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EXPLORATION OF A ROCK SALT MINE FOR NOVEL MICROBES WITH INDUSTRIAL POTENTIAL

Ashutosh Malhotra (041520)

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Submitted in partial fulfillment of Degree of Bachelor of Technology

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JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT (SOLAN)

JAYPEE UNIVERSITY OF INFORMATION AND TECHNOLOGY

CERTIFICATE

This is to certify that the work entitled, "Exploration of Rock Salt Mine for Novel Microbes with Industrial Potential" submitted by **Ashutosh Malhotra** in partial fulfillment for the award of degree of Bachelor of Technology in Bioinformatics of Jaypee University of Information and Technology has been carried out under my Supervision .This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

PROJECT SUPERVISOR

Dr Rajinder Singh Chauhan

Professor & Head,

Department of Biotechnology & Bioinformatics

Jaypee University of Information and Technology

ACKNOWLEDGMENTS

No venture can be completed without the blessing of almighty. I consider it my bounded duty to bow to almighty whose kind blessings always inspire me to walk on right path of life.

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Ashutosh Malhotra

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List of abbreviations

CTAB: Hexa decyl methyl ammonium Bromide

EDTA: Ethylene diamine tetra acetic acid

NaCl: Sodium Chloride

PCR: Polymerase chain reaction

rpm: Rotations per minute

SDS: Sodium Dodecyl Sulphate

U.V: Ultraviolet

pH: Potential of Hydrogen

mM: Milli molar

MW: Molecular weight

IPTG Isopropyl β thiogalactopyranoside

DNA: Deoxy ribo nucleic acid

µM: Micromolar

EtOH: Ethanol

TLC: Thin layer chromatography

mg: Milli gram

SD: Standard deviation

LB: Luria broth

abstract

> Basic research is like shooting an arrow into the air and, where it lands, painting a target.

Abstract

High salinity represents an extreme environment that relatively few organisms have been able to adapt to and occupy. Halophiles represent class of microbes which can survive and flourish in this environment that limit growth of most other microorganisms and this characteristic makes them paragon for use in various industrial applications. The purpose of this study was to explore all halotolerant microbial population inhabiting in rock salt mine of Darang village in Mandi district of Himachal Pradesh which is the only rock salt mine of India and has not been yet explored. We are first to report the isolation of 11 new halophilic strains with varying levels of Halotolerance ranging from slight halophilic to extreme halophilic concentration of salt from this site. In our study we also proceeded for identification of these halophilic strains using 16S ribosomal RNA primers and further these isolated microbial strains can be explored to discover large variety of stable and unique biomolecules that may be useful for practical applications.

Aim and objectives

Aim- Exploration of Rock Salt Mine for Novel Microbes with Industrial Importance.

Objectives

1. Isolation of all culturable halophiles present in rock salt mine.
2. Identification and characterization of Halophiles present in rock salt mine.

CHAPTER-1
introduction

> Men love to wonder, and that is the seed of science

INTRODUCTION

Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria. Examples of well-adapted and widely distributed extremely halophilic microorganisms include archaeal *Halobacterium* species, cyanobacteria such as *Aphanothece halophytica*, and the green alga *Dunaliella salina*. Although salts are required for all life forms, halophiles are distinguished by their requirement of hypersaline conditions for growth. They may be classified according to their salt requirement: slight halophiles grow optimally at 0.2–0.85 mol L^{-1} (2–5%) NaCl; moderate halophiles grow optimally at 0.85–3.4 mol L^{-1} (5–18%) NaCl; and extreme halophiles grow optimally above 3.4–5.1 mol L^{-1} (19–30%) NaCl. In contrast, non halophiles grow optimally at less than 0.2 mol L^{-1} NaCl. Halotolerant organisms can grow both in high salinity and in the absence of a high concentration of salts. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with requirement or tolerance for salts sometimes depending on environmental and nutritional factors. High osmolarity in hypersaline conditions can be deleterious to cells since water is lost to the external medium until osmotic equilibrium is achieved. To prevent loss of cellular water under these circumstances, halophiles generally accumulate high solute concentrations within the cytoplasm (Galinski, 1993). When an iso-osmotic balance with the medium is

achieved, cell volume is maintained. The compatible solutes or osmolytes that accumulate in halophiles are usually amino acids e.g. glycine, betaine, ectoine, sucrose, trehalose and glycerol, which do not disrupt metabolic processes and have no net charge at physiological pH. A major exception is for the halo bacteria and some other extreme halophiles, which accumulate KCl equal to the external concentration of NaCl. Halotolerant yeasts and green algae accumulate polyols, while main halophilic and halotolerant bacteria accumulate glycine betaine and ectoine. Compatible solute accumulation may occur by biosynthesis, de novo or from storage material, or by uptake from the medium. The extrcemely halophilic archaea, in particular, are well adapted to saturating NaCl concentrations and have a number of novel molecular characteristics, such as enzymes that function in saturated salts, purple membrane that allows phototrophic growth, sensory rhodopsins that mediate the phototactic response, and gas vesicles that promote cell flotation. Halophiles are found distributed all over the world in hypersaline environments, which includes hypersaline brines in arid, coastal, and even deep sea locations, as well as in rock salt mines.

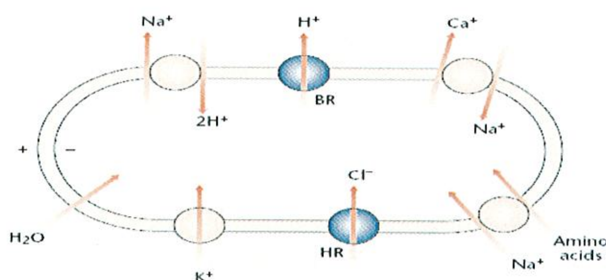


Figure 1: Flux of ions and molecules across the membrane of halobacteria. The light-driven proton and chloride pumps, bacteriorhodopsin (BR) and halorhodopsin (HR) (respectively), are shown in blue, and the sodium/ proton antiporter, potassium uniporter, sodium/amino acid symporter and calcium/sodium antiporter are shown in pink.

CHAPTER-2

review of literature

Observations always involve theory

REVIEW OF LITERATURE

Halophilic bacteria constitute a heterogeneous physiological group of microorganisms which belong to different genera. The halophilic property is probably widespread in the bacterial domain and may occur in a variety of morphological and physiological types but current knowledge of the taxonomic status of these bacteria contrasts with early studies, since in 1980 only seven halophilic species were included in the *Approved Lists of Bacterial Names* (1) and most strains used in physiological and biochemical studies were isolated from salted cured foods or unrefined salt or were even laboratory culture contaminants. These halophiles were *Vibrio* (*Salinivibrio*) *costicola* (2), *Micrococcus* (*Nesterenkonia*) *halobius* (3), *Paracoccus* (*Halomonas*) *halodenitrificans* (4), *Flavobacterium* (*Halomonas*) *halmephilum* (5), *Planococcus* (*Marinococcus*) *halophilus* (6), and *Spirochaeta halophila* (7).).Halophiles diversity varies from unicellular to multicellular organisms which include repective prokaryotes and eukaryotes:

2.1: Eukaryotic Halophiles

Multicellular eukaryotes

Few such organisms can tolerate hypersaline conditions and the highest salinity at which any vertebrates have been observed (e.g. *Tilapia* species) is about 1 mol L^{-1} NaCl. A variety of obligate and facultative halophytic plants, e.g. *Atriplex halimus* and *Mesembryanthemum crystallinum*, can survive in moderately high saline soils. There are also a surprising number of invertebrates that can survive in hypersaline environments. Some examples are rotifers such as *Brachionus angularis* and

Keratella quadrata, tubellarian worms such as *Macrostomum* species, copepods such as *Nitocra lacustris* and *Robertsonia salsa*, ostracods such as *Cypridis torosa*, *Paracyprideinae* spp., *Diacypris compacta*, and *Reticypris herbsti*. Some insects from hypersaline environments include brine flies *Ephydra hians* and *E.gracillis* and brine shrimp *Artemia franciscana* and related species. Some hypersaline environments help to support many birds, one of the most spectacular of which is the pink flamingo.

2.2: Algae

At moderately high salinities (1–3.5 mol L^{-1} NaCl) dense populations of green algae (> 10^5 Mol L^{-1}), are supported. These are obligatory aerobic, photosynthetic, unicellular eukaryotic microorganisms, some species of which produce large quantities of orange-coloured b-carotene at high salinities. Green algae of the genus *Dunaliella*, e.g. *Dunaliella salina*, *D. parva*, and *D. viridis*, are ubiquitous and are the main source of food for brine shrimps and the larvae of brine flies. Most species of green algae are moderate halophiles, with only a few extremely halophilic species, e.g. *Dunaliella salina* and *Asteromonas gracilis*, which can grow even in saturated NaCl. Algae predominantly use polyols as compatible solutes. In *Dunaliella salina*, glycerol is synthesized in response to osmotic stress. The cytoplasmic concentration of glycerol can reach 7 mol L^{-1} when grown in medium containing 5 mol L^{-1} NaCl and can constitute over 50% of the dry weight of the cells. The intracellular sodium concentration has been reported to be less than 100 mMol L^{-1} over a wide range of external salt concentrations. During moderate dilution stress, glycerol does not leak out of cells but is metabolized and transformed into osmotically inactive reserve material. Diatoms are algae surrounded by silica cell walls and are commonly found

but rarely abundant in hypersaline environments. A variety of species have been found at about 2 mol L^{-1} NaCl, although the upper limit for diatom growth is about 3 mol L^{-1} NaCl. Examples of common diatoms include *Amphora coffeaeformis*, *Nitzschia* and *Navicula* species. Although osmoregulation has not been studied extensively in diatoms, accumulation of proline and oligosaccharides has been reported in some species.

2.3: Protozoa

A large variety of protozoa, which are cell wall-less chemo heterotrophic protists that ingest algae and bacteria, have been described from hypersaline environments. Identified species include the moderate halophile Fabrea salina from a west Australian lake (8), and the extreme halophile *Porodon utahensis* from the Great Salt Lake. Although protozoa are known to regulate osmotic pressure in freshwater with contractile vacuoles that expel water, their mechanism of osmoregulation in hypersaline brine has not been investigated.

2.4: Fungi

Yeasts and other fungi are chemo heterotrophic cell-walled eukaryotes, some of which are well adapted to tolerate hypersaline environments. They grow best under aerobic conditions on carbohydrates at moderate temperatures and acidic to neutral pH. *Debaromyces hansenii* is halotolerant yeast, isolated from sea water that can grow aerobically up to salinities of 4.5mol L^{-1}NaCl. It produces glycerol as a compatible solute during the logarithmic phase and arabitol in the stationary phase. A saprophytic hyphomycete, *Cladosporium glycolicum*, was found growing on submerged wood panels at a salinity exceeding 4.5 mol L^{-1} NaCl in the Great Salt

Lake. Halophilic fungi, e.g. *Polypaecilum pisce* and *Basipetospora halophila*, have also been isolated from salted fish.

2.5: Prokaryotic Halophiles

Cyanobacteria

Cyanobacteria (or 'blue-green algae') are bacterial prokaryotes that are characterized by the presence of chlorophyll a and phycobilin pigments and carry out oxygenic photosynthesis. They dominate the planktonic biomass and form microbial mats in many hypersaline lakes. The top brown layer of microbial mats contains a common unicellular cyanobacterial species, *Aphanothece halophytica*.It can grow over a wide range of salt concentrations, from 2–5 mol L^{-1} NaCl, is an extreme halophile with a salt optimum of 3.5 mol L^{-1}, and lyses in distilled water. It uses glycine betaine as the major compatible solute, which it can take up from the medium or synthesize from choline. A. halophytica and similar unicellular cyanobacteria have been described from the Great Salt Lake, Dead Sea, Solar Lake and artificial solar ponds. Aplanktonic cyanobacterium reported from the Great Salt Lake is *Dactylococcopsis salina*. A variety of filamentous cyanobacteria, e.g. in the order Oscillatoriales, such as *Oscillatoria neglecta*, *O. limnetica, O. salina* and *Phormidium ambiguum*, have also been described that develop in the green second layer of mats in hypersaline lakes. These are more moderate halophiles, usually growing optimally at 1–2.5 mol L^{-1} NaCl, and form heterocysts that fix nitrogen. Another common species in the same

family is *Microcoleus chthonoplastes*.The diversity of cyanobacteria occurring in hypersaline environments has not been studied extensively.

2.6- Other phototrophic bacteria

Phototrophic bacteria occur beneath the cyanobacterial layers in anaerobic but lighted zones in hypersaline microbial mats. They usually grow anaerobically by an oxygenic photosynthesis, although many also have the capacity to grow aerobically as heterotrophs. They can use reduced sulfur (hydrogen sulfide, elemental sulfur), organic compounds or hydrogen as electron donors. They include green and purple sulfur and non-sulfur bacteria that are characterized by bacteriochlorophyll pigments. The green sulfur bacteria, such as the slight to moderately halophilic *Chlorobium limicola* and *C. phaeobacteriales*, deposit elemental sulfur granules outside their cells and are capable of nitrogen fixation. *Chlorobium limicola* can take up glycine betaine from the environment and synthesize trehalose for use as an osmolyte. The moderately halophilic, filamentous green non-sulfur bacteria such as *Chloroflexus aurautiacus* are also slightly thermophilic. Halophilic purple sulfur bacteria such the *Chromatiaceae*, deposit sulfur granules inside cells, include mainly moderate halophiles, e.g. *Chromatium glycolicum*, which grows photoorganotrophically using glycolate and glycerol, *C. violescens* and *C. salexigens*. They synthesize N-acetylglutaminylglutamine amide as a minor component of their compatible solute and use sucrose and glycine betaine from their environment. The moderate halophiles *Thiocapsa roseoparsarcina* and *T. halophila* from *Guerrero Negro* both synthesize sucrose and take up glycine betaine from the environment. *T. halophila* also synthesizes glycine betaine and N-acetylglutaminylglutamine amide for

osmoprotection.The moderately halophilic purple nonsulfur bacterium *Rhodospirillum salexigens* from evaporated seawater pools and *R. salinarum* from a saltern both use glycine betaine and *R. salexigens* also uses ectoine as an osmolyte.The purple sulfur bacteria, *Ectothiorhodospira* species,dominate alkaline soda lakes in Egypt and Central Africa. The moderate halophile *Ectothiorhodospira marismortui* is a strict anaerobe and uses carboxamines as compatiblesolutes and uses the osmolyte N-a-carbamoyl-l-glutamine- 1-amide. The extreme halophile *Ectothiorhodospira halochloris* isolated from Wadi Natrun, was the first bacterium shown to synthesize and accumulate ectoine, a cyclic amino acid, which it uses along with glycine betaine and trehalose as compatible solutes.

2.7- Sulfur-oxidizing bacteria

Below the cyanobacteria and the phototrophic bacteria in microbial mats, halophilic, filamentous, carbon dioxide fixing bacteria oxidize hydrogen sulfide (and elemental sulfur) to sulfate. Examples include the filamentous *Achromatium volutans* from Solar Lake, *Beggiatoa alba* from *Guerrero Negro*, and *B. leptiformis* from Solar Lake. A unicellular halophilic, chemoautotrophic sulfur-oxidizing bacterium, *Thiobacillus halophilus*, from a hypersaline western Australian lake.

2.8- Anaerobic bacteria and archaea

A large variety of facultative and strictly anaerobic bacteria and archaea inhabit the bottom layers of microbial mat communities and sediment in hypersaline lakes. These include fermentative bacteria, homoacetogenic bacteria, sulfate-reducing bacteria and methanogenic archaea. Fermentative anaerobic bacteria that grow at saturated NaCl concentrations have been described. One example is *Haloanaerobacter*

chitinovorans, isolated from a saltern, which is capable of fermenting chitin contained in brine shrimp and brine flies. Other more moderate halophilic isolates are *Haloanaerobacter saccharolytica*, which ferments carbohydrates, Halobacterioides acetoethylicus, from an oil well, and Halocella cellulolytica, which ferments carbohydrates including cellulose. *Sporohalobacter lorretii* and *S. marismortui* are sporogenous and ferment carbohydrates. Several homoacetogens, strict anaerobes that produce acetate from oxidation of sugars or amines, have been described. For example, *Haloincola saccharolytica* ferments carbohydrates and N-acetylglucosamine and can grow at a wide range of NaCl concentrations. *Acetohalobium arabaticum*, which grows from 1– 4.5 mol L^{-1} NaCl, grows on glycine betaine and trimethylamine. A. arabaticum, Isolated from Lake Sivash, also has the ability to reduce carbon dioxide to acetate and is a likely competitor of sulfate-reducing bacteria for hydrogen. Sulfate-reducing bacteria use sulfate as the terminal electron acceptor, although many can also utilize other sulfur compounds, nitrate, and fumarate. They differ in their ability to oxidize different compounds, though most use low-molecular weight organic species such as lactate, pyruvate, ethanol and volatile fatty acids or hydrogen as electron donors. A few can use carbon dioxide as the sole carbon source. Although many slightly halophilic sulfate reducers have been isolated, mostly from marine environments, relatively few that can survive at an extremely high salinity have been cultured. *Desulfohalobium retbaense*, isolated from Lake Retba, Senegal, and *Desulfovibrio halophilus*, from Solar Lake, are two moderately halophilic sulfate-reducing species that have been described. These can grow at up to 4 mol L^{-1} NaCl, but only relatively slowly. Another isolate, from the deep-sea

hypersaline pools in the Red Sea, is similar to D. halophilus. The osmoregulation of sulfate-reducing bacteria has not been studied extensively; preliminary indications are that they do not synthesize compatible solutes but accumulate salts internally. Methanogens from hypersaline environments generally use methyl tropic substrates rather than carbon dioxide, acetate and hydrogen, and are strict anaerobic archaea. Several, mostly moderate halophilic, methanogens have been identified, including *Methanohalophilus halophilus* from a microbial mat, M. muhii from the Great Salt Lake, and *M. portucalensis* from a saltern. The slight halophile *Methanosalsus zhilinac* is also an alkaliphile and a slight thermophile. The extremely halophilic methanogen, *Methanohalobium evestigatum*, with an NaCl optimum of 4.5 mol L^{-1}, is also a thermophile with a temperature optimum of 50°C. Methanogenesis has also been reported from deep-sea brine pools in the Gulf of Mexico that contain moderately high salinity. Methanogens use bamino acids (b-glutamine, N-e-acetyl-b-lysine) as compatible solutes and also play an important role in the anaerobic degradation of glycine betaine in their environments. Their intracellular salt concentration is somewhat higher than that of most bacteria, about 0.6 mol L^{-1} KCl, but is significantly lower than for the halophilic archaea (halobacteria)

2.9- Aerobic and facultative anaerobic Gram-negative bacteria

Many moderately halophilic, heterotrophic Gram-negative bacteria belonging to the *Halomonas* and *Chromohalobacter* genera have been described. Other genera with halophilic representatives include *Salinovibrio, Arhodomonas,Dichotomicrobium*, *Pseudomonas*, *Flavobacterium,Alcaligenes, Alteromonas, Acinetobacter*, and *Spirochaeta*.Most of these are heterotrophs, and include *Arhodomonas aqueoli*,

isolated from a subterranean brine associated with an oil field and capable of nitrate reduction; *Chromohalobacter marismortui* from the Dead Sea, also capable of nitrate reduction; *Pseudomonas beijerinckii* from salted beans preserved in brine; *Pseudomonas halophila* from the Great Salt Lake; and Salinovibrio costicola, originally isolated from Australian bacon. Several Halomonas species are capable of nitrate reduction, including H. elongata, isolated from a solar saltern, and *H. halodenitrificans*, isolated from meat-curing brines. Others include *H. eurihalina*, isolated from saline soil, which produces an extracellular polysaccharide; *H. halodurans*, from estuarine waters, which is capable of degrading aromatic compounds; *H. halophila*, from saline soil; *H. panteleriense*, from alkaline saline soil, which grows at a pH optimum of 9; H. salina, from saline soil; and *H. subglaciescola*, from beneath the ice of Organic Lake in Antarctica. These organisms use primarily glycine betaine and ectoine as the compatible solutes. Genes for uptake of glycine betaine from the medium (betT) and for its synthesis from choline (betI, A, and B) and for synthesis of ectoine (ectA, B, and C) have been cloned from *Halomonas* species and some other halophiles. Among spirochaetes, the moderate halophile *Spirochaeta halophila*, found in Solar Lake, is a chemolithotroph capable of iron and manganese oxidization. The flavobacteria *Flavobacterium gondwanese* and *F. salegens*, are psychrotolerant halophiles isolated from Antarctic Lakes.

2.10- Gram-positive bacteria

This group includes moderately halophilic species of the genera *Halobacillus, Bacillus*, *Marinococcus, Salinococcus, Nesterenkonia*, and *Tetragenococcus*. They include cocci such as *Nesterenkonia halobia*, isolated from salterns, which produce

yellow-red carotenoid pigments; *Tetragenococcus* halophilus, from fermented soy sauces and squid liver sauce, and from brine for curing anchovies, which are capable of lactic acid fermentation; and several *Salinococcus* species from salterns. Other examples include *B. diposauri*, from the nasal cavity of a desert iguana; *B. haloalkaliphilus*, from Wadi Natrun; and *B. halodenitrificans*, from a solar saltern in southern France. *Halobacillus litoralis* and *H. trueperi* are found in the Great Salt Lake. *Sporosarcina halophila* is an endospore-forming bacterium, from which the compatible solute N-e-acetyl-lysine was originally isolated. Many of these organisms use n -proline, ectoine or N-acetylated diamino acids, which they are capable of synthesizing, as a compatible solute. Actinomycetes from saline soils include *Actinopolyspora halophila*, which grows best at moderate NaCl concentrations and is one of the few heterotrophic bacteria that can synthesize the compatible solute glycine betaine, and *Norcardopsis halophila*, which uses a hydroxy derivative of ectoine and b-glutamate as compatible solutes.

2.11- Halobacteria

These extreme halophiles grow best at the highest salinities (3.4–5 mol L^{-1} NaCl) forming dense blooms and resulting in the red colour of many brines. Common species of halobacteria are rod-, cocci- or disc-shaped, although triangular and even square-shaped species exist. Many are pleiomorphic, especially when the ionic conditions of the media are altered, and most lyse below 1–1.5 mol L^{-1} NaCl. Halobacteria are classified as archaea (and are also called halophilic archaea or haloarchaea) and belong to the family Halobacteriacea. Ten genera have been reported, *Halobacterium, Haloarcula, Halococcus, Haloferax, Halorubrum,*

Halobaculum, Natronobacterium, Natronococcus, Natrialba and Natromonas, and an eleventh genus,

Haloterrigena, has recently been proposed. The first microbiological analysis was conducted on several closely related Halobacterium strains (originally designated *H. halobium, H. salinarium, and H. cutirubrum*) isolated in the mid-twentieth century from salted fish and meat from northern Europe and North America. These are generally amino acid-utilizing facultative aerobes that require a number of growth factors and slightly elevated temperatures (38–45°C) for optimal growth. Most have distinctive features such as gas vesicles, purple membrane and red-orange carotenoids. Many have the ability to grow in the absence of oxygen via dissimilatory nitrate reduction and denitrification, fermentation of different sugars, breakdown of arginine, and use of light energy mediated by retinal pigments. Several more recently isolated species will oxidize carbohydrates, e.g. *Haloarcula marismortui, Haloarculav llismortis*, and *Haloferax volcanii* from the Dead Sea; *Haloferax mediterranei* and *Halorubrum saccharovorum* from salterns; and Halorubrum lacusprofundi, a psychrotolerant species from Deep Lake, Antarctica. Glucose is oxidized by a modified Entner–Doudoroff pathway and the pyruvate resulting is further oxidized by pyruvate oxidoreductase and the tricarboxylic acid cycle. Several strains are capable of growth on single carbon sources such as sugars, glycerol and acetate. Some halobacterial species growing in alkaline lakes are alkaliphilic and others are rather acid-tolerant. Natronobacterium pharaonis from Wadi Natrun, and *Natronococcus occultus* from Lake Magadi, have pH optima in the range 9.5–10 and do not grow below pH 8.5. Slight acidophiles, such as *Haloferax volcanii* and

Haloferax mediterranei, grow at pH values as low as 4.5. The intracellular salt concentration of halobacteria has been measured to be extremely high and it is generally assumed that organic compatible solutes are not accumulated in these extreme halophiles. Potassium ions are accumulated internally up to 5 mol L^{-1} concentration. In addition, the content of sodium ions appears to be in the molar range, although the ratio of cytoplasmic potassium to sodium is high. The potassium gradient is maintained by the combination of an electrogenic sodium ion/proton antiporter and a potasium ion uniporter. Amino acid uptake is carried out by a sodium ion/amino acid symporter. Proteins of halobacteria are either resistant to high salt concentrations or requires salts for activity. As a group, they contain an excess ratio of acidic to basic amino acids, a feature likely to be required for activity at high salinity. This characteristic is shared with proteins from some halophilic bacteria. Surface negative charges are thought to be important for solvation of halophilic proteins, and to prevent the denaturation, aggregation and precipitation that usually results when nonhalophilic proteins are exposed to high salt concentrations. The structure of one enzyme of halobacteria, malate dehydrogenase, has been halobacteria is the purple membrane, specialized regions of the cell membrane that contain a two-dimensional crystalline lattice of a chromoprotein, bacteriorhodopsin. Bacteriorhodopsin contains a protein moiety (bacterioopsin) and a covalently bound chromophore (retinal) and acts as a light-dependent transmembrane proton pump (10). The membrane potential generated can be used to drive ATP synthesis and support a period of phototrophic growth. Bacteriorhodopsin is induced by low oxygen tension and high light intensity and can cover more than 50% of the surface of the

cells. Halobacteria produce large quantities of red-orange carotenoids. Carotenoids have been shown to be necessary for stimulating an active photo repair system for repair of thymine dimers resulting from ultraviolet radiation. The most abundant carotenoids are C-50 *bacterioruberins*, although smaller amounts of biosynthetic intermediates such as b-carotene and lycopene are also present. Retinal is produced by oxidative cleavage of b-carotene, a step that requires molecular oxygen. Several retinal proteins, in addition to bacteriorhodopsin, are also produced by halobacteria, including halorhodopsin, which is an inwardly directed light-driven chloride pump, and two sensory rhodopsins, which mediate the phototactic response. Halobacteria and methanogens are phylogenetically distinct from both bacteria and eukaryotes, and are classified as archaea. As such, they exhibit features characteristic of the archaea, including eukaryotic-like transcription and translation machinery, ether-linked lipids and, like some bacteria, a cell wall S-layer composed of a glycoprotein.

CHAPTER-3

biological techniques

Research is what I'm doing when I don't know what I'm doing.

Biological Techniques

Various Biological techniques are used in the present study:

1. Pour Plate Technique.
2. Spread Plate Technique.
3. Serial Dilution.
4. Streaking.
5. DNA isolation.
6. Polymerase chain reaction.
7. Gel electrophoresis.
8. Ligation
9. Cloning
10. Plasmid Isolation.
11. Carotene Extraction and quantification

CHAPTER-4

material & methods

If we wish to make a new world we have the material ready. The first one, too, was made out of chaos.

4.1: Materials:

Collection of Soil Samples: The soil samples from the rock salt mine were collected using quadrant method i.e. were collected from different areas in the sites and were then pooled together so that final sample represents the whole area. These samples were then put in aseptic plastic bags and were then taken to laboratory for further processing.

Chemicals: The chemicals used in the present study were obtained from S.D fine chemicals Limited, Merck Limited and Qualigens fine chemicals Limited. The various media used in the study namely Nutrient Broth, Nutrient Agar and Bushnell-Hass were manufactured by HiMedia Laboratories Pvt. Ltd.

4.2: Methods:

Media preparation

Nutrient agar and Nutrient broth was used as solid or liquid media for growth of halophiles which can be prepared by mixing a desired concentration of media in water which is necessarily followed by autoclaving in order to avoid any contamination.

Differential media:

Certain reagents such as salt are incorporated into culture media, which allows differentiation of various kinds of bacteria. Incorporation of salt into media will allow differentiation of halophiles which are adapted to survive in salt.

Streak- plate technique:

By means of a transfer loop, a portion of the mixed culture is placed on the surface of an agar medium and streaked across the surface. This manipulation thins out the bacteria on the agar surface so that some individual bacteria are separated from each other.

Pour plate technique:

In the pour plate method the mixed culture is diluted directly in tubes of liquid agar medium. The medium is maintained in a liquid state at a temperature of 45°C to allow distribution of innoculum. The inoculated medium is dispensed into Petri dishes, allowed to solidify, and then incubated.

Spread Plate technique:

In the spread plate method the mixed culture is not diluted in the culture medium; instead it is diluted in a series of tubes containing sterile liquid which is usually water.

4.3: Isolation of halophiles from air

A less tedious and newer method of isolating halophile strains directly from air was employed. The experiment was set up in 250 ml flasks containing 100 ml nutrient broth media, supplemented with increasing concentrations of salt (20% to 25%) and left open for isolating strains from air against a blank which was well covered. The flasks were left open for 15 days and manual shaking was done at regular intervals. These cultures were then plated on nutrient agar and incubated at 28°C overnight after which several isolates were obtained.

4.4: Bacterial DNA Isolation

Halophile strains were grown in rich broth culture overnight.4 ml of each of these cultures is taken up and spin for 2 min .Supernatant is decanted .Pallet is further resuspended in 1ml TE buffer by repeated pipetting. 30μl of 10 % SDS is added to tube along with 20 mg/ml Proteinase K, mix and incubate 1hr at 37°C.Add equal volume of phenol/chloroform and mix well but very gently to avoid shearing DNA by inverting the tubes until the phases are completely mixed. Carefully transfer DNA/phenol mixture to another tube and spin at 12,000 RPM for 10 min. Transfer upper layer to a new tube and add 1/10 volume of sodium acetate mix it and further add 0.6 volume of iso-propanol and mix gently until DNA precipitate.

16S rRNA Primers

Bacteria (source paper AEM Sept. 2006)

Forward primer, 8F, 5'-GGATCCAGACTTTGATYMTGGCTCAG

Reverse primer, 907R, (5'-CCGTCAATTCMTTTGAGTTT) 95-4min, 94-30sec, 50-54-40sec, 72-70sec, 72-20min, total 30 cycles.

Bacteria (source paper AEM 62 (1996)

D30F PCR, sequencing ATTCCGGTTGATCCTGC 6–22 Forward

B36R Sequencing GGACTACCAGGGTATCTA 789–806 Reverse

X10R Sequencing ACGGGCGGTGTGTRC 1392–1406 Reverse

D56R PCR, sequencing GYTACCTTGTTACGACTT 1492–1509 Reverse95-5min,94-45sec,50-45sec,72-1.5min,72-15min,35 cycles

Bacteria (source paper AM Biotech 2007)

GM5F 5'-CCTACGGGAGGCAGCAG-3'

DS907R 5'-CCCCGTCAATTCMTTTGAGTTT-3'same as above

Bacteria (source paper Biomed R.M.U. 2005)

Outer forward UNI_OL 5'-GTGTAGCGGTGAAATGCG-3',

Outer reverse UNI_OR 5'-ACGGGCGGTGTGTACAA-3',

Inner forward UNI_IL 5'-GGTGGAGCATGTGGTTTA-3',

Inner reverse UNI_IR 5'-CCATTGTAGCACGTGTGT-3'.94-5min,94-1min,55-1min,72-1min,72-10min.total 30 cycles.

Bacteria (source paper J. Clinic. Microb. 38; 2000)

U1: 5'-CCAGCAGCCGCGGTAATACG-3'

U2: 5'-ATCGG(C/T)TACCTTGTTACGACTTC-3' 94-10min,94-1min,55-1min,72-2min,72-10min,total 35 cycles.

4.5: Amplification of 16S RNA Gene Sequences

DNA amplification was carried out in a 25 µl reaction volume containing 20ng template DNA, 0.2µm of each dNTP, 1.5 mM MgCl2, 1X PCR buffer (10mM Tris-HCl, 50 Mm KCl, pH 8.3), 1 unit Taq polymerase (Sigma) and 0.5 µl of forward and 0.5 µl of reverse primer. PCR amplification was carried out in a Thermocycler (Applied Biosystem and BioRad) using the specific temperature profile for Specific primer pairs.

4.6: Cloning of the amplified PCR product

The amplified PCR product was cloned into linearised T vector using TA cloning Kit (Genei)

4.7: Ligation

The ligation reaction was set up in a total volume of 10µl.The ligation mixture was prepared by adding 0.5µl PCR product, 1.0µl ligation buffer (2X),0.5µl linearised T vector(supplied by Bangalore Genei), 0.5µl T4 DNA ligase and to this 7.5 µl sterile water was added to get a final volume of 10 µl. The ligation mix was incubated overnight at 16°C.

4.8: Transformation

For transformation of competent *E.coli* cells, 100 µl of suspension of competent cells (DH5α) strain of was mixed with 5 µl ligated product in chilled eppendorff tube, contents were mixed by swirling gently and the tube was incubated on ice for 30 min (Maniatis *et al.*, 1982). Heat shock treatment was given to the tube at 42 °C for 90 seconds and was then allowed to chill on ice for 1-2 minutes. The contents of the tube were then transferred to sterile screw capped tube and 800 µl of Luria broth was added. The culture was incubated at 37 °C for 1.5 hrs with shaking at 190 rpm in order to allow the bacteria to express antibiotic resistant marker. LB agar plates containing Ampicillin (50 µg/ml) were prepared .Plates were spread with 40 µl X-gal (20 mg/ml stock) followed by 7µl IPTG (1M stock) and were allowed to dry up. Then 100 µl of culture was transferred and spread on to the plates. When the liquid was completely absorbed, plates were incubated at 37 °C, overnight in inverted position. After 12 – 16 hrs screening of the transformed colonies was done. The white colonies i.e. the ones which had transformed successfully, were picked up and plasmid was isolated from them.

4.9: Plasmid isolation from the transformed colonies

Plasmid isolation was done using alkaline lysis miniprep method (Maniatis *et al.*, 1982). Single colony of transformed bacteria was picked and inoculated in 2ml Luria broth and was allowed to grow overnight at 37 °C with vigorous shaking.1.5 ml of culture was taken and centrifuged at 10,000 rpm for 30 sec. Supernatant was discarded, pellet was allowed to dry and was then resuspended in 100μl of ice cold alkaline lysis solution 1(50 mM glucose, 25 mM Tris-Cl (pH 8), 10mM EDTA(pH 8)).To this tube alkaline lysis solution2(0.2 N NaOH, 1% w/v SDS) was added, contents of the tube were mixed rapidly and tube was stored on ice.150 μl of ice cold sol 3 (5M potassium acetate(60 ml), glacial acetic acid(11.5 ml), H2O(28.5 ml)) was added to the tube, contents were mixed by inverting the tube and was stored on ice for 3-5 minutes followed by centrifugation at 10,000 rpm for 5 minutes and supernatant was transferred to new tube. An equal volume of phenol: chloroform (1:1) was added to the supernatant, phases were mixed by vortexing followed by centrifugation at 10,000 rpm for 2 minutes. Upper aqueous layer was again transferred to a new tube. In order to precipitate the DNA, 2 volumes of ethanol were added to the supernatant; solution was mixed and allowed to stand for 2 minutes. Precipitated DNA was collected by centrifugation at 10,000 rpm for 5 minutes. Supernatant was discarded and pellet was allowed to dry up. Pellet was then washed with 70% ethanol and DNA was dissolved in 50 μl TE (containing 20 μg/ml DNAse free RNase A, pH 8.0). Solution was vortexed gently and stored at -20°C.

4.10: Carotene Extraction

Protocol for extraction of Carotene in Methanol-

Perform the methanol extraction separately for each sample.

1. Weigh approximately 30 mg of Halophile powder directly into Tubes. Record the weight.
2. 2.5 ml DMSO to each tube and go for Sonication.
3. Tightly cap the tubes and vortex them briefly for 30 seconds.
4. Place tubes into a 50 C degree water bath for 30 minutes. Every 10 minutes remove tubes from the water bath and vortex them for 30 seconds.
5. After 30 minutes in the water bath, remove the tubes.
6. Add 5 ml of methanol to each tub, cap the tubes and vortex them vigorously for 30 seconds. Centrifuge the tubes at 4200 rpm for 3 minutes.
7. With a Pipette, draw the supernatant from each tube and into volumetric flasks.
8. Add 4 ml of methanol, cap the tubes, vortex them for 15-30 seconds and centrifuge them again for three minutes. Collect the supernatant into the volumetric flasks.
9. Add just enough methanol to cover the glass beads. Cap the tubes and vortex them vigorously for 30 seconds. Remove the caps and add 4 ml of methanol. Recap the tubes and vortex them for 30 seconds.
10. Continue to add methanol, vortex, and centrifuge and collect supernatant in the volumetric flask until the methanol is absolutely clear. It is important to note that any color left in the methanol is beta-carotene so it is extremely important to

extract all the pigment from the pellet even if it means an extra extraction for verification.

4.11: Thin layer Chromatography:

TLC Silica gel 60 F 254 (Catalogue number-1.05554.0007) was used for performing Thin layer chromatography procedure for quantification of carotene. Solvent system used contains Petroleum ether, Acetone and Diethyl ether in the ratio of 4:1:1 and detection was done under waveleght of 254 nm.

CHAPTER-5

results & discussions

Nothing shocks me. I'm a scientist

5.1: Selection of Halophile Inhabiting Site

Rock and Soil Samples were collected from Salt mine at Darang in Mandi district of Himachal Pradesh which is the only salt mine in India.

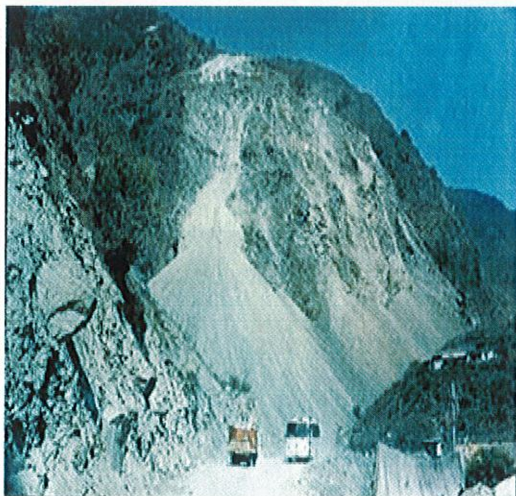


Figure2a: Salt mine at Darang village

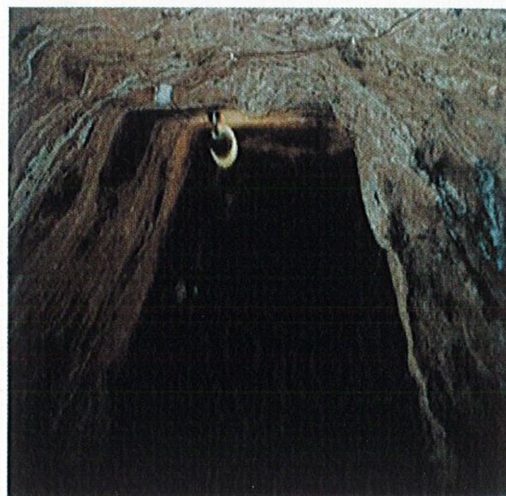


Figure2b: Inside view of Salt mine

5.2: Isolation of halophiles present rock salt mine

To extract out halophiles we supplemented agar with 3% salt and after 48 hrs of incubation at 37°C we got various strains on a master plates which we named as H1, H2, and H3. After 14 days of incubation we got some new strains on PDA plates which we named as A1 , A2 ,A3, A4, A5 and further after 26 days of incubation another strains grew which we named as A6 ,A7,A8,A9,A10.

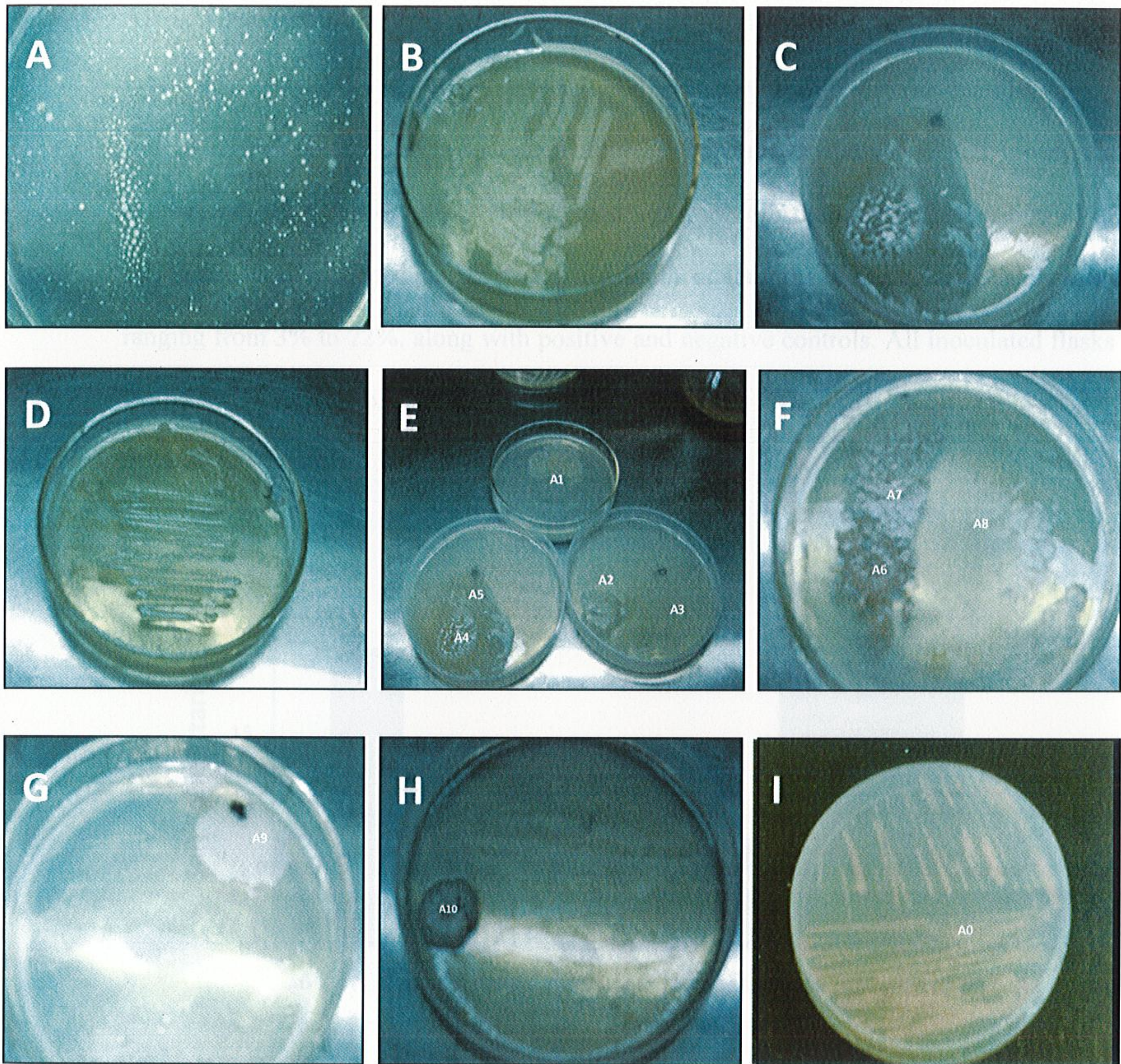
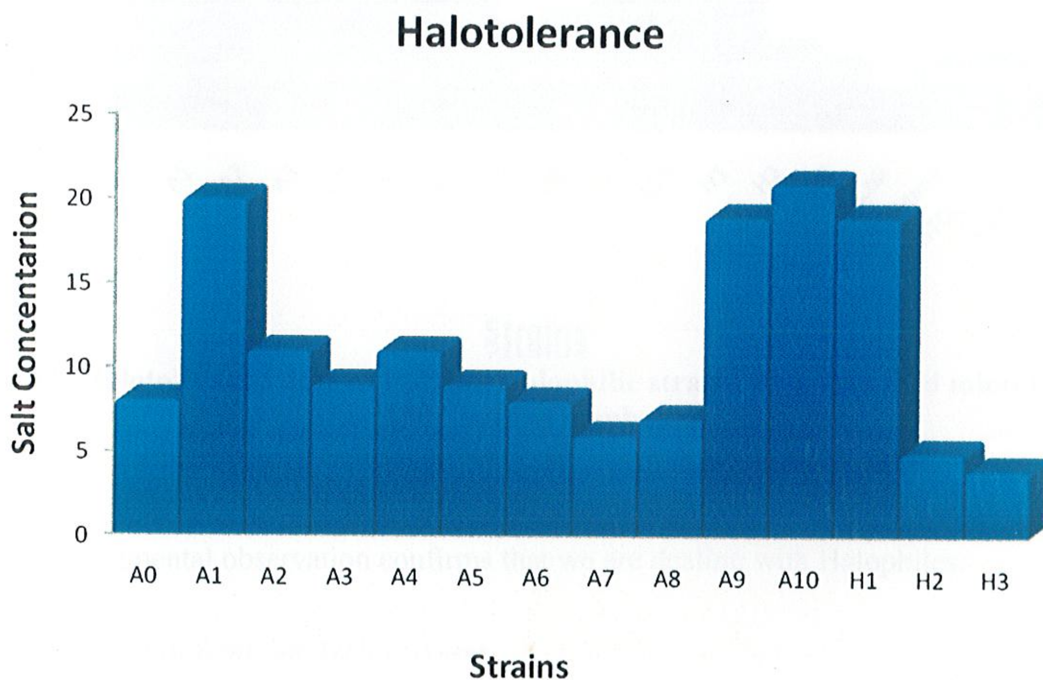


Figure 3- (A) Master Plate (B) H1 Strain (C) H2 Strain (D) H3 Strain (E) Contain Strains A1, A2, A3, A4, A5 (F) Contain Strains A6, A7, A8 (G) A9 Strain (H) A10 Strain (I) A0 Strain

5.3: Halotolerance of isolated Halophile strains

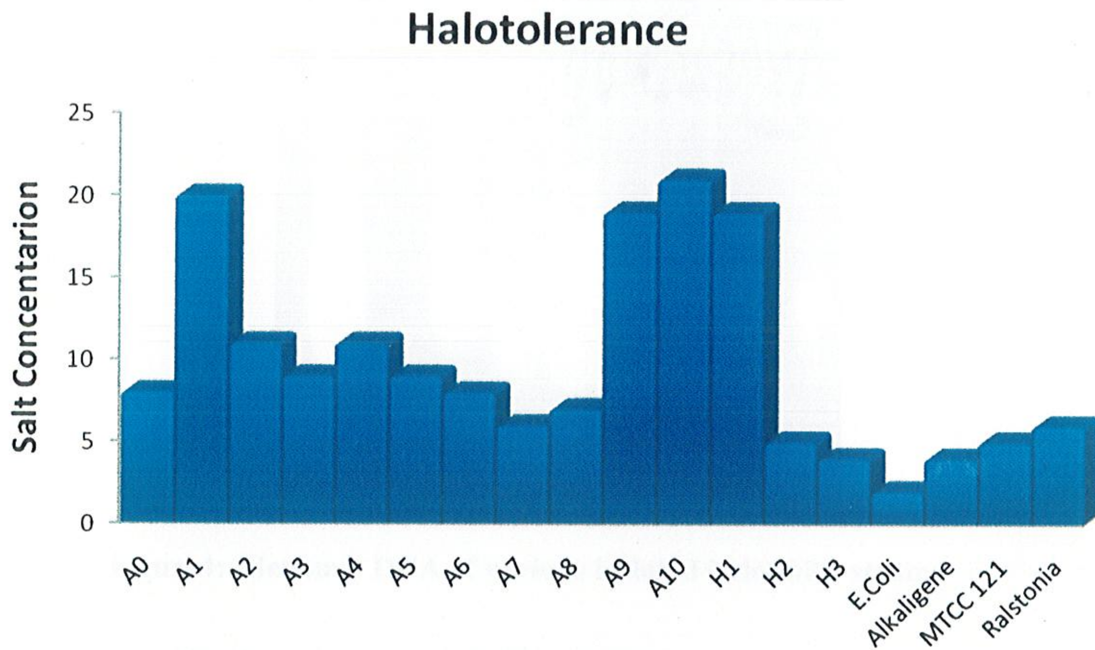
All isolated strains ranging from' from A0 to A10 along with H1, H2 and H3 was inoculated into 50 ml of nutrient broth. Initial seed was prepared by inoculating a loop full of inoculums into broth and then after 24 hrs 1ml of the inoculated broth was centrifuged and washed with .9 N NaCl and OD was adjusted to .875 further 500 μl of this innoculum was inoculated into 50 ml broth containing desired salt concentration ranging from 3% to 22%. along with positive and negative controls. All inoculated flasks were kept for incubation at 35°C at 120 Rpm.



Graph1: Halotolerance data of various isolated halophilic strains after 150 hours of incubation.

Result- A1, A9, A10 and H1 are extreme halophiles while all others are moderate Halophiles.

Further to cross validate data for salt tolerance and to check if we are dealing with true Halophiles or not we checked growth of ***E.coli, Ralstonia, MTCC 121, Alkaligenes*** strains with our isolated strains.



Graph2: Halotolerance data of isolated Halophilic strains with standard microbes after 150 hours of incubation.

Result- Experimental observation confirms that we are dealing with Halophiles.

5.4: DNA isolation from bacterial strains

Isolated DNA fragments from Halophilic strains were subjected to Gel electrophoresis in 1.2 % agarose gel. The gel was stained with ethidium bromide was visualized under UV light using Geldoc software.

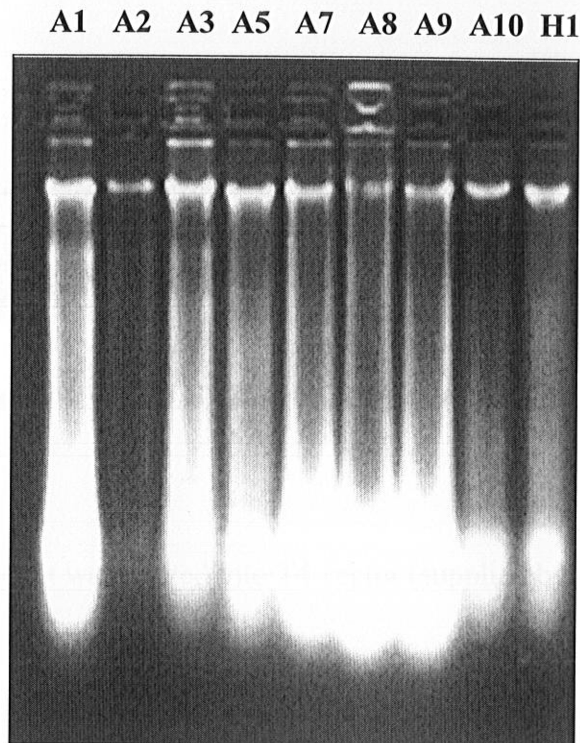


Figure4: Genomic DNA of various isolated halophilic strains

5.5: PCR amplifications by using 16S RNA primers

We obtained best single band amplification with U1and U2 primer set where as no amplification or multiple band amplification was observed with most of other primer sets. This might be due to the presence of certain inhibitory components in the extracted DNA samples which inhibit key enzymatic processes in molecular biological methods (Miller D.N, 2000). These contaminants can be humic acids which are the most widely reported contaminant in soil (Wilson, 1997), or certain metals and polysaccharides which can also inhibit molecular reactions (Straub *et al.*, 1995; Tsai and Olson, 1992.)A0 and A9 strains showed no amplification with any listed primer which indicates that these may not be bacterial halophiles.

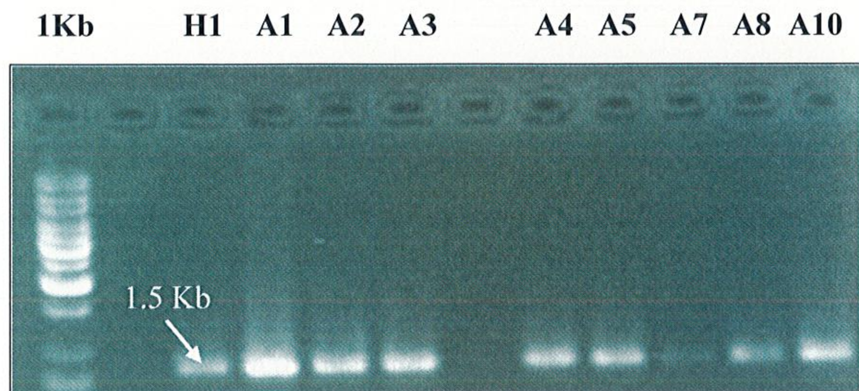


Figure 5: PCR amplification of 16S RNA gene sequences with U1 and U2 primer set

5.6: Cloning in *E.coli*

Ligation: Amplified product was ligated into T4 vector (supplied by Bangalore Genei) using T4 DNA ligase

Transformation: Ligated product was transformed in DH5α strain of *E.coli* and positive transformants were identified by Blue white screening.

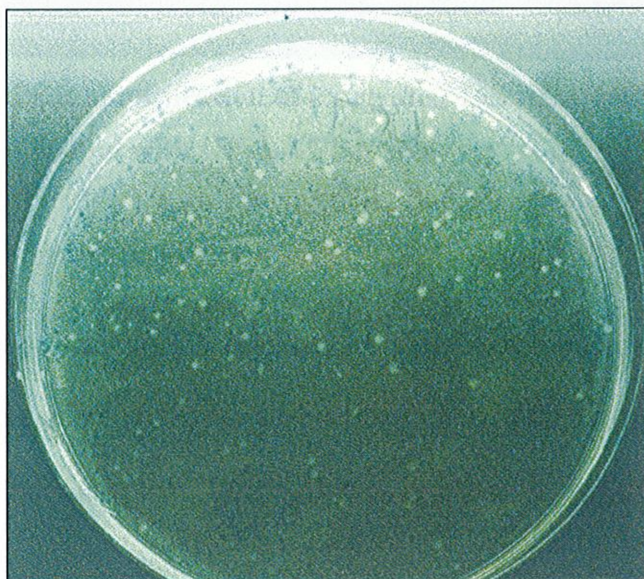


Figure 6: LB plate containing Blue white colonies

5.7: Plasmid Isolation:

Plasmid was isolated from the positive (white) colonies using Alkaline lysis Miniprep method. Presence of insert in the plasmid was confirmed by PCR using the insert specific primers(U1 and U2) under standard stationary conditions.

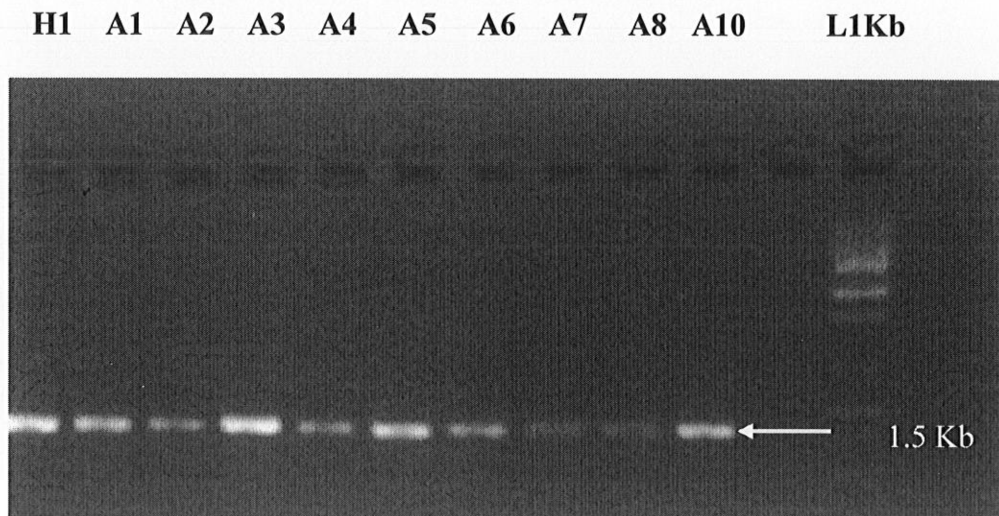


Figure7: PCR amplification of integrated 16S RNA gene sequences in Plasmid with U1 and U2 primer set

5.8: Thin layer Chromatography:

The crude extract of carotenoids obtained from orange colored halophilic bacteria, when run on TLC plates, showed a distinct band that corresponded to the band of β carotene standard run on the same plate .Thus this observation leads to the inference that the crude sample most probably contains considerable amount of β carotene.

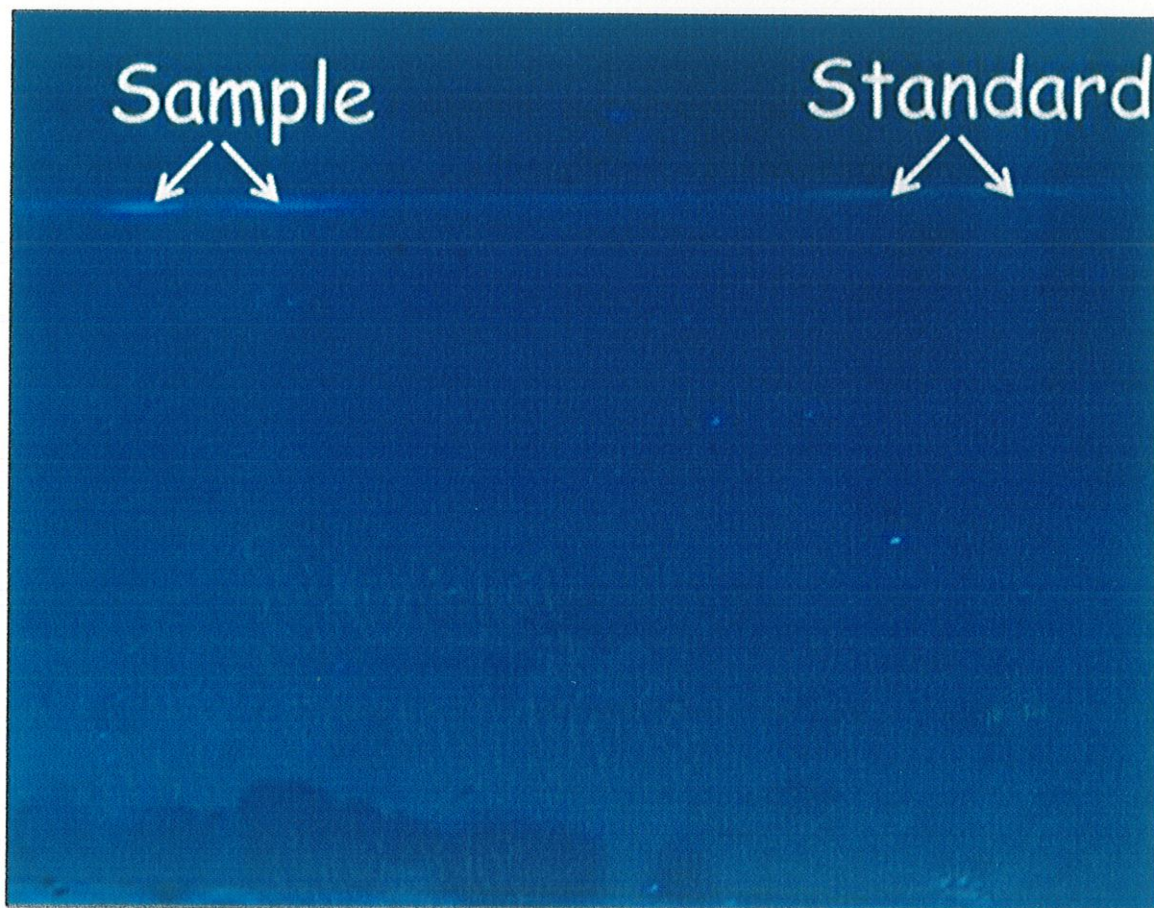
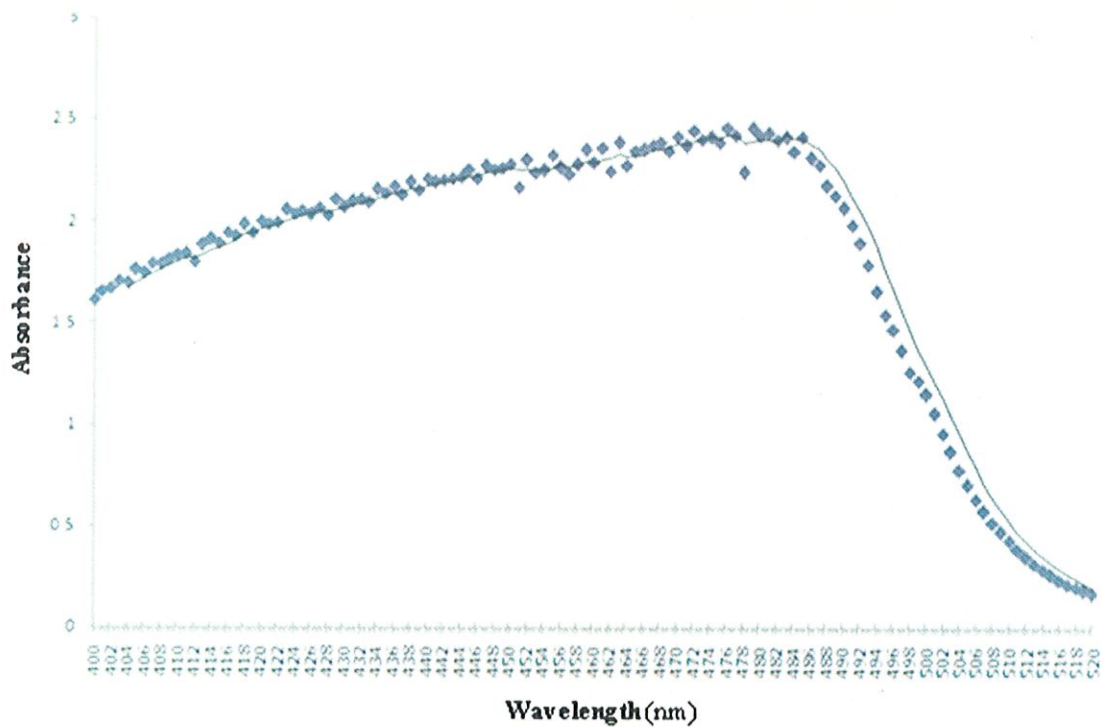


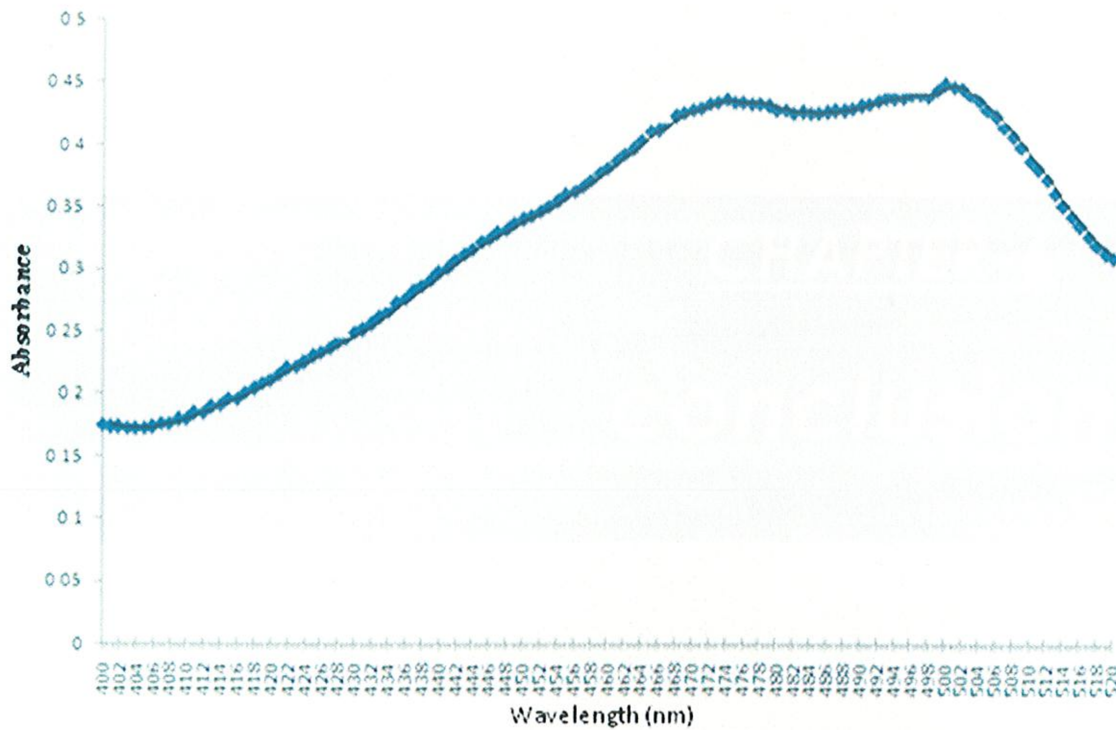
Figure 8: Thin layer chromatogram showing crude carotenoid extract and standard β Carotene.

Spectrum Analysis

OD was taken for Standard and Sample from 400 to 520 nm and OD was plotted in form of a graph to compare the two samples as each compound has a specific absorbance so this property can be used to compare two samples.



Graph 3: Spectrum analysis of β carotene standard in methanol



Graph 4: Spectrum analysis of sample in methanol

CHAPTER-6

conclusion

I have had my results for a long time: but I do not yet know how I am to arrive at them.

CONCLUSION

In the present study, by using enrichment culture technique we were able to isolate a large number of Halophile strains from rock salt mine at Darang which is the only salt mine in India. Our experimental observations validate our isolation of Halophiles as all these isolated microbial strains grew at variable salt concentration ranging from 3 % to 20% which is the distinct characteristic of halophilic microorganisms. The isolation of halophiles from air has proved to be a new and simple method as compared to the conventional method of isolating such bacteria from soil in salt mine. This is because time taken to obtain the potential isolates is comparatively lesser and the risk of loss of certain strains due to serial dilutions as in the case of enrichment culture technique is not there. The air isolate was able to grow on 20 % concentration of salt which is quite high salt concentration. Halophiles are likely to provide significant opportunities for biotechnology. As a result of natural and man-made global changes, hypersaline environments are on the increase. Moreover, hypersaline environments may easily be created by the concentration of sea water in arid environments, two commodities in plentiful supply. These facts, together with the occurrence of novel and stable biomolecules in halophiles, suggest that these organisms will prove even more valuable in the future.

bibliography

In Science the credit goes to the man who convinces the world, not to the man to whom the idea first occurred

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