mM). The overall growth pattern of *Haloferax* strain BBK2 upon exposure to 0.5 mM and 1 mM Cd was similar to that of the control (Fig. 5.3A). The control reached a maximum OD600 of 2.0 in 7 days. However, it took 8 days in 0.5 mM Cd and 10 days in 1 mM Cd for *Haloferax* strain BBK2 to reach comparable growth density. In presence of Cd, lag of around 1 day was observed in comparison with the control. The generation time was 2.207 days in case of 0.5 mM Cd and 2.132 in case of 1 mM Cd. There was no significant difference from the generation time of control (1.968 days). In NGSM medium, the maximum OD600 attained by the culture was around 1.3 on the 4th day of growth (Fig. 5.3B).

¶ This work is published as <u>Das, D.</u>, Salgaonkar, B. B., Mani, K., & Braganca, J. M. (2014). Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2. *Chemosphere*, *112*, 385-392.

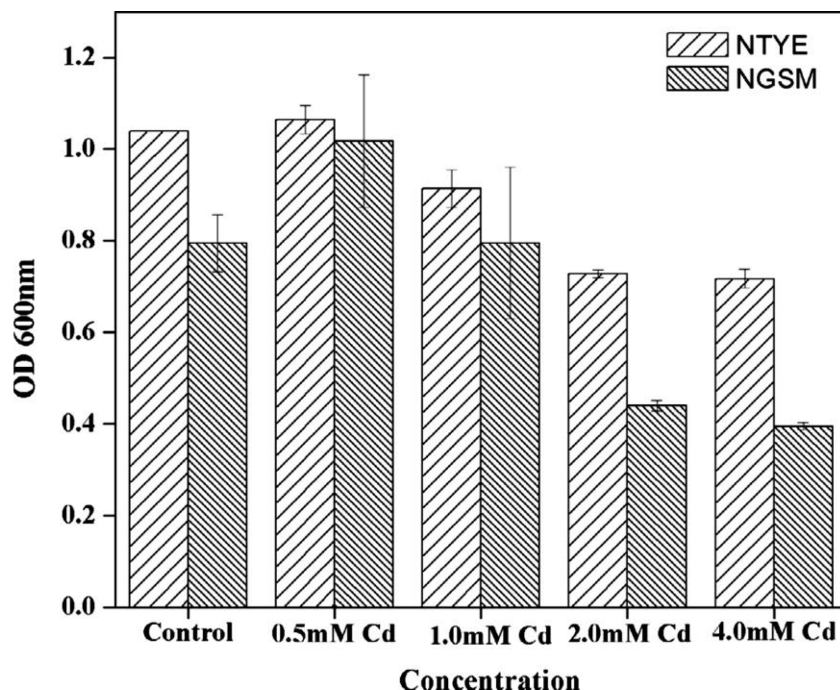


Fig 5.2: Effect of varying Cd concentration on *Haloferax* strain BBK2 grown in NTYE and NGSM media.

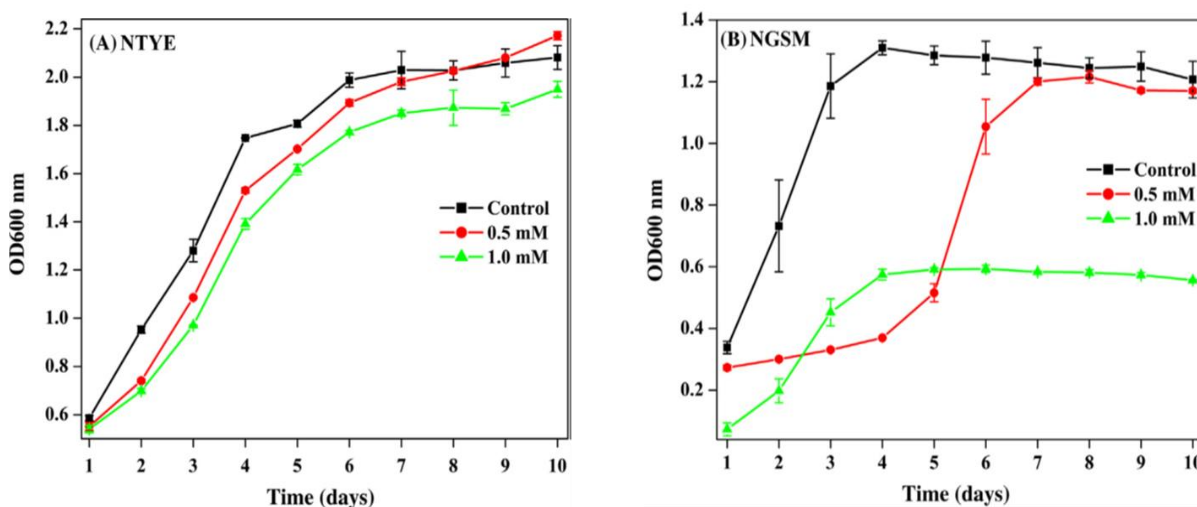


Fig 5.3: Growth profile of *Haloferax* strain BBK2 grown in (A) complex (NTYE) and (B) minimal (NGSM) media with (0.5 mM and 1 mM) and without Cd.

The *Haloferax* strain BBK2 in 0.5 mM Cd showed a prolonged lag phase of around 4 days. The culture reached the maximum OD_{600} of around 1.2 on the 7th day of growth. *Haloferax* strain BBK2 grown in presence of 1 mM Cd showed a lag of 1 day after which the growth increased progressively till 4th day and started decreasing slowly after the 7th day.

There was a short lag phase and the growth density was less when compared with the control and culture in grown in 0.5 mM Cd.

(ii) In varying salt concentrations and pH:

Haloferax strain BBK2 was able to grow in a salt concentration range of 5–30% NaCl with the optimum being 30% NaCl. In the presence of 1 mM Cd, the culture grew optimally at 25% NaCl. Though at 30% NaCl the culture grew optimally in absence of Cd, its growth drastically decreased in presence of Cd (Fig. 5.4).

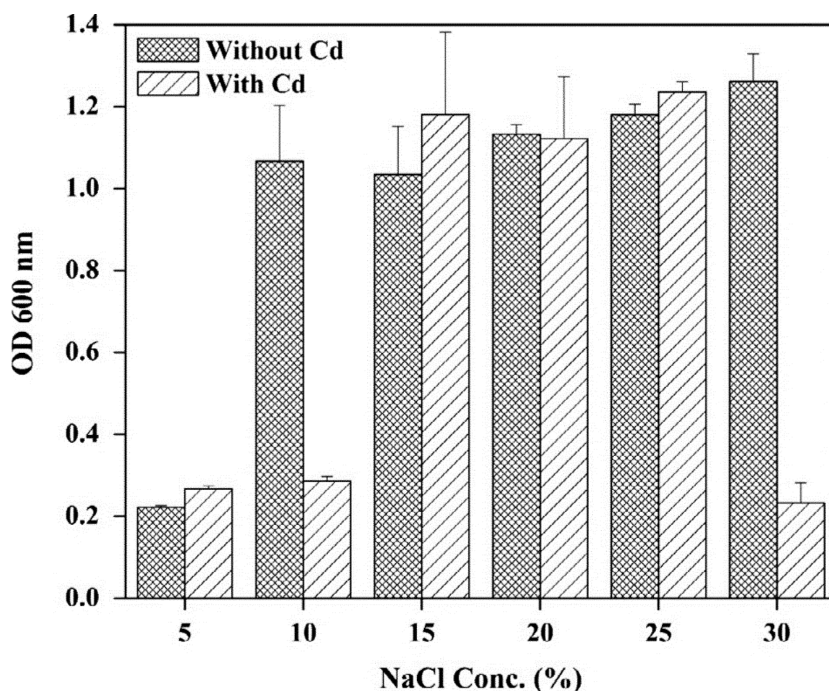


Fig. 5.4: Effect of varying NaCl concentration on *Haloferax* strain BBK2 grown in minimal media with and without Cd.

Xenobiotics which are released into the environment distribute themselves between the components of the eco system such as water, sediment and biota. The degree of metal toxicity depends on its remobilization from these components which in turn depends on the changes in environmental conditions such as pH, redox potential and salinity (Jain et al., 2007). *Haloferax* strain BBK2 was able to adjust to a significant range of NaCl concentration even in the presence of 1 mM Cd. This adaptability augments their viability for long periods in metal contaminated aquatic environments (Massadeh et al., 2005). Presence of sodium and potassium have been reported to enhance metal tolerance in halophilic *Bacilli* (Amoozegar et al., 2005).

A reduction in toxicity of Cd with increasing salt concentration has been reported in *Pseudomonas* sp. (Onishi et al., 1984). Interestingly, the same trend was seen in BBK2 with an exception at 30% NaCl concentration wherein the metal resistance was highly diminished.

Haloferax strain BBK2 showed good growth at a pH range of 7.0–9.0 with the optimum being pH 8. However, in the presence of Cd, the culture also showed good growth in a pH range of pH 7.0–9.0 with the best being pH 9.0 (Fig. 5.5). Growth density of the cultures with metal was comparable with their respective controls. At acidic pH, there was very faint growth and pigmentation. However, at alkaline pH (pH 9 and pH 10), the culture pigment was visually intense as compared to neutral pH 7.0.

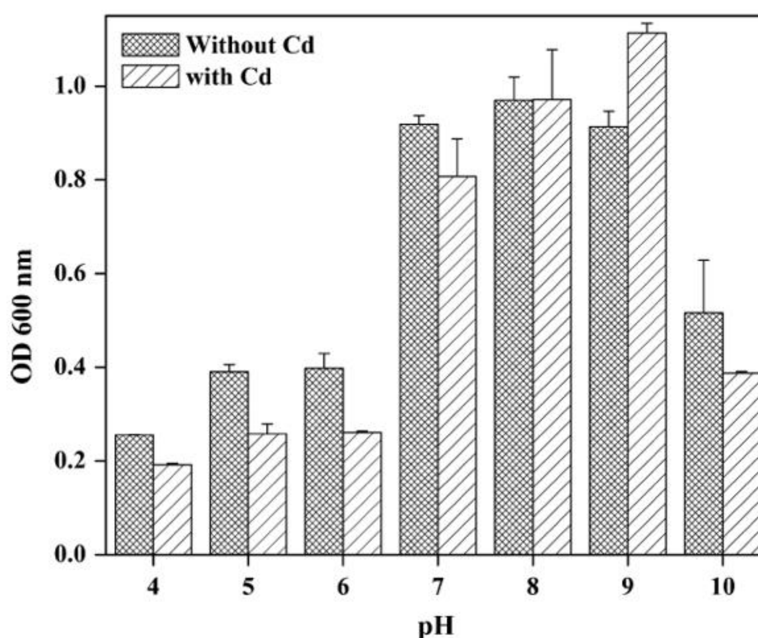


Fig. 5.5: Effect of varying pH on *Haloferax* strain BBK2 grown in minimal media with and without Cd.

pH affects the bioavailability of the metal ions in solution as well as the metal binding sites on the cell surface (Zouboulis et al., 2004; Amoozegar et al., 2012). Hence, pH is a major deciding factor in metal tolerance. *Haloferax* strain BBK2 was able to tolerate 1 mM Cd at a pH range of 6.0 – 9.0.

5.3.2.3 Chemotaxonomic characterization:

Haloferax sp. strain BBK2 showed orange pigmentation in NTYE and pinkish mauve

pigmentation in NGSM. The chloroform-methanol extracts showed characteristic peaks at 388, 467, 495 and 526 indicative of bacterioruberin pigmentation. (Stan-Lotter et al., 2002; Wang et al., 2007; Mani et al., 2012). There was no difference in the pigment peaks of the culture grown in the two media with or without Cd. However, it was seen that in the presence of cadmium, pigment production decreased in complex while it increased in minimal medium (Fig. 5.6).

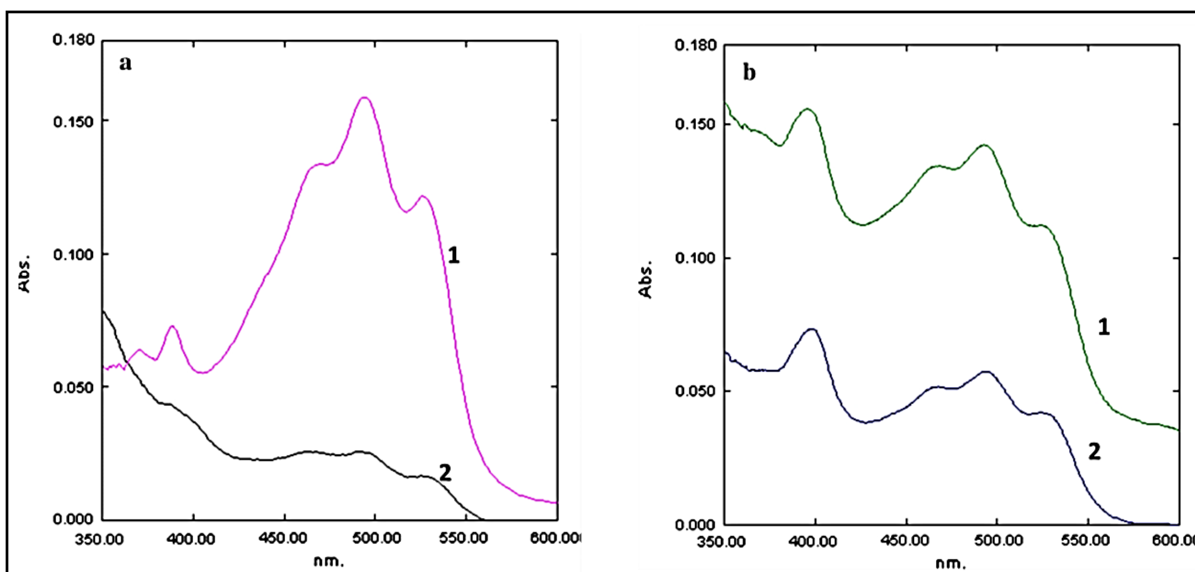


Fig. 5.6: Pigment profiles of *Haloferax* strain BBK2 grown in (a) NTYE and (b) NGSM media. 1- control without Cd (pigment diluted 8 times); 2- with 1 mM Cd.

An increase in concentration of Cd in the growth medium exerted a significant inhibitory effect on the pigmentation of BBK2. In NGSM, the intensity of peaks in the 450–600 nm region reduced whereas the intensity of peak at 400 nm was much more in comparison with that of NTYE. Even though the pigment production was not abolished as in the case of NTYE, the growth was much less in comparison. Such effects on pigmentation in halophilic archaea under hydrocarbon stress have been previously reported by Raghavan and Furtado (2005).

There was a decrease in the overall protein production corresponding to the increased concentration of Cd in NTYE media while in NGSM, the protein profile showed a change in the expression of some specific proteins (Fig. 5.7). The relative molecular mass of the overexpressed proteins was estimated to be approximately 74.14 and 40 kDa.

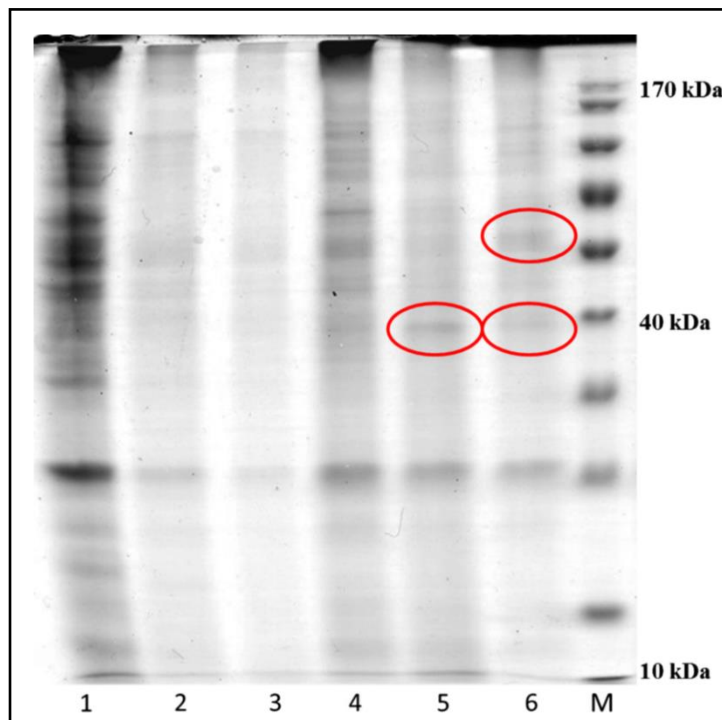


Fig. 5.7: Whole cell protein profile of *Haloferax* strain BBK2 grown in NTYE and NGSM media with varying Cd concentrations. Lanes: 1- control in NTYE, 2- 0.5 mM Cd in NTYE, 3- 1 mM Cd in NTYE, 4- control in NGSM, 5- 0.5 mM Cd in NGSM, 6- 1 mM Cd in NGSM, M- Marker.

Total protein production in BBK2 decreased considerably in the presence of Cd. Similar observation was made in the case of *Sulfolobus metallicus* where several proteins decreased to nondetectable levels in the presence of Cd (Orell et al., 2013). As the concentration of Cd increased, some specific proteins were overexpressed. Similar transcriptomic response has been reported in *Thermococcus gammatolerans* exposed to Cd. (Lagorce et al., 2012). The overexpressed proteins could be ATP synthases to meet the increased energy needs of the stressed cells, chaperons such as HSP70, HSP60 etc. as was observed in *Ferroplasma acidarmanus* exposed to As(III) and Cd (Orell et al., 2013) or proteasomes which destroy the incorrectly folded proteins. Increased levels of proteins involved in biosynthesis of amino acids have been reported in *S. metallicus* and *S. sulfataricus* in response to Cd (Orell et al., 2013). Upregulation of proteins required for the synthesis of sulfur containing amino acids after Cd exposure has been reported in *Saccharomyces*

cerevisiae by Vido et al. (2001). Metal stress response has also been documented to be mediated directly by specific metal binding proteins (O'Halloran, 1993).

5.3.2.4 AAS, SEM EDS, FTIR and XRD analysis:

Various mechanisms are used by microbes to combat metal toxicity. The most common ones are selective uptake, efflux, biotransformation into less toxic forms, and sequestration or a combination of these (Kaur et al., 2006; Osman et al., 2010). Analysis by Atomic Absorption Spectroscopy (AAS) revealed the accumulation of Cd by *Haloferax* strain BBK2. The cells showed a maximal accumulation of 21.08% in the presence of 0.5 mM Cd and 15.19% in presence of 1 mM Cd (Fig. 5.8).

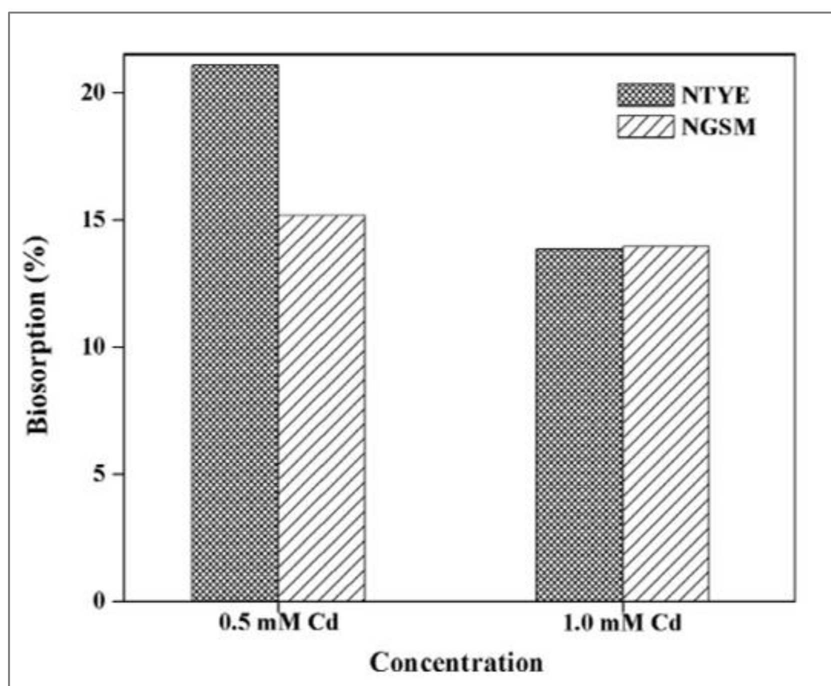


Fig. 5.8: Accumulation of (%) of Cd by *Haloferax* strain BBK2 grown in NTYE and NGSM media with 0.5 and 1.0 mM Cd.

AAS studies confirmed that Cd was indeed bound to the cells. Also, the accumulation by cells grown in nutrient medium was found to be more than that in minimal media. The amount of Cd accumulated decreased with the increasing Cd concentration in the media. Similar trend was reported by Williams et al., 2013 for haloarchaeal isolates such as *Halobacterium saccharovorum*, *Hb salinarium*, and *Natronobacterium gregoryi*. *N. gregoryi* showed maximum resistance of up to 0.001 mM Cd with 39.8% biosorption. Studies on Cd

resistance and bioaccumulation in eubacteria both Gram negative (*Brevundimonas* sp. ZF12 strain accumulating 49.01 mg $g1^{-1}$) and Gram positive (*Streptomyces pimprina* accumulating 30.4 mg $g1^{-1}$) have been reported (Puranik et al., 1995; Masoudzadeh et al., 2011).

SEM EDX analysis was carried out on the surface of intact *Haloferax* cells as well as on cells disrupted by dialysis. No Cd was detected on intact cell surface whereas the cells disrupted by dialysis showed a 1.97 weight% of Cd (Fig. 5.9).

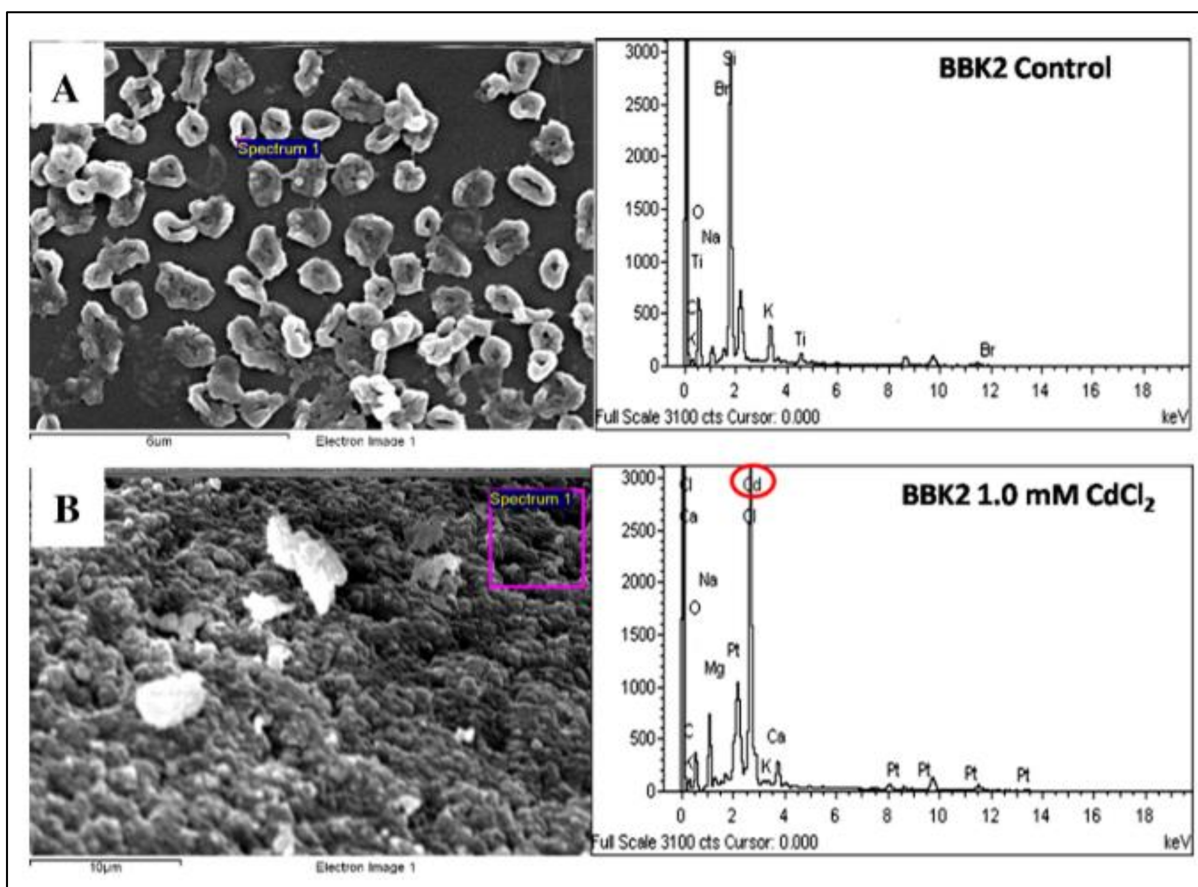


Fig. 5.9: Scanning electron microscopy and energy-dispersive X ray analysis of the *Haloferax* strain BBK2 grown in media with 1 mM Cd. (Area marked with square is used for EDS analysis). A- Intact cells, B- disrupted cells.

SEM EDX analysis showed that Cd was not sorbed on to the outer surface of the cell but has been bound to some inner cell component or accumulated intracellularly. This is in agreement with the previous report of accumulation of Cd in the cytoplasm as well as between the cell wall and cell membrane in halophilic organisms by Massadeh et al., 2005.

The bioaccumulation capacity of the organism was further confirmed by XRD and FTIR. The FTIR spectrum was recorded in the range of 4000–400 cm^{-1} for *Haloferax* strain BBK2 cells grown in minimal media. Spectral shifts were observed in the range of 3300–3500 cm^{-1} belonging to the stretching of N-H from amino group and a bonded hydroxyl group. For cells grown in presence of Cd, a peak shift at 1083 cm^{-1} was observed along with broadening of the peak region. Interaction between lipid and Cd was observed by the functional group shift in the range of 2900–3000 cm^{-1} representing the C–H of the –CH2 and –CH3 groups of the lipids (Fig. 5.10).

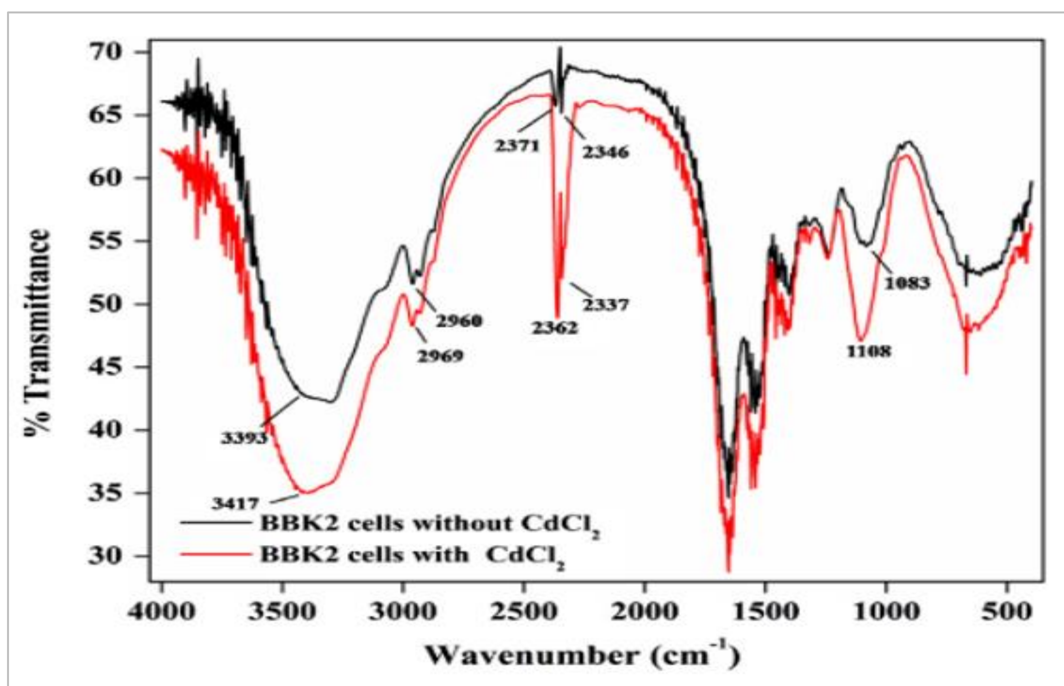


Fig. 5.10: FTIR spectra of cells of *Haloferax* strain BBK2 grown in presence and absence of Cd.

The FTIR spectra showed clear difference between the control cells and the cells grown with Cd. The changes in the functional groups indicate the complex interaction of Cd with the cell biomolecules like carbohydrates, proteins and lipids. Spectral range from 950 to 1150 cm^{-1} indicate the absorbance of the functional group belonging to alcohol (PO_2, C–OH and C–C groups) and polysaccharides (C–O–P and C–O–C groups). The shift observed in the spectra between the cells grown in presence and absence of Cd demonstrated the possible interaction of Cd with the phosphate and polysaccharide groups. The FTIR analysis indicates the complex interaction of the Cd with biomolecules like polysaccharide, lipids and proteins. However, the

most prominent interaction was observed in the functional group belonging to polysaccharide suggesting their potential role in the adsorption of Cd (Parikh and Chorover, 2006; Eboigbodin and Biggs, 2008; Masoudzadeh et al., 2011).

The XRD analysis of the powdered crystalline cells containing Cd were performed and compared with cells without Cd as control. A broad prominent peak was obtained at 24.8° which indicated the presence of CdS nanoparticles. However, peaks obtained in the range of 20° to 60° determined the complexity of cells grown in presence of Cd. (Fig. 5.11).

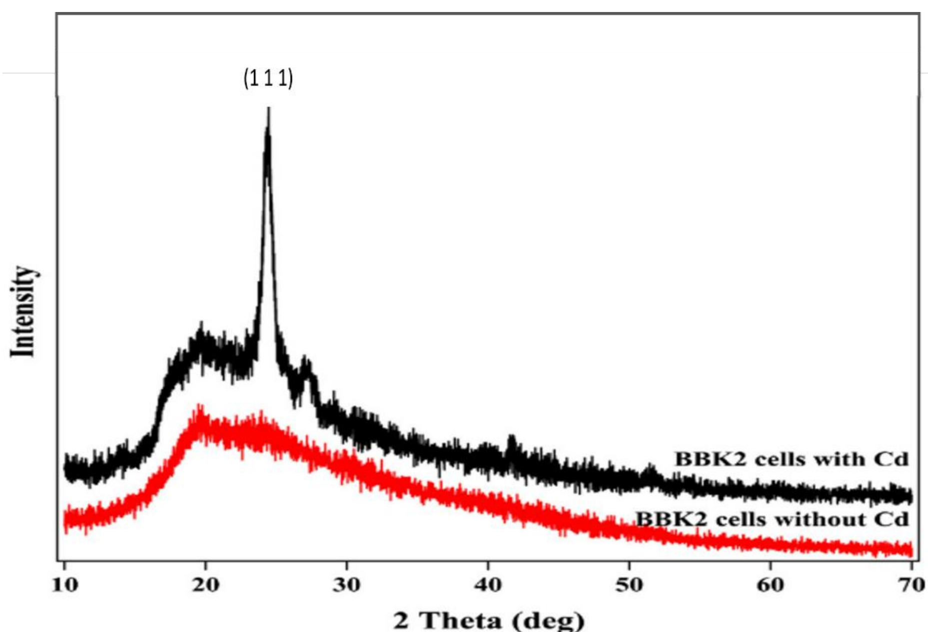


Fig. 5.11: X-ray diffraction profiles of powdered cells of *Haloferax* strain BBK2 grown in presence and absence of Cd.

The XRD analysis of the cells grown in the presence of Cd showed peaks between 18° and 52° indicating the difference in crystallinity due to the presence of Cd. The broadening of the peak (111) obtained at 24.8^o indicated the amorphous nature of the sample and presence of CdS nanoparticles (JCPDS card File no: 41-1049). Absence of peaks at 37° and 48° indicated that the CdS nanoparticles are cuboid in shape (Bandaranayake et al., 1995). The presence of other peaks between $20^o$and 60° indicated the complex nature of the cells in the presence of Cd. These results compared well with the study by Masoudzadeh et al. who investigated the biosorption potential of cadmium by Gram negative Bacillus, *Brevundimonas* sp. ZF12 strain (Masoudzadeh et al., 2011).

Even though release of Cd into the environment has decreased significantly due to the regulations in its use, the amount which has already been released into the environment needs to be detoxified. The utilization of different selected or genetically modified strains with high metal resistance as well as the capability to survive in multiple stress conditions may play an important role in the industrial bio mining in the coming years (Orell et al., 2010). An insight of the microbes' environmental adaptations will go a long way in understanding and exploiting their metal resistant properties.

5.3.3 Resistance of extremely halophilic archaea to zinc and zinc oxide nanoparticles [§]:

5.3.3.1 Minimal Inhibitory Concentration (MIC) of Zn and ZnO NPs

The MIC of Zn and ZnO NPs on extremely halophilic archaea was assessed as the minimum Zn concentration that inhibits the growth. The MIC results in NTYE/NT and NGSM are represented in Table 5.2.

Table 5.2 Minimal inhibitory concentration (MIC) of $ZnCl_2$ (heavy metal) and ZnO NPs (metal nanoparticles) on four extremely halophilic archaeal genera *Halococcus, Haloferax, Halorubrum* and *Haloarcula* grown in complex (NTYE/NT) and minimal (NGSM) media.

	Extremely halophilic archaeal cultures							
	Halococcus strain BK6		*Haloferax* strain BBK2		*Halorubrum* strain BS17		*Haloarcula* strain BS2	
Growth media	NTYE	NGSM	NTYE	NGSM	NT	NGSM	NT	NGSM
Heavy metal ($ZnCl_2$) mM								
Control	+++	+++	+++	+++	+++	+++	+++	+++
0.1	+++	+++	+++	+++	++	++	++	++
0.5	+++	++	++	++	+	+	+	±
1	++	++	+	+	−	−	−	−
2	+	−	+	−	−	−	−	−
4	±	−	±	−	−	−	−	−
Metal nanoparticles (ZnO NPs) mM								
0.1	+++	+++	+++	+++	++	++	++	+
0.5	+++	++	++	+	+	+	−	−
1	++	++	+	−	−	−	−	−
2	+	−	+	−	−	−	−	−
4	±	−	±	−	−	−	−	−

+++ very good growth, ++ good growth, + growth, ± not sure, − no growth

[§] **This work is published as Salgaonkar, B. B., Das, D., & Bragança, J. M. (2016). Resistance of extremely halophilic archaea to zinc and zinc oxide nanoparticles.** ***Applied Nanoscience*, *6*(2), 251-258.**

The $ZnCl_2$ resistance in complex/minimal media was seen as *Halococcus sp.* strain BK6 (2.0/1.0 mM) >*Haloferax sp.* strain BBK2 (2.0/1.0 mM) >*Halorubrum sp.* strain BS17 (0.5/0.5 mM) > *Haloarcula sp.* strain BS2 (0.5/0.1 mM) whereas for ZnO NPs resistance was BK6 (2.0/1.0 mM) >BBK2 (2.0/0.5 mM) >BS17 (0.5/ 0.5 mM) >BS2 (0.1/0.1 mM).

Zinc (Zn) is essential for proper functioning of large number of metalloproteins (Zn-binding proteins) and is required by organisms of all three domains of life (archaea, bacteria and eukaryote) thereby making it one of the key metals of life (Andreini et al. 2006). However, metals in excess are detrimental and cause cellular damage (Bini 2010). Zn is toxic to cells due to the formation of reactive oxygen species (ROS) thereby inhibiting some of the vital enzymes like endonucleases, DNA glucosylases, etc. Acosta et al. (2011) and Zhao et al. (2013) reported that increase in ionic strength (salinity) increases the concentration of metal (Pb, Cu, Cd, Zn and Mn) released. High concentration of $MgCl_2$ and NaCl in hypersaline environment leads to an increased release of Zn from the sediments thereby increasing its mobility and bioavailability. Presence of high concentrations of NaCl increases the toxicity of Zn due to the formation of $ZnCl^-$ species which is more toxic than the cationic Zn^{2+} (Nieto et al. 1987).

Among all haloarchaeal strains screened, the genera *Halococcus* and *Haloferax* showed the best resistance and tolerated highest amount of both Zn and ZnO NPs in complex NTYE medium and the minimal medium NGSM. Nieto et al. (1987) in his study of haloarchaeal susceptibility to different heavy metals found that the MIC of Zn was 0.05–0.5 mM. Williams et al. (2013) reported that haloarchaeal strains *Halobacterium saccharovorum* can tolerate only up to 0.01 mM of Zn. The MIC of Zn of *Haloferax* strain BBK2 in NTYE and NGSM media (2.0/ 1.0 mM) was much higher as compared to reports by Popescu and Dumitru (2009) which was 0.5–1.0 mM of Zn. Gunalan et al. (2012) and Premanathan et al. (2011) reported MIC values of ZnO NPs for Gram-positive organisms like *Staphylococcus aureus* is in the range of 0.8–1.5 mM where as that of Gram-negative bacteria like *E. coli* and *Pseudomonas aeruginosa* to be 6.1 mM. In metal microbe interactions, the cell wall is the first part of microbe which will contact and interact with the metal. Studies on metal resistance in halophilic bacteria done by Al-Momani et al. (2007) indicated that the metal was accumulated on the cell wall, plasma membrane as well as in the cytoplasm. Li et al. (2011) studied the susceptibility of Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas putida* and *E. coli*) bacteria to

ZnO NPs and found that Gram-negative organisms were more resistant to ZnO NPs. The cell wall of Gram-positive bacteria comprises of thicker peptidoglycan as compared to their Gram-negative counterparts which have an outer membrane. The outer membrane acts as impermeable lipid barrier and hence most of Gram-negative bacteria are resistant to most of the toxic substances like antibiotics, metals, etc. Increased concentrations of zinc results in inhibition of the electron transport chain where as ZnO NPs results in the formation of reactive oxygen species (ROS) and lipid peroxidation resulting in apoptosis in human myeloblastic leukemia cells—HL60 (Premanathan et al. 2011). The toxicity of the ZnO NPs and their bulk counterparts on eukaryotes like zebrafish is found to be in the range of 0.04–0.099 mM (Xiong et al. 2011).

5.3.3.2 Effect of Zn and ZnO NPs on growth of halophilic archaea

Growth kinetics were studied in NGSM medium (Fig. 5.12; Table 5.2) and the concentration of $ZnCl_2$/ZnO NPs was selected based on the MIC results for the respective haloarchaeal strains. *Haloferax* strain BBK2 in presence of 0.5 mM of ZnCl2/ ZnO NPs reached its maximum OD of 1.31/1.4 in 7 days which was almost same when compared to the control, i.e., 1.36 in 6 days. The culture grew with doubling time of 37.9 h which increased with $ZnCl_2$/ZnO NPs to 47.0/47.5 (Fig. 5.12).

Halococcus strain BK6 in presence of 0.5 mM of $ZnCl_2$/ ZnO NPs reached its maximum optical density (OD at 600 nm) of 1.22/0.69 in 7 days which was little lower when compared to control, i.e., 1.44 in 3 days (Fig. 5.12). The culture grew with doubling time of 16.1 h which increased with $ZnCl_2$/ZnO NPs to 53.6/72.2. Growth of *Halorubrum* strain BS17 was very slow with maximum OD of 0.87/0.92 in 7 days in presence of 0.1 mM $ZnCl_2$/ZnO NPs which was comparable to the control, i.e., 0.84 in 7 days (Fig. 5.12). The culture grew with doubling time of 92.4 h which decreased with bulk $ZnCl_2$ to 86.4 h and increased with ZnO NPs to 97.6 h. *Haloarcula* strain BS2 in presence of 0.1 mM of $ZnCl_2$/ ZnO NPs reached its maximum OD of 1.15/1.19 in 7 days which was almost same when compared to the control, i.e., 1.09 in 7 days. The culture grew with doubling time of 49.9 h which surprisingly decreased with $ZnCl_2$/ZnO NPs to 35.7/35.0 (Table 5.3).

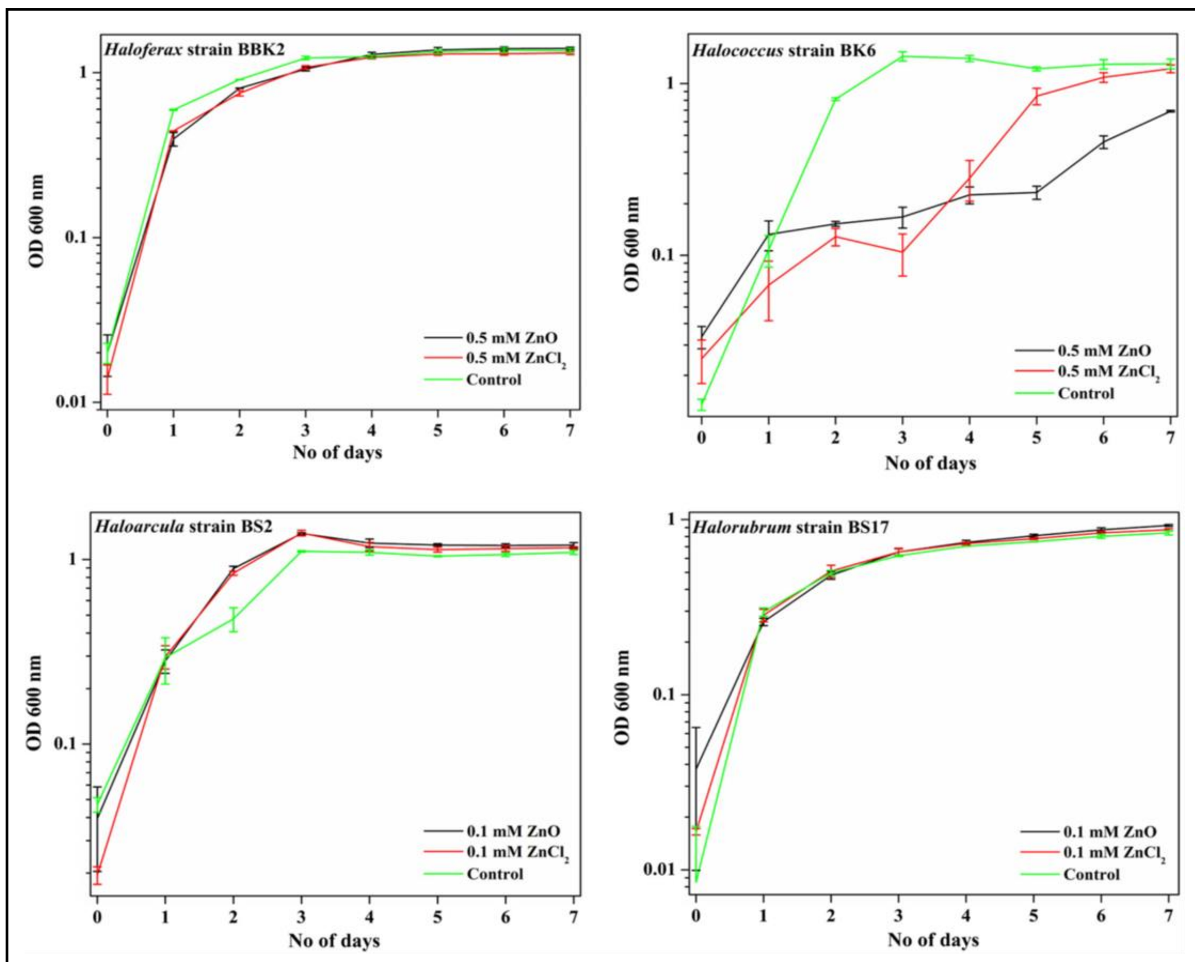


Fig 5.12: Growth profile of the extremely halophilic archaeal cultures *Haloferax* strain BBK2, *Halococcus* strain BK6, *Halorubrum* strain BS17 and *Haloarcula* strain BS2 grown in NGSM with $ZnCl_2$ and ZnO NPs.

Table 5.3 Comparative study of the effect of $ZnCl_2$ (heavy metal) and ZnO NPs (metal nanoparticle) on the growth profile of the haloarchaeal genera during growth in NGSM.

Haloarchaeal isolates	Control			$ZnCl_2$			ZnO		
	λ (h)	μ (h^{-1})	t (h)	λ (h)	μ (h^{-1})	t (h)	λ (h)	μ (h^{-1})	t (h)
Halococcus strain BK6 (1.0 mM)	29.7	24.7	16.1	70.5	7.4	53.6	110.4	5.5	72.2
Haloferax strain BBK2 (0.5 mM)	–	10.4	37.9	–	8.4	47.1	–	8.3	47.5
Haloarcula strain BS2 (0.1 mM)	–	7.9	49.9	–	11.1	35.7	–	11.3	35.0
Halorubrum strain BS17 (0.1 mM)	–	4.3	92.4	–	4.5	86.4	–	4.0	97.6

λ lag phase time, μ growth rate, t doubling time

The four haloarchaeal genera *Halococcus, Haloferax, Haloarcula* and *Halorubrum* when grown in presence of Zn and ZnO NPs showed varying resistance in both complex (NTYE/NT) and minimal medium. The cultures exhibited increased resistance in complex media when compared with the minimal medium. This may be due to the complex formation by the media ingredients with the metal which decreases the availability of metal and/or metal NPs to the microorganisms. Similar observation was made by of haloarchaea increased in presence of $ZnCl_2$ and further increased in ZnO NPs. Interestingly, *Haloarcula* strain BS2 showed better growth in presence of bulk as well as Zn NPs.

5.3.3.3 Pigment analysis

The haloarchaeal strains when grown in NGSM showed pink or mauve pigmentation (Fig 5.13). The pigment was unaltered during growth in the presence of both bulk Zn or ZnO NPs. Extraction of the pigments in acetone showed characteristic peaks at 389, 471, 496 and 528 nm corresponding to bacterioruberins. Interestingly, a shift in the peaks (323, 394, 477, 503 and 536) was observed when pigments were extracted in chloroform:methanol (2:1 v/v).

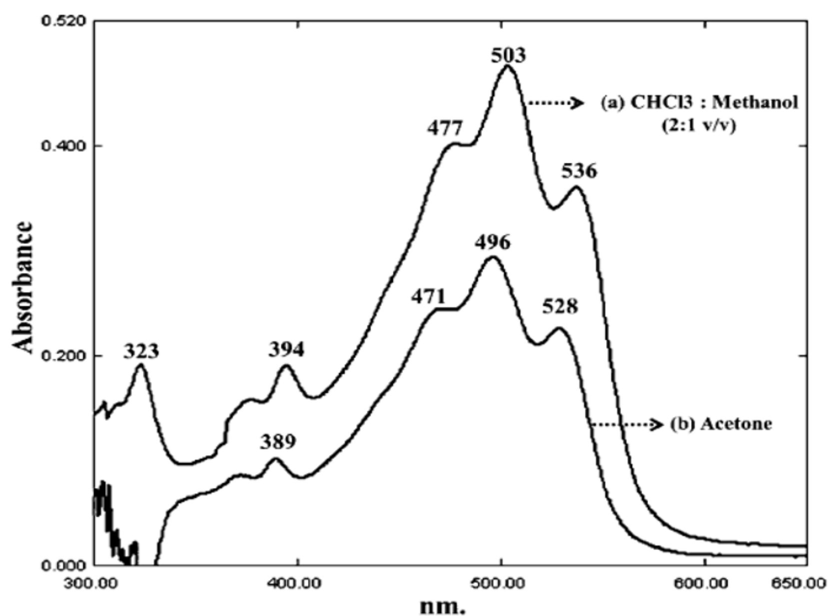


Fig 5.13: UV-visible spectrophotometric scans of pigments from *Halorubrum* strain BS17 grown in NGSM containing 0.1 mM $ZnCl_2$ and extracted using (a) chloroform: methanol (2:1 v/v) and (b) acetone

5.3.3.4 Zn accumulation studies:

The amount of Zn accumulated by whole cells of each genus varied when grown in $ZnCl_2$ and ZnO NPs. The $ZnCl_2$ accumulation analyzed by atomic absorption spectroscopy was seen as *Haloferax* strain BBK2 (287.2 mg g^{-1}), *Halococcus* strain BK6 (165.9 mg g^{-1}), *Haloarcula* strain BS2 (93.2 mg g^{-1}), *Halorubrum* strain BS17 (29.9 mg g^{-1}), whereas for ZnO NPs accumulation was *Haloferax* strain BBK2 (549.2 mg g^{-1}), *Halococcus* strain BK6 (388.5 mg g^{-1}), *Haloarcula* strain BS2 (28.5 mg g^{-1}). *Halorubrum* strain BS17 (16.2 mg g^{-1}) (Table 5.3).

Haloarchaeal strains	Bioaccumulation (mg g^{-1})	
	$ZnCl_2$	ZnO NPs
Haloferax strain BBK2	287.2	549.6
Haloarcula strain BS2	93.2	28.5
Halorubrum strain BS17	29.9	16.2
Halococcus strain BK6	165.9	388.5

Table 5.3 Bioaccumulation of $ZnCl_2$ and ZnO NPs by the haloarchaeal strains estimated using atomic absorption spectrophotometry

Among the four genera, *Haloferax* showed a higher Zn accumulation although both *Halococcus* and *Haloferax* were grown in 0.5 mM of $ZnCl_2$ and 0.5 mM of ZnO NPs. Interestingly, the amount of ZnO NPs accumulated was more as compared to $ZnCl_2$ as revealed by AAS. However, *Haloarcula* strain BS2 and *Halorubrum* strain BS17 grown in 0.1 mM of $ZnCl_2$ and 0.1 mM of ZnO NPs showed higher accumulation of $ZnCl_2$ than ZnO NPs. Recent study by Das et al. (2014) indicated that *Haloferax* strain BBK2 was also resistant to cadmium (Cd) and accumulated 21.08 and/or 15.19 % of Cd in the presence of 0.5/1 mM Cd. *Haloferax* is known to produce exopolysaccharide (EPS) which protects the cells from direct contact with the metals and hence its resistance (Poli et al. 2011).

Halococcal cells resist lysis when suspended in low-osmolarity solutions (3.5 % NaCl) (Mani et al. 2012; Legat et al. 2013). The cell wall of *Halococcus* is composed of heteropolysaccharide with acetylated amino sugars unlike glycoprotein S-layer in genus *Haloferax* (Schleifer et al. 1982; Kandler and Konig 1998). This could be a contributing factor for higher metal resistance of these organisms. On the other hand, Al-Mailem et al. (2011)

studied the resistance and mercury (Hg) volatilization (Hg^{2+} to Hg^{o}) and oil consumption capability of haloarchaea viz *Haloferax, Halobacterium* and *Halococcus* and found that genus *Halococcus* was the most efficient in Hg volatilization as compared with the other genera. The haloarchaeal cells grown in NGSM medium with $ZnCl_2$/ZnO NPs showed peaks for Zn on the cell surface when examined by SEM–EDX. However, the amount (percent) of Zn sorbed on the surface of cells of *Haloferax* strain BBK2 grown in the presence of $ZnCl_2$ was greater (21.77 %) than cells grown in presence of ZnO NPs (14.89 %) (Fig. 5.14).

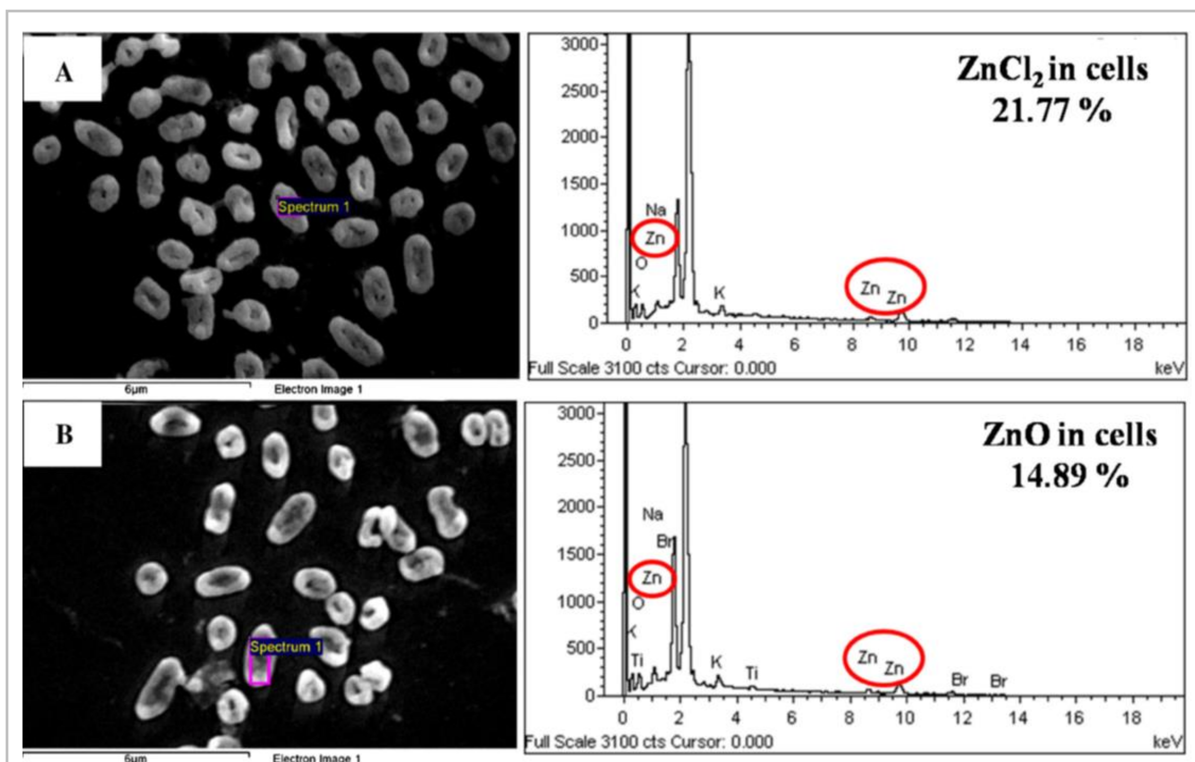


Fig 5.14 Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analysis of haloarchaeon *Haloferax* strain BBK2, grown in NGSM containing 0.1 mM $ZnCl_2$ (A) and ZnO nanoparticles (B).

Peaks of K, Mg, Ca, which are components of the growth medium, were also detected in SEM– EDX analysis. Recent study by Williams et al. (2013) investigated the ability of halophilic archaeon *Halobacterium saccharovorum* to tolerate up to 0.01 mM Zn with maximum of 68.6 % biosorption, while at higher Zn concentration (0.1 mM) only 19.5 % of Zn was biosorbed.

The XRD analysis of the cells showed peaks between 22° and 57° for cells grown in presence of $ZnCl_2$. This indicated the difference in crystallinity due to the sorption of bulk $ZnCl_2$ by the haloarchaeal cell components. At 2ϴ of 28.8, weak reflection was seen which could be attributed to the reflection of ZnS sphalerite phase (111) as reported by Dedova et al. 2007 for $ZnCl_2$ sample (Fig 5.15).

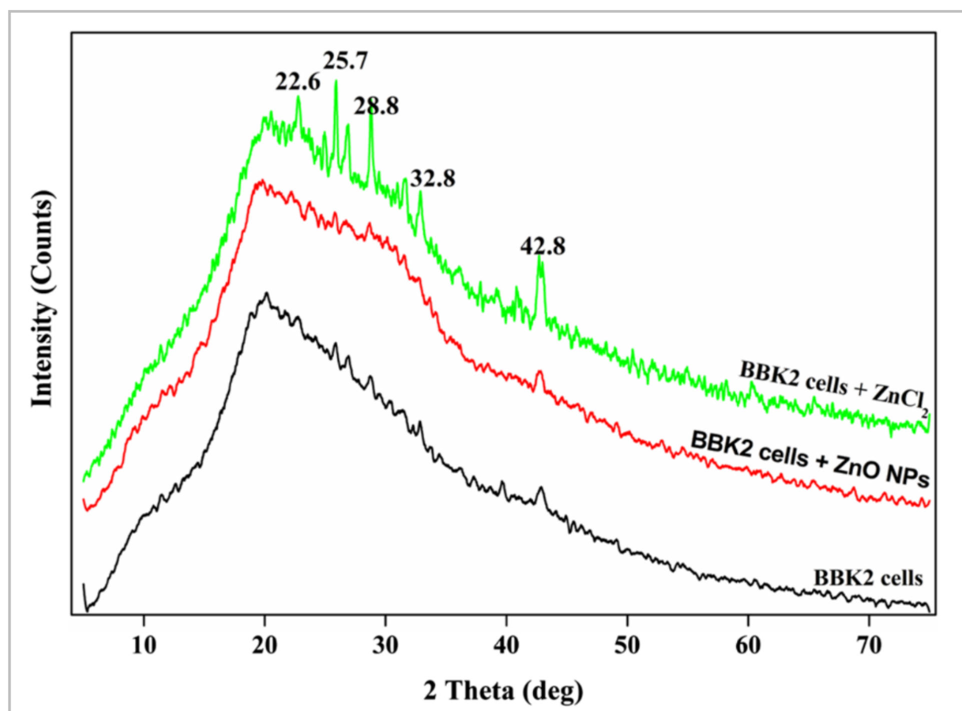


Fig 5.15: The XRD pattern of dialyzed cells of *Haloferax* strain BBK2 grown in presence of $ZnCl_2$ and/ ZnO NPs.

The broadening of the peaks indicated the amorphous nature of the sample. However, XRD profile of BBK2 cells grown in presence of ZnO NPs and without Zn showed no significant difference.

5.3.4 Platinum Resistance studies in *Haloarcula* sp. strain E2

5.3.4.1 *Haloarcula* sp. strain E2

The extremely halophilic archaea, *Haloarcula* sp. strain E2 (Accession number: AB904832), was isolated from the solar salterns of Siridao, Goa, India. It is an orange-red pigmented Gram-negative organism, grown and maintained in EHM media (Table 2.1). Total lipid analysis showed the presence of phospholipid PGP-Me and glycolipid S DGD. Pigment

analysis revealed the presence of bacterioruberin and carbohydrate utilization studies showed the ability of E2 to utilize various sugars such as glucose, fructose, sorbitol, mannitol, sucrose and lactose. Detailed description of E2 is given in chapter 2.

5.3.4.2 Effect of Pt on the growth of *Haloarcula* sp. strain E2

On solid EHM medium, E2 was able to grow till 1mM Pt but in liquid EHM broth, the maximum concentration of Pt which allowed growth was 0.5mM Pt.

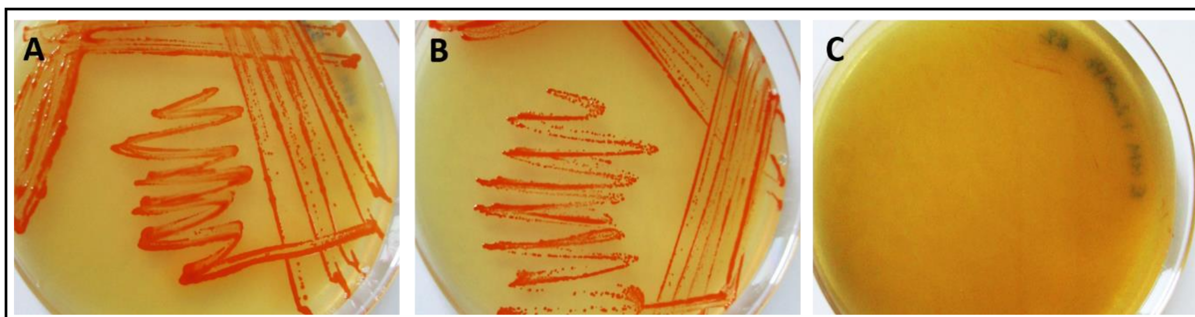


Fig 5.16: *Haloarcula* sp. strain E2 grown in EHM agar media containing 0.5mM (A), 1.0 mM (B) and 2mM (C) platinum.

The control cells took 7-8 days to reach the stationary phase whereas the cells grown in Pt took 28-30 days to reach a maximum OD of 1.5 (Fig 5.17).

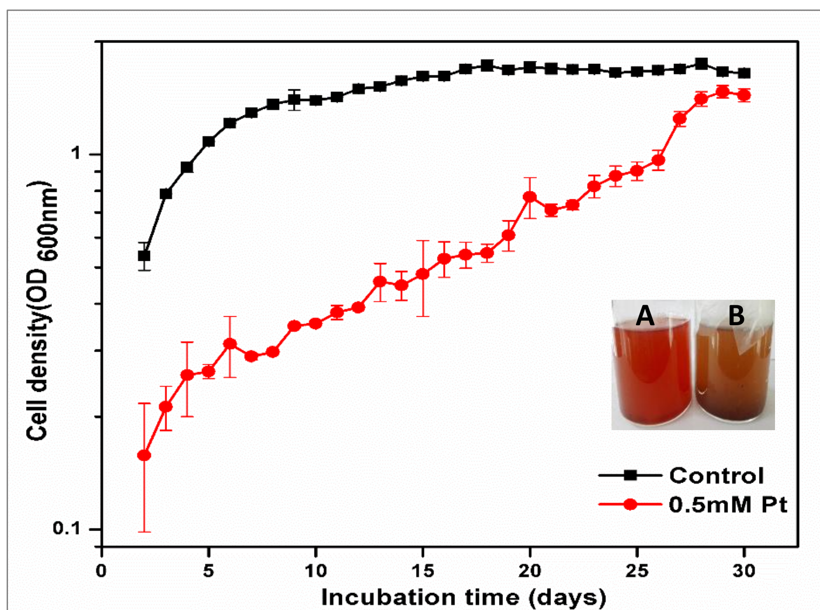


Fig 5.17: Growth profile of *Haloarcula sp*. strain E2 grown in EHM broth in the absence and presence (0.5mM) of Pt. Inset shows the visual difference in colour between control E2 culture (without Pt) and E2 grown in presence of Pt

Specific growth rate, calculated according to Berney et al. (2006), was found to be 28.0 day^{-1} for the control culture and 0.07 $days^{-1}$ for the E2 strain grown in Pt. The brownish colour in of the culture grown in the presence of Pt is indicative of the formation of platinum nanoparticles (Castro et al., 2015).

5.3.4.3 Pt accumulation studies by AAS, FTIR, XRD, SEM EDX and TEM analysis

The Pt accumulation in *Haloarcula* sp. E2 cells quantified by atomic absorption spectroscopy showed an accumulation of platinum 208.42 ±18.3 mg g^{-1} which accounts for around 20.84% of the wet weight of the E2 cells.

The FTIR spectrum was recorded in the range of 4000-500cm^{-1} for *Haloarcula* sp. strain E2. The shift in the peaks show the complex interaction of various biomolecules in the cells with the Pt. The interaction between proteins (amine group) and Pt is indicated by the peak shift in 3300-3500cm^{-1}. The shift in amine group is also observed in 1030 -1230cm^{-1}. The functional group shift at 2900cm^{-1} to 3000cm^{-1} revealed the interaction between lipids and Pt.

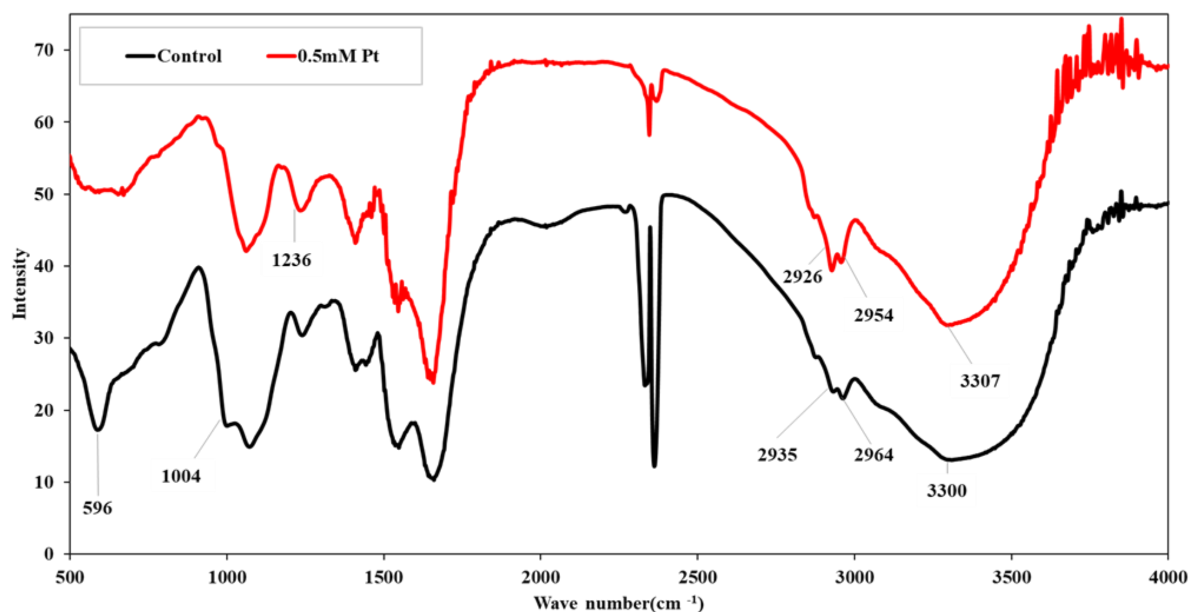


Fig 5.18: FTIR spectra of cells of *Haloarcula* sp. strain E2 grown in presence and absence of Pt

Spectral range from 950 to 1150 cm^{-1} indicate the absorbance of the functional group belonging to alcohol (PO2, C–OH and C–C groups) and polysaccharides (C–O–P and C–O–C groups). The shift observed in the spectra between the cells grown in presence and absence of

Pt demonstrated the possible interaction of Pt with the phosphate and polysaccharide groups. However, the most prominent interaction was observed in the functional group belonging to amines (N-H) suggesting their potential role in the adsorption /accumulation of Pt (Parikh and Chorover, 2006; Eboigbodin and Biggs, 2008; Masoudzadeh et al., 2011). The 1650 and 1536 cm−1 bands are assigned to the amide I and II bonds of proteins, respectively (Ahmad et al, 2003). Free amine groups or cysteine residues in the proteins bind to platinum nanoparticles and could aid in the stabilization of the platinum nanoparticles by surface-bound proteins. The fungus Fu*sarium oxysporum* is known to produce and secrete protenaceous substances which cap the Pt nanoparticle thereby stabilizing them (Syed and Ahmad (2012).

XRD analysis of platinum nanoparticles was carried out by depositing them as a biofilm on a glass substrate. There were no sharp Bragg reflections except for a broad peak at the 2θ angles of 15–35°, indicating that the reaction product was not a crystalline phase (Li et.al., 2007). The broad peak may also be due to the amorphous organic matter covering the accumulated platinum.

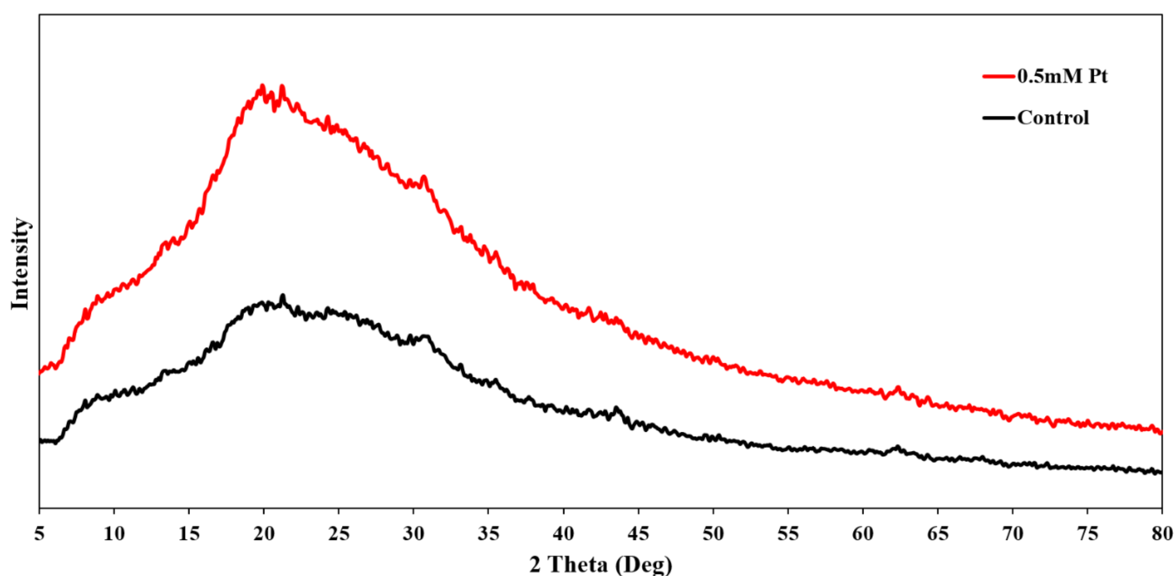


Fig 5.19: X-ray diffraction profiles of powdered cells of *Haloarcula* strain E2 grown in presence and absence of Pt.

SEM EDX analysis was carried out on the surface of *Haloarcula* sp. strain E2 showed that Pt was not sorbed on the surface of cells but was bound to some intracellular component

as inferred from the presence of platinum in the dialyzed/disrupted cells of *Haloarcula* sp. strain E2.

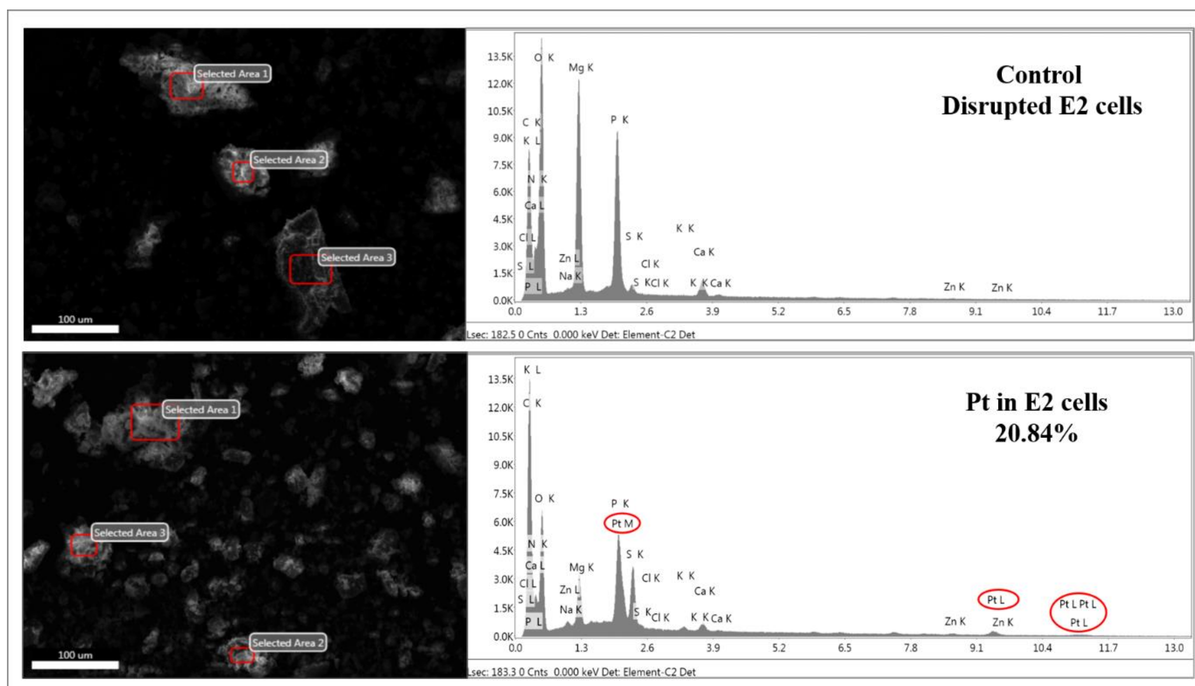


Fig 5.20: Scanning electron microscopy and energy-dispersive X ray analysis of the disrupted cells of *Haloarcula* sp. strain E2 grown in absence of Pt (Control) and with 0.5mM Pt. (Area marked with squares have been used for EDS analysis).

Both intracellular and extracellular accumulation of Pt has been observed in halophilic mixed bacterial cultures and their application in the reduction of Pt salts and removal from the aqueous solution by accumulation was also explored (Maes et. al., 2016). However, Pt accumulation in halophilic archaea have not been explored till date. Their interaction with protein molecules (Section5.4.4.3 (ii)) might be a stabilizing factor in the intracellular Pt.

TEM analysis of platinum accumulated in the *Haloarcula* sp. E2 was carried out to determine the size and morphology of the bioaccumulated platinum particles. The transmission electron micrographs (Fig 5.21) shows the formation of nanoparticles in size range of 5–30 nm. Platinum nanoparticles exhibited irregular shapes but were predominantly spherical in shape with an average size of 5-10nm (calculated from the TEM images). The irregularity of the nanoparticle shape may be attributed to organic matter containing sulphur. Sulphur atom has a huge affinity for noble metals and this property has been exploited to produce surface-modified noble metal nanoparticles (Takami, 2012). Syed and Ahmad (2012) reported Pt NPs

in the size range of 5-20nm synthesized by fungus *Fusarium oxysporum*. Pt nanoparticles synthesized using *Diopyroskaki* leaf extract also showed similar mixed morphology and these nanoparticles are presumed to be stabilized by some metabolites like terpenoids (Song et al., 2010).

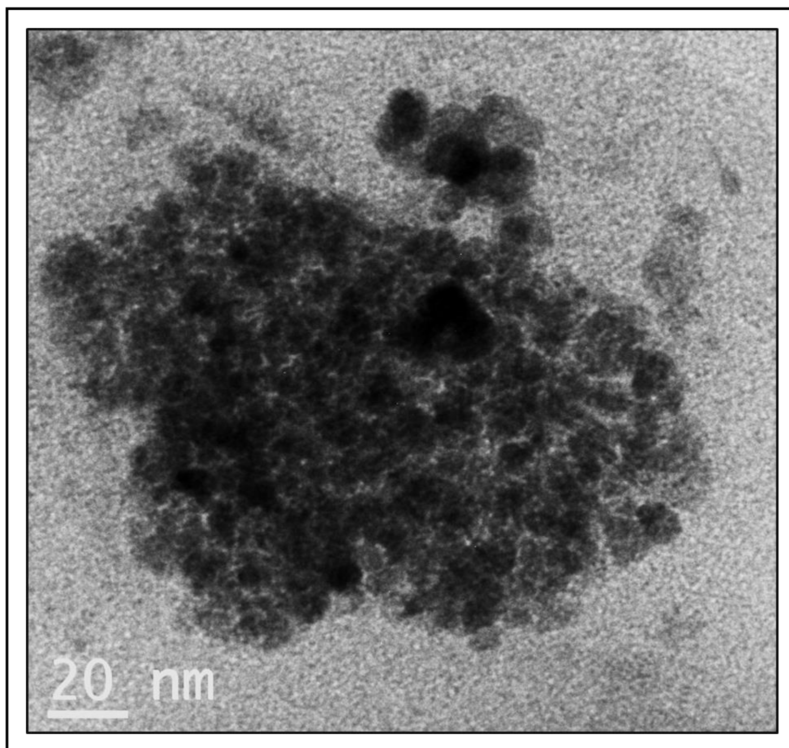


Fig 5.21: TEM micrograph of a drop-cast film of the platinum-nanoparticles synthesized by cells of *Haloarcula* sp. strain E2 grown in presence of H_2PtCl_6.

5.4 SUMMARY AND CONCLUSION

Haloarchaeal strains belonging to four different genera (viz. *Haloferax* sp., *Halorubrum* sp., *Haloarcula* sp., and *Halococcus* sp.) were screened for resistance/ tolerance against metals/ metalloids (viz. Cd, Cu, Pt, Zn, Se and Te).

The growth of the extremely halophilic archaeon *Haloferax* strain BBK2 in presence of Cadmium ($CdCl_2.2H_2O$) was studied in detail. BBK2 cells can grow in the presence of Cd in both complex and minimal media with NaCl concentration varying from 15% to 25%. The cells showed a maximal accumulation of Cd of 21.08% in the presence of 0.5 mM Cd and 15.19% in presence of 1 mM Cd. Interaction between polysaccharides, proteins, lipids and Cd was observed by FTIR spectroscopy. The SEM EDX and XRD analysis proved that the Cd

was accumulated by intracellular cell components probably as CdS nanoparticles. This is the first report of Cadmium accumulation in *Haloferax* sp.

Four representative genera *Halococcus, Haloferax, Halorubrum and Haloarcula* studied were able to tolerate and grow in complex as well as minimal media in presence of Zn and ZnO NPs. The $ZnCl_2$ resistance in complex/minimal media was seen as *Halococcus sp.* strain BK6 (2.0/1.0 mM) >*Haloferax sp.* strain BBK2 (2.0/1.0 mM) >*Halorubrum sp.* strain BS17 (0.5/0.5 mM) > *Haloarcula sp.* strain BS2 (0.5/0.1 mM) whereas for ZnO NPs resistance was BK6 (2.0/1.0 mM) >BBK2 (2.0/0.5 mM) >BS17 (0.5/ 0.5 mM) >BS2 (0.1/0.1 mM). The pigment was unaltered during growth in the presence of both bulk Zn or ZnO NPs. Among the four genera, *Haloferax* showed a higher Zn accumulation as seen by AAS analysis. The amount (percent) of Zn sorbed on the surface of cells of *Haloferax* strain BBK2 grown in the presence of $ZnCl_2$ was greater (21.77 %) than cells grown in presence of ZnO NPs (14.89 %). XRD studies showed the accumulation of Zn in crystalline form in the cells grown with $ZnCl_2$.

Haloarcula sp. strain E2 was investigated for Pt resistance and accumulation. On solid EHM medium, E2 was able to tolerate 1mM Pt but in liquid EHM broth, the MIC was 0.5mM Pt. AAS analysis and SEM EDX proved that Pt was accumulated intracellularly (20.84% of wet weight of cells). Organic matter (especially protein) interaction with platinum could be inferred from FTIR spectrum. TEM analysis showed irregular shaped and spherical nanoparticles of the size ranging from 5-30 nm. This is the first study showing platinum resistance and accumulation in haloarchaeal cells.

High metal resistance of various haloarchaeal strains may be useful in bioremediation of various heavy metals such as cadmium and zinc. Green synthesis of platinum nanoparticles by halophilic archaea was studied which could have potential applications in electrocatalysts, magnetic nanopowders, polymer membranes, coatings, nanofibers, etc.

Summary of Results and Conclusions

The family *Halobacteriaceae* is rapidly expanding with the discovery of more and more halophilic archaeal genera and species. Halophilic archaea are known for their robustness and their ability to survive multiple stress conditions such as UV exposure, high temperature, high metal content, variations in pH, etc. in their environment. Hence, halophilic archaea and their metabolites are demanding the attention of biotechnologists across the world especially in the last two decades. This study was aimed at isolating halophilic archaea from the solar salterns of India and studying them for various biotechnological prospects.

Extremely halophilic isolates obtained from the various solar salterns of Goa and Tamil Nadu, lining the east and west coast of India were cultured in several nutrient rich halophilic media. Visually different cultures were purified using repeated streaking and were characterized using various morphological, biochemical and molecular methods and their identity was established using sequence comparison. The halophilic isolates belonged to 6 different genera of extremely halophilic archaea (*Haloarcula, Halococcus, Halorubrum, Halogeometricum, Haloterrigena, Haloferax*) and one extremely halophilic bacterium belonging to the genus *Halomonas*. M1, BBK2 and E5 belonged to *Haloferax* sp., M2, BS17 and M5 were seen to be closely related to *Halorubrum* sp., M3, BK6 and E4 were *Halococcus* sp., E1, BS2 and E2 were related to *Haloarcula* sp. whereas E3 belonged to *Halogeometricum* sp. and J1 belonged to *Haloterrigena* sp.

Halophilic archaea isolated from the solar salterns of Goa and Tamil Nadu, India were screened for the production of various hydrolytic enzymes and 7 amylase producers, 3 protease producers, 1 lipase producer, 6 esterase producers and 5 pectinase producers were obtained in the study. Cellulase producers were not seen in the screened halophilic archaeal isolates.

Protease was produced by *Halococcus* sp. strain E4 in Norberg-Hofstein (NH) medium supplemented with 1% skimmed milk (SM). The optimum pH and temperature for production of protease was pH 7.0-8.0 and 37 ^{0}C respectively. E4 protease was concentrated and partially purified using ultrafiltration and gel chromatography and a yield of 32% and a purification fold of 6.39 was obtained. The molecular weight of the protein fractions was found by native PAGE to be around 67 kDa and 46 kDa. The protease was found to be metalloprotease and the optimum conditions were pH 8 and Temp 60^{0}C. Metal ions Ca^{2+}, Fe^{2+}, and Mg^{2+} contributed to an increase in activity. The enzyme was able to

tolerate 1% (v/v) non-ionic detergents such as Triton X-100, Tween 80 and was active in organic solvents such as methanol, ethanol and acetone. This is the first report on production and characterization of a protease from *Halococcus* sp.

Amylase from *Halogeometricum* sp. E3 was concentrated 1.64 folds by ultrafiltration using a 10 kDa membrane. Optimum amylase production was observed in NH medium supplemented with 1% starch in the stationary phase. The initial pH of the medium was 7-8 and temperature 37^0C. Though purification was attempted using triple phase partitioning and gel filtration chromatography, presence of starch interfered with the purification techniques used. It was active from pH 5.0 to pH 10.0 and optimum pH was at 8-10. The amylase from *Halogeometricum* sp. E3 was active from 25^0C to 80^0C while its temperature optimum was at 60^0C. It was active in the presence of 1% non-ionic detergents Triton X-100 and Tween 80 and organic solvents such as ethanol, methanol, acetone and butanol. Metals Ca^{2+}, Mg^{2+}, Zn^{2+} increased the activity whereas metals Mn^{2+}, Ba^{2+}, Co^{2+} decreased the activity of the enzyme. The polyextremophilic amylase was found to be α amylase and this is the first report of an amylase from *Halogeometricum* sp.

Pigments from orange coloured, extremely halophilic archaea, *Haloarcula* sp. (strain BS2 and strain E2) as well as *Halorubrum* sp. (strain M5) were characterized using various techniques such as UV Visible spectroscopy, Thin layer chromatography, Raman spectroscopy, FTIR and LCMS. The main component was found to be bacterioruberin. Other derivatives of bacterioruberin such as mono-anhydrobacterioruberin, bis-anhydrobacterioruberin, and 2-isopentenyl-3,4-dehydrorhodopin were also detected in the study. Minor carotenoids of lycopene and β carotene was identified in both *Haloarcula* and *Halorubrum* pigments.

Photoprotective activity of the haloarchaeal pigment was evaluated by challenging keratinocyte cell line (HaCaT) and cervical carcinoma cell line (HeLa) with UV after exposure to the pigment. It was seen that in the conditions studied, the pigment extract did not offer any photoprotection. However, it was noted that, the pigment extracts from both *Haloarcula* sp. (HA) and *Halorubrum* sp. (HR) exhibited a protective antioxidant effect, comparable to that of vitamin E, against other oxidative stresses such as H_2O_2 and arachidonic acid. Hence, the study concluded that the halophilic archaea isolated from the solar salterns of India has a potential to open new promising opportunities for the development of bioactive agents.

Haloarchaeal strains belonging to four different genera (viz. *Haloferax* sp., *Halorubrum* sp., *Haloarcula* sp., and *Halococcus* sp.) were tested for resistance/ tolerance against metals/ metalloids (viz. Cd, Cu, Pt, Zn, Se and Te).

The growth of the extremely halophilic archaeon *Haloferax* strain BBK2 in presence of Cd was studied in detail. BBK2 cells can grow in the presence of Cd in both complex and minimal media with NaCl concentration varying from 15% to 25%. The cells showed a maximal accumulation of Cd of 21.08% in the presence of 0.5 mM Cd and 15.19% in presence of 1 mM Cd. Interaction between polysaccharides, proteins, lipids and Cd was observed by FTIR spectroscopy. The SEM EDX and XRD analysis proved that the Cd was accumulated by intracellular cell components probably as CdS nanoparticles.

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Haloarcula sp. strain E2 tolerated and grew in 0.5mM/1mM platinum (Pt) in EHM broth/ agar. AAS analysis and SEM EDX proved that Pt was accumulated intracellularly upto 20.84% of wet weight of cell. Organic matter, especially protein interaction (amine group), with platinum could be inferred from FTIR spectrum. TEM analysis showed irregular shaped and spherical nanoparticles of the size ranging from 5-20 nm. This is the first study showing Pt resistance and accumulation in haloarchaeal cells.

This study concluded that halophilic archaea from Indian solar salterns can serve as a source for production of various polyextremophilic hydrolytic enzymes and potent antioxidants. High metal resistance and metal accumulation in the form of nanoparticles

were observed in several strains of halophilic archaea making them potential candidates for bioremediation and green synthesis of nanoparticles.

FUTURE SCOPE OF WORK

1. In this study, enzyme production from various halophilic archaea was studied. Optimization of production conditions was carried out for protease and amylase, obtained from novel sources *Halococcus* sp. and *Halogeometricum* sp. respectively. Protease from *Halococcus* sp. E4 was partially purified and characterized. The properties of amylase from *Halogeometricum* sp. E3 was also evaluated. These enzymes need to be produced in larger quantities and their application in various industries needs to be explored.

2. Pigments from halophilic archaea *Halorubrum* sp. M5 and *Haloarcula* sp. strains E2 and BS2 were characterized. The photoprotective activity should be analysed by different ways of administration of these pigments. Specialized formulations, micellar form, microemulsions, water-dispersible beadlets, enriched bovine serum, artificial liposomes, or nanoparticles should be explored in order to stabilize the pigments which may enable it to show improved photoprotective activity.

3. Halophilic pigments were found to provide effective protection against specific oxidative stresses such as arachidonic acid and hydrogen peroxide. A selective protection was offered for HaCaT cells in comparison to HeLa cells. Though it is known that carotenoid protection differs with the cell line used, bacterioruberin had not been explored in this aspect. In future, the mechanism of selection needs to be elucidated. Also, the use of bacterioruberin as an antioxidant must be evaluated *in-vivo.*

4. Metal accumulation was observed in many halophilic archaea. In the future, mechanism of metal tolerance needs to be studied. Platinum was accumulated in nanoparticle form. The characterization of these nanoparticles and their applications need to be studied further.

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<u>Appendix I</u> Gram staining protocol

1. The halophilic cells, suspended in a drop of sterile 15% (w/v) NaCl solution, was used to make a thin smear on a clean glass slide and allowed to air dry.
2. The smear was fixed and desalted using 2% acetic acid (v/v). The smear is allowed to air dry.
3. The dried smear is stained with Crystal violet (primary stain) for 1'.

   <u>Primary stain (Crystal violet): 100 ml</u>

Crystal Violet	1 g
Ethanol	10ml
Distilled water	90 ml

4. Rinse with distilled water
5. Smear was covered with Gram's Iodine (Mordant) for 30". Rinsed with distilled water.

   <u>Gram's Iodine (Mordant): 100ml</u>

Iodine	1 g
Potassium Iodide	2 g
Distilled water	100 ml

6. 70% (v/v) ethanol was used for decolourization (30"). Rinse with distilled water.
7. Smear was counterstained with safranin for 1'. Rinsed with distilled water.

   <u>Safranin (Counter stain): 100ml</u>

Safranin	0.5 g
Distilled water	100 ml

8. Slide was dried and examined under oil immersion objective (100 X) of phase contrast microscope (Olympus BX41, Japan)

<u>Appendix II</u> Sample preparation for SEM microscopic analysis

1. 50-100 µl culture was suspended in 15% sterile NaCl was taken on a clean cover slip
2. Air dried / oven dried (at 60 ^{0}C for 15 min) till sample is completely dry
3. Desalted with 2% acetic acid till all salt crystals are removed.
4. Washed with distilled water (3 times).
5. Fixed with 2% glutaraldehyde (overnight fixation)
6. Washed thoroughly with distilled water (on a rocker) for 10 min (3 times).
7. Post fixe with OSO_4 for 1 hour at RT or overnight at 4 ^{0}C
8. Note: OSO4 is light sensitive and toxic. To be handled in the fume hood with protective goggles and gloves
9. Washed these cells thoroughly with distilled water (on a rocker) for 10 min (3 times)
10. Exposed to a series of acetone concentrations (10%, 30%, 50%, 70%, 90%) in glass petriplates
11. Exposed to 100% acetone for 10 min
12. Started Critical point drying (CPD).

Appendix III Reagents for Genomic DNA Extraction and PCR Amplification

SET (Sucrose EDTA Tris) buffer

Sodium chloride	75.0 mM
EDTA	25.0 mM
Tris	20.0 mM
pH 7.5	

TE Buffer

Tris (pH 7.3)	10.0 mM
Sodium-EDTA	1.0 mM

Phenol-chloroform-isoamyl alcohol (PCI)

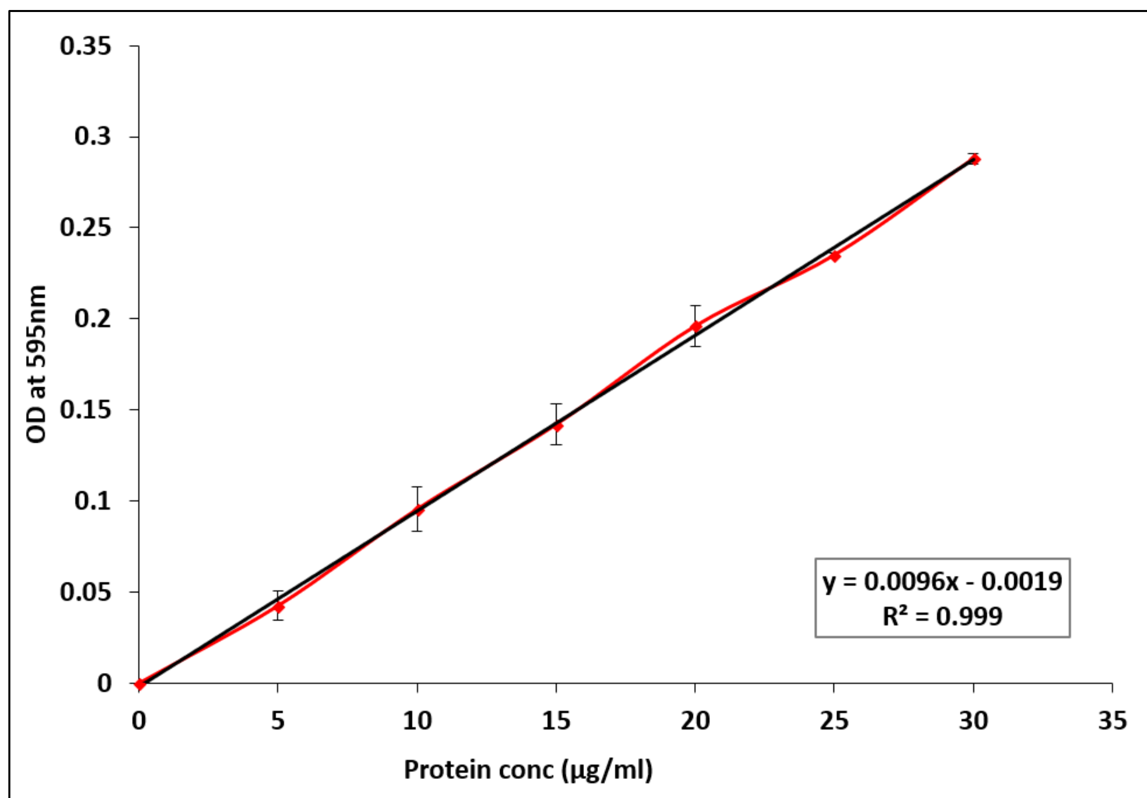
Phenol (pH 8.0)	25.0 ml
Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

Chloroform-isoamyl alcohol (CI)

Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

Appendix IV Protein quantification by Bradford assay

Eight hundred microliters of different concentrations of Bovine Serum Albumin (BSA) (5, 10, 15, 20, 25 and 30µg/mL) was added to 200µL 5X Bradford reagent. The mixture was incubated at room temperature for 5 min. OD was measured at 595nm (Bradford, 1976).



Calibration curve of Bovine serum albumin (BSA) for quantification of proteins

<u>Appendix V</u> Quantification of reducing sugar by Dinitro salicylic acid (DNS) method

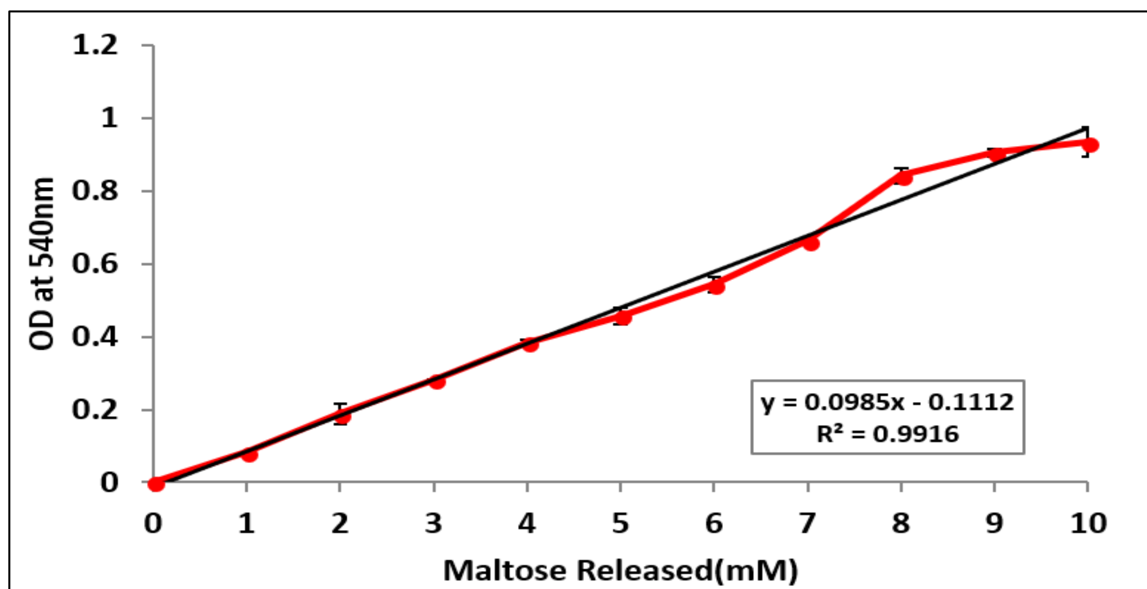
<u>DNS Reagent</u>

DNS	1% (w/v)
Sodium Potassium Tartatate	30% (w/v)
NaOH	0.4 M

For 100 ml DNS reagent, DNS is dissolved in 50mL Distilled water. Sodium Potassium Tartarate is added in small amounts to obtain a homogeneous milky yellow solution. Addition of NaOH gives a clear solution of DNS reagent. It is stored at room temperature in amber coloured bottle.

<u>Procedure:</u>

1ml of standard solution with concentrations varying from 0mM-10mM maltose was added to equal volume of DNS reagent (1mL) and boiled for 10 min. Once the samples cooled to room temperature, OD was taken at 540 nm. Dilutions were performed wherever required (Miller, 1959; Moshfegh et al., 2013).



Calibration curve of maltose for the estimation of amylase activity by DNS method (Miller, 1959)

Appendix VI SDS (Denaturing) PAGE and Non-Denaturing PAGE Protocol

The acrylamide percentage in SDS PAGE gel depends on the size of the target protein in the sample.

Acrylamide %	M.W. Range
7%	50 kDa - 500 kDa
10%	20 kDa - 300 kDa
12%	10 kDa - 200 kDa
15%	3 kDa - 100 kDa

5 ml stacking gel:

H_2O	2.970 ml
0.5 M Tris-HCl, pH 6.8	1.250 ml
10% (w/v) SDS	0.050 ml
Acrylamide/Bis-acrylamide (30%- 29.2/0.8 (w/v))	0.670 ml
10% (w/v) ammonium persulfate (APS)	0.050 ml
TEMED	0.005 ml

10ml separating gel:

Acrylamide percentage	**6%**	**8%**	**10%**	**12%**	**15%**
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris (pH 8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v) SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) APS	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

Note: APS and TEMED must be added right before each use.

5X Sample buffer (loading buffer):

SDS	10 % (w/v)
Dithiothreitol/ beta-mercapto ethanol	10 mM
Glycerol	20 % (v/v)
Tris-HCl, pH 6.8	0.2 M
Bromophenol blue	0.05 % (w/v)

1x Running Buffer:

Tris-HCl	25 mM
Glycine	200 mM
SDS	0.1% (w/v)

SDS (Denaturing) PAGE Protocol

- **Separating gel**: Set up the SDS PAGE unit. Prepare the gel solution (as described above) in a separate small beaker. Swirl the solution gently but thoroughly. Pipet appropriate amount of separating gel solution into the gap between the glass plates. To make the top of the separating gel be horizontal, fill in water (either isopropanol) into the gap until overflow. Wait for 20-30min to let it polymerize.
- **Stacking gel**: Discard the water. Pipet in stacking gel until overflow. Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it polymerize. Take out the comb. Pour the running buffer (electrophoresis buffer). Prepare the samples: Mix the samples with sample buffer (loading buffer). Heat them in boiling water for 5-10 min. Load the prepared samples into wells. Load protein marker into the first lane. Then cover the top and connect the anodes. Set an appropriate volt and run the electrophoresis. Once the dye front reaches the bottom, stop the run. Remove the gel and stain it afterwards for visualization of protein bands.

Non-Denaturing PAGE Protocol

The protocol followed is the same except that the sample buffer does not contain β mercapto-ethanol/ dithiothreitol and the sample is not boiled. Preferably, non-denaturing PAGE was carried out without the stacking gel.

<u>Appendix VII</u> Zymogram

Zymogram can be performed in three ways.

- Co polymerization with substrate
- Incubation of gel in the substrate solution
- Gel overlay assay

<u>Co polymerization with substrate</u>: During polymerization of separating gel, gelatin (for protease) or starch (for amylase) was incorporated. Once the gel run was over, the gel was incubated in 50mM Tris buffer pH 8 containing 4M NaCl and 10 mM $CaCl_2$ at 37^0C for 12hrs/ 24hrs. Gels for protease detection was stained with Coomassie Brilliant Blue and Gels for amylase detection were immersed in Iodine solution. Clearance, in each case, indicated the presence of the respective enzymes.

<u>Incubation of gel in the substrate solution</u>: The gel, after completion of the electrophoresis run, was immersed in a series of solutions in the following order.

1. Distilled water containing 2.5% (v/v) Triton X-100 for 10 min (2 times)
2. 50mM Tris buffer (pH 8) containing 4M NaCl, 10 mM $CaCl_2$ and 2.5% (v/v) Triton X-100 for 10 min (2 times)
3. for 10 min (3 times)
4. 50mM Tris buffer (pH 8) containing 4M NaCl, 10 mM $CaCl_2$ and 0.25-1%(w/v) substrate for 6-24 hrs.

The gel is washed in distilled water for 1 min and stained as mentioned earlier (in the previous section).

<u>Gel overlay assay</u>: 1% (w/v) agar and is added to 50mM Tris buffer (pH 8) containing 4M NaCl, 10 mM $CaCl_2$ and 1% (w/v) substrate. This is heated at 60^0C and then poured onto plates. The PAGE gel, after electrophoresis run, is overlaid on the substrate plate and incubated at $37\text{-}40^0C$ for 12-24 hrs. The position of gel is marked and gel is removed and the plate is stained as mentioned earlier.

LIST OF PUBLICATIONS

Publications:

- **Deepthi Das**, Bhakti B. Salgaonkar, Kabilan Mani, and Judith M. Braganca. "Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2." *Chemosphere* 112 (2014): 385-392.

- Salgaonkar, Bhakti B., **Deepthi Das**, and Judith Maria Bragança. "Resistance of extremely halophilic archaea to zinc and zinc oxide nanoparticles." *Applied Nanoscience* 6, no. 2 (2016): 251-258.

- Mani, Kabilan, Bhakti B. Salgaonkar, **Deepthi Das**, and Judith M. Bragança. "Community solar salt production in Goa, India." *Aquatic biosystems* 8, no. 1 (2012): 30.

Oral Presentation:

- **Deepthi Das** and Judith M. Braganca (2015) Screening and characterization of halophilic archaea from Indian salt pans for the production of hydrolytic enzymes. State level seminar on 'Archaea: Microbes of the third Domain of Life' at PES's RSN College of Arts and Science, Goa, India on September 28, 2015.

Poster Presentation:

- **Deepthi Das**, B.B. Salgaonkar, Kabilan M. and Judith M. Braganca (2013) Resistance of extremely halophilic archaea to metals. International Conference "Halophiles 2013' at University of Connecticut, Storrs, USA in June 23- 27, 2013.

- Isha Kalra, **Deepthi Das** and Judith M. Braganca (2013) Isolation and characterization of hydrolytic enzyme producing *Halogeometricum* sp. E3. International Conference "Halophiles 2013' at University of Connecticut, Storrs, USA in June 23-27, 2013.

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Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2

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HIGHLIGHTS

- *Haloferax* strain BBK2 grew in media supplemented with up to 4 mM Cadmium.
- *Haloferax* grew at a pH range of 6–10 and salinity range of 10–25% NaCl in 1 mM Cd.
- The cells showed a maximal accumulation of 15.19% in presence of 1 mM Cd.

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ABSTRACT

Halophilic archaea are prevalent in highly saline habitats. *Haloferax* strain BBK2 is an orange pigmented, exopolysaccharide (EPS) producing extremely halophilic archaeon, isolated from solar salterns of Ribandar, Goa, India. It grew in varying pH (5–10) and NaCl concentration (10–30%). The isolate grew well in complex (NTYE) and minimal media (NGSM) in presence of heavy metal cadmium (Cd) up to 4.0 mM (805.28 mg L^{-1}) concentration. The optimum growth in the presence and absence of Cd was seen at a pH range of 7–9 and salinity of 15–25%. The growth kinetics of the isolate in NTYE showed a specific growth rate (μmax) of 0.352 with generation time of 1.968 days. In presence of 1 mM Cd, the μmax was 0.325 day^{-1} and generation time was 2.132 days. In NGSM, the μmax decreased from 0.517 day^{-1} (in control) to 0.265 day^{-1} in 1 mM Cd while, the doubling time increased from 1.34 days in control to 2.615 days in presence of 1 mM Cd. SDS PAGE of the whole cell protein extracts showed overexpressed proteins of 74.14 and 40 kDa. The scanning electron microscopy, energy dispersive X-ray spectroscopy (SEM–EDX) analysis of the intact cells and cells disrupted by dialysis revealed that Cd was bound onto the cells, which was further confirmed by AAS, FTIR and XRD analysis.

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1. Introduction

Metals are ubiquitous in the environment due to natural processes like weathering and/or erosion. However, most of the toxic metals are released into the water bodies as a result of anthropogenic activities such as sewage and industrial disposals, agricultural waste and mining. There have been world-wide reports on metal (Cd, Cr, Cu, Mn, Pb, Zn, Ni and Fe) contamination of soil (sediments) and water bodies like rivers, lakes, estuaries, etc. due to industrial processes (Jain et al., 2007; Amin et al., 2009; Kaushik et al., 2009; Rath et al., 2009; Uluturhan, 2010).

Cadmium is released in the environment as a byproduct of the zinc and lead mining industries. It is also used as pigment in paints, in electroplating and galvanizing, in batteries, etc. It is a non-essential element, highly toxic, highly water soluble heavy metal, which can accumulate in living tissues and hence can easily enter the food chain resulting in bioaccumulation (Gonzalez et al., 2006; Garcia et al., 2008; Khan et al., 2008; Yang et al., 2009). Because of its toxic, persistent and xenobiotic nature it is a potential threat to both environment and human health (Raju et al., 2011). Although most of the microorganisms are susceptible to Cd, a number of bacteria, fungi and mosses are capable of tolerating Cd (Green-Ruiz et al., 2008; Sari et al., 2008; Xiao et al., 2010; Saluja et al., 2011; Rehman and Anjum, 2011; Zhou et al., 2013).

Halophilic (salt loving) archaea belong to the family *Halobacteriaceae* and are the predominant microorganisms of the extreme hypersaline environments like the Great Salt Lake, Dead Sea, crystallizer ponds of solar salterns, etc. These halophilic organisms

Abbreviations: EPS, Extracellular polysaccharide; SEM, scanning electron microscopy; EDX, energy dispersive X-ray spectroscopy; XRD, X ray diffraction; NTYE, NaCl Tryptone Yeast Extract; NGSM, NaCl Glucose Synthetic Medium.

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ORIGINAL ARTICLE

Resistance of extremely halophilic archaea to zinc and zinc oxide nanoparticles

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Abstract Industrialization as well as other anthropogenic activities have resulted in addition of high loads of metal and/or metal nanoparticles to the environment. In this study, the effect of one of the widely used heavy metal, zinc (Zn) and zinc oxide nanoparticles (ZnO NPs) on extremely halophilic archaea was evaluated. One representative member from four genera namely *Halococcus, Haloferax, Halorubrum* and *Haloarcula* of the family *Halobacteriaceae* was taken as the model organism. All the haloarchaeal genera investigated were resistant to both $ZnCl_2$ and ZnO NPs at varying concentrations. *Halococcus* strain BK6 and *Haloferax* strain BBK2 showed the highest resistance in complex/minimal medium of up to 2.0/1.0 mM $ZnCl_2$ and 2.0/1.0–0.5 mM ZnO NP. Accumulation of $ZnCl_2$/ZnO NPs was seen as *Haloferax* strain BBK2 (287.2/549.6 mg g^{-1}) > *Halococcus* strain BK6 (165.9/388.5 mg g^{-1}) > *Haloarcula* strain BS2 (93.2/28.5 mg g^{-1}) > *Halorubrum* strain BS17 (29.9/16.2 mg g^{-1}). Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM–EDX) analysis revealed that bulk $ZnCl_2$ was sorbed at a higher concentration (21.77 %) on the cell surface of *Haloferax* strain BBK2 as compared to the ZnO NPs (14.89 %).

Keywords Halophilic · Archaea · *Halococcus* strain BK6 · *Haloferax* strain BBK2 · *Halorubrum* strain BS17 · *Haloarcula* strain BS2 · $ZnCl_2$ · ZnO nanoparticles · Metal tolerance · Growth kinetics

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Introduction

Rapid industrialization and other anthropogenic activities have resulted in drastic environmental pollution. Natural processes like surface runoffs, weathering and/or erosion and anthropogenic activities like mining, industrial effluents, agricultural runoffs and sewage have led to the accumulation of toxic metals and their derivatives like nanoparticles in the environment (Paula et al. 2013; Zhao et al. 2012). Zinc and zinc oxide nanoparticles (ZnO NPs) are of high concern because of their increasing demand in electroplating, galvanization, cosmetics, sunscreens, paints, food industry, anticancer drugs, antimicrobials, ceramics, and semiconductors (Monteiro et al. 2011; Li et al. 2011).

ZnO NPs have been extensively studied for their antifungal (*Candida albicans*), antibacterial (*Escherichia coli*) and antiviral (bacteriophages MS2) effect (Lipovsky et al. 2011; You et al. 2011). There are numerous reports on resistance and/or tolerance of metals by bacterial strains either individually or in consortium (non halophilic) (Gadd 2009). Various mechanisms of Zn resistance such as physical bioadsorption/sorption, ion exchange, bioprecipitation and intracellular accumulation in microorganisms such as bacteria (Gram-positive and Gram-negative), cyanobacteria (*Microcystis aeruginosa*) archaea (*Halobacterium saccharovorum*) and eukarya (diatoms) have been revealed (Gadd 2009; Green-Ruiz et al. 2008; Zeng and Wang 2009; Guine et al. 2006; Mangold et al. 2013; Williams et al. 2013; Gelabert et al. 2006).

Li et al. (2011) studied the antibacterial activity of ZnO NPs on bacterial cells and found that Gram-negative bacteria (*Pseudomonas putida* and *E. coli*) are more resistant than Gram-positive bacteria (*Bacillus subtilis*). On the other hand, Sinha et al. (2011) investigated the toxic effect of silver and zinc oxide nanoparticle on mesophilic and

AQUATIC BIOSYSTEMS

REVIEW Open Access

Community solar salt production in Goa, India

Kabilan Mani, Bhakti B Salgaonkar, Deepthi Das and Judith M Bragança*

Abstract

Traditional salt farming in Goa, India has been practised for the past 1,500 years by a few communities. Goa's riverine estuaries, easy access to sea water and favourable climatic conditions makes salt production attractive during summer. Salt produced through this natural evaporation process also played an important role in the economy of Goa even during the Portuguese rule as salt was the chief export commodity. In the past there were 36 villages involved in salt production, which is now reduced to 9. Low income, lack of skilled labour, competition from industrially produced salt, losses incurred on the yearly damage of embankments are the major reasons responsible for the reduction in the number of salt pans.

Salt pans (*Mithagar or Mithache agor*) form a part of the reclaimed waterlogged *khazan* lands, which are also utilised for aquaculture, pisciculture and agriculture. Salt pans in Goa experience three phases namely, the ceased phase during monsoon period of June to October, preparatory phase from December to January, and salt harvesting phase, from February to June. After the monsoons, the salt pans are prepared manually for salt production. During high tide, an influx of sea water occurs, which enters the reservoir pans through sluice gates. The sea water after 1–2 days on attaining a salinity of approximately 5ºBé, is released into the evaporator pans and kept till it attains a salinity of 23 - 25ºBé. The brine is then released to crystallizer pans, where the salt crystallises out 25 - 27ºBé and is then harvested.

Salt pans form a unique ecosystem where succession of different organisms with varying environmental conditions occurs. Organisms ranging from bacteria, archaea to fungi, algae, etc., are known to colonise salt pans and may influence the quality of salt produced.

The aim of this review is to describe salt farming in Goa's history, importance of salt production as a community activity, traditional method of salt production and the biota associated with salt pans.

Keywords: Salt pan, Goa, Estuary, Community, *Khazan*, Tidal influx, India, Salt production

Background

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 [1]. Solar salt production in Goa had been an important activity through its history.

Goa experiences a tropical monsoon climate with hot summers followed by long monsoons from June to October. Goa has 9 rivers, most of them forming estuaries, the major being river Mandovi and Zuari. These rivers experience high tidal influx during summers and therefore the salinity varies during monsoon (2–3ºBé) and non-monsoon times (4–5ºBé) [2]. Various factors such as favourable climatic conditions and easy accessibility to sea water have aided salt production through natural evaporation in Goa.

Today, solar salt production has become a declining industry due to low income generated, competition from industrially produced iodized salt, yearly damage and repairs of the embankments and pollution. Currently there are 9 villages producing salt each having a few operational salt pans.

Historical background

Solar salt production in Goa, described as a traditional village industry, has been practiced for the past 1,500 years by various communities [3,4]. Since most of the rivers form estuaries and experience tidal influxes, salt production was started mainly in the coastal villages. Salt served as an important trade commodity too, playing an important role in the economy of Goa. The salt

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No. of publications: Three

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. Prof. 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. She has mentored three Ph.D students besides numerous thesis, dissertation and project students.

Besides teaching, Prof. Bragança has been associated with BITS Pilani administration assuming multifarious responsibilities, mainly as Head of Department - Department of Biological Sciences, 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.