Ph.D. Thesis

P. KARTHIKEYAN

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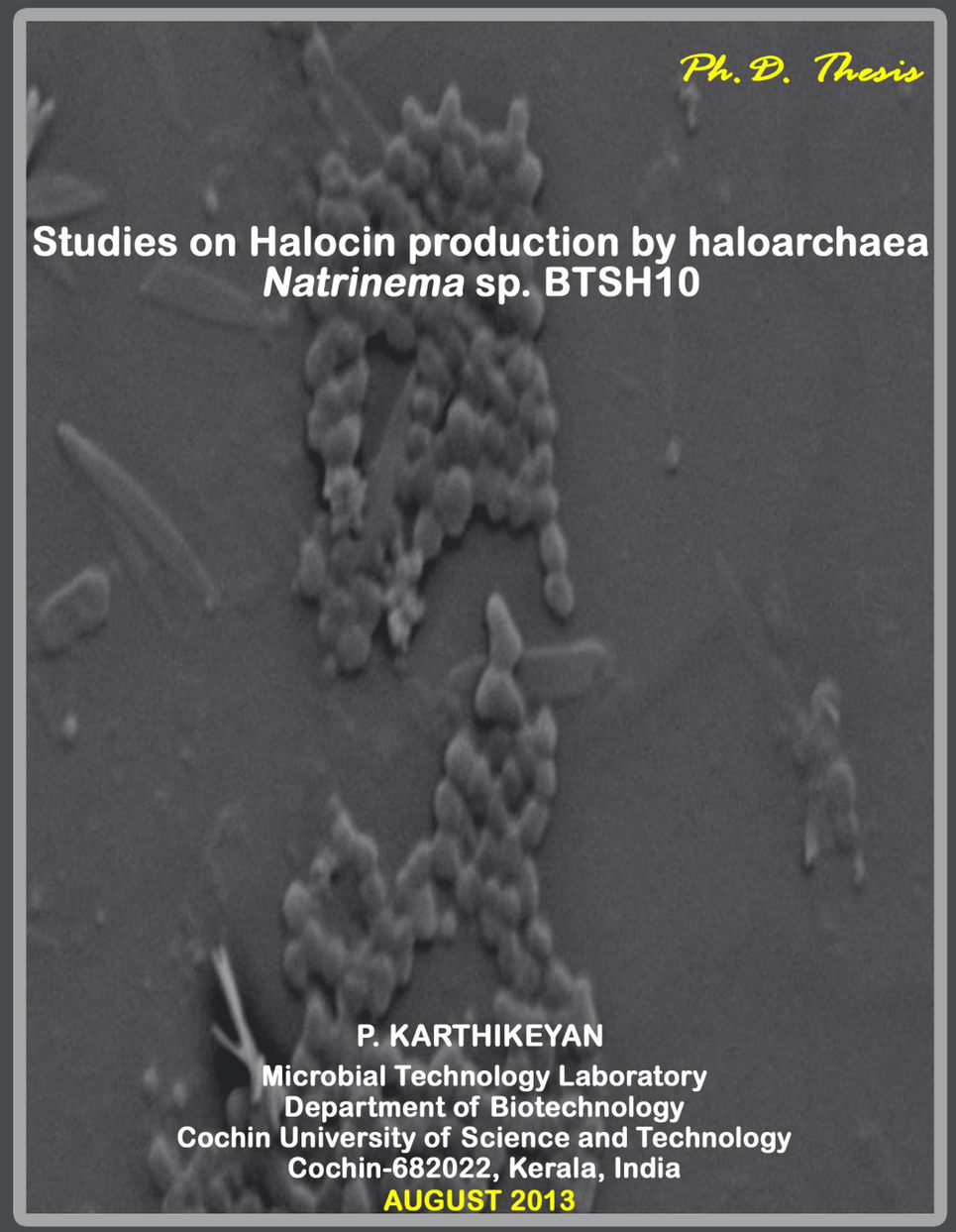

# Studies on Halocin production by haloarchaea *Natrinema* sp. BTSH10

*Thesis submitted to the*
*Cochin University of Science and Technology*
*under the Faculty of Science*
*in partial fulfillment of the requirements*
*for the degree of*

**DOCTOR OF PHILOSOPHY**

**IN**

**BIOTECHNOLOGY**

***By***


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# CERTIFICATE

This is to certify that the research work presented in this thesis entitled "**Studies on Halocin production by haloarchaea *Natrinema* sp. BTSH10**" is based on the original research work carried out by Mr. P.Karthikeyan under the guidance and supervision of Dr. M. Chandrasekaran and my Co-guidance at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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## DECLARATION

I hereby declare that the thesis entitled **"Studies on Halocin production by haloarchaea *Natrinema* sp. BTSH10 "** is based on the original research work carried out by me for my doctoral degree, under the supervision and guidance of Dr. M. Chandrasekaran, Professor, Department of Biotechnology, Cochin University of Science and Technology and co-guidance of Dr. Sarita G. Bhat, Associate Professor and Head, Department of Biotechnology, Cochin University of Science and Technology and the thesis or no part thereof has presented for the award of any degree, diploma, associateship or other similar titles or recognition.

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***P.Karthikeyan***

*Dedicated to*

*my beloved parents*

## ABBREVIATIONS

| | | |
|---|---|---|
| % | - | Percentage |
| °C | - | Degree Celcius |
| µg | - | microgram |
| µL | - | microliter |
| µM | - | micromole |
| $^{13}C$ | - | Radioactive Carbon |
| A | - | Adenine |
| ACN | - | Acetonitrile |
| AES | - | Atomic Emission Spectrometer |
| ANOVA | - | Analysis of Variance |
| APS | - | Ammonium per sulphate |
| ATP | - | Adenosine-5'-triphosphate |
| AU | - | Arbitrary units |
| BLAST | - | Basic Local Alignment Search Tool |
| BSS | - | Basal Salt Solution |
| bp | - | base pair |
| C | - | Cytosine |
| cfu | - | Colony forming units |
| DLA | - | Dalton’s Lymphoma Ascities |
| DMEM | - | Dulbecco's Modified Eagle's medium |
| DNA | - | Deoxyribonucleic acid |
| dNTP | - | Deoxyribonucleotide triphosphate |
| DTA | - | Differential Thermal Analysis |
| DW | - | Distilled water |
| EDTA | - | Ethylene diamine tetra acetic acid |

| | | |
|---|---|---|
| EtBr | - | Ethidium bromide |
| FAME | - | Fatty acid methyl esterase |
| FBS | - | Fetal bovine serum |
| Fig. | - | Figure |
| FT-IR | - | Fourier-Transform infra red |
| FU | - | Flurouracil |
| G | - | Guanine |
| GC | - | Gas chromatography |
| g | - | gram |
| h | - | hours |
| HBBS | - | Hanks balanced salt solution |
| *Hbt.* | - | *Halobacterium* |
| *Hfx.* | - | *Haloferax* |
| HPLC | - | High Performance Liquid Chromatography |
| kbp | - | Kilo base pair |
| kDa | - | Kilo Dalton |
| L | - | liter |
| MEM | - | Minimal essential medium |
| MS | - | Mass spectrometry |
| m | - | meter |
| M | - | Molar |
| MALDI | - | matrix-assisted laser desorption ionization |
| MAR | - | Multiple Antibiotic Resistance |
| min. | - | minutes |
| mL | - | milliliter |
| MLSA | - | Multilocus sequence analysis |
| mm | - | millimeter |

| | | |
|---|---|---|
| mM | - | millimolar |
| MTT | - | 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide |
| N | - | Normality |
| NaCl | - | Sodium chloride |
| NBCS | - | New born calf serum |
| ng | - | nanogram |
| NIR | - | Near Infra-Red |
| nm | - | nanometer |
| NMR | - | Nuclear Magnetic Resonance |
| No. | - | Number |
| O.D. | - | Optical density |
| PAGE | - | Poly acrylamide gel - electrophoresis |
| ppm | - | parts per million |
| PR | - | Phenol red |
| rDNA | - | Ribosomal Deoxyribonucleic acid |
| rpm | - | revolution per minute |
| RT | - | Room Temperature |
| s | - | seconds |
| SDS | - | Sodium dodecyl sulphate |
| S. No. | - | Serial number |
| sp. | - | species |
| T | - | Thymine |
| TAE | - | Tris-EDTA |
| Taq | - | *Thermus aquaticus* |
| TE | - | Tris-EDTA |
| TEMED | - | N-N-N'-N'-tetramethyl ethylene diamine |

| | | |
|---|---|---|
| TFA | - | Trifluroacetic acid |
| TLC | - | Thin Layer Chromatography |
| TG | - | Thermo gravimetric |
| TOF | - | Time-of-flight |
| UV | - | Ultraviolet |
| V | | Volts |
| v/v | - | volume/volume |
| w/v | - | weight/volume |

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# *Chapter 1*

## INTRODUCTION

*Archaea*, prokaryotic microorganisms, live in marine and terrestrial ecosystem. A characteristic feature of these *Archaea* is that they survive in extreme conditions of temperature, salt and pH. They are considered as a separate branch of ancient organisms evolved under conditions of high temperature, anaerobic atmosphere and high salinity. It is also believed that they play a major role in global energy cycles since they live in extreme conditions. Hence, it is important to study the molecular mechanism of these organisms with regard to their survival and metabolism at such extreme conditions.

India is the third largest salt producer in the world next to China and USA. Salt is the common name for the substance sodium chloride (NaCl), which occurs in the form of transparent cubic crystals. Salt, harvested through solar evaporation from seawater or salt lakes, is used for countless purposes. One of the uses is that it is used as a preservative in food industries to avoid microbial growth and contamination. The organisms which love to colonize in higher concentration of NaCl are known as halophiles.

Halophiles are known as salt lovers and these organisms live at a very high concentration of sodium chloride. They live in salt pans and hypersaline lakes such as the Great Salt Lake and Dead Sea where the NaCl concentration is above 4.5M (saturated). Their populations provide the red colouration to the salt pans. In order to maintain osmoregulation and to maintain stability and activity of intracellular and extracellular proteins, the halophiles accumulate K+ ions intracellularly.

Extensive studies on extremophiles which began only in 1970's have shown that these organisms are treasures, that provide a wide range of biotechnological products such as enzymes, bacteriorhodopsin, halorhodopsin, compatible solutes, biopolymers, biosurfactants, exopolysacchrides, polyhydroxy alkaloids, flavouring agents, antibacterial compounds, cosmetics, nutritional supplements, molecular probes, osmoprotectants, preservatives and fine chemicals.

Enzymes which are salt tolerant and having application in food processing and chemical industries have also been studied. Amylase, protease, lipase, gelatinase, cellulase, β-galactosidase, isomerase, nucleases etc., are some of the enzymes which were isolated and characterized from halophiles. These enzymes have diverse effect in fish and meat processing industry and in pickling industry where the waste water contains more concentration of NaCl (Kargi *et al.*, 2000). Apart from these enzymes, peptidyl prolyl *cis-trans* isomerase an enzyme used for production of recombinant proteins, protein stabilization, and in regeneration of denatured proteins, have been isolated and studied. The production of a novel cyclophilin type peptidyl prolyl *cis-trans* isomerase, using a gene amplified from the genome of *Halobacterium salinarum* has been patented (Iida *et al*., 1997). Chymotrypsinogen B-like protease, which is pH tolerant (active at pH 10), has been isolated from *Natronomonas pharainis*, and it functions well at higher temperature (61°C) (Stan-Lotter *et al*., 1999).

Bacteriorhodopsins are purple pigments synthesized by certain species of extreme halophiles in order to trap sunlight and proceed with photosynthesis. This shares the same functional and structural similarities to visual pigment of the eye rhodopsin and also known as retinal (chromophore). The chromophore is covalently bound to a protein moiety and functions as a light dependent transmembrane protein pump (Krebs and Khorona, 1993).

Bacteriorhodopsins have a broad range of applications (Table 1.1) based on the photochemical, photoelectric, and proton motive properties of this molecule. They can be used as an optoelectronic material for holographic image storage where the interference patterns are registered as purple or yellow areas. These films are nowadays explored to be used as bio-electronic element in computer memories and information processing units. This has led to the development of new generation computers. They also have a role in bio-photoelectrochemical reactor for photochemical hydrogen production. A chloride-sensitive biosensor using immobilized membrane vesicles containing halorhodopsin has been developed to find out the concentration of chloride (Seki *et al*., 1994).

C50 and C30 bacterioruberin and its derivatives are the main carotenoids of the halobacteriaceae having economic value (Margesin and Schinner, 2001). They give red or orange coloration to the salt pans. Halophilic ketocarotenoid canthaxanthin is commercially produced by companies. These pigments harvest light and support the light mediated ATP synthesis. It is also suggested that the red *Archaea* and the β-carotene rich strains of *Dunaliella*, and red halophilic bacteria of the genus *Salinibacter* raise the temperature of the salterns by trapping solar radiation which ultimately increases the rate of evaporation thereby increasing salt production.

Salted fishes and meat are an important economic resource in Northern countries such as Canada and Norway. The studies conducted on spoilage of these food products upon storage showed the presence of true red halophilic *Archaea*. These halophiles developed red microbial mats on hides commonly known as red heat or pink heat. This is due to the growth of the *Archaea* such as *Halobacterium*

or *Halococcus* and the halophile populations in these products are due to the usage of raw salt for storage.

**Table 1.1 Potential biotechnological uses of bacteriorhodopsin (adopted from Hampp, 2000).**

| Category | Application |
|---|---|
| Proton Transport | ATP generation |
| | Desalination of seawater |
| | Conversion of sunlight to electricity |
| Photoelectrism | Ultrafast light detection |
| | Artificial retinas |
| | Motion detection |
| Photochromism-optical storage | 2-D storage |
| | 3-D storage |
| | Holographic storage |
| | Associative memories |
| Photochromism-optical | Optical bistability/light switching |
| processing | Optical filtering |
| | Signal conditioning |
| | Neural networks |
| | Spatial light modulators |
| | Phase conjugation |
| | Pattern recognition |
| | Interferometry |
| Miscellaneous | Second harmonic generation |
| | Radiation detection |
| | Biosensor application |

The property of spoilage of food products by halopiles upon storage has been taken as an advantage and it is used to prepare certain traditional fermented foods. “Nam pla” is a traditionally made fermented fish sauce in Thailand. Microbiological survey upon this food showed the presence of the red halophilic *Archaea*, *Halobacterium* and *Halococcus*. In Japan fermented salted puffer fish ovaries in rice bran known as “fugunoko nukazuke,” also known as “Nakazuke”, is produced by fermentation process at 10-30% salinity for one to two years. The Korean fermented seafood “jeotgal” is produced due to fermentation by *Halomonas alimentaria* (Yoon *et al*., 2002).

“*Micrococcus varians* subsp.halophilus” halophilic nuclease H is used to produce food flavouring agents such as 5’-guanylic acid (5’- GMP) and 5’-inosinic acid from RNA. This enzyme degrades RNA at 60°C and 120g $L^{-1}$ NaCl.

Halophilic *Archaea* produce more amount of lipids as they grow in medium containing higher concentration of sodium chloride. Investigation of these lipids has shown the presence of isoprenoid diether which could be used as food additive, to serve as emulsifier and/or as a low calorie fat substitute.

Halophilic *Archaea* play a major role in production of biological polyesters also known as the thermoplastics (biodegradable plastics). These thermoplastics are similar to polypropylene that has a high melting point. Halophiles have shown the presence of exopolysacchrides and poly-β-hydroxyalkanote, a copolymer of β-hydroxybutyrate and β-hydroxyvalerate. Exopolysacchrides are used as gelling agents, for viscosity stabilization as thickening agents, to modify rheological properties of aqueous system, microbially enhanced oil recovery and as emulsifiers. *Natrialba aegyptiaca* (aegyptia) produces poly (γ-D-glutamic acid) which has a major role as a drug

carrier in some food and pharmaceutical industries, humectants and biodegradable thickener.

The biodegradation potential of halophilic *Archaea* was studied and degradation potential of these organisms have been explained. These organisms have the capacity of degrading aromatic hydrocarbons such as anthracene, acenapthene, 9-methylanthracene and phenanthrene. Crude oil degrading *Haloferax* strain has been isolated which have the potential to grow on aromatic compounds such as benzoate, cinnanate and 3-phenylpyronate. *Haloarcheon* growing on saturated concentration of tetradecane, hexadecane, eicosane, heneicisane and pristine are reported (McMeekin *et al*., 1993). Halogenated hydrocarbons such as trichlorophenols or the insecticides lindane and DDT degrading haloarchaeal strains of the genera *Haloarcula*, *Halobacterium* and *Haloferax* have been subjected to patent selection process (Oesterhelt *et al*., 1998).

*Halomonas* sp. tolerant to mercury, cadmium, copper, chromium, and/or zinc besides having the capacity to biotransform and bioremediate selenium and uranium has been studied (D'Souza *et al*., 2001;Francis *et al*., 2000).

Ecotine and hydroxyecotine are tetrahydropyrimidines synthesized in *Haloarchaea* which has the ability to protect whole cells against salt stress by stabilizing enzymes (increase shelf life of commercially available enzyme) and DNA membranes. They also protect thermal denaturation, desiccation and freezing of cells. So these tetrahydropyrimidines can be used in storage of whole cells as they keep them intact from stress. They play major role in cosmetic industries where they are used in production of moisturizers for the care of aged, dry or irritated skin with respect to the immune system of the Langerhans cells, formation of heat shock proteins and protection of membrane integrity. They protect cells from sunburns due to exposure to UV radiation (Bunger *et al*., 2000).

Genes encoding synthesis of gas vesicles have been isolated from *Halobacterium salinarum* and cloned in a vector. The gas vesicles thus produced are used as an antigen epitope display and delivery system. Gas vesicles coupled to haptens such as trinitrophenol or small peptides when injected to mice showed excellent immunological response. Halophilic *Archaea* also prove suitable for the expression of certain human membrane proteins. The human $\beta_2$ – adenoreceptor, gene has been cloned and expressed in *Halobacterium salinarium* using an expression vector for bacterio-opsin modified to express the adenoreceptors under the control of bacterio-opsin regulatory element (Sohlemann *et al*., 1997). This has proved that halophilic *Archaea* are suitable for expression of certain human proteins. Production and purification are easy by bioprocess methods as these cells lyse when comes in contact to water.

In diagnosis of cancer an archaeal 84kDa protein from *Halobacterium salinarum* could be used as an antigen to detect antibodies against the human c-*myc* as they share common epitopes with human c-*myc* protein which is an oncogene product in the serum of certain cancer patients (Ben – Mahrez *et al*., 1988, 1991). The epipodophyllotoxin VP16, an eukaryotic DNA topoisomerase II inhibitor when treated with *Halobacterium* cells induced DNA strand breaks and DNA Protein covalent linkage. This suggested that halophilic *Archaea* can be used to prescreen antitumor drugs active on eukaryotic cells. The stimulatory effect of *Halococcus morrhuae* on human lymphocytes was judged by $^{3}H$ – thymidine uptake (Montes *et al*., 1999).

Halophilic *Archaea* are inhibited by several drugs which has the property to interact with eucaryote, tubulin, actomyosin and DNA topoisomerase II. So it is possible that those proteins which may be the target of such drugs are present in *Archaea* and hence they could be used as a source of drug testing. Archaeal ether

lipid liposomes ("archaeosomes") have been tested as delivery system for vaccine and drugs (Patel and Sprott, 1999).

The halocin produced by *Haloferax gibbonsii* (halocin H6/H7) functions as haloarchael $Na^+/H^+$ antiporter inhibitor. This was reported to decrease infract size and ectopic beats after myocardial reperfusion in dogs and decreased A – V intrinsic nodal conduction and heart rate in isolated rabbit hearts. This characteristic of the halocin protect the myocardium against ischemia and reperfusion injury and reduce injury during organ transplantation (Alberola *et al.*, 1988).

Halophilic *Archaea* are considered as an excellent source of biomolecules of commercial importance. However problems are associated with large scale production with respect to fermentation process development, derivation of new and novel proteins and metabolites, and their purification processes. To overcome these problems it is necessary to have intensive research in this field. Genome sequencing and molecular biology techniques have to be customized and new designs of experiments to be programmed and executed to explore this less explored halophilic *Archaea*. In this context in the present study an attempt was made to explore the prospects of deriving halocins that have wide range of applications.

## OBJECTIVES OF THE PRESENT STUDY

In spite of the fact that halocin production is widespread among the *Halobacteriaceae*, no information is available on their ecological significance. Whereas, halocins may play a role in the interspecies competition between different types of halobacteria in saltern crystallizer ponds inhabited by dense communities of these red halophiles. The haloarchaea bacteria are anticipated to synthesise novel and potential bioactive substances that may have pharmaceutical applications besides other applications. Further studies pertaining to microbiology of saltern ponds of South India which contribute to significant amount of commercial salt production has not been made adequately. In this context the present study was aimed to explore the prospects of harnessing halophilic bacteria for possible bioactive substances.

### 1.2 Specific objectives of the present study include:

1. Screening for halocin producing haloarchaea from salt pans of Kanyakumari, Tamilnadu, South India.
2. Characterization of halocin producing bacteria employing phenotypic and genotypic approaches.
3. Optimization of bioprocess variables for halocin production by selected strain of halophilic bacteria.
4. Purification and characterization of halocin produced by selected strain of halophilic bacteria.
5. Application studies of halocin produced by selected strain of halophilic bacteria.

# *Chapter 2*

## REVIEW OF LITERATURE

### 2.1 Halophiles

Archaebacteria (ancient bacteria) – prokaryotic organisms that lives in abnormal conditions which could be a normal environment for those organisms such as salt evaporation ponds, deep sea hydrothermal vents, such as "black smokers", hot springs, the Dead Sea, acid lakes, etc., which are considered to be extreme for life. These organisms shows similarity with eukaryotes but are entirely different from existing bacteria and this is proved by biochemical analysis and the nucleic acid sequencing studies. Archaebacteria include halophiles that inhabit saline environments.

Based on NaCl requirement for survival, the microbes inhabiting saline environment are divided into four groups (Larsen, 1962) given in Table 2.1

**Table 2.1 Larsen's four division of microbes based on NaCl requirement**

| | |
|---|---|
| non-halophiles | < 2% NaCl |
| slight halophiles | 2 – 5% NaCl |
| moderate | 5 – 20% NaCl |
| extreme halophiles | 20 – 30% NaCl |

Kushner (1993) expanded Larsen's (1962) definition, and proposed the five groups of halophiles (Table 2.2).

**Table 2.2 Kushner's five division of microbes based on NaCl requirement**

| | |
|---|---|
| non-halophilic | <0.2M (1%) NaCl |
| slight halophiles | 0.2 – 0.5M (1 – 3%)NaCl |
| moderate halophiles | 0.5 – 2.5M (3 – 15%)NaCl |
| borderline extreme halophiles | 1.5 – 4.0M (9 – 23%) NaCl |
| extreme halophiles | 2.5 – 5.2M (15 – 32%) NaCl |

Halotolerant microbes are another group which grow best in media containing less than 0.2 M (1%) salt and also can survive/tolerate high concentrations of salt (Arahal and Ventosa, 2002; Yoon *et al*., 2003). Halobacteria belongs to the domain Archaea and they require 4-5M NaCl for survival below which they could not survive and is characterized by cell lysis (Meral *et al.*, 2007).

Extreme environments such as salt marshes, hypersaline lakes, saline ponds, hot springs and salt deposits are distributed throughout the world. These environments are also characterized by their varying pH ranging between 6 to 11. Salt evaporation ponds/solar salterns are regions for salt extraction for commercial purpose. They are considered as natural resources for studying salt precipitation and related biogeochemical processes (Blatt *et al*., 1980). Halophilic microorganisms living in these environments are distributed among all three domains of life. (Benlloch *et al*., 2002; DasSarma and DasSarma, 2006). The dominant microbial population was found in the *Halobacteriaceae* families which are especially reported in halites that contain more than 32% (w/v) NaCl (Oren, 2002; Mani *et al.*, 2012). These hypersaline lakes are generally red in colour which is due to the presence of pigmented halophilic archaea (Yildiz *et al*., 2012) and the eukaryote *Dunaliella* (Mancinelli, 2005). Studies on halophilic archaea have become a hot area of research as more understanding is required about their

metabolism as the environment which they survive are extreme. (Bonete, 2008; Falb *et al*., 2008).

## 2.2 *Halobacteriaceae*

The species belonging to *Halobacteriaceae* family is classified based on polyphasic taxonomy (Oren *et al*., 1997). Evaluation of properties such as cell morphology, growth characteristics, chemotaxonomic traits and nucleic acid sequence analysis included main characteristics. Chemotaxonomic traits included the isolation, characterization, examination and identification of polar lipids, as many genera have a distinctive polar lipid signature (Kamakura, 1998, 1999; Torreblanca *et al.*, 1986).

### 2.2.1 Molecular taxonomy

In recent years molecular characterization of *Archaea* by the comparison of 16S rRNA gene sequences has contributed much to identification and classification of the species within the *Halobacteriaceae*. Molecular characterization has also led to creation of new genera, splitting of existing genera into a number of new genera and also identification of genera which were classified earlier as separate genera (Kamakura, 1998, 1999; Oren *et al*., 2001).

The studies conducted on 23S rDNA on taxonomy showed that the halobacterial genes have been diverged over a relatively short time interval (Lodwick *et al*., 1994). Briones and Amils (2000) reported that the topology of phylogenetic tree made from 23S rRNA sequences may be completely different from that of 16S rRNA sequences.

Multilocus sequence analysis (MLSA) is a method that uses more information regarding the phylogenetic information which could be obtained than the 16S rRNA gene sequence comparison studies (Oren & Ventosa, 2010; Arahal *et al*., 2011). Papke *et al*., (2011) pointed out that few housekeeping genes contribute to MLSA based on a study conducted with 33 species belonging to 14 genera (Table 2.3).

**Table 2.3 Genes that contribute to MLSA**

| Gene | Function |
|---|---|
| *atpB* | ATPase subunit |
| EF-2 | protein synthesis elongation factor |
| *radA* | DNA repair |
| *rpoB* | RNA polymerase subunit |
| *secY* | protein export through the membrane |

The comparison studies made with 16S rRNA, *atpB*, *bop*, EF-2 and *radA* as marker genes between *Halorubrum* strains isolated from Spanish saltern and a salt lake in Algeria explained the relationships between different isolates belonging to a single genus. *Halobacteriaceae* family was further split into two families based on the presence of two clusters of the *pyrD* gene (coding for dihydroorotate oxidase), the *lpdA* gene (dihydrolipoamide dehydrogenase) immediately upstream to 16S rRNA gene of the rRNA operon. It was said that based on single genotypic property, splitting of the family could not be done and it could be done only based on phenotypic properties.

### 2.2.2 Chemotaxonomy

The membrane lipids in *Archaea* are specific to some genus of extreme halophiles and so they play a major role in differentiation between genera. This

specificity of lipids has lead scientists to use core lipids, phospholipids and glycolipid structures as molecular markers in taxonomic classification (Grant and Larsen, 1988; Kamekura, 1999).

The difference in the cell wall composition is also used in taxonomic classification. The glycoprotein subunits of the family *Halobacteriaceae* (Guan *et al*., 2011), repeating units of a poly (L-glutamine) glycoconjugate the cell wall of *Natronococcus occultus* (Niemetz *et al*., 1997) and complex polysaccharide wall of the genus *Halococcus* (Schleifer *et al*., 1982) play a major role in taxonomic classification. In *Haloferax volcanii* the NaCl concentration has a direct impact on the S – layer glycoprotein N-glycosylation in the cell wall (Guan *et al*., 2011).

### 2.2.3 Lipid core structural diversity

The family *Halobacteriaceae* has been studied well for the structure and diversity of membrane core lipids (Gambacorta *et al*., 1994; Grant *et al*., 1998; McGenity *et al*., 1998). $C_{20}C_{20}$ lipid (sn–2,3–diphytanylglycerol) core was found specifically in *Halobacterium*, *Haloarcula*, *Haloferax*, *Halobaculum* and *Halorubrum* (Kamekura and Kates, 1999). $C_{20}C_{20}$ and $C_{20}$ $C_{25}$ (sn–2 sesterterpanyl–3–phytanyl–glycerol) were present in *Halococcus*, *Natronobacterium*, *Natronococcus*, *Natronomonas*, *Natrialba*, *Natrinema*, *Haloterrigena* and few more.

#### 2.2.3.1 Polar lipid structural diversity

##### 2.2.3.1.1 Phospholipids

*Halobacterium*, *Haloarcula*, *Haloferax*, *Halorubrum*, *Halobaculum*, *Halococcus*, *Natranobacterium*, *Natranococcus*, *Natranomonas*, *Natrialba*,

*Halogeometricum*, *Natrinema* and *Haloterrigena* contain Phosphatidylglycerol phosphate – methyl ester (PGP – Me), Phosphatidyl glycerol (PG) and Phosphatidic acid (PA) (Ross *et al*., 1985; Tindall, 1992; Kates, 1995). *Natranococcus occulutus* contains PGP – 1',2'–cyclic phosphate (PL2) (Lanzotti *et al*., 1989) as well as PG and PGP–Me . A monoisoprenyl analogue of PGP–Me with both $C_{20}C_{20}$ and $C_{25}C_{25}$ lipid cores have been identified in *Halococcus saccharolyticus* (Moldoveanu *et al*., 1990).

### 2.2.3.1.2 Glycolipids

Along with 16S rRNA gene molecular characterization the glycolipid composition has given a strong base to identify *Archaea* especially *Halobacteriaeae* family.

**Table 2.4 Glycolipid composition of few Halobacterial genera**

| S.No | Halobacterial genera | Glycolipid found |
|---|---|---|
| 1 | *Halobacterium* | S–TGD ,S–TeGD |
| 2 | *Haloarcula* | TGD–2 ,DGD–2 |
| 3 | *Haloferax* | S–DGD–1 |
| 4 | *Halococcus* | S–DGD–1 ,P–TGD (Moldoveanu *et al*.,1990) |
| 5 | *Halobaculum* | S–DGD–1 AND UNIDENTIFIED GLYCOLIPIDS |
| 6 | *Natronobacterium* | NO GLYCOLIPIDS (Tindall, 1992; Ross *et al*., 1985) |
| 7 | *Natranococcus* | NO GLYCOLIPIDS |
| 8 | *Natrialba* | $S_2$–DGD–1 (Matsubara *et al*., 1994) |

| | | |
|---|---|---|
| 9 | *Natranomonas* | NO GLYCOLIPIDS (Grant *et al*., 1985; Kamekura, 1997) |
| 10 | *Halogeometricum* | UNIDENTIFIED NON–SULFATE (Rodriguez *et al*., 1998) |
| 11 | *Haloterrigena* | $S_2$–DGD–1 AND UNIDENTIFIED GLYCOLIPIDS (Ventosa *et al*., 1999) |

**S-TGD:** 3-$HSO_3$-tri glycosyl-glycodiether; **S-TeGD:** 3-$HSO_3$-*Galp*-$\alpha$(1→2)-*Glcp*-$\alpha$(1→1)-*sn*-glyceroldiether; **TGD-2:** *Glcp*-$\beta$(1→6)- *Manp*-$\alpha$(1→2)-*Glcp*-$\alpha$(1→1)-*sn*-glyceroldiether; **DGD-2:** Man-Glc,glycerol-diether; **S-DGD-1:** 6-$HSO_3$-*Manp*-$\beta$(1→6)-[*Galf*-$\alpha$(1→3)]- *Manp*-$\beta$(1→2)-*Glcp*-$\alpha$(1→1)- *sn*-glyceroldiether; **P-TGD:** phospho-triglycosyl-glyceroldiether; **$S_2$-DGD-1:** 2,6-$HSO_3$-*Manp*-$\alpha$(1→2)-*Glcp*-$\alpha$(1→1)-*sn*-glyceroldiether

. Techniques such as the characterization of cellular protein patterns by gel electrophoresis (Hasselberg and Veerland, 1995; Zvyagintseva *et al*., 1999) and antigenic fingerprinting (Conway de Marico *et al*., 1986) have also proven useful in taxonomic characterizations, but they have not been extensively used.

The strains *Halobacterium halobium, Halobacterium salinarum and Halobacterium cutirubrum* are now unified in a single species, *Halobacterium salinarum* based on antigenic fingerprinting (Oren and Ventosa, 1996).

Apart from the above said the chemical structures of quinones and polyamines could also be taken for taxonomic classification (Oren, 2006). In many prokaryotes these polyamines can be used as chemotaxonomic markers but the *Halobacteriaceae* cells contain very low amounts of polyamines, near or below detection level. Polyamines such as putrescine, spermidine, spermine and agmatine (Cateni-Farina *et al*., 1985; Hamana *et al*., 1985, 1995; Kamekura *et al*., 1986) have been detected so far. MK-8 and MK-(8-VIII-H2) (Collins *et al*., 1981)

are the most abundant isoprenoid quinones found in the family *Halobacteriaceae*. The concentration of these two varies according to the cultures age and the growth mediums salinity (Tindall *et al*., 1991).

### 2.3 Distribution of *Haloarchaea*

Haloarchaea are distributed in saltern ponds and have been isolated and characterized. These include saltern ponds worldwide: Great Salt Lake, the Dead Sea (Arahal *et al*., 1996; Bodaker *et al*., 2010), saline lakes in Inner Mongolia (Pagaling *et al*., 2009), African soda lakes, deep-sea brines (Wielen *et al*., 2005;Das Sharma and Das Sharma, 2012), Sereflikochisar salt lake in central Turkey (Birbir and Sesel, 2003), Salt mine in Central Anatolia ,Turkey (Yildiz *et al*., 2012), Salar de Atacama, Chile (Catherine *et al*.,2001), Ayvalik Saltern, Turkey (Elevi *et al*., 2004), Goa salt pans, India (Mani *et al*., 2012), Secovlje solar saltern, Slovenia (Pasic *et al*., 2005), Tuz Lake, Turkey (Mehmet *et al*., 2008),Tunisian multipond solar saltern, Tunisia (Baati *et al*., 2008) and many others.

About $10^4$-$10^6$ colony-forming units of extremely halophilic bacteria per gram salt and $10^3$-$10^5$ colony-forming units of extremely halophilic bacteria per mL were found in the samples collected from Sereflikochisar salt lake in central Turkey (Birbir and Sesel 2003). Dombrowski (1961, 1963) and Reiser and Tasch (1960) reported the presence of viable micro organisms in rock salt. Rippel (1935) demonstrated that bacteria-like rods were present in dissolved rock salt residues and thin sections of rock salt, but he failed to culture these viable microorganisms (Bien, 1965). Later these organisms were cultured which were rod-shaped and coccoid neutrophilic cells, but some species were pleomorphic. Walsby (1980)

observed flat and square boxes to rectangular shaped organisms for the first time and named them *Haloquadratum walsbyi*.

Recently it was reported that the family *Halobacteriaceae* consists of nearly 130 species with validly published names (Oren, 2012). Metagemomics is a culture independent method which gives a clear idea that there are many new species yet to be isolated and characterized (Bodaker *et al*., 2010; Narasingarao *et al*., 2011; Ghai *et al*., 2011).

*Archaea* isolated from saltern ponds include the type strains of *Halorubrum trapanicum* (Elazari-Volcani,1957), *Halorubrum saccharovorum* (Tomlinson and Hochstein, 1972), *Halomonas elongate* (Vreeland *et al*., 1980), *Haloferax mediterranei* (Rodriguez-Valera, 1980), *Haloarcula californiae* (Javor *et al*., 1982), *Haloarchula hispanica* (Juez *et al*., 1986), *Haloferax gibbonsii* (Juez *et al*., 1986), *Haloferax denitrificans* (Tomlinson *et al*., 1986), *Halococcus saccharolyticus* (Montero *et al*., 1989), *Haloarcula japonica* (Takashina *et al*., 1990), *Halomonas salina* (Valderrama *et al*., 1991), *Halorubrum coriense* (Nuttall and Dyall-Smith, 1993), *Natrialba taiwanensis* (Kamekura and Dyall-Smith,1995; Hezayen *et al*., 2001), *Halogeometricum borinquense* (Montalvo-Rodriguez *et al*., 1998), , *Natrinema pellirubrum* (McGenity *et al*., 1998), *Haloterrigena thermotolerans* (Montalvo-Rodriguez *et al*., 2000), *Natrinema versiforme* (Xin *et al*., 2000), *Natrialba aegyptiaca* (Hezayen *et al*., 2001), *Salanibacter ruber* (Anton *et al*., 2002), *Natrinema altunense* (Xu *et al*., 2005), *Natrinema ejinorense* (Castillo *et al*., 2006), *Halomarina oriensis* (Inoue *et al*., 2010). The phylogenetic tree representing the classification of family *halobacteriaceae* based on 16S rRNA gene sequence (fig 4.57-APPENDIX) and the position of Halobacteria in the Euryarcheota phylum is represented as cladogram (fig. 4.58-APPENDIX).

## 2.4 Halophilism

Haloarchaea produce organic solutes which maintain the concentration of ions both inside and outside the cell in order to keep themselves intact and survive in high saline environments (Gonzalo *et al*., 2002; Jan *et al*., 2007; Torsten *et al*., 2007). To survive in such conditions, the halophilic *Archaea* accumulate potassium ions inside their cells to balance the high salt content of the environment. This mechanism is named as the "salt-in-cytoplasm mechanism", which was first observed and discovered in *Halobacteria.* This is considered as a typical archaeal strategy of osmoadaptation. The halophilic environment contains saturated concentrations of NaCl but halophilic *Archaea* keeps the cytoplasm free of sodium ions. They accumulate potassium in the cell through an energy-dependent potassium uptake system, together with its counter ion $Cl^-$. Thus it increases the concentration of potassium ions upto 100 times greater inside the cell with respect to its surrounding environment. This was clearly explained in *Haloanaerobium praevalens* which showed a higher rate of $K^+$ ions with respect to $Na^+$ ions during the exponential phase and $Na^+$ ions replaces $K^+$ ions when the cells entered stationary phase (Oren *et al*., 1997). Studies conducted on *Haloanaerobium acetoethylicum* showed that $Na^+$ could be the major cation in stationary cells as well as in exponentially growing cells (Mermelstein *et al*., 1998). Those studies conducted clearly indicated that the accumulation of potassium and/or sodium in the cytoplasm takes place only when they are exposed to an ionic environment. The Halophilic archaea habituating saturated salt concentrations outcompete organic-osmolyte producers, proving members of the "salt-in-cytoplasm mechanism" as *extreme* halophiles (Kunte *et al*., 1999). This ability differentiates them from halophilic bacteria that usually accumulate compatible solutes (betaine, ectoine) to counteract the high external salt concentrations (da Costa *et al*., 1998; Ochsenreiter *et al*., 2002; Roberts, 2005).

They are often considered as dependable source for deriving novel enzymes, novel genes, bioactive compounds and other industrially important molecules. Protein antibiotics have potential for application as preserving agents in food industry, leather industry and in control of infectious bacteria and hence there is great interest in isolation of potential proteinaceous bioactive substances.

## 2.5 Haloarchaea and halite precipitation

There are several possible processes by which brines form: (i) natural recrystallization of hydrated minerals, forming residual brines; (ii) influx of surface water or ground water; (iii) condensation of air used for ventilation; (iv) locally introduced water, spills, etc.; and (v) water deliberately injected into salt horizons for extraction as brine (solution mining) (McGenity *et al*., 2000).

Evaporation of sea water from salt pans brings precipitation of salt to its saturation point and crystallizes the salt. The calcium ion are precipitated in the form of gypsum which results in formation of concentrated sea water, which is now shifted to the next crystallizer pond where NaCl precipitate as crystals (Javor, 2002). The importance of microbes in contribution towards salt formation is of greater importance and studies conducted revealed few facts. It is reported that the microorganisms in the ponds contribute to NaCl crystallization by heating the ponds to higher temperatures rapidly. This was confirmed when the studies on the pigment carotenoid, a light harvesting molecule was conducted (Jones *et al.*, 1981; Javor, 1989)

The salt crystal thus formed 'the halite' is loaded with halobacterial cells inside. The group colonizing inside the halite may be haloarchaea which requires a minimum of 2.5M NaCl for survival (Grant *et al*., 1989). When the concentration

of NaCl increased above 20% (w/v) up to halite saturation [< 32% (w/v)], haloarchaea became the dominant microorganisms (Benlloch *et al*., 1996). This life inside the halite is possible by the formation of larger fluid inclusions (Norton and Grant, 1988). Brian *et al*. (1990) demonstrated that haloarchaea survives in halite for more than 30000 years. He proved the presence of motile haloarchaeal cells inside the halite after several weeks which remained viable inside fluid inclusions for 6 months and more (Norton and Grant, 1988). Bain *et al*. (1958) recovered viable `pink bacteria', haloarchaea, from solar salt that had been in storage for 4 years, while Dombrowski (1966) recultivated bacteria encased in salt after 5 years.

Lopez-Cortes *et al*. (1994, 1998) have conducted experiments and substantiated the role of Halobacteria in halite formation. They inoculated haloarchaea with NaCl in medium and had a control only with NaCl, uninoculation. The strains used were *Halobacterium halobium* NRC-817, *Haloarcula vellismortis* ATCC29252 and *Haloarcula* sp. 8807. The results showed that halite formation occurred in all the conditions including control but there was a difference in the halite size. Inoculated medium showed a larger size of crystal comparison to the control. Control yielded 1-8mm size halite where as the halite size was 1-25mm in the presence of *Halobacterium halobium* NRC-817, 1-15mm in the presence of *Haloarcula vellismortis* ATCC29252 and *Haloarcula* sp. 8807. The S-Layer in the halophiles plays a major role in formation of salt crystals. The S-Layer gets modified in the presence of excess NaCl and leads to the precipitation of ions on the S-Layer, may be due to bio-mineralization (Maria *et al*., 2004) or biosorption (Tsezos, 1985; Tsezos and Deutschmann, 1990) and forms dendritic crystals and cubic crystals. Due to this mineralization/ biosorption process, a honey comb like structure formation occurs which indicates the

reassembling of S-layer. These reassembled units are suspected to act as template for crystal nucleation and growth (Maria *et al*., 2004).

The haloarchaeal population affects crystallization in several ways: it leads to an increase in the size and number of crystals (Lopez-Cortes *et al.*, 1994); results in bigger fluid inclusions (Norton and Grant, 1988); encourages dendritic crystals (Norton and Grant, 1988; Lopez-Cortes *et al*., 1994); accelerates crystal formation (Norton and Grant, 1988) and sometimes encourages the formation of salt ooids (Castanier *et al*., 1999).

Halophiles such as bacteria (*Salinibacter* sp.), *Archaea* (*Halobacterium* sp.) and eukaryotes like fungi (*Hortaea* sp.) and algae (*Dunaliella* sp.) might thrive symbiotically (Bardavid, 2008) and give pink or red coloration to the salt pans (Oren, 1994, 2002, 2008, 2009) due to the presence of carotenoids and bacteriorhodopsin pigments in the brines due to high halophile densities. These pigments act as a protectant to the cells from the harmful effects of ultraviolet light (Shahmohammadi *et al*., 1998) and also encourage evaporation by trapping solar radiation. This evaporation leads to NaCl precipitation due to which the haloarchaea gets trapped inside fluid inclusions that can constitute 2 ± 6% (w/w) of freshly harvested solar salt (Lefond, 1969). This precipitation phenomenon is universal (Norton and Grant, 1988; Castanier *et al*., 1999). In order to study and understand the role of halophiles in the salt evaporates and their survival inside salt crystals a clear understanding of the geology and geochemistry of evaporates as well as information about isolated microorganisms are necessary (McGenity *et al*., 2000).

## 2.6 Archeocins

Bacteriocins are compounds produced by bacteria showing antagonistic property against micro organisms. They may also be termed as 'bacteriocin-like substances' as they might be proteins or complexes of proteins, and they are not active against the producer strain. The bacteriocin-like substances produced by the domain *Archaea* are known as archeocins and are of two types, the halocins produced by haloarchaea and the sulfolobicins produced by sulfolobous.

Tamar and Oren (2000) conducted experiments to test whether halocins play a role in the interspecies competition between different types of halobacteria in saltern crystallizer ponds inhabited by dense communities of red halophiles. They assayed halocins activity against a variety of halobacteria in salterns from different locations worldwide. No halocin activity was detected in any of the brines examined but the isolates of these brines showed halocin activity against sensitive halophiles upon culturing. This showed that the halocin do not play any major role in the competition between the halobacteria in hypersaline aquatic environment.

### 2.6.1 Sulfolobicin

Prangishvili *et al*. (2000) were the first to report sulfolobicins by *Sulfolobous islandicus*. The sulfolobicin inhibited *S. solfataricus* P1, *S. shibatae* B12, and six non-producing strains of "*S. islandicus*". Activity of sulfolobicins was reported to be archaeocidal but not archaeolytic. It did not inhibit *S. acidocaldarius* DSM639, *Halobacterium salinarum* R1 or *Escherichia coli* (Prangishvili *et al*., 2000). As observed with other bacteriocins, sulfolobicin was apparently not released from the producer cells in soluble form in liquid medium

but remained bound to the membranes of the cells or of cell-derived S-layer-coated membrane vesicles. This characteristic makes this a special type of archeocin. The supernatant of the grown organism did not show any antagonistic activity but upon concentrating it 100 folds it showed antagonism. Purification of sulfolobicin was achieved by harvesting late stationary phase cells followed by sonication. The cell debris was precipitated by high speed centrifugation and the release of sulfolobicin was attained by addition of TritonX100 as they were membrane bound. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by size exclusion chromatography (30 - 40kDa) showed that the sulfolobicin was 20kDa size protein. Activity of the purified sulfolobicin remained stable for 6 months at 4°C and at 85°C it remained active for 5 days and pH 3.5 to 6.5 (Prangishvili *et al*., 2000). The chemical nature of the sulfolobicin was studied by incubating the purified compound with glycolytic, lipolytic and proteolytic enzymes. No decrease of sulfolobicin activity was detected after treatment with glycolytic or lipolytic enzymes. Incubation of proteolytic enzymes tested led to the complete loss of sulfolobicin activity, indicating that an intact protein is required for activity. (Prangishvili *et al.*, 2000).

Sulfolobicins exhibit some classical bacteriocin characteristics, as they are proteinaceous and are directed against strains that are closely related to the producer. Although some of the producer strains contain conjugative plasmids, neither sulfolobicin production nor immunity was transferred to non-producer strains, suggesting that the genes for these traits may be located on the chromosome (Prangishvili *et al*., 2000).

### 2.6.2 Halocin

Haloarchaea were the first members of *Archaea* found to produce proteinaceous bacteriocins which are released into the environment and it is a

universal characteristic of halophilic bacteria (Rodrı´guez-Valera *et al*., 1982). These proteinaceous bacteriocins, termed as halocins, act against related species and are universally produced by halophilic *Archaea* (Torreblanca *et al*., 1994). Halocin could be classified into two types

i. Microhalocin (peptides) - ≤10kDa size
ii. Protein halocin - ≥10kDa size

Eventhough several halophilic *Archaea* are being explored for halocin production, only very few halocins have been studied up to their molecular level. Their mode of action against halophiles as a defense is yet to be understood clearly (Tamar and Aharon, 2000; Meseguer and Rodrı´guez Valera, 1986). Moreover, all haloarchaea are not sensitive to any particular halocin, and a ‘‘sensitive’’ strain is the one which elaborates a zone of inhibition on a double - agar overlay plate in response to the presence of halocin (Paši *et al*., 2008).

Halocin A4, G1, R1, H1, H2 (O’Connor and Shand, 2002); H3, H5 (Rodrı´guez-Valera *et al*., 1982; O’Connor and Shand, 2002); H4 (Sun *et al*., 2005; Gonzalo *et al*., 2002; O’Connor and Shand, 2002); H6/H7 (O’Connor and Shand, 2002; Li *et al*., 2003); S8 (O’Connor and Shand, 2002); C8 (Li *et al*., 2003, Sun *et al*., 2005), Sech7a (Paši *et al*., 2008) and KPS1 (Kavitha *et al*., 2011) are few halocins reported till date and only few of them are studied up to molecular level (Table 2.5) .

**2.6.2.1 Halocin assay**

The halocin activity is determined using serial two-fold critical end point dilutions to extinction (Meseguer *et al*., 1986) and expressed as arbitrary units

(AU), (Figure 2.1) which are defined as the reciprocal of the first dilution at which all trace of inhibitory activity disappears (Cheung *et al*., 1997). Thus the expressed AU are standard values. The activity of halocin is expressed in terms of centimeters, for halocins that showed low level of activities, according to the following expression: Halocin activity (cm) = (inhibition halo diameter - well diameter)/2 (Torreblanca *et al*., 1989).

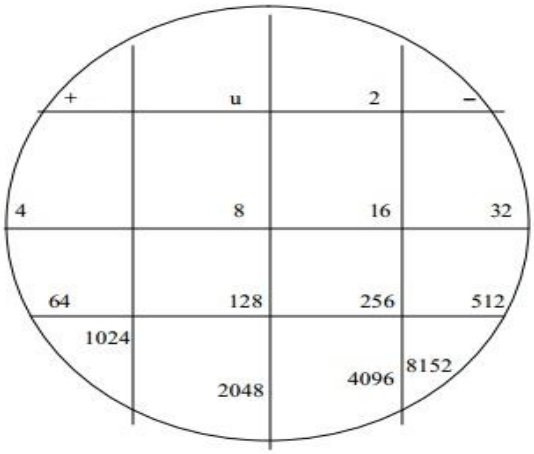



**Figure 2.1 Twofold serial dilution method for halocin assay** (Meseguer *et al*., 1986)

### 2.6.2.2 Halocin H4

Halocin H4 (HalH4) produced by *Haloferax mediterranei* R4 (ATCC 33500) isolated from solar saltern lakes of Spain, was the first halocin discovered (Rodrı´guez-Valera *et al*., 1982). The molecular mass of this halocin was determined as 28kDa by SDS- PAGE (Meseguer and Rodrı´guez Valera, 1985) and as 34.9kDa by cloning and expressing the gene (Cheung *et al*., 1997). This 34.9kDa HalH4 was reported to be processed from a preprotein of 39.6kDa which contains the 46 aminoacid N-Terminal Tat signal sequence (Cheung *et al*., 1997).

Halocin H4 is encoded by a 1,080-bp open reading frame that corresponds to a polypeptide of 359 residues (Cheung *et al*., 1997). It was also found that the HalH4 contains a hydrophobic region which is said to have a major role in binding to the target site (Shand *et al*., 1999). The actual target site of HalH4 is not determined (Meseguer *et al*., 1995). Upon exposure, the sensitive cells *Halobacterium salinarum* swells and becomes spherical due to ionic imbalance and lyse (Meseguer and Rodrı´guez Valera, 1986).

### 2.6.2.3 Halocin S8

Halocin S8 (HalS8) is produced by haloarcheon S8a, isolated from the Great Salt Lake, Utah (Price and Shand, 2000). This contains only 36 aminoacids and is called as a microhalocin. This inhibits *Hbt. salinarum* NRC817, *Halobacterium* sp. strain GRB and *Hfx. gibbonsii* (Price and Shand. 2000). HalS8 is resistant to heat, trypsin digestion and organic solvent (Price and Shand, 2000; Shand *et al*., 1999). Its molecular weight was determined as 3.58kDa (Price and Shand, 2000). The halocin S8 is encoded by 933bp open reading frame which yields the preproprotein of 311 aminoacids which has to undergo processing at two different sites to yield three peptides (i) a 230 aminoacid N-Terminal protein containing the Tat signal peptide (ii) a 45amino acid C-Terminal peptide and (iii) a 36 aminoacid mature halocin (in between the both peptides) which is unusual and this is attained by some uncharacterized haloarcheal protease (Price and Shand, 2000). This preproprotein contains 230 aminoacids in the N-Terminal region, 45 amino acids in the C-Terminal region and this plays major role in the halocin immunity, induction, translocation and regulation (Price and Shand, 2000). Edman degradation of purified HalS8 revealed a 47% hydrophobic, 36-amino- acid peptide and BLAST searches of HalS8 revealed no homology with any protein in the database (Price and Shand, 2000). The gene for HalS8 has been

cloned and sequenced, and contour - clamped homogeneous gel electrophoresis analysis shows that it is located on a 200kbp megaplasmid. Aminoacid sequence reported for HalS8: SDCNINSNTAADVILCFNQVGSCALCSPTLVGGPVP (Price and Shand, 2000)

#### 2.6.2.4 Halocin H6/H7

Halocin H6 (HalH6) is produced by *Haloferax gibbonsii* strain Ma2.39. It was first isolated from solar saltern, Alicante, Spain (Torreblanca *et al*., 1989). HalH6 was purified using hydroxylapatite chromatography, gel filtration chromatography on sephadex G50 and HPLC on Spherogel column and the molecular weight of HalH6 was determined as 32kDa from SDS-PAGE (Torreblanca *et al*., 1989). Halocin H6 retained 100% activity upon exposure to 90°C for ten minutes and retained 50% activity upon exposure to 100°C for 10 minutes but lost its whole activity upon autoclaving (Torreblanca *et al*., 1989). It was resistant to trypsin and sensitive to pronase. Unlike other antibiotics the production of HalH6 was not inducible by UV light or acridine orange. The halocin was actually a 3kDa protein which was combined with a carrier protein and showed the molecular mass of 32kDa. Upon denaturation conditions the 3kDa mature protein was released. This halocin was named as halocin H7 (HalH7) and characterized as microhalocin (Shand and Kathryn, 2007).

#### 2.6.2.5 Halocin C8

Halocin C8 (HalC8) is produced by *Halobacterium* strain AS7092 isolated from the Great Chaidan Salt Lake in Qinghai province in the People's Republic of China (Li *et al*., 2003; Sun *et al.*, 2005). It inhibited 16 haloarchaeon strains, which included three alkaliphilic halobacteria that grow optimally at pH

9.5. It was concentrated by Tangential flow filtration (TFF) and purified using sephadex G50 Gel filteration column and DEAE-sepharose fast flow chromatography. The gel filtered elutes were subjected to Tricine SDS PAGE. The molecular weight of this halocin was found to be 6.3kDa and it was found to be a single peptide (Li *et al*., 2003).

Cloning and sequence analysis of the HalC8 gene showed that this microhalocin was 7.44kDa in size and constituted with 76 aminoacids. This is rich in cysteine and contains 283 amino acids in the C-terminal region which is also known as ProC8 and the processing of this HalC8 starts at this region. The N-Terminal region contains the Tat leader sequence followed by a 207 amino acid known as the HalI which is rich in hydrophilic aminoacids and this region confers immunity. This was the first report of a single gene encoding for an antimicrobial peptide and immunity (Sun *et al*., 2005).

**2.6.2.6 Halocin A4**

Halocin A4 (HalA4) was isolated from uncharacterized haloarchael strain TuA4 isolated from Tunisian saltern. It has a broad spectrum of antagonist activity and inhibited crenarchael hyperthermophile *S.solfataricus* and mutants of *S.solfataricus* has also been isolated which is resistant to HalA4 (Haseltine e*t al*., 2000). HalA4 was purified by gel filtration chromatography and RP HPLC (Shand, 2006). It has a molecular mass of 7,435Da determined by mass spectrometry and was found to have both acidic and hydrophobic nature (Shand and Leyva, 2007).

### 2.6.2.7 Halocin H1

Halocin H1 (HalH1), obtained from the supernatant of *H. mediterranei* M2a (previously known as *H. mediterranei* Xia3) was initially described by Rodrı´guez-Valera *et al*. (1982). This halocin was found to have a broad inhibitory spectrum among different haloarchaea. It was initially purified by column chromatography using CNBr-Sepharose 4B and hydroxylapatite Bio-Gel HTP. The molecular weight was determined to be 31kDa by SDS PAGE. It is salt dependent and required a minimum of 5% of NaCl to retain its activity (Platas *et al*., 2002). HalH1 is heat labile (Platas, 1995) and the production was increased when grown in N-Z amine E. The halocin yield was 1,280AU/ml whereas in the presence of other nutrients the production fluctuated between 0-320AU/ml (Platas, 1995; Shand and Leyva, 2007). HalH1 is effective against haloarchael organisms classified within the same genus than the producer strain. The mechanism of action of HalH1 is still unclear but preliminary experiments showed that its effects on *H. salinarum* NRC 817, were similar to those observed for halocin H4 (Platas *et al*., 2002).

### 2.6.2.8 Halocin R1

Halocin R1 (HalR1) is the second microhalocin to be characterized. It is produced by *Halobacterium. salinarum* GN101 isolated from a solar saltern in Guererro Negro, Mexico by Barbara Javor (Ebert e*t al.*, 1986). Its molecular weight was determined to be 3.8kDa. Rdest and Sturm (1987) reported that Halocin R1 was 6.2kDa and later it was found that this 3.8kDa protein is associated with a carrier protein which gets detached upon heating and gel filtration (Shand *et al*., 1999; O'Connor, 2002). This microhalocin has been fully sequenced by Edman degradation and was found to have very close similarity and identity to HalS8. (Price and Shand, 2000; O'Connor, 2002; O'Connor and

Shand, 2002). The Aminoacid sequence reported for HalR1 is as follows: LQSNININTAAAVILIFNQVQVGALCAPTPVSGGGPPP (O'Connor, 2002)

### 2.6.2.9 Halocin Sech7a

Halocin Sech7a is produced by an extremely halophilic haloarchaeon Sech7a isolated from Secˇovlje solar salterns crystallizers in Slovenia (Pasic *et al.*, 2008). The isolate was phylogenetically related to *Haloferax mediterranei* and its halocin showed activity against *Halobacterium salinarum* NRC817. Purification was done by using 3kDa molecular weight cut off membrane followed by ultracentrifugation and ion exchange chromatography using DEAE Sephacel column. Its molecular weight was determined as 10.7kDa using SDS-PAGE. Sech7a was found to be stable over a wide range of pH (2–8) and was thermostable at temperatures between 20°C–80°C. This was salt dependent where it loses its activity when exposed to lower salt concentrations. Similar to other halocin H4, H6, and C8 (Meseguer *et al*., 1985; Torreblanca *et al*., 1989; Li *et al.*, 2003) this also shared the property of lysing the sensitive cells by initial swelling.

### 2.6.2.10 Halocin KPS1

Halocin KPS1 was produced by *Haloferax volcanii* KPS1 isolated from solar salterns of Kanyakumari, South India. This was the first halocin reported to show antimicrobial activity against gram positive and gram negative human pathogens (Kavitha *et al*., 2011). It also inhibited *Halobacterium sodomens*. This was found to be pH stable (3–9) and was thermolabile above 80°C. The molecular weight has not been determined and it lost its activity when incubated with 1mg/mL proteinase K and trypsin for 10 min.

**2.6.2.11 Halocin gene and its expression**

To date, all of the known halocin genes are encoded on megaplasmids (> 100kbp) and possess typical haloarcheal TATA and BRE promoter regions. Halocin transcripts are leaderless and using the twin arginine translocation (Tat) pathway the translated preproteins or preproproteins are exported. The Tat signal motif (two adjacent arginine residues) is present within the amino terminus of the protein sequence. The expression of halocin genes are at the transition between exponential and stationary phases of growth. Halocin H1 gene is induced during exponential phase which is an exception (Platas *et al*., 1996). The larger halocin proteins are generally salt dependent and are heat-labile. In an earlier study halocin production by haloarchaeal strain Sech7a was reported to be growth dependent (Paši *et al*., 2008). Halocin Sech7a activity was observed in the early exponential phase of growth, but only during the stationary phase peak activity was observed (Paši *et al*., 2008) in contrast to most other halocins which were first detected when the bacteria entered the stationary phase of growth (Shand *et al*., 1999; O'Connor and Shand, 2002). HalS8 activity was reported only when the culture began the transition into stationary phase and were undetectable before that (Price and Shand, 2000; Shand *et al*., 1999) and later the activity reached a maximum within 10h and was stable for greater than 80h after reaching maximum values.

Halocins are usually produced when cultures enter stationary phase (Cheung *et al*., 1997; Price and Shand, 2000; Li *et al*., 2003). Halocin H4 consists of a polypeptide of 359 residues and is encoded by a 1,080bp open reading frame (Cheung *et al*., 1997). In accordance with these observations, the expression of HalH4 and HalS8 genes was identified as growth-stage dependent, during exponential growth the concentration of transcripts low, but steadily reached maximal levels upon entering stationary phase (Cheung *et al*., 1997; Price and

Shand, 2000). As the gene expression was found to be at its peak, halocin genes were believed to be good models for the study of stationary-phase gene regulation, which has become an important focus in haloarchaeal research (Price and Shand, 2000).

Halocin S8 gene was found to be encoded by a 200bp megaplasmid. A 2,873bp fragment containing the entire HalS8 gene was amplified by PCR using two degenerate primers and probes designed from the halocin S8 protein sequenced by Edman degradation. The 933bp open reading frame containing a 36-amino-acid region (HalS8) was identified. The gene was cloned and expressed which showed 97% identity to the sequence for HalS8 determined by Edman degradation. The derived sequence showed a change in single residue at $34^{th}$ position which was found to be a proline instead of a glycine. It was the first microhalocin discovered with a molecular mass of 3580Da.The transcription start site was leaderless and upstream to HalS8 230 aminoacids were found and 45 amino acids downstream (DasSarma *et al*., 1984; Blank and Oesterhelt, 1987; Ruepp and Soppa, 1996; Cheung *et al*., 1997). Upstream to the transcription start site a haloarchaeal promoter hexamer was located from 229 to 224bp. This sequence matched the haloarchaeal consensus sequence (229 TTTWWW 224) in five of six residues (Soppa, 1999a, b). In addition, a transcription factor B recognition element was also present 5bp upstream of the promoter at positions 234 and 235 (Price and Shand, 2000).

HalC8 sequence (DIDITGCSACKYAAG) was determined by Edman degradation (Li *et al*., 2003). Two degenerate primers (Sun *et al*., 2005) and the HalC8 RNA probe were designed to identify the halocin C8 gene from *Halobacterium* sp. AS7092 by polymerase chain reaction (PCR). A 44bp DNA fragment was amplified and cloned into pUCm-T vector. Upon Hybridization a

6.3kb *Bam* HI restriction fragment of the AS7092 genome was isolated and a plasmid library was constructed, sequenced, and HalC8 gene was then recovered. The *halC8*gene encodes a prepro-protein of 283-amino-acid residues (ProC8, 29.38kDa). This protein contained the mature 15-amino-acid region (amino acids 208–222) which was fully identical to the N-terminal sequence of the purified mature peptide halocin HalC8. Agar well diffusion method shows that ProC8 and its 207-amino-acid N-terminal peptide (HalI) inhibited the activity of HalC8 *in vitro*. This clearly indicates thata single gene encode both peptide antibiotic and immunity protein (Sun *et al.*, 2005). The transcription start site of *halC8* was found to be at the sixth base (G) upstream of the ATG start. Haloarchaeal TATA was found to be located at 23-18bp upstream to the transcription start site. This sequence had 100% match to the haloarchaeal promoters (Soppa, 1999a,b; Reeve, 2003) and 3bp upstream to the promoter B recognition element (BRE) was found .

The production of this halocin was controlled through both transcription regulation and protein processing: the HalC8 transcripts and HalC8 activity rapidly increased to maximal levels upon transition from exponential to stationary phase. However, while HalC8 transcripts remained abundant, the HalC8 processing was inhibited during stationary phase (Sun *et al.*, 2005).

**2.6.2.12 Mode of action of halocin**

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

*gibbonsii* was reported to inhibit Na+/H+ exchanger (NHE) in mammalian cells (Meseguer *et al*., 1995).

Halocins are generally produced at the mid exponential phase (O'Connor and Shand, 2002; Paši *et al*., 2008). Studies have been conducted up to gene level for halocins H4 (Cheung *et al*., 1997), C8 (Sun *et al*., 2005), and S8 (Price and Shand, 2000), and identification of the mRNA responsible for production of Halocin (Cheung *et al*., 1997, Price and Shand, 2000).

**2.6.2.13 Common features of halocins** (Adopted from Shand and Leyva, 2007)

Halocins are peptides (≤ 10kDa; "microhalocins") or protein (>10kDa) antibiotics produced by members of the archaeal family Halobacteriaceae. The halocin genes are induced at the transition between exponential and stationary phases where as halocin H1 is induced during exponential phase (Platas *et al*., 1996). They are located on megaplasmids ("mini-chromosomes") which are about 100kb in size. They have typical haloarchaeal TATA boxes and TFB recognition elements (BRE), the TATA box element for halocin C8 is close to the transcription start site than usual (18bp rather than 22–25bp (Sun *et al*., 2005). Halocin transcripts are "leaderless", where the transcriptional start site is either coincident with or only a few bps upstream of the translational start codon ATG. Halocin preproteins appear to be exported by the twin arginine translocation (Tat) pathway, as all have a Tat signal motif at their amino terminus, mature halocins are inactivated by one or more proteases, confirming their proteinaceous nature; microhalocins are hydrophobic and are robust, and they can be desalted without losing activity and are resistant to organic solvents such as acetonitrile and acetone, are heat stable (halocin R1 is the most sensitive, but can withstand heating at 60ºC for 1h without losing activity, [O'Connor, 2002]). They can be

stored at 4ºC for prolonged periods (as long as 7 years for halocin R1; [O'Connor, 2002]) without significant loss of activity, and protein halocins (halocins H1 and H4) are heat sensitive and lose activity when desalted below 5% (w/v) NaCl, although halocin H4 can be desalted to 10mM Na+ with only a twofold loss in activity (Perez, 2000). However, desalting to this level decreases the length of time halocin H4 can be stored at 4ºC.

**2.6.2.14 Application studies**

According to Alberola *et al*. (1998) halocins may have a role in human medicine since they have been reported to show antimicrobial activity against gram positive and gram negative human pathogens (Kavitha *et al*., 2011). Hence halocins could be used as alternatives for antibiotics as there is a need for new antibiotics to manage the existing antibiotic resistant pathogens.

Halocins are of different types and each species differ in their type and so they are proposed to be used to learn about halophiles in general (Soppa *et al*., 1989). Halocins are antimicrobial proteins which could be used in leather industries to control spoilage (Rdest and Sturm 1987; Shand and Perez 1999; Shand *et al*., 1999; Shand and Leyva, 2008). Crude salt from the solar salterns harbour excess of lipolytic and proteolytic extremely halophilic microorganisms which when applied on hides causes damage to leather. Halobacterial growth on hides could be prevented with natural antimicrobial compound such as halocins (Birbir and Eryilmaz, 2005).

Halocin H6/H7 from haloarchaea *Haloferax gibbonsii* inhibits $Na^+/H^+$ exchanger (NHE) in mammalian cells. Its cardio-protective efficacy on the ischemic and reperfused myocardium has been evaluated. Halocin H6 was applied on cell lines of mammalian origin (HEK293, NIH3T3, Jurkat and HL-1) and on

primary cell culture from human skeletal muscle (myocytes and fibroblasts) which showed inhibition in NHE activity which was measured by flowcytometry (Lequerica *et al*., 2006). When an ischemia-reperfusion model in dogs by coronary arterial occlusion was treated with halocin H6, *in vivo*, a reduction in premature ventricular ectopic beats and infarct size were noted with normal blood pressure and heartbeat. This explains that halocin H6 is the only biological molecule exerts a specific inhibitory activity in NHE of eukaryotic cells (Meseguer *et al*., 1995; Alberola *et al*., 1998; O'Connor and Shand, 2002).

**Table 2.5 Halocin characteristics** (adapted from O'Connor and Shand, 2002; Shand and Leyva, 2007)

| Halocin | Producer (strain) | Size | Thermal stability | Salt dependent | Activity Spectrum | Mechanism |
|---|---|---|---|---|---|---|
| A4 | Strain TuA4 (solar saltern, Tunisia ) | <5kDa | ≥1 week | No | Broad *Sulfolobus* sp. | ND |
| C8 | Halobacterium stain AS7092(Great Chaidan Salt lake, Cina) | 7,427Da | >60min at 100°C | No | Broad | ND |
| G1 | Halobacterium strain GRB (solar saltern, France) | ND | ND | ND | Broad | ND |
| H1 | *Hfx. Mediterranei* M2a (previously Xai3; solar saltern, Spain ) | 31kDa | <50°C | Yes | Broad | Membrane permeability |

| | | | | | | |
|---|---|---|---|---|---|---|
| H2 | Haloarcheon GLA22 (solar saltern, Spain ) | ND | ND | ND | Broad | ND |
| H3 | Haloarcheon Gaa 12(solar saltern Spain) | ND | ND | ND | Broad | ND |
| H4 | *Haloferax mediterranei* R4(solar saltern,Spain) | 34.9kDa | <60°C | Partially | Broad | Proton flux |
| H5 | Haloarcheon Ma2.20(solar saltern, Spain) | ND | ND | ND | Narrow | ND |
| H6/H7 | *Haloferax gibbonsii* Ma2.39(solar saltern, Spain) | ~3kDa | ≥90°C | No | Broad | Na+/H+ antiporter inhibitor |
| R1 | Halobacterium strain GN101 (solar saltern, | 3.8kDa | 60°C | No | Broad, *Sulfolobus* sp., | ND |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Mexico) | | | | *M.thermophila* | |
| S8 | Haloarchaeon S8a(Great Salt Lake,Utah) | 3.58kDa | ≥24h at boiling | No | Broad, *Sulfolobus* sp. | ND |
| Sech A | *Haloferax mediterranei* SechA | 10.7kDa | 80°C | Partially | Narrow | swelling and lysis of sensitive cells |
| KPS1 | *Haloferax volcanii* KPS1 | - | >80°C | ND | Broad | ND |

# *Chapter 3*

## MATERIALS AND METHODS

### 3.1 SCREENING OF HALOCIN PRODUCING HALOARCHAEA

#### 3.1.1 Source of bacteria

Haloarchaeal bacterial strains capable of halocin production were isolated from soil samples from salt pans of Kanyakumari, Tamilnadu, South India.

#### 3.1.2 Collection of Samples

Soil samples from the salt crystallizer ponds were collected in sterile polythene bags and transported under iced condition to the laboratory. The concentration of different salt forming cations ($Na^+$, $K^+$, $Mg_2^+$, $Ca_2^+$, $HCO_3^-$, $Cl^-$, $So_4^{2-}$) present in the samples were analysed in a soil testing laboratory, at Tirunelveli, Tamilnadu. After reaching the laboratory they were immediately processed for bacteriological analysis.

#### 3.1.3 Inoculum preparation

One gram of soil was mixed with 10mL of saturated solution of sodium chloride, homogenized and 100µL aliquot of the prepared sample was spread plated on medium containing 3M sodium chloride and incubated at 42°C for 7 days.

### 3.1.4 Medium

Medium that supported maximal biomass and halocin production was selected from among six different media namely Eimhjellen medium (EM) (Catherine *et al.*, 2001), Sehgal and Gibbons (SG) (Sehgal and Gibbons, 1960), MH medium (Ventosa *et al*., 1982), HE medium (Torreblanca *et al*., 1986; Catherine *et al.*, 2001) DSM 97(Das Sarma *et al*., 1995) and Zobell's medium (Hi-media). The compositions of the different media are as given below:

**3.1.4.1 Eimhjellen medium (modified)**: Yeast extract-5g, $MgSO_4.7H_2O$ - 2.0g, $CaCl_2.2H_2O$ – 0.5g, NaCl – 3.0M, Distilled water – 100mL (Catherine *et al*., 2001).

**3.1.4.2 Sehgal and Gibbons medium (modified):** Yeast extract – 1g, Casamino acids -0.75g, Sodium citrate – 0.3g, $MgSO_4.7H_2O$ – 2.0g, KCl – 0.2g, $FeCl_2$ – 0.0023g, NaCl – 3.0M, Distilled water – 100mL (Sehgel and Gibbons, 1960).

**3.1.4.3 DSM 97**: Casamino acids – 7.5g, KCl – 2g, NaCl – 3.0M, Trisodium citrate – 3g, $MgSO_4$ - 20g, MnSO4 – 0.05g, Ferrous sulphate – 0.5g, Yeast extract – 10g, Agar – 10g, Distilled water – 1000mL (DasSarma *et al*., 1995).

**3.1.4.4 MH medium (modified**): Protease peptone – 0.5g, Yeast extract – 1.0g, Glucose – 0.1g with 25% (w/v) of total salts (Ventosa *et al.*, 1982).

**3.1.4.5 HE medium (modified)***:* Yeast extract – 0.5g, Glucose – 0.1g with 25% (w/v) of total salts (Torreblanca *et al*., 1986).

The **total salts solution** was prepared with NaCl – 3.0M, $MgCl_2.6H_2O$ – 4.2g, $MgSO_4.7H_2O$ – 6.0g, $CaCl_2.2H_2O$ – 0.1g , KCl – 0.6g, $NaCO_3H$ – 0.02g, NaBr – 0.07g, $FeCl_3$ – 0.0005g, Distilled water – 100mL (Subov ,1931).

**3.1.4.6 Zobell's Medium**: Peptic digest of animal tissues – 5g, Yeast extract – 1g, Ferric citrate – 0.1g, NaCl – 3.0M, $MgCl_2.6H_2O$ – 8.8g, Sodium sulphate – 3.24g, $CaCl_2.2H_2O$ – 1.8g, KCl -0.55g, $NaCO_3H$ – 0.16g, KBr – 0.08g, Strontium chloride – 0.034g, Boric acid – 0.022g, Sodium silicate – 0.004g, Sodium fluorate – 0.0024g, Ammonium nitrate –0.0016g, Disodium phosphate – 0.008g, Distilled water – 1000mL supplemented with NaCl (4.5M).

Irrespective of the medium, the final concentration of sodium chloride in the medium was adjusted to 3M unless otherwise specified. The pH of the medium was maintained at 7.4 ± 2. Solid medium was prepared by the addition of 2% (w/v) of agar (Hi-media) to broth.

### 3.1.5 Preliminary screening of halocin activity

All the halophilic bacterial strains isolated from the salt ponds were screened for halocin activity by testing each strain against the other strain. The presence of zone of inhibition on double layer agar plates was used as indicator for halocin production (Shand *et al*., 1999; O'Connor and Shand, 2002). 10µL aliquots of broth culture of each strain grown in Zobell's medium for 48h at 37$^{o}$C were spotted onto 24h grown top-agar lawn culture of the other halophilic strains and incubated at 42$^{o}$C for 7 days. Appropriate controls were maintained using uninoculated media. Cultures that showed inhibition were selected and subjected to further studies.

### 3.1.6 Selection of Potential strain

Cultures that showed inhibition of halobacteria were selected and the potential strain that showed higher zone of inhibition against larger population of halophiles was selected. The haloarchaeal isolate with code No. BTSH10 showed maximal inhibition and hence was selected as the potential halocin producing strain.

### 3.1.7 Selection of indicator strain

Among the different strains tested the haloarchaeal isolate with code No. BTSH03 which showed higher percentage of sensitivity against the bioactive compound produced by BTSH10 was selected as the indicator strain.

### 3.1.8 Maintenance of Culture

The halophilic bacterial cultures were maintained as mentioned below

**I. Glycerol stock** - Halophiles grown for 48h in Zobell's broth were dispensed as 1mL aliquot in 2mL cryovial and added with equal volume of 40% sterile glycerol, mixed well and stored in -80°C deep freezer.

**II. Saturated salt tablets** (Oren, 2002; Tindall, 1992) – saturated salt solutions were prepared and sterilized. Concentrated cells were mixed with it and the mixture was poured to into microtitre plate and kept in sterile condition so that they form dried tablets. These tablets obtained were stored aseptically in room temperature.

## 3.2 IDENTIFICATION OF SELECTED STRAINS

Both the strains that produced bioactive substance (BTSH10) and that showed inhibition (BTSH03) were identified based on polyphasic taxonomy which included morphological, biochemical and physiological characteristics (Bergey's Manual of Systematic bacteriology) and molecular ribotyping by amplification and sequencing 16S rDNA ( Silva *et al.*, 2001). All the media used were supplemented with 15% sodium chloride.

### 3.2.1 Molecular ribotyping

Halobacteria universal 16S rDNA primer (Vidyasagar *et al.*, 2009) was used. The primer sequence (Sigma Aldrich-India) was as follows.

**Table.3.1 Primers used for amplification of 16S rDNA of Halobacteria**

| | |
|---|---|
| 16F27 | 5' CCAGAGTTTGATCM*TGGCTCAG 3' |
| 16R1 525XP | 5' TTCTGCAGTCTAGAAGGAGGTGW*TCCAGCC 3' |

*M – A/C; W- A/T

#### 3.2.1.1 Colony PCR (Polymerase Chain Reaction)

Colony PCR was performed in Bio-Rad thermal cycler under the following standardized conditions per reaction.1X PCR buffer, 2mM DNTP's, 1.5mM $MgCl_2$, 10µM forward primer, 10µM reverse primer, 1U/reaction Taq DNA polymerase and a single colony was finally inoculated before subjecting to Thermal cycler with the following conditions.

| | | | |
|---|---|---|---|
| 1. | Initial Denaturation | - | 94°C for 5 min |
| 2. | Denaturation | - | 94°C for 30 sec |
| 3. | Annealing | - | 55°C for 1 min |
| 4. | Primer extension | - | 72°C for 1 min |
| 5. | Repeat steps 2 - 4 for 35 cycles | | |
| 6. | Final extension | - | 72°C for 10 min |
| 7. | Hold | - | 4°C forever |

**3.2.1.2 Agarose gel electrophoresis**

Agarose gel electrophoresis was carried out in order to check the amplified 16S rRNA gene.

1. 0.8% agarose gel was prepared using 1XTAE buffer and the gel was casted on gel casting platform with its comb.
2. After polymerization the comb was removed and the gel with the platform was placed in the electrophoresis tank filled with buffer.
3. 10µL of the amplicon was loaded in the well along with loading dye which was at the cathode.
4. The electrophoresis was carried out at 80V and was stopped when the tracking dye reached two third distance of the gel.
5. Lambda DNA EcoRI + HindIII (Fermentas) double digest was used as marker to find the molecular mass of the amplicon.
6. The gel was stained in 0.5µg/mL ethidium bromide solution for 15 min, followed by destaining in sterile MilliQ water for 10 min and visualized under UV transilluminator at 254nm wavelength (Bio-Rad).

### 3.2.1.3 DNA sequencing

Nucleotide sequencing of the PCR amplicon was performed with ABI 3730*xl* cycle sequencer, using the big dye Terminator kit (Applied Biosystems) at SciGenom Labs Pvt Ltd, Cochin, India.

### 3.2.1.4 Sequence alignment

Forward and the reverse sequences thus obtained after sequencing were aligned to a single length using BioEdit software.

### 3.2.1.5 DNA BLAST (Basic Local Alignment Search Tool)

The aligned sequences were subjected to homology search by comparison with the available nucleotide database in NCBI. The query sequence showing maximum identity with a particular sequence from the database also reveals the organism name as well.

### 3.2.1.6 NCBI genbank submission

The single length aligned sequences were made as a sequin format which is accepted by NCBI. The sequences thus submitted were assigned with NCBI Genbank Accession numbers. The sequences deposited are available in DDJB (DNA Data Bank of Japan) and EMBL (European Molecular Biology Laboratory) databases.

### 3.2.1.7 Phylogenetic tree construction

Phylogentic tree was constructed using similar 16S rRNA gene sequences obtained from NCBI. The partial sequences were aligned using Multiple alignment Clustal X2 programme and neighbour hood joining tree using MEGA 4 software.

## 3.3 GROWTH STUDIES

### 3.3.1 Inoculum preparation

A preculture of the selected bacterial strain was prepared initially by growing the strain in 10mL of Zobell's medium at 37°C for 24h. After growth the culture obtained was centrifuged at 10000 rpm at 4°C for 15 min, under aseptic conditions, and the cells were harvested, washed with brine solution (15% NaCl), and suspended in the same solution. The concentration of the cell suspension was adjusted to 0.2 OD at 600nm and used as inoculums of 2% (v/v).

### 3.3.2 Optimum NaCl concentration, pH and temperature determination

The optimal conditions for maximal growth of the isolates and the minimum and maximum range for these conditions for the isolates to survive were determined by growing the isolates at different conditions (NaCl concentration from 0.5M to 5M, pH between 2-12 and temperatures from 20°C to 60°C) and checking OD at 600nm in a UV-Visible spectrophotometer (Shimadzu, Japan) after 120h of incubation (4 days). Uninoculated medium was maintained as control.

### 3.3.3 Growth curve

100mL of Zobell's medium taken in 250mL conical flask was inoculated with the prepared inoculum and incubated at 42°C which was found to be the optimum temperature. At regular intervals the samples were drawn and growth curve of the isolate BTSH10 was determined by measuring OD at 600nm in a UV-Visible spectrophotometer (Shimadzu, Japan) after 120h incubation (4 days). Uninoculated medium was maintained as control. The generation time and specific growth rate (μ) were calculated (Friedrich, 2010).

## 3.4 ENZYME PROFILING FOR THE CULTURE

Both the cultures BTSH10 and BTSH03 were checked for their enzyme profile as detailed below

### 3.4.1 Protease

Proteolytic activities of the cultures were evaluated in skim milk agar containing 1% (w/v) skim milk, 2% (w/v) agar on Zobell's medium. The culture was inoculated as a spot. Clear zones around the growth after 7 days incubation at 42°C were taken as evidence of proteolytic activity. An uninoculated plate was maintained as control (Kim and Hoppe, 1986).

### 3.4.2 Amylase test

Amylase production was checked according to Kim and Hoppe (1986) on Zobell Marine agar added with 1% (w/v) soluble starch as substrate. The prepared plates were spot inoculated with the prepared inoculum. After 7 days of incubation

at 42°C the plate was flooded with Iodine reagent. A clearing zone formation around the bacterial colony indicate amylase positive. An uninoculated plate was maintained as control.

### 3.4.3 Lipase

Lipase activity was checked according to Kim and Hoppe (1986) on Zobell's Marine agar medium supplemented with 1% Tween 20 (Sorbitol monooleate; w/v). The prepared plates were spot inoculated with the prepared inoculum and incubated for 7 days at 42°C. A clearing zone formation around the bacterial colony indicate lipase positive. An uninoculated plate was taken as control.

### 3.4.4 Gelatinase

Gelatinase activity was tested in Zobell's agar medium containing 0.8% gelatine and 5% agar. Prepared plates were spot inoculated and incubated at 42°C for 7 days. 10% trichloroacetic acid (TCA) was added to the gelatine plate which resulted in very clear halo indicating gelatinase positive. An uninoculated plate was maintained as control (Kim and Hoppe, 1986).

## 3.5 ANTIBIOTIC SENSITIVITY PROFILING

Antibiotic sensitivity of the isolates was determined on Zobell's agar medium using antibiotic discs (Himedia octadiscs). The isolates were checked with 30 different antibiotics and the sensitivity and resistance profile was recorded. Five different octadiscs were used in the study. Cultures were swabbed on the surface of the agar medium and the antibiotic discs were placed on the plates which were incubated at 42°C for 7 days.

### 3.5.1 Multiple antibiotic resistances (MAR) index

Multiple antibiotic resistance index (MAR) was used to check the antibiotic resistance. This calculation was performed according to Saba Riaz et al., (2011). In this study, 31 antibiotics were used and represented as "b", while the number of antibiotics for which the isolate shown resistance was represented as "a" then its MAR is calculated as

$$MAR = a/b$$

**Table.3.2 Octadiscs Antibiotics and concentration**

| S.No | Cat.No | Antibiotics(µg) |
|---|---|---|
| 1 | OD 020 | Cephalothin(30),Clindamycin(2),Co-Trimoxazole(25), Erythromycin(15), Gentamycin(10) , Ofloxacin(1), Penicillin-G(10units),Vancomycin(30) |
| 2 | OD 038 | Bacitracin(10units), Chloramphenicol(30), Penicillin-G(10units) , Co-Trimoxazole(25), Polymyxin-B(300units), Gentamycin(10), Neomycin(30), Tetracyclin(30) |
| 3 | OD 042 | Ceftazidime(30),Ciprofloxacin(30),Cephotaxime(30),Nalidixic acid(30), Nitrofurantoin(30), Norfloxacin(10), Netillin(30), Ofloxacin(5) |
| 4 | OD 258 | Impeneme(10),Meropeneme(10),Ciprofloxacin(5), Tobromycin(10), Moxifloxacin(5), Ofloxacin(5), Sparfloxacin(5) ,Levfloxacin(5) |
| 5 | OD 271 | Ciprofloxacin(5),Ofloxacin(5), Sparfloxacin(5), Gatifloxacin(5), Aztreonam(30) , Azithromycin(15), Vancomycin(30), Doxycycline hydrochloride(30) |

*Numbers in antibiotics indicate concentration in µg

## 3.6 LIPID PROFILING OF THE PRODUCER STRAIN

### 3.6.1 Isolation of Cell wall lipid (Bligh and Dyer, 1959)

1. 5mL of the bacterial culture was pelleted out and the cells were concentrated to 1mL. The cells were transferred to a glass tube and added with 3.75mL of methanol: chloroform (2:1-v/v) mixture, followed by vortexing.
2. 1.25mL chloroform was added, vortexed and then added with 1.25mL distilled water followed by vortexing.
3. The tube was then centrifuged at 1000 rpm for 5 min at room temperature to give a two phase system (upper aqueous phase and bottom organic phase).
4. The bottom phase was recovered using Pasteur pipette without the interference of the aqueous phase.
5. The organic phase was then concentrated and subjected to TLC.
6. The concentrated sample was also purified by using authentic upper phase.

### 3.6.2 Preparation of authentic upper phase for clean preparation of lipids

1. The above procedure was performed using distilled water in place of sample in large and multiple glass tubes. The upper phase was collected and stored.
2. The organic phase obtained from step 4 (section **3.6.1**) was mixed with the authentic upper phase in the concentration in the ratio of 1mL of sample: 2.25mL of authentic upper phase.
3. The tubes were vortexed, centrifuged and the organic phase thus obtained was collected, concentrated and subjected to TLC.

### 3.6.3 Thin Layer Chromatography

Thin layer chromatograpy for the isolated lipid was performed on Silica gel 60 aluminium sheets 20x20 (Merck, Germany). 10µL of the lipid sample was spotted onto the TLC plate about 0.5cm above the lower edge so that the solvent did not touch the sample loaded region. Chloroform: methanol (1:4) was the solvent used with two drops of water mixed to it. Initially after preparation of the solvent front the container was kept close so that the whole container became saturated with the solvent. Later the TLC plate spotted with the sample and dried was kept on the solvent front. Due to capillary action the solvent moved towards the top of the TLC plate where it also moved the samples and got separated according to migration capacity. The plate was dried and subjected to iodine vapours and the $R_f$ values were calculated by

$$R_f \text{ value} = \text{Migration of spot/Migration of solvent}$$

### 3.7 FAME ANALYSIS

The Fatty acid methyl esterase (FAME) analysis was done by using the Sherlock MIS Software with the Agilent technologies 6890 series gas chromatographs which is uniquely designed for FAME analysis and a 25m x 0.2mm phenyl methyl silicone fused silica capillary column. The temperature program ramped from 170°C to 270°C at 5°C per minute where the total GC run time was for 22 min. The detector used was a flame ionization detector which provided more sensitivity and allowed for a larger dynamic range. The carrier gas used was hydrogen whereas nitrogen was used as the “makeup” gas, and air was used to support the flame. The electronic signal from the GC detector was converted into a chromatogram which indicated the ECL values. Initially a

standard mixture of straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxy acids was used to calibrate the Sherlock MIS system. These compounds functioned quality control checks and the retention time data obtained from injecting the calibration mixture was converted to Equivalent Chain Length (ECL) data for bacterial fatty acid naming. The ECL value for each fatty acid was derived as a function of its elution time in relation to the elution times of a known series of straight chain fatty acids.

### 3.7.1 Reagents for FAME analysis

**Reagent 1**

45g sodium hydroxide

150mL methanol

150mL distilled water

**Reagent 2**

325mL 6.0N hydrochloric acid

275mL methyl alcohol

**Reagent 3**

200mL hexane

200mL methyl tert-butyl ether

**Reagent 4**

10.8g sodium hydroxide

900mL distilled water.

### 3.7.2 Sample processing

**3.7.2.1 Harvesting:** A 4mm loop was used to harvest about 40mg of bacterial cells from the third quadrant (second or first quadrant if slow growing) of the quadrant streaked plate. The cells were placed in a clean 13x100 culture tube.

**3.7.2.2 Saponification**: 1.0mL of Reagent 1 was added to each tube containing cells. The tubes were securely sealed with teflon lined caps, vortexed briefly and heated in a boiling water bath for 5 min, at which time the tubes were vigorously vortexed for 5-10 sec and returned to the water bath to complete the 30 min heating.

**3.7.2.3 Methylation:** The cooled tubes were uncapped and 2mL of Reagent 2 was added. The tubes were capped and briefly vortexed. After vortexing, the tubes were heated for 10 ± 1 min at 80 ± 1°C. (This step is critical in time and temperature.)

**3.7.2.4 Extraction**: Addition of 1.25mL of Reagent 3 to the cooled tubes was followed by recapping and gentle tumbling on a clinical rotator for about 10 min. The tubes were uncapped and the aqueous (lower) phase was pipetted out and discarded.

**3.7.2.5 Base Wash:** About 3mL of Reagent 4 was added to the organic phase remaining in the tubes. The tubes were recapped and tumbled for 5 min. Following uncapping, about 2/3 of the organic phase was pipetted into a GC vial which was capped and ready for analysis.

**3.7.3 Sample analysis**

GC, computer analysis and ECL values were derived from the chromatogram obtained.

## 3.8 CHARACTERIZATION OF SALT CRYSTAL FORMED BY BTSH10

The bacteria showed formation of salt crystals of different size and shapes during growth in the medium. The salt crystals were washed with alcohol and then with water aseptically, and inoculated in Zobell's medium and incubated at 42°C for 7days. Growth of BTSH10 in medium confirmed that bacteria were embedded inside the crystal. The salt crystal formed by the bacteria was characterized.

### 3.8.1 Scanning electron microscopy (SEM)

Scanning electron microscopy was performed in order to confirm the presence of haloarchaea inside the crystal.

### 3.8.2 Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES)

The elemental analysis of the crystal was carried out using Thermo Electron IRIS INTREPID II XSP DUO analyzer since the spectra obtained will show clearly the presence of different element and its concentration.

### 3.8.3 Powder X-ray diffraction analysis

Powder X-ray diffraction studies were carried out to demonstrate the crystallinity using Rich-Seifert X-ray diffractometer employing Bruker AXS D8 Advance with Ni filtered Cu-Kα radiations of Wavelength (1.5406 Å) in the range 10–80°C with a scan speed of 2°min.

### 3.8.4 FTIR Study

Fourier transform infrared radiation of crystal was carried out in the middle IR between 4000 and 400cm$^{-1}$ using a Thermo Nicolet, Avatar 370 Spectrophotometer.

### 3.8.5 UV-vis-NIR study

To determine the transmission range and to know the suitability of the crystals for optical applications, the UV–vis–NIR spectra were recorded in the range of 190–2500nm.

### 3.8.6 TG/DTA - Thermogravimetric and Differential Thermal Analysis

The thermal behavior of grown sample was studied using TA instrument Q600 SDT and Q20 DSC model and the melting point and thermal stability of the salt crystals were determined.

## 3.9 OPTIMIZATION OF BIOPROCESS VARIABLES FOR HALOCIN PRODUCTION BY STRAIN BTSH10.

### 3.9.1 Halocin assay

The halocin activity was determined using serial two-fold critical end point dilutions to extinction (Meseguer *et al.*, 1986) and expressed as arbitrary units (AU), which are defined as the reciprocal of the first dilution at which all trace of inhibitory activity disappears (Cheung *et al*., 1997). The two fold dilution

ratio of halocin follows a geometric progression where the halocin activity can be calculated by

$$a_n = a_1.q^{n-1}$$

where "a" denotes the scale factor, "q" is the common ratio and "n" being the first dilution at which all trace of inhibitory activity disappears.

### 3.9.2 Selection of media for halocin production

Medium that supported maximal halocin production was selected from among six different media namely Eimhjellen medium (EM) (Catherine *et al*., 2001), Sehgal and Gibbons (SG) (Sehga l and Gibbons, 1960), MH medium (Ventosa *et al*., 1982), HE medium (Torreblanca *et al*., 1986; Catherine *et al*., 2001) DSM 97 (DasSarma *et al*., 1995) and Zobell's medium (Hi-media).

### 3.9.3 Medium preparation

An aliquot of 40.25g of Zobell's medium was mixed with 800mL of distilled water in a beaker and the final Sodium Chloride concentration was adjusted to 15%. Total volume of medium was made upto to 1000mL and dispensed as 100mL in a 250mL Conical flasks and autoclaved.

### 3.9.4 Inoculum preparation

A preculture of the selected bacterial strain that showed halocin activity was prepared initially by growing the strain in 10mL of Zobell's medium at 37°C for 24h. After growth the culture obtained was centrifuged at 10000 rpm at 4$^{o}$C for 15 min, under aseptic conditions, and the cells were harvested, washed with brine solution (15% NaCl), and suspended in the same solution. The concentration of

the cell suspension was adjusted to 0.2 OD at 600nm and used as inoculums of 2% (v/v).

### 3.9.5 Optimization of variables

Various constituents of the selected medium and process parameters that influence halocin production by BTSH10 was optimized by adopting 'one factor at a time' approach. Strategy adopted for the optimization was to evaluate the effect of each variable for its optimum level for maximal halocin production, and incorporate the same variable at its optimized level in the subsequent experiment while evaluating the next variable. The variables studied included the following in the sequential order: Incubation temperature (27°C - 47°C), pH (2 - 13), NaCl concentration (0.5M - 4M), carbon sources at 0.1M concentrations (dextrin, galactose, fructose, lactose, sucrose, sorbitol, xylose, maltose and glycerol), nitrogen source at 1% (w/v) concentration (peptone, yeast extract, malt extract, soybean meal, tryptone, casein, urea and beef extract) and different inorganic salts at 0.1M concentration (ammonium nitrate, sodium fluorate, sodium silicate, potassium chloride, magnesium chloride, calcium chloride, sodium bicarbonate, potassium bromide and strontium chloride), agitation (50 rpm- 250 rpm), and incubation time (0-144h). Preparation of inoculum, inoculation and culture conditions were same as mentioned earlier unless otherwise mentioned.

#### 3.9.5.1 Optimization of Incubation temperature

Optimal incubation temperature for maximal halocin production was determined by incubating the inoculated medium at various temperatures *viz.*, 27°C, 32°C, 37°C, 42°C, 47°C at pH 7.4, 3M NaCl, 150 rpm, 2% (v/v) inoculum concentration and 96h of incubation.

### 3.9.5.2 Optimization of pH

Optimal pH for maximal halocin production was determined by adjusting the pH of the medium to various levels (i.e. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) and incubating the inoculated medium at 42°C, 3M NaCl, 150 rpm, 2% (v/v) inoculum concentration and 96h of incubation.

### 3.9.5.3 Optimization of NaCl concentration

Optimal NaCl concentration for maximal halocin production was determined by addition of 0.5M, 1M, 1.5M, 2M, 2.5M, 3M, 3.5M and 4M NaCl to the media and incubating the inoculated medium at 42°C, pH 8.0, 150 rpm, 2% (V/V) inoculum concentration and 96h of incubation.

### 3.9.5.4 Effect of different carbon source

The effect of different carbon sources on halocin production was determined by addition of 0.1M concentrations dextrin, galactose, fructose, lactose, sucrose, sorbitol, xylose, maltose and glycerol to the medium and incubating the inoculated medium at 42°C, pH 8.0, 3M NaCl, 150 rpm, 2% (v/v) inoculum concentration and 96h of incubation.

### 3.9.5.5 Effect of different nitrogen source

The effect of different nitrogen sources on halocin production was determined by addition of 1% (w/v) concentration peptone, yeast extract, malt extract, soybean meal, tryptone, casein, urea and beef extract to the medium and

incubating the inoculated medium at 42°C, pH 8.0, 3M NaCl, galactose, 150 rpm, 2% (v/v) inoculum concentration and 96h of incubation.

**3.9.5.6 Effect of different inorganic salts**

The effect of different inorganic salts on halocin production was determined by addition of 0.1M concentration of ammonium nitrate, sodium fluorate, sodium silicate, potassium chloride, magnesium chloride, calcium chloride, sodium bicarbonate, potassium bromide and strontium chloride at 42°C, pH 8.0, 3M NaCl, galactose, beef extract, 150 rpm, 2% (v/v) inoculum concentration and 96h of incubation.

**3.9.5.7 Effect of agitation**

The effect of agitation rates on halocin production was determined by incubating the the inoculated medium on a orbital shaker at different rpm (50, 100, 150, 200, 250) at 42°C, pH 8.0, 3M NaCl, galactose, beef extract, calcium chloride, 2% (v/v) inoculum concentration and 96h of incubation.

**3.9.5.8 Time course experiment for halocin production**

A time course experiment was performed in order to ascertain the rate of halocin production at optimized conditions which included at 42°C, pH 8, 3M NaCl, galactose, beef extract, calcium chloride, 2% (v/v) inoculum cocnentration and 200 rpm. Samples were drawn at 8h inverval and assayed for halocin production by two fold serial dilution method and the cell concetration was checked by measuring OD at 600nm in a UV-VIS Spectrophotometer (Schimadzu, Japan).

## 3.10 PURIFICATION AND CHARACTERIZATION OF HALOCINSH10

### 3.10.1 Acetone precipitation

Culture supernatant obtained after centrifugation at 10000 rpm for 10 min at 4$^{o}$C was subjected to acetone precipitation initially. Four volumes of ice cold acetone was added to one volume of supernatant and incubated at -20$^{o}$C (in a deep freezer) and then the contents were centrifuged at 10000 rpm for 15 min at 4$^{o}$C to separate the precipitate as pellet. After decanting the supernatant, the above said step was repeated with the pellet. Later the pellets in the tubes were air dried and dissolved with 0.05M Tris-HCl pH 8.0. The dissolved content was checked for halocin activity.

### 3.10.2 Fractionation by molecular weight cut off centrifugal concentration

The acetone precipitated product was further subjected to fractionation with 30kDa molecular weight cut off centrifugal concentration using Amicon ultra tubes (Millipore, Ireland. Ltd) towards concentrating size specific biomolecules present in the acetone precipitated product. Both the supernatants obtained in the upper and the lower phases were checked for halocin activities and the positive phase was taken for further purification by gel filtration chromatography.

### 3.10.3 Gel filtration Chromatography

Gel filtration chromatography was performed using sephadex G50 (Sigma-Aldrich) column packed in Bio-Rad column of 55cm height and 1cm diameter.

### 3.10.3.1 Preparation of column

a. 5g of sephadex G50 (Sigma-Aldrich) was suspended in 0.05M Tris-HCl pH 8.0 and hydrated for 15 min at 80°C in a water bath and the fine particles were removed.
b. To the hydrated sephadex suspension some more buffer was added.
c. The glass column was filled with the column material without air bubble formation maintaining a slow flow rate so that the column settled under gravity.
d. The column was equilibrated by allowing 450mL of 0.05M Tris-HCl pH 8.0 buffer to pass through the column.

### 3.10.3.2 Sample preparation and application on the column

A total volume of 1.6mL of concentrated 30kDa molecular weight cut off centrifugal tube subjected active sample was loaded on to a packed column (55cmx1cm). The sample was completely dissolved and applied on column. Initial 10ml of the buffer was collected as a single fraction and then fractions of 1 mL/4 min were collected and OD of the fractions were checked at 280nm. Halocin activities in the fractions were checked and all the fractions having the activities were pooled together. Later the pooled fractions were once again subjected to acetone precipitation towards concentrating the final sample to a reduced volume. One part was taken for HPLC analysis for checking the purity and the other for N-terminal protein sequencing.

### 3.10.4 High Performance Liquid Chromatography (HPLC)

The acetone precipitated culture supernatant, amicon ultra molecular weight cut off centrifugation subjected sample and the pooled fractions obtained after gel filtration chromatography were subjected to HPLC to check the purity of the positive fractions. HPLC was performed in Shimadzu preparative HPLC unit with C18 column. Acetonitrile (ACN) /Water were used as gradient with 0.1% Trifluroacetic acid (TFA). Further, samples obtained after initial acetone precipitation of crude supernatant and that obtained after fractionation by 30kDa molecular weight cut off centrifugal concentrator were also subjected to HPLC in order to check their purity by comparison of the chromatograms obtained.

### 3.10.5 FTIR study

Fourier transform infrared radiation of acetone precipitated Zobell's broth and acetone precipitated culture supernatant was performed in the middle IR between 4000 and 400$cm^{-1}$ using a Thermo Nicolet, Avatar 370 Spectrophotometer.

### 3.10.6 TRICINE SDS-PAGE

The purified halocin samples were further subjected to Tricine SDS PAGE (Schägger and von Jagow, 1987) performed on Bio-Rad protein gel electrophoresis system. Acetone precipitated and gel filtration fractions were mixed separately with loading dye containing sample buffer (prepared with Glycerol, SDS, β-mercaptoethanol, Tris-HCl and Bromophenol blue). The prepared samples were heated and loaded on to 15% Tricine Polyacrylamide gel, and the molecular weight and the purity of the loaded samples were determined. Staining was performed using Coomassie brilliant blue stain. The protein bands

were analysed by subjecting the gel to Quantity One software in Bio-Rad densitometer (GS800).

**3.10.6.1 Reagents for Tricine-SDS PAGE**

Glycerol (SRL)

Tetramethylethylenediamine (TEMED; Sigma)

Mercaptoethanol (Sigma)

Ammonium persulfate (APS; Sigma)

**Stock Acrylamide solution(AB solution)**

48g of acrylamide and

1.5g of bisacrylamide

100mL of water

Stored at 4°C in amber coloured bottle

**Reducing sample buffers**

**Buffer A**

12% SDS (w/v)

6% mercaptoethanol (v/v)

30% glycerol (w/v)

0.05% Coomassie blue G-250

150mM Tris/HCl (pH 7.0)

**Buffer A/4**

Buffer A diluted with 3 volumes of water (for dissolving protein pellet)

**Non-reducing sample buffers**

**Buffer B**

12% SDS (w/v)

30% glycerol (w/v)

0.05% Coomassie blue G-250

150mM Tris/HCl (pH 7.0)

**Buffer B/4**

Buffer B diluted with 3 volumes of water (for dissolving protein pellet)

**Protein staining solution**

100mg Coomassie brillinant blue (0.1%)

40mL methanol (40%)

10mL Glacial acetic acid

50mL distilled water

**Destaining solution**

40mL methanol (40%)

10mL Glacial acetic acid (10%)

50mL distilled water

**Fixing solution**

50% methanol

10% acetic acid

100mM ammonium acetate

**Electrode and gel buffers for Tricine–SDS-PAGE**

**Anode buffer (10X)**

1M Tris

0.225M HCl

pH 8.9

**Cathode buffer (10X)**

1M Tris

1M Tricine

1% SDS

pH ~8.25

**Gel Buffer (3X)**

3M Tris

1M HCl

0.3% SDS

pH 8.45

**Gel preparation**

**Stacking gel (4%)**

1mL AB solution

3mL Gel buffer (3X)

12mL distilled water

90µL APS (10%)

9µL TEMED

**Separating gel (16%)**

10mL AB solution

10mL Gel buffer (3X)

3g glycerol

30mL distilled water

100µL APS (10%)

10µL TEMED

**Protein Markers for SDS-PAGE**

**Low molecular weight protein marker (Bio-Rad)**

| **Components** | | **MW** |
|---|---|---|
| Rabbit muscle phosphorylase b | - | 97.4kDa |
| Bovine serum albumin(BSA) | - | 66.2kDa |
| Hen egg white oavlbumin | - | 45.0kDa |
| Bovine carbonic anhydrase | - | 31.0kDa |
| Soybean trypsin inhibitor | - | 21.5kDa |
| Hen eggwhite lysozyme | - | 14.4kDa |

**3.10.6.2 PROCEDURE**

1. The gel plates were cleaned and assembled.
2. *Resolving gel*- All components required for stacking gel were mixed except APS and TEMED which were mixed finally and immediately poured into the cast and overlaid with water and left to stand for at least 30 min for polymerizing and the water was removed before casting with separating gel.
3. *Separating gel*- The polymerized resolving gel was overlaid with separating gel which was prepared in a beaker by finally adding APS and TEMED. Soon after pouring the separating gel a comb was inserted between the glass plates. It was left for at least 30 min for polymerization.
4. Gel was placed in the electrophoresis apparatus, and then the upper and lower reservoirs were filled with buffer for Tricine SDS-PAGE.
5. The gel was pre run for 1h at 80V.
6. The protein sample was loaded in the gel and was run at 80V till the dye front entered the resolving gel thereafter the gel was run at 100V.
7. The electrophoresis was stopped when the dye front reached 1cm distance from the lower end of the plate.
8. The gel was removed from the cast and incubated in fixing solution for 30 minutes.
9. It was subjected to staining for at least one hour in staining solution (twice the length of time used for fixing).
10. The gel was destained in destaining solution until clear bands were visible and observed under transilluminator.

**3.10.7 Bioautography assay**

Purified halocin samples were also subjected to bioautographic assay in order to reconfirm their halocin activity. The unstained Tricine SDS PAGE gel

after completing the electrophoretic separation was subjected to bioautography assay. The gel was first washed with sterile 0.1% TritonX100 solution to remove SDS from the gel and then aseptically overlaid with top agar (Zobell's medium with 15% NaCl and half strength agar) containing the indicator strain. The plates were incubated at 42°C and checked for zone formation. The results were compared with that of the gel which was stained.

### 3.10.8 Cell lysis assay

Activity of halocin SH10 on the cells of indicator bacteria *Halorubrum* sp BTSH03 was studied by monitoring the cell lysis under phase contrast microscope. Stationary phase cells of indicator strain BTSH03 was mixed with Halocin SH10 (1024 AU/mL) on a microtitre plate and incubated at different time intervals. Samples were drawn at regular intervals and observed under phase contrast microscope.

### 3.10.9 MALDI

MALDI analysis of the purified halocin sample was performed on an Ultraflex TOF/TOF model, Bruker Daltonics Germany with Flex Analysis 2.0, Bruker software in linear mode with N2 Laser, 337nm, 50Hz and average of 800 shots at Centre for DNA and fingerprinting and diagnosis (CDFD), Hyderabad, India. Saturated solution of matrix (Sinnapinic Acid) was prepared with 50% ACN /water with 0.1% TFA. Equal volume of sample (trypsin digested protein gel) and matrix (1µL each) were mixed on a centrifuge tube cap and spotted on a MALDI plate (MTP 384 ground steel, Target plate-Bruker). The Protein finger print was obtained on applying the m/z values obtained to MASCOT analysis.

### 3.10.10 Total amino acid analysis

The total aminoacid content of halocin SH10 was determined using Shimadzu LC-2010HT system. 10µL of the concentrated sample was loaded on the column. The chromatogram obtained was compared with the standard amino acid chromatogram and the presence and concentration of different amino acids were noted.

### 3.10.11 NMR of halocin SH10

1mg of the precipitated protein sample was mixed with 0.5mL $D_2O$ and the samples were subjected to NMR analysis. NMR experiments were performed on a Bruker AVANCE III 400 spectrometer operating at a proton frequency of 400.12MHz and a $^{13}C$ frequency of 100.62 MHz in $CDCl_3$. Proton acquisition were recorded with 900 pulse of 4 us,64 scan. $^{13}C$ experiment was recorded with a 900 pulse of 8.5 us, 1024 scan.

### 3.10.12 N-Terminal protein sequencing

Once the purity of the samples was confirmed by HPLC they were subjected to N-Terminal Protein sequencing. Automated Edman Degradation was carried out using an Applied Biosystems 494 Procise Protein Sequencing system at National facility for protein sequencing, Indian Institute of Technology, Mumbai, India.

### 3.10.13 Thermostability of halocin

The stability of halocin at different temperatures was checked by incubating 4096AU of halocin for one hour at different temperatures *viz.*, 4, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C and testing its residual activity.

### 3.10.14 pH stability of halocin

The stability of halocin at different pH was checked by incubating 4096AU of halocin for one hour in different buffers with different pH. The halocin was initially acetone precipitated and the pellet obtained was mixed with different buffer systems of different pH ranging from 2-10. HCl-KCl buffer-pH 2.0, citrate-phosphate buffer-pH 3.0 to 6.0, phosphate buffer-pH 7.0, Tris-HCl buffer- pH 8.0 & 9.0 and carbonate-bicarbonate buffer- pH 10.0

### 3.10.15 Solvent stability of halocin

Stability of halocin in different solvents were studied using acetone, ethanol, methanol, DMSO, hexane, benzene, butanol, acetonitrile, isopropanol and chloroform at various concentrations (10%, 20% and 30% (v/v)). The solvent was mixed with 4096AU halocin and incubated at room temperature for an hour. The tubes were then centrifuged and the pellet was dissolved with buffer and the activity of halocin was checked by two fold serial dilution method.

## 3.11 APPLICATION STUDIES

### 3.11.1 Storage of Raw Hides

Raw hides are stored by applying raw salts before processing. When they are stored for a long time the halophilic /salt tolerant lipolytic and proteolytic bacteria colonize the hides and form small pores and also lead to putrefaction. Formation of such pores on the hides does not contribute to the production of quality leather products. Application of halocin as a preservative agent may reduce the number of bacteria on the hides and will help in storage of raw hides for a long time and to maintain quality in leather. Hence the study was conducted.

#### 3.11.1.1 Evaluation of halocin SH10 as preservative for leather hides

Evaluation of halocin SH10 as preservative for storage of leather hides was done by subjecting the leather hides to halocin treatment. Hides were cut in to 4 pieces of 1cmx1cm size. One piece was incubated in 30% brine solution for 30minutes with constant shaking and 100µL was plated on Zobell's agar plate supplemented with a total of 15% NaCl. Remaining three pieces were incubated in halocin for 3h, 6h and 12h respectively and later analysed for CFU on Zobell's agar medium. The number of CFU formed on the Zobell's agar medium (15% NaCl) was compared with that of the CFU formed from the hide without halocin treatment. The plates were wrapped inside a plastic cover in order to maintain humidity and incubated at 42°C for 10 days. This temperature was selected because when hides are stored one on the other it generates heat and the temperature is around 43°C which is an apt temperature for the bacteria to divide.

### 3.11.2 *In vitro* anticancer studies of halocin SH10

#### 3.11.2.1 *In vitro* short term toxicity studies

Dalton's Lymphoma Ascities (DLA) method (Unnikrishnan and Ramadasan Kuttan, 1988) was used to study the short term toxicity study. This test relies on a breakdown of membrane integrity determined by the uptake of a dye such as tryphan blue, erythorisin and nigrosin to which the cell is normally impermeable.

##### 3.11.2.1.1 Procedure

DLA cells (1.0 x $10^5$ cells/mL) were injected intraperitoneally to mice and cultured. After 12-15 days of culturing the peritoneal fluid was drawn out using sterile syringe and washed with Hanks balanced salt solution (HBSS) and centrifuged for 10-15 min at 1,200 rpm. The cells were washed thrice and finally the pellet was resuspended in known quantity of HBSS so that the cell count is adjusted to $2x10^6$ cells/mL. 0.1 mL of cells was aliquoted into eppendorf tubes and halocin was added at different concentration as 32AU, 64AU, 128AU, 256AU, 512AU, 1024AU, 2048AU and 4096AU and incubated at 37ºC for 3h. After incubation equal volume of tryphan blue (0.4%) was mixed and left for one minute. It was then loaded onto a haemocytometer and the viable and non-viable cells were counted and recorded. Viable cells seem to be colourless as they have intact cell membrane, whereas dead cells take up colour and appear as a dot. The percentage growth inhibition was calculated and $CTC_{50}$ value was generated from the dose-response curves.

$$\% \text{ growth inhibition} = 100 - \left[ \frac{\text{Total cells - Dead cells}}{\text{Total cells}} \right] X\ 100$$

### 3.11.2.2 *In vitro* cytotoxicity studies

A novel anticancer drug should not possess cytotoxicity against normal cell lines even at higher concentrations and should possess cytotoxicity at low concentration against cancerous cell lines (Masters, 2000). All the proteins isolated were tested for cytotoxicity by MTT assay. The experiments were performed at Department of Virology, Kings Institute of Health Sciences, Chennai, Tamilnadu, India. Testing for cytotoxicity in human cells lines for new drugs started since 1946 when chemotherapy began. Each and every drug that are told to have anticancerous activity is checked on 60 different human tumor cell lines (pre-clinical test) at the National Cancer Institute before being introduced as drug.

#### 3.11.2.2.1 Sample Preparation

Halocin SH10 was diluted to 10mL with MEM/DMEM, at pH 7.4 and supplemented with 2% inactivated FBS/NBCS (maintenance medium) and maintained as stock. It was filter sterilized and stored at -20ºC till use. Serial two fold dilution of the sample was prepared from the stock solution to obtain lower concentrations.

### 3.11.2.2.2 Cell culture maintenance

HBL100 (Normal Cell line), HeLa (Cervical Cancer), A549 (Lung carcinoma), Hep2 (Larynx Carcinoma) and OAW42 (Ovary cancer Cells) were the cell lines used and they were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. They were maintained in Dulbecco's Modified Eagles medium supplemented with 2mM L-glutamine and Earle's BSS containing 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids and 1.0mM of sodium pyruvate. Penicillin and streptomycin (100 IU/100µg) were adjusted to 1mL/L. Cells were maintained at $37^0$C with 5% $CO_2$ atmosphere.

### 3.11.2.2.3 Cell viability

The viability of the cells was assessed by trypan blue dye exclusion test (Chakraborty *et al.*, 2004). The numbers of stained (non-viable) and unstained (viable) cells were counted using a haemocytometer (Improved Neubauer Brightline, USA).

### 3.11.2.2.4 Determination of mitochondrial synthesis by MTT assay

This assay is based on the reduction of tetrazolium salt, 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), to an insoluble blue formazan (purple) derivative by living cells products. The MTT is cleaved by mitochondrial enzyme succinate dehydrogenase. The number of cells is known to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986). MTT assays are usually done in the dark since the reagents is sensitive to light.

### 3.11.2.2.5 Procedure

The monolayer cell culture was trypsinized and the cell count adjusted to 1.0 x $10^5$ cells/ mL using MEM/DMEM medium containing 10% NBCS. To each well of the 96 well microtitre plate, 0.1mL of the diluted cell suspension (approximately 10,000 cells) was added in triplicate. After 24h, when a partial monolayer was formed, it was washed twice with medium and remaining supernatant was discarded. Halocin SH10 (100µL each) was added to the microtitre plates containing monolayer and the plates were incubated at 37ºC for 3 days in 5% $CO_2$ atmosphere. The cells were observed every 24 hours for 72 hours under microscope and observations were noted. 50µL of MTT in MEM – PR (Minimum essential medium without phenol red) was added to each well after removing the excess supernatant. The plates were gently shaken and incubated for 3h at 37ºC in 5% $CO_2$ atmosphere. 50µL of propanol was added removing the additional supernatant present in the well, the plates were gently shaken to solubilize the formed formazan. The absorbance was read using ELISA multiwell plate reader (Thermo Multiskan EX, USA) at 540nm. The percentage growth inhibition was calculated using the following formula and $IC_{50}$ (concentration of drug or test sample needed to inhibit cell growth by 50%) values were generated from the dose-response curves for each cell line. The relative viability of the treated cells as compared to the control cells was expressed as the % cytoviability, using the following formula (Sukirtha *et al.,* 2011).

$$\text{Percentage of viability} = \left[ \frac{\text{OD of experimental sample}}{\text{OD of experimental control(untreated)}} \right] \text{X } 100$$

$IC_{50}$ was then determined by the corresponding dose response curve.

### 3.11.3 *In vivo* anticancer studies

#### 3.11.3.1 Selection and maintenance of animals

Healthy adult Swiss albino mice weighing 25-30g were obtained from the animal house of J.S.S. College of Pharmacy, Ootacamund, India, and were maintained under standard environmental conditions (22-28°C, 60-70% relative humidity 12h dark/light cycle) and fed with standard rat feed (Amrut Rat Feed, Nav Maharasthra Chakan Oil Mill Ltd., Pune, India) and water *ad libitum*. The animals were housed in large spacious hygienic cages during the course of the experimental period. The experiments were performed at the Department of Pharmacology, JSS College of Pharmacy, Ootacamund, India. as per the guidelines of CPCSEA, Chennai, India. (Approval no: JSSCP/IAEC/Ph.D., / PHYTOPHARM /01/2010-11)

#### 3.11.3.2 Preparation of standard and sample

Standard 5-Flurouracil (5-FU) was suspended in distilled water using sodium carboxy methyl cellulose (0.3%) and Halocin SH10 was used as sample.

#### 3.11.3.3 Effect of halocin SH10 on mice bearing Dalton's Lymphoma Ascities (DLA) cells.

DLA (Dalton's Lymphoma Ascites) cells were procured from Amala Cancer Institute, Amala nagar, Trissur, Kerala, India. The cells were maintained *in vivo* in Swiss albino mice intraperitoneally. Swiss Albino mice were divided into nine groups with six animals in each group. All the animals were injected with DLA cells ($1 \times 10^6$ cells) intraperitoneally except the normal group. This was

taken as day zero. Group I served as normal control and group II as tumor control. These two groups received sodium CMC suspension (0.3%) administered orally. Group III served as a positive control and was treated with standard 5-Flurouracil (5-FU) at 20 mg/kg p.o. Group IV and V were treated with halocin SH10 at a dose of 1024 and 512AU respectively. The treatments were given 24h after the DLA cells were inoculated, once daily for 10 days. On day11, blood was collected from the animals by retro-orbital puncture under mild anesthesia and was subjected to hematological parameters such as RBC, total WBC, differential count and hemoglobin estimation.

**3.11.3.4 Statistical Analysis**

The significance of all the *in vivo* data was analyzed by One-Way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Value of ($p < 0.05$) was considered as statistically significant.

# *Chapter 4*

## RESULTS

### 4.1 SCREENING AND SELECTION OF HALOCIN PRODUCING HALOARCHAEA

#### 4.1.1 Screening and selection of halocin producer and the indicator

Soil samples obtained from a salt pan from Thamaraikulam, Kanyakumari district, Tamilnadu were screened to isolate halocin producing haloarchaea and an indicator strain. The salt pan soil samples were analysed for their ionic composition. It was observed that the soil samples in general contained high levels of ions of various salts as shown in Table 4.1.

From the salt pan soil samples 57 isolates were obtained and all of them were purified and assigned with the identification code BTSH series 1 to 57. Pure cultures of the isolates were stored under sterile mineral oil as stock culture at room temperature ($28 \pm 2^{o}C$) until their use for further studies. Preliminary screening enabled selection of four isolates namely BTSH02, BTSH06, BTSH10 and BTSH15 as potential halocin producers.

**Table 4.1. Ionic composition of the saltpan sample used to isolate halocin producing strain**

| Ionic composition | Concentration ±SD(mg/mL) |
|---|---|
| $Na^+$ | 62.1 ± 0.57 |
| $K^+$ | 2.022 ± 0.05 |
| $Mg_2^+$ | 19.4 ± 0.63 |
| $Ca_2^+$ | 1.2 ± 0.25 |
| $HCO_3^-$ | 2.1 ± 0.39 |
| $Cl^-$ | 30.8 ± 0.72 |
| $So_4^{2-}$ | 4.1 ± 0.03 |
| Total soluble salts | 126 ± 0.93 |
| pH | 7.6 |
| EC (dS/m) | 139.80 |

Among the four isolates obtained as halophiles from the saltern pond the Haloarchaeal strain BTSH10 was found to show strong halocin activity and hence it was selected for further studies. The selected strain was observed to require high concentration of NaCl for its growth and hence the medium was supplemented with 15% NaCl after standardization of optimal NaCl for growth. Initially BTSH10 was found to produce halocin when the bacterial cells were incubated in Zobells agar medium supplemented with 15% NaCl at 37°C for 96h.

It was noted that screening by spot inoculation method and agar well diffusion method facilitated maximum zone of inhibition of halophiles by the halocin. The culture whose supernatant recorded maximal inhibition was selected as the producer strain. The strain BTSH03 was found to record maximal

sensitivity against the halocin produced by BTSH10 and hence was considered as the indicator organism.

Halocin production by the strain BTSH10 was reconfirmed after recording the inhibition zone of the indicator bacteria on addition of 10µL of culture supernatant on top of the double agar containing indicator strain. Further to confirm the activity, 80µL of BTSH10 cell free supernatant was added in a agar well on a Zobell's agar plate layered with indicator strain BTSH03 on the top and incubated at 42℃ for 7 days. This resulted in the formation of 3.6cm zone of inhibition (Fig 4.1). Further the halocin produced by BTSH10 also showed activity against other halophilic strains *Halobacillus sp.* HUC 1, *Thallassobacillus sp*. HUC7, *Marinobacter sp.* HUC8, *Salimicrobium sp*. BTMT10, *Chromohalobacter sp.* BTMT11 and *Halomonas sp*. BTMT12 which were isolated from tannery effluents and edible salt.

Out of the six different media used for isolation, Zobell's medium was selected as the best since it showed maximal biomass and halocin production when compared to other media used in the study. The halocin produced by BTSH10 was named as halocin SH10.

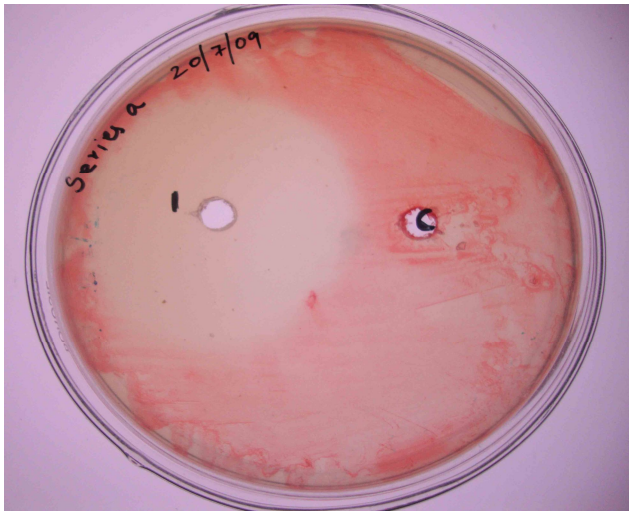



**Fig 4.1. Preliminary screening on Zobell's agar medium (15% NaCl):** 1. BTSH10 cell free supernatant in well show zone of inhibition against BTSH03. C.Control well contained sterile Zobell's broth.

### 4.1.2. Identification of the halocin producer and indicator strain

The halocin producing strain BTSH10 and the indicator strain BTSH03 were identified respectively as *Natrinema* sp. and as *Halorubrum* sp. based on their morphological, biochemical, physiological characteristics and molecular ribotyping. Characteristics of strains BTSH10 and BTSH03 are presented in Table 4.2

**Table 4.2. Characteristics of strains BTSH10 and BTSH03**

| Characteristic | BTSH10 | BTSH03 |
|---|---|---|
| Colony shape | Round ,smooth | Round ,smooth |
| Pigmentation | Pale orange | Dark red |
| Cell shape | Pleomorphic rods | Pleomorphic |
| Grams nature | Negative | Negative |
| Motility | Non-motile | Non-motile |
| Minimum NaCl concentration required for survival | 1.0M | 1.0M |
| Optimal NaCl concentration required for growth | 3.0M | 3.0M |
| NaCl range supporting growth | 1.0M - 5.2M | 1.0M - 5.2M |
| pH range | 5.5-9.0 | 5.0-9.5 |
| pH optimum | 6.0-8.0 | 6.0-8.0 |
| Temperature range(°C) | 20°C-70°C | 20°C-60°C |
| Temperature optimum(°C) | 32°C-47°C | 32°C-47°C |
| Indole | – | – |
| Nitrate reduction | + | + |
| Citrate Utilization | – | – |
| Catalase | + | + |
| Oxidase | + | + |
| Hydrolysis of | | |
| Tween 20 | + | + |
| Tween 80 | + | + |
| Gelatin | + | + |
| Starch | + | + |

| | | |
|---|---|---|
| Casein | + | + |
| Carbohydrate utilization | | |
| Glucose | + | - |
| Mannose | - | - |
| Galactose | + | - |
| Fructose | - | - |
| Maltose | + | - |
| Sucrose | + | - |
| Lactose | - | - |
| Glycerol | + | - |

- = negative

+= positive

#### 4.1.2.1 Single colony visualization under scanning electron microscopy (SEM)

The single cells of the strains BTSH10 (Fig 4.2) and BTSH03 (Fig 4.3) were visualized under scanning electron microscopy and the cell shapes were noted. The SEM photomicrographs also clearly showed excess amount of lipid accumulation around the cells besides accumulation of salts as crystals.

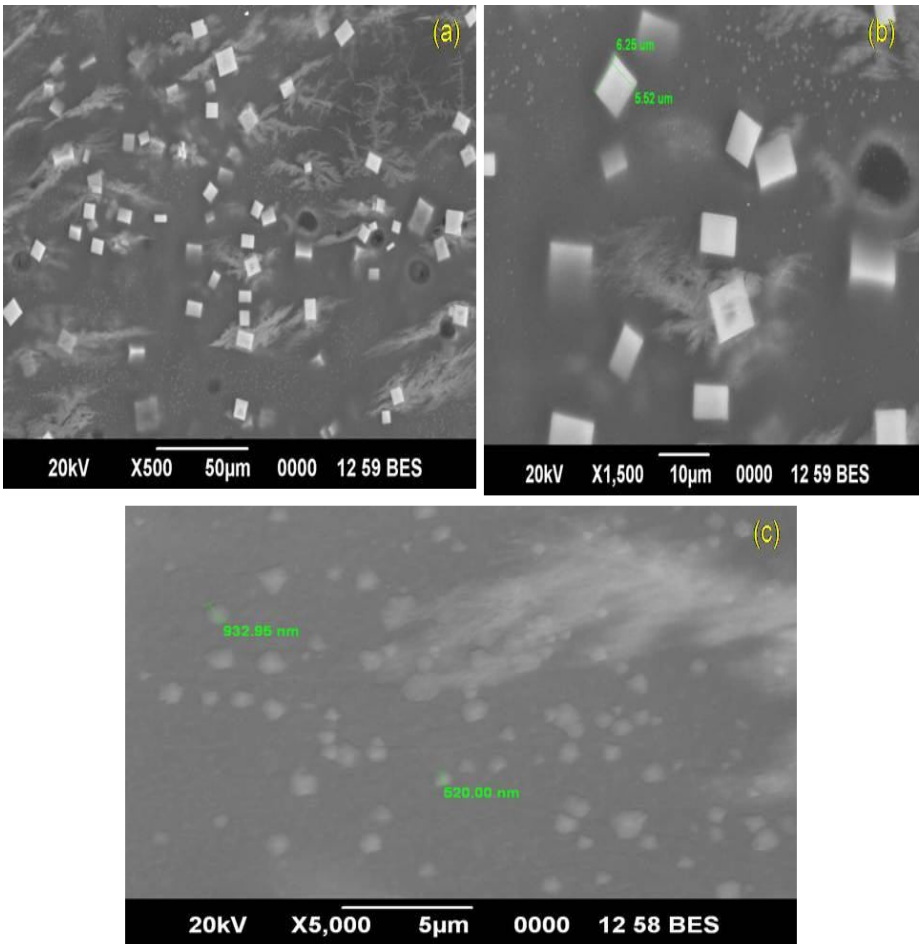



**Fig. 4.2 Photomicrograph showing Scanning Electron Microscopy (SEM) of single cells of *Natrinema* sp. BTSH10**

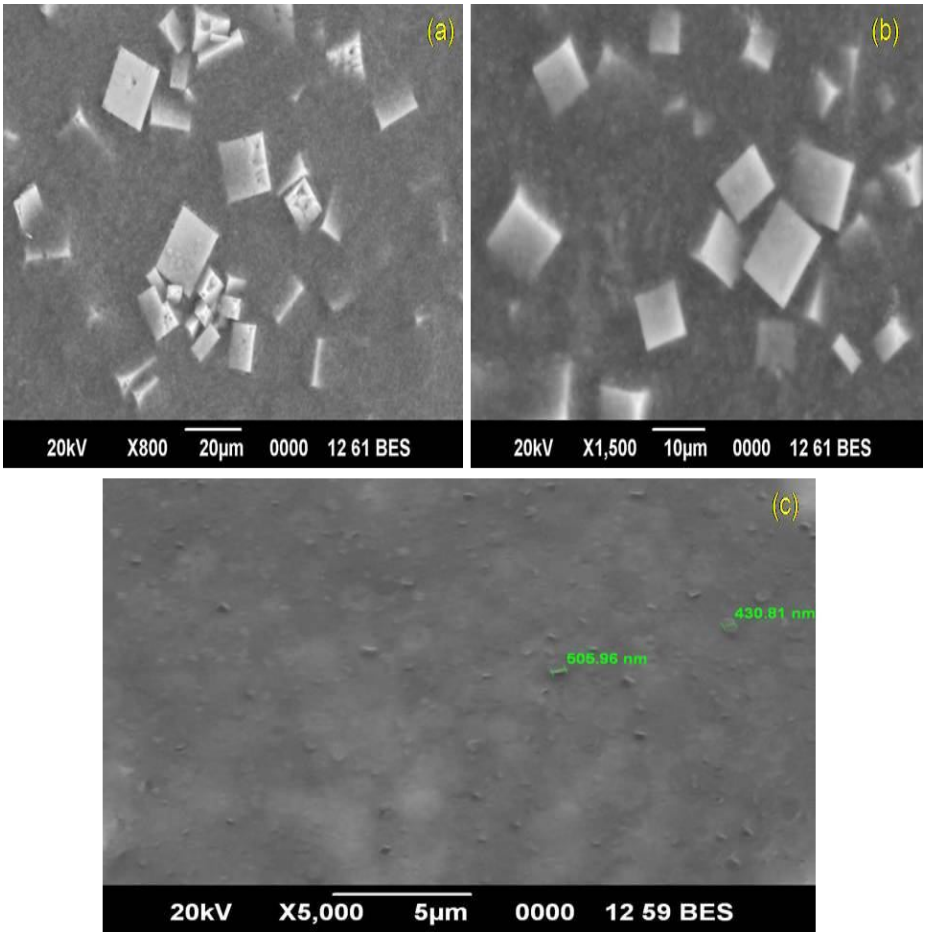



**Fig. 4.3 Photomicrograph showing Scanning Electron Microscopy (SEM) of single cells of *Halorubrum* sp. BTSH03**

It was also observed that along with the increase in incubation period there was solid salt crystal formation on plates. These photomicrographs (Fig 4.2 and 4.3) provide clear evidence that salt crystals were accumulated by these organisms and there was increase in size of the crystal over the period.

#### 4.1.2.1.1 Bacterial growth curve

Bacterial growth curves of *Natrinema* sp. BTSH10 and *Halorubrum* sp. BTSH03 were determined and the results are presented in Fig 4.4 and 4.5. The growth curve showed that the haloarchaea *Natrinema* sp. BTSH10 (Fig 4.4) entered the stationary phase after 102h and initially there was a longer lag phase. This is a specific characteristic feature of halophiles. Significant growth (1.0 OD) was attained only after 80h of incubation. *Halorubrum* sp. BTSH03 was observed to have almost the same growth pattern as that of *Natrinema* sp. BTSH10, but they entered the stationary phase only around 112h (Fig 4.5).The specific growth rate and generation time for BTSH10 was calculated as 0.042 and 24 hours, for BTSH03 it was found to be 0.0219 and 16 hours.

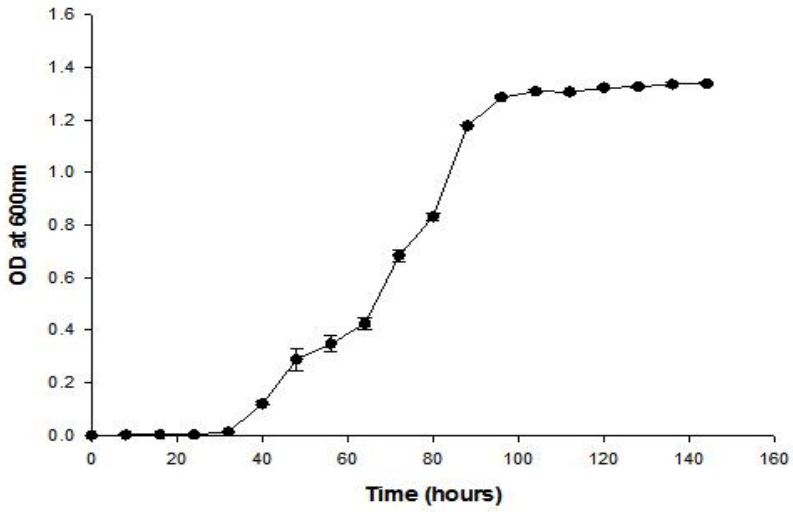



**Fig. 4.4 Growth curve of haloarchaeon *Natrinema* sp.BTSH10**

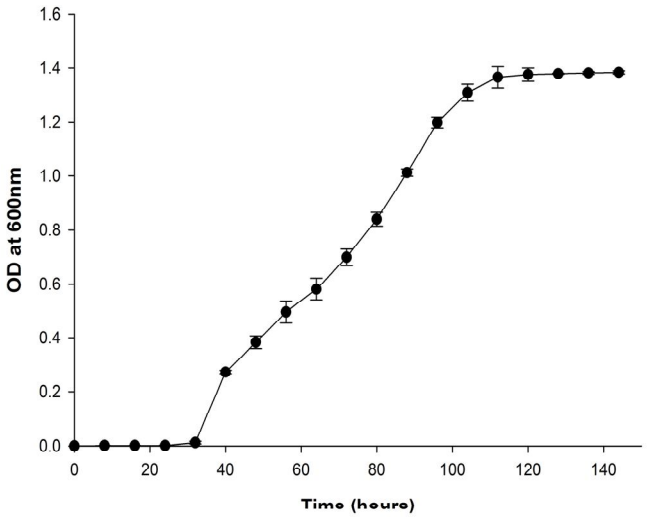



**Fig. 4.5 Growth curve of haloarchaeon *Halorubrum* sp. BTSH03**

### 4.1.2.2 Molecular identification of the strains by 16S rRNA gene

The molecular identification of the strains was done by amplification and sequencing of partial nucleotide sequences of 16S rRNA gene. The partial nucleotide sequences obtained are presented in Fig 4.7 and 4.9. PCR amplification showed that the amplicon was approximately 1000bp (Fig 4.6). Further the nucleotide sequences of the amplicon obtained were compared with Genbank entries in NCBI database, using BLAST and the identity of the strains were confirmed.

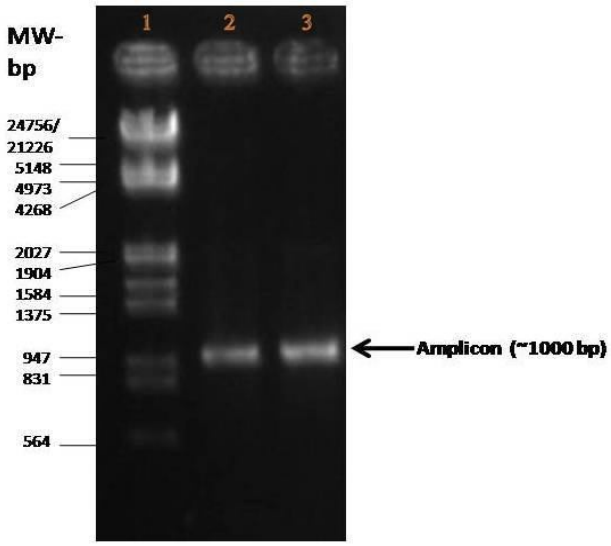



**Fig. 4.6 The PCR amplicon of 16S rRNA gene**

Lane 1 Lambda DNA Eco R1+ Hind III digest (Molecular weight marker)
Lane 2 Amplified 16S rRNA gene of *Natrinema* sp. BTSH10
Lane 3 Amplified 16S rRNA gene of *Halorubrum* sp. BTSH03

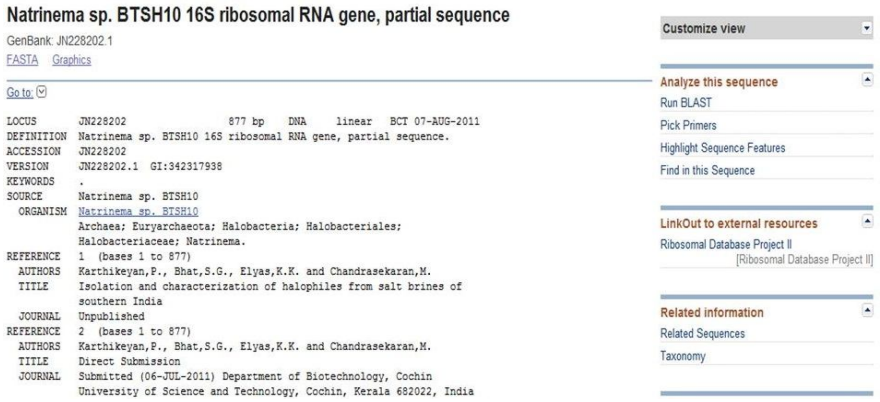
#### 4.1.2.2.3 Partial 16S rRNA gene sequences

The partial gene sequences of 16S rRNA were submitted to Genbank using SEQUIN programme at the NCBI site (www.ncbi.nlm.nih.gov). The submitted sequences were provided with accession number. The partial sequences of the two strains are shown in Fig 4.7 and 4.9

AAGCGTCTGTAGCTGGGCCACGCAAGTCTATCGGGAAATCCGCGCGCT
AAACGCGCGGGCGTCCGGTGGAAACTGCGTGGCTTGGGACCGGAAGA
CCAGAGGGGTACGTCCGGGGTAGGAGTGAAATCCCGTAATCCTGGACG
GACCACCGGTGGCGAAAGCGCCTCTGGAAGACGGATCCGACGGTGAG
GGACGAAAGCTCGGGTCACGAACCGGATTAGATACCCGGGTAGTCCGA
GCTGTAAACGATGTCTGCTAGGTGTGGCACAGGCTACGAGCCTGTGCT
GTGCCGTAGGGAAGCCGTGAAGCAGACCGCCTGGGAAGTACGTCCGC
AAGGATGAAACTTAAAGGAATTGGCGGGGGAGCACTACAACCGGAGG
AGCCTGCGGTTTAATTGGACTCAACGCCGGACATCTCACCAGCATCGAC
AATGTGCAGTGAACGTCAGGTTGATGACCTTACTGGAGCCATTGAGAG
GAGGTGCATGGCCGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGT
CAGGCAACGAGCGAGACCCGCACTCCTAATTGCCAGCAACACCCTTGC
GGTGGTTGGGTACATTAGGAGGACTGCCAGTGCCAAACTGGAGGAAGG
AACGGGCAACGGTAGGTCAGTATGCCCCGAATGTGCTGGGCGACACGC
GGGCTACAATGGCCGAGACAGTGGGATGCAACCCCGAAAGGGGACGC
TAATCTCCGAAACTCGGTCGTAGTTCGGATTGAGGGCTGAAACTCGCCC
TCATGAAGCTGGATTCGGTAGTAATCGCGCCTCAGAAGGGCGCGGTGA
ATACGTCCCTGCTCCTTGCACACACCGCCCGTCAAAGACCCGAGTGGG
GTCCGGATGAGGCCGA

**Fig. 4.7 The partial 16S rRNA gene sequence obtained for *Natrinema* sp. BTSH10**

*Natrinema* sp. BTSH10 was provided with the accession number JN228202 and the NCBI page with the allotted accession number is shown in Fig 4.8



Natrinema sp. BTSH10 16S ribosomal RNA gene, partial sequence

GenBank: JN228202.1

FASTA Graphics

Go to:

```
LOCUS       JN228202               877 bp    DNA     linear   BCT 07-AUG-2011
DEFINITION  Natrinema sp. BTSH10 16S ribosomal RNA gene, partial sequence.
ACCESSION   JN228202
VERSION     JN228202.1  GI:342317938
KEYWORDS    .
SOURCE      Natrinema sp. BTSH10
  ORGANISM  Natrinema sp. BTSH10
            Archaea; Euryarchaeota; Halobacteria; Halobacteriales;
            Halobacteriaceae; Natrinema.
REFERENCE   1  (bases 1 to 877)
  AUTHORS   Karthikeyan,P., Bhat,S.G., Elyas,K.K. and Chandrasekaran,M.
  TITLE     Isolation and characterization of halophiles from salt brines of
            southern India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 877)
  AUTHORS   Karthikeyan,P., Bhat,S.G., Elyas,K.K. and Chandrasekaran,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-JUL-2011) Department of Biotechnology, Cochin
            University of Science and Technology, Cochin, Kerala 682022, India
```

Customize view

Analyze this sequence

Run BLAST

Pick Primers

Highlight Sequence Features

Find in this Sequence

LinkOut to external resources

Ribosomal Database Project II

[Ribosomal Database Project II]

Related information

Related Sequences

Taxonomy

**Fig. 4.8 NCBI Gen bank file data of *Natrinema* sp. BTSH10 with the allotted accession number**

GCGTCCGTACTGGCCGCGCAAGTCCATCGGGAAATCCACCTGCTCAA
CAGGTGGGCGCCCGGTAGAAACTGCGTGGCTTGGGACCGGAAGGCG
CGACGGGTACGTCCGGGGTAGGAGTGAAATCCCGTAATCCTGGACGG
ACCGCCGATGGCGAAAGCACGTCGCGAGAACGGATCCGACAGTGAG
GGACGAAAGCCAGGGTCTCGAACCGGATTAGATACCCGGGTAGTCCT
GGCCGTAAACAATGCCTGCTAGGTGTGGCTCCCACTACGAGTGGGTG
CTGTGCCGTAGGGAAGCCGCTAAGCAGGCCGCCTGGGAAGTACGTCC
GCAAGGATGAAACTTAAAGGAATTGGCGGGGGAGCACTACAACCGG
AGGAGCCTGCGGTTTAATTGGACTCAACGCCGGACATCTCACCAGCA
TCGACTGTAATAATGACGACCAGGTTGATGACCTTGTCCGAGTTTCAG
AGAGGAGGTGCATGGCCGCCGTCAGCTCGTACCGTGAGGCGTCCTGT
TAAGTCAGGCAACGAGCGAGACCCGCATCCTTACTTGCCAGCAGTAC
CGCGAGGTAGCTGGGGACAGTAGGGAGACCGCCGTGGCTAACACGG
AGGAAGGAACGGGCAACGGTAGGTCAGTATGCCCCGAATGTGCTGG
GCAACACGCGGGCTACAATGGTCGAGACAAAGGGTTCCAACTCCGA
AAGGAGACGGTAATCTCAGAAACTCGATCGTAGTTCGGATTGTGGGC
TGCAACTCGCCCACATGAAGCTGGATTCGGTAGTAATCGCGTGTCAC
AAGCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCTGTC
AAAGCACCCTGAGTGAGGTCCGGATGAGGCGTCCA

**Fig. 4.9 The partial 16S rRNA gene sequence obtained for *Halorubrum* sp. BTSH03**

*Halorubrum* sp. BTSH03 was provided with the accession number JF830242 and the NCBI page with the allotted accession number is shown in Fig 4.10

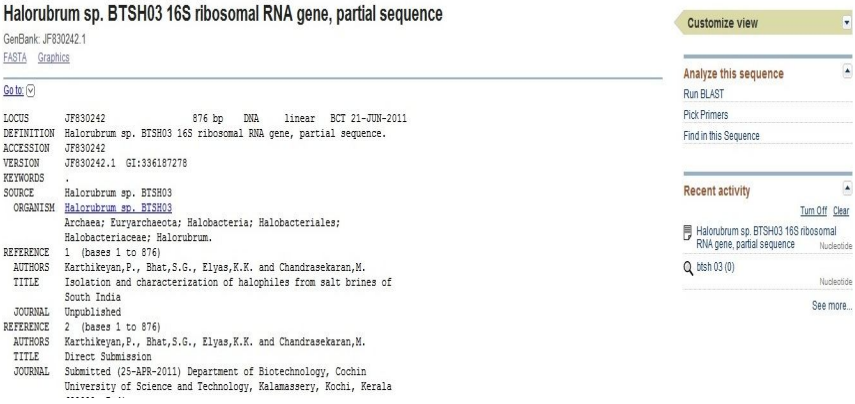



**Fig. 4.10 NCBI Gen bank file data of *Halorubrum* sp. BTSH03 with the allotted accession number**

### 4.1.2.3 Phylogenetic tree construction

#### 4.1.2.3.1 Phylogenetic tree analysis of *Natrinema* sp. BTSH10

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.04566946 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches (Fig 4.11). 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. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 869 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

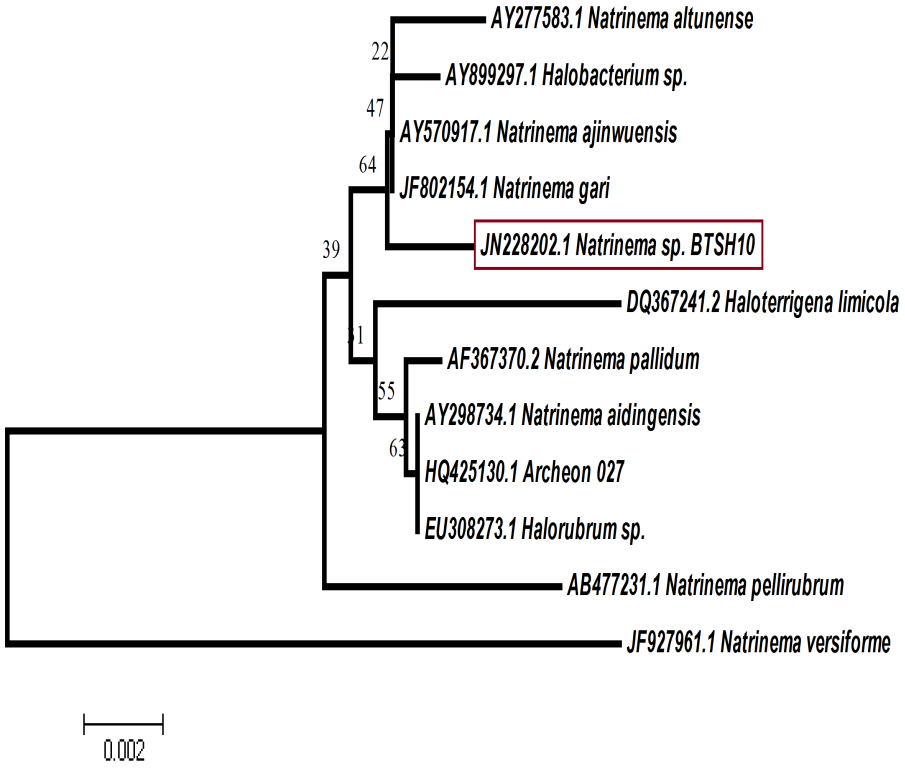



**Fig. 4.11 Phylogram of *Natrinema* sp. BTSH10 showing evolutionary relationships of 12 taxa (linearized) based on 16S rRNA gene.**

#### 4.1.2.3.2 Phylogenetic tree analysis of *Halorubrum* sp.BTSH03

The evolutionary history of *Halorubrum* sp. BTSH03 was inferred using the Neighbor-Joining method (Fig 4.12). The optimal tree with the sum of branch length = 0.05391769 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. 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. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 871 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

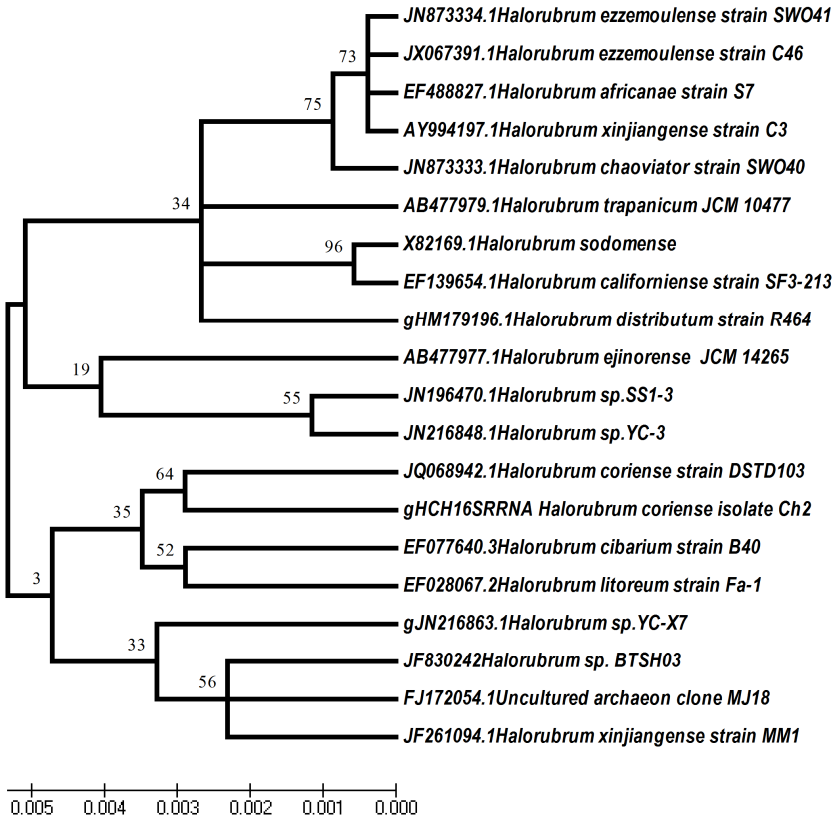



**Fig. 4.12 Phylogram of *Halorubrum* sp. BTSH03 showing evolutionary relationships of 20 taxa (linearized) based on 16S rRNA gene.**

#### 4.1.2.2.1 Taxonomic hierarchy of *Natrinema* sp.

Taxonomic hierarchy of *Natrinema* sp. BTSH03 is presented in Table 4.3

**Table 4.3. Taxonomic hierarchy of *Natrinema* sp. BTSH10**

| | |
|---|---|
| **Domain** | Archaea |
| **Phylum** | Euryarchaeota |
| **Class** | Halobacteria class. nov |
| **Order** | Halobacteriales |
| **Family** | Halobacteriaceae |
| **Genus** | *Natrinema* |

#### 4.1.2.2.2 Taxonomic hierarchy of *Halorubrum* sp.

Taxonomic hierarchy of *Halorubrum* sp. BTSH03 is presented in Table 4.4

**Table 4.4. Taxonomic hierarchy of *Halorubrum* sp. BTSH03**

| | |
|---|---|
| **Domain** | Archaea |
| **Phylum** | Euryarchaeota |
| **Class** | Halobacteria class. nov |
| **Order** | Halobacteriales |
| **Family** | Halobacteriaceae |
| **Genus** | *Halorubrum* |

## 4.2 ANTIBIOTIC SENSITIVITY PROFILING AND MULTIPLE ANTIBIOTIC RESISTANCE (MAR) INDEX FOR THE STRAINS

Antibiotic sensitivity profiling showed that the *Natrinema* sp. BTSH10 was resistant to 12 antibiotics which included ciprofloxacin, aztreonam, vancomycin, impeneme, neomycin, tetracycline, bacitracin, chloramphenicol, cotrimoxazole, cephalothin, cephotaxime and norfloxacin. Accordingly the MAR value was calculated as 0.3870. (Table 4.11-Appendix)

*Halorubrum* sp. BTSH03 was sensitive to most of the antibiotics and showed resistance to only three antibiotics namely impeneme, cotrimoxazole and netillin out of the 31 used. Its MAR index was calculated as 0.09677. (Table 4.11-Appendix)

## 4.3 LIPID PROFILING OF THE STRAINS

Total cell wall lipids from both the strains were isolated and subjected to Thin layer chromatography using chloroform : methanol (1:4). TLC analyses showed three bands each for the two strains. For *Natrinema* sp. BTSH10 the solvent front was measured as 7.5cm from the initial sample spot and the bands appeared at a distance of 5.6cm, 6.0cm and 6.9cm and the $R_f$ values were calculated as 0.746, 0.8 and 0.92 respectively (Fig 4.13).

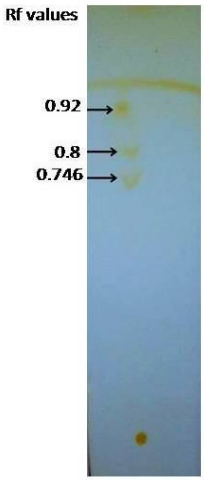



**Fig 4.13 Thin layer chromatography of cell wall lipids of *Natrinema* sp. BTSH10 on silica gel.**

### 4.3.1 GC-MS profile of *Natrinema* sp. BTSH10 lipid

GC-MS analysis of the lipids showed 10 peaks which were separated at different time intervals within the 25 min of the total run (Fig 4.14). The peaks were at 12.48, 14.27, 15.85, 17.31, 18.63, 19.86, 20.01, 22.09, 23.26, and 24.65 min. The individual peaks obtained at different periods were split and scanned again for finding the m/z value of each peak so that the lipid could be identified based on those obtained values. The results are interpreted in Table 4.5 presented below. The results clearly indicated the presence of new lipids which need to be explored.

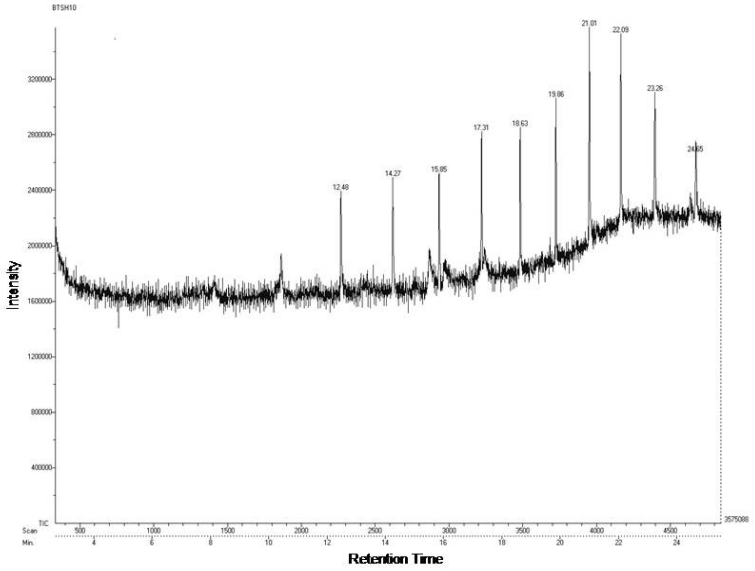



**Fig. 4.14 GC-MS separation of the lipids isolated from *Natrinema* sp. BTSH10**

**Table 4.5. Lipids present in the cell wall of *Natrinema* sp. BTSH10**

| S.No | Lipid peak separated (min) | Identification |
|---|---|---|
| 1 | 12.48 | D-acetoxy 10 hydroxy glycon/Oleprin |
| 2 | 14.27 | D-deconoic acid/Capric acid |
| 3 | 15.85 | Hexesterol |
| 4 | 17.31 | Tri butyl phosphate($C_{12}H_{27}O_4P$) |
| 5 | 18.63 | Unidentified |
| 6 | 19.86 | Unidentified |
| 7 | 21.01 | Unidentified |
| 8 | 22.09 | Unidentified |
| 9 | 23.26 | Unidentified |
| 10 | 24.65 | Unidentified |

### 4.3.2 Fatty acid methyl esterase (FAME) analysis of *Natrinema* sp. BTSH10

Fatty acid methyl esterase analysis showed that the organism is made up of both saturated and unsaturated fatty acids. The chromatogram presented as Fig 4.15 shows the presence of different fatty acids which were separated at different time periods. The separated peaks and the concentration of the peaks presented in the Table 4.6 given below showed that 27.76% was composed of 16:0 fatty acid which was identified as palmitic acid and 11.13% was composed of 18:0 fatty acid identified as stearic acid. Other fatty acids such as 10:0-capric acid, 12:0-lauric acid, 14:0 myristic acid, 16:1-palmetolic acid, 18:2-linoleic acid, 18:1- oleic acid and 19:1-non adeceonic acid which were present were in low concentrations. Some of the fatty acids shown in the Table 4.6 could not be identified and hence reported as unidentified fattyacids.

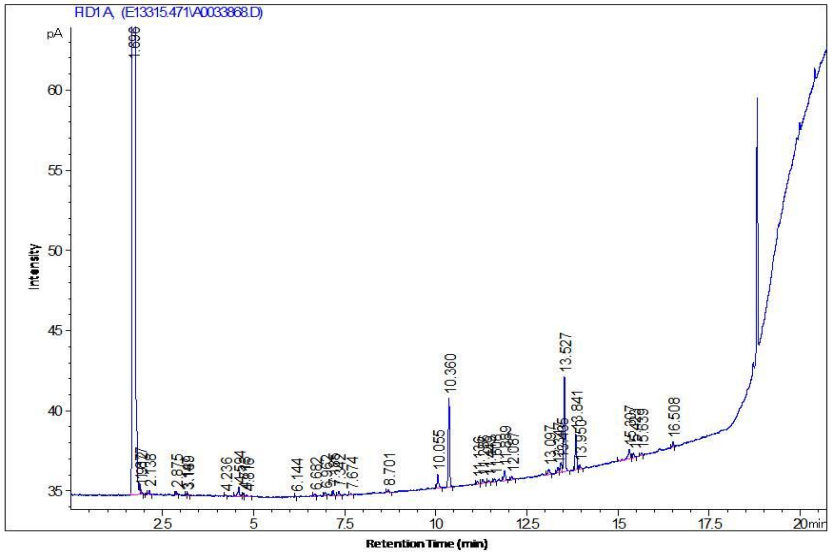



**Fig. 4.15 Fatty acid profile of *Natrinema* sp.BTSH10 obtained after FAME analysis**

**Table 4.6. Fatty acid content of *Natrinema* sp. BTSH10**

Volume: DATA File: E133154.71A Samp Ctr: 3 ID Number: 3868
Type: Samp Bottle: 2 Method: TSBA6
Created: 3/15/2013 12:06:30 PM Created By: admin
Sample ID: BTSH10 CUSAT

| RT | Response | Ar/Ht | RFact | ECL | Peak Name | Percent | Comment1 | Comment2 |
|---|---|---|---|---|---|---|---|---|
| 1.696 | 4.166E+8 | 0.025 | ----- | 7.022 | SOLVENT PEAK | ----- | < min rt | |
| 1.877 | 1115 | 0.022 | ----- | 7.394 | | ----- | < min rt | |
| 1.912 | 726 | 0.036 | ----- | 7.465 | | ----- | < min rt | |
| 2.138 | 833 | 0.027 | ----- | 7.929 | | ----- | < min rt | |
| 2.875 | 1065 | 0.036 | ----- | 9.442 | | ----- | | |
| 3.147 | 241 | 0.025 | 1.200 | 10.001 | 10:0 | 0.28 | ECL deviates 0.001 | Reference 0.003 |
| 3.189 | 588 | 0.028 | ----- | 10.063 | | ----- | | |
| 4.236 | 347 | 0.031 | ----- | 11.465 | | ----- | | |
| 4.594 | 3832 | 0.047 | ----- | 11.864 | | ----- | | |
| 4.713 | 748 | 0.034 | 1.071 | 11.997 | 12:0 | 0.78 | ECL deviates -0.003 | Reference -0.002 |
| 4.816 | 497 | 0.038 | 1.067 | 12.089 | 11:0 iso 3OH | 0.52 | ECL deviates 0.000 | |
| 6.144 | 277 | 0.026 | ----- | 13.224 | | ----- | | |
| 6.682 | 606 | 0.036 | 0.998 | 13.624 | 14:0 iso | 0.59 | ECL deviates 0.005 | Reference 0.004 |
| 6.962 | 1415 | 0.041 | ----- | 13.832 | | ----- | | |
| 7.185 | 1626 | 0.038 | 0.985 | 13.998 | 14:0 | 1.57 | ECL deviates -0.002 | Reference -0.003 |
| 7.342 | 1418 | 0.038 | 0.981 | 14.101 | 13:0 iso 3OH | 1.36 | ECL deviates -0.008 | |
| 7.674 | 349 | 0.032 | ----- | 14.319 | | ----- | | |
| 8.701 | 1208 | 0.050 | 0.955 | 14.994 | 15:0 | ----- | ECL deviates -0.006 | |
| 10.055 | 5019 | 0.044 | 0.936 | 15.814 | Sum In Feature 3 | 4.59 | ECL deviates -0.008 | 16:1 w7c/16:1 w6c |
| 10.360 | 30471 | 0.044 | 0.932 | 15.998 | 16:0 | 27.76 | ECL deviates -0.002 | Reference -0.005 |
| 11.136 | 810 | 0.035 | ----- | 16.449 | | ----- | | |
| 11.286 | 1475 | 0.044 | 0.923 | 16.536 | 17:1 anteiso A | 1.33 | ECL deviates -0.004 | |
| 11.449 | 399 | 0.029 | 0.921 | 16.631 | 17:0 iso | 0.36 | ECL deviates 0.001 | Reference -0.003 |
| 11.608 | 1139 | 0.045 | 0.920 | 16.723 | 17:0 anteiso | 1.02 | ECL deviates 0.000 | Reference -0.003 |
| 11.839 | 3923 | 0.046 | 0.917 | 16.886 | 17:0 cyclo | 3.52 | ECL deviates -0.002 | |
| 12.087 | 1437 | 0.044 | 0.916 | 17.001 | 17:0 | 1.29 | ECL deviates 0.001 | Reference -0.003 |
| 13.097 | 2193 | 0.048 | 0.909 | 17.576 | 18:3 w6c (6,9,12) | 1.95 | ECL deviates -0.001 | |
| 13.347 | 2050 | 0.040 | 0.907 | 17.719 | Sum In Feature 5 | 1.82 | ECL deviates -0.001 | 18:2 w6,9c/18:0 ante |
| 13.435 | 3874 | 0.044 | 0.907 | 17.769 | 18:1 w9c | 3.43 | ECL deviates 0.000 | |
| 13.527 | 35348 | 0.048 | 0.906 | 17.821 | Sum In Feature 8 | 31.30 | ECL deviates -0.002 | 18:1 w7c |
| 13.841 | 12588 | 0.045 | 0.904 | 17.999 | 18:0 | 11.13 | ECL deviates -0.001 | Reference -0.005 |
| 13.950 | 1343 | 0.036 | ----- | 18.062 | | ----- | | |
| 15.307 | 4505 | 0.060 | 0.898 | 18.839 | Sum In Feature 7 | 3.95 | ECL deviates -0.007 | un 18.846/19:1 w6c |
| 15.423 | 1648 | 0.046 | 0.898 | 18.905 | 19:0 cyclo w8c | 1.45 | ECL deviates 0.003 | |
| 15.639 | 1004 | 0.043 | ----- | 19.029 | | ----- | | |
| 16.508 | 1722 | 0.039 | ----- | 19.529 | | ----- | | |
| ----- | 5019 | --- | ----- | ----- | Summed Feature 3 | 4.59 | 16:1 w7c/16:1 w6c | 16:1 w6c/16:1 w7c |
| ----- | 2050 | --- | ----- | ----- | Summed Feature 5 | 1.82 | 18:2 w6,9c/18:0 ante | 18:0 ante/18:2 w6,9c |
| ----- | 4505 | --- | ----- | ----- | Summed Feature 7 | 3.95 | un 18.846/19:1 w6c | 19:1 w6c/.846/19cy |
| ----- | ----- | --- | ----- | ----- | | ----- | 19:0 cyclo w10c/19w6 | |
| ----- | 35348 | --- | ----- | ----- | Summed Feature 8 | 31.30 | 18:1 w7c | 18:1 w6c |

ECL Deviation: 0.004 Reference ECL Shift: 0.004 Number Reference Peaks: 9
Total Response: 123957 Total Named: 111205
Percent Named: 89.71% Total Amount: 103459

## 4.4 CHARACTERIZATION OF SALT CRYSTAL FORMED BY *Natrinema* sp. BTSH10

Haloarchaea *Natrinema* sp. BTSH10 showed characteristic salt crystal formation and the size of the crystals formed varied from few millimeters upto 2cm.

### 4.4.1 Crystal formation and its size

The crystals formed in the presence of bacteria were of different shapes and different sizes (Fig 4.16).

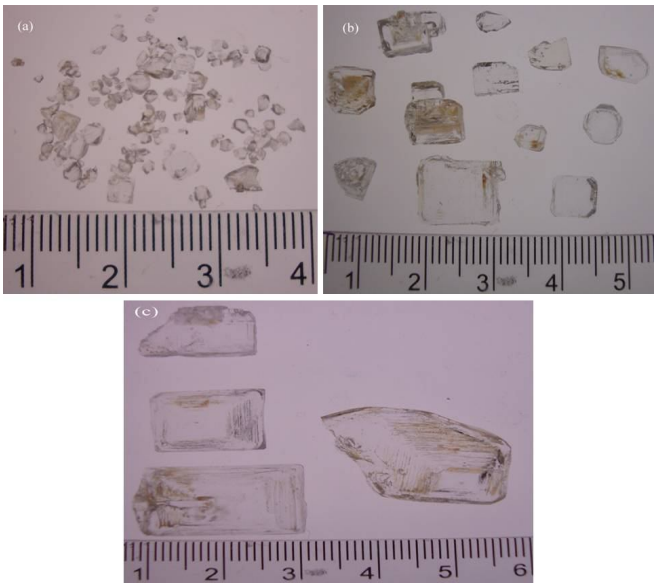



**Fig. 4.16 Salt crystals formed in Haloarchaea growth medium**
(a) Control-Crystal growth in medium without *Natrinema* sp. BTSH10.
(b) Crystal growth in medium in the presence of *Natrinema* sp. BTSH10.
(c) Increased crystal size upon storage of formed crystal with bacteria in broth under refrigeration.

The size of the crystals varied between 0.2mm to1cm in length and approximately 0.3mm in diameter compared to the the size of crystal formed in control medium was few mm. It was also noted that the size of the crystal increased when the medium with the crystal was stored under refrigeration. The size increased upto 2cm in length.

### 4.4.2 Scanning Electron Microscopy (SEM)

The colonization of bacteria inside the halite was confirmed using Scanning electron microscopy (Fig 4.17). It was observed that the haloarchaea *Natrinema* sp. BTSH10 grew and colonized inside the halite crystal, which was very well visible in few crystals. Growth of *Natrinema* sp. BTSH10 inside the crystal was confirmed upon dissolving the crystal in Zobell's broth.

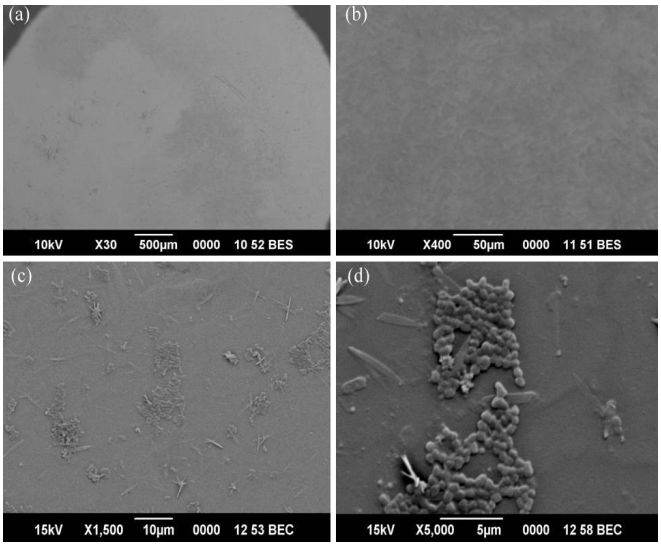



**Fig. 4.17 Scanning Electron Microscope images of the crystal (a, b, c, d) at different magnification**

### 4.4.3 Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES)

Results obtained for the ICP-AES analysis of the salt crystal formed by *Natrinema* sp. BTSH10 is presented in Table 4.7. From the data presented in the table it was noted that elements namely Fe, K, Mg, Na, Sr were present in the salt crystal. The concentration of sodium ion (Na) was maximum (29.65%) followed by Fe (4015.20ppm), K (770.10ppm), Mg (346.57ppm), and Sr (24.65ppm)

**Table 4.7. ICP-AES analysis of the salt crystal formed by *Natrinema* sp. BTSH10**

| Sample No | Sample Name | Element Measured | | | | |
|---|---|---|---|---|---|---|
| | | Fe2599 (ppm) | K_7664 (ppm) | Mg2852 (ppm) | Na5895 (%) | Sr3464 (ppm) |
| 1 | BTSH-10 | 4015.20 | 770.10 | 346.57 | 29.65 | 24.65 |
| 2 | AR | 1855.99 | 607.46 | 192.25 | 31.06 | 61.20 |
| Detection Limit in ppm | | 0.01 | 0.10 | 0.01 | 0.10 | 0.10 |

### 4.4.4 Powder X-ray diffraction analysis

Powder X-ray diffraction studies were carried out to demonstrate the crystallinity of the formed salt crystal and the spectrum obtained and displayed in the Figure 4.18 showed high intensity peak. The presence of such peaks indicates a high degree of crystallinity similar to that of a laboratory synthesized crystal.

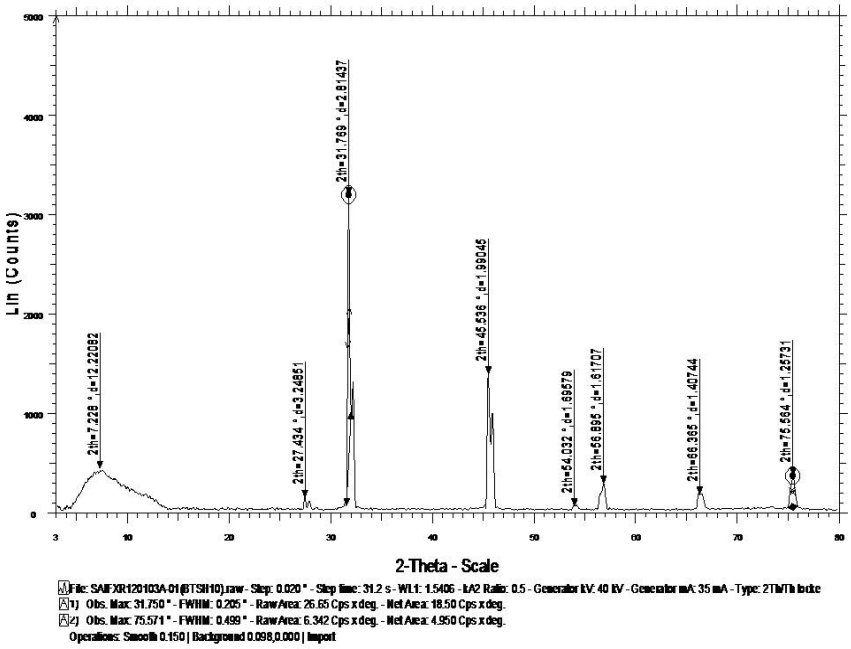



**Fig. 4.18 The powder X-ray diffraction spectrum of the crystal formed by *Natrinema* sp. BTSH10**

### 4.4.5 FTIR Study

FTIR analysis of the crystal presented in Fig 4.19 showed a broad band at 3439cm$^{-1}$ which corresponds to –OH stretching vibrations. The presence of unprotected C=C group attributed to its stretching frequency around 1637cm$^{-1}$. The C-N stretching vibration was assigned at 1122cm$^{-1}$.

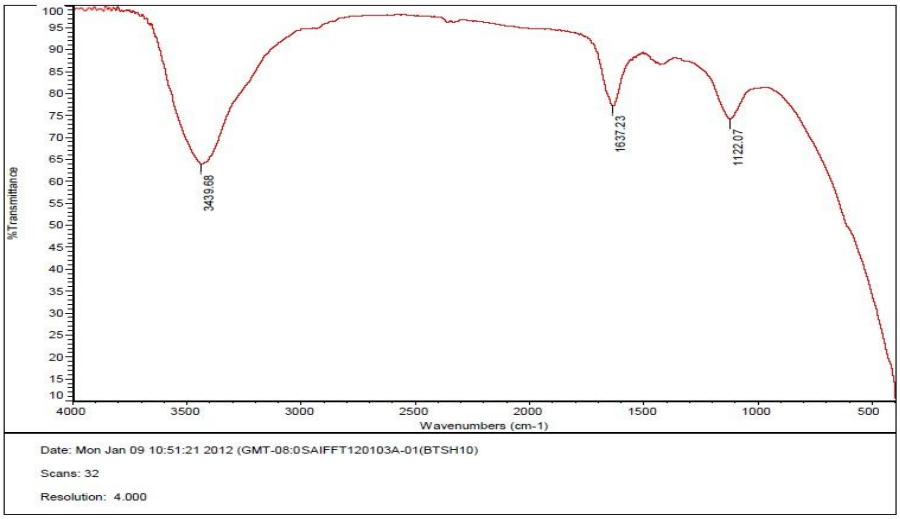
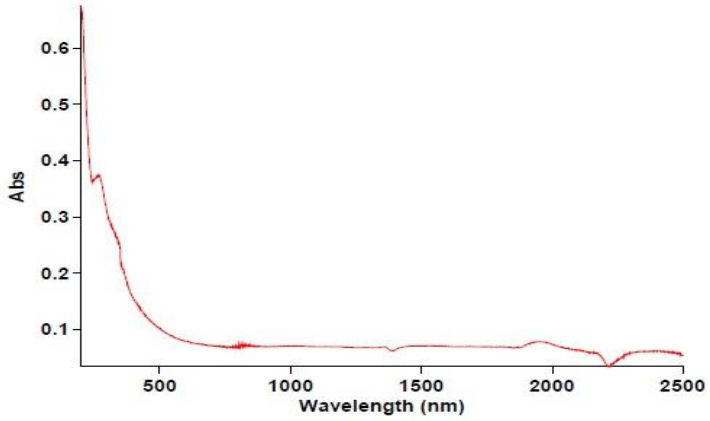



**Fig. 4.19 FTIR spectrum of the crystal formed by *Natrinema* sp. BTSH10**

### 4.4.6 UV-VIS-NIR study

The UV–VIS–NIR spectra were recorded in the range of 190–2500nm in order to determine the transmission range and know the suitability of the crystals for optical applications. This spectrum gives information about the structure of the molecule since the absorption of UV and visible light involves promotion of the electron in the σ and π orbital from the ground state to higher states. Data

presented in the absorption spectrum as Fig 4.20 showed that the absorption was minimum in the entire UV-VIS-NIR region and the cut off wavelength was around 230nm, closer to UV range. Thus it was inferred that among the various analogs used, the compound has the superior cut off wavelength and good transparency in blue region.




Scan Analysis Report
Sample Name: SAIFUV120103A-01(BTSH10)

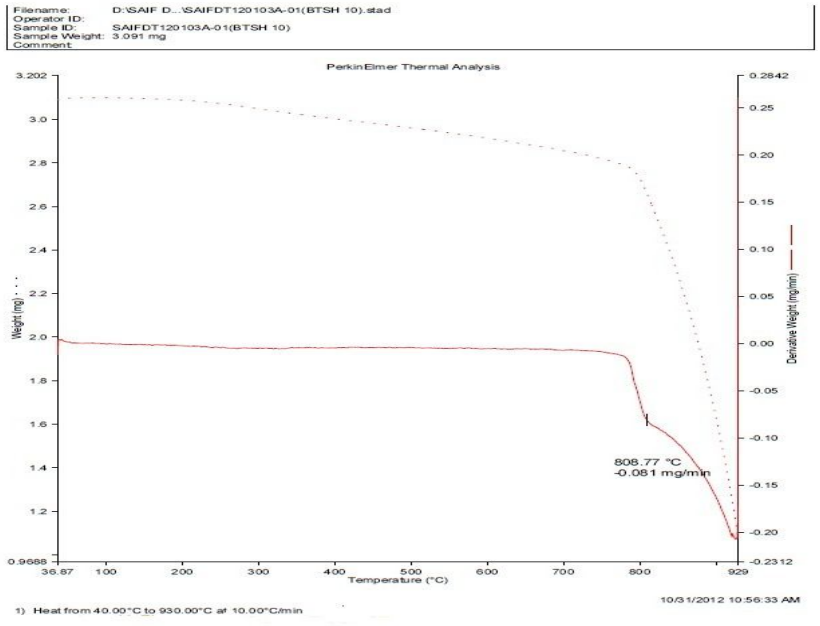
| | |
|---|---|
| Peak Table | |
| Peak Style | Peaks |
| Peak Threshold | 0.0100 |
| Range | 2500.000nm to 200.000nm |

| Wavelength (nm) | Abs |
|---|---|
| 1945.000 | 0.079 |
| 266.000 | 0.375 |

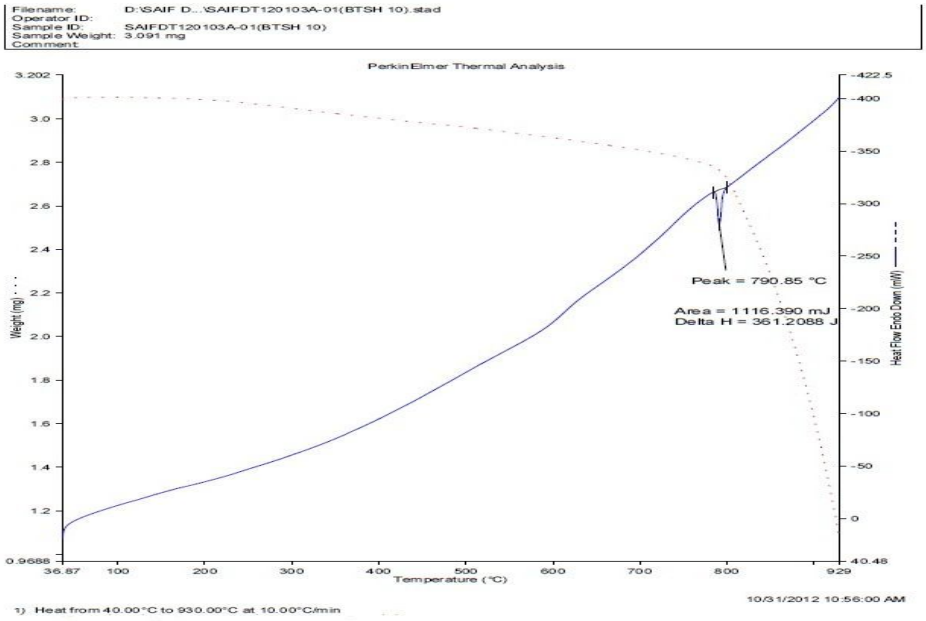
**Fig. 4.20 UV –VIS- NIR spectrum of the crystal formed by *Natrinema* sp. BTSH10**

### 4.4.7 Thermal analysis

The thermal behavior of grown halite (salt crystal) sample was studied by using TA instrument Q600 SDT and Q20 DSC model. To determine the melting point and thermal stability of the crystal it was subjected to TGA-DTA analysis and the results are shown in Fig 4.21. The sample (3.091mg) was taken for TG analysis. The curve showed that there was a gradual weight loss which occurred in the temperature range of 36-750℃ followed by a rapid weight loss between 750-929°C due to the liberation of volatile substances like CO, $CO_2$, $NH_3$ etc (Fig 4.21 a).




(a)




(b)

**Fig. 4.21 TGA-DTA analysis of the crystal formed by *Natrinema* sp. BTSH10**
(a) gradual weight loss of crystal at temperature range of 36-750°C
(b) sharp endothermic peak at 790.85°C showing the stability of the crystal

From the DTA curve, it was also observed that there was a sharp endothermic peak at 790.85°C corresponding to the stability of the crystal and there was no phase transition before it (Fig 4.21 b). A small endothermic hump around 345°C corresponds to the loss of moisture available on the surface was also observed.

## 4.5 OPTIMIZATION OF BIOPROCESS VARIABLES FOR HALOCIN PRODUCTION BY *NATRINEMA* SP. BTSH10

Various constituents of the selected medium and process parameters that influence halocin production by BTSH10 was optimized by adopting 'one factor at a time' approach. Strategy adopted for the optimization was to evaluate the effect of each variable for its optimum level for maximal halocin production, and incorporate the same variable at its optimized level in the subsequent experiment while evaluating the next variable. The variables studied included the following in the sequential order: Incubation temperature (27°C - 47°C), pH (2 - 13), NaCl concentration (0.5M - 4M), carbon sources at 0.1M concentrations (dextrin, galactose, fructose, lactose, sucrose, sorbitol, xylose, maltose and glycerol), nitrogen source at 1% (w/v) concentration (peptone, yeast extract, malt extract, soybean meal, tryptone, casein, urea and beef extract) and different inorganic salts at 0.1M concentration (ammonium nitrate, sodium fluorate, sodium silicate, potassium chloride, magnesium chloride, calcium chloride, sodium bicarbonate, potassium bromide and strontium chloride), agitation (50 - 250 rpm), and incubation time (0 - 144h). Preparation of inoculum, inoculation and culture conditions were same as mentioned earlier unless otherwise mentioned.

### 4.5.1 Optimization of incubation temperature

From the results documented in Fig 4.22 it was inferred that 42°C was the optimum for maximum halocin production (1024AU) by *Natrinema* sp. BTSH10 although enhanced level of halocin could be recorded at both 37$^{o}$C and 47$^{o}$C while the pH of the medium was 7.4, NaCl concentration was 3M maintained at 150 rpm for 96h. Nevertheless appreciable levels of halocin activities could be recorded at

other temperatures. Halocin productions at high levels at 42°C, and relatively at lesser levels at lower and at slightly higher temperatures (47ºC) were observed.

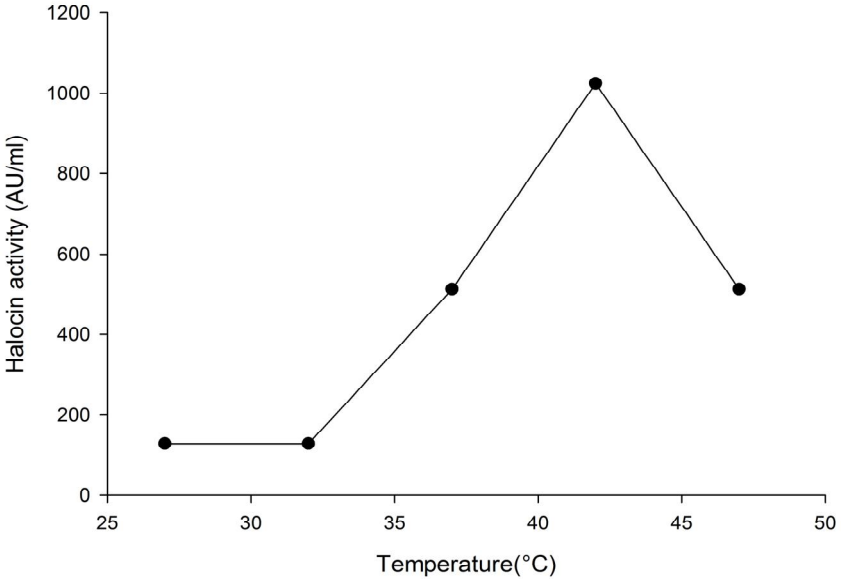



**Fig. 4.22 Effect of different incubation temperatures on halocin production by *Natrinema* sp. BTSH10 in Zobell's medium at pH 7.4, 3M NaCl, 150 rpm, 2% (V/V) inoculum concentration and 96h of incubation (adapted from Karthikeyan *et al*.,2013)**

### 4.5.2 Optimization of pH

Results presented in Figure 4.23 showed that the halobacteria *Natrinema* sp. BTSH10 could produce halocin in media with a pH varying between pH 5 and 9 although maximum halocin production was recorded at pH 7.0- 8.0 (1024AU). However, the bacteria did not produce halocin under acidic conditions.

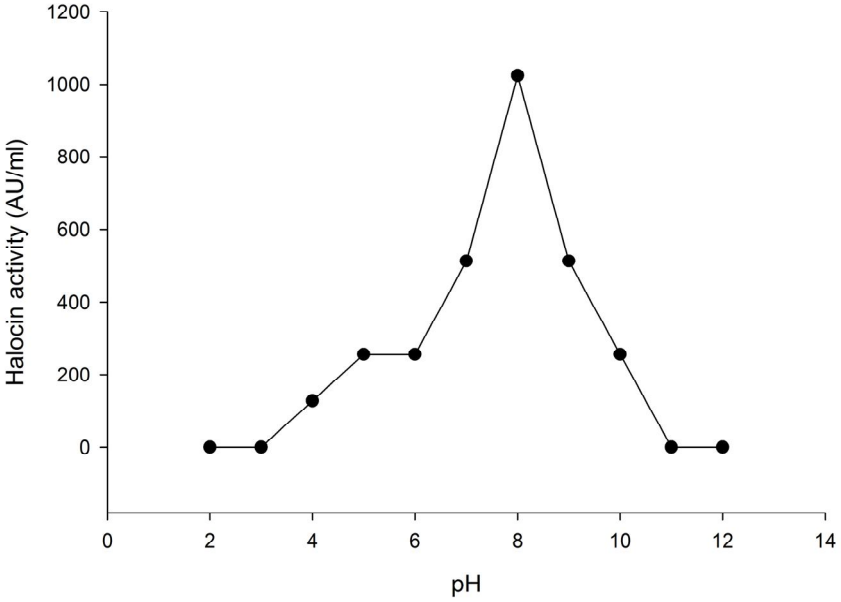



**Fig. 4.23 Effect of different pH on halocin production by *Natrinema* sp. BTSH10 in Zobell's medium at 42°C, 3M NaCl, 150 rpm, 2% (V/V) inoculum concentration and 96h of incubation. (adapted from Karthikeyan *et al.*,2013)**

### 4.5.3 Optimization of sodium chloride concentration

Data presented in Fig 4.24 indicated that *Natrinema* sp. BTSH10 required 3M NaCl for recording maximum (1024AU) halocin production although NaCl concentration ranging from 2.5M – 4M in the medium supported enhanced production of halocin. It was also noted that a minimum of 1.5M NaCl was required for growth, and lesser concentrations did not even support survival of the bacterium.

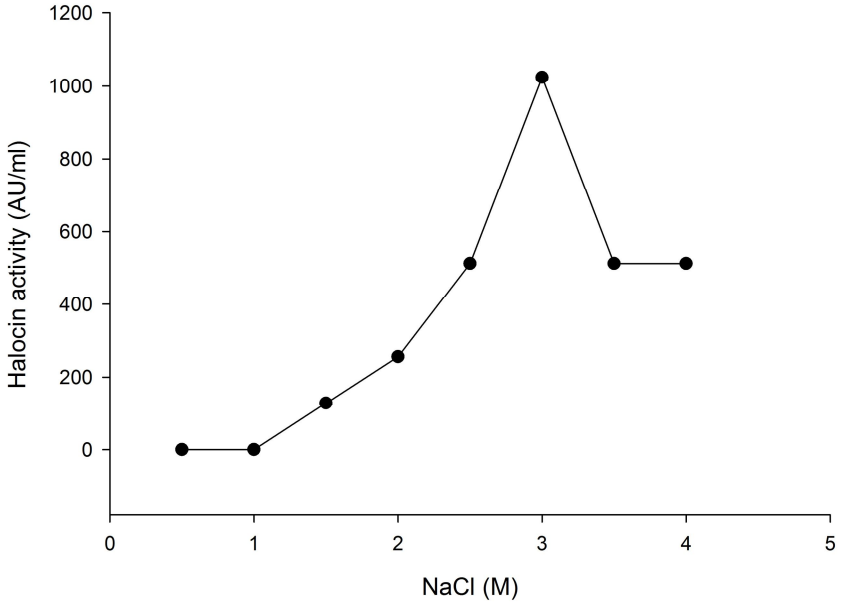



**Fig 4.24 Effect of different concentrations of NaCl on halocin production by *Natrinema* sp. BTSH10 in Zobell's medium at 42°C, pH 8.0, 150 rpm, 2% (V/V) inoculum concentration and 96h of incubation. (adapted from Karthikeyan *et al*.,2013)**

### 4.5.4 Optimization of different carbon source

From the results presented in Fig 4.25 it was inferred that maximal halocin production by *Natrinema* sp. BTSH10 was supported by the medium supplemented with galactose (2048AU) followed by sorbitol, maltose, glycerol, glucose, fructose, and lactose, Whereas, medium supplemented with dextrin, sucrose and xylose supported reduced levels of halocin production. Galactose was observed to enhanced halocin production in the medium compared to other carbon sources.

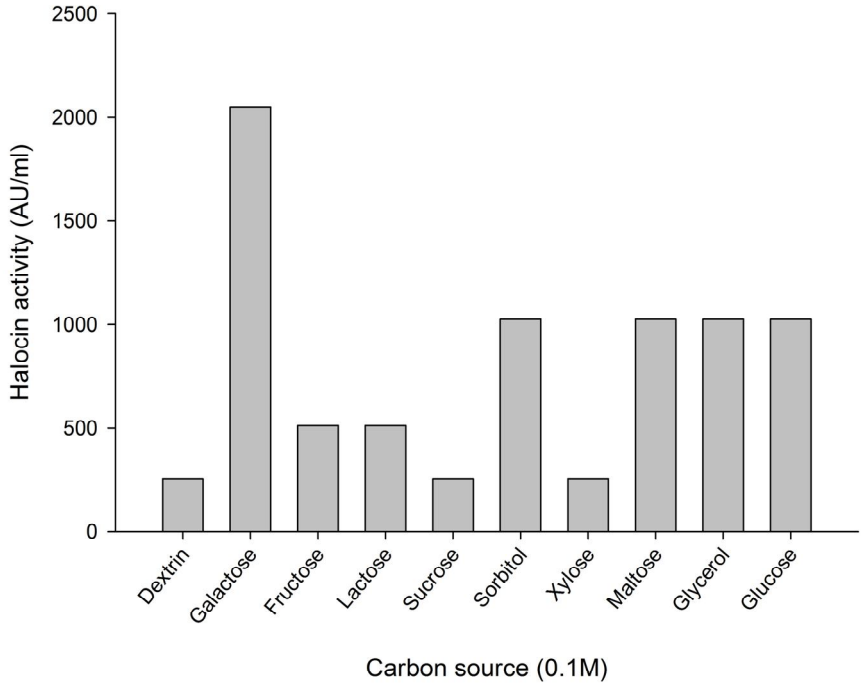



**Fig. 4.25 Effect of different carbon sources on halocin production by *Natrinema* sp. BTSH10 in Zobell's medium at 42°C, pH 8.0, 3M NaCl, 150 rpm, 2% (V/V) inoculum concentration and 96h of incubation. (adapted from Karthikeyan *et al.*,2013)**

### 4.5.5 Optimization of different nitrogen source

Results documented in Fig 4.26 indicated that *Natrinema* sp. BTSH10 could produce maximal halocin in the presence of beef extract (2048 AU) in the medium followed by soybean meal, malt extract, tryptone, peptone, yeast extract, casein and gelatin. Urea did not support halocin production.

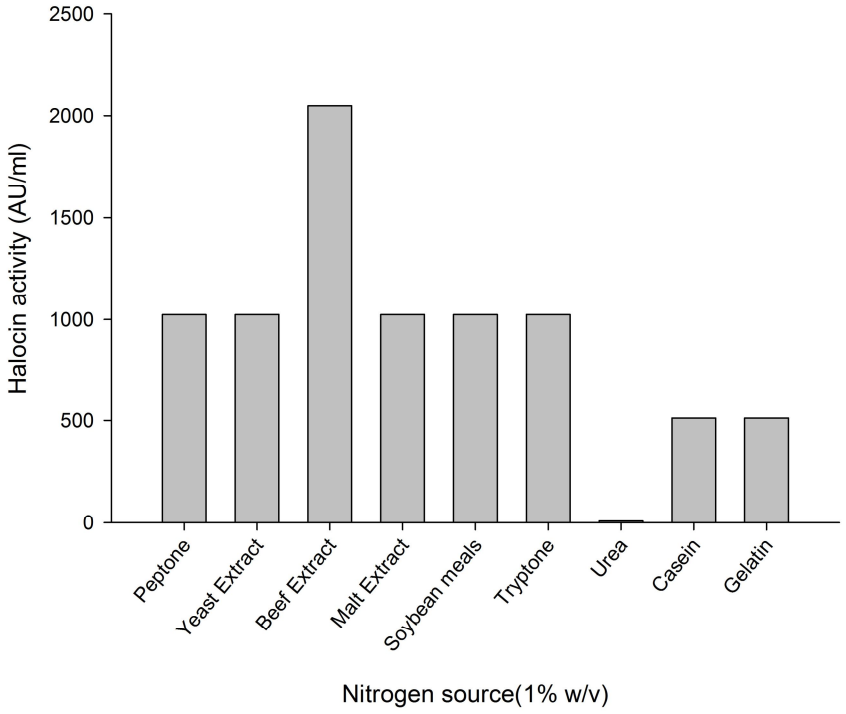



**Fig. 4.26 Effect of different nitrogen sources on halocin production by *Natrinema* sp. BTSH10 in Zobell's medium at 42°C, pH 8.0, 3M NaCl, galactose, 150rpm, 2% (V/V) inoculum concentration and 96h of incubation. (adapted from Karthikeyan *et al*.,2013)**

### 4.5.6 Optimization of different inorganic salt

Additional salts in the medium were found to exert influence on halocin production by *Natrinema* sp. BTSH10. Among the inorganic salts used for supplementation of the medium as additional salts calcium chloride (2048AU) was found to support maximal halocin production in the medium followed by magnesium chloride, sodium fluorate, potassium chloride, and sodium bicarbonate (Fig 4.27). Whereas aluminium nitrate, potassium bromide, strontium chloride and

sodium silicate led to a much reduced level of halocin production when compared to the levels noted with Zobell's medium in the absence of these particular salts.

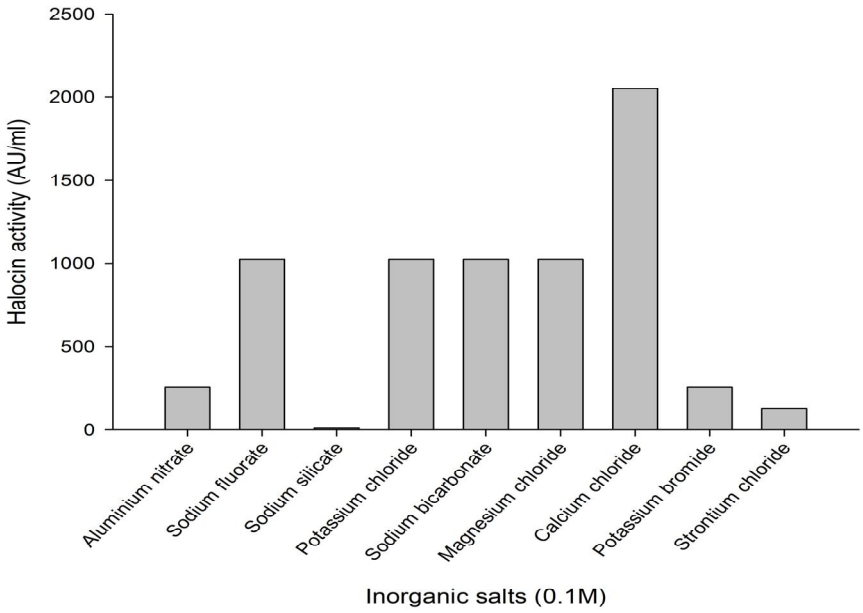



**Fig 4.27 Effect of different inorganic salts on halocin production by *Natrinema* sp. BTSH10 in Zobell,s medium at 42°C, pH 8.0, 3M NaCl, galactose, beef extract, 150 rpm ,2% (V/V) inoculum concentration and 96h of incubation. (adapted from Karthikeyan *et al.*,2013)**

### 4.5.7 Effect of agitation on halocin production

Data documented in Fig 4.28 indicated the influence of agitation on the rate of halocin production by *Natrinema* sp. BTSH10. It was found that higher agitation rate led to enhanced bacterial growth and production of halocin compared to lesser agitation rates. A maximum of 4096AU was recorded at 200

and 250 rpm although lower agitation rates (50 rpm to 150 rpm) also supported considerable levels of halocin production.

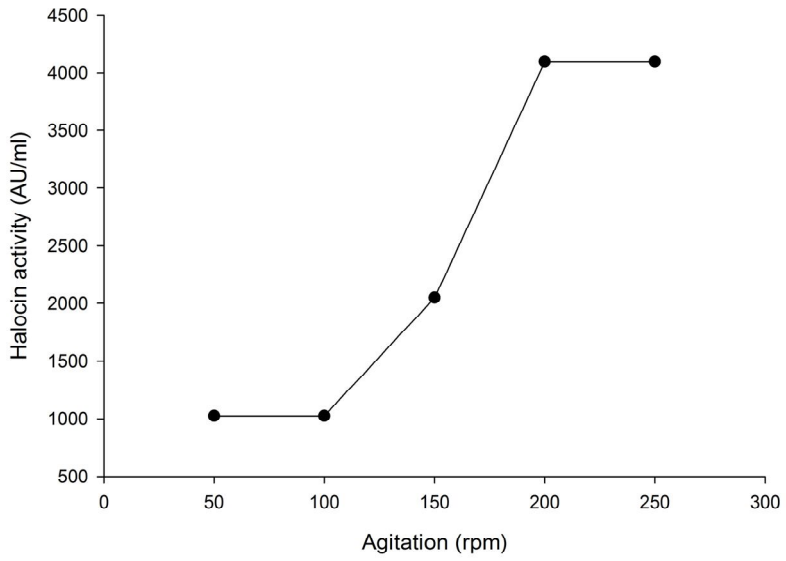



**Fig. 4.28 Effect of different agitation rates on halocin production by *Natrinema* sp. BTSH10 in Zobell's medium at 42°C, pH 8.0, 3M NaCl, galactose, beef extract, calcium chloride, 2% (V/V) inoculum concentration and 96h of incubation. (adapted from Karthikeyan *et al.*,2013)**

### 4.5.8 Time course experiment for halocin production

Results depicted in Fig 4.29 very clearly indicated that *Natrinema* sp. BTSH10 produce halocin at enhanced levels only during the stationary phase of growth although significant levels of halocin could be noted during late exponential phase. Maximum (8192AU) production of halocin was observed at 104h, during the stationary phase. Nevertheless the halocin activity in the medium was also noted even after 144h.

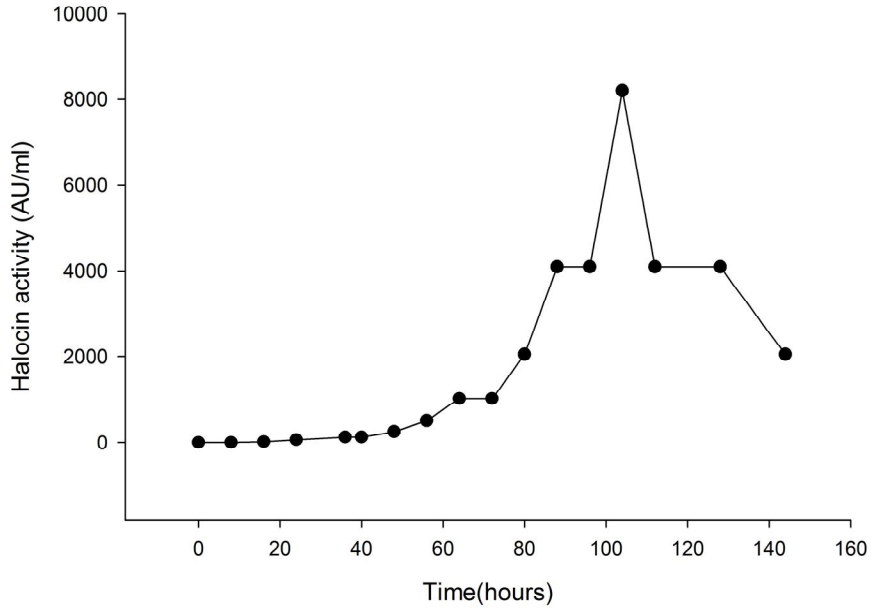



**Fig. 4.29 Time course experiment on halocin production by *Natrinema* sp BTSH10 in Zobell's medium at 37°C, pH 8, 3M NaCl, galactose, beef extract, calcium chloride, 2% (V/V) inoculum concentration and 200 rpm. (adapted from Karthikeyan *et al.*,2013)**

## 4.6 PURIFICATION OF HALOCIN PRODUCED *NATRINEMA* SP.BTSH10

### 4.6.1 Acetone precipitation and fractionation by molecular weight cut off centrifugal concentration

Initially the 100mL of culture filtrate that showed activity was concentrated to 25mL employing acetone precipitation, followed by fractionation

with 30kDa molecular weight cut off centrifugation and its activity was determined as 16384 $AUmL^{-1}$.

### 4.6.2 Gel filtration chromatography

The gel filtration chromatography using sephadex G50 column gave active fractions in tubes numbered 3 to 9 (Fig 4.30) and all the fractions were pooled together and the total volume of 7mL was concentrated to 1.5mL which recorded 8192$AUmL^{-1}$ halocin activity.

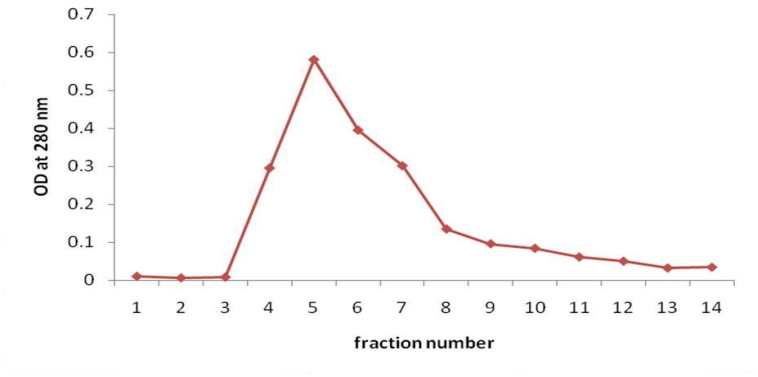



**Fig. 4.30 Gel filtration chromatography elution profile of halocin that showed halocin activity**

### 4.6.3 HPLC

Confirmation of the purity of the prepared halocin was done using HPLC. The HPLC profile presented in Fig 4.31 showed that a single peak could be obtained in all the samples exactly between 11 and 12 min [Fig 4.31 (a) Acetone precipitated; Fig 4.31 (b) 30kDa molecular weight cut –off centrifugation tube subjected ; Fig 4.31 (c) Sephadex G 50 column purified] where the ACN/WATER

volume was calculated as 32% : 68%. The purity of the halocin was confirmed based on comparison of the profiles.

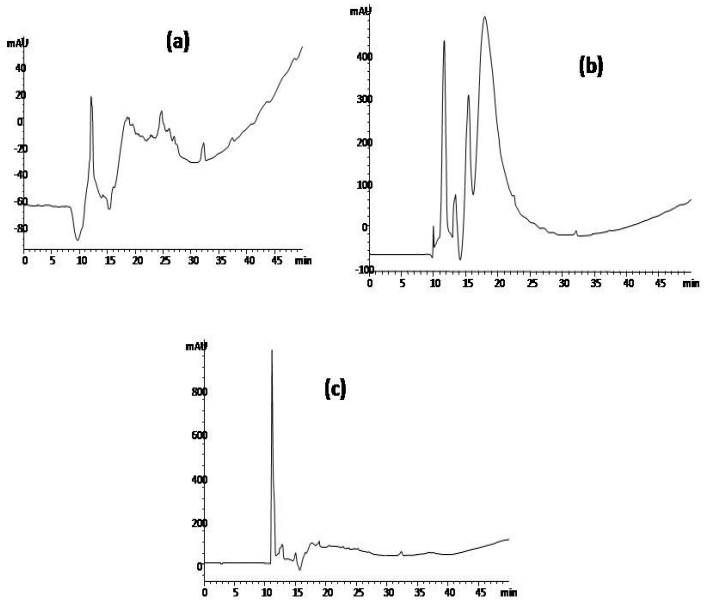



**Fig. 4.31 HPLC profile showing purity of Halocin during different stages of purification** (a)Acetone precipitated (b)30kDa molecular weight cut – off centrifugation tube subjected (c)Sephadex G 50 column purified

### 4.6.4 Tricine-PAGE and bioautography assay

The purity and the molecular weight of the halocin SH10 was further determined by Tricine PAGE. From the results presented in Fig 4.32.a, it was inferred that the halocin obtained was pure since a single band appeared on Tricine PAGE just below the 21.5kDa region of the marker protein. The molecular mass was calculated as 20kDa based on the analysis of the gel with Quantity One software in Bio-rad densitometer (GS800). The present study indicated that the

halocin is relatively larger in size than the microhalocin and smaller than the halocins reported from other halobacteria. To confirm whether the visible single band is halocin, an autobiography assay was performed on the unstained gel by agar overlay method which showed a clearing zone (Fig 4.32.b) exactly at the 20kDa single band protein region corresponding to the coomassie brilliant blue stained gel.

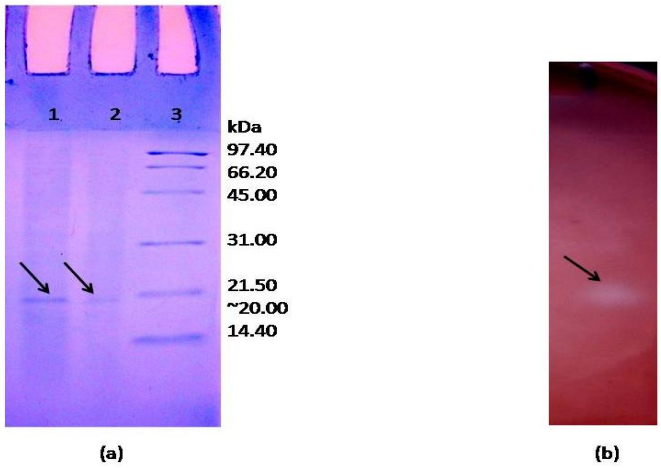



**Fig. 4.32 (a) Purified halocin on Tricine-PAGE showing a single band corresponding to approx 20kDa.(Lane 1 & 2)and Lane 3 Marker (b) Bioautography assay on Tricine-PAGE - Region marked with arrow showing clearing zone around the single band obtained on Tricine-PAGE confirming halocin activity.**

### 4.6.5 Cell lysis assay

The results presented as photomicrographs in Fig 4.33 indicated the activity of halocin SH10 on the indicator strain *Halorubrum* sp. BTSH03 clearly evidenced cell lysis. Initially *Halorubrum* sp. did not show any change on exposure to halocin SH10 (Fig 4.31a). However, after 3h the cells showed shrinkage and were found as a floc forming small islands /colonies (Fig 4.31b).

After 6h the cells were found to appear bulged and showed signs of lysis (Fig 4.31c). On further incubation for 12h complete lysis of the cells resulting in cell debris (Fig 4.31d) was observed. These observations not only confirmed the antibacterial activity of halocin SH10 against other halophiles but also suggest cell lysis as the possible mechanism of action of halocin SH10.

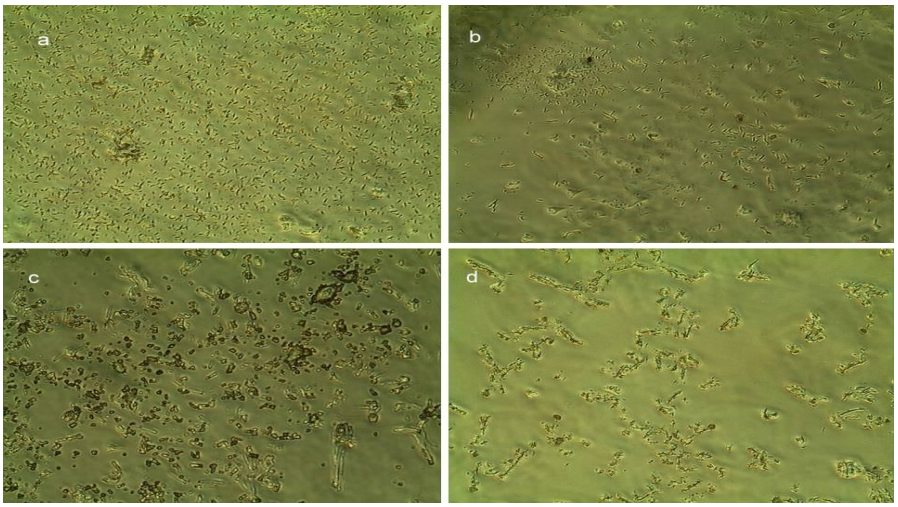



**Fig. 4.33 Action of halocin SH10 on indicator strain *Halorubrum sp*. BTSH03 (Phase contrast microscopic view- adapted from Karthikeyan *et al*.,2013)**

(a) BTSH03 normal cells soon after SH10 treatment
(b) 3 hours after treatment -cells shrinks and forms islands
(c) 6 hours after treatment -cells appear bulged
(d) 12 hours after treatment -cells completely lysed

## 4.7 CHARACTERIZATION OF HALOCIN PRODUCED *NATRINEMA* SP. BTSH10

### 4.7.1 N-TERMINAL SEQUENCING

First five amino acids were detected by N-Terminal protein sequencing. They were "ala – pro – phe –tyr - ile (APFYI)". Out of the five N-Terminal aminoacids of SH10 four were non-polar hydrophobic aminoacids.

### 4.7.2 MALDI and MASCOT analysis

MALDI was performed for the 20kDa protein and the m/z value obtained (Fig 4.34) was used to check the protein similarity by MASCOT analysis. This protein similarity search (Fig 4.35) indicated that the halocin shared sequence similarity with 50S ribosomal protein L3P of *Methanosarcina acetivorans* (strain ATCC 35395).

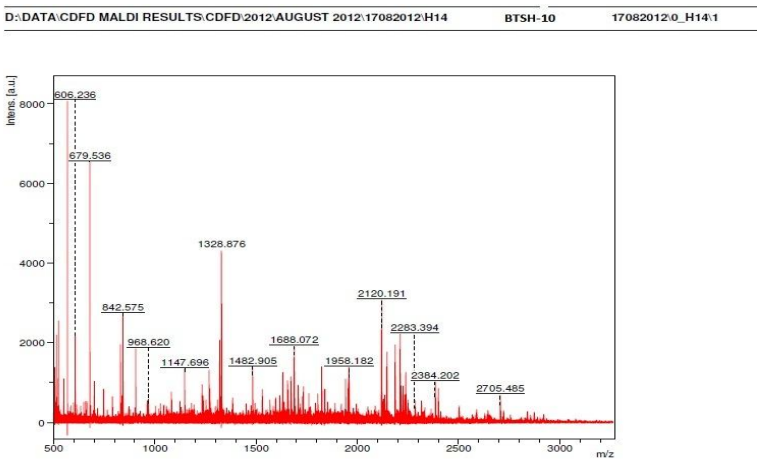



**Fig. 4.34 MALDI profile of the 20kDa halocin**

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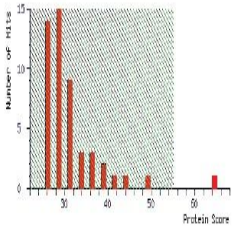
Search title :
MS data file : peaklist.xml
Database : SwissProt 2012_07 (536789 sequences; 190518892 residues)
Taxonomy : Archaea (Archaeobacteria) (18914 sequences)
Timestamp : 1 Sep 2012 at 08:38:39 GMT
Top Score : 64 for RL3_METAC, 50S ribosomal protein L3P OS=Methanosarcina acetivorans (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A) GN=rpl3p PE=3 SV=1

**Mascot Score Histogram**

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 55 are significant (p<0.05).




**Protein Summary Report**

Format As | Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

**Index**

| | Accession | Mass | Score | Description |
|---|---|---|---|---|
| 1. | RL3_METAC | 36870 | 64 | 50S ribosomal protein L3P OS=Methanosarcina acetivorans (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A) GN=rpl3p PE=3 SV=1 |
| 2. | PUR2_ARCFU | 52117 | 48 | Phosphoribosylamine--glycine ligase OS=Archaeoglobus fulgidus (strain ATCC 49558 / VC-16 / DSM 4304 / JCM 9628 / NBRC 100126) GN=purD PE=3 SV=1 |
| 3. | SYFB_METTH | 62050 | 44 | Phenylalanine--tRNA ligase beta subunit OS=Methanothermobacter thermautotrophicus (strain ATCC 29096 / DSM 1053 / JCM 10044 / NBRC 100330 / Delta H) GN |
| 4. | THII_ARCFU | 42530 | 42 | Probable tRNA sulfurtransferase OS=Archaeoglobus fulgidus (strain ATCC 49558 / VC-16 / DSM 4304 / JCM 9628 / NBRC 100126) GN=thiI PE=3 SV=1 |
| 5. | HIS8_PYRFU | 39233 | 39 | Histidinol-phosphate aminotransferase OS=Pyrococcus furiosus (strain ATCC 43587 / DSM 3638 / JCM 8422 / Vc1) GN=hisC PE=3 SV=1 |
| 6. | RL10_PYRAR | 20204 | 39 | 50S ribosomal protein L10e OS=Pyrobaculum aerophilum (strain ATCC 51768 / IM2 / DSM 7523 / JCM 9630 / NBRC 100827) GN=rpl10e PE=3 SV=1 |
| 7. | QUEC_PYEHO | 26605 | 38 | 7-cyano-7-deazaguanine synthase OS=Pyrococcus kodakaraensis (strain ATCC BAA-918 / JCM 12380 / KOD1) GN=queC PE=3 SV=1 |
| 8. | DAPAL_THENV | 32213 | 37 | Uncharacterized DAPA-like lyase Tneu_1812 OS=Thermoproteus neutrophilus (strain DSM 2338 / JCM 9278 / VC45ta) GN=dapAL PE=3 SV=1 |
| 9. | MOAA_METMA | 35979 | 36 | Probable molybdenum cofactor biosynthesis protein A OS=Methanothermobacter marburgensis (strain DSM 2133 / 14651 / NBRC 100331 / OCM 82 / Marburg) GN=m |
| 10. | CHEB_PYCHO | 40302 | 33 | Chemotaxis response regulator protein-glutamate methylesterase OS=Pyrococcus horikoshii (strain ATCC 700860 / DSM 12428 / JCM 9974 / NBRC 100139 / OT-3 |
| 11. | Y3388_METAC | 48570 | 33 | Uncharacterized serpin-like protein MA_3388 OS=Methanosarcina acetivorans (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A) GN=MA_3388 PE=3 SV=1 |

**Fig. 4.35 MASCOT search for the 20kDa halocin showing similarity results**

### 4.7.3 NMR of halocin SH10

NMR spectrum of halocin SH10 showed intense peaks in the aliphatic range (Fig 4.36) clearly indicating that the aliphatic chain containing aminoacids were present in numerous counts. It is well known that aliphatic R groups are non polar and hydrophobic.

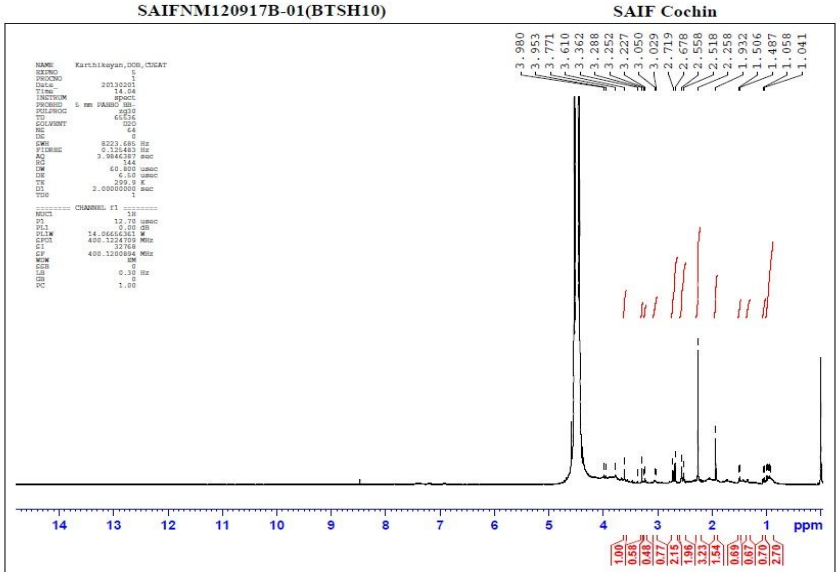



**Fig. 4.36 NMR spectrum of Halocin SH10 showing the peaks at aliphatic range**

### 4.7.4 Total amino acid analysis of SH10

The total aminoacid content of halocin SH10 was performed and the results showed that the glycine was the dominant aminoacid (12.74%) followed by asparigine(10.35%), glutamine (9.78%), valine (6.05%), alanine (3.94%), serine (3.69%), histidine (3.43%), threonine (3.11%). Other amino acids were found in

trace amount ranging from 0.1% to 1.3%. This showed the presence of hydrophobic amino acids in higher percentage when compared to other amino acids (Table 4.8).

**Table. 4.8 Concentration of different aminoacid content of halocin SH10 and its properties**

| S. No | Amino acid | Percentage of Amino acid (%) | Amino acid property |
|---|---|---|---|
| 1 | Asp | 10.35 | Polar Acidic |
| 2 | Thr | 3.11 | Polar Neutral |
| 3 | Ser | 3.69 | Polar Neutral |
| 4 | Glu | 9.78 | Polar Acidic |
| 5 | Pro | 0.10 | Non-Polar Hydrophobic |
| 6 | Gly | 12.74 | Non-Polar Hydrophobic |
| 7 | Ala | 3.94 | Non-Polar Hydrophobic |
| 8 | Cys | 0.46 | Polar Neutral |
| 9 | Val | 6.05 | Non-Polar Hydrophobic |
| 10 | Met | 0.26 | Non-Polar Hydrophobic |
| 11 | Ile | 0.95 | Non-Polar Hydrophobic |
| 12 | Leu | 1.29 | Non-Polar Hydrophobic |
| 13 | Phe | 0.83 | Non-Polar Hydrophobic |
| 14 | His | 3.43 | Polar Basic |
| 15 | Lys | 0.19 | Polar Basic |
| 16 | Arg | 0.80 | Polar Basic |

### 4.7.5 Halocin stability assay

The stability of halocin at different temperatures, pH and in different solvents were studied and the results are presented below

#### 4.7.5.1 Temperature stability studies

The halocin SH10 was thermostable. Results presented in Fig 4.37 suggested that the halocin was stable over a range of temperature from 4°C to 40°C (4096AU) without any loss of activity which however got declined to 1024AU at 60°C and 70°C and 128AU at 80°C and then lost activity at higher temperatures.

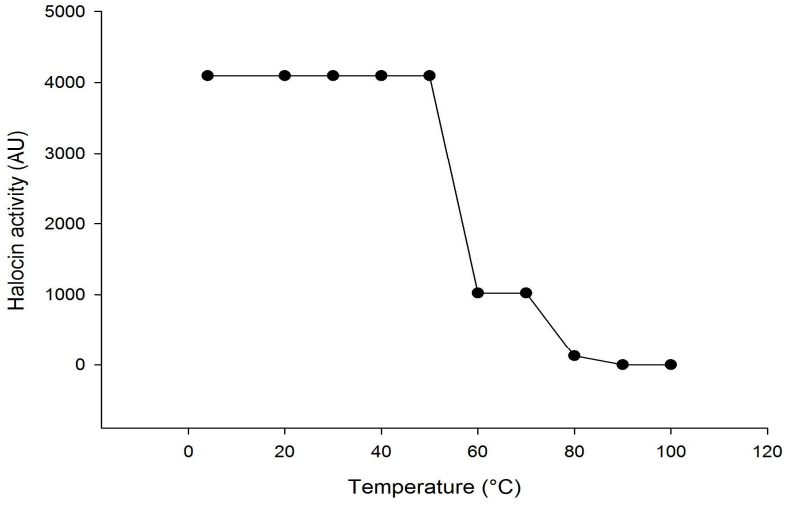



**Fig. 4.37 Thermostability of halocin SH10.**

### 4.7.5.2 pH stability studies

With respect to pH stability it was noted that the halocin was stable (Fig 4.38) only at pH 6-8 (4096AU) and at pH 9 (2048AU) the activity got declined.

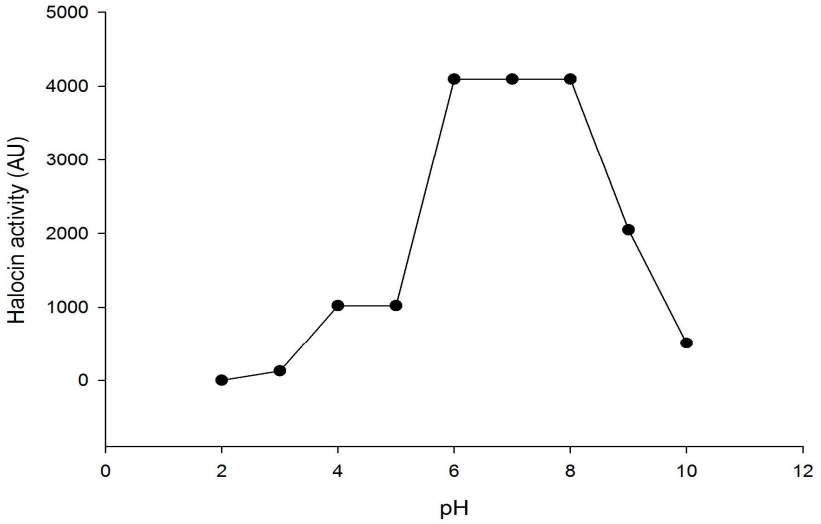



**Fig. 4.38 pH stability of halocin SH10.**

### 4.7.5.3 Organic solvent stability

From the results depicted in Fig 4.39 it was evident that the halocin SH10 could retain its activity (4096AU) when incubated with 10% of organic solvents. However upon incubation with 20% solvent the halocin showed reduction in its activity (2048AU) with ethanol and lost more than 50% activity in methanol (1024AU) and chloroform (64AU). Whereas in

30% acetone, acetonitrile and isopropanol the halocin was observed to retain its 4096AU even after one hour of incubation.

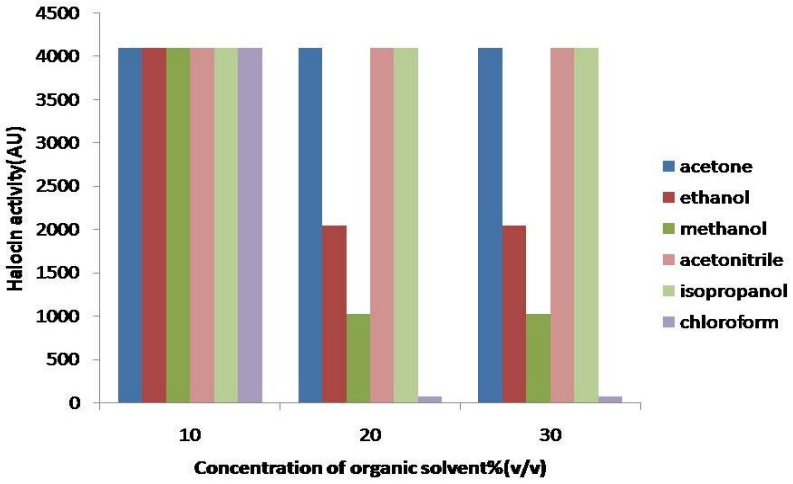



**Fig. 4.39 Effect of organic solvents on halocin SH10.**

## 4.8. APPLICATION OF HALOCIN SH10 PRODUCED BY *NATRINEMA* SP. BTSH10

### 4.8.1. Evaluation of halocin SH10 as preservative for leather hides

Evaluation of halocin SH10 as preservative for storage of leather hides was done by subjecting the leather hides to halocin treatment. From the results obtained and present in Fig 4.40 it was observed that halocin treatment for 12 hours could inhibit halophilic bacterial growth on hides. In raw hides 67 ± 2 CFU were obtained. Whereas in the raw hides treated with Halocin for3 hours, 6h and 12h, the CFU was found to get drastically decreased.

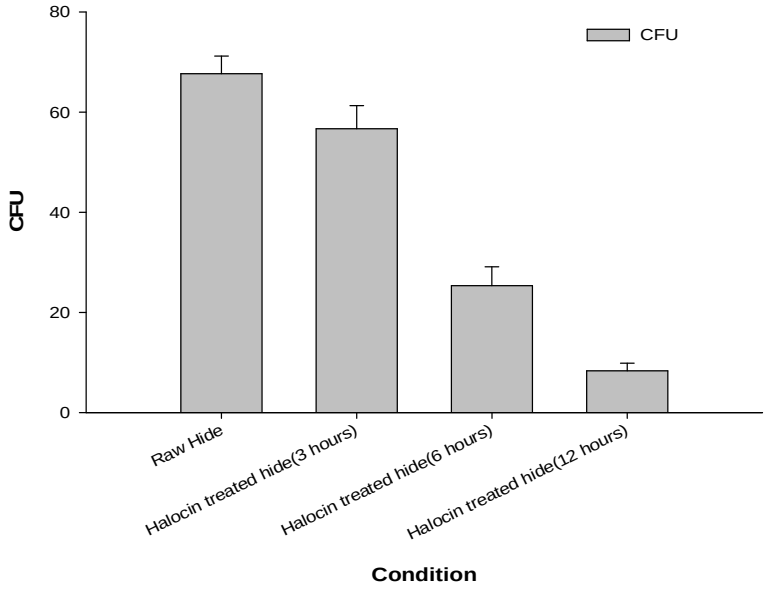



**Fig. 4.40 Bacterial load (CFU) on hides upon treatment with Halocin SH10**

### 4.8.2. Preliminary *in vitro* short term toxicity studies of the samples

*In vitro* short term toxicity studies were carried out with halocin SH10 against Dalton's Lymphoma Ascitis (DLA) cells using standard procedures. The data presented in Table 4.9 evidenced that halocin SH10 has a good activity against DLA cells. The samples that had $CTC_{50}$ values greater than 500 showed either very less activity or no activity. Halocin SH10 also showed very good cytotoxicity. Lower concentrations of halocin (ie., 2AU and 4AU ) had high $CTC_{50}$ valued indicating lesser activity or no activity. Remaining all other concentrations showed $CTC_{50}$ value less than 500 and hence was considered for further experiments.

**Table 4.9. *In vitro* short term toxicity studies of halocin SH10 against DLA cells**

| S.No | Sample concentration (AU) | % viability | % growth inhibition | $CTC_{50}$ * |
|---|---|---|---|---|
| 1 | 2 | 97.92 | 2.08 | 2971.69±14.74 |
| 2 | 4 | 92.96 | 7.04 | 905.48±13.69 |
| 3 | 8 | 83.12 | 16.88 | 400.37±13.34 |
| 4 | 16 | 78.74 | 21.26 | 325.92±09.81 |
| 5 | 32 | 70.28 | 29.72 | 250.02±07.20 |
| 6 | 64 | 64.58 | 35.42 | 211.22±10.91 |
| 7 | 128 | 58.17 | 41.83 | 184.82±12.53 |
| 8 | 256 | 52.94 | 47.06 | 168.62±11.32 |
| 9 | 512 | 39.05 | 60.95 | 139.08±13.68 |
| 10 | 1024 | 31.53 | 68.47 | 128.09±11.16 |
| 11 | 2048 | 17.43 | 82.57 | 112.99±14.53 |
| 12 | 4096 | 10.96 | 89.04 | 107.52±09.08 |

* Average of three independent experiments ± S.E.M.

### 4.8.3. *In vitro* cytotoxicity studies

*In vitro* cytotoxic properties of halocin SH10 was evaluated on several cancer and normal cell line cultures using standard microculture tetrasolium (MTT) assay. HBL100 was the normal cell line used to test the cytotoxicity of SH10. Results presented in Figure 4.41 shows the percentage viability of cells against the cell line HBL100 (normal cell). The $IC_{50}$ value of the sample was determined as 4096AU.

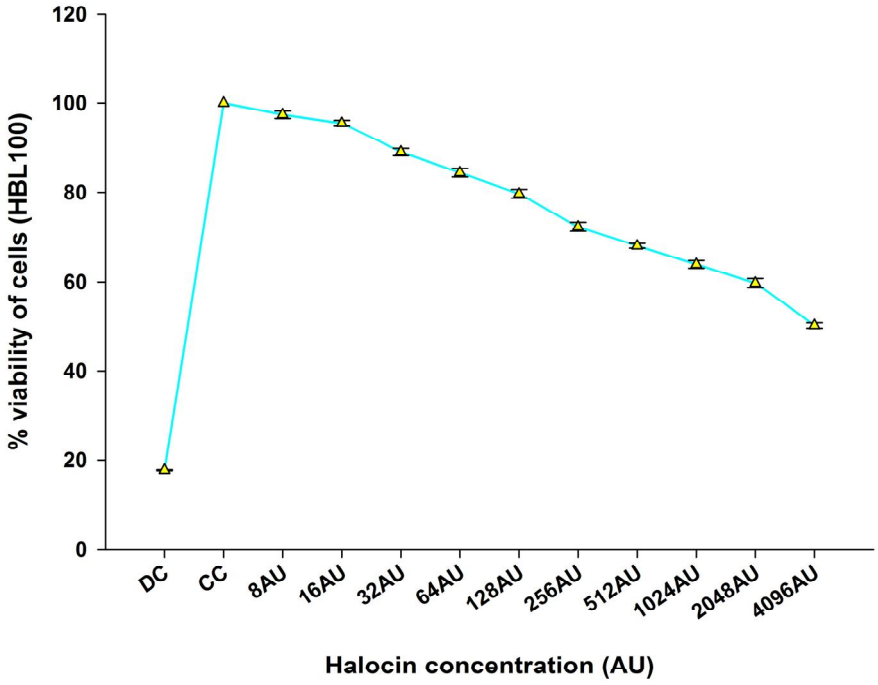



**Fig.4.41 *In vitro* anticancer activity of the halocin SH10 against HBL100 cell lines**

Figure 4.42(a) shows the normal cell line control and Fig 4.42 (b) shows the distorted cells after SH10 activity. This clearly indicated that halocin SH10 has cytotoxity activity but at a lower concentration. It was also observed that cell lysis occurred in normal cell lines when the concentration was increased. Hence, further studies against cancer cell lines were made based on the $IC_{50}$ value of SH10 towards HBL100 cell lines.

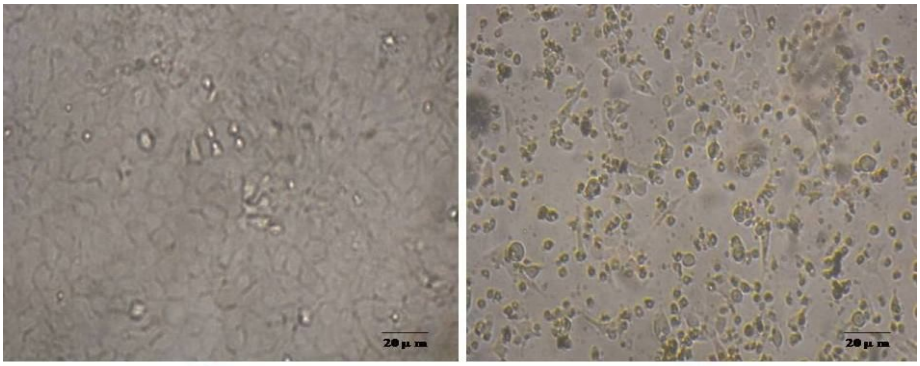



**Fig.4.42 Cytotoxicity of halocin SH10 against HBL100 cell lines. (a)Normal cell line (b) Cytotoxocity caused by halocin SH10**

Halocin SH10 activities against HeLa (cervical cancer cells) cell lines were observed. It was noted that there was distortion of the cells and the $IC_{50}$ was found to be 1024AU (Fig. 4.43). The results clearly evidenced cytotoxicity effect of the halocin SH10 against the cancer line. Activity of halocin SH10 on the HeLa cell line is presented in Fig. 4.44

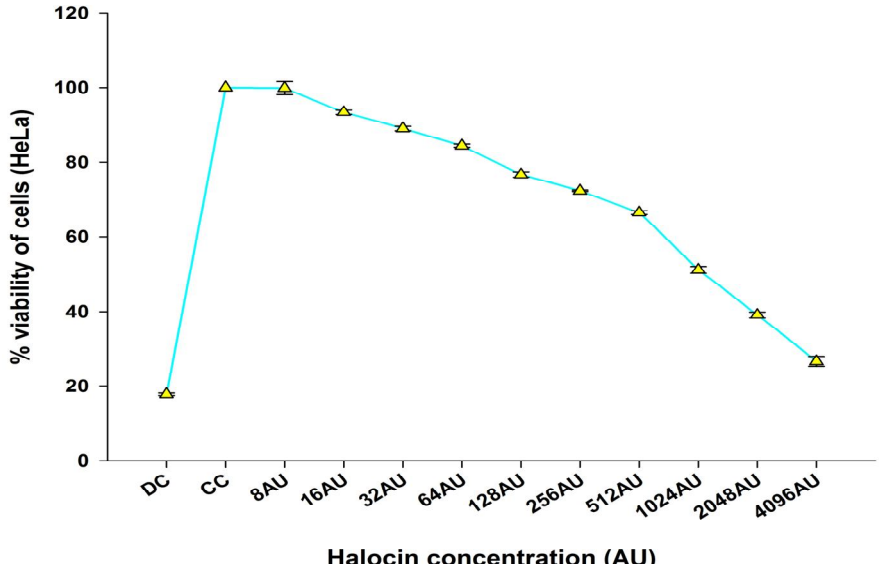



**Fig.4.43 *In vitro* anticancer activity of the halocin SH10 against HeLa cell lines**

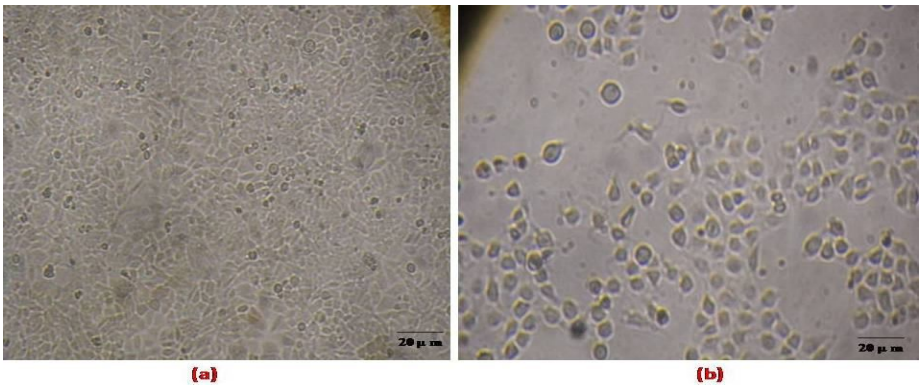



**Fig.4.44 Cytotoxicity of halocin SH10 against HeLa cell lines. (a)Normal cell line (b) Cytotoxicity caused by SH10**

A549 cells are adenocarcinomic human alveolar basal epithelial cells also known as the lung cancer cell line. Results obtained for the *in vitro* anticancer activity of the halocin SH10 against A549 cell lines is presented in Fig 4.45 and the $IC_{50}$ was determined as 512AU. From the results depicted in Fig 4.46 it was inferred that halocin SH10 has cytotoxicity against A549 cell lines.

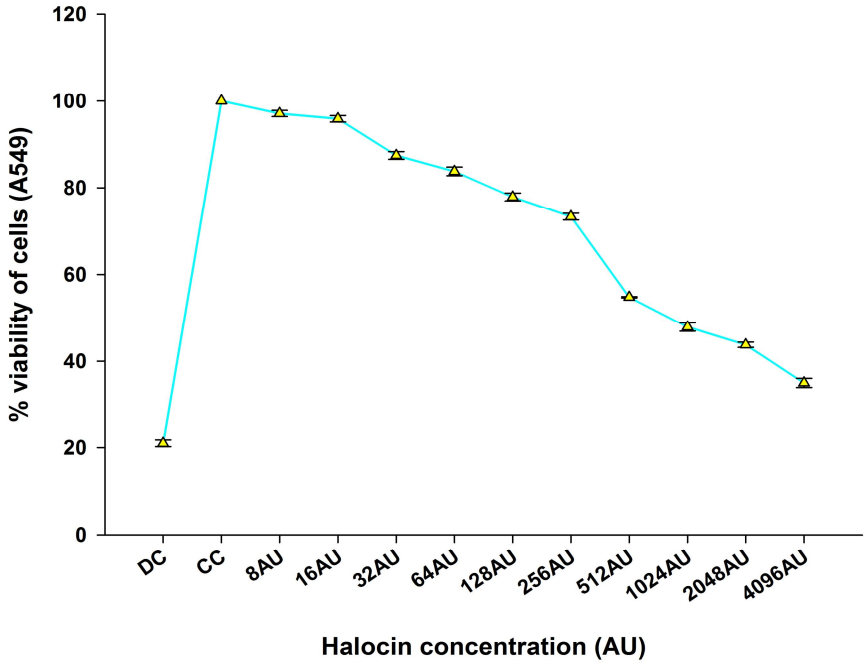



**Fig.4.45 *In vitro* anticancer activity of the halocin SH10 against A549 cell lines**

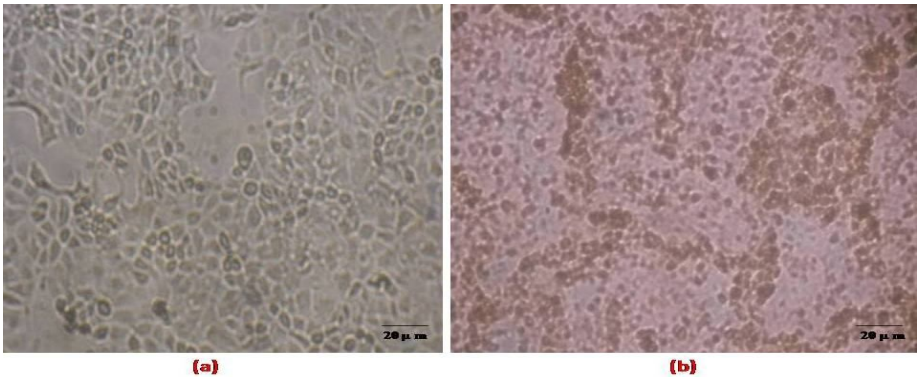



**Fig. 4.46 Cytotoxicity of halocin SH10 against A549 cell lines. (a)Normal cell line (b) Cytotoxicity caused by halocin SH10**

The OAW42 cell line was established from the ascites of a patient with ovarian cystadenocarcinoma also known as the ovarian cell line. Results obtained for the *in vitro* anticancer activity of the halocin SH10 against OAW42 cell lines is presented in Fig 4.47 and the $IC_{50}$ was determined as 512AU. From the results depicted in Fig 4.48 it was inferred that halocin SH10 has cell cytotoxicity against OAW42 cell lines

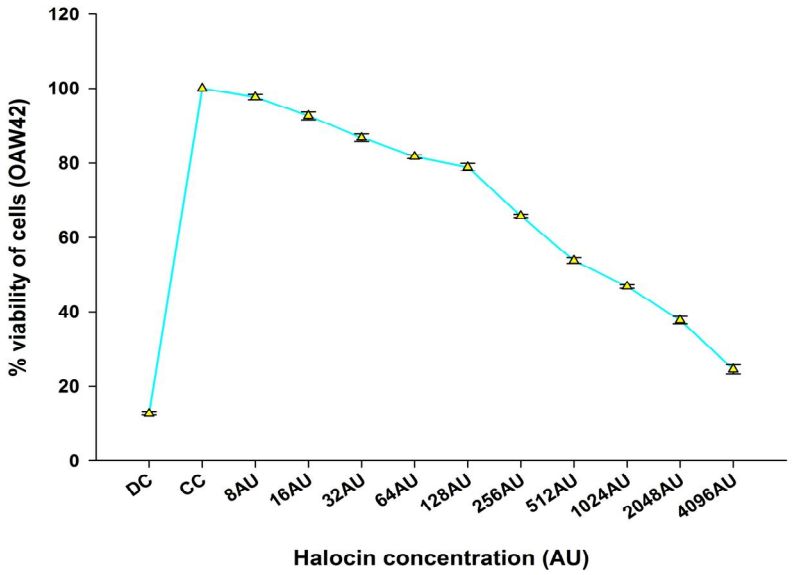



**Fig. 4.47 *In vitro* anticancer activity of the halocin SH10 against OAW42 cell lines**

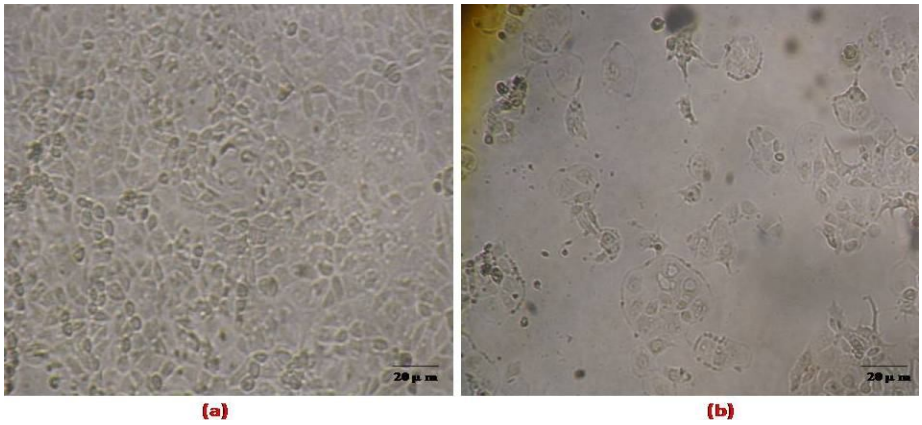



**Fig.4.48 Cytotoxicity of halocin SH10 against OAW42 cell lines. (a)Normal cell line (b) Cytotoxicity caused by halocin SH10**

Human epidermoid carcinogenic cell line (Hep2 cell line) has been described to originate from tumours which were produced in irradiated-cortisonised weanling rats after injection of epidermoid carcinoma tissue isolated from the larynx. Results obtained for the *In vitro* anticancer activity of the halocin SH10 against HEp2 cells is presented in Fig 4.49 and the $IC_{50}$ was determined as 512AU. From the results depicted in Fig 4.50 it was inferred that halocin SH10 has cell cytotoxicity against HEp2 cells.

These results clearly showed that halocin SH10 has anticancer activity. Halocin SH10 has IC50 value of 4096AU against HBL100 cell line which is a normal cell line. In the other cancer cell lines such as HeLa, A549, OAW42 and HEp2 the IC50 values were determined as 1024AU, 512AU, 512AU and 512AU respectively. These observations indicated that damage is caused to normal cell line only at higher concentrations of halocin SH10 while cytotoxicity against cancer cell lines could be recorded at a lower concentration suggesting the possible use of halocin SH10 as a effective drug against cancer.

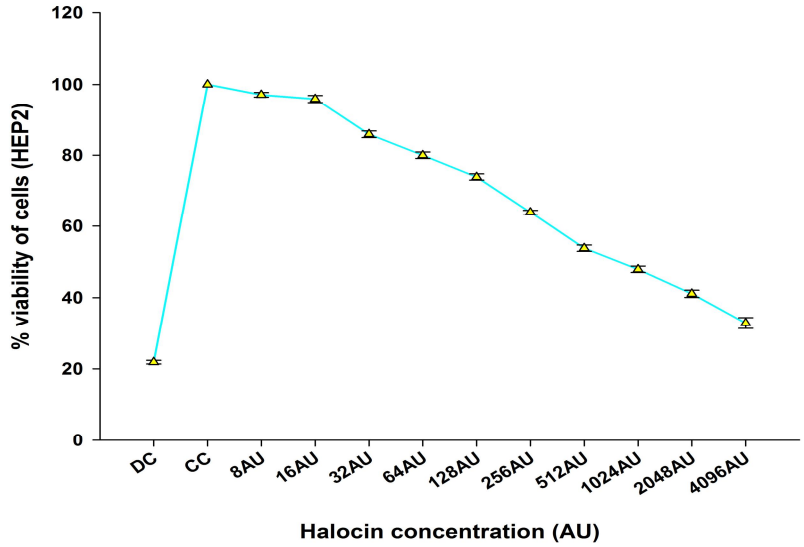



**Fig.4.49 *In vitro* anticancer activity of the halocin SH10 against HEp2 cell lines**

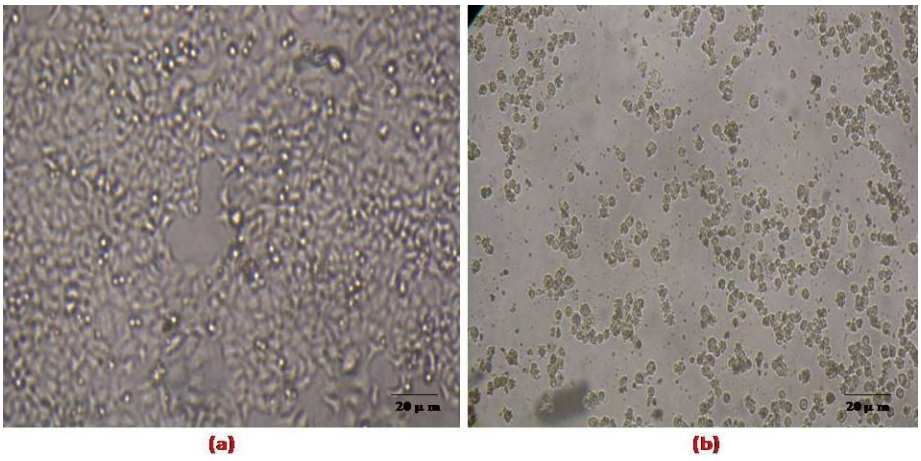



**Fig.4.50 Cytotoxicity of halocin SH10 against HEp2 cell lines. (a)Normal cell line (b) Cytotoxicity caused by halocin SH10**

### 4.8.4. *In vivo* cytotoxicity studies

The results for the studies on the effect of halocin SH10 on DLA induced ascitic tumor are given in Table 4.10 and in Figures 4.51 – 4.56. From the data presented the following informations were inferred:

#### 4.8.4.1. Antitumour parameters

The treatment with halocin SH10 at 0.1mL/kg of 512AU significantly ($p<0.05$) increased the average life span (ALS) of DLA bearing mice from 17.35 ± 0.56 to 23.52 ± $1.05^b$ days, when compared to DLA tumor control group. The standard 5-FU at 20mg/kg, significantly ($p < 0.001$) increased the ALS to 29.24 ± 1.37days. 1024AU of halocin SH10 at 0.1 ml/kg showed increase ($p<0.05$) in ALS namely 27.72 ± 1.21. The increase in body weight of DLA bearing mice was 34.23 ± 0.62. Treatment with 1024AU halocin SH10 showed a significant reduction in percent increase in body weight ($p<0.001$) when compared to DLA control. The standard 5-FU, however, was more potent in inhibiting the increase in body weight of tumor bearing mice. 1024AU halocin SH10 also showed a significant reduction in percent increase in body weight, when compared to DLA control.

#### 4.8.4.2. *Hematological Parameters*

Inoculation of DLA cells resulted in a significant increase in the levels of total WBC, neutrophils and eosinophils and a significant decrease in the levels of RBC, haemoglobin and lymphocytes in DLA control group when compared to normal group of animals ($p< 0.001$). 1024AU of halocin SH10 at 0.1mL/kg was found to significantly ($p< 0.001$) decrease the levels of total WBC, neutrophils and

eosinophils and also led to significant ($p < 0.001$) increase in the levels of RBC, haemoglobin and lymphocytes when compared to DLA control group. The standard 5-FU, however, was found to be more potent than halocin SH10 causing reversal of the haematological parameters towards normal values.

**Table 4.10. Effect of halocin SH10 on antitumor parameters of DLA bearing mice**

| Parameter | Normal | DLA | DLA+5FU | DLA+0.1ml 512 AU | DLA+0.1ml 1024 AU |
|---|---|---|---|---|---|
| **Average life span** | - | 17.35±0.56 | 29.24±1.37[d] | 23.52±1.05[b] | 27.72±1.21[b] |
| **% increase in life span** | - | - | 63.42±1.26 | 26.42±1.13 | 45.17±1.21 |
| **% increase in body weight** | - | 34.23±0.62 | 11.25±1.32[d] | 24.43±1.35[b] | 20.85±1.73[d] |
| **Total WBC($1x10^3$ $mm^3$)** | 10.41±0.46 | 19.58±1.22[a] | 10.77±0.32[d] | 15.19±0.51[d] | 13.36±0.45[d] |
| **Total RBC ($1x10^6$ $mm^3$)** | 11.10±0.38 | 5.52±0.47[a] | 9.45±0.29[d] | 8.15±0.31[d] | 8.75±0.16[d] |
| **Haemoglobin(g/dl)** | 11.10±0.38 | 8.64±0.68[a] | 13.43±0.57[d] | 11.02±0.79[c] | 12.35±0.72[d] |
| **Differential WBC** | | | | | |
| **Lymphocytes** | 66.36±0.84 | 34.55±0.83[a] | 64.28±0.92[d] | 57.97±1.28[d] | 59.82±1.27[d] |
| **Neutrophils** | 29.18±0.57 | 62.23±0.82[a] | 32.68±1.62[d] | 36.42±1.10[d] | 33.46±1.52[d] |
| **Eosinophils** | 4.07±0.13 | 5.85±0.27[a] | 4.52±0.22[d] | 5.78±0.26 | 5.01±0.28[c] |

The results are Mean ± S.E.M. (n=5), a= $p<0.001$, between normal and tumor control group, b= $p<0.05$, c= $p<0.01$, d= $p<0.001$, between tumor control and treated groups

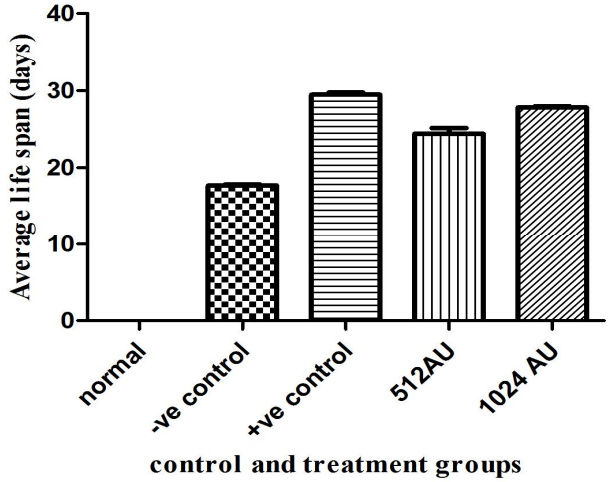



**Fig.4.51 Effect of halocin SH10 on average life span of DLA tumor bearing mice**

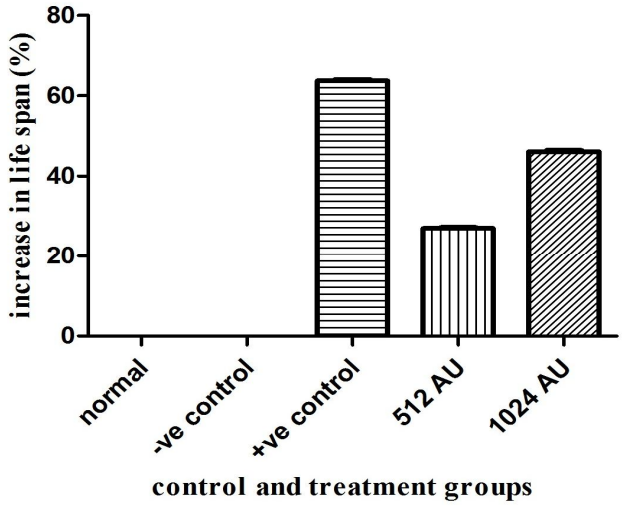



**Fig.4.52 Effect of halocin SH10 on % increase of average life span of DLA tumor bearing mice**

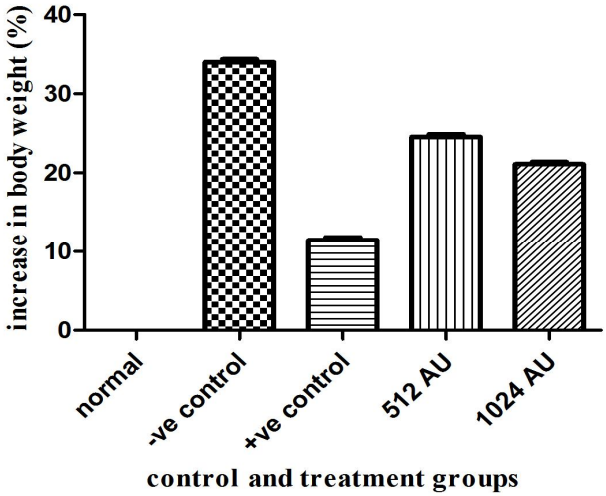



**Fig. 4.53 Effect of halocin SH10 on % increase on body weight of DLA tumor bearing mice**

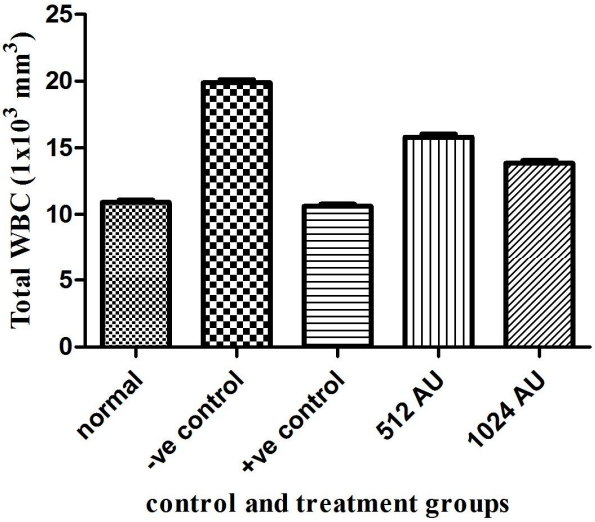



**Fig.4.54 Effect of halocin SH10 on total WBC count of DLA tumor bearing mice**

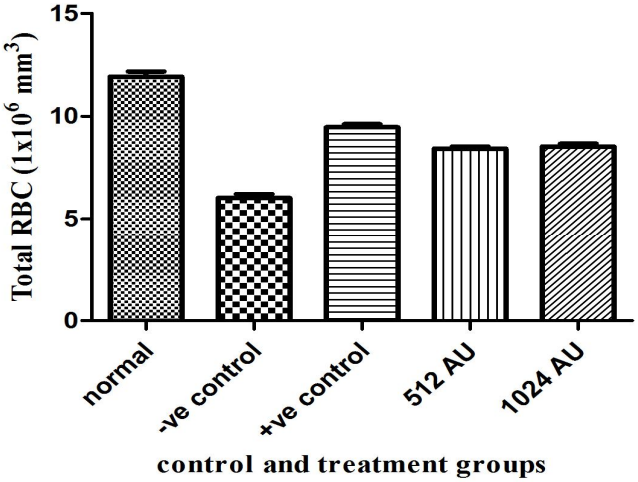



**Fig. 4.55 Effect of halocin SH10 on total RBC count of DLA tumor bearing mice**

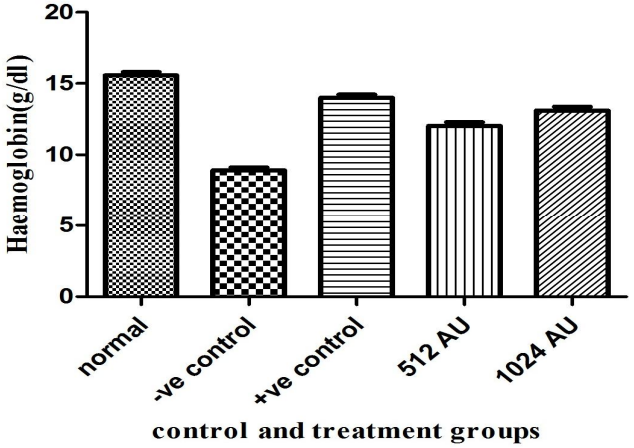



**Fig. 4.56 Effect of halocin SH10 on haemoglobin content of DLA tumor bearing mice**

# *Chapter 5*

## DISCUSSION

### 5.1 Isolation of potential halocin producing haloarchaea

The *Halobacteriaceae* are extremely halophilic archaeobacteria that inhabit aquatic hypersaline environments. They produce a wide variety of antagonistic substances termed halocins (Rodriguez *et al.,* 1982; Meseguer *et al.,* 1986) which resemble bacteriocins of eubacteria (Reeves, 1965). Many representatives of the family *Halobacteriaceae* (halobacteria) excrete halophilic bacteriocins (halocins) that inhibit the growth of other halobacteria. In spite of the fact that halocin production is widespread among the *Halobacteriaceae,* no information is available on their ecological significance. Halocins may play a role in the interspecies competition between different types of halobacteria in saltern crystallizer ponds inhabited by dense communities of these red halophiles. A study of antagonisms among 79 strains of halobacteria showed that probably at least 15 different groups of halocins are present (Meseguer *et al.,* 1986). The mechanism of action of this halocin seems to involve the disruption of ion-gradients across the membrane of target cells. The halocin causes cell-death and lysis that follow 'single-hit'-type kinetics (Meseguer and Rodriguez, 1986). Halocin production has been shown to be a near-universal feature of haloarchaeal rods (Torreblanca *et al.*, 1994) and, based on antagonism studies, hundreds of different types have been found to exist (Meseguer *et al*., 1986, Torreblanca *et al.*, 1994). In order to explore halocin diversity at both the protein and gene expression levels and to exploit halocins as models for stationary-phase gene regulation, many more halocins need to be characterized (Price and Shand, 2000).

Multi-pond solar salterns, which are used worldwide for salt production along tropical and subtropical coastal areas, present an environment with increasing salt concentrations, from seawater to NaCl saturation. Characteristic salt-adapted microbial communities are found along the salinity gradient. However studies pertaining to microbiology of saltern ponds of South India which contribute to significant amount of commercial salt production have not been made adequately. In this context the present study addressed the prospects of deriving haloarchaeal bacteria which may return potential halocins that may hold scope for applications in several industries.

In the present study two potential haloarchaeal strains BTSH10 and BTSH03 were isolated from salt pans of Kanyakumari, Tamilnadu, India. Among the two, the strain BTSH10 showed strong halocin activity against the strain BTSH03 which showed sensitivity against the halocin produced by BTSH10. The halocin producing strain BTSH10 and the indicator strain BTSH03 were identified as *Natrinema* sp. BTSH10 and *Halorubrum* sp. BTSH03, respectively. The genus *Natrinema* was proposed by McGenity *et al*., 1998. The genus *Halorubrum* was proposed by Mc Genity and Grant to accommodate *Natrinema pellirubrum* (formerly *Halobacterium salinarum* NCIMB 786T) and *Natrinema pallidum* (formerly *Halobacterium halobium* NCIMB 777T). In a phylogenetic tree based on 16S rRNA gene sequences, *Natrinema* species formed an independent cluster with respect to *Halobacterium* species. *Natrinema* species could be cultured at low salt concentrations, and possessed a specific protein profile and polar lipid composition. Subsequently, a novel species of this genus, *Natrinema versiforme*, was described (Xin *et al*., 2000).

From the results obtained for the standardization of optimal cultivation media for halocin production, it was inferred that Zobell's medium supported enhanced growth and halocin production compared to other media evaluated and

hence the same was selected for further cultivation of the halophiles. Zobell's medium with varied inorganic salts was originally designed for isolation of marine bacteria and perhaps that is the reason why halophiles from saltern ponds found this medium as optimal medium for enhanced growth.

Since the halocin was produced by *Natrinema* sp. BTSH10 it was named as halocin SH10 for easy reference and to make a difference from other reported halocins. Interestingly *Natrinema* sp.BTSH10 showed resistance to 12 antibiotics. Perhaps this antibiotic resitance could be attributed to a possible presence of large megaplasmids in this bacteria (Argandona *et al*., 2003). Further FAME analysis has also shown that *Natrinema* sp. BTSH10 contain a number of fatty acids both saturated and unsaturated; many of them are identified and few are still unidentified (Pabba *et al*., 2011)

### 5.2 Characterization of salt crystal formed by *Natrinema* Sp. BTSH10

*Natrinema* sp.BTSH10 showed characteristic salt crystal formation during their growth in cultivation medium. The salt crystal thus formed known as the halite was loaded with halobacterial cells which were present inside. The group colonizing inside the halite may be haloarchaea which requires a minimum of 2.5M NaCl for survival (Grant *et al*., 1989). This life inside the halite was possible by the formation of larger fluid inclusions (Norton and Grant, 1988). The haloarchaea can survive inside the crystals for years together and Brian *et al*., (2009) have demonstrated that haloarchaea survives in halite for more than 30000 years. This sounds to be a method to store haloarchaea strains for several years.

Further the present results clearly indicated that the bacteria might act as a nucleus around which the trace elements get attached which gives a crystalline nature and structure. The formation of the crystal starts only after a weeks' time.

By this time there could be depletion of nutrients in medium and hence bacteria in order to overcome the stressed condition bacteria may accumulate the salts towards formation of such type of crystal. Perhaps haloarchaea in specific may show this type of response during nutrient limited condition. Stress genes may get activated which produces some stress related proteins that might accumulate the salts around it. The increase in crystal size noted during refrigeration which was not so in normal conditions could explain this phenomenon. Earlier studies reported that dendritic crystals and cubic crystals are formed by S-Layer in the presence of NaCl. This biomineralization (Maria *et al*., 2004) or biosorption (Tsezos, 1985; Tsezos and Deutschmann, 1990) forms a honey comb like structure reassembling the S-layer. These reassembled units are suspected to act as template for crystal nucleation and growth

FTIR analysis of the crystal could be used to derive the molecular structure of this compound. The presence of different functional groups could attribute to the arrangement of different ions in the crystal. The crystal absorbs light at 230 nm and it can be used for filters and microlaser waveguides (Kumaresan *et al*., 2008). This new organic nonlinear optical crystal, grown by culturing bacteria could be made use in optical studies. Thermal analysis shows that the halite could be used in applications where the crystal is required for high temperature up to 790.85 °C (Kumaresan *et al*., 2008; Sankar *et al*., 2008)

These crystals formed may be categorized as biological hard materials and could be used as filters, cavities or microlaser waveguides, immunoassays, cell sorting (Kiyotaka 2001; Mattoussi *et al*., 2000; Sun *et al*., 2001; Marek *et al*., 2007).

The carotenoids and bacteriorhodopsin pigment producing organisms in the Brine (Oren, 2008) protect cells from the harmful effects of ultraviolet light

(Wu *et al*.,1983;Shahmohammadi *et al*., 1998) and also encourage evaporation by trapping solar radiation. This evaporation leads to NaCl precipitation due to which the haloarchaea gets trapped inside fluid inclusions, which can constitute 2±6% (w/w) of freshly harvested solar salt and this phenomenon is universal (Castanier *et al*., 1999).

## 5.3 Optimization of bioprocess variables for halocin production by *Natrinema* sp.BTSH10

From the results documented it was inferred that 42°C was the optimum temperature for maximum halocin production (1024AU). Nevertheless appreciable levels of halocin activities could be recorded at other temperatures. Halocin production observed at high levels at 42°C, and relatively at lesser levels at lower and at slightly higher temperatures (47°C) indicated that the halocin gene expression might be either temperature dependent or specific temperature of the cultivation medium might act as an antagonist (stress) to the organism that induce this gene expression (Christine *et al*., 2008; Mirko *et al*., 2012). Haloarchaeon Sech7a was reported to be thermophilic in character with optimal growth occurring at 45°C, although the temperature in its native solar saltern crystallizer rarely exceeds 32°C (Pasic *et al*., 2005). Consistent with the physicochemical properties of a crystallizer, the optimal growth of haloarchaeon Sech7a was observed at pH 8, yet the halocin production reached maximum at neutral pH (Paši *et al*., 2008). *Natrinema* sp.BTSH10 could produce halocin in media with a pH varying between pH 4 and pH 10 although maximum halocin production was recorded at pH 8.0 (1024AU). However, the bacteria did not produce halocin under acidic conditions.

*Natrinema* sp. BTSH10 required 3M NaCl for recording maximum (1024AU) halocin production although NaCl concentration ranging from 1.5M –

4M in the medium supported enhanced production of halocin. It was also noted that a minimum of 1.5M NaCl was required for growth, and lesser concentrations did not even support survival of the bacterium. These observations testified the halophilic nature of the isolated bacterium and the impact of higher concentrations of sodium chloride for halocin activity. The halocin of haloarchaeon Sech7a was observed to remain active over wide NaCl concentration range (0.02M–5.2M) with highest production observed in high salt media containing 3.4M NaCl (Paši *et al*., 2008). These observations made with *Natrinema* sp. BTSH10 was in agreement with the earlier reports for other species of haloarchaeon although there were marginal differences in optimal temperature and sodium chloride concentrations. It was also noted that sodium chloride concentration was found to have strong influence on the halocin activity of the halobacteria. Another specific observation made during the study was that the maximal halocin activity observed with these three factors namely temperature, pH and NaCl were almost same. This observation could be attributed to the fact that the control medium had 3M NaCl and pH of the medium was 7.4 which were almost identical with the optimum pH and NaCl concentrations. Hence there was no marked enhancement in halocin activity after optimization of these 3 variables. Further the results also indicated that these three factors are independent in exerting their influence on halocin production by the bacteria.

Maximal halocin production was supported by the medium supplemented with galactose (2048AU) followed by sorbitol, maltose, glycerol, glucose, fructose, and lactose. Whereas, medium supplemented with dextrin, sucrose and xylose supported reduced levels of halocin production. Galactose was observed to enhance halocin production in the medium compared to other carbon sources. The bacteria could produce maximal halocin in the presence of beef extract (2048 AU) in the medium followed by soybean meal, malt extract, tryptone, peptone, yeast extract, casein and gelatin. Urea did not support halocin production. In fact it was

reported earlier that the algae *Dunaliella* sp. (Avinash *et al*., 2011) that exist in the natural salt pan ecosystem provides galactose for the halobacteria and thus the strain recorded maximal halocin in response to supplementation of galactose under laboratory conditions. In a similar fashion maximal growth rate and halocin activity by haloarchaeon Sech7a was observed in media supplemented with glycerol and yeast extract (Paši *et al.*, 2008). It must be noted that in solar salterns, glycerol produced by blooms of unicellular green algae, *Dunaliella* is considered the most important source of organic carbon for the heterotrophic prokaryotes (Bardavid *et al*., 2008).

Additional salts in the medium were found to exert influence on halocin production by the archaebacteria. Among the inorganic salts used for supplementation of the medium as additional salts, calcium chloride (2048 AU) was found to support maximal halocin production in the medium followed by magnesium chloride, sodium fluorate, potassium chloride, and sodium bicarbonate. Whereas aluminium nitrate, potassium bromide, strontium chloride and sodium silicate led to a much reduced level of halocin production when compared to the levels noted with Zobell's medium in the absence of these particular salts. These observations indicate that the members of archaea have a special nutritional requirements and critical life style in the salt pan environment which needs to be investigated further to have a better understanding of their physiology in hyper saline environments.

It was found that higher agitation rate led to enhanced bacterial growth and production of halocin compared to lesser agitation rates. A maximum of 4096AU was recorded at 200 rpm and 250 rpm although lower agitation rates (50 rpm to 150 rpm) also supported considerable levels of halocin production.This might be due to better mixing of the medium which facilitates better mass transfer and also does not support the adherence of the microbes to the surface of the flask.

These observations strongly suggested that aerobic conditions are required for enhanced halocin production by the halophilic *Natrinema* sp. Generally the dissolved oxygen is low in aqueous medium at higher concentrations of NaCl and hence aerobic organisms need provision of adequate oxygen for enhanced electron transport and consequent growth and halocin production. Normally the agitation process facilitates infusion of atmospheric air into the growth medium and provides required oxygen for the bacterium (Feng *et al*., 2003; Hay *et al*., 2012).

*Natrinema* sp. BTSH10 produce halocin at enhanced levels only during the stationary phase of growth although significant levels of halocin could be noted during late exponential phase. Maximum (8192 AU) production of halocin was observed at 104 hours, during the stationary phase. Nevertheless the halocin activity in the medium was also noted even after 144h. The halocin production was reported to register an increase when the culture entered the exponential phase and continued to increase during the course of exponential phase although maximal level was attained during the stationary phase (Price and Shand, 2000; O'Connor and Shand, 2002). These observations made in the present study indicated that in the case of *Natrinema* sp. BTSH10, halocin production is growth associated which gets accumulated in the cell and released during stationary phase. In an earlier study halocin production by haloarchaeal strain Sech7a was reported to be growth dependent (Paši *et al*., 2008). Although the onset of halocin activity was observed in the early exponential phase of growth, the halocin Sech7a peak activity was observed as the bacteria entered the stationary phase of growth (Paši *et al.*, 2008) in contrast to most other halocins which were first detected when the bacteria entered the stationary phase of growth (Shand *et al*., 1999; O'Connor and Shand, 2002). Further HalS8 activity was reported to be undetectable in culture supernatants until the culture began the transition into stationary phase (Price and Shand , 2000; Shand *et al*., 1999) and later the activity reached a maximum within 10h of onset and was stable for greater than 80h after

reaching maximum values (Price and Shand, 2000). The results observed in the present study for *Natrinema* sp. BTSH10 were very similar to that observed for most other haloarchaeal bacteria in terms of halocin synthesis during growth in the production medium. It may be noted that during the process of optimization of variables, one after another, the halocin content showed increase and reached a maximum under optimized culture conditions. This observation strongly indicated the need for optimization of production medium towards enhanced level of halocinSH10 production by *Natrinema* sp. BTSH10 and the significant role of media constituents in inducing halocin synthesis by the bacterium.

## 5.4 Purification and characterization of halocin produced by *Natrinema* sp.BTSH10

The cell free supernatant obtained after molecular weight cut off fractionation with 10kDa and then with 30kDa molecular weight cut off centrifugation, showed halocin activity. In fact the results noted for the fractions obtained with the below 30kDa range but above 10kDa indicated that the product size must be between 10kDa to 30kDa.

The gel filtration chromatography using Sephadex G50 column gave 7 peaks with halocin activity. The purity and the molecular weight of the halocin SH10 was further determined by Tricine PAGE. The single band appeared on Tricine PAGE confirmed that the molecular mass of halocin is 20kDa. The present study indicated that the halocin is relatively larger in size than the microhalocin and smaller than the halocins reported from other halobacteria. The size of halocin SH10 was found to be very different when compared with the size of halocins reported so far in the literature. Thus size of halocins such as A4 is <5 kDa (Haseltine *et al*., 2001), C8 is ~31.1kDa (Li *et al*., 2003, Sun *et al*., 2005), H1 is 31kDa ( Platas *et al*., 2002), H4 is 34.9kDa (Rodrı´guez *et al*., 1982), H6/H7is

~3kDa (Rodrı´guez-Valera *et al*., 1982, Meseguer *et al*., 1986, Torreblanca *et al* .,1989), R1is 3.8 kDa (Haseltine *et al*., 2001; O'Connor, 2002), S8 is 3.58kDa (Shand *et al*., 1999; Price and Shand 2000; Haseltine *et al*., 2001) and Sech A is10.7kDa (Pasic *et al*., 2008). The HPLC profile obtained showed that a HalocinSH10 is exactly separated between 11 and 12 min where the ACN/WATER volume was calculated as 32%: 68%. The purity of the halocin was confirmed based on comparison of the profiles. It was also inferred that the halocin is a hydrophilic protein since it was eluted in the presence of more water.

First five amino acids detected by N-Terminal protein sequencing showed the protein sequence as ala – pro – phe –tyr - ile (APFYI). Comparison of halocin SH10 with already reported protein sequences of halocin H4, S8 and C8 (http://bactibase. pfba-lab-tun.org/bacteriocinslist.php?q=halocin) showed that, out of the five N-Terminal amino acid of SH10 four are non-polar hydrophobic amino acids. This shows similar character to halocin H4 and S8. It was reported that halocin S8 contain 47% hydrophobic aminoacid (Price and Shand, 2000) and halocin H4 contain a hydrophobic segment between aminoacid region 178- 209 (Cheung *et al* .,1997). NMR spectrum of halocin SH10 showed intense peaks in the aliphatic range indicating clearly the presence of aliphatic chain containing amino acids which are numerous in count. It is well known that Aliphatic R groups are non polar and Hydrophobic. Total amino acid profile showed the presence of hydrophobic amino acids in higher percentage when compared to other amino acids. This observation adds evidence to the fact that halocin SH10 shares a similar character with that of halocin H4 and S8. MALDI was performed and the m/z value obtained was used to check the protein similarity by MASCOT analysis. This protein similarity search showed that the halocin shared sequence similarity with 50S ribosomal protein L3P of *Methanosarcina acetivorans* (strain ATCC 35395).

In the present investigation it was found that halocin SH 10 produced by *Natrinema* sp. BTSH10 could kill the indicator strain *Halorubrum* sp. BTSH03 by cell swelling followed by cell lysis. Halocins have been reported to generally kill the indicator organisms by cell swelling followed by cell lysis (O'Connor and Shand, 2002; Sun *et al*., 2005; Paši *et al*., 2008). Similar action has been observed with halocin Sech7a (Pasic *et al*., 2008), H4 (Meseguer and Rodrı´guez, 1985), H6 (Torreblanca *et al*., 1989), and C8 (Li *et al*., 2003). Torreblanca *et al*., (1989) also reported that due to halocinH6 action the intracellular volume of sensitive cells increased followed by cell swelling and lysis. They also suggested that these intracellular changes might have happened due to the action of halocin H6 on the cell membrane. Following this lead, experiments that measured changes in cell volume, internal pH, membrane potential, proton motive force and sodium and proton flux in response to HalH6 were conducted and the results showed that the $Na^+/H^+$ antiporter is the target of this halocin (Meseguer *et al*., 1995).

## 5.5 Halocin as a preservative for leather

Leather industry makes use of hides and skins. Once the hide is removed from the animal, the inner surface of the hide becomes contaminated, bacteria penetrate the hide and their action rapidly overtakes that of autolysis. Hide putrefaction is essentially attributed to degradation of heterogeneous fibrous matrix. In most of the occasions, aerobic and facultative anaerobic organisms were found to be associated with the cattle hide, which were found to cause only partial hydrolysis of hide proteins. Hence raw hides before processing are stored by applying raw salts. When they are stored for a long time the halophilic /salt tolerant lipolytic and proteolytic bacteria colonize the hides and form small pores and also lead for putrefaction. Formation of such pores on the hides does not contribute for the production of quality leather products. Applying the halocin as an agent to reduce to number of Bacteria on the hides will help in storage of raw

hides for a long time and to maintain quality in leather. In this context the studies conducted with hides using halocin SH10 as a possible preservative indicated that halcoin treated hides had relatively very low bioburden compared to control which did not receive halocin treatment. Halocin treatment for 12h could inhibit halophilic bacterial growth on hides during incubation for 10 days compared to other period of treatment. Probably this halocin might have acted as a regulatory protein since the halocin is an extracellular protein it might have entered the cells of the indicator organism and acted as a repressor. Thus this halocin SH10 could have become effective.

Earlier studies on halophiles showed that brine cured hides processed in different countries had extremely halophilic *Archaea* (Bailey and Birbir, 1993,1996; Birbir, 1997) mostly contributed by the salt used in brine curing of hide. In the US, 131 brine cured hides were tested for extremely halophilic *Archaea* and 98% of them contained these microorganisms. Further, presence of proteolytic and lipolytic halophilic strains in salt affect hide quality adversely and every brine curing raceway that produces hide contains a significant number of extremely halophilic bacteria that contaminate almost every hide (Kallenberger, 1988; Birbir *et al*., 2002; Birbir and Sesal, 2002; Birbir, 2004). Considerable attempts have been made to use bactericides during brine curing of hides (Vivian, 1969; Hendry *et al*., 2001; Birbir and Bailey, 2000). Although effective bactericides have been recommended (Birbir and Bailey, 2000; Weiss and Thornton, 1984; Lollar and Kallenberger, 1986), in recent years the uses of bactericides have been questioned due to their toxicity and bacterial resistance to them on repeated use. Whereas, halobacterial growth on hides can be prevented by using natural antimicrobial compounds such as halocins produced by extreme halophiles. Thus antiarchaeal substances have drawn the attention towards prevention of haloarchaeal damage on brine cured or salt packed skins and hides. Though several species of Haloarchaeon were studied for halocin production,

reports on halocin production by *Natrinema* sp. are rather very limited. In this context the present study indicate scope for possible application of halocin SH10 as a preservative for hides in tanneries and leather processing industries.

### 5.6 Anticancerous activity of halocin SH10

Most of the cancer cell lines HeLa has the characteristic feature of abnormal cell proliferation and an active version of telomerase during cell division, which prevents the incremental shortening of telomeres that is implicated in aging and eventual cell death. In this way the cells circumvent the Hayflick Limit, which is the limited number of cell divisions that most normal cells can later undergo before becoming senescent (Ivanković *et al*., 2007). HeLa cell line was derived for use in cancer research. In the present study it was observed that halocin SH10 led to the lysis of the cancer cells indicating prospective application as anticancer agent.

A549 cells are reported to synthesize excess lecithin with a high percentage of disaturated fatty acids utilizing the cytidine diphosphocholine pathway. This process of phospholipid synthesis is responsible for pulmonary surfactant synthesis. This human surfactant thus synthesized and secreted interferes with therapeutics during pulmonary disease treatment (Michael *et al*., 1976). So the anticancer drugs could not function properly over these cell lines. In the present study it was observed that halocin SH10 could negotiate with these surfactants produced and was active against these tumor cell lines. Perhaps it could have also interfered in reducing the production of lecithin through which the entire process of surfactant synthesis and secretion may be restricted.

OAW42 - human ovarian carcinoma lines are generally multi drug resistance and due to the exposure of increasing concentrations of doxorubicin.

These cells show resistance to doxorubicin (Wilson, 1984; Redmond *et al*.,1993), vincristine, etoposide, tenoposide and also to cisplatin but not to 5-fluorouracil. This resistance characteristic is due to the overexpression of P-glycoprotein (Redmond *et al*., 1993). This situation necessitated requirement for new antibiotics to overcome this sort of resistance. In this context the results of the present study with halocin SH10 indicate scope for use as an alternate to such antibiotics since halocin showed effective cytotoxicity. This might reduce the expression of P-glycoprotein in these cells and so the multi drug resistance of this cell line could be reverted.

In DLA tumor bearing mice shows, a regular and rapid increase in ascitic tumor volume has been reported (Badami *et al*., 2003). This ascitic fluid is essential for tumor growth, as it constitutes nutrition directly to tumor cells (Gupta *et al.*, 2004). Due to the increase in fluid there is an increase in local inflammatory reactions which results ultimately in vascular permeability which further results in cellular migration and intense oedema (Gupta *et al*., 2004). After treatment with halocin SH10 there was a decrease in the body weight probably as a result of reduction in ascitic fluid volume. Thus decrease in the ascetic fluid is a good sign that the tumor is reverted and mice were turning normal. Generally the cancer patients are anemic (De Vita *et al*., 1993) due to the decrease in haemoglobin. Whereas, due to the increase in inflammation, there could be an increase in WBC in tumor bearing mice. The tumor bearing organism does not have a site specific function. Even though the tumor is site specific the functions of vital organs especially the liver is affected to the maximum (De Wys, 1982). The present study also showed a significant decrease in the total WBC, neutrophils and eosinophils count and increase in the RBC, hemoglobin and lymphocytes towards the normal values compared to DLA tumor control. These observations suggest that there was a reversal of hematological parameters near to normal control mice. Further the results also indicate that halocin SH10 may possess protective action on the

haemopoietic system. Such results have been reported earlier (Chandrasekar *et al*., 2006**,** Gupta *et al*., 2004, Kumar and Kuttan, 2005).

# *Chapter 6*

## SUMMARY

Multi-pond solar salterns are used worldwide for salt production along tropical and subtropical coastal areas and characteristic salt-adapted microbial communities are found along the salinity gradient in these saltern ponds. Further, in spite of the fact that halocin production is widespread among the *Halobacteriaceae*, no information is available on their ecological significance. Whereas, halocins may play a role in the interspecies competition between different types of halobacteria in saltern crystallizer ponds inhabited by dense communities of these red halophiles.

Research investigations pertaining to microbiology of saltern ponds of South India which contribute to significant amount of commercial salt production has not been made adequately. In this context the present investigation addressed the prospects of deriving haloarchaeal bacteria which may return potential halocins that may hold scope for applications in several industries.

A potential halocin producing haloarchaeal strain *Natrinema* sp BTSH10 and an indicator strain *Halorubrum* sp. BTSH03 were isolated from salt pan of Kanyakumari, Tamilnadu, India. They were identified based on polyphasic taxonomy characteristics. The 16S rRNA partial gene sequences were submitted to GenBank (JN228202-*Natrinema* sp.BTSH10, JF830242-*Halorubrum* sp.BTSH03) through Bioedit programme, at NCBI site. From the phylogenetic tree constructed, it was inferred that the *Natrinema* sp. is found in a separate branch indicating that this might

be a new strain which has to be further characterized and confirmed. *Halorubrum* sp. showed very close similarity with an uncultured archeon and *Halorubrum xinjiangense.*

The halocin produced by *Natrinema* sp.BTSH10 was named as halocin SH10 after the name of the producing organism. The activity of the halocin against the sensitive strain *Halorubrum* sp.BTSH03 was confirmed by agar overlay method.

Antibiotic sensitivity studies were done using 31 antibiotics and *Natrinema* sp. showed multi drug resistance and the MAR index was calculated as 0.3870. *Halorubrum* sp. showed resistance to 3 antibiotics and its MAR index was calculated as 0.09677.

Lipid analysis of *Natrinema* sp. showed presence of 10 different lipids out of which only four were identifiable and remaining 6 were unidentified. Fatty acid methyl esterase (FAME) analysis showed the presence of many unidentifiable fatty acids.

*Natrinema* sp. BTSH10 formed halites which were of different sizes varying from few millimeters to upto 2cm length. SEM confirmed the colonization of these haloarchaea inside the crystal. ICP-AES analysis showed the presence of Fe, K, Mg, Na, Sr in the halite. Powder XRD studies, FTIR analysis, UV-VIS-NIR analysis and thermal analysis confirmed the crystallinity of the sample and also the thermal stability of the crystal.

Various bioprocess variables were optimized for halocin production by *Natrinema* sp.BTSH10. The initial medium used for production was Zobell's broth. Strategy adopted for optimization was 'one-factor-at-a-time' method where the optimized variable will be constant in the forthcoming optimizations.

Optimization of incubation temperature for halocin showed that maximum halocin production was observed at 42°C which showed 1024AU of halocin. 37°C and 47°C also showed enhance in halocin production but only at lower levels.

Halocin production was observed over a broad pH range from pH 5 to pH 9 although maximum halocin production was recorded at 8.0 (1024AU). However, the bacteria did not produce halocin under acidic conditions.

Sodium chloride concentration was considered to be a key factor for halocin production. *Natrinema* sp. BTSH10 required 3M NaCl for maximum (1024AU) halocin production. NaCl concentration ranging from 2.5M – 4M (512AU) in the medium also supported enhanced production of halocin. *Natrinema* sp.BTSH10 required a minimum of 1.5M NaCl for its survival in the medium.

Among the different carbon sources used maximal halocin production was supported by the medium supplemented with galactose (2048AU) followed by sorbitol, maltose, glycerol, glucose (1024AU) and fructose, lactose (512AU). Whereas, medium supplemented with dextrin, sucrose and xylose supported reduced levels of halocin production. Galactose was observed to enhanced halocin production in the medium compared to other carbon sources.

Among the different nitrogen sources tested, the presence of beef extract (2048AU) in the medium supported maximal halocin by bacteria followed by soybean meal, malt extract, tryptone, peptone, yeast extract (1024AU) and casein, gelatin (512AU). Urea did not support halocin production.

Among the different inorganic salts used in halocin producing medium calcium chloride (2048AU) was found to support maximal halocin production by bacteria followed by magnesium chloride, sodium fluorate, potassium chloride, and sodium bicarbonate (1024AU). Whereas aluminium nitrate, potassium bromide, strontium chloride and sodium silicate led to a much reduced level of halocin production.

Agitation influenced halocin production. Maximum halocin production (4096AU) was observed at 200 rpm and 250 rpm. Lower agitation rates 50 rpm to 150 rpm also supported considerable levels of halocin production.

The time course experiment was conducted over a total period of 144h. Maximum (8192AU) production of halocin was observed at 104h, during the stationary phase. Halocin production was observed at considerable levels even after 88, 96, 112 and 128h where the halocin production was noted to be 4096AU. The halocin activity was found to be 2048AU after incubation for 80 and 144h.

Halocin SH10 was purified employing standard purification protocols which included ethanol precipitation of sample followed by molecular weight cut off centrifugation by 30kDa cut off membrane and gel filtration chromatography.

Fraction with halocin activity obtained after separation with molecular cut-off membrane confirmed that the molecular mass of the halocin is below 30kDa.

Sephadex G50 column was used for gel filtration chromatography and the elution was done using 50mM Tris-HCl buffer pH 8.0. A single peak was obtained for the elutants having halocin activity.

The purity of the halocin was confirmed by HPLC. The crude supernatant, 30kDa cut off centrifugation subjected supernatant and the gel filtration elute showed a single peak exactly between 11 and 12 min where the ACN/WATER volume was calculated as 32% : 68%.

Tricine PAGE was performed to confirm the purity of the halocin and determine its molecular mass. The molecular mass of halocin was calculated as 20kDa.

Bioautography assay was also performed on the unstained protein gel (Tricine PAGE) by agar overlay method which showed a clearing zone exactly at the 20kDa region comparing to the stained gel.

Cell lysis assay performed using halocin against *Halorubrum* sp.BTSH03 showed cell shrinkage and formation of small islands /colonies after 3h of incubation. Cell bulging and signs of cell lysis appeared after 6h and complete cell lysis after 12h of treatment.

N-Terminal sequencing enabled detection of first five amino acid which was found to be APFYI. MALDI and MASCOT analysis of the 20kDa halocin SH10 showed that it has a very close sequence similarity with 50S ribosomal protein L3P of *Methanosarcina acetivorans* (strain ATCC 35395).

NMR spectrum showed that halocin SH10 has excess of aliphatic chain containing aminoacids which are generally non polar and Hydrophobic. Total aminoacid analysis showed the presence of glycine (12.74%), asparigine (10.35%), glutamine (9.78%), valine (6.05%), alanine (3.94%), serine (3.69%), histidine (3.43%), threonine (3.11%) which confirms the NMR spectrum.

The halocin SH10 was thermostable up to 40°C (4096AU) without any loss of activity which however got declined to 1024AU at 60°C and 70°C and 128AU at 80°C and then lost activity at higher temperatures.

Halocin SH10 was stable at pH 6.0-8.0 (4096AU) and at pH 9.0 (2048AU) the activity got declined. At pH below 6.0 there was a rapid decrease in halocin activity.

The halocin SH10 retained its activity (4096AU) when incubated with 10% of organic solvents, upon incubation with 20% and 30% solvent halocin retained its activity with acetone, acetonitrile and isopropanol but lost activity with chloroform ,methanol and ethanol after one hour incubation.

HalocinSH10 was found to have greater importance in leather industry. Halocin treatment for 12h could inhibit halophilic bacterial growth on hides. In raw

hides 67 ± 2 CFU were obtained and after treating with halocin for 3, 6 and 12h, CFU decreased considerably.

Halocin SH10 also found to have anticancerous activity which was confirmed by short time cell cytotoxicity studies conducted on DLA cells where the $CTC_{50}$ value was found to be 256AU. MTT assay carried out on cell lines to study anticancer activity of halocin *in vitro,* which showed that it had $IC_{50}$ value for HBL100 cell line was 4096AU which clearly indicated that it does not have a toxicity effect towards normal cell line. Cell cytotoxicity was shown towards other cancer cell lines used and $IC_{50}$ values were calculated as 1024AU for HeLa cell lines and 512AU for A549, OAW42 and HEp 2 cell lines. Accordingly 1024AU and 512AU concentration was used for *in vivo* anticancer study.

Halocin SH10 when treated towards DLA bearing mice showed that the parameter reverted to near normal comparing to normal DLA and DLA+5FU with the halocin SH10. This reversion is indicated by reduction in the body weight (grams) to 20.85 ± 1.73 after treatment of DLA mice from 34.23 ± 0.62 with halocin which may be a reason for the decrease in tumor cells. In the same way the increase in WBC ($1x10^3$ $mm^3$) from 10.41 ± 0.46 in normal to 19.58 ± 1.22 in DLA mice was reduced to 13.36 ± 0.45. RBC ($1x10^6$ $mm^3$) level also decreased to 5.52 ± 0.47 in DLA mice which was 11.10 ± 0.38 in normal mice and the level increased to 8.75 ± 0.16 upon treatment with halocin SH10. Hemoglobin (g/dl) level increased to 12.35 ± 0.72 upon treatment of DLA mice bearing 8.64 ± 0.68 which was lesser in content comparing to normal mice (11.10 ± 0.38). The differential WBC count also showed reversal of cell number near equal to normal mice after treatment with halocin SH10.

# *Chapter 7*

## CONCLUSION

Based on the results obtained in the present study it is concluded that haloarchaea holds a treasure of bioactive substances, particularly halocins which have immense scope for several applications. To the best of our knowledge this is the first halocin reported for *Natrinema* sp. Further this is the first time a halocin from a halocbacteria *Natrinema* sp. is shown to have anticancerous activity against major cancer cells concerned with humans. The results obtained for the characterization of this halocin showed that the halocin is stable at wide range of pH, organic solvents besides being thermostable. These characteristics add more importance to this halocin and indicate scope for other applications as a preservative in food and in leather industries. This halocin SH10 can also be used in leather industry for storing the leather, free from bacterial community which damages the hide and decreases the quality of the hide. There is ample scope for further research to be conducted on halocin such as biochemical characteristics, elucidation of structure.

This study on halocin production by *Natrinema* sp.BTSH10 indicate the prospects for intensive research which could lead to discovery of novel halocins which could have far reaching impact in biopharmaceutical industry particularly as anticancer drug. It is also anticipated that further research on this halocin could lead towards development of novel anticancer drug and new era in pharmaceutical biotechnology. There is no doubt that haloarchaea from saltern ponds have immense potential to return novel and valuable drugs and bioactive substances.

# *Chapter 8*

## REFERENCES


Alberola, A., Meseguer, I., Torreblanca, M., Moya, A., Sancho, S., Polo, B., and Such, L. (1988): Halocin H7 decreases infract size and ectopic beats after myocardial reperfusion ion dogs. *J. Physiol. London.* **509**:148.

Anton, J., Oren, A., Benlloch, S., Rodriguez-Valera, F., Amann, R., and Rossello-Mora. R. (2002): *Salanibacter ruber* gen. nov., sp.nov., a novel extreme halophilic bacterium from saltern crystallizer ponds. *Int. J. Syst. Evol. Microbiol.* **52**: 485-491.

Arahal, D.R., and Ventosa, A. (2002): Moderately halophilic and halotolerant species of *Bacillus* and related genera. In *Applications and Systematics of Bacillus and Relatives*. Edited by Berkeley,R., Heyndrickx,M., Logan,N., and De Vos,P. Oxford: Blackwell. 83-99.

Argandoña, M., Fernando, M.C., Inmaculada, L., Emilia, Q., and Ana, D.M. (2003): Megaplasmids in Gram-negative, moderately halophilic bacteria. FEMS *Microbiol. Lett.* **227:** 81–86.

Avinash, M., Kumari, K., and Bhavanath, J. (2011): Characterization of extracellular polymeric substances produced by micro-algae *Dunaliella salina*. *Carbo. Poly.* **83**:852-857.

Baas Becking, L.G.M. (1931): Historical notes on salt and salt manufacture. *Sci. Month.* **32**:434- 446.

Baati, H., Guermazi, S., Amdouni, R., Gharsallah, N., Sghir, A., and Ammar, E. (2008): Prokaryotic diversity of a Tunisian multipond solar saltern. *Extremophiles*. **12**:505-518.

Badami, S., Aneesh, R., Sankar, S., Sathishkumar, M.N., Suresh, B., and Rajan, S. (2003): Antifertility activity of *Derris brevipes* var *coriacea*. *J. Ethnopharmacol.* **84**:99-104.

Bailey, D.G., and Birbir, M. (1993): A study of the extremely halophilic microorganisms found on commercially brine-cured cattle hides. *J. Am. Leather. Chem. Assoc*. **88**:285-293.

Bailey, D.G., and Birbir, M. (1996): The impact of halophilic organisms on the grain quality of brine cured hides. *J. Am. Leather. Chem. Assoc.* **91**:47-51.

Bain, N., Hodgkiss, W., and Shewan, J.M. (1958): The bacteriology of salt used in fish curing. In the microbiology of fish and meat curing brines. Proceedings of the 2nd international symposium on food microbiology. Eddy, B.P. (ed.). *London: HMSO*. **1**:11.

Bardavid, E.R., Khristo, P., and Oren, A. (2008): Inter-relationships between Dunaliella and halophilic prokaryotes in saltern crystallizer ponds. *Extremophiles.* **12(1)**:5-14.

Benlloch, S., Acinas, S.G., Martinez-Murcia, A.J., and Rodriguez-Valera, F. (1996): Description of prokaryotic biodiversity along the salinity gradient of a multi pond saltern by direct PCR amplification of 16S rDNA. *Hydrobiologica.* **329**:19-31.

Benlloch, S., Lo´pez-Lo´pez, A., Casamayor, E.O., Ovrea˚s, L., Goddard, V., Daae, F.L., Smerdon, G., Massana, R., Joint, I., Thingstad, F., Pedro´sAlio, C., and Rodrıguez-Valera, F. (2002): Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern. *Environ. Microbiol.* **4**:349-360.

Ben-Mahrez, K., Sorokine, I., Thierrry, D., Kawasumi, T., Ishii, S., Salmon, R., and Kohiyama, M. (1991): An archaebacterial antigen used to study immunological human response to *c-myc* oncogen product. In Rodriguez-Valera,F.(Ed). General and applied aspects of halophilic microorganisms.

Plenum Press,Newyork. 367-372.

Ben-Mahrez, K., Thierry, D., Sorokine, I., Danna-Muller, A., and Kohiyama, M. (1988): Detection of circulating antibiotics against *c-myc* protein in cancer patient sera. *Brit. J. Cancer*. **57**:529-534.

Bien, E., and Schwartz, W. (1965): Geomikrobiologische Untersuchungen.VI. UÈ ber das Vorkommen konservierter toter und lebender Bakterienzellen in Salzgesteinen. *Z. Allg. Mikrobiol.* **5**, 185-205.

Birbir, M. (1997): Investigation of salted-cured France and Russian hides for halophilic bacteria. *J. Turk. Microbiol. Soc.* **27**, 68-73.

Birbir, M. (2004): 1st National leather symposium book, Say.Cilt. sf 39-40.

Birbir, M. (2004): Examination of amylase, caseinase and cellulase enzymes production of extremely halophilic strains isolated from Tuz lake, Kaldırım and Kayacık Salterns and Tuzköy Salt Mine. *Marine Bacteriology Congress Proceedings*. Say. Cilt . sf. 25-28.

Birbir, M. (2004): Examination of proteolytic and lipolytic bacteria in salt used in hide conservation. *1st National Leather Symposium Book.* Say. Cilt. sf. 39-49.

Birbir, M., and Bailey, D.G. (2000): Controlling the growth of extremely halophilic bacteria on brine cured cattle hides. *J. Soc. Leather. Technol. Chem.* **84**, 201-203.

Birbir, M., and Sesal, C. (2002): Extremely halophilic bacterial communities in Şereflikoçhisar salt Lake in Turkey. *Turk. J. Biol.* **27**, 7-22.

Birbir, M., Eryılmaz, S., and Ogan, A. (2004): Prevention of halophilic bacterial damage on brine cured hide with halocins. *J. Soc. Leather. Technol. Chem.* **88(3)**, 99-104.

Birbir, M., Kall, N., and Johansson, C. (2002): The examination of salt quality of Sereflikochisar Lake used in Turkish Leather Industry. *J. Soc. Leather Technol. Chem*. **86**, 112.

Blank, A., and Oesterhelt, D. (1987): The halo-opsin gene. II. sequence, primary structure of halorhodopsin and comparison with bacteriorhodopsin. *EMBO. J.* **6**, 265-273.

Blatt, H., Middleton, G., and Murray, R. (1980): Origin of sedimentary rocks. Englewood Cliffs, NJ: Prentice Hall.

Bligh, E.G., and Dyer, W.J. (1959): A rapid method of total lipid extraction and purification. *Can J. Biochem. Physiol.* **37**, 911-917.

Bodaker, I. (2010): Comparative community genomics in the dead sea: an increasingly extreme environment. *ISME J.* **4**, 399-407.

Bonete, M.J., Martínez-Espinosa, R.M., Pire, C., Zafrilla, B., and David, J. (2008): Richardson. Nitrogen metabolism in haloarchaea. S*aline Sys.* **4**, 9-20.

Brian, A., Schubert, Tim, K., Lowenstein, Michael, N. Timofeeff and Matthew A. Parker (2009). How do prokaryotes survive in fluid inclusions in halite for 30 k.y.? **37**. 1059-1062.

Briones, C., and Amils, R. (2000): Nucleotide sequence of the 23S rRNA from *Haloferax mediterranei* and phylogenetic analysis of halophilic archaea based on LSU rRNA. *Syst. Appl. Microbiol.* **23(1)**, 124-31.

Bunger, J., Axt, A., Zur, L., Fritz, A., Degwert, J., and Driller, H. (2000): The protection function of compatible solute ecotine on the skin, skin cells and its biomolecules with respect to UV-radiation,immune-suppression and membrane damage. In proceedings of the XXI$^{st}$ IFSCC international congress, Berlin. 359-365.

Carteni-Farina, M., Porcelli, M., Cacciapuoti, G., DeRosa, M., Gambacorta, A., Grant, W.D., and Ross, M.N.M. (1985): Polyamines in halophilic agrobacteria. *FEMS Microbiol. Lett.* **65**, 269-274.

Castanier, S., Perthuisot, J.P., Matrat, M., and Morvan, J.Y. (1999): The salt ooids of Berre salt works: the role of bacteria in salt crystallization. *S. ed. Geol.* **125**, 9-23.

Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., and Ventosa, A. (2006): *Halostagnicola larsenii* gen. nov., sp. nov., an extremely halophilic archaeon isolated from a saline lake in inner Mongolia, China. *Int. J. Syst. Evol. Microbiol*. **56(7)**, 1519-1524.

Catherine, L., Mercedes, M.S., Bernardo, P., Alberto, R.C., Jurgen, W., and Victoriano, C. (2001): Taxonomic study of extreme halophilic archaea isolated from the "Salar de Atacama",Chile. *Syst. Appl. Microbiol.* **24,** 464-74.

Chakraborty, S., Roy, M., Taraphdar, A. K., and Bhattacharya, R. K. (2004): Cytotoxic effect of root extract of *Tiliacora racemosa* and oil of *Semecarpusanacardium* nut in human tumor cells. *Phytother. Res.* **18**, 595

Chandrasekar, M.J.N., Praveen, B., Nanjan, M.J., Suresh, B. (2006): Chemo protective effect of *Phyllanthes maderaspatensis* in modulating cisplastin- induced nephrotoxicity and genotoxicity. *Pharma. Bio.* **2**, 100-106.

Cheung, J., Danna, K.J., O'Connor, E.M., Price, L.B., and Shand, R.F. (1997): Isolation, sequence, and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon *Haloferax mediterranei* R4. *J. Bacteriol.* **179**, 548-551.

Christine, A., White-Ziegler., Suzin, U., Natalie, M.P., Abby, L.B., Amy, J.M., and Sarah, Y. (2008): Low temperature (23°C) increases expression of biofilm- cold-shock- and RpoS-dependent genes in *Escherichia coli* K-12. *Microbiolog.* **154**, 148-166.

Collins, M.D., Ross, H.N.M., Tindall, B.J., and Grant,W.D.( 1981): Distribution of isoprenoid quinones in halophilic bacteria. *J. Appl. Bacteriol.* **50**, 559-565.

Conway de Macario, E., Konig, H., and Macario, A.J.L. (1986): Immunological distinctiveness of archaebacteria that grow in high salt. *J. Bacteriol*. **168,** 425-427.

D'Souza, M.P., Amini, A., Dojka, M.A., Pickering, I.J., Dawson, S.C., Pace, N.R., and Terry, N. (2001): Identification and characterization of bacteria in a selenium- contaminated hypersaline evaporation ponds. *Appl. Environ. Microbiol*. **67**, 3785-3794.

Da Costa, M.S., Santos, H., and Galinski, E.A. (1998): An overview of the role and diversity compatible solutes in Bacteria and Archaea, pp.117-153. In: Antranikian,G. (Ed), Biotechnology of extremophiles. Springer- Verlag, Berlin.

DasSarma, P., and DasSarma, S. (2008): On the origin of prokaryotic ''species'' the taxonomy of halophilic archaea. *Saline Systems*. **4**, 5.

DasSarma, S., and Fleischmann, E.M. (1995): Halophiles. Cold spring harbor laboratory press, Plainview, N.Y.

DasSarma, S., RajBhandary, U.L., and Khorana, H.G. (1984): Bacterio-opsin mRNA in wild-type and bacterio-opsin-deficient *Halobacterium halobium*strains. *Proc. Natl. Acad. Sci. USA*. **81**, 125-129.

DasSarma, S., and DasSarma, P.(2012): Halophiles. In: eLS. John Wiley & Sons, Ltd: Chichester.

DeVita, V., Hellman, S., and Rosenberg, S. (1993): 4th ed. Cancer principles and practice of oncology. Philadelphia: Lippincott.

DeWys, W.D. (1982): Pathophysiology of cancer cachexia: current understanding and areas for future research. *Cancer Res*. **42**, 721-725.

Dombrowski, H.J. (1963): Bacteria from palaeozoic salt deposits. *Ann. New York Acad. Sci.* **108**, 477-484.

Dombrowski, H.J. (1966): Geological problems in the question of living bacteria in Paleozoic salt deposits. In 2nd symposium on salt, Rau,J.L. (ed). Cleveland, Ohio: Northern Ohio Geological Society. **1**, 215-219.

Ebert, K., Goebel, W., Rdest, U., and Surek, B. (1986): Genes and genome structures in the archaebacteria. *Syst. Appl. Microbiol.* **7**, 30 – 35.

Eimhjellen, K. (1965): Zentralblatt für bakteriologie, parasitenkunde, infektionskrankheiten und hygiene. I, 126-137.

Elazari-Volcani, B. (1957): Genus XII. Halobacterium. In:Breed, Murray,R.S. EGD and Smith,N,R. (Eds), Bergey's manual of determinative bacteriology.7th. ed. Willams and Wilkins, Baltimore. 207-212.

Falb, Müller, Königsmaier, Oberwinkler, Horn, von Gronau, Gonzalez, Pfeiffer, Bornberg-Bauer, and Oesterhelt. (2008): Metabolism of halophilic archaea. *Extremophiles*. **12**, 177-96.

Feng, Y., He, Z., Ong, S.L., Hu, J., Zhang, Z., and Ng, W.J. (2003): Optimization of agitation, aeration, and temperature conditions for maximum β-mannanase production. *Enz. Microbial. Tech.* **32(2)**, 282-289.

Francis, D., Rita, L. (1986): Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods*. **89**, 271-7.

Francis, A.J., Dodge, C.J., Gillow, J.H., and Papenguth, H.W. (2000): Biotransformation of uranium compound in high ionic strength brine by a halophilic bacteria under denitrifying conditions. *Environ. Sci. Technol.* **34**, 2311-2317.

Gambacorta, A., Gliozzi, A., and DeRosa, N. (1995): Archaeal lipids and their biotechnological applications. *World. J. Microbiol and Biotechnol.* **11**, 115-131.

Ghai, R., Pasic, L., Ferna´ndez, A.B., Martin-Cuadrado, A., Mizuno, C.M., Katherine, D., McMahon, R., Papke, T., Stepanauskas, R., Rodriguez-Brito, B., Rohwer, F., Sa´nchez-Porro, C., Ventosa, A., and Rodrı´guez-

Valera, F. (1997): New abundant microbial groups in aquatic hypersaline environments. *Sci. reports.* **1**, 135-145.

Gonzalo, P., Inmaculada, M., and Ricardo, A. (2002): Purification and biological characterization of halocin H1 from *Haloferax mediterranei* M2a**.** *Int. Microbiol.* **5**, 15-19.

Grant, and Larsen, (1988): Grant, W.D., and Larsen, H. (1989): Group III. Extremely halophilic archaeobacteria, Order Halobacteriales ord. nov.. In: Staley,J.T., Bryant,M.P., Pfennig,N., Holt,J.G. (eds) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, M.D. 2216-2233.

Grant, W.D., Gemmell, R.T., and McGenity, T.J. (1998): Halophiles. In: extremophiles: Microbial life in extreme environments (Horikoshi,K., and Grant,W.D., Eds.), Wiley-Liss, Inc., 93-132.

Guan, Z., Naparstek, S., Calo, D., Eichler, J. (2012): Protein glycosylation as an adaptive response in Archaea: growth at different salt concentrations leads to alterations in Haloferax volcanii S-layer glycoprotein N-glycosylation. *Environ. Microbiol.* **14**, 743-53.

Gupta, M, Mazumder, U.K., Kumar, R.S., Thangavel, S.K. (2004): Antitumor activity and antioxident role of *Bauhinia racemosa* against Ehrlich ascites carcinoma in Swiss albino mice. *Acta. Pharmacol. Sin.* **25 (8)**, 1070-1076.

Hamana, K., Matsuzaki, S., Niitsu, M., and Samejima, K. (1989): Polyamine distribution and the potential to form novel polyamines in phytopathogenic agrobacteria. *FEMS Microbiol. Lett.* **65**, 269-274.

Hamana, K., Matsuzaki, S., Niitsu, M., and Samejima, K. (1990): Synthesis of novel polyamines in paracoccus, rhodobacter and micrococcus. *FEMS Microbiol. Lett.* **67**, 267-274.

Hampp ,N., (2000): Bacteriorhodopsin: mutating a biomaterial into an optoelectronic material. *Appl. Microbiol. Biotechnol.* **53**, 633-639.

Haseltine, C., Hill, T., Montalvo-Rodriguez, R., Kemper, S.K., Shand, R.F and Blum, P. (2001): Secreted euryarchaeal mictohalocins kill hyper thermophilic crenarchaea. *J. Bacteriol.* **183**, 287-291.

Hay, J.X.W., Wu, T.Y., Teh, C.Y., and Jahim, J.M. (2012): Optimized growth of *Rhodobacter sphaeroides* O.U.001 using response surface methodology (RSM). *JSIR*. **71(02)**, 149-154.

Hendry, M.F., Cooper, D.R., and Woods, D.R. (2001): The microbiology curing and tanning process, Part IV, The laboratory screening of antiseptics. *J. Amer. Leather. Chem. Ass*. **66**, 31.

Iida, T., Furyiya, M., Suzuki, K., Iwabuchi, N., and Maruyama, T. (1997): Cyclophilin type PPI-ase gene originating from halophilic arechaebacerium. Patent JP9313184. September 12.

Inoue, K., Itoh, T., Ohkuma, M., Kogure, K. (2011): *Halomarina oriensis* gen.nov., sp. nov., a halophilic archaeon isolated from a seawater aquarium. *Int. J. Syst. Evol. Microbiol.* **61**, 942– 946.

Ivankovic, M., Cukusic, A., Gotic, I., Skrobot, N., Matijasic, M., Polancec, D., and Rubelj, I. (2007): Telomerase activity in Hela cervical carcinoma cell line proliferation. *Biogerontology*. **8(2)**, 163-72.

Jan, B., Antonio, J.P., Nathalie, P., and Erhard, B., (2007): Osmotically induced synthesis of the compatible solute hydroxyectoine is mediated by an evolutionarily conserved ectoine hydroxylase. *J. Biol. chem.* **282**, 31147-31155.

Javor, B. (1989): Hypersaline environments. *Microbiol. Biogeochem*. Springer-Valera, Berlin.

Javor, B., Requadt, C., and Stoeckenius, W. (1982): Box-shaped halophilic bacteria. *J. Bacteriol.* **151**, 1532-1542.

Javor, B.J. (2002): Industrial Microbiology of Solar salt production. *J. Ind. Microbiol. Biotechnol.* **28**, 42-27.

Jones, A.G., Ewing, C.M., and Melvin, M.V. (1981): Biotechnology of solar salt fields. *Hydrobiologia*. **82**, 391-406.

Juez, G., Rodriguez-Valera, F., Ventosa, A., and Kushner, D.J. (1986):*Haloarcula hispanica* spec.nov. and *Haloferax gibbonsii* spec.nov., two new species of extremely halophilic archaebacteria. *Syst. Appl.Microbiol*. **8**, 75-79.

Kallenberger, W.E. (1988): Halophilic bacteria in brine curing. *J. Amer Leather Chem Ass*. **79**, 04-11.

Kamekura, M. (1998): Diversity of extremely halophilic bacteria. *Extremophiles*. **2**, 289-295.

Kamekura, M., and Dyall-Smith, M.L. (1995): Taxonomy of the family Halobacteriaceae and the description of two new genera *Halorubrobacterium* and *Natrialba*. *J. Gen. Appl. Microbiol.* **41**, 333-350.

Kamekura, M., and Kates, M. (1999): Structural diversity of membrane lipids in members of Halobacteriaceae. *Biosci. Biotechnol. Biochem*. **63**, 969-972.

Kargi, F., and Dincer, A.R. (2000): Use of halophilic bacteria in biological treatment of saline wastewater by fed batch operation. *Water. Ebviron. Res*. **72**, 170-174.

Kargi, F., Dincer, A.R., and Pala, A. (1996): Characterization and biological treatment of pickle industry waste water. *Bioprocess. Engin.* **23**, 371-374.

Karthikeyan, P., Bhat, S.G., and Chandrasekaran, M. (2013): Halocin SH10 production by an extreme haloarchaeon *Natrinema* sp. BTSH10 isolated from salt pans of South India. *S. J. Biol. Sci.* **2**, 205-212.

Kates, M. (1995): Adventures with membrane lipids. *Biochem. Soc. Trans.* **23**, 679-709.

Kavitha, P., Lipton, A.P., Sarika, A.K., and Aishwarya, M.S. (2011): Growth characteristics and halocin production by a new isolate *Haloferax volcanii* KPS1 from Kovalam solar saltern (India). *Res. J. Biol. Sci.* **6,** 257-262.

Kim, S.J., and Hoppe, H.G. (1986): Microbial extracellular enzyme detection on agar plates by means of methylum-belliferyl-substrates. In: GERBAM-Deuxieme Colloque International de Bacteriologie Marine, Actes de Colloque. REMER, Brest. 175-181.

Krebs,M.P., and Khorona,H.G. (1993): Mechanism of light dependent photon translocation by bacteriorhodopsin. *J. Bacteriol.* **175**:1555-1560.

Kumar, K.B.H., and Kuttan, R. (2005): Chemoprotective activity of an extract of *Phyllanthus amarus* against cyclophosphamide induced toxicity in mice. *Phytomedicine.* **12**, 494-500.

Kumaresan, P.S., Moorthy Babu, S., and Anbarasan, P.M. (2008): Thermal, dielectric studies on pure and amino acid ( L-glutamic acid, L-histidine, L-valine) doped KDP single crystals. *Optical Materials.* **30**, 1361-1368.

Kushner, D.J. (1993): Growth and nutrition of halophilic bacteria. *In* R. Vreeland, H., and Hochstein, L.I. (ed.), The biology of halophilic bacteria. *CRC Press, Boca Raton, Fla*. 87-103.

Lanzotti, V., Trincone, A., Nicolaus, B., Zillig, W., DeRosa, M., and Gambacorta, B. (1989): Complex lipids of Pyrococcus and AN1, thermophilic members of archaebacteria belonging to the Thermococcales. *Biochim. Biophys. Acta*. **1004**, 44-48.

Larsen, H. (1962): Halophilism in the Bacteria. *Academic Press, New York.* **4**, 297-342.

Lefond, S.J. (1969): Handbook of world salt resources. Plenum Press, New York

Lequerica, J.L., O'Connor, J.E., Such, L., Alberola, A., Meseguer, I., Dolz, M., Torreboanca, M., Moya, A., Colom, F., and Soria, B. (2006): A halocin acting as Na+/H+ ecchange of haloarchaea as a new type of inhibitor in NHE of mammals. *J. Physiol. Biochem.* **62**, 253-262.

Li, Y., Xiang, H., Liu, J., Zhou, M., and Tan, H. (2003): Purification and biological characterization of halocin C8, a novel peptide antibiotic from halobacterium strain AS7092. *Extremophiles.* **7(5)**, 401-407.

Lollar, M.R., and Kallenberger, E.W. (1986): Halophilic bacteria thrive in seasonal cycles. *J. Amer. Leather. Chem. Ass.* **81**, 248.

Lopez, A., and Ochoa, J.L. (1998): Adsorption and its application in industry and environmental pollution-edited by A.Dabrowski. *Studies in surface science and Catalysis*. **120**, 903-923.

Lopez, A., Jose, L., and Rafael, V. (1994): Participation of halobacteria in crystal formation and the crystallization rate of NaCl. *Geomicrobiology journal.* **12**, 69-80.

Mancinelli, R.L. (2005): Microbial life in brines, evaporites and saline sediments: the search for life on Mars. In: Tokano T (ed) Water on Mars and life. Springer, Berlin. 277-297.

Mani, K., Salgaonkar, B.B., Das, D., and Bragança, J.M. (2012): Community solar salt production in Goa, India. *Aqua. Biosystems.* **8**, 30.

Margesin, R., and Schinner, F. (2001): Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles.* **5**, 73-83.

Martin,B.D., and Natarajan, S. (2008): Growth and characterization of two new NLO materials from the amino acid family: L-Histidine nitrate and L-Cysteine tartrate monohydrate. *Optics Communications.* **281**, 457-462.

Mattoussi, H., Mauro, J.M., Goldman, E.R., Anderson, G.P., Sundar, V.C., Mikulec, F.V., and Bawendi, M.G. (2000): *J. Am. Chem. Soc.* **122**:12142-12250.

McGenity, T.J., Gemmell, R.T., and Grant, W.D. (1998): Proposal of a new halobacterial genus *Natrinema* gen. nov., with two species *Natrinema pellirubrum* nom. nov. and *Natrinema pallidum* nom. nov. *Int. J. Syst. Bacteriol.* **48(4)**, 1187-1196.

McGenity, T.J., Renia, T., Gemmell., William, D., and Stan-Lotter, G.H. (2000): Origins of halophilic microorganisms in ancient salt Deposits. *Environ. Microbiol*. **2(3)**, 243- 250.

McMeekin, T.A., Nichols, P.A., Nichols, S.T., Juhasz, A., and Franzmann, P.D. (1993): Biology and biotechnological potential of halotolerant bacteria from Antartic saline lakes. *Experimentia*. **49**, 1042-1046.

Mehmet, B. M., Manuel, M.G., Fernando, S., Arantxa, P., Kiymet. G., and Josefa, A. ( 2008). Prokaryotic diversity in Tuz Lake, a hypersaline environment in Inland Turkey. *FEMS Microbiol. Ecol.* **65**, 474–483.

Meral, B., Baris, C., Bulent, M., Rahel, E.B., Aharon, O., Mehmet, N.O., and Ayse, O. (2007): Extremely halophilic archaea from Tuz Lake,Turkey, a nd the adjacent Kaldirim and Kayacik salterns. *World. J. Microbiol Biotechnol*. **23**, 309-316.

Meseguer, I., and Rodrı´guez, V.F. (1985): Production and purification of halocin H4. *FEMS Microbiol. Lett.* **28(2)**, 177-182.

Meseguer, I., and Rodriguez, V.F. (1986): Effect of halocin H4 on cells of *Halobacterium halobium*. *J. Gen. Microbiol.* **132**, 3061-3068.

Meseguer, I., Rodrı´guez, V.F., and Ventosa, A. (1986): Antagonistic interactions among halobacteria due to halocin production. *FEMS Microbiol Lett.* **36(2-3)**, 177-182.

Meseguer, I., Torreblanca, M., and Konishi, T. (1995): Specific inhibition of the halobacterial Na+/H+ antiporter by halocin H6. *J. Biol. Chem.* **270(12)**, 6450-6455.

Mirko, B., Junsong, S., and Michael, W.W.A. (2012): Engineering a hyper thermophilic archaeon for temperature dependent product formation. *ASM journals.* **3(2)**, 1-8.

Moldoveanu, M., Kates, M., Montero, C.G., and Ventosa, A. (1990): Polar lipids of non-alkalophilic *Halococci. Biochim.BIophy.Acta.* **1046,** 127-135.

Montalvo-Rodriguez, R., Lopez-Garriga, J., Veerland, R.H., Oren, A., Ventosa,A., and Kamakura. (2000): *Haloterrigena thermotolerans* sp. nov., a halophilic archeon from Puerto Rico. *Int. J. Syst. Bacteriol.* **50**, 1065-1071.

Montalvo-Rodriguez, R., Veerland, R.H., Oren, A., Kessel, M., Betancourt, C., and Lopez-Garriga, J. (1998): *Halogeometricum borinquense* gen. nov., a novel halophilic archeon from Puerto Rico. *Int. J. Syst. Bacteriol.* **48**, 1305-1312.

Montero, C.G., Ventosa, A., Rodriguez-Valera, F., Kates, M., Moldoveanu, N., and Ruiz-Berraquero, F. (1989): *Halococcus saccharolyticus* sp.nov., a new species of extremely halophilic non-alkaliphilic cocci. *Syst. Appl. Microbiol.* **12**, 167-171.

Montes, M.J., Abadia-Molina, A.C., Monteolia-Sanchez, M., Ramos-Cornenzana, A., and Ruiz, C. (1999): Effect of *Halococcus morrhuae* and *Halobacterium saccharovorum* on the activation of human peripheral blood lymphocytes. *Microbes.* **98**, 141-147.

Narasingarao, P., Podell, S., and Ugalde, J.A. (2011): De novo metagenomic assembly reveals abundant novel major lineage of archaea in hypersaline microbial communities. *The ISME Journal. doi.* **10**,1038-78.

Niemetz, R., Karcher, U., Kandler, O., Tindall, B.J., and König, H. (1997): The cell wall polymer of the extremely halophilic archaeon *Natronococcus occultus*. *Eur.J. Biochem.* **249**, 905-911.

Norton, C.F., and Grant, W.D. (1988): Survival of halobacteria within fluid inclusions in salt crystals. *J. Gen. Microbiol*. **134**, 1365-1373.

Nuttall, S.D., and Dyall-Smith, M.L. (1993): Ch2, a novel halophilic archeon from an Australian solar saltern. *Int. J. Syst. Bacteriol.* **43**, 729-734.

O'Connor, E.M., and Shand, R.F. (2002): Halocins and sulfolobicins: The emerging story of archaeal protein and peptide antibiotics. *J.Ind. Microbiol. Biotech*. **28**, 23-31.

Ochsenreiter, T., Pfeifer, F., and Schleper, C. (2002): Diversity of archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies. *Extremophiles*. **6**, 267-274.

Oesterhelt, D., Patzelt, H., and Kesler, B. (1998): Decomposition and halogenated hydrocarbos by halophilic bacteria. Patent DE19639894,April 9.

Oren, A., Arahal, D. R., and Ventosa, A. (2009): Emended descriptions of genera of the family Halobacteriaceae. *Int J Syst Evol Microbiol*. **59**, 637–642.

Oren, A. (2008): Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline. Syst.* **4**, 2.

Oren, A. (1994): The ecology of the extremely halophilic archaea. *FEMS Microbiol. Rev*. **13**:415- 440.

Oren,A. (2000): The order Halobacteriales. in: the prokaryotes: an evolving electronic resource for the microbiological community (Dworkin,M., Falkow, S., Rosenberg, E., Schleifer, K.H., and Stackebrandt, E., Eds), 3rd Edition, release 3.2, Springer, NY.

Oren, A. (2002): Diversity of halophilic microorganisms: environments, phylogeny, physiology and applications. *J Ind. Microbiol. Biotechnol.* **28**, 56-63.

Oren, A. (2002): Halophilic microorganisms and their environments. Kluwer Academic Publisher, Netherlands.

Oren, A. **(**2012a): Two centuries of microbiological research in the Wadi Natrun, Egypt: a model system for the study of the ecology, physiology,and taxonomy of haloalkaliphilic microorganisms. *In* Seckbach,J., Oren,A., Stan-Lotter,H. (ed), Polyextremophiles- organisms living under multiple stress, in press. Springer, Dordrecht, the Netherlands.

Oren, A.(2012b). Taxonomy of the family Halobacteriaceae:a paradigm for changing concepts in prokaryote systematics. *Int. J. Sys. Evol. Microbiol*. **62**, 263–271.

Oren, A., and Ventosa, A. (1996): A proposal for the transfer of *Halorubrobacterium distributum* and *Halorubrobacterium coriense* to the genus *Halorubrum* as *Halorubrum distributum* comb. nov. and *Halorubrum coriense* comb. nov., respectively. *Int. J. Syst. Bacteriol.* **46**, 1180.

Oren, A., and Ventosa, A. (2010): International committee on systematics of prokaryotes subcommittee on the taxonomy of *Halobacteriaceae* and subcommittee on the taxonomy of *Halomonadaceae*. Minutes of the joint open meeting, 30 June 2010, Beijing, China. *Int. J. Syst. Evo.l Microbiol.* **60**, 2257-2259.

Oren, A., Ventosa, A., and Grant, W.D. (1997): Proposed minimal standards for description of new taxa in the order Halobacteriales. *Int. J. Sys. Bacteriol.* **47**, 233-238.

Pabba, S.K., Burra, S.B., Ram, P.M., Nidadavolu, S.H., Singara Chary, M. A. (2011): Fame Fingerprints For Identification Of Marine Red Pigmented Bacteria Isolated From Nellore Costal Region Of Andhra Pradesh. *J.Rec.Adv.Appl.Sci.* (Jraas) 2637-42

Pagaling, E., Wang, H., Venables, M., Wallace, A., Grant, W.D., and Cowan,D.A. (2009): Microbial biogeography of six salt lakes in Inner Mongolia, China, and a salt lake in Argentina. *Appl. Environ. Microbiol.* **75**, 5750-5760.

Pasic, L., Galan,B.S., Poklar, U.N., Grabnar, M., and Herzog, V.B. (2005): Diversity of halophilic archaea in the crystallizers of an Adriatic solar saltern. *FEMS Microbiol. Ecol.* **54(3)**, 491-498.

Pasic, L., Velikonja, B.H., and Ulrih, N.P. (2008): Optimization of the culture conditions for the production of a bacteriocin from halophilic archaeon Sech7a. *Prep. Biochem. Biotechnol.* **38(3)**, 229-245.

Patel, G.B., and Sprott, G.D. (1999): Archaeobacterial ether lipid liposome (archeosomes) as a novel vaccine and drug delivery systems. *Crit. Rev. Biotechnol.* **19**, 317-357.

Platas, G. (1995): Caracterizacíon de la actividad antimicrobiana de la haloarquea *Haloferax mediterranei* Xia3. Madrid, Spain: Universidad Autónoma de Madrid. pp. Doctoral Thesis.

Platas, G., Meseguer, I., and Amils, R. (1996): Optimization of the production of a bacteriocin from *Haloferax mediterranei* Xia3. *Microbiologia.* **12**, 75-84.

Platas, G., Meseguer, I., and Amils, R. (2002): Purification and biological characterization of halocin H1 from *Haloferax mediterranei* M2a. *Int. Microbiol.* **5**, 15-19.

Prangishvili, D., Holz, I., Stieger, E., Nickell, S., Kristjansson, J.K., and Zillig, W. (2000): Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus *Sulfolobus*. *J. Bacteriol.* **182**, 2985-2988.

Price, L.B., and Shand, R.F. (2000): Halocin S8: a 36-amino-acid microhalocin from the haloarchaeal strain S8a. *J. Bacteriol.* **182**, 4951-4958.

Rdest, U., and Sturm, M. (1987): Bacteriocins from halobacteria. In *Protein Purification*: *M Icro To Macro,* New York: Alan R. Liss. 271-278.

Redmond, A., Moran, E., and Clynes, M. (1993): Multiple drug resistance in the human ovarian carcinoma cell line OAW42-A. *Eur. J. Cancer*. **29**, 1078-1081.

Reeves, P. (1965): The Bacteriocins. *Bacteriological reviews* **29**, 24-45.

Reiser, R., and Tasch, P. (1960): Investigation of the viability of osmophile bacteria of great geological age. *Trans Kans Acad Sci*. **63**, 31-34.

Riaz, S., Faisal, M., and Hasnain, S. (1998): Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species in Pakistan. *Af. J. Biotechnol*. **10**, 6325-6331.

Michelle,M,R., Bennett, A.F., and Long, A.D. (2005): *Phy. Biochem. Zoo.* **78**, 299-315.

Rippel, A. (1935): Fossile Mikroorganismen in einem permischen Salzlager. *Arch.f.Mikrobiol*. **6**, 350-359.

Maria,.A,R., Jesus P.A., Delgado, R., Ramos-Cormenzana, A., and Delgado, G. (2004): Biomineralization of carbonates by *Halobacillus trueperi* in solid and liquid media with different salinities. *FEMS Microbiol. Eco*. **48,** 39-46.

Roberts, M.F. (2005): Organic compatible solutes of halotolerant and halophilic microorganisms. *Saline Sys.* **1**, 5-35.

Rodriguez-Valera, F., Juez, G., and Kushner, D. (1982): Halocins: salt-dependent bacteriocins produced by extremely halophilic rods. *Can. J. Microbiol.* **28**, 151–154.

Rodriguez-Valera, F., Ruiz-Berraquero, F., and Ramos-Cormenzana, A. (1980): Isolation of extremely halophilic bacteria able to grow in defined inorganic media with single carbon sources. *J. Gen. Microbiol.* **119**, 535-538.

Ross, H.M., Grant, W.D., and Harris, J.E. (1985): Lipids in archaeabacterial taxonomy. In: Goodfellow,M., Minnikin,D.E. editors. Chemical Methods in Bacterial Systematics. Academic Press; London. 289-299.

Ruepp, A., and Soppa, J. (1996): Fermentative arginine degradation in *Halobacterium salinarium* (formerly *Halobacterium halobium*): genes, gene products, and transcripts of the *arcRACB* gene cluster. *J. Bacteriol.* **178**, 4942-4947.

Sankar, R., Muralidharan, R., Raghavan, C.M., and Jayavel, R. (2008): Structural, thermal, mechanical and optical properties of l-arginine di-iodate crystal: A new nonlinear optical material. *Mat. Chem. Phy.* **107**, 51-56.

Kiyotaka,S. (2001): Crystal engineering of biological soft materials. *Adv. Cry. Gro. Res.* 401-419.

Schägger, H., and von Jagow, G. (1987): Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-79.

Schleifer, K.H., Steber, J., and Mayer, H. (1982): Chemical composition and structure of the cell wall of *Halococcus morrhuae. Zentrbl Bakteriol. Mikrobiol. Hyg.I. Abt. Orig.* **3**, 171-178.

Sehgel, S.N., and Gibbons. (1960): Effect of some metal ions on the growth of *Halobacterium cutirubrum. Can. J. Microbiol.* **6**, 165-169.

Seki, A., Kubo, I., Sasabe, H., and Tomioka, H. (1994): A new anion-sensitive biosensor using an ion-sensitive field effect transistor and a light-driven chloride pump, halorhodopsin. *Appl. biochem. Biotechnol.* **48**, 205-211.

Shahmohammadi, H.R., Asgarini, E., Terat, H., Saito, T., Ohyama, Y., and Gekko, K. (1998): Protective roles of bacterioruberin and intracellular KCl in the resistance of *Halobacterium salinarium* against DNA-damaging agents. *J. Radiat. Res.* **39**, 251-262.

Shand, R.F. (2006): Detection, quantification and purification of halocins:peptide antibiotics from haloarchaeal extremophiles. In Extremophiles- methods in microbiology. Rainey, F.A., and Oren, A. eds. Amsterdam: Elsevier / Academic Press. **35**, 703-718.

Shand, R.F., and Leyva, K.J. (2008): Archaeal antimicrobials: an undiscovered country. In Blum,P. ed. *Archaea: new models for prokaryotic biology*. Caister Academic Press, Norfolk, U.K.. 233–243.

Shand, R.F., and Leyva K.J. (2007): Peptide and protein antibiotics from the domain archaea: halocins and sulfolobicins. In Riley, M.A., and Chavan, M.A. eds. *Bacteriocins: ecology and evolution*. Springer, New York. 93-109.

Shand, R.F., and Perez, A.M. (1999): Haloarchaeal growth physiology. In Seckback. *J. Enigmatic Organisms and Life in Extreme Environments*. Dordrecht, The Netherlands: Kluwer Acadademic Press. 413-424.

Shand, R.F., Price, L.B., and O'Connor, E.M. (1999): Halocins: protein antibiotics from hypersaline environments. In: Oren A (ed) microbiology and biogeochemistry of hyper saline environments. CRC Press, Boca Raton. 295-306.

Silva, Z., Borges, N., Martins, L.O., Wait, R., daCosta, M.S., and Santos, H. (1999): Combined effect of the growth temperature and salinity of the medium on the accumulation of compatible solutes by *Rhodothermus marinus* and *Rhodothermus obamensis*. *Extremophiles.* **3**, 163-172.

Silverstein, R.M., Bassler, G.C., and Morrill, T.C. (1981): Spectrometric identification of organic compounds. 4th ed. New York: John Wiley and Sons.

Maresk, S., DeYoreo, J.J., and Wang, C.A. (2007): Perspectives on inorganic, organic, and biological crystal growth from fundamentals to applications, 13th international summer school on crystal. *Ame. Ins. Phy*,1 edition.

Sohlemann, P., Soppa, J., Oesterhelt, D., and Lohse, M.J. (1997): Expression of $\beta_2$-adenoreceptors in halobacteria. *Naynyn-Schmiedeberg's Arch.Pharmacol*. **355**, 150-160.

Soppa, J. (1999a): Transcription initiation in archaea: facts, factors and future aspects. *Mol. Microbiol*, **31**, 1295-1305.

Soppa, J. (1999b): Normalized nucleotide frequencies allow the definition of archaeal promoter elements for different archaeal groups and reveal base-specific TFB contacts upstream of the TATA box. *Mol. Microbiol*, **31**, 1589-1592.

Stan-Lotter, H., Doppler, E., Jarosch, M., Radax, C., Gruber, C., and Inatomi, K. (1999): Isolation of chymotripsinigen-B like enzyme from the archeon *Natronomonas pharaonis* and other halobacteria. *Extremophiles*. **3**, 153-161.

Subov, N.N. (1931): Oceanographical tables. USSR Oceanographic Institute of Hydrometeorological Commission, Moscow.

Sukirtha, R., Priyanka, K., Antony, J.J., Kamalakkannan, S., Thangam, R., Gunasekaran, P., and Achiraman, S. (2011): Cytotoxic effect of green synthesized silver nanoparticles using Melia azedarach against in vitro HeLa cell lines and lymphoma mice model. *Process. Biochem.* **47**, 273-279.

Sun, C., Li, Y., Mei, S., Lu, Q., Zhou, L., and Xiang, H. (2005): A single gene directs both production and immunity of halocin C8 in a haloarchaeal strain AS7092. *Mol. Microbiol.* **57**, 537-49.

Sun, W., Xie, G., Yi, D., Chen, Y., Zhou, J., and Cheng. (2001): Microminiaturized immunoassays using quantum dots as fluorescent label by laser confocal scanning fluorescence detection. *J.Immuno. Methods.* 249, 85-89.

Takashina, T., Hamamoto, T., Otozai, K., Grant, W.D., and Horikoshi, K. (1990): *Haloarchaea japonica* sp.nov.,a new triangular halophilic archaebacterium. *Syst. Appl. Microbiol.* **13**, 177-181.

Tamar, K.P., and Aharon, O. (2000): Halocins: are they involved in the competition between halobacteria in saltern ponds? *Extremophiles.* **4**, 35-41.

Tamura K, Dudley J, Nei M & Kumar S (*2007*) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599. (http://www.kumarlab.net/publications)

Thongthai, C., Mcgenity, T.J., Suntinanalert, P., and Grant, W.D. (1992): Isolation and characterization of an extremely halophilic archaeobacterium from traditionally fermented Thai fish sauce (nam pla) *Letters in Applied Microbiology*. **14**, 111-114.

Tindall, B.J. (1992): The family *Halobacteriaceae*. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (eds) The prokaryotes. A handbook of bacteria: ecophysiology, isolation, identification, applications, Springer, New York. **1**, 768-808.

Tindall, B.J., Amendt, B., and Dahl, C. (1991): Variation in lipid composition of aerobic, halophilic archaeobacteria. In: Rodriguez-Valera, F. (Ed), General and applied aspects of halophilic microorganisms. Plenum Press, New York. 199-205.

Tomlinson, G.A., and Hochstein, L.I. (1972): Isolation of carbohydrate-metabolizing, extremely halophilic bacteria. *Can.J.Microbiol.* **18**, 698-701.

Tomlinson, G.A., and Hochstein, L.I. (1976): *Halobacterium saccharovorum* sp. nov., a carbohydrate-metabolizing, extremely halophilic bacterium. *Can. J. Microbiol.* **22**, 587-591.

Tomlinson, G.A., Jahnke, L.L., and Hochstein, L.I. (1986): *Halobacterium denitrificans* sp.nov halophilic denitrifying bacterium. *Int.J.Syst.Bacteriol.* **36**, 66-70.

Torreblanca, M., Meseguer, I., and Rodrı´guez, F.V. (1989): Halocin H6, a bacteriocin from *Haloferax gibbonsii*. *J. Gen Microbiol.* **135**,2655-2661.

Torreblanca, M., Meseguer, I., and Ventosa, A. (1994): Production of halocin is a practically universal feature of archaeal halophilic rods. *Lett. Appl.Microbiol.* **19**, 201-205.

Torreblanca, M., Rodríguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M., and Kates, M. (1986): Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of *Haloarcula* gen. nov and *Haloferax* gen. nov. *Syst. Appl. Microbiol.* **8**, 89-99.

Torsten, S., Thomas, M., Dirk, B., Hauke, H., and Uta, B. (2007): Continuous synthesis and excretion of the compatible solute ectoine by a transgenic, non halophilic bacterium. *Applied and environmental microbiology*. **73**, 3343-3347.

Tsezos, M. (1985): The selective extraction of metals from solution by micro-organisms. A brief overview. *Can. Metal. Quart.* **24**, 141-144.

Tsezos, M., and Deutschmann, A.A. (1990): An investigation of engineering parameters for the use of immobilised biomass particles in biosorption. *J. Chem. Technol. Biotechnol*. **48**, 29-39.

Unnikrishnan, M.C., and Kuttan, R. (1998): Cytotoxicity of extracts of spices to cultured cells. *Nutrition and Cancer*. **11**, 251-257.

Valderrama, M.J., Quesada, E., Bejar, V., Ventosa, A., Gutierrez, M.C., Ruiz-Berraquero, F., and Ramos-Cormenzana, A. (1991): *Deleya salina* sp.nov., a moderately halophilic Gram-negative bacterium. *Int.J.Syst.Bacteriol.* **41**, 377-384.

Ventosa, A., Quesada, F., Rodrı´guez, F.V., Ruiz, B.Q.F., and Ramos, C.A.`(1982): Numerical taxonomy of moderately Gram negative rods. *J. Gen. Microbiol.* **128**, 1959-1968.

Vidyasagar, M., Prakash, S., Mahajan, V., Shouche, Y.S, and Sreeramulu, K. (2009): Purification and characterization of an extreme halothermophilic

protease from a halophilic bacterium *Chromohalobacter* sp. TVSP101. *Braz. J. Microbiol.* **40**, 12-19.

Vivian, G.W. (1969): The Preservation of hides and skins against bacterial damage. *J.Amer. Leather Chem. Ass.* **64**, 489.

Vreeland, R.H., Litchfield, C.D., Martin, E.L., and Eliot, E. (1980): *Halomonas elongate*, a new genus and species of extremely salt-tolerant bacteria. *Int. J. Syst. Appl. Bacteriol.* **30**, 485-495.

Walsby, A.E. (1980): A square bacterium. *Nature.* **283**, 69-71.

Weiss, E.F., and Thornton, R.L. (2005): Growth and control of microbiological activity during hide curing process. Buckman Laboratories Inc, 1984, 18.

Wilson. (1984): Characterization of a cell line derived from the ascites of a patient with papillary serious cystadenocarcinoma of the ovary. *J. Natl. Cancer Inst.* **72**, 513-21.

Wu, L.C., Chow, K.C., and Mark, K.K. (1983): Role of pigments in *Halobacterium cutirubrum* against UV irradiation. *Microbiol. Lett.* **24**, 85-90.

www.ncbi.nlm.nih.gov

Xin, H., Itoh, T., Zhou, P., Suzuki, K., Kamekura, M., and Nakase, T. (2000): *Natrinema versiforme* sp. nov., an extremely halophilic archaeon from Aibi salt lake, Xinjiang, China. *Int. J. Syst. Evol .Microbiol.* **50**, 1297-1303.

Yildiz, E., Ozcan, B., and Caliskan, M. (2012): Isolation, characterization and phylogenetic analysis of halophilic archaea from a salt mine in central Anatolia (Turkey). *Pol. J. Microbiol.* **61**, 111-117.

Yoon, J.H., Lee, K.C., Weiss, N., Kang, K.H. and Park, Y.H. (2003): *Jeotgalicoccus halotolerans* gen. nov., sp. nov. and *Jeotgalicoccus psychrophilus* sp. nov., isolated from the traditional Korean fermented seafood jeotgal. *Int. J. Syst. Evol. Microbiol.* **53**, 595-602.

Yoon, J.H., Lu, K.C., Kho, Y.H., Kang, K.H., Kim, C.J and Park, J.H. (2002): *Halomonas alimentoria* sp.nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int.J.Syst.Evol.Microbiol.* **52**, 123-130.

Zvyagintseva, I.S., Kostrikana, N.A., and Belyaev, S.S. (1998): Detection of halophilic archaea in an upper devonian oil field in Tatarstan. *Microbiology* (Russia). **67**, 688-691.

## APPENDIX


**Fig 4.57.** Evolutionary relationships of 44 taxa of *Halobacteriaceae* family inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.99715993 is shown. The percentage of replicate trees in which

the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4.

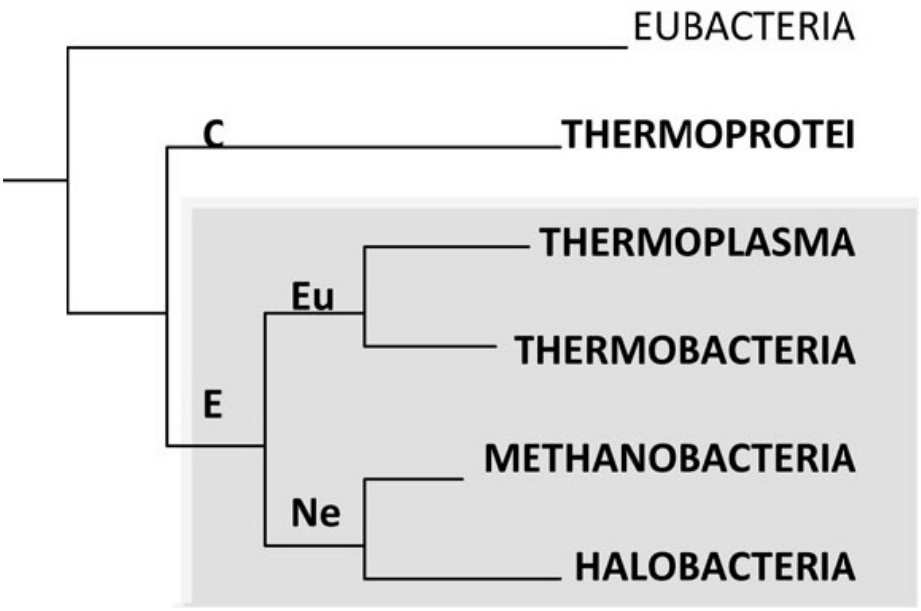



**Fig 4.58.** Cladogram that highlights the phyla of the kingdom Euryarchaeota. The general organization of the cladogram according to - The All-Species Living Tree Project (Woese et al., 1990). C = Crenarchaeota; E = Euryarchaeota; Eu = Eurythermea; Ne = Neobacteria.

**Table 4.11 Antibiotic sensitivity and resistance profile of *Natrinema* sp. BTSH10 and *Halorubrum* sp. BTSH03**

| S.No | Antibiotic | *Natrinema* sp. BTSH10 | *Halorubrum* sp. BTSH03 |
|---|---|---|---|
| 1 | Ciprofloxacin | R | S |
| 2 | Norfloxacin | R | S |
| 3 | Cephotaxime | R | S |
| 4 | Cephalothin | R | S |
| 5 | Cotrimoxazole | R | R |
| 6 | Chloramphenicol | R | S |
| 7 | Bacitracin | R | S |
| 8 | Tetracycline | R | S |
| 9 | Neomycin | R | S |
| 10 | Imipenem | R | R |
| 11 | Vancomycin | R | S |
| 12 | Aztreonam | R | S |
| 13 | Oflaxacin | S | S |
| 14 | Penicillin G | S | S |
| 15 | Azithromycin | S | S |
| 16 | Meropenem | S | S |
| 17 | Tobromycin | S | S |
| 18 | Moxifloxacin | S | S |
| 19 | Sparfloxacin | S | S |
| 20 | Levofloxacin | S | S |
| 21 | Amoxicillin | S | S |
| 22 | Polymixin B | S | S |
| 23 | Gentamycin | S | S |
| 24 | Erythromycin | S | S |
| 25 | Clindamycin | S | S |
| 26 | Ceftazidime | S | S |
| 27 | Netillin | S | R |
| 28 | Doxycycline hydrochloride | S | S |
| 29 | Nalidixic acid | S | S |
| 30 | Nitrofurantoin | S | S |
| 31 | Gatifloxacin | S | S |

R – Resistance; S - Sensitive

## List of Publications

**Peer reviewed**

- Halocin SH10-A novel bacteriocin from an extreme haloarchaeon *Natrinema* sp. BTSH10 isolated from salt pans of South India- **P.Karthikeyan,** Sarita G Bhat, and Chandrasekaran M.- **Saudi Journal of Biological Sciences (2013) 20, 205–212.**

- Halophiles and Halozymes from Tannery effluent as well as food grade table salt crystals- Manjula R, **Karthikeyan P**, Cikesh PC, Bindhiya ES, Sarita G Bhat and Chandrasekaran M - **The journal of Pure and Applied Microbiology-Accepted for publication.**

**Conference Proceedings**

- Microflora of heavily rain flooded salt pans in South India - **P.Karthikeyan**, Lineesha K.A, Divya T.N, Sarita G.Bhat, Chandrasekaran.M - Proceedings of 31st Symposium of the Malaysian Society for Microbiology, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia, December 13-15, 2012. Pg 298-301.

**NCBI submissions**

1. GenBank accession number - JN228202 – *Natrinema* sp.BTSH10- **Karthikeyan,P**., Bhat,S.G., Elyas,K.K. and Chandrasekaran,M
2. GenBank accession number - JF830251 - *Marinobacter* sp.HUC12- **Karthikeyan,P**., Divya,T.N., Lineesha,K.A., Cikesh,P.C., Bhat,S.G. and Chandrasekaran,M.

3. GenBank accession number - JF830252 - *Halobacillus* sp.HUC13- **Karthikeyan,P**., Divya,T.N., Lineesha,K.A., Cikesh,P.C., Bhat,S.G. and Chandrasekaran,M.
4. GenBank accession number - JF830253 - *Halobacillus* sp.HUC14- **Karthikeyan,P**., Divya,T.N., Lineesha,K.A., Cikesh,P.C., Bhat,S.G. and Chandrasekaran,M.
5. GenBank accession number - JF830254 - *Halomonas* sp.HUC17- **Karthikeyan,P**., Lineesha,K.A., Divya,T.N., Cikesh,P.C., Bhat,S.G. and Chandrasekaran,M.
6. GenBank accession number - JF830255 - *Halomonas shengliensis HUC17*- **Karthikeyan,P**., Lineesha,K.A., Divya,T.N., Cikesh,P.C., Bhat,S.G. and Chandrasekaran,M.
7. GenBank accession number - JF830256 - *Marinobacter* sp.HUC18- **Karthikeyan,P**., Lineesha,K.A., Divya,T.N., Cikesh,P.C., Bhat,S.G. and Chandrasekaran,M.
8. GenBank accession number - JF830242 - *Halorubrum* sp.BTSH03- **Karthikeyan,P**., Bhat,S.G., Elyas,K.K. and Chandrasekaran,M.
9. GenBank accession number - JF830243 - *Halobacillus trueperi HUC01*- **Karthikeyan,P.**, Divya,T.N., Lineesha,K.A., Bhat,S.G. and Chandrasekaran,M.
10. GenBank accession number - JF830248 - *Halobacillus trueperi* HUC06- Karthikeyan,P., Lineesha,K.A., Divya,T.N., Bhat,S.G. and Chandrasekaran,M.
11. GenBank accession number - JN228201 - *Staphylococcus arlettae* BTMT04- Manjula,R., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.

12. GenBank accession number - JN228200 - *Staphylococcus arlettae.* BTMT02- Manjula,R., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
13. GenBank accession number - JN228199 – *Salimicrobium* sp. BTMT08- Manjula,R., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
14. GenBank accession number - JN228198 – *Salimicrobium* sp. BTMT10 -Manjula,R., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
15. GenBank accession number - JN228197 – *Oceanobacillus* sp. BTMT03 -Manjula,R., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
16. GenBank accession number - JN228196 – *Chromohalobacter salaxigens.* BTMT09- Manjula,R., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
17. GenBank accession number - JF830244 – *Halobacillus* sp.HUC02- Divya,T.N., Lineesha,K.A., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
18. GenBank accession number - JF830245 – *Staphylococcus* sp.HUC03- Divya,T.N., Lineesha,K.A., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
19. GenBank accession number - JF830246 – *Halomonas* sp.HUC04 - Divya,T.N., Lineesha,K.A., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
20. GenBank accession number - JF830247 – *Halomonas* sp.HUC05- Lineesha,K.A., Divya,T.N., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.

21. GenBank accession number - JF830249 – *Marinobacter* sp.HUC08-Lineesha,K.A., Divya,T.N., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
22. GenBank accession number - JF830250 – *Halobacillus* sp.HUC11-Lineesha,K.A., Divya,T.N., **Karthikeyan,P**., Bhat,S.G. and Chandrasekaran,M.
23. GenBank accession number - JX975062 - *Halomonas* sp. BTMT13-Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
24. GenBank accession number - JX975063 - *Halomonas elongate* BTMT07-Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
25. GenBank accession number - JX975064 - *Chlorohalobacter* sp.BTMT11 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
26. GenBank accession number - JX975065 - *Halomonas* sp.BTMT12 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
27. GenBank accession number - JX975066 - *Oceanobacillus* sp.BTMT01 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
28. GenBank accession number - KC019170 - *Halomonas* sp.BTMT06 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
29. GenBank accession number - KC019171 - *Halomonas* sp. BTMT05 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
30. GenBank accession number - JX975062- *Halomonas elongate*.BTMT13 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.

31. GenBank accession number - JX975063 - *Halomonas elongate BTMT07* Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
32. GenBank accession number - JX975064- *Chromohalobacter* sp. BTMT11 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M
33. GenBank accession number - JX975065 - *Halomonas elongate.BTMT12* Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.
34. GenBank accession number - JX975066- *Oceanobacillus* sp.BTMT01 Manjula,R., **Karthikeyan,P**., Sarita,G.B. and Chandrasekaran,M.

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ORIGINAL ARTICLE

# Halocin SH10 production by an extreme haloarchaeon *Natrinema* sp. BTSH10 isolated from salt pans of South India

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**Abstract** Halobacteria, members of the domain Archaea that live under extremely halophilic conditions, are often considered as dependable source for deriving novel enzymes, novel genes, bioactive compounds and other industrially important molecules. Protein antibiotics have potential for application as preserving agents in food industry, leather industry and in control of infectious bacteria. Halocins are proteinaceous antibiotics synthesized and released into the environment by extreme halophiles, a universal characteristic of halophilic bacteria. Herein, we report the production of halocin (SH10) by an extremely halophilic archeon *Natrinema* sp. BTSH10 isolated from salt pan of Kanyakumari, Tamilnadu, India and optimization of medium for enhanced production of halocin. It was found that the optimal conditions for maximal halocin production were 42 °C, pH 8.0, and 104 h of incubation at 200 rpm with 2% (V/V) inoculum concentration in Zobell's medium containing 3 M NaCl, Galactose, beef extract, and calcium chloride as additional supplements. Results indicated scope for fermentation production of halocin for probable applications using halophilic archeon *Natrinema* sp. BTSH10.



## 1. Introduction

Halobacteria, members of the domain Archaea, live under extremely halophilic conditions (4–5 M NaCl) and lower concentrations of sodium chloride generally cause cell lysis (Meral et al., 2007). They produce organic solutes which maintain the concentration of ions inside and outside the cell in order to keep themselves intact and survive in high saline environments (Gonzalo et al., 2002; Jan et al., 2007; Torsten et al., 2007). They are often considered as dependable source for deriving novel enzymes, novel genes, bioactive compounds and other industrially important molecules. Protein antibiotics have potential for application as preserving agents in food

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industry, leather industry and in control of infectious bacteria and hence there is great interest in isolation of potential proteinaceous bioactive substances. Haloarchaea were the first members of Archaea found to produce proteinaceous bacteriocins which are released into the environment, a universal characteristic of halophilic bacteria (Rodríguez et al., 1982). These proteinaceous bacteriocins, termed as halocins, act against related species and are universally produced by halophilic archaea (Torreblanca et al., 1994). In spite of the fact that several halophilic archaea are being explored, only very few halocins have been studied up to their molecular level and their mode of action whether they kill or inhibit other haloarchaeons as a defence is yet to be understood clearly (Tamar and Aharon, 2000; Meseguer and Rodríguez, 1986). Moreover, all haloarchaea are not sensitive to any particular halocin, and a "sensitive" strain is the one which elaborates a zone of inhibition on a double-agar overlay plate in response to the presence of halocin (Paši et al., 2008).

Halocins have been reported to generally kill the indicator organisms by cell swelling followed by cell lysis (O'Connor and Shand, 2002; Sun et al., 2005; Paši et al., 2008). Halocin A4, G1, R1, H1, H2 (O'Connor and Shand, 2002); H3, H5 (Rodríguez et al., 1982; O'Connor and Shand, 2002); H4 (Sun et al.,2005; Gonzalo et al., 2002; O'Connor and Shand, 2002); H6/H7 (O'Connor and Shand, 2002; Li et al., 2003); S8 (O'Connor and Shand, 2002); C8 (Li et al., 2003; Sun et al., 2005) and Sech7a (Paši et al., 2008) are few halocins reported till date and only few of them are studied up to molecular level. They are generally produced at the mid exponential phase (O'Connor and Shand, 2002; Paši et al., 2008). The mechanism of action of halocin may involve modification of cell permeability or inhibition of $Na^+/H^+$ antiporter and Proton flux. Few halocins are said to be salt dependent since the protein loses its activity when the concentration of salts decreases beyond a minimum level (Rodríguez et al., 1982; Price and Shand, 2000). Halocin H6 produced by haloarchaea *Haloferax gibbonsii* was reported to inhibit $Na^+/H^+$ exchanger (NHE) in mammalian cells (Meseguer et al., 1995). Studies have been conducted up to gene level for halocins H4 (Cheung et al., 1997), C8 (Sun et al., 2005), and S8 (Price and Shand, 2000), and for identification of the mRNA responsible for production of halocin (Cheung et al., 1997; Price and Shand, 2000).

Though several species of Haloarchaeons were studied for halocin production, reports on halocin production by *Natrinema* sp. are rather limited. In this context, herein we report the production of halocin by *Natrinema* sp. an extreme halophile isolated from saltern ponds of south India, which are well known as source of salt prepared for human consumption.

## 2. Materials and methods

### 2.1. Microorganisms

Soil samples were collected from salt pans of Kanyakumari district, Tamilnadu, India. One gram of soil was mixed with 10 ml of saturated solution of sodium chloride, homogenized and 100 μl of the prepared sample was spread plated on Zobell's agar medium containing 3 M sodium chloride and incubated for a week at 37 °C. Later, the single cell colonies formed on the plates were picked, purified and stored as glycerol stocks in −80 °C deep freezer. All the isolated cultures were tested for production of antimicrobial compounds (halocins) against other halophilic bacterial cultures. Potential strains that showed intense bioactivities were screened against selected halobacteria isolated from saltern pond. Both strains that produced bioactive substances and showed inhibition were identified based on their morphological, biochemical, physiological characteristics (Bergey's Manual of Systematic bacteriology) and 16S ribotyping.

### 2.2. Media for halocin production

Medium that supported maximal biomass and halocin production was selected from among six different media namely Eimhjellen medium (EM) (Catherine et al., 2001), Sehgal and Gibbons (SG) (Sehgel and Gibbons, 1960), MH medium (Ventosa et al., 1982), HE medium (Torreblanca et al., 1986; Catherine et al., 2001) DSM 97 (ATCC Manual) and Zobell's medium (Hi-media). The compositions of the media are as given below:

#### 2.2.1. Eimhjellen medium (modified)

Yeast extract – 5 g, $MgSO_4{\cdot}7H_2O$ – 2.0 g, $CaCl_2{\cdot}2H_2O$ – 0.5 g, NaCl – 3.0 M, Distilled water – 100 ml (Catherine et al., 2001).

#### 2.2.2. Sehgel and gibbons medium (modified)

Yeast extract – 1 g, Casamino acids – 0.75 g, Sodium citrate – 0.3 g, $MgSO_4{\cdot}7H_2O$ – 2.0 g, KCl – 0.2 g, $FeCl_2$ – 0.0023 g, NaCl – 3.0 M, Distilled water – 100 ml (Sehgel and Gibbons, 1960).

#### 2.2.3. DSM 97

Casamino acids – 7.5 g, KCl – 2 g, NaCl – 3.0 M, Trisodium citrate – 3 g, $MgSO_4$ – 20 g, $MnSO_4$ – 0.05 g, Ferrous sulphate – 0.5 g, Yeast extract – 10 g, Agar – 10 g, Distilled water – 1000 ml (ATCC manual).

#### 2.2.4. MH medium (modified)

Protease peptone – 0.5 g, Yeast extract – 1.0 g, Glucose – 0.1 g with 25% (w/v) of total salts (Ventosa et al.,1982).

#### 2.2.5. HE medium (modified)

Yeast extract – 0.5 g, Glucose – 0.1 g with 25% (w/v) of total salts (Torreblanca et al., 1986).

The total salts solution was prepared with NaCl – 3.0 M, $MgCl_2{\cdot}6H_2O$ – 4.2 g, $MgSO_4{\cdot}7H_2O$ – 6.0 g, $CaCl_2{\cdot}2H_2O$ – 0.1 g, KCl – 0.6 g, $NaCO_3H$ – 0.02 g, NaBr – 0.07 g, $FeCl_3$ – 0.0005 g, Distilled water – 100 ml (Subov, 1931).

#### 2.2.6. Zobell's medium

Peptic digest of animal tissues – 5 g, Yeast extract – 1 g, Ferric citrate – 0.1 g, NaCl – 3.0 M, $MgCl_2{\cdot}6H_2O$ – 8.8 g, Sodium sulphate – 3.24 g, $CaCl_2{\cdot}2H_2O$ – 1.8 g, KCl – 0.55 g, $NaCO_3H$ – 0.16 g, KBr – 0.08 g, Strontium chloride – 0.034 g, Boric acid – 0.022 g, Sodium silicate – 0.004 g, Sodium fluorate – 0.0024 g, Ammonium nitrate – 0.0016 g, Disodium phosphate – 0.008 g, Distilled water – 1000 ml supplemented with NaCl (4.5 M).

Irrespective of the medium, the final concentration of sodium chloride in the medium was adjusted to 3 M unless otherwise specified. The pH of the medium was maintained

at 7.4 ± 2. Solid medium was prepared by the addition of 2% (w/v) of agar (Hi-media) to broth. The bacterial growth was measured in terms of OD at 600 nm in a UV–Visible spectrophotometer (Shimadzu Model 160A).

### 2.3. Preliminary screening of halocin activity

All the halophilic bacterial strains isolated from the salt ponds were screened for halocin activity by testing each strain against the other strain. The presence of zone of inhibition on double layer agar plates was used as indicator for halocin production (Shand et al., 1999; O'Connor and Shand, 2002). 10 μl aliquots of broth culture of each strain grown in Zobell's medium were spotted onto top-agar lawn culture of the other halophilic strains and incubated at 42 °C for 7 days. Appropriate controls were maintained using uninoculated media. Cultures that showed inhibition were selected and subjected to further studies.

### 2.4. Culture conditions

A preculture of the selected bacterial strain that showed halocin activity was prepared initially by growing the strain in 10 ml of Zobell's medium at 37 °C for 24 h. After growth the culture obtained was centrifuged at 10,000 rpm at 4 °C for 15 min, under aseptic conditions, and the cells were harvested, washed with brine solution (15% NaCl), and suspended in the same solution. The concentration of the cell suspension was adjusted to 0.2 OD at 600 nm and used as inoculum at 2% (V/V) level. 100 ml of Zobell's medium taken in a 250 ml conical flask was inoculated with the prepared inoculums and incubated for 96 h. Similarly the selected indicator organism was also grown in Zobell's medium and a cell suspension was prepared in the same manner as was done with the halocin producing strain.

### 2.5. Halocin production and assay

Zobell's medium was used for the production of halocin. The sterile medium was inoculated with the selected strain BTSH10, incubated at 37 °C for 96 h, and the bacterial free supernatant was used for halocin assay after centrifugation (10,000 rpm for 10 min) of the culture broth.

Halocin activity was checked by agar well diffusion method (John et al., 1966) using the culture supernatant. The indicator strain BTSH03 was grown up to 0.3 OD at 600 nm and 200 μl of the indicator strain was mixed with 20 ml of Zobell's medium containing half strength (1%) agar, and overlaid on Zobell agar plates containing 2% agar. Halocin activity was checked by the addition of 50 μl of cell free supernatant of BTSH10 into the well (0.5 cm diameter) made on the plate containing the top agar and performing the halocin assay after incubation for 72 h.

The halocin activity was determined using serial twofold critical end point dilutions to extinction (Meseguer et al., 1986) and expressed as arbitrary units (AU), which are defined as the reciprocal of the first dilution at which all traces of inhibitory activity disappears (Cheung et al., 1997). The two fold dilution ratio of halocin follows a geometric progression where the halocin activity can be calculated by

$$a_n = a_1 \cdot q^{n-1}$$

where "$a$" denotes the scale factor, "$q$" is the common ratio and "$n$" being the first dilution at which all traces of inhibitory activity disappears.

### 2.6. Cell lysis assay

Activity of halocin SH10 on the cells of indicator bacteria BTSH03 was studied by monitoring the cell lysis under phase contrast microscope. Stationary phase cells of indicator strain *Halorubrum* sp. BTSH03 was mixed with halocin SH10 (1024 AU/ml) on a microtitre plate and incubated for different time intervals. Samples were drawn at regular intervals and observed under the microscope. Images were captured and presented as photomicrographs.

### 2.7. Optimization of medium and process variables for halocin production

Various constituents of the selected medium and process parameters that influence halocin production by BTSH10 was optimized by adopting 'one factor at a time' approach. Strategy adopted for the optimization was to evaluate the effect of each variable for its optimum level for maximal halocin production, and incorporate the same variable at its optimized level in the subsequent experiment while evaluating the next variable. The variables studied included the following in the sequential order: incubation temperature (27–47 °C), pH (2–13), NaCl concentration (0.5–4 M), carbon sources at 0.1 M concentrations (dextrin, galactose, fructose, lactose, sucrose, sorbitol, xylose, maltose and glycerol), nitrogen source at 1% (w/v) concentration (peptone, yeast extract, malt extract, soybean meal, tryptone, casein, urea and beef extract) and different inorganic salts at 0.1 M concentration (ammonium nitrate, sodium fluorate, sodium silicate, potassium chloride, magnesium chloride, calcium chloride, sodium bicarbonate, potassium bromide and strontium chloride), agitation (50–250 rpm), and incubation time (0–144 h). Preparation of inoculum, inoculation and culture conditions were same as mentioned earlier unless otherwise mentioned.

## 3. Results and discussion

### 3.1. Identification of halophiles

The halophilic bacterial strain BTSH10 that showed strong halocin activity from among the isolates obtained as halophiles from the saltern pond was selected for further studies. Strain BTSH03 which showed sensitivity against halocin SH10 was considered as the indicator organism for determining halocin activity during the course of the studies. Based on the morphological, biochemical, physiological characteristics, and molecular 16S ribotyping (data not shown) the halocin producing strain BTSH10 and the indicator strain BTSH03 were identified as *Natrinema* sp. BTSH10 and *Halorubrum* sp. BTSH03, respectively. The nucleotide sequences obtained for the 16S rDNA of the selected bacterial strains were submitted to NCBI and the allotted accession numbers are JN228202 and JF830242, for *Natrinema* sp. BTSH10 and *Halorubrum* sp BTSH03, respectively. The genus *Natrinema* was proposed by McGenity et al., 1998) to accommodate *Natrinema pellirubrum* (formerly *Halobacterium salinarum* NCIMB 786T) and

*Natrinema pallidum* (formerly *Halobacterium halobium* NCIMB 777T). In a phylogenetic tree based on 16S rRNA gene sequences, *Natrinema* species formed an independent cluster with respect to *Halobacterium* species. *Natrinema* species could be cultured at low salt concentrations, and possessed a specific protein profile and polar lipid composition. Subsequently, a novel species of this genus, *Natrinema versiforme*, was described (Xin et al., 2000). From the results (data not shown) obtained for the standardization of optimal cultivation media for halocin production, it was observed that Zobell's medium supported enhanced growth and halocin production compared to other media evaluated and hence the same was selected for further cultivation of the halophiles. Further the halocin was named as halocin SH10 since it was produced by *Natrinema* sp. BTSH10.

The results presented as photomicrographs in Fig. 1 showing the activity of halocin SH10 on the indicator strain *Halorubrum* sp. BTSH03 clearly evidence cell lysis. Initially *Halorubrum* sp. did not show any change on exposure to halocin SH10 (Fig. 1a). However, after 3 h the cells showed shrinkage and were found as a floc forming small islands/colonies (Fig. 1b). After 6 h the cells were found to appear bulged and showed signs of lysis (Fig. 1c). On further incubation for 12 h complete lysis of the cells resulting in cell debris (Fig. 1d) was observed. These observations not only confirmed the antibacterial activity of halocin SH10 against other halophiles but also suggest cell lysis as the possible mechanism of action of halocin SH10. Exposure of sensitive cells to HalH6 was reported to cause increase in the intracellular volume and cellular swelling followed by lysis, suggesting that HalH6 may act at the level of the cell membrane (Torreblanca et al., 1989). The present observations on morphological changes of sensitive cells upon exposure to halocin and swelling of the cells and cell lysis corroborate well with those observations reported in previous studies on halocins Sech7a, H4, H6, and C8, (Meseguer and Rodrıguez, 1985; Torreblanca et al., 1989; Li et al., 2003).

### 3.2. Optimization of media and process variables

#### 3.2.1. Effect of incubation temperature, pH and NaCl concentration on halocin production

From the results documented in Fig. 2 it was inferred that 42 °C was the optimum temperature for maximum halocin production (1024 AU). Nevertheless appreciable levels of halocin activities could be recorded at other temperatures. Halocin production at high levels observed at 42 °C, and relatively at lesser levels at lower and at slightly higher temperatures (47 °C) indicated that the halocin gene expression might be either temperature dependent or specific temperature of the cultivation medium might act as an antagonist (stress) to the organism that induces this gene expression (Christine et al., 2008; Mirko et al., 2012). Haloarchaeon Sech7a was reported to be thermophilic in character with optimal growth occurring at 45 °C, although the temperature in its native solar saltern crystallizer rarely exceeds 32 °C (Pasić et al., 2005). Consistent with the physicochemical properties of a crystallizer, the optimal growth of haloarchaeon Sech7a was observed at pH 8, yet the halocin production reached maximum at neutral pH (Paši et al., 2008). Results presented in Fig. 3 show that the halobacteria could produce halocin in media with a pH varying between pH 4 and pH 10 although maximum halocin production was recorded at pH 8.0 (1024 AU). However, the bacteria did not produce halocin under acidic conditions.

Results presented in Fig. 4 indicate that *Natrinema* sp. BTSH10 required 3 M NaCl as optimum concentration for maximum (1024 AU) production of halocin although NaCl concentration ranging from 1.5 to 4 M in the medium supported enhanced production of halocin. It was also noted that a minimum of 1.5 M NaCl was required for growth, and lesser concentrations did not even support survival of the bacterium. These observations testified the halophilic nature of the isolated bacterium and the impact of higher concentrations of sodium chloride for halocin activity. The halocin of haloarchaeon Sech7a was

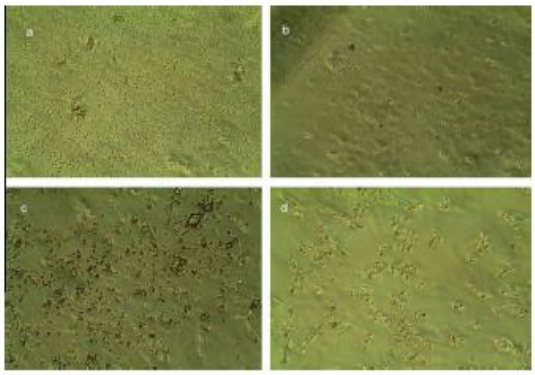


**Figure 1** Photomicrographs showing activity of halocin SH10 on indicator strain *Halorubrum* sp. BTSH03 (a) Normal cells of *Halorubrum* sp BTSH03 soon after (0 h) halocin SH10 treatment (b) Shrinked cells forming islands after 3 h of treatment (c) Bulged cells after 6 h of treatment (d) Completely lysed cells after 12 h of treatment.

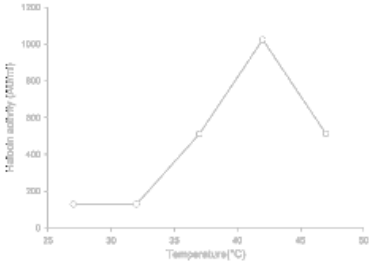


**Figure 2** Effect of different incubation temperatures on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at pH 7.4, 3 M NaCl, 150 rpm, 2% (V/V) inoculum concentration and 96 h of incubation.

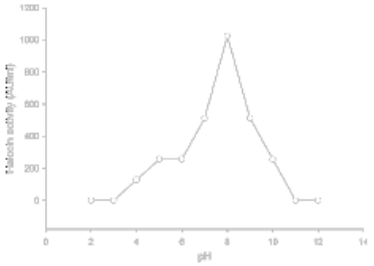


**Figure 3** Effect of different pH on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at 42 °C, 3 M NaCl, 150 rpm, 2% (V/V) inoculum concentration and 96 h of incubation.

observed to remain active over a wide NaCl concentration range (0.02–5.2 M) with the highest production observed in high salt media containing 3.4 M NaCl (Paši et al., 2008). The observations made with *Natrinema* sp. BTSH10 was in agreement with these earlier reports for other species of haloarchaeon although there were marginal differences in optimal temperature and sodium chloride concentrations. It was also noted that sodium chloride concentration was found to have strong influence on the halocin activity of the halobacteria. Another specific observation made during the study was that the maximal halocin activity observed with these three factors namely temperature, pH and NaCl were almost same. This observation could be attributed to the fact that the control medium had 3 M NaCl and pH of the medium was 7.4 which were almost identical with the optimum pH and NaCl concentrations. Hence there was no marked enhancement in halocin activity after optimization of these three variables. Further the results also indicated that these

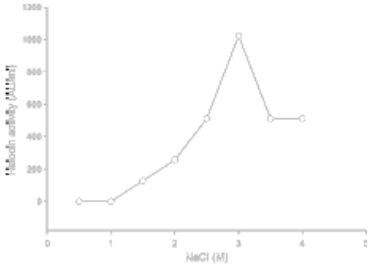


**Figure 4** Effect of different concentrations of NaCl on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at 42 °C, pH 8.0, 150 rpm, 2% (V/V) inoculum concentration and 96 h of incubation.

three factors are independent in exerting their influence on halocin production by the bacteria.

### *3.3. Effect of different carbon and nitrogen sources and inorganic salts in the medium on halocin production*

From the results presented in Fig. 5 it was inferred that maximal halocin production was supported by the medium supplemented with galactose (2048 AU) followed by sorbitol, maltose, glycerol, glucose, fructose, and lactose. Whereas, the medium supplemented with dextrin, sucrose and xylose supported reduced levels of halocin production. Galactose was observed to enhance halocin production in the medium compared to other carbon sources. Results documented in Fig. 6 indicate that the bacteria could produce maximal halocin in the presence of beef extract (2048 AU) in the medium followed by soybean meal, malt extract, tryptone, peptone,

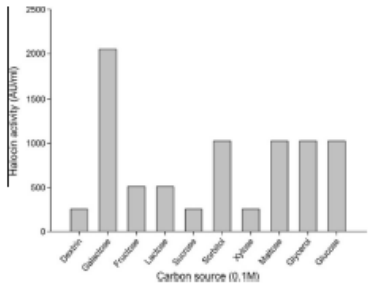


**Figure 5** Effect of different carbon sources on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at 42 °C, pH 8.0, 3 M NaCl, 150 rpm, 2% (V/V) inoculum concentration and 96 h of incubation.

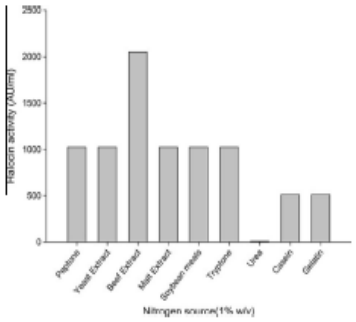


**Figure 6** Effect of different nitrogen sources on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at 42 °C, pH 8.0, 3 M NaCl, Galactose 150 rpm, 2% (V/V) inoculum concentration and 96 h of incubation.

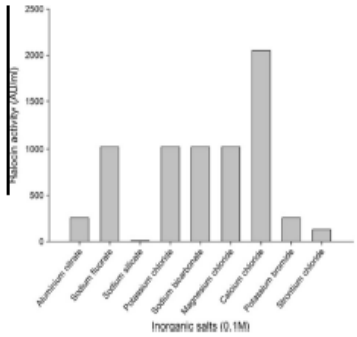


**Figure 7** Effect of different inorganic salts on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at 42 °C, pH 8.0, 3 M NaCl, Galactose beef extract, 150 rpm, 2% (V/V) inoculum concentration and 96 h of incubation.

yeast extract, casein and gelatin. Urea did not support halocin production. In fact it was reported earlier that the algae *Dunaliella* sp. (Avinash et al., 2011) that exist in the natural salt pan ecosystem provides glycerol and galactose for the halobacteria and thus the strain recorded maximal halocin in response to supplementation of galactose under laboratory conditions. In a similar fashion maximal growth rate and halocin activity by haloarchaeon Sech7a was observed in media supplemented with glycerol and yeast extract (Paši et al., 2008). It must be noted that in solar salterns glycerol produced by blooms of unicellular green algae, *Dunaliella* is considered the most important source of organic carbon for the heterotrophic prokaryotes (Bardavid et al., 2008). Additional salts in the medium were found to exert influence on halocin production by the archaebacteria. Among the inorganic salts used for supplementation of the medium as additional salts calcium chloride (2048 AU) was found to support maximal halocin production in the medium followed by magnesium chloride, sodium fluorate, potassium chloride, and sodium bicarbonate Fig. 7. Whereas aluminium nitrate, potassium bromide, strontium chloride and sodium silicate led to a much reduced level of halocin production when compared to the levels noted with Zobell's medium in the absence of these particular salts. These observations indicate that the members of archaea have a special nutritional requirements and critical life style in the salt pan environment which needs to be investigated farther to have a better understanding of their physiology in hyper saline environments.

### *3.4. Effect of agitation on halocin production*

Data documented in Fig. 8 indicate the influence of agitation on the rate of halocin production. It was found that higher agitation rate led to enhanced bacterial growth and production of halocin compared to lesser agitation rates. A maximum of 4096 AU was recorded at 200 and 250 rpm although lower

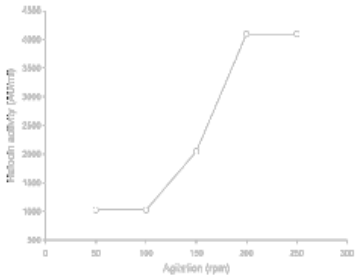


**Figure 8** Effect of different agitation rates on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at 42 °C, pH 8.0, 3 M NaCl, Galactose, beef extract, calcium chloride, 2% (V/V) inoculum concentration and 96 h of incubation.

agitation rates (50–150 rpm) also supported considerable levels of halocin production. This might be due to better mixing of the medium which facilitates better mass transfer and also does not support the adherence of the microbes to the surface of the flask. These observations strongly suggested that aerobic conditions are required for enhanced halocin production by the halophilic *Natrinema* sp. Generally the dissolved oxygen is low in aqueous medium at higher concentrations of NaCl and hence aerobic organisms need provision of adequate oxygen for enhanced electron transport and consequent growth and halocin production. Normally the agitation process facilitates infusion of atmospheric air into the growth medium and provides required oxygen for the bacterium (Feng et al., 2003; Hay et al., 2012).

### 3.5. Time course experiment for halocin production

Results depicted in Fig. 9 very clearly indicate that *Natrinema* sp. BTSH10 produces halocin at enhanced levels only during the stationary phase of growth although significant levels of halocin could be noted during late exponential phase. Maximum (8192 AU) production of halocin was observed at 104 h, during the stationary phase. Nevertheless the halocin activity in the medium was also noted even after 144 h. The organism *Natrinema* sp. is an extreme haloarcheon which produces halocin SH10. The halocin production was reported to register an increase when the culture entered the exponential phase and continued to increase during the course of exponential phase although the maximal level was attained during the stationary phase (Price and Shand, 2000; O'Connor and Shand, 2002). The observations made in the present study indicated that in the case of *Natrinema* sp. BTSH10 the halocin production is growth associated which gets accumulated in the cell and released during stationary phase. In an earlier study halocin production by haloarchaeal strain Sech7a was reported to be growth dependent (Paši et al., 2008). Although the onset of halocin activity was observed in the early exponential phase of growth, the halocin Sech7a peak activity was observed as the bacteria entered the stationary phase of growth (Paši et al., 2008) in contrast to most other halocins which were first detected when the bacteria entered the stationary phase of growth (Shand et al., 1999; O'Connor and Shand, 2002). Further HalS8 activity was reported to be undetectable in culture supernatants until the culture began the transition into stationary phase (Price and Shand, 2000; Shand et al., 1999) and later the activity reached a maximum within 10 h of onset and was stable for greater than 80 h after reaching maximum values (Price and Shand, 2000). The results observed in the present study for *Natrinema* sp. BTSH10 were very similar to those observed for most other haloarchaeal bacteria in terms of halocin synthesis during growth in the production medium. It may be noted that during the process of optimization of variables, one after another, the halocin content showed increase and reached a maximum under optimized culture conditions. This observation strongly indicated the need for optimization of production medium towards enhanced level of halocin SH10 production by *Natrinema* sp. BTSH10 and the significant role of media constituents in inducing halocin synthesis by the bacterium.

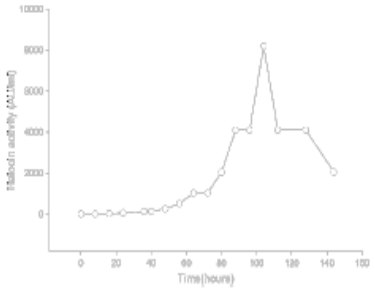


**Figure 9** Time course experiment on halocin production by *Natrinema* sp. BTSH10 in Zobells medium at 37 °C, pH 8, 3 M NaCl, Galactose beef extract, calcium chloride, 2% (V/V) inoculum concentration and 200 rpm.

## 4. Conclusion

Based on the results obtained in the present study it is concluded that *Natrinema* sp. BTSH10, relatively a new genus of archaebacteria that is less explored, exists as a native flora in the highly saline salt pan of coastal areas of southern India known for the harvest of commercial salt used for human consumption. This bacterium is capable of synthesizing halocin SH10 which has scope for probable application as preservative against those bacteria that cause spoilage in food products, leather products during processing and in control of infectious bacteria and few other applications. Results of the present study indicated that halocin could be easily produced under submerged fermentation. Further studies could reveal the mode of gene expression under the influence of various nutrients and inorganic salts which were found to induce halocin under differential environmental conditions.

## Acknowledgements

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## References

Avinash, M., Kumari, K., Bhavanath, J., 2011. Characterization of extracellular polymeric substances produced by micro-algae *Dunaliella salina*. Carbohydr. Polym. 83, 852–857.

Bardavid, E.R., Khristo, P., Oren, A., 2008. Interrelationships between *Dunaliella* and halophilic prokaryotes in saltern crystallizer ponds. Extremophiles 12 (1), 5–14.

Catherine, L., Mercedes, M.S., Bernardo, P., Alberto, R.C., Jurgen, W., Victoriano, C., 2001. Taxonomic study of extreme halophilic archaea isolated from the "Salar de Atacama", Chile. Syst. Appl. Microbiol. 24 (3), 464–474.

Cheung, J., Danna, K.J., O'Connor, E.M., Price, L.B., Shand, R.F., 1997. Isolation, sequence, and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon haloferax mediterranei R4. J. Bacteriol. 179, 548–551.

Christine, A., White-Ziegler, Suzin, U., Natalie, M.P., Abby, L.B., Amy, J.M., Sarah, Y., 2008. Low temperature (236 °C) increases expression of biofilm- cold-shock- and RpoS-dependent genes in *Escherichia coli* K-12. Microbiology 154, 148–166.

Feng, Y., He, Z., Ong, S.L., Hu, J., Zhang, Z., Ng, W.J., 2003. Optimization of agitation, aeration, and temperature conditions for maximum β-mannanase production. Enzyme Microb. Technol. 32 (2), 282–289, 2003.

Gonzalo, P., Inmaculada, M., Ricardo, A., 2002. Purification and biological characterization of halocin H1 from haloferax mediterranei M2a. Int. Microbiol. 5, 15–19.

Hay, J.X.W., Wu, T.Y., Teh, C.Y., Jahim, J.M., 2012. Optimized growth of Rhodobacter sphaeroides O.U.001 using response surface methodology (RSM). JSIR 71 (02), 149–154.

Jan, B., Antonio, J.P., Nathalie, P., Erhard, B., 2007. Osmotically induced synthesis of the compatible solute hydroxyectoine is mediated by an evolutionarily conserved ectoine hydroxylase. J. Biol. Chem. 282, 31147–31155.

John, V.B., Jean, L.B., Ernest, J.B., Kirby, W.M.M., 1966. Simplified, accurate method for antibiotic assay of clinical specimens. Appl. Microbiol. 14 (2), 170.

Li, Y., Xiang, H., Liu, J., Zhou, M., Tan, H., 2003. Purification and biological characterization of halocin C8, a novel peptide antibiotic from Halobacterium strain AS7092. Extremophiles 7 (5), 401–407.

McGenity, T.J., Gemmell, R.T., Grant, W.D., 1998. Proposal of a new halobacterial genus *Natrinema* gen nov., with two species *Natrinema pellirubrum* nom.nov and *Natrinema pallidum* nom.nov. Int. J. Syst. Bacteriol. 48, 1187–1196.

Meral, B., Baris, C., Bulent, M., Rahel, E.B., Aharon, O., Mehmet, N.O., Ayse, O., 2007. Extremely halophilic archaea from Tuz Lake, Turkey, and the adjacent Kaldirim and Kayacik salterns. World J. Microbiol. Biotechnol. 23, 309–316.

Meseguer, I., Rodriguez, V.F., 1985. Production and purification of halocin H4 28 (2), 177–182.

Meseguer, I., Rodriguez, V.F., 1986. Effect of halocin H4 on cells of halobacterium halobium. J. Gen. Microbiol. 132, 3061–3068.

Meseguer, I., Rodriguez, V.F., Ventosa, A., 1986. Antagonistic interactions among halobacteria due to halocin production. FEMS Microbiol. Lett. 36 (2–3), 173–182.

Meseguer, I., Torreblanca, M.T., Konishi, 1995. Specific inhibition of the halobacterial Na + /H + antiporter by halocin H6. J. Biol. Chem. 270 (12), 6450–6455.

Mirko, B., Junsong, S., Michael, W.W.A., 2012. Engineering a hyperthermophilic archaeon for temperaturedependent product formation. ASM J. 3 (2), 1–8.

O'Connor, E.M., Shand, R.F., 2002. Halocins and sulfolobicins: the emerging story of archaeal protein and peptide antibiotics. J. Ind. Microbiol. Biotechnol. 28 (1), 23–31.

Pašić, L., Velikonja, B.H., Ulrih, N.P., 2008. Optimization of the culture conditions for the production of a bacteriocin from halophilic archaeon Sech7a. Prep. Biochem. Biotechnol. 38 (3), 229–245.

Pašić, L., Galan, B.S., Poklar, U.N., Grabnar, M., Herzog, V.B., 2005. Diversity of halophilic archaea in the crystallizers of an adriatic solar saltern. FEMS Microbiol. Ecol. 54 (3), 491–498.

Price, L.B., Shand, R.F., 2000. Halocin S8: a 36-amino-acid microhalocin from the haloarchaeal strain S8a. J. Bacteriol. 182, 4951–4958.

Rodríguez, F.V., Juez, G., Kushner, D.J., 1982. Halocins: salt dependent bacteriocins produced by extremely halophilic rods. Can. J. Microbiol. 28 (1), 151–154.

Sehgal, S.N., Gibbons, 1960. Effect of some metal ions on the growth of halobacterium cutirubrum. Can. J. Microbiol. 6 (2), 165–169.

Shand, R.F., Price, L.B., O'Connor, E.M., 1999. Halocins: protein antibiotics from hypersaline environments. In: Oren, A. (Ed.), Microbiology and Biogeochemistry of Hypersaline Environments. CRC Press, Boca Raton, pp. 295–305.

Subov, N.N., 1931. Oceanographical tables. USSR Oceanographic Institute of Hydrometerological Commission, Moscow.

Sun, C., Li, Y., Mei, S., Lu, Q., Zhou, L., Xiang, H., 2005. A single gene directs both production and immunity of halocin C8 in a haloarchaeal strain AS7092. Mol. Microbiol. 57 (2), 537–549.

Tamar, K.P., Aharon, O., 2000. Halocins: are they involved in the competition between halobacteria in saltern ponds? Extremophiles 4 (1), 35–41.

Torreblanca, M., Rodríguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M., Kates, M., 1986. Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of halorcula gen. nov and haloferax gen. nov. syst. Appl. Microbiol. 8, 89–99.

Torreblanca, M., Meseguer, I., Rodríguez, F.V., 1989. Halocin H6, a bacteriocin from haloferax gibbonsii. J. Gen. Microbiol. 135 (10), 2655–2661.

Torreblanca, M., Meseguer, I., Ventosa, A., 1994. Production of halocin is a practically universal feature of archaeal halophilic rods. Lett. Appl. Microbiol. 19, 201–205.

Torsten, S., Thomas, M., Dirk, B., Hauke, H., Uta, B., 2007. Continuous synthesis and excretion of the compatible solute ectoine by a transgenic, nonhalophilic bacterium. Appl. Environ. Microbiol. 73, 3343–3347.

Ventosa, A., Quesada, F., Rodríguez, F.V., Ruiz, B.Q.F., Ramos, C.A., 1982. Numerical taxonomy of moderately gram negative rods. J. Gen. Microbiol. 128, 1959–1968.

Xin, H., Itoh, T., Zhou, P., Suzuki, K., Kamekura, M., Nakase, T., 2000. *Natrinema* versiforme sp. nov., an extremely halophilic archaeon from Aibi salt lake, Xinjiang, China. Int. J. Syst. Evol. Microbiol. 50 (3), 1297–1303.

# Natrinema sp. BTSH10 16S ribosomal RNA gene, partial sequence

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