# Biochemical characterization of nucleoid from Halophilic archaeon *Halobacterium salinarium H3*

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**By**

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This is to certify that I, **Kotipatruni R. Prasadarao** have carried out the research work embodied in the present theses entitled " **Biochemical characterization of nucleoid from Halophilic archaeon *Halobacterium salinarium H3*** " and submitted for the award of degree of Doctor of Philosophy was accomplished for the full period prescribed under PhD ordinances of the University, under the supervision of Prof. T. Suryanarayana, in the Department of Biochemistry, School of Life Sciences, University of Hyderabad.

I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award of research degree of any University.

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## Contents

## Introduction

The present thesis deals with characterization of nucleoid from halophilic archaeon *Halobacterium salinarium H3.* Archaea constitute a separate domain of life distinct from bacteria and eukarya. Archaea live in some of the most extreme environments. Some live near rift in the deep sea at temperatures higher than 100 centigrade. Others live in hot springs in extremely abundant in environments that are hostile to all other forms of life. However, archaea are not restricted to extreme environments, new research is showing that archaea are also quite abundant in the plankton of the open sea, exist in soils, fresh water and almost all places. Archaea has been given a status of separate kingdom by Woese *et al.,* (1990) based on 16S rRNA sequence homologies and the presence of ether-linked lipids in their membrane. Since many of archaea are extremely difficult to culture, a comparative analysis of small subunit ribosomal RNA (ss rRNA) genes obtained directly from the environments is used to characterize the newly discovered species. Archaeal features can be classified as bacteria-like, eukaryotic-like and those unique to the archaea. As much as a third of the archaeal genome consists of genes found uniquely in archaea (Woese and Gupta, 1983).

## Classification of Archaea:

The classification of archaea into sub kingdoms is based on small subunit rRNA sequences and several other macro molecular components of the transcription and translation. On the basis of ssrRNA analysis, the Archaea consist of three phylogenetically distinct groups: **Crenarchaeota, Euryarchaeota** and **Korarchaeota**. Three sub groups or kingdoms – Euryarchaeotes, crenarchaeotes and nanoarchaeota have been grown as culture. Small subunit rDNA sequence data from uncultured organism indicate existence of a fourth subgroup korarchaeota.

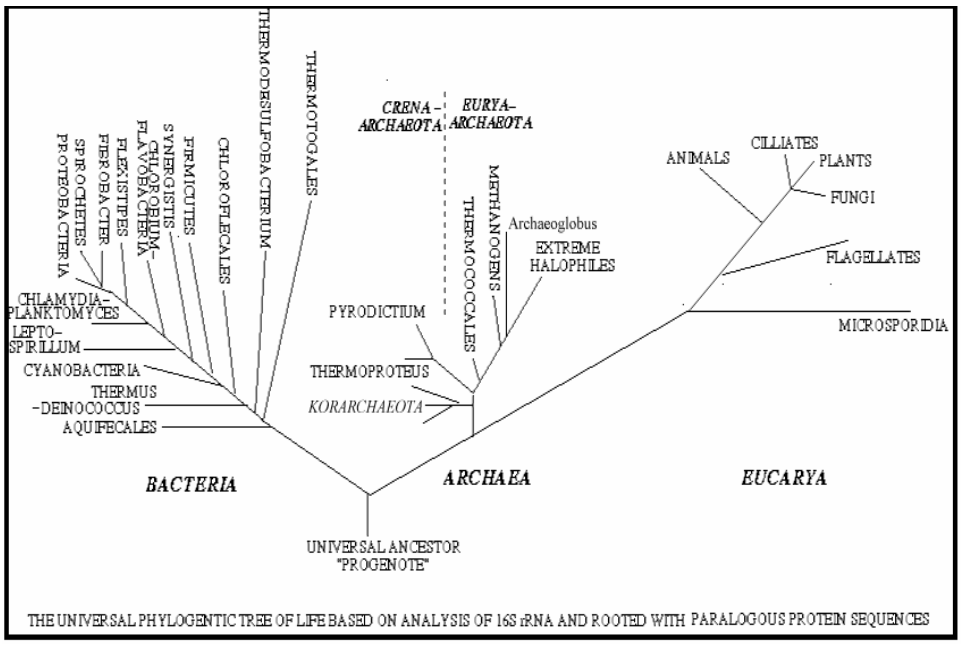


Fig: Prokaryotic organisms were demarcated into Archaea and Eubacteria based on rRNA sequence analysis.

Euryarchaea comprise halophiles, methanogens, hyperthermophilic sulfate reducers (e.g., *Archaeoglobus*), hyperthermophilic heterotrophes (e.g., *Pyrococcus*) and some thermoacidophiles (e.g., *Thermoplasma*).

The axenically cultured Crenarchaeotes are all thermophilic or hyperthermophilic Mesophilic crenarchaeotes have been detected living as obligate symbionts to a marine sponge. Crenarchaeotes have also been found in cold terrestrial and marine environments.

For the Korarchaeota, only their nucleic acids have been detected, and no organism has been isolated or cultured so far. SS rRNA of the Korarchaeota has been obtained from hyperthermophilic environments like volcanic hot springs. They have been detected in mixed cultures using fluorescent DNA probes. These organisms probably have similar metabolisms to other Archaea from extreme environments. The branch Korarchaeota is near the division between bacteria, archaea and eukaryotes.

**Euryarchaeota**

There are three main groups of euryarchaeotes

**1. Methanogens**

These are found living in such anaerobic environments as the muck of swamps and marshes the rumen of cattle (where they live on the hydrogen and CO2 produced by other microbes living along with them) sewage sludge the gut of termites. They are autotrophic; using hydrogen as a source of electrons for reducing carbon dioxide to food and giving off methane ("marsh gas", $CH_4$) as a byproduct. $4H_2 + CO_2 \longrightarrow H4 + 2H_2O$.

**2. Thermoacidophiles**

As their name suggests, these like it hot and acid (but not as hot as some of the Crenarchaeota!). They are found in such places as acidic sulfur springs (e.g., in Yellowstone National Park) and under sea vents ("smokers").

**3. Halophiles**

These are found in extremely saline environments such as the Great Salt Lake in the U.S. and the Dead Sea. They maintain osmotic balance with their surroundings (nearly NaCl-saturated concentration) by building up the solute concentration within their cells. This group of organisms is known as “extreme Halophiles”.

Halophiles are salt-loving organisms that inhabit hypersaline environments. Halophiles can be loosely classified as slightly, moderately or extremely halophilic, depending on their requirement for NaCl. The extremely halophilic archaea, in particular, are well adapted to saturating NaCl concentrations and have a number of novel molecular characteristics, such as enzymes that function in saturated salts, purple membrane that allows phototrophic growth, sensory rhodopsins that mediate the phototactic response, and gas vesicles that promote cell flotation. Halophiles are found distributed all over the world in hypersaline environments, many in natural hypersaline brines in arid, coastal, and even deep sea locations, as well as in artificial saltterns used to mine salts from the sea. These extreme halophiles grow best at the highest salinities (3.4 – 5 mol/L NaCl)

forming dense blooms (up to 108 cells ml21), and resulting in the red colour of many brines. Common species of halobacteria are rod-, cocci-or disc-shaped, although triangular and even square shaped species exist. Many are pleiomorphic, especially when the ionic conditions of the media are altered, and most lyse below 1– 1.5 mol/L NaCl. Halobacteria are classified as archaea (and are also called halophilic archaea or haloarchaea) and belong to the family Halobacteriacea(Das Sarma, 2002) Ten genera have been reported, *Halobacterium, Haloarcula, Halococcus, Haloferax, Halorubrum, Halobaculum, Natronobacterium, Natronococcus, Natrialba and Natromonas,* and an eleventh genus, *Haloterrigena,* has recently been proposed.

The first microbiological analysis was conducted on several closely related Halobacterium strains (originally designated *H. halobium, H. salinarium, and H. cutirubrum*) isolated in the mid-twentieth century from salted fish and meat from northern Europe and North America. These are generally amino acid-utilizing facultative aerobes that require a number of growth factors and slightly elevated temperatures (38 – 45ºC) for optimal growth. Most have distinctive features such as gas vesicles, purple membrane and red-orange carotenoids. Many have the ability to grow in the absence of oxygen via dissimilatory nitrate reduction and denitrification, fermentation of different sugars, breakdown of arginine, and use of light energy mediated by retinal pigments. Several more recently isolated species will oxidize carbohydrates, e.g. *Haloarcula marismortui, Haloarcula vallismortis,* and *Haloferax volcanii* from the Dead Sea; *Haloferax mediterranei* and *Halorubrum saccharovorum* from saltterns; and *Halorubrum lacusprofundi*, a psychrotolerant species from Deep Lake, Antarctica. Glucose is oxidized by a modified Entner–Doudoroff pathway and the resulting pyruvate is further oxidized by Pyruvate oxidoreductase and the tricarboxylic acid cycle. Several strains are capable of growth on single carbon sources such as sugars, glycerol and acetate.

*Halobacterium salinarium H3* is a member of the Archaebacteria, recognized as a phylogenetically separate group of organisms that are distinct from other eubacteria and from eukaryotes. Some of their biochemical properties are unique while others are shared with eubacteria or with eukaryotes. Archaebacterial lipids and membranes,

Transcriptional and Translational machinery, enzymes and cofactors show certain unusual features. The intracellular salt concentration is found to be very high in extreme Halophiles. As a consequence, universally conserved molecules like proteins and nucleic acids might have also adapted to function at such a high salt concentration (Eisenberg, 1992).

**Biochemistry and Molecular biology of Archaea:**

The lipids of archaea are chemically unique. Archaea utilize glycerolipids with ether linkages in their membrane structure. But lipids in the bacteria and eukarya utilize ester linkages. It has lipid monolayer composed of glycerol di ethers and glycerol tetra ethers, which is resistant to high temperatures. The lipids of Eocytes (*Sulfolobus* and *Thermoplasma*) contain high amount of tetra ethers in contrast to other archaebacteria, which contain relatively less or none. The tetra ether membrane lipids of Eocytes contain cyclopentanol C-40 biphytanol chains. In addition their neutral lipids also contain branched alkyl benzenes. The central metabolic pathways are glycolytic pathway, pentose phosphate pathway and citric acid cycle. In many molecular properties archaea resemble eukaryotes. Their DNA dependent RNA polymerases are composed of protein subunits with molecular weights and immunological properties that resemble those of eukaryotic polymerase A (Huet et al. 1983). Of 10 smaller protein subunits of RNA polymerase, six are homologues of eukaryote specific subunits shared in some combination among eukaryotic RNA polymerases. S. *acidocaldarius* transcription also shows homology to eukaryotes (Zillig et al., 1985).

Among the major cellular functions to be characterized in archaea are chromosome replication, nucleoid segregation and cell division and the coordination of these processes with cellular growth. Analysis of the archaeal genome sequences has revealed that the replication, transcription and translation machineries of archaea are similar to those of eukaryotic organisms, while genes involved in the metabolic processes show more similarity to their bacterial counterparts. For example, the gene products involved in the chromosome segregation show similarity to both eukaryal and bacterial proteins whereas the cell division apparatus is mostly of bacterial type. (Bernander, 1998). Since the

archaea cell cycle contains a mixture of features from both eukarya and bacteria they may contribute to a better understanding of the evolution of the process. There is speculation that the eukaryotes may have originated from a fusion event between a bacterium and an archaeon (Gupta and Golding, 1996; Martin and Muller, 1998; Moneira and Lopez-Garcia, 1998) which implies that the eukaryal cell cycle characteristics may be derived from those of an archaea like ancestor.

Certain features however are characteristic of archaea like their ribosomal RNA sequences, the lipid composition (ether glycerol lipids) and organization of the cytoplasmic membrane (bi or mono layer) and also particular modes of energy metabolism, such as methanogenesis and bacterio rhodopsin – driven photosynthesis (Jones et al., 1987).

The cell envelope of *Halobacterium* NRC-1 consists of a single lipid bilayer membrane surrounded by an S-layer assembled from the cell-surface glycoprotein (Kushner, 1985). Although the cytoplasm is in osmotic equilibrium with the hypersaline environment, the cell maintains a high (≈4 M) intracellular $K^+$ concentration that is equivalent to the external $Na^+$ concentration. The passive permeability of the membrane to $K^+$ and $Na^+$ ions is low (Stoeckenius, *et al.,* 1979) so active transport is required to maintain the ionic distribution. Accordingly, NRC-1 has multiple active $K^+$ transporters, including KdpABC, an ATP-driven $K^+$ transport system, and TrkAH, a low-affinity $K^+$ transporter driven by the membrane potential. Active $Na^+$ efflux is probably mediated by NhaC proteins, which likely correspond to the unidirectional $Na^+/H^+$ antiporter activity described previously (Lanyi, 1978). Interestingly, KdpABC, TrkA (three of five copies), and NhaC (one of three copies) are coded by pNRC200.

At least 27 members of the ABC transporter super family are present in *Halobacterium* NRC-1. Among active transporters for nutrient uptake identified were those for cationic amino acids (Cat) and proline (PutP), dipeptides (DppABCDF), oligopeptides (AppACF), and a sugar transporter (Rbs). Among small-ion transporters, most were closely related to bacterial proteins. Genes for exporting heavy metals (arsenite and cadmium) and other

toxic compounds (multidrug-resistance homologs) are present. For polypeptide translocation across the membrane, the general secretory (Sec) machinery for *Halobacterium* NRC-1 appears to be a hybrid of the eukaryotic and bacterial systems (Pohlschroder, *et al.,* 1997).The core components, Sec61α/SecY and Sec61γ/SecE, as well as those of the signal recognition particle, SRP54/Ffh and its 7S RNA scaffold, are related to the corresponding eukaryotic factors. The SRP complex also includes SRP19, a subunit found in eukaryotes but not in bacteria. On the other hand, like bacteria, NRC-1 contains the universally conserved SRP-receptor subunit SRPα/FtsY and lacks the eukaryotic ß -subunit homolog. The bacterial translocase protein homologs SecD and SecF are also present, but the essential bacterial ATPase SecA is absent. In addition, a gene closely related to *tat*C of *A. fulgidus* (Berks, *et al.,* 2000) was found, suggesting the presence of the twin-arginine protein export pathway.

**DNA replication machinery:**

The *Halobacterium* genome was originally studied in the 1960s and found to be composed of two components, a GC-rich (68%) major fraction and a relatively AT-rich (58% GC) satellite ( Joshi, *et al.,* 1963 and Moore and McCarthy, 1969). Satellite DNA corresponded to the presence of large and variable covalently closed extra chromosomal circles and a large number of transposable insertion sequence (IS) elements, which explained the observed genetic plasticity of halophiles (Charlebois, *et al.,* 1989 and Das Sarma, 1993). For *Halobacterium* NRC-1, 3 circular replicons were mapped, a ≈2-Mbp chromosome and 2 large replicons, pNRC200 and pNRC100, about 350 and 200 Kbp in size (Bobovnikova, *et al.,* 1994; Hackett, *et al.,* 1994; Ng, 1991, 1994).

The *Halobacterium* NRC-1 genome revealed three DNA polymerase types (Cann and Ishino, 1999) two family B polymerases (one coded by pNRC200), a bacteriophage-like family A polymerase, as well as the heterodimeric family D polymerase. The large subunit of the latter contains an intein similar to the hyperthermophilic archaeon *Pyrococcus horikoshii.* Additional proteins that may be active at the replication fork include a putative DNA ligase, primase, type I topoisomerase (TopA), and two type II topoisomerases (GyrA and B, and Top6A and B). The presence of PCNA, sliding clamp,

RFC, clamp loader, and RPA, replication protein A involved in single-strand DNA binding, Mcm minichromosome maintenance protein, and Orc/Cdc6, origin recognition complex proteins has also been reported (Cann and Ishino, 1999). Nine copies of origin sequences are present including three scattered on the large chromosome, suggesting the possibility of multiple replication origins.

For DNA repair, *Halobacterium* NRC-1 possesses two of the three genes involved in the guanine oxidization pathway, *mut*T and *mut*Y. In addition, both the nucleotide and base excision pathways appear to be complete as copies of the *uvr*ABC nuclease and *uvr*D helicase, and endonucleases and glycosylase genes are present. Two of the three genes of methyl-directed mismatch repair were found, *mut*L and *mut*S (three copies), but the nuclease gene *mut*H was missing. The *E. coli*-type *dam* methylase (recognizing GATC) is absent in NRC-1. However, a putative CTAG-specific methylase gene is present, which has also been found in *Methanobacterium thermoformicicum* (Nolling, 1992).
Repair genes similar to those in yeast are present in *Halobacterium* NRC-1, including *rad2, rad3*, *rad24,* and *rad25.* Several of these proteins appear to be active in the excision repair pathway. Products of *rad3* and *rad25* have been identified as repair helicases and Rad2 is a single-stranded DNA endonuclease. This suggests that *Halobacterium* NRC-1 has developed multiple pathways to repair UV-induced damage as a means for survival. Cell-cycle genes in *Halobacterium* NRC-1 include five copies of *cdc48*.

**Transcription and translation machinery**

The basal transcription machinery in archaea has been suggested to be a simplified version of the eukaryotic RNA polymerase II machinery (Reeve, *et al.,* 1997; Soppa, 1999a; Van der Oost, *et al.,* 1998, and Zillig, *et al.,* 1993). Both archaeal and eukaryal RNA polymerase II are multisubunit complexes. For example, both *Sulfolobus acidocaldarius* and yeast RNA polymerase consists of 12 subunits, in contrast to *E. coli* where the holoenzyme consists of only 4 subunits.

**Table 1: Transcription in the three domains of life**

| Characteristic | Bacteria | Eukarya | Archaea |
|---|---|---|---|
| **RNA polymerase** | Single 4 subunits(2α,β,β´) | Three types Multisubunit (12 subunits in yeast) | Single Multisubunit (12 subunits in *Sulfolobus*) |
| **Mode of RNA polymerase binding to promoter** | Recognition and binding dependent on σ factor | Recognition and binding dependent on TATA-binding protein (TBP) | Recognition and binding dependent on TBP |
| **Promoter core elements** | -35 box*;* -10 box | RNA polymerase II: TATA box, Initiator element (INR),Downstream promoter element (DPE), TFIIB recognition element | TATA box, TFB recognition element (similar to promoter elements for RNA polymerase II) |
| **Transcription initiation factor** | Usually not required | Transcription factor IIB (TFIIB) and other factors | TFIIB homologue (TFB) |
| **Other transcription factors** | Multiple | Multiple | Eukaryotic and bacterial-like factors |
| **m7Gppp cap** | Absent | Present | Absent |
| **Polyadenylation** | Long polyA tail absent | Long polyA tail present | Long polyA tail absent |
| **mRNA** | Absent | Present | Absent |

As in most eukaryotic genes, the archaeal TATA box is localized about 30 bp from the transcription initiation start (Bell and Jackson, 1998). Archaea contain a homolog of eukaryotic TATA binding protein (TBP), which specifically recognizes and binds to the TATA box in the promoter region before loading the RNA polymerase (van der Oost, *et al.,* 1998). In eukaryotes, TBP is essential for initiation of transcription in all three RNA polymerase systems (White and Jackson, 1992). All archaeal species with the exception of halophilic ones contain a single TBP.

The TFB recognition element (BRE) is a binding site for transcription factor B (TFB). The archaeal BRE consists of a minimum of two adenines located around position -34 to -33 with respect to the transcription initiation site (Soppa, 1999b). Upon binding to the BRE, the TFB forms a complex with the TBP that is necessary for the initiation of transcription. In some euryarchaea there are multiple TFB proteins (Thompson, *et al.,* 1999). In addition to TBP and TFB, archaea contain many other eukaryotic and bacterial-like transcription factors (Kyrpides and Ouzounis, 1999) that are required for the efficient regulation of archaeal transcription.

**Translation.** The translation process in archaea appears to have traits from both bacteria and eukaryotes (Table 3). Initiator tRNA is non formyl-methionine specific. M7Gppp cap is absent SD-like sequences are present in many archaeal genes and are localized 3 - 10 nucleotides in front of the initiation codon (Amils, *et al.,* 1993; Dennis, 1997). Recent experimental studies with *Sulfolobus solfataricus* demonstrated that the Shine-Dalgarno is essential for the initiation of translation, at least in crenarchaea (Condo, 1999). A SD-independent initiation of translation could proceed, but only after the artificial removal of the 5′ untranslated mRNA leader region in front of the initiation codon. In archaeal genes that lack SD, translation was suggested to start from the first initiation codon located downstream of the transcription initiation start (Dennis, 1997). The process of translation initiation of such genes in *Sulfolobus* might be enhanced by yet an unidentified sequence elements, located downstream of the initiation codon, inside a coding sequence (Tolstrup, 2000). As in bacteria, archaeal ribosomes are composed of 30S and 50S subunits. Archaeal rRNA further parallels bacterial rRNA since it also consists of 16S, 23S, and 5S

RNAs. Furthermore, archaea possess both bacterial-like and eukaryotic-like factors (Kyrpides and Woese, 1998) required for different stages of the translation process.

**Table 3: Translation in the three domains of life**

| Characteristic | Bacteria | Eukarya | Archaea |
|---|---|---|---|
| **rRNA** | | | |
| **Ribosomes (subunits)** | 70S (30S + 50S) | 80S (40S + 60S), 70S in organelles | 70S (30S + 50S |
| **Shine-Dalgarno (SD)** | Present | Absent | Present |
| **Translation initiation codons** | Mainly AUG, Other initiation codons (GUG, UUG etc.) also used to minor extent | Initiation essentially restricted to AUG | Mainly AUG, Other initiation codons (GUG, UUG etc.) also used to minor extent |
| **Translation factors** | Multiple initiation, elongation and release factors | Multiple initiation, elongation and release factors | Both eukaryal and bacterial-like factors present. |
| **Initiator tRNA** | Formyl-methionine | Methionine | Methionine |
| **$m^7$Gppp cap** | Absent | Present | Absent |

*Halobacterium* NRC-1, like other archaea, drives regulated transcription by using a single version of a eukaryotic RNA polymerase II-like transcription system. The information for the multisubunit RNA polymerase II is coded by 12 genes located at 6 loci. Multiple copies of TBP and TFB transcription factor genes. Five complete *tbp* genes and one partial gene that have one-half of the two stirrups were identified. Four of the six *tbp* genes were reported previously. The possibility of a novel regulatory system involving up to 42 different TBP-TFB combinations has been discussed recently (Baliga,

2000). 27 transcriptional regulators were also identified. Transcription factors known to be required for polymerase II transcription in other systems (TFIIF, TFIIH, and TFIIE) were not evident. A TFIIE homolog, termination/antitermination factor homologs NusA and NusG was identified (Bateman, *et al*, 2000, Bermudez-Cruz, *et al*, 1999).

Translational components of *Halobacterium* NRC-1, like other archaea, have both bacterial and eukaryotic homologs. 47 tRNA genes for all 20 amino acids and all 61 possible codons were identified (Lowe and Eddy, 1997) including tRNAs with 44 unique anticodons, 1 methionine initiator tRNA, 1 redundant tRNA (Ala-CGC), and 1 tRNA (anticodon CAU), which is predicted to be converted from methionine to isoleucine specificity posttranscriptionally as in *E. coli* (Muramatsu, *et al*, 1988). Aminoacyl tRNA synthetases are present for all amino acids except asparagine and glutamine, which likely require amidotransferases (Tumbula, *et al*, 1999). The single-copy rRNA operon is bacterial-like in its organization and gene content: 5' 16S, tRNA (Ala-UGC), 23S, 5S, tRNA (Cys-GCA) (Hui, and Dennis, 1985). The RNA component but not protein components of RNaseP was detected. Genes coding homologs of the eukaryotic nucleolar proteins fibrillarin and Nop56/58 were also identified in NRC-1. The occurrence of these proteins in other archaea and the recent identification of C/D box snoRNAs in thermophilic archaea (Omer, *et al*, 2000) suggest that the snoRNA-mediated 2-*O*-methylribose modification system is generally present.

Generally, the protein components of the translation apparatus of archaea resemble more closely those of eukaryotes than those of bacteria (Dennis, 1997). Total twenty five 30 S subunit and twenty eight 50 S subunit r-proteins have been enumerated by purification, partial or complete amino acid sequence analysis, and gene sequence analysis (Engelmann, *et al*, 1995) and the crystal structure of the 50S subunit has been determined. Another unique feature of halophilic ribosomes in the preponderance of acidic ribosomal proteins. Genes coding homologs of eukaryotic eIF1A, eIF2 , ß , and subunits, eIF4, eIF5, and eIF2B and are also present (Shimmin, *et al.,* 1989).

**Cell cycle characteristics of Archaea**

The cell cycle characteristics of archaea show similarities with both prokarya and eukarya. Growth phase dependent modification of nucleoid size and a long gap period between the termination of nucleosome replication and completion of nucleoid partition, similar to the G2 phase in eukaryotic cells were observed in the euryarchaeon *Thermococcus kodakaraensis* (Jeon, *et al*, 2001). And the crenarchaeon *Sulfolobus acidocaldarius* (Poplawski and Bernander., 1997). The period of visible constriction, however was found to be similar to that of prokaryotes. The separation of nucleoids to opposite cell halves before division is similar to a recent model proposed for nucleoid partition in eubacteria (Wheeler and Shapiro, 1997) in which the replication origins become attached to the cell pole before division. During the exponential phase the nucleoids are highly organized but have an unstructured appearance during stationary phase. The structure of *E .coli* nucleoid has been suggested to be highly dependent on the transcriptional activity through coupled transcription and translation of membrane proteins which would anchor transcription complexes and associated DNA in the membrane and thereby extend the nucleoid. (Woldringh and Nanninga, 1985, Woldringh, *et al*, 1995). If coupled transcription, translation and membrane insertion also occurs in archaea, the relaxed nucleoid structure in stationary phase may at least in part due to reduced transcriptional activity.

Genome projects have revealed the presence of eukarya – like regulators for cell division, like CDC proteins, in archaea. (Bult, *et al*, 1996; Kawarabayasi, *et al*, 1998; Klenk, *et al*, 1997). One of the CDC 48 homologues is shown to be functional in archaeal cells (Jeon, 1999). A Cdc6-like factor has been purified and characterized from *Sulfolobus acidocaldarius.* (De Felice, *et al*, 2003).

*Sulfolobus acidocaldarius* exhibits an unusual cell cycle, with a short prereplication period and long post replication period. Also in stationary phase the cell rests with two fully replicated chromosomes (Poplawski and Bernander, 1997). The tightly regulated chromosome replication, nucleoid organization, nucleoid partition and cell division have been shown to be inhibited or uncoupled by mutation (Bernander, 2000). The *Sulfolobus*

nucleoid shows a high level of organization at exponential phase, at stationary phase, cells are more transparent than exponential phase cells nucleoid has more relaxed organization. Nucleoid was observed to occupy more space at stationary phase as compared to exponential phase (Poplawski and Bernander, 1997).

**Chromosomes and extra chromosomal elements of Archaea**

Chromatin or nucleoid is commonly defined as the complex of DNA and proteins in the nucleus that condenses into chromosomes during mitosis. This definition is eukaryote-specific and originates from light microscopy of eukaryotic cells; however, the term 'chromatin' is frequently used with 'archaeal' and 'bacterial' modifiers to describe the dynamic complex formed by proteins bound to prokaryotic genomes. Genome sequencing has revealed that archaeal chromosomes resemble most bacterial genomes, in being circular DNA molecules, accompanied occasionally by circular extra chromosomal elements (plasmids), with genome lengths that range from 2-10 Mbp comprising a single circular chromosome complexed with DNA binding proteins. The G+C content of these organisms ranges from 21-68 %. Archaeal genome size correlates approximately with metabolic diversity, but in all cases genome length exceeds cell length, necessitating packaging. The concentration of DNA within a prokaryotic cell exceeds 80 mg/ml (Bohrmann, *et al*, 1993) and at such concentrations *in vitro* protein-free DNA condenses to form an ordered aggregate. For gene expression, this must be prevented *in vivo*, and although chromatin proteins are often described as facilitating genome compaction, they also have essentially the opposite function of preventing total DNA collapse (Sandman, *et al*, 1998). The term 'macromolecular structure' evokes an image of a large complex with a defined three-dimensional structure and precise spatial coordinates, but the 'structure' of chromatin is dynamic and is dependent on three factors: macromolecular crowding, DNA supercoiling and protein binding. Given the high intracellular concentration of macromolecules and the low water content, cytoplasm is a 'poor solvent' for DNA (Minsky, 2004) and favors condensation. DNA supercoiling, resulting from both local DNA–protein interactions and the global topology mediated by topoisomerases, also promotes condensation, whereas DNA binding by chromatin proteins counteracts this condensation. In all three biological domains, distinct families of

small, basic and abundant chromatin proteins bind DNA with little or no sequence specificity. Chromosomal DNA in *Methanococcus jannaschii*, a euryarchaeote is organized into large network like structures and 3-10 copies of the chromosome are present during exponential phase and 1-5 during stationary phase. The chromosome in *Sulfolobus acidocaldarius*, a crenarchaeon is highly structured and chromosome alignment has also been observed before nucleoid partition.1-2 copies of the chromosome are present during exponential phase and 2 copies are present during stationary phase (Sandman and Reeve, 2005).

Archaeal genes are arranged in polycistronic operons. Archaeal introns have mostly been observed as stable rRNA genes. Large free introns derived from pre rRNAs have been observed as stable and abundant circular RNAs in certain Crenarchaeota (Salgia et al., 2003). Introns in protein encoding genes are ubiquitous in eukaryotic cells, but pre m-RNA splicing is yet to be reported in archaeal and its viral genomes. Recently introns were reported in genes encoding a homolog of eukaryotic Cbf5p (centromere binding factor 5), a subunit of a small nucleolar ribonucleoprotein, from *Aeropyrum premix, Sulfolobus solfataricus* and *Sulfolobus tokodaii* (Watanabe et al., 2002). Plasmids isolated from hyperthermophilic archaea, Crenarchaeota (*Thermoproteales* and *Sulfolobales*) and the orders *Thermococcales* and *Thermoplasmales* in the kingdom Euryarchaeota, were found to range in size from 4.5 kb to about 40 kb. Most of them occur in high copy number. These plasmids are from relaxed to positively super coiled in contrast to the negatively super coiled state of DNA molecules in mesophiles, bacteria, euryarchaea or eukaryotes.

Bult et al (1996) determined the complete 1.66 mega base pair genome sequence of *Methanococcus jannaschii* and its 58 kb and 16 kb extra chromosomal elements. Only 38% of the predicted proteins encoding regions have been assigned cellular roles. Sequencing of the *Sulfolobus solfataricus* genome has shown the clustering of genes by function (Charlebois et al., 1996).

Plasmids initially were found in Halobacterium based upon the observation that many species contained satellite DNA with a different mol% G1C from that in genomic DNA. In Halobacterium salinarium strain 5, a 66.7kb plasmid was found to be associated with gas vacuole production. This strain also contained two additional plasmids of 39.4 kb and 130.3 kb that were never linked to a phenotype. Gas vacuole production was also linked to the 150 kb plasmid pHH1 from *Halobacterium halobium* strain NRC817. This plasmid was maintained at four copies per chromosome (Encyclopedia of life sciences 2001). Other H. halobium strains also contained plasmids with homology to pHH1. *H. halobium* strain DSM670, *H. halobium* strain DSM671 and Halobacterium cutirubrum maintained plasmids that varied in size from 75.8 kb to 150 kb with homology to pHH1. These strains have not been characterized further (Hackett, 1990).

**Chromatin organization in Eubacteria**

The organization of the chromatin in prokaryotes is not completely understood. The genome is present as a tightly condensed structure, the nucleoid, within the cell (Kornberg et al., 1974; Materman and Van Gool, 1978). By electron microscopy it has been shown that the bacterial DNA from gently lysed *E.coli* is organized into a 12 nm filament having an axial repeat of about 13 nm (Griffith, 1976). The nucleoid DNA has been shown to be in a highly super coiled state (Materman and Van Gool, 1978). Micrococcal nuclease digestion resulted in the formation of 120 bp DNA fragments bound to histone like proteins (Varshavsky, *et al*, 1977). However regular ladders of protected fragments such as those found with eukaryotic nucleosomes were not observed (Pettijohn, 1988, Sinden and Pettijohn 1981). It has been shown that several different protein components are associated with the nucleoid including RNA polymerase, core enzyme, DNA polymerases, several DNA binding proteins and outer and inner membrane proteins both by 2- D gel analysis and by functional assays (Stonington and Pettijohn, 1971; Portalier and Worcel, 1976; Moriya, *et al*, 1981; Lossius, *et al*, 1984; Gaziev, *et al*, 1986). Folded chromosomes have been isolated from *Bacillus subtilis* (Guillen et al., 1978), *E.coli* (Worcel and Burgi, 1972), *Streptomyces hygroscopicus* (Sarfert, *et al*, 1983) and their protein composition was analyzed.

**Nucleoid associated proteins in Eubacteria**

The genome of *Escherichia coli* is associated with about 10 DNA-binding structural proteins, altogether forming the nucleoid. The nucleoid proteins play some functional roles, besides their structural roles, in the global regulation of such essential DNA functions as replication, recombination, and transcription. The intracellular concentrations of 12 species of the nucleoid protein in *E. coli* W3110, including CbpA (curved DNA-binding protein A), CbpB (curved DNA-binding protein B, also known as Rob [right origin binding protein]), DnaA (DNA-binding protein A), Dps (DNA-binding protein from starved cells), Fis (factor for inversion stimulation), Hfq (host factor for phage $Q_{ß}$), H-NS (histone-like nucleoid structuring protein), HU (heat-unstable nucleoid protein), IciA (inhibitor of chromosome initiation A), IHF (integration host factor), Lrp (leucine-responsive regulatory protein), and StpA (suppressor of *td* mutant phenotype A) Talukder and Ishihama, 1999).

Four proteins HU, H-NS, HLP1 and H have been identified as eubacterial histone – like proteins (Drlica and Rouviere –Yaniv, 1987) HU (9KDa) is a basic protein which stabilizes the DNA against thermal denaturation. Rouviere-Yaniv et al., (1979) reported that HU protein wraps DNA into nucleosomes like structures having 8-10 dimers of HU and 275 bp of DNA. H-NS is a neutral protein which binds intrinsically curved AT rich DNA with slight sequence specificity (Zuber et al., 1994; Falconi et al., 1996). It does not wrap DNA but influences the initiation of replication (Katayama et al., 1996), acts as a transcription repressor (Ueguchi et al., 1996) and also plays a role in post transcriptional regulation of rpo-s gene. HLP1 (17 KDa) encoded by fir A gene interacts with RNA polymerase and is involved in transcription and maintenance of individual domains of supercoiling (Schaffer and Zilling, 1973; Lathe et al., 1980;Sinden and Pettijohn, 1981). H (28KDa) is a basic protein which binds DNA, inhibits DNA synthesis in vitro and has an amino acid sequence similar to eukaryotic histones (Hubscher et al., 1980).

**Chromatin organization in Eukarya**

Chromatin structure and regulation of gene expression determined in part by protein – DNA interactions. Histones, nuclear matrix and chromosomal scaffold proteins and enzymes involved in replication, repair and recombination contribute to chromatin structure, function and regulation.

Eukaryotic chromatin consists of DNA – proteins complexes, nucleosomes where two turns of DNA are wound around an octamer of histones. The nucleosome core particle consists of 146 bp DNA wrapped around an octamer of the core histones (H3+H4)2 and H2A – H2B dimmers. Nucleosome cores are connected by a linker DNA associated with a single H1 histone molecule. H1 serves to condense the polynucleosomal DNA into higher order structures (Igo-Kemenes 1982).

**Chromatin organization in archaea**

Chromatin organization in archaea has both eukaryal and eubacterial characteristics. Euryarchaea contain histones that have primary sequences in common with eukaryal nucleosome core histones. They also form a histone fold that facilitates DNA wrapping into nucleosome –like structures. Archaeal nucleosomes comprising an archaeal histone tetramer circumscribed by ~80 bp of DNA, similar to the eukaryal tetrasome (H3 + H4)2 with ~80 bp of DNA have been reported in this organism (Pereira *et al.,* 1997; Sandman and Reeve 1999). Archaeal nucleosomes in *Methanobacterium Thermoautotrophicum* and *Methanobacterium fervidus* protect ~60bp of DNA and multiples of 60 bp from Micrococcal nuclease digestion. DNA structure which is dependent on DNA sequences directs the assembly of archaeal nucleosomes. The repeat sequence features are similar to the eukaryal nucleosome positioning elements. Repeated sequences like phased oligo (dA) tracts, 5' – (G/C) 3NN (A/T) 3NN-3' and CTG repeats direct both archaeal and eukaryal nucleosome positioning (Bailey and Reeve, 1999; Sandman and Reeve, 1999). HMfB from *Methanobacterium fervidus* selectively incorporates GC, AA and TA dinucleotides at ~10 bp intervals into archaeal nucleosomes. These molecules direct the positioned assembly of archaeal nucleosomes. Fourier analysis also reveals an enrichment of the AA dinucleotide in the eukaryal and euryarchaeal genomes. Histone

packaging of the genomic DNA apparently imposes constraints on genome sequence evolution (Bailey, et al., 2000).

The chromosome in *Sulfolobus acidocaldarius* is highly structured. One to two copies of the chromosome are present during exponential phase and two copies are present during stationary phase. The sheared chromatin of *Sulfolobus acidocaldarius* isolated at exponential phase resolves into two peaks during gel filtration. Peak I consists of high molecular weight and an abundance of low molecular weight proteins, mainly HSNP C', DBNP B, HSNP A, along with proteins in the molecular weight range of 45 KDa, 25KDa and 15 KDa (Jaya 1998) . Electron microscopic studies have shown the exponential phase nucleoid to be highly organized with a structured appearance, occupying a relatively small part of the cell. At stationary phase the nucleoid shows a relaxed organization, is unstructured and occupies a larger part of the cell (Poplawski and Bernander, 1997).

**Genome structure in halobacteria**. The genomes of *H.halobium* and related organisms (i.e., organisms capable of producing the bacterio-opsin-containing purple membranes) exhibit several interesting structural features. Their cellular DNAs can be separated into two fractions by either CsCl buoyant density gradient centrifugation or by malachite green bisacrylamide column chromatography (Ebert, and Goebel, 1985). The major fraction representing 70 to 90% of the cellular DNA has a G+C content of 68% and represents almost exclusively chromosomal, single-copy DNA sequences. The minor fraction representing 10 to 30% of the cellular DNA is more heterogeneous, has a G+C content of 60% or less, and includes a variety of different covalently closed, circular plasmid DNAs as well as A+T-rich island from the chromosomal DNA. Repetitive sequence families clustered in the A+T rich regions are associated with high-frequency genomic rearrangements (Sapienza and Doolittle 1982). One A+T rich island 70 kb in size has been analyzed and shown to contain a variety of different insertion elements and related repetitive sequences that are also found on plasmid DNA but only rarely, if at all, in G+C-rich single-copy genomic DNA (Pfeifer, and Bethack, 1985). For Halobacterium NRC-1, 3 circular replicons were mapped, a '2-Mbp chromosome and 2

large replicons, pNRC200 and pNRC100, about 350 and 200 Kbp in size (Hackett, *et al.*, 1994).

The chromatin in Halobacterium salinarium has been reported to consist of regions of DNA associated with protein and regions of DNA free protein. These regions are interchangeable depending on the phase of the growth (Shioda, *et al.*, 1992). Electron microscopy of the two types of the nucleoid fractions showed more nucleosome like structure for the protein free DNA (Shioda *et al.,* 1989). Sheared chromosomes obtained from the late exponential phase cells resolved into two peaks on a sucrose density gradient. The peak I consists of protein free DNA and peak II consists of rugged fibers consisting of nucleosome like structures as seen under microscope Most of the DNA is in peak I form during exponential phase and in peak II form during stationary phase. The transition between the two forms occurs during the late exponential phase (Shioda *et al.*, 1992).

**Nucleoid associated proteins in archaea**

Hyperthermophilic archaea grow at extreme conditions of temperature, pH and anaerobicity (Fiaia and Stetter, 1986; Bouthier *et al*., 1990). However their chromosomal G+C content is less than 40 mol%, *Pyrococcus* (38-45%), *Thermococcus* (38 -57%) and Methanothermus (33%). This demonstrates that a high G+C content is not essential for maintaining the stability of the genomic DNA *in vitro* at high temperatures. Many extremophiles produce low molecular weight compounds or compatible solutes to protect macromolecules especially proteins against denaturation by heat, oxidation and dryness. Compatible solutes stabilize the native tertiary structure of the protein, preventing protein aggregation. Mannosylglycerate and Mannosylglyceramide from *Rhodothermus marinus*, cyclic diphosphoglycerate from *Methanothermus fervidus,* di-myo-inosytol phosphate from *Pyrococcus furiosus*, and diglycerol phosphate from Archaeoglobus fulgidus are a few examples of such compatible solutes. These solutes also protect the proteins against proteolytic degradation by stabilizing the folded state of the protein thus making the proteolytic sites less accessible to proteases (Bagyan *et al.*, 2003).

Polyamines which are positively charged at physiological conditions stabilize the DNA against thermal denaturation by binding to the negatively recharged phosphate groups (Tomita *et al.*, 1989; Tabor and Tabor 1984; Morgan *et al.*, 1987). Linear, tertiary branched and quarternary branched polyamines have been detected in hyperthermophilic archaea (Hamana et al., 1994). High intracellular salt concentration also plays a role in maintaining double stranded DNA. Methanococcus fervidus and Pyrococcus woesei have 1 to 0.6 M potassium ion concentrations (Scholz *et al.*, 1992; Bowater *et al*., 1994).

Several small basic proteins have been isolated from euryarchaea which bind to DNA without sequence specificity. They have core domain structure similar to that of eukaryotic histones. Archaeal histones identified in euryarchaea are the structural homologues of the eukaryal nucleosome core histones. Archaeal histones are not present in Crenarchaeota (sulfur dependent hyperthermophiles and acidophiles) but are present in most euryarchaeota (methanogens, halophiles) except *Thermoplasma* (Ruepp, *et al*., 2000). The combination of histones of the archaeal nucleosomes varies with the growth conditions similar to the changes observed in the histone like proteins in the nucleoids of bacteria at different growth phases (Claret, Rouviere –Yaniv, 1997).

HTa (9.9 KDa) from Thermus acidophilum, was the first archaeal histone – like protein identified (Searcy 1975, Stein and Searcy, 1978). It is a basic protein which exists as a tetramer in solution (Searcy and Stein 1980) and protects DNA against thermal denaturation. It has a greater homology to HU than to histones H2A and H3 (Drlical and Rouviere – Yaniv, 1987; DeLange *et al*., 1981).

*Sulfolobus* genus does not have histone proteins, but it has a number of small basic and abundant DNA binding proteins of 7 KDa, 8KDa and 10 KDa species which play important roles in DNA compaction, helix stabilization and DNA processing mechanisms (Kimura *et al*., 1984; Choli, et al., 1988 Reddy and Suryanarayana, 1988; Reddy and Suryanarayana 1989).

The 7 KDa classes of proteins comprise five isoforms, 7a to 7e. The 8 KDa and 10 KDa class of proteins comprise two proteins respectively, 8a, 8b and 10a 10b. Electron microscopy of the protein – DNA complexes of 7 KDa, 8 KDa and 10 KDa proteins show that they bind ss and ds DNA to form compact structures (Lurz *et al*., 1986).

The 7d protein (known as Sso 7d, Sac 7d, and Ssh 7d depending on species of origin) and Alba (known as Sso 10b, Sac 10b, and Ssh 10b depending on species of origin) are two highly abundant DNA binding proteins have been characterized. The non-sequence specific DNA binding protein Sso7d protects DNA against thermal denaturation and promotes the renaturation of complimentary DNA strands at temperatures above the melting point of the duplex in a strictly homology dependent manner (Guagliardi, *et al*., 1997). Sso7d is a 62 residue basic protein. At neutral pH it denatures at about 100 $^{o}$C. Binding of multiple Sso7d molecules to short DNA fragments induces significant curvature and residues the stiffness of the complex. It induces negative supercoiling in DNA molecules of any topology – relaxed, positively or negatively super coiled DNA. It also inhibits the positive supercoiling activity of the thermophilic specific enzyme, reverse gyrase (Napoli, *et al*., 2002).

Archaeal proteins of Sac 10b family (conserved only in thermophilic archaea) are thought to affect the topology of the chromosomal DNA in thermophilic archaea. Ssh 10b constrains negative DNA supercoils in a temperature dependent manner (Xue *et al*., 2000). Studies have shown that two forms of Ssh 10b homodimers coexist in solution and the slow *cis-trans* isomerization of the Leu61-Pro62 peptide bond accounts for the conformational heterogeneity of the homodimers. The *trans* form (T-form) is dominant at high temperatures and the cis form (C-form) at lower temperatures. Both forms have the same DNA binding site but different conformational features which are responsible for the temperature dependent nature of Ssh 10b-DNA interaction. (Cui, *et al.*, 2003). The Alba proteins coat the ds DNA without significantly compacting it protecting it from nuclease digestion (Lurz *et al*., 1986). Recent reports show that Ssh 10b binds with similar affinity to ds DNA, ssDNA and RNA *invitro* and binds exclusively to RNA in *Sulfolobus shibitae* cells *in vivo* (Guo *et al.*, 2003).

Reddy and Suryanarayana, (1998) purified four soluble low molecular weight proteins from the nucleoid of Sulfolobus acidocaldarius, HSNP (Helix stabilizing nucleoid A (12 KDa), C (9KDa), C' (7KDa) and DBNP (DNA binding nucleoid protein) B (10KDa). The HSNP proteins stabilize the DNA helix and protect DNA against thermal denaturation. They are present exclusively on the genomic DNA as revealed by Immuno gold labeling electron microscopy of the nucleoid and could be responsible for DNA compaction and genome organization (Bohrmann et al., 1994). HSNP C' is identical to Sac 7d (Gauri 1997) and DBNP B is identical to Sac 10b (Sreenivas 1994 and Jaya, 1998). DBNP B binds ds and ss DNA strongly but does not protect against thermal denaturation. It forms different types of complexes of different protein to DNA ratios (Sreenivas *et al.*, 1998). DBNP – B (Sac 10 b) shows structure specific binding of circular DNA. HSNP A strongly binds native DNA and RNA, weakly to denatured DNA and ribosomes. It protects the DNA against thermal denaturation but not RNA.

MC1 (Hmb), a 14KDA abundant histone like protein was isolated from Methanosarcina species (Chartier *et al.*, 1985).It preferentially binds ds DNA and protects it against thermal denaturation (Chartier *et al.*,1988) and radiolysis by fast neurons (Isabelle *et al.*, 1993). It stimulates transcription (Chartier *et al*., 1989) though it does not form nucleosomes; it preferentially binds negatively supercoiled DNA and bends the DNA upon binding (Teyssier *et al*., 1996). The amino acid sequence of this protein does not share homology with any of the histones or histone like proteins.

In *Methanococcus fervidus,* proteins like HMfA and HMfB compact the DNA and protect it against thermal denaturation in vitro. DNA-binding and nuclease-protection properties of the HMf histones from the hyperthermophilic archaeon *Methanothermus fervidus* have been shown to be consistent with the formation of nucleosome-like structures (NLS). These proteins bind to DNA fragments resulted from Micrococcal nuclease (MNae) digestion that are approximately 30 bp, 60 bp and multiples of 60 bp in length. DNA structure determined by DNA sequence plays an important role in positioning and such repeated sequences as phased oligo (dA) tracts, 5'-(G/C)3NN(A/T)-

3’ and CTG repeats direct both archaeal and eukaryal nucleosome positioning (Sandman *et al.*, 1999). HpkA and HpkB from *Pyrococcus kodakaraensis* KOD1 also compact DNA and protect against thermal denaturation (Higashibata *et al.*, 1999).

*Methanobacterium formicum* has three HMF like proteins, HFoA1, HFoA2 and HFoA3. They bind and compact DNA into nucleosome like structures similar to those formed by HMf proteins (Darcy *et al.*, 1995). No nucleoid associated proteins or histone like protein have been isolated to date from any Halobacteria.

**Growth-dependent changes in the content and levels of proteins associated with nucleoid**

Structural and functional modulations occur in the nucleoid of the prokaryotes and chromatin of eukaryotes during the transition from exponential growth to stationary phase. Cells enter into stationary phase upon sensing an impending saturation level of their population density.

Nucleoid proteins play functional roles besides their structural roles, in the regulation of essential DNA functions such as replication, recombination and transcription. Studies on the composition of these nucleoid associated proteins show that a variation in protein contents during the stationary phase is accompanied by compaction of the genome DNA and silencing of the genome functions.

The intracellular concentrations of 12 species of the nucleoid protein in *E. coli* W3110, including CbpA (curved DNA-binding protein A), CbpB (curved DNA-binding protein B, also known as Rob [right origin binding protein]), dnaA (DNA-binding protein A), Dps (DNA-binding protein from starved cells), Fis (factor for inversion stimulation), Hfq (host factor for phage $Q_{ß}$), H-NS (histone-like nucleoid structuring protein), HU (heat-unstable nucleoid protein), IciA (inhibitor of chromosome initiation A), IHF (integration host factor), Lrp (leucine-responsive regulatory protein), and StpA (suppressor of *td* mutant phenotype A) were found to vary in a growth phase dependent manner ( Talukder, 1999).

Growth phase dependent expression and degradation of histones has been observed in the thermophilic archaeon *Thermococcus zilligi,* the levels of certain proteins decreased dramatically on entry into stationary phase. The HTz protein could not be detected by late stationary phase (Dinger et al., 2000).*Methanothermus fervidus* cells have been shown to contain HMfA and HMfB homodimers and HMfA-HMfB heterodimers. The HMfA/HMfB ratio and the relative amounts of homodimers and heterodimers is growth phase regulated. HMfA is more abundant in exponential phase and HMfB during the stationary phase (Sandman, *et al*., 1994).

In present study detailed characterization of Halobacterium salinarium H3 nucleoid was under taken to understand the organization of the intracellular DNA in this euryarchaeote. Studies were also carried out on an abundant nucleoid associated protein to characterize its biochemical properties and analyze its DNA binding properties.

## OBJECTIVES AND SCOPE OF THE PRESENT INVESTIGATION

The present study is an attempt to characterize the nucleoid from extreme halophilic organism *Halobacterium salinarium*. ***Haloarchaea*** are a group of organisms that are found in adverse environmental conditions like high salinity, nearly saturated-NaCl concentration. This group of organisms is known as **"**extreme halophiles"**.** The intracellular salt concentration is found to be very high in extreme halophiles. As a consequence, universally conserved molecules like proteins and nucleic acids may have also adapted to function at such a high salt concentration (Eisenberg et al., 1992).

*H. salinarium* is a euryarchaeote requiring at least 3.5 M NaCl (Maximum 5.2 M NaCl) for optimal growth. It has high intracellular salt concentrations (>4 M), which is normally detrimental to the structural and functional integrity of majority of the mesophilic proteins. High negative DNA supercoiling ($\sigma$ =0.7), high G+C content (~67%) and thick (17-20 nm) rugged nucleosome like DNA – protein structures as seen under electron microscopy (Shioda, *et al*., 1989) interspersed with stretches of protein free DNA are the reported characteristics of the genomic DNA of this organism (Shioda, *et al.,* 1992).

As already presented in the Introduction, DNA is compacted into nucleosome-like particles by proteins of HMf family, which have a structure and function similar to eukaryal histones in Methanogenic archaea (Reeve, *et al.,* 1990, 2000). In thermophilic archaea, the organization of intracellular DNA is more like bacterial nucleoid with no apparent eukaryotic chromatin like organization. Many helix stabilizing, DNA binding proteins associated with nucleoid were identified in thermophiles. In the case of halobacteria, the nucleoid organization and nucleoid associated DNA binding proteins have not yet been studied.

The objective of the present investigation to characterize the Haloarchaeal nucleoid with respect to its associated proteins and the mode of compaction, whether it is like nucleosomal type as in methanogenic archaea or non nucleosomal type as in thermophilic archaea and mesophilic bacteria. We found that the nucleoid from this Halobacterium is

nonnucleosomal type condensed structure. The study also showed the growth phase dependent variation in nucleoid fractions.

Another objective of the present study is to purify any abundant protein associated with the nucleoid from this organism. A 30 kDa protein which was present in all nucleoid fraction was chosen for purification. Characterization of this protein indicated that it is a DNA unwinding protein. Peptide Mass Fingerprint identified the protein as Mn2+-Super Oxide Dismutase. Association of $Mn^{2+}$-SOD with the nucleoid is interesting as it may have a possible role in DNA metabolism. The protein may be utilized for unwinding the duplex for DNA replication, transcription and repair like ssb protein and at the same time for protecting the DNA against oxidative damage. Further work can be carried out to characterize the interaction of $Mn^{2+}$-SOD with DNA.

## MATERIALS AND METHODS:

### Materials:

Bis -Tris, DNase I, BSA, NP-40, Sucrose, Agarose, Coomassie brilliant blue R-250, Poly (U), Poly (A), Poly dG-dC, ATP, Pancreatic RNase, PEG-20,000, Sodium Deoxy-cholate and EtBr were obtained from SIGMA chemical company, U.S.A. Poly dA, Poly dT, Poly dC, Poly dA-dT from Miles Laboratories, Elkhart, Indiana. Triton –X-100 from Fluka chemicals, U.S.A. DEAE cellulose from Whatman chemical company, England. Sephacryl S-1000, Sephacryl S-200, Sephadex G-100, Acrylamide, Tris, DTT were obtained from Amersham Pharmacia chemical company, U.S.A. SDS, N-N' methylene bis acrylamide were purchased from Serva chemical company, Germany. Yeast extract, Casein acid Hydrolysate were obtained from Hi-media chemical company Mumbai, India. TEMED, Folin's reagent, distilled phenol, Magnesium acetate was purchased from SRL chemicals Mumbai, India. Protein Molecular weight markers, Lambda DNA were purchased from Bangalore GENEI chemicals, Bangalore, India. Sodium chloride, Potassium chloride, Magnesium chloride, Magnesium Sulphate, Calcium chloride, Glycine, Ferrous Sulphate, Manganese chloride, Isoamyl alcohol, Chloroform, Methanol, Glacial acetic acid and other chemicals were purchased from Qualigens, Glaxo laboratories, India.

*Halobacterium salinarium H3* strain was obtained from National Collection of Industrial Microorganism Pune, India.

### Growth and harvesting:

*H. Salinarium* H3 was grown in a medium containing 0.75 % Casein acid Hydrolysate, 0.2% KCl, 2 % Magnesium sulfate 0.005% Ferrous sulfate, 40 µg of Manganese chloride and 3.418 M Sodium Chloride, pH adjusted to 7.0 with 2N NaOH. The cells were harvested by centrifugation at 6000 rpm at 4º C for 20 minutes. The cell pellet was washed by resuspending in solution containing 50 mM KCl and 3.14 M NaCl followed by centrifugation at 6000 rpm at 4 ºC.

**Isolation of nucleoid:** Two different methods were employed to isolate nucleoid chromatin from *Halobacterium salinarium H3*

**Sucrose density gradient centrifugation for the isolation of (Chromatin or nucleoid) from *H. salinarium H 3***

The procedure was described by Shioda, *et al* (1992). Freshly harvested mid logarithmic phase and stationary phase cells were suspended gently in 6 ml of buffer containing 50 mM Tris-HCl pH 7.6, 1 mM $Na_2$-EDTA, 1 mM Magnesium acetate,7 mM 2-mercapto ethanol, 3.4 M NaCl and cells were allowed to lyse. The resulting viscous lysate was gently applied on 5 – 20 % sucrose gradient containing the above buffer solution. The samples were centrifuged in Sorvall ultra Centrifuge at 105,000 g /1 hr. After fractionation (each 1.5 ml from the top) of the gradient, $A_{260}$ and $A_{280}$ was measured, protein and nucleic acid in the fractions were analyzed on SDS - PAGE and agarose gel electrophoresis.

**Isolation of Nucleoid:**

Nucleoid from *Halobacterium salinarium H3* was isolated according to a procedure developed in our laboratory. Freshly harvested cells (4g) were suspended in 12 ml of nucleoid buffer containing 20 mM Tris-HCl pH 7.6, 0.5 M NaCl, 3.4 M KCl, 0.1 M Mg-Ac, 1 mM $Na_2$ – EDTA, 7 mM 2 - mercaptoethanol and 1.5 ml of lysis mix was added and kept for 1 hr at 4˚C by constant stirring (Lysis mix – NP – 40, 1 % Triton – X –100 and 0.1 % sodium Deoxy-cholate). The lysate was centrifuged at 1000 rpm/20 mins at 4˚C.The supernatant was collected and loaded onto 30 % sucrose cushion buffer pH 7 and centrifuged at 10,000 rpm/1 hr/ 4˚C. The nucleoid pellet was collected and resuspended in nucleoid buffer, again the pellet was loaded on a 20% sucrose cushion and centrifuged at 30,000 rpm/ 1hr in 70 Ti rotor. The nucleoid pellet was collected and used for further studies.

**Sephacryl S-1000 column chromatography:**

Purified nucleoid fraction was also obtained by Sephacryl S – 1000 column chromatography of nucleoid isolated by sucrose cushion procedure. Sephacryl S -1000 matrix was packed to a bed volume of about 100 ml in a column measuring 55 cm x 1.6

cm and equilibrated with the buffer containing 20 mM Tris-HCl, 3 M KCl, 0.5 M NaCl, 0.1 M Magnesium – acetate, 1 mM $Na_2$ – EDTA and 7 mM 2- mercaptoethanol. Nucleoid (2 ml) was gently sonicated and clarified by centrifugation at 1500 rpm at 4 º C for 10 minutes and loaded onto the Sephacryl S – 1000 column. Elution was done with 3 bed volumes of reverse salt gradient range between 3 M – 0.05 M potassium chloride and 3 ml fractions were collected at the rate of 24 ml per hour. Fractions collected were analyzed by measuring absorbance at 260 and 280 nm. Protein, DNA and RNA were estimated in the column fractions. SDS-PAGE analyses for protein, agarose gel electrophoresis for nucleic acids were also performed. Nucleoid containing fractions were pooled and used for further studies.

**Micrococcal nuclease (MNase) digestion of chromatin from *Halobacterium salinarium H3***

This was performed following the procedure of Owens Huges and Workman (1996) Initially nucleoid was dialyzed against the MNase digestion buffer containing 20 mM Tris-HCl pH 7.6, 50 mM NaCl, 50 mM $MgCl_2$, 1 mM $CaCl_2$. After dialysis, nucleoid fraction (1µg) was incubated with 0.1 unit of MNase in MNase digestion buffer for different time intervals (1min, 3 min, 5 min, 10 min, 20 min, and 30 min). Reaction was stopped by adding 1% SDS and 25 mM EDTA to a final concentration. The reaction products were analyzed on a 0.8 % agarose gel.

**Isolation of MNase digested products of nucleoid by Sephacryl S – 200 column chromatography**

Sephacryl S – 200 matrix was packed to a bed volume of 100 ml in a column measuring 55 cm x 1.6 cm and equilibrated with the buffer containing 20 mM Tris-HCl pH 7.6, 250 mM NaCl, 1 mM $Na_2$ – EDTA, 7 mM 2- mercaptoethanol. Nucleoid or chromosomal fraction (2 ml) was digested with MNase (0.1 U/1µg DNA) as described above. The sample was dialyzed against buffer containing 20 mM Tris-HCl pH 7.6, 250 mM NaCl, 1 mM $Na_2$ – EDTA, 7 mM 2- mercaptoethanol. MNase digested products of chromosomal fractions peak I and peak II obtained through sucrose density gradient centrifugation of stationary phase cells and MNase digested products of nucleoid from 5 days and 7 days

cell culture isolated by ultra centrifugation using 30 % sucrose cushion were chromatographed on S- 200 column chromatography. Elution was done with 2 bed volumes of the buffer and 3 ml fractions were collected at the rate of 24 ml per hour. Fractions were collected and analyzed by measuring absorbance at 260 and 280 nm, 18% SDS-PAGE and 0.8% agarose gel analysis of nucleoprotein complexes, DNA and RNA.

**DEAE- cellulose column chromatography of sephacryl S -1000 Peak nucleoid fractions:**

Preswollen DEAE-cellulose was suspended in a buffer containing 20 mM Tris-HCl pH 7.6, 1 mM Magnesium – acetate, 7 mM 2- mercaptoethanol (column buffer). The gel was packed into a column of 10 ml bed volume and equilibrated with the column buffer. Sephacryl S -1000 peak fractions were pooled and dialyzed against column buffer with out salt overnight. Sample was loaded onto the DE-52 column, washed with 3 bed volumes of the column buffer. Elution was carried out with increasing linear salt gradient from 0.05 M – 0.75 M NaCl. Fractions (1ml) were collected and the $A_{280}$ was measured. Peak fractions were analyzed on an 18 % SDS-PAGE; DNA concentration was estimated by diphenylamine method.

**DNase I digestion of DEAE – cellulose nucleoid fraction:**

DNase I digestion of the nucleoid fractions, obtained from the DEAE- cellulose column was performed by incubating with DNase I at a ratio of 10:1 (DNA: DNase I) in 10 mM Tris-HCl pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT at 37º C for 30 seconds. The reaction was terminated by the addition of SDS and EDTA to 1 % and 25 mM final concentrations respectively.

**Gel filtration chromatography of purified nucleoid protein:**

Preswollen Sephadex G-100 was deaerated and packed into a thin long column of 40cm × 0.8cm and equilibrated with 20 mM Tris-Cl pH 7.6**,** 1 mM $Na_2$ – EDTA, 7 mM 2-mercaptoethanol and 150 mM NaCl. HS30K protein (100µg) was loaded on to the column and eluted with the same buffer. Fractions (1ml) were collected from the column at a flow rate of 5 ml/hr and alternate fractions were analyzed for the presence of the

protein by both SDS –PAGE and absorbance measurements at 280 nm. Simultaneously protein molecular weight markers *viz.* Bovine serum albumin (66 KDa), Ovalbumin (45KDa) and Cytochrome C (12 KDa) were also chromatographed on the Sephadex G-100 column for determination of the native molecular weight of HS30K protein.

**Ultraviolet absorption spectra of the proteins:**

The U. V. absorption spectra and the fourth derivative spectra of the purified protein was recorded at 25º C in Thermo spectronic Biomate spectrophotometer. Protein solutions (1mg /ml) were in 20 mM Tris-HC1, pH 7.6, 1 mM Magnesium acetate and 500 mM NaC1.

**Fluorescence spectra of the nucleoid protein:**

Fluorescence intensity and emission spectra were recorded in a JASCO FP-777 spectrophotometer with a cuvette of 1 cm path length. All measurements were made with excitation and emission bandwidths of 5 nm and 10 nm respectively, using a single photomultiplier. The measurements were performed in 20 mM Tris-HCl pH 7.6 and different NaCl concentrations at room temperature (25ºC). The excitation wavelength was 280 nm. Emission spectra were recorded between 290nm and 330 nm. They were baseline corrected for the corresponding buffer.

**Circular Dichroic Spectra**:

CD spectra of the nucleoid protein (in 20 mM Tris-HC1 pH 7.6, 50 mM or 2 M NaCl) were recorded in a Jasco-810 scanning spectropolarimeter. The instrument was calibrated using an aqueous solution of d-10-camphorsulfonic acid. The spectrometer was continuously purged with dry nitrogen before and during the experiment. CD spectra of HS nucleoid protein (2 sec response time, scan rate 100 nm/min, 8 scan average,) at concentrations of 1 mg/ml and at 20º C temperature in a buffer containing 20 mM Tris-HCl pH 7.6, 50 mM NaCl or 2 M NaCl were obtained using 1 mm path length. Experimental data were converted to units of differential molar circular dichroic absorption. All CD spectral data were plotted as molar ellipticity *(8)* using the formula:

$$Ø = \frac{\textbf{(ø) obs} \times 100 \times MRW}{C \times l}$$

The spectra were deconvolved using the Dichro Web CD software package.

Where **(ø) obs** = ellipticity measured in degrees, **c** = concentration of the protein sample in grams/ml, 1 = path length in centimeters, MRW = mean residue molecular weight. The units of [e] are degrees.cm$^2$. Decimole".

**Gel mobility shift assay:**

Gel mobility shift analysis of HS30K protein – DNA complexes was done as described by (Lohman et al., 1986). Protein –DNA complexes were allowed to form in 20 mM Tris-HCl pH 7.6, 1 mM sodium EDTA, 7 mM 2- mercaptoethanol and with magnesium (5 mM) or without magnesium in presence of different salt concentrations at different DNA to protein ratios. Samples were incubated at 37º C for 20 – 30 minute and analyzed by electrophoresis on 0.8% agarose gel in TAE buffer (40 mM Tris – acetate pH 7.8 and 1 mM sodium- EDTA. Gels were stained with 0.5 µg/ml ethidium bromide and visualized under UV illumination.

**Intrinsic fluorescence titrations:**

All fluorescence measurements were obtained using JASCO FP-777 spectrophotometer. The measurements were performed in 20 mM Tris-HCl pH 7.6 and different NaCl concentrations at room temperature (25ºC). The band width was 5 nm and 10 nm respectively. After each addition of nucleic acid to protein, the contents were mixed gently and allowed to stand for 3 minutes. The fluorescence emission intensities were recorded after the fluorescence signal was stabilized. The fluorescence titrations were performed by adding lattice (nucleic acid) to the ligand (protein) (reverse titration) or ligand to lattice (direct titration).

**Binding of the Proteins to DNA as Measured *by* Intrinsic Fluorescence Quenching:**

Binding of DNA to the nucleoid protein was measured by this assay in a reaction mixture of 1 ml containing 20 mM Tris-HCI, pH 7.6, 50 mM – 2 M NaCl and 25 µg of each protein and increasing amounts of either native or denatured DNA (1µg/ ml). Excitation and emission band widths were 5 nm and 10 nm respectively. After each addition of nucleic acid to protein, the contents were mixed gently and allowed to stand for 3

minutes. The fluorescence emission intensities were recorded after the fluorescence signal was stabilized. The decrease in the fluorescence intensity at emission maximum was measured at 25º C in a FP-77 Jasco Spectrofluorimeter. The average of three readings with in an interval of 60 seconds was taken for each titration point. The fluorescence units (arbitrary units) were corrected for dilution.

**Reverse Titrations:**

Aliquots of nucleic acids or other quenchers were added to a fixed concentration of HS30K protein (25 µg) in 1 ml of reaction buffer (20 mM Tris – HCl pH 7.6 and 50- 2M NaCl) and the decrease in fluorescence intensity was measured. The initial protein fluorescence was taken to be 100 % and all other measurements were made with reference to the initial fluorescence of the protein. Titrations were also performed with different mononucleotides.

**Direct fluorescence titrations:**

Small volumes of protein solution was added to constant amount of double stranded DNA and the fluorescence emission of the protein was measured as above. In this mode of titrations, initially the quenched fluorescence of the bound protein was measured and after saturation, the fluorescence intensity of both the bound and free protein was measured.

**Circular dichroism measurement of HS30K protein – DNA complex**

CD titrations were performed by the addition of aliquots of concentrated protein solution to a 1mm path length cuvette containing known concentrations of DNA. Forward titrations carried out with Lambda DNA (25 µg) and pBR 322 (5 µg) with increasing concentrations of protein at increasing salt concentrations. The spectra were scaled to delta € of the DNA.

**Trypsin digestion and MALDI – TOF /TOF**

Tryptic digestion and peptide mass finger printing was kindly performed at Molecular Biophysics Unit (IISc, Bangalore) and CCMB, Hyderabad (Dr. Ravi Sir Deshmukh Lab).

We are grateful to both Dr. Ravi Sir Deshmukh and Dr. Prakash of CCMB, and IISc respectively for the MALDI analysis

Samples were resolved through 1-D gel electrophoresis. Proteins spots were excised from Coomassie stained gels and washed with milli-Q water and twice with 50 % acetonitrile for 15 min. Gel pieces were then washed with a 1:1 solution of 0.1 M $NH_4HCO_3$ and acetonitrile for 15 min. For destaining, gel pieces were incubated in 10 mM DTT/0.1 M $NH_4HCO_3$ for 45 min at 56 °C to reduce the protein, followed by incubation in 55 mM iodoacetamide/0.1 M $NH_4HCO_3$ for 30 min at room temperature in the dark for alkylation. Supernatants were discarded and gel pieces were washed with 100 µl $NH_4HCO_3$, followed by two washes (5 min each with vortexing and brief centrifugation) with 100µl (or enough to cover) of 25 mM $NH_4HCO_3$ in 50% acetonitrile. The gel particles were dehydrated in a Speed Vac (Thermo Savant) to complete dryness and rehydrated with trypsin digestion buffer (50 mM $NH_4HCO_3$, 5 mM CaCl2). For trypsin digestion, 12.5 ng/µl of porcine trypsin (Promega, Madison, USA) was added in a final volume of 25 µl. Tubes were incubated on ice for 45 min, after which 25 mM $NH_4HCO_3$ was added and tubes were further incubated overnight at 37 °C. The supernatant was removed into a clean siliconized tube and extracted twice in 50 % acetonitrile and 5 % formic acid and acetonitrile. The mixture was vortexed 20-30 min and centrifuged. Supernatant was pooled into a separate tube and the volume was reduced to 10 µl using a Speed Vac. Subsequently, the digested mixture was passed through a C18 ZipTip (Millipore). Peptides were eluted into a siliconized tube with 3 µl of 5 % formic acid prior to analysis by MALDI. The digests of individual spots were analyzed by Ultra flex MALDI TOF/TOF (Bruker Daltonics) (IISc, Bangalore) and an in house constructed Applied Biosystems 4700 Proteomics Analyzer 90(CCMB, Hyderabad). Peptide Mass Fingerprint data was used to search different databases. The data bases used in the search were MASCOT search software (Matrix Science USA) and ProFound – Prowl search software and MS – fit software.

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**Fluorescence titration of HS30 K nucleoid protein with Manganese ion**

Aliquots of Manganese chloride ( 1 µl each equal to 1 mM $MNCl_2$ ) were added to a fixed concentration of HS30K protein (25 µg) in 2 ml of reaction buffer (20 mM Tris – HCl pH 7.6 and 500 mM - 2M NaCl) and the change in fluorescence intensity was measured between 285 nm to 340 nm.

**Assay of superoxide dismutase ( SOD) activity by gel electrophoresis**

Electrophoresis under nondenaturing was carried out at 4°C according to a modified procedure of Gabriel (1971) with 1.5 mm of 12.5 % polyacrylamide mini-slab gel in standard tris-glycine buffer (pH 8.3). Samples were loaded into each well and then electrophoresed at 80 V through the stacking gel for 15 min and 120 V through the separating gel for 60 min. After electrophoresis, SOD activity was localized on the gel according to Schmidt *et al* (1999). The gel was first soaked in 25 ml of 1.23 mM NBT for 30 min, briefly washed with buffer, then soaked in the dark in 30 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 24 µM riboflavin for another 30 min. The gel was briefly washed again, and then illuminated on a light box with a light intensity of 30 $m\text{Em}^{-2}\text{s}^{-1}$ (measured by LI-COR LI 1000) for 15 min to initiate the photochemical reaction. All the procedures were carried out at room temperature.

**Isolation of pBR322 super coiled DNA:**

pBR322 DNA was isolated as described by Sambrook et al. (1994) from *E. coli* cells. LB plates containing 25 µg/ml ampicillin were streaked with *E. coli* containg pBR322 plasmid and grown for 24 hrs at $37^{o}$ C. A single colony was used to inoculate 25 ml LB medium, supplemented with 1 % glucose and 25 µg/ml ampicillin. The culture was grown with vigorous aeration at 37º C to OD of 0.3 at 550 nm and used as inoculum. 250 ml LB media was inoculated with 1 % of inoculum and grown at $37^{o}$ C for 16 hrs. Cells were harvested by centrifugation at 5000 rpm for 10 minutes at $4^{o}$ C. The cell pellet was resuspended in 10 ml of ice cold solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM $Na_2$ - EDTA pH 8.0). To the cell suspension, 20 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, mixed rapidly and incubated on ice for 10 minutes. Then 15 ml of ice cold solution III (3M potassium acetate, 11.5 % glacial acetic acid) was

added and mixed gently and stored on ice for 3-5 minutes. The suspension was centrifuged at 12000 g for 5 minutes at 4 $^{o}$C and the supernatant was transferred to a fresh tube. Equal volume of phenol: chloroform: isoamylalcohol (24: 1: 1) was added, mixed gently and centrifuged at 12000 g for 5 minutes at 4 $^{o}$C. The aqueous phase was collected and two volumes of absolute alcohol were added. DNA was left to precipitate overnight at -20$^{o}$ C. The DNA pellet was washed with 70 % ethanol and dissolved in TE buffer (10 mM Tris –HCl pH 8.0 and 1 mM sodium EDTA, pH 8.0) containing 2 µg/ml DNase free RNase stored at -80 $^{o}$C and reprecipitated with ethanol.

**Agarose gel electrophoresis:**

DNA and nucleic acid: protein complexes were electrophoresed on 0.8 % agarose gels. Agarose gels were formed in 1X TAE (40 mM Tris-acetate pH 7.8 and 1 mM sodium EDTA) buffer. Samples were mixed with 6X buffer containing 0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol to a final concentration of 1 X. Electrophoresis was done in 1X TAE buffer at 40 V till the bromophenol blue dye reached 3 quarters of the gel. The gel was stained with 0.5 µg/ml ethidium bromide for 30 mins, destained with water for 1 hour and visualized by UV illumination.

**Isoelectric focusing:**

Isoelectric focusing was performed according to the method of 'O' Farrell (1975) with some modifications. Here we avoided using the agarose to seal the one dimension gel stacking of second dimension gel slab. All the protein samples were prepared in lysis buffer containing 9.5 M urea, 2% (w/v) NP-40, 2% Ampolines (comprised of 1.6 % pH range 5 to 7 and 0.4% pH range 3 to 10) and 5% 2- Mercaptoethanol.

**One dimensional electrophoresis (IEF):**

Polyacrylamide gel electrophoresis in presence of 2% Ampolines was performed using gel mixture composed of 4% acrylamide/bisacrylamide. The gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 60 min. Sample were applied on the top of the gels and electrophoresed at 400 V for 16 hrs, using 0.01 M $H_3PO_4$ as upper electrode buffer. The gels were removed and equilibrated with equilibration buffer.

**Second Dimension:**

After the run in first dimension, the gels were removed and equilibrated with the buffer containing Tris-HCl pH 6.8, Glycerol, 1 M DTT and 10% SDS and placed 1.5 cm above 11.75 % polyacrylamide/ bisacrylamide gel slab in 0.55 M Tris-HCl pH 8.8 and 0.12% SDS. A stacking gel of 4.75% polyacrylamide/bisacrylamide in 0.125 M Tris-HCl pH 6.8 and 0.1% SDS was formed between the first dimensional gel and the slab gel. Electrophoresis in the second dimension was performed at 15 mA for 2 hrs and 20 mA for another 2 ½ hours. The running buffer contained 0.025 M Tris base, 0.192 M glycine and 0.1% SDS. After the electrophoresis the slab gel was removed and stained by the Coomassie blue staining method.

**SDS – polyacrylamide gel electrophoresis:**

SDS –PAGE was performed using 18 % gel as described by Thomas and Kornberg (1975). The ratio of acrylamide to bisacrylamide was 40: 0.4. The 18% separating gel was in 0.75 M Tris – Cl pH 8.8, 0.02 M sodium EDTA and 0.1% SDS and the stacking gel was of 5 % polyacrylamide in 0.08 M Tris – HCl pH 6.8, 0.02 M sodium ETDA and 0.1% SDS. Protein samples were treated with 0.1 % SDS and 1 % 2 - mercaptoethanol at 65 $^{o}$ C for 15 mins. Electrophoresis was performed at 9 V/cm for 4 hrs for mini gels and 10 hrs for standard gels. Electrode buffer used was 0.05 M Tris, 0.38 M glycine and 0.1% SDS.

**Staining of polyacrylamide gels:**

**Coomassie blue staining:**

Gels were fixed for 1 hour in 50: 50: 10 methanol: water: glacial acetic acid and then stained with Coomassie blue R -250 (0.1% in 50 % methanol, 7.5% acetic acid) for 1 hour and destained in 5% methanol, 7.5% acetic acid.

**Silver staining:**

Gels were stained according to Blum and Gross (1987). Gels were fixed in 50 % methanol, 12.5 % acetic acid and 0.02% formaldehyde for 1 hour, rinsed with water and washed three times with 50% ethanol for 20 minutes each. Gels were rinsed with water treated with 0.02% hypo for 1 minute and washed with water three times for 20 seconds each. Gels were soaked in 0.2% silver nitrate solution containing 0.03% formaldehyde for 20 minutes. Gel was rinsed with water and developed with 0.2% hypo, 6% sodium carbonate, 0.02% formaldehyde for 10 minutes. Reaction was terminated by addition of 50% methanol and 12.5% acetic acid. Gels were stored in 10% methanol.

**Nucleic acid estimation:**

Nucleic acid content was determined according to Morgan *et al*., (1979). Increasing amounts of calf thymus DNA (10 µg, 20 µg, 40 µg, 80 µg, 120 µg, 160 µg) were added to 1 ml of buffer containing 0.5 µg/ml ethidium bromide, 5 mM Tris-HCl pH 8.1., 0.5 mM sodium EDTA and the fluorescence emission was measured at 600nm (excitation at 525 nm) with the maximum slit width in a JASCO Spectrofluorimeter. 50µl of the sample was added to 1 ml of buffer and fluorescence was measured as before. The amount of nucleic acid in the sample was calculated from standard graph.

**DNA estimation**

DNA was estimated by diphenylamine method as described by Burton *et al.,* (1956). Increasing amounts of calf thymus DNA (10µg, 20 µg, 40 µg, 80 µg, 120 µg, 160 µg) were taken in 2 ml water and mixed with 4 ml of diphenylamine reagent containing 0.1% diphenylamine, 2.5 % sulfuric acid and 97.5% glacial acetic acid. The samples were kept in a boiling water bath for 10 minutes, cooled and absorbance was measured at 595 nm. Protein sample (1 ml) was diluted with 1 ml of water, mixed with 4 ml of DPA reagent and absorbance measured as before. The amount of DNA in the sample was calculated from the standard graph.

**Protein estimation:**

Protein concentration was estimated by Lowry *et al.,* (1951) with Folin-Ciocalteau reagent using bovine serum albumin as a standard.

**RNA estimation:**

RNA was estimated by orcinol method as described by Ceriotti (1955). Increasing amounts of *E. coli* t RNA (10µg, 20 µg, 40 µg, 80 µg, 120 µg, 160 µg) were taken in 2 ml water and mixed with 3 ml of orcinol reagent containing per 100 ml, 0.1g ferric chloride, 2.5 ml 6% w/v orcinol in alcohol and 97.5 ml concentrated hydrochloric acid. The samples were kept in a boiling water bath for 20 minutes, cooled and absorbance was measured at 665 nm. 1 ml sample was diluted with 1 ml of water, mixed with 3 ml of orcinol reagent and absorbance measured as before. The amount of RNA in the sample was calculated from the standard graph.

**TCA/Acetone precipitation**

The following procedure is used to precipitate protein from freshly prepared cell lysates. This allows for optimal protein recovery for SDS – PAGE. The acetone used in this procedure is prechilled and stored at -20 °C and kept on ice during the entire procedure.

The following solutions were mixed in a 1:8:1 ratio in the following order (mixing the contents after adding each component): 1 ml cell lysate, 8 ml 100% ice-cold acetone, 1 ml 100% trichloroacetic acid (100% TCA) and incubated at -20 °C for 1 hr. The c precipitation was collected by centrifugation at 11,500 rpm (18,000 x g) for 15 min at 4 °C in a micro centrifuge. The supernatant was discarded and the precipitate was washed with 1 ml ice-cold acetone and recollected by centrifuging at 11,500 rpm for 15 min at 4 °C. The protein pellet was dissolved in the appropriate volume of buffer.

# RESULTS

## Biochemical characterization of Haloarchaeal nucleoid from *Halobacterium salinarium H3*

### 3. Growth phase dependent variation of the nucleic acid and protein composition of the *H. salinarium H3* nucleoid.

**Growth of Halobacterium salinariumH3:**

*H. salinarium* was grown at 37ºC as described in methods section Fig: 1 shows the growth curve of *H. salinarium*. Midlogarithmic phase cells (5 days) and Stationary phase cells (7 days) were used in all studies.

**3.1: Isolation of nucleoid by sucrose density gradient centrifugation**

Centrifugation of lysates obtained from midlogarithmic phase and stationary phase cells on sucrose gradient resulted in resolution of nucleoid into two components, peak I and peak II (rapidly sedimenting fraction). The overall yield of the two peaks showed growth phase dependent variation. Peak I was higher than peak II in midlogarithmic phase (Fig 2A ) whereas peak II was higher than peak I in stationary phase (Fig 2B ) i.e. there was a decrease in peak I in stationary phase as compared to peak I in midlogarithmic phase. Both nucleic acid content and protein content in the two fractions were analyzed. There was reduction of both protein and nucleic acid (DNA) content in peak I obtained from stationary phase cells and there was an increase in both protein and nucleic acid (DNA) contents in peak II obtained from stationary phase cells. Protein and nucleic acid concentrations of nucleoid fractions at various growth phases were presented in table-1

SDS - PAGE was performed to analyze protein present in peak I and peak II of midlogarithmic and stationary phase cells. SDS-PAGE analysis of peak I and peak II nucleoid fractions of exponential phase ( 5 days) cells collected from the gradient showed presence of both high and low molecular weight proteins ranging from 10 KDa to 110 KDa in both the peak fractions of nucleoid (Fig 3A). The top fractions of the gradient are peak I containg high molecular weight proteins and low amount of low molecular weight

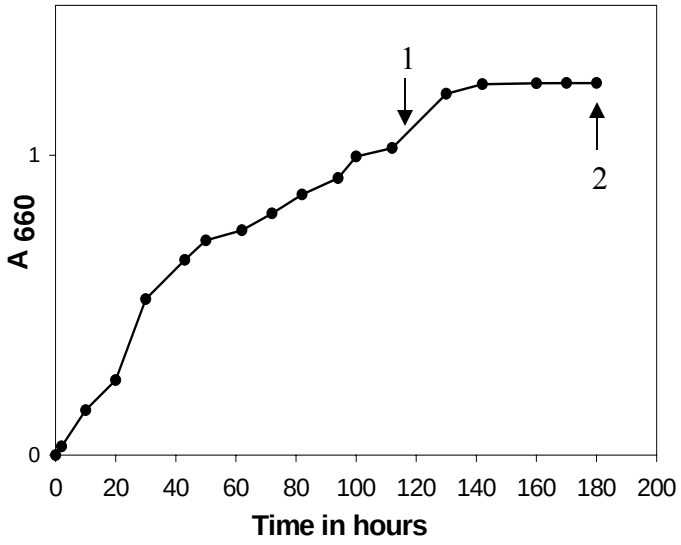



**Fig: 1. Growth curve of Halobacterium salinarium H3.**

The bacteria were grown as described under materials and methods section up to 180 hrs at 37 ºC and absorbance was measured at 660 nm.
1. Midlogarithmic phase
2. Stationery phase

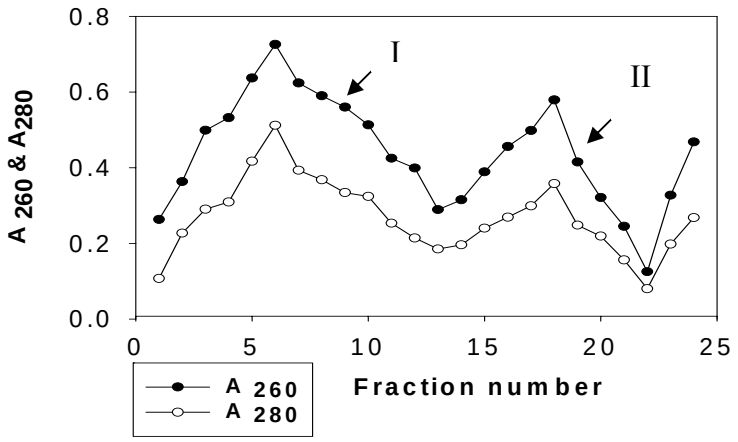



**Fig 2A: 5 – 20 % sucrose density gradient centrifugation profile of chromosomal fractions from midlogarithmic phase cells (5 days culture).**

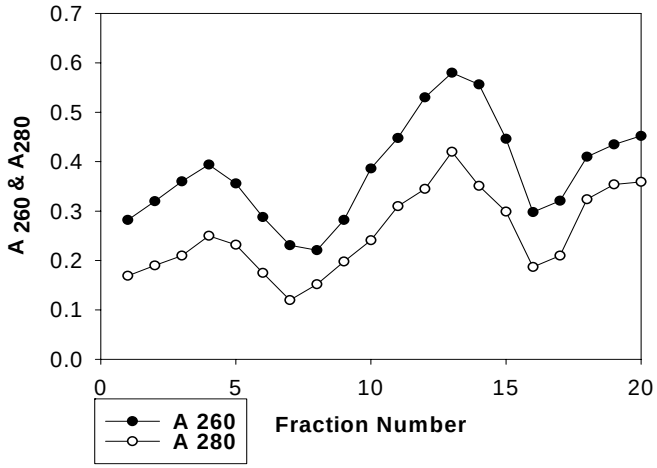



**Fig 2B: 5 – 20 % sucrose density gradient centrifugation profile of chromosomal fractions from Stationary phase cells (7 days culture).**

proteins, (lanes 1 – 8) followed by fractions towards bottom corresponding to peak II which have similar protein banding pattern as seen in peak I, lane 9 – 16.

SDS – PAGE analysis of the nucleoid or chromosomal fractions of peak I and peak II ( Fig 3 B ) obtained from the stationary phase ( 7 days ) cells also showed both high and low molecular weight protein. Top fractions of gradient (peak I) showed proteins in the range of 10 KDa – 110 KDa, (lane 1 – 8), followed by fractions towards bottom are peak II, (lane 9 – 16). Peak I from stationary phase contained fewer proteins in decreased amounts. Peak II fractions from midlogarithmic and stationary phase cells showed similar SDS-PAGE pattern.

Agarose gel analysis of nucleoprotein complexes (Fig. 4A) and deproteinised samples of the gradient fractions of peak I and peak II from midlogarithmic (5 days) phase cells showed thick high molecular weight DNA bands in the initial fractions, lanes 2- 7 (Fig 4A) and lanes 1 -6 (Fig 4B) along with low molecular weight smear of DNA in the both peak I and peak II fractions, Lanes 8 -13 (Fig 4A) lanes 8 – 12 (Fig 4B). The results obtained indicate that peak I has greater amount of high molecular weight DNA than peak II in nucleoid isolated from midlogarithmic phase cells.

As seen in (Fig 5A) nucleo protein complexes and nucleic acid content (Fig 5B) of the gradient peak I fraction from stationary phase cells showed low concentrations of high and low molecular weight DNA bands in the initial fractions (lanes 2 – 7), whereas peak II fractions contained thick higher concentrations of both high and low molecular weight DNA bands (lanes 8 – 12).

Nucleic acid and protein content of chromatin or nucleoid isolated from late stationary phase (10 days) cells by5-20 % sucrose density gradient centrifugation were also estimated. Nucleoid component was not resolved into two peaks. There was almost uniform distribution of U V absorbing material throughout the gradient. SDS - PAGE analysis of fractions obtained by sucrose density gradient centrifugation of chromosomal fractions from late stationary phase cells (Fig. 6B) showed lower amounts of proteins in

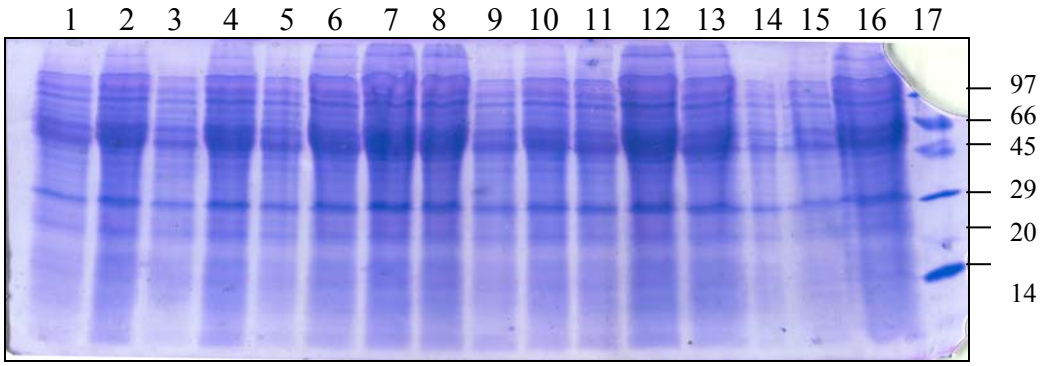



**Fig 3A: SDS-PAGE analysis of fractions obtained from sucrose density gradient centrifugation of chromosomal fractions from midlogarithmic phase cells**

Nucleoid was isolated from exponential phase cells by sucrose density gradient (5 - 20 %) centrifugation of the gently lysed cell lysates. Fractions (100 µl) were TCA precipitated and analyzed on an 18% SDS-Polyacrylamide gel followed by coomassie blue staining.
Lane 1 -8: Peak I fractions 3 to 10 respectively.
Lane 9 -16: Peak II fractions 15 to 20 respectively.
Lane 17: Molecular weight markers

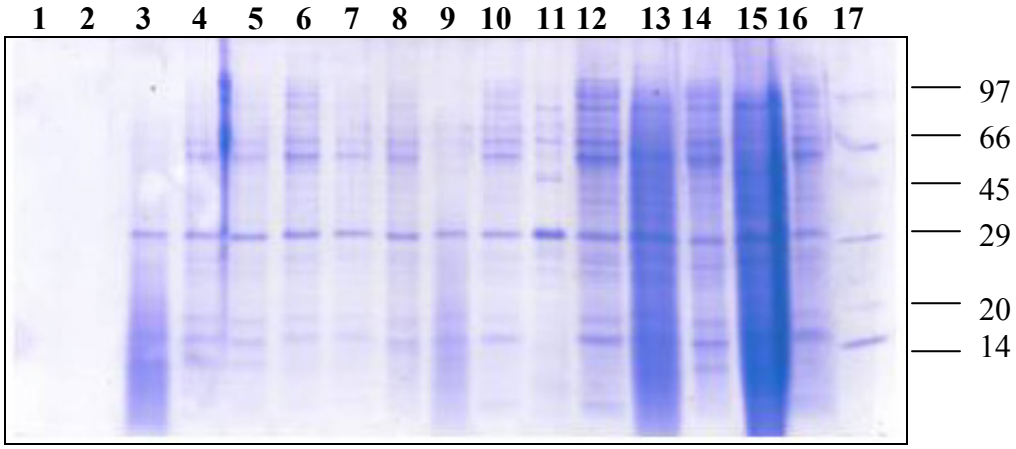



**Fig 3 B: SDS-PAGE analysis of fractions obtained from sucrose density gradient centrifugation of chromosomal fractions from Stationary phage cells**

Nucleoid was isolated from exponential phase cells by sucrose density gradient (5 - 20 %) centrifugation of the gently lysed cell lysates. Fractions (100 µl) were TCA precipitated and analyzed on an 18% SDS-Polyacrylamide gel followed by coomassie blue staining.
Lane 1 -8: Peak I fractions 1 to 8 respectively.
Lane 9 -16: Peak II fractions 11 to 17 respectively
Lane 17: Molecular weight protein marker

**Fig 4: Agarose gel electrophoresis of the nucleoprotein complex and nucleic acids in chromosomal fractions peak I and peak II from midlogarithmic phase cells.**

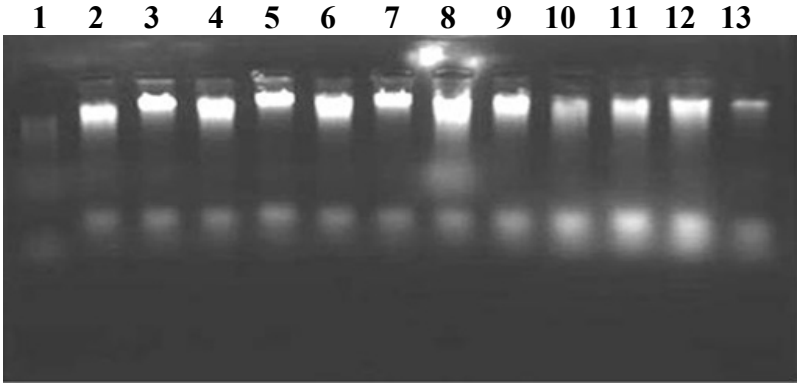



**Fig 4 A: Nucleoprotein complex profile of fractions obtained from sucrose density gradient of *H. salinarium H3* lysates.**

Fractions (50µl) from density gradient centrifugation of nucleoid isolated from the mid logarithmic phase cells were analyzed on a 0.8% agarose gel.
Lane 1: Total peak I and peak II pool fraction.
Lane 2 – 7: Peak I fractions 3 - 8.
Lane 8 -13: Peak II fractions 15 – 20.

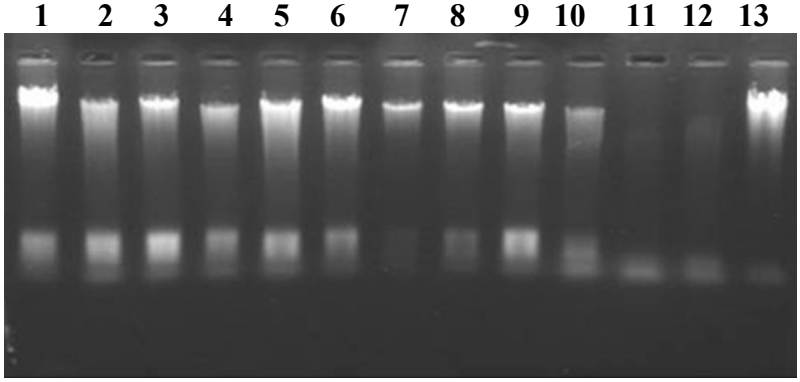



**Fig 4 B: Nucleic acid profile of fractions obtained from sucrose density gradient of nucleoid from *H. salinarium H3* lysates.**

Fractions (50µl) from density gradient centrifugation of nucleoid isolated from the mid logarithmic phase cells were phenolized, nucleic acids were precipitated with ethanol and analyzed on a 0.8% agarose gel.
Lane 1-6: Peak I fractions 3 - 8.
Lane 8 -12: Peak II fractions 15 – 20.
Lane 13: Total DNA of peak I and peak II pool

**Fig 5: Agarose gel electrophoresis of the nucleoprotein complex and nucleic acids in chromosomal peak I and peak II from stationary phase cells.**

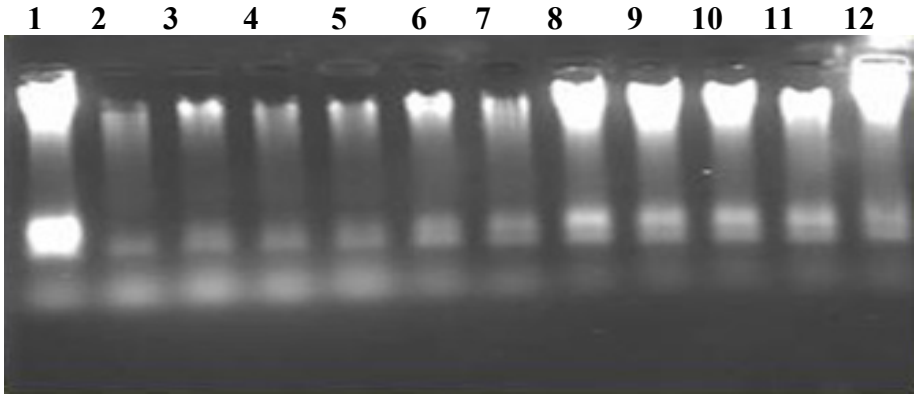



**Fig 5 A: Nucleoprotein complex profile of fractions obtained from sucrose density gradient of nucleoid from *H salinarium H3*.**

Fractions (50µl) from density gradient centrifugation of nucleoid isolated from the stationary phase cells were analyzed on a 0.8% agarose gel.
Lane 1: Total peak I and peak II pool fraction.
Lane 2 – 7: Peak I fractions 2 - 7
Lane 8 -12: Peak II fractions 11 – 15.

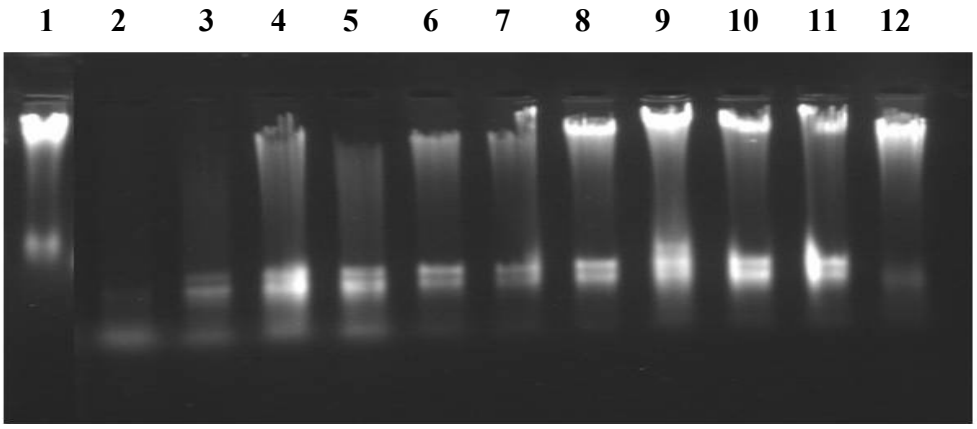



**Fig 5 B: Nucleic acid profile of fractions obtained from sucrose density gradient of nucleoid from *H salinarium H3.***

Fractions (50µl) from density gradient centrifugation of nucleoid isolated from the stationary phase cells were phenolized, nucleic acids were precipitated with ethanol and analyzed on a 0.8% agarose gel.
Lane 1: Total peak I and peak II pool fraction.
Lane 2 – 7: Peak I fractions 2 - 7
Lane 8 -12: Peak II fractions 11 – 15.

**Table 1: Total nucleic acid and protein ratio of Nucleoid from *Halobacterium salinarium* cells at different growth phases.**

| | | Mid logarithmic phase cells | | | Stationary phase cells | | |
|---|---|---|---|---|---|---|---|
| | | DNA mg/ml | Protein mg/ml | P/D ratio | DNA mg/ml | Protein mg/ml | P/D ratio |
| Nucleoid 5 – 20 % Sucrose density gradient | Peak I | 1.09 | 2.3 | 2.1 | 0.8 | 1.4 | 1.75 |
| | Peak II | 0.5 | 0.25 | 0.5 | 1.2 | 2 | 1.67 |
| Nucleoid 30 %Sucrose cushion | | 3.4 | 4.7 | 1.38 | 4.16 | 6.37 | 1.553 |
| S – 1000 Nucleoid | | 1.5 | 2.5 | 1.67 | 2 | 3.4 | 1.7 |

**Fig 6: Sucrose density gradient centrifugation of nucleoid from *H salinarium* cell lysate (10 days cell culture).**

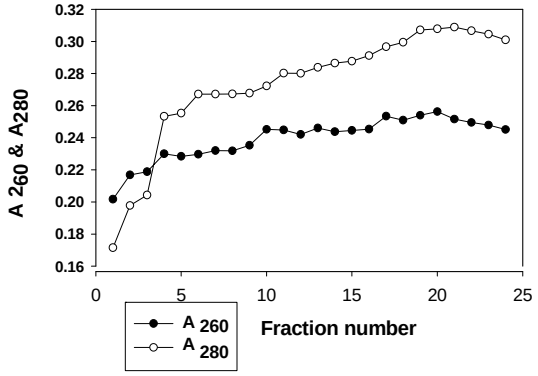



**Fig 6A: 5 – 20 % sucrose density gradient centrifugation profile of chromosomal fractions from late Stationary phage cells**

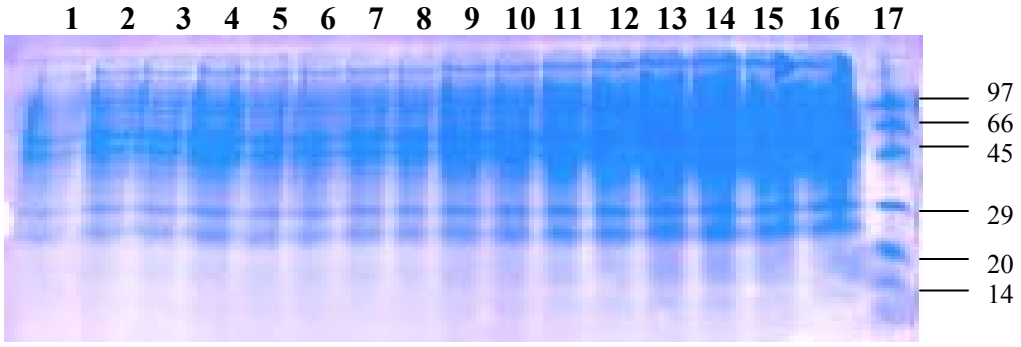



**Fig 6 B: SDS-PAGE analysis of fractions obtained from sucrose density gradient centrifugation of chromosomal fractions from late Stationary phage cells**
Nucleoid was isolated from exponential phase cells by sucrose density gradient (5 - 20 %) centrifugation of the gently lysed cell lysates. Fractions (100 µl) were TCA precipitated and analyzed on an 18% SDS-Polyacrylamide gel followed by coomassie blue staining.

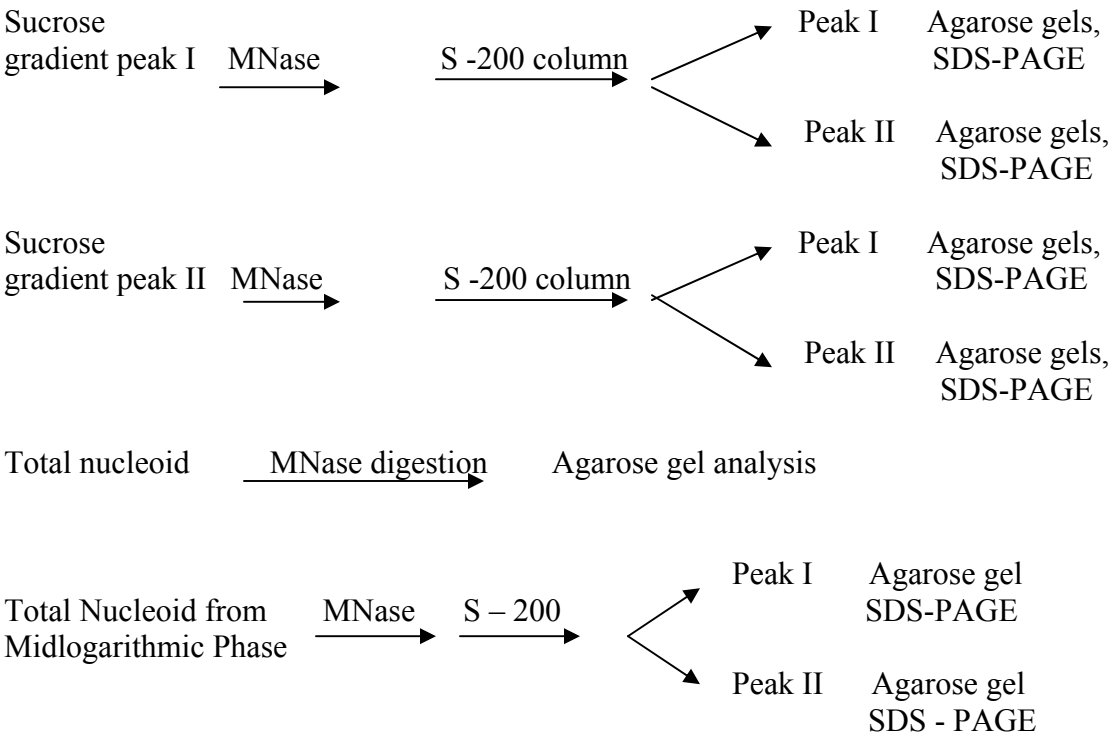
Lane 1 -8: fractions 3-10 respectively
Lane 9 -16: fractions 14 -21 respectively
Lane 17: Protein molecular weight marker.

the initial fractions of the gradient (lanes 1 – 8), followed by higher amounts of both high and low molecular weight proteins in the range of 150 KDa to 10 KDa, (lanes 9 -16) in the bottom fractions.

Agarose gel electrophoresis of the nucleoprotein and nucleic acid content of gradient fractions (Fig 7A, Fig 7 B) showed a smear of heterogenous size DNA throughout the gradient fraction. The later fractions contained high amount of smear type DNA (lanes 7 – 12). Protein and nucleic acid contents are increasing towards the bottom of the gradient. Overall protein content is higher than nucleic acid in the nucleoid from late stationary phase (10 days) cells as indicated by higher absorbance at 280 nm. In all the above cases nucleoid DNA is in fragmented state which could be due to shear form during isolation of the nucleoid.

**Different fractions of nucleoid were analyzed by Micrococcal nuclease digestion.**

1. Sucrose gradient Peak I fraction from stationary phase
2. Sucrose gradient peak II fraction from stationary phase



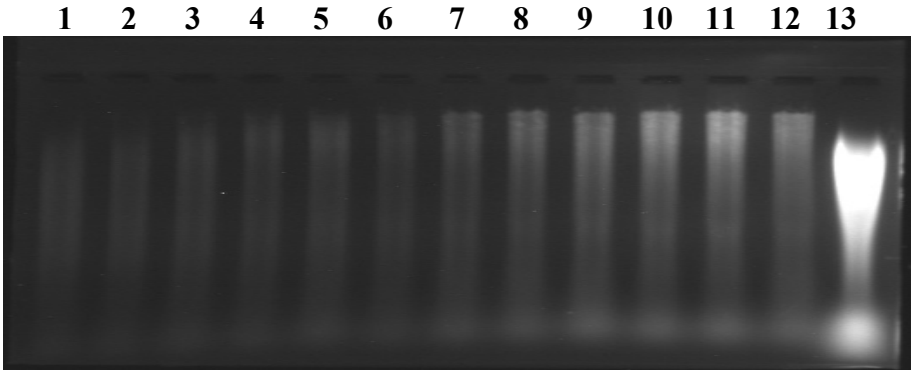



**Fig 7A: Agarose gel nucleoprotein complex profile of fractions obtained from sucrose density gradient of nucleoid from Halobacterium salinarium H3 (late stationary phase, 10 days cell culture).**

Fractions (50µl) from density gradient centrifugation of nucleoid isolated from the late stationary phase cells were analyzed on a 0.8% agarose gel.

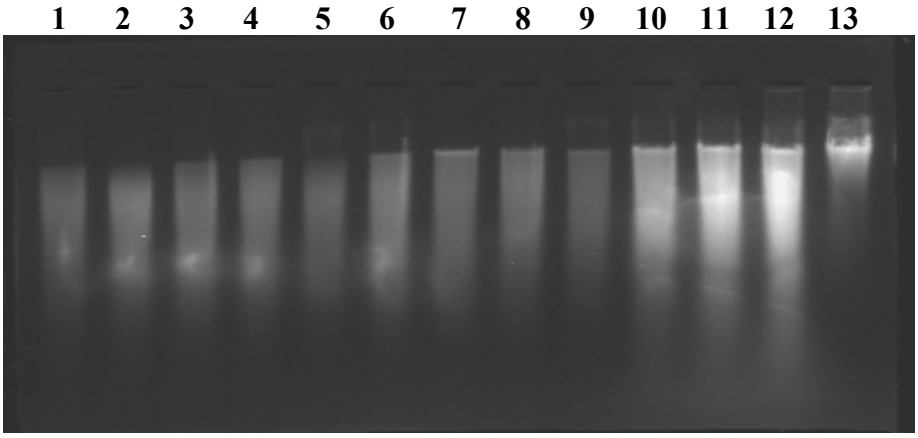
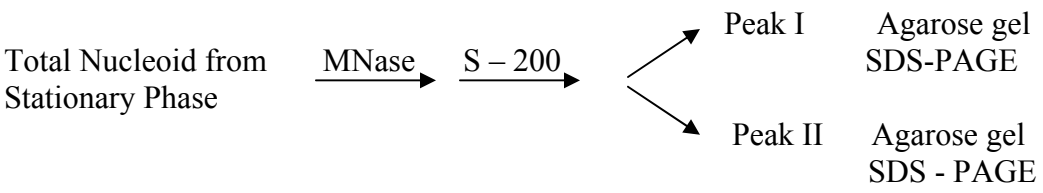



**Fig 7B: Agarose gel nucleic acid profile of fractions obtained from sucrose density gradient of nucleoid from Halobacterium salinarium H3 (10 days cell culture).**

Fractions (50µl) from density gradient centrifugation of nucleoid isolated from the late stationary phase cells were phenolized precipitated with ethanol and analyzed on a 0.8% agarose gel.




**3.1.1: Isolation of MNase digested products of nucleoid by Sephacryl S – 200 column chromatography.**

Peak I and peak II fractions obtained by sucrose density centrifugation of nucleoid from stationary phase cells were individually treated with Micrococcal nuclease. The MNase digested products were chromatographed on Sephacryl S – 200 column.

Sephacryl S- 200 column elution profile of *H. salinarium H3* nucleoid (or) chromosomal peak I fraction digested with micrococcal nuclease from stationary phase was resolved into two peaks , a smaller peak in the initial fractions and a bigger peak in the later fractions (Fig 8 A). SDS – PAGE analysis (Fig 8 B) showed high molecular weight proteins ~ 60 kDa to 45 kDa and a few proteins of ~ 30 kDa to 20 kDa in the peak I fractions (Lanes 4 – 7). The later fractions of S – 200 peak II mainly comprised of abundant amount of ~ 30 kDa proteins and a few other proteins in the molecular weight range of 20 KDa and 14 KDa (lanes 8 – 13).

Agarose gel analysis (Fig 9) showed that S – 200 peak I fraction contained much less DNA than peak II (which contained DNA fragments of 200 bp).

Sucrose gradient chromosomal peak II fraction from stationary phase cells was also digested with micrococcal nuclease and the digest was chromatographed on Sephacryl S - 200. Chromatography resulted in the elution of two distinct peaks. (Fig.10A). SDS-PAGE analysis (Fig 10B) showed high molecular weight proteins ~ 60 kDa to 45 kDa and a few proteins of ~30 kDa to 20 kDa proteins (lanes 4 – 7) in the S – 200 peak I fractions. The later fractions of S – 200 peak II mainly comprised of abundant amount of ~ 30 KDa protein along with a few proteins of 20 KDa and 14 KDa (lanes 8 – 12).

Agarose gel analysis of extracted nucleic acid (Fig. 11) present in the sephacryl S- 200 peak I (lane 4 -7) and peak II (lanes 8 – 11) fractions showed the smear like

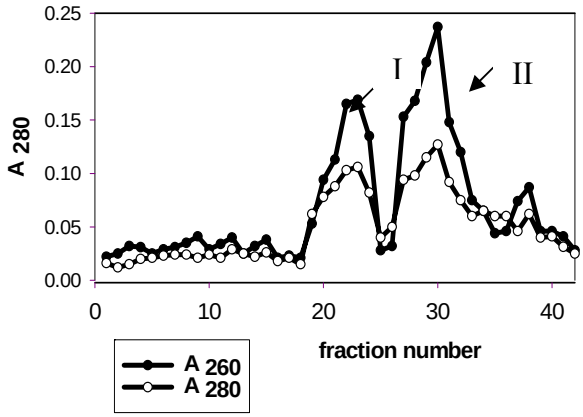



**Fig 8 A: Sephacryl S- 200 column elution profile of *H salinarium H3* gradient chromosomal peak I fraction digested with micrococcal nuclease from stationary phase (7 days cell culture).**

Absorbance was measured at 260 and 280 nm of fractions obtained from sephacryl S-200 column chromatography of chromosomal peak I fraction obtained through sucrose density centrifugation ( 5-20 %) from cells harvested at stationary phase and digested with micrococcal nuclease was measured

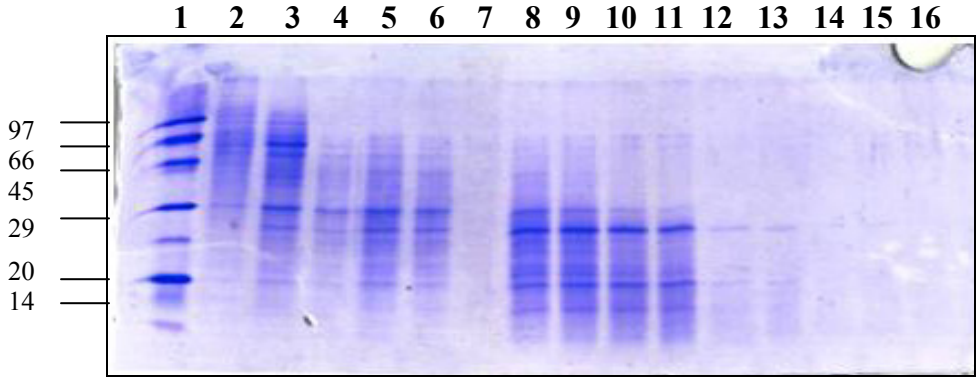



**Fig 8 B: SDS – PAGE analysis of fractions obtained from Sephacryl S-200 column chromatography of chromosomal peak I fraction digested with micrococcal nuclease.**

Chromosomal peak I fraction obtained through sucrose density centrifugation (5-20 %) was digested with micrococcal nuclease and chromatographed on S-200 column. Fractions 60 µl were analyzed on 18% SDS- Polyacrylamide

Lane 1: protein marker

Lane2 and 3: fractions 6 and 19

Lane 4 -7: Peak I fractions 20, 21, 22 and 24 respectively

Lane 8 – 13: Peak II fractions 27, 29, 30, 32, 33 and 35 respectively

Lane 14 – 16: fractions 37, 38 and 40 respectively.

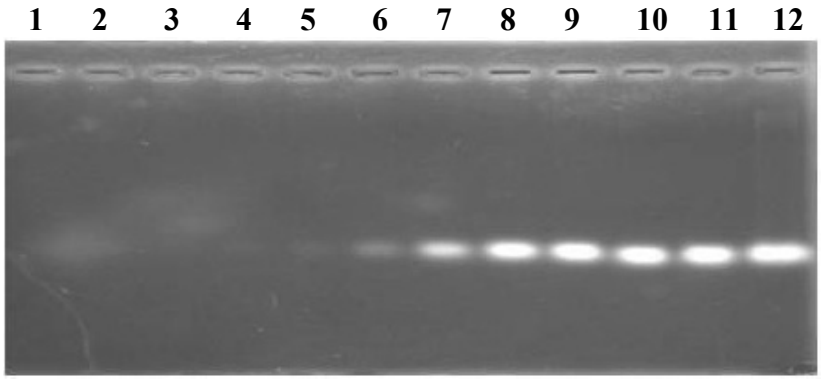



**Fig 9: Nucleic acid profile Peak I and peak II Fractions obtained from S-200 column chromatography of MNase digested chromosomal peak I fraction which is obtained by sucrose density gradient centrifugation.**

Fractions (60 µl) from the sephacryl S-200 column chromatography of MNase digested chromosomal peak I fraction from stationary phase cells were phenolized precipitated with ethanol and analyzed on 0.8% agarose gel.
Lane 1-3: fractions 4, 8 and 16 respectively
Lane 4 - 7: peak I fractions 20 21, 22 and 24 respectively
Lane 8 -11: Peak II fractions 29, 30, 32 and 33 respectively
Lane 12: Total nucleic acid extracted from peak I pool fraction.

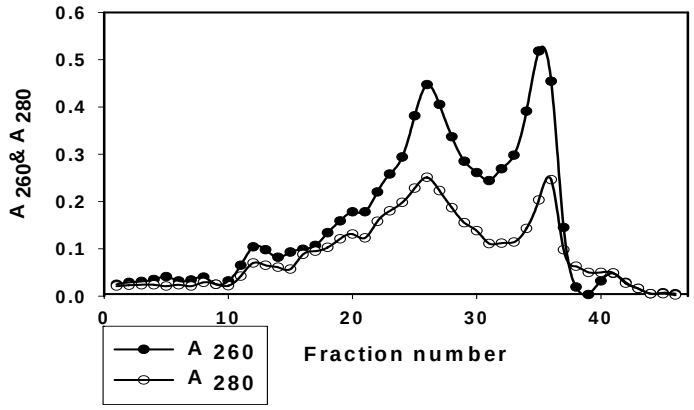



**Fig 10 A: Sephacryl S- 200 column elution profile of *H. salinarium H3* chromosomal peak II fraction digested with micrococcal nuclease from stationary phase( 7 days cell culture).**

Absorbance was measured at 260 and 280 nm of fractions obtained from sephacryl S-200 column chromatography of nucleoid or chromosomal peak II fraction obtained through sucrose density centrifugation ( 5-20 %) from cells harvested at stationary phase and digested with micrococcal nuclease.

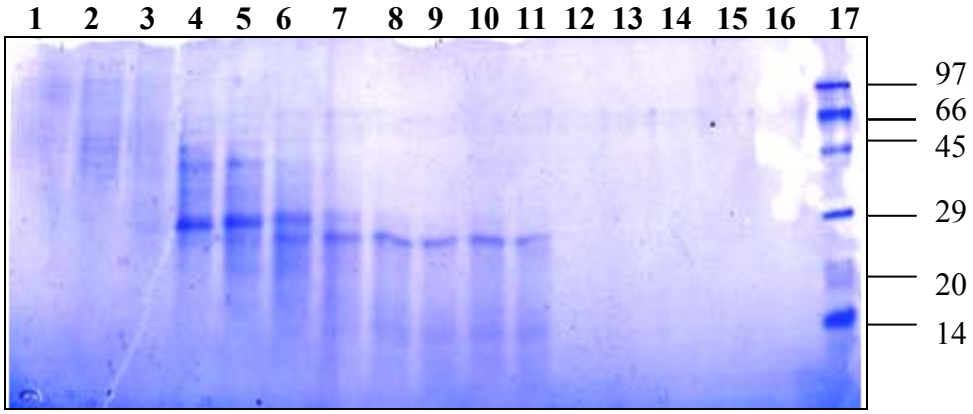



**Fig 10 B: SDS – PAGE analysis of fractions obtained from Sephacryl S-200 column chromatography of chromosomal peak II fraction digested with micrococcal nuclease.**

Chromosomal peak I fraction obtained through sucrose density centrifugation (5-20 %) was digested with micrococcal nuclease and chromatographed on S-200 column.
Fractions 60 µl were analyzed on 18% SDS- Polyacrylamide.
Lane1-3: fractions 5, 10 and 15 respectively.
Lane 4 -7: Fractions 22, 24, 26 and 28 respectively.
Lane 8 - 12: fractions 35, 36, 37 and 38 respectively
Lane 13-16: fractions 40 to 43 respectively.
Lane 17: Protein molecular weight marker.

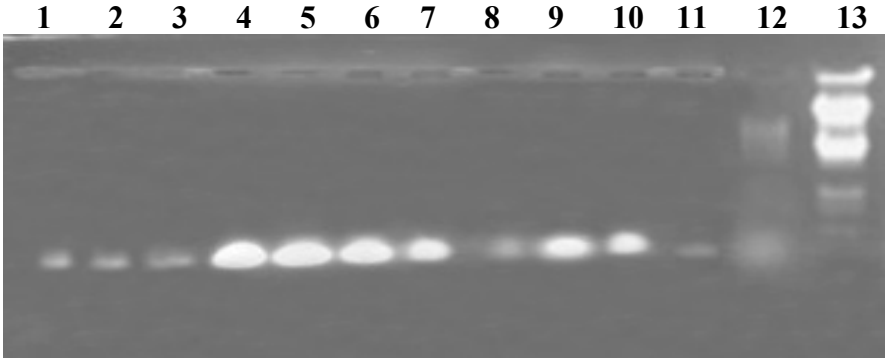



**Fig11: Nucleic profile of Peak I and peak II Fractions obtained from S-200 column chromatography of MNase digested chromosomal peak II fraction which is obtained by sucrose density gradient centrifugation.**

Fractions (60 µl) from the sephacryl S-200 column chromatography of MNase digested chromosomal peak II fraction from stationary phase cells were phenolized precipitated with ethanol and analyzed on 0.8% agarose gel.
Lane1-3: fractions 5, 10 and 15 respectively.
Lane 4 -7: Fractions 22, 24, 26 and 28 respectively
Lane 8 – 11: fractions 35, 36, 37 and 38 respectively
Lane 12: Total Nucleic acid extracted from Peak II pool fraction
Lane 13: Lambda DNA Hind III/Eco I digest.

nucleoprotein aggregates and DNA fragments of the size of 200 bp migrating towards the bottom of the gel.

### 3.1.2: Micrococcal nuclease digestion of total nucleoid and analysis of the products

Micrococcal nuclease digestion of 30 % sucrose cushion isolated total nucleoid from both midlogarithmic phase and stationary phase cells was carried out and digestion products were analyzed by agarose gel electrophoresis. Agarose gel analysis of MNase digested products of nucleoid from 5 days cells (Fig 12A) showed smear like DNA bands with decreasing size with increasing incubation time (lanes 2 -7). After deproteinisation of MNase digested products, DNA bands of approximately 200 bp were observed in all fractions (Fig 12B lanes 2- 7). MNase digested products of nucleoid from stationary phase cells also showed (Fig 13A and 13 B) similar results as in midlogarithmic phase cells.

### Sephacryl S -200 column chromatography of MNase digested products of total nucleoid

Sephacryl S -200 column chromatography of MNase digested products of total nucleoid isolated by ultra centrifugation using 30 % sucrose cushion and further purified by centrifugation through 20% sucrose cushion from midlogarithmic phase (Fig14 A) and stationary phase (Fig 16A) cells also resolved into two peaks (peak I and peak II). Protein and DNA were analyzed by SDS – PAGE and agarose gel electrophoresis respectively. Similar results were obtained as described before for MNase digestion of peak I and Peak II chromosomal fraction isolated by sucrose gradient centrifugation.

SDS –PAGE analysis of peak I fractions obtained from sephacryl S-200 column chromatography of total nucleoid digested with MNase showed ( Fig 14 B and Fig 16B ) high molecular weight proteins in the range of ~100 kDa to ~ 35 kDa and a few low molecular weight proteins. In peak II fractions few high molecular weight proteins~ 60 KDa to 43 KDa and several low molecular weight proteins in the range of 30 kDa to 14 kDa are present. 30KDa protein is present predominantly in all fractions of peak I and peak II. High concentration of 30 KDa protein was observed in peak II fractions of

nucleoid in stationary phase cells (Fig 16B). Peak I in both the cases showed relatively higher molecular weight proteins in greater amounts than peak II.

Nucleoprotein complexes in the column fraction from peak I were analyzed by 0.8% agarose gel electrophoresis. Peak I fractions contained high molecular weight aggregates migrating towards the middle of the gel ((Fig 15A and Fig 17A lanes 3 -8), along with 200 bp length small nucleoprotein bands in all fractions peak I. However peak II fractions showed only a ~ 200 bp length DNA bands in lesser amounts (lanes 9 – 13).

RNase treated nucleic acid extracts from peak I and peak II were also analyzed by 0.8% agarose gel electrophoresis (Fig 15 B and Fig 17 B) . In initial fractions of peak I (lane 3 – 8), the DNA migrated as a smear of 2kbp ~ 4 kbp and ~200 bp length DNA bands. DNA in later fraction migrated towards the bottom. Peak II fractions contained mostly (lanes 9 – 12) ~ 200 bp length DNA.

In all the cases peak I fractions of S – 200 column contained heterogenous higher molecular weight DNA and peak II fractions has low molecular weight DNA. S – 200 peak I and peak II fractions showed difference in the protein profile as visualized by SDS – PAGE. These results obtained by MNase digestion of nucleoid fraction from Halobacterial cells suggest that the nucleoid contained different region of DNA bound by different set of proteins with no nucleosomal organization.

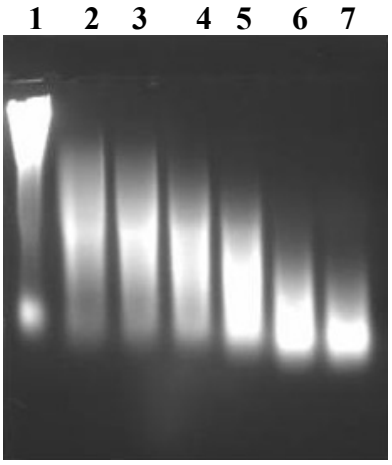



**Fig12A: Agarose gel electrophoresis of micrococcal nuclease digest of *H.salinarium* total nucleoid from midlogarithmic phase cells (5 days).**
Nucleoid (10 µg) was digested with 1 U of MNase and nucleoprotein complexes were analyzed on 0.8% agarose gel as described.
Lane 1: Nucleoid (control)
Lane 2 – 7: 10 µg of nucleoid incubated with 1 U of MNase (0.1 U/ 1µg DNA) at 3 **7**ºC for 1 mins, 3, 5, 10, 20, 30 mins respectively

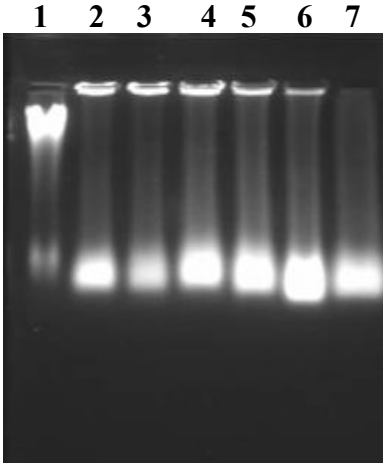



**Fig12 B: Micrococcal nuclease digestion on *H. salinarium* total nucleoid from midlogarithmic phase cells (5 days) and analysis of extracted nucleic acid by agarose gel electrophoresis.**
Nucleoid (10 µg) was digested with 1 U of MNase and deproteinised nucleic acids were analyzed on 0.8% agarose gel as described.
Lane 1: Nucleoid (control)
Lane 2 – 7: Phenolized Nucleic acid extracts of MNase digested Nucleoid samples at 37ºc for 1 mins.3, 5 10, 20, 30 mins respectively.

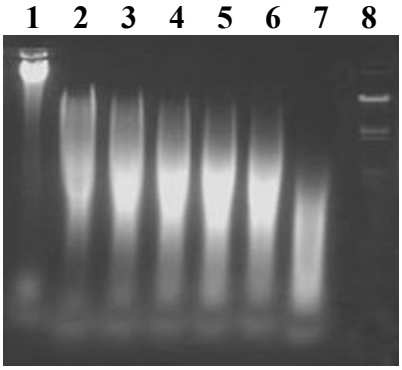



**Fig13A: Agarose gel electrophoresis of Micrococcal nuclease digestion of *H. salinarium* total nucleoid from stationary phase cells (7 days).**
Nucleoid (10 µg) was digested with 1 U of MNase and nucleoprotein complexes were analyzed on 0.8% agarose gel as described
Lane 1: Nucleoid (control)
Lane 2 – 7: 10 µg of nucleoid incubated with 1 U of MNase (0.1 U/ 1µg DNA) at 37 ºC for 1 mins, 3, 5, 10, 20, 30 mins respectively.
Lane 8: Lambdas DNA with Hind III digest.

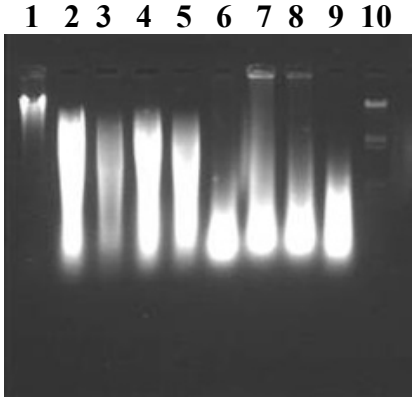



**Fig13 B: Effect of Micrococcal nuclease digestion on *H. salinarium* total nucleoid from stationary phase cells (7 days).**
Nucleoid (10 µg) was digested with 1 U of MNase and deproteinised nucleic acids were analyzed on 0.8% agarose gel as described.
Lane 1: Nucleoid (control)
Lane 2 – 9: Phenolized Nucleic acid extracts of MNase digested
Nucleoid samples at 37ºc for 1 mins.3, 5 10, 20, 30, 45, 60mins respectively.
Lane 10: Lambdas DNA with Hind III digest

**Fig 14: Sephacryl S-200 column chromatography of MNase digested products of nucleoid isolated by ultra centrifugation using 30 % sucrose cushion from midlogarithmic phase cells.**

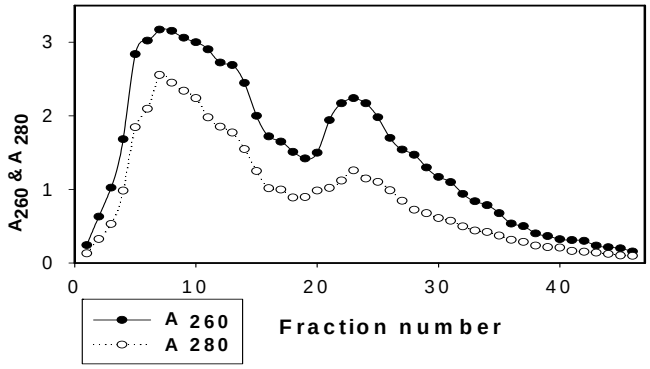



**Fig 14 A: Sephacryl S-200 column elution profile of *Halobacterium salinarium* nucleoid digested with micrococcal nuclease at midlogarithmic phase.**

Absorption at 260 and 280 nm of fractions obtained from the sephacryl S-200 column chromatography of nucleoid isolated from cells harvested at mid logarithmic phase and digested with micrococcal nuclease was measured.

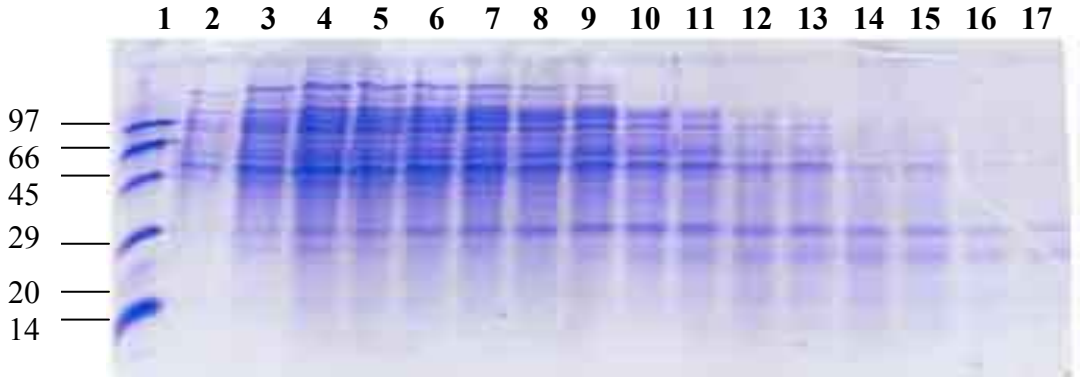



**Fig 14 B: SDS – PAGE analysis of fractions obtained from sephacryl S-200 column chromatography of nucleoid digested with micrococcal nuclease.**

Nucleoid isolated from midlogarithmic phase cells was digested with micrococcal nuclease and chromatographed on a sephacryl S-200 column. Fractions (60µl) were analyzed on an 18% SDS –Polyacrylamide gel followed by coomassie blue staining.
Lane 1: Protein molecular weight marker.
Lane 2-9: fraction numbers 7 – 14 respectively
Lane 10-17: fraction numbers 21 – 28 respectively.

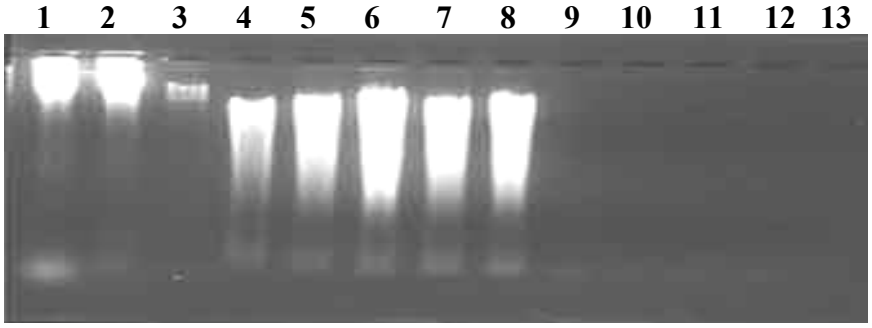



**Fig 15A: Nucleoprotein complex profile of Peak I and peak II Fractions obtained from S-200 column chromatography of MNase digested total nucleoid from midlogarithmic phase cells obtained by ultra centrifugation using 30% sucrose cushion.**

Fractions (50 µl) from the sephacryl S- 200 column chromatography of nucleoid digested with MNase were analyzed on a 0.8% agarose gel.
Lane 1: Total P I and P II pool fraction
Lane 2: nucleoid from mid log phase cells.
Lane 3 – 8: fractions 7 – 12 respectively.
Lane 9 – 13: fractions 21 -25 respectively.

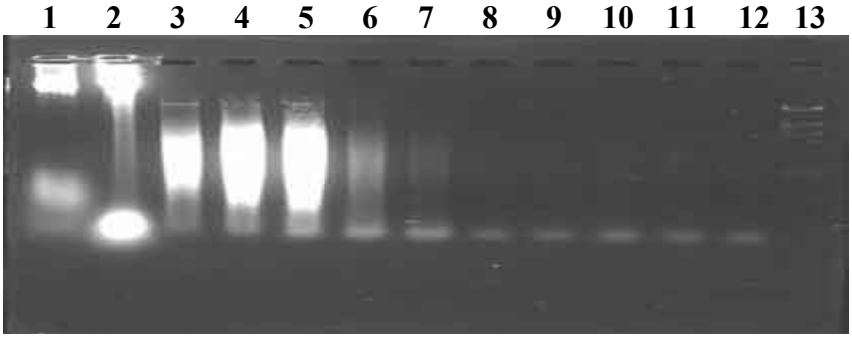



**Fig 15B: Nucleic acid profile of Peak I and peak II Fractions obtained from S-200 column chromatography of MNase digested total nucleoid from midlogarithmic phase cells obtained by ultra centrifugation using 30% sucrose cushion.**

Fractions (50 µl) from the sephacryl S- 200 column chromatography of MNase digested nucleoid isolated from the midlogarithmic phase cells were phenolized precipitated with ethanol and analyzed on 0.8% agarose gel analyze on a 0.8% agarose gel
Lane 1: Total Nucleic acid from PI and p II pool fraction
Lane 2: Direct nucleoid (control)
Lane 3 –8: fractions 7 – 12 respectively.
Lane 9 – 12: fractions 21 – 24 respectively.
Lane 13: Lambda Hind III digest

**Fig 16: Sephacryl S-200 column chromatography of MNase digested products of nucleoid isolated by ultra centrifugation using 30 % sucrose cushion from stationary phase cells.**

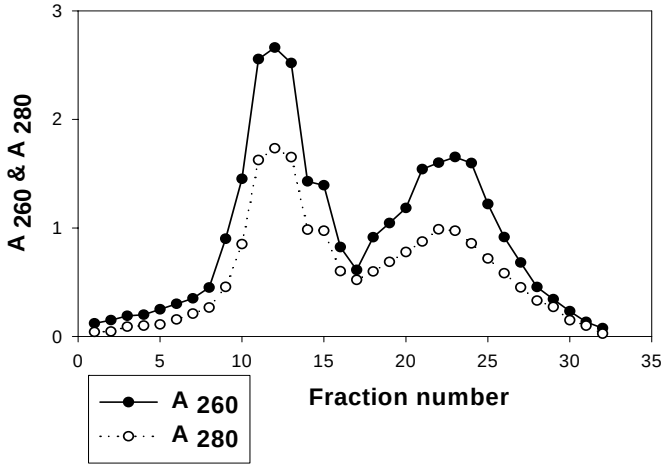



**Fig 16 A: Sephacryl S-200 column elution profile of *Halobacterium salinarium* total nucleoid digested with micrococcal nuclease at stationary phase.**

Absorption at 260 and 280 nm of fractions obtained from the sephacryl S-200 column chromatography of nucleoid isolated from cells harvested at stationary phase and digested with micrococcal nuclease was measured.

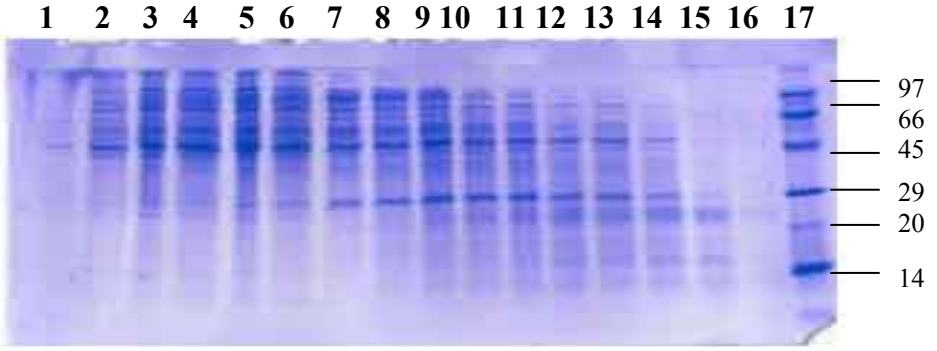



**Fig 16 B: SDS – PAGE analysis of fractions obtained from sephacryl S-200 column chromatography of total nucleoid digested with micrococcal nuclease.**

Nucleoid isolated from midlogarithmic phase cells was digested with micrococcal nuclease and chromatographed on a sephacryl S-200 column. Fractions (60µl) were analyzed on an 18% SDS –Polyacrylamide gel followed by coomassie blue staining.
Lane 1-8: Fraction number 8 – 15 respectively.
Lane 9 -16: fraction number 19 – 26 respectively
.Lane 17: Protein molecular weight marker.

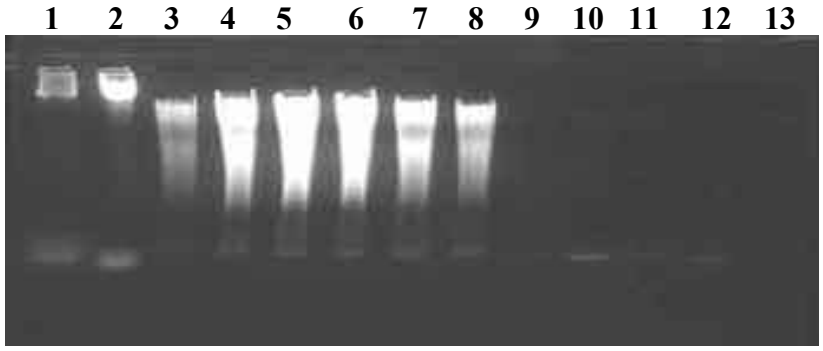



**Fig 17A: Nucleoprotein complex profile of Peak I and peak II Fractions obtained from S-200 column chromatography of MNase digested total nucleoid from stationary phase cells obtained by ultra centrifugation using 30% sucrose cushion.**
Fractions (50 µl) from the sephacryl S- 200 column chromatography of nucleoid isolated from the stationary phase cells and digested with MNase were analyze on a 0.8% agarose gel.
Lane 1: Total peak I and peak II of S – 200 pool fraction.
Lane 2: Total nucleoid of stationary phase cells.
Lane 3 – 8: fractions 10 – 15 respectively.
Lane 9 – 13: fractions 20 - 24 respectively.

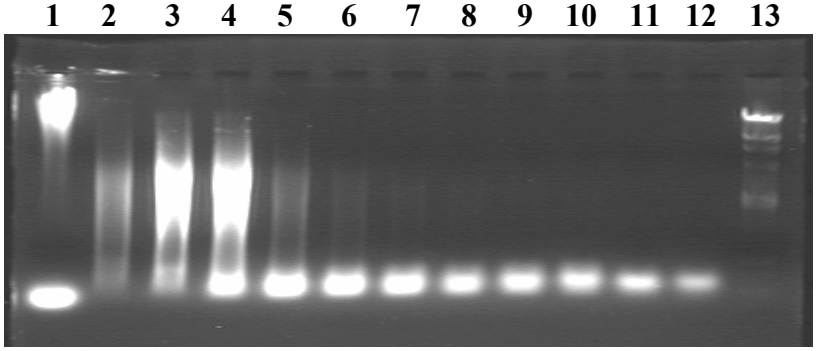



**Fig 17B: Nucleic acid profile of Peak I and peak II Fractions obtained from S-200 column chromatography of MNase digested total nucleoid from stationary phase cells obtained by ultra centrifugation using 30% sucrose cushion.**

Fractions (50 µl) from the sephacryl S- 200 column chromatography of MNase digested nucleoid isolated from the stationary phase cells were phenolized precipitated with ethanol and analyzed on 0.8% agarose gel analyze on a 0.8% agarose gel
Lane 1: Total nucleic acid of S-200 pool.
Lane2 – 7: Fractions 10 – 15 respectively
Lane 8-12: fractions 20 – 24 respectively
Lane 13: Lambda DNA Hind III digest.

## 3. 2: Purification of 30 KDa nucleoid protein and characterization of its DNA binding properties

A 30 kDa protein was present along with DNA in all the nucleoid fractions isolated. This protein was purified to homogeneity and characterized.

**Isolation of nucleoid**

Nucleoid was isolated from freshly harvested midlogarithmic phase cells using non ionic detergents by centrifugation through a 30 % sucrose cushion. Freshly harvested cells (4 g) were suspended in 12 ml of nucleoid buffer containing 20 mM Tris-HCl pH 7.6, 0.5 M NaCl, 3.4 M KCl, 0.1 M Mg-Ac, 1 mM $Na_2$ – EDTA, 7 mM 2- mercaptoethanol and 1.5 ml of lysis mix was added and kept for 1 hr at 4˚C by constant stirring (Lysis mix – NP – 40, 1 % Triton – X –100 and 0.1 % sodium Deoxy-cholate). Lysate was centrifuged at 1000 rpm/20 mins at 4˚C. The supernatant was collected and loaded onto 30 % sucrose cushion buffer pH 7 and centrifuged at 10,000 rpm/1 hr/ 4˚C. The nucleoid pellet was collected and resuspended in nucleoid buffer, again loaded on 20% sucrose cushion and centrifuged at 30,000 rpm/ 1hr in 70 Ti rotor. The nucleoid pellet was collected after second centrifugation and protein and nucleic acid contents were measured.

### 3.2.1: Purification of nucleoid by Sephacryl S- 1000 column chromatography:

The nucleoid isolated on 30 % sucrose cushion was sheared by sonication and chromatographed on a sephacryl S – 1000 column, and the column was eluted with reverse salt gradient range between 3 M – 0.05 M potassium chloride. As seen in fig.18 nucleoprotein complexes were resolved into a broad single peak (Fig.18) Absorption spectra analysis indicates a higher protein to DNA ratio. Amount of protein, DNA, RNA present in S – 1000 nucleoid was in the ratio of 1: 0.6: 0.048 (Fig.19A, 19B and 19C) Total nucleoid has 2.5 mg of protein, 1.5 mg of DNA and 120 µg of RNA per ml of fraction. SDS – PAGE electrophoresis of peak fractions showed (Fig. 20) presence of protein in molecular weight range from 60 kDa to 10 kDa.

**Fig 18: Purification of nucleoid by sephacryl S -1000 column chromatography**

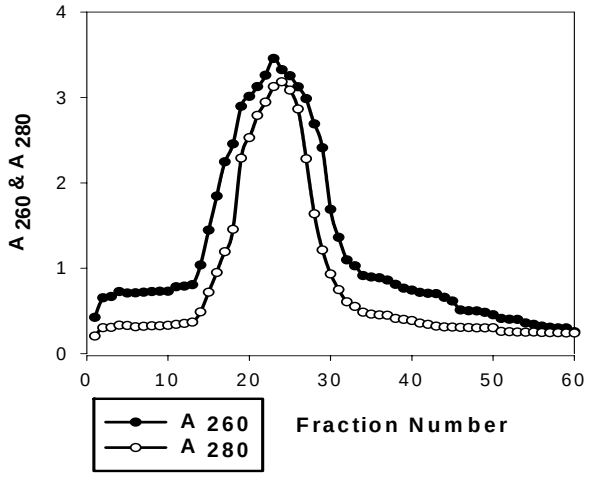



**Fig18: Sephacryl S- 1000 column elution profile of *H. salinarium* nucleoid isolated from midlogarithmic phase cells. Total nucleoid was chromatographed on S – 1000 column. The column was eluted with reverse salt gradient (see methods). Fraction was analyzed for absorbance at 260 nm and 280 nm.**

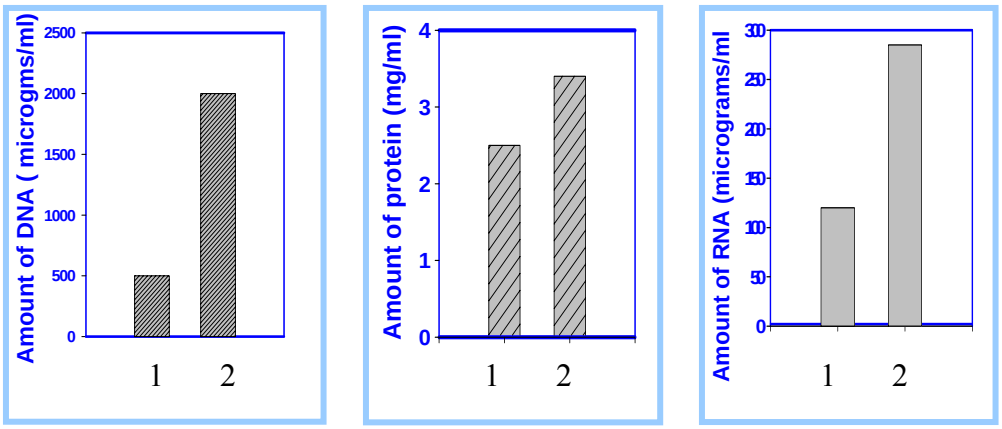



**Fig 19: DNA, Protein and RNA content of the peak pool fraction eluted from sephacryl S-1000 column chromatography of nucleoid from midlogarithmic and stationary phase cells**

A. DNA content of midlogarithmic phase (1) and stationary phase (2) nucleoid
B. Protein content of midlogarithmic phase (1) and stationary phase (2) nucleoid.
C. RNA content of midlogarithmic phase (1) and stationary phase (2) nucleoid.

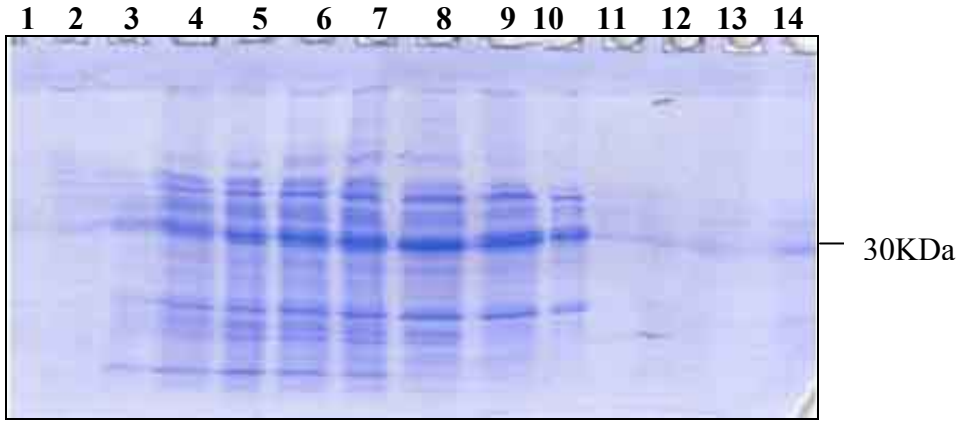



**Fig 20: SDS – PAGE analysis of fractions obtained from sephacryl S-1000 column chromatography of nucleoid.**

Nucleoid isolated from midlogarithmic phase cells was chromatographed on a sephacryl S-1000 column. Fractions (200 µl) were TCA precipitated and analyzed on an 18% SDS-Polyacrylamide gel followed by coomassie blue staining.
Lane 1- 14: Fraction number 18 – 31 respectively.

Nucleoprotein complexes from S – 1000 column nucleoid fractions were analyzed by agarose gel electrophoresis. Heterogenous DNA fragment of size of about 300 bp aggregates were observed (fig. 21 A). Agarose gel analysis of RNase treatment did not result in any change in the mobility of DNA. However total nucleoid (Fig. 21A lane 13) contained a faster migrating band which could be RNA since this band disappeared after RNase digestion (Fig 21 B lane 13). RNA might have eluted in later fraction of S – 1000 column chromatography. S-1000 nucleoid was also isolated from the stationary phase cells. Protein and nucleic acid contents were analyzed as before. S-1000 nucleoid of stationary phase cells contained 2 mg of DNA, 3.4 mg of protein and 285 µg of RNA per ml of peak fraction. Nucleic acids and protein concentrations are higher than the S – 1000 nucleoid of midlogarithmic phase cells. The 30 KDa nucleoid protein was dominantly present in all fractions.

### 3.2.2: DE -52 column chromatography of Sephacryl S-1000 nucleoid proteins

Sephacryl S – 1000 nucleoid pool fraction was desalted by dialysis and chromatographed on DE – 52 anionic exchange column. Elution was carried out with linear increasing salt gradient from 0.05 M – 0.75 M NaCl (Fig 22 A). SDS –PAGE analysis of column peak fractions showed (Fig. 22 B) elution of the 30 kDa Protein at 0.5 M NaCl concentration. The protein (HS30 K) was purified by chromatography on a second DE – 52 column after DNase treatment. Purified 30 kDa protein was again eluted (Fig 23 A) at 0.5 M NaCl during the 0.05 M to 0.75 M NaCl linear salt gradient elution.

## 3.3: Characterization of HS30K protein

### 3.3.1: Molecular weight determination of HS30K nucleoid protein

Molecular weight determination of *H. salinarium* nucleoid protein (HS30 K) was carried out by electrophoresing on 18 % polyacrylamide gel in the presence of SDS as described by Thomas and Kornberg (1974). SDS – PAGE analysis of the purified 30 kDa proteins showed (Fig 23 B) a single band in the gel (lanes 22 – 28). HS30K protein migration corresponded to a molecular weight of 30 kDa. However the estimation of molecular weight could be on the higher side. This could be due to anomalous migration of the protein in SDS gels, because Mass spectrum of the intact protein showed a molecular

**Fig 21: Agarose gel electrophoresis of the nucleoprotein complex, nucleic acids DNA and RNA of S – 1000 nucleoid fractions.**

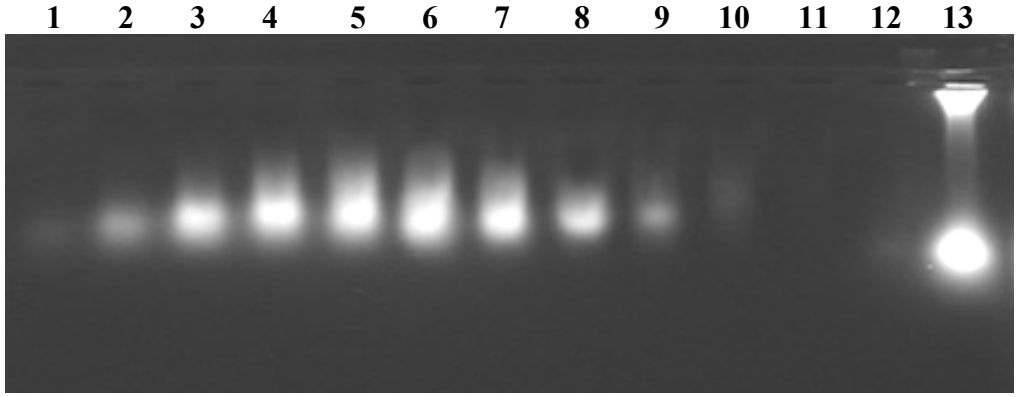



**Fig 21A: Nucleoprotein complex profile of nucleoid chromatographed on sephacryl S – 1000 column.**
Fractions (60µl) from the sephacryl S -1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells were analyzed on a 0.8% agarose gel.
Lane 1- 12: S-1000 peak fractions 20-31 respectively.
Lane 13: Total nucleoid.

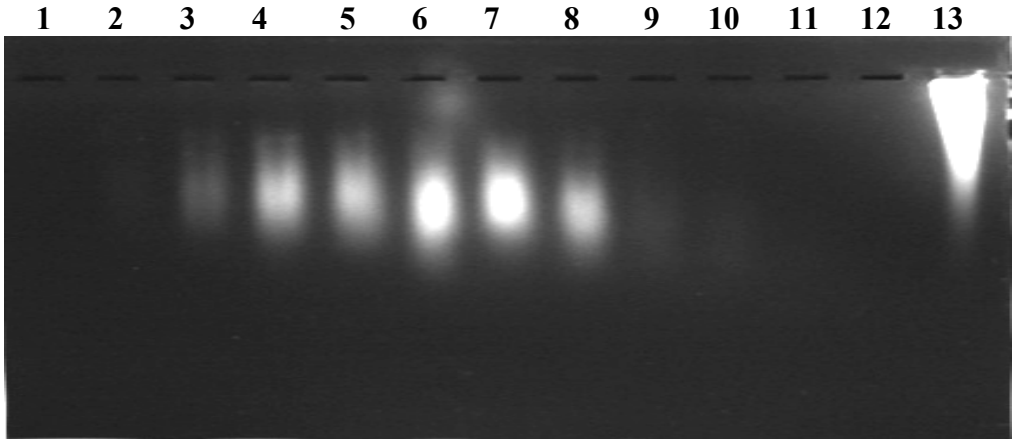



**Fig 21 B: Nucleic acid profile of nucleoid chromatographed on sephacryl S – 1000 nucleoid**
Fractions (60µl) from sephacryl S -1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells were phenolized and precipitated with ethanol and analyzed on a 0.8% agarose gel.
Lane 1-12: S-1000 peak fractions 20-31 respectively.
Lane 13: Total nucleic acid extracted from nucleoid

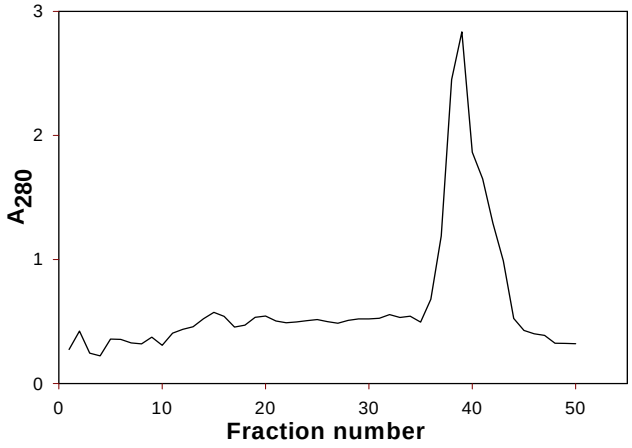



**Fig 22A: DEAE – cellulose column elution profile of S-1000 nucleoid proteins.**
S – 1000 nucleoid proteins were chromatographed on a 10 ml DE-52 cellulose column as described in materials and methods. The fractions collected were checked for absorbance at 280 nm.

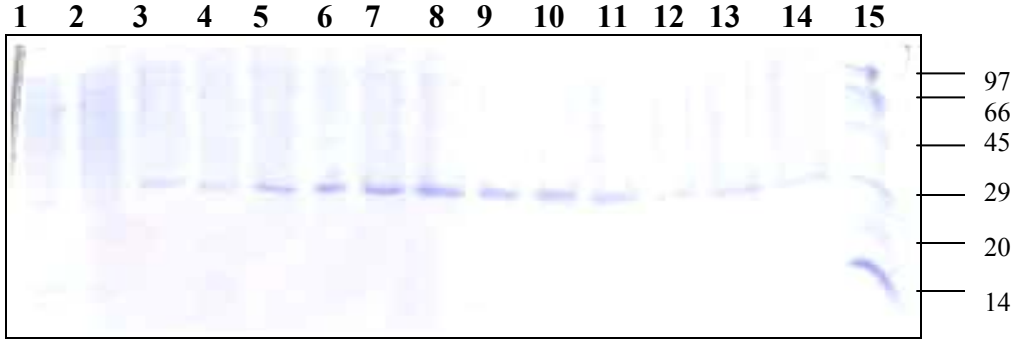



**Fig 22 B: SDS-PAGE analysis of fractions from DEAE – cellulose column chromatography of nucleoid protein on an 18% gel**
S-1000 nucleoid pool fraction was chromatographed on DEAE – cellulose column, 1 ml fractions were collected and peak fractions (50µl) analyzed on 18 % SDS Polyacrylamide gels and visualized by coomassie blue staining.
Lane 1-14: Fractions 35 – 48 respectively.

**Fig 23: DEAE – cellulose column chromatography of DNase I digestion of nucleoid protein**

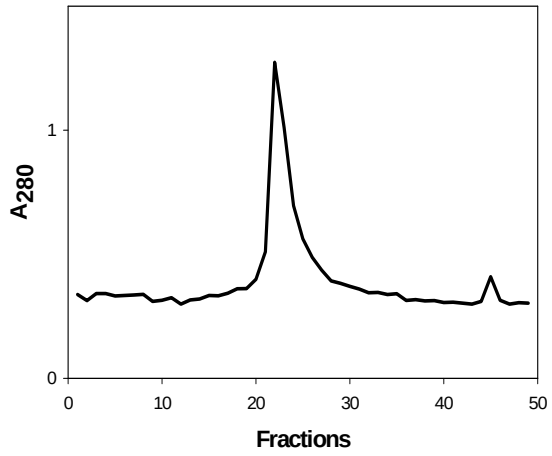



**Fig 23A: DE-52 cellulose column elution profile of DNase I digestion of DEAE – cellulose nucleoid fraction**

DNase I digested DEAE- cellulose peak pool fraction was again chromatographed on to the DEAE cellulose column. 1 ml fractions were collected, peak fractions (50µl) analyzed on 18% SDS polyacrylamide gels and visualized by coomassie blue staining.

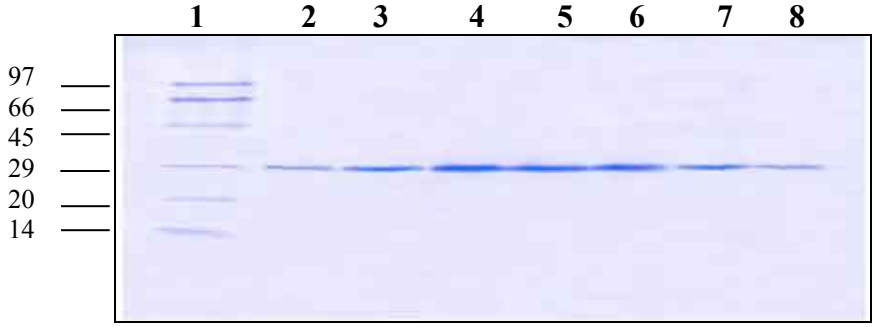



**Fig 23B: SDS-PAGE analysis of fractions from DEAE – cellulose column chromatography of nucleoid protein on an 18% gel.**

Dnase I digested DEAE- cellulose peak pool fraction was again chromatographed on DEAE – cellulose column, 1 ml fractions were collected and peak fractions (50µl) analyzed on 18 % SDS Polyacrylamide gels and visualized by coomassie blue staining.
Lane 1: Protein molecular weight marker.
Lane 2: fractions 22 – 28 respectively

weight of 22000. Gel permeation chromatography was performed to determine the molecular weight of the purified nucleoid protein in solution. Purified protein was loaded and eluted with same buffer. Similarly standard proteins were also chromatographed on Sephadex G – 100 column. Purified nucleoid protein eluted (Fig 24) at a volume corresponding to a molecular weight of approx ~30,000, indicating monomeric state of the protein in solution.

To check the homogeneity of the protein, we have performed isoelectric focusing followed by two dimensional gel electrophoresis. Protein migrated as a single spot with the pI value between 4.1 – 4.3 on 2-D gel electrophoresis (fig. 25).

### 3.3.2: Spectroscopic properties of (HS30 K) protein

UV absorption spectrum of 30 kDa protein was recorded between 230 nm – 330 nm. The protein showed an absorption maximum (Fig. 26) of 280 nm. Fourth derivative U V absorption spectra of HS30K protein showed (Fig 27) contribution of all aromatic amino acid to absorption with preponderance of phenylalanine and tyrosine residues.

Fluorescence excitation and emission spectra of HS30K protein were recorded. The excitation spectrum showed as excitation maximum of 274 nm with emission wavelength fixed at 320 nm. The emission spectrum showed a broad peak with emission maximum in the region of 300 – 310 nm with excitation fixed at 284 nm (Fig. 28 A and 28 B). Fluorescence emission spectrum of HS30K protein was also recorded at different salt concentrations (100mM, 500 mM, 1 M, 2 M). The intrinsic fluorescence intensity was gradually decreased (Fig.29) indicating conformational change (compact folding) of the protein at high salt concentration.

### 3.3.3: CD spectra of purified HS30K protein

CD spectra of HS30K protein were recorded in a Jasco spectropolarimeter at 25 ºC as described in methods section, CD spectra of the HS30K protein was recorded using 1 mm path length cuvette in buffers containing 50 mM and 2 M NaCl. CD spectra analysis of protein in the presence of low salt (fig 30 A) and high salt (fig. 30 B) showed difference

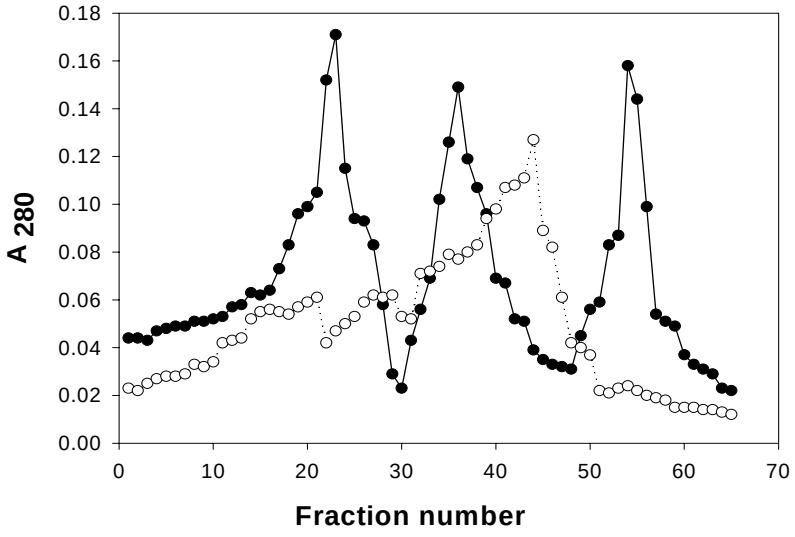



**Fig 24: Gel filtration chromatography of purified H.s nucleoid protein**

30 KDa nucleoid protein (100µg) and Molecular weight markers were chromatographed on Sephadex G-50 as described in the methods section. Fractions were analyzed at 280 nm. (-•- Molecular weight standards Peak I 66 KDa, Peak II 45 KDa and peak III 14 KDa.) (-o- 30 KDa nucleoid protein).

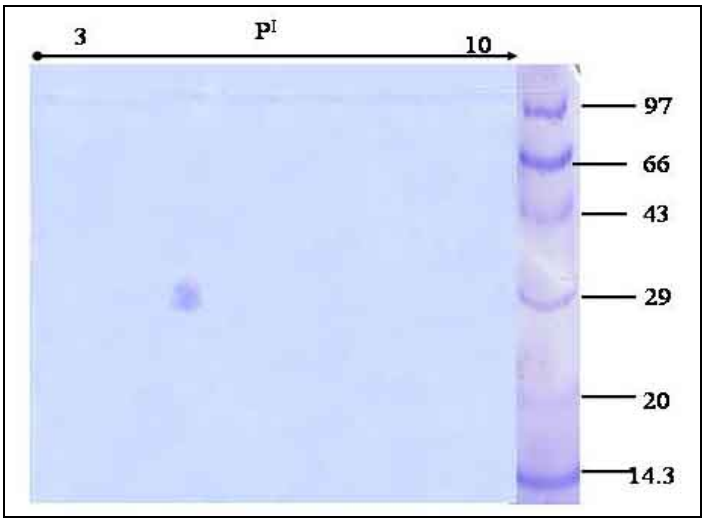



**Fig25: Two – dimensional gel electrophoresis of *H.salinarium H3* S nucleoid protein**
~ 30 KDa purified nucleoid protein was analyzed on 2D gel.

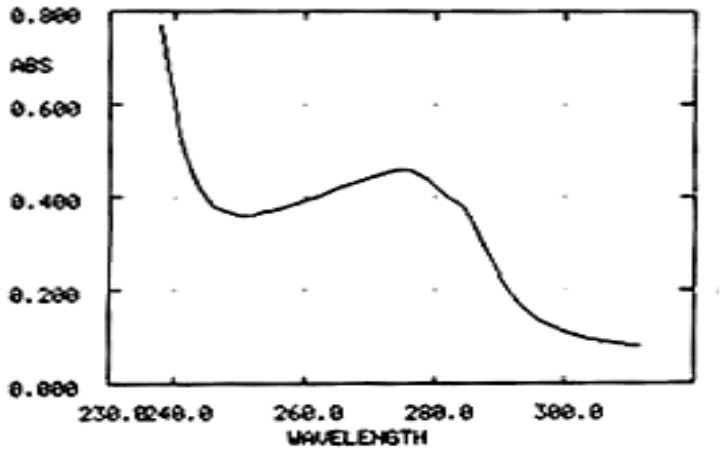



**Fig 26: U V absorption spectrum of H.s nucleoid protein recorded between 230 nm to 330 nm in 20 mM Tris – Cl pH 7.6 and 25 mM NaCl.**

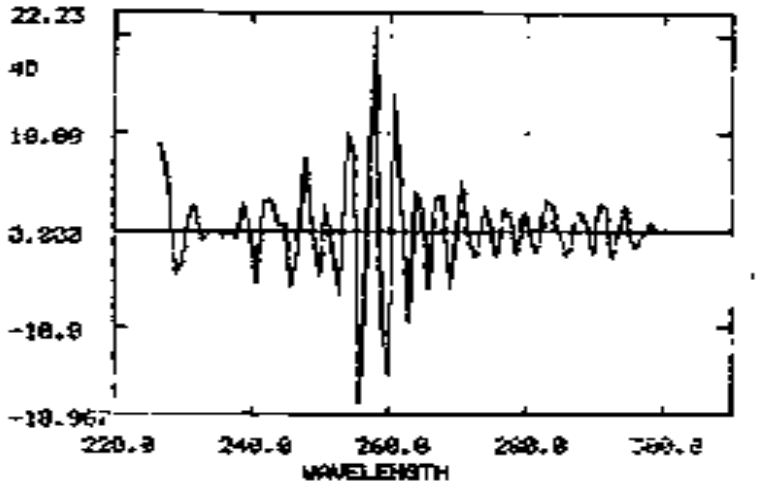
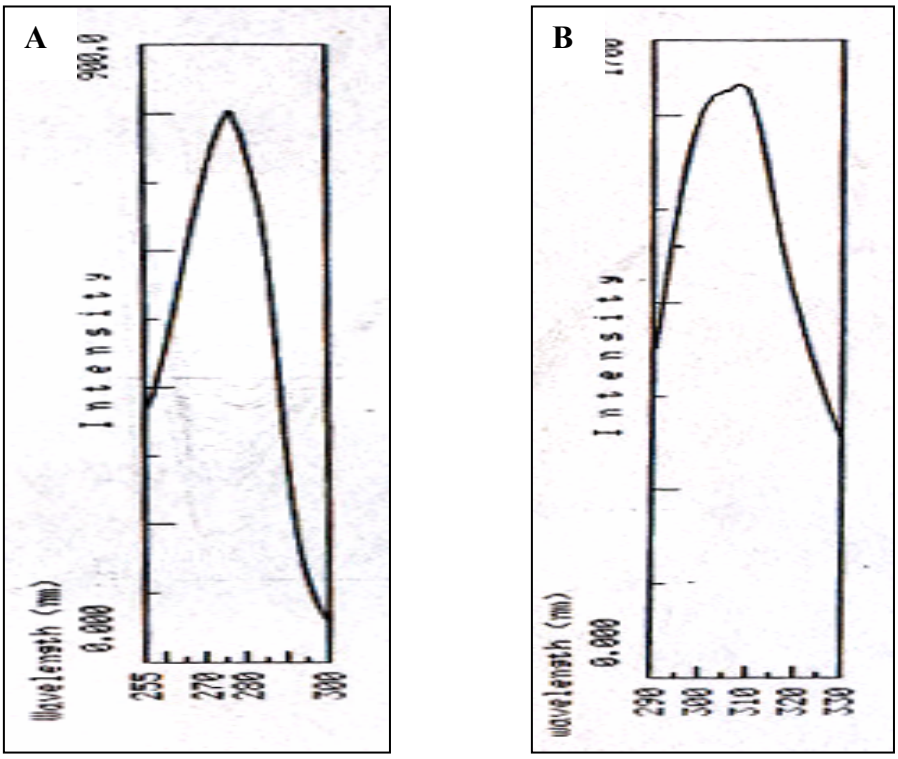



**Fig 27: Fourth derivative spectra of Purified 30 KDa nucleoid protein**

**Fig 28: Intrinsic Fluorescence spectra of purified nucleoid protein**

**A. Excitation spectrum of 30 KDa Protein.**
**B. Emission spectrum at 310 nm.**



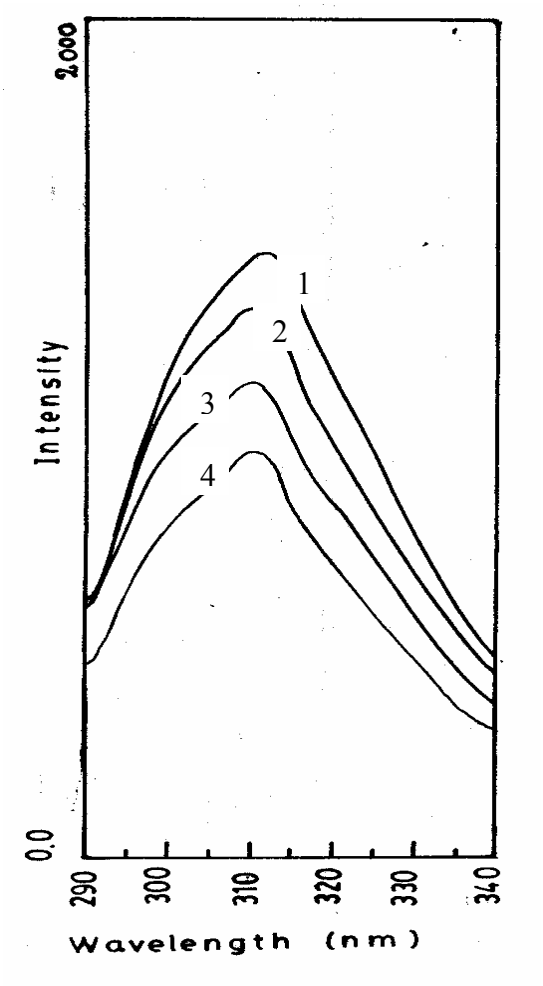



**Fig 29: Fluorescence emission spectrum of 30KDa nucleoid protein at 20 mM Tris – Cl pH 7.6 (1) 100 mM NaCl (2) 500 mM (3) 1M (4) 2M NaCl.**

**Fig 30: CD Spectroscopy of 30 KDa nucleoid protein**

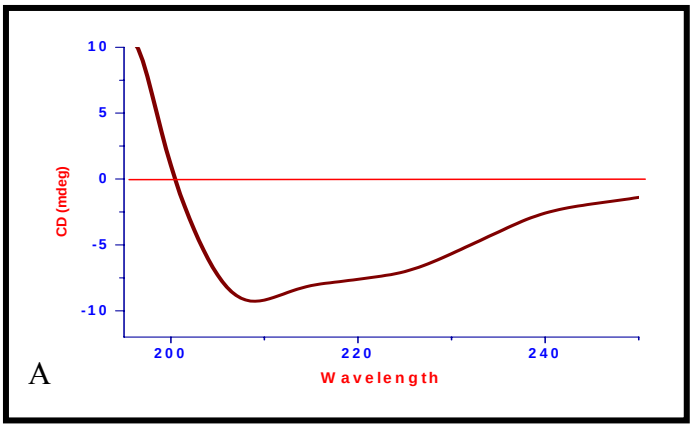



Fig 30 A: CD spectrum of 30 KDa nucleoid protein at 50 mM NaCl salt concentration.

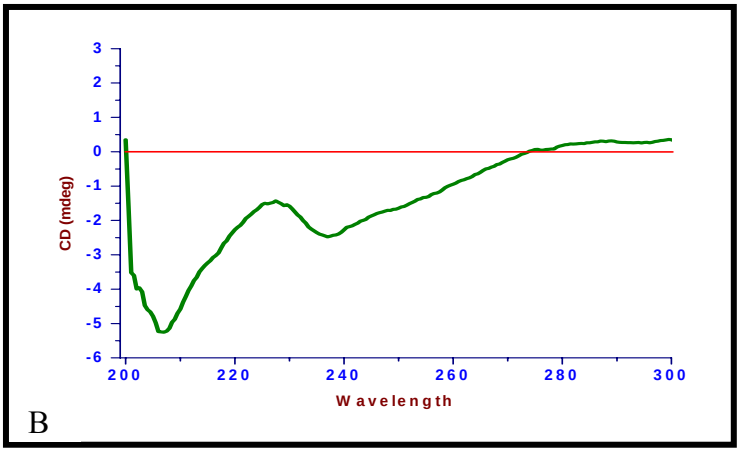



Fig 30 B: CD spectrum of 30 KDa nucleoid protein at 2 M NaCl salt concentration.

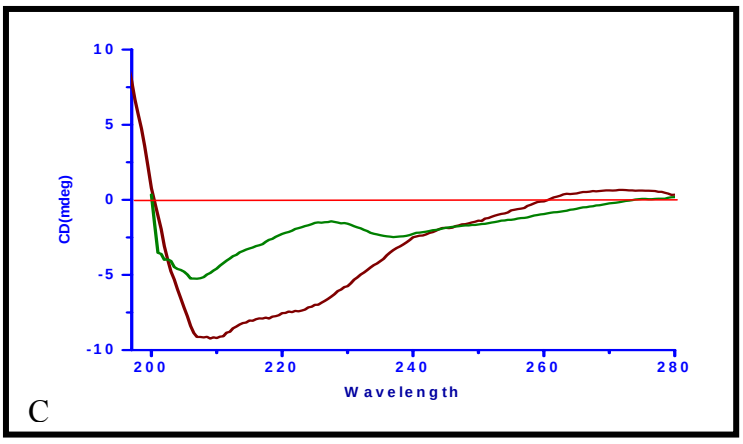



Fig 30 C: Overlapping picture of CD spectrum of 30 KDa nucleoid protein at 50 mM and 2 M NaCl salt concentration.

with pronounced CD bands at high salt concentration indicating change in secondary structure of the protein. The overlapped far- UV CD spectra of nucleoid protein in high and low NaCl are presented in Fig 30 C. In 50 mM NaCl, a double minima was observed near 208 nm and 224 nm indicated significant œ – helical content in the secondary structure of the protein. However a different spectrum was obtained for the protein at high salt concentrations (fig. 30 B). The overall shape of the CD spectrum of HS30K protein with minima of molar ellipticity at 207 nm and 237 nm. The CD spectra of HS30K protein at high and low salt concentrations were analyzed for secondary structural elements by using the Dicroweb software. The predicted secondary structures at High and low salt concentrations are presented in table-2. There was an increase in ß sheet structure with decrease in œ helical content of the protein at high salt concentration

**Table 2: The distribution of secondary structures in the nucleoid protein obtained by evaluation of CD spectra through the DICROWEB –CD software analysis programme Selcon3 and K2D Methods**

| Protein | α- helix (%) | β –sheet (%) | Random coil (%) |
|---|---|---|---|
| **HS dbnp 30KDa Presence of low salt (0.05 M NaCl** | 13.5 | 32.5 | 52.9 |
| **HS dbnp 30 KDa Presence of high salt (2M NaCl)** | 5 | 47 | 48 |

### 3.3.4: Gel mobility shift assays

Gel mobility shift assays were performed with protein – DNA complexes obtained after incubating pBR322 plasmid DNA with increasing concentrations of protein as described in methods at four different salt concentrations (100 mM, 500 mM, 1 M, 2 M and 3 M NaCl). The nucleoprotein complexes were electrophoresed on 0.8 % agarose gels. HS30K protein bounds to both super coiled and relaxed forms of pBR322 and the mobility of pBR322 DNA were retarded indicating binding of the protein to DNA. As seen in Fig 31 A, mobility shift assays were performed at 100 mM NaCl concentration, 500 mM ( Fig 31 B), 1M NaCl ( Fig 31 C), 2M NaCl ( Fig 31 D) and 3M NaCl ( Fig 31 E). Increasing amounts of nucleoid protein added to pBR322 DNA progressively, (Fig 31 A – E, lanes 2 -7) showed progressive increase in retardation of the DNA. The retardation increased with increasing salt concentration.

Binding of nucleoid protein with lambda DNA was also analyzed by mobility shift analysis on 0.8% agarose gel. The retardation of nucleoprotein complexes increases with increasing amounts of protein. As seen in Fig 32 A, mobility shift assays were performed at 100 mM NaCl concentration, 500 mM ( Fig 32 B), 1M NaCl ( Fig 32 C), 2M NaCl ( Fig 32 D). Increasing amounts of HS30K protein added to lambda DNA (Fig 32 A – D, lanes 2 -6 and 9-13) resulted in progressive increase in the retardation of the DNA. However, with lambda DNA the retardation was maximally seen at 500 mM NaCl. Presence or absence of Mg2+ did not change the retardation pattern at all salt concentrations. Gel mobility shift assay confirmed that the purified HS30K protein is a DNA binding protein and showed strong binding at high salt concentrations.

## 3.3.5: Fluorescence titrations:

**Direct fluorescence titrations:**

Nucleic acid binding properties of HS30K protein was studied by fluorescence titrations. Binding site size of the interaction at 100 mM NaCl was obtained by the method of Schwarz and Watanabe, (1983) for the binding of large ligands to long lattice structure like nucleic acids. In this case direct fluorescence titrations were performed with CT DNA and increasing concentrations of protein at 50 mM (Fig. 33 A) and 2 M NaCl (Fig.

**Fig 31: Gel Mobility shift assays:**

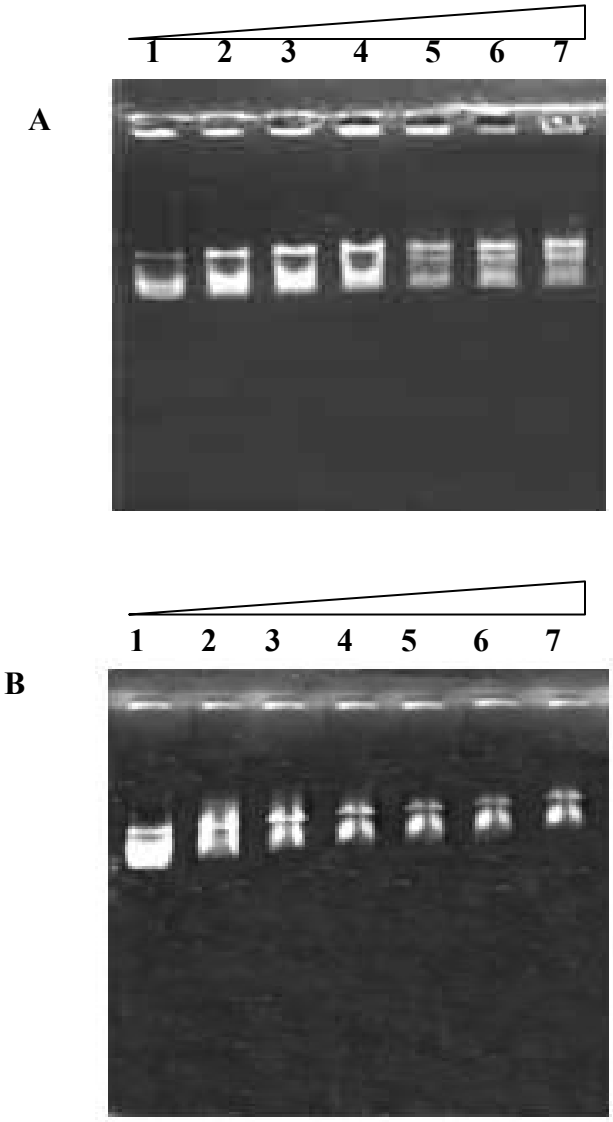



**Fig 31: Gel Mobility shift analysis of 30 KDa nucleoid protein –DNA complexes. pBR 322 (1µg) was incubated with increasing amounts of Protein in a buffer containing A) 100mM NaCl B) 0.5 M NaCl and analyzed on 0.8 % agarose gel.**
Lane1 : pBR 322 (control)
Lane 2- 7: pBR 322 DNA incubated with 1µg, 2µg, 3µg, 4µg, 5µg and 6µg respectively.

**Fig 31: Effect of 30 KDa nucleoid protein on the mobility of pBR 322 (1µg) containing supercoiled relaxed and linear forms at 37ºC.**

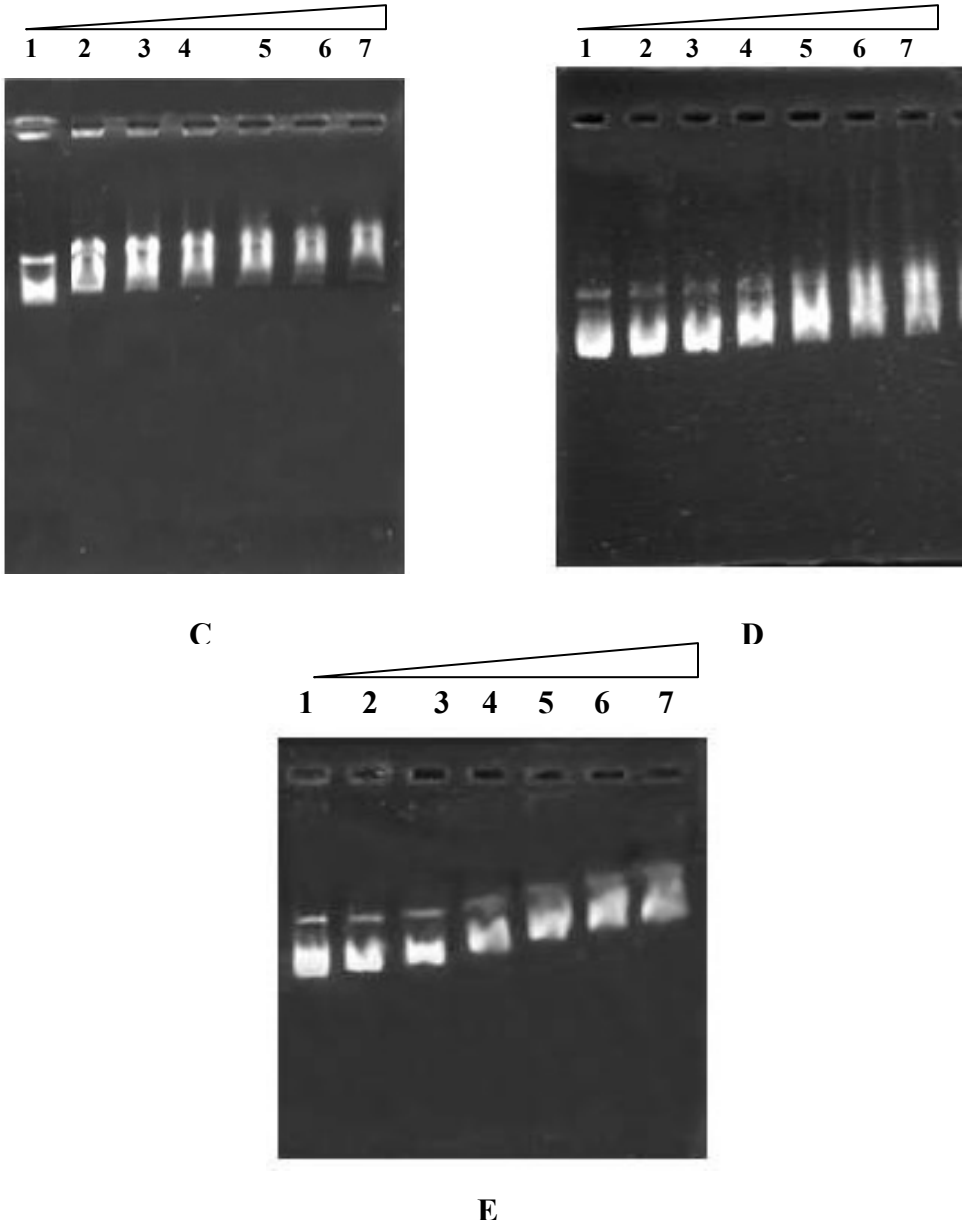



**Fig: Gel Mobility shift analysis of 30 KDa nucleoid protein –DNA complexes .pBR -322 (1 µg) was incubated with increasing amounts of Protein in a buffer containing A) 1 M NaCl B) 2 M NaCl and C) 3M NaCl and analyzed on 0.8 % Agarose gel.**

Lane 1 : pBR 322 (control)

Lane 2 – 7: pBR 322 DNA incubated with 1µg, 2µg, 3µ 4 µg, 5µg, and 6 µg of Protein respectively

**Fig 32: Mobility shift of Lambda DNA by 30 KDa H.s. nucleoid protein**

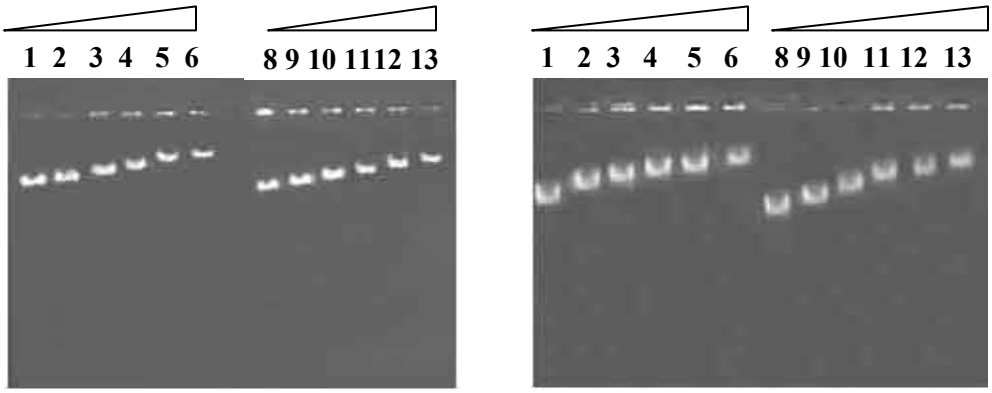



**Fig 32: Gel Mobility shift analysis of 30 KDa nucleoid protein –DNA complexes. Lambda DNA500 ng was incubated with increasing amounts of Protein present in a buffer containing A)100mM NaCl B) 0.5 M NaCl described below and analyzed on 0.8 % agarose gel.**

Lane 1and 8: 500 ng of Lambda DNA

Lane 2 – 6 and 9 - 13: Lambda DNA incubated with 2µg, 4µg, 8µg, 12µg and 16 µg of protein respectively.

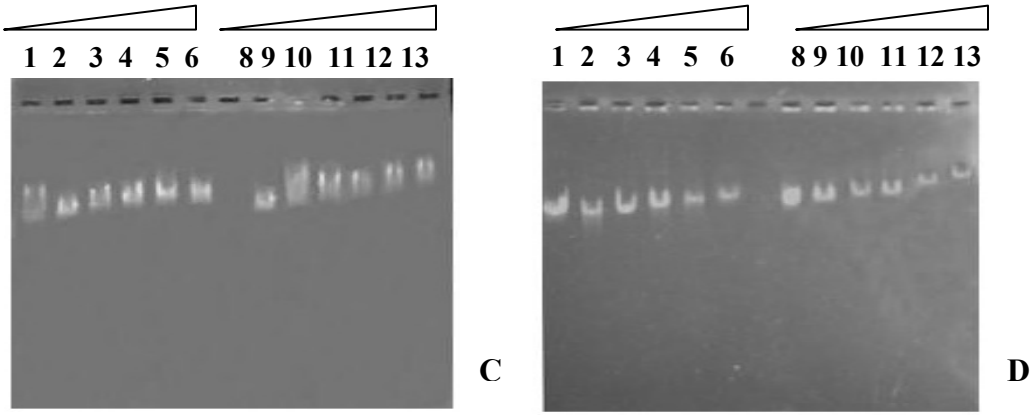



**Fig 32: Gel Mobility shift analysis of 30 KDa nucleoid protein – DNA complexes. Lambda DNA500 ng was incubated with increasing amounts of Protein present in a buffer containing C) 1M NaCl D) 2 M NaCl described below and analyzed on 0.8 % agarose gel. Lane 1 -6 assays are performed without Magnesium ions.**
**Lane 8-13: assays are performed in the presence of 5 mM $MgCl_2$.**

**Lane 1 and 8:** 0.5µg of Lambda DNA and Lane 2 – 6 and 9 - 13: Lambda DNA incubated with 2µg, 4µg, 8µg, 12µg and 16µg of protein respectively.

**Fig 33: Binding of the Proteins to DNA as Measured by Intrinsic Fluorescence Quenching: direct fluorescence titrations.**

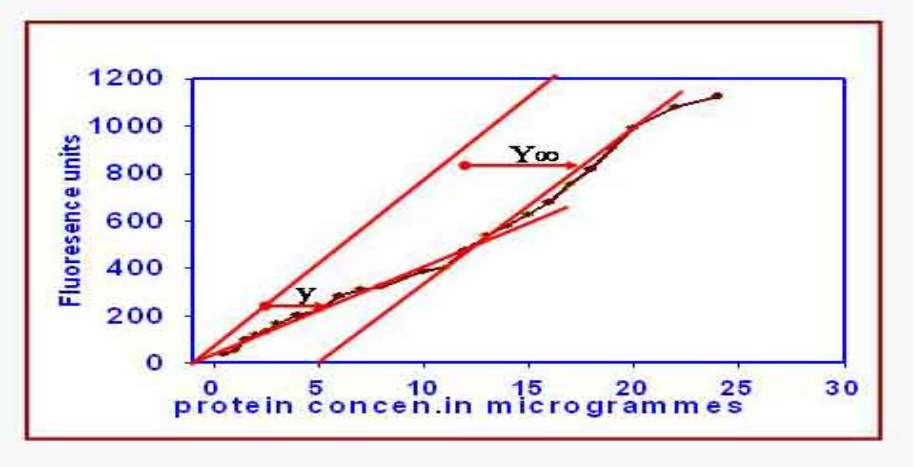



**Fig33 A: Direct fluorescence titration of Calf thymus DNA (10µg) with nucleoid protein Aliquots of protein ((1µg) were added to constant amount o CT DNA in 50 mM NaCl buffer and intensity of fluorescence emission at wavelength 310 nm was measured. (Ex 284 nm). Fluorescence intensity (arbitrary units) was plotted against concentration of nucleoid protein.**

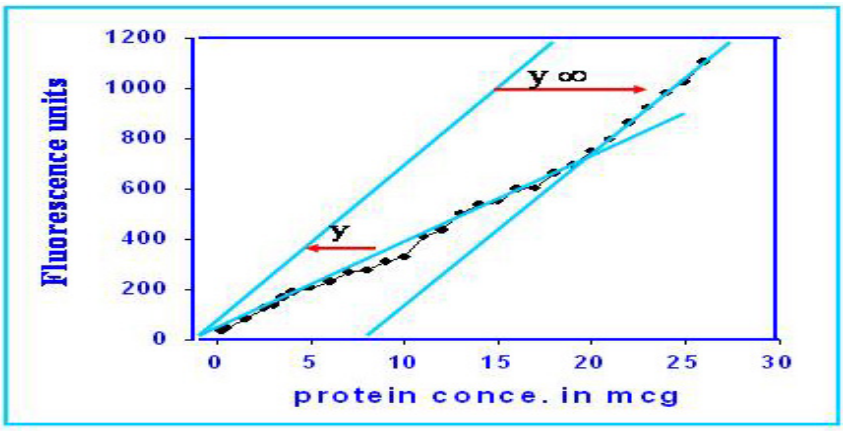


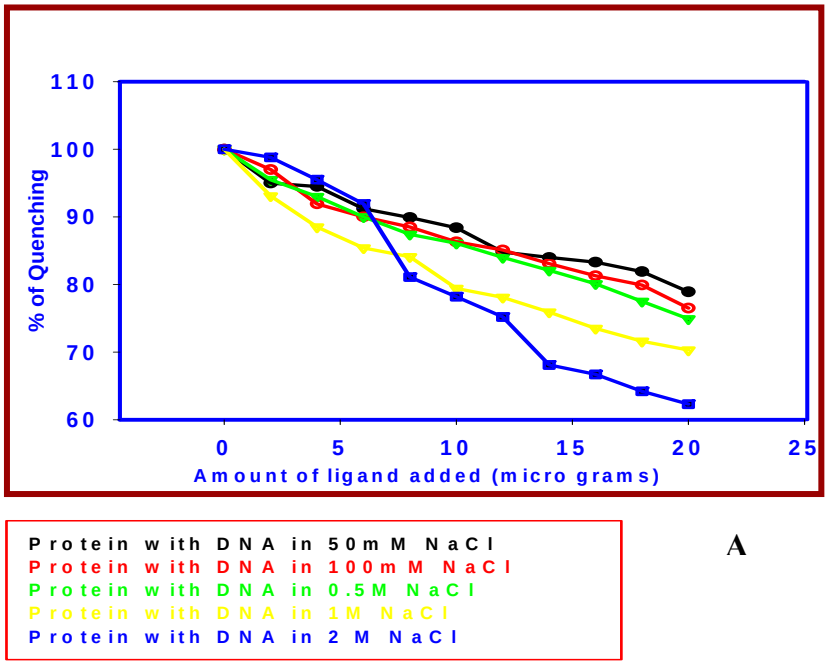

**Fig33 B: Direct fluorescence titration of Calf thymus DNA (10µg) with nucleoid protein Aliquots of protein ((1µg) were added to constant amount o CT DNA in 2 M NaCl buffer and intensity of fluorescence emission at wavelength 310 nm was measured. (Ex 284 nm). Fluorescence intensity (arbitrary units) was plotted against concentration of nucleoid protein.**

33 B) concentrations showed more amount of protein is required to saturate the DNA at high salt concentration. At saturation a linear asymptote was reached. This asymptote was parallel to the straight line obtained for free protein. A site size of 75 nucleotides (37.5 bp) and 47 nucleotides (23.5bp) were obtained at 50 mM NaCl and 2 M NaCl respectively taking the molecular weight of protein as 30 kDa as determined by SDS – PAGE. However, the protein molecular weight was determined as 22,000 by Mass spectrum of intact protein. Hence the actual site size could be 27 bp and 17 bp at 0.05 M NaCl and 2 M NaCl respectively.

**3.3.6: Reverse fluorescence titrations:**

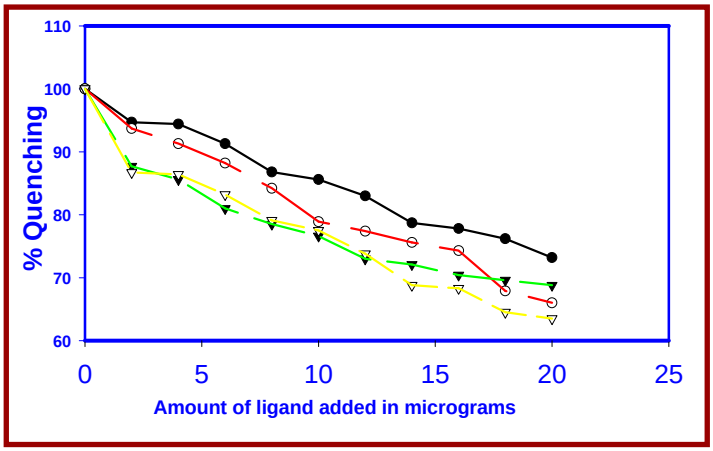
Reverse titrations were also performed by adding nucleic acids and synthetic polynucleotides to constant amount of protein and measuring the extent of fluorescence quenching. The quenched fluorescence spectrum were obtained by adding nucleic acids and polynucleotides (CT DNA, poly A, poly dA, poly C, poly dG, poly U, poly dT, poly dA –dT, poly dG- dC). All individual reverse titrations were carried out in the presence of the buffer containing Tris-HCl pH 7.6 and different salt concentrations of 100 mM NaCl, 0.5 M NaCl, 1 M NaCl and 2 M NaCl. As seen in figure 34 A, quenching of fluorescence with CT DNA increased from 100 mM NaCl to 2M NaCl concentration, comparatively more quenching was obtained at higher salt concentration. As explained in the methods section, reverse titrations were carried out with different synthetic polynucleotides like poly A and poly dA (fig 34 B and C), poly C and poly dC (fig 34 D and E), poly U and poly dT ( fig 34 F and G) and finally dA-dT and poly dG-dC (Fig 34 H and I). The data with amount of ligand added vs % of quenching showed that more quenching was obtained with poly dA, poly dC and poly dT compared to poly A and poly C and poly U indicating strong binding of the protein to the single stranded deoxyribo nucleic acids.

**Fig 34: Quenching of intrinsic fluorescence of purified 30 KDa nucleoid protein by nucleic acids.**

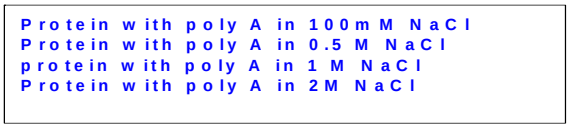


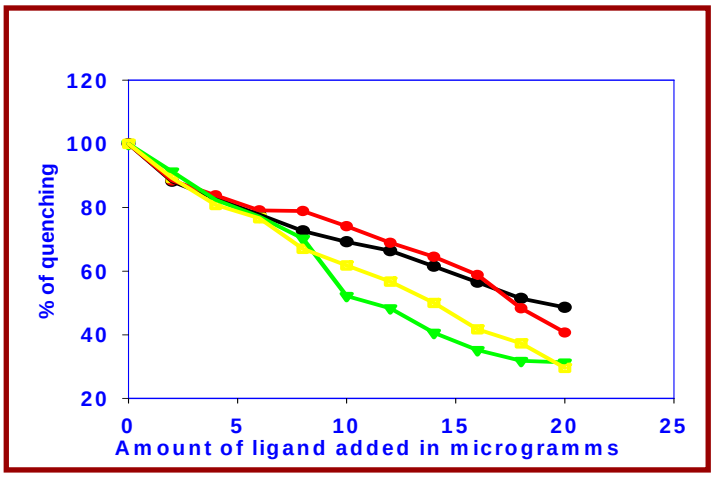

**A**

A: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by calf thymus DNA. All individual reverse titrations were carried out in the presence of the buffer containing Tris-HCl pH 7.6 and different salt concentrations of 100 mM NaCl, 0.5 M NaCl, 1 M NaCl and 2 M NaCl




B




C

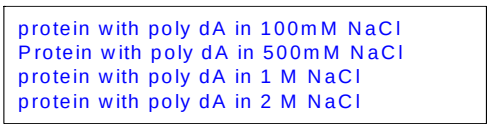
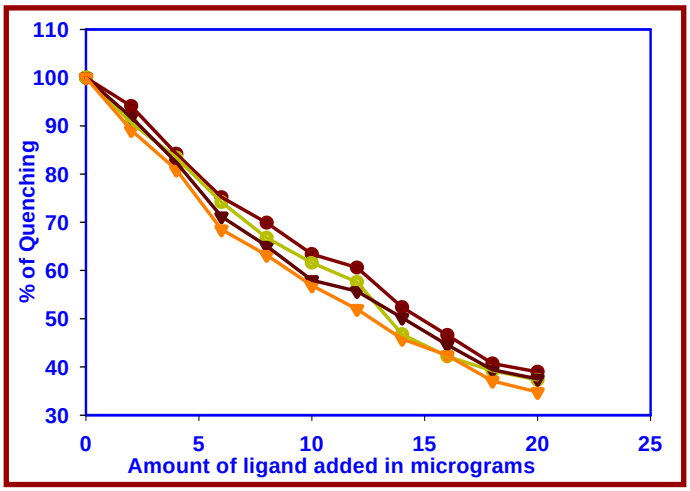
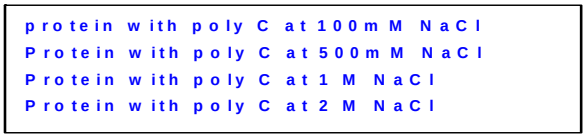


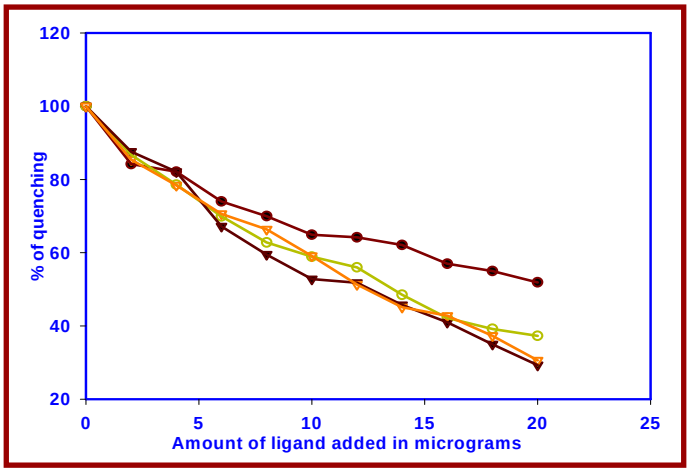

**Fig 34 B and C: Fluorescence titration of Halobacterial 30 KDa nucleoid protein with PolyA and Poly dA at different salt concentrations.**

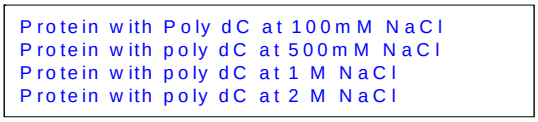
B: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly A.
C: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly dA.




**D**

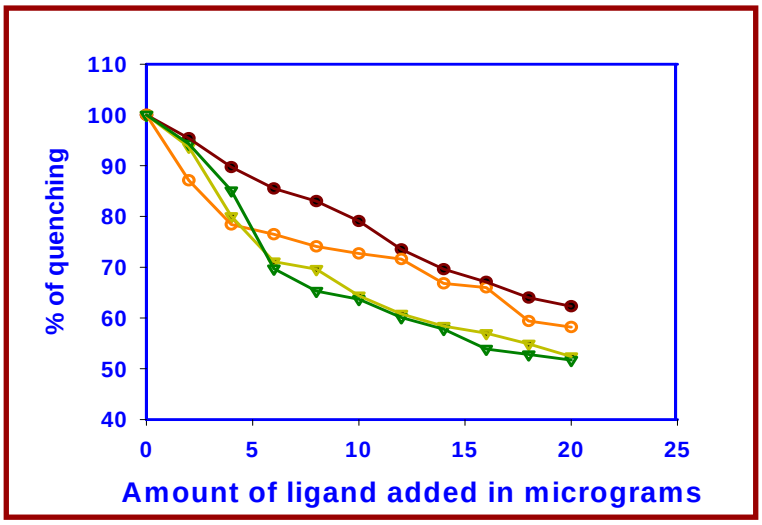



**E**

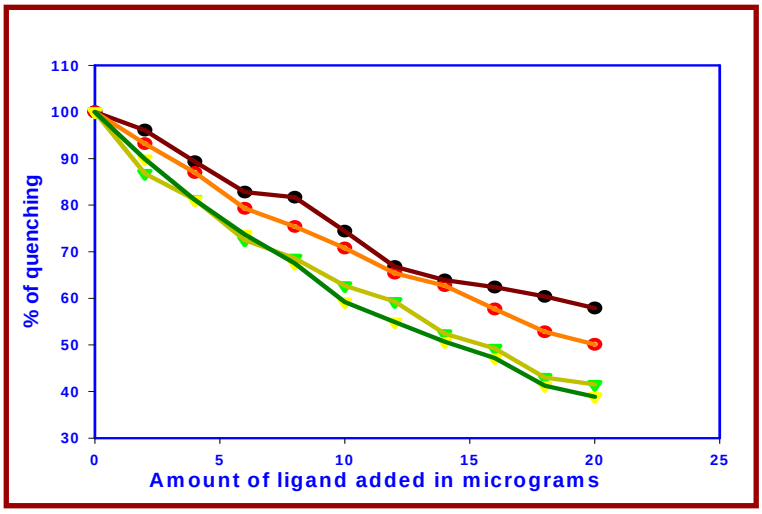
**Fig 34 D and E: Fluorescence titration of Halobacterial 30 KDa nucleoid with Poly C and Poly dC at different salt concentrations.**

D: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly C.
E: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly dC.




Protein with poly U at 100mM NaCl
Protein with poly U at 500mM NaCl
Protein with poly U at 1 M NaCl
Protein with poly U at 2 M NaCl

F




Protein with poly dT at 100mM NaCl
Protein with poly dT at 0.5 M NaCl
Protein with poly dT at 1 M NaCl
Protein with poly dT at 2 M NaCl

G

**Fig 34 F and G: Fluorescence titration of Halobacterial 30 KDa nucleoid with Poly U and Poly dT at different salt concentrations.**

F: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly U.

G: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly d T

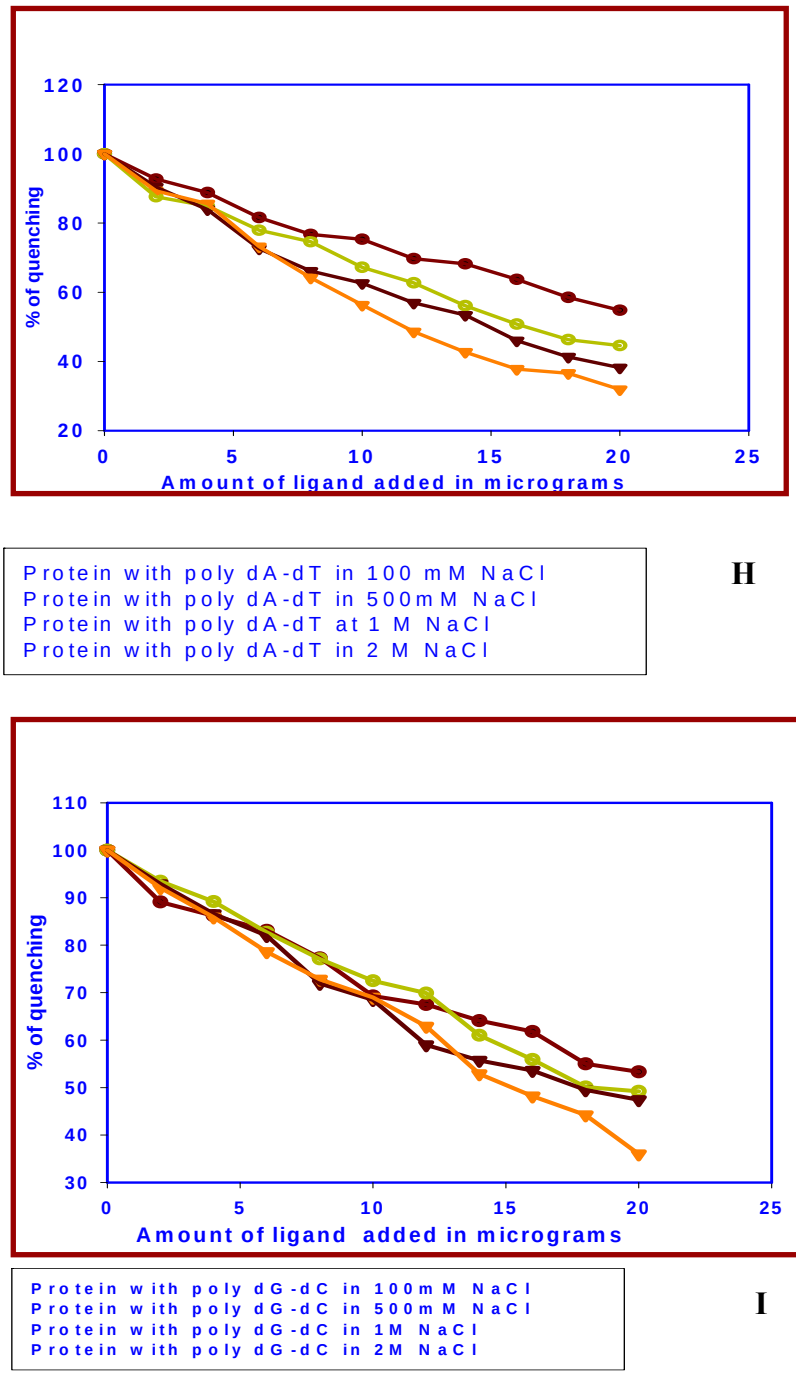



**Fig 34 H and I: Fluorescence titration of Halobacterial 30 KDa nucleoid with Poly dA- dT and Poly dG – dC at different salt concentrations.**

H: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly dA - dT.
I: Quenching of intrinsic fluorescence of 30 KDa nucleoid protein by Poly dG - dC

### 3.3.7: Circular dichroic spectra analysis of protein – DNA complexes:

Forward titrations of lambda DNA and pBR322 with increasing concentrations of HS30K protein resulted in alteration of the CD spectrum of DNA with increasing salt concentrations. The far UV – CD spectra of pBR322 DNA- HS30K protein complex in low and high NaCl are presented in fig. 35. The C D spectrum of pBR322 (5µg) is significantly altered ( Fig. 35) as the nucleic acid is titrated separately with 15 µg of HS30K protein at 50 mM NaCl and 2 M NaCl. In the presence of high salts, negative peaks were observed at 209 nm, 230 nm. At low salt concentrations however, these ellipticity values are lower than the CD spectra of pBR322-protein complex at high NaCl. In near UV-CD spectra of pBR322- protein complex showed positive peak at 265 nm in the both low and high NaCl. But in the presence of low NaCl decrease in the ellipticity was observed. The CD spectra showed isodichroic points at 245 nm and 280 nm suggests that there are two predominant DNA conformations (one free DNA and the other DNA bound by HS30K protein)

Fig 36 shows a forward titration of lambda DNA (25 µg) with HS30K protein monitored by CD at 50mM NaCl. The spectra are scaled to the molar CD of the DNA component. The increase in the CD at shorter wavelengths (< 240nm) reflects increasing contributions from protein (5 µg, 25µg and 50 µg) while the changes at long wavelengths are dominated by DNA and reflect alterations in the structure of DNA upon protein binding. As shown in fig. 37 forward titration of lambda DNA was carried out with increasing concentrations of protein (5 µg, 25µg and 50 µg) monitored by CD at 2M NaCl. These CD spectra of protein – DNA complexes were compared with CD spectra of free protein and free DNA. As seen in fig 37, less drastic changes were observed with lambda DNA in 2M NaCl with decrease in the intensity of the positive wavelengths. In the presence of 50 mM NaCl (fig 36) slight increases were observed in the long wavelength positive CD band of lambda DNA. The negative CD band (fig 37) at 210 nm increased with increasing the protein concentration (5 µg, 25µg and 50 µg) indicating the strong binding of the protein to DNA at high salt concentrations

**Fig 35: CD spectroscopic analysis of pBR 322 DNA – nucleoid protein complexes.**

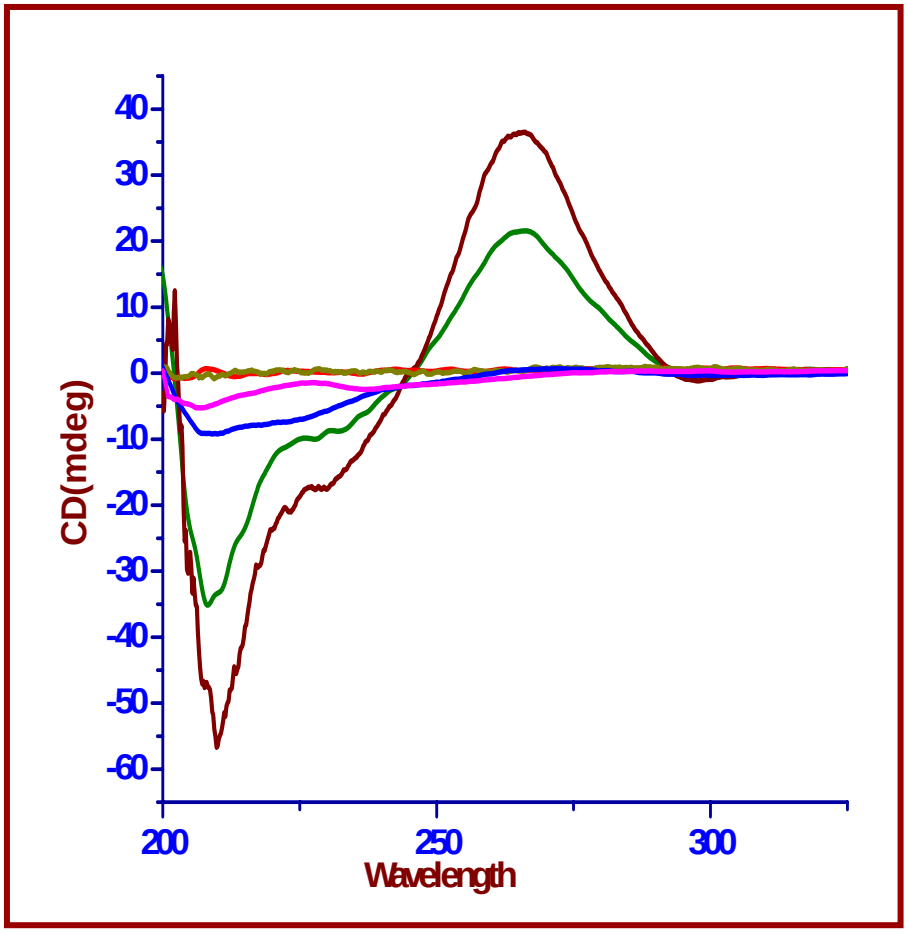



**Fig 35 A: Titration of pBR 322 DNA with nucleoid protein followed by circular dichroism measurements. The initial CD spectrum in the absence of protein. The initial concentration of pBR 322 (0.3534µM). The nucleoid protein concentration is 33.3µM. CD spectra of nucleoid protein alone; Lambda DNA alone and protein with plasmid DNA were recorded using 1 mm path length cuvette and the buffer containing 50 mM and 2 M NaCl.**

— 15 mcg of protein+5 mcg of pBR 322 at 50 mM NaCl

— 15 mcg of protein+5 mcg of pBR 322 at 2 M NaCl

— pBR 322 at 50 mM NaCl

— pBR 322 at 2 M NaCl

— Protein only at 50mM NaCl

— Protein only at 2 M NaCl

**Fig 36: CD spectroscopic analysis of Lambda DNA – nucleoid protein complexes**

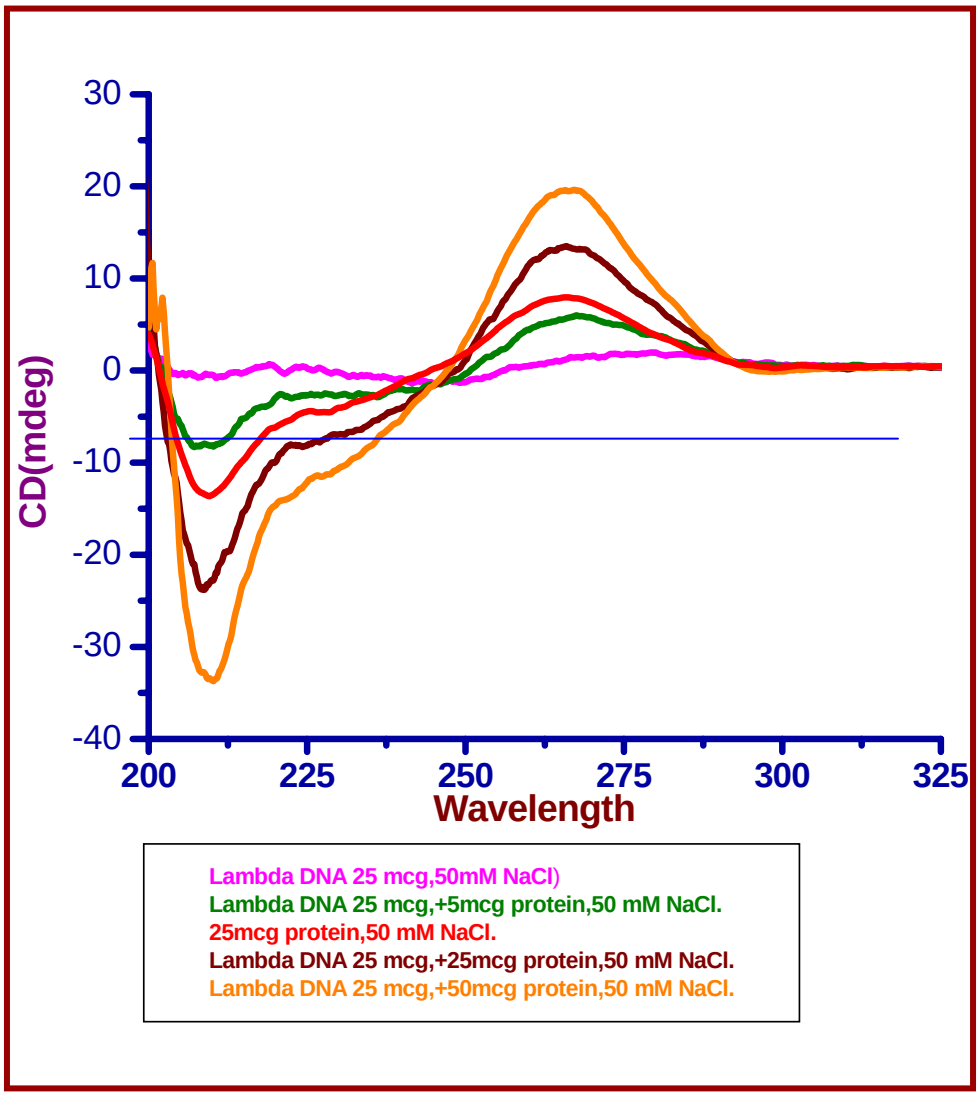



**Fig 36: Titration of Lambda DNA with nucleoid protein followed by circular dichroism measurements. The initial CD spectrum in the absence of protein. The initial concentration of Lambda (25µg). The nucleoid protein concentration is 25µg. CD spectra of nucleoid protein alone, Lambda DNA alone and protein with Lambda DNA were recorded using 1 mm path length cuvette and the buffer containing 50 mM NaCl.**

**Fig 37: CD spectroscopic analysis of Lambda DNA – nucleoid protein complexes**

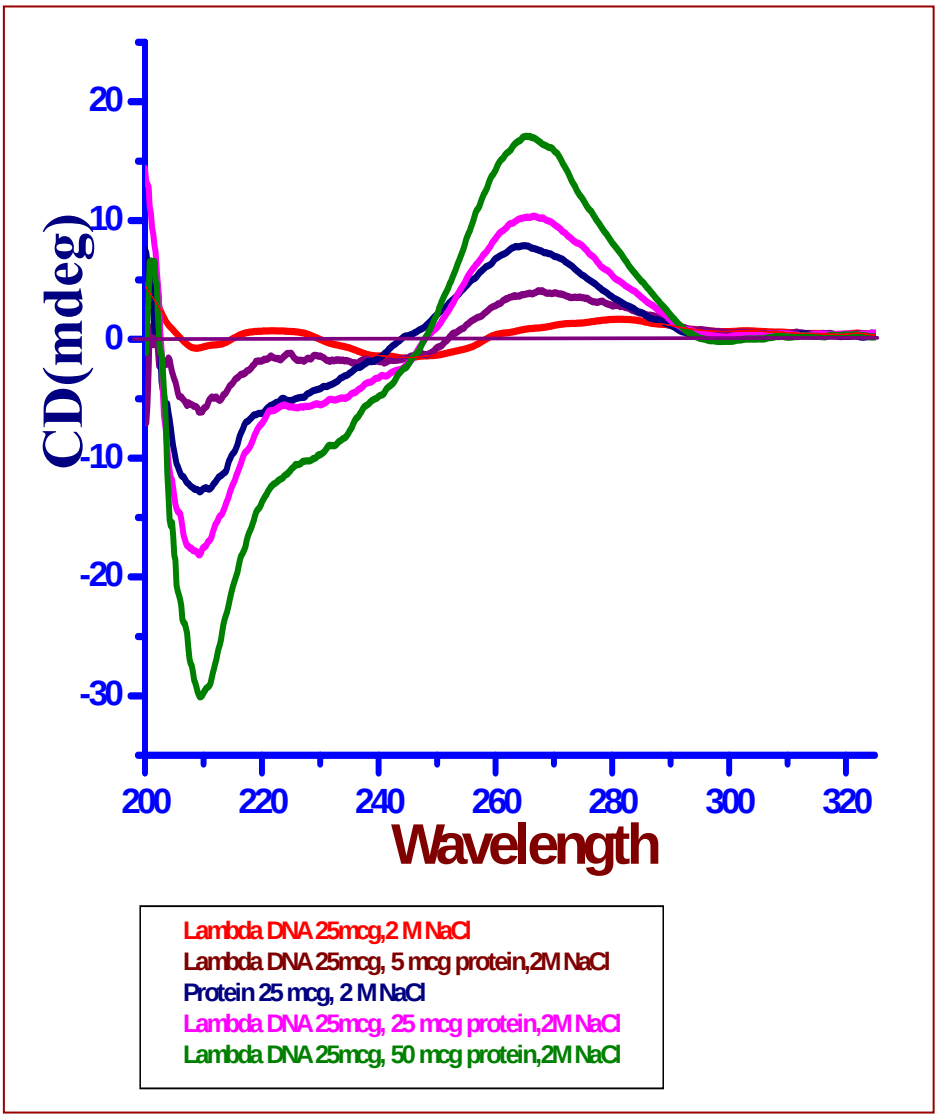



**Fig37: Titration of Lambda DNA with nucleoid protein followed by circular dichroism measurements. The initial CD spectrum in the absence of protein. The initial concentration of Lambda (25µg). The nucleoid protein concentration is 25µg. CD spectra of nucleoid protein alone, Lambda DNA alone and protein with Lambda DNA were recorded using 1 mm path length cuvette and the buffer containing 2 M NaCl.**

### 3.3.8: DNA unwinding studies of HS30K nucleoid protein

Spectroscopic experiments have also been carried out on the binding of protein with DNA. UV absorption spectra of CT DNA and CT DNA incubated with increasing HS30K protein (5µg, 15µg and 25µg) (fig. 38) were recorded between 230 nm to 320 nm in 20 mM Tris – HCl pH 7.6 and 25 mM NaCl. There was increase in $A_{260}$ absorbance indicating progressive unwinding of DNA of lambda DNA (25 µg). Titrations of lambda DNA (25 µg) (fig. 39) with increasing concentration of protein at different salt concentrations (50 mM, 1 M NaCl and 2 M NaCl) were carried out. The extent of unwinding increased with increasing salt concentration, (more unwinding occurred at 2M NaCl and in the presence of 10mM ATP) as indicated by increase in $A_{260}$ absorbance. These results showed that the protein has strong DNA unwinding activity which may have important physiological role. The present study may help in understanding the interaction of proteins with DNA at high salt concentration.

### 3.3.9: MALDI analysis and identification of HS30 KDa protein

HS30 K was subjected to MALDI- TOF analysis to obtain peptide mass fingerprint. The analysis was carried out with purified protein at Indian Institute of Science, Bangalore, India and Centre for Cellular and Molecular Biology, Hyderabad. The data obtained with peptide Mass fingerprint (M 2 values) were used to search databases (MSDB and NCBI Nr) using software MASCOT search software (Matrix Science USA) and ProFound – prowl search software (Rockefeller University) as well as M S fit software (University of California) also identified the protein as $Mn^{2+}$– Superoxide dismutase ($Mn^{2+}$ – SOD). In all cases there was indication that the protein peptide masses corresponded partly (5 peptide match) with $Mn^{2+}$ – SOD of *H. salinarium* and *Halobacterium NRC-1*. The score was highest with $Mn^{2+}$ – SOD. Similarity to SOD seemed more possible after close examination of SOD sequence from the database and the peptide mass finger print of HS30K protein (see discussion). The results of the peptide mass fingerprints (fig. 40) (IISc) data base search with the Mascot search (both MSDB, NCBI Nr) ProFound –Prowl and MS fit are presentated in (fig. 41, 42 and 43).

**Fig 38: Absorption spectra of DNA with increasing concentration of 30 KDa protein.**

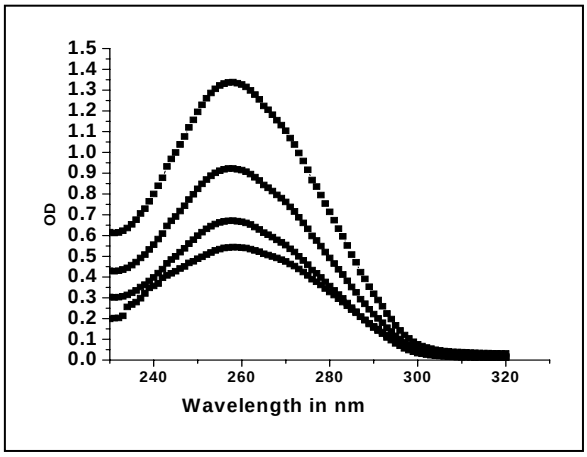



**Fig 38: UV absorption spectra of CT DNA with increasing concentrations of H.s 30 KDa nucleoid protein. CT DNA incubated with the nucleoid protein 5µg, 15µg and 25µg separately and UV absorption spectrum of CT DNA recorded between 230 nm to 320 nm in 20 mM Tris – Cl pH 7.6 and 25 mM NaCl.**

**Fig 39: DNA unwinding profile of H.s nucleoid protein**

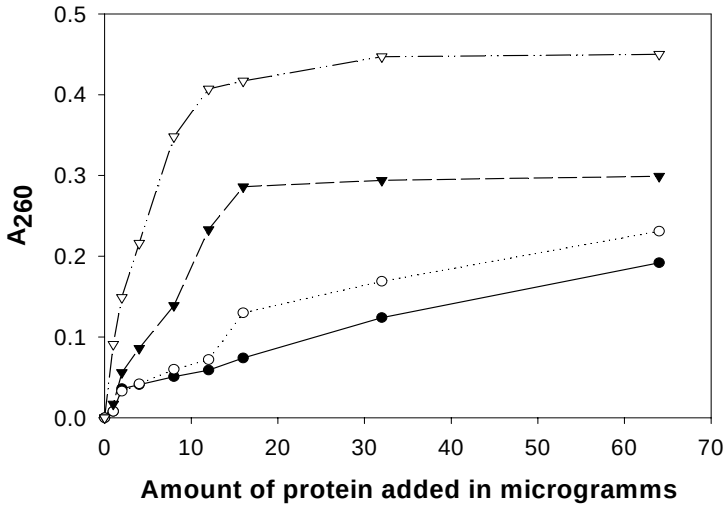
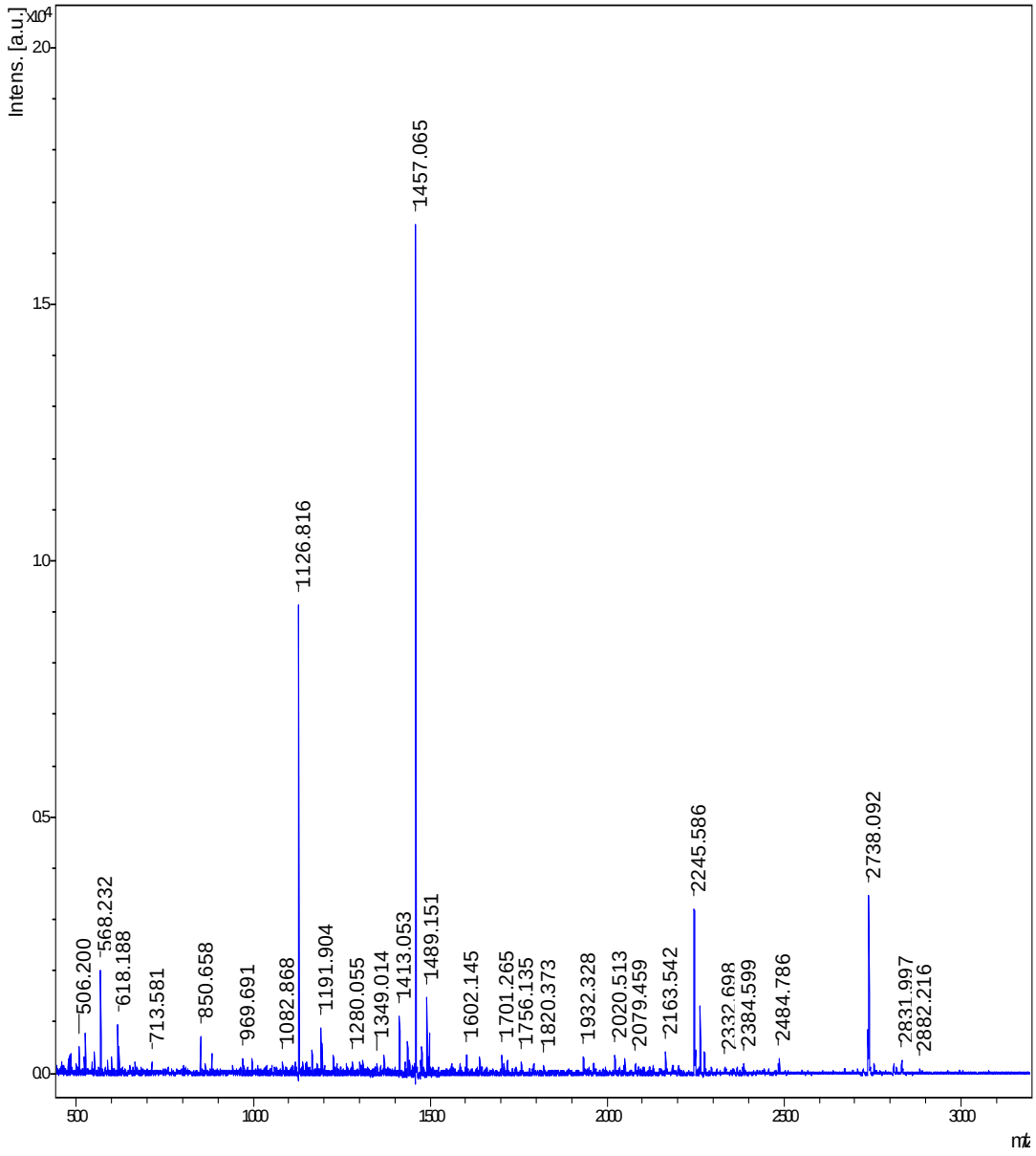



**Fig 39. Increase in $A_{260}$ absorbance of Lambda DNA with the addition of 30 KDa protein at different salt concentration.**

Direct titration curves of Lambda DNA with increasing concentration 30 KDa nucleoid protein. Each time 5 µg of protein was added to Lambda DNA (25µg) in a buffer containing 20 mM Tris-HCl pH 7.6 1mM Magnesium acetate, 1mM $Na_2$ EDTA, and different salt concentrations. 50 mM NaCl, 1M NaCl, and 2 M NaCl was used for each reaction. The increase in UV absorption after each addition was measured ($A_{260}$). Observed absorption changes were plotted against concentration of protein.
(-•- DNA + protein at 50 mM NaCl), (-o- DNA + protein at 1 M NaCl)
(-▼- DNA + protein at 2 M NaCl), ( - ▽ - DNA + protein + 10 µM ATP)

**Fig. 40: Ingel-digestion - Peptide mass fingerprint**




| m/z | SN | Quality Fac. | Res. | Intens. | Area |
|---|---|---|---|---|---|
| 445.055 | 11.1 | 311 | 3161 | 131.61 | 28 |
| 457.159 | 17.5 | 564 | 3166 | 207.59 | 41 |
| 478.167 | 23.4 | 904 | 3195 | 275.27 | 59 |

| | | | | | |
|---|---|---|---|---|---|
| 480.176 | 28.7 | 2546 | 3327 | 336.40 | 70 |
| 481.180 | 9.7 | 282 | 3408 | 113.63 | 22 |
| 483.172 | 31.1 | 1258 | 3091 | 362.90 | 80 |
| 485.372 | 12.8 | 266 | 3125 | 149.51 | 38 |
| 506.200 | 46.4 | 1123 | 3552 | 529.71 | 107 |
| 520.195 | 17.9 | 949 | 3425 | 204.47 | 48 |
| 522.197 | 26.5 | 750 | 3014 | 302.90 | 76 |
| 524.211 | 67.7 | 3793 | 3643 | 777.52 | 168 |
| 526.240 | 19.7 | 359 | 3447 | 227.72 | 51 |
| 543.219 | 11.9 | 286 | 3491 | 139.78 | 38 |
| 544.263 | 17.0 | 260 | 2383 | 199.79 | 63 |
| 550.213 | 35.5 | 2758 | 3552 | 420.99 | 99 |
| 562.255 | 8.6 | 292 | 2544 | 102.93 | 36 |
| 568.232 | 170.1 | 7703 | 3347 | 2038.90 | 521 |
| 587.272 | 23.3 | 577 | 2971 | 273.76 | 82 |
| 599.499 | 27.0 | 2372 | 3858 | 314.86 | 73 |
| 617.180 | 82.8 | 4636 | 3364 | 946.24 | 290 |
| 618.188 | 20.5 | 265 | 4559 | 233.85 | 66 |
| 622.159 | 15.4 | 840 | 3583 | 175.70 | 58 |
| 650.148 | 12.6 | 250 | 3757 | 138.87 | 43 |
| 666.156 | 21.3 | 681 | 3133 | 231.04 | 93 |
| 672.257 | 8.9 | 275 | 2613 | 95.37 | 46 |
| 713.581 | 20.6 | 833 | 3037 | 210.08 | 77 |
| 758.621 | 13.5 | 359 | 4294 | 133.64 | 37 |
| 802.614 | 14.5 | 469 | 4193 | 146.38 | 46 |
| 850.658 | 66.3 | 6322 | 3399 | 713.25 | 316 |
| 863.705 | 14.3 | 694 | 4036 | 157.31 | 60 |
| 882.765 | 35.5 | 1407 | 3897 | 400.57 | 160 |
| 940.766 | 13.4 | 366 | 4049 | 165.22 | 71 |
| 969.691 | 22.6 | 968 | 4222 | 291.33 | 143 |
| 973.793 | 11.1 | 562 | 4646 | 143.05 | 54 |

| | | | | | |
|---|---|---|---|---|---|
| 996.826 | 21.6 | 988 | 4777 | 288.59 | 123 |
| 1011.845 | 14.6 | 299 | 3365 | 197.72 | 100 |
| 1051.927 | 10.3 | 573 | 3332 | 152.89 | 85 |
| 1082.868 | 11.7 | 733 | 3082 | 192.68 | 129 |
| 1126.816 | 545.8 | 14924 | 3420 | 9566.78 | 6682 |
| 1138.895 | 13.3 | 436 | 5009 | 233.04 | 108 |
| 1165.976 | 24.8 | 993 | 4199 | 433.04 | 262 |
| 1179.930 | 12.3 | 609 | 4099 | 211.75 | 127 |
| 1191.904 | 54.3 | 4299 | 3794 | 915.72 | 622 |
| 1201.966 | 9.2 | 484 | 4926 | 153.37 | 89 |
| 1225.986 | 24.2 | 2984 | 3923 | 382.37 | 264 |
| 1229.931 | 8.5 | 493 | 3495 | 131.93 | 99 |
| 1235.928 | 11.5 | 653 | 4495 | 176.66 | 106 |
| 1245.941 | 10.4 | 389 | 3580 | 156.41 | 118 |
| 1263.963 | 11.9 | 427 | 4898 | 186.05 | 107 |
| 1280.055 | 13.9 | 1109 | 4295 | 227.52 | 144 |
| 1309.013 | 13.6 | 745 | 4880 | 238.96 | 152 |
| 1349.014 | 7.7 | 2758893656 | 1863255 | 303.00 | 0 |
| 1359.060 | 7.8 | 365 | 5538 | 146.13 | 90 |
| 1368.997 | 21.8 | 1985 | 3963 | 411.29 | 350 |
| 1411.032 | 14.7 | 371 | 4498 | 309.41 | 243 |
| 1413.053 | 49.6 | 5453 | 4127 | 1053.92 | 892 |
| 1429.046 | 9.7 | 1298 | 5254 | 227.27 | 161 |
| 1435.988 | 24.9 | 1611 | 3825 | 612.23 | 540 |
| 1457.065 | 660.0 | 9097 | 3642 | 18229.37 | 18252 |
| 1473.087 | 9.0 | 625 | 3635 | 268.49 | 235 |
| 1476.128 | 16.3 | 1073 | 4221 | 495.34 | 435 |
| 1489.151 | 55.3 | 7913 | 3964 | 1626.82 | 1570 |
| 1494.119 | 12.0 | 652 | 4066 | 342.12 | 315 |
| 1497.122 | 27.7 | 6382 | 3979 | 771.74 | 704 |
| 1560.174 | 11.8 | 438 | 4483 | 198.54 | 175 |

| | | | | | |
|---|---|---|---|---|---|
| 1585.157 | 9.7 | 763 | 4540 | 158.02 | 144 |
| 1602.145 | 21.5 | 1311 | 4317 | 347.58 | 360 |
| 1639.277 | 18.3 | 1014 | 3805 | 294.71 | 347 |
| 1701.265 | 25.0 | 3905 | 4123 | 376.33 | 467 |
| 1708.231 | 10.9 | 541 | 4440 | 163.65 | 173 |
| 1717.269 | 17.1 | 1745 | 4736 | 252.82 | 254 |
| 1756.135 | 14.9 | 356 | 4937 | 209.73 | 233 |
| 1792.199 | 11.5 | 1011 | 4146 | 152.54 | 194 |
| 1820.373 | 9.6 | 374 | 4355 | 121.55 | 125 |
| 1932.328 | 24.5 | 689 | 4760 | 301.05 | 410 |
| 1961.449 | 15.0 | 1852 | 4883 | 186.51 | 244 |
| 1965.369 | 6.6 | 277 | 4354 | 82.72 | 113 |
| 2020.513 | 28.5 | 2987 | 4343 | 351.60 | 527 |
| 2033.498 | 8.5 | 474 | 7395 | 104.56 | 98 |
| 2048.476 | 19.5 | 2040 | 4985 | 231.26 | 316 |
| 2079.459 | 14.6 | 851 | 4933 | 169.15 | 223 |
| 2102.515 | 9.8 | 570 | 5865 | 110.13 | 131 |
| 2119.539 | 9.5 | 630 | 5328 | 108.94 | 136 |
| 2129.609 | 11.4 | 983 | 4997 | 132.43 | 176 |
| 2163.542 | 26.1 | 1987 | 5311 | 313.90 | 422 |
| 2167.542 | 6.7 | 673 | 4897 | 80.56 | 113 |
| 2184.605 | 10.5 | 429 | 5047 | 126.15 | 201 |
| 2201.596 | 9.0 | 533 | 6267 | 112.91 | 146 |
| 2245.586 | 211.6 | 24348 | 4556 | 2543.88 | 4151 |
| 2261.710 | 88.7 | 21693 | 4608 | 1059.73 | 1695 |
| 2273.674 | 25.9 | 501 | 5528 | 294.33 | 503 |
| 2293.643 | 7.7 | 1061 | 6098 | 91.18 | 125 |
| 2332.698 | 9.5 | 744 | 5646 | 100.98 | 137 |
| 2384.599 | 15.7 | 1242 | 6337 | 142.88 | 201 |
| 2484.786 | 30.8 | 3678 | 5522 | 219.03 | 346 |
| 2738.092 | 377.9 | 42341 | 5339 | 2294.26 | 4188 |

| | | | | | |
|---|---|---|---|---|---|
| 2754.050 | 17.2 | 1039 | 6352 | 102.57 | 156 |
| 2809.134 | 22.0 | 2148 | 5950 | 121.42 | 205 |
| 2831.997 | 33.0 | 3778 | 5969 | 174.06 | 311 |
| 2882.216 | 12.3 | 544 | 7661 | 57.91 | 102 |

**Intact-HS30kda**

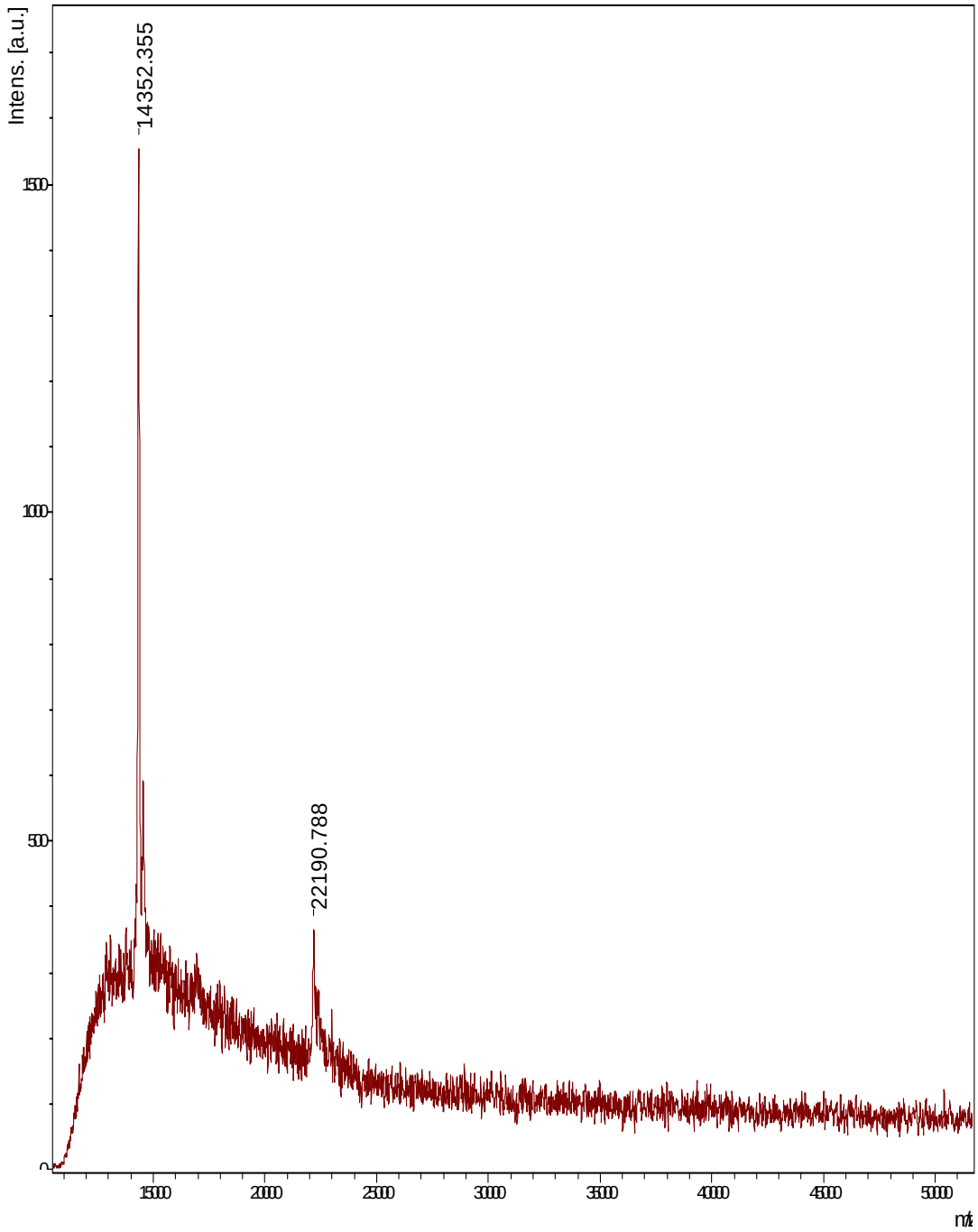


**Fig. 41:**

# MATRIX SCIENCE Mascot Search Results

**User : T Suryanarayana**

**Email : tssl@uohyd.ernet.in**

**Search title : hsk**

**Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)**

**Taxonomy : Archaea (Archaeobacteria) (79478 sequences)**

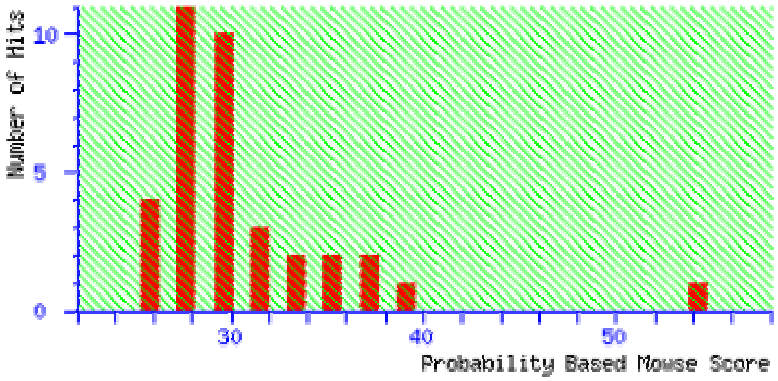
**Timestamp : 23 Oct 2006 at 06:17:38 GMT**

**Top Score : 54 for T50043, superoxide dismutase (EC 1.15.1.1) (Mn) [similarity] - Halobacterium sp.**

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.

Protein scores greater than 62 are significant (p<0.05).




Top of Form

Concise Protein Summary Report

| | | |
|---|---|---|
| Format As | Concise Protein Summary | Help |
| | Significance threshold p< 0.05 | Max. number of hits 20 |

Bottom of Form

Top of Form

Re-Search All

1. T50043 Mass: 22399 Score: 54 Expect: 0.3 Queries matched: 5
superoxide dismutase (EC 1.15.1.1) (Mn) [similarity] - Halobacterium sp.

SODM1_HALSA Mass: 22240 Score: 41 Expect: 6.5 Queries matched: 4
Superoxide dismutase [Mn] 1 (EC 1.15.1.1).- Halobacterium salinarium (Halobacterium halobium).

A34319 Mass: 22371 Score: 41 Expect: 6.5 Queries matched: 4
superoxide dismutase (EC 1.15.1.1) (Mn) [validated] - Halobacterium salinarum

Q4J989_SULAC Mass: 51700 Score: 27 Expect: 1.5e+02 Queries matched: 4
Homocitrate synthase (EC 4.1.3.14).- Sulfolobus acidocaldarius.

---

2. Q2NFL1_METST Mass: 54297 Score: 40 Expect: 7.9 Queries matched: 6
ProS (EC 6.1.1.15).- Methanosphaera stadtmanae (strain DSM 3091).

Q41MN4_METBU Mass: 18691 Score: 28 Expect: 1.3e+02 Queries matched: 3
Appr-1-p processing.- Methanococcoides burtonii DSM 6242.

---

3. Q8ZY97_PYRAE Mass: 23819 Score: 37 Expect: 15 Queries matched: 4
Hypothetical protein PAE0886.- Pyrobaculum aerophilum.

Q2Y4I4_9ARCH Mass: 3789 Score: 27 Expect: 1.7e+02 Queries matched: 2
Hypothetical protein C3_0061.- uncultured archaeon.

---

4. Q1ER93_9CREN Mass: 14199 Score: 37 Expect: 17 Queries matched: 4
Hypothetical protein HGP-33.- uncultured crenarchaeote 31-F-01.

Q8ZYY3_PYRAE Mass: 3000 Score: 30 Expect: 74 Queries matched: 2
Hypothetical protein PAE0551.- Pyrobaculum aerophilum.

---

5. Q8PWT9_METMA Mass: 43291 Score: 35 Expect: 23 Queries matched: 5
Methyltransferase (EC 2.1.1.-).- Methanosarcina mazei (Methanosarcina frisia).

Q6LXN8_METMP Mass: 5432 Score: 27 Expect: 1.5e+02 Queries matched: 2
Ferredoxin.- Methanococcus maripaludis.

Q5JIK7_PYRKO Mass: 18032 Score: 26 Expect: 1.9e+02 Queries matched: 3
RNA-binding protein, containing THUMP domain.- Pyrococcus kodakaraensis (Thermococcus kodakaraensis).

A72676 Mass: 13207 Score: 26 Expect: 2.1e+02 Queries matched: 3
probable GAGE-2 protein APE0829 - Aeropyrum pernix (strain K1)

---

6. A69215 Mass: 21136 Score: 35 Expect: 23 Queries matched: 4
conserved hypothetical protein MTH861 - Methanobacterium thermoautotrophicum (strain Delta H)

---

7. Q46CM4_METBF Mass: 13764 Score: 34 Expect: 32 Queries matched: 4
Transposase.- Methanosarcina barkeri (strain Fusaro / DSM 804).

---

8. F64370 Mass: 74407 Score: 33 Expect: 44 Queries matched: 5
ferrous iron transport protein B - Methanococcus jannaschii

---

9. E69250 Mass: 22570 Score: 32 Expect: 46 Queries matched: 4
hypothetical protein AF0005 - Archaeoglobus fulgidus

Q18KM4_9EURY Mass: 13484 Score: 28 Expect: 1.4e+02 Queries matched: 3
Hypothetical protein.- Haloquadratum walsbyi.

Q6LZB0_METMP Mass: 15543 Score: 27 Expect: 1.4e+02 Queries matched: 3
Predicted transcriptional regluator.- Methanococcus maripaludis.

---

10. F71076 Mass: 32347 Score: 31 Expect: 58 Queries matched: 4
hypothetical protein PH0878 - Pyrococcus horikoshii

---

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Search Parameters
Type of search          : Peptide Mass Fingerprint
Enzyme                  : Trypsin
Mass values             : Monoisotopic
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Protein Mass : Unrestricted

Peptide Mass Tolerance : ± 1 Da

**Peptide Charge State : 1+**

**Max Missed Cleavages : 1**

**Number of queries : 12**

**Mascot:** http://www.matrixscience.com/

# Mascot Search Results

**Protein View**
**Top of Form**

Match to: **gi|148820** Score: **54** Expect: **0.34**
**Mn-superoxide dismutase**

Nominal mass ($M_r$): **22399**; Calculated pI value: **4.33**
NCBI BLAST search of gi|148820 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Halobacterium sp. GRB

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **12**
Number of mass values matched: **5**
Sequence Coverage: **33%**

Matched peptides shown in **Bold Red**

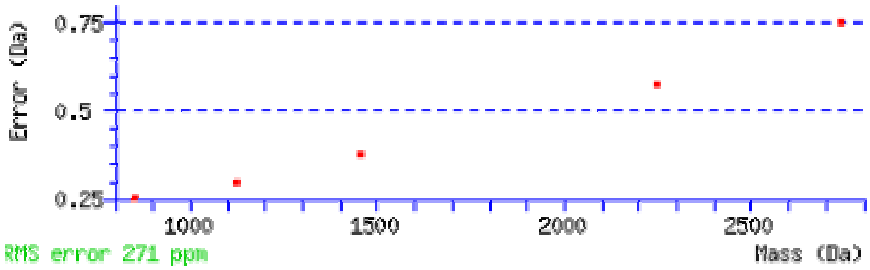
**1** MSEYELPPLP YDYDALEPHI SEQVLTWHHD THHQGYVNGW NDAEETLAEN
**51** RETGDHASTA GALGDVTHNG SGHILHTLFW QSMSPAGGDE PSGALADR**IV**
**101** **ADFGSYENWR AEFEAAASAA SGWALLVYDS HSNTLR**NVAV DNHDEGALWG
**151** SHPILALDVW EHSYYYDYGP DR**GSFVDAFF EVVDWDEPTE RFEQAAERFE**
**201**

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

| Start - End | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Sequence |
|---|---|---|---|---|---|---|
| 99 - 110 | 1457.0650 | 1456.0577 | 1455.6782 | 0.3795 | 0 | R.IVADFGSYENWR.A |
| 111 - 136 | 2738.0720 | 2737.0647 | 2736.3143 | 0.7505 | 0 | R.AEFEAAASAASGWALLVYDSHSNTLR.N |
| 173 - 191 | 2245.5860 | 2244.5787 | 2244.0011 | 0.5777 | 0 | R.GSFVDAFFEVVDWDEPTER.F |
| 192 - 198 | 850.6580 | 849.6507 | 849.3980 | 0.2527 | 0 | R.FEQAAER.F |
| 192 - 200 | 1126.8160 | 1125.8087 | 1125.5090 | 0.2997 | 1 | R.FEQAAERFE.- |

**No match to:** 568.2320, 618.1880, 1191.9040, 1413.0530, 1489.1510, 1602.1450, 2261.7100




Bottom of Form

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LOCUS AAA73373 200 aa linear BCT 21-MAY-1993
DEFINITION Mn-superoxide dismutase.
ACCESSION AAA73373
VERSION AAA73373.1 GI:148820
DBSOURCE locus HALSODC accession M97484.1
KEYWORDS .
SOURCE Halobacterium sp. GRB
ORGANISM Halobacterium sp. GRB
Archaea; Euryarchaeota; Halobacteria; Halobacteriales;
Halobacteriaceae; Halobacterium.
REFERENCE 1 (residues 1 to 200)
AUTHORS Joshi,P. and Dennis,P.P.
TITLE Characterization of paralogous and orthologous members of the
superoxide dismutase gene family from genera of the halophilic
archaebacteria
JOURNAL J. Bacteriol. 175 (6), 1561-1571 (1993)
PUBMED 8449865
COMMENT Method: conceptual translation.
FEATURES Location/Qualifiers
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/function="dismutation of superoxide free radicals"
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Region 3..84
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/db_xref="CDD:40182"
Region 89..192
/region_name="Sod_Fe_C"
/note="Iron/manganese superoxide dismutases, C-terminal domain; p fam02777" /db_xref="CDD:42733"

CDS 1..200
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/standard_name="'superoxide dismutase gene'"
/coded_by="M97484.1:175..777"
/transl_table=11

**Mascot:** http://www.matrixscience.com/

**Fig.42: Laboratory of Mass Spectrometry and Gaseous Ion Chemistry**

**PROFOUND - PROWL**


Top of Form

Protein Candidates

| Rank | Expectation | Protein Information and Sequence Analyse Tools (T) | % | pI | kDa | R |
|---|---|---|---|---|---|---|
| 1 | 0.15 | gi\|148820\|gb\|AAA73373.1\| Mn-superoxide dismutase | 33 | 4.3 | 22.39 | |

NOTE:

1. To search again using unmatched masses, click the symbol .

Input Summary

| | |
|---|---|
| Search id | B5EE2521-04D0-BFD2A4A3 |
| Sequences | 1941819 |
| Date & Time | Fri Oct 27 09:49:18 2006 UTC (Search Time: 16.27 sec.) |
| Sample ID | hs30k [Pass: 1] |
| Database | NCBInr [..\databases\nr] |
| Taxonomy | All-taxa |
| Mass Range | 0 - 35 kDa |
| pI Range | 0.0 -6.0 |
| Digestion | Trypsin |
| Missed Cuts | 1 |
| Modifications | +O@M(Partial); |
| Charge State | MH+ |
| Masses (avg) | |
| Tolerance (avg) | 1.00 Da |
| Masses (mon) | 568.232 850.658 1126.816 1165.976 1225.986 1411.032 1435.988 1457.065 1473.087 1476.128 1489.151 1497.122 1639.277 1701.265 1717.269 1756.135 1932.328 2020.513 2048.476 2079.459 2163.542 2245.586 2261.710 2273.674 2384.599 2738.092 2809.134 2831.997 |
| Tolerance (mon) | 1.00 Da |
| Number of Peptides | 28 |

# Fig. 43:MS-Fit Search Results

## Parameters

Database searched: **SwissProt.2005.01.06**
Digest Used: **Trypsin**
Max. # Missed Cleavages: **1**
Cysteine Modification: **acrylamide**
Instrument Name: **MALDI-TOF**
Sample ID (comment): **Magic Bullet digest**
Minimum Matches: **4**
Sort Type: **Score Sort**
Considered modifications: | **Oxidation of M** |
Min Parent Ion Matches: **1**
MOWSE On: **1**
MOWSE P Factor: **0.4**

## Pre Search Results

Number of entries in the database: **161074**
Molecular weight search (**20000 - 35000 Da**) selects **41644** entries.
pI search (**4.00 - 6.00**) selects **64000** entries.
Combined molecular weight and pI searches select **16859** entries.
Pre searches select **16859** entries.

## Data Set 1 Result

MS-Fit search selects **123** entries (results displayed for top **5** matches).

### Results Summary

| | MOWSE Score | #/22(%) Masses Matched | % Cov | % TIC | Mean Err Da | Data Tol Da | MS-Digest Index # | Protein MW (Da)/pI | Accession # | Species | Protein Name |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 546 | 5 (22) | 33.0 | 22.7 | 0.456 | 0.430 | | 22413/4.3 | | HALSG | Superoxide dismutase [Mn] 1 |
| 2 | 269 | 4 (18) | 21.8 | 18.2 | 0.110 | 1.26 | | 30204/5.6 | | RHISN | Hypothetical 30.2 kDa protein y4oV |
| 3 | 183 | 4 (18) | 27.0 | 18.2 | 0.475 | 0.487 | | 22385/4.3 | | HALN1 | Superoxide dismutase [Mn] 1 |
| 4 | 133 | 4 (18) | 17.1 | 18.2 | 0.139 | 1.00 | | 30119/5.6 | | DEIRA | Pantoate--beta-alanine ligase (Pantothenate synthetase) (Pantoate |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Activating enzyme) |
| 5 | 126 | 5 (22) | 26.4 | 22.7 | -0.111 | 0.964 | 26459/5.4 | HUMAN | N-terminal acetyltransferase complex ARD1 subunit homolog |

**144679** . x x . . . x . . . . . . . . . . x . . . x
**156133** . . . . . . . . . . . . . . x . . x x . . x
**144492** . x x . . . . . . . . . . . . . x . . . x
**92390** . . . . x . . . . . x . . . . x . . x . . .
**148622** . . . x . x . . . . . . . . . . x x x . .

---

## Detailed Results

**1.** 5/22 matches (22%).
**Acc. #:** **Species:** HALSG **Name:** Superoxide dismutase [Mn] 1
**Index:** **MW:** 22413 Da **pI:** 4.3

| m/z Submitted | $MH^+$ Matched | Delta Da | Modifications | Start | End | Missed Cleavages | Sequence | |
|---|---|---|---|---|---|---|---|---|
| 850.6580 | 850.4054 | 0.25 | | 192 | 198 | 0 | (R) | (F) |
| 1126.8160 | 1126.5164 | 0.30 | | 192 | 200 | 1 | (R) | (-) |
| 1457.0650 | 1456.6856 | 0.38 | | 99 | 110 | 0 | (R) | (A) |
| 2245.5860 | 2245.0084 | 0.58 | | 173 | 191 | 0 | (R) | (F) |
| 2738.0920 | 2737.3216 | 0.77 | | 111 | 136 | 0 | (R) | (N) |

The matched peptides cover **33.0%** (66/200AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #):

### 3.4: Activity assay for $Mn^{2+}$- SOD with HS30 K protein by fluorescence titration

To confirm that HS30 K protein corresponds to $Mn^{2+}$- SOD experiments have been carried to see the binding of $Mn^{2+}$ to HS 30 K protein and to assay the activity for Mn – SOD by gel method. Since it is known that $Mn^{2+}$ –SOD binds manganese ion, fluorescence titration of HS30 K protein with increasing concentration of $Mn^{2+}$ was carried out to see if binding of $Mn^{2+}$ will change fluorescence emission property of HS30K protein results presented in fig.44 and 45 showed that increasing concentration of $Mn^{2+}$ ions resulted in dramatic increase of fluorescence emission by the protein. These result indicate that HS30 K protein not only binds $Mn^{2+}$ions but also that the binding of $Mn^{2+}$ induces large conformational change in the protein.

#### 3.4.1: Gel assay for $Mn^{2+}$ – SOD with H.s 30 K protein

SOD gel assay was carried out as described in methods section. The incubation of the gel in assay buffer showed presence of clear zone of the band at the place of HS30 K protein (Fig. 46) confirming that HS30K protein with DNA binding activity corresponds to $Mn^{2+}$ – SOD of Halobacterium.

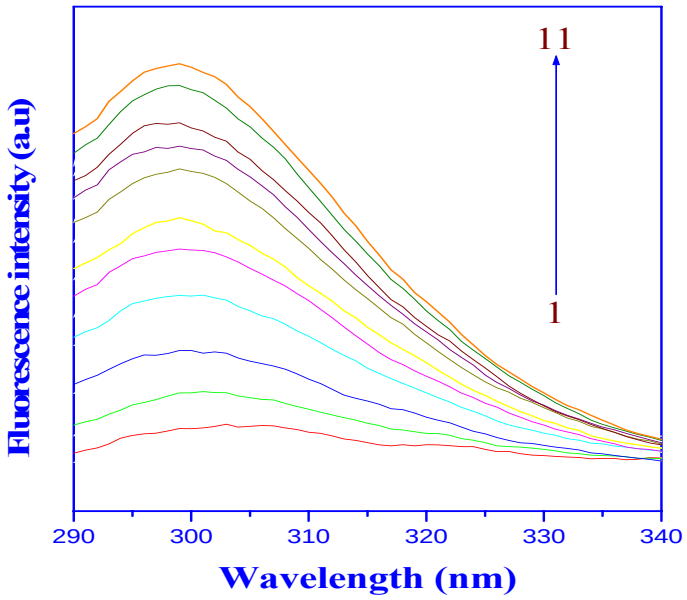


**Fig. 44: Activity assay for $Mn^{2+}$- SOD with HS30 K protein by fluorescence titration**
Aliquots of Manganese chloride ( 1 µl each equal to 1 mM $MNCl_2$ ) were added to a fixed concentration of HS30K protein (25 µg) in 2 ml of reaction buffer (20 mM Tris – HCl pH 7.6 and 500 mM ) and the change in fluorescence intensity was measured between 285 nm to 340 nm.

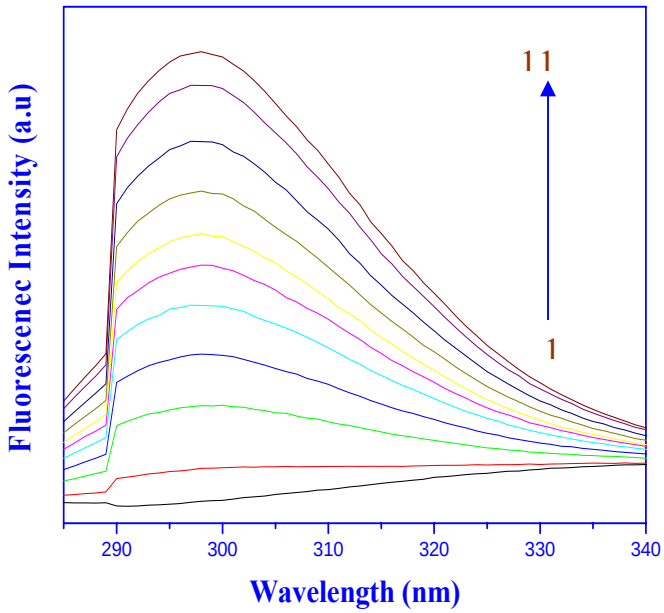


**Fig. 45: Activity assay for $Mn^{2+}$- SOD with HS30 K protein by fluorescence titration**
Aliquots of Manganese chloride ( 1 µl each equal to 1 mM $MNCl_2$ ) were added to a fixed concentration of HS30K protein (25 µg) in 2 ml of reaction buffer (20 mM Tris – HCl pH 7.6 and 2 M ) and the change in fluorescence intensity was measured between 285 nm to 340 nm

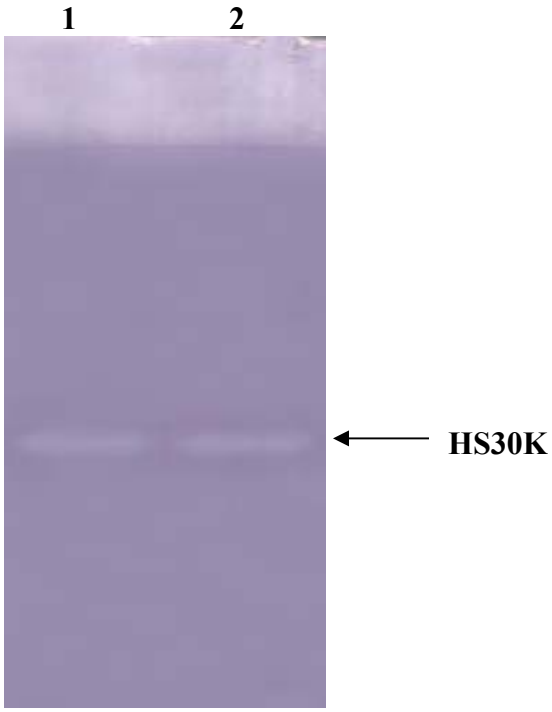


**Fig. 46: Gel assay for $Mn^{2+}$ – SOD activity with H.s 30 K protein**

Poly acrylamide gel analysis of superoxide dismutase activity associated with HS30K protein. Protein sample examined for superoxide dismutase activity by the NBT gel assay.

Lane1 and 2: Equal amounts (5 µg) of the protein loaded on the polyacrylamide gel under non denaturing conditions.

## DISCUSSION

The present investigation deals with the studies on nucleoid from Halophilic euryarchaeota, *Halobacterium salinarium.* The characterization of nucleoid (chromatin) from this Halophilic archaeon was carried because of the reported chromatin like structure of part of the nucleoid from this organism (Shioda *et al 1989).* There are no reports on the biochemical characterization of the nucleoid from halophilic archaea. Hence we wanted to see whether Halobacterium possess protein with histone domain like those seen in *Methanococcus fervidus,* HMf A and HMf B (Sandman et al 1990) or the organization is more like nucleoid of bacteria and other thermophilic archaea. Intracellular DNA in all cell types is in condensed state with the help of several proteins. However, the type of condensation is different in different type of cells. In eukaryotic cells the DNA is organized into chromatin with repeating nucleosomal structure (Kornberg, 1974, Arents and Moudrianakis, 1993). This feature is invariably present in all the eukaryotic cells studied. Intracellular DNA in prokaryotes is organized into ill defined rapidly sedimenting condensed structure called nucleoid. Several studies on *E.coli* and other bacteria showed lack of nucleosomal structure in bacterial nucleoid, but contain different domains condensed by variety of different DNA binding proteins (Gaziev et al, 1986) and also by macromolecular crowding (Zimmerman 2002 and Zimmerman 2004). However no defined organization can be described for bacterial nucleoid and no distinct role of different proteins in DNA condensation is reported.

In case of archaea it has been reported that euryarchaeote *Methanococcus fervidus* contained proteins named HMf A and HMfB contain histone domain and interact with DNA to form nucleosomal structure (Sandman *et al*. 1990 and Reeve 1999). In Crenarchaeota like thermophilic *Sulfolobus*, no histone homologue have been found in spite of the presence of small DNA binding protein which compact DNA ( Kimura *et al.,* 1984, Reddy and Suryanarayana 1989). The organization of intracellular DNA in thermophilic Crenarchaea is nucleoid – like as in bacteria (Zeenath 2005).

Nucleoid (Chromatin) was isolated from *H.salinarium* by the method described by Shioda *et al.* (1989) as well as the method developed in our laboratory for the isolation of thermophilic archaeal nucleoid (Reddy and Suryanarayana, 1988). These nucleoid preparations were analyzed for the protein and nucleic acid composition and by sensitivity to micrococcal nuclease.

Sucrose gradient centrifugation of lysates resolved into two nucleoid components as described by Shioda *et al*. (1989). The nucleoid components showed growth phase dependent variation in the yield and in nucleic acid and protein composition. Peak I fraction was more with more amount of protein and nucleic acid than peak II (rapidly sedimenting) from midlogarithmic phase cells on the contrary peak II fraction from stationary phase cells was more than peak I fraction with higher amounts of the protein and DNA. Substantially low amounts of both protein and nucleic acids were seen in peak I fraction from stationary phase cells. The peak I fraction may represent membrane associated nucleoid fraction where greater amount of DNA may be in association with membrane in the case of rapidly dividing midlogarithmic phase cells. Preliminary results showed presence of membrane proteins in peak I nucleoid fraction. Both these peak fractions were subjected to agarose gel electrophoresis for nucleic acid analysis and SDS – PAGE for protein composition. Nucleoid from stationary phase cells showed presence of some additional proteins in the lower molecular weight region (14 to 16 kDa). An interesting feature was seen in case of nucleoid from late stationary phase cells, the nucleoid did not resolve into two fractions. There was overall decrease in the amount of nucleic acid and protein in the nucleoid from late stationary phase (resting cells). Low molecular weight protein (< 20 kDa) was totally absent in nucleoid fraction from late stationary phase cells.

Nucleoid fractions obtained from the sucrose density gradient centrifugation and sucrose cushion centrifugation were subjected to MNase digestion. Total nucleoid and both the gradient nucleoid fraction produced two components which can be resolved by S -200 gel filtration chromatography.

Micrococcal nuclease digestion was performed on different nucleoid fractions viz. sucrose gradient peak I and peak II nucleoid fraction. In each case micrococcal nuclease digest was fractionated by gel filtration as sephacryl S – 200 column. The elution profile showed resolution into two peaks (S - 200 peak I and S - 200 peak II) with both the micrococcal nuclease digests (of peak I nucleoid fraction and peak II nucleoid fraction). S – 200 peak I fraction obtained by MNase digestion of gradient peak I and peak II chromosomal fraction contained different sets of proteins: a 30 kDa protein and some higher molecular weight proteins in peak I and decreased 30 kDa protein and a 29 kDa protein along with lower molecular weight proteins respectively. Lower molecular weight DNA bands were present in both the peaks ( S – 200 peak I and S-200 peak II) obtained after MNase digestion of chromosomal peak I and peak II fractions. However, there was differential distribution of this low molecular weight DNA in S – 200 peak I and peak II fraction. There was decreased amount of DNA in S -200 peak I fraction obtained after MNase digestion of chromosomal peak I fraction compared to S – 200 peak I fraction obtained after MNase digestion of chromosomal peak II fraction. Similarly S -200 peak II from MNase digestion of gradient chromosomal peak II contained decreased DNA fragments than S-200 peak II obtained from MNase digestion of chromosomal peak I fraction. Differential distribution of DNA and proteins in the different MNase digestion products indicate that different proteins bound to different regions of DNA in the nucleoid. This interpretation was confirmed by MNase digestion of total nucleoid isolated from both midlogarithmic and stationary phase cells.

MNase digest of total nucleoid also resolved into two peaks upon Sephacryl S - 200 column chromatography. S – 200 peak I fraction in both cases contained higher molecular weight DNA and peak II fraction low molecular weight (200 bp) DNA fragments. S - 200 peak II from stationary phase total nucleoid has more amounts of DNA fragments than S -200 peak II from midlogarithmic phase total nucleoid. S – 200 peak I fraction from both types of cells has higher molecular weight proteins in abundance and S – 200 peak II fraction contained low molecular weight proteins in abundance along with 30 kDa protein ( HS30K protein) which was present in both peaks with higher amount in the peak II fraction. These results also suggest difference in the

distribution of proteins associated with the DNA. In eukaryotic cells MNase digestion results in production of different sized DNA fragments all of which are associated with histone proteins in constant amount. These results also suggest lack of histone like proteins. *H. salinarium* nucleoid did not contain any acid extractable low molecular weight basic proteins (results not shown).

We have purified a 30 kDa protein because it is present in different nucleoid fractions as one of the major protein. The protein has molecular weight of 30 kDa as determined by SDS – PAGE and pI of 4.3. In the purified nucleoid fraction obtained by reverse salt gradient on S – 1000 column, the 30 kDa (HS30K) protein is present in larger amounts than other nucleoid associated proteins.

The HS30K protein is a major nucleoid associated protein. The protein was characterized and its nucleic acid binding properties were studied. The U.V absorption spectral properties suggest that the protein has all the three aromatic amino acids with greater contribution of phenylalanine and tyrosine to the absorbance as indicated by derivative spectrum. Fluorescence emission intensity measurements also showed the same result. The protein undergoes salt dependent conformational changes. The fluorescence emission decreased with increasing salt concentration. C. D spectroscopy of the protein also showed salt dependent conformational change as indicated by alteration in secondary structural elements. The œ helical structure which was dominant at low salt concentration decreased at high salt concentration. The ß sheet structure was the major secondary structural element at higher salt concentration.

The purified protein binds double stranded DNA, single stranded DNA and single stranded RNA as measured by gel mobility shift analysis and fluorescence titration experiments. At higher salt concentration there was an increase in the affinity of the protein as seen by increased retardation in mobility shift as well as increased fluorescence quenching by the nucleic acid. Overall the protein has greater affinity to single stranded DNA than double stranded DNA. The protein binds synthetic RNA with lower affinity than ss DNA and ds DNA.

We have also tested the ability of HS30 K protein to unwind ds DNA since it showed greater affinity to single stranded DNA. U. V. absorbance of nucleic acids with increasing protein at low and higher salt showed increase in the U. V absorption (hyperchromicity) of the nucleic acid. The hyperchromicity was greater at higher salt (2 M NaCl) than low salt concentration which indicates the ability of the protein to unwind the duplex DNA at higher salt concentration. The unwinding property of the HS30K protein is very similar to T 4 gene 32 protein and *E. coli* ssb protein (Weaver, 2003) with the difference that the protein can unwind at very high salt concentration (2M NaCl).

In order to identify the protein we performed MALDI – TOF analysis after trypsin digestion. The peptide mass finger printing data was used to search different data bases to identify the protein, if possible. We were surprised that the protein showed similarity to $Mn^{2+}$ - Superoxide dismutase of *H. salinarium* and *Halobacterium NRC – 1*. The results showed matching of 5 major peptides obtained with data bases (MSDB, NCBI nr). Mascot, Profound Prowl and MS fit search results showed similarity to Mn 2+ - SOD. The experimentally determined pI exactly matches with Mn – SOD. The molecular weight from mass spectrum data with intact protein was 22190 which is exactly same as molecular weight of $Mn^{2+}$ - SOD from Halobacteria. The mobility corresponding 30 kDa in our SDS – PAGE could be due to anomalous behavior of the protein during SDS – PAGE. The sequence of $Mn^{2+}$ - SOD showed that the protein with 200 amino acids has characteristic distribution of basic amino acids (R, K). There are only two R residues from the N – terminal to 98 amino acid residue. Cleavages at these two R would have produced very large peptides of mass 5,500 which are out of the range in our present PMF. The major peptides matched cover the most of the remaining half of the protein (from residue no 98 to 200). We wanted to see if the protein corresponds to $Mn^{2+}$ - SOD. Experiments were carried out with purified HS30 K protein to see if the protein has binding sites for $Mn^{2+}$ and has SOD activity. The results showed unambiguously that the protein binds strongly to manganese ion and that the protein indeed has SOD activity as shown by activity gel assay. So the $Mn^{2+}$-SOD of *Halobacterium* is DNA binding protein with unwinding property. These results also suggested that the HS30K is $Mn^{2+}$- SOD and it is associated with halobacterial nucleoid. It is appropriate to mention here that

Steinman *et a*l. (1994) have reported that the $Mn^{2+}$ – SOD of *E. coli* K-12 associates with DNA *in vitro* and that the enzyme is co localized with *E. coli* nucleoid. $Mn^{2+}$ – SOD associated in greater amounts with the nucleoid in aerobically grown *E. coli* and may be protecting the DNA against oxidative damage (Steinman *et al* 1994). The high salt dependent DNA unwinding activity of HS30K protein may be physiologically relevant to unwind the DNA and stabilize the single stranded DNA for DNA replication and transcription at the same time protecting the DNA from oxidative damage in Halobacteria.

## SUMMARY

Thesis includes 6 chapters.

**Chapter 1: Introduction**

A review of the cell and molecular biology of the archaea, their classification and characteristics followed by a review of the histone like nucleoid proteins present in the eubacteria and archaea is covered in this chapter. This is followed by objectives and scope of present investigation.

**Chapter 2: Materials and Methods**

This chapter lists the materials used in the study and a brief description of the methods used during the course of the investigation. Isolation and purification protocol for the nucleoid, nucleoid associated DNA binding proteins are presented. The nucleoid was investigated and its components were determined and analyzed by Micrococcal nuclease digestion. The changes in the nucleoid composition during the growth phases were determined. The nucleic acid binding properties of 30 KDa nucleoid protein were studied by spectrophotometry, fluorimetry, gel mobility shift assay, and CD spectroscopy.

## Chapter 3: Results

**Biochemical characterization of Haloarchaeal nucleoid from *Halobacterium salinarium H3.***

This chapter deals with the results were obtained in the present investigation.

**Growth phase dependent variation of the nucleic acid and protein composition of the *Halobacterium salinarium H3.***

**Isolation of nucleoid by sucrose density gradient centrifugation**

Centrifugation of lysates obtained from midlogarithmic phase and stationary phase cells on sucrose gradient resulted in resolution of nucleoid into two components peak I and peak II (rapidly sedimenting fraction). The overall yield of the two peaks showed growth phase dependent variation. Peak I was higher than peak II in midlogarithmic phase whereas peak II was higher than peak I in stationary phase i.e. there was a decrease in peak I in stationary phase as compared to peak I in midlogarithmic phase. Both nucleic

acid content and protein content in the two fractions were analyzed. There was reduction of both protein and nucleic acid (DNA) content in peak I obtained from stationary phase cells and there was an increase in both protein and nucleic acid (DNA) contents in peak II obtained from stationary phase cells.

SDS - Polyacrylamide gel electrophoresis was performed to analyze protein present in peak I and peak II of midlogarithmic and stationary phase cells. Nucleoprotein complexes and nucleic acids from peak I and II were analyzed by agarose gel electrophoresis. Proteins in the molecular weight range 10 KDa to 110 KDa were present in both the peak fractions of the nucleoid. Peak I from stationary phase contained fewer proteins in decreased amounts. Higher molecular weight DNA along with low molecular weight smear of DNA was present in both the peak fractions.

**Micrococcal nuclease digestion of nucleoid**

Micrococcal nuclease digestion of 30 % sucrose cushion isolated nucleoid from both midlogarithmic phase and stationary phase cells was carried out and digestion products were analyzed by agarose gel electrophoresis. Agarose gel analysis of MNase digested products of nucleoid from 5 days cells showed that smear like DNA bands with decreasing in size were observed with increasing incubation time. After deproteinisation of MNase digested products, DNA bands of approximately 200 bp length DNA were observed in all lanes. MNase digested products of nucleoid from stationary phase cells also showed similar results as in midlogarithmic phase cells but deproteinised samples showed decrease in size of the DNA bands.

**Isolation of MNase digested products of nucleoid by Sephacryl S – 200 column chromatography**

Peak I and peak II fractions obtained by sucrose density centrifugation of nucleoid from stationary phase cells were treated with Micrococcal nuclease. The MNase digested products were chromatographed on Sephacryl S – 200. The chromatography of these fractions on S – 200 resulted in resolution again into two peaks. S – 200 column fractions were analyzed by SDS – PAGE for protein and agarose gel electrophoresis for nucleic

acids. Peak I faction in both the cases contain several proteins in the range of 60 KDa – 14 KDa and peak II fraction contained the abundant amount of 30 KDa. DNA content in all these fractions analyzed by agarose gel electrophoresis which showed in the presence of DNA fragments in the size of 200 bp.

Sephacryl S -200 column chromatography of MNase digested products of nucleoid isolated by ultra centrifugation using 30 % sucrose cushion from midlogarithmic phase and stationary phase cells also showed two peaks (peak I and peak II). Protein and DNA were analyzed by SDS – PAGE and agarose gel electrophoresis. Similar results were obtained as described before for MNase digestion of peak I and Peak II fraction isolated by sucrose gradient centrifugation. However the initial eluted fractions of S – 200 column contained heterogenous higher molecular weight DNA. These results indicated that both the nucleoid fraction from Halobacterial cells have no nucleosomal structural organization and have no histone protein homologues.

## Purification of 30 KDa nucleoid protein and characterization of its DNA binding properties

A 30 KDa protein was present along with DNA in the entire nucleoid fraction isolated. This protein was purified to homogeneity and characterized.

**Isolation of nucleoid**

Nucleoid was isolated from freshly harvested midlogarithmic phase cells using non ionic detergents by centrifugation through a 30 % sucrose cushion.

**Purification of nucleoid by Sephacryl S- 1000 column chromatography:**

The nucleoid isolated on 30 % sucrose cushion was sheared by sonication and chromatographed on a sephacryl S – 1000 column, and the column was eluted with reverse salt gradient range between 3 M – 0.05 M potassium chloride. Nucleoprotein complexes were resolved into a broad single peak. Absorption spectra analysis indicates a higher protein to DNA ratio. Amount of protein, DNA, RNA present in S – 1000

nucleoid was in the ratio of 1: 0.6: 0.048. Total nucleoid has 2.5 mg of protein, 1.5 mg of DNA and 120 μg of RNA per ml of peak fraction. SDS – polyacrylamide gel electrophoresis of peak fractions showed presence of high and low molecular weight proteins ranging from 110 KDa to 10 KDa.

Nucleoprotein complexes from S – 1000 column nucleoid fractions were analyzed by agarose gel electrophoresis. High molecular weight aggregates which were slightly migrated as a smear from the wells and small smear like bands were observed at end of the gel indicates the presence of RNA in the nucleoid. Agarose gel analysis of RNase treated nucleic acid extracts from the peak fractions showed high molecular weight DNA bands migrated towards the middle of the gel. S -1000 nucleoid was also isolated from the stationary phase cells. Protein and nucleic acid contents were analyzed as before. S-1000 nucleoid of stationary phase cells contain 2 mg of DNA, 3.4 mg of protein and 285 μg of RNA per ml of peak fraction. Nucleic acids and protein concentrations are higher than the S – 1000 nucleoid of midlogarithmic phase cells. The 30 KDa nucleoid protein was present in all fractions hence we have choosen this 30 KDa protein for purification and characterization.

Sephacryl S – 1000 nucleoid pool fraction was desalted through dialysis and chromatographed on DE – 52 anionic exchange column. Elution was carried out with linear increasing salt gradient from 0.05 M – 0.75 M NaCl. SDS –PAGE analysis of column peak fractions showed the 30 KDa Protein eluting at 0.5 M NaCl concentration. The protein was purified by chromatography on a second DE – 52 column. Purified 30 KDa protein was again eluted at 0.5 M NaCl during the 0.05 M to 0.75 M NaCl increasing linear salt gradient elution. SDS – PAGE analysis of the purified 30 KDa proteins showed a single band on gel. To check the homogeneity of the protein we have performed isoelectric focusing and two dimentional gel electrophoresis. This protein has showed the P I value between 4.1 – 4.3 and gave single spot on 2-D gel electrophoresis.

**Molecular weight det ermination of H S nucleoid protein**

Molecular weight determination of *Halobacterium salinarium* nucleoid protein was carried out by electrophoresing on 18 % polyacrylamide gel in the presence of SDS as described by Thomas and Kornberg (1974). *Hs* nucleoid protein migrated corresponding a molecular weight of 30 KDa. Gel permeation chromatography was performed to determine the molecular weight of the purified nucleoid protein in solution. Purified protein loaded and eluted with same buffer. Similarly standard proteins were also chromatographed on Sephadex G – 100 column. Purified nucleoid protein was eluted at a volume corresponding to a Molecular weight of ~30,000, indicating monomeric state of the protein in solution.

**Spectroscopic properties of nucleoid protein**

Spectroscopic properties of the protein were also studied. UV absorption spectrum of 30 KDa protein was recorded between 230 nm – 330 nm. Absorption maximum showed of the protein was 280 nm. Fourth derivative U V absorption spectra of 30 KDa protein showed contribution of all aromatic amino acid to absorption with preponderance of phenylalanine and tyrosine residues.

Fluorescence emission spectrum of 30 KDa protein showed a broad spectrum from 295 nm to 330 nm with the maximum 310 nm, with the excitation wave length of 280 nm. Fluorescence emission spectrum of 30 KDa protein was measured at different salt concentrations (100mM, 500 mM, 1 M, 2 M) by increasing the salt concentration. The intrinsic fluorescence intensity has gradually decreased indicating conformational change of the protein at high salt concentration.

**CD spectra of purified DNA binding protein**

CD spectra of nucleoid protein were recorded using 1 mm path length cuvette and the buffer containing 50 mM and 2 M NaCl. CD spectra analysis of protein in presence of low salt and high salt showing change in secondary structure, at high salt concentration percent of œ helix is decreased with increase in ß sheet structure.

**Gel mobility shift assays**

Gel mobility shift assay were performed with protein – DNA complexes obtained after incubating pBR 322 plasmid DNA and lambda DNA with increasing concentrations of protein at four different salt concentrations (100 mM, 500 mM, 1 M, 2 M). The nucleoprotein complexes were electrophoresed on 0.8 % agarose gel electrophoresis. 30 KDa nucleoid protein binds to both super coiled and relaxed forms of pBR 322 and the mobility of nucleoid protein - pBR 322 DNA complexes were retarded in the gel. The retardation increases with increasing salt concentration. Gel mobility shift assay confirmed that the purified 30 KDa protein is a DNA binding protein and showed strong binding at high salt concentrations. Gel mobility shift analysis of 30 KDa nucleoid protein was also carried out with lambda DNA.

**Fluorescence titrations**

Nucleic acid binding properties of 30 KDa nucleoid protein was studied by fluorescence titrations. Direct fluorescence titrations were performed with CT DNA and increasing concentrations of protein at 50 mM and 2 M NaCl concentrations showed that more amount of protein is required to saturate the DNA at high salts indicating strong DNA binding of the protein. Reverse titrations were also performed by adding nucleic acids and synthetic polynucleotides to constant amount of protein and measuring the extent of fluorescence quenching. The quenched fluorescence spectrum were obtained by adding nucleic acids and polynucleotides (CT DNA, ss DNA, poly A, poly dA, poly C, poly dG, poly U, poly dT, poly dA –dT, poly dG- dC). The data with amount of ligand added vs % of quenching showed that more quenching was observed with poly dA, poly C and poly dC indicating strong binding of the protein to the single stranded nucleic acids.

**Circular dichroic spectra analysis of protein – DNA complexes**

Forward titrations of Lambda DNA and pBR 322 with increasing concentrations of HS nucleoid protein resulted in alteration of the CD spectrum of DNA with increasing salt concentrations. Indicating the protein more strongly binding to DNA at high salt concentrations and is showing it is stable at high salts. MALDI - TOF- TOF analysis is being carried out for the identification of the DNA binding protein. Spectroscopic experiments have also been carried out on the binding of protein with DNA. These results showed that the protein has strong DNA unwinding activity which may have important physiological role. The present study may help in understanding the interaction of proteins with DNA at high salt concentration.

**DNA unwinding studies of HS30K nucleoid protein**

Spectroscopic experiments have also been carried out on the binding of protein with DNA. UV absorption spectra of CT DNA and CT DNA incubated with increasing HS30K protein (5µg, 15µg and 25µg) were recorded between 230 nm to 320 nm in 20 mM Tris – HCl pH 7.6 and 25 mM NaCl. Titrations of lambda DNA (25 µg) (fig. 39) with increasing concentration of protein at different salt concentrations (50 mM, 1 M NaCl and 2 M NaCl) were carried out. These results showed that the protein has strong DNA unwinding activity which may have important physiological role. The present study may help in understanding the interaction of proteins with DNA at high salt concentration.

**MALDI analysis and identification of HS30 KDa protein**

HS30 K was subjected to MALDI- TOF analysis to obtain peptide mass fingerprint. The analysis was carried out with purified protein at Indian Institute of Science, Bangalore. The data obtained with peptide Mass fingerprint (M 2 values) were used to search databases (MSDB and NCBI Nr) using software MASCOT search software (Matrix Science USA) and ProFound – prowl search software (Rockefeller University) as well as M S fit software (University of California) also identified the protein as $Mn^{2+}$– Superoxide dismutase ($Mn^{2+}$ – SOD).

**Activity assay for $Mn^{2+}$- SOD with HS30 K protein by fluorescence titration**

To confirm that HS30 K protein corresponds to $Mn^{2+}$- SOD experiments have been carried to see the binding of $Mn^{2+}$ to HS 30 K protein and to assay the activity for Mn – SOD by gel method. These result indicate that HS30 K protein not only binds $Mn^{2+}$ions but also that the binding of $Mn^{2+}$ induces large conformational change in the protein.

**Gel assay for $Mn^{2+}$ – SOD with H.s 30 K protein**

SOD gel assay was carried out as described in methods section. The incubation of the gel in assay buffer showed presence of clear zone of the band at the place of HS30 K protein (Fig. 46) confirming that HS30K protein with DNA binding activity corresponds to $Mn^{2+}$ – SOD of Halobacterium.

**Chapter 4: Discussion**

This chapter deals with the conclusions derived from and the implications of the results observed during the course of the investigation. Halobacterium Salinarium is an extreme Halophilic Archaebacterium was chosen to analyze its Chromatin structure and composition. On the basis of the chromosomal fraction by SDG centrifugation we conclude chromosomal structure of the bacteria in late exponential phase seem to be represent a form of chromosome that is in transit from non protein or low protein to protein associated form. In sucrose density gradient chromosomal fractions from 5 day cell cultures High MWt protein associated with DNA fragments were eluted in Peak I fraction and High MWt free DNA and Low molecular weight proteins were eluted in Peak II fractions. A 30 KDa nucleoid protein was predominately eluted in all fractions of MNase digested S-200 column and SDG centrifugation. Micrococcal nuclease digestion studies indicated that the nucleoid in H.Salinarium is occurred in condensed structure with no repetitive nucleosome like structure. DNA fragments in size of 200 bp are obtained when digested with nucleases which correspond to the DNA length in the MNase digested core. Halobacterial proteins have been notoriously difficult to study because many of the conventional separation methods do not perform well at high salt. While small number of Halobacterial proteins can be purified in the presence of salt allowing a wide range of separation techniques. We could isolate the nucleoid and purify

a 30 KDa DNA binding protein by S-1000 column chromatography eluted by reverse salt gradient range between 3M – 0.07M NaCl followed by DE-52 column chromatography. Gel mobility shift assay confirmed that the purified 30 KDa protein is a DNA binding protein and showed strong binding at high salt concentrations. Fluorescence Spectroscopic analysis suggests that this protein is rich in tryptophan residues as it emission maximum at 310 nm. Quenching of Intrinsic fluorescence experiments showed that HS nucleoid protein binds to ss DNA and ds DNA with comparable affinity. Forward titration of Lambda DNA with HS nucleoid protein followed by UV absorption at A260 showed that DNA unwinding property. CD spectroscopic analysis of the protein in the presence of low salt and high salt showing change in the second order structure, decreasing in % of Alfa helix, and increasing in % of beta sheet. The unwinding property of the HS30K protein is very similar to T 4 gene 32 protein and *E. coli* ssb protein (Weaver, 2003) with the difference that the protein can unwind at very high salt concentration (2M NaCl). In order to identify the protein we performed MALDI – TOF analysis after trypsin digestion. The peptide mass finger printing data was used to search different data bases to identify the protein, if possible. The results showed unambiguously that the protein binds strongly to manganese ion and that the protein indeed has SOD activity as shown by activity gel assay. So the $Mn^{2+}$-SOD of *Halobacterium* is DNA binding protein with unwinding property. These results also suggested that the HS30K is $Mn^{2+}$- SOD and it is associated with halobacterial nucleoid. $Mn^{2+}$ – SOD associated in greater amounts with the nucleoid in aerobically grown *E. coli* and may be protecting the DNA against oxidative damage (Steinman *et al* 1994). The high salt dependent DNA unwinding activity of HS30K protein may be physiologically relevant to unwind the DNA and stabilize the single stranded DNA for DNA replication and transcription at the same time protecting the DNA from oxidative damage in Halobacteria.

**Chapter 5: Summary**

It includes the summary of the each chapter.

**Chapter 6: References**

This chapter includes list of references cited in different chapters in an alphabetical order

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