STUDIES
ON
ADAPTATION OF *HALOBACTERIUM* STRAIN R_1 TO FLUCTUATING PHYSICO-CHEMICAL CONDITIONS AND HEAVY METAL IONS

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the corrections / suggestions. * referees have been incorporated in the thesis

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DECLARATION

I hereby declare that this thesis submitted for the Ph.D degree on "STUDIES ON ADAPTATION OF *HALOBACTERIUM* STRAIN R_1 TO FLUCTUATING PHYSICO-CHEMICAL CONDITIONS AND HEAVY METAL IONS" represents research work carried out by me during the period of study and that it has not been submitted to any other University or Institute for the award of any Degree, Diploma, Associateship, Fellowship or any other such titles.

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Candidate

Date: 29.10.2001.

Place: Goa.

CERTIFICATE

I hereby certify the above and state that the thesis is a record of research work done by the candidate under my guidance.

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Suneeta

Abbreviations

PCR	-	Polymerase chain reaction
ATP	-	Adenosine tri phosphate
RNA	-	Ribonucleic acid
DNA	-	Deoxyribonucleic acid
NAD	-	Nicotinamide adenine dinucleotide
NADP	-	Nicotinamide adenine dinucleotide phosphate
SDS	-	Sodium dodecyl sulphate
TEMED	-	*N, N, N', N'* -Tetramethylethylenediamine
sec	-	Seconds
min	-	Minutes
h	-	Hours
d	-	Day / s
RT	-	Room temperature

CONTENTS

CHAPTER 1: INTRODUCTION

The idea that entire life can be represented by either of the two kingdoms - Prokaryota or Eukaryota (1, 2) was modified with the use of 16S rRNA sequencing as molecular chronometer for measuring phylogenetic relationships among the biota (3,4). Organisms are now regrouped under three distinct forms: namely Bacteria, Archaea and Eukarya (5,6) (Fig.1). Archaeal organisms, considered to be primitive forms (6, 7), grow in extreme conditions of temperature (5,8-11), anaerobiosis and or toxic gases (12-14) and high concentrations of sodium chloride (15), that were presumably present during the early stages of life on earth. However, Polymerase Chain Reaction (PCR) amplifications of RNA sequences from water and sediment samples from mesophilic environments such as temperate, marine coastal waters (16), freshwater lakes, marine planktons (17) and even marine sponges (18) revealed the presence of archaeal species indicating their ubiquity (19). Based on the sequences of universally present proteins, Archaea were positioned on the branch leading to Eukarya (20). However, recent studies on protein phylogeny (7) suggest that Archaea show relationship closer to Gram-positive bacteria and that the eukaryotic cell possibly originated from a unique fusion between a thermoacidophilic archaebacterium and a Gram-negative eubacterium.

According to the rRNA phylogenetic tree, there are two groups (11) within the Archaea - the kingdoms of Crenarchaeota and Euryarchaeota (Fig. 1 & 2) Crenarchaeota consists of hyper thermophiles and thermoacidophiles (e.g. *Sulfolobus*, *Desulphurococcus*, *Pyrodictium*, *Thermoproteus* and *Thermofilum*) (5, 8, 9, 11) while Euryarchaeota includes hyperthermophiles (e.g. *Pyrococcus* and *Thermococcus*); methanogens (e.g. *Methanosarcina*); halophiles (e.g. *Halobacterium* and *Haloferax*) and even thermophilic methanogens (e.g. *Methanothermus*, *Methanobacterium* and *Methanococcus* (21). The PCR surveys of hot spring microbiota detected new archaeal RNA sequences that branch just

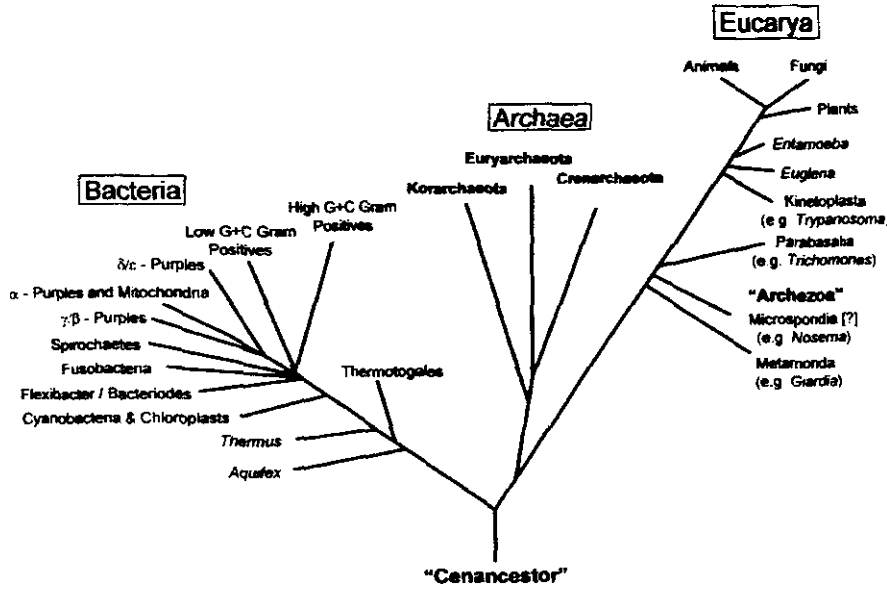


Fig.1. Schematic drawing of universal rRNA tree (6)

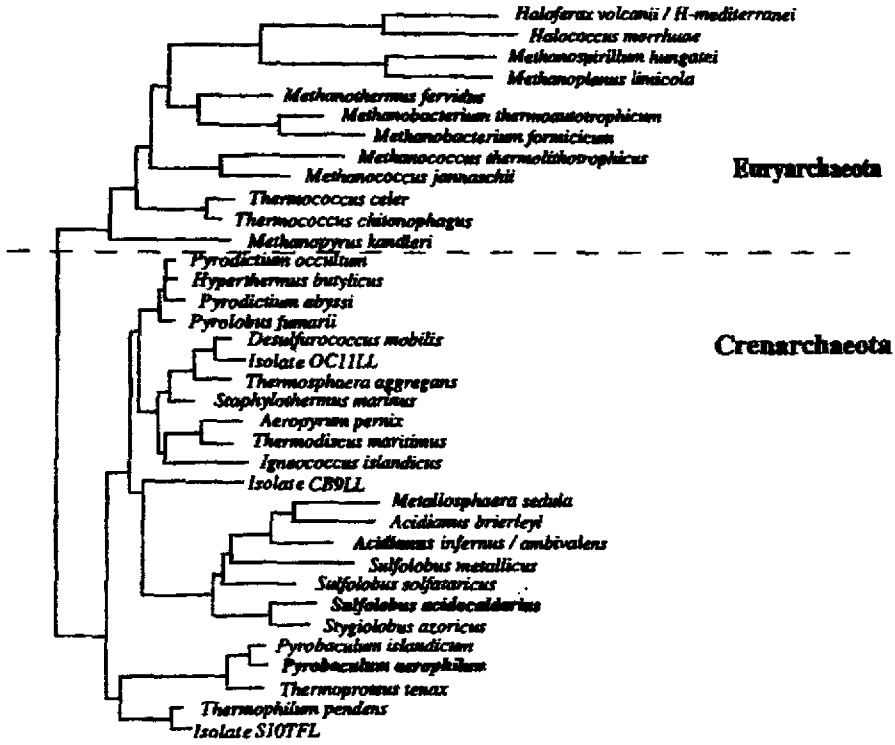


Fig. 2. Dendrogram showing the branching in Archaea (148)

below Crenarchaeota – Euryarchaetoa divergence (22, 23) resulting in a further division of Archaea - into a third kingdom, Koraarchaeota (Fig.1).

1.1 UNIQUE FEATURES OF ARCHAEA

Archaea exhibit certain unique structural and physiological characters, distinct from eubacterial and eukaryotic cells as listed below.

Cell envelopes of Archaea are characterised by the absence of eubacterial murein components and display a diverse chemical nature (24) as shown in Table 1.1.

Cell membranes of Archaea possess special glycerol isoprenoid diether lipids in halophiles (38), tetraether lipids in methanogens (39) and macrocyclic ether lipids in thermophiles (40-42), instead of fattyacyl glycerol ester lipids of Eubacteria and Eukarya. The stereospecificity of glycerol in Archaea is 2,3-*sn*- glycerol, which differs from the 1,2-*sn*-glycerol of the other two domains (43,44).

Archaea also possess characteristic modifications in base sequences of t-RNA molecules and RNA polymerase sub-units (45-47).

Central metabolic pathways in Archaea are unique and deviate from the conventional ones observed in bacteria and eukaryotes (48-51) For instance, the thermoacidophilic species of *Thermoplasma* and *Sulfolobus* catabolise glucose via a modified Entner-Doudroff (E-D) pathway in which glyceraldehyde and pyruvate can be produced from glucose without phosphorylation (49). These and various other unique metabolic pathways proposed by Danson (52) for Archaea are detailed in Table 1.2.

Table 1.1 Diversity in chemical nature of archaeal cell envelopes

Archaeal Organism	Chemical constituent of Cell envelope	Reference
Methanobacterium	Pseudomurein	25,26,27
Methanothermus	Pseudomurein with protein subunits	28
Methanosarcina	Methanochondriotin	29
Halococcus	Sulphated acidic heteropolysaccharide	30,31,32
Methanoplanus	Surface layer consisting of Glycoprotein	33
Thermococcus		34
Halobacterium		35,36
Methanospirillum	Proteinacious sheath	37

Table 1.2 Central metabolic pathways of Archaea

Organism	Glucose catabolism	Gluconeogenesis	Citric acid cycle
1.Haloarchaea			
Halobacterium saccharovorum	Modified E-D pathway	Reverse Embden Meyerhof pathway	Complete oxidative
Halobacterium halobium	-do-	-do-	-do-
2.Thermophiles			
Sulfolobus Sp	Non-phosphorylated E-D pathway	Unknown	Complete reductive (autotrophic)
Thermoplasma acidophilum	-do-	-do-	Complete oxidative
Thermoproteus neutrophilus	Unknown	-do-	Complete reductive (autotrophic), Incomplete oxidative (heterotrophic)
3.Methanogens			
Methanobacterium thermoautotrophicum	Embden Meyerhof pathway	Reverse Embden Meyerhof pathway	Incomplete reductive
Methanosarcina barkeri	Unknown	Unknown	Incomplete oxidative

Source: (52)

Table 1.3 Physical and chemical characteristics of haloarchaeal habitats

Parameter	Dead Sea (Israel)	Great Salt Lake (U.S.A.)	Wadi Natrun (Egypt)	Marine Saltern (Spain)	Atacama Salar (Chile)	Sea Water
pH	5.9 - 6.3	7.7 - 8.4	11	6.8 - 8.1	7.2 - 8.6	6.8 - 8.2
Temperature (^{0}C)	21 - 36	-5 - 30	-	22 - 42	12 - 43	24 - 32
Total salts (g/l)	299 - 332	113 - 332	394	140 - 260	137	31.15
Cations (g/l)						
Na^+	39	105	142	87	38	10.56
Mg^{2+}	40.7	11	0	8	-	1.269
Ca^{2+}	17	0.3	0	-	0.5	0.404
K^+	7.3	6.7	2.3	-	0.6	0.391
Anions (g/l)						
Cl^-	212	181	155	169	59	18.97
Br^-	5.1	-	-	-	-	0.066
SO_4^{2-}	0.5	27	22.6	22	2.4	2.66
HCO_3^-	-	0.01	67.2	-	0.06	0.402

Compiled from (15,73 &75)

Table 1.4 Genera and species of Haloarchaea

Genus and species	Source
1. Genus Halobacterium	
Halobacterium salinarium	Salted hides, salted fish
Halobacterium halobium	Salted fish
Halobacterium cutirubrum	Salted buffalo hide
Halobacterium distributum	Salted fish
2. Genus Halorubrum	
Halorubrum saccharovorum	Saltern, California
Halorubrum sodomense	Dead sea
Halorubrum lacusprofundi	Deep lake, Antarctica
Halorubrum trapanicum	Solat salt
3. Genus Haloarcula	
Haloarcula vallismortis	Salt pools, Death Valley
Haloarcula marismortui	Dead Sea
Haloarcula japonica	Saltern, Japan
Haloarcula hispanica	Saltern, Spain
Haloarcula californiae	Saltern, California
Haloarcula sinaiiensis	Salt pool, Sinai
4. Genus Haloferax	
Haloferax volcanii	Dead sea
Haloferax denitrificans	Saltern, California
Haloferax gibbonsii	Saltern, Spain
Haloferax mediterranei	Saltern, Spain
5. Genus Halococcus	
Halococcus morrhuae	Salted fish
Halococcus saccharolyticus	Saltern, Spain
Halococcus turkmenicus	Saline soil
6. Genus Natronobacterium	
Natronobacterium pharaonis	Wadi Natrun, Egypt
Natronobacterium magadii	Lake Magadi, Kenya
Natronobacterium gregoryi	Saltern Lake, Magadi
Natronobacterium vacuolatum	Lake Magadi, Kenya
7. Genus Natronococcus	
Natronococcus occultus	Lake Magadi, Kenya

Compiled from (77, 78)

Apart from the metabolic pathways, Archaea also possess typical enzymes. The phosphofructokinases in Archaea are either adenosine tri phosphate (ATP) or pyrophosphate dependent, where as Eubacteria and Eukarya use both the types. Also, the pyruvate dehydrogenase complex of Eubacteria and Eukarya is replaced by Pyruvate: ferredoxin oxidoreductase in Archaea (53,54). Moreover, the nicotinamide nucleotide dependent dehydrogenases of Eubacteria or Eukarya show characteristic specificity for either NAD or NADP (55, 56), while a number of archaeal dehydrogenases have dual co-factor specificity (57-61).

1.1.1 Typically eubacterial or eukaryotic features of Archaea

Eubacterial characters (6, 46,62-64)

a) General cell size of 1-2 by 1- 4 µm
b) Absence of defined nuclear membrane and organelles
c) Presence of single circular chromosome
d) Presence of small circular plasmids
e) Single replication initiation site 'Ori C'
f) Presence of topoisomerases, gyrases
g) Clustering of genes into Operons
h) Absence of 5'end caps for mRNA
i) Presence of Shine-Dalgarno ribosome binding site on mRNA

Eukaryotic characters (63,64-72)

a) Resistance to streptomycin
b) Sensitivity to anisomysin, aphidicolon
c) Similarities in DNA replication, transcriptional and translational components

d) Homology between DNA polymerases
e) Presence of 7 subunits in RNA polymerases
f) Presence of TATA-box like binding sites
g) Presence of HMG CoA (3 hydroxy, 3-methyl glutaryl coenzyme A for isoprenoid biosynthesis)
h) Similarities in primary sequence and three-dimensional structure of DNA binding proteins.

1.2 HALOARCHAEA

The haloarchaea have been isolated from hyper saline environments such as Great Salt Lake, USA; Dead Sea, Israel; Wadi Naturn, Egypt; Lake Magadi, Kenya; and Solar salterns (15, 73) apart from marine waters (74). Some typical characteristics of these environments are compiled and presented in Table 1.3.

1.2.1 Taxonomy of Haloarchaea

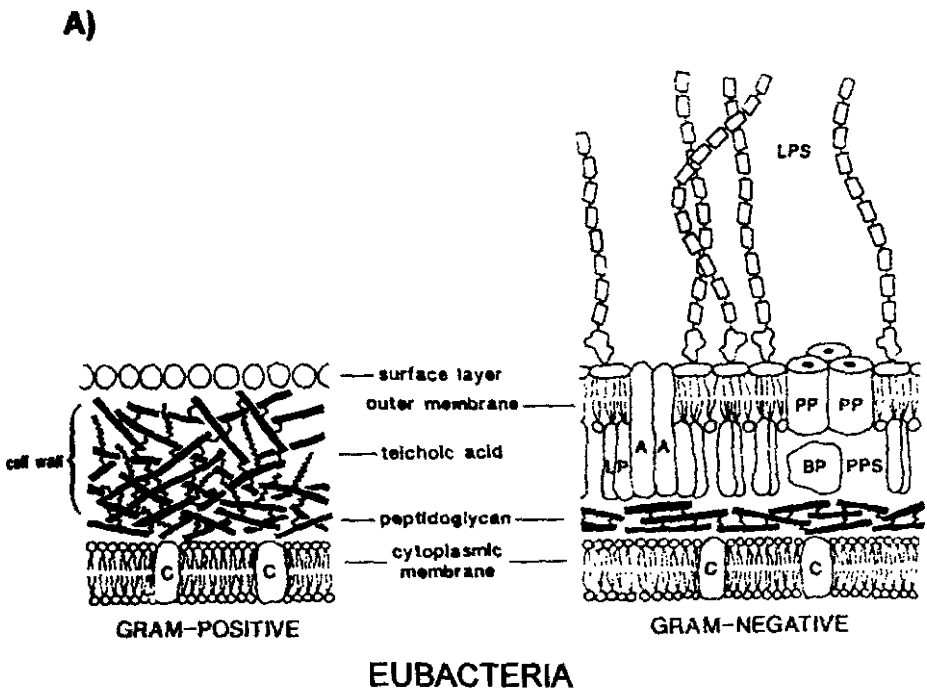
Isolates of Haloarchaea, currently assigned to a single family of Halobacteriaceae within the order halobacteriales (76) and the validly published genera have been listed in Table 1.4 (77). The genera *Halobacterium*, *Haloarcula*, *Halorubrum* and *Haloferax* are rod shaped or pleomorphic and the genus *Halococcus* comprises of coccid bacteria. *Natronobacterium* and *Natronococci* are haloalkalophiles growing at high (~11) values of pH (77).

1.3 CELLULAR FEATURES OF HALOARCHAEA

1.3.1 Cell Envelopes of Haloarchaea

Morphological and chemical analysis of cell envelopes of haloarchaea show that they lack the peptidoglycan present in Gram-positive eubacteria and an outer membrane containing lipopolysaccharide, typically seen in Gram-negative eubacteria (35, 79-81 and Fig. 3). Houwink pointed that the cells of *Halobacterium halobium* were surrounded by a single surface layer (S-layer) of hexagonally arranged sub-units (82) Based on X-ray diffraction studies, Blaurock, et al. in 1976 pointed out the presence of two protein layers and assumed that the space between the two layers could be the periplasmic space (83). Robertson, et al. also considered the presence of two layers which were 3 and 5 nm wide and separated by 5 nm wide zone (84). However, Kandler and Konig. on comparison of micrographs of haloarchaeal cell cross-sections with those of other archaeal forms, possessing S-layers suggested that. the above assumed extracytoplasmic layers may actually be the inner and outer surface of the S-layer (24). They further suggested that, these being more osmophilic, would be more electron dense than the central portion. The S-layer is very tightly joined to the cytoplasmic membrane, as is generally observed in Archaea. thus leaving no place for a periplasmic space. The isolated S-layers of *Halobacterium salinarium,* contain an extremely acidic glycoprotein of molecular weight ~ 200 KDa. with 10-12% of carbohydrate consisting of neutral hexoses. amino sugars and uronic acids (85). Mescher and Strominger (1976) demonstrated the covalent linkage of the carbohydrate to peptide moiety of glycoprotein by digestion with trypsin and pronase (85).

Wieland, et al. (86,87) reported the presence of a high molecular weight sulphated glycoprotein in cell envelopes of *Halobacterium halobium*, similar to animal mucins (87), rather than a



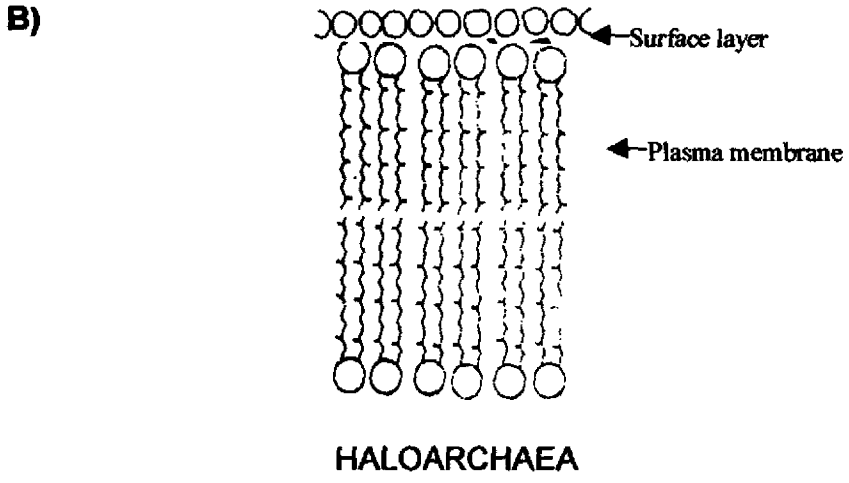


Fig.3. Schematic presentation of the cell envelope:
A) Gram - positive and Gram - negative Eubacteria
B) Haloarchaea

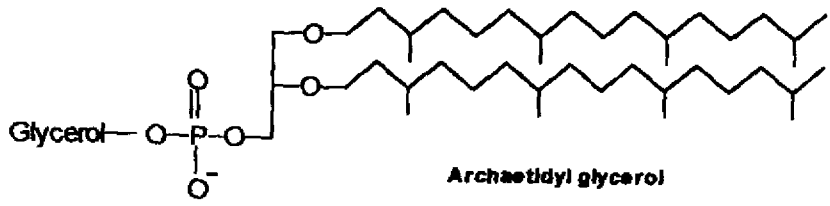
(96) (Fig. 4), now proposed to be renamed by Nishihara et.al. (97) as described in Table 1.5. Haloarchaeal membranes also possess non polar lipids comprising of the red C_{50} carotenoid pigments such as bacterioruberins, C_{40} phytoenes, lycopenes, β-carotene, C_{30} squalenes, C_{20} retinal as well as vitamin MK-8 (98-100) (Fig. 5).

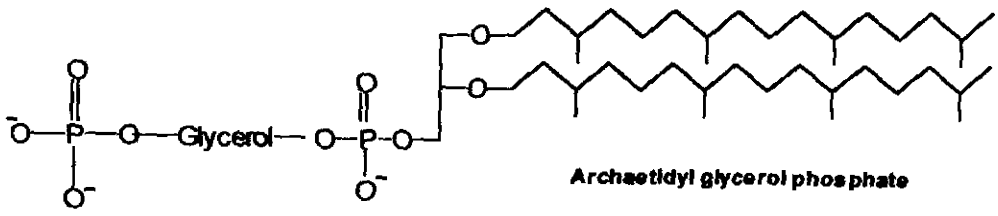
Genesis of Haloarchaeal Lipids

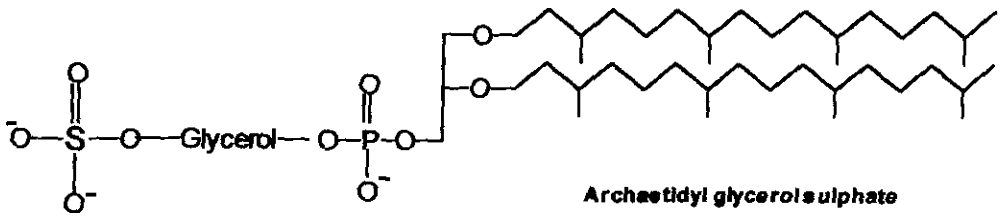
Koga, et al. in 1993, hypothesized that during the early stages of the origin or evolution of life under anoxic atmosphere, earlier to the generation of atmospheric oxygen on Earth, isoprenoid hydrocarbon chains prevailed as the lipid components of biomembranes. They further state that, under anoxic conditions, isoprenoid alcohols could readily get reduced to saturated isopranoid alcohols rather than oxidized to isoprenoid carboxylic acids. As a result, isoprenoid alcohols probably produced ether linkages with glycerol forming polar lipid (101).

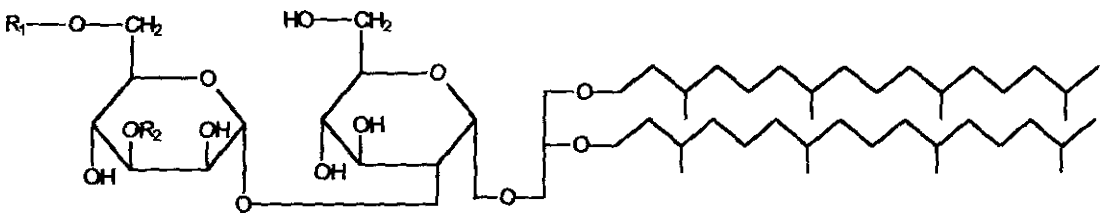
1.3.3 Purple Membrane and other Rhodopsins

The purple membrane isolated from *Halobacterium halobium* (102) contained ~ 25% lipid and 75% protein (103-104). Later it was demonstrated that, a retinal chromophore (Fig. 6) attached to this protein gives rise to the purple colour. This retinylidene protein was named as Bacteriorhodopsin because of its resemblance to the vertebrate rhodopsins (105). Three other bacterial rhodopsins were discovered during the elucidation of the proton pumping mechanism of Bacteriorhodopsin (BR), which are functionally quite distinct (106-108). Halorhodopsin (HR), like BR, is an inwardly directed chloride ion pump. The 2 other bacterial rhodopsins, Sensory rhodopsins SR I and SR II, are photoreceptors that trigger the photophobic and / or photo attractive response of bacteria.









Diglycosyl archaeol	R_1= H	R_2 = H
Sulphated diglycosyl archaeol	R_1= -SO_2OH	R_2 = H
Triglycosyl archaeol	R_1= β-Gal*p*	R_2 = H
Sulphated triglycosyl archaeol	R_1= 3-SO_3H-β-Gal*p*	R_2 = H
Tetraglycosyl archaeol	R_1= β-Gal*p*	R_2 = α-Gal*f*

Fig. 4 Polar lipids of Haloarchaea

Table 1.5 Haloarchaeal lipids

Old name	Proposed new name
2,3-di-o-phytanyl-sn-glycerol	Archaeol
Di ether analogue of phosphatidyl glycerol	Archaetidylglycerol
Di ether analogue of phosphatidyl glycerol phosphate	Archaetidylglycerol phosphate
Di ether analogue of phosphatidyl glycerol sulphate	Archaetidylglycerol sulphate
Diglycosyl diether	Diglycosly archaeol
Triglycosyl diether	Triglycosly archaeol
Tetraglycosyl diether	Tetraglycosly archaeol

Source: (97)

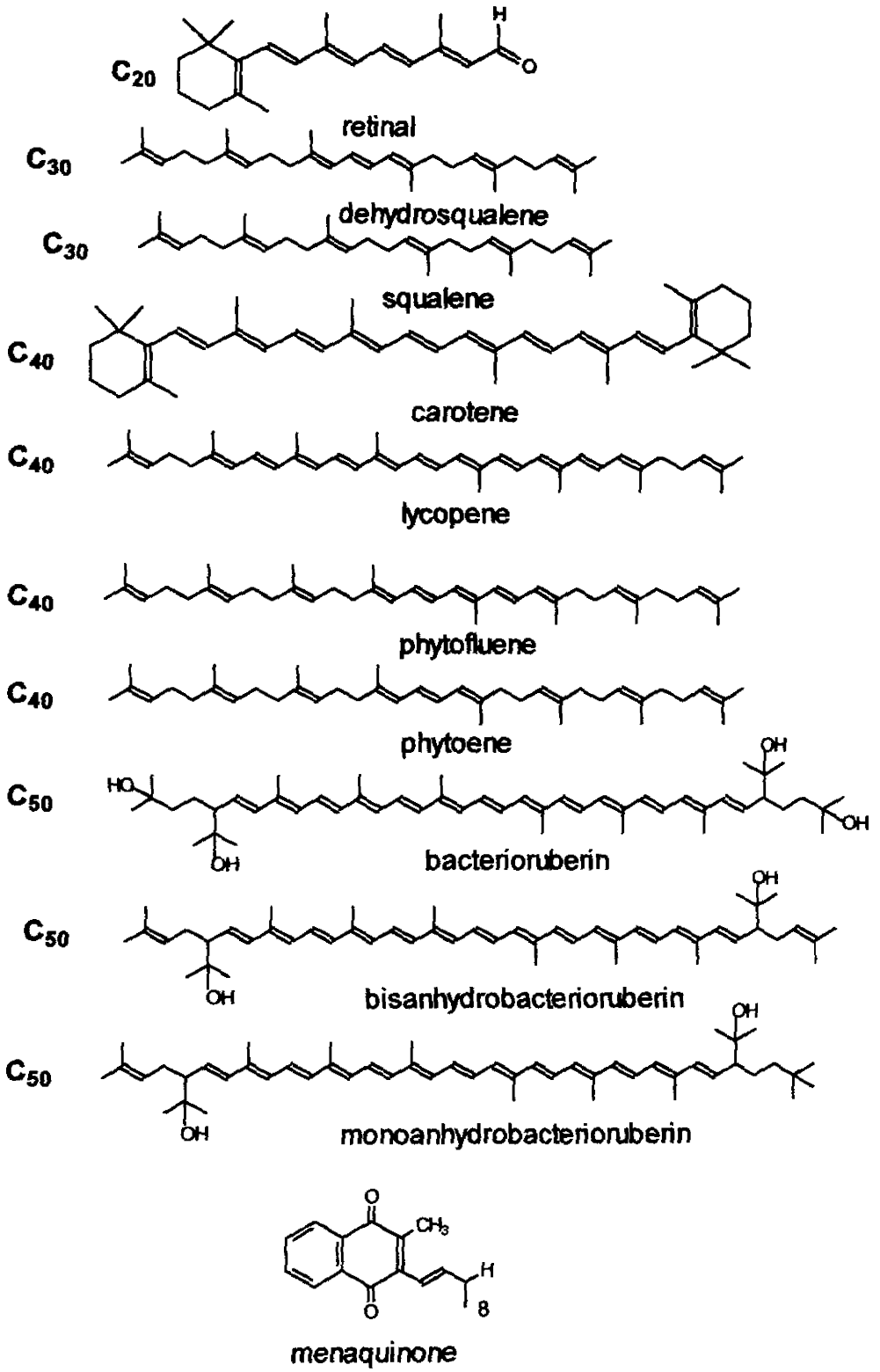


Fig. 5. Non polar lipids of Haloarchaea

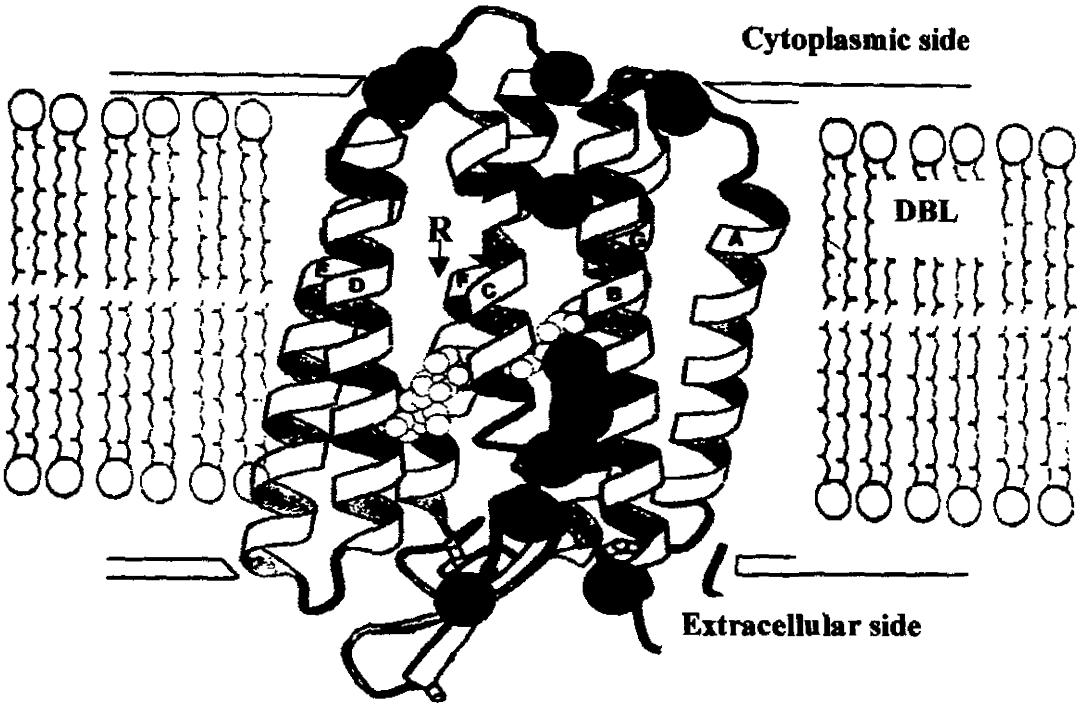


Fig.6. Structural model of Bacteriorhodopsin (148)

R – retinal
DBL- diether bilayer

1.3.4 Genome of Haloarchaea

The genomic size of 2.3 x 10^9 Da observed in *Halobaterium halobium* & *Halococcus morrhuae* (109) is comparable to that of 2.5 x 10^9 Da generally seen in Eubacteria (110). The haloarchaea (111-112) are characterized by the presence of extra chromosomal DNA, termed satellite DNA consisting of plasmids, minor circular DNA and DNA containing several A + T rich regions (112,113) with G + C content varying from 57 to 60 % as compared to the chromosomal DNA with a G + C content of 66 to 68 % (111). A mega plasmid (95 Mdal) designated as pHH1 was detected in *Halobaterium halobium*, which codes for gas vacuoles and probably for bacteriorhodopsin and bacterioruberin (114). Pfeifer, et al. in1981, demonstrated that the A + T rich region of the satellite DNA, based on restriction mapping, to be indistinct from the plasmid pHH1 isolated from these species (115). A large number of megaplasmids have been found to be harboured by haloarchaea and are summarized in Table 1.6.

1.4 DEPENDENCE OF HALOARCHAEAL CELLULAR ARCHITECTURE ON SODIUM CHLORIDE

The shape of haloarchaea is maintained by the envelope, which requires high concentration of NaCl (4 M) for being intact. The Na^+ ions, however, can be replaced by K^+, Mg^{+2} or Ca^{+2} ions to some extent (121,122). At lower concentrations of NaCl, haloarchaea other than halococci form spheres and lyse (123). Lowering the salt concentration easily solubilizes isolated S layers of *Halobacterium salinarium* and the components can be characterized by treatment with SDS and mercaptoethanol (124).

Clues on membrane architecture were obtained by dispersion studies of the total lipids and of the isolated polar lipids from *H. cutirubrum*

Table 1.6 Megaplasmids in Haloarchaea

Organism	Number of plasmids	Size of the plasmid (M Dal)	Reference
Halobacterium	2	115, <100	119
H.halobium CCM 2090	1	133	118
H.halobium NRC 1	1	100	116
H.halobium NRC 817	1	24	117
H.halobium pHH 4	3	233, 106, 47	120
H.halobium CCM 2090	3	167. 106. 47	119
H.halobium CECT 396			
H.saccharovorum ATCC 29252	2	125, <100	119
H.vallismortis ATCC 29715	2	260, 110	119
H.mediterranei ATCC 33500	3	300, 245, 125	119
H.volcanii DS 2	2	275. 120	119
Haloarcula			
H.hispanica ATCC 33960	3	266, 246, 120	120
H.vallismortis ATCC 29215	4	266, 187, 93, 87	120
H.sinaiiensis ATCC 33800	5	300,250, 215, 185,100	119
Haloferax			
H.volcanii NCMB 2092	3	453, 273, 60	120
H.gibbonsii ATCC 33959	3	287, 233, 100	120

(125). These experiments suggest that lipid hydrocarbon chains packed themselves as bilayers with a good deal of kinking. Further, squalene, if added, was seen to be accommodated in the hydrocarbon region of the bilayer perpendicular to the plane of the membrane. Furthermore, these polar lipids from *H. cutirubram* could combine with Mg^{+2} or Ca^{+2} only in the presence of squalene. Thus, squalene is believed to be required to space the polar lipid molecules sufficiently apart so as to permit the entry of the cations and enable them to reach the charged phosphate groups. These effects were observed at high salt concentrations in haloarchaea, thereby suggesting an important physiological role for squalene (125,126). Highly methyl-branched, saturated isoprenoid hydrocarbon chains such as squalene are bulky and are reported to have a phase transition temperature ideal for these cells to function properly under their physiological conditions (127).

The polar lipids of haloarchaea are also reported to be unusually acidic, containing few or no basic groups and are suggested to offer important binding sites for cations (128-131). Russel, in 1989, pointed out that haloarchaea have a high charge density on their membranes (157). According to Kates, the advantage would be that, the negative charges on these acidic polar head groups are shielded by the high ionic concentration thus preventing disruption of the lipid bilayers due to charge repulsive forces and also to provide a charge stabilized lipid bilayer (133).

Evidence came through the studies of Yamauchi, et al. in 1992, who compared the stability of haloarchaea like ether lipids with bacterial like phospholipids. The ether phytanyl lipids were stable and could form a concentrated suspension of liposomes at a wide range of salt concentrations. In contrast, the bacteria like lipids were found to be less stable (134). The high stability of the haloarchaeal phytanyl chain is proposed to result from the limited segmentary motion of tertiary carbon

atoms (135). This restriction may enhance the stability of the haloarchaeal lipids at high salt concentrations and keep the permeability low. Thus the phytanyl chain is an important determinant for the exceptional properties of the haloarchaeal lipids. Jack et al. (1999) studied the influence of pH and the salt concentrations on the proton and Na ion permeability of liposomes formed from haloarchaeal and *E.coli* lipids and concluded that, the haloarchaeal membranes are stable at high salt concentrations up to 4M and at elevated pH values up to 9 in contrast to the eubacterial liposomes (136).

The four pigments i.e. BR; HR; SR I and SR II are membrane proteins of ~ 26 KDa (108,137). In each case, the polypeptide chain folds into seven membrane spanning helices 'A to G' (Fig. 6) with the 'C' terminus positioned on the cytoplasmic side. The retinal chromophore is attached in the all-trans configuration to a lysine residue via a protonated Schiff's base in the C-terminal helix G (137-140).

Upon light activation, a proton is transferred from the inside of the cell to the extracellular medium across the membrane through a series of conformational changes in the rhodopsin moiety (141). As a consequence, the inside of the cell becomes negatively charged creating an electric potential difference. This electric potential difference together with that generated by the respiratory chain is thereby utilized by *H. salinarium* for the synthesis of ATP (142). The reversible molecular events associated with this include the isomerisation of the retinal chromophore from all-trans to 13-cis, deprotonation of the Schiff's base and conformational changes of backbone. All these light triggered thermal reactions are reversible, as, on completion of cycle, the protein regains its original state (139, 140,141,143,144).

The ion pumps return to the original state in milliseconds (145,146) with SRs requiring maximum time ~ 100 milliseconds (147). The reason for the difference is proposed to be related to the functions of these proteins. The functional efficiency of the ion pumps (HR and BR) is dependent on a high turn over rate, while the SRs have to remain in the signalling state, long enough to relay the signal to cytoplasmic proteins (148).

The presence of high intracellular solute concentration is generally devastating to proteins and other macromolecules by virtue of their interference with electrostatic interactions between and within macromolecules. This reduces the availability of free water causing aggregation or structural collapse, or both (149,150). Unlike this, the haloarchael proteins show unique molecular stability under high salt concentration, which is the essential requirement for normal existence (150-152).

A number of haloarchaeal enzymes studied showed the requirement of at least 1 M monovalent salt for stability and maximal activity at 2.4 M concentration of salt. For e.g. the aspartate transcarbamoyl transferase of *Halobacterium cutirubrum* (153) became irreversibly denatured within minutes of reduction of salt to less than 2 M. Other enzymes with reported high salt requirement are malate dehydrogenase, isocitrate dehydrogenase (154,155), pyruvate kinase (156), citrate synthase (150,157), alanine dehydrogenase (158,159), etc The structural stability of 70S, 50S and 30S ribosomes required salt concentrations up to 3 M NaCl. The proteins from the subunits were lost at lower concentrations (126,160,161).

Baxter (1959) suggested that salts support the activity of halophilic enzymes by virtue of their cations, screening negatively charged groups and by preventing mutual repulsion of these charges from distorting

protein confirmation (162). Though the charge-shielding action of salts must be very important, Lanyi (1974) pointed out that this action cannot account for the very high concentration of salts that are generally required and that maximal shielding would be reached in about 0.1 M NaCl and in much lower concentrations of salts of divalent cations (150).

Bulk cytoplasmic proteins, ribosomal proteins, and many isolated enzymes of several haloarchaea are more acidic and contain fewer amounts of non-polar amino acids than non-halophilic bacteria (150,163,164). The properties of amino acids in proteins of haloarchaea are compared with those of non-halophilic eubacteria in Table 1.7. The lower frequency of non-polar amino acid residues resulting in weaker hydrophobic bonds (126) suggests that high concentration of salt may be required to produce the necessary hydrophobic bonding, as is generally the case with stability of any protein (150). Lanyi (1974) suggested that. new hydrophobic bonds are created (150) at such ionic strengths involving marginally hydrophobic groups such as serine and threonine (165), which are normally not well suited for non-polar interaction. Thus high concentrations of salt actually required by haloarchaea, is possibly for stabilizing hydrophobic bonds (150.166.167).

Presence of glutamate and aspartate (being acidic amino acid residues) at an increased frequency in haloarchaeal proteins is most effective with respect to hydration capacity (168). These amino acids bind 6.0 – 7.5 mol water per mol. residue. compared to the 3.0 – 4.5 mol water per mol basic amino acid residue (169). Their frequent occurrence. therefore, confers organization of a hydrated salt ion network at the surface of the protein (170). The high water binding capacity of malate dehydrogenase from *H. marismortui* in its native configuration is lost in its unfolded configuration at low salt concentration (171,172). The replacement of aspartate by glutamate residues at the *n*-terminus of

Table 1.7 Amino acid contents of haloarchael and non-halophilic bacterial proteins

Material	Bacteria	Amino acids (mol %)		
		Acidic	Basic	Excess acidic
Cytoplasmic proteins	*Halobacterium salinarium*	26.8	9.7	17.1
	Halococcus	27.8	9.9	17.9
	Pseudomonas fluorescens	20.9	13.8	7.1
	Sarcina lutea	21.2	12.6	8.6
Ribosomes	*H.cutirubrum*	26.6	13.8	13.5
	E.coli	18.4	18.2	0.2
Cell envelope	*H.cutirubrum*	27.6	6.9	20.7
	H.halobium	28.0	7.3	20.7
Gas vacuole	*H.halobium*	21.2	10.9	10.3

Source: (126)

ferredoxins from *H.halobium* and *H marismortui* (165), resulted in higher water binding ability and prevented structural collapse or aggregation (150, 173,174). Further, the acidic residues help in forming salt bridges providing structural rigidity and are important determinants in stabilization of three-dimensional structure of haloarchaeal proteins (175) i.e. the confirmation at secondary, tertiary and quaternary level (171,176).

The genome of the Haloarchaea is extremely unstable (116, 177-179). This genomic instability is brought by the very frequently occurring rearrangements, insertions and deletions mainly in the AT rich region of plasmid DNA; i.e. pHH1 plasmid in the case of *Halobaterium halobium* (116). These rearrangements / insertions / deletions are reported to be due to the presence of a number of repetitive sequences that may be similar to the transposable elements of Eubacteria and Eukarya (180,182). Deletion events occurring spontaneously in case of 36 kbp, pHH4 plasmid of *Halobacterium halobium*, were due to deletions of insertional elements (IS) namely ISH 2 and ISH 27 (117).

Genomic studies of purple membrane and gas vesicles of haloarchaea have also shown that, spontaneous insertion mutations occur frequently in these genes (183,184). Three genes important for the purple membrane synthesis have been identified on the *Halobaterium halobium* chromosome i.e. *bop* (185) for purple membrane apoproterin, and *brp* and *bat* genes with possible regulatory functions (186). Insertions into *brp* or *bat* regions resulted in the loss of *bop* gene expression (187). The presence of insertion elements in these genes, which are located on the chromosomes, indicates that the movement of IS elements is not exclusively limited to the A+T rich, plasmid region (177).

1.5 STRATEGIES OF OSMOTIC BALANCE IN HALOARCHAEA

The haloarchaea that crucially require very high salt concentrations for survival and multiplication, attain the osmotic balance by Salt-in strategy, a mechanism of maintaining high intracellular salt concentration (150,188-191), equivalent to the external conditions. Although, the internal salt concentration in haloarchaea is equivalent to the external medium (188) it is generally different in composition involving gradients of Na^+ and K^+ ions (192-195). Analysis of cell associated KCl of haloarchaea have indicated concentrations of up to 5M, which represents K^+ concentration gradient inside/outside of more than 1000 fold (188,193,196). Such Na^+ / K^+ gradients between the outside and inside are thought to be under a great physiological strain but it has been realized that these gradients may instead confer a physiological advantage. Skulachev and collaborators (197) have proposed that in many bacteria the Na^+ / K^+ gradient has a proton-motive force (PMF), which causes buffering. According to this theory when PMF is low, K^+ will diffuse out of the cells making a change in membrane potential causing a simultaneous inflow of sodium into the cell to maintain electro-neutrality, which in turn leads to the extrusion of H^+ via Na^+ / H^+ antiport and a consequent increase in the PMF (197,198). One particular haloalkalophile *Natranococcus ocultus*, however, was reported to produce and accumulate 2-sulfotrehalose, an organic osmotic solute to maintain osmotic balance (199).

1.6 HALOPHILIC EUBACTERIA

The halophilic eubacteria are the microorganisms that grow at physiological concentrations of 0.85% salinity. These, however, unlike non-halophilic microorganisms have capabilities to conditionally adapt, survive and multiply at salinity ranges of 1 to 3.5 M and unlike the Haloarchaea do not require the presence of a minimum of 1.5 M NaCl

concentration for their growth (200,201). The maximum salt tolerance level of these microorganisms depends on growth conditions such as temperature and availability of nutrients (202,203). For e.g. salt requirement for growth of *S. costicola* in synthetic medium with glucose, decreased to 0.3 M (NaCl) from 0.8 to 1.5 M NaCl, observed in nutrient rich medium (204). A shift in growth temperature of *H. halophila* from 42°C to 22°C also decreased the salt requirement for growth from 7.5 to 5% (205).

1.6.1 Adaptation mechanisms of halophilic eubacteria to sodium chloride

Non-halophilic microorganisms exposed to NaCl concentration higher than physiological concentrations are subject to osmotic imbalance that leads to loss of water from cellular cytoplasm into the surrounding milieu and results in self-destruction (206,207). The halophilic eubacteria living in 1 to 3.5 M NaCl adapts to the osmotic balance between the internal and external surroundings of its cytoplasm through four mechanisms (208), i.e. (1) Accumulation of salts, (2) Accumulation of organic molecules, (3) A combination of accumulation of salt and organic molecules, (4) Controlled movements of water in and out of the cytoplasm (209-211).

The intracellular salt concentrations of eubacterial halophiles (compiled in Table 1.8) are reported to increase with increase in external salt concentration of the medium (211,212,214). These, although not equivalent to those of haloarchaea, are fairly high, as compared to the non-halophiles. Based on available data, Kushner et al. concluded that the intracellular salt concentrations alone are generally insufficient to provide the required osmotic balance.

Table 1.8 Intracellular ionic concentrations of halophilic eubacteria

Species	Medium Concentration (M)		Intracellular Concentration (M)		Reference
	Na^+	K^+	Na^+	K^+	
Halomonas elongata	0.06 1.38 3.4	0.02 0.02 0.01	0.04 0.31 0.63	0.002 0.02 0.02	211
Halomonas canadensis	0.6 4.4	0.04 0.04	0.05 0.62	0.34 0.58	212
Halomonas israelensis	2.0	-	1.14	-	213
Halomonas halodenitrificans	1.0 3.0	0.04 -	0.31 1.07	0.47 0.12	214
Pseudomonas halosaccharolytica	1.0 2.0 3.0	0.006 0.006 0.006	0.90 1.15 1.04	0.71 0.89 0.67	215
Micrococcus varians var. halophilus	1.0 4.0	- -	1.17 2.11	0.03 0.03	216
Bacillus haloalkaliphilus	3.4	-	3.52	0.31	217

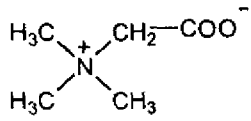
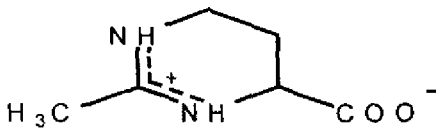
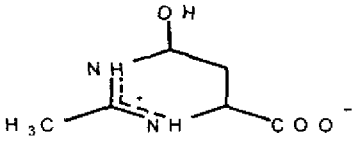
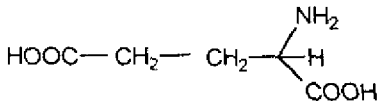
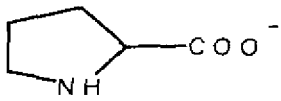
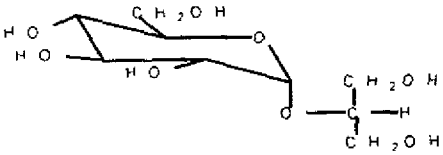
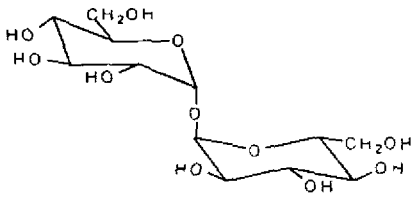
The osmotic balance in these halophiles such as *Halomonas halophila, Salinococcus roseus* is achieved by simultaneous accumulation of low molecular weight, polar, highly soluble (219-221) organic molecules termed as compatible solutes (Table 1.9) that are either synthesized or taken up from the growth medium. Ectione and its beta-hydroxy derivative are recently recognized as the most widely occurring compatible solutes in halophilic bacteria (208). The compatible solutes provide osmotic balance, without interfering with the metabolic functions of the bacterial cell (131,222,223). These compatible solutes form strong water structures around themselves and are excluded from the hydration shell of proteins This phenomenon of non-specific exclusion minimises entropy and reinforces hydrophobic effects thus preventing unfolding and denaturation of macromolecules (224-226).

In some halophilic eubacteria such as *V. costicola* (209) and *H. elongata* (218,219), the intra-cellular environment is hypo-osmotic with mechanisms to control water movements against its own gradient, by interaction with membranous and proteinous surfaces as suggested by Vreeland et al. (210). Alternatively, Patlack (220) and Stein (221) proposed independently the presence of two-membrane system of which one is osmotically tight, selectively permeable and the other, a loose osmotic membrane, permitting passage of water into a chambered region. Hydrostatic pressure built up in the chamber between the membranes ultimately forces the water against its own concentration gradient

1.6.2 Adaptations in cellular Proteins / Lipids of halophilic eubacteria

The halophilic eubacteria, besides adjusting the cytoplasmic osmotica, is also seen to modify its cellular protein, lipid components in response to extracellular sodium chloride.

Table 1.9 Compatible solutes in halophilic eubacteria

Compatible solute	Structure	*Organism*
Glycine Betaine		*Salinococcus roseus,* *Halomonas euryhalina* *Salinivibrio costicola*
Ectoine		*Micrococcus halobius,* *Marinococcus halophilus,* *Halomonas halophila* *Halomonas salina* *Pseudomonas* *Halosaccharolytica*
Hydroxy Ectione		*Micrococcus halobius* *Marinococcus albus*
Glutamate		*Halomonas halophila* *Halomonas euryhalina*
Proline		*Planococcus citreus* *Salinococcus hispanicus*
Glucosyl glycerol		*Pseudomonas mendocina*
Trehalose		*Micrococcus halobius* *Halomonas variabilis*

Proteins

The halobacterial proteins are similar to haloarchaeal proteins having moderately high content of acidic amino acids (227-229), which are intermediate to those in *E. coli* (non halophile) and *Haloferax mediterranei* (a haloarchaeon). Ventosa et al. (208) who studied the adaptation of halophilic eubacteria, divided their enzymes into three categories, based on their location in cell and exposure to salt concentration: 1) The intracellular enzymes such as pyruvate kinase (230), glycerol dehydrogenase, and malate dehydrogenase (231,232) which sense the true intracellular environment, show optimal activity at low salt concentration of 0.1 to 0.5 M, but are inactive above 1 M salt concentration. 2) The membrane bound transport proteins and ATPases (233,234) exhibit optimum activity at 2 M KCl or NaCl. Other membrane bound enzymes activated by high salt concentration, are lactate dehydrogenase and alkaline phosphatase (235). 3) The extracellular amylases (236,237) and nucleases (238), which are exposed to the external high salt concentration, show optimal activity between 1.4 to 3.2 M NaCl or KCl and are inactive below these salt concentrations.

Lipids

With increase in salt concentration from 0.5M to 3M, the amount of phosphotidyl ethanolamine (PE) in eubacterial membrane was reported to decrease with a concomitant increase in the amounts of negatively charged phosphotidyl glycerol (PG) and / or diphosphosphotidyl glycerol (CL) in *S.costicola* and *H.elongata* (210.239.240). This high content of negatively charged phospholipids is believed to occur at the expense of neutral phospholipids (239).

To explain the shift toward a higher negative charge density on the membrane, at increasing salt concentrations, it was postulated that the increase in the amount of anionic lipids served to allow charge balance at the membrane surface exposed to high Na^+ concentration (241). Another idea proposed was, the high content of negatively charged phospholipids might contribute to the regulation of the selective permeability of the membrane to cations (242). It is now assumed that the change in polar lipid composition provides a mechanism for preserving the membrane bilayer structure (208). PE containing unsaturated fatty acids tends to form non-bilayer-phase, while PG forms bilayers (243). A functional membrane has a suitable proportion of bilayer and non-bilayer forming lipids. Modification of the membrane phospholipid ratio is necessary to preserve the integrity of the membrane as PE has an increased tendency to form non-bilayer phases as a consequence of raised external salinity (244). The tendency to form non-bilayer-phase is expected to activate specific phospholipid synthesizing enzymes, which are located in the membrane, resulting in an increase in the proportion of lipids such as PG, to counteract the disruptive forces of the non-bilayer-phase forming lipids (240).

The adaptation of Gram-negative halophilic bacteria to growth in increasing concentrations of salt is also reflected in increase of cyclopropane fatty acids and unsaturated fatty acids with a decrease in the abundance of branched chain fatty acids (242,245-247). *H. halophila* showed an increase of 7 to 25% cyclopropane fatty acids with increase in salt concentration from 0.5 to 3.5 M, with a corresponding decrease in the content of monounsaturated fatty acids (246). An increase in unsaturated and cyclopropane fatty acids is expected to cause an increase in the membrane fluidity (243).

1.7 RESPONSE OF MICROORGANISMS TO HEAVY METALS

Urbanization and industrialisation of coastal environments resulted in contamination of these regions with a number of toxic heavy metal ions such as Hg^{+2}, Co^{+2}, Pb^{+2}, Cu^{+2}, Cd^{+2}, Zn^{+2}, Cr^{+6}, etc., due to atmospheric deposition and / or release of municipal and industrial wastes (248-250). The microorganisms present in these eco-niches are subject to intoxication and hence, the microflora either succumb or survive (251). Decrease in the number of microbial species have been noted in soils from sites, heavily contaminated with Cd^{+2}, Pb^{+2}, Zn^{+2} and Cu^{+2} as compared to soil from non-contaminated sites (252-255) of the same region and also in laboratory studies using specific microorganisms (255-257). Nieto et al. surveyed the metal tolerance in moderately halophilic eubacteria for Ag^{+}, As^{+5}, Cd^{+2}, Cu^{+2}, Co^{+2}, Cr^{+6}, Hg^{+2}, Ni^{+2}, Pb^{+2} & Zn^{+2}, and reported Minimum Inhibitory Concentration (MIC) values ranging from 0.01 mM (Hg^{+2}) to 20 mM (As^{+5}) (258). A number of culture collection strains and fresh isolates of haloarchaea were also reported to be resistant to heavy metals mentioned above. The MIC values ranged between 0.05 mM (Hg^{+2}) and 20 mM (As^{+5}) for Halococci (259), while with Halobacteria the range is between 0.01mM (Hg^{+2}) to 20mM (As^{+5}) (260). Biochemical activities of microorganisms such as photosynthesis (261,262); respiration (263); N_2 fixation (264,265); de-nitrification (266) and nitrification (267,268) are adversely affected by heavy metal pollutants. Various studies carried out in eubacterial forms at laboratory level, have indicated these eubacteria to resist metals via mechanisms of 1) efflux systems, 2) enzymatic detoxification, 3) bioaccumulation, 4) sequestration, etc (269). These generalised modes, however, exhibit metal specific modification in different bacteria, which are mediated either through chromosome or plasmid as listed in Table 2.0 (245).

Table 2.0 Mechanisms of copper resistance in Eubacteria

Species	Mechanism of resistance
a) Chromosome mediated	
Desulfovibrio sps	Complexing via production of sulphide
Klebsiella pneumoniae	Transient physiological adaptation
E.coli	Lack of a major outer *membrane protein* Binding and efflux
Vibrio alginolyticus	Chelation by secreting Cu binding proteins
K.aerogenes	Binding via capsular polysaccharide
Cyanobacteria	Metallothionin like protein
b) Plasmid mediated	
E.coli	Decreased OMP F protein Reduced Cu accumulation
Ps.syringae	Chelation by Cu binding proteins
Ps.syringae	Cu binding protein
Mycobacterium scrofulaceum	SO_4 dependent Cu precipitation

Compiled from (345)

1.7.1 Mechanisms of resistance of Cu^{+2} and Zn^{+2}

In *Alcaligenes eutrophus*, a soil bacterium, divalent cations of Zn^{+2}, besides Cd^{+2} and Co^{+2} (270-272) are resisted through plasmid-mediated system called 'Czc system' which is an efflux pump (270). This system consists of 3 units Czc A, Czc B and Czc C (Fig. 7a). The Czc A, an intermembrane transport protein is involved in pumping out the toxic ions, through a membrane fusion protein Czc B and an outer membrane protein Czc C (270).

Another efflux system '*Cut* system' is reported to be involved in Zn^{+2} as well as in Cu^{+2} resistance (273). It consists of two membrane bound uptake proteins *Cut A* and *Cut B*, two intracellular copper storage / carrier proteins *Cut E* and *Cut F* suspected to be responsible for protecting the bacterial cell from Cu^{+2} toxicity and for delivering Cu^{+2} to the sites of synthesis of copper proteins (Fig. 7b). *Cut C* and *Cut D* proteins believed to be ATPases, play a role in Cu efflux (274).

Recently, 'copper chaperone' coded by *Cop* operon, has been identified, to transport copper to the site of utilization in *Enterococcus hirae* (275). These copper chaperones prevent inappropriate Cu^{+2} interactions with other cellular components and ensure cellular safety (276,277).

Expression of the *Cop operon* is regulated by concerted action of the *Cop Y* repressor and *Cop Z*, a Cu^{+2} binding chaperone protein. When the cytoplasmic Cu^{+2} concentration rises, 2 *Cop Z* molecules specifically deliver their Cu^{+2} to *Cop Y*. The Cu^{+2} replaces the structurally required Zn^{+2}, thereby releasing *Cop Y* from the DNA (Fig. 8) and induces *Cop* operon (278). Copper delivery by chaperones appears to be a specific process involving a key-lock: *chaperone-target protein interaction*, which is transitory (278).

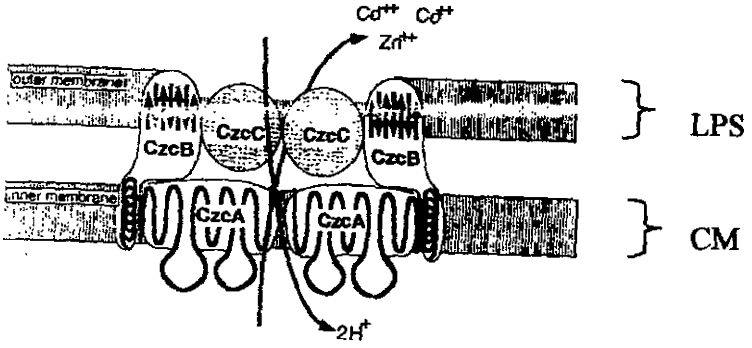


Fig.7a. Czc model for Cd^{+2}, Zn^{+2}, Co^{+2}, efflux system (270)

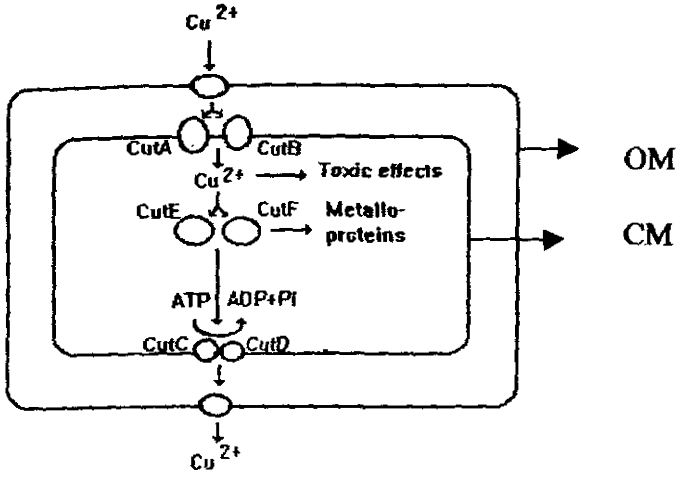


Fig.7b. Model for the functioning of chromosomal (*cut* genes) products involved in copper metabolism in *E.coli* (274)

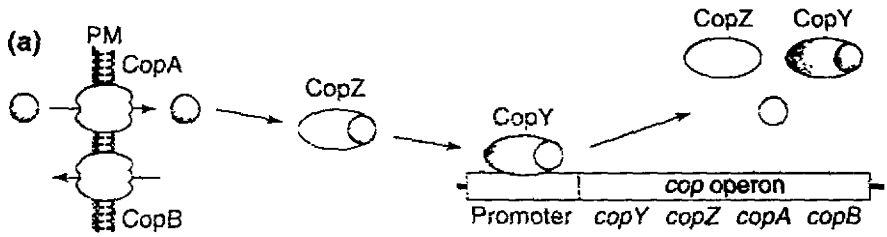


Fig.8. Copper chaperone functions in Cu^{+2} homeostasis in *Enterococcus hirae* (278)

AIM & SCOPE

Haloarchaea occur predominantly in salt marshes, salt lakes and salt pans (15,76). These estuarine regions experience natural fluctuations in prevailing physico- chemical conditions such as temperature, salinity, pH, availability of O_2, and nutrients etc (337) due to changes brought about by the periodic inundations of the estuarine reaches by the alternating flood and ebb waters. Further, Estuarine regions are increasingly being polluted especially with heavy metal ions due to heavy industrialization and by anthropogenic activities (248). Haloarchaeal inhabitants of these niches, therefore, would be exposed to and in turn respond to these changes.

Haloarchaeal isolates have been largely studied at 20% to 25% NaCl concentrations and at neutral pH (15). The strategies and the mechanisms employed by the haloarchaeal microorganisms to establish themselves under varying physico-chemical conditions and metal stress conditions have not been investigated at laboratory settings. A few studies (259,260) have reported the tolerance of haloarchaea to heavy metal ions.

For the first time in India, a microorganism capable of growing in media with 30% solar salt was isolated from an estuarine salt pan in Goa (340). Based on preliminary studies the culture was designated as *Halobacterium* strain R_1 (340 a). This culture was deposited in Microbial Type Culture Collection (MTCC), Chandigarh, India, as *Halobacterium* strain R_1 MTCC 3265. The culture shows the ability to utilize glucose (341) and also sodium benzoate as sole source of carbon (342) in mineral salts medium containing 20 % NaCl and devoid of any growth factors *Halobacterium* strain R_1 also is tolerant to a wide range of hydrocarbons such as benzene, toluene, xylene, hexane and crude oil components (343) and a number of heavy metals i.e. Cd^{+2}, Co^{+2}, Ni^{+2}, Fe^{+2}, As^{+5} etc (344).

The work presented in this thesis was directed at: determining the haloarchaeal growth and cellular characteristics of *Halobacterium* strain R_1 MTCC 3265 (HR), investigation of its cellular adaptation to set fluctuations in physico-chemical conditions of consecutive growth cycles and the evaluation of its response to metal ions.

CHAPTER 2: HALOARCHAEAL CHARACTERISTICS OF *HALOBACTERIUM* STRAIN R_1 MTCC 3265

Halobacterium strain R_1 MTCC 3265 was originally isolated by culturing a salt pan sediment in Tryptone Yeast Extract medium containing 30% solar salt (340) All microorganisms have inherent growth and cellular characteristics. Studies pertaining to elucidation optimal physico-chemical conditions for growth of *Halobacterium* strain R_1 MTCC 3265 and the determination of the cellular characteristics such as instability in water, presence or absence of glycerol diether moieties, diaminopimelic acid and bacterioruberin pigments, identifying its archaeal nature are presented in this chapter.

METHODOLOGY

2.1 CULTURE

2.1.1 Source and Maintenance

The estuarine Halophilic strain *Halobacterium strain* R_1, isolated and designated in the laboratory in an earlier study (340), was used. This culture is deposited in Microbial Type Culture Collection (MTCC, at IMTECH, Chandigarh, India) as *Halobacterium strain* R_1 (MTCC 3265) and referred as HR, hereafter

This culture was routinely maintained on agar slants of Tryptone Yeast Extract (TYE) medium containing 25% solar salt (NTYE) (Appendix I), at room temperature (RT, 28 – 30^{0}C) Hereafter, in this thesis, solar salt has been referred as salt.

2.1.2 Preparation of seed culture of HR

Seed culture of HR was prepared by inoculating a loop full of 6 d old culture from NTYE slants into 25 ml NTYE liquid medium in 150 ml Erlenmeyer flask and incubated at RT, at 150 rotations per min (rpm) on a rotary shaker. The orange-red growth obtained by 5th d was routinely used as 5 % inoculum for experimental work.

2.2 GROWTH OF HR

A 250 ml Erlenmeyer flask containing 50 ml of sterile NTYE broth was inoculated with 2.5 ml seed culture of HR. The flask was incubated at RT, on a rotary shaker (Remi, model RS 24), at 150 rpm. Growth was monitored by withdrawing aliquots under sterile condition at pre-decided time intervals and measuring the absorbance at 600 nm using a double beam UV - visible spectrophotometer (Shimadzu, model UV-240), against a medium blank.

2.2.1 Optimisation of physico-chemical conditions for growth of HR

Growth was also studied in the same medium but having pH of 4 / 5 / 6 / 7 / 8 / 9 / 10; or having 5 / 10 / 15 / 20 / 30% salt, or at different incubation temperature(s) of 37 / 45 / 55°C (maintained by Orbitek-LE environmental shaker equipped with a thermostat and a cooler). The generation times of the culture growing at each condition was calculated (279), separately.

2.2.2 Preparation of resting cells of HR

HR growing in 100 ml of NTYE medium was harvested after 96 h by centrifuging the culture broth at 8000 rpm for 20 min. at 10^0C using Remi cooling centrifuge, model C-24. The pellet obtained was separated from the debris of media, dispersed in 25 ml 20% NaCl mixed on cyclo-mixer to uniform suspension and centrifuged, as before. To ensure removal of growth medium components, the pellet was once again suspended in 25 ml of 20% NaCl and the process was repeated to obtain the cell pellet. This was re-suspended in 20% NaCl to uniform suspension of absorbance 1 or 2 at 600 nm. These resting cells were stored at 10^0C and used as and when required within 4 d.

2.3 DETERMINATION OF CELLULAR CHARACTERISTICS OF HR

2.3.1 Gram reaction of HR

Gram reaction of HR was studied by modified Gram staining method for halophiles (280). An even smear made on a clean, grease free, dry, glass slide was air dried and fixed in 2% acetic acid for 3 min. The fixed smear was air dried and flooded for 30 sec with 2% crystal violet solution (Appendix II). Subsequently, it was replaced with Grams iodine (Appendix II) for next 30 sec. thereafter, the smear was decolourised with 70% absolute alcohol and exposed for 1 min to 0.5% saffranine (Appendix II), washed and air-dried. The slide was then observed under oil immersion lens using a phase contrast microscope (Olympus model BX 40).

2.3.2 Stability of whole cells of HR in water

Aliquots of 2 ml of resting cells (A_{600} ~1) were taken in an eppendorf tube and centrifuged in Beckman microfuge at, 14,000 rpm at, 4^0C for 10 min to obtain a tight pellet. The pellet was then suspended in 2 ml deionized water, mixed on cyclo-mixer, transferred to a spectrophotometric cuvette rapidly within 10 sec and the absorbance was monitored at 600 nm, over a period of 10 min at intervals of 15 sec.

2.3.3 Detection of Diaminopimelic acid (DAP) in cells of HR

Hydrolysis

100 ml of resting cells of HR (A_{600}~ 2) were centrifuged at 8000 rpm for 20 min to obtain a pellet. The pellet was suspended in 3 ml of 6 N HCl in a glass Pyrex tube with stopper and was hydrolysed at 100^0C in a boiling water bath for a period of 16 h (282). Resting cells *E.coli* and *S.aureus* of absorbance 2 at 600 nm obtained by growing separately in nutrient broth were treated in a similar way to serve as controls.

Ascending paper chromatographic analysis of hydrolysates

Presence of DAP was sought specifically by analysing the hydrolysates by paper chromatography using the solvent system of Rhuland et al. (283). Each of the hydrolysate was spotted, separately at a distance of 2 cm from one of the long end of a rectangular chromatographic paper, 160 X 100 X 3 mm (thickness) using fresh glass capillaries of 1mm bore size.

The paper was suspended into Methanol : Water : 10 N HCl : Pyridine (80 : 17.5 : 2.5 : 10 v/v/v/v) contained in a rectangular glass chamber of 200 X 100 X 150 mm and allowed to run for ~40 min. Care was taken to ensure the pre-saturation of the chamber and that the hydrolysate spots were not immersed into the solvent system. The air-dried, chromatogram was then sprayed with 0.1% ninhydrin in acetone and heated in the oven, at 110^0C to develop the spots, if any.

2.3.4 Determination of hydrophobicity of whole cells of HR

Hydrophobicity of HR cells was determined by measuring the percent (%) affinity to hydrocarbon as employed in the Microbial Adhesion To Hexadecane (MATH) assay of Rosenberg et al (281). Resting cells of HR were taken in a flat-bottomed cuvette of a colorimeter (Elico) and adjusted to optical density (OD) 1 using filter No. 60. This OD was designated as A_1. The cuvette was taken out of sample chamber of the colorimeter and the column of cell suspension was layered with 0.3 ml of n-hexadecane, mixed thoroughly on a cyclo-mixer for a min and allowed to stand at RT for 30 min. On separation of the phases (visually), the cuvette was transferred to the sample chamber, with maximum care and minimum disturbance of phases. The OD reading was recorded as A_2. Hydrophobicity of HR cells was inferred using the generalization

$$\text{Percent decrease in absorbance} = \frac{A_1 - A_2}{A_1} \times 100 = \% \text{ Hydrophobicity}$$

2.3.5 Whole cell acid methanolysis

200 mg of freeze dried HR or *E.coli* cells (Labconco 4.5 Freeze Drier) were mixed with 6.2 ml of methanol, toluene and concentrated H_2SO_4 mixture (3 : 3 : 0.2 v\v\v) in separate. Teflon coated, screw capped glass tubes and heated in a water bath at 56^0C for 18 h. 1.5 ml aliquots of hexane was then added to each of the tube, contents transferred to a separating funnel, and mixed vigorously by shaking. The hexane layers were collected on phase separation and concentrated separately under a stream of N_2 gas. Each of the hexane concentrates were spotted separately on a silica gel H plate and developed in Petroleum ether (boiling range 60^0C to 80^0C): Diethyl ether (85 : 15), air dried and visualized by iodine vapours.

2.3.6 Analysis of pigment of HR

Extraction of the pigment

10 ml of resting cells (A_{600} ~ 2) of HR were centrifuged as described earlier. The pellet thus obtained was dispersed into 3 ml of 20% NaCl and transferred to a 25 ml glass beaker along with 5 ml of acetone. The beaker was placed in an ice bath and the cells were sonicated at 100 mA (8 pulses of 15 sec duration, with 15 sec cooling time) using a sonicator (Vibronics).

Spectrophotometric analysis of the pigment extract

The acetone extract free of cell debris was scanned between 190-700 nm against an acetone blank, using UV-visible spectrophotometer

2.3.7 Isolation and characterisation of lipids of HR

Large scale preparation of whole cells

Seven Erlenmeyer flasks of 2 L capacity each, containing 700 ml NTYE liquid medium were grown for 96 h and pelleted as described earlier. After determining the wet weight, the entire pellet was used for extraction of lipids.

Extraction of lipids

From the HR cells, whole cell lipids were extracted following the method of Bligh and Dyer, modified for halophiles (284) as presented schematically in (Fig. 9).

Separation of component lipids by column chromatography

Preparation of column: Silicic acid of mesh size 100 - 325 (Hi-media) was taken in a dry beaker and activated in the oven at 100^0C for 4 h, and cooled in a desiccator. 4 g of this was mixed with n-hexane in a 50 ml beaker to form slurry. The slurry was poured into a clean dry glass column of 300 x 15 (mm). Uniform packing of silicic acid was ensured by gently tapping the sides of the column with a rubber rod. The packed column was then equilibrated by passing n-hexane through it for 3 h at a flow rate of 1.7 ml / min.

Loading and elution of samples: The lipid sample of 98 mg dissolved in 0.5 ml $CHCl_3$ was loaded on to the column using a Pasteur pipette, to form a uniform band and n-hexane (100 ml) continued as mobile phase. This was followed by 100 ml benzene, 50 ml chloroform, 75 ml chloroform methanol (2:1v\v), and finally 50 ml methanol. Fractions each of 3 ml were collected and checked for the presence of carbon by spotting on silica gel and exposing to iodine vapours. Fractions showing the presence of carbon

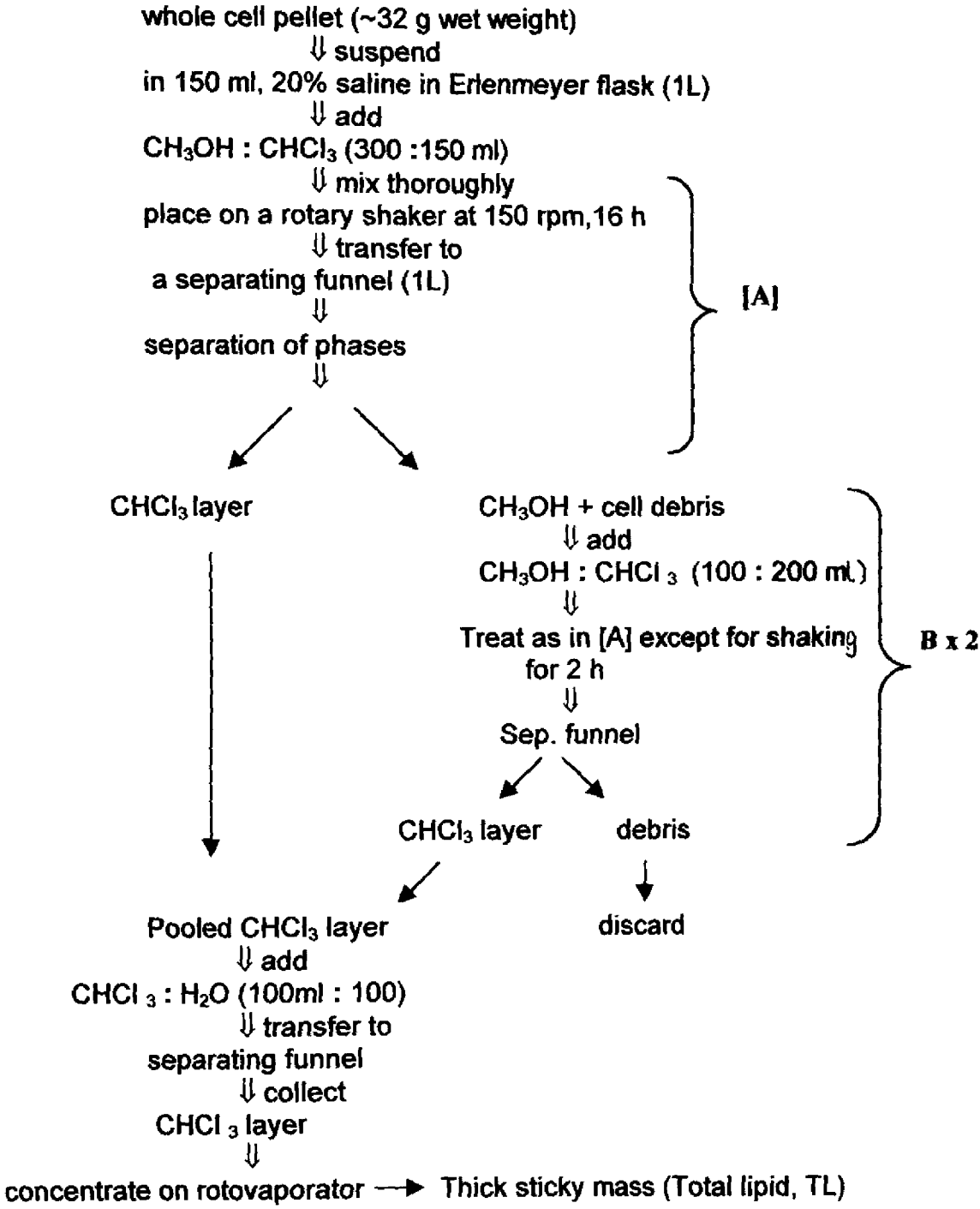


Fig 9. Extraction of whole cell lipids of HR

were retained and analysed individually by TLC. The fractions showing similarity, with respect to the spots and their Rf, were pooled and concentrated by evaporating the solvent with N_2 gas, re-checked for purity by TLC before further analysis.

Analysis of components of lipid/s by TLC:

Samples were applied using fine capillaries, at a distance of ½ cm above the edge of the long side of rectangular plates, quoted with silica (Appendix-III) and were developed in pre-saturated rectangular solvent chambers containing appropriate solvent systems The developed chromatograms were air-dried and the separated components were visualized either on exposure to iodine vapours or by using specific spray reagents. The preparation of spray reagents is described in the Appendix-III.

Spectral analysis of lipid/s:

The TLC-wise pure components were analysed for their characteristics in UV region by Shimadzu UV-240, UV-visible spectrophotometer and in the IR region by IR spectrophotometer Shimadzu FTIR 8101A.

RESULTS

2.4 GROWTH OF HR IN NTYE MEDIUM

HR, on inoculation from a slant into NTYE agar, grew as reddish orange culture by 6 d (Plate 2.1) Inoculation of culture into NTYE broth resulted in increase in turbidity, reaching an absorbance of 1.82 at 600 nm and attained a light orange tinge after 3 d of incubation. The light orange tinge increased in intensity and became dark orange-red on incubation up to 6 d (Plate 2.2).

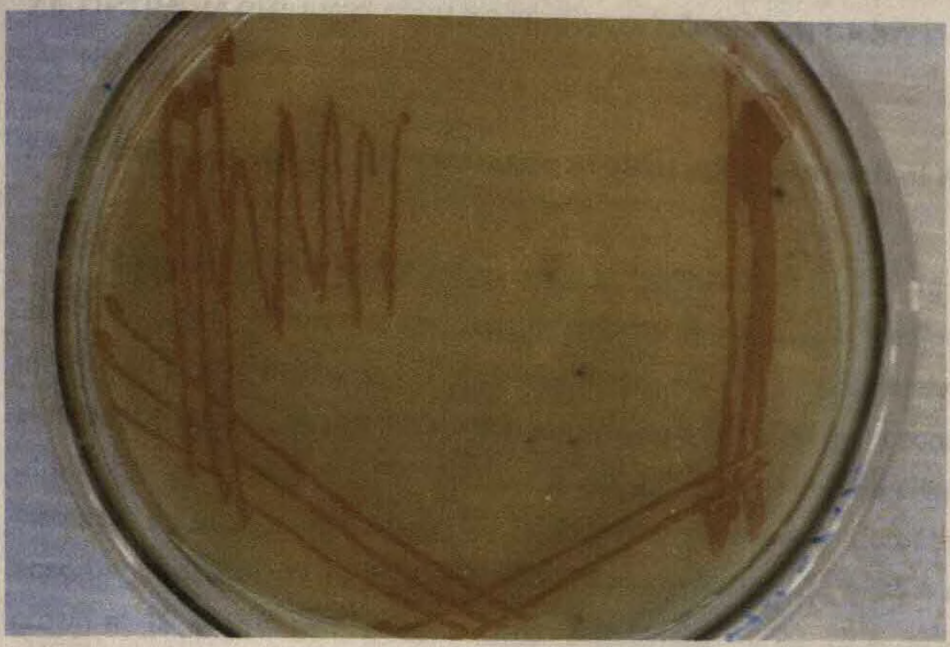


Plate 2.1. Growth of HR on NTYE agar

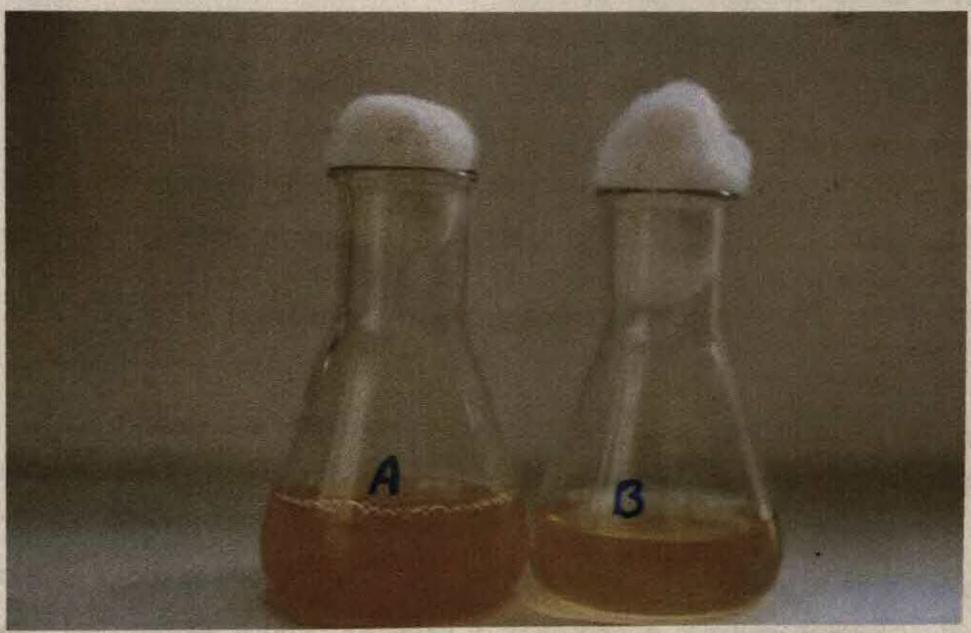


Plate 2.2. Growth of HR in NTYE liquid medium
A) HR culture (6 d)
B) NTYE (uninoculated)

2.5 OPTIMUM PHYSICO-CHEMICAL CONDITIONS FOR GROWTH OF HR

2.5.1 Effect of varying concentration of solar salt on growth of HR

Inoculation of seed culture of HR into medium with 5 / 10 / 15 / 20 / 25 / 30% solar salt showed changes in turbidity of the culture medium, on incubation at RT, at 150 rpm. Aliquots of these, withdrawn separately from individual flasks at regular time intervals, showed changes in absorbance at 600 nm as depicted in Fig. 10. At 5% salt concentrations there was no increase in absorbance up to 6 d The culture showed increases in absorbance from 0.11 to 1.28 and from 0.12 to 1.28 by 120 & 96 h when grown at 10 & 15% salt concentrations respectively. At 20, 25 & 30% salt concentrations, the absorbance increased from 0.12 to 1.98; 0.12 to 2.01 & 0.14 to 1.74 respectively. As recorded, in Table 2.1 HR grew with an initial lag of 36 & 24 h in NTYE with 10% and 15% salt concentration respectively. At 20, 25 or 30 % salt, the culture grew within first 12 h of incubation. HR as depicted in Fig. 10, grew exponentially up to 108 h at 10%; 96 h at 15, 20 & 30% and 72 h in case of 25% salt. The culture had a minimal doubling time of 14.6 h at 25% and a maximum of 22.8 h at 10% salt concentration as recorded in Table 2.1.

2.5.2 Effect of pH of medium on growth of HR.

To study the response of culture to pH of growth medium, HR was inoculated separately into NTYE medium, either at pH 4 / 5 / 6 / 7 / 8 / 9 / 10. At pH 4 or 10, there was no increase in absorbance up to 120 h in culture broth of HR. At pH 5 or 9, HR showed a lag phase of 48 h, which was followed by a rapid increase in absorbance reaching a maximum of 1.24 & 1.602 by 108 and 120 h respectively (Fig.11). A minimal incubation of 12 h was required for initiation of growth at pH 6 or 7 where as, at pH 8, initiation of growth was by 36 h. As depicted in Fig. 11, the exponential

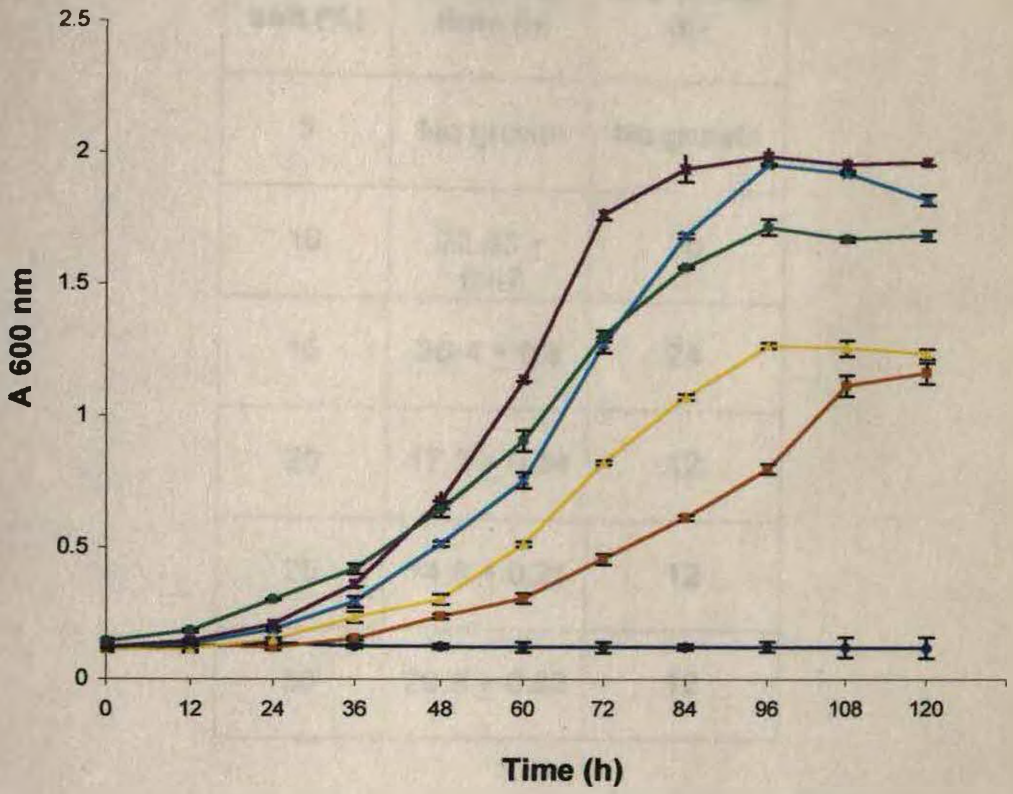


Fig. 10 Growth of HR in NTYE (pH 7; RT) with solar salt

Table 2.1 Effect of concentration of solar salt on growth of HR in NTYE

Solar salt (%)	Doubling time (h)	Lag phase (h)
5	No growth	No growth
10	22.83 ± 0.47	36
15	20.4 ± 0.8	24
20	17.3 ± 0.34	12
25	14.6 ± 0.25	12
30	20.8 ± 0.82	12

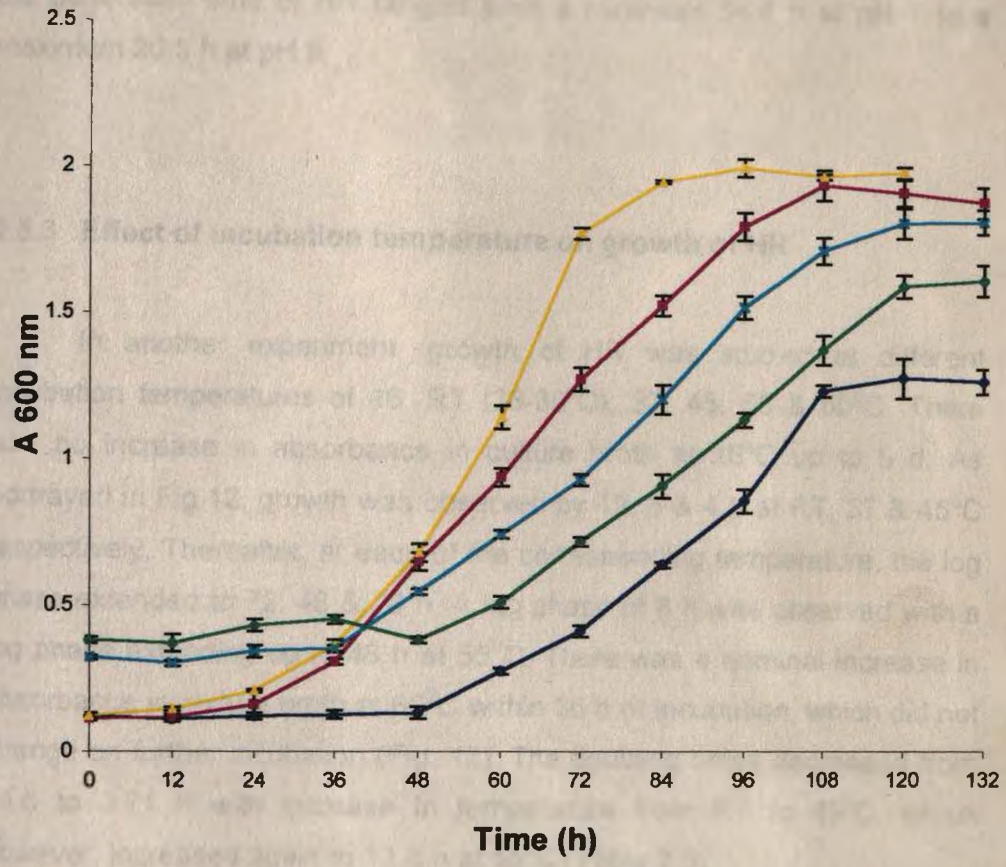


Fig. 11 Growth of HR in NTYE (25% salt; RT) at pH

—◆— 5, —■— 6, —▲— 7, —✕— 8, —◆— 9

phase extended up to 96, 72 & 108 h reaching an absorbance of 1.80, 1.852 & 1.752 at pH 6, 7 & 8 respectively. Further, as noted in Table 2.2, the generation time of HR ranged from a minimum 14.6 h at pH 7 to a maximum 26.5 h at pH 9.

2.5.3 Effect of incubation temperature on growth of HR

In another experiment, growth of HR was studied at different incubation temperatures of 16, RT (28-30°C), 37, 45, 55 & 60°C. There was no increase in absorbance in culture broth at 16°C up to 5 d. As portrayed in Fig.12, growth was observed by 12, 8 & 4 h at RT, 37 & 45°C respectively. Thereafter, at each of the corresponding temperature, the log phase extended to 72, 48 & 28 h. A lag phase of 8 h was observed with a log phase extending up to 48 h at 55°C. There was a nominal increase in absorbance in culture broth at 60°C within 36 h of incubation, which did not change on further incubation (Fig. 12). The doubling times decreased from 14.6 to 3.71 h with increase in temperature from RT to 45°C, which however, increased again to 11.8 h at 55°C (Table 2.3).

2.6 HALOARCHAEAL CELLULAR CHARACTERISTICS OF HR

Six d old cells of HR, grown at RT, in NTYE medium having 25% salt concentration and pH 7 were used to determine the cellular characteristics such as Gram character, morphology, hydrophobicity, effect of water, pigmentation, and whole cell lipids. The observations and data obtained thereof, is described below.

Table 2.2 Effect of pH on growth of HR in NTYE

pH	Doubling time (h)	Lag phase (h)
4	No growth	No growth
5	20.02 ± 0.94	48
6	14.9 ± 0.45	12
7	14.6 ± 0.25	12
8	20.99 ± 0.18	36
9	26.46 ± 0.41	48
10	No growth	No growth

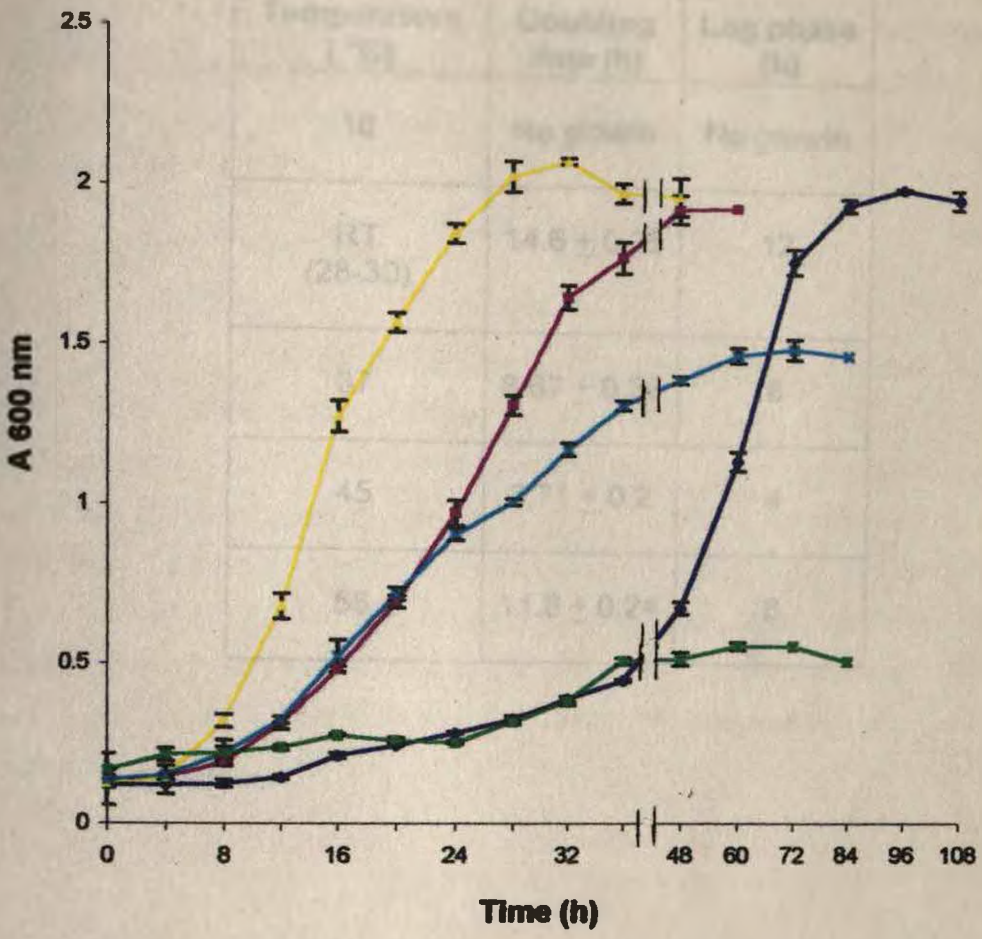


Fig. 12 Growth of HR in NTYE (25 % salt; pH 7) at

RT 37°C, 45°C, 55°C, 60°C

Table 2.3 Effect of temperature on growth of HR in NTYE

Temperature (°C)	Doubling time (h)	Lag phase (h)
16	No growth	No growth
RT (28-30)	14.6 ± 0.25	12
37	8.67 ± 0.39	8
45	3.71 ± 0.2	4
55	11.8 ± 0.24	8

2.6.1 Morphological characteristics

Cells of HR suspended in 20 % NaCl and observed under phase contrast microscope appeared to be short rods. The same on staining by the modified Gram staining procedure for halophiles appeared to be Gram-negative (Plate 2.3).

2.6.2 Lysis of HR in water

Cells of HR suspended in deionized water showed an immediate, rapid decline in absorbance at 600 nm. As seen in Fig. 13, absorbance declined from 1 to 0.028 within the first 15 sec of exposure to deionized water and remained almost the same up to 10 min. The first reading of absorbance was after 15 sec of exposure to deionized water, as this was the time period standardized to suspend the pellet and transfer to the spectrophotometric cuvette after mixing for 10 sec on a cyclo-mixer. Cells suspended in 20% NaCl to an absorbance 1 showed no change in absorbance at RT.

2:6.3 DAP analysis

Acid hydrolysates of Cells of HR on analysis by paper chromatography and visualization with ninhydrin at 110^0C gave a dark purple spot at Rf 0.71 and two light purple spots at Rf 0.63 & 0.44 Hydrolysates of cells of *E.coli* and *S.aureus*, used as eubacterial controls showed an elongated olive green spot at Rf 0.27 in addition to the 3 spots shown by hydrolysate of HR Authentic DAP run on the same chromatogram resolved at 0.27 Rf as an elongated olive green spot which turned yellow on cooling and is seen in Plate 2.4.This spot at Rf 0.27. corresponding to DAP is absent in the hydrolysate of HR (Plate 2.4).

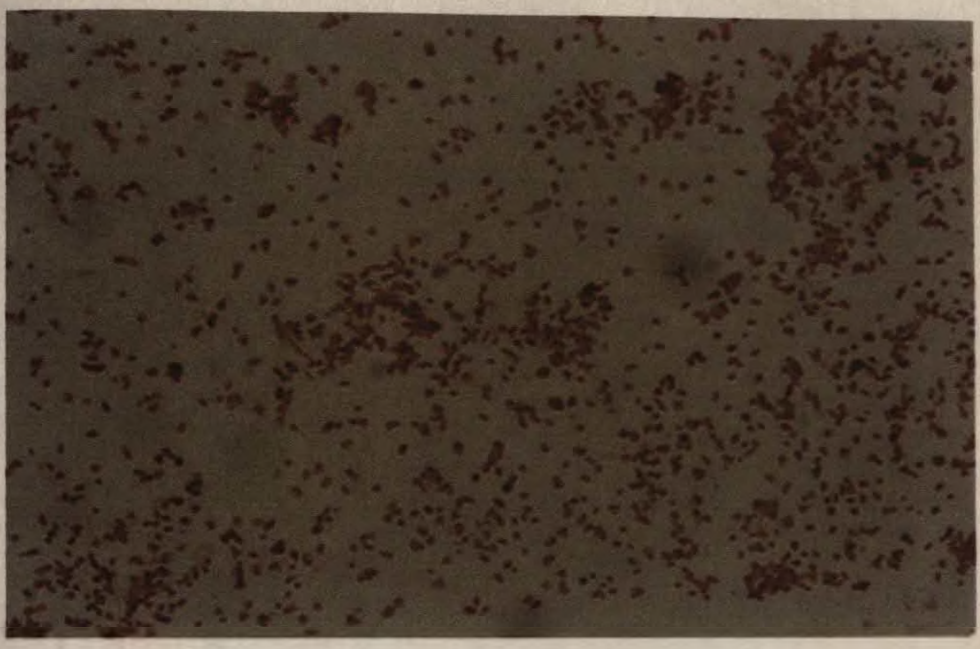


Plate 2.3. Morphology and Gram character of HR (1250 *x*)

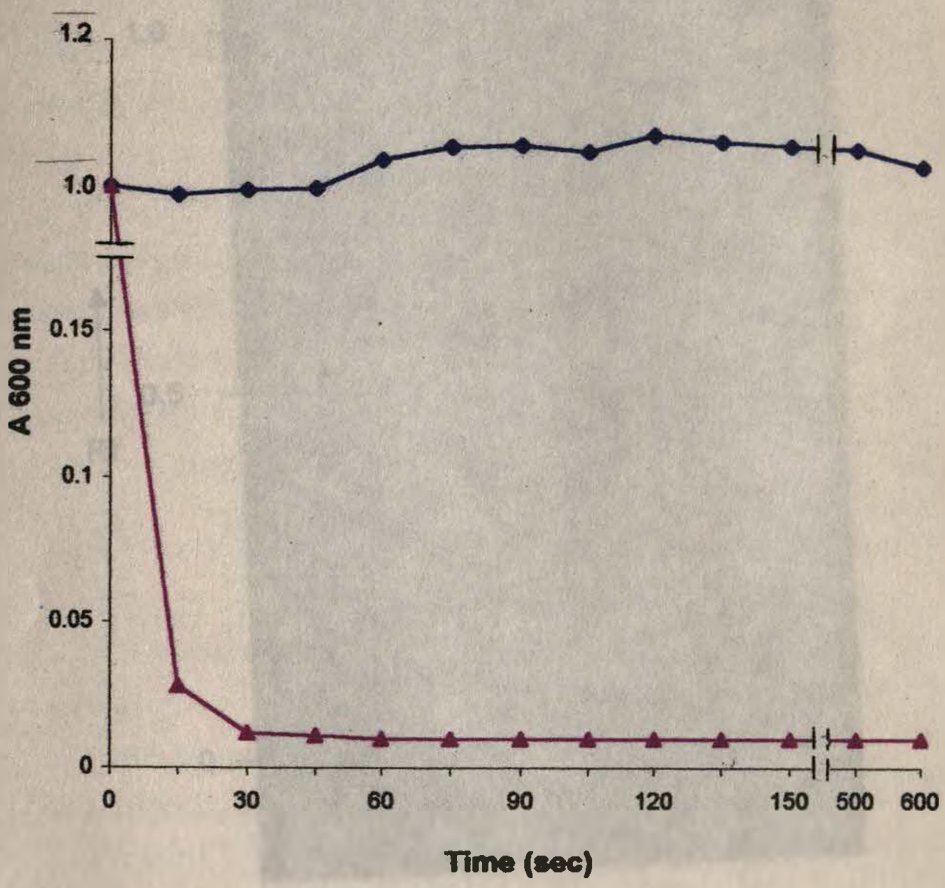


Fig. 13 Absorbance of resting cells of HR suspended in:

—●— 20% NaCl, —▲— water

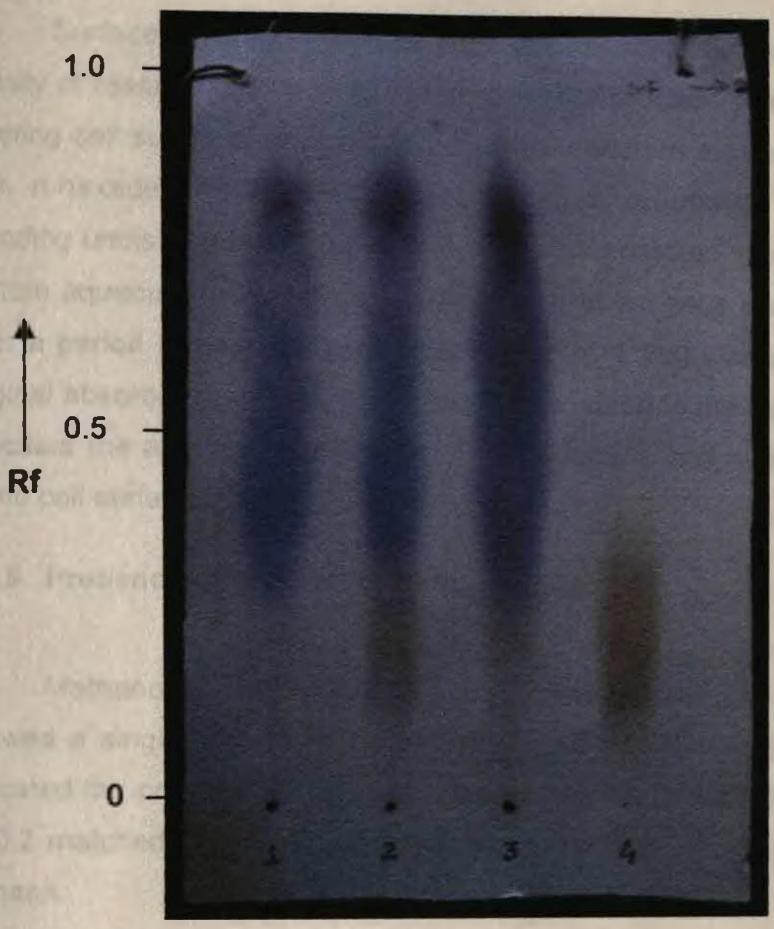


Plate 2.4. Paper chromatogram of whole cell hydrolysates of

1. **HR**
2. ***E.coli***
3. ***S. aureus***
4. **Authentic DAP**

2.6.4 Surface hydrophobicity

Surface hydrophobicity of HR was studied by determining the affinity of resting cells to n-hexadecane, by the procedure of MATH assay. Resting cell suspension of HR in 20% NaCl taken in a tube when layered with n-hexadecane on mixing, resulted in a column of emulsion. On standing undisturbed for 20 min, the contents separated into 2 phases: the bottom aqueous and top organic. Monitoring of aqueous phase at 600 nm over a period of time, gave an absorbance of 1 that was identical to the original absorbance of resting cell suspension used in the experiment. This indicates the absence of affinity to n- hexadecane and hydrophilic nature of the cell surface of HR.

2.6.5 Presence of glycerol diether moieties

Methanolic hydrolysate of whole cells of HR on TLC analysis showed a single spot at Rf 0.2. Methanolysate of *E coli* used as control indicated the presence of one single spot of Rf 0.8 (Plate 2.5). The spot at Rf 0.2 matched with reported Rf for glycerol diether moieties observed in archaea.

2.6.6 Pigment analysis

NTYE liquid medium inoculated with seed culture of HR attained an orange tinge after 72 h of incubation at RT at 150 rpm and intensified to dark orange colour by 120 h (Plate 2.2). Centrifugation of the culture broth at 8000 rpm for 20 min at 16°C resulted in an orange coloured pellet of cells of HR and a clear yellowish supernatant, which resembled the NYTE medium.

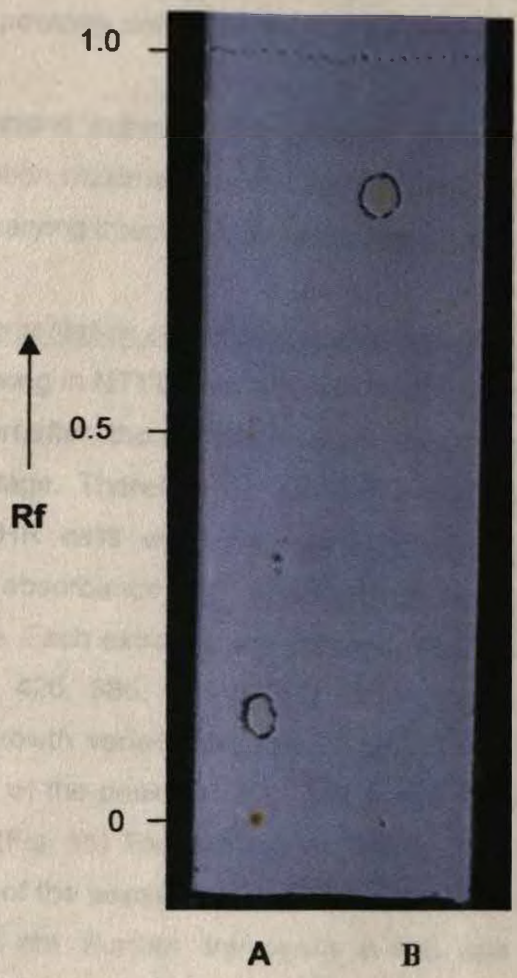


Plate 2.5. Thin layer chromatogram of acid methanolysates of whole cells :
A) HR
B) *E. coli*

The cell pellet on dispersal into acetone and subjection to sonication resulted in release of orange colour into the organic solvent, with a whitish powdery debris settling at the bottom of beaker.

The acetone extract when scanned between 190-700 nm gave 8 distinct absorption maxima at 600, 528, 494, 468, 426, 386, 368 & 350 nm (Fig. 14) with varying intensities as listed in the Table 2.4.

Progress in pigmentation of HR cells during growth

HR growing in NTYE medium was visually colourless up to 72 h and orange red thereafter, though the medium became fully turbid with growth prior to this stage. Therefore, to study the progress of development of pigmentation, HR cells were harvested at every 24 h during growth, adjusted to an absorbance of 2, at 600 nm and individually extracted with 5 ml of acetone. Each extract scanned separately, showed 8 peaks at 600, 528, 494, 468, 426, 386, 368 & 350 nm. However, the intensity of the peaks during growth varied. Extracts of cells, up to 48 h of age showed higher intensity of the peaks at 350, 368 & 386 nm compared to the 468, 494 & 528 nm (Fig. 15) The extracts of cells of 72 & 96 h of age, showed higher intensity of the peaks at 468, 494 and 528 nm compared to those at 350, 368 & 386 nm. Further, the peaks at 468, 494 & 528 nm gradually increased in intensity, in extracts of 120 & 144 h old cells as compared to those in extracts of 96 h old cells (Fig. 15).

2.6.7 Lipid analysis

Total lipids

HR culture grown in NTYE medium, on extraction by the modified Bligh and Dyers method and recovery in chloroform yielded a total lipid of 293 mg corresponding to 3.9 % on whole cell dry weight basis.

Fig. 14 Spectrum of acetone extract of HR cells grown in NTYE

Table 2.4 Intensities of peaks of pigment of HR grown in NTYE

Peak λ (nm)	Absorbance
600	0.04
528	0.28
494	0.35
468	0.29
426	0.19
386	0.22
368	0.2
350	0.18

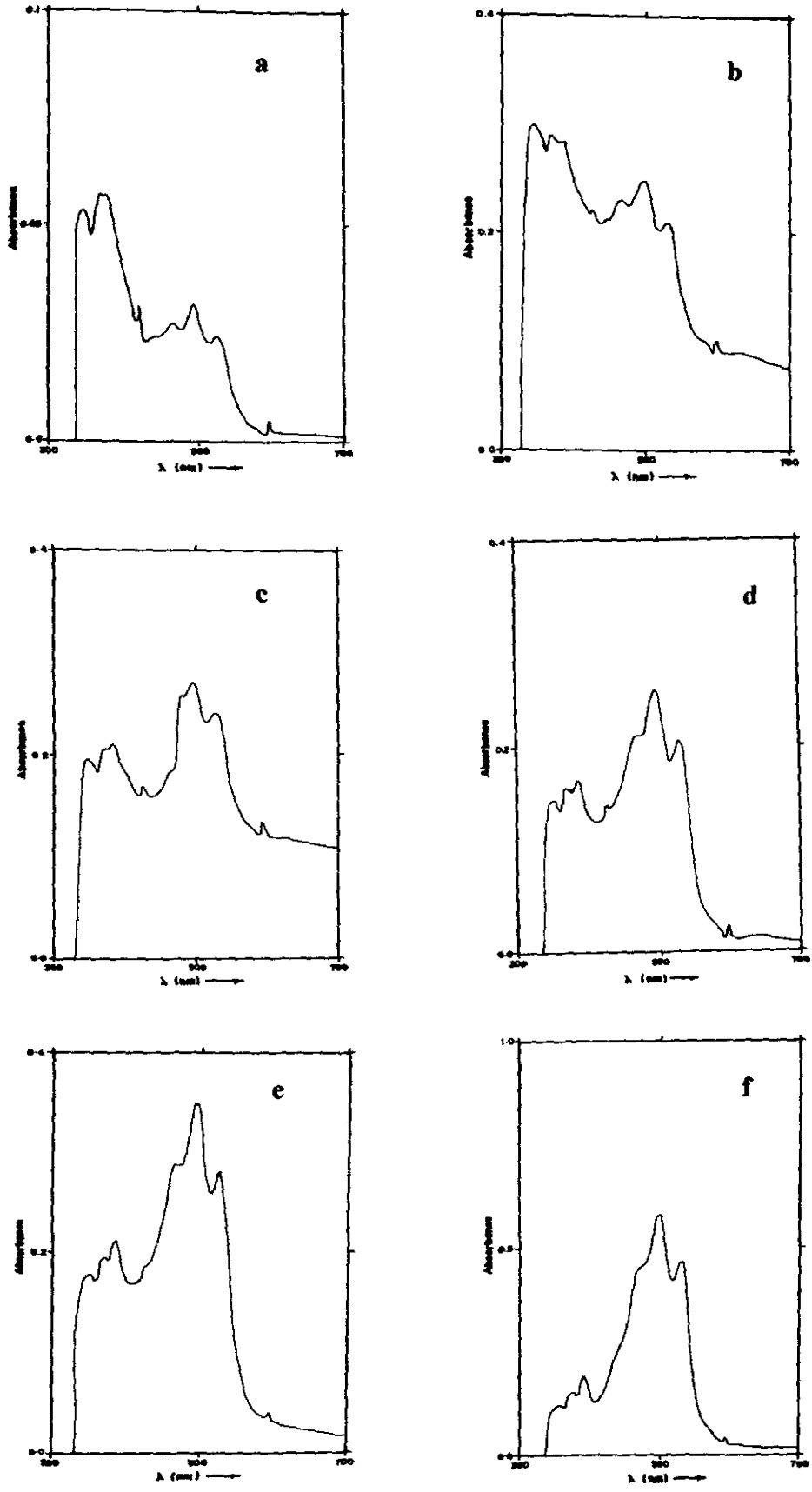


Fig.15. Pigment profiles of HR cells at *different* stages of growth
a) 24 h b) 48 h c) 72 h d) 96 h e) 120 h f) 144 h

Chloroform extract of total lipids (TL) spotted on a silica gel H plate and developed in Petroleum ether : Diethyl ether : Acetic acid (90 : 10 : 1 v/v/v) system resolved into 6 spots at Rf 0.82, 0.71, 0.54, 0.32, 0.2, and 0.1 on exposure to iodine vapours (Fig. 16). Spectrophotometric analysis of chloroform extract of TL revealed 7 prominent peaks at 520, 496, 462 386, 368, 330 & 270 nm as recorded in Fig.17.

Addition of chilled acetone to chloroform extract of TL, at a ratio of 10:1, resulted in a clear orange-red acetone layer with a yellowish particulate, precipitate remaining behind in the lower chloroform layer, in 16 h at -20^{0}C. The orange fraction in acetone, on concentration, weighed 98 mg and was designated as A_1 fraction.

The yellowish particulate precipitate washed with chilled acetone, weighed 184 mg and was designated as A_2 fraction.

Spectrophotometric characterization of crude A_1 and A_2

The UV spectrum of A_1 in chloroform (Fig. 17), showed 6 peaks at 520, 496, 462, 386, 368 & 330 nm respectively. The IR spectral analysis of A_1, as seen in Fig. 18, showed peaks at 2850 to 2960 cm^{-1} & 1315 and 1460 cm^{-1} of alkyl groups; at 1700 cm^{-1} of carbonyl groups; at 1600 cm^{-1} of C=C groups; at 1260 cm^{-1} of aromatic groups; at 1020 to 1100 cm^{-1} of ortho substituted aromatic groups and at 800 cm^{-1} of olefinic C=CH groups

The A_2 fraction showed a broad absorption peak between 260 - 300 nm on UV spectral analysis (Fig. 17). The IR spectrum of A_2 in Fig. 19, revealed broad band between 3600 - 3200 cm^{-1} of hydroxyl groups; 2850 to 2960 cm^{-1} & 1315 and 1460 cm^{-1} of alkyl groups; 1120 cm^{-1} of ether linkages; 1220 cm^{-1} of P=O groups; 1060 cm^{-1} of P-O-C groups and weak absorption bands at 1250 for SO_4 groups and 760 to 810 cm^{-1} of olefinic C=CH, groups respectively.

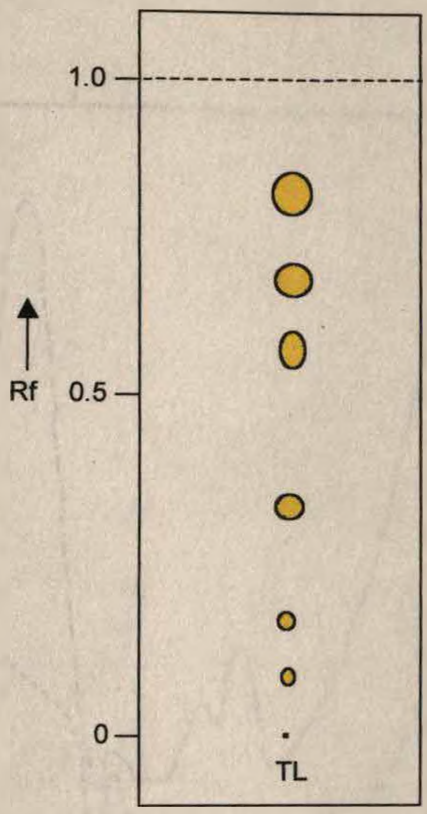


Fig. 16 Thin layer chromatogram of total lipids

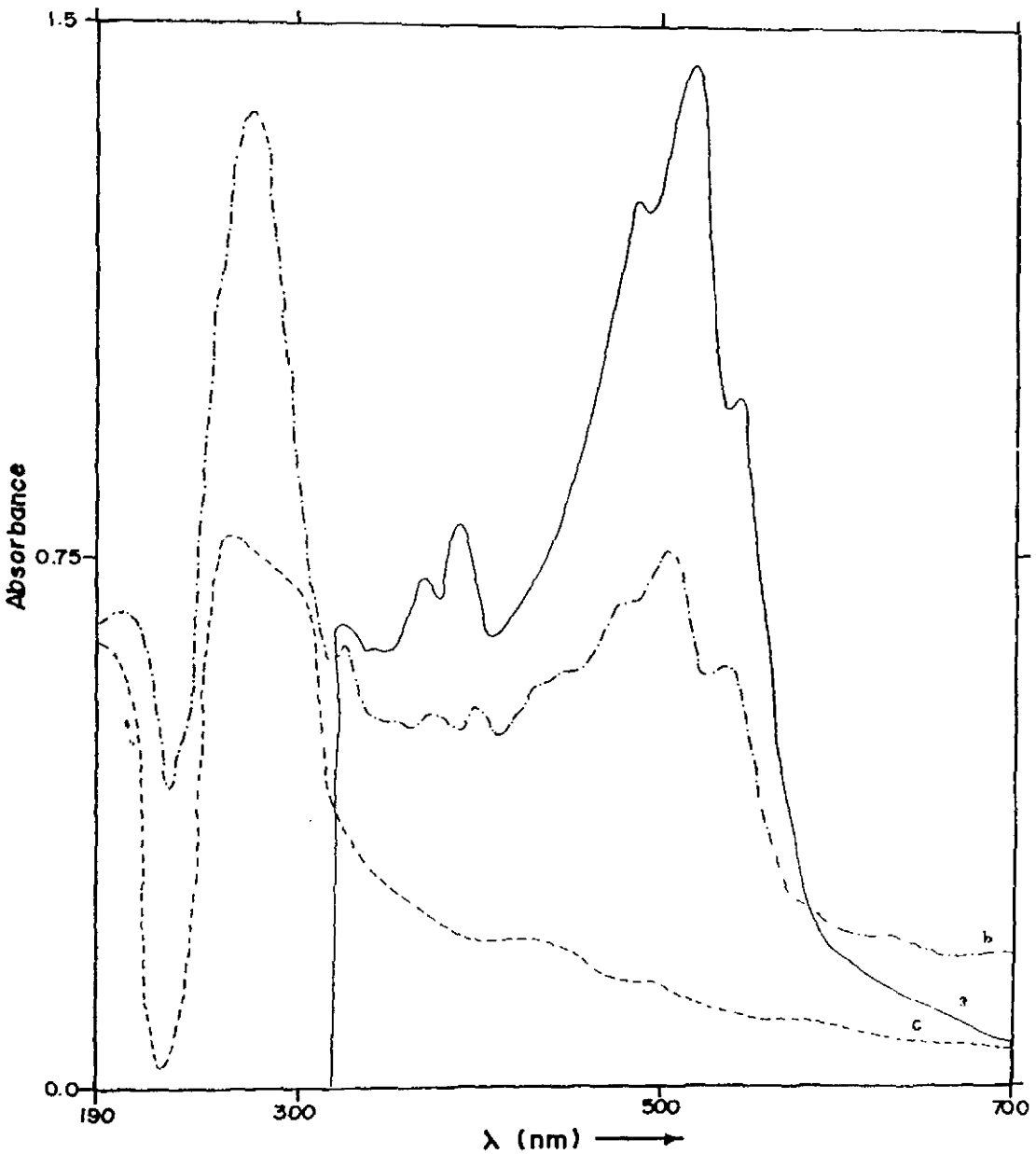


Fig. 17 Spectra of total, acetone soluble and insoluble lipid fractions of HR
a) Total lipids b) A_1 fraction c) A_2 fraction

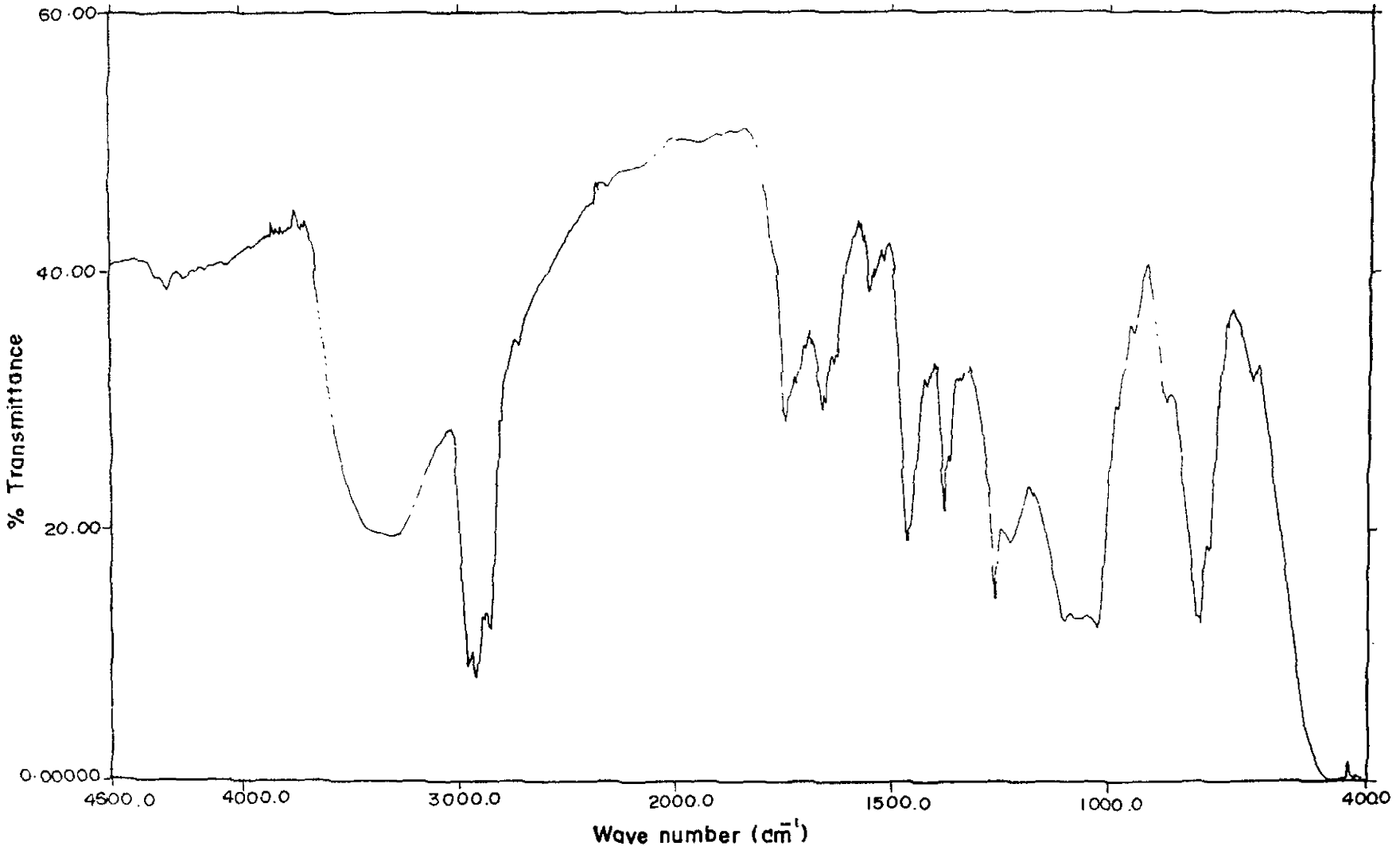


Fig. 18 IR Spectrum of A_1 fraction of total lipids

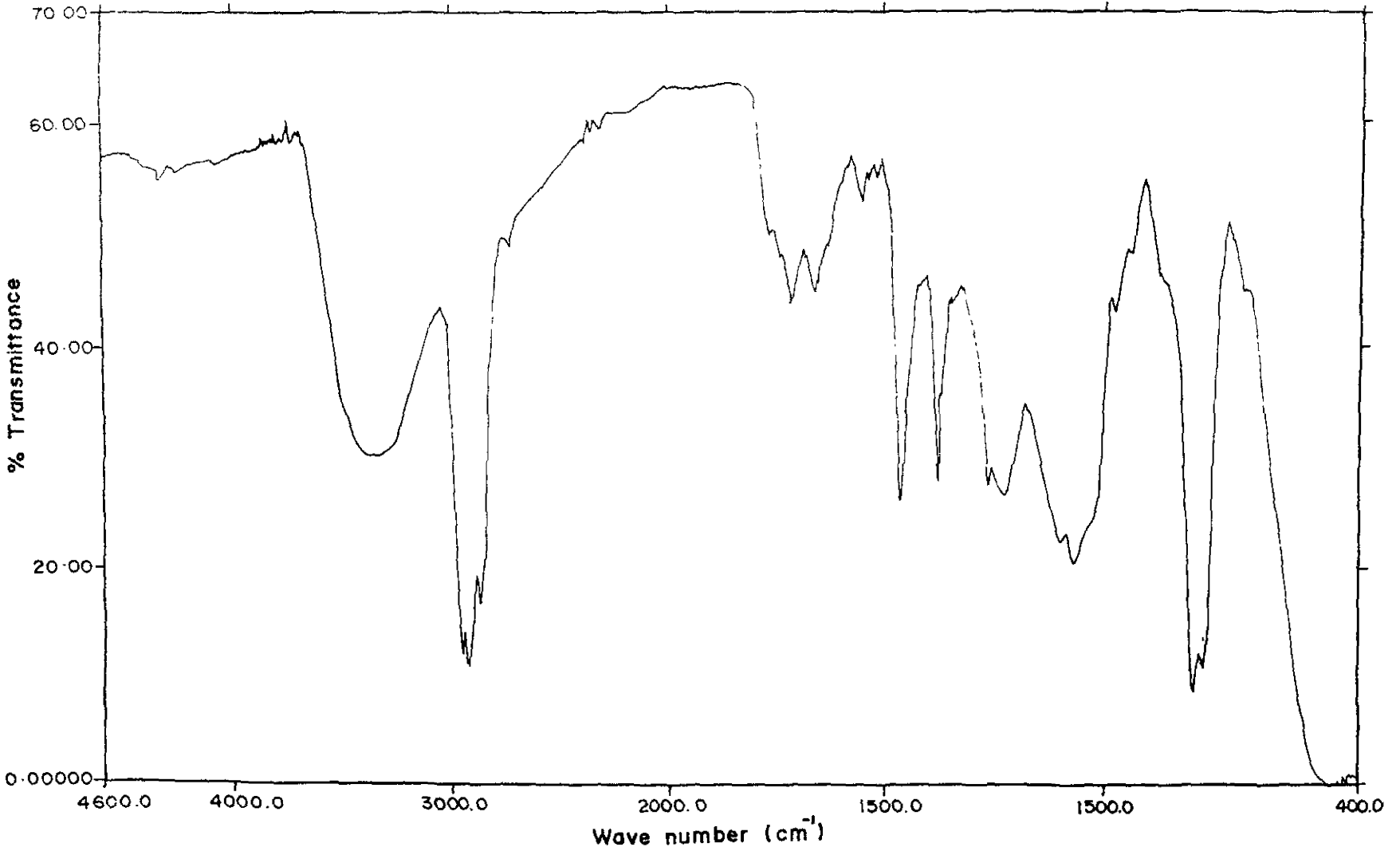


Fig. 19 IR Spectrum of A_2 fraction of total lipids

Thin layer Chromatographic analysis of A_2

The A_2 spotted on silica gel 'G' plates and developed in a chloroform : methanol : acetic acid : H_2O (80 :22.5 : 10 : 4 v/v/v/v) on exposure to iodine vapours revealed four spots designated as P_1 at Rf 0.78; P_2 at Rf 0.64; P_3 at Rf 0.56 and P_4 at Rf 0.46 (Fig. 20).

The four components developed a bluish green colour on spraying with dodecamolybdic acid reagent (Appendix-II) and heating at 110°C for 10 min. P_1 and P_3 closely matched with Archaetidylglycerol and Archaetidylglycerol phosphate respectively in their Rf value.

On spraying with α-naphthol followed by concentrated sulphuric acid in ethanol and heating in the oven at 110°C, the P_2 and P_4 developed as pink coloured spots and matched in their Rf values to Diglycosyl archaeol and Sulphated diglycosyl archaeol, respectively.

Thin layer chromatography of A_1

Development of A_1 on silica gel plates in Petroleum ether : Diethyl ether : Acetic acid (90:10:1v/v/v) followed by exposure to iodine vapours resolved it into a total of 6 spots. 3 major spots of Rf values 0.84, 0.74 and 0.56 were designated as S_1, S_2 and S_3 of which Rf values of S_1 and S_3 corresponded to those of Squalene and Menaquinone, respectively. The remaining 3 minor spots were designated as X_1 (Rf 0.31), X_2 (Rf 0.2) and X_3 (Rf 0.08), (Fig. 21).

Column separation of components of A_1

The A_1 fraction loaded on to silicic acid column was eluted out as S_1, S_2 and S_3 components. The component S_1 eluted in first 50 ml of hexane followed by S_2 and S_3, which co-eluted in 75 ml of Benzene Further elution using chloroform up to 50 ml did not elute any components.

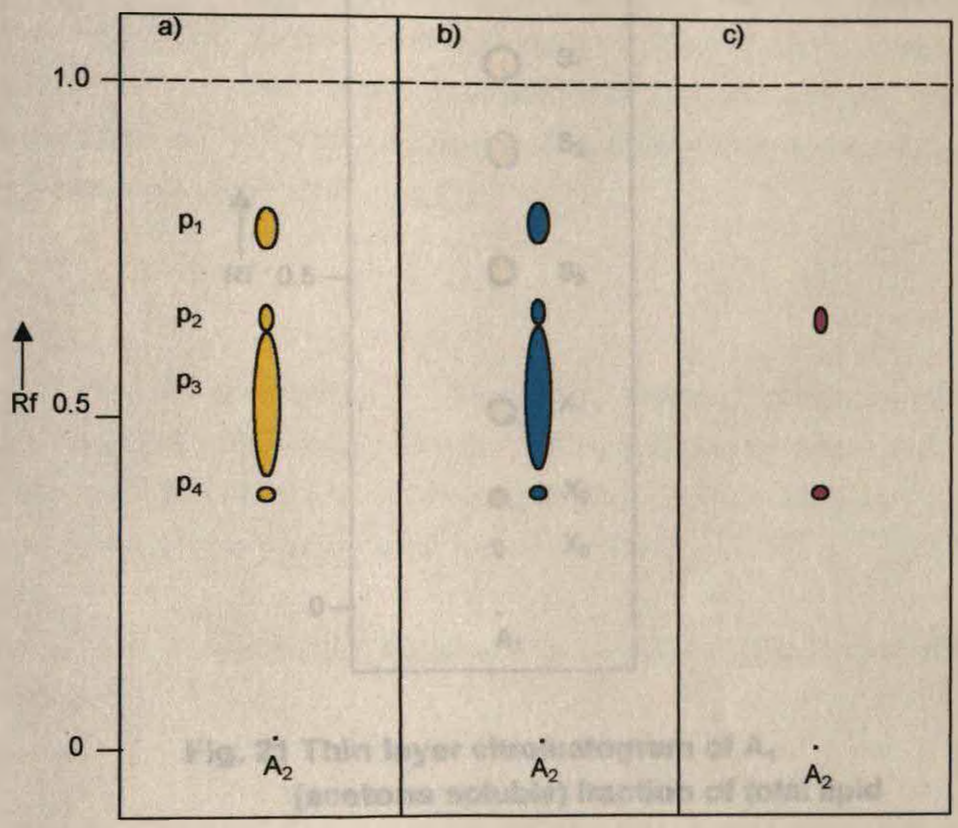


Fig.20 Thin layer chromatogram of A_2 (acetone insoluble) fraction of total lipid Visualised by : (a) I_2 (b) Dodecamolybdate (c) α- Naphthol

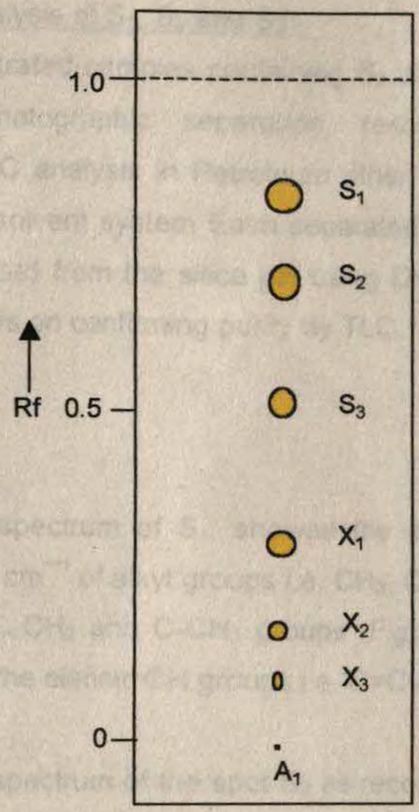


Fig. 21 Thin layer chromatogram of A_1 (acetone soluble) fraction of total lipid

The slow moving components of X_1, X_2 and X_3 co-eluted out in 50 ml of a mixture of 2 : 1 v/v chloroform-methanol.

IR spectral analysis of S_1, S_2 and S_3

Concentrated samples containing S_2 and S_3 which co eluted during column chromatographic separation, resolved into two spots, on preparative TLC analysis in Petroleum ether : Diethyl ether : Acetic acid (90:10:1v/v/v) solvent system Each separated spot was scrapped and the component eluted from the silica gel using Diethyl ether and subjected to spectral analysis on confirming purity by TLC.

S_1 & S_2:

The IR spectrum of S_1, showed the presence of peaks at 2960, 2920 and 2850 cm^{-1} of alkyl groups i.e. CH_3, CH_2 and CH and at 1460 and 1375 cm^{-1} of C-CH_2 and C-CH_3 groups (Fig 22). The peaks at 790-800 cm^{-1} represent the olefinic CH groups i.e. C=C-H.

The IR spectrum of the spot S_2 as recorded in (Fig. 23) was similar to that of S_1.

S_3:

As seen in Fig. 24, IR spectrum of spot S_3 indicated the presence of C=0 stretching of quinone ring (intense peak at 1660 cm^{-1}); C=C of conjugated aromatic ring (1598, 1110 to1020 cm^{-1}); characteristic quinone groups (1330,1295 & 1250 cm^{-1}) and olefinic C=CH groups (760 to 810 cm^{-1}). The peaks between 2860-2970 cm^{-1} showed the presence of alkyl groups of CH_3, CH_2 and CH and the peaks between 1455-1375 cm^{-1} represent the C-CH_3, C-CH_2 groups of the isoprenoid side chain of Menaquinone structure.

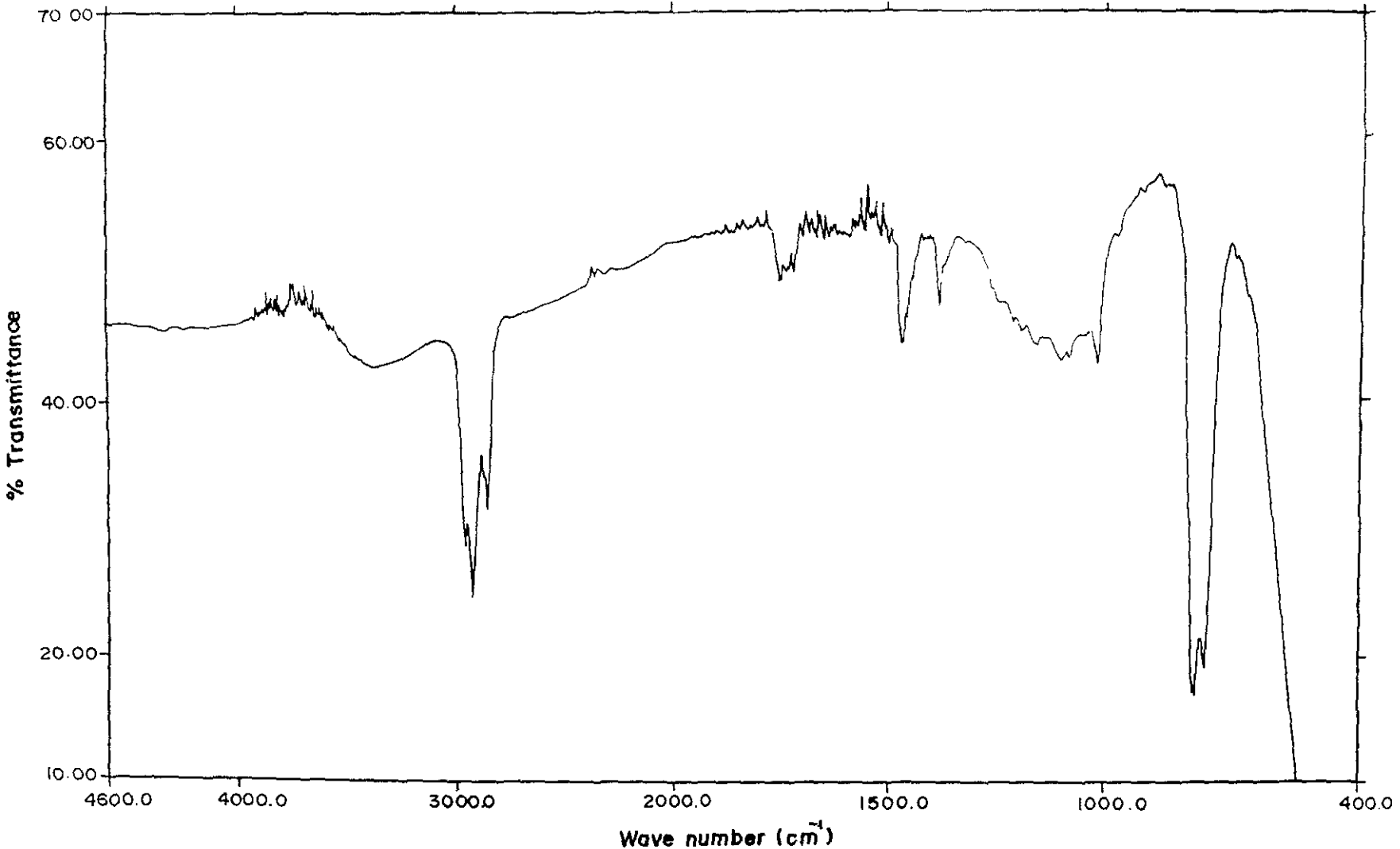


Fig. 22 IR Spectrum of S_1

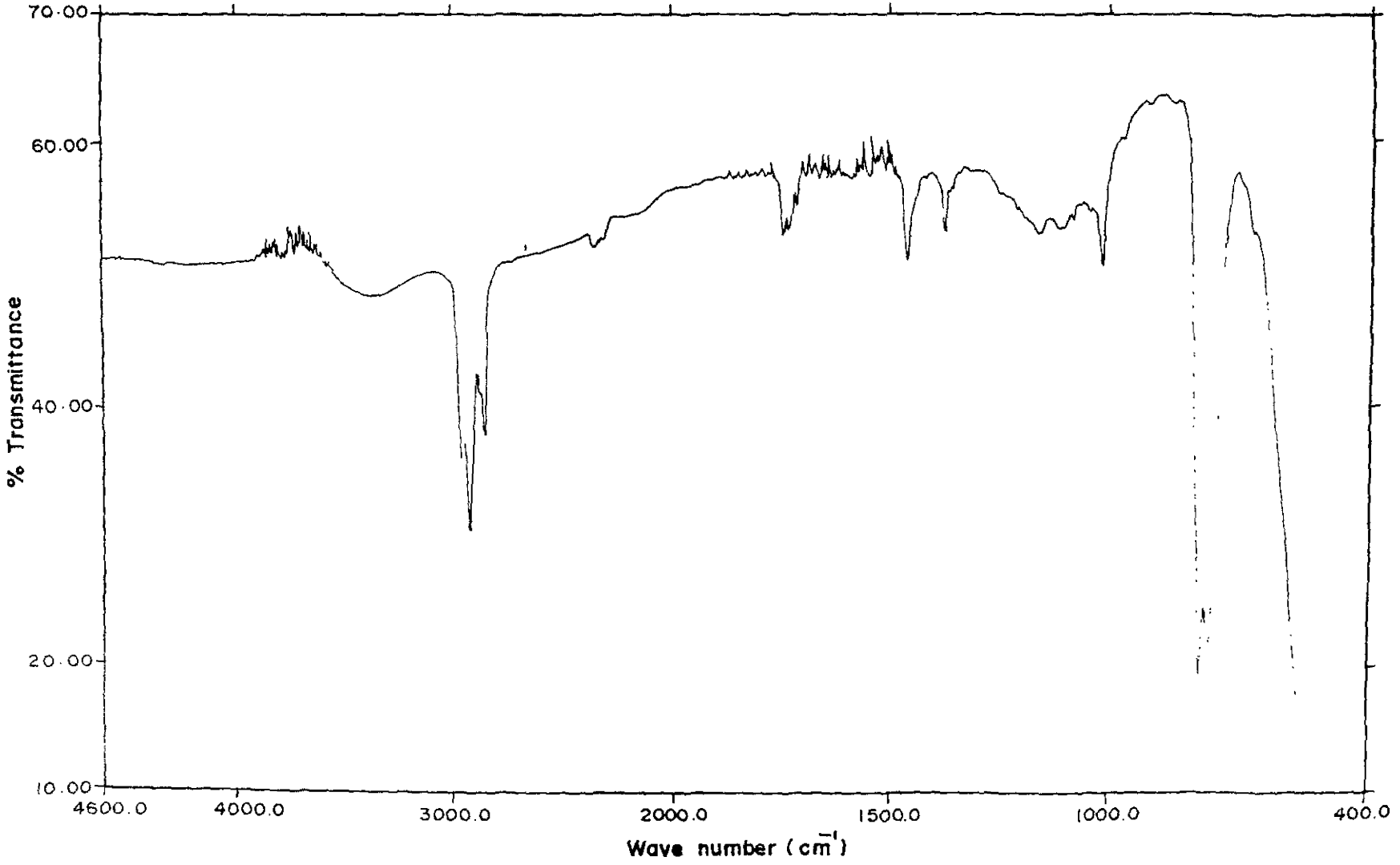


Fig. 23 IR Spectrum of S_2

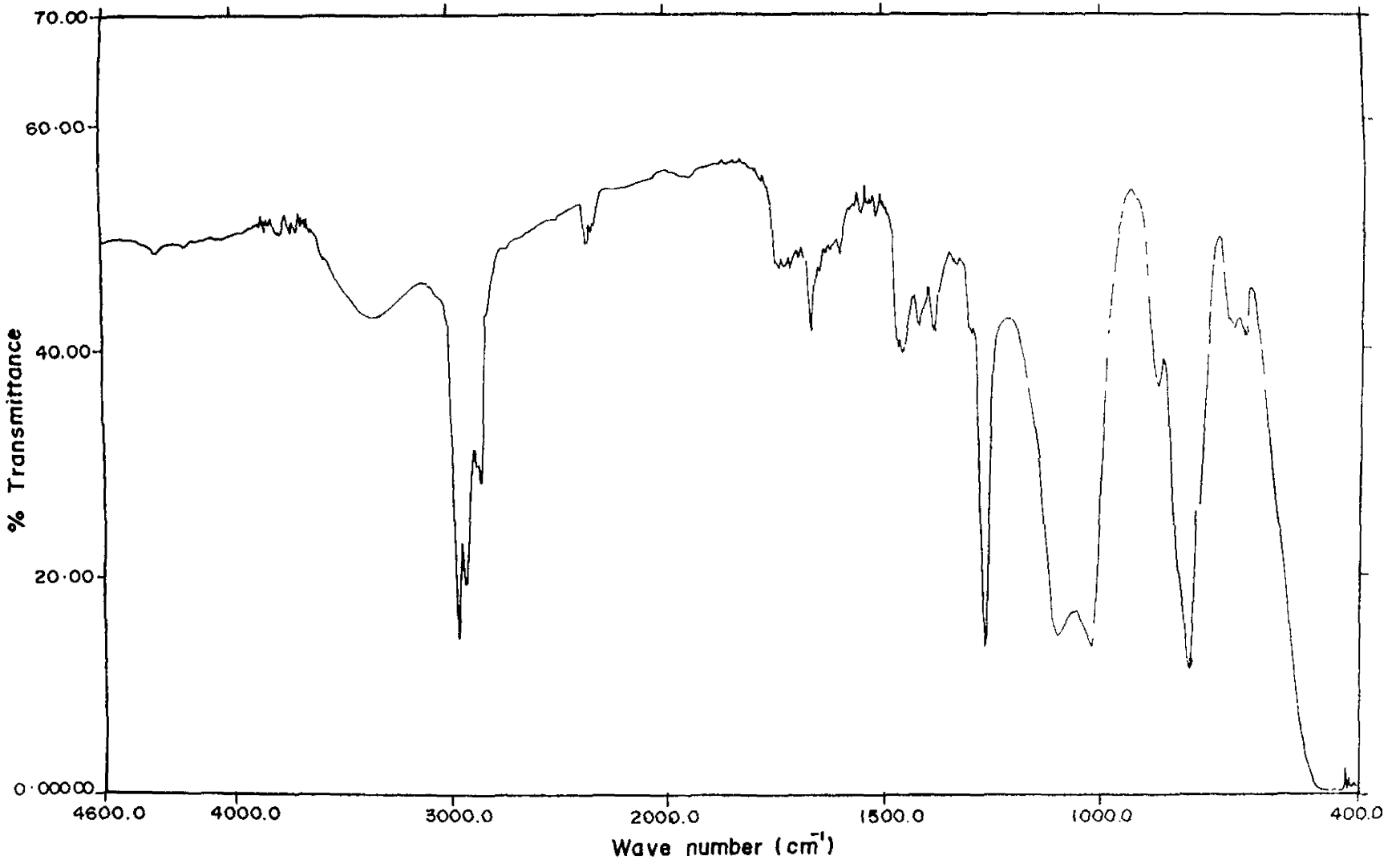


Fig. 24 IR Spectrum of S_3

UV spectrum of S_3:

S_3 recovered from TLC and dissolved in 2 ml ethanol gave absorption maxima between 243–270 nm (Fig. 25), which is characteristic of quinone structures. Incubation of the ethanol extract with 1g of sodium borohydride, at RT, for 30 min resulted in disappearance of peaks between 260 to 270 nm (Fig. 25).

DISCUSSION

HR was isolated as a predominant culture from a salt pan (situated near Ribandar, along the southern bank of Mandovi estuary, Goa), in an earlier study (340). HR grew luxuriantly in NTYE agar and liquid medium containing 25% solar salt at pH 7 and RT (28 –30°C) attaining a dark orange-red pigment as seen in Plate 2.1 & 2.2 respectively. The low lying estuarine lands used for salt farming, from which the bacterial strain was originally isolated are subject to tidal reaches (285) and hence generally experience fluctuations in the prevailing physico-chemical parameters such as temperature, salinity, pH etc. (337). In view of this, experiments were carried out, to determine the effect of salt concentration, pH and temperature, on growth of HR in laboratory medium, namely NTYE. As seen in Fig. 11, HR grew in NTYE at solar salt concentration from 10% up to 30% but did not grow at 5%. This indicates the true halophilic nature of the isolate and ascribes it to the category of extreme halophiles as classified by Kushner (126), based on the response of different microorganisms to salt. However, many of the more recently isolated organisms belonging to this group such as genus *Haloferax*, have been shown to grow at much lower concentrations of NaCl (286,287). Increased doubling times and longer lag phases observed when HR was grown at 10, 15, 20 or 30% solar salt as against those observed during growth at 25 % salt, indicated that, HR grows optimally at 25% concentration with a minimal doubling time. Two other extremely halophilic bacteria *H*

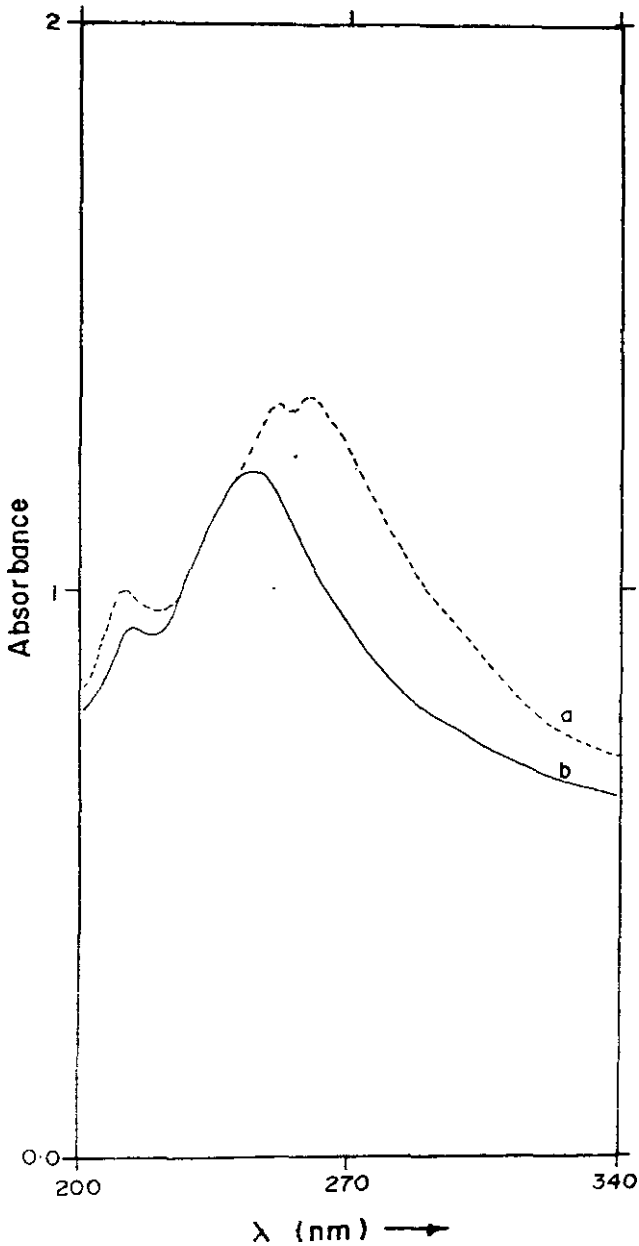


Fig. 25 UV Spectrum of ethanolic solution of:

a) S_3

b) S_3 reduced by sodium borohydride

cutirubrum and strain R_4 were reported to grow to a lesser extent when grown at 15% and 35% NaCl respectively (338). The growth rate of another facultatively halophilic bacterium showed an increasing trend up to 1M, which decreased with further increase in NaCl concentration (288), when grown at 25 ^{0}C.

Solar salt concentration of 25% was chosen to study the effect of pH or temperature on growth of HR as it was found to be optimal. The pH range for growth of HR was found to be 5 to 9, with no growth at pH 4 or 10. As seen in Fig. 12 & Table 2.2, pH 5, 8 or 9 supported the growth of HR to a lesser extent and increased the generation times. pH 6 or 7 supported the growth of HR optimally with no significant difference in the generation times. Most of the halobacterial strains are reported to grow at neutral pH (15), except, the haloalkalophiles, which grow optimally at alkaline conditions of pH 9 or 10 (76).

Salts were seen to precipitate during autoclaving of the medium, having pH 9. The precipitate disappeared with progress in growth of HR.

Increase in incubation temperature up to 45^{0}C, increased the growth rate of HR, as seen in Fig 13. HR grew at 45^{0}C, with a minimum lag period of 4 h, reaching a maximum absorbance of 2 within 28 h indicating the optimal temperature for growth. Most of the extremely halophilic bacteria are known to have temperature optima between 40 to 50^{0}C (289), with no growth below 10^{0}C. However, a novel extremely halophilic bacterium *Halobacterium lacusprofundi* isolated from Deep lake in Antarctica, where the temperature varies between 0 to 11.5^{0}C, was reported to grow at temperature as low as 4^{0}C, although with a low rate (290). Growth at temperature other than optima results in decreased growth rate and is postulated to be a consequence of the biosynthetic

reactions that do not keep pace with catabolic reactions, a condition called energy uncoupling (291).

Extremely halophilic bacteria have been included in the kingdom Archaea based on their unique physiological characteristics and phylogenetic studies using 16 S rRNA (11). However, some of the halophilic eubacteria have also been reported to grow essentially at concentrations as high as 30% (292,293,294). In the light of this, to ensure the archaeal identity, HR was studied for select characteristics, which are known to be unique to haloarchaea.

HR cells when suspended in deionized water, showed an immediate drop in absorbance at 600 nm (Fig. 13) The suspension, immediately became viscous indicating the lysis of cells. Further an attempt to sediment the cells by centrifugation at 8000 rpm for 20 min., which was routinely used, did not yield any cell pellet, confirming that cells were lysed.

This characteristic feature of immediate lysis on exposure to water distinguishes halophilic archaea from extremely halophilic eubacteria (15) Studies done a way back in 1963 on *Halobacterium salinarium* by Mohr and Larsen, indicated that the extreme susceptibility of these bacteria to low ionic conditions might be due to the cell wall being held together by hydrogen bonds and electrostatic forces which could be stabilized only in the presence of high salt concentration (295). Later, Stoeckenius et al also confirmed that halophilic archaea contain a very fragile envelope which is highly susceptible to breakage, on exposure to water due to lowering of ionic conditions (81, 296).

During an earlier study (297), HR was observed to be resistant to Penicillin, indicating the possible absence of peptidoglycan. As seen in

Plate 2.4, the failure to demonstrate the presence of diaminopimelic acid, one of the characteristic components of peptidoglycan, upon whole cell hydrolysis of HR further supports the absence of peptidoglycan. The detection of diaminopimelic acid in hydrolysates of Eubacteria, namely *E.coli* (Gram-negative) and *S. aureus* (Gram-positive), which served as controls, ensured the effectiveness of the hydrolysis process and that of the separation system used. This clearly demonstrates the uniqueness of cell envelope of HR. The presence or absence of eubacterial cell wall components such as diaminopimelic acid and or muramic acid has been widely made use of in confirming the archaeal nature of isolates and understanding the nature of cell envelopes of Haloarchaea (79, 298).

It is a well-known fact that hydrophobic interactions play a major role in stabilizing the bonds of biological structures (150). However, HR did not display surface hydrophobicity as measured in terms of affinity to n-hexadecane

Whole cell acid methanolysates of HR followed by TLC analysis indicated the presence of Glycerol Diether Moieties (GDEM), a characteristic spot of low Rf (0.2) seen in Plate 2.5, as against the fast moving (Rf 0.8), Fatty Acyl Methyl Esters (FAME) of *E.coli*. Presence of GDEMs in whole cell acid methanolysates has been proposed as a rapid identification feature for Archaea by Ross et.al. (94).

NTYE liquid broth inoculated with HR attained orange-red colouration after an incubation period of 120 h (Plate 2.2). Separation of the broth into an orange coloured pellet and clear supernatant resembling NTYE medium, upon centrifugation indicated the presence of an intracellular pigment. UV spectral analysis of acetone extracts of pigment revealed it to possess a number of components such as bacterioruberin (peaks at 528 and 494 nm), lycopene (468 nm) and carotene (426 nm).

retinal (386 nm) and phytofluene (368 & 350 nm). (98,299,300). Majority of the haloarchaea are characterized by red, pink, vermilion, orange-red or mauve red pigments although occasionally colourless strains are also reported (301).

During the growth of the culture in NTYE medium, it was observed, that dark orange pigmentation was attained only after an incubation period of 96 h, although a tinge of orange colour appeared at the end of 72 h. During the early phases, although increase in growth could be seen in the form of turbidity, pigmentation could not be detected with the naked eye. However, it was not clear if the cells attained pigmentation at a certain stage during growth or they grew as pigmented cells, but escaped detection by naked eye as the number of cells were less during the earlier stages of growth. In order to verify this, an equal quantity of cells at different stages of growth was extracted with fixed volume of acetone. The UV spectral analysis as seen in Fig. 15, revealed an interesting trend in the intensity of various components of the pigment during growth. During first 24 h of growth, where the visual pigmentation could not be detected, the components of retinal (peak at 386 nm) and phytofluenes (368 and 350 nm) were in higher concentration as compared to the components bacterioruberin (494 and 528 nm) and lycopene (468 nm). During the following 24 h, components of bacterioruberin (494 and 528 nm) and lycopene (468 nm) were seen to enhance. Visual detection of the pigment at the end of 72 h coincides with an increase in bacterioruberin and lycopene components and a decrease in retinal and phytofluenes components. Gradual increase in the orange-red pigmentation of the culture during the period of 72 to 120 h corresponds to a further gradual increase in the bacterioruberin and lycopene components with no significant changes in the retinal and phytofluenes components. Gradual decrease in the phytofluenes and retinal components coinciding with a gradual increase in the bacterioruberin and lycopene strongly supports the

view that they may be serving as precursors to bacterioruberin and lycopene suggested by Langworthy (302). Individual carotenoid pigments of *H. cutirubrum* were reported to show a decrease in their content, with increasing glycerol concentration in the growth medium (303).

Whole cell lipid extraction using Bligh and Dyers method as modified for halophiles by Kates et. al. yielded a total lipid content of 3.9% on percent dry weight basis. As seen from Fig.18, acetone could separate the total lipids into 2 fractions. Solvent fractionation has been widely used by many workers to separate the total lipids into individual components (90.304,305).

The IR analysis of the A_2 fraction (Fig. 20) indicated the presence of PO_4 (1220 cm^{-1}) and SO_4 (1250 cm^{-1}) functional groups. TLC analysis of this fraction followed by the use of specific spray reagents was helpful in further assigning the functional groups to the isolated components. α-Naphthol followed by ethanol in sulphuric acid has been used to specifically detect the presence of sugar moieties in lipid analysis (306). Lipids containing sugar moieties such as glycolipids stain pink with this spray reagent. Another spray reagent dodeca phosphomolybdic acid was used to detect the phosphate groups, which stain bluish-green. However, this spray reagent did not prove to be highly specific since it stained all the four components in A_2 fraction, bluish-green, including those of the sugar moieties. Thus the A_2 fraction of total lipids showed the presence of 2 phospholipid moieties P_1 Archaetidylglycerol (Rf 0.78); P_3 Archaetidylglycerol phosphate Rf (0.56), and 2 sugar positive glycolipid moieties Diglycosyl archaeol (P_2, Rf 0.64) and Sulphated diglycosyl archaeol (P_4 , Rf 0.46). Many of the haloarchaea are reported to possess Archaetidylglycerol as the major phospholipid along with Archaetidylglycerol phosphate (306) and glycolipids in sulphated as well as

non-sulphated forms (308). Minor quantities of Archaetidylglycerol sulphate have been reported in *H. cutirubrum* and *H. Salinarium* (96).

The A_1 fraction of total lipids on TLC analysis showed the presence of 3 major spots S_1, S_2 and S_3 of which S_1 and S_3 matched with the Rf values of Squalene and Menaquinone. Very close movement of S_2 to S_1 which sometimes merged together on TLC, suggested that S_2 could also be of Squalene nature. In the absence of specific spray reagents, these components have been further purified by column chromatography and preparative TLC. The IR spectra of the spots S_1 and S_2 were identical and the presence of alkyl groups (Fig. 22 & 24) showed good agreement with the long chain isoprene units of Squalene. The IR spectrum of the S_3 spot revealed a number of functional groups such as stretching of quinone ring, conjugated and ortho substituted aromatic ring, characteristic quinone groups, which confirm its Menaquinone nature. Further the peak between 260-269 nm seen on UV spectrum of S_3 (Fig. 25) reduced on treatment with borohydride, which is also a typical characteristic of quinone structures (309). The other 3 minor components of A_1 fraction, designated as X_1 X_2 and X_3 could not be studied further due to paucity of sample. However, their slow movement on TLC during the development in non polar solvent system of Petroleum ether : Diethyl ether : acetic acid (90:10:1 v/v/v) suggested that they may be the components of the orange-red pigment, which extracts more readily into polar solvents such as acetone and methanol. (310,311).

Halobacterium strain R_1 MTCC 3265, grows optimally in NTYE liquid medium containing 25 % solar salt, pH 7 and 45°C but fails to grow in absence of NaCl. HR is a Gram-negative short rod. Cells of culture lyse on exposure to water, are devoid of DAP, but have glycerol diether moieties. The orange red pigment of HR is extractable into acetone and consists of bacterioruberin, lycopene, β-carotene, retinal, and phytofluenes. The major lipids of HR are Archaetidylglycerol, Archaetidylglycerol phosphate, Diglycosyl archaeol, Sulphated diglycosyl archaeol, Squalene, Dehydrosqualene and Menaquinone.

Haloarchaeal nature of *Halobacterium* strain R_1 MTCC 3265 is substantiated by the growth and cellular characteristics.

CHAPTER 3: CELLULAR ADAPTATION OF HR TO SINGLE STEP FLUCTUATION IN PHYSICO-CHEMICAL CONDITIONS OF GROWTH

Microorganisms generally employ various response mechanisms when encountered with physico-chemical conditions that do not support optimal growth. These are reflected as changes in growth patterns and in cellular features. *Halobacterium* strain R_1 MTCC 3265 was isolated from an estuarine region, which undergoes fluctuations in physico-chemical settings of pH and salinity due to periodic inundation by alternating flood and ebb waters. The ambient temperature in Goa, (India) over a large part of the year range between 28-30°C. HR grown at RT (28 – 30°C), optimal salt concentration and pH is evaluated for the adaptive changes in cellular proteins, lipids, pigment etc occurring in response to set fluctuations in pH / salinity / temperature of the consecutive growth cycle, and detailed in this chapter.

METHODOLOGY

3.1 DETERMINATION OF THE EFFECT OF GROWTH CONDITIONS ON CELLULAR FEATURES OF HR

Cells of HR growing in NTYE with 25% salt, pH 7 at RT were transferred to fresh, identical medium except for variation in either solar salt concentration (10 - 30%); pH (5 - 9) or temperature (37- 55°C). Resting cells of HR obtained in each case, were evaluated for their protein profiles, susceptibility to water, surface hydrophobicity, pigment and lipids.

3.2 SDS – POLYACRYLAMIDE GEL ELECTROPHORESIS OF WHOLE CELLS OF HR

3.2.1 Preparation of HR cell lysates

10 ml of resting cells HR (A_{600} ~2) was centrifuged at 8000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 0.5 ml of 20% saline. From this, 100 µl were taken in an eppendorf tube, to which, 10 µl of 25% SDS was added and boiled for 3 min 50 µl of treatment buffer (Appendix III) was then added, again boiled for 2 min. cooled to RT and mixed with 20 µl 0.1% bromophenol blue solution (Appendix III). Of this, 60 µl was loaded into the wells of polyacrylamide gel (Appendix IV).

3.2.2 Preparation of cell envelopes of HR

Resting cells of HR (A_{600}~2), grown in NYTE were centrifuged to obtain a pellet. The supernatant was discarded and the pellet was dispersed into 250 ml of 20% NaCl and sonicated using an ultrasonicator (Labsonic) for 10 min (pulses of 30 sec duration and with a cooling time of 15 sec). The efficiency of sonication was monitored by observing the extent of cell lysis through use of phase contrast microscope. The sonicated suspension was centrifuged at 5000 rpm for 20 min at a temperature of 4°C to remove the unbroken cells, if any. The supernatant was once again centrifuged at 27,000 g at 4°C for 1 h to sediment the envelopes (35). The cell envelopes pellet thus obtained, was washed and re-suspended in 10 ml of 20% NaCl and treated as given above (3.2.1).

3.2.3 Isolation of the cell membrane of HR

Resting cell suspension of HR (A_{600}~2) was centrifuged at 10,000 rpm for 30 min to obtain a tight pellet. The pellet was then dispersed into a 200 ml solution of 0.02 M, pre-chilled, $MgCl_2$ and incubated at 4 °C (312). On overnight incubation, an aliquot of 10 µl of DNAse (Sigma, USA) was added and the incubation at 4 °C was continued for an additional 2 h. The suspension was first centrifuged at 5000 rpm for 30 min to remove any unbroken cells. The supernatant obtained thereof, was centrifuged again at 17,000 g, at 4°C for 1h. The cell membrane pellet thus obtained was washed and re-suspended in 10 ml of 20% NaCl and treated as given above (3.2.1).

3.2.4 Electrophoresis and visualization

Electrophoresis of the lysate mixtures of cellular fractions was carried out at a constant current of 30 mA in the case of 1.5 mm thick gel. till the dye reached bottom of the gel. The plates were disassembled and the gel was put in appropriate staining solution or fixative, for the required time and then put into the destaining solution I for 1 h and later to the destaining solution II, till the bands were visible (Appendix IV), wherever necessary.

3.3 RESPONSE OF HR TO ELEVATED TEMPERATURES

3.3.1 Preparation of cell suspension

Ten ml of 96 h old HR culture grown in NTYE medium at RT was pelleted at 8000 rpm for 20 min and the pellet thus obtained was washed twice with 20% saline and dispersed into PUM–NaCl buffer, (pH 7.1) (Appendix -II) to a final absorbance of 2 at 600 nm.

3.3.2 Exposure of HR to elevated temperatures

Five ml aliquots of above cell suspension were taken in three different sets of boiling tubes. Of the three, two sets were incubated at 60°C or 80°C and were transferred to RT at the end of 10, 30 and 120 min. In a second experiment, a set of boiling tubes, containing 5 ml aliquots of cell suspension each, were incubated at 60°C. One tube was transferred to RT and the remaining tubes were incubated at 80°C, after 30 min. These were transferred to RT at the end of 10, 30 & 120 min. On cooling at RT, the cells from each treatment were centrifuged separately, at 8000 rpm for 20 min, re-suspended in a 5 ml fresh PUM–NaCl buffer and monitored for absorbance at 600 nm. Viability of cells was checked by the TTC reduction method (313), described below.

3.3.3 2,3,5 Triphenyl Tetrazolium Chloride (TTC) dye reduction assay

To a 2 ml mixture containing 12 mM TTC and 0.1% glucose, 1 ml of the above cell suspension was added and the time course of reduction of tetrazolium to formazan was followed at 450 nm. Appropriate control without reagent / cells was maintained separately and monitored likewise.

RESULTS

3.4 RESPONSE OF HR TO ONE POINT FLUCTUATION IN CONSECUTIVE GROWTH CYCLES

Changes in cellular features caused by the one step physico-chemical fluctuation from 25% salt, pH 7 at RT to either salt concentration (10 - 30%); pH (5 - 9) or temperature (37- 55°C) are described below.

3.4.1 Proteins

Effect of solar salt concentration

SDS–PAGE of whole cells grown in NTYE containing 20 – 30% salt showed 24 distinct bands (varying between 11.5 to 93.1 KDa) on staining with coomasie blue (Plate 3.1,lanes c, d & e). Cells grown at 10% salt (lane a) showed protein bands in the region corresponding to 11.5 to 42.4 KDa. Approximately 10 faint protein bands, not seen in cells grown at 10% were observed to be present in the region corresponding to 97 to 43 KDa (lane b). Of the 24 bands observed in cells grown at 20, 25 or 30% salt, 9 bands were very intense (lanes c, d & e). Based on their relative position, these 9 protein bands of molecular weight 93.1; 42.4; 34; 32; 26; 22; 20; 18.7& 12.8 KDa were designated as P_1 through P_9 respectively. Majority of these protein bands were found to be absent or faintly present when the cells were grown at 10 or 15% salt concentration. Also, two other protein bands of molecular weight 14 & 16 Kda, respectively were absent and were faintly present in cells grown at 10% (lane a) & 15% (lane b) salt respectively, which attained prominence in cells grown at salt concentration of 20, 25 or 30%.

Effect of growth pH on whole cell proteins of HR

Cells grown in NTYE at pH 5 or 6 on electrophoresis showed 23 bands (Plate 3.2, lanes a & b) as compared to the 24 bands observed in cells which were grown at a pH of 7, 8 & 9 (lanes c, d & e). The additional band designated as Px was of 13.1 KDa (lanes c, d & e.). The intensity of the bands at 14 & 16 KDa (Px_1 & Px_2), increased by two fold in cells grown at a pH 6 and by four fold in cells grown at pH 7, 8 or 9 as compared to those of the cells grown at pH 5 (lanes a to e). Of the nine protein bands P_1 through P_9, seen in cells grown at pH 7, only P_3 (34 KDa); P_5 (26 KDa); P_6 (22 KDa) & P_9 (12.8 KDa) were prominent and the protein bands P_1 (93.1 KDa) ; P_2 (42.4 KDa) ; P_4 (32 KDa) & P_8 (18.7 KDa) were of lesser

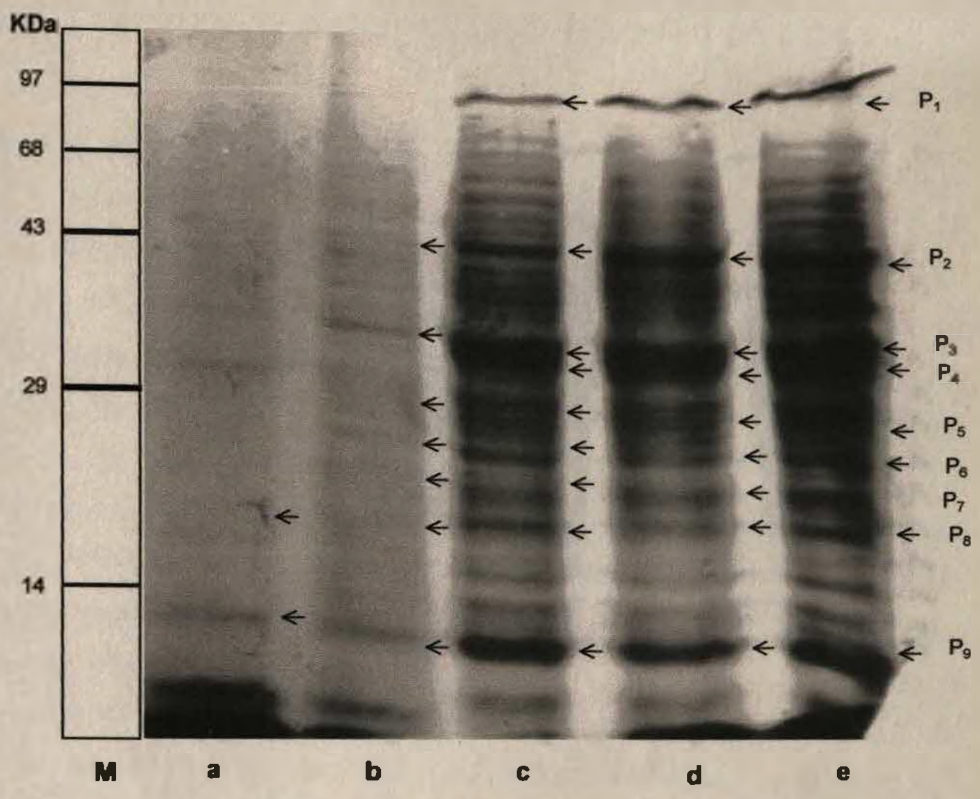


Plate 3.1. SDS-PAGE of whole cells of HR grown in NTYE containing solar salt : 10% (a); 15% (b); 20% (c); 25% (d); 30% (e) along with molecular weight of standard markers (M).

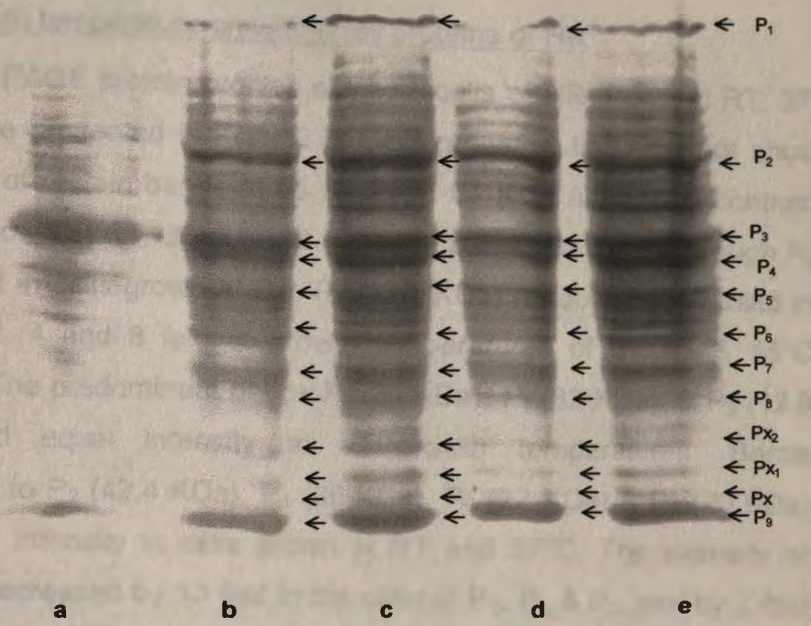


Plate 3.2. SDS - PAGE of whole cells of HR grown in NTYE at different pH
pH 5 (a); pH 6 (b); pH 7 (c); pH 8 (d); pH 9 (e)

intensity than the former ones in cells grown at pH 5. All bands between P_1 through P_9 showed equal intensity in the cells grown at pH 6, 7, 8 or 9.

Effect of growth temperature on whole cell proteins of HR

SDS - PAGE protein profiles of whole cells of HR grown at RT; 37; 45 & 55°C are presented in Plate 3.3. Cells grown at 55°C did not show the presence of protein bands at 66 KDa and 64 KDa (lane d) in contrast to the cells grown at RT, 37 or 45°C (lanes a, b &c). Of the P_1 through P_9 bands present in cells grown at RT, P_1 (93.1 KDa) gradually decreased in intensity by 2, 4 and 8 fold at growth temperatures of 37, 45 & 55°C respectively. The predominant bands P_3 (34 KDa), P_4 (32 KDa), & P_9 (12.8 KDa) showed equal intensity at all growth temperatures. Bands corresponding to P_2 (42.4 KDa), P_5 (26 KDa), P_6 (22 KDa) & P_7 (20 KDa) were of equal intensity in cells grown at RT and 37°C. The intensity of these bands decreased by 10 fold in the case of P_2, P_6 & P_7, and by 2 fold in the case of P_5 when cells were grown at 45 or 55°C. The band at 18.7 KDa corresponding to P_8 showed equal intensity in cells grown at RT; 37 & 45°C but decreased by 10 fold in cells grown at 55°C.

3.4.2 NaCl dependent stability of whole cell proteins of HR

Disintegration of whole cell proteins in absence of NaCl

Resting cells, obtained by growing HR in NTYE containing 25% solar salt, pH 7, at RT and maintained at 20% NaCl, when pelleted out and exposed overnight to distilled water, lysed. These cell lysates on SDS - PAGE, as seen in Plate 3.4, lane a, showed only two protein bands corresponding to 12.8 & 11.2 KDa as against those shown by cells maintained at 20% NaCl (Plate 3.4, lane d). Cells separately exposed to 5% or 10% NaCl, showed presence of protein in region from 22 - 13 KDa which did not resolve into any distinct bands (lane b & c) and was followed

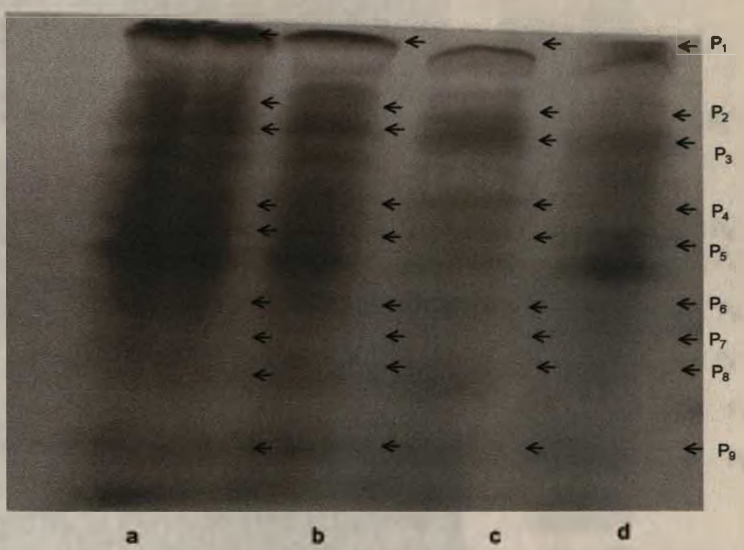


Plate 3.3. SDS-PAGE of whole cells of HR grown in NTYE at different temperatures

RT (a); 37°C (b); 45°C (c); 55°C (d)

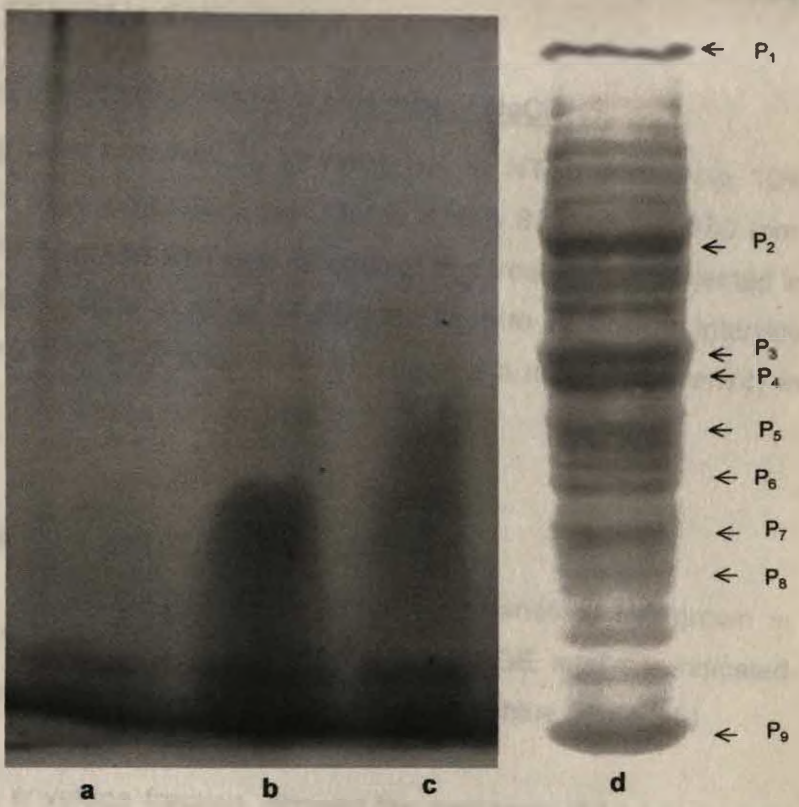


Plate 3.4. SDS - PAGE profiles of resting cells of HR incubated in NaCl:
0% NaCl (a); 5% Nacl (b); 10% NaCl (c); 20%NaCl (d)

by the presence of 12.8 & 11.2 KDa bands equal in intensities, as those seen in cells exposed to distilled water.

Amplification of whole cell proteins in presence of NaCl

Resting cells obtained by growing HR in NTYE containing 10% solar salt were exposed to 30% NaCl for 2, 4, 6 & 8 h at RT at 150 rpm. The SDS – PAGE analysis of cells of each of this treatment is projected in Plate 3.5. Protein bands of 42 to 14 KDa are seen to increase in intensity gradually after 2 h of incubation (lane b), attaining a maximum intensity in 8 h (lane e).

Cellular localisation of salt dependent proteins

Isolated cell envelopes and plasma membranes of HR grown in NTYE with 25% salt, pH7, at RT on SDS – PAGE analysis indicated presence of protein bands on staining with coomasie blue (Plate 3.6).

The cell envelope fraction, showed the presence of 12 bands with molecular weights 93.1, 66, 64, 42 39, 34, 32, 26, 22, 20, 13 & 11.2 KDa respectively (lane a). The stability of all these proteins except that at 11.2 KDa depends on salt as seen in Plate 3.4, lane a. The membrane fraction was resolved into 5 protein bands of 42, 34, 32, 29 & 26 KDa (Plate 3.6, lane b) which disintegrate in cells exposed to water (Plate 3.4, lane a)

3.4.3 Susceptibility to water

HR grown in NTYE at 30% or 25% salt concentration (pH 7 & RT), when suspended in de-ionized water with 0% NaCl, showed an immediate decline in absorbance within the first 15 sec as depicted in Fig 26, corresponding to a percent decline of 96.3 and 97.2 (inset), respectively, which remained constant thereafter up to 10 min. Cells grown at 20, 15 or 10% salt concentration, displayed a decline in absorbance from 1 to 0 073.

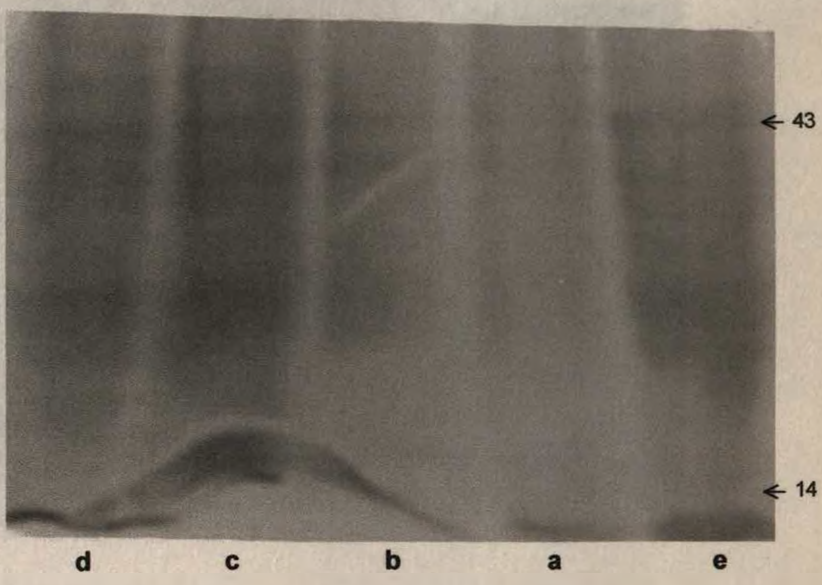


Plate 3.5. SDS - PAGE of resting cells of HR grown at 10% solar salt and exposed to 30% NaCl for various time intervals: 0 h (a); 2 h (b); 4 h (c); 6 h (d); 8 h (e)

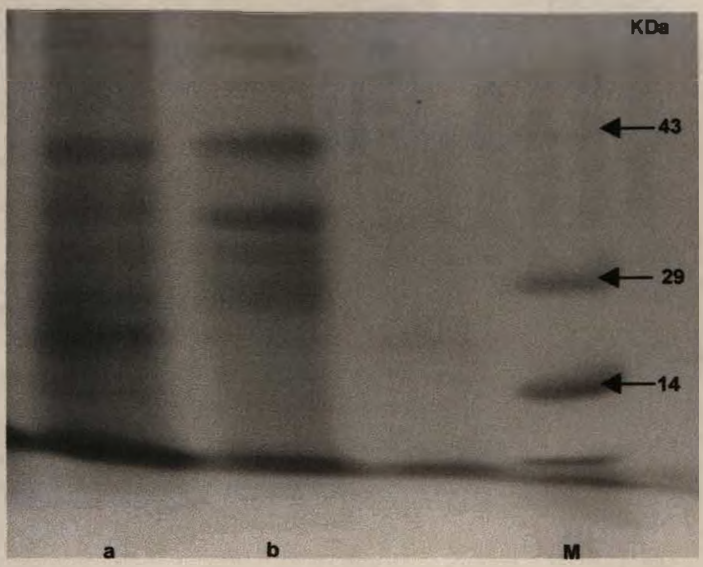


Plate 3.6. SDS - PAGE of cellular fractions of HR grown in NTYE:
cell envelope (a); plasma membrane (b); molecular weight marker (M).

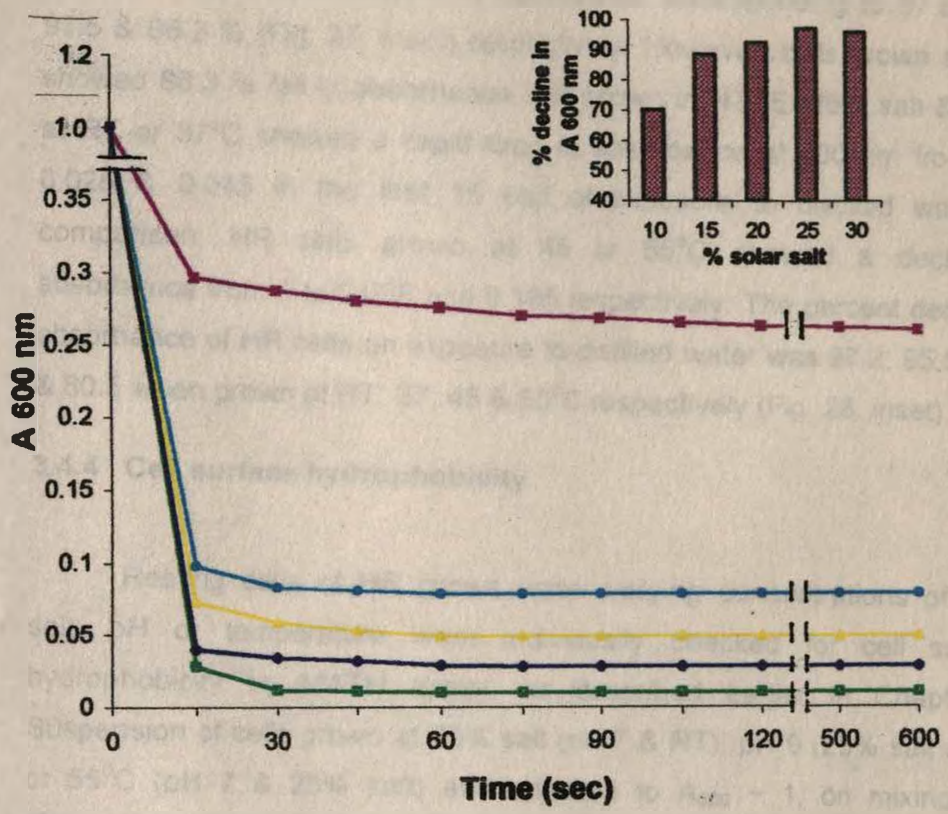


Fig. 26 Effect of water on resting cells of HR grown in NTYE (pH 7 ; RT) with solar salt

10%, 15%, 20%, 25%, 30%

0.114 and 0.297 within 15 sec corresponding to 92.7; 88.6 & 70.3 % decline. Similarly, HR cells grown in NTYE (25% salt & RT), at pH 6, 7, 8 or 9 showed a rapid decline in absorbance corresponding to 97.5; 97.2; 97.5 & 96.2 % (Fig. 27, inset) respectively. However, cells grown at pH 5 showed 88.3 % fall in absorbance. HR grown in NTYE (25% salt & pH 7) at RT or 37°C showed a rapid drop in absorbance at 600 nm from 1 to 0.028 & 0.045 in the first 15 sec of exposure to distilled water. In comparison, HR cells grown at 45 or 55°C showed a decline in absorbance from 1 to 0.078 and 0.195 respectively. The percent decline in absorbance of HR cells on exposure to distilled water was 97.2; 95.5; 92.5 & 80.5 when grown at RT; 37; 45 & 55°C respectively (Fig. 28, inset).

3.4.4 Cell surface hydrophobicity

Resting cells of HR grown under varying concentrations of solar salt; pH or temperature were individually checked for cell surface hydrophobicity by MATH assay, as described earlier in Chapter 2. Suspension of cells grown at 30% salt (pH 7 & RT); pH 9 (25% salt & RT) or 55°C (pH 7 & 25% salt) and adjusted to A_{600} ~ 1, on mixing with hexadecane and phase separation showed a drop in absorbance corresponding to 12.3%; 7.0% & 6 0% hydrophobicity, respectively. The resting cells of HR grown under other conditions of salt concentration, pH and temperature, however, did not display any change in absorbance (Table 3.1), indicating absence of cell surface hydrophobicity.

3.4.5 Pigmentation

During the growth of HR in NTYE medium, under varying conditions of: percent solar salt or pH or temperature, the culture was observed to attain only a dull yellowish orange colour when grown at 10% salt (pH 7& RT); pH 5 (25% salt & RT) or at 55°C (pH 7 & 25% salt). This colour did not darken even after 6 d of incubation as against the bright orange red

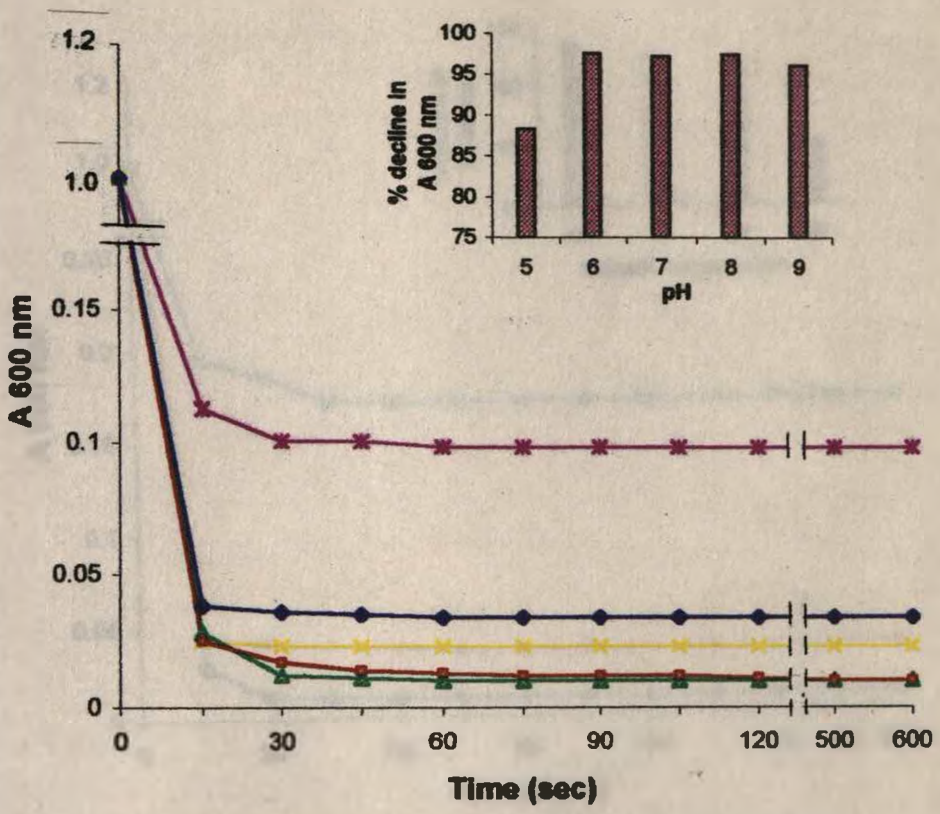


Fig. 27 Effect of water on resting cells of HR grown in NTYE (25 % salt ; RT) with pH

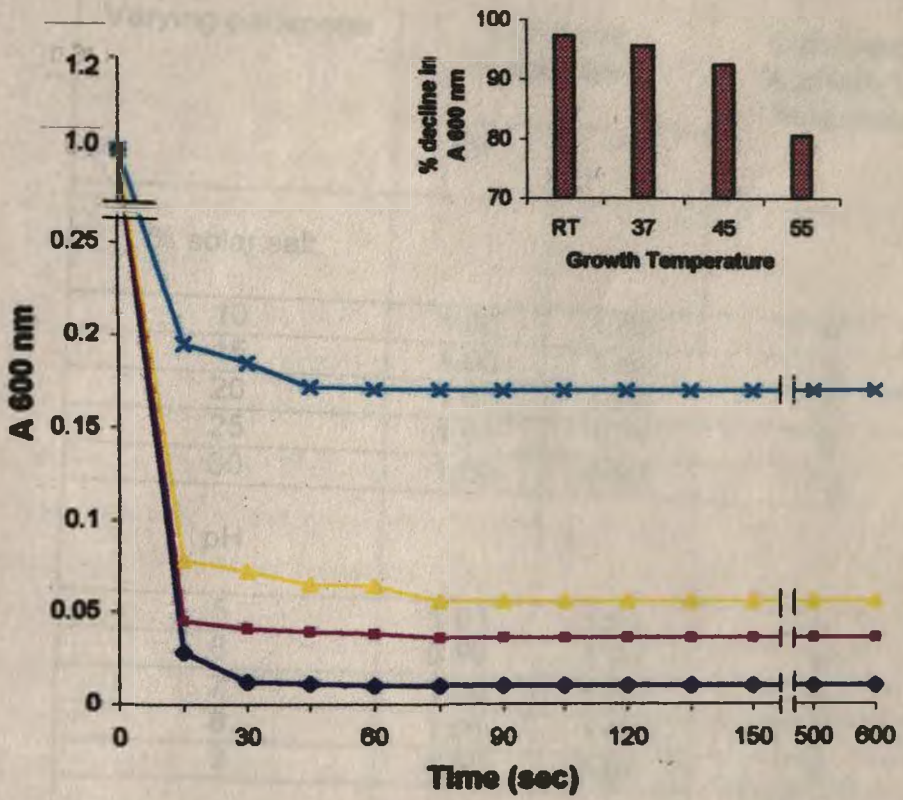


Fig. 28 Effect of water on resting cells of HR grown in NTYE (25% salt ; pH 7) at

RT, 37°C, 45°C, 55°C

Table 3.1 Hydrophobicity of HR grown in NTYE at different Conditions of salinity, pH and temperature

Varying parameter	OD of cell suspension		Calculated % affinity to n- hexadecane
	A_1	A_2	
% solar salt			
10	1.00	1.00	0
15	1.00	1.00	0
20	1.00	1.00	0
25	1.01	1.01	0
30	1.00	0.88	12
pH			
5	1.01	1.01	0
6	0.99	1.00	0
7	1.00	1.00	0
8	1.01	1.00	0
9	1.00	0.94	6
Temperature (°C)			
RT (28-30)	1.00	1.00	0
37	1.00	1.00	0
45	1.00	0.99	0
55	1.00	0.93	7

A_1: Initial OD of HR cell suspension in PUM-NaCl buffer

A_2: OD of HR cell suspension after interaction with n-hexadecane

pigmentation observed when culture grew at 15 - 30% salt, pH 6 - 9 or at RT to 45 °C. Spectral analysis of acetone extracts of pigment as seen in Fig. 29, 30, & 31 showed the presence of peaks at 350; 368; 386; 426; 468; 496; 528 & 600 nm corresponding to phytofluenes, retinal, β-carotene, lycopene and bacterioruberin under all the varying conditions. However, these peaks varied in intensities, with varying growth conditions. For e.g. with increases in salt concentration from 15 to 30% in the medium, the intensities of the peaks of bacterioruberin (528 & 496 nm) and lycopene (468 nm) increased gradually, the maximum being shown by HR cells, grown at 30% salt (Table 3.2 and Fig. 29). The peaks corresponding to β carotene (426 nm), retinal (386 nm) and phytofluenes (368 & 350 nm) did not reveal any particular trend, under these conditions However, a distinct difference observed in cells grown at 10% salt, was the higher intensity of the peaks corresponding to retinal and phytofluenes as compared to those of lycopene and bacterioruberins. (Fig. 29). In case of cells grown at varying pH, the intensities of all the components increased gradually with increase in pH of the growth medium from 6 to 9 as depicted in Fig 30 and detailed in Table 3.3, except for phytofluenes which showed same intensity at pH 8 & 9. However, the cells grown at pH 5 showed a high intensity of β carotene, (426 nm), retinal (386 nm) and phytofluenes (368 & 350 nm) compared to the cells grown at pH 6 to 9. (Fig. 30).

Similarly with increase in growth temperature from RT to 37 °C, the intensity of all the peaks increased as seen in Fig. 31 & Table 3.4. However, on further increase in growth temperature from 37 - 45°C to 55°C, the bacterioruberin and lycopene peaks decreased in intensity, while the peaks of β carotene (426 nm), retinal (386 nm), and phytofluenes (368 & 350 nm) increased in intensity, as seen in Fig. 31.

Table 3.2 Intensities of peaks of pigment of HR grown in NTYE with different salt concentrations

% Solar salt in NTYE	Absorbance of pigment at λ(nm)							
	600	528	494	468	426	386	368	350
10	0.34	0.42	0.48	0.49	0.51	0.54	0.56	0.59
15	0.02	0.4	0.51	0.39	0.18	0.18	0.16	0.12
20	0.06	0.44	0.54	0.44	0.28	0.24	0.22	0.22
25	0.02	0.46	0.58	0.46	0.2	0.2	0.16	0.12
30	0.21	0.64	0.76	0.66	0.48	0.47	0.46	0.45

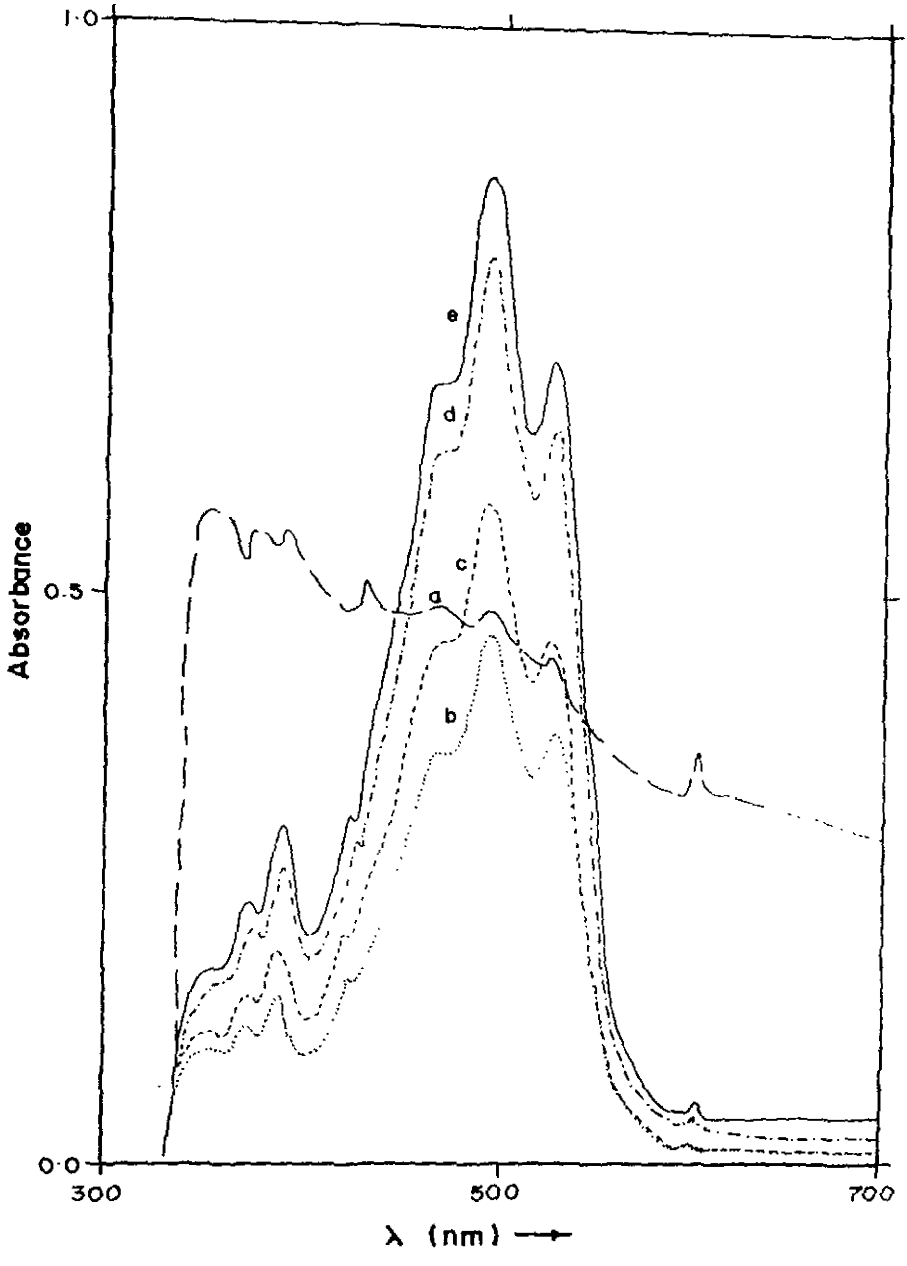


Fig. 29 Pigment profiles of cells of HR cells grown in NTYE with different concentrations of solar salt (at pH 7 & RT)
a) 10% b) 15% c) 20% d) 25% e) 30%

Table 3.3 Intensities of peaks of pigment of HR grown in NTYE with different pH

pH of NTYE	Absorbance of pigment at λ nm							
	600	528	494	468	426	386	368	350
5	0.36	0.42	0.46	0.46	0.51	0.55	0.56	0.58
6	0.02	0.38	0.47	0.36	0.16	0.15	0.12	0.11
7	0.02	0.46	0.58	0.46	0.2	0.2	0.16	0.12
8	0.04	0.66	0.8	0.63	0.28	0.28	0.22	0.18
9	0.05	0.7	0.86	0.68	0.3	0.3	0.22	0.18

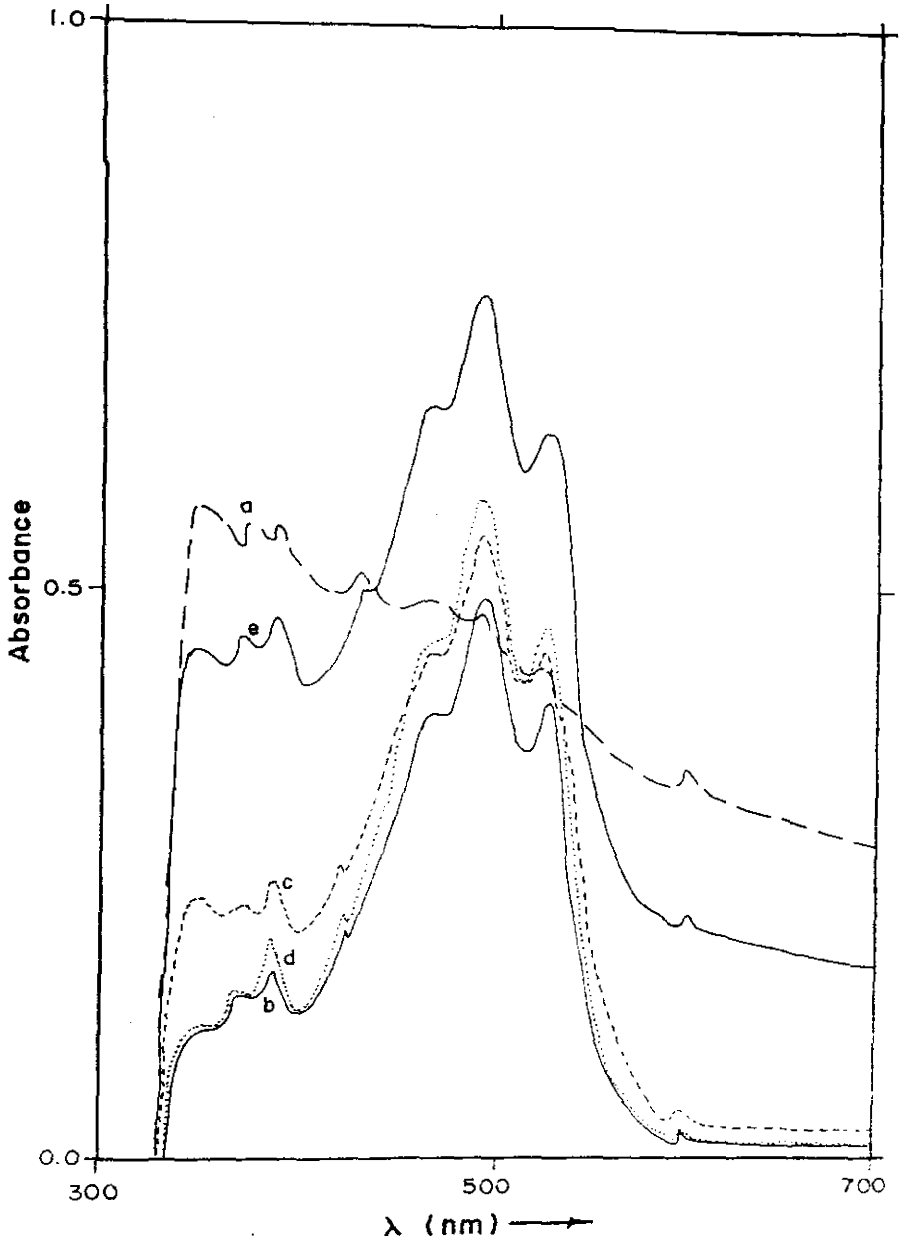


Fig. 30 Pigment profiles of HR cells grown at RT in NTYE with 25% solar salt and at
a) pH 5 b) pH 6 c) pH 7 d) pH 8 e) pH 9

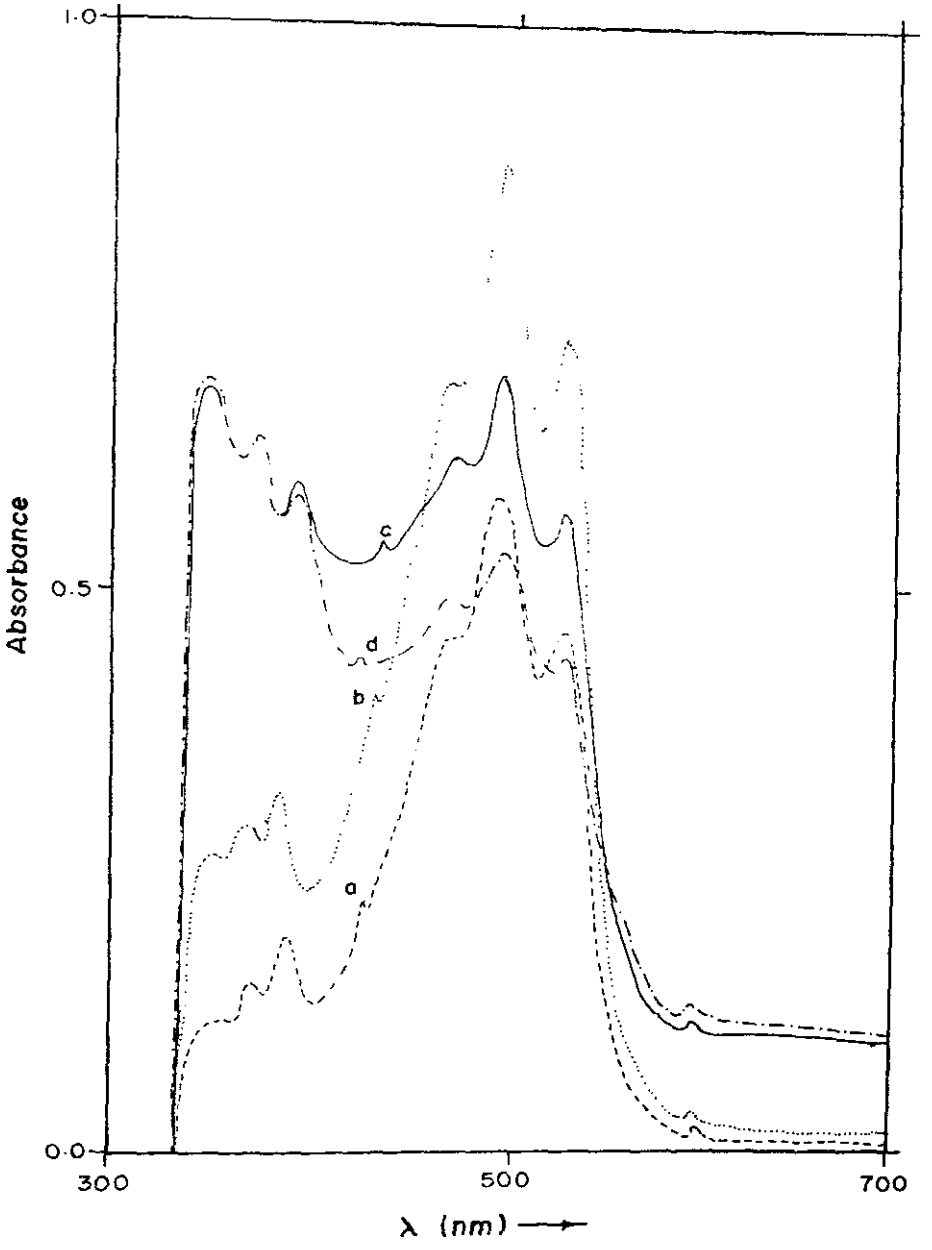


Fig. 31 Pigment profiles of HR cells grown in NTYE with 25% solar salt and pH 7 at
a) RT b) 37°C c) 45°C d) 55°C

Table 3.4 Intensities of peaks of pigment of HR grown in NTYE at different temperature

Temperature (°C)	Absorbance of pigment at λ nm							
	600	528	494	468	426	386	368	350
RT (28-30)	0.02	0.46	0.58	0.46	0.2	0.2	0.16	0.12
37	0.04	0.72	0.88	0.68	0.42	0.3	0.28	0.26
45	0.12	0.56	0.69	0.62	0.54	0.6	0.64	0.69
55	0.14	0.44	0.54	0.5	0.44	0.59	0.64	0.7

3.4.6 Lipids

As recorded in Table 3.5, the total lipids of HR (mg / L of resting cells) were similar when grown under varying conditions of salt concentration of 15% to 25% (pH 7 & RT), pH 6 – 8 (25% salt & RT) or temperature of RT to 45°C (pH 7 & 25% salt) which however increased, when HR cells were grown at either 10% or 30% salt (pH 7 & RT); pH 5 or 9 (25% salt & RT) and at 55°C (pH 7 & 25% salt). TLC analysis of A_1 fractions of total lipids of cells grown at each of the conditions in the solvent system Petroleum ether : Diethyl ether : Acetic acid (90:10:1v/v/v) followed by exposure to iodine vapours indicated the presence of 6 spots at R_f of 0.86; 0.76; 0.57; 0.33; 0.18 and 0.09 comparable to the cells grown at 25% salt, pH 7 and at RT (Fig. 21). Three of the major spots corresponded to those designated as S_1, S_2 & S_3 accordingly identified as Squalene, Dehydrosqualene and Menaquinone respectively. However, an additional spot was visualized at R_f of 0.67 in cells grown at 10 or 30% salt concentration (pH 7 & RT); pH 5 or 9 (25% salt & RT) as depicted in Plate 3.7 & 3.8 respectively.

Thin layer chromatograms of A_2 fractions, visualized with dodecaphospho-molybdic acid revealed two spots at R_f 0.78 & 0.56 corresponding to Archaetidylglycerol and Archaetidylglycerol phosphate respectively under all growth conditions. On spraying separate chromatograms, with α - naphthol spray reagent, two pink spots corresponding to Diglycosyl archaeol (R_f 0.66) and Sulphated diglycosyl archaeol (R_f 0.47) were also observed under all growth conditions. However, when the culture was grown at 55°C, the A_2 fraction showed the presence of an extra lipid spot (R_f 0.15), which got stained pink on spraying with α – naphthol spray reagent (Plate 3.9).

Table 3.5 Total lipid content of HR grown in NTYE at different conditions A) Solar salt concentration, B) pH, C) Temperature

A		B		C	
% Solar salt	Total lipid (mg/L)	pH	Total lipid (mg/L)	Temperature (°C)	Total lipid (mg/L)
10	67.8	5	72.4	RT (28-30)	58.6
15	58.2	6	59.2	37	56.8
20	57.4	7	58.6	45	57.9
25	58.6	8	57.5	55	71.3
30	69.4	9	63.2		

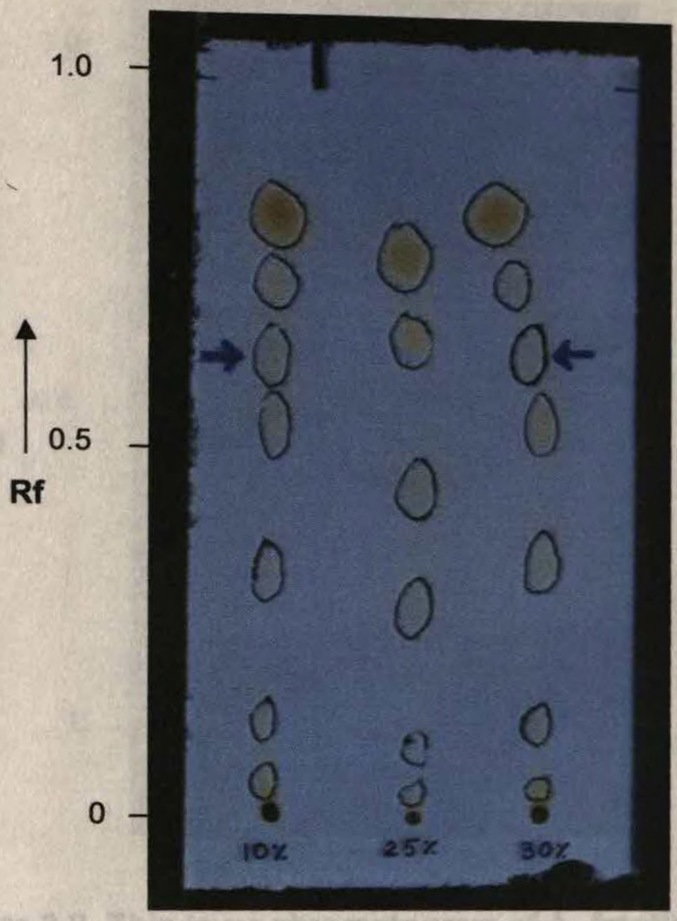


Plate 3.7. Thin layer chromatogram of A_1 fraction of total lipids of HR grown in NTYE with different concentrations of solar salt

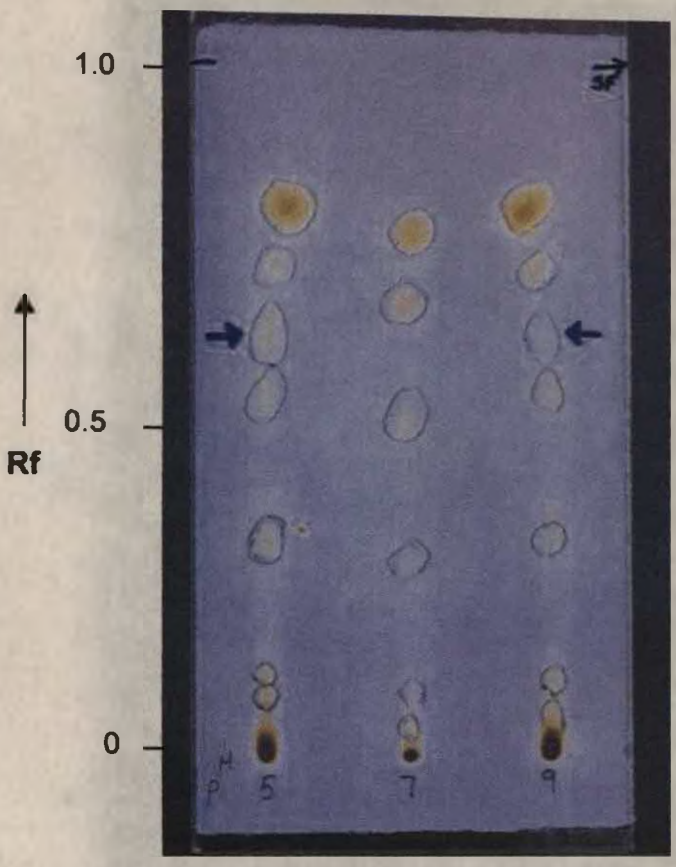


Plate 3.8. Thin layer chromatogram of A_1 fraction of total lipids of HR grown in NTYE with varying pH

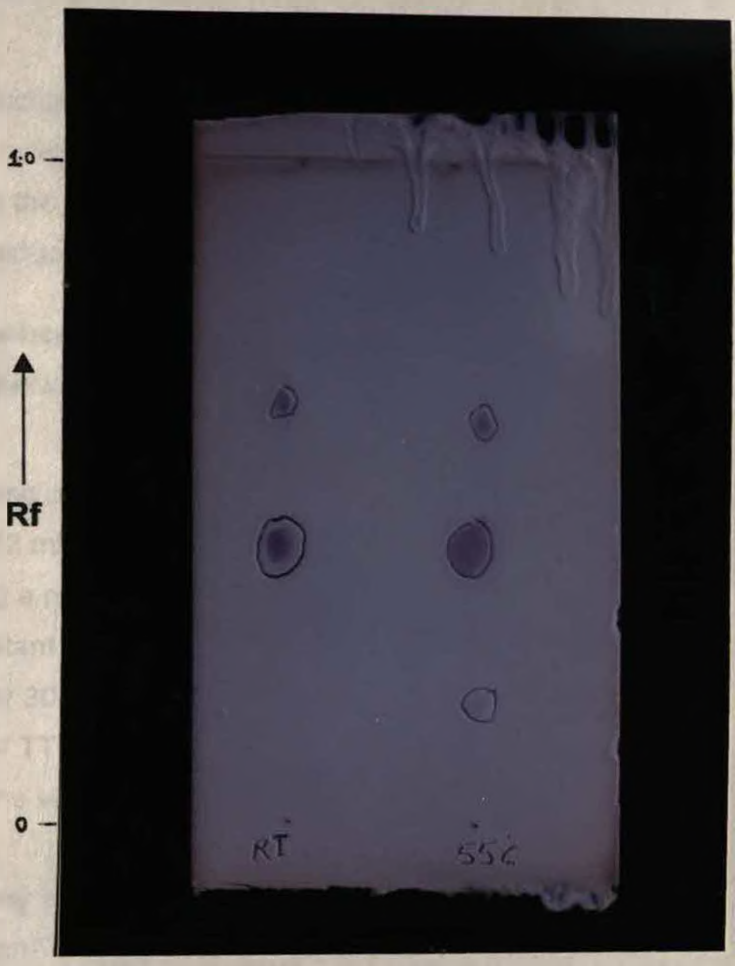


Plate 3.9. Thin layer chromatogram of A_2 fraction of total lipids of HR grown at RT and 55oC visualised with glycolipid specific spray.

3.5 RESPONSE OF RESTING CELLS OF HR TO ELEVATED TEMPERATURES

Reduction of the dye "TTC" has been used as an indicator of the viability of cells (313). The viability of HR cells, exposed to temperatures higher than the growth temperature, was checked by evaluating the ability of cells to reduce TTC.

3.5.1 Influence of growth temperature on response of HR to elevated temperatures

Resting cells of HR (A_{600}~2), when incubated with 0.1% glucose along with 12 mM TTC at RT, showed an increase in absorbance at 450 nm reaching a maximum of 0.88 within 10 min and thereafter remained almost constant up to 30 min as depicted in Fig. 32 (inset). Addition of glucose after 30 min showed no further increase in absorption at 450 nm. Reduction of TTC occurring within 20 min was therefore selected for evaluating the viability of resting cells of HR in further experiments.

Resting cells of HR grown at RT, on exposure to 60°C or 80°C directly showed a decrease in viability as assessed by TTC reduction as depicted in Fig. 32 However cells exposed to 80°C after a prior incubation at 60°C showed better response to heat, in terms of TTC reduction as compared to the cells directly exposed to 80°C (Fig. 32).

In a similar experiment, resting cells of HR grown at different temperatures were exposed to 80°C, either directly or stepwise with a prior incubation at 60°C for 30 min. Increase in growth temperature up to 45°C, resulted in increasing resistance of cells to heat in terms of TTC reduction, as seen in Fig. 33.

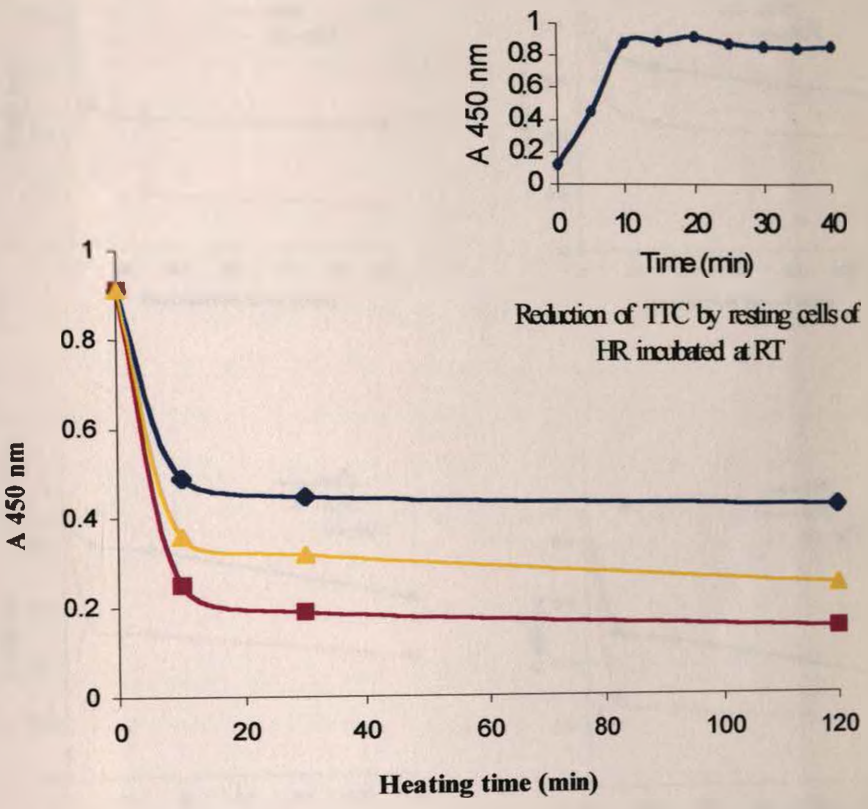


Fig. 32 TTC reduction by HR grown at RT and exposed to elevated temperature (^{o}C)

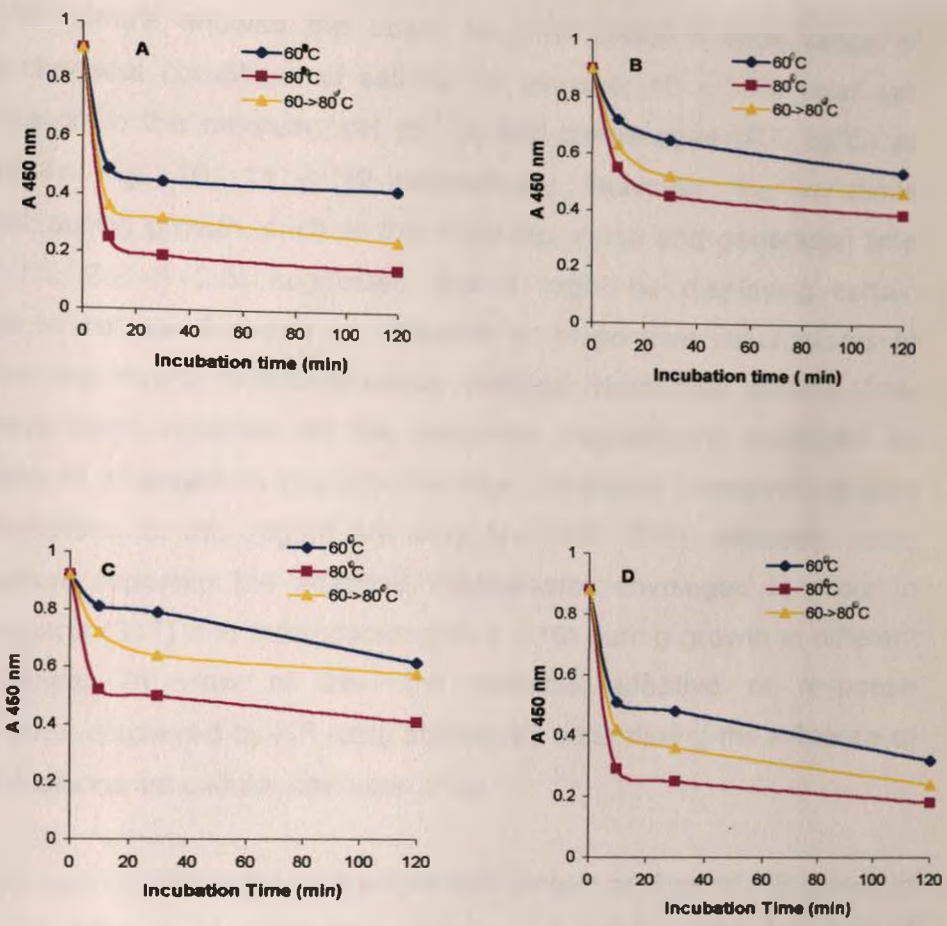


Fig. 33 Effect of growth temperature on reduction of TTC by HR cells exposed to elevated temperatures

A) cells grown at RT **B) cells grown at 37° C**

C) cells grown at 45°C **D) cells grown at 55° C**

DISCUSSION

HR culture showed the ability to grow under a wide range of physico-chemical conditions of salinity (in terms of 10 - 30% solar salt concentration in the medium); pH (5 - 9) and temperature (RT- 55°C) as indicated in Fig. 10, 11 & 12 respectively. However, the variations exhibited, during growth, such as the initial lag phase and generation time (Table 2.1, 2.2 & 2.3) suggested that it might be displaying certain changes in cellular features as adaptive or responsive mechanisms to these varying invitro physico-chemical settings. Numerous studies (314-316) have been reported on the response mechanisms employed by eubacteria to changes in physico-chemical conditions. However, studies on haloarchaea in this regard are very few (338, 339), although, some investigators reported the adaptive mechanisms envisaged to occur in methanogens (317) and thermoacidophiles (318) during growth at different temperatures. In view of this, the possible adaptive or response mechanisms displayed by HR were studied by determining the influence of these conditions on cellular characteristics.

As seen in Plate 3.1, the whole cell protein profiles of HR grown in NTYE with different concentrations of solar salt, indicated the presence of 9 unique bands, designated as P_1 to P_9 which showed high intensity in cells grown at 20, 25% or 30% salt but were faintly present or absent when grown at 10% or 15% salt concentration. The absence or low intensity of these protein bands in cells grown at relatively less salt concentration, i.e at 10% or 15%, compared to optimal salt concentration of growth (25%) suggests that, the salt requirement of at least 15% for moderate production of these proteins. Further, the proteins observed in cells grown at optimal salt concentration of 25%, could not be seen if these cells were incubated in water prior to electrophoresis (Plate 3.4) suggesting that these proteins possibly disintegrated in the absence of salt. However, as

seen in Plate 3.4, cells incubated at 5% or 10% NaCl, although did not resolve into distinct bands, showed the presence of protein suggesting a lesser degree of disintegration as compared to the disintegration of proteins of the cells suspended in water. This indicates the true halophilic requirement of the cellular proteins P_1 through P_8 for structural stability. This was further confirmed by an other experiment, in which cells grown at 10% salt on incubation in 30% NaCl, showed increases in intensities with incubation time and attained maximum intensity, within 8 h. Furthermore, protein profiles of isolated envelopes showed the presence of the salt-requiring proteins P_1 to P_7 & P_9 and the membrane fraction showed the presence of P_2 to P_5 & P_6 proteins. This is in keeping with Haloarchaea, which have been well established in their essential requirement of high salt for structural stability of proteins (35), especially of envelope and membrane that provide cell shape and structural rigidity.

HR cells grown in NTYE with different pH or at different temperatures showed interesting variations in their protein profiles Although, the salt concentrations in NTYE medium was maintained at 25% under these varying conditions, the P_1 to P_9 bands were either absent or faintly present at pH 5 Similarly, with increase in growth temperature up to 45^0C or 55^0C, these bands showed progressive decrease in intensity indicating that, although, higher salt concentration is the prime requirement for these proteins, other physico-chemical conditions such as pH and temperature in this case, do have an influence on the cellular proteins. Most of the studies in haloarchaeal field have primarily focused on the salt requiring aspects of these bacteria and influence of other physico-chemical conditions, under the presence of salt have not been addressed so far

Exposure of cells grown at RT in NTYE with 25% solar salt was observed to result in lysis of the cells (Chapter 2). However, exposure HR

cells grown in NTYE with 10% salt to water, on the other hand, showed relatively less susceptibility to lysis, as depicted in Fig. 26. Interestingly, HR cells grown in NTYE with 25% salt, at pH 5 or at 55°C also showed a similar trend, although, the salt concentration during growth was optimal. An interesting correlation between the protein bands of HR cells especially in P_1 to P_9 and effect of water on HR cells was observed. Cells grown in NTYE with 10% salt (pH 7 & RT); pH 5 (at RT & 25% salt), or at 55°C (25% salt & pH 7) which showed less susceptibility to lysis (Fig. 26, 27 & 28), also had low intensity of P_1 to P_9 protein bands suggesting that, the protein bands of P_1 to P_9 if produced at higher intensity, being obligate salt requiring, may possibly disintegrate immediately upon lowering the salt concentration, thus leading to immediate lysis. In contrast, HR cells having P_1 to P_9 at negligible level could resist the exposure to low ionic conditions to some extent.

HR cells displayed a surface hydrophobicity of 12.3%, 7% & 6% when grown at 30% salt (pH 7 & RT); pH 9 (25% salt & RT) and 55°C (25% salt & pH 7) respectively. as compared to the other growth conditions. in which HR did not show any hydrophobicity. Increased cell surface hydrophobicity results in decreased permeability of cell membrane and plays an important role in retention of the cytoplasmic water. especially when exposed to salt concentrations. higher than the optimal conditions (210). Pashley et al. have reported that the strength of interaction between hydrophobic surfaces and water may be up to 100 times stronger than that arising from the interaction between hydrophobic surfaces and water (319).

HR cells when grown under different physico-chemical conditions. also resulted in distinct variations in their pigment profiles. Cells grown at 10% salt (pH 7 & RT): pH 5 (25% salt & RT) or at 55 °C (25% salt & pH 7) showed higher intensity of retinal and phytofluenes as compared to

bacterioruberins (Fig. 29, 30 & 31). As discussed in Chapter 2, in HR cells the abundance of retinal and phytofluenes was higher during log phase of cells, which declined at stationary phase with an increase in the other two components i.e. lycopene and bacterioruberin. However, the high abundance of retinal cells and phytofluenes in cells grown at 10% salt, at pH 5 or at 55°C suggests that these components could be also involved in adaptation of these cells to these non-optimal conditions. Also, the intensity of the bacterioruberin and lycopene components increased with increase in salt concentration from 15 to 30% and with increase in pH from 6 to 9, as depicted in Fig 29 & 30. Similar variations were observed by Kushwaha et al. in the pigmentation of *H.cutirubrum*, which did not produce bacterioruberins when grown at 15% salt concentration, although it was strongly pigmented at 20% and higher concentration of salt (338). In contrast, certain Spanish haloarchaea were reported to show decrease in bacterioruberin components with increase in salt concentration (320,321). However, the comparative proportion of these to lycopene or phytofluene has not been reported.

HR cells grown at varying conditions also differed in their lipid moieties. Cells grown at 10 or 30% (pH 7 & RT); pH 5 (25% salt & RT) or pH 9 (25% salt & pH 7) as seen in Plate 3.7 & 3.8, showed the presence of an extra lipid moiety in A_1 fraction and another extra lipid moiety was seen in A_2 fraction during growth at 55°C (Plate 3.9). Kushwaha et al. in 1982 reported changes in the lipid composition of haloarchaeal cells as a response towards changes in salt concentration during growth (338). However, induction of extra lipid moieties, as an adaptive mechanism to changes in the external conditions has not been reported in haloarchaea. Many of the eubacteria have been well established in their response to physico-chemical conditions in terms of alteration of lipids components, especially of fatty acids (322, 323). In the absence of fatty acids, haloarchaea may respond to physico-chemical conditions by either

producing extra lipid moieties or by abolishing the existing ones. Such alterations are believed to result in membranes with physical characteristics that would facilitate membrane functions such as membrane fluidity (324) under those physico-chemical conditions.

Growth of HR under a wide arrange of temperatures prompted the study of their response to elevated temperatures. The dye, 2,3,5 tri-phenyl tetrazolium chloride was used to study the response of HR cells to heat Tetrazolium salts have been widely used as indicators of bacterial viability (325, 326). These compounds accept electrons and hence, are reduced by metabolically active cells to a coloured compound called formazan. Thus the metabolic activity of HR cells after exposure to heat was assessed by their capacity to reduce TTC to formazan. As indicated in Fig. 32, the prior incubation of HR cells at 60°C for 30 min. before exposing to 80°C resulted in higher reduction of TTC, as compared to the cells directly exposed to 80°C. This suggests that the cells might have developed a degree of thermo-tolerance during their initial exposure to 60°C. Similar acquisition of thermo-tolerance on stepwise exposure to heat was observed in thermophilic archaea e.g. *Sulfolobus* and *ES4* and also in eukaryotes such as *Saccharomyces cereviceae* and fruit fly *Drosophila Melanogaster* (327,328). Interestingly, with the increase in growth temperature of HR up to 45°C, the cells showed better response to elevated temperature of 80°C either directly or step wise (Fig. 33) as assessed by TTC reduction, indicating that growth temperature itself confers certain thermo-stability to the cells

Halobacterium strain R_1 MTCC 3265 subjected to one step fluctuation of pH, temperature and salt concentration during subsequent growth cycle exhibited variations in cellular features. Cells grown at 25% salt, pH 7 and RT showed 9 prominent proteins namely P_1 to P_9 HR cells.

grown at set fluctuation of 10% salt (pH 7 & RT); pH 5 (25% salt & RT) or at 55°C (25% salt & pH 7) had majority of the P_1 to P_9 bands faint. Cells grown at pH 5 / 6 lacked protein band Px of 13.1 KDa and showed faint bands of Px_1 & Px_2 at 14 & 16 KDa. Growth temperature had no effect on P_3, P_4 or P_9 bands. Exposure of whole cells of HR to water disintegrated majority of proteins including P_1 to P_9. Of the P_1 to P_9 bands, P_1 to P_7 & P_9 were found to be located in the envelope and P_2 to P_5 & P_6 in the membrane fraction. The cell surface hydrophobicity of HR cells was 12.3; 7 & 6% when grown at 30% salt (pH 7 & RT); pH 9 (25% salt & RT) and at 55°C (25% salt & pH 7), respectively as compared to the cells obtained using other growth conditions that showed no hydrophobicity. HR cells, grown at 10% salt (pH 7 & RT); pH 5 (25% salt & RT) or at 55°C (25% salt & pH 7) displayed a degree of tolerance on exposure to water. The intensity of the bacterioruberin peaks increased with increase in salt concentration (15% to 30%) and pH (6-9) during growth. The retinal and phytofluene components showed higher abundance in cells grown at 10% salt (pH 7 & RT); pH 5 (25% salt & RT) or at 55°C (25% salt & pH 7) as compared to the bacterioruberin and lycopene components. Additional lipid moieties at Rf 0.67 & 0.15 were seen during growth at 10 & 30% salt (pH 7 & RT); pH 5 & 9 (25% salt & RT) or at 55°C (25% salt & pH 7). Growth temperature of HR cells influenced the cell's acquisition of thermo-tolerance.

One step fluctuation in physico-chemical conditions of consecutive growth cycles of HR alters the proteins, lipids, pigments, susceptibility to water and surface hydrophobicity of the cells. Response of whole cells of HR to elevated temperatures is governed by the growth temperature.

CHAPTER 4: RESPONSE OF HR TO Zn^{+2} AND Cu^{+2}

Globally, urbanisation and rapid industrialisation in coastal countries has lead to the pollution of the environment. Estuarine regions, in particular act as sinks to heavy metal ions. A few reports indicated tolerance of haloarchaea to heavy metal ions (259,260). Studies evaluating the effect of heavy metal ions of Cu^{+2} and Zn^{+2} on growth and cellular characteristics of HR are discussed in this chapter.

METHODOLOGY

4.1 DETERMINATION OF GROWTH OF HR IN MINERAL SALTS MEDIUM CONTAINING GLUCOSE AS SOLE SOURCE OF CARBON

Seed culture of HR was inoculated to a final concentration of 5% into a 150 ml flask containing 50 ml sterile mineral salts medium (NSM) (Appendix I) containing 20% NaCl, pH 7 and 0.2% glucose (NGSM) The flask was incubated at RT on a rotary shaker at 150 rpm. The changes in absorbance were monitored by withdrawing aliquots at pre-decided intervals of time and observing the absorbance at 600 nm. using a Shimadzu UV-240 spectrophotometer.

4.1.1 Effect of Cu^{+2} / Zn^{+2} on growth of HR in NGSM

To study the effect of heavy metal ions of Cu^{+2} / Zn^{+2} on growth of HR, metal salts of $CuSO_4.5H_2O$ / $ZnSO_4.5H_2O$ were weighed according to the required concentration and dissolved in sterile mineral medium before inoculation of the culture.

4.1.2 Effect of continuous subculture in Cu^{+2} / Zn^{+2} on growth of HR

Seed culture of HR was inoculated into NGSM containing Cu^{+2} or Zn^{+2} at 1mM and the amount of growth was measured by determining the absorbance at 600 nm at the end of 7 d, during the stationary phase of growth. The effect of serial exposure to metal ions on the growth was studied by serially transferring HR grown in the presence of metal ions, to NGSM containing Cu^{+2} / Zn^{+2}, at 1 mM, for four subsequent subcultures.

4.2 DETERMINATION OF CHEMICAL COMPOSITION OF CELL ENVELOPES AND MEMBRANES OF HR GROWN IN PRESENCE OF METAL IONS

4.2.1 Isolation of cell envelopes and cell membranes of HR grown in NGSM with and without Cu^{+2} / Zn^{+2}

Resting cells of HR (A_{600}~2) were prepared from culture grown in NGSM with or without the metal ions for 7 d, and used to prepare envelopes and membranes fractions, as described earlier. The envelope or membrane preparation in each case, was suspended in 10 ml of 20% NaCl and used for further determination of chemical constituent analysis.

4.2.2 Protein

To 100 µl of envelope or membrane sample, 1 N NaOH was added to make the volume up to 1 ml. 5 ml of Folin reagent 'C ' (Appendix II) was then added and the tubes were incubated at RT for 20 min (329). 0.5 ml of Folin-Ciocalteaux reagent (Appendix II) was then added and the tubes were incubated in dark at RT for 15 min and the absorbance was measured at 660 nm against a reagent blank. The protein content was determined from a standard curve prepared using bovine serum albumin as a standard.

4.2.3 Lipid

Lipids were extracted from 1 ml of the envelope or membrane sample using 5 ml Chloroform: Methanol (2 : 1 v/v) mixture (79). The chloroform layer was separated using a separating funnel after extraction, and placed in a clean pre-weighed glass flask, and was evaporated to dryness using N_2 gas and the weight of the lipid determined.

4.2.4 Total Carbohydrate

100 µl of envelope or membrane samples was taken in a test tube and the volume was made to 1 ml using distilled water. It was then mixed thoroughly with 50 µl of 80% of aqueous phenol (330). After an addition 5 ml of concentrated H_2SO_4 the tubes were incubated at RT for 5 min. cooled under running tap water and the absorbance was measured at 480 nm. The total carbohydrate content was measured from a standard curve prepared using D-glucose as a standard (Appendix V).

4.3.4 Hexosamine

1 ml of envelope or membrane sample was hydrolysed using 2 ml of 6 N HCl in a glass stoppered Pyrex tube at 100°C for 12 h. The HCl was then evaporated to dryness using a sand bath and the volume was made to 1 ml with distilled water. To 400 µl of the above hydrolysate, was added 0.05 ml Reagent A (Appendix II) mixed and allowed to stand at RT for 5 min (331). An aliquot of 0 15 ml of Reagent B (Appendix II) was then added, the tubes stoppered and heated in a boiling water bath for 3 min and allowed to cool at RT. Thereafter, 0.3 ml of Reagent C (Appendix II) and 2.7 ml of glacial acetic acid were added and the tubes were incubated at 37°C for 20 min. Absorbance was measured at 585 nm, on cooling the

reaction mixture at RT. The hexosamine content was obtained from a standard curve obtained using glucosamine as a standard (Appendix V)

4.3.5 Total Sulphate

One ml samples of envelope or membrane were hydrolysed using 6 N HCl for 12 h. The hydrolysate was evaporated on a sand bath and the volume was made to 1 ml with distilled water. To 1 ml of the above samples, 1.4 ml of 4% TCA and 0.5 ml barium chloride-gelatin reagent were added. The tubes were allowed to stand for 20 min and the absorbance was measured at 360 nm (332) and the sulphate content was determined from a standard curve using K_2SO_4 as a standard (Appendix V).

4.3 DETERMINATION OF Cu^{+2} / Zn^{+2} IONS IN CELLS OF HR

4.3.1 Incubation of HR with metal ions

HR cells grown in NGSM were harvested at the late log phase and the pellet obtained was washed with 20% NaCl The washed pellet was re-suspended in 20% NaCl to an absorbance of 2 at 600 nm To 100 ml of the above cell suspension metal ions of Cu^{+2} / Zn^{+2} in the form of $CuSO_4.5H_2O$ / $MgSO_4.H_2O$ were added to a final concentration of 5 mM. Also, in a separate set, HR cells having absorbance of 2 at 600 nm were incubated with 0.5 mm of Cu^{+2} / Zn^{+2} for 30 min at RT on a rotary shaker at 150 rpm before the addition of Cu^{-2} / Zn^{+2} at 5 mM concentration 5ml samples from each flask were immediately withdrawn and the flasks were incubated at RT on a rotary shaker at 150 rpm. Aliquots of 5ml were removed after intervals of 0.5, 1, 2, 4, 8 and 16 h from individual flasks, centrifuged separately at 12,000 rpm for 10 min. The pellet thus obtained in each case was washed in mineral salts with 20% NaCl and analysed for metal ions.

4.3.2 Atomic absorption spectrophotometric analysis of Cu^{+2} / Zn^{+2} in cell digests

Wet digestion of cell pellet

The washed pellet, dispersed in 2 ml of de-ionized water was mixed with 5ml of HNO_3 : H_2SO_4 mixture (2:1 v/v) in glass stoppered test tube and digested in a boiling water bath for 18 h or till the suspension became clear.

Estimation of metal ions

The digested samples were made up to 10 ml with de-ionized water and were analysed by the atomic absorption spectrophotometer (GBC 932 AA) for the content of Cu^{+2} or Zn^{+2} ions.

4.4 SCREENING FOR PRESENCE OF PLASMID IN HR

HR.cells grown in NTYE, NGSM and in NGSM with Cu^{+2} / Zn^{+2} (1mM) individually, were harvested at the late log phase and each cell mass was washed with 20 % NaCl. The washed cells were suspended in 20 % NaCl to an absorbance of 2 at 600 nm.

4.4.1 Alkali-lysis method

4 ml of each of the above cell suspension was centrifuged at 10.000 rpm at 4°C for 20 min and the supernatant was decanted. To the pellet was added 100 µl of ice-cold solution I (Appendix II) at RT and mixed by vigorous vortexing. 200 µl of freshly prepared solution II (Appendix II) was then added and mixed the contents by gently inverting the eppendorf tube 5 to 6 times. The tubes were incubated at 0°C for 10 min and 150 µl of ice-cold solution III (Appendix II) was then added. The contents were thoroughly mixed by gently shaking the tube. The tubes were then

incubated 0°C for 10 to15 min. The supernatant was decanted after centrifuging the tubes at 12000 rpm for 10 min into a clean, dry eppendorf tube and 2 volumes of chilled absolute alcohol (900 µl) was added The tubes were incubated at –70°C for 10 min and were centrifuged at 12,000 rpm for 20 min at 0°C to precipitate the plasmid DNA. Alcohol was removed using a micropipette and the pellet was air-dried for 30 min The pellet was then dissolved in 50 µl of TE buffer (Appendix II) and checked for absorbance at 260; 256 and 280 nm. 50 µl of the dissolved sample along with 2 µl of the dye solution (Appendix II) was loaded on an agarose gel (0.8%) prepared in T.A.E. buffer (pH_8.0) (Appendix II) and the electrophoresis was carried out at 70 volts for 8 h. The gel was observed. for the presence of plasmid on UV Photodyne and photographed

4.4.2 Slot lysis method

2 ml each of the above cell suspension described above was centrifuged at 12.000 rpm. for 10 min at 4°C. The pellet was then mixed with 30 µl of lysis solution (Appendix II) and loaded into the wells of 0.5% agarose gel with a double comb system (Appendix IV). Electrophoresis was carried out at 8 mA for 1.5 h and at 40 mA for 15 h (119)

RESULTS

4.5 GROWTH OF HR WITH GLUCOSE AS SOLE SOURCE OF CARBON

HR on inoculation into NGSM with 20% NaCl and 0.2% glucose and incubation at RT at 150 rpm. grew with an initial lag of one d. and reached a maximum absorbance of 1.3 at 600 nm by the 5th d at a doubling time of 1.1 d. Stationary phase followed thereafter and persisted till 7th d, with no

change in absorbance as depicted in Fig. 34. During this growth the culture attained a pink pigmentation, by the 4th d. The pink colour darkened with further incubation up to 6 d. Colonies on NGSM agar were translucent and attained pink pigmentation by the 6th d (Plate 4.1).

4.6 TOLERANCE TO Zn^{+2} DURING GROWTH OF HR

During the growth of HR in NGSM containing different concentration of Zn^{+2}, the turbidity of HR steadily increased with time at 1, 2 or 3 mM as see in Fig. 35. However, no increase in absorbance was observed at 4 or 5 mM concentration. HR grew with a time lag of one d at 1mM concentration, reaching a maximum absorbance of 0.96 within 6 d, with a doubling time of 1.4 d. A two-d lag period was observed during growth at concentration of 2 mM or 3 mM that reached a maximum absorbance of 0.45 and 0.3 respectively on the 5th d. with no further increase on incubation up to 7 d. The doubling times were 2 and 2.5 d during growth of HR at concentration of 2 / 3 mM of Zn^{+2} respectively.

4.7 TOLERANCE TO Cu^{+2} DURING GROWTH OF HR

As seen from the Fig. 36, at concentration of 1 or 1.5 mM of Cu^{+2} the turbidity increased with time, where as no increase was observed at concentration of 2.5 or 3 mM up to 7 d of incubation. During the growth of HR at a concentration of 2 mM of Cu^{+2}, there was an initial increase in absorbance after 2 d, which, however, declined on the 3rd d and did not increase further, as seen in Fig. 36. HR grew with an initial time lag of 1 and 2 d reaching a maximum absorbance of 0.76 and 0.51 on the 5th d with doubling times of 1.25 and 1.5 d at concentration of 1 and 1.5 mM respectively. No increase in absorbance was observed when HR was inoculated into NGSM containing Cu^{+2} at concentrations of 2.5 or 3 mM.

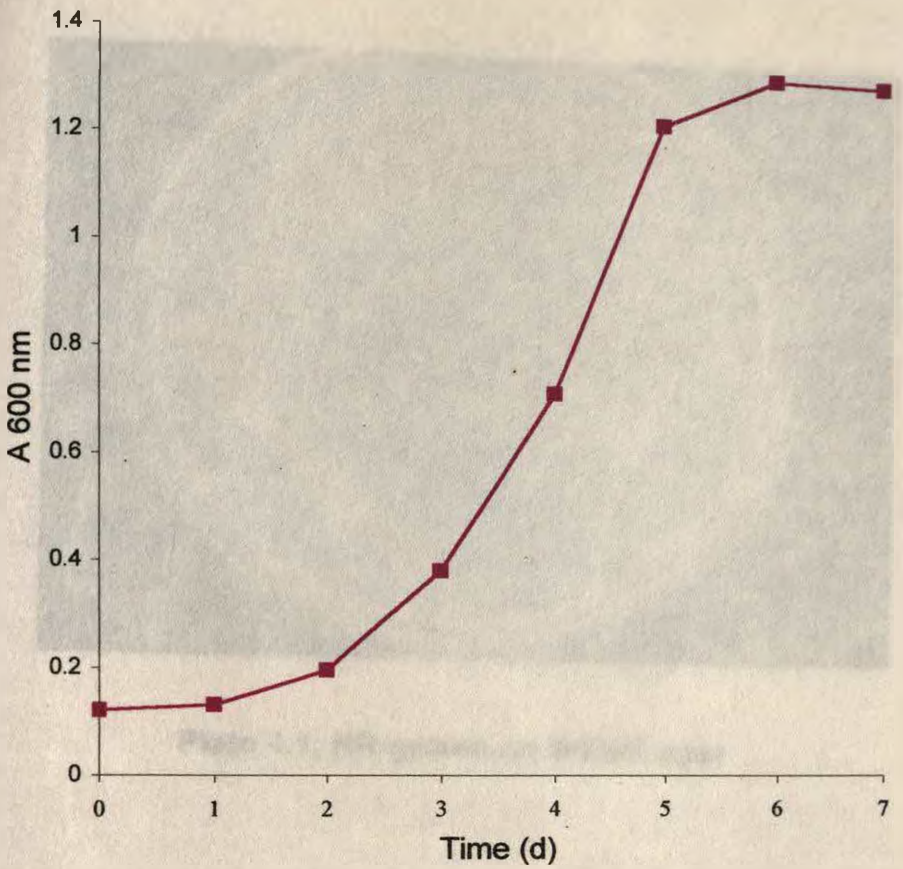


Fig. 34 Growth of HR in NGSM

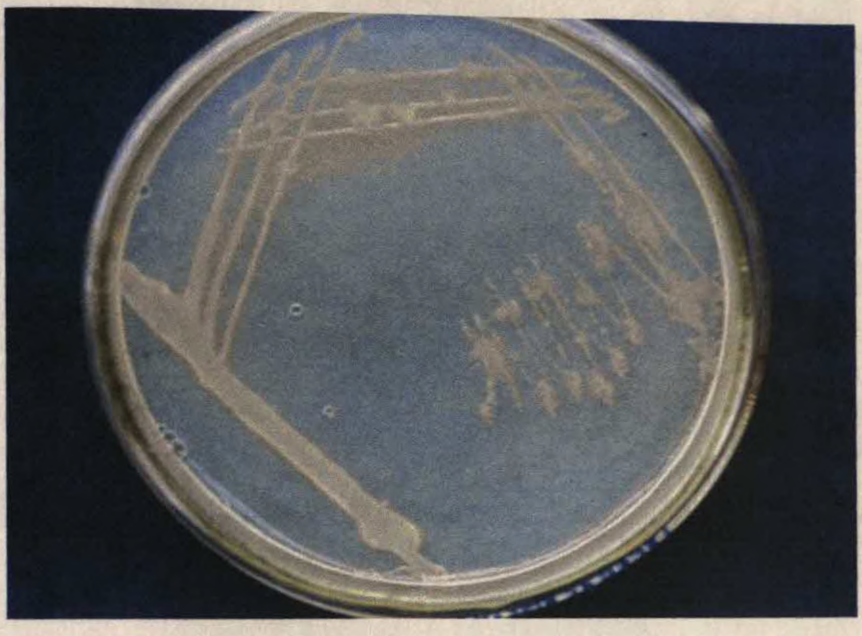


Plate 4.1. HR grown on NGSM agar

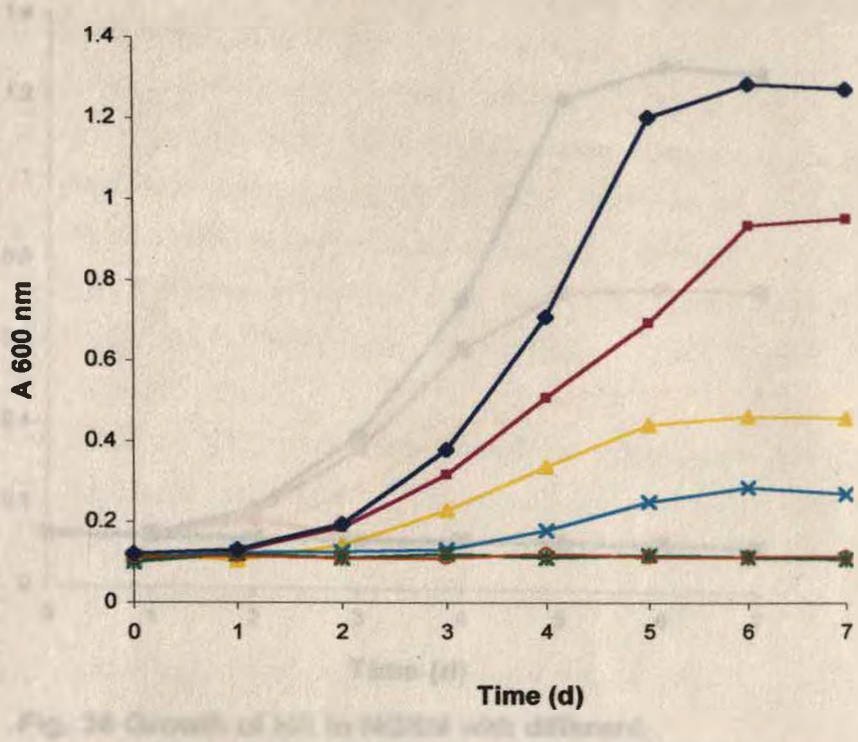


Fig. 35 Growth of HR in NGSM with different concentrations of Zn^{+2} ($ZnSO_4.5H_2O$)

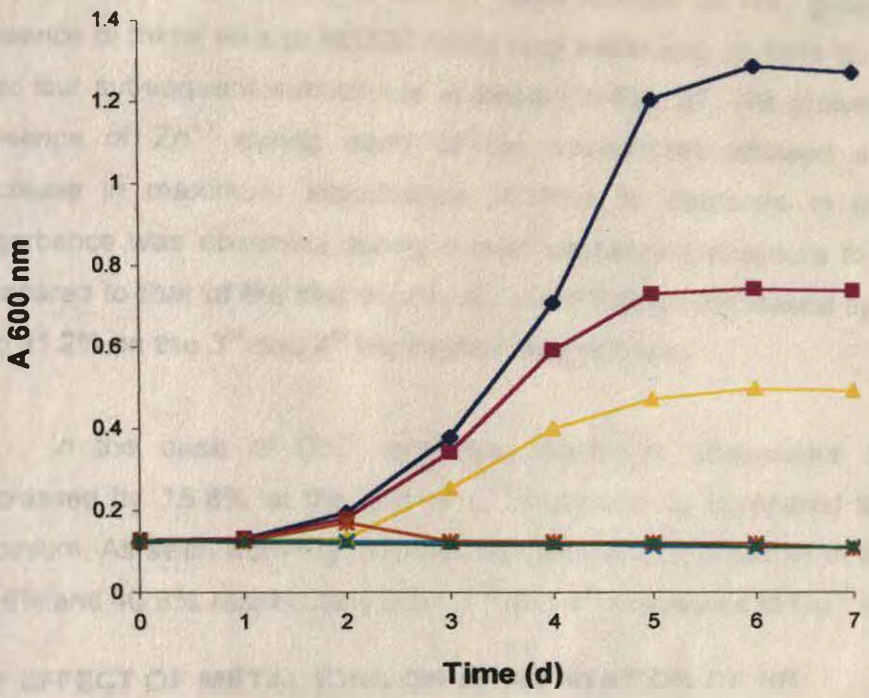


Fig. 36 Growth of HR in NGSM with different concentrations of Cu^{+2} ($CuSO_4.5H_2O$)

4.8 RESPONSE OF HR TO CONTINUOUS SUBCULTURE IN Cu^{+2} / Zn^{+2}

Growth response obtained on serial transfer of HR, grown in the presence of metal ions to NGSM containing metal ions of 1mM Cu^{+2} / Zn^{+2} over four subsequent subcultures is shown in Fig. 37. HR growing in the presence of Zn^{+2} during each of the subcultures showed a steady decrease in maximum absorbance. A 16.1 % decrease in maximum absorbance was observed during growth on second exposure to Zn^{+2} as compared to that of the first exposure, which further decreased by 24.5 % and 31.2% on the 3rd and 4th exposures, respectively.

In the case of Cu^{+2} ions, the maximum absorbance obtained decreased by 15.8% at the end of 2nd exposure as compared to the 1st exposure. As seen from Fig. 37, this was followed by a further decrease of 31.6% and 40.8% respectively after 3 rd and 4th exposures to Cu^{+2} ions

4.9 EFFECT OF METAL IONS ON PIGMENTATION OF HR

During growth in NGSM liquid medium without metal ions, HR developed faint pink pigmentation on the 4th d, which darkened on further incubation up to 6 d. Also, a dull pink pigmentation was observed when grown in NGSM containing Zn^{+2} at 1 mM concentration on the 4th d, which did not intensify on further incubation up to 6 d to the extent seen in HR growing in glucose alone. (Plate 4.2)

In the case of HR grown in the presence of Cu^{+2} (1 mM) in NGSM there was no visual discernable pigmentation. NGSM with Cu^{+2}, however, had a light blue colouration brought about by the Cu^{+2} ions (Plate 4.2) Sedimentation of the cells by centrifugation at 8000 rpm for 20 min revealed the presence of whitish cells with a faint blue tinge. On NGSM agar containing 1 mM Cu^{+2} HR grew as translucent colonies by the end of 3 d. at RT. These colonies turned light orange by 6 d; dull orange in 8 d

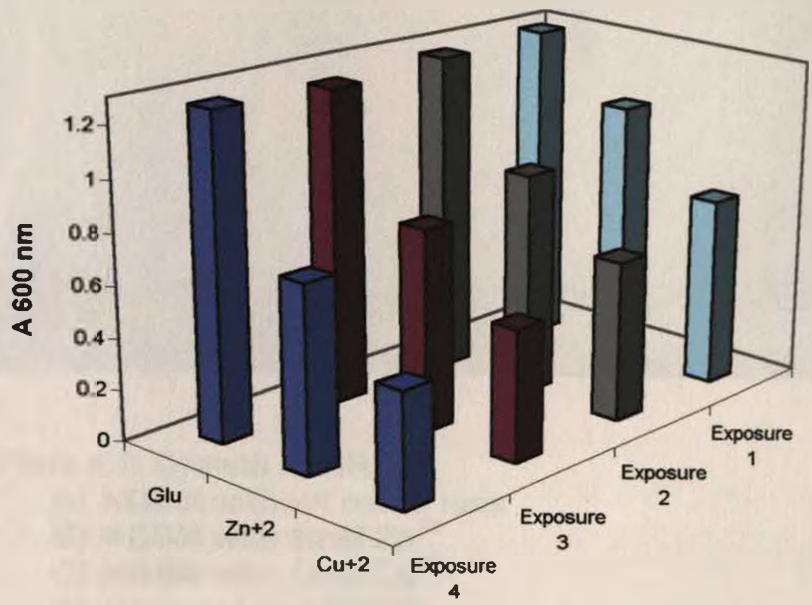


Fig. 37 Growth of HR on serial exposure to Zn^{+2} (1 mM) or Cu^{+2} (1 mM)

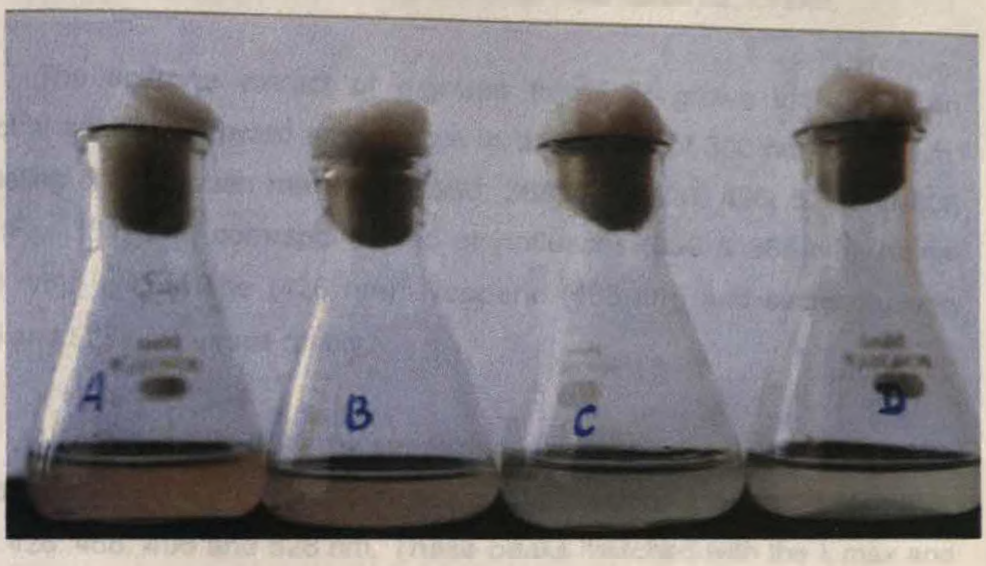


Plate 4.2. Growth of HR

A) NGSM without metal ions
B) NGSM with 1mM Zn^{+2}
C) NGSM with 1mM Cu^{+2}
D) Uninoculated NGSM

and creamish thereafter. Finally, after 12 d colonies attained a bluish tinge (Plate 4.3). The dull orange colonies present at the end of 6 d. when subcultured on to NGSM agar containing 1 mM Cu^{+2}, grew with a light orange tinge and attained creamish colour at the end of 10 d. However, these creamish colonies failed to grow when sub-cultured, further.

The acetone extract of pigment from HR grown in NGSM, on spectral analysis showed absorbance in the region of 300 nm to 600 nm, revealing 8 absorption maxima at 350; 368; 386; 426; 496; 528 and 600 nm (Fig. 38a) that corresponded to phytofluenes (350 & 368 nm); retinal (386 nm); β- carotene (426 nm); lycopene (468 nm) and bacterioruberin (496 and 528 nm), respectively.

As seen in Fig. 38b, the spectral analysis of cell extracts grown with metal indicated the presence of 8 peaks of absorption maxima at 350; 368; 386; 426; 468; 496 and 528 nm. These peaks matched with the λ max and some of them differed in their intensities with the peaks observed for pigment of HR cells grown in the absence of metal ions (Fig. 38a). For example, the intensity of the peak at 600 nm increased by 33% and peaks at 528; 496; 468 and 426 nm decreased by 15.6; 15.8; 15.1 and 4.2% respectively in the case of acetone extracts of HR cells grown in the presence of Zn^{+2}, as compared to those of the cells grown without metal ions.

Spectral analysis of acetone extracts of HR grown in the presence of Cu^{+2} revealed four additional absorption maxima at 660; 640; 560 and 540 nm beside the 6 peaks, normally observed between 300 – 700 nm when HR was grown in glucose alone. The peak at 600 nm in Cu^{+2} showed a 62.5% increase in intensity while the peaks at 426; 386; 368 and 350 nm increased by 40; 37.5; 39.1 & 65.2% in comparison with those of the cells grown without metal ions. The peaks at 528 nm and 468 nm were

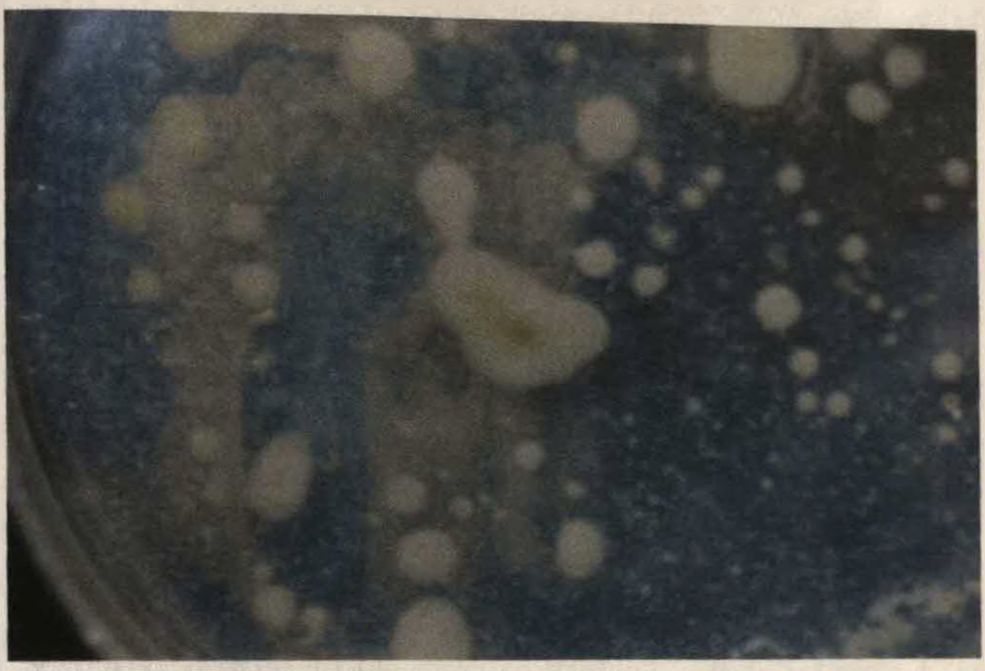


Plate 4.3. HR grown on NGSM agar with 1mM Cu^{+2}

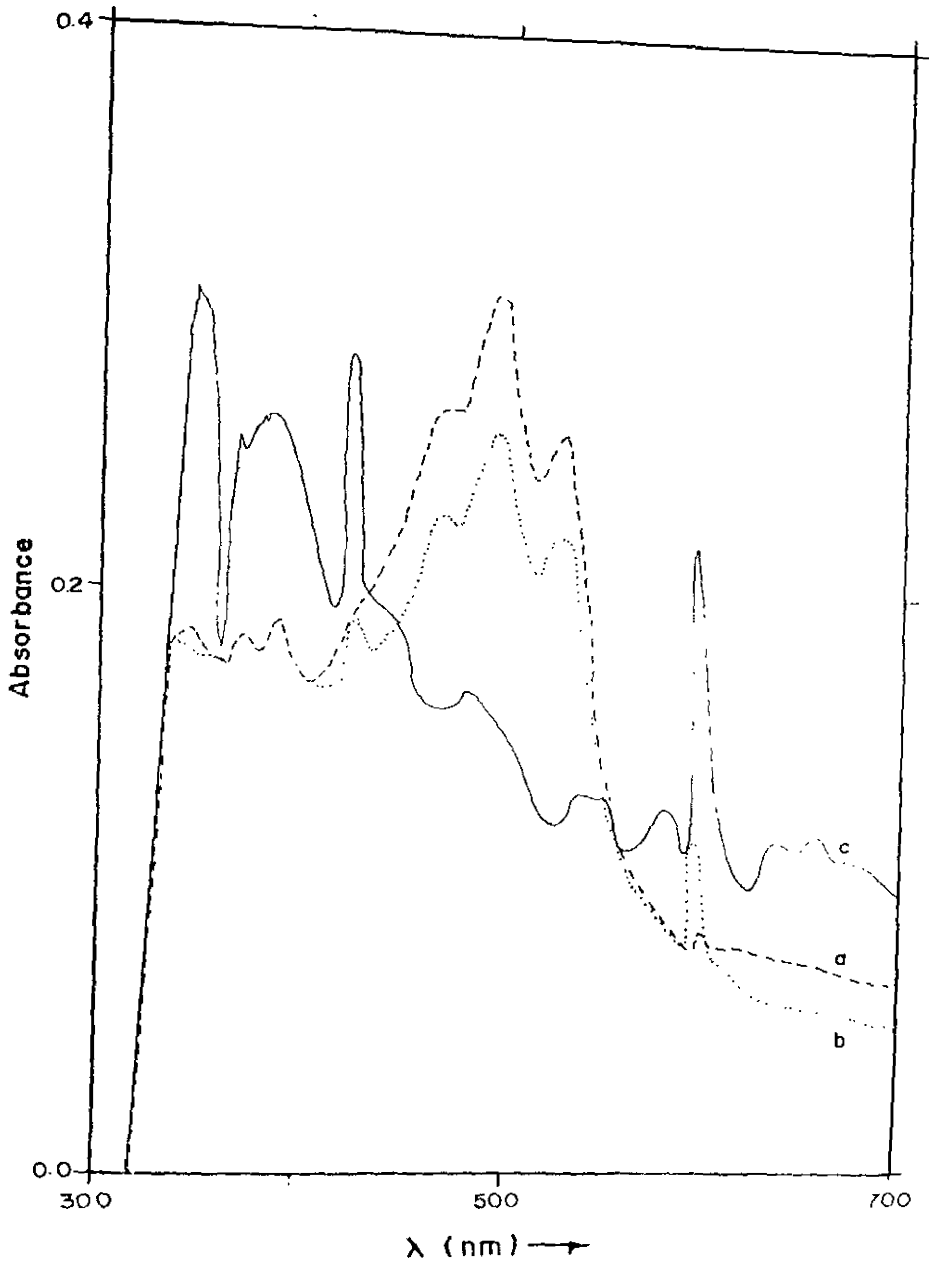


Fig. 38 Pigment profiles of HR cells grown in
a) NGSM b) NGSM with 1 mM Zn^{+2}
c) NGSM with 1 mM Cu^{+2}

absent in acetone extracts of HR grown in the presence of Cu^{+2} ions (Fig 38c).

4.10 CHANGES IN CELL SURFACE HYDROPHOBICITY OF HR GROWN WITH Zn^{+2} / Cu^{+2}

Resting cells of HR grown in NGSM with either 1 mM Zn^{+2} / Cu^{+2} when mixed with n-hexadecane for MATH assay, moved to hexadecane phase the absorbance of aqueous layer declined by 50 - 58% as seen in Table 4.1. In contrast, the resting cells of HR prepared by growing in NGSM without Zn^{+2} / Cu^{+2} and treated likewise, showed a very little difference (Table 4.1). The percent hydrophobicity calculated on the basis of absorbance were 3, 58 and 50 % for the cells grown in NGSM and in the presence of Zn^{+2} / Cu^{+2} respectively.

4.11 CHANGES IN CELLULAR LIPIDS OF HR GROWN WITH Zn^{+2} / Cu^{+2}

HR grown in NGSM gave a total yield of 52.1mg/L of whole cell lipids by modified Bligh & Dyers method. The yield of total lipids decreased to 40.4 mg/L and 38.2.mg/L in cells grown in the presence of Zn^{+2} and Cu^{+2}, respectively.

The total lipids, on fractionation yielded A_1 & A_2 fractions in a ratio of 1:2.The A_1 fraction on TLC analysis in Petroleum ether : Diethyl etner Acetic acid (90 : 10 : 1v/v/v) in case of HR grown in NGSM without Cu^{+2} / Zn^{+2} , resolved into a total of 6 spots with Rf values of 0.86, 0.76, 0.57, 0.36, 0.12 and 0.05 that corresponded to S_1, S_2, S_3, X_1, X_2 and X_3 respectively of the A_1 fraction of cells grown in NTYE medium (Fig. 39). However, only five spots corresponding to S_1, S_3, X_1, X_2 and X_3 respectively were observed on TLC analysis of A_1 fractions from HR cells grown in the presence of either Cu^{+2} or Zn^{+2} ions (Fig. 39).

Table 4.1 Hydrophobicity of HR grown in NGSM with and without metal ions

Growth condition	OD of cell suspension		Calculated % affinity to n- hexadecane
	A_1	A_2	
NGSM with 20% NaCl	1.00	0.97	3
NGSM with 20% NaCl and 1 mM Cu^{+2}	1.00	0.50	50
NGSM with 20% NaCl and 1 mM Zn^{+2}	1.00	0.42	58

A_1: Initial OD of HR cell suspension in PUM-NaCl buffer

A_2: OD of HR cell suspension after interaction with n-hexadecane

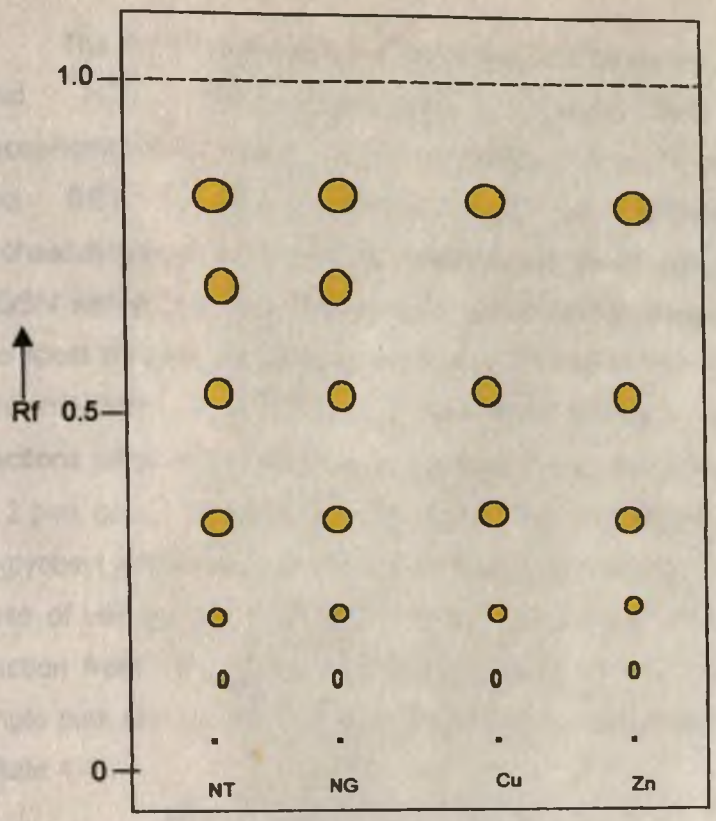


Fig. 39 Thin layer chromatogram of A1 fraction of Lipid of HR grown in NTYE (NT), NGSM (NG), NGSM + Cu^{+2} (Cu) and NGSM + Zn^{+2} (Zn)

The A_2 fractions on TLC analysis in Chloroform : Methanol : Acetic acid : H_2O (80 : 22.5 : 10 : 4 v/v/v), and on spraying with phosphomolybdate, showed the presence of 2 spots with Rf values of 0.84 and 0.61, that corresponded to Archaetidylglycerol and Archaetidylglycerol phosphate respectively in all cases i.e. HR grown in NGSM with and without metal ions and in NTYE. However, the intensity of the spots decreased by approximately 10 fold in the cells grown in NGSM and in NGSM with Cu^{+2} / Zn^{+2}. Duplicate thin layer chromatogram of A_2 fractions when sprayed with α – naphthol reagent revealed the presence of 2 pink coloured spots with Rf values of 0.66 and 0.47 that correspond to Diglycosyl archaeol and Sulphated diglycosyl archaeol, respectively in the case of HR grown in NGSM with or without Cu^{+2} ions. Whereas, the A_2 fraction from HR grown in NGSM containing Zn^{+2} ions, showed only a single pink spot at Rf 0.47, corresponding to Sulphated diglycosyl archaeol (Plate 4.4).

4.12 CHANGES IN CHEMICAL COMPOSITION OF CELL ENVELOPE AND PLASMA MEMBRANE OF HR GROWN WITH Zn^{+2} / Cu^{+2}

As depicted in Fig. 40, cell envelopes of HR contained 61% protein, 30% lipid and 4.4% carbohydrate on per cent dry weight basis. Minor quantities of hexosamine and sulphate were also found to be present. However, when grown in presence of Zn^{+2} or Cu^{+2}, the protein content of envelopes increased by 14.5 & 16.4% and the lipid content decreased by 16.2 & 19.6% respectively as seen in Fig. 41 with no significant changes in the other components.

The plasma membranes of HR cells grown in NGSM consisted of 56% protein, 37% lipid and 2% carbohydrate with minor quantities of sulphate and hexosamine, as depicted in Fig. 42. The variations observed in case of cells grown with metal ions, in protein content (12.0 & 14.2%

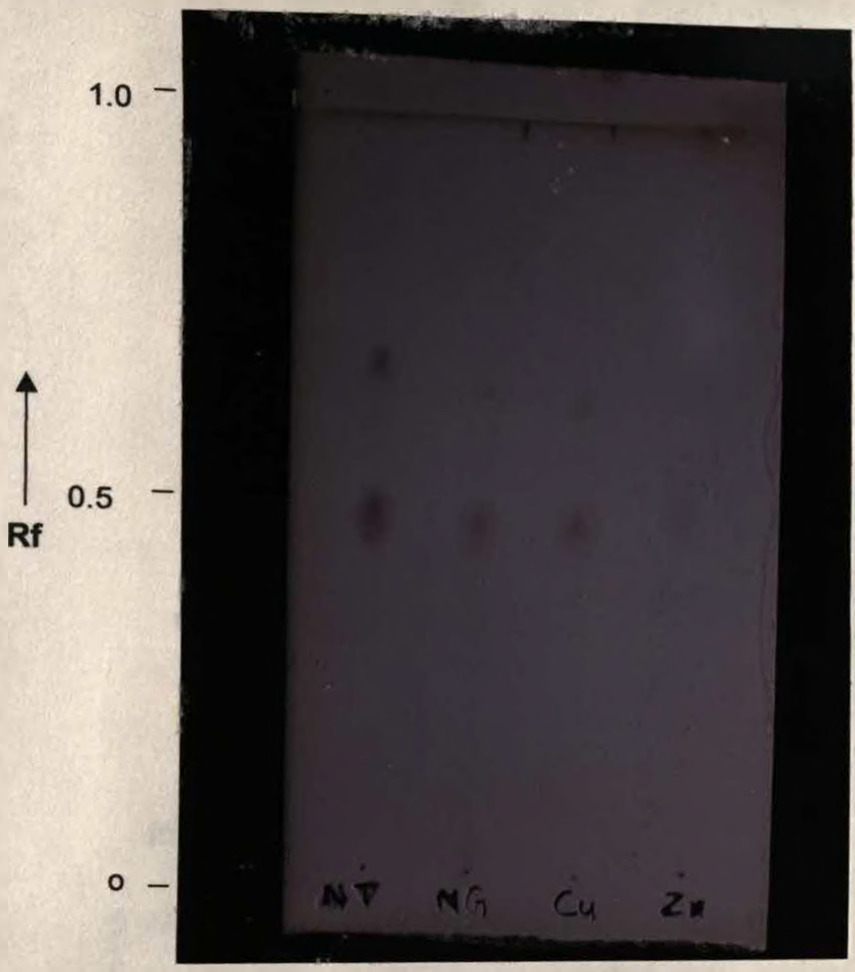


Plate 4.4. Thin layer chromatogram of A_2 fraction of HR grown in NTYE (NT); NGSM (NG); NGSM+Cu^{+2} (Cu) and NGSM+Zn^{+2} (Zn) on spraying with α-napthol

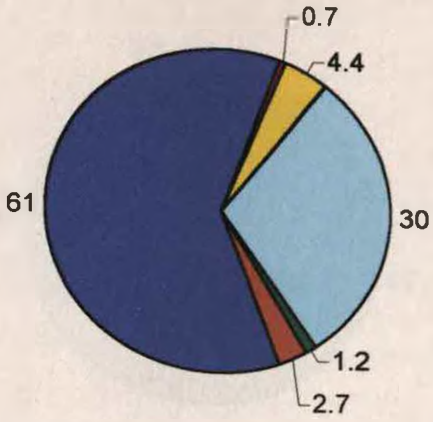


Fig. 40 Composition of cell envelopes of HR grown in NGSM (as mg \ 100 mg dry weight)

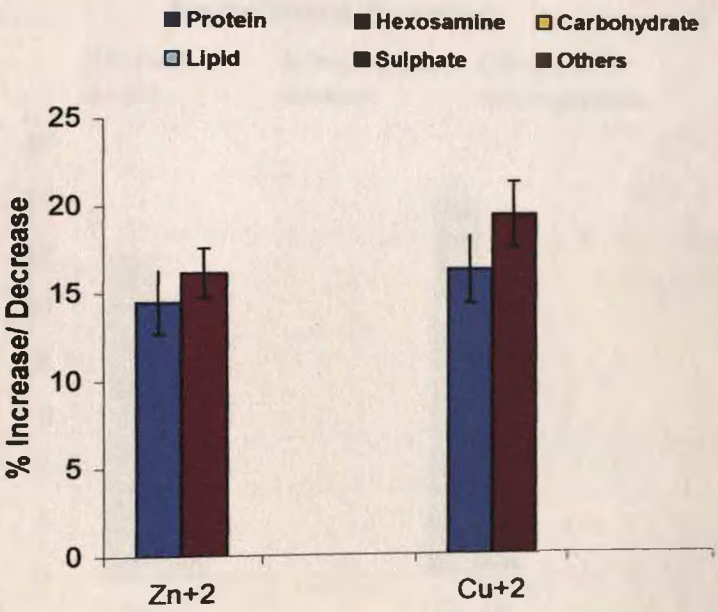


Fig. 41 Variation in cell envelope composition of HR grown in presence of metal ions

Increase in protein
Decrease in lipid

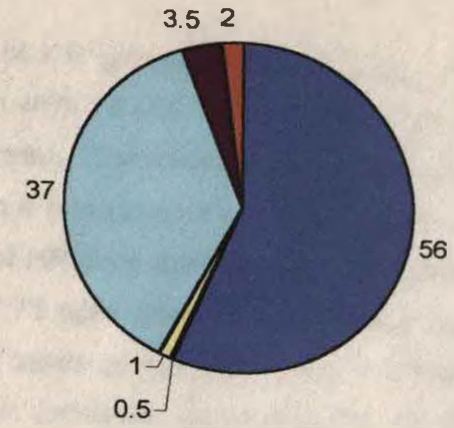


Fig. 42 Composition of plasma membranes of HR grown in NGSM (as mg/100mg dry weight)

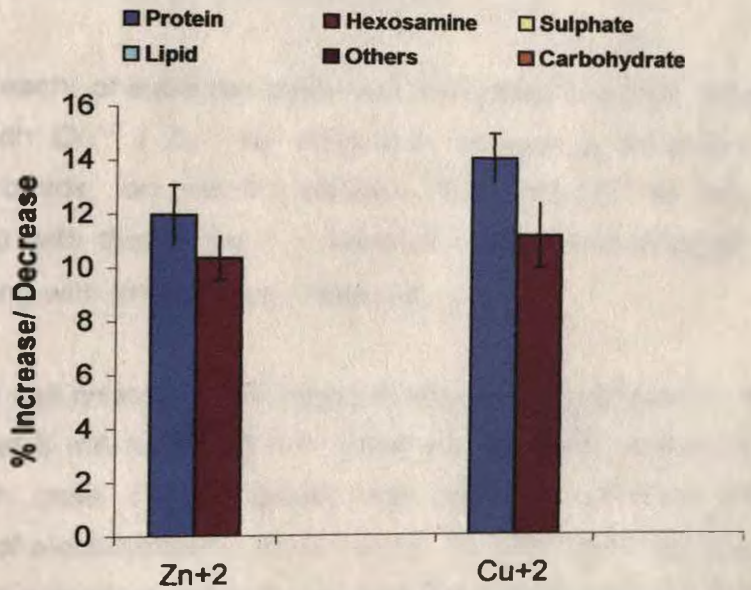


Fig. 43 Variation in plasma membrane composition of HR grown in presence of metal ions

■ Increase in protein
■ Decrease in lipid

increase for Zn^{+2} and Cu^{+2} respectively) and the lipid content (10.4 & 11.3% decrease in Zn^{+2} and Cu^{+2} respectively) are presented in Fig. 43.

4.13 ACCUMULATION OF Zn^{+2} / Cu^{+2} BY RESTING CELLS OF HR

Resting cells of HR grown in NGSM were used either directly or after a pre incubation with 0.5 mM Zn^{+2} / Cu^{+2}, for studying the sorption of individual ions, separately. The initial uptake of 31 ppm of Zn^{+2}, remained almost constant up to 4 h in case of HR cells directly incubated with metal or up to 8 h in case of HR cells that were prior incubated with metal ion. A maximum sorption of 75 ppm after 16 h of incubation with the metal ions was observed in both cases as depicted in Fig. 44 Resting cells took up to 60 ppm of Cu^{+2} in 16 h. However, as seen in Fig. 45, the uptake of Cu^{+2} by the cells pre-incubated with Cu^{+2} was 44 ppm in 16 h.

4.14 OCCURRENCE OF PLASMID IN HR

60 µl each, of extracted DNA from HR grown in NTYE, NGSM and in NGSM with Cu^{+2} / Zn^{+2} by alkali-lysis, showed a single band with Ethidium bromide on electrophoresis. The position of this band corresponded with that of the first band of *λ DNA/Hind III digest* (23.13 Kbp), run along with the samples (Plate 4.5).

Whole cell lysates of HR, when directly electrophoresed on a 0.5% agarose gel at 8 mA for 1h 30 min, gave a single band very close to the wells in each case, i.e. HR grown with and with out metal ions On continuation of electrophoresis at 40 mA for 15 h, this band resolved into 2 faint bands at a position about 8 mm from the loaded well The first band of λ DNA/Hind III digest (23.13 Kbp) and the band observed in alkali lysis DNA extract, run on the same gel moved further, to ~ 3 cm from the bottom of the gel as depicted in Fig. 46.

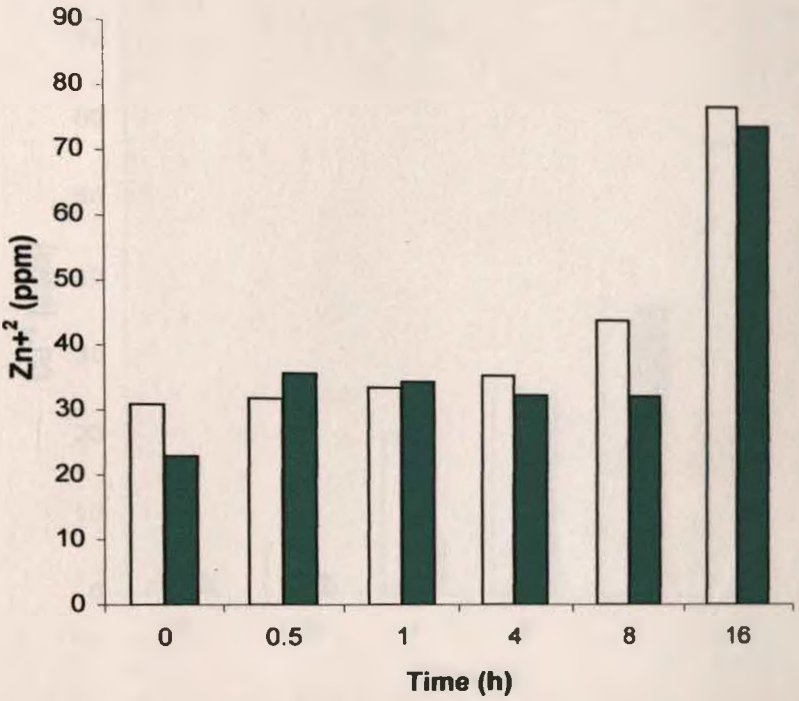


Fig. 44 Sorption of Zn^{+2} by HR

□ **Resting cells**

■ **Resting cells prior incubated with 0.5 mM Zn^{+2}**

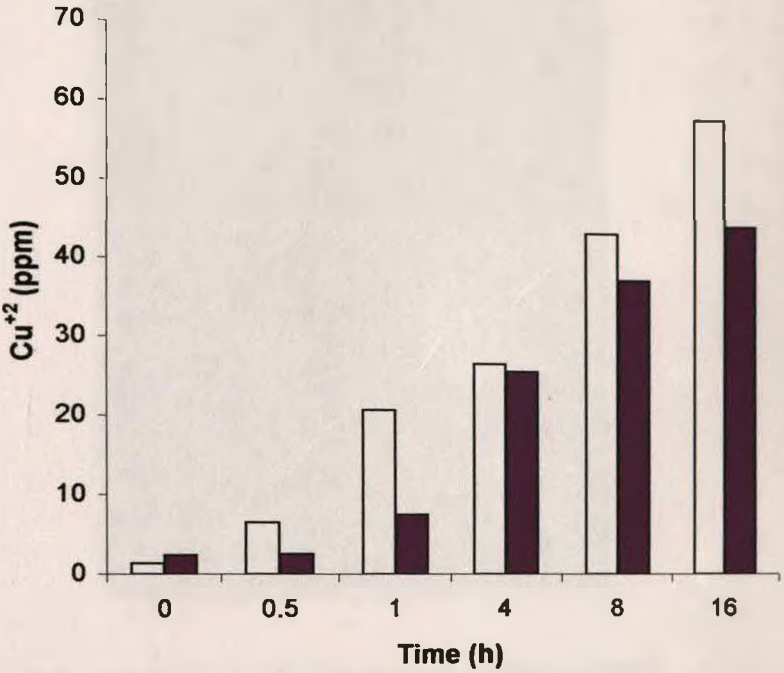


Fig. 45 Sorption of Cu^{+2} by HR

□ Resting cells

■ Resting cells prior incubated with 0.5 mM Cu^{+2}

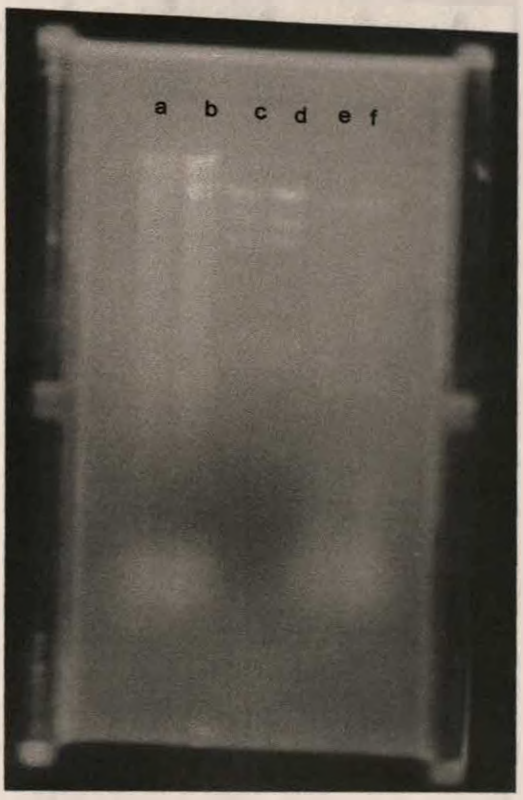


Plate 4.5. Agarose gel electrophoresis of DNA extracted by alkali-lysis from HR grown in: a: NTYE b: NGSM e: NGSM with Zn^{+2} f: NGSM with Cu^{+2} and c,d: λ DNA/Hind III-digest

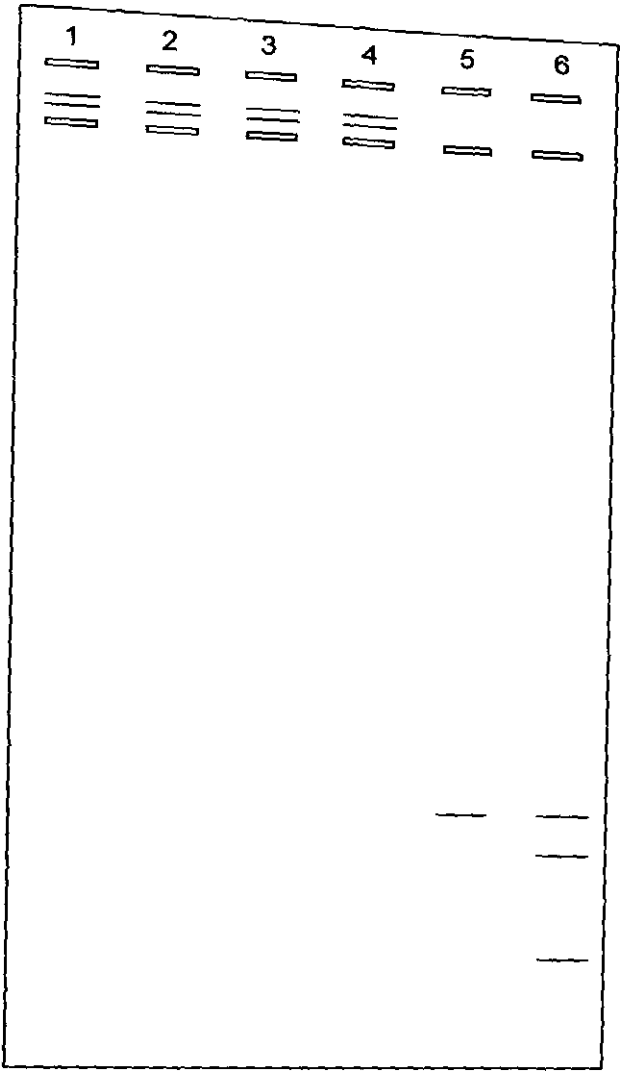


Fig. 46 Agarose gel electrophoresis of whole cell lysates of HR grown in:
1)NTYE 2)NGSM 3)NGSM with Cu^{+2} 4) NGSM with Zn^{+2} and
5) DNA extracted by alkali lysis method
6) Hind III digest of λ DNA

DISCUSSION

Environmental Pollution arising from heavy industrialisation and other anthropogenic activities has been a major concern in recent times through out the world (248-250). Estuarine regions, especially act as sinks for these pollutants due to the discharge of un / ill-treated effluents containing organic and heavy metal toxicants of industrial and domestic origin (343). Microflora, resident to these eco-niches, therefore would be exposed to these toxicants and often develop resistance mechanisms to survive the stress. HR, originally isolated from an estuarine salt pan showed resistance to a number of heavy metals during an earlier study in the laboratory (344).

Heavy metal ions of Cu^{+2} and Zn^{+2} play an important role as co-factors for many enzymes and are essentially required in nanomolar concentrations for growth. Yet, at higher concentrations, these metal ions are highly toxic to microbial cells causing alteration of enzyme active sites, oxidation of membrane components etc. (334).

In view of this, effect of heavy metal ions of Cu^{+2} and Zn^{+2} on HR was studied, herein. Presence of nutrient rich growth factors such as peptone and yeast extract have been shown to bind the metal ions in the medium and does not allow the true interaction of organisms with the metal ions (14). Hence, the response of HR to Cu^{+2} / Zn^{+2} has been studied in mineral salts medium (NGSM) containing 20% NaCl and 0.2% Glucose. Growth of HR in presence of Cu^{+2} / Zn^{+2} at 1 mM (Plate 4.2) indicates the tolerance of HR to these metal ions. However, increase in concentration of metal ions, above 1mm, decreased the growth of HR as seen in Fig. 35 for Zn^{+2} and Fig. 36 for Cu^{+2} and resulted in total inhibition of growth at 4mm for Zn^{+2} and 2mm for Cu^{+2}, thus indicating the MIC (minimum inhibitory concentration) levels for growth of HR. Studies done

on some other haloarchaea, reported the MIC levels of 0.5 & 2.5 mM of Zn^{+2} and Cu^{+2} respectively (260).

Although, HR grew fairly well at 1mm concentration of Cu^{+2} / Zn^{+2}, repeated exposure of these metal ions, decreased the growth as seen in Fig. 37, suggesting that continuous exposure to metal ions, if results in accumulation in the cell, may possibly reach toxicity levels affecting the metabolism and decreasing the growth. Heavy metal ions are largely reported to interfere with metabolic activities such as photosynthesis, N_2 fixation, and denitrification in microbiota (261,264), thus establishing toxicity. In the light of this, response of to heavy metal ions was studied by determining the cellular characteristics of HR, grown in presence of Cu^{+2} / Zn^{+2} in glucose mineral salts medium. Although, some workers reported the MIC levels of heavy metals to haloarchaea (260, 261), no reports are available on the effect of these heavy metal ions on cellular features of haloarchaea

As indicated in Plate 4.2, HR grew as dull pink pigmented cells in case Zn^{+2}, in contrast to the bright pink pigmentation observed in mineral salts medium without metal ions. The UV spectra of acetone extracts of these cells, indicated variations in intensities (in case of Zn^{+2}), and / or absorption maxima (in case of Cu^{+2}), as compared to those of cells grown without metal ions (Fig 38). However, the increase in intensities of phytofluenes (350 & 368 nm), retinal (386 nm) and of an unidentified component (600 nm) in HR cells grown with Cu^{+2} ions. (Fig. 38) as compared to their intensities in HR grown without metal ions, indicate that these components may possibly be involved in resistance mechanism employed by HR to, subvert the stress exerted by Cu^{+2} ions. Such reports of metal ion induced changes in pigmentation of haloarchaea have not been reported. Cd^{+2} ions, however, are known to delay the pigmentation in the eubacterium *S. marcescens* (257).

The high increase in surface hydrophobicity of HR cells grown in the presence of Zn^{+2} or Cu^{+2} ions, accounting to 50-58% as compared to the 3% of cells grown without metal ions, indicates the importance of surface hydrophobicity, especially under conditions of stress. Increase in surface hydrophobicity, as discussed by Vreeland (210), generally decreases the cell permeability and may possibly be involved in decreasing the influx of metal ions into the cell cytoplasm to some extent. Further, decreases in cell permeability resulting from an increase in cell surface hydrophobicity, also increases the thermodynamic energy required to push molecules across the cell envelope into the cytoplasm, thus, making it an energetically expensive process (210).

Presence of metal ions of Cu^{+2} / Zn^{+2} during growth of HR, decreased the total lipid content of HR. Further the absence of Dehydrosqualene (in both Cu^{+2} / Zn^{+2}) and Sulphated diglycosyl archaeol in case of cells grown with Zn^{+2} suggest that HR may possibly be responding to the metal ion stress by alterations at the cell envelope level. In order to verify this, the chemical composition of isolated cell envelopes and plasma membranes of HR cells grown in NGSM with and without metal ions was investigated. Chemical analysis of cell envelopes and plasma membranes of HR cells grown in NGSM with and without metal ions indicated alterations in protein and lipid composition in both cases as seen in Fig. 41 & 43. A number of studies (335,336), indicate that microorganisms generally respond to environmental stress conditions, primarily by alterations at the cell surface level, since this is in direct contact with the outer environment.

Growth of HR on NGSM agar incorporated with copper (1 mm) resulted in formation of pink colonies, which eventually turned creamish and bluish on further incubation, indicating that metal ions possibly

accumulated in the cells of HR, thus giving a bluish white tinge (Plate 4.3) In view of this, sorption of metal ions by resting cells of HR was studied As depicted in Fig. 45, resting cells of HR grown in NGSM, on incubation with Cu^{+2} ions, showed 60 PPM in the cells, after 16 h. Similarly, HR cells also showed an uptake of Zn^{+2}, up to 78 ppm in 16 h of incubation. Many microorganisms display metal resistance, mediated through plasmids (269) HR when screened for presence of plasmid by alkali lysis method. showed a single band, positioned close to the 23 Kbp band of λ DNA/Hind III digest. However, on screening for plasmid by the slot lysis method. recommended for megaplasmids generally seen in haloarchaea (119), the band of 23 Kbp was not detected. Instead, 2 other bands. were seen very close to the wells loaded with lysates of HR cells grown with and without metal ions (Fig 46) The position of these bands very close to the well even after electrophoresis for 15 h at 40mA. suggests that. they could be of megaplasmids. and the absence of the band seen in alkali lysis method. suggests that it could be a sheared part of the chromosome. possibly extracted during the alkali lysis. It is also possible that. the DNA may have got sheared giving a band on electrophoresis. However, the presence of these megaplasmids. although very widely reported in haloarchaea (119,120) may not be mediating the metal resistance of HR. as they are seen to be present in cells grown without metal ions also

Halobacterium strain R_1 MTCC 3265 grows in mineral salts medium containing 20% NaCl and 0.2% glucose as sole source of carbon The toxicity of Cu^{+2} and Zn^{+2} ions to HR increases with increase in their concentration The MIC values of Cu^{+2} and Zn^{+2} to HR are 2 & 4 mM respectively Continuous presence of metal ions of Cu^{+2} / Zn^{+2} at sub-lethal concentration of 1 mM. decreases the growth of HR at every consecutive growth cycle

Presence of metal ions of Cu^{+2} / Zn^{+2} during growth alters the pigment profile between 540 and 680 nm; chemical composition of cell envelopes and plasma membranes; increases the cell surface hydrophobicity by 50 – 58% and abolishes the lipid moieties of Dehydrosqualene and Diglycosyl archaeol. Lysates of resting cells of HR incubated with metal ions showed an accumulation of 60 ppm (Cu^{+2}) and 75 ppm (Zn^{+2}). Plasmid screening by slot lysis method, showed the presence of two bands, which could be mega plasmid in nature, in HR grown with or with out metal ions.

Halobacterium strain R_1 MTCC 3265 is tolerant to Cu^{+2} and Zn^{+2} and accumulates the two metal toxicants via alteration of cellular features

CHAPTER 5: SUMMARY

HR (*Halobacterium* strain R_1 MTCC 3265) an extremely halophilic bacterium, isolated from an estuarine salt pan in Goa (340), was able to grow at solar salt concentration ranging from 10 to 30%, pH 5 to 9 and at temperatures of RT (28 - 30°C) to 55°C. The optimal conditions for growth were 25% salt, pH 7 and 45°C. HR displayed distinct archaeal characters such as absence of Diaminopimelic acid, presence of glycerol diether moieties, and lysis on exposure to water.

The acetone extract of the orange-red pigment of HR, on spectral analysis, indicated the presence of bacterioruberins, lycopene, carotene, retinal and phytofluenes. The progressive decrease in retinal and phytofluene peaks, coinciding with increase in lycopene and bacterioruberin peaks indicated that retinal and phytofluenes could be the precursors for lycopene and bacterioruberin. Whole cell lipids of HR consisted of the non-polar lipids of Squalene, Dehydrosqualene Menaquinone and the polar lipids of Archaetidylglycerol, Archaetidylglycerol phosphate, Diglycosyl archaeol and Sulphated diglycosyl archaeol.

Growth of HR at 10% salt (RT & pH 7); pH 5 (25% salt & RT) or at 55°C (25% salt & pH 7), decreased the intensity of salt dependent P_1 to P_9 cellular proteins seen prominently in cells grown at 15 – 30% salt, pH 6 – 9 or at RT – 45°C. Of the P_1 to P_9 bands, P_1 to P_7 & P_9 were found to be located in the envelope and P_2 to P_5 & P_6 in the membrane fraction. Cells grown at pH 5 / 6 lacked protein band Px of 13 1 KDa and showed faint bands of Px_1 & Px_2 at 14 & 16 KDa. Cells grown at 10% salt (RT & pH 7), pH 5 (25% salt & RT) or at 55°C (25% salt & pH 7) were also, less susceptible to water as compared to the other growth conditions. Further, HR cells grown at 30% salt (pH 7 & RT), pH 9 (25% salt & RT) or at 55°C displayed cell surface hydrophobicity of 12.3, 7.0 and 6.0% respectively as compared to its absence under other conditions of growth. HR showed

progressive increase in intensities of bacterioruberin and lycopene components of the pigment with increase in salt concentration from 15 to 30%, pH 6 to 9 and at temperature from RT to 37°C. Cell grown under these conditions showed a higher intensity of these components as compared to the retinal and phytofluenes. In contrast, cell grown at 10% salt (pH 7 & RT): pH 5 (25% salt & RT) or 55 °C (pH 7 & 25% salt) showed increased abundance of phytofluene and retinal components compared to bacterioruberin and lycopene. Cells grown at 10 or 30% salt (pH 7 & RT), pH 5 or 9 (25% salt & RT) or at 55 °C (25% salt & pH 7), showed increased total lipid content in cells and showed an extra lipid moiety in A_1 and A_2 fractions of total lipids, apart from the moieties of Squalene, Dehydrosqualene, Menaquinone, Archaetidylglycerol, Archaetidylglycerol phosphate, Diglycosyl archaeol and Sulphated diglycosyl archaeol.

Minimum inhibitory concentration levels of Cu^{+2} and Zn^{+2} to growth of HR were 2 and 4 mM respectively. Serial exposure to either Cu^{+2} or Zn^{+2} (at 1 mM) resulted in progressive decrease in growth of HR, with each exposure. Pigment components of phytofluenes and retinal and an un-identified peak at 600 nm showed increased intensity in cells grown in presence of Cu^{+2} as compared to the cells grown without metal ions. Cell surface hydrophobicity of HR cells increased by 58 & 50% in Zn^{+2} and Cu^{+2} respectively as compared to glucose grown cells. Presence of Cu^{+2} ions during growth of HR, abolished Dehydrosqualene lipid moiety and Zn^{+2} abolished Diglycosyl archaeol and Dehydrosqualene lipid moieties.

Presence of Zn^{+2} ions during growth, increased the protein content by 14.5% and 12.4% and decreased the lipid content by 16.2 and 10.4% in cell envelopes and membranes respectively, while the presence of Cu^{+2} ions during growth resulted in 16.4% and 14.2% increase in protein content and in 19.6% and 11.3% decrease in lipid content in cell envelopes and membranes, respectively. HR cells incubated with metal

ions showed an accumulation of 75 ppm of Zn^{+2} and 60 ppm of Cu^{+2} on 16 h of incubation. Plasmid screening by slot lysis method showed the presence of 2 bands, which could be of mega plasmid size.

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APPENDICES

Appendix I

Media

a) NTYE (Tryptone Yeast Extract medium containing 25% Solar salt)

$MgSO_4.7H_2O$	20.0 g
KCl	5.0 g
$CaCl_2.2H_2O$	0.2 g
Yeast Extract	3.0 g
Tryptone	5.0 g
Crude salt	250.0 g
Distilled water	1000.0 ml
Adjust pH to neutrality with 1 M NaOH	
For solid media: (Agar)	20.0 g

Digest for 30 minutes. sterilise at 121°C and 15 lbs pressure for 20 minutes.

b) NGSM (Mineral salt medium containing 20% NaCl and 0.2% Glucose)

NaCl	200.0 g
$MgCl_2.6H_2O$	13.0 g
$CaCl_2.6H_2O$	1.0 g
KCl	4.0 g
$NaHCO_3$	0.2 g
NH_4Cl	2.0 g
$FeCl_3.6H_2O$	0.005 g
KH_2PO_4	0 5 g
Distilled water	1000.0 ml
*Glucose solution	100.0 ml
Adjust pH to neutrality with 1 M KOH	
For solid media: (Agar)	20.0 g

Digest for 30 minutes. sterilise at 121°C and 15 lbs pressure for 20 minutes

*2% Glucose solution

Glucose	2.0 g
Distilled water	100 0 ml

Glucose solution is separately sterilised at 121 °C and 15 lbs pressure for 10 minutes. This is then added to sterilised liquid medium or solid medium.

Appendix II

Stains, Buffers and Reagents

1. TTC dye reduction:

PUM-NaCl buffer (Phosphate Urea Magnesium-NaCl buffer)

$K_2HPO_4.3H_2O$	2.220 g
KH_2PO_4	0.726 g
$MgSO_4.7H_2O$	0.020 g
Urea	0.180 g
NaCl	20.0 g
Distilled water	100.00 ml
pH	7.1

2. Gram stains

1) Crystal violet
A) 2 g crystal violet in 200 ml methylated spirit
B) 8 g ammonium oxalate in 800 ml distilled water
mix A and B to make up to 1 litre

2) Grams Iodine

Iodine	10 g
Potassium iodide	20 g
Distilled water	1000 ml

3) Saffranine

Saffranine	500 mg
Distilled water	100 ml

3 Protein estimation

Reagent A
2% (w/v) Sodium carbonate in 0 1N Sodium hydroxide

Reagent B
0.5% (w/v) Copper sulphate in1% (w/v) Sodium potassium tartarate

Reagent C
Reagent A 50ml + Reagent B 1ml

Reagent D
Folin- Ciocalteaux solution: Distilled water (1:3)

4. Hexosamine estimation

Reagent A : 1.5% Acetic anhydride in dry acetone
Reagent B : 0.7M Potassium tartarate
Reagent C : 10% (w/v) Dimethylaminobenzaldehyde

5. Sulphate estimation

Barium chloride-gelatin reagent
Dissolve Diffco Bacto Gelatin (1.5 g) in 300 ml of water at 60-70°. Leave at 4°C overnight. After warming to RT, add barium chloride (1g) to 200 ml of this solution, stir to dissolve And leave to stand for 2 - 3 hours.

6. Plasmid extraction

(a) Alkali lysis

(i) Solution I
50 mM Glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)

(ii) Solution II
0.2N Sodium hydroxide
10% (w/v) Sodium dodecyl sulphate

(iii) Solution III

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

(iv) TE buffer (pH 8.0)
10 mM Tris.Cl
1mM EDTA

(v) 5x Tris Acetate buffer (pH 8.0)

Tris. Base	2.42 g
Glacial acetic acid	0.57 ml
0.5M EDTA	1 0 ml

(vi) Tracking dye
0.25 % (w/v) Bromophenol blue

40 % (w/v) Sucrose
Store at 4°C

(b) Slot lysis

i) Tris - borate buffer

89 mM Tris base, 2.5 mM Di Sodium EDTA, 8.9 mM Boric acid

Tris	1.078 g
Di Sodium EDTA	0.0930 g
Borate	0.275 g
Distilled water	100 ml

ii) Lysis solution

Ficoll	0.104 g/ml,
Lysozyme -	0.83 mg /ml,
RNAse -	0.166 mg /ml
Bromophenol blue	0.25 mg /ml in tris -borate buffer.

Appendix III

Preparation of thin layer chromatography plates

Thin layer Silica gel plates were prepared by dissolving silica gel G / H [Acme / Qualigens] in distilled water to obtain slurry of desired thickness. The slurry was spread evenly using a glass rod on to clean dry grease free glass plates placed side-by-side and fixed by means of leucoplast. The air-dried plates were then activated in the oven at 110^0C for 30 to 40 minutes and used as and when needed

1. *Phosphate spray reagent*

Dodeca phosphomolybdic acid	5 g
Ethanol	100 ml

2. α- Naphthol spray reagent
   1) 0.5 % α- naphthol in 50 % methanol in water
   2) 5 % Sulphuric acid in ethanol

Appendix IV

Preparation of Polyacrylamide gel:

Two pre-cleaned and dried glass plates were placed one against the other, separated by 1.5 mm spacing and were fixed using plastic clips. The two vertical edges and the lower edge were sealed using 1% agar solution. In a 150 ml vacuum flask, the separating gel solutions were mixed (Appendix IIc) except for ammonium per sulphate and TEMED and vacuum was applied for 4 to 5 minutes while stirring on a magnetic stirrer. TEMED and ammonium per sulphate were then added and the flask was shaken thoroughly taking care that air bubbles did not generate. The contents were then quickly introduced into the glass plate sandwich to a level of 4 cm from the top surface. It was over layered with distilled water to obtain a uniform upper surface and was allowed to set for about an hour. After setting of the separating gel, the distilled water was poured off. The stacking gel mixture was prepared in a similar way to separating gel mixture and poured in the sandwich of glass plates. A comb was quickly introduced in the sandwich to obtain the wells and the gel was allowed to set for 1-2 hours. On setting, the comb was gently removed and the wells were rinsed with distilled water.

Running of polyacrylamide gel electrophoresis

The plates were assembled in the electrophoretic unit. Each well was filled with the tank buffer. The upper and lower tanks were also filled with the tank buffer. The lid was placed on the unit and the electrodes were connected to the power pack. The power supply was set at 120 volts and 30 mA for 1.5 mm thick gel. It was pre-run for 30 minutes. The samples were then loaded and the electrophoresis was carried out.

i) Monomer solution (30% T, 2.7% C)

Acrylamide	58.4 g
Bis acrylamide	1.6 g
Distilled water	to 200 ml

ii) Separating gel buffer (1.5 M tris, pH 8.8)

Tris	36.3 g
Adjust pH to 8.8 with HCl	
Distilled water	200 ml

iii) Stacking gel buffer (0.5 M tris, pH 6.8)

Tris	3 g
Adjust pH to 6.8 with HCl	
Distilled water	50 ml

iv) Treatment buffer (0.125 M tris, pH 6.8, 4% SDS, 20% glycerol and 10% 2-Mercaptoethanol)

Tris (stacking gel buffer)	2.5 ml
10% SDS	4 ml
Glycerol	2 ml
2-Mercaptoethanol	1 ml

v) Tank buffer (0.025 M tris, pH 8.3, 0.192 M Glycine and 0.1% SDS)

Tris	12 g
Glycine	57.6 g
10% SDS	40 ml
Distilled water	To 4 litres

vi) Dye solution

Bromophenol blue	0.1 g
Sucrose solution (50%)	100 ml

vii) Staining solution (0.025% Coomasie blue R-250, 40% methanol, 7% SDS)

Coomasie blue R-250	0.5 g
Methanol	800 ml
Stir until dissolved	
Acetic acid	140 ml
Distilled water	to 2 litres

viii) Destaining solutions

Destaining solutions	I / II
Methanol	500 ml/500 ml
Acetic acid	100 ml/700 ml
Distilled water	to 1 litre/10 litres

ix) Separating gel (10% T, 2.7% C)

Monomer solution (i)	20 ml
Separating gel buffer (ii)	15 ml

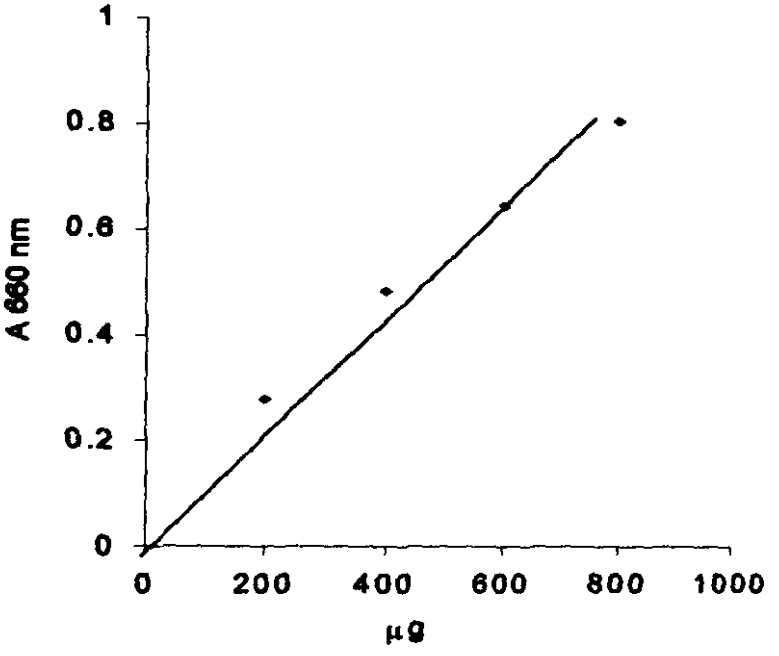
10% SDS	0.6 ml
Distilled water	24.1 ml
Ammoniumper sulphate (10%)	300 µl
TEMED	20 µl

x) Stacking gel (4% T, 2.7% C)

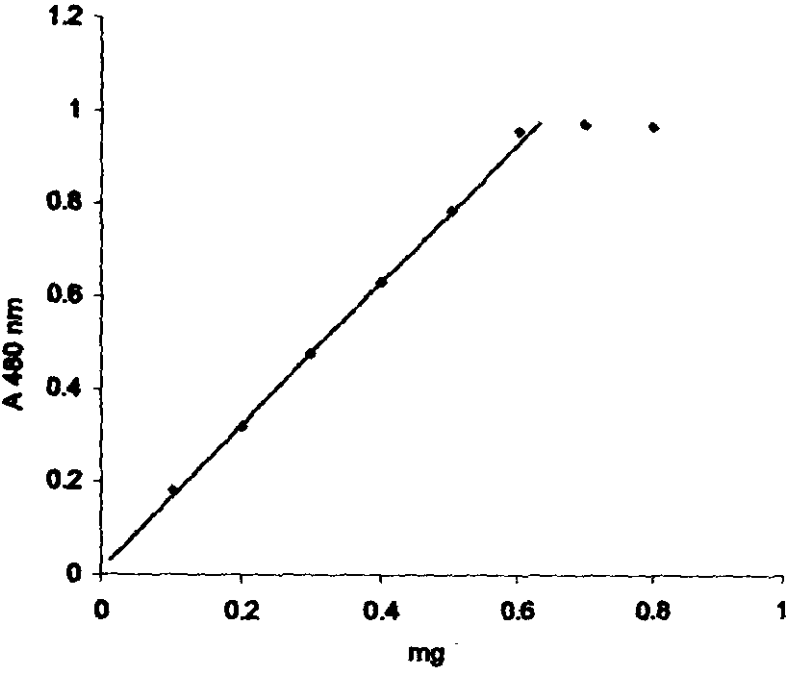
Monomer solution (i)	2.66 ml
Stacking gel buffer (iii)	5 ml
10% SDS	0.2 ml
Distilled water	12.2 ml
Ammoniumper sulphate (10%)	100 µl
TEMED	10 µl

Preparation of agarose gel with a double comb :

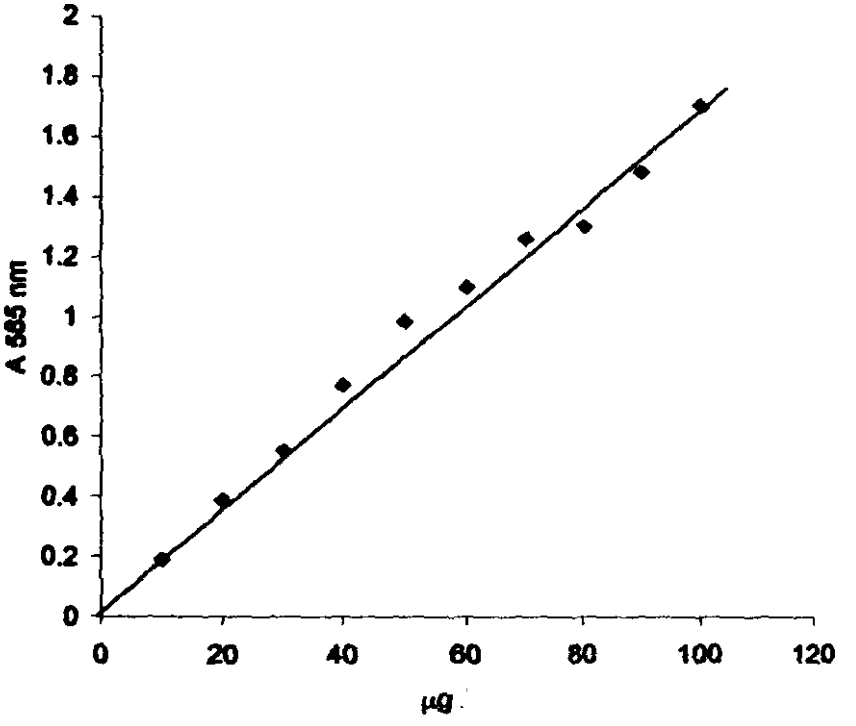
In a 250 ml flask, 50 ml of 0.5% agarose in Tris borate buffer (Appendix II) was prepared. Agarose was dissolved by keeping the flask in microwave oven for one minute. The solution was cooled to 45°C. Ethidium bromide solution (5 µg/ml) was added and was immediately poured into the gel platform. Two combs were quickly inserted taking care that the wells of the two combs were aligned with each other. After the gel was set, second comb was removed and the wells were filled with 0.4% agarose solution containing 1% SDS. After agarose in the wells of the second comb was solidified, the first comb was removed and the samples were loaded and the electrophoresis was carried out



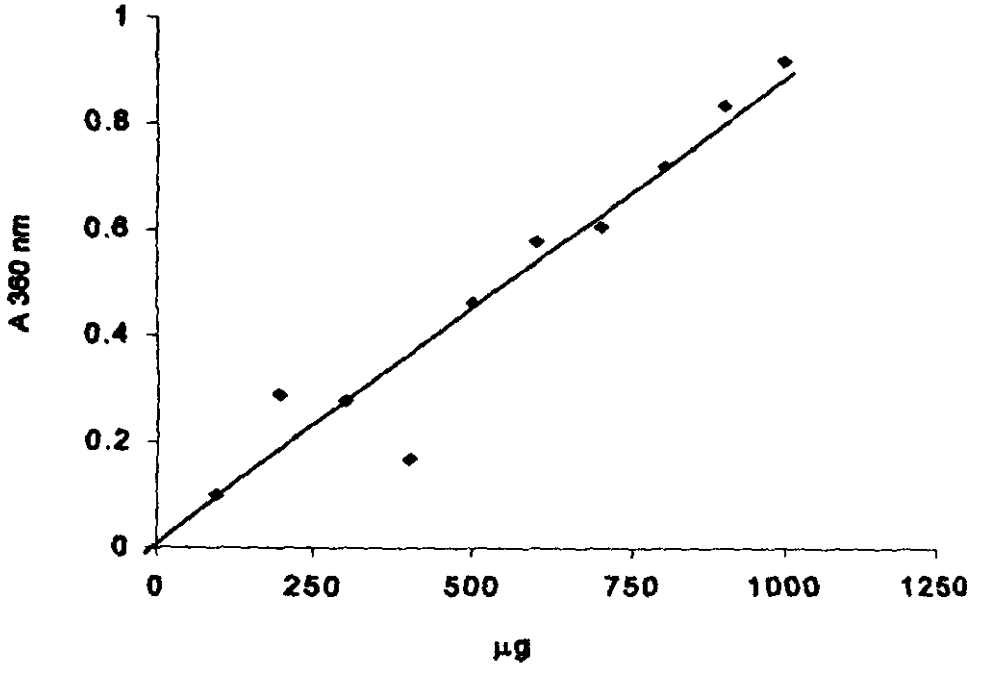
Standard graph for protein estimation



Standard graph for total sugar estimation



Standard curve for hexosamine estimation



Standard curve for sulphate estimation