Chapter - 3

Tolerance of Haloarchael cultures to Arsenic and Cadmium
Urbanisation and industrialization in coastal countries results in the release of effluents containing toxic metals such as As$^{+3}$, As$^{+5}$, Cd$^{+2}$, Cr$^{+6}$ etc. into open rivers and estuarine econiches. These recalcitrant metal ions exert toxic effects on the flora and fauna of the region. Interaction of various eubacteria with metals has formed the content of many studies (13, 15, 17, 48). Halophilic archaea are prevalent in marine ecosystems and are believed to posses inherent resistance mechanisms to overcome stress. The screening of twenty one haloarchaeal cultures (identified to genus level in Chapter 2) for their tolerance to As$^{+3}$, As$^{+5}$ and Cd$^{+2}$ is detailed in the first part of this chapter. Elucidation of the mechanism employed by one of the haloarchaeal cultures namely, Halobacterium strain R1 MTCC 3265 (GUSF), to tolerance of As$^{+3}$, As$^{+5}$ and Cd$^{+2}$ and determination of the fate of metal during growth of the culture forms the second part of the chapter.

**METHODOLOGY**

**3.1 EVALUATION OF RESPONSE OF HALOARCHEAL CULTURES TO As$^{+3}$, As$^{+5}$ AND Cd$^{+2}$**

**3.1.1 NTYE medium**

To evaluate the response of haloarchaeal cultures to heavy metal ions of As$^{+3}$/As$^{+5}$/Cd$^{+2}$, 1 M stock solution of the metal was prepared in sterile distilled water. Aliquots of the stock solution was added to a set of boiling tubes containing 5 ml of NTYE, to a concentration of 1, 2, 4, 8, 16,
32, 60, 70, 80 and 90 mM of As\(^{+3}\) / As\(^{+5}\) / Cd\(^{+2}\). Tubes were incubated on a rotary shaker (Orbitek, India) at 150 rpm, RT (27 – 30°C) and growth or no growth was monitored visually for five days.

3.1.2 NGSM medium

Response of the haloarchaeal cultures to As\(^{+3}\) / As\(^{+5}\) / Cd\(^{+2}\) was studied by using aliquots of the metal stock solution to a set of tubes containing 5 ml of NGSM to a concentration of 1, 2, 4, 8, 16, 32, 60, 70, 80 and 90 mM of As\(^{+3}\) / As\(^{+5}\) / Cd\(^{+2}\). Tubes were incubated on a rotary shaker (Orbitek, India) at 150 rpm and growth or no growth was monitored visually for five days.

3.2 DETERMINATION OF MAXIMUM TOLERANCE CONCENTRATION OF As\(^{+3}\), As\(^{+5}\) AND Cd\(^{+2}\) FOR HALOBACTERIUM STRAIN R1 MTCC 3265 (GUSF)

GUSF was selected primarily because it utilized a wide range of carbon substrates, produced PHA (polyhydroxyalkanoate) and exhibited tolerance to 1 mM As\(^{+3}\), 80 mM As\(^{+5}\) and 2 mM Cd\(^{+2}\) in synthetic medium (NGSM).

To triple sets of 250 ml Erlenmeyer flasks, each containing 100 ml of NTYE / NGSM medium, was added aqueous aliquots of 1 M Cd\(^{+2}\) (CdCl\(_2\)) or 1 M As\(^{+3}\) (NaAsO\(_2\)) to obtain a final concentration of 0.5, 1.0, 1.5, 20, 2.5, 3.0, 4.0, 4.5, 5.0, 5.5 and 6.0 mM of Cd\(^{+2}\) / As\(^{+3}\) ions. Also
aliquots of 1M As\textsuperscript{+5} (Na\textsubscript{2}HAsO\textsubscript{4}.7H\textsubscript{2}O) was added to NTYE / NGSM medium to obtain a final concentration of 20, 30, 40, 50, 60, 70, 80 mM As\textsuperscript{+5}. Individual flasks were then inoculated with three day old Halobacterium strain R\textsubscript{1}, MTCC 3265 (GUSF) grown in the same medium without metal. Flasks, were incubated at R.T on a rotary shaker at 160 rpm. Absorbance of culture broth from each flask was monitored at 600 nm every 8 h using a spectrophotometer (Shimadzu UV-240, Japan) against uninoculated medium maintained at identical conditions.

3.3 EFFECT OF CHEMICAL AND PHYSICAL FACTORS ON RESISTANCE OF GUSF TO As\textsuperscript{+3} / As\textsuperscript{+5} / Cd\textsuperscript{+2}.

3.3.1 Determination of the effect of pH of growth medium.

To triple sets of flasks containing 25 ml of NGSM of pH 4, 5, 6, 7, 8 and 9, As\textsuperscript{+3} was incorporated to a final concentration of 1 mM or As\textsuperscript{+5} to 30 mM or Cd\textsuperscript{+2} to 1mM. Flasks were inoculated with 3 day old GUSF grown in NGSM pH 7 without any cadmium. Flasks were incubated and monitored as described in Section 3.2. Appropriate control sets were maintained without any metal, under identical conditions.

3.3.2 Evaluation of effect of salinity of growth medium.

Triple sets of flasks containing 25 ml of NGSM medium having a final concentration of 0%, 5%, 10%, 15%, 20%, 25% and 30%. NaCl were taken. As\textsuperscript{+3} was incorporated to a final concentration of 1 mM or As\textsuperscript{+5} to 30 mM or Cd\textsuperscript{+2} to 1mM. Flasks were inoculated with 3 day old culture grown in NGSM (20% NaCl) incubated and monitored as described in Section 3.2. Control sets were maintained without any metal.
3.3.3 Determination of effect of temperature of growth medium

Triple sets of flasks containing 25 ml of NGSM, 20% NaCl, pH 7, were inoculated with 3 day old GUSF grown in NGSM at R.T As$^{+3}$ was incorporated to a final concentration of 1 mM or As$^{+5}$ to 30mM or Cd$^{+2}$ to 1 mM. Flasks were incubated at RT, 37°C, 45°C and 55°C. Appropriate control sets were maintained without any metal.

3.3.4 Selection of inoculum type

To 4 sets of flasks having 4 flasks each containing 25 ml of NGSM were incorporated with 1 mM As$^{+3}$, 30 mM A$^{+5}$, 1 mM Cd$^{+2}$ and a control flask without any metal. One set of flasks were inoculated with 3 day old GUSF grown in NGSM, the second with GUSF grown in NGSM + 1 mM As$^{+3}$ the third with GUSF grown in NGSM + 30 mM As$^{+5}$ and the fourth with GUSF grown in NGSM + 1mM Cd$^{+2}$. Flasks were incubated at R.T on a rotary shaker at 160 rpm and absorbance of culture broth monitored at A$^{600}$ against an uninoculated blank.

3.3.5 Evaluation of the effect of type of salt of As$^{+3}$ / As$^{+5}$ / Cd$^{+2}$.

To triple sets of flasks containing 25 ml of NGSM, Cd$^{+2}$ was incorporated to a final concentration of 1 mM from 1 M stock solution of cadmium as Cd(NO$_3$)$_2$ or CdCl$_2$. Flasks were inoculated and monitored as described previously.
3.4 EVALUATION OF THE MECHANISM OF TOLERANCE OF GUSF TO METAL IONS.

3.4.1 Standardisation of estimation methods for metals

3.4.1.1 Volumetric Methods

i) Iodometric Estimation of As$^{+3}$ (249).

5 ml of sample / culture supernatant was taken in a 25 ml flask. To this was added one drop of methyl red indicator. If yellow, 50% HCl was added dropwise until the colour changed to red. If red, 2 N NaOH was added till yellow and then 50% HCl added until colour returned to red. To the mixture was added 5 ml of distilled water and 0.5 ml of 1% starch solution and titrated with standard 0.05 N iodine solution (Appendix Ig) till the appearance of blue coloration.

ii) Iodimetric estimation of As$^{+5}$ (249)

5 ml of sample / culture supernatant was taken in a 250 ml glass stoppered flask. To this was added one drop of methyl red indicator. If yellow, 50% HCl was added dropwise until a colour changed to red. If red, 2 N NaOH was added till yellow and then 50% HCl added until colour returned to red. 10 ml of 1:1 HCl was added and warmed to 70°C. A pinch of NaHCO$_3$ was added and swirled gently, followed by the addition of 1g of potassium iodate. Flask was immediately stoppered with a tight fitting stopper, allowed to stand for 10 minutes, cooled and titrated with 1 N thiosulfate solution (Appendix Ig) till pale yellow, followed by the addition of 1 ml starch and titration continued till colorless.
3.4.1.2 Chromatographic separation of As$^{+3}$ and As$^{+5}$

Five ml of culture supernatant was taken and concentrated to 1 ml in boiling water bath, 15 μl of this was spotted onto 0.5 mm silica gel F$_{254}$ thin layer chromatographic glass plates and developed in methanol - ammonium hydroxide - 10 % TCA - water (65:15:5:15 by volume). The developed chromatogram was dried in air and sprayed with 1 % silver nitrate and heated in an oven at 80°C till the appearance of coloured spots.

3.4.1.3 Colorimetric methods

i) Estimation of cadmium by dithizone method (250).

One ml of the test solution / culture supernatant was taken in a 100 ml separating funnel and diluted with 9 ml of deionised water. 5 ml of 25% v/v potassium sodium tartarate was added dropwise till the red colour of the indicator turned to yellowish orange. To this 2 ml of NaOH – potassium cyanide solution (Appendix I h) was added, followed by 1 ml of hydroxylammonium chloride solution and 10 ml of dithizone solution. The reagents were added in the same order an mixed thoroughly after each addition. The separating funnel was allowed to stand at R.T. for phase separation. The solvent phase was transferred into a glass cuvette (3 ml) and measured at 530 nm against a blank of deionised water treated in the same manner. A standard curve (Appendix VII) was obtained using a stock of 1 μg Cd$^{+2}$ solution.
ii) Estimation of Cadmium by modified dithizone method

15 µl of 0.02 % Cadion 2 B (Appendix Ih) was spotted onto a Whatman No 1 filter paper. 15 µl of test solution acidified with 2 M acetic acid was spotted onto the reagent. While still moist 15 µl of 2 M KOH was then spotted over the test solution and colour observed.

iii) Estimation of arsenite and arsenate using KMnO₄

To 200 µl of 0.01 M KMnO₄, 100 µl of culture supernatant of GUSF growing in presence of arsenite or arsenate was added, mixed by gentle swirling and colour observed.

3.4.1.4 Gravimetric estimation of cadmium

Five ml of culture supernatant / test solution was taken in 25 ml flask. H₂S was passed into the solution through a Kipps apparatus for 5 min. The contents of the flasks were then transferred to a pre - weighed silica crucible, dried in the oven at 100°C for 4 h and weighed.

3.4.1.5 Estimation of cadmium by Atomic absorption spectroscopy.

Acid Digestion

Cell pellets were heated with a mixture HNO₃ - H₂SO₄ (3:1 v/v) on a heating mantle in a fuming cupboard until a clear solution was obtained. The volume was brought up to 5 ml using deionised water cadmium was estimated in this digest after appropriate dilution using GBC Atomic Absorption spectrophotometer and / or by the dithizone colorimetric method.
**Instrumentation**

Set the instrument to a wavelength of 228.8 nm using a cadmium hollow cathode lamp. The digested sample (after appropriate dilution) was aspirated into air – acetylene flame. Standard curve was obtained using a stock of 10 µg / ml Cd\(^{+2}\) solution.

### 3.4.1.6 X-ray diffraction analysis

Cells of GUSF were harvested by centrifuging 700 ml of 4 day old NGSM / NGSM + 1 mM Cd\(^{+2}\) broth at 13,000 rpm for 10 minutes at 10\(^{0}\)C in a high speed centrifuge (Haerus, Germany). This ensured a tight cell pellet. Supernatant was decanted by inverting the centrifuge tube onto tissue paper. The cell pellet was scooped with the help of a spatula and transferred to a clean, acid rinsed petriplates and dried in an oven at 100\(^{0}\)C for 3 hours with occasional sifting, taking care not to burn / char the cells. The dried cells were loosened and powdered using a mortar and pestle and coated onto a 20 mm x 15 mm area, on a tray of aluminium alloy and put in the diffractometer and X-rayed over 10\(^{0}\) - 70\(^{0}\) @ 3 on a X-ray generator (Phillips PW 1729).

### 3.4.2 Detection of metal in cells and cell supernatant.

#### 3.4.2.1 Cadmium

200 ml of N\(_4\)GES medium containing 1mM Cd\(^{+2}\) was inoculated with 3 day old GUSF and incubated at R.T on a rotary shaker at 160 rpm. At fixed time intervals, 5ml of the culture broth was removed, absorbance measured at A\(_{600nm}\), pH recorded using a pH meter (Labindia, India) and centrifuged at 8000 rpm, 4\(^{0}\)C to obtain cell pellet and supernatant. The
cell pellets and cell free supernatants were acid digested prior to estimation of cadmium.

3.4.2.2 As$^{+3}$

200 ml of NGSM medium containing 1mM As$^{+3}$ was inoculated with 3 days old GUSF and incubated at R.T on a rotary shaper at 160 rpm. At fixed time intervals, 10 ml of culture broth was removed, absorbance measured at $A_{600nm}$, pH recorded using a pH meter and centrifuged to obtain cell pellet and supernatant. As$^{+3}$ was estimated in cells after acid digestion and in supernatants using TLC and volumetric methods.

3.4.2.2 As$^{+5}$

200 ml of NGSM medium containing 30 mM As$^{+5}$ was inoculated with 3 day old GUSF and incubated on a rotary shaker at 160 rpm. At fixed time intervals 5 ml of culture broth was removed, absorbance measured at $A_{600nm}$, pH recorded and centrifuged to obtain cell pellet and supernatant. As$^{+5}$ was estimated in cells after acid digestion and supernatants using TLC and volumetric methods.

3.4.3 Determination of Biological factor involved in resistance

Estimation of extracellular protein

100 ml of culture supernatant of GUSF grown in NGSM in the presence or absence of metal was extracted with 200 ml of absolute ethanol and kept o/n in the cold room at 12°C. Protein content of ethanol precipitate, if any or 1ml of supernatant was estimated by Lowry method (317). To 1 ml of sample, 1ml of reagent C was added (Appendix Ic). After
10 min, 3 ml of Folin Ciocalteau reagent (1:10 dilution) was added and left for 30 min. Absorbance values were read at 640 nm on spectrophotometer (Shimadzu UV-240, Japan). Bovine serum albumin (1mg / ml) was used as standard.

3.5 DETERMINATION OF METAL BINDING COMPONENTS IN / ON CELLS OF GUSF

3.5.1 Localisation of Cd$^{+2}$ in cells of GUSF

Cells were harvested from a total of 2 L of NGSM flask of 2 L capacity. The cell pellet obtained was resuspended into minimal quantity of NSM (10 ml).

Disruption of GUSF cells by sonication

Cell suspension of GUSF was taken in a 25ml glass beaker and kept in an ice bath. The suspension was sonicated using the flat medium probe for 3 min using a duty cycle of 0.5 sec with a sonicator (Labsonic B. Braun, Germany). Sonication was repeated 4 times till complete disruption of cells occurred (cell suspension of disrupted cells showed broken cells under light microscope and did not form colonies on NTYE agar).

Preparation of cell envelopes (226).

The sonicated cell suspension was centrifuged at 5000 rpm to remove whole cells, if any and cell debris. The supernatant was decanted and re-centrifuged at 17,000 rpm for 1 h at 4°C cooling centrifuge (Remi C24, India). The sedimented cell envelopes and supernatants were collected separately and frozen at −76°C in a freezer (Denley, India) and used within a day of preparation.
Cadmium was estimated in the cell envelopes and cytoplasm fractions.

3.6.2 SDS-PAGE

GUSF grown in NGSM / NGSM + 1 mM Cd⁺² / 1 mM As⁺³ / 30 mM As⁺⁵ was used for whole cell protein analysis by SDS-PAGE. Electrophoretic pattern of proteins of cell envelope and cytoplasmic fractions of GUSF grown in the presence and absence of metal were also studied.

a) Preparation of protein lysates

3 ml of culture was centrifuged and washed twice with 20% NaCl. To this 10 µl of 20% SDS was added and boiled for three minutes followed by addition of 50 µl of treatment buffer (Appendix IIIId) and again boiled for 5 min. Finally 20 µl of dye solution was added and loaded into the wells of a SDS – polyacrylamide gel.

b) Preparation of the gel

Glass plates and spacers were cleaned with acetone swab. Teflon spacers were placed on three sides except on top and clipped with clamps. Molten agar (1 % w/v) was poured to seal the three sides from inside. Separating gel mixture was prepared according to Appendix III j. TEMED and APS were added last, solution swirled gently and poured into the glass plate sandwich to a level of about 4 cm from the top and overlaid with distilled water for uniform setting. After one hour, the water was drained. Stacking gel was prepared according to Appendix and poured on top of the separating gel. Teflon comb was inserted in the gel fluid immediately and allowed to set.
c) Electrophoresis

The comb was removed on setting of the gel, 30 µl of protein samples were loaded into each well and the lower and upper tanks filled with the running buffer (Appendix IIIe). Power supply was set to a constant current of 30 m amps or 100 volts. When the tracking dye reached the bottom of the gel in about 4 h power supply was turned off. Plates were separated carefully and gel was stained.

d) Staining and destaining

i) Coomassie blue staining

Gel was stained with coomassie blue (Appendix IIIf) for twelve hours and destained on a gel rocker (Orbitek, India), using destain solution 1 (Appendix IIIg) for 1 h and then transferred into destaining solution 2 (Appendix IIIg) till clear bands appeared.

ii) Periodic acid schiff reagent (251).

The gel was fixed overnight in 200 ml of PAS fixative (Appendix IIIf). The fixing solution was poured off and the gel treated with 0.7% periodic acid for 2-3 h, the gel was then treated with 0.2% sodium metabisulfite for half an hour and then transferred to a fresh solution of 0.2% sodium metabisulfite for 2 h. Gel was then stained with Schiffs reagent, till the appearance of pink bands.
RESULTS

3.6 RESPONSE OF HALOARCHEAL CULTURES TO METALS TO As$^{+3}$, As$^{+5}$ AND Cd$^{+2}$

3.6.1 NTYE medium

Cultures showed a high tolerance to As$^{+3}$, As$^{+5}$ and Cd$^{+2}$ ions. In nutrient rich medium all cultures were resistant to 50 mM of As$^{+5}$ (Figure 16). GUBF 5 and GUBF 10 were resistant to 4 mM As$^{+3}$, all other cultures were resistant to 2 mM As$^{+3}$ and cultures GUBF 1, GUBF 2, GUBF3, GUBF 6, GUBF 7, GUBF8 and GUBF 17 resistant to 1 mM of As$^{+3}$ as observed in Figure 15. All the cultures were resistant to 4 mM Cd$^{+2}$ with the exception of GUBF7 and GUBF 11 which were resistant to 1 mM Cd$^{+2}$ (Figure 17).

3.6.2 NGSM medium

In NGSM medium all the cultures were resistant to upto 80 mM As$^{+5}$. Above this concentration, addition of As$^{+5}$ to the NGSM medium formed a white precipitate due to which growth of the culture was not visible. Cultures GUBF 5 and GUBF 10 were resistant to 2 mM As$^{+3}$, while all the other cultures were resistant to 1 mM As$^{+3}$ with the exception of cultures GUBF 7, GUBF 8 and GUBF 11 which were resistant to 0.5 mM As$^{+3}$. Cultures GUBF 1, GUBF 2, GUBF 3, GUBF 6 and GUBF 12 failed to grow in NGSM containing even 0.1 mM As$^{+3}$. 
Figure 15: Tolerance of Haloarchaeal cultures to arsenite (As\textsuperscript{3+})

Figure 16: Tolerance of Haloarchaeal cultures to arsentate (As\textsuperscript{5+})
Figure 17: Tolerance of Haloarchaeal cultures to cadmium (Cd$^{2+}$)
As seen in Figure 17 all the cultures were resistant to 2 mM of Cd$^{+2}$ with the exception of GUBF 7, GUBF 8, GUBF 9, GUBF 11, GUBF 12, GUBF 19, GUBF 20, GUBF 22 and GUBF 23 which were resistant to 1 mM Cd$^{+2}$.

3.7 MAXIMUM TOLERANCE OF As$^{+3}$, As$^{+5}$, AND Cd$^{+2}$ FOR HALOBACTERIUM STRAIN R1 (GUSF)

3.7.1 NTYE medium

GUSF grew optimally in NYTE containing 25% NaCl, pH 7 without a lag reaching the stationary phase on day 4 with a maximum absorbance of 2.2 at 600nm. The orange red pigmentation seen by the second day increased in visual intensity with progress of growth. Addition of Cd$^{+2}$ affected initiation of growth (Figure 18A). Growth occurred after a lag of 4, 6, and 12 h with a maximum absorbance of 1.5, 1.4 and 1.2 in presence of 1 mM, 2 mM and 3 mM concentration respectively. GUSF did not grow at 5 mM concentration.

As seen in Figure 18B, at a concentration of 1 and 1.5 mM As$^{+3}$ the turbidity increased after a lag of 12 h with a maximum absorbance of 0.8 and 0.48 whereas no increase was observed at 2.0 and 2.5 mM upto seven days of incubation.

In presence of As$^{+5}$, culture grew with a maximum absorbance of 1.0 on the third day with a lag of 12 h at 30, 40 mM concentration (Figure 18c). At concentration above 45 mM concentration extensive precipitation of salts was seen and difficult to measure absorbance.
Figure 18A: Effect of cadmium on the growth of GUSF in NTYE.
3.7.2 NGSM medium

The growth of culture in mineral salts medium occurred after a lag of 16 h, attained a mauve pigmentation and reached a maximum absorbance of 1.4 at 600nm. In presence of upto 1mM Cd\(^{2+}\), culture grew the same way, except that the lag increased by 4 h. At 1.5 mM growth occurred after a days lag and attained a maximum absorbance of 0.65. With further increase to 2.0 mM, the lag correspondingly increased to 2 days with a maximum absorbance of 0.57. Culture failed to grow at a concentration of 2.5 mM Cd\(^{2+}\) (Figure 19A).

Growth of GUSF in NGSM containing As\(^{3+}\) as seen in Figure 19B, occurred after a lag of 1 day at 0.5 and 1 mM concentration attaining a maximum absorbance of 0.88 and 0.8 on the 3\(^{rd}\) day of growth. No increase in absorbance was observed in NGSM containing As\(^{3+}\) at a concentration of 1.5 and above.

During the growth of GUSF in NGSM containing different concentration of As\(^{5+}\), the turbidity of the culture steadily increased with time at 30, 40, 50 and 60 mM of As\(^{5+}\) as seen in Figure 19C, attaining a maximum absorbance of 1.2, 1.0 and 1.0 on the 3\(^{rd}\) day of growth. In NGSM medium containing 50 and 60 mM As\(^{5+}\) a white crystalline precipitate formed initially turned into a bright gelatinous precipitate with progress of growth. This pink gelatinous precipitate also appeared during growth of GUSF at 30 and 40 mM on the 4\(^{th}\) day.
Figure 19A: Effect of cadmium on the growth of GUSF in NGSM
3.8 PHYSICAL AND CHEMICAL FACTORS ON RESISTANCE OF HALOBACTERIUM STRAIN R1 to As¹⁺³, As¹⁺⁵ AND Cd¹⁺².

3.8.1 pH

GUSF grew over a pH range of 5 to 9 in NGSM. At pH 5, 6, 7 and 8 the culture grew with a mauve pigment and attained a maximum absorbance of 0.55, 0.95, 1.42 and 1.28 after a lag period of 24 h, 16 h, 16 h and 14 h respectively. At pH 9, the culture showed an intense pink pigmentation and achieved a maximum absorbance of 1.0 on the fourth day of growth (Figure 20A).

In the presence of 1 mM Cd¹⁺² at pH 5, GUSF grew with the lag of two days but attained a maximum absorbance of 0.5 by the 5th day of growth. At pH 6 and 7, culture grew with a maximum of 0.6 and 1.0 respectively. At pH 8, it attained a maximum absorbance of 0.88 and failed to grow at pH 9 (Figure 21A).

In the presence of As¹⁺³, as seen in Figure 22A, GUSF grew over a pH range of 5 to 8. At pH 5 and 6, growth occurred after a days lag reaching a maximum absorbance of 0.6 and 0.7 respectively. At pH 7 too growth occurred after a days lag with a maximum absorbance of 0.8. At pH 8 growth occurred after 32 h with a maximum absorbance of 0.6.

In the presence of As¹⁺⁵, GUSF grew over a pH range of 5 to 9. At pH 5 and 6, growth occurred after a lag 16 h and attained a maximum absorbance of 0.5 and 0.9. At pH 7 and 8, growth occurred with a lag of 16 h and reached a maximum absorbance of 1.3 and 1.0 respectively. At pH 9 growth occurred after a lag of 16 h with a maximum absorbance of 0.8 (Figure 23A).
Figure 20A: Growth of GUSF in NGSM at varying pH

Figure 20B: Growth of GUSF in NGSM at varying NaCl concentration
Figure 21A: Growth of GUSF in NGSM containing 1mM Cd^{2+} at varying pH

Figure 21B: Growth of GUSF in NGSM containing 1mM Cd^{2+} at varying NaCl concentration
Figure 22A: Growth of GUSF in NGSM containing 1mM As$^{3+}$ at varying pH

Figure 22B: Growth of GUSF in NGSM containing 1mM As$^{3+}$ at varying NaCl concentration
3.8.2 Salinity

GUSF failed to grow in NGSM having a NaCl concentration of 10% or less. At 15% NaCl, GUSF grew with a lag of 1 day and attained a maximum absorbance of 0.7. At 20%, the lag decreased to 16 h and attained a maximum absorbance of 1.43. At 25% and 30% NaCl concentration too, a sixteen h lag was observed and the culture attained a maximum absorbance of 1.1 and 1.0 respectively (Figure 20B).

In presence of Cd$^{2+}$, also GUSF could not grow at 10% or less. At 15% NaCl, GUSF grew with a lag of 18 h and attained a maximum absorbance of 0.75 (Figure 21B). At 20% and 25% NaCl, the culture grew with a lag of 20 h and attained a maximum absorbance of 1.0. At 30% NaCl, the culture attained a maximum absorbance of 0.88 after a lag of 12 h.

In presence of As$^{3+}$, as seen in Figure 22B GUSF failed to grow in NGSM, having a NaCl concentration of 15% or less. At 20 and 25% NaCl, GUSF grew with a lag of 1 day and achieved a maximum absorbance of 0.8. At 30% NaCl, the lag increased by 2 days and attained a maximum absorbance of 0.5.

In presence of As$^{5+}$ as seen in Figure 23B, GUSF grew at 15 to 30%, NaCl concentration. At 15% NaCl, GUSF grew with a lag of 24 h and attained a maximum absorbance of 0.9. At 20 and 25%. GUSF grew with a maximum absorbance of 1.3 and 1.0 after a lag of 16 h. At 30% NaCl concentration the culture grew with a lag of 16 h and attained a maximum absorbance of 0.9.
3.8.3 Temperature

As seen in Figure 24, the culture grew with a maximum absorbance of 1.4 after a lag of 16 h at RT. At 37°C the culture attained a maximum absorbance of 1.6 at 45°C. Culture grew to an absorbance of 1.0 on the 2nd day after which there was a clumping of the cells into aggregates.

In presence of Cd²⁺, the maximum absorbance attained by the culture was 1.0 at R.T. and 1.2 at 37°C and 0.92 at 45°C on the 3rd day after which clumping occurred.

In As³⁺, the maximum absorption attained by GUSF was 0.8 at R.T. and 0.6 at 37°C with no growth at 45°C.

In presence of As⁵⁺, GUSF attained a maximum absorbance of 1.3 on the 3rd day at R.T, 37°C and 45°C.

3.8.4 Selection of inoculum type

GUSF pregrown in NGSM containing 1 mM As³⁺, when subsequently subcultured in NGSM or NGSM containing metals, GUSF grew with a lag of 24 h in NGSM and NGSM containing 1 mM As³⁺. However good growth was seen in the presence of As⁵⁺. A maximum absorbance of 0.5 was attained in presence of cadmium (Figure 25).

When pre grown in As⁵⁺ was used as inoculum, good growth was observed in NGSM, NGSM containing As³⁺ and NGSM containing As⁵⁺. No lag was seen during growth in As³⁺. No growth was observed in presence of Cd²⁺ (Figure 26).

When GUSF pregrown in Cd²⁺ was used as inoculum, as seen in Figure 27, growth of culture preceded with a lag of 24 h in presence of As⁵⁺. Prolonged / extended log phase was observed for the culture in
Figure 24: Effect of temperature on the growth of GUSF in presence of metals A) R.T. B) 37°C C) 45°C
Figure 25: Growth of GUSF (pregrown in NGSM containing 1mM of As$^{+3}$) in presence and absence of metals

- NGSM
- 1mM As$^{+3}$
- 30mM As$^{+5}$
- 1mM Cd$^{+2}$
Figure 26: Growth of GUSF (pregrown in NGSM containing 30mM As$^{+5}$) in presence and absence of metals

Figure 27: Growth of GUSF (pregrown in NGSM containing 1mM Cd$^{+2}$) in presence and absence of metals
NGSM and in NGSM containing \( \text{As}^{+5} \) or \( \text{Cd}^{+2} \). Culture failed to grow in presence of \( \text{As}^{+3} \).

### 3.8.5 Type of Metal Salt

In presence of 1mM, \( \text{Cd}^{+2} \) (CdCl\(_2\)), GUSF attained a maximum absorbance of 1.0 after a lag of 20 h. So also when \( \text{Cd(NO}_3\)\(_2\) was used the culture behaved in the same way attaining a maximum absorbance of 1.0 (Figure 28).

### 3.9 ELUCIDATION OF THE MECHANISM OF TOLERANCE IN *HALOBACTERIUM* STRAIN R\(_1\) (GUSF)

#### 3.9.1 Detection of Cadmium in Cells and cell supernatant

In presence of \( \text{Cd}^{+2} \), the exponential phase began after a lag of 20 h. The dividing cells began accumulating \( \text{Cd}^{+2} \) on the third day of growth (2 ppm), which increased progressively to 18 ppm on the 5\(^{th}\) day of growth and remained constant thereafter (Figure 29). A decrease in pH was observed in the growth from pH 7 to pH 5.2.

When grown in NGSM containing 1mM \( \text{Cd}^{+2} \), adjusted to varying pH, GUSF accumulated 12 ppm of \( \text{Cd}^{+2} \) at pH 5, 18 ppm at pH 6 and 7 and 16 ppm of \( \text{Cd}^{+2} \) at pH 8 (Figure 30A).

\( \text{NaCl} \) concentration in the growth medium also affected the accumulation of \( \text{Cd}^{+2} \) by GUSF as seen in Figure 30B. At 15, 20, 25 and 30% \( \text{NaCl} \) concentration, GUSF accumulated 18, 18, 20 and 23 ppm of \( \text{Cd}^{+2} \), respectively.

Accumulation of cadmium in cells was estimated using the atomic absorption spectroscopy. However cadmium could not be estimated in
Figure 28: Effect of type of cadmium salt on the growth of GUSF

CdCl₂ - Cd(NO₃)₂ - control
Figure 29: Concentration of Cd²⁺ accumulated by growing cells of GUSF
Figure 30: Effect of A) pH and B) concentration of NaCl on accumulation of cadmium by growing cells of GUSF
supernatants by either AAS or the Dithizone method due to interference of high NaCl concentration (25%).

When cells of GUSF were treated using the modified dithizone method, cells grown in the presence of Cd\(^{2+}\) showed a magenta pink coloration as seen in Plate 14 while the cells grown in NGSM (absence of Cd\(^{2+}\)) showed a yellowish coloration.

Acetone extracts of the GUSF grown in NTYE, NGSM and NGSM containing 1 mM Cd\(^{2+}\) were also treated using this method. The magenta pink coloration occurred only in the acetone extract of the GUSF grown in NGSM containing 1mM Cd\(^{2+}\) (Plate 16).

When cells of GUSF grown in NGSM and NGSM containing 1 mM Cd\(^{2+}\) were treated individually using the Cadion 2B reagent, a pink colour was obtained from the cells of GUSF grown in NGSM and 1mM Cd\(^{2+}\) (plate 15), while a blue coloration was observed in cells grown in NGSM.

3.9.1.1 Localization of Cd\(^{2+}\) in the Cells of GUSF

GUSF grown in presence of Cd\(^{2+}\) was fractionated into cell envelope and cytoplasmic fractions. 60% of the accumulated cadmium was found in the cell envelope and 15% of Cd\(^{2+}\) was found in the cytoplasmic fraction. The cell envelopes and the cytoplasmic fractions stained magenta pink with the formation of cadmium dithizonate and is depicted in Plate 17.

Wide angle X-ray diffraction pattern of cells grown in the presence and absence of cadmium obtained using a copper target revealed various peaks. 2θ values were assigned to the peaks and using
Plate 14: Demonstration of magenta pink cadmium dithizonate complex in
1) 1mM Cd^{2+} in water 2) 1mM Cd^{2+} in NSM 3) cells of GUSF grown in NSM 4) cells of GUSF grown in NSM containing 1 mM Cd^{2+}

Plate 15: Detection of cadmium with cadion 2B.
1) water (negative control) 2) NSM (negative control) 3) NSM + 1mM Cd^{2+} (positive control) 4) supernatant of GUSF grown in NGSM 5) cells of GUSF grown in NGSM 6) supernatant of GUSF grown in NGSM + 1mM Cd^{2+} 7) cells of GUSF grown in NGSM + 1mM Cd^{2+}

Plate 16: Detection of cadmium dithizonate in acetone extracts of GUSF grown in 1) NTYE 2) NGSM 3) NGSM containing 1mM Cd^{2+}.
Braggs equation, \( 2d \sin \theta = n \lambda \), the interplanar distance 'd' was calculated taking \( n = 1 \), \( \lambda \) = wavelength of the copper target = 1.5418. The peaks at 2θ 11.2, 21.2, 25.4, 26 and 34.5 having a d of 7.89, 4.18, 3.50, 3.42 and 2.59 respectively, detected in GUSF grown in NGSM containing 1 mM Cd\(^{+2} \) were absent in the diffractogram of GUSF grown in the absence of cadmium (Figure 31) and were matching with those of CdPO\(_4\).

3.9.2 Detection of arsenite in cells and cell supernatant of GUSF

When GUSF was grown in presence of 1mM As\(^{+3} \), thin layer chromatogram of culture supernatant (Plate 18) showed the disappearance of As\(^{+3} \) (yellow spot) with simultaneous appearance of As\(^{+5} \) (mauve spots) from the third day onwards. This was also demonstrated using qualitative KMnO\(_4\) test. Culture supernatant on the day of inoculation on treatment with KMnO\(_4\) gave a yellow coloration while on the 5\(^{th} \) day of growth gave a pink coloration (Plate 19). As\(^{+5} \) does not react with KMnO\(_4\) and the resulting solution is pink in colour. As\(^{+3} \) reacts with permanganate resulting in clear or yellowish solution.

This decrease in arsenite was quantitated iodimetrically. A decrease in As\(^{+3} \) concentration from 1 mM (75 ppm) to 0.8 mM (60 ppm) was seen on the 3\(^{rd} \) day of growth and a simultaneous increase in As\(^{+5} \) was seen. A lowest concentration of 0.5 mM (37.5 ppm) As\(^{+3} \) and a maximum concentration of 0.4 mM (30 ppm) As\(^{+5} \) was detected in the culture supernatant on the 6\(^{th} \) day of growth (Figure 32). No cell bound arsenite was detected but 0.02 mM As\(^{+5} \) was found on the cells at the end of the growth period. A basal concentration of 0.1 mM As\(^{+5} \) was always detected even in control flask containing 1mM As\(^{+3} \).
Figure 31: X-ray diffractogram of cells of GUSF grown in a) NGSM
b) NGSM containing 1 mM Cd$^{+2}$
Plate 18: Thin layer chromatogram of supernatant of GUSF at different intervals of growth in NGSM + 1mM As$^{+3}$ taken
1) before inoculation  2) after inoculation
3) 1 day  4) 2 day  5) 3 day  6) 4 day  7) 5 day
Plate 19: Demonstration of arsenic in culture supernatant of GUSF using KMnO$_4$. 1) As$^{+3}$ in water (control); 2) As$^{+5}$ in water (control); 3) As$^{+3}$ in NSM (control); 4) As$^{+5}$ in NSM control; 5) culture supernatant of GUSF (NGSM + 1mM As$^{+3}$) 0 day; 6) 5th day; 7) culture supernatant of GUSF (NGSM + 30mM As$^{+5}$) 0 day; 8) 5th day.
Figure 32: Concentration of $\text{As}^{+3}$ and $\text{As}^{+5}$ in cell-free supernatant during growth of GUSF in NGSM containing 1mM of $\text{As}^{+3}$
3.9.3 Detection of arsenate in cells and cell supernatant of GUSF

Thin layer chromatogram of supernatants of GUSF growing in 30 mM As\(^{+5}\) showed a decrease in the As\(^{+5}\) concentration from the 2\(^{nd}\) day of growth (Plate 20), with no appearance of As\(^{+3}\).

The qualitative KMnO\(_4\) test of culture supernatant showed a pink coloration on 0 day as well as on the 5\(^{th}\) day of growth (Plate 19).

Iodometric estimation of culture supernatant showed a decrease in As\(^{+5}\) concentration from 26 mM (1950 ppm) to 24 mM (1800 ppm) on the second day of growth which further decreased to 20 mM on the 3\(^{rd}\) day after which it remained the same (Figure 33). Cells of GUSF accumulated 2.2 mM (150 ppm) of As\(^{+5}\) on the 5\(^{th}\) day of growth. A gelatinous pink precipitate of As\(^{+5}\) and cells was visually observed. FTIR of this pink precipitate revealed a broad band at 800 to 900 cm\(^{-1}\) with a weak absorption band at 1220 cm\(^{-1}\) indicative of arsenic and P=O groups respectively (Figure 34).

3.10 EFFECT OF As\(^{+3}\), As\(^{+5}\) AND Cd\(^{+2}\) IONS ON CELLULAR COMPONENTS OF GUSF

3.10.1 Pigments

GUSF grew as small orange colonies on NTYE agar and as pink colonies on NGSM agar medium (Plate 21A, B). Scanning electron microscopy of these cells revealed cup shaped morphology (Plate 21C). Intense pigmentation was observed when the culture was grown in the presence of 1 mM As\(^{+3}\) / 30 mM As\(^{+5}\) / 1 mM Cd\(^{+2}\) (Plate 22). Acetone extract of culture grown in each of the above metal showed the seven
Plate 20: Thin layer chromatogram of supernatant of GUSF at different intervals of growth in NGSM + 30 mM As\textsuperscript{+5} taken 1) before inoculation; 2) after inoculation; On incubation for: 3) 1 day; 4) 2 day; 5) 3 day; 6) 4 day; 7) 5 day.
Figure 33: Concentration of As\(^{+5}\) in cell free supernatant during growth of GUSF in NGSM containing 30 mM of As\(^{+5}\).
Figure 34: FTIR spectrum of the gelatinous pink precipitate obtained during growth of GUSF in NGSM containing 30 mM As$^{+5}$
Plate 21: *Halobacterium* strain R₁, MTCC 3285 (GUSF) growing on A) NTYE agar  B) NGSM agar  C) Scanning electron micrograph of GUSF
Plate 22: Growth of GUSF in 1) NTYE; 2) NGSM; 3) NGSM with 1 mM As$^{3+}$; 4) NGSM with 30 mM As$^{5+}$; 5) NGSM with 1 mM Cd$^{2+}$. 
peaks observed in culture grown in the absence of metal at 528, 494, 468, 426, 386 and 350 nm, however the intensity of the peaks varied as seen in Figure 35.

3.10.2 Proteins

SDS-Polyacrylamide gel electrophoresis, of whole cells and cell envelopes on specific staining with PAS reagent revealed a single glycoprotein band of approximate 93 KDa (Plate 23). On coomassie blue staining a number of bands were seen in addition to the 93 KDa protein band. A 22 KDa band was seen in whole cells and cytoplasmic fraction of the GUSF grown in the presence of Cd$^{2+}$. As seen in Plate 24, the band stained pink when stained by Periodic Schiff staining indicating the glycoprotein nature.

Amplification of 68 KDa protein was observed in GUSF grown in NGSM containing 1 mM As$^{3+}$ and amplification of 62 KDa band was seen in GUSF grown in presence of As$^{5+}$ (Plate 25).

3.10.3 Lipids

Lipid analysis of optimally growing GUSF, indicated that the culture possesses PG and PGP type of phospholipids and SDGD-1 and DGD-1 type of glycolipids in Plate 10 and 11 in 1 dimensional and 2 dimensional TLC (Plate 26). FTIR (Shimadzu FTIR 8101 A) spectrum of total lipids (Figure 36) showed no ester absorption in the range of 1735 to 1750 cm$^{-1}$. Strong absorption bands indicative of long chain alkyl groups (phytanyl chain $\text{CH}_2$, $\text{CH}_3$) were seen at 2960, 2920, 2850, 1460 and 1375 cm$^{-1}$. The presence of alkyl phosphate P=O band at 1260 - 1220 cm$^{-1}$ and at
Figure 35: Spectrum of acetone extract of GUSF grown in 1) NGSM
2) NGSM containing 1 mM Cd$^{2+}$
3) NGSM containing 1 mM As$^{3+}$
4) NGSM containing 30 mM As$^{5+}$
Plate 23: SDS-PAGE of 1) whole cells of GUSF  2) cell envelopes of GUSF with subsequent staining with Periodic Schiff reagent, along with standard molecular weight markers (M).
Plate 17: Detection of cadmium in cells of GUSF. 1) water (control); 2) NGSM (control); 3) water + 1 mM Cd^{2+} control; 4) NGSM + 1 mM Cd^{2+} control; 5) NGSM grown cells; 6) NGSM + 1 mM Cd^{2+} grown cells; 7) NGSM grown cell pellet; 8) NGSM + 1 mM Cd^{2+} grown cell pellet; 9) cell envelopes of NGSM grown cells; 10) cytoplasm of NGSM grown cells; 11) cell envelopes of NGSM + Cd^{2+} grown cells; 12) cytoplasm of NGSM + Cd^{2+} grown cells.
Plate 24: SDS-PAGE of GUSF (A): grown in 1) NGSM; 2) NGSM with 1 mM Cd\textsuperscript{2+}; 3) cell envelope and 4) cytoplasmic fraction of GUSF grown in NGSM with 1 mM Cd\textsuperscript{2+}; M) molecular weight marker and stained with coomasie blue. (B): 1) cell envelope and 2) cytoplasmic fraction of GUSF grown in NGSM; 3) cell envelope and 4) cytoplasmic fraction of GUSF grown in NGSM with 1 mM Cd\textsuperscript{2+} stained with Periodic Schiff reagent.
Plate 25: SDS-PAGE of GUSF grown in 1) NGSM; 2) NGSM with 1 mM As$^{3+}$; 3) NGSM with 30 mM As$^{5+}$; M) molecular weight marker, stained with coomassie blue.
Plate 26: Polar Lipid profiles of A) GUSF  B) *Halobacterium salinarium* ATCC 33171. The plates were developed once with chloroform-methanol-water 65:25:4 by volume) (1st dimension) chloroform-methanol-acetic acid-water (80:12:15:4) in the second dimension as indicated by the arrows. Lipids were visualised on exposure to iodine.
Figure 36: FTIR spectrum of total lipids of GUSF grown in NGSM
1650 cm$^{-1}$ attributed to P–OH groups, OH absorption band at 3300 cm$^{-1}$ and an ether absorption C–O–C at 1120 cm$^{-1}$ were observed.

Long phytanyl chain was detected by proton magnetic resonance spectroscopy (Bruker WT 300 MHz FTNMR). The spectrum showed methyl signals (phytanyl chain) at $\delta$ 0.75 to 0.95 ppm, a methylene envelope / cluster at $\delta$ 0.95 to 1.70 ppm and several sharp methoxy signals at 3.25 and 3.75 ppm and methyl groups on quinone ring at 7.5 and 7.7 ppm (Figure 37). C$^{13}$ NMR also showed CH$_3$, CH$_2$ signals at 18 – 22 ppm, CH$_2$ at 35 – 41 ppm and CH$_2$-O at 66 – 71 ppm (Figure 38).

No change in lipid profile was observed in culture grown in the presence and absence of metals (Plate 27). Spraying the developed plates with ammoniacal silver nitrate and heating resulted in a dark brown spot of SDGD-1 in the lipid extract of GUSF grown in control condition as well as that of arsenite and arsenate. The spots of PG and PGP stained yellow to light brown only in the lipid extract of GUSF grown in presence of arsenite and arsenate (Plate 28). FTIR spectrum of lipid extract of GUSF grown in presence of arsenite and arsenate was identical to that of control, with the exception that in the presence of As$^{+3}$ and As$^{+5}$ the band at 1230 cm$^{-1}$ (alkyl phosphate P=O) was seen as a broad band and the band at 1650 cm$^{-1}$ (P-OH) was absent (Figure 39).
Figure 37: $^1$H NMR (300 MHz) spectrum of total lipids of GUSF in $[^2$H $] $ chloroform.

Figure 38: $^{13}$C NMR (300 MHz) spectrum of total lipids of GUSF
Plate 27: Thin layer chromatogram of polar lipids of GUSF pregrown in 1) NGSM
2) NGSM + 1mM As^+\text{III} 3) NGSM + 30mM As^+\text{V} 4) NGSM + 1mM Cd^{2+}
The silica gel plate was developed once with chloroform - methanol - acetic acid - water (85:22.5:10:4 by volume) and A) was visualised on exposure to iodine;
B) sprayed for glycolipids;  C) developed twice and sprayed for glycolipids.
Figure 39: FTIR spectrum of total lipids of GUSF grown in NGSM containing 1 mM As$^{+3}$ or 30 mM As$^{+5}$
Plate 28: Thin layer chromatogram of polar lipids of GUSF grown in 1) NGSM 2) NGSM + 1mM As\(^{3+}\) 3) NGSM + 30mM As\(^{5+}\) on spraying with ammoniacal silver nitrate.
DISCUSSION

Metal pollution is on the rise in the estuarine econiches, due to surface water traffic of barges, trawlers, passenger ferries etc. as well as release of effluents from industries dealing with pesticides, fertilizers etc. Thus the estuarine network is exposed to constant anthropogenic flux. Salt marshes and estuarine regions are thus considered as good geochemical recorders of pollutant input (252).

Salinity being an inherent characteristic of this econiche, the halophilic Archaebacteria represents one of the dominant microflora and hence possibly play a major role in the geochemical cycles of the estuaries.

Tolerance of microorganisms to metals has been widely studied using eubacteria (13, 15, 17). During this study 21 haloarchaeal cultures isolated and characterized and detailed in the previous chapter, were studied for their response to As$^{+3}$, As$^{+5}$ and Cd$^{+2}$.

As estuarine environment undergoes transient phases of nutrient high and limitation, we attempted to study all the 21 haloarchaeal in nutrient rich NTYE medium and synthetic medium with 0.2% glucose as sole source of carbon. All the 21 haloarchaeal isolates were resistant to As$^{+5}$ upto 50 mM in nutrient rich NTYE medium. Surprisingly all the isolates could tolerate upto 80 mM As$^{+5}$ in NGSM medium. A 30% increase in tolerance levels is of significance as cultures growing in minimal medium are reported to be more susceptible to metal toxicants than those cultures growing in nutrient rich conditions. Cultures varied in
their response to arsenic in the +3 state in NTYE. Cultures GUBF 5 and GUBF 10 were resistant to 2 mM while all the cultures were resistant to 2 mM with the exception of cultures GUBF1, GUBF 2, GUBF 3, GUBF 6, GUBF 7, GUBF 8 and GUBF 17 were resistant to 1 mM As$^{+3}$. Interestingly arsenite was toxic at as low a concentration of 1 mM in synthetic medium as against the resistance exhibited even at 80 mM concentration of As$^{+5}$. The resistance of the haloarchaeal cultures to cadmium decreased by 50% in NGSM as compared to NTYE. Nieto et al 1987, 1989 (198, 199) reported an MIC of 20 mM of As$^{+5}$ for Halococci and Halobacteria in nutrient rich medium, whereas that of 0.5 to 2.5 mM Cd$^{+2}$ for Halobacteria and 2.5 mM Cd$^{+2}$ for Halococci.

The availability of metal in a medium is known to depend upon the concentration of organic material such as yeast extract, peptone etc. which have a capacity to adsorb heavy metals and mask their toxicity (253).

Halobacterium strain R$_1$ MTCC 3265 (GUSF), utilize a wide range of carbon substrates, produced PHA and was resistant to 1 mM As$^{+3}$, 80 mM As$^{+5}$ and 2 mM Cd$^{+2}$ in NGSM and was therefore selected for further studies. The culture grew with a bright orange pigment in NTYE (Plate 21A, B) and a mauve pigmentation in NGSM medium. Scanning electron microscopy revealed cup shaped morphology characteristic of the genus Haloferax (231, 232). All members of the Halobacteriaceae are known to lack murein in their cell wall but instead possess a high molecular weight glycoprotein outer membrane whereas that of Halococci posses a hetropolysaccharide (222). As seen in Plate 23, whole cell and cell envelopes of GUSF showed the presence of 93.1 KDa glycoprotein on
specific staining with PAS reagent. Cell walls of many archael species are composed of a single S layer glycoprotein (254). Surface layer protein (S layer) of the halophilic archaelon *Halobacterium halobium* was the first glycoprotein to be described in detail (255).

GUSF possesses ether linked phosphatidyl glycerol (PG), phosphatidyl glycerol phosphate (PGP), sulfated diglycosyl diether S-DGD-1 and the unsulfated diglycosyl diether DGD-1 type of polar lipids. Infra red spectrum of the total lipids confirmed the absence of fatty acids ester groups as it showed no ester absorption in the expected range 1735 to 1750 cm$^{-1}$ and presence of ether C-O-C at 1120 cm$^{-1}$. Long phytanyl chains were detected by proton resonance magnetic spectrum (Figure 37, 38). Archael lipid components are known to be unusual in that they contain no fatty acid ester groups but instead have long chain alkyl groups joined by ether linkages (256).

Visual intensity of the mauve pigmentation of GUSF increased in presence of As$^{+3}$, As$^{+5}$ and Cd$^{+2}$. Small variations in growth medium can have considerable effect on pigmentation of Halobacterium (145).

Toxicity of Cd$^{+2}$ ions to the growth of GUSF in NGSM doubled with a corresponding 50% decline in the maximum tolerance level (MTC) as compared to that in NTYE. Toxicity of As$^{+3}$ too, increased in NGSM with a MTC of 1 mM while As$^{+5}$ tolerated was higher in NGSM as compared to NTYE. Thus the maximum concentration of metal tolerated by GUSF in NGSM is 2 mM Cd$^{+2}$, 1 mM As$^{+3}$ and upto 80 mM As$^{+5}$. Physico chemical parameters such as pH, temperature and salinity also had an effect on the resistance of GUSF to As$^{+3}$, As$^{+5}$ and Cd$^{+2}$. The toxicity of arsenite and arsenate to GUSF increased with increase in pH being maximum at an
alkaline pH of 9. The toxicity of cadmium too, increased with an increase in pH and failed to grow at pH 9. These findings are in agreement with those of eubacteria as the CdOH⁻ species which appear at pH above 7.5 are more toxic to eubacteria and eukaryotes (257).

Variations in salinity too had an effect on the toxicity of As⁺³, As⁺⁵ and Cd⁺². The toxicity of metals increased with an increase in salinity resulting in a decrease in maximum absorbance of the culture at 30% salinity (Figure 20, 21, 22, 23). Optimum growth of culture in the presence and absence of metals was seen at 20% NaCl concentration. At 15% NaCl concentration better growth was observed in presence of Cd⁺² and As⁺⁵ as compared to that without metal. This disagrees with the results obtained by Onishi et al. 1984 (312) wherein increasing concentration of NaCl increased the tolerance of the moderately halophilic Pseudomonas to cadmium.

GUSF pregrown in presence of As⁺³ imparted tolerance or resistance to As⁺⁵, but grew slowly in control conditions or in subsequent growth in presence of As⁺³. Similarly GUSF pregrown in As⁺⁵ developed resistance for growth in presence of As⁺³. Pregrowth in presence of Cd⁺², extended the log phase of culture under control conditions as well as in presence of As⁺⁵ and Cd⁺² with no growth in presence of As⁺³.

GUSF grew very rapidly at higher growth temperatures. At 45°C, GUSF reached the stationary phase within two days and thereafter a clumping of cells resulted. Similar case was evident in presence of Cd⁺². GUSF failed to grow in presence of As⁺³ at elevated temperatures of 37°C and 45°C. However good and sustained growth was observed in presence of As⁺⁵ even at 55°C. Arsenate is known to induce heat shock
protein capable of imparting thermotolerance (258) in mammalian (259) and yeast cells (76).

Type of metal salt that is CdCl₂ or Cd(NO₃)₂ had no effect on the resistance or sensitivity of GUSF to cadmium. This is in disaccord with Babich and Stotzky 1980 (1) where they found that cadmium as Cd(NO₃)₂ was more toxic than other forms.

Metals in general, are known to be toxic to microorganisms and is evident from the above that cadmium and arsenic at certain concentration is toxic to GUSF. It is therefore of interest to understand the interaction between metal and growth kinetics, biochemical activities and synthesis of important cellular constituents such as proteins and lipids.

Exposure of GUSF to cadmium during growth resulted in accumulation of 18 ppm of Cd⁺² by the cells, at the end of the growth phase. No extra cellular protein or polysaccharide was produced in response to cadmium.

Fractionation of the cells into cell envelopes and cytosol revealed that 60% of the accumulated cadmium was bound to the cell envelope and low levels of 15% cadmium was present in the cytosol. Sites for metal binding are present on the cell walls of different microorganisms, bacteria and fungi. A similar trend in the cellular partitioning was reported for E. coli (261), when adapted to the presence of Cd⁺², Pseudomonas putida (262) and in Alcaligens eutrophus (263). However Joho et al 1985 (264) have reported that a large proportion of cellular Cd⁺² was found in the cytosol in the resistant strain of Saccharomyces cereviisea 301N. Such a metal sorption, onto bacterial cells has been studied using chemical equilibrium modeling techniques (265).
SDS-PAGE of the cell envelope and cytosol revealed a 22 KDa glycoprotein band in the cytosol of GUSF grown in the presence of cadmium. Cells grown in the presence of cadmium, on X ray analysis revealed the presence of CdPO₄. This indicates that the cell envelope is the first line defence of GUSF and sequesters cadmium, probably as CdPO₄ thus making it unavailable to the cells. Simultaneously there is an induction of 22 KDa protein to bind any incoming metal and thus render it inactive. Several membrane glycoproteins, in addition to the surface layer glycoprotein have been reported in the archaeon *Haloferax volcanii* (260). In *Hb. halobium*, glycosylation of the S layer protein is responsible for the cells rod shape (226). *Saccharomyces cerevisiae* 301 N accumulated cadmium with the concomitant synthesis of cadmium binding protein (8.2 KDa) when grown in Cd⁺² containing medium (271). Metallothionein has been reported in cadmium resistant mouse cells (272).

Accumulation of cadmium by the cells was qualitatively shown with the modified dithizone method. A magenta pink cadmium dithizonate complex was formed on the cells pre grown in cadmium. Interestingly the acetone extract of GUSF grown in presence of Cd⁺² also showed a magenta pink coloration with the modified dithizone reagent (Plate 16). Cadion 2B too could effectively detect cadmium in the supernatant and cells with the formation of pink colour in presence of cadmium.

No change in the lipid profile was observed when GUSF was grown in the presence of cadmium. Lipid profile of the haloarchaea is fairly stable in fluctuating physico chemical conditions and therefore used as a chemotaxonomic marker (127).
Growth of GUSF in presence of arsenite resulted in a decrease in As$^{+3}$ concentration with a simultaneous appearance of As$^{+5}$ in the growth medium, from the third day onwards (Plate 18). No cell bound arsenite was detected but cell associated As$^{+5}$ was present. The decrease in arsenite concentration indicates that oxidation occurred during the late exponential phase, once the culture had attained a higher cell density. Such extracellular accumulation, of breakdown or transformation products have been reported in Haloferax spp. D1227 during growth in presence of 3-phenyl propionic acid (162).

GUSF grown in the presence of As$^{+3}$ showed a lag period of 24 h before entering the exponential growth phase. The overall cell yield was also significantly lower as compared to control. This suggests that As$^{+3}$ is toxic to GUSF. Consistent with this hypothesis, growth of GUSF is not inhibited by up to 80 mM of As$^{+5}$. This clearly indicates that for GUSF, arsenite is more toxic than arsenate and the transformation of arsenite to the less toxic form is a mechanism of resistance and detoxification. This is contrast with the arsenic resistance associated with the reduction of arsenite to arsenate and subsequent efflux of arsenate from the cell (68). Arsenite oxidation has been reported in strains of Alcaligenes faecalis (65, 66) mediated by an oxomolybdenum enzyme, arsenite oxidase located in the periplasmic space of the bacterial cell (67). Arsenite oxidation proceeds via electron transfer chain with arsenite oxidase as the first protein. The respiratory system in Halobacterium halobium contains a predominant complex of b-type cytochromes, a low level of c-type cytochromes and two co-reactive haemproteins o and a1 (266).
Growth in presence of $\text{As}^{+5}$ resulted in a decrease in the residual arsenate concentration from 27 mM to 18 mM of $\text{As}^{+5}$ with precipitation of arsenate as a gelatinous precipitate. No arsenite appeared in the growth medium. Arsenate seems to be relatively less toxic to GUSF and cells of GUSF show an ability to accumulate arsenate. Arsenate is a structural analogue of phosphate. GUSF has two phospholipids; PG and PGP. Growth in presence of arsenic did not change the lipid profile of the culture. However when sprayed with ammoniacal silver nitrate and heating, as seen in Plate 28, the glycolipid SDGD-1 stained a dark brown in the lipid extract of GUSF grown in NGSM as well as NGSM containing arsenite or arsenate. However PG and PGP spots stained yellow to light brown only in the lipid extract of GUSF grown in the presence of arsenite or arsenate, suggesting that arsenate was incorporated in the phospholipid component of GUSF. The FTIR spectrum of the lipids of GUSF grown in the presence of $\text{As}^{+3}$/ $\text{As}^{+5}$ (Figure 39), were identical to that of NGSM (Figure 36). However the alkyl phosphate group having an absorption of 1230 cm$^{-1}$ was seen as a broad band in the lipid extract of GUSF grown in presence of arsenite and arsenate, while the band at 1650 cm$^{-1}$ was absent. This indicates that GUSF possibly substitutes $\text{As}^{+5}$ for $\text{PO}_4$ during growth in presence of arsenic.

The haloarchaeal cultures were resistant to $\text{As}^{+3}$, $\text{As}^{+5}$ and $\text{Cd}^{+2}$ in nutrient rich NTYE and synthetic NGSM medium. Culture GUSF was selected and further characterized for its archaeal nature. Physico chemical parameters of pH, temperature and salinity affected the
resistance of the culture to As$^{+3}$, As$^{+5}$ and Cd$^{+2}$. Five day old cells of GUSF accumulated 18 ppm of Cd$^{+2}$ at the end of growth phase. 60% of the accumulated cadmium was found to be localized in the cell envelopes. A 22 KDa glycoprotein was induced in the cytosol. Growth in presence of As$^{+3}$ resulted in the formation of As$^{+5}$ and its accumulation in the growth medium. Whereas growth in As$^{+5}$ resulted in a consistent decline in arsenate concentration. Presence of As$^{+3}$ and As$^{+5}$ resulted in amplification of 6E and 612 KDa proteins respectively. GUSF possibly substitutes As$^{+5}$ for PO$_4$ during growth in presence of arsenic.