International Standard Serial Number (ISSN): 2249-6807

*International Journal of Institutional Pharmacy and Life Sciences 5(3): May-June 2015*

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

**Life Sciences** **Research Article……!!!**

Received: 29-04-2015; Revised: 30-04-2015; Accepted: 01-05-2015

# OSMOLARITY STUDIES IN HALOPHILIC BACTERIUM *HALOBACTERIUM* STRAIN R1

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**Keywords:**

Halophiles, *Halobacterium*, saltern, hypotonic, lysis, bactopeptone Halophiles, *Halobacterium*, saltern, hypotonic, lysis, bactopeptone

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## ABSTRACT

*Halobacterium* strain R1, used in the present study, is an extremely halophilic archaea isolated from the saltpans of Goa. It has rod shaped pleomorphic morphology. Apart from being able to grown in presence of 25% NaCl concentration, the culture was able to grow at elevated temperatures. The red coloured halophile lost its colour at higher temperature, and turned mauve. Growth profiles in the presence of antibiotics were studied which prove its archael nature. The behaviour of the strain in distilled water *i.e.* in absence of salts was studied in R. T. and elevated temperature grown culture.

## INTRODUCTION

Kingdom Archaebacteriaceae comprises of microorganisms found in extreme environments such as thermophiles, the methanogens and the halophiles. Halophilic archaebacteria grow at very high salt concentration i.e. more than 15 – 35% NaCl. The halophiles are found in two types of environments: Thallasohaline environment, where $Na^+$ ions are predominant and Athallasohaline in which ions other than Na+ are predominant[1]. Based on the salt requirements, the halophiles are further grouped as non-halophiles (<0.2M), halotolerant (can tolerate >10%), slight halophile (0.2 – 0.8 M), moderate halophile (0.8 – 3.4 M), borderline extreme halophile (1.5 – 4 M), extreme halophile (2.5 – 5.2 M)[2,3].

Halophilic archaebactria found in natural environments are responsible for red coloration of the surroundings. They are endowed with three pigments, bacterioruberin associated with the red membrane, bacteriorhodopsin that is associated with purple membrane and halorhodopsin involved in phototaxis. Pigments function in the protection of bacteria from light and also help in the absorption of light as energy source[4-7].

High salt concentration is detrimental to the cells nutrient uptake, enzyme activities and cell surface stability[8]. Halobacteria are found to maintain a very high internal solute concentration unlike the eubacteria. Halobacteria can carry out all its metabolic functions in the presence of high salt concentration and the enzymes which are stable in high salt concentration get inactivated in its absence. Extremely halophilic archaebacteria are rod shaped in high salt concentration. When the salt concentration is lowered, the cells swell and become irregular in shape. The outer layer becomes frayed as the envelope disintegrates and intracellular substances are released [9,10]. If NaCl is absent completely, the outer membrane dissolves and cytoplasmic membrane disintegrates into tiny flakes.

Presence of certain lytic substances can lead to cell lysis, Bactopeptone is one such substance that even in the presence of NaCl, causes lysis. The lytic factor is the bile acids. Extremely halophilic bacteria are more sensitive to lysis by bile acids. This is due to their cell membrane composition *i.e* the presence of external layer of hexagonally arranged glycoproteins which form the binding site for the bile acids[11,12]. The action of bile acids can be countered by addition of starch and bentolite. The lytic property can be used to estimate the halophilic archaebacterial count of any ecosystem in salterns[13].

The present work focuses on the behaviour of the halophilic archaea *Halobacterium* strain R1 in the presence of various physiological parameters, which prove its nature as an archaea and its ability to combat with stress conditions.

## MATERIALS AND METHODS

### Culture and maintenance

*Halobacterium* strain R1, an isolate from salt pan in Goa was used for the study. The culture was maintained on NaCl Tryptone Yeast Extract (NTYE) media containing 25% crude salt. The composition of the media is $MgSO_4.7\ H_2O$ (20g/L), KCl (5g/L)**,** $CaCl_2.H_2O$ (0.2g/L), Tryptone (5g/L), Yeast extract (3g/L), NaCl (250g/L), agar (20g/L), distilled water (1000ml), pH 7.

### Working culture

*Halobacterium* strain R1, grown in NTYE for 3 days was used routinely as inoculum.

### Growth of Halobacterium strain R1 at varying temperatures

5% inoculum of working culture was inoculated in set of four sterile 250ml flasks, each containing 100 ml of NTYE medium. The flasks were incubated at R.T., 37 $^oC$, 45 $^oC$ and 60 $^oC$. Increase in absorbance was monitored every 24 hours using Shimadzu UV-240 spectrophotometer at 600nm.

### Growth of Halobacterium strain R1 in the presence of antibiotics

5% inoculum of working culture grown for 3 days was inoculated in set of 12 sterile 250ml flasks, each containing 50 ml of NTYE medium and different antibiotics. The flasks were incubated at R.T. on shaker after taking 0 hr reading. Growth was monitored every 24 hrs at 600 nm on Shimadzu UV-240 spectrophotometer.

### Pigment analysis of culture grown in NTYE under different growth conditions

Culture grown at varying temperature as well as in the presence of different antibiotics were analysed for pigmentation. Cells in stationary phase were harvested by centrifugation at 5000rpm for 10 mins. The cell pellet was collected in 25 ml clean glass beaker and suspended in 5 ml cold acetone. Cell suspension was sonicated for 2 min at 100 mV using Vibronics sonicator and a flat probe, under ice cold conditions. The supernatant was filtered through Whatman filter paper no. 1 and scanned from 300-700 nm using Shimadzu UV-240 spectrophotometer.

### Osmotic instability of Halobacterium strain R1

50ml of four day old liquid culture grown in NTYE at R.T. was centrifuged at 5000 rpm for 20 mins. The pellet was washed with 25% NaCl and resuspended either into 25% NaCl or distilled water to a final absorbance of 1.0 at 600 nm. The tubes were incubated at different temperatures and decrease in absorbance was monitored. Suspension of *E.coli* in distilled water was used as control. Similarly, *Halobacterium* strain R1 culture grown at different temperatures was also checked for osmotic instability.

**Cell lysis of Halobacterium strain R1 in 1% Bactopeptone solution**

50ml of four day old liquid cultures grown in NTYE at R.T., 37$^{o}$C, 45 $^{o}$C and 60 $^{o}$C, was centrifuged separately at 5000 rpm for 20 mins. The pellet obtained was washed with 25% NaCl and resuspended into 25% NaCl to a final absorbance of 2.0 at 600 nm. A 5ml aliquot of this suspension was taken in glass cuvette and 0.5ml of 10% bactopeptone solution was added. Absorbance was monitored at intervals of 1 min for 10 mins on spectrophotometer.

**RESULTS AND DISCUSSION**

*Halobacterium* strain R1, an isolate from the saltpans of Goa, grew very well in NTYE containing 25% salt. It is an extremely halophilic archaeabacteria, rod shaped and with pleomorphic morphology. It is oval in shape which is comparable to halobacteria[14]. *Halobacterium* strain R1 grew luxuriantly at R.T. with a lag phase of one day and log of four days, followed by stationary phase. At elevated temperatures there was a slight increase in the lag phase. The lag phase of culture incubated at 37 $^{o}$C was 12 hrs while at 45 $^{o}$C, it was 6 hrs. While the log phase were of 2 days and 1 day at 37 $^{o}$C and 45 $^{o}$C, respectively. The culture grown in NTYE showed a variation in growth rates at varying incubation temperature. The growth rates were 1.74, 0.8 and 0.5 per day at R. T., 37 $^{o}$C and 45 $^{o}$C, respectively. The growth at 60 $^{o}$C was very insignificant. The growth curves are depicted in Fig. 1. The ability of the culture to grow at higher incubation temperatures is possibly due to inherent characteristics of the culture or may be as a consequence of adaptation to the ever changing conditions in saltpan ecosystem from where the culture was obtained[1].

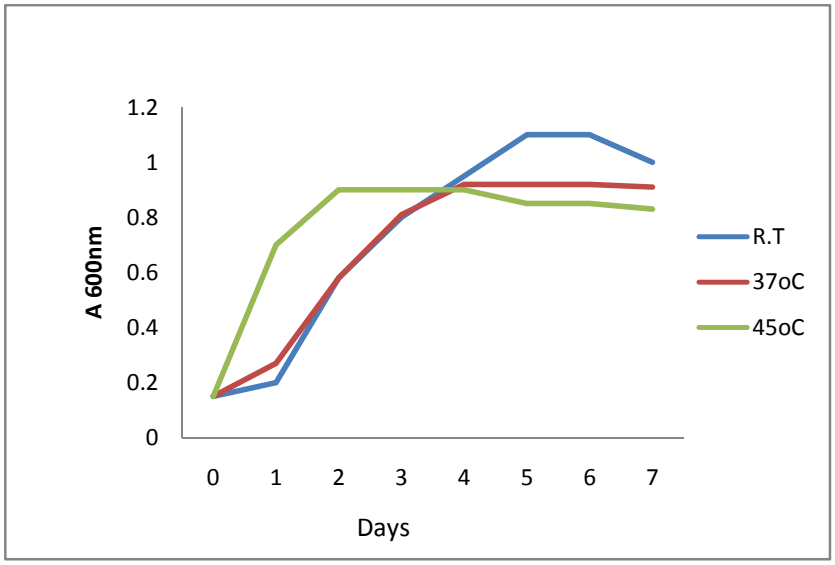


**Figure 1. Growth of *Halobacterium* strain R1at varying temperature**

During growth of the culture in NTYE at R.T., the culture is orange red in colour. The extracted pigment showed absorption peaks at 345, 370, 390, 470, 490 and 530 nm (Fig.2). Peaks at 470 and 490 nm identifies the pigment with bacteriorubirin[7]. Culture grown at 37 $^{o}$C, was mauve in colour and showed additional absorption peak at 430 nm. Although the absorption peaks of cells grown at R. T. and 37 $^{o}$C are almost similar, there is visible difference in the pigmentation of cultures grown at these two temperatures. At 45 $^{o}$C, absorption peaks at 345, 370, 430, 500, 530 and 595 nm were seen. Acetone extract pigment from cells grown at R.T. in NTYE in the presence of various antibiotics showed absorption peaks at 345, 370, 390, 470, 490, 530 and 595 nm. The pigment is unaffected during growth at 37 $^{o}$C and in the presence of antibiotics. At 45 $^{o}$C, the peak at 470 nm is lost.

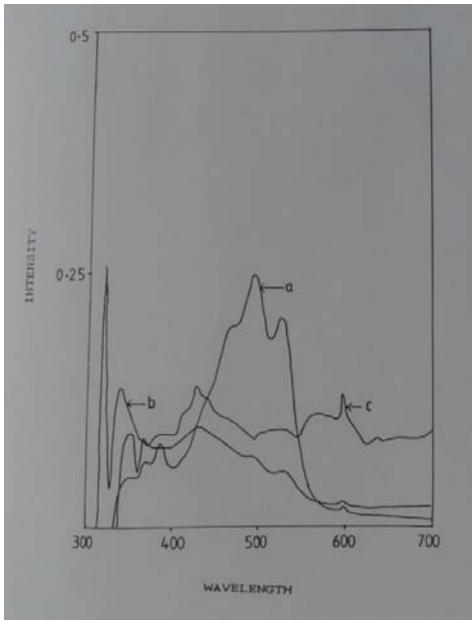


**Figure 2. Pigment profile of *Halobacterium* strain R1 grown in NTYE at (a)R.T.; (b) 37 $^{o}$C ;(c) 45 $^{o}$C**

*Halobacterium* strain R1 showed a varied response when grown in NTYE in the presence of different antibiotics (Fig.3a,3b). Chloramphenicol, cyclohexamide, ampicillin, polymixin B and Penicillin G failed to inhibit the growth and pigmentation of *Halobacterium* strain R1. Tetracycline and cycloserine inhibited only pigmentation, while the culture was sensitive to novobiocin, rifampicin, kanamycin and bacitracin. Polymixin, chloramphenicol and cycloheximide normally known to inhibit Gram negative bacteria, failed to inhibit growth of *Halobacterium* strain R1. Similarly, penicillin and ampicillin inhibiting the Gram positive bacteria, did not affect the growth of this strain. However, the culture growth was inhibited by kanamycin, rifampicin, bacitracin and novobiocin. Archael halobacteria are inhibited by these antibiotics and thus proves the archael nature.

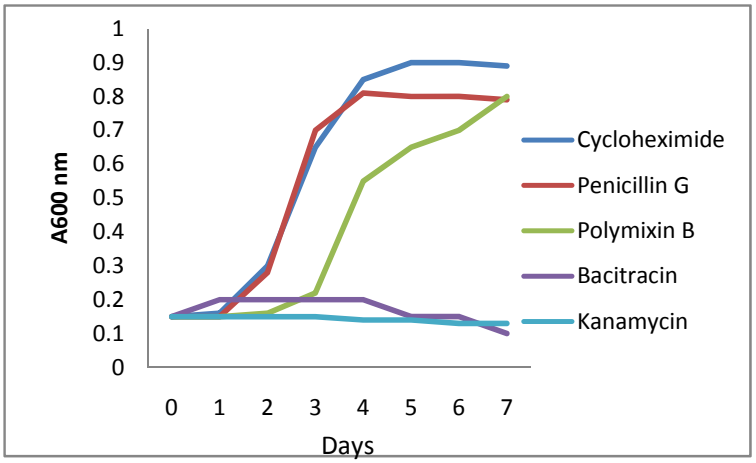


**Figure 3a. Growth profile of *Halobacterium* strain R1 in NTYE at R.T. in presence of cyclohexamide, Penicillin G, polymixin B, bacitracin and kanamycin.**

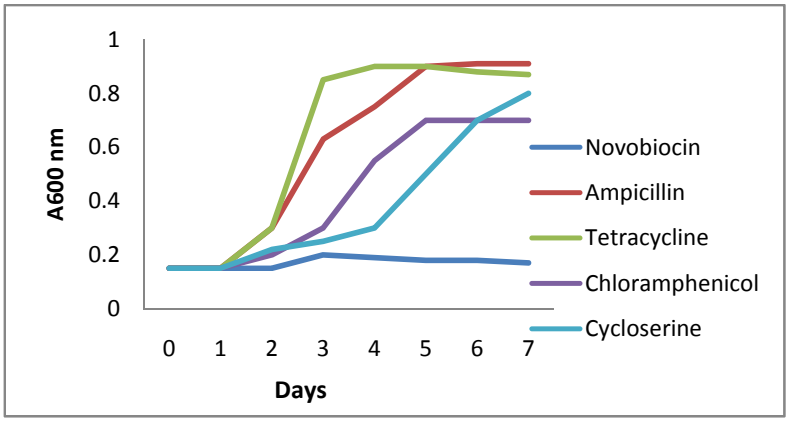


**Figure 3b. Growth profile of *Halobacterium* strain R1 in NTYE at R.T. in presence of novobiocin, ampicillin, tetracycline, chloramphenicol and rifampicin.**

The effect of absence of salts was tested on the grown culture. Cell lysis was observed when the cells of *Halobacterium* strain R1 were suspended in distilled water. This method of cell lysis is used to separate vesicles from Halobacteria[15]. The absorbance decreases sharply in the first 15 mins and then reduces slowly. The rate of lysis increased with increase in incubation temperature (Fig. 4). When the cell suspension is incubated at 37 $^{o}$C, the decrease in absorbance is more than at R.T. and similarly at 45 and 60 $^{o}$C.

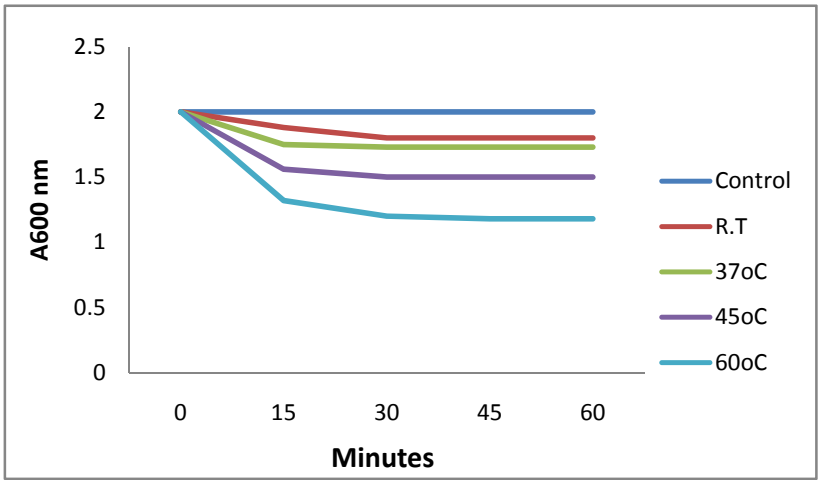


**Figure 4. Effect of distilled water on *Halobacterium* strain R1grown in NTYE at R.T. and incubated at different temperatures.**

However, when cells of *Halobacterium* strain R1grown at elevated temperature were subjected to distilled water, showed resistance to lysis (Fig.5). Lysis of cells suspended in distilled water is a characteristic feature of halophilic archaea[16].

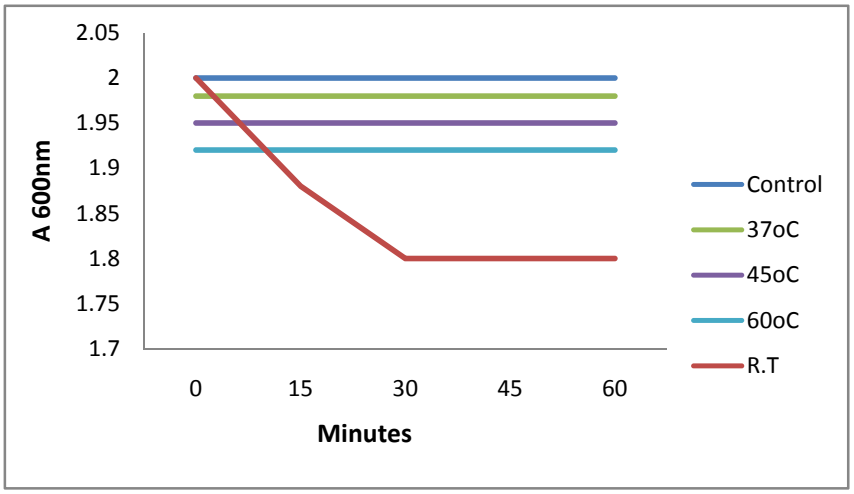


**Figure 5. Effect of distilled water on *Halobacterium* strain R1grown in NTYE at different temperatures.**

Bactopeptone is reported to cause lysis of archaebacterial cells when the cells are suspended in 1% bactopeptone solution[12]. With R.T grown culture, lysis was observed, however culture grown at higher temperatures showed resistance to lysis (Fig. 6). Thus, the lysis of cells of *Halobacterium* strain R1 in the presence of distilled water and bactopeptone is due to presence of bile acids and proves the archael nature of the strain.

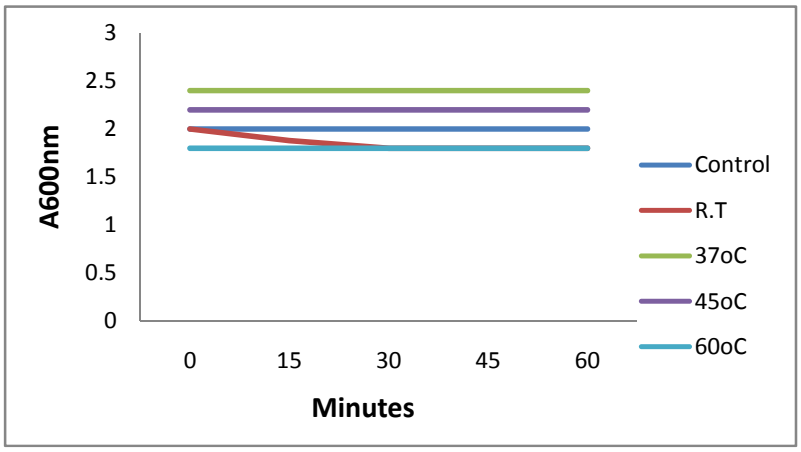


**Figure 6. Effect of bactopeptone solution on *Halobacterium* strain R1grown in NTYE at different temperatures.**

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