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 R1 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 (A₆₀₀ ~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 IV) was then added, again boiled for 2 min, cooled to RT and mixed with 20 μl 0.1% bromophenol blue solution (Appendix IV). 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 (A₆₀₀~2), 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 P1 through P9 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 (Px1 & Px2), 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 P1 through P9, seen in cells grown at pH 7, only P3 (34 KDa); P5 (26 KDa); P6 (22 KDa) & P9 (12.8 KDa) were prominent and the protein bands P1 (93.1 KDa); P2 (42.4 KDa); P4 (32 KDa) & P8 (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 P₁ through P₉ 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 P₁ through P₉ bands present in cells grown at RT, P₁ (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 P₃ (34 KDa), P₄ (32 KDa), & P₉ (12.8 KDa) showed equal intensity at all growth temperatures. Bands corresponding to P₂ (42.4 KDa), P₅ (26 KDa), P₆ (22 KDa) & P₇ (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 P₂, P₆ & P₇, and by 2 fold in the case of P₅ when cells were grown at 45 or 55°C. The band at 18.7 KDa corresponding to P₉ 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 3.2, 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, 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 hexasdecane 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

- 5, 6, 7, 8, 9
Flg. 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

<table>
<thead>
<tr>
<th>Varying parameter</th>
<th>OD of cell suspension</th>
<th>Calculated % affinity to n-hexadecane</th>
</tr>
</thead>
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<tr>
<td></td>
<td>$A_1$</td>
<td>$A_2$</td>
</tr>
<tr>
<td>% solar salt</td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>15</td>
<td>1.00</td>
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<td>1.01</td>
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<tr>
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</tr>
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<td>7</td>
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<tr>
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<td>1.00</td>
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</tr>
<tr>
<td>Temperature (°C)</td>
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<td>1.00</td>
</tr>
<tr>
<td>37</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>45</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td>55</td>
<td>1.00</td>
<td>0.93</td>
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$A_1$: 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

<table>
<thead>
<tr>
<th>% Solar salt in NTYE</th>
<th>Absorbance of pigment at λ (nm)</th>
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<td></td>
<td>600</td>
</tr>
<tr>
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<tr>
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<tr>
<td>25</td>
<td>0.02</td>
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<tr>
<td>30</td>
<td>0.21</td>
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</tbody>
</table>
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) 16%  c) 20%  d) 25%  e) 30%
Table 3.3 Intensities of peaks of pigment of HR grown in NTYE with different pH

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<tr>
<th>pH of NTYE</th>
<th>600</th>
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<th>494</th>
<th>468</th>
<th>426</th>
<th>386</th>
<th>368</th>
<th>350</th>
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<td>5</td>
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<td>0.22</td>
<td>0.18</td>
</tr>
<tr>
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<td>0.70</td>
<td>0.86</td>
<td>0.68</td>
<td>0.30</td>
<td>0.30</td>
<td>0.22</td>
<td>0.18</td>
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</table>
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

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Absorbance of pigment at λ nm</th>
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<td>600</td>
</tr>
<tr>
<td>RT (28-30)</td>
<td>0.02</td>
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<tr>
<td>37</td>
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<tr>
<td>45</td>
<td>0.12</td>
</tr>
<tr>
<td>55</td>
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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 A₁ 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 Rf 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 S₁, S₂ & S₃ accordingly identified as Squalene, Dehydrosqualene and Menaquinone respectively. However, an additional spot was visualized at Rf 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 A₂ fractions, visualized with dodecaphospho-molybdic acid revealed two spots at Rf 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 (Rf 0.66) and Sulphated diglycosyl archaeol (Rf 0.47) were also observed under all growth conditions. However, when the culture was grown at 55°C, the A₂ fraction showed the presence of an extra lipid spot (Rf 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

<table>
<thead>
<tr>
<th>% Solar salt</th>
<th>Total lipid (mg/L)</th>
<th>pH</th>
<th>Total lipid (mg/L)</th>
<th>Temperature (°C)</th>
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<tbody>
<tr>
<td>10</td>
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<td>RT (28-30)</td>
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<tr>
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<td>37</td>
<td>56.8</td>
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<td>69.4</td>
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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 A1 fraction of total lipids of HR grown in NTYE with varying pH
Plate 3.9. Thin layer chromatogram of A2 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<sub>600</sub>~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 (°C)

Reduction of TTC by resting cells of HR incubated at RT

- 60
- 80
- 60->80
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 invtro 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 P₁ to P₉ 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 P₁ through P₉ for structural stability. This was further confirmed by another 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 P₁ to P₇ & P₉ and the membrane fraction showed the presence of P₂ to P₅ & P₆ proteins. This is in keeping with Haloarchaeota 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 P₁ to P₉ bands were either absent or faintly present at pH 5. Similarly, with increase in growth temperature up to 45°C or 55°C, 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 P₁ to P₉ 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 P₁ to P₉ protein bands suggesting that, the protein bands of P₁ to P₉ 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 P₁ to P₉ 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 phytofluones 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 A₁ fraction and another extra lipid moiety was seen in A₂ 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 R1 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 P1 to P9 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 P₁ to P₉ bands faint. Cells grown at pH 5 / 6 lacked protein band Px of 13.1 KDa and showed faint bands of Px₁ & Px₂ at 14 & 16 KDa. Growth temperature had no effect on P₃, P₄ or P₉ bands. Exposure of whole cells of HR to water disintegrated majority of proteins including P₁ to P₉. Of the P₁ to P₉ bands, P₁ to P₇ & P₉ were found to be located in the envelope and P₂ to P₅ & P₆ 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.