A study on the salt adaptation strategies in a halophile, *Rhodovibrio salinarum* JA137

A thesis submitted to the University of Hyderabad for the award of

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By

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DECLARATION

I **Paramata Bhavani Devi**, here by declare that this thesis entitled **"A study on the salt adaptation strategies in a halophile, *Rhodovibrio salinarum* JA137**" submitted by me under the guidance and supervision of **Dr. J. S. S. Prakash** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"A study on the salt adaptation strategies in a halophile, *Rhodovibrio salinarum* JA137"** is a record of bonafide work done by **Ms. Paramata Bhavani Devi**, a research scholar for Ph.D. programme in Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma

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- *My Master*


Dedicated to
****
my Husband
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TABLE OF CONTENTS

Abstract

The objective is to comprehend, how an environmental disturbance would influence the behavioral patterns of an organism at the molecular level, its cellular networks and ultimately its phenotype. However this would be an outstanding possibility in near future with the sophisticated insights gained into the unique ways of how the genome of an extremophile, dynamically ensembled into biological circuits in accordance to the ways it interacts with the environmental perturbations.

The objective is to exploit the information gained through the study of the adaptive strategies of extremophiles for engineering new biotechnological solutions to diverse problems spanning bioenergy, bioremediation and agriculture. In principle, the prime focus is to re-engineer novel capabilities by selectively combining otherwise unique biochemical potentials encoded in diverse genomes of extremophiles.

Rhodovibrio salinarum JA137 represents a class of potential halophiles with unexplored salt adaptive strategies and as such provides an explicit demonstration of how these endeavors can be used to investigate its response mechanisms. As we anticipate that this approach would provide a unique window into molecular mechanisms underlying fascinating response physiologies in the environmental extremes of salinity.

In particular, an outstanding trait exhibited by *Rhodovibrio salinarum* JA137 is its immense potential to thrive in an environment of very high salinity and can be expected to provide insights into evolutionary adaptation for survival in high salinity, which precludes growth and survival of other organisms. Based on its ability to thrive in unpredicted changes in environment, it is expected, to have regulatory circuits that quite efficiently negotiate these multifarious and often stressful surroundings. Consequently, we explored a significant fraction of the intercoordination of physiological processes in strain JA137 in differing salinity conditions.

The diverse array of life forms surviving because of their unique survival strategies has become the center stage of focus today in science. The diverse life forms in different adversities are making survival possible. This is gaining impetus with increasing environmental demands coming to the fore. The consortia of microorganisms displaying their outstanding capabilities to survive the extremities of nature has nurtured hope which can be well exploited for engineering molecular capacities in the life forms at the optimum parameters of survival. Exploring into their extreme possibilities of survival, helps us gain knowledge and insights into the molecular evolutionary

patterns, protein signatures, structural and genetic makeup. Elucidation of their adaptive strategic details might pave the way for avenues of that knowledge, exploited for an array of scientific endeavors striving for excellence and bridge many gaps in areas of agriculture and industries.

Our study on the salt adaptive strategies of this halophile *Rhodovibrio salinarum* JA137 is also an endeavor embarked on to gain insights into evolutionary molecular patterns shaped up to survive the salt stress. We investigated the difference in the modes of survival by the strain JA 137 at such high salinity conditions when compared to an array of survival strategies exhibited by the huge consortia of microorganisms already unravelled and yet to be explored. Our prime focus is on the aspects of synchronization that is unique to this organism during the stress episode among the cellular, molecular, structural and physiological compartments. The aspect which stimulates are the different tactics employed for thriving in high salt. Therefore, we have attempted to investigate the survival tactics of the strain JA137 at molecular level by cloning of the chaperonins *GroESL* to make comparative study of the molecular chaperones and chemical chaperones of halophiles. The response physiology of this organism was explored by studying its growth patterns at different salt concentrations, and in the presence of osmolytes, biofilm in varying salinity ranges, halites, and the influence of the salinity on the morphology in different salt concentrations. Examined the impact of high salt on the integrity of photosystem, lipid and fatty acid profiles, ROS (reactive oxygen species) and investigated the impact of osmolytes on these aspects. Observations of recovery of the high salt stressed cells of the strain JA 137 when the medium was supplemented with osmolyte glycine betaine, during the process of investigation of morphology, growth, photosystems became evident. This observation had left with clues of its inherent molecular potential to cope with high salt and its ability to cope with stress. We observed distinct patterns of growth with varying medium salinity and recovery from stress as reflected in their recovered morphology, growth in the presence of glycine betaine, carotenoids, recovery of the photosystem integrity which emphasized its innate abilities of adaptations.

Genome walking methodology resulted in cloning the partial length of the *GroESL* gene of *Rhodovibrio salinarum* JA137 which showed almost 70% similarity with the *GroESL* gene sequence of the close relative *Rhodospirillum rubrum*. Our conclusion is that might be the *groESL* of *Rhodovibrio salinarum* JA137might be a shorter fragment or the *GroEL* C- terminal end is missing not showing match with *groEL* of *Rhodospirillum rubrum* which can be unravelled only with further insights with complete genome sequencing of *Rhodovibrio salinarum* JA137.

ABBREVIATIONS

ATP	Adenosine triphosphate
Da	Dalton
DEAE	Diethyl amino ethyl cellulose
ABRC	Anaerobic Bacterial Resource Center
APB	Anoxygenic phototrophic bacteria
ATCC	American Type Culture Collection
BChl	Bacteriochlorophyll
BLAST	Basic Local Alignment Search Tool
DMSO	Dimethylsulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EMBL	European Molecular Biology Laboratory
ICM	Intracytoplasmic membrane
JCM	Japan Collection of Microorganisms
MEGA	Molecular Evolutionary Genetic Analysis
MLSA	Multilocus sequence analysis
NBRC	NITE Biological Resource Center
NCBI	National Center for Biotechnology Information
PHYLIP	Phylogeny inference package
PNSB	Purple non-sulfur bacteria
PSB	Purple sulfur bacteria
Q	Ubiquinone
RC	Reaction center
RQ	Rhodoquinone
TLC	Thin layer chromatography
TMAO	Trimethylamine-N-oxide
SEM	Scanning Electron Microscopy
CLSM	Confocal Laser Scanning Microscopy
PSI	Photosystem I
PSII	Photosystem II

INTRODUCTION

1.0 Introduction

1.1 Extremophiles

Life flourishes in chemical and physical extremes. Environmental conditions beyond the optimal range of existence are considered as extremes and the organisms which thrive under such extreme conditions are called as extremophiles. Extremophiles include thermophiles, alkaliphiles, halophiles, barophiles, thermoacidophiles confined to the environmental extremes of temperature, pH, salt, pressure, which precludes normal life (Satyanarayana et al., 2005). The extremophiles distribution in varied environmental conditions implies their diversity in molecular and metabolic adaptive strategies. The extremophiles diversity also extends categorically to span all the three domains of life, Archea, Bacteria and Eukarya (Oren, 2008). However extreme conditions are dominated by the microbial world. These microbes tolerate a wide range of environmental extremities distinct from other life forms (Satyanarayana et al., 2005). The extreme conditions are optimal for extreme life forms but they are stressful for non extremophiles. This implies that the extremophiles have evolved the machinery in their cellular, molecular, structural compartments to survive the extremes. The exotic environments are detrimental for moderate life forms as they have their cellular and molecular machinery tuned stringently in accordance to the normal parameters of survival.(Fig .2) The extremophiles have unique physiologies that have been evolved to meet the challenges poised by the extremes. Microbes in adverse environmental conditions have to contend with an array of challenges which includes protein stability, function, fluctuating osmotic gradients, membrane fluidity, oxidative and DNA damage and cell lysis (Singh and Gabani, 2011). For example the extensively studied extremophiles like the Halophiles and thermophiles have been reported to have unique membrane compositions evolved to cope with the temperature and salty extremes (Albers et al., 2000). For example the thermopiles face the temperature extremes which lead to the damage of membranes and DNA. High temperatures lead to DNA denaturation and a temperature drop leads to damage of the cellular membranes as they serve as surfaces for life's myriad reactions to occur and also contain cell's content. Temperature is a critical parameter as the change in temperature's, like high temperature would lead to irreversible alterations in the structures of biomolecules and increased membrane fluidity (Averhoff and Muller, 2010). However numerous examples have signified that they have evolved enzymatic machinery to combat the extreme temperatures. *Pyrococcus furiosis* signifies an example of a thermophile living in temperature extremes above 98ºC. The polymerase chain reaction of molecular biology would not have been

possible without the enzyme DNA polymerase from a thermophile *Thermus aquaticus* (Rampelotto, 2010). Thermophiles have been reported to have developed discrete ways of heat stabilizing the proteins for survival by minimizing the surface energy of the protein, and by strategic packing of the hydrophobic groups of the protein, and increase in salt bridges and with an increase of chaperonin proteins (Singh et al.,2012).

Alkaliphiles survive the extremes of pH by maintaining near-neutral pH of their surroundings because pH 7 is optimal for biological reactions. The other common challenges which all kinds of extremophiles have to overcome and survive, are the protein conformations and membrane integrity in extremes of pH, temperature, radiation and salt (Reed et al., 2013). The extremophiles ought to maintain the surface of the proteins with a negative charge by a high ratio of acidic to basic amino acids in order to prevent denaturation, aggregation of proteins, thus helps establish a solvated protein environment in high salt (Song and Gunner, 2014) The extraordinary tolerance mechanism accomplished by a unique repair mechanism to repair its damaged DNA exposed to high radiation by *Deinococcus radiodurans* serves as excellent example of an extremophile (Harada et al., 2003). Temperature, pH, osmotic balance all vital determinants of survival as any fluctuation might impair key physiological functions and thereby the cumulative effects of these changes metabolically lead to reduction in viability (De Champdore et al., 2007; Reed et al., 2013). Therefore the stress disturbance brings about a series of changes across the cell and its various compartments molecular, structural and cellular as a hint to facilitate the organism to cope accordingly with the kind of stress. The organism under stress responds by means of changes brought about by activation of stress genes, chaperones, osmolytes, upregulation and down regulation of genes having contributions in the maintenance of membrane integrity, the respective protein conformations, required lipid synthesis (Fig.2). The extremophiles survive in similar conditions as they harbour an array of inherent resistance mechanisms that facilitate such coordinations leading to overall homeostasis in hostile conditions. Deciphering such aspects of stress and stress responses in extremophiles provides with an overview of the diversity of adaptive tactics which open up avenues that can be exploited for commercial implications.

The biggest challenge for microbiologists is to explore their vast diverse genetic potential and comprehend and harness it for commercial applications.

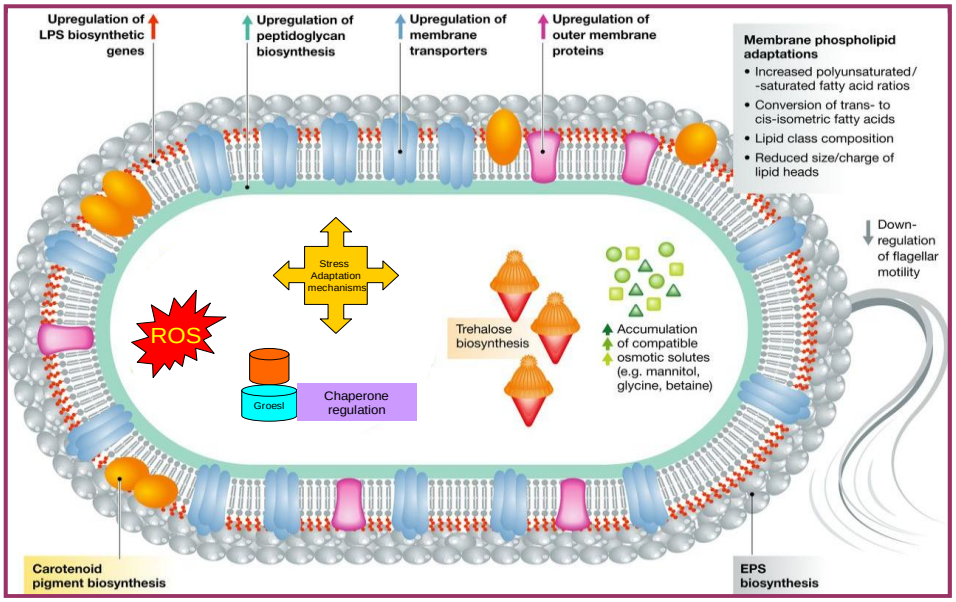

Fig. 1 Survival strategies of an extremophile (De Maayer et al., 2014)

Figure shows how a typical extremophile, responds to stress in synchrony with all the different compartments of the cell at cellular, molecular, structural to serve a common purpose of surviving the externalities of the environment. The changes facilitating adaptation to stress are brought out by the accumulation of compatible osmotic solutes, carotenoid biosynthesis, chaperone regulation, down regulation of flagellar mobility, membrane phospholipids adaptations, upregulation of outer membrane proteins, EPS biosynthesis.

Extremophilic bacteria display improved cellular robustness under extreme conditions probably because of their adaptive strategies (Zhu et al.,2012). The significance of extremophiles not only emphasizes the importance of their diversity structurally and biochemically but also their enormous potential serving as sources of enzymes with many commercial implications (Gabani and Singh, 2013). The extremozymes from extremophiles have the potential to catalyze reactions in non standard conditions. However, enzymes from non-extremophiles have reduced activity, and denature in such non standard conditions. Extremozymes establish a tight hydration shell thus being active in low water limiting conditions thus enabling them to function optimally in high salinity and pH, temperature extremes(Karan et al.,2012). Amylases, catalases, xylanases, lipases and cellulases from extremophiles serve as typical examples of great industrial interest.

Extremophiles serve as a rich source of biocatalysts with unique properties that function under extreme conditions comparable to those in various industrial processes (Kumar et al., 2011). Some of the radiation resistant extremophiles produce extremolytes i.e. novel metabolites or compounds which are protective against the radiation, having potential biotechnological and therapeutic applications (Singh and Gabani, 2011). Extremolytes were reported to serve as useful drugs, antibiotics, anticancer drugs and also as agricultural products of commercial interest(Kumar et al.,2010,Singh and Gabani, 2011). The carotenoids, bacterioruberin from *Rubrobacter radiotolerans* and *Halobacterium salinarum* were proposed to be potential extremolytes to be used as anticancer agents in treating skin cancer in humans (Singh and gabani,2011).

By exploring their characteristic environment, new insights may be gained that are important to obtain an overview of ecological, evolutionary relationships among organisms, emergence of new species, which compensate certain environmental externalities (Kumar et al., 2011). Extremophiles from diverse habitats can be studied for novel metabolic pathways or metabolites unique to them that are probable avenues with bioremediation potential (Oarga, 2009; Sorokin et al., 2014; Tazi et al., 2014). Research on extremophiles is a fascinating research endeavor as they provide with a glimpse of the breadth of strategies employed to survive the extremities which can be eventually exploited for engineering resistant recombinants of non extremophiles (Ma et al., 2010).

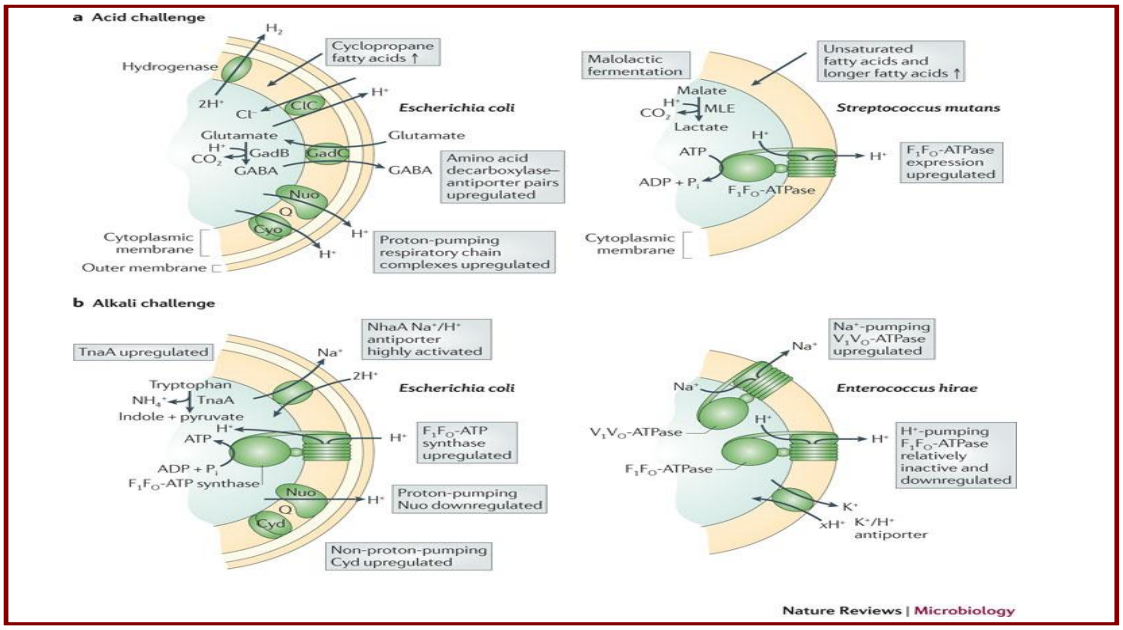

Fig. 2 Different stress response mechanisms in various bacteria in diverse adversities (Krulwich et al., 2011)

Adaptations by neutralophilic bacteria to tolerate acid or alkali conditions. a) *Escherchia coli* adaptations of acid tolerance while passing through the stomach and *E. coli* adaptations supporting alkali tolerance are shown on left; b) strategies proposed for the non-respiratory oral bacterium *Streptococcus mutans* are shown; Adaptations of the non-respiratory species *Enterococcus* supporting alkali tolerance are shown on right; Cyd, cytochrome bd; Cyo, cytochrome bo; GABA, gamma-aminobutyric acid; Gadb, glutamate decarboxylase-beta; Gadc, glutamate/y-amino butyrate antiporter; MLE, malolactic enzyme; NhaA, Na+/H+ antiporter; Nuo, NADH Ubiquinone; Tna, tryptophan deaminases.

Among all the extremophiles, halophiles which are abundantly found, have their major contributions in terms of the diverse adaptive strategies, commercially important implications from the salt tolerant enzymes, osmolytes like glycine betaine, trehalose, ectoine, proline serving as biocatalysts. And additionally, relevance of halophiles, lies in the fact that the greatest part of the biosphere is saline (99%) (Das Sarma et al.,2012) which inhabits a large diversity of halophiles found in various habitats like salt marshes, subterranean salt deposits, dry soils, salted meats, hypersaline seas, and salt evaporation ponds (Zhu et al., 2010). Therefore salinity is a major environmental parameter which deserves the focus of the ecologist, microbial physiologist and the biotechnologist as the adaptive mechanisms evolved in halophiles might have potential commercial applications (Deole R et al., 2013).

1.2 Halophiles

The moderate environments are known to be dominated by the other life forms like the mesophiles, however many examples of life surviving the extreme environmental externalities such as halophiles have recently attracted the attention of the scientists all over the world. Halophiles occupy a unique niche as organisms that require high salt for their survival. Halophiles' being categorized based on their salt requirement is an intriguing aspect. Halophiles are reported to survive the presence of extreme salt concentrations with the presence of vitally curious niches of delicately balanced osmoticum, vital protein-nucleic acid interactions and distinct molecular signatures (Deole et al.,2013).

The halophiles are challenged to establish an osmotic balance across the cell as they are in a hypertonic solution because of which, water diffuses out of the cells and thus cells shrivel up reducing the survival possibilities. However the halophiles are reported to maintain equilibrium across the cellular environments by an intake of sodium ions and create an osmotic equilibrium by accumulating potassium ions and expel sodium at high concentrations of salt. Halophiles also accumulate aminoacids, glycine betaine ecotine, as survival tactics in the supersaturating salt environments (Das Sarma et al., 2013).

The genome and proteomic analysis in *Salinibacter ruber* have revealed that they have an excess of acidic to basic amino acids which serves as the vital requirement for protein activity in high salt. This strategy also serves to prevent protein denaturation, aggregation and precipitation which are the common consequences when non halophilic proteins are exposed to high salt, are

examples worth mentioning here in this context (Das Sarma et al.,2012). They also exploit bacteriorhodopsin and retinal for maintaining membranes in high salt (Das Sarma et al.,2012). Therefore these halophiles can be exploited for enzymes and bacteriorhodopsins which contribute towards adaptations to survive high salt conditions (Oren et., 2010). The chromoproteins from these halophiles are extensively exploited for diverse applications like biocomputing, as a neurological probe and in the medical field for the treatment of blindness. Halophilic oraganisms are a potential source for many useful enzymes like DNases, lipases, amylases and proteases. The compatible solutes are used for enhancing hydration properties. They can be exploited to address issues of bioremediation as most of the industrial effluents are contaminated with brine (Das Sarma et al., 2012).

The halophiles were reported to serve as the first example of a vegetative cell, able to survive exposure to the space environments. Therefore, halophiles equipped with such potential might be serving as the probable avenue for the key aspects of abiogenesis. Examples of halophiles signify, that the unique art of DNA repair might be serving as their possibility for surviving the Dead Sea's briny water which is ten times saltier than normal seawater (DiRuggiero et al., 2002). The mechanisms of adaptations are quite diverse in accordance to the diversity of the halophiles and still remain to be explored.

1.3 Halophiles and their diversity

Halophiles are salt loving organisms that thrive in saline environments (Das Sarma 2012). Based on their requirement for sodium chloride they were categorized as slightly, moderately, and extremely halophilic with their characteristic ability to survive in hypersaline environments with salinity higher than the sea (marine salinity approximately 0.6M) (Das Sarma et al.,2013) However most of the marine organisms are classified as slight halophiles. The hypersaline environments are found distributed all over the world that include deep sea, salterns, arid coastal sea locations. Halophilic microbes include heterotrophs, phototrophs, methanogens in the archeal domain, lithotrophs, photosynthetic and heterotrophic bacteria, photosynthetic and heterotrophic eukaryotes. They also include cyanobacteria green and purple bacteria, sulphur oxidizing bacteria, anaerobic bacteria, sulphate reducing bacteria, Gram-negative and Gram-positive heterotrophic bacteria. Additionally the stretch also extends to cover some algae, protozoa, fungi and higher organisms. *Halobacterium* sp. NRC-1 serves as an example of widely distributed extreme halophilic archea

(Deole et al., 2013). *Dunaliella salina* serves as another example of well studied halophiles (Oren, 2005).

Halophiles span all the three domains of life (Archaea, Bacteria and Eukarya) (Fig. 2). In the domain, Bacteria, halophiles are reported to be distributed in the phyla Cyanobacteria, Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes and Bacteroidetes (Oren, 2008). The anaerobic fermentative bacteria of orders Halanaerobiales (phyla- Firmicutes) consists of halophiles only, similar to the Halobacteriaceae family in Archaea. The halophilic cyanobacterial genera, widely studied include Aphanothece, *Spirulina* and *Dactylococcopsis.* The hypersaline lakes are reported to be dominated by the planktonic mass of cyanobacteria (Das Sarma etal.,2012). Halophilic actinomycetes have been discovered to be harboring saline soils of diverse locations (Solanki and Kothari, 2012).

The Proteobacteria, the sulfur reducers, the purple sulfur bacteria and Halomonads have been extensively studied. The moderate halophiles which belong to the family Chromatiaceae mainly consist of halophilic purple sulfur bacteria that are reported to deposit sulfur granules inside their cells.

Hyper saline environments depend on the geology, geography and topography of the areas in which they grow and also are under the influence of seawater that contain 35 g/L dissolved salts. The most extreme environments are dominated by the hyper saline environments, with salinities far above the typical seawater salinities, originated as a consequence of evaporation of seawater (Das Sarma et al., 2103). Hyper saline bodies derived from the evaporation of seawater retaining the salts in the sea and therefore termed thalassic. Athalassic waters are those in which salts are derived from non marine and very uncommon. Thalassic brines (3-3.5 M NaCl possible for the survival of few extremophiles) harbour a huge diversity of microbes. Examples include *Halobacterium, Dunaliella.* Therefore Halophiles are suggested to thrive at salt concentrations of approximately 100 g per liter (1.7 M NaCl) (Deole et al.,2013).

The Great Salt lake and the Dead sea (*Haloarcula marismortui, haloarcula vallismortis*) are the well studied hypersaline lakes. The Wadi Natrun lakes of Egypt, Lake Magadi, Great Basin lakes of Western United states are the examples of hypersaline lakes. Additionally numerous examples of artificial solar salterns also serve as examples for hypersaline environments (Das Sarma et al.,2012).

The alkaline soda lakes of Egypt and Central Africa are known to be dominated by halophilic anoxygenic photosynthetic sulfur bacteria of the *Halorhodospira-Ectothiorhodospira* group (Schwibbert et al., 2011).

The bulk of prokaryotic organisms in hypersaline environments are dominated by the members of *Haloarchaea*, which forms a discrete evolutionary branch in the Archaeal domain. They belong to the family *Halobacteriaceae*, which includes 30 genera.

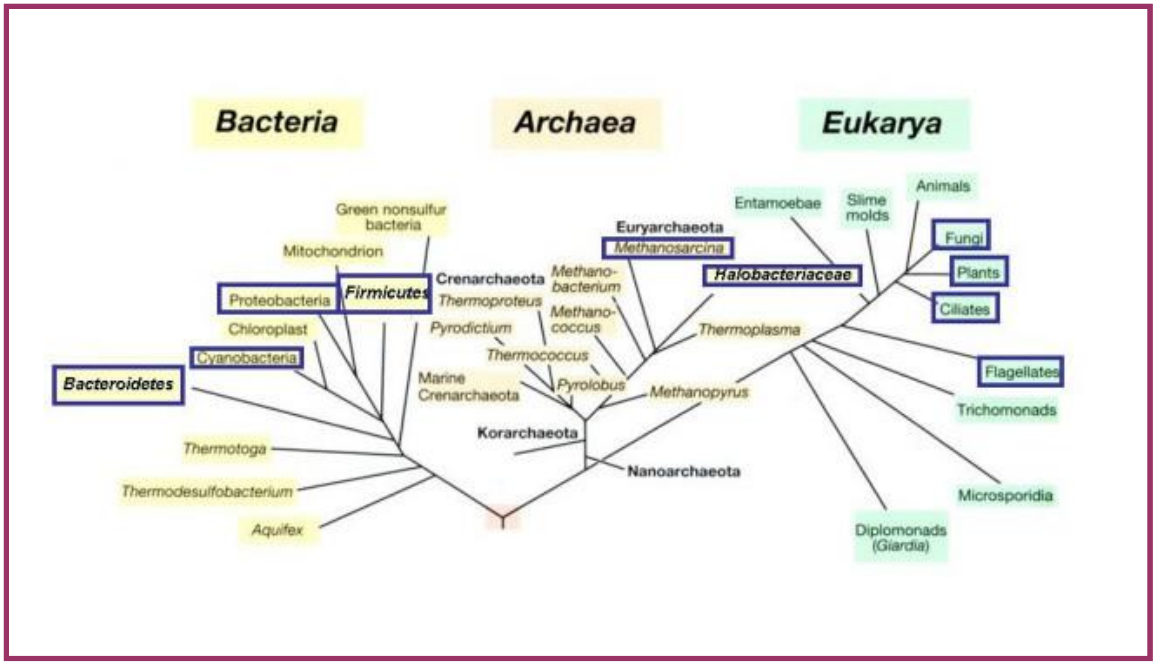

Fig. 3 Phylogenetic tree of microorganisms showing the distribution of extremophiles

Groups marked with purple boxes contain at least one halophilic representative (e.g. *Salinobacter ruber* within the Bacteriodetes). The tree is based on Fig. 11.16 in Madigan and Martinko, 2006 (78) (Oren, 2008)

1.4 Categories of halophiles

All life forms require salt, however halophiles stand distinct by their requirement for high salt to survive. Different classification schemes have been devised to portray microorganisms according to their behavior towards salt. The most extensively followed classification of halophiles based on the requirement of salt is that of Dr Donn Kushner who defined moderate halophiles as organisms growing optimally between 0.5 and 2.5 M salt (Table 1). Organisms able to grow in the

absence of salt as well as in the presence of comparatively high salt concentrations are classified as halotolerant (or extremely halotolerant if growth extends above 2.5 M). Microorganisms that can grow above 2.5 M salt concentration but not in the absence of salt are classified as extreme halophiles (Deole et al., 2013).

Table 1: Classification of halophiles based on salt requirement (Oren, 2006)

Category	Optimum salt requirement(M)	Examples
Non halophiles	<0.2	Most freshwater bacteria
Slightly halophilic	0.2-0.5	*Demequina aestuarii, Shewanella gaetbuli*
Moderate halophiles	0.5-2.5	*Saccharomonospora saliphila, Salinivibrio costicola*.
Borderline extreme halophiles	1.5-4.0	*Streptomyces tritolerans, Halorhodospira halophila*
Extreme halophiles	2.5-5.2	*Actinopolyspora halophila, Halobacterium salinarum*
Halotolerant	Non-halophilic which can tolerate salt (>2.5M)	*Staphylococcus aureus*

.

The physiological properties of these halophilic microbes are suggested to also fluctuate significantly with varied metabolic types designed accordingly to function in high salt conditions (Horikoshi, 2010; Seth-Pasricha et al., 2013). The distinct physiological characteristics and evolutionary position of *Haloarchaea* have lead to an immense deal of interest to sequence their genomes to gain more insights of their genetic make up. Most of them are reported to grow best at salinities 3.4-5 M NaCl and require 1-1.5 M NaCl (DasSarma and DasSarma, 2012). The various morphological features exhibited by these halophiles include rods, cocci, disc-shaped, triangular and even square-shaped (Zhuang et al., 2011) which may be different adaptive modes, so evolved to facilitate survival in the supersaturating salt environments.

Elucidating the limits of biological adaptations to different environmental conditions is very important because this kind of research may reveal the evolutionary aspects that include phenotypic and genotypic changes that might have taken place in the life forms. The focus is particularly on how biological machinery which has been delicately tuned to a given set of conditions, can adapt to a drastic change(s), that necessitate an adjustment of metabolic machinery, in order to establish a new state of homeostasis (Mittler et al., 2006).

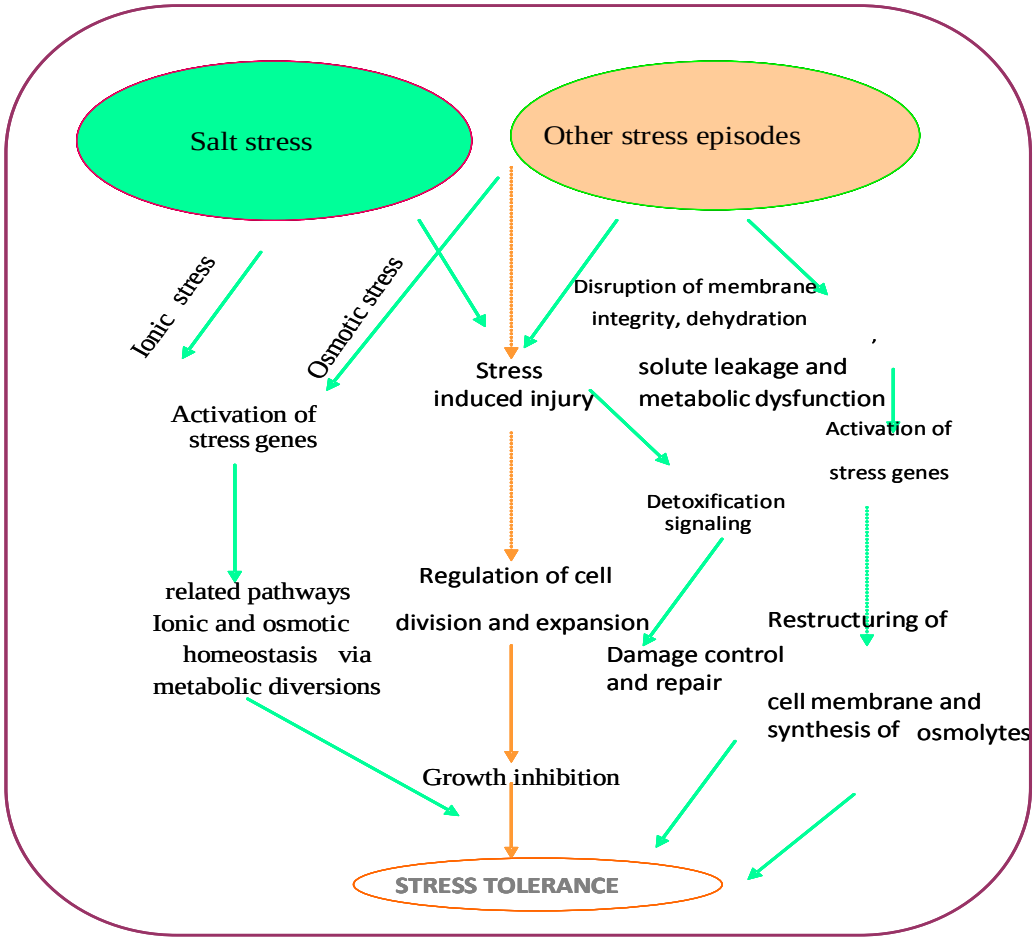

Fig. 4 Stress tolerance strategies during salt and other stress episodes (Mahajan et al., 2005; Miller et al., 2009)

Stress triggers the cellular components to respond, by activation of stress genes, metabolic diversions, regulation of cell division and expansion, growth inhibition, ROS management, cell membrane and osmolyte synthesis and regulation. All these regulatory aspects are activated leading to stress tolerance by the cell to establish homeostasis.

1.5 The adaptive styles

The array of survival tactics displayed by the consortia of halophiles in order to survive hypersaline conditions, clearly elucidates the facts of diverse adaptation mechanisms operating to establish overall cellular homeostasis. Proteins of *Haloarchaea* have been reported to require the presence of high salt concentrations for their optimal activity. The intracellular K^+ concentration of these organisms has been found to be tremendously high, up to 5 M. Halotolerant and halophilic actinomycetes have been reported for extensive adaptability to Na^+, K^+ and Mg^{2+} (Tang et al., 2009). Other unique features are their modes of replication, transcription and translation machinery and their cell membrane composition all delicately tuned for survival in the salt extremes. Pigmentation seen in numerous halophiles imparts color to their habitat where they thrive. The Bacterioruberin pigment in the membranes of red halophilic archaea of the family *Halobacteriaceae* is responsible for pigmentation of brines. The pigment bacteriorhodopsin is known to contribute to the purple color of the brines (Antunes et al., 2008; Oren, 2012). Halorhodopsin is proposed to be an inward directed chloride pump driven by light and sensory rhodopsins which mediates photo taxis to pump protons across the membrane and flush them out of the cell (Songa et al., 2014). Another example of adaptation style displayed is the production of buoyant gas vesicles that are hollow proteinaceous structures surrounding a gas-filled space which facilitates the floating of cells to more oxygenated surface layers in low oxygen surroundings. Discovery of halophilic adaptation tactics of extreme halophile *Salinibacter rubber* of the phylum Bacteriodetes in the domain bacteria, has garnered a great deal of interest in halophilic adaptations that accumulate compatible solutes as their osmoprotectants (Deole et al., 2013). There are many illustrations of adaptive styles displayed by these organisms. However the interesting thing yet to be explored is the innate intercoordinating network signature, operating in accordance to the different environmental conditions.

All these examples of unique adaptation strategies, these organisms have evolved to thrive in the adverse conditions of salinity is a clear elucidation of many facts like, the existence of diverse strategies in accordance to the demand of the environmental conditions, at various levels like molecular level, cellular level and at the metabolic level which function in synchrony for a survival possibility.

1.6 Mechanisms of salt tolerant adaptation strategies

The high salinity conditions in their living environments triggers many metabolic diversions thereby the consequences of corresponding changes in their molecular machinery so as to establish metabolic homeostasis across the cell (Tazi et al.,2014). The occurrence of high intracellular salt, demands the requirement for adaptations of the entire enzymatic machinery of the cell. The halophiles have been reported to have the cytoplasmic accumulation of molar concentrations of KCl consequently resulting in a sharp decrease in the distribution of protein isoelectric points facilitating a balance of the osmoticum(Das Sarma et al., 2012). Compared with the other organisms, the halophiles confer many advantages being potential candidates for genetic manipulation, feasible to be maintained in the laboratory with a minimum necessity for aseptic conditions (Ratnakar, 2013) (Fig. 4).

1.6.1 Structural adaptations

The Haloarchaeal cell membranes serve as the best examples of structural adaptations, the extremophiles opt for in order to thrive in the environmental extremities (Song and Gunner, 2014). They are reported to be structurally equipped with phospholipids which are composed of branched isoprene units linked to glycerol by an ether group and comparatively distinct, the bacterial and eukaryal membranes have fatty acids linked to glycerol by an ester bond. The membranes of halophiles are so designed that they are less permeable to ions and more resistant to high salts which implies a survival strategy (Ratnakar, 2013).These examples signify the strategic adjustments in the membranes made by the organisms living in the environmental extremes.

The halophiles contend the salinity phase through changes in their cell membrane compositions. Changes in the composition of bacterial membranes which might be triggered by environmental factors are proposed to act as an adaptive response to maintain membrane stability and function. In fact, structural adaptations of membranes primarily involve alterations in the composition and synthesis of proteins, lipids and fatty acids (Vreeland, 2012). Lipids are known to play many significant roles as esters of fatty acids and alcohols that comprise a large group of structurally distinct organic compounds including fats, waxes, phospholipids, glycolipids (Deole et al., 2013; Al-Beloshei et al., 2014). Lipids being the most effective source of storage energy, also function to provide insulation to the delicate internal machinery and serve as the structural constituents of the cellular membranes. The unsaturation of fatty acids to counteract water or salt

stress is reported to be displayed by halophiles. This is evident from previous reports that the percentage of saturated fatty acids and unsaturated fatty acids is affected with the concentration of NaCl in micro algae (Yatsunami et al., 2014). The percentage of saturated fatty acids decreased as the concentration of NaCl increased, while the percentage of highly unsaturated fatty acid increased (Kirroliaa et al., 2011; Asulabh et al., 2012).

The mechanism of desiccation tolerance is proposed to depend on phospholipid bilayers which are stabilized during stress by sugars, especially by trehalose. Previous evidences have suggested that sugars, particularly trehalose, play a vital role during the salinity phase by preventing damage from dehydration by inhibiting fusion between adjacent membrane vesicles, by maintaining membrane lipids in the fluid phase in the absence of water (Leslie et al., 1994). Molecular modeling studies have clearly indicated how trehalose displays its protective role by fitting between the phosphate of adjacent phospholipids. There are reports of a direct interaction between the sugar and lipid exhibited especially at low trehalose/lipid ratios, when trehalose is not available to bind water (Rudolph et al., 1990). All these evidences clearly elucidate how lipids become a part of the adaptive strategies the organism opts for during stress and how it encounters stressful environment by exhibiting its mechanisms possible in its cellular and molecular compartments. To any environmental stress the organism shows its own unique way of response by a change vital to the membrane integrity. By an alteration in the lipid content of membranes it is able to acclimatize to the respective change in its surroundings (Ritter and Yopp, 1993). The exciting aspect is the diversity in the mechanisms by which different organisms under different stressful conditions maintain the membrane integrity resulting in tolerance (Fig. 4,5). The central mechanisms of the survival strategies of the bacteria at environmental extremes of salinity had been membrane fluidity and lipid composition (Oliver et al., 1998). An example worth mentioning is the rehydration of dry mats of *Scytonema geitleri* that resulted in a slight increase in the amount of total lipids (Singh, 2001). The tolerance mechanisms of many bacteria have been reported to operate on the basis of the saturation and unsaturation of the fatty acids in membrane lipids in coordination with other mechanisms of adaptation(Fig. 3) (Hagemann, 2011). In this context it is worth mentioning the example of the studies on cyanobacteria which have elucidated that an increase in the unsaturation of fatty acids in membrane lipids enhances the tolerance to salt stress of the photosynthetic and Na^+/H^+ antiport systems of *Synechococcus* (Allakhverdiev et al., 1999). These studies suggest that such changes like the unsaturation of fatty acids in membrane lipids

might stimulate and influence the activity of the Na^+/H^+ antiporter system by enhanced fluidity of the membrane with consequent protection of PSII and PSI activities. The components of the antiport system might result in a decrease in the concentration of Na+ in the cytosol, resulting in the protection of PSII and PSI against NaCl-induced destabilization and hence accelerate the recovery of PSII and PSI activities, thereby leading to the maintenance of cellular homeostasis (Allakhverdiev et al., 2001). There are also reports about the activities of several membrane bound enzymes being influenced by changes in membrane fluidity which is a necessary survival response exhibited by the organism under stress. There are evidences from previous literature reports which have emphasized the facts of how there is a huge diversity in the protective signals exhibited by different bacteria with respect to the environmental change and the examples worthy enough to be mentioned here, are the Gram positive and the Gram negative bacteria displaying diversity in their usage of different compounds as signal molecules. Many Gram-negative bacteria use N-acylhomoserinelactones (AHLs) as signal molecules, and other fatty acid derivatives such as 3-hydroxypalmitic acid methyl ester and cis-unsaturated fattyacids forming protective signals and in quite contrast to this the Gram-positive bacteria use aminoacids or peptides as signal molecules (Twigg et al., 2014).

The comparative studies of the genomes and proteomes of halophiles and non-halophiles (Paul et al., 2008) emphasized the fact that halophilic proteins have been designed in such a way so as to survive the salinity by low hydrophobicity, increased number of acidic amino acids, especially aspartic acid, under-representation of cystine, limited helix formation and higher occurrence of coiled structures and the dinucleotide abundance profiles of halophilic genomes have some distinct characteristics, explicit with DNA salt adaptation signatures. Thus, it may be that halophiles have unique membrane proteins and internal proteins all in concert to facilitate survival possibilities in high salty environments (Reinhardt et al., 2010). However, synchronization processes contributing towards adaptation, directed towards the protection of the organism being influenced by environmental extremities remains unclear Fig. 3, 4).

1.6.2 Cellular adaptations

The halophiles must maintain their cytoplasm iso osmotic with their surrounding medium in order to thrive in high salinity. Salt tolerance requires the accumulation of compatible solutes in the cytosol where these facilitate in osmotic adjustment and osmoadaptation (Becker et al., 2014). Some are essential elemental ions, such as K+, but the majority are organic solutes (Fig. 4, 5).There

are two fundamentally different strategies employed by halophilic microorganisms to maintain their osmoticum with their surrounding environment (Zahran, 1997). Each mechanism operates with its own interesting aspects so organized and designed, all well synchronized for the ultimate survival of the organism to the respective environmental demands.

(1) The salt-in-strategy called so as it involves the accumulation of molar concentrations of potassium and chloride facilitating balance of the osmoticum within their cellular environment. The significant aspect about salt-in-strategy is that the system is well designed and evolved with extensive adaptation of the intracellular enzymatic machinery to the presence of salt as it is vital for the proteins to maintain their respective conformations and functional activity at such high saturating salt concentrations (Singh et al.,2014). Reports have elucidated that the proteome of such organisms is highly acidic, and most proteins denature when suspended in low salt. The "salt-in-strategy" is reported to be the most energy efficient than the "compatible solute" strategy, as it operates by hydrolyzing only two ATP molecules for every three molecules of potassium chloride accumulated (Shivanand et al., 2013). The aerobic, extremely halophilic archaea and anaerobic bacteria exploit this strategy. Microorganisms employing such strategy generally cannot survive in low salt media. Though this strategy is energetically less costly to the cell, it is not widely used among the different phylogenetic and physiological groups of halophiles. Examples of organisms employing this strategy are *Halobacterium salinarum, Haloarcula marismortui* (Baxter et al., 2014).

(2) Second strategy is based on the biosynthesis and/or accumulation of organic osmotic solutes. Cells utilizing this strategy exclude salt from their cytoplasm as much as possible. The high concentrations of organic 'compatible' solutes do not greatly interfere with normal enzymatic activity. Organisms employing this strategy can often adapt to a surprisingly broad range of salt concentration which stimulates many important intriguing questions still to be explored (Roberts, 2005). Compatible solute accumulation as a response to osmotic stress is an ubiquitous process in organisms as diverse as bacteria to plants and animals. However, the solutes that accumulate vary with the organism and even between species (Singh et al., 2014). The intriguing aspect about these osmolytes is their vast diversity with respect to their chemical nature and their strategic distribution in accordance to the situational demand by the organism and its surroundings. This is clearly elucidated by literature of the diverse categories of organic osmotic solutes all serving the purpose of surviving stress acting in concert to the available metabolic machinery. The examples that

signify are the switch in osmolyte strategies with increasing salt concentrations, contributions made by more than one osmolyte, requirement of chloride for osmolyte synthesis (Muller 2008).

Many organic compounds (amino acids, glycine betaine, ectoine, hydroxyectoine etc.) serve as osmotic solutes in halophilic microorganisms, in both prokaryotes and eukaryotes. Most compatible solutes are based on amino acids and amino acid derivatives, sugars, or sugar alcohols, and are either uncharged or zwitterionic (Kurz, 2008).

The "salt-out strategy" of haloadaptation with accumulation of organic osmotic solutes is reported to be widespread in the small subunit rRNA sequence-based phylogenetic tree of life. All groups of halophiles have not yet been examined for the occurrence and distribution of organic solutes (Wang et al., 2008). The osmotic solutes may be either produced by de novo synthesis or the organism may accumulate it from the surroundings. The later mechanism is preferred over de novo synthesis (Irwin and Baird, 2004). Energetically synthesizing solutes is proposed to be an expensive process.

The intracellular accumulation of compatible solutes as an adaptive strategy in extreme environments is evolutionarily well-conserved in Bacteria, Archaea, and Eukarya (DasSarma and DasSarma, 2012). Ectoines represent the prime class of osmolytes accumulated by halophiles. *Halomonas elongata* strain KS3 has been reported to accumulate the osmolytes, ectoine and hydroxyectoine (Oren, 2008). Occurrence of certain solutes in halophilic/halotolerant prokaryotes is reported to be correlated with their position in the phylogenetic tree of life, that is few solutes in the domain Archaea have not been yet detected elsewhere within the tree. Reports of Halophilic methanogens like *Methanohalophilus* species containing, in addition to glycine-betaine that is reported to be most prevalent in nature, have β-aminoacids and rare derivatives β-glutamine, β-glutamate, and Nε-acetyl-β-lysine distributed in other groups (Das Sarma et al.,2012). Studies have suggested of Sulfotrehalose being found only in a few alkaliphilic members of the Halobacteriaceae, and it is accumulated in substantial concentrations (up to 1 M) in addition to KCl which serves as the main osmotic solute like their neutrophilic relatives (Oren, 2008). Many elucidations clearly make it evident of how these osmolytes facilitate osmoprotection in different ways in accordance to varied environmental conditions. For example, the vital roles they play during the high saline phase, extreme temperatures, desiccation, scavenging the reactive oxygen species generated as a consequence of stress that disturb membrane integrity, and thereby dysfunction (DasSarma and DasSarma, 2012). The essential feature about these osmosolutes is that they facilitate protection at

all levels without hindering intracellular biochemistry and thus preserve and maintain their activity intact (Fig. 3). They are able to play roles vital to the organisms survival as they show minimal affect on their cytoplasmic pH and also on the luminal compartments of the various organelles of the organism and this is made possible by the metabolic diversion of the vital intermediary metabolites into unique biochemical reactions. Stress probably facilitates the trigger for this metabolic diversion. Much research is devoted to some of the major tolerance mechanisms, including ion transporters, osmoprotectants, free-radical scavengers, stress genes and factors involved in signaling cascades and transcriptional control (Wang et al., 2003). All these evidences leave us with clues of their protective roles they play but how they synchronize with the wide array of adaptive mechanisms across the cell and its various compartments remains elusive.

1.6.3 Molecular adaptations

Molecular chaperones are the well known key components contributing to cellular homeostasis in cells under both optimal and adverse growth conditions. They are reported to be responsible for protein folding, assembly, translocation and degradation across an array of normal cellular processes. They also function in facilitating the stabilization of proteins and membranes, and can assist in protein refolding during the stress episodes. Many molecular chaperones are stress proteins and many of them were originally identified as heat-shock proteins (Hsps). They are referred as Hsps/chaperones following their early nomenclatures (Hänelt and and Müller, 2013).

Literature suggests five major families of Hsps/chaperones that includes Hsp70 (DnaK), the chaperonins (GroEL and Hsp60), the Hsp90, the Hsp100 (Clp), and the small Hsp (sHsp), Chaperonins (Hsp60). Major examples of this class of Hsps/chaperones include the prokaryotic GroEL and the eukaryotic equivalent Hsp60. In prokaryotes, Group I chaperonin (e.g. GroEL of *E. coli*) consists of two distinct family members, chaperonin 60 (Cpn60) and chaperonin 10 (Cpn10) as co-chaperone, which function together in an ATP-dependent manner. They are double-ring assemblies composed of back-to-back stacked rings of identical or closely related rotationally symmetrical subunits (Wang et al., 2004).

It is a well known fact that during stress, many enzymes and structural proteins undergo deleterious structural and functional changes. The protective response of chaperones can be attributed to the network of the chaperone machinery, in which many kinds of chaperones act in concert (Hanelt and Muller, 2013). Therefore, maintaining functional conformations, preventing aggregation of non-native proteins, refolding of denatured proteins to regain their functional

conformation and removal of potentially harmful polypeptides (arising from denaturation or aggregation) are particularly significant for cell survival under stress. Thus, the different classes of chaperones in synchrony, play complementary and often overlapping roles contributing to the protection of the cell (Wang et al., 2003).

Chaperones, as regulatory molecules, participate in stress sensing, signal transduction and transcriptional activation of stress genes needs further insights (Kumazaki et al., 2014). Most of the current research in organisms is devoted to detecting changes in chemical/biological chaperones and their ways of coordination operating, during stress. Moreover, studies of the changes in one or more stress response mechanisms are investigated simultaneously with changes in chemical/biological chaperones (Shukla, 2006) (Fig. 4). Anticipating active research into the crosstalk between chemical/biological chaperones and other stress-response mechanisms will provide a further understanding of acquired stress tolerance (Fukuda et al., 2002). Individual members of each class of biological /chemical chaperones have particular functions, but the co-operation between different biological/ chemical chaperone networks appears to be a central principle of the integrated chaperone machinery (Shukla, 2006). Under normal growth conditions or during and after stress, the fate of a particular denatured or non-native protein is determined by the entire chemical/biological chaperone system. Many questions about this issue have still to be answered like the existence of a central determinant of the chemical/biological chaperone network, cross-talk between Hsps/chaperones and other stress response mechanisms. There is wealth of information already available on different chaperones, their functions, however the dearth is regarding how they act in synchrony with other stress response and adaptive mechanisms available and how they act in cooperation towards a common goal of survival (Shivanand and Mugeraya, 2011).

Abiotic stress evokes multiple responses that involve a series of physiological, biochemical and molecular eventualities (Chakraborty et al., 2011). Stress tolerance is often a result of various stress-response mechanisms that act synergistically to prevent cellular damage and to re-establish cellular homeostasis (Wang et al., 2003). An increasing number of studies suggest that the chaperones interact with other stress-response mechanisms (Fig. 4). In *E. coli,* it was suggested that the osmolytes (glycine-betaine, glycerol, proline and trehalose) can also play the role as ‘chemical chaperones’ by increasing the stability of native proteins and assisting in the refolding of unfolded polypeptides (Levy-Sakin et al., 2014). Reports suggest that cells specifically control

protein stability and chaperone mediated disaggregation and refolding by modulating the intracellular levels of different osmolytes (Diamant et al., 2001). These studies might give new insights into the cross-talk between biological/ chemical chaperones and other stress-protective mechanisms.

1.7 Novel adaptations

1.7.1 Halites

Microbes can persevere in various quiescent forms for extended periods of time. Sporulation is perhaps the best-known strategy for dormancy, a tactic used by some bacteria to transcend hostile conditions (Sankaranarayanan et al., 2011). Cysts and spores thus signify an example of convergent evolution in which distinct processes arise to serve a similar purpose of long-term survival in an adverse environment (Ma et al., 2010) Studies provide yet another fascinating example of the strategies that microbes have evolved to combat hostile environments, and that is by the phenomenon of formation of halites. Halite, a novel form of dormancy, evolved as a specific adaptation for long-term survival and persistence in the halite environment (Vreeland, 2012). Halites are the mineral form of the salt sodium chloride (NaCl) and are abundantly produced in terrestrial sedimentary environments when salty water evaporates. Mars Exploration Rover reported of halites in Mars and similar crystals with fluid inclusions found in billion-year-old meteorites originating from other places in the universe. These ancient crystals are proposed to be widespread and probably harboring alien life (Schubert et al., 2010).

In 2010, Schubert and collaborators reported of spherical cells in 34,000 year-old halite crystals and were able to resuscitate them in growth media. The evidences provided by many researchers on halites has emphasized the fact of a unique phenomenon of adaptation by halophiles similar to the formation of spores and cysts. The halophiles are reported to occur at such high cell densities that they cause brines to turn red, which facilitates evaporation by trapping solar radiation. The red colour is caused by carotenoid pigments that protect cells from the harmful effects of ultraviolet light.

The phenomenon of NaCl precipitation entraps the halophiles inside fluid inclusions. This phenomenon had been reported to be observed in solar salterns throughout the world (DasSarma and DasSarma, 2012). This phenomenon of halite formation as a novel adaptive response is a very interesting aspect observed only in halophiles. This is proposed to be similar to the formation of

cysts and spores which are well known dormancy phases of many bacteria in adverse conditions. Moreover, it had been reported that this novel phenomenon of entrapment affects crystallization in several ways, it leads to an increase in the size and number of crystals, results in bigger fluid inclusions, encourages dendritic crystals accelerates crystal formation, and sometimes encourages the formation of salt ooids (Castanier et al., 1999). Thus these are clear elucidations of the fact that these organisms are known to promote crystal formation of halites acting as seeds or nuclei exhibiting a morphological transition (Lopez-Cortes et al., 1994). The fact known is that most of the cellular components (enzymes, DNA, etc.) require interactions with water molecules for their optimal cellular operations to be carried out. Bacteria, for example, require a water activity of 0.9 and anything above and below this, is suggested to inhibit microbial growth. Studies suggest that probably the shift from rods to spherical shapes reflect the transition from an active vegetative state to a dormant form that could withstand the low water activity in high salt conditions (Fig. 5). Further evidences suggest that, as the number of rod cells decreased, the number of small spheres increased in the ratio of 3-4 spherical cells per initial rod cell. This suggested that the morphological shift resulted from the division of individual rod cells into several (3-4) small spheres (Baxter et al., 2014) . Studies suggest that the spheres, the apparent dormancy phase shifted to their original rod shape in favorable conditions. Hence, probably the spheres remained viable and retained the potential to revert to their ancestral rod morphology (Oren et al.,2012). Reports suggested that although the spheres seemed to be physiologically similar to spores, they were not spores in the typical sense but might be a temporary phase of adaptation (Conner and Benison, 2013) (Fig. 5).

The possibility of the halophilic microbes to survive in a state of dormancy over geological time periods remains still undeciphered and is in its infancy. Such approaches might serve to address in a unique way, many interesting questions about evolutionary rates and physiological adaptation. These revived microorganisms could perhaps be used as living biomarkers to determine palaeotemperatures. The long-term novel dormancy cannot definitely be ruled out, and the Mars exploration programme continues to give this aspect an impetus beyond the usual scientific curiosity. Biological explanations for long-term survival could, in principle, provide credible explanations for the hypothesis of longevity (Terry et al., 2000).

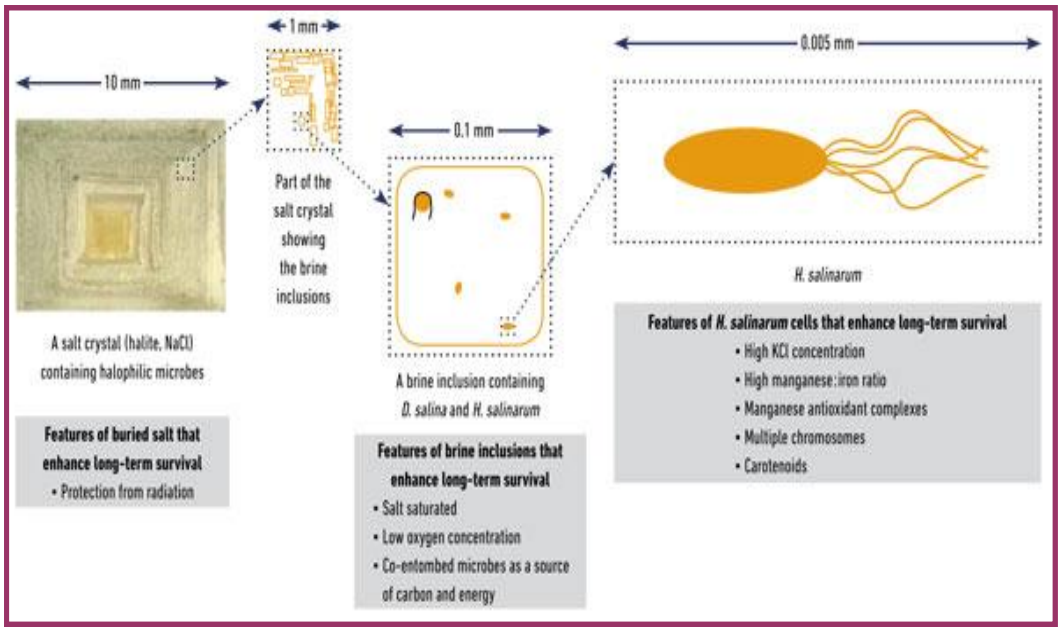

Fig. 5 Illustration of how halophiles survive in halites (salt crystals), (Oren, 2012)

A salt crystal containing halophilic microbes entrapped, also shown the brine inclusions. The brine inclusions facilitate long-term survival because of the low oxygen concentrations, salt saturation and the co-entombed microbes as source of carbon and energy. High KCL concentrations high manganese iron ratios, multiple chromosomes and carotenoids are features facilitating the long term survival of *Halobacterium salinarum* is also shown in this picture.

1.7.2 Biofilm formation

In nature, biofilm, a protective niche constitutes a growth modality allowing bacteria to survive in hostile environmental conditions. Biofilm is defined as a well organized three dimensional polymeric matrix produced by bacterial communities attached to an inert surface. Biofilm formation as a protective response in accordance to the environmental stress, creates protective niches for bacterial thriving (Qurashi and Sabri, 2011). They serve as the best examples of highly ordered structures formed by bacteria permeated by channels facilitating the circulation of and exchange of signals, water, nutrients, and as well to discard toxic metabolites and thus synchronize metabolic cooperativity. Biofilm composed of exopolysaccharides, the major

constituents display their protective role by forming a protective physical barrier conferring protection against stress factors such as UV radiation, pH changes, and osmotic imbalance (Gilbert and Knox, 1997). In addition to exopolysachharides, biofilm consists of proteins and nucleic acids which also influence function of biofilm in different organisms (Branda et al., 2005).

These reports are the clear elucidations of the facts that the biofilm is a well organized cooperating community of microorganisms attached to the surfaces. All these interesting features of a biofilm emphasize the fact that these assemblages of microorganisms at an interface and typically attached to an abiotic or biotic surface reflect a perfect example of a highly evolved system of adaptive response to stress (Chimileski et al., 2014). There are many factors to be considered due to which microbes have tendency to form biofilm to tolerate biotic and abiotic stress conditions. Nutritional stress, high pressure and temperature, osmotic stress, hypo/hyper saline conditions induce biofilm formation for protection (Al-Beloshei et al., 2014).Studies on the different aspects of biofilm from different organisms elucidates the significant potential latent in the different adaptive strategies of an organism and gaining an insight into their potential strategies gives us an overview of their molecular evolution. Biofilms have great potential in serving as sensitive bioindicators of petrochemical pollution, ecotoxicology tests, bioremediation, biohydrogen production and soil improvement (Chaillan et al., 2006).

In addition, another upcoming potential benefit of EPS produced by algae and cyanobacteria is that it can be exploited to improve the soil water-holding capacity and prevent erosion (Redmile-Gordon et al., 2014). Studies showed that addition of 0.5 mg of *Microcoleus* sp. EPS per gram of sand retained approximately 30% of the water-holding capacity of the soil (Fischer et al., 2012). It has been shown that inoculation of soil surfaces with a suspension of halotolerant cyanobacteria leads to a salinity reduction (Singh et al., 2013). This amelioration of soil salinity is reported to be probably caused by entrapment of Na+ ions in cyanobacterial EPS sheaths, resulting in a restricted Na+ influx in the plant roots (Ashraf et al., 2006; Roeselers et al., 2008). These studies have offered important clues of how biofilm can be exploited to transcend the environmental adversities and how much far, are we from understanding its biology in detail and what are the various pathways that have to be explored in order to gain insights into the biofilm physiology and how they have synchronized with various compartments of the cell to serve a common purpose of adaptation.

1.8 Reactive oxygen species (ROS)

Reactive oxygen species seem to be playing a role in mediating or as signals to hint the cellular mechanisms about and how to combat stress either in a constructive way or destructive way. Playing a role as signal molecules they seem to hint the system of the metabolic diversion the organism's cellular machinery has to take, to counter act the stress disturbance. The ROS accumulation and scavenging mechanisms seem to balance all the pathways involved or influenced by stress. A gain of knowledge how they operate or form a part of those that cause stress and help in combating stress are very important questions that remain elusive. Therefore studies on different model organisms of how their system tackles stress, what are the different reactive oxygen species generated after stress would definitely leave us with clues of the various metabolic diversions the organism has taken during a stressful condition. And how each type of ROS, is able to play a role to encounter the kind of stress and how they are able to operate in concert with the adaptation network inside the organism. Numerous studies have shown that the reactive oxygen species are potentially strong enough to not only cause oxidative damage to cells during stress but also play a role as signal molecules during environmental stress, cell death and different growth stimuli (Torres et al., 2006). The rapid ROS accumulation is an essential prerequisite for the cell and many of its metabolic operations, as studies have revealed the facts that during stress, ROS accumulation primarily depends on the balance between ROS produced and ROS scavenged (Kwak et al., 2006). This balance is critically dependent on various factors like alterations in growth conditions, the stress duration, severity and also the ability of the molecular machinery and system to rapidly acclimatize itself to the energy imbalance. All this knowledge leaves us with clues and also different stimulating questions of the acclimations at the cellular and molecular level (Yatsunami et al., 2014).

1.9 Rationale behind the study

A deeper insight into the latent molecular mechanisms of salt stress tolerance not only contributes to a better conceptual understanding of adaptations to environmental changes, but also provides researchers a valuable tool to proficiently improve the bioproductive capacity of crop plants under various adverse conditions. This is a huge concern for attaining self-sufficiency in food production for the ever-increasing world population because halophytes constituting only 1% of the world's flora are native in saline environment, whereas glycophytes cannot tolerate salt level to the

same degree as halophytes. Most of the terrestrial plants are glycophytes with varying levels of salt tolerance. Majority of them being crop plants are very sensitive to salt stress. Reports propose that universally no toxic substance restricts plant growth more than does salt. Moreover, the loss of cultivable land, due to expansion of salinity areas through poor irrigation practices, is likely to impinge on world food supplies. The United Nations Environment Program estimated that approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed.

However, the daunting task is the development of tolerance in non halophiles (Becker et al., 2014). Such a scenario makes it vitally necessary to explore other avenues with naturally evolved diverse salt adaptive strategies. Therefore this attempt to study halophiles which are the potential examples of organisms equipped with the latent molecular machinery evolved for adaptation to stress. Thus exploit the facts for an engineering eventuality with enhanced salt tolerance. Since it has been shown that inoculation of soil surfaces with a suspension of halotolerant cyanobacteria leads to a salinity reduction (Singh et al., 2013). In addition, another upcoming potential conferred by halophiles is the benefit of EPS production that can be exploited to improve the soil water-holding capacity and prevent erosion as reported in algae and cyanobacteria (Redmile-Gordon et al., 2014). The unique adaptive strategies exhibited like high salt tolerance, osmolytes, chemical and biological chaperones confer adavantages that can be exploited in the areas of bioremediation, xeriscaping. Halophilic organisms like *Pseudomonas aeruginosa, Bacillus flexus, Staphylococcus aureus* signify their contributions in areas of bioremediation and in biodegradation.

These halophiles with unique features and propensity to live in supersaturating salt conditions have their contributions in astrobiology also as test organisms (Deole et al., 2013). Thus an approach to explore such potential halophilic microbes would contribute to gain an overview of their unique physiology, their cellular machinery evolved and adapted to environmental extremes.

Rhodovibrio salinarum JA137 being a halophile might be a potential microbe expected to have unexplored mechanisms modulating efficiently the complex physiology in high salt. Therefore this approach, to comprehend the crucial interactions among the physiological, molecular, structural levels taking place in fluctuating salt conditions in this halophile.

Hence attempted to study the salt adaptive strategies of this halophile *Rhodovibrio salinarum* JA137. Strain JA137 is a gram negative, photosynthetic purple non sulphur, Alpha Proteobacterium. The solar saltern is the natural habitat of this halophile and was isolated from a saltern in western coast of India. The cells contain vesicle type intracytoplasmic membranes.

Absorption spectra show bacteriochlorophyll a with absorption maxima at (380, 595, 800 and 870 nm) and Spirilloxanthin at 550nm (Imhoff et al., 1998). *Rhodovibrio salinarum* JA137 formerly called as *Rhodospirillum salinarum*. The cells are rod or vibroid in shape having a width of approximately 0.3µm, length is usually 1-3µm. It is motile by means of a pair of flagella at one end of each cell. The cells multiply by binary fission. Photoheterotrophic growth anaerobically gave rise to bright red colonies. Its DNA base composition is 68.1% guanine plus cytosine (Imhoff et al., 1998).

The strain JA137 represents a potential halophile with regard to its uniquely designed molecular chaperones and chemical chaperones evolved accordingly, to withstand high salt and as such might serve as an explicit demonstration to elucidate its response physiologies in high salt. Therefore we made an attempt to clone *groESL* genes of this halophile *Rhodovibrio salinarum* JA 137 in order to explore the facts that while these biological chaperones perform the same function as their non halophilic counterparts, their ways of coordinating reactions in high salt conditions in concert to the chemical chaperones. We attempted to study the response physiology in high salt to elucidate the adaptive strategies displayed by this halophile *R. salinarum* JA137. The outstanding trait exhibited by strain JA137 was its ability to tolerate high salt which clearly indicates its latent potential with strategically designed molecular machinery for salt adaptations. Such approach to examine the salt adaptive mechanisms employed might serve to address in a unique way, many issues about salt tolerance.

1.10 Objectives

- Physiological responses of *Rhodovibrio salinarum* JA137 at varied salt concentrations
- Role of osmoprotectants, the chemical chaperones on *Rhodovibrio salinarum* JA137 during salt stress
- Effect of salt stress on photosystem integrity and pigment composition in *Rhodovibrio salinarum* JA137
- Isolation and cloning of genes coding for molecular chaperonins, GroES and GroEL of *Rhodovibrio salinarum* JA137

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Chemicals

All the chemicals used in the present study were of analytical grade from Sigma-Aldrich, Lancaster, Himedia, Qualigens, Merck, Genei and GE Health Care, BioRad.

2.2 Determination of pH

pH was determined using a digital pH meter (Digisun Electronics, India model DI-707) and pH meter was often calibrated with standard buffer solutions.

2.3 Buffers and standard solutions

Deionised and sterile water (Milli Q) was used for the preparation of buffers. Buffers were prepared according to standard protocols and pH was adjusted at room temperature unless otherwise stated.

2.3.1 General buffers

Phosphate buffer: 50 mM potassium, (pH range 5.5-7.5).

Borate buffer: 50 mM borate, (pH10).

Tris buffer 100 mM Tris/HCl (pH7.8).

2.4 Fixation/staining solution

2.4.1 Coomasie brilliant blue-250mg dissolved in 45/45/10 Methanol/ distilled water /acetic acid (v/v/v).

2.4.2 Destaining solution-12.5/77.5/10 Isopropanol/distilled water/acetic acid (v/v/v).

2.5 Dyes for confocal microscopy

2.5.1 Stock solutions

Acridine orange were prepared in DMSO (dimethyl sulfoxide) (1 $mg.ml^{-1}$), solutions were prepared in sterile Milli-Q water, SYTOX Green (5 mM) in DMSO solution was from Invitrogen. All dyes were stored at -20º C.

2.6 Sterilization

Sterilization of the culture media and glassware was done by autoclaving at 15 lbs 120º C for 15 minutes. Heat labile compounds were sterilized by filtration (Millipore) of their aqueous solutions through a 0.45 μm cellulose acetate membrane.

2.7 Purity of cultures

Culture was checked for purity before and after experimentation by streaking on nutrient agar plates (peptone-10 g l^{-1}, NaCl- 5 g l^{-1}, yeast extract-3 g l^{-1} and agar-20 g l^{-1}) and incubated under illumination (2,400 lx) at 30-32 °C.

2.8 Growth and Biomass

Growth was measured turbidometrically (OD_{660nm}) and the biomass as dry weight. Dry weight was calculated by empirical formula generated from plot of OD_{660nm} against dry weight $0.1(OD_{660nm}) = 0.15$ mg dry wt.ml^{-1}

2.9 Maintenance of stock cultures

2.9.1 Agar stabs

Stock culture of the purified isolate was maintained as agar stabs or as broth cultures. Stabs were prepared using growth medium (Table 4) with 2 % (w/v) agar as solidifying agent, filled to ¾ volume of 5 ml capacity screw cap test tubes. The culture was stabbed into the agar deeps and incubated under illumination (2,400 lx) at 30 - 32 °C. After 6-8 days of growth, the stab cultures were preserved under refrigeration at 4°C until further use. The stabs were sub-cultured and contamination from other bacteria was checked periodically by microscopic examination and by streaking on nutrient agar plates (g l^{-1}: peptone-5, Yeast extract-3 and agar-15) supplemented with 2 % NaCl (w/v) both under aerobic and anaerobic conditions.

2.9.2 Agar slants

Stock culture of the isolate was maintained as agar slants. Slants were prepared by using 2 % (w/v) agar solidified nutrient broth filled to 1/10 volume in test tubes in slanting position. Culture was taken onto the inoculation loop and streaked onto the slant. After 48 h of incubation, slant cultures were preserved under refrigeration at 4 ºC until further use. The slants were sub cultured and purity was checked periodically by streaking onto nutrient agar plates.

2.10 Mineral media composition

Table 2: Composition of mineral medium (Lakshmi *et al.*, 2009) used for the growth of purple non sulfur bacteria.

Ingredient	g l^{-1}
KH_2PO_4	0.25
$MgSO_4.7H_2O$	0.5
NaCl	20
NH_4Cl	0.34
$CaCl_2.2H_20$	0.25
Pyruvate	3.0
Yeast Extract	0.4
Ferric citrate solution (0.1 %, w/v)	1.5 ml
*Micronutrient solution (SL7)	1 ml
**$Na_2S·9H_2O/Na_2S_2O_3.5H_2O$	2 ml
***Vitamin B_{12} (2 mg/100 ml, w/v)	1 ml

*SL7: (mg l^{-1}): HCl (25%, v/v)–1 ml; $ZnCl_2$ (70); $MnCl_2.4H_2O$ (100); H_3BO_3 (60); $CoCl_2.6H_2O$ (200)$NiCl_2.6H_2O$(20);$Na_2MoO_4.2H_2O$(40);$CuCl_2.2H_2O$ (20); **$Na_2S·9H_2O$ solution $Na_2S·9H_2O$ (2.4 g) was dissolved in deionized water (10 ml) in 15ml screw cap test tube and flushed with nitrogen gas for 2-3 minutes to replace the air in the tube with nitrogen gas and closed tightly and was autoclaved.***Vitamin B_{12} (2 mg/100 ml, w/v) was dissolved in distilled water and filter sterilized by using 0.2 μm pore sized Millipore cellulose acetate membrane filters into a sterile screw cap tube. Final pH of the medium was adjusted to 6.8 -7.0 with sterile HCl (1 N)/ NaOH (1 N).

2.11 Nutrient Agar (ingredients $g.l^{-1}$).

NaCl-5, Peptone-10, yeast-3.5 and agar 20 g, pH7.0.

2.11.1 Luria and Bertani medium (L.B. MEDIUM)

Tryptone (10 gm), Yeast extract (5 gm), NaCl (10 gm) and pH adjusted to 7-7.2. All these ingredients were added to 950ml of double distilled water (DDW). The pH was adjusted to 7.2 with 1 N HCl/1 N NaOH and DDW was added to make up to 1 liter. In order to prepare LB Agar, 1.5% of agar agar (w/v) was added to the LB solution. This is then sterilized by autoclaving at 15psi pressure for 15-20 minutes. In case of LB Agar the media was allowed to cool to 50°C before adding appropriate antibiotics. About 25 ml of media was poured in 90 mm Petri plate and allowed it to solidify. These solidified LB plates were stored at 4 °C.

2.12 Kits, enzymes, chemicals and reagents

Molecular biology kits and enzymes were purchased from MBI Fermentas (Germany) and Takara bio (Japan). Sigma-Aldrich (USA), Qiagen (Germany), the manufacturers precautions were followed. The analytical grade chemicals and reagents were purchased from Sigma-Aldrich (USA), GE health care (USA), Fermentas (Germany), Agilent technologies (USA) Himedia (India), SRL (India) and Qualigens fine chemicals (India).

2.13 Model Organism

Rhodovibrio salinarum JA137 (=ATCC 35394 =DSM 9154) strain was given by Prof.Ch.Venkata Ramana's research group (University of Hyderabad) and this strain was used for all the experiments. *Escherichia coli* (DH5α) was used for the cloning experiments.

2.14 Photoheterotrophic (Anaerobic) growth

Rhodovibrio salinarum JA137 (ATCC 35394 =DSM 9154) was grown photoheterotrophically (anaerobic,30-32ºC;light,2400lux) on minimal media (KalyanChakravarthy et al., 2007) supplemented with pyruvate (22 mM) as carbon source and ammonium chloride (7 mM) as nitrogen source in fully filled screw cap tubes (10x100 mm)/reagent bottles (250 ml) at pH 7.0. Culture was grown anaerobically on mineral medium in 250ml conical flasks at 150 rpm in orbital shaker at 30ºC. Growth of the culture was monitored by measuring optical density at 660 nm using a Systronics make (Model 112) calorimeter (Filter 8) against an un-inoculated medium as blank. For growth experiments at different salt concentrations and with osmolytes, culture grown to log phase (an absorbance of ~0.6 OD at 660 nm) at 30 - 32 ºC was used.

2.15 Extractions

2.15.1 Preparation of cell free extracts

Seventy two hour old culture was centrifuged (14,000 rpm for 10 min), washed with buffer (0.02 M K-phosphate, pH 7) and the pellet was sonicated (6-8 cycles,probe MS 72 in Bendelin sonoplus sonicator). The sonicated sample was centrifuged (14000rpm for 10min) and the supernatant was used for further studies.

2.15.2 Total lipid extraction

Total lipids were extracted from frozen and thawed cells using the Bligh and Dyer method (methanol/chloroform/water:2:1:0.8, v/v as modified for extreme halophiles (Aruna sri et al.,2010).The extracts were dried in (liquid nitrogen) before weighing and then dissolved in chloroform**.**

2.15.3 Pigment extraction

Pigments were extracted from the bacterial cell pellet by using solvent mixture of acetone:methanol in the ratio 7:2.

2.16 Microscopic techniques

2.16.1 Confocal laser scanning microscopy analysis

Purified EPS or cells or halites were used in confocal microscopy. EPS or cells were stained with acridine orange. Five microliter of acridine dye (1 $mg.ml^{-1}$) was added to EPS/cells. After 20 minutes incubation in the dark, excess of the dye was washed with sterile Milli-Q water. Stained samples were mounted on to glass slide and visualized by confocal laser microscopy.

Confocal laser microscopic analysis of samples was performed on Zeiss microscope equipped with blue, green, and red lasers. Images were taken by 10X and 40X objectives. Probes were visualized at their respective emission wavelengths. Acridine orange $_{Ex}500/_{Em}526$ nm. Five random places were visualized for one sample.

2.16.2 Scanning electron microscopy (SEM)

Control or exposed cells as described were used for SEM analysis. Cells were harvested by centrifugation (4 ºC, 10,000 rpm, 5 min) and supernatant was discarded, the pellet was washed several times with autoclaved double distilled water to remove all traces of salt and media, pellet was suspended in 0.1 M phosphate buffer saline (PBS, pH 7.2).

Phosphate buffer saline suspended cells were centrifuged, PBS was discarded and the cells were prefixed in a mixture of glutarldehyde (2.4% final concentration),ruthenium red (0.01%) and

incubated for 30min. After prefixation, cells were removed by centrifugation (4 ºC, 10,000 rpm, 5 min) and suspended in 2.4% glutarladehyde and 0.01 % ruthenium red fixation for overnight at 4 ºC. Cells were removed by centrifugation (4ºC, 10,000 rpm, 6 min) from fixation solution, washed with PBS three times and finally cell pellet obtained was post-fixed in 1% osmiumtetraoxide solution for 2 h at 4 ºC. After post fixation, cells were harvested by centrifugation (4 ºC, 10,000 rpm,6 min) and washed with PBS for three times and the cells were dehydrated by series of ethanolic washes starting from 20%, 30%, 50%, 70%, 90% and 100% ethanol twice, incubated for 20 min. After dehydration cells were mounted on to cover slips(0.5x0.5cm) and dried by critical point dryer using standard protocol, dried samples were fixed to SEM stubs and coated with gold. The specimens were examined by using SEM (PhilipsXL30 series) and at different magnification ranges.

2.17 Analytical methods

2.17.1 FAME (Fatty acid methyl ester) analysis

Fatty acids of the samples were identified by FAME analysis was outsourced at Royal Life Sciences Pvt. Ltd. Fatty acids were saponified, methylated and extracted by using the protocol of the Sherlock microbial identification system (MIDI Inc, Sasser,1990). FAME's were analysed by gas chromatography**.** Photoheterotrophically grown cultures under different concentrations of NaCl were used for the analysis. The culture pellets were lyophilized and about 500 mg of bacterial cell pellet was taken and processed further for analysis.

2.17. 2 Thin Layer Chromatographic analysis (TLC) of polar lipids

Total lipid extracts were analysed by TLC on silica 60A plates (Merck, 20x10 cm, layer thickness 0.2 mm) in aluminium sheets. The plates were washed twice with chloroform/methanol (1:1, v/v) and activated at 180 ºC before use. Polar lipids were eluted with solvent A (chloroform/methanol/acetic acid /water85:15:10:3.5, v/v). The following stainings were performed in order to identify the lipid classes present in the TLC bands. (i) molybdophosphoric acid spray reagent (sigma) specific for phospholipids (Lobasso et al.,2012).

2.17.3 Thin Layer Chromatographic analysis (TLC) of Carotenoids

The pigment extracts were analysed by TLC on silica 60A plates (Merck, 20x10 cm, layer thickness 0.2 mm) in aluminium sheets. The mobile phase used for the TLC analysis was petroleum ether: Acetone in the ratio 7:2 (v/v).

2.18 Whole cell absorption spectrum

Absorption spectrum of whole cells was measured by the sucrose method (Arunasri etal., 2005). To 3.5 ml of the liquid culture, 5 g of sucrose was added and mixed thoroughly on a vortex spinner. The absorption spectrum from 300-1100 nm was measured on a Spectronic Genesys 2 spectrophotometer using sucrose in the medium as blank.

2.19 Saline requirement

To the prepared mineral medium (Table 4) with NH_4Cl (0.07 %, w/v) as nitrogen source, different concentrations (0%, 2%, 5%, 10%, 15%, 20%, 25%, 30%,) of sodium chloride were added before autoclaving. Media with out NaCl served as control. One percent of the culture was inoculated and incubated under phototrophic conditions at 2,400 lx and at 30-32 °C.

2.20 Biofilm quantification

The Quantification of biofilm was carried out using a standard microtiter plate assay, a static biofilm quantification protocol using crystal violet as the dye. Fresh culture which was inoculated into a culture flask was allowed to grow to 0.3 OD. This culture was reinoculated into another sterilized flask with fresh media and was grown to an OD of 0.3. This culture of 300 microlitre volume was inoculated into each well of the microtitre plate and grown until it reaches an OD of 0.6. After it reached the maximum OD, the media from each well is carefully aspirated with a pipette and the wells with the biofilm on their walls is washed with a buffer twice and dried for 15 minutes. Crystal violet dye mixed in 100% ethanol is added to each well and incubated for half an hour. After incubation the absorbance readings are taken at an OD of 600nm, thus the biofilm is quantified.

2.21 Sucrose Gradient Centrifugation for Photosystems

2.21.1 Preparation of membranes, pigment-protein solubilisation and fractionation

Photosynthetic membranes were extracted from cells by sonicating the cell pellet after centrifugation. Pigment proteins were solubilised from these membranes by a protocol using 4% n-dodecyl-β-D-maltoside (DDM). Solubilised pigment proteins were fractionated by ultracentrifugation on (w/v) five step (0, 0.25, 0.5, 1, 1.5% sucrose in buffer A, at 250,000 g at 4 °C for 18 h) five-step sucrose density gradients. After photography, sucrose gradients were fractionated for analysis by absorbance spectroscopy.

2.22 Reactive oxygen species evaluation

ROS (reactive oxygen species) studies were carried out with H_2DCFDA which was added to the culture and incubated for an hour and then the fluorescence readings were taken at λex-485 nm/λem-530 nm. The cells are to be treated with various concentrations of test compounds/standard/ for 1 h. For the estimation of intracellular ROS, the supernatant in the microtitre plate is replaced with DCFH-DA (100 μM) (a cell-permeable non-fluorescent substance that undergoes intracellular oxidation in the presence of ROS to the highly fluorescent dichlorofluorescein) and incubated for 1 h. The wells are then washed with freshly prepared salt solution and fluorescence intensity measured using a fluorescence microplate reader (FLx800, Biotek Instrument Inc., Winooski, VT, USA) at λex-485 nm/λem-530 nm.

2.23 Colorimetric Analysis

2.23.1 Estimation of Glycine betaine

The estimations of the osmolyte glycine-betaine is performed according to the standard protocol of Bhaskaran et al., 1985. The assay is based on the principle that at low temperature betaine makes a betaine-periodite complex with iodide in acidic medium, which absorbs at 360 nm in UV range. According to this method of estimation cold potassium iodide-iodine solution: Iodine (15.7 g) and potassium iodide (20 g) were dissolved in 100 ml of water (distilled water) and kept in refrigerator at 4ºC. Concentrated Sulphuric acid: Fifty-five ml of sulphuric acid (2N) is dissolved in distilled water and the volume is made up to 1 liter. Sonicated culture of (50 ml) was mixed with 2 ml of deionized water . The samples were then filtered through Whatman No. 1 filter paper (Schleicher & Schuell, cat#1001917) and the filtrates were used for analysis. The extract was diluted with 2 N Sulphuric acid, (1:l v/v) and 0.5 ml of the acidified extract was cooled in ice water for 1 h. Later, 0.2 ml of cold potassium tri-iodide solution was added and mixed in a vortex mixture and the tubes were stored at 4 ºC for 15

2.23.2 Estimation of Proline

Measurement of cellular proline pools.

The intracellular proline contents of *Rhodovibrio salinarum* JA137 strain were determined by a colorimetric assay detecting proline as a colored proline-ninhydrin complex that can be quantified by measuring the absorption of the solution at 520 nm. Cells of the *R. salinarum* JA137

studied were grown in in the absence or presence of proline, using NaCl and proline concentrations specified in the figures. The cells (8 ml) were then harvested by centrifugation and were frozen at 20°C until further use. The soluble cellular content was extracted, and the amount of proline was assessed according to the procedure detailed by Bates et al., 1973. Acid ninhydrin was prepared by dissolving in 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M H_3PO_4. The soluble cellular content was extracted with 0-5 ml acid ninhydrin and 0.5 ml glacial acetic acid at 100 °C for 1 h. Samples were then plunged into ice and the colour extracted by mixing vigorously with 2 ml toluene. After 15 min the absorbance was read at 520 nm against a toluene blank and the amount of proline was assessed according to the procedure detailed by Bates et al. Proline concentrations were determined by establishing a standard curve with L-proline for the colorimetric proline assay, and intra cellular proline concentrations of *R.salinarum* JA137 were then calculated with readings at an OD_{520nm} unit of cell culture.

2.24 Molecular biology protocols

All the Plasmid and genomic DNA isolations, restriction digestions, agarose gel electrophoresis, ligation, competent cell preparation and transformation were performed according to Sambrook et al., (1989) and/or as per manufacturers' protocol.

2.24.1 Plasmid DNA vectors

Commercially available T-vector of Fermentas /Bangalore genei was used for simple TA-cloning experiments and blue white screening.

2.24.2 Plasmid isolation

Plasmid isolation was done through conventional alkaline lysis method (Sambrook Russell, Molecular cloning, a laboratory manual).

2.24.3 Genomic DNA isolation

2.24.4 DNA extraction and purification

The culture was harvested by centrifugation (10,000 rpm for 15 minutes) and their genomic DNA was isolated by the modified method of Marmur (Srinivas et al., 2007). Except the solvents used, glassware, buffers, solutions were all sterilized by autoclaving. DNA was dissolved in a concentrated buffer solution or milliQ water and the absorption at 260 nm was noted using a UV-VIS spectrophotometry.

2.24.5 Agarose gel electrophoresis

Genomic DNA 1 µl and 6 µl of standard genomic DNA (as marker) were electrophoresed (Bangalore GENEI) in 0.8 % (w/v) horizontal agarose gel in TAE buffer at 15 V cm^{-1}, stained in 0.5 µg ml^{-1}ethidium bromide and visualized on UV transilluminator (Bangalore GENEI).

2.24.6 PCR amplicon purification

The amplified product was purified by using the QIAquick PCR Purification Kit (Cat. No.28104), the quality and concentration of the purified product was checked by agarose gel electrophoresis as described previously

2.24.7 Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from the culture pellet of *R. salinarum* JA137 by the modified method of Marmur. PCR amplification was performed using designed primer sets and amplification of *groESL* gene was performed accordingly as mentioned with few modifications. The band with expected length was excised from the gel, purified using a QIAGEN gel purification kit and sequenced. The PCR was carried out with genomic DNA, primers, dNTPs, taq polymerase, $MgCl_2$, buffers, PCR grade water as the ingredients with a specific programme set in the thermocycler**.**

2.24.8 PCR amplification of *groESL* gene and sequencing

Culture (250 ml) was harvested after 84 h by centrifugation (10,000rpm for 10 min). Genomic DNA was extracted from the pellet by the modified method mentioned above. PCR amplification was performed using primer sets listed in Table 3, designed from alignments of known sequences (EMBL accession numbers). PCR amplification for *groESL* gene was performed according to the instructions of the manufacturer with few modifications and parameters were mentioned in Table 4. The products were pooled up and run on a 1.5-2 % agarose gel. On an agarose gel more than one band was observed, the band of the expected length was excised from the gel and purified using a QIAGEN gel purification kit (QIAGEN,) and sequenced, (which was outsourced).

2.24.9 Oliginucleotides and sequencing

All DNA oligonucleotides were synthesized by either Sigma Aldrich or MWG Biotech (Eurofins). Nucleic acid sequencing was carried out by Eurofins according to the requirement. All the clones were confirmed by sequencing.

Table 3: Primer sets designed from alignments of known (*Rhodospirillum rubrum*) *groESL* sequences

Primer sets	Sequence (5'–3')	Annealing temp (°C)
1) GroES- R5'-CCGCAGTGATGTCGCGCATTCTCCTTCATGAT- 3'		63
2) GroES- F5'-GACACCGCCAAGGAAAAGCC- 3'		60
3) GroEL- R5'-CAGACGCTCCTGCAGCTTCTC- 3'		63
4) GroEL- F5'-GACAAGTTCGAGAACATGGGC- 3'		60

Table 4: PCR cycling parameters (modified according to primers and according to manufacturer's instructions) used to amplify *groESL* in *Rhodovibrio salinarum* JA137

Steps	Temp (^{o}C)	Time (min)	Cycles
Initial denaturation	94	4 (sec)	
Denaturation	94	1	
Annealing	65	1	30
Elongation	72	1	
Final extension	72	5	

2.25 BLAST search

The single contig of sequence of length approximately 1350 to 1450 bp was submitted to the NCBI-BLAST search in order to know the nearest phylogenetic relative. EzTaxon server (web based database of 16S rRNA gene sequences-easiest way to the accurate identification of prokaryotes) is more useful for comparison of 16S rRNA gene sequences.

2.25.1 Collection of 16S rRNA gene sequences of the type strains

Based on the blast search results, sequences of the closely related members and an out group sequence were obtained in FASTA format from National Center for Biotechnology Information (NCBI) - Nucleotide search.

2.25.2 16S rRNA gene Sequence Alignment

Sequences (all the closely related type strain sequences along and the sequence to be analyzed) were aligned using the CLUSTAL X program (Thompson et al., 1997).

min and centrifuged at 10,000 rpm for 15 min. The supernatant was aspirated with a fine-tipped glass tube. The per iodide crystals were dissolved in 9 ml of 1,2-dichloroethane with vigorous shaking. After 2.5 h, the absorbance was measured at 365 nm in a Spectrophotometer. The glycine betaine content was determined from a standard curve prepared with glycine betaine in 1 N suluphuric acid, and expressed in µg/g.dw. Potassium tri-iodide reagent: 15.7 g of iodine and 20 g of potassium iodide were dissolved in 100 ml of distilled water and stirred in a vortex mixture.

2.26 Designing degenerate primers

Rhodovibrio salinarum JA137 genome is not yet fully sequenced, therefore, using NCBI blast search, retrieved 16s rRNA sequences of all the closely related organisms of *R. salinarum* (*Rhodospirillum rubrum*-very closely related to *Rhodovibrio salinarum* JA137). Clustal aligned all the sequences retrieved. Also retrieved *groES* and *groEL* sequences of all the closely related organisms of *R.salinarum* JA137 using NCBI blast search and performed clustal alignment to get the conserved regions. Then taking the conserved regions from the clustal aligned sequences designed degenerate primers for both *groES* and *groEL*. Then performed PCR amplification using these degenerate primers with taq polymerase and Pfu polymerase. GroESL band (amplified) was selected and after gel extraction and purification, was sequenced. The sequencing results were checked for similarity with the cpn10-cpn60 (i.e.*groESL*) of *Rhodospirillum rubrum* and then designed primers based on the similarity (gene specific primers for genome walking). Genomic DNA of *R. salinarum* JA137 was checked for purity by agarose gel electrophoresis, first digested with Dra-1 to check for the purity of genomic DNA after getting a good smear with Dra 1 digestion, digested with stu1, EcorV, PvuII, and purified and ligated with adaptors as suggested in the genome walking protocol. The sequencing results were clustal aligned for finding the high similarity match and selected the high similarity sequence 79% (percentage of similarity with *Rhodospirillum rubrum*) and 75% with *groELR* (reverse) and *groESF* (forward) respectively for designing gene specific primers for performing primary and secondary PCR subsequently. The secondary PCR products are gel extracted, purified, TA cloned and sequenced to obtain the whole length gene of *groESL* cloned.

2.27 Genome walking

Genome Walker DNAwalking is a simple method for finding unknown Genomic DNA sequences adjacent to a known sequence such as a cDNA using the method of (Siebert et al.,1995). Using the genomic DNA isolated from the halophile *Rhodovibrio salinarum* JA137, the first step followed was to construct pools of uncloned, adaptor ligated genomic DNA fragments, which are referred to for convenience as Genome Walker "libraries." The starting DNA was ensured to be very pure and has a high average molecular weight, requiring a higher quality preparation than the minimum suitable for conventional PCR. To ensure that the genomic DNA isolated is of adequate quality, the controls from the kit are used for comparison. Separate aliquots of DNA are completely digested with different restriction enzymes that leave blunt ends. The Genome Walker Universal Kit provides with a set of four restriction enzymes. Each batch of digested genomic DNA was then ligated separately to the Genome Walker Adaptor. After the libraries have been constructed, as per the protocol two PCR amplifications per library were performed. The first or primary PCR was performed with the outer adaptor primer (AP1) provided in the kit and an outer, gene-specifc primer (GSP1) designed in the lab. The primary PCR mixture is then diluted and used as a template for a secondary or "nested" PCR with the nested adaptor primer (AP2) and a nested gene-specifc primer (GSP2). This resulted in the production of a single, major PCR product from the four libraries. Each of the DNA fragments which began in a known sequence at the 5' end of GSP2 and extended into the unknown adjacent genomic DNA, was cloned and further analyzed by sequencing.

The following reagents are required for performing genome walking.

Phenol, Chloroform, Glycogen (10 µg/µl),3 M sodium acetate, 95% ethanol, 80% ethanol, TE 10 mM Tris, 0.1 mM EDTA (10/0.1, pH 7.5), TE 10 mM Tris, 1 mM EDTA (10/1, pH 7.5), 0.5X TBE buffer or 1X TAE buffer, Advantage 2 Polymerase Mix (50X).

We used a Taq-based 50X polymerase mix suitable for PCR. Conventional PCR with a single polymerase did not produce a band in most of the Genome Walker experiments. This protocol has been optimized with the Advantage 2 Polymerase Mix (Cat. No. 639201). This enzyme mix was specifically developed for PCR amplifications of genomic DNA templates of all sizes. The adaptor is ligated to both ends of the genomic DNA fragments to create Genome Walker libraries. The structure of the adaptor primers is shown below.

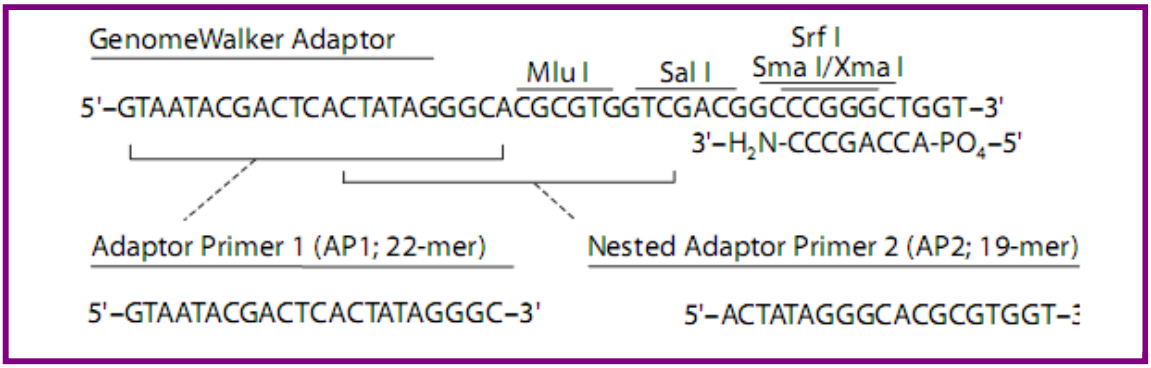

Fig. 10 The sequences of the adaptor primers supplied by the manufacturer of the genome walking kit.

The following reagents are required for performing PCR in genome walking.

50X Advantage 2 Polymerase Mix	30 µl
10X Advantage 2 PCR Buffer	200 µl
10X Advantage 2 SA PCR Buffer	200 µl
50X dNTP Mix (10 mM each)	50 µl
Control DNA Template (100 ng/µl)	30 µl
Control Primer Mix (10 µM)	30 µl
PCR-Grade Water	2.5 ml

2.27.1 Construction of the GenomeWalker libraries

Restriction enzymes and buffers: Dra I (10 units/µl),10X Dra I Restriction Buffer, EcoR V (10 units/µl), 10X EcoR V Restriction Buffer, Pvu II (10 units/µl),10X Pvu II Restriction Buffer, Stu I (10 units/µl), 10X Stu I Restriction Buffer, Control Human Genomic DNA (0.1 µg/µl),T4 DNA Ligase (6 units/µl), 10X Ligation Buffer, Genome Walker Adaptor (25 µM),Adaptor Primer 1 (AP1; 10 µM), Nested Adaptor Primer 2 (AP2; 10 µM), Genome Walker Human Positive Control Library. Positive Control tPA Primer (PCP1; 10 µM), Positive Control tPA Nested Primer (PCP2; 10 µM).

The following protocol was designed for the construction of four libraries from experimental genomic DNA and one Pvu II library from positive control human genomic DNA (provided in kit).

2.27.2 Quality check of Genomic DNA

1. Checked the size of genomic DNA on a 0.6% agarose/EtBr gel. Loaded 1 µl of experimental genomic DNA (0.1 µg/µl) and 1 µl of control genomic DNA (0.1 µg/µl) on a 0.6% agarose/EtBr gel in 1X TAE, along with DNA size markers, such as a 1 kb ladder or λ/Hind III digest.

2. Checked the purity of genomic DNA by Dra I digestion. In a 0.5-ml reaction tube, combined the following; 5 µl Experimental genomic DNA; 1.6 µl Dra I (10 units/µl); 2 µl 10X Dra I Restriction Buffer; 11.4 µl Deionized H2O; Also set up a control digestion without enzyme; Mixed gently by inverting tube; Incubated at 37°C overnight, Ran 5 µl of each reaction on a 0.6% agarose/EtBr gel along with 0.5 µl of experimental genomic DNA as a control.

2.27.3 Digestion of Genomic DNA

For each library construction, set up a total of five reactions. For experimental genomic DNA, set up four blunt-end digestions one for each blunt-end restriction enzyme provided. Additionally, set up one Pvu II digestion of human genomic DNA as a positive control. Labelled five 1.5-ml tubes: DL1, DL2, DL3, DL4, and positive control. For each reaction, combined the following in 1.5-ml tube; 25 µl Genomic DNA (0.1 µg/µl); 8 µl Restriction enzyme (10 units/µl);10 µl Restriction enzyme buffer (10X);57 µl Deionized H2O. Mixed gently by inverting tube; Incubated at 37°C for 2 hr; Vortexed the reaction at slow speed for 5–10 sec; Incubated at 37°C overnight (16–18 hr); From each reaction tube, removed 5 µl and ran on a 0.6% agarose/EtBr gel to determine whether digestion is complete. Saved an additional aliquot of each sample to run on the gel.

2.27.4 Purification of DNA

To each reaction tube, added an equal volume (95 µl) of phenol.; Vortexed at slow speed for 5–10 sec; Spun briefy at room temperature to separate the aqueous and organic phases;Using a pipet, transferred the upper (aqueous) layer into a fresh 1.5-ml tube; Discarded the lower (organic) layer properly into the chlorinated hazardous waste; to each tube, added an equal volume (95 µl) of chloroform; Vortexed at slow speed for 5–10 sec; Spun briefy at room temperature to separate the aqueous and organic phases; using a pipet, transferred the upper (aqueous) layer into a fresh 1.5-ml tube; Discarded the lower (organic) layer properly into the chlorinated hazardous waste.

To each tube, added 2 volumes (190 µl) of ice cold 95% ethanol, 1/10 volume (9.5 µl) of 3 M NaOAc (pH 4.5), and 20 µg of glycogen;Vortexed at slow speed for 5–10 sec; Centrifuged at 14,000 rpm for 15 min at 4°C;Decanted the supernatant and washed the pellet in 100 µl of ice cold 80% ethanol; Centrifuged at 14,000 rpm for 10 min; Decanted the supernatant and air dried the pellet.; Dissolved pellet in 20 µl of TE (10/0.1, pH 7.5); Vortexed at slow speed for 5-10 sec; From each reaction tube, removed 1 µl and run on a 0.6% agarose; EtBr gel to determine the approximate quantity of DNA after purification.

2.27.5 Ligation of GenomeWalker™ Adaptors to Genomic DNA

For each library construction, set up a total of five ligation reactions. Obtained four blunt-end digestions of the experimental genomic DNA and one positive control Pvu II digestion of human genomic DNA. From each tube, transferred 4 µl of digested, purifed DNA to a fresh 0.5-ml tube. To each, added the following; 1.9 µl GenomeWalker Adaptor (25 µM); 1.6 µl 10X Ligation Buffer;0.5 µl T4 DNA Ligase (6 units/µl);Incubated at 16°C overnight; A PCR thermal cycler holds a very constant temperature and so used this in place of a water bath for this reaction; to stop the reactions, incubated at 70°C for 5 min; to each tube, added 72 µl of TE (10/1, pH 7.5);vortexed at slow speed for 10–15 sec.

2.27.6 Designing of the Gene Specific Primers

Two gene-specifc primers, one for primary PCR (GSP1) and one for secondary PCR (GSP2) were designed . The nested PCR primer was designed to anneal to sequences beyond the 3’ end of the primary PCR primer (i.e., upstream of the primary PCR primer when walking upstream and downstream of the primary PCR primer when walking downstream). The primers are designed in such a way, so that the outer and nested primers did not overlap; when overlapping primers were used, it was ensured that the 3’ end of the nested primer had as much unique sequence as possible. The gene-specifcprimers were derived from sequences as close to the end of the known sequence as possible. For walking upstream from the DNA sequence, the primer was ensured to be as close to the 5’ end as possible. Gene-specifc primers were 26–30 nucleotides in length and had a GC content of 40–60%. (Even if the Tm’s seem high, designed primers were not shorter than 26 bp. This was ensured so that the primers will effectively anneal to the template at the recommended annealing and extension temperature of 67°C. Primers should not be able to fold back and form intramolecular hydrogen bonds, and sequences at the 3’ end of the primers should not be able to anneal to the 3’ end of the adaptor primers. There were no more than three

G's and C's in the last six positions at the 3' end of the primer. (Five restriction sites have been incorporated into the Genome Walker Adaptor—Sal I (cohesive ends), Mlu I (cohesive ends), and overlapping Adaptor—Sal I (cohesive ends), Mlu I (cohesive ends), and overlapping Srf I (cohesive ends), Sma I (blunt ends), and Xma I (cohesive ends) sites. The sites in the Adaptor Primer allow easy insertion of PCR products into commonly used promoter reporter vectors) (Fig.1) according to the manufacturer.

2.27.7 Procedure for PCR-based DNA Walking in Genome Walker Libraries

The Genome Walker DNA walking protocol consists of eight primary and secondary PCR amplifcations: four experimental libraries, two positive controls (Genome Walker Human Positive Control Library and one positive control library constructed from Control Human Genomic DNA), and two negative controls (without templates). For both positive controls, used the positive control gene-specifc primers, PCP1 and PCP2 (provided). For primary PCR, used 1 µl of each library. For secondary PCR, used 1 µl of a 50X dilution of the primary PCR product. All GenomeWalker PCR steps have been optimized with the Advantage 2 Polymerase Mix, which includes TaqStart Antibody for automatic hot start PCR.

RESULTS AND DISCUSSION

3.0 Results and Discussion

3.1 Growth physiology of *Rhodovibrio salinarum* JA137

Halophiles are well distinguished from other life forms by their requirement for high salt for their survival and growth (DasSarma and DasSarma, 2012). Although most of the marine organisms can be categorized as slight halophiles, the moderate and extreme halophiles are specialized microbes, as they have unique genetic makeup to flourish in hypersaline environments with salinity higher than in the sea (Shivanand and and Mugeraya, 2011). Moderate halophiles are reported to have their optimal salt range of 5 - 15% of NaCl (Irshad et al., 2014). The adaptation strategies and the unique physiologies of halophiles are being explored for gaining insights into their genetic makeup and the evolution of the molecular mechanisms of adaptations to salt stress. The model system of this work, *Rhodovibrio salinarum* JA137 is a photosynthetic, anoxygenic purple nonsulphur bacterium, isolated from salterns of western India. Based on its natural habitat probably it is a halophile with high genetic potential that can be explored. Moreover, salt tolerance mechanism of anoxygenic purple non sulphur bacteria were not well studied. Therefore the influence of salt (NaCl) concentrations on the growth patterns of *Rhodovibrio salinarum* JA137 was investigated.

3.1.1 Growth patterns of strain JA137 at different salt concentrations

Growth analysis was done to identify the tolerance range of strain JA137 and salt concentrations used were from 0 M to 5 M of NaCl, in the modified Biebel and pfennings medium. Log phase culture growing in (0.8 M) NaCl was used as starter culture and inoculated in fresh media with different concentrations of salt to an initial cell density of OD_{660} (0.04) and allowed to grow photoheterotophically as mentioned in materials and methods. Growth in terms of cell density OD_{660} was measured at regular intervals of time. Interestingly, this strain requires a minimum concentration of 0.35 M in the external medium for survival and growth. At concentrations lower than 0.35 M NaCl no increase in cell density was observed even after 120 h of growth. At 30% NaCl (5 M) no increase in cell density was observed even after 60 h of growth but cells started dividing after 72 h of growth reaching to the final cell density of OD_{660nm} 0.18 and reached OD_{660nm} 0.25 after 84 h. Despite of its natural saline habitat and wide range of salt tolerance from 0.35 M to 5 M of salt in external medium, strain JA137 exhibited relatively slow growth at high salt

concentrations and different growth profiles at different salt concentrations (Fig. 6). The generation time of the cells grown at 5 M is 9 h, and at 1.73, 2.6 M is 14 h. However the generation time for cells grown at high salt concentrations (3.5 M, 4.3 M, 5 M) could not be determined as a clear log phase was not observed. From the figure. 6, it is evident that from 0.35 to 5 M is its growth range including suboptimal and hyper salt stress conditions and salt required for optimal growth is in the range of 0.8 to 2.6 M (Fig. 7).

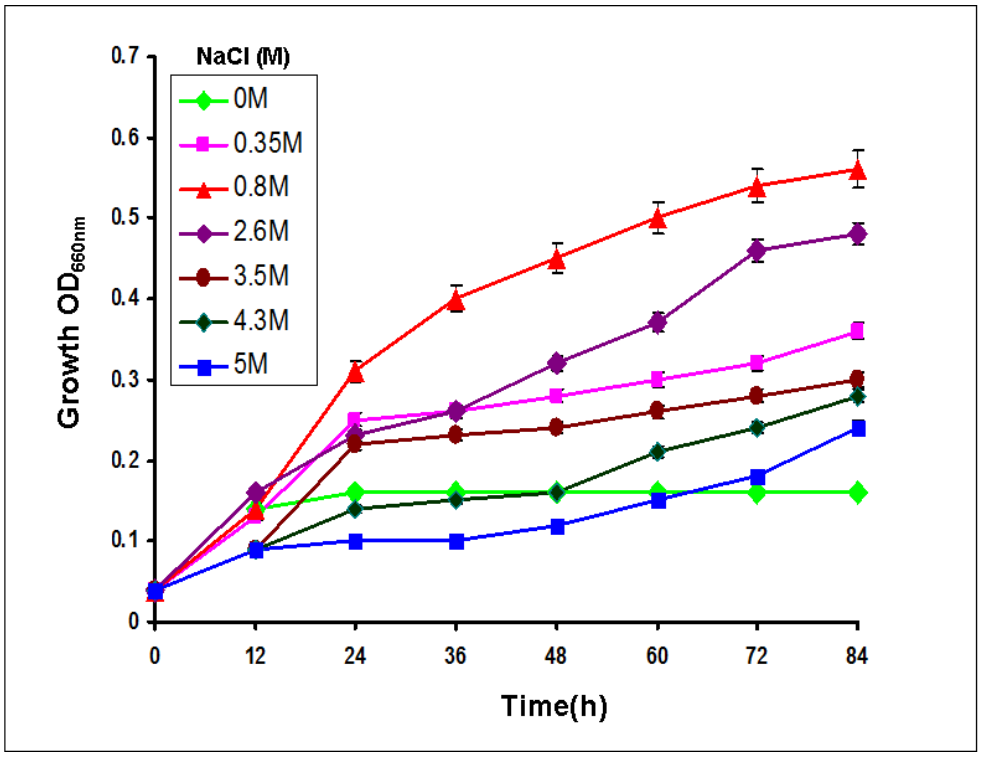

Fig. 6: Photoheterotrophic growth of *Rhodovibrio salinarum* JA137 at different NaCl concentrations.

The growth anlaysis of *R. salinarum* JA137 at different concentrations (0 M, 0.35 M, 0.8 M, 2.6 M, 3.5 M, 4.3 M and 5 M) of NaCl was shown in the figure. The strain JA137 was grown as mentioned in the methods section (2.8). Cell density in terms of O.D was recorded at regular time intervals and the graph represents the average of three biological replicates with (standard deviation) as error bars of three independent experiments.

3.1.2 *Rhodovibrio salinarum* JA137 is a moderate halophile

There were differences in the biomass of the strain *R. salinarum* JA137 when grown under different salt concentrations ranging from 0 M, 0.35 M, 0.8 M, 2.6 M, 3.5 M, 4.3 M and 5 M. Optimum with growth $O.D_{660\ nm}$ 0.58-0.48 was observed at 0.8 -2.6 M NaCl concentration and reaches logarithmic phase within 24-72 h with $O.D_{660\ nm}$ 0.58-0.6, whereas no growth was observed when grown without NaCl (0%) and reduced at high NaCl concentrations (30%). However, strain JA137 at high salt concentration (30%), with poor growth($O.D_{660\ nm}$ 0.18), remained in this phase till 72 h and thereby reached log phase very slowly ($O.D_{660\ nm}$ 0.25), whereas no growth was observed when grown without NaCl (0%) even after 84 h also ($O.D_{660\ nm}$ 0.1). This indicates that the strain JA137 tolerates high salt concentrations (30%) and requires minimum salt concentration to survive. From the above observations, strain JA137 required optimum NaCl concentration i.e., 5% (0.8-2.6 M) for its growth (Fig. 7). The biomass after 72 h in 0.8 M is 0.8 mg.drywtml^{-1} is represented here in (Fig.7). However the biomass after 72 h in 1.73 M and 2.6 M was around 0.8 mg.drywtml^{-1}. The maximum biomass reached was 1.5 mg. drywtml^{-1} in 0.8 M after 84 h.

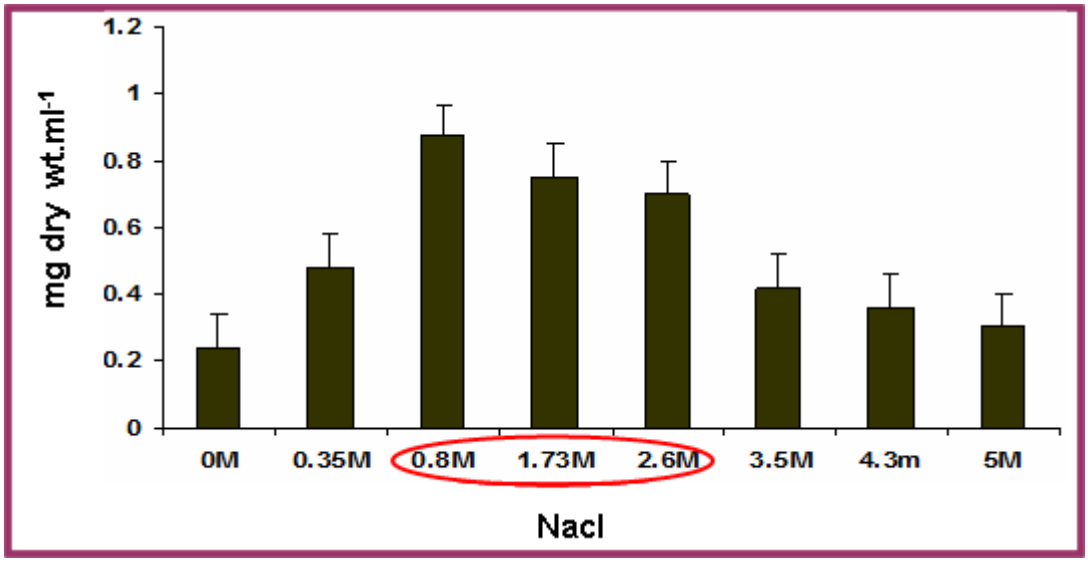

Fig. 7: The biomass as mg dry weight at 72 h (early stationary phase with reference to 0.8 M) of inoculation.

The growth anlaysis of *R. salinarum* JA137 at different concentrations (0M, 0.35M,0.8M, 1.73M, 2.6M, 3.5M, 4.3M and 5Mw/v) of NaCl was shown in the figure and the culture was grown as mentioned in methods section (2.8). Biomass in terms of mg.drywtml^{-1} and the graph represents the average of three biological replicates with (standard deviation) as error bars of three independent experiments. The concentrations (0.8, 1.73, 2.6 M) at which the optimum biomass was observed after 72 h is represented by a red circle.

The cultures grown at the 0.8-2.6 M salt range showed biomass of 0.9-0.68 mg dry.wt.ml^{-1}. Cells grown at 0.35 M showed biomass (0.45 mg.drywt ml^{-1}) and cells grown at 3.5 -5 M showed an yield with a biomass of 0.4-0.28 mg drywtml^{-1}. The maximum biomass was observed at 0.8 M (0.85-1 mg dry.wt.ml^{-1}). This result indicated that the optimum range of NaCl concentration for growth is 0.8-2.6 M (Fig. 7). The optimum salt concentration (0.8-2.6 M) for growth of strain JA 137 falls within the range reported in other moderate halophiles (Oren et al., 2006), suggesting that the strain JA137 is a moderate halophile.

Strain JA137 grows in presence of NaCl between 0.35 M (2%) and 2.6 M (15%), however there is a gradual decrease in $OD_{(660\ nm)}$ with increasing salt concentration and even can tolerate upto 5.0 M (30%) NaCl (Fig. 6, 7). Therefore this organism may be termed a moderate halophile with a minimum requirement of salt for its survival and an optimum range of NaCl concentration from 5 - 15% (Oren, 2008, 2013) according to classification of halophiles.

3.1.3 Growth of strain JA137 at different salt concentrations and osmolytes

The growth experiments indicated that strain JA137 requires minimum of 0.35 M NaCl in the external medium for its survival and it is also capable of tolerating high salt concentrations which made it evident that it is well equipped with the molecular machinery to adapt accordingly and thus survive the high salt concentrations in its surroundings. Bacteria combat salinity stress by increasing the pool size of the osmolytes, switch the osmolyte strategy with increasing medium salinity (Das Sarma et al., 2012). Therefore with an anticipation to gain an overview of such aspects in strain JA137. Also we performed growth analysis of strain JA137 by supplementing with osmolytes, glycine betaine and proline along with varied salt concentrations (Fig. 8) and the endogenous levels of the osmolytes at different salt concentrations were measured (Fig. 9).

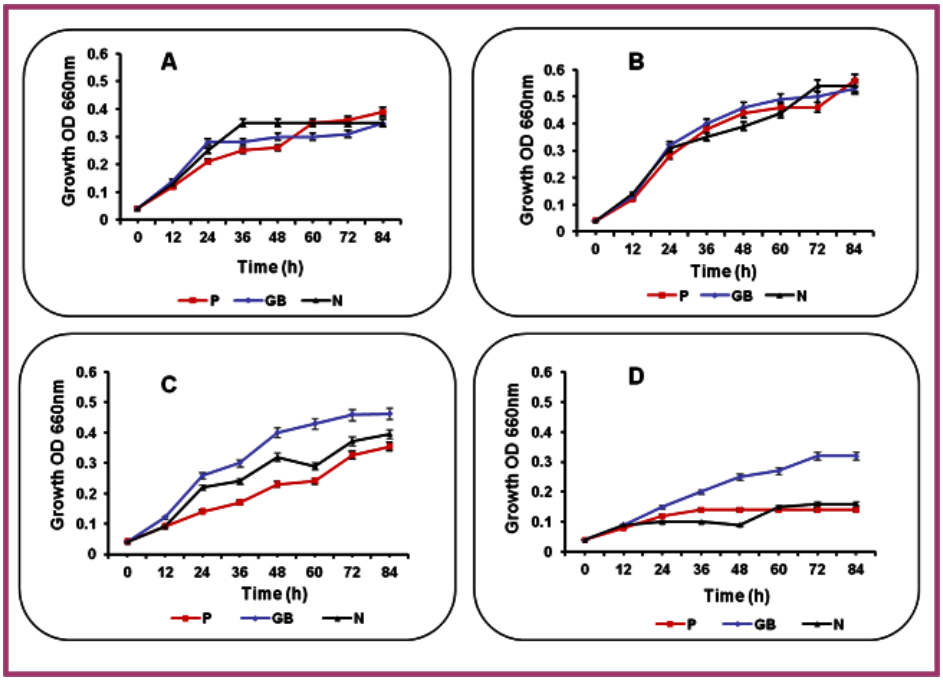

Fig. 8 Growth profiles of the strain JA137 with varied salt concentrations and osmolytes supplementation. Concentration of NaCl (N) in the external medium is 0.35 M (A); 0.8 M (B) ; 3.5 M(C); 5 M(D).

One mM of final concentration of Glycine-Betaine (GB) and proline (P) were added to the medium. Represented values are average of three individual experiments.

Our primary focus being, to unravel *R. salinarum* JA137 potential to survive 30% salt and if it is so, its growth response in presence of glycine-betaine and proline in the media. Whether this organism is able to utilize its latent molecular potential to exploit the osmoprotectants and able to flourish high salt 5 M (30%) also. Enhanced growth was observed at 30% with an OD_{660nm} (0.35) with glycine-betaine and but not significantly with proline (OD_{660nm} (0.2) which is a remarkable observation particularly at high salt. However this was not observed with low salt (0.35 M) grown cells of strain JA137. However 0.35 M (2%) salt grown cells showed improved OD_{660nm} (0.4) growth with proline supplementation interestingly.

Growth of strain JA137 in presence of varied NaCl concentrations [0.35 (2%), 0.8 (5%), 3.5 (20%), 5 M (30%)] supplemented with osmolytes, Glycine-betaine (1 mM) and proline (1 mM)

was performed. Growth of stain JA137 was not significantly influenced at 5% NaCl concentration even though supplemented with glycine-betaine and proline (Fig. 8B), however slight decrease in the growth was observed in strain JA137 when supplemented with Glycine-betaine and proline at 2% NaCl concentration (Fig. 8A). Growth was observed to be significantly enhanced in the presence of glycine betaine particularly at high NaCl concentrations (20% and 30%). Comparatively it was observed that proline showed no such significant enhancement in growth particularly at high salt concentrations. (Fig. 8C, D). From the above results growth in strain JA137 was observed to be enhanced with glycine-betaine supplementation. Inline with previous reports which also suggested that a moderate halophilic bacterium *Tetragenococcus halophila*, when supplemented with 2 mM of Glycine-betaine or choline or carnitine showed enhanced growth not only at high salt concentrations (up to 2.5 M) but also in the absence of NaCl (Robert et al., 2000) which suggests that glycine-betaine promotes growth reverting the influence of salt on the growth of the organism.

3.1.4 Endogenous levels of osmolytes (Glycine-betaine and Proline) at different salt concentrations

The experimental analysis of the levels of osmolytes glycine betaine and proline endogenous levels with the osmolytes was performed. In order to examine the response of the organism towards the availability of the osmolytes and how better they were utilized to withstand the salt stress. Previous reports proposed that the halophiles prefer absorption of the osmolytes from the surroundings that the denovo synthisesis as it an expensive process for them and it is taxing for the cell already under stress. Therefore the organism prefers the other mode of taking osmolytes from the surroundings and utilize it for its adaptive strategies and other metabolic functions accordingly. Through our limted analysis done in examining the endogenous levels we have observed that the glycine betaine content increased (200µg/gm.dry.wt) in the case of 2% salt and (260 µg/gm.dry.wt) in case of 5%salt and (250 µg/gm.dry.wt) in case of 30% salt when endogenous levels were compared. When we considered proline levels at 2% salt (15µg/gm.dry.wt), in case of 5% (38µg/gm.dry.wt) and 30% (28µg/gm.dry.wt) was observed (Fig. 9). The slight increase in the exogenous levels of osmolytes can be interpreted as that the supplementation of osmolytes helped the organism to execute its other adaptive mechanisms with minimum energy at its disposal and thus facilitate survival at high salt.

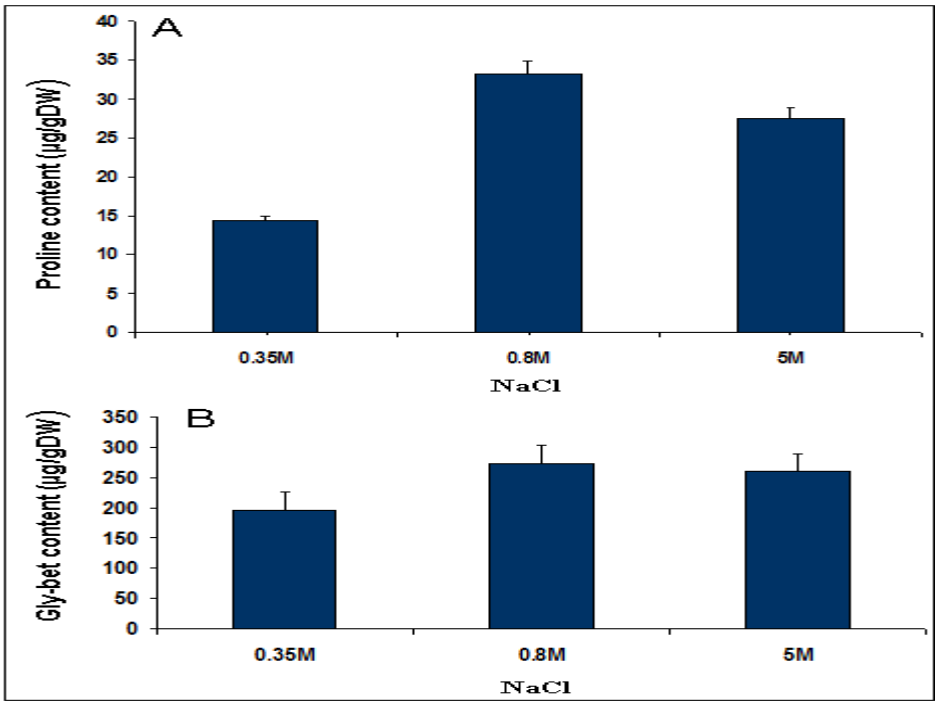

Fig. 9 The endogenous levels of the osmolytes glycine-betaine and proline in *Rhodovibrio salinarum* JA137. A-B depict the endogenous levels µg/ gm.dry.wt of the osmolytes glycine-betaine and proline respectively, at varied NaCl concentrations.

Experiment was done as mentioned in methods section (2.23) in biological triplicates.

3.1.5 High Salt stress leads to elongated cell phenotype in *Rhodovibrio salinarum* JA137

Studies on high salinity stress in *Bacillus cereus* revealed filamentous morphology in cells under the salt influence (Den Besten et al., 2009). During the process of investigation of the organisms response towards salinity we also observed phenotypic and morphological changes in the strain JA137. We confirmed our observations through scanning electron microscopic analysis. The SEM analysis revealed interesting morphological changes under the influence of salinity in the strain JA137. We observed that strain JA137 exhibited small rod shaped to vibroid shaped cells at the optimum salt concentration (5% NaCl) (Fig. 10A) and coccoid shape at low (2% NaCl) (Fig. 10B) and elongated cells at high salinity (20 and 30% NaCl) (Fig. 10C, D). Graphical representation of the change in the morphological phenotype of strain JA137 at different salt concentrations is shown below (Fig. 10E).

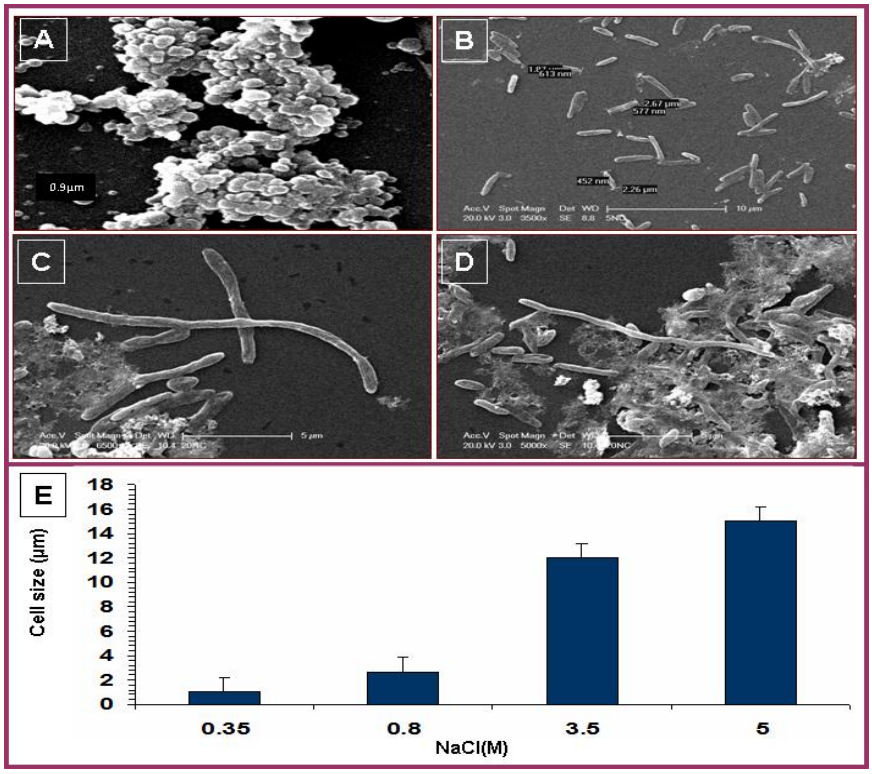

Fig. 10 Scanning Electron Microscopic images of the morphological changes of the cells of *Rhodovibrio salinarum* JA137 grown at different NaCl concentrations. (A) 0.35 M (2%); (B) 0.8 M (5%); (C) 3.5 M (20%); (D) 5 M (30%); (E) Cell length of strain JA137 grown at the represented concentrations of salt,

Experiment was done according to the protocol as mentioned in materials and methods section (2.16.2). Through SEM analysis observed the changes in the phenotype morphologically. It was observed that the increase in cell length was proportionate to the salt concentration. The bar graph represents average cell length with SD as error bar. The statistically significant observation is depicted in the above figure.

Cells at optimum concentration of salt were having vibroid-rod morphology but the cells grown at high salt concentrations of 3.5M and 5M salt exhibited elongated morphology. Minimum of 200 cells were studied and the average cell size was observed to be increasing in proportion to the salt concentration. The cells at optimum salt concentration were of 1.8-2.8 µm where as the cells grown at high salt range exhibited 15-18 µm size (Fig. 10).

3.2 Osmolytes influence on morphology of strain JA137 under the salinity stress

Earlier studies on moderate halophiles reported the protective impact of osmolytes on the growth and morphology of bacteria(Vreeland et al.,2013). Similarly, during the process of investigation of the organisms response towards salinity we also observed a bleached phenotype displayed by this strain JA137 in high salt conditions (30%). However, it not only showed increased biomass formation but also increased pigmentation on supplementation of glycine-betaine to the medium. Hence an attempt was made to elucidate the phenotypic variations in the strain JA137 when supplemented with glycine-betaine at different salt concentrations.The interesting observation as revealed from SEM analysis was that salinity effects the morphology of strain JA137. The cells grown at high salt concentrations exhibited elongated morphology and the cells at the optimum salt range (0.8-2.5M)exhibited vibroid morphology and interstingly the cells grown at low salt (0.35M) exhibited coccoid or spherical morphology. However, it was also observed that when the media was supplemented with glycine betaine, organism could regain its vibroid morpholog y (2.5µm) from an elongated morphology (16 µm) as observed at the optimum salt range, an interesting phenotype displayed by this halophile which brought the evident of two factors under the influence of salt, one is the change in growth and morphology and reverting back to optimum parameters by the protective impact of the osmolyte glycine betaine (Fig. 11). It was observed that on glycine-betaine supplementation,the cells exposed to high salt reverted to the rod-vibroid shape and the length also resumed to normal size 2.6 µm as in the optimum. It was also observed that low salt exposed cells did not show much difference as such.

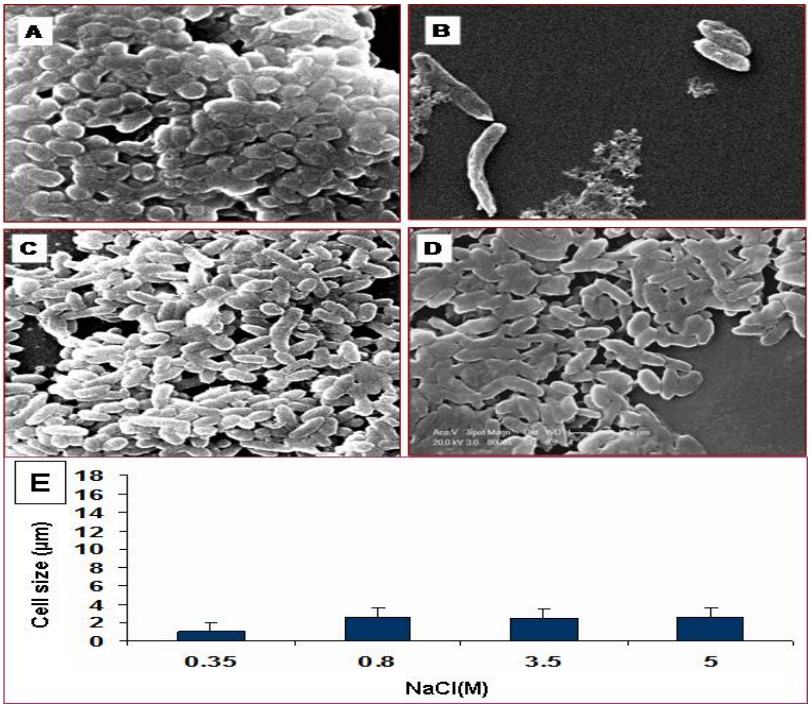

Fig. 11 Scanning Electron Microscopic images representing the morphological changes of the cells of *Rhodovibrio salinarum* JA137 grown in presence of glycine-betaine (1 mM) at different NaCl concentrations . (A) 0.35 M (2%); (B) 0.8 M (5%); (C) 3.5 M (20%); and (D) 5 M (30%) NaCl concentrations; (E) Graphical representation of the change in the morphological phenotype.

Experiment was done according to the protocol mentioned in materials and methods section (2.16.2). It was observed that on glycine-betaine supplementation, the cells exposed to high salt reverted to the rod- vibroid shape and the length (15 µm) also resumed to normal size 2.6 µm as in the optimum. It was also observed that low salt exposed cells did not show much difference as such and they retained their spherical morphology even on supplementation with glycine-betaine also.

3.2.1 Biofilm formation in *Rhodovibrio salinarum* JA137 under different salinity conditions

During the course of our study, on growth of the strain JA137 under salt stress, we observed the formation of biofilm as a slimy structure with cells entrapped in it. Anticipating biofilm formation to be an adaptive strategy employed by strain JA137 an attempt was made to study the biofilm formation in strain JA137 under high and low salt concentrations.

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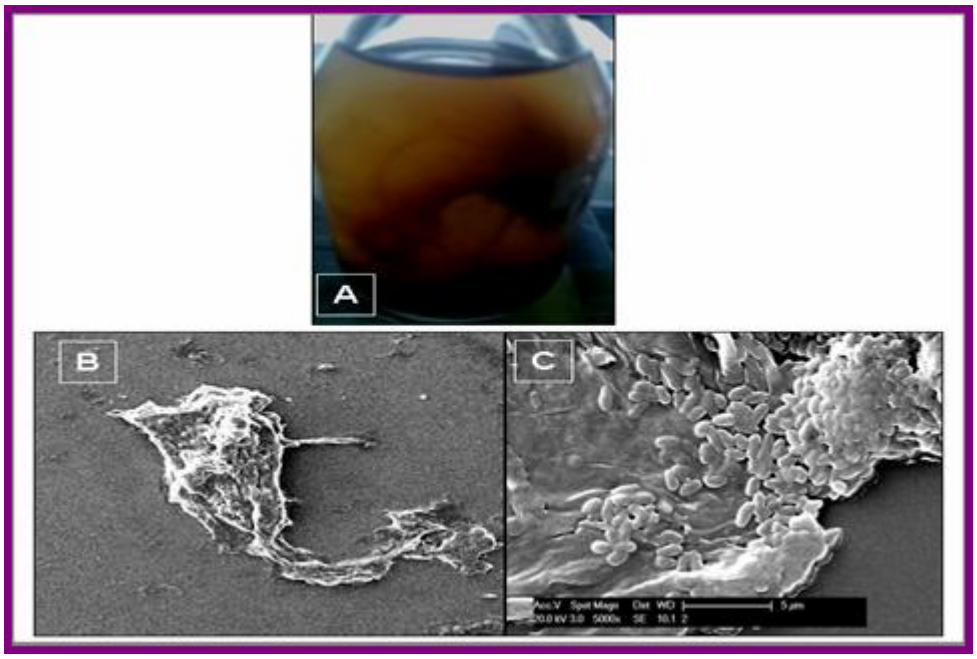

Fig. 12 Biofilm production in *Rhodovibrio salinarum* JA137 and scanning electron microscopic examinations of the biofilm produced by *R. salinarum* JA137.

(A) shows the culture of *R. salinarum* JA137 within suspended biofilm. (B) depicts the scanning electron microscopic images of the biofilm produced by *R. salinarum* JA137, (C) depicts the SEM image of the cells entrapped within the biofilm matrix taken at 5000x magnification.

Culture of strain JA137 was grown as mentioned in the materials and methods and the SEM images were recorded according to the methodology (2.16.2).

In line with previous reports, biofilm formation is a usual protective mechanism exhibited by many bacteria under adverse conditions (Qurashi and Sabri, 2011). Recently, salinity level modulating the biofilm formation in few rhizospheric bacterial genera, *Bacillus* and *Paenibacillus* were reported (Sa et al., 2014). During the experimental analysis of this strain JA137 biofilm formation was observed. The biofilm was observed to be suspended in the culture bottle and also some part of it is settled at the bottom and on the walls. (Fig. 12A). The strain JA137 was grown at different NaCl concentrations (2%, 5%, 10%, 20%, and 30%) was observed to be forming biofilm showing slimy nature adhering to the walls of the culture flask. Sometimes even under optimum salinity conditions also strain JA137 forms biofilm on long term revealing that the viable growing and dead cells were entrapped in the biofilm. The thin layer of biofilm was represented using the scanning electron microscopy (Fig. 12B). It is also confimed that the cells were entrapped and aggregated within the biofilm through scanning electron microscopic analysis (Fig. 12C). Further confirmation of the viability status of the cells trapped in biofilm was confirmed using confocal microscopy. It was observed that at low salt concentrations there were aggregates of cells. Extra polymeric substance formation and its distribution as a slimy substance and forming aggregates of *R. salinarum* JA137 was confirmed by scanning electron microscopy.

3.2.2 Biofilm quantification in strain JA137 under different salt concentrations

Much of the current literature suggests that biofilm exhibits unique patterns of protection that dictate behaviours including resistance to stress. Reports of the halotolerant strains *Halomonas variabilis* (HT1) and *Planococcus rifietoensis* (RT4) form biofilm with increasing salt concentrations (Qurashi and Sabri, 2012). Similarly, physical and chemical stress (NaCl stress) induced biofilm formation in MRSA and Methicillin sensitive *Staphylococcus aureus* (Mirani et al., 2011) and some of the staphylococcal biofilms were also reported from the pathogenic bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* (Asai et al., 2014). Halophilic bacteria produce bioflm under salt stress which is one of the adaptive strategies to survive in salt stress. Hence an attempt was made to demonstrate the biofilm quantification under the influence of increasing salt concentrations based on this knowledge and observations during the process of examining the strain JA137. Biofilm quantification in strain JA137 was done and results suggested that biofilm formation increases with increase in the salt concentrations. Strain JA137 produced high amount of biofilm (0.9 $O.D_{600\ nm}$) at high concentrations 30% (5 M) NaCl compared to

optimum concentration 5% (0.65 O.D $_{600\ nm}$) NaCl (Fig. 13). Strain JA137 probably employed this adaptive strategy of forming biofilm to survive under high salt conditions. This study on biofilm formation in strain JA137 under different salt concentrations is an interesting aspect which implies that bacteria can form biofilm and thereby withstand the salt stress (Fig. 13) and the increasing amount of biofilm probably an adaptive strategy exhibited by *R. salinarum* JA137 was evident from this study. Overall biofilm formation appears to be an adaptive strategy for coping with the salt stress by this strain JA137.

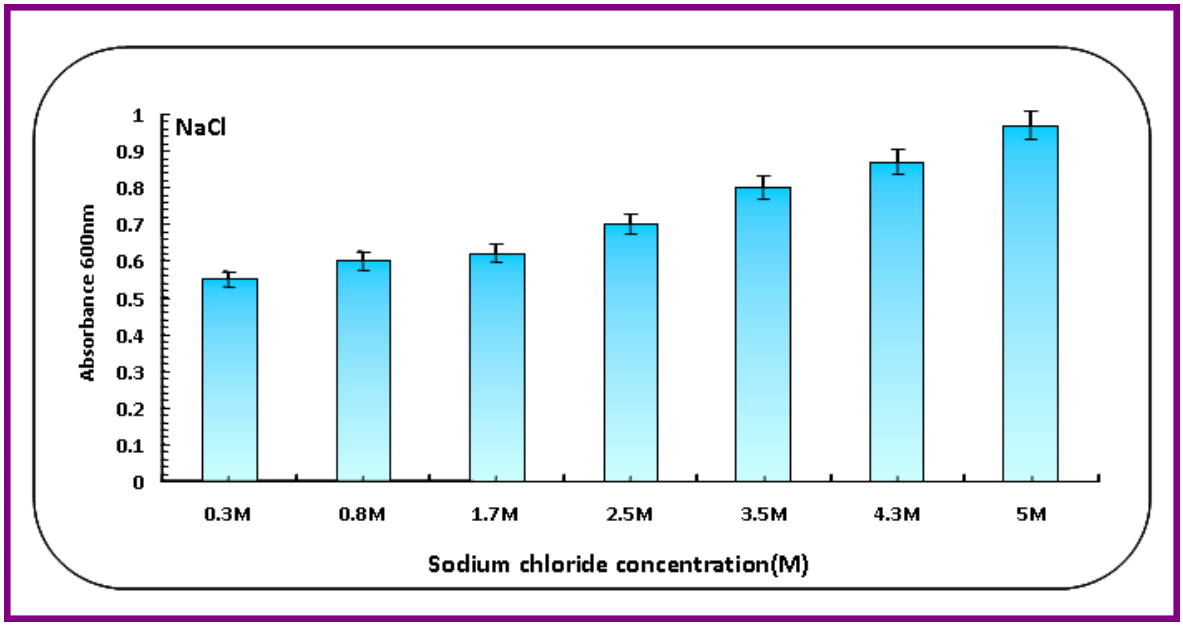

Fig. 13 Biofilm formation by *Rhodovibrio salinarum* JA137 at various salt concentrations.

Biofilm quantification was done as mentioned in the materials and methods section (2.20). The experiment was done in biological replicates. The quantification of biofilm was done with cultures grown at varied 0.3-5 M NaCl concentrations The absorbance is directly proportional to the quantity of biofilm formed. This demonstrates the increase of biofilm with increasing salt concentrations.

3.2.3 Influence of osmolytes on biofilm formation in *Rhodovibrio salinarum* JA137 at different salt concentrations

Osmolytes play an important role in growth of the organism under salinity stress. Anticipating their probable role in the biofilm formation of bacteria under different salt concentrations, an attempt was made to explore the effect of osmolytes such as glycine-betaine, proline on biofilm formation in strain JA137 under different salt concentrations.

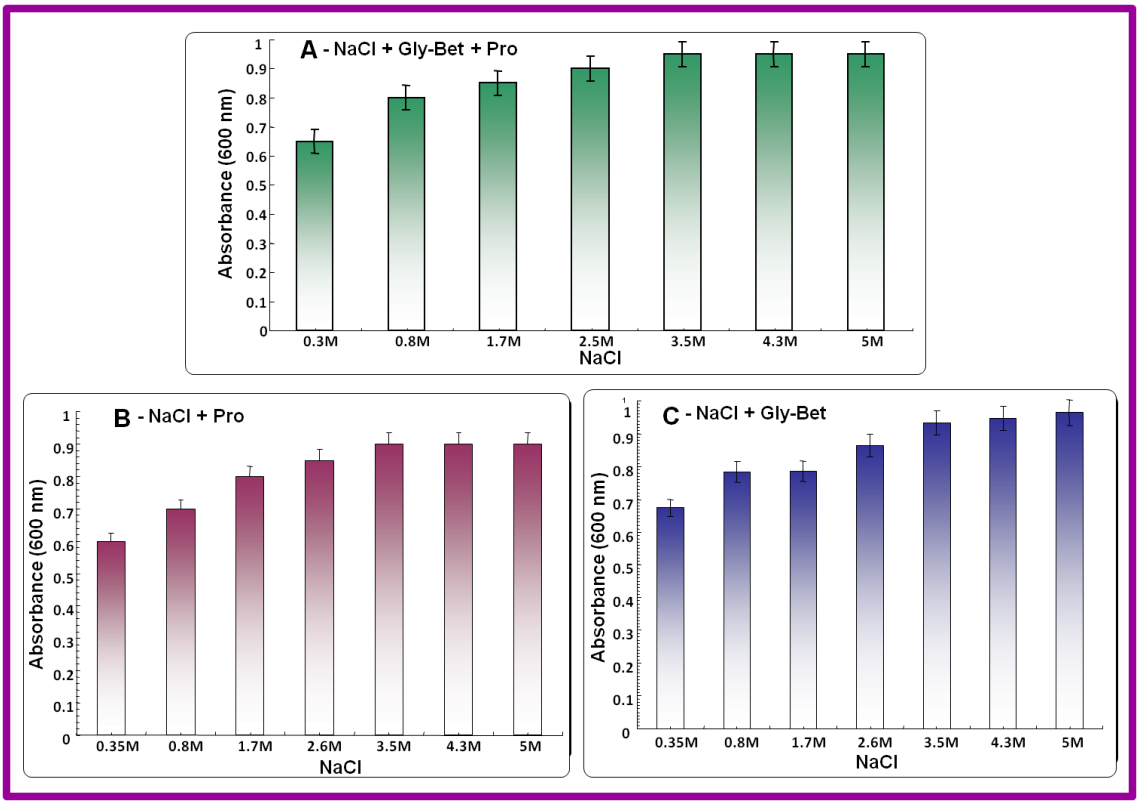

Fig. 14 Biofilm quantification at various NaCl concentrations with the supplementation of osmolytes in the growth medium. (A) depicts the cells grown in NaCl with proline and glycine-betaine supplementation. (B) depicts the cells grown in presence of NaCl and 1mM Proline, (C) depicts the cells grown in NaCl and 1 mM glycine-betaine,

The quantification of the biofilm formation by *R. salinarum* JA137 is performed by a standard microtiter plate assay as mentioned in materials and methods section. (2.20).

Biofilm formation in strain JA137 was observed at different salt concentrations when supplemented with glycine-betaine and proline separately and also with the combination of glycine-betaine and proline. The quantity of the biofilm formed by strain JA137 at different salt concentrations is done by a standard method and the absorbance measured at 600 nm is directly proportional to the quantity of biofilm formed in strain JA137 (Fig. 14). In this context it is worth mentioning the reports of the osmolyte-dependent effects of biofilm formation in *Salmonella typhimurium* (Van Houdt and Michiels, 2010). Based on this knowledge we attempted to investigate whether these adaptive responses of osmolytes and biofiln formation have any crosstalk to act in concert to protect the bacterium from stress or they have their own individual roles independent to play. Therefore this attempt to study the biofilm formation under the influence of varied salt concentrations and osmolytes. Production of biofilm in strain JA137 slightly enhanced in the osmolyte supplemented cells (Fig. 14) compared to the cells without osmolytes (Fig. 13) at different salt concentrations. However biofilm formation is not significantly influenced with glycine-betaine supplemented in the cells of strain JA137 (Fig. 14C), proline (Fig. 14B), glycine-betaine and proline (Fig. 14A). Interestingly we observed the slight increase in the biofilm formation when supplemented with osmolytes in comparison to without osmolytes at different salt concentrations depict that biofilm plays an important role as one of the adaptive strategies employed by the strain JA137.

3.3 Halites –a novel form of dormancy

Halite formation by strain JA137 is an interesting phenomenon observed as one of the adaptive response employed to over come the high salinity stress which is in line to earlier reports. However we wanted to elucidate the viability status of the cells entrapped in side the salt crystal (halite) because we noticed the cells inside the salt crystals to retain their pigmentation even after prolonged periods. Therefore we inoculated the salt crystals into a fresh medium and analysed its growth (Fig.15A). Although the cells took long time for revival, they were able to recover back to normal growth and also retained the pigment color when transferred into the fresh mineral media. The halite formed consists of trapped cells, where as in control no cells were observed. It took ~60 days for its complete recovery (Fig. 15B). Strain JA137 reached early log phase within 40days and grown exponentially in logarithmic phase and reached late log phase within 60 days (Fig. 15C). These observations are in line to the previous reports which provided with evidences of many

halophiles entrapped in salt crystals, were revived back to their optimum even after many years very much like bacteria in spores and cysts (Lopez-Cortes et al., 1994). This is an interesting observation even in *R. salinarum* JA137 forming halites. Through scanning electron microscopy and Confocal Microscopic studies, observed the morphological transitions from rods to spheres of the cells entrapped inside the salt crystals the aggregates of cells within a biofilm, cells in the fluid inclusions inside the halite (Fig. 15, 16, 17) which were also reported previously suggesting that this is a novel survival strategy displayed by halophiles to combat salt stress(Conner and Benison, 2013; Baxter et al., 2014).

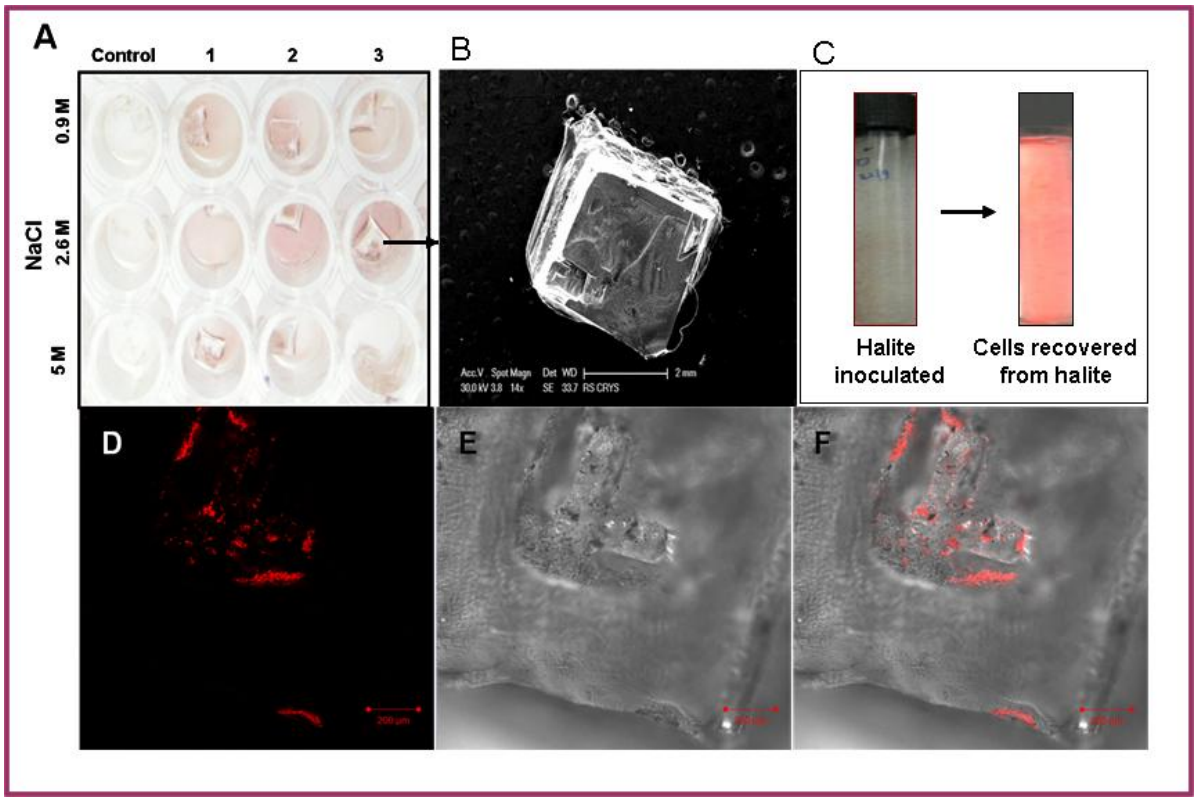

Fig. 15 Halite formation and growth recovery of *Rhodovibrio salinarum* JA137 from the halite. (A) depicts the halites formation by strain JA137 in a microtiter plate, 1, 2, 3 are the triplicates of cells grown at 0.8 M, 2.6 M and 5 M with a control in the first lane; (B) depicts scanning electron microscopic image of halite; (C) Recovery of viable cells of strain JA137 from halite; (D, E, F) acridine orange stained cells inside the halite.

Experiment was done as represented in the methodology section (2.16)

3.3.1 Scanning electron microscopic and confocal microscopic analysis of halite formed in strain JA137

The scanning electron microscopy and confocal Microscopic analysis of the salt crystals the strain JA137 made it evident that the difference in the morphology of the cells present on the surface of the halite and inside the fluid inclusions of the halites. (Fig. 16A, B). Some of the cells of strain JA137 were elongated and found entrapped inside the biofilm. (Fig. 16). Our observations were in accordance to previous of *Salinococcus halitifaciens* a moderate halophilic bacterium which forms halites with cell aggregates in groups at 5% NaCl and also these aggregated cells form filamentous branching at 20% of NaCl (Ramana et al., 2013). Cells were observed to be present on the surface and edges of the halite (Fig. 16A, C),

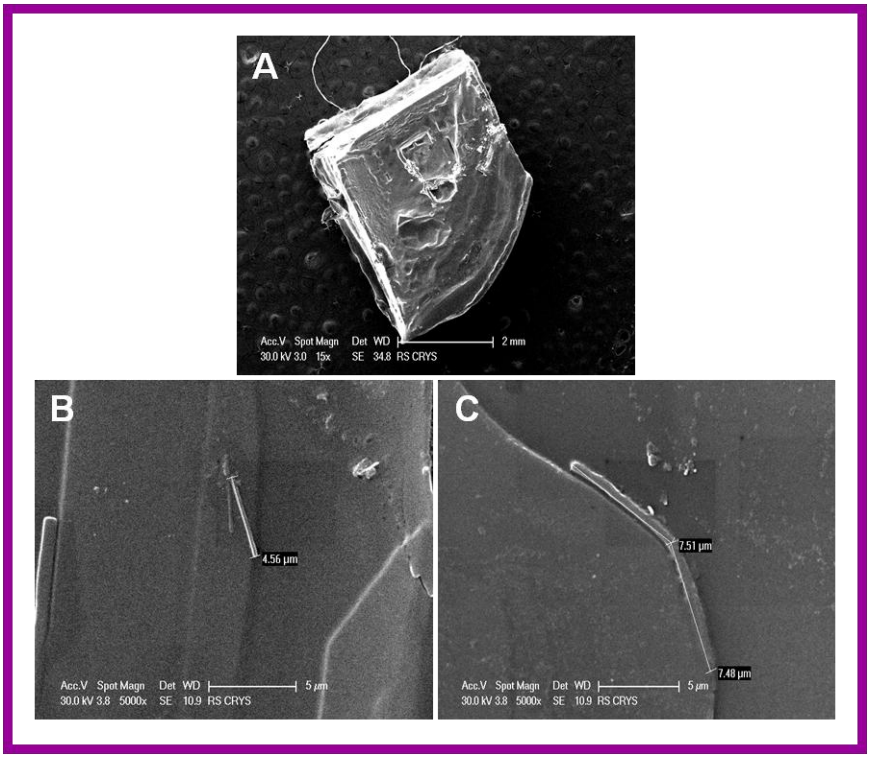

Fig. 16 Scanning electron microscopic examination of halites formed by *Rhodovibrio salinarum* JA137 and the cells trapped in the crystal.

Panel (A) depicts the image of salt crystal taken at 5000x magnification; panel(B)depicts the bacterial cell on the surface of a salt crystal; panel (C) depicts the image of an elongated cell on the surface of a salt crystal.

SEM analysis was carried out as mentioned in the materials and methods (2.16.2).

Confocal microscopic analysis also revealed the existence of live cells aggregated within the salt crystals formed at high salinity conditions in strain JA137 (Fig. 17, 18). The morphological variations of aggregated cells of strain JA137 such as elongated, rod shaped, coccoid , vibriod (Fig. 18) were observed through confocal microscopy. Cells were even observed within the halite and crushed salt crystal (Fig. 17) with scattered cells (Fig. 17,18), aggregates (Fig. 18) and inside fluid inclusions (Fig.17)

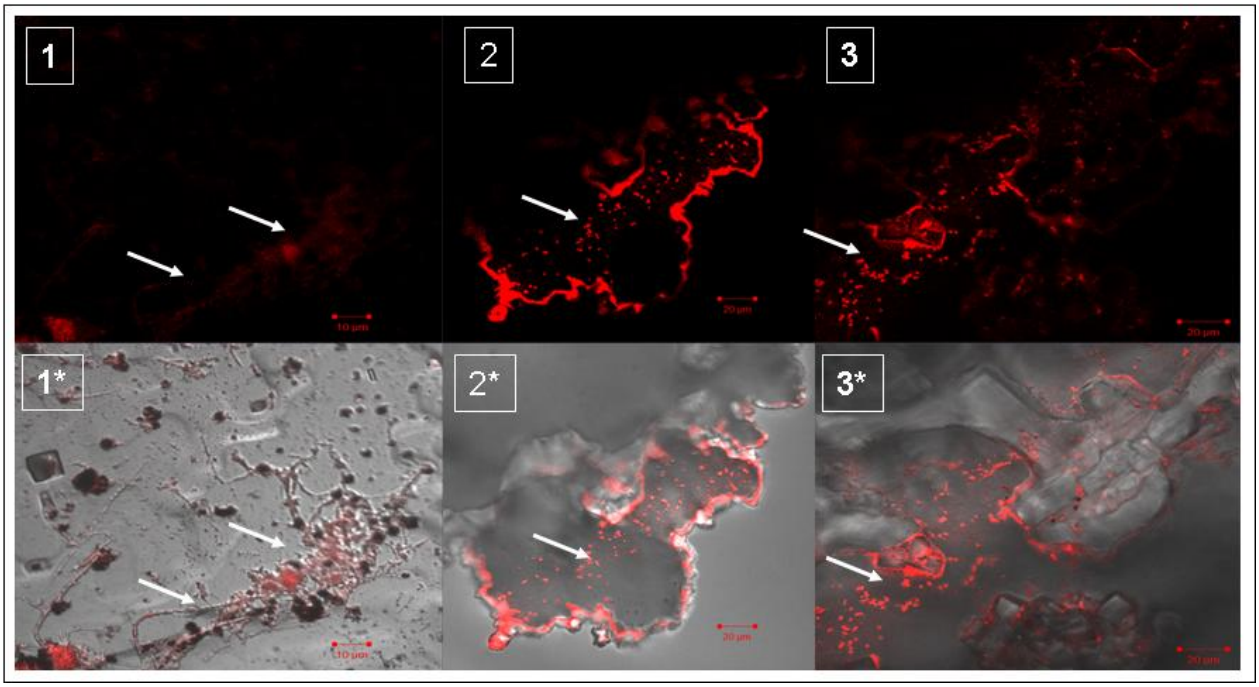

Fig. 17 The confocal microscopic examination of the halites of *Rhodovibrio salinarum* JA137 stained with acridine orange.

Panels (1-1*, 2-2*, 3-3*) depict the acridine orange stained and the phase contrast images of cells respectively,panel 1-1* shows the biofilm entrapped cells on the halite surface, panel 2-2* shows the cells inside a fluid inclusion of the halite,panel 3-3* shows the cells inside a crushed salt crystal. White arrows indicate the elongated cells entrapped inside the biofilm as in1* spherical cells inside a fluid inclusion as in2*-3*.

Confocal microscopy was performed as mentioned in methods (2.16.1).

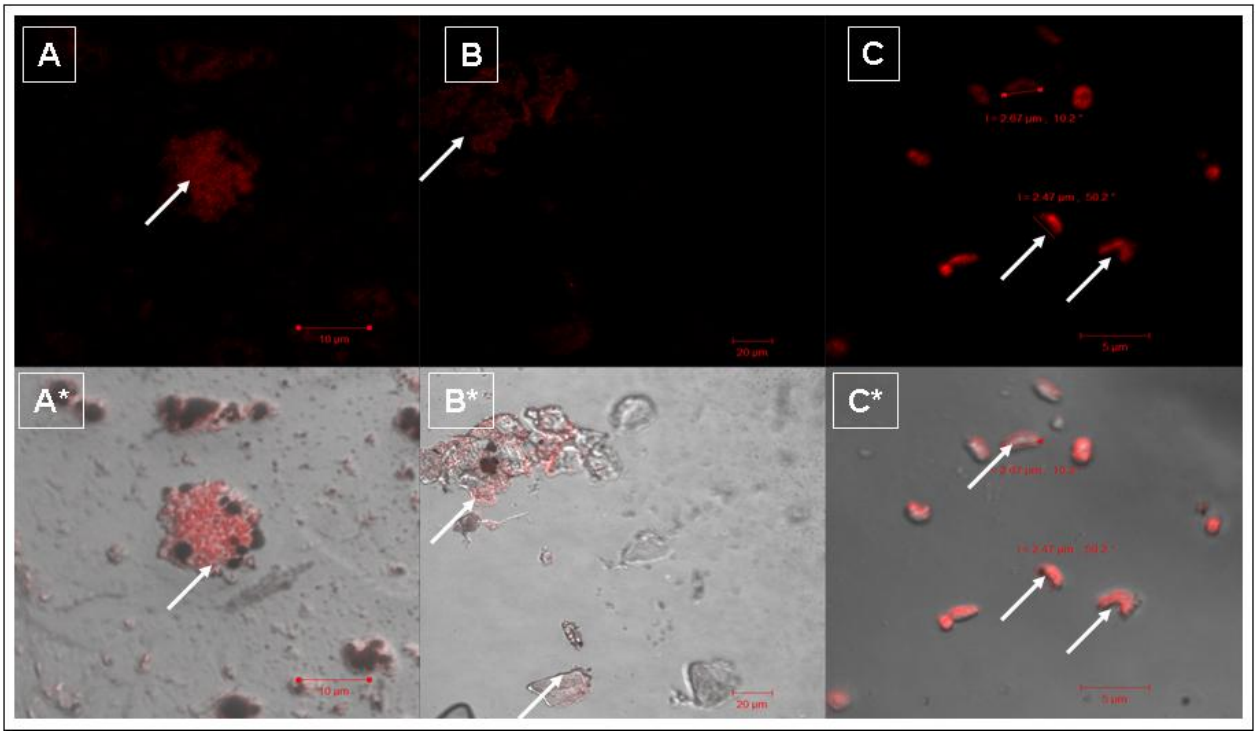

Fig. 18 Confocal microscopic examination of the cells of *Rhodovibrio salinarum* JA137 stained with acridine orange inside a salt crystal.

panels(A-A*,B-B*,C-C*) depict the stained(acridine) and Phase contrast images respectively indicated by star, panel A-A* shows the aggregates of cells inside the salt crystal; panel B-B* shows the cells entrapped inside a fluid inclusion, panel C-C* shows the coccoid. vibriod, rod shaped cells found in halite. White arrows show the cell aggregates, coccoid, vibriod, rod-shaped cells. Confocal microscopy was performed as mentioned in methods section (2.16.1).

These observations are in line with literature reports of halite formation by many halophiles as a strategic survival response exhibited by many bacteria in high salt environments which is similar to the formation of spores and cysts by many bacteria to encounter the adverse conditions by being dormant inside them and wait for favorable conditions to return. It had been reported that formation of halites and the phenomenon of entrapment of the bacteria inside the salt crystals, their morphological transition from rods to spheres and surviving inside fluid inclusions for long time which may be months to years is a novel phenomenon of adaptation by many halophiles. Many details like how they nucleate the formation of halites, how they are able to survive inside the halites, yet to be explored (Roldan et al., 2014). Strain JA137 was observed to respond to high salt by means of forming halites and exhibited the features of forming halites, showing morphological transition of rods to spheres inside the halites. This phenomenon of halite formation in strain JA137 is not yet reported till now. We are the first to report in this bacteria about this adaptive strategy displayed in high salinity and our observations of their viability status inside the halites was also confirmed through these microscopic analysis and recovery analysis.

3.4 Impact of salinity on the photosystem integrity of *Rhodovibrio salinarum* JA137

The photosynthetic apparatus of purple bacteria like *Rhodovibrio salinarum* JA137 were reported to consist of a series of light harvesting (LH) and reaction centre (RC) complexes which are strategically and dynamically interconnected, so that energy migrates from the periphery where the LH II complexes are located, to the core where RC's are surrounded by LH I complexes (Van Grondelle, et al.,1994). Small intrinsic membrane proteins constitute both LH I and LH II complexes which span the membrane, and binds at least one molecule of bacteriochlorophyll (BChl). The migration of excitations towards the RC is facilitated by the absorbance properties of the LH complexes. Thus, the BChls in the LH II complex absorb near 800 and 850 nm, whereas the BChls of the LH I complex lie at a lower energy, absorbing near 875 nm (Vermeglio and Joliot, 1999).

All three complexes of the photosynthetic unit contain the pigment bacteriocholrophyll a (Bchl a), however within these three complexes, this shared cofactor bacteriochlorophyll a gives rise to absorption bands whose maxima span a range from 800-875 nm. The means by which the protein environment tunes the absorption bands of this cofactor has been a topic of intensive study (Cogdell et al., 2006). The various pigment–protein complexes of the photosynthetic apparatus are

involved in sunlight capture, energy transfer, and final conversion of electronic excitation energy to separated charges. Detailed structural and conceptual understanding of those pigment-protein complexes would contribute to an overall picture of photosynthesis and its dynamics during stress (Vermeglio and Joliot, 1999). This photosynthetic process relies on the light-absorbing pigment molecules that are arranged at well-defined positions and angles in the dynamic complexes to facilitate energy transfer (Blundell, 2005; Zhang et al., 2013). However, these high order complexes are reported to be very sensitive towards stress in the environment and any such disturbances are reflected in the photosynthetic efficiency of the organism. Nevertheless, there are many studies regarding the aspects of photosynthetic units being affected by stress like salinity in halophiles, but no reports are available on the photosynthetic issues in high salinity in strain JA137. The growth investigations lead us to many observations of huge differences in the phenotype of this halophile particularly at high salt conditions which lead us to hypothesize the influence of high salt on the photosynthetic efficiency of this strain JA137. Therefore we investigated the photosynthetic integrity using spectroscopic analysis and how halophiles are able to conserve their redox activity in high salt.

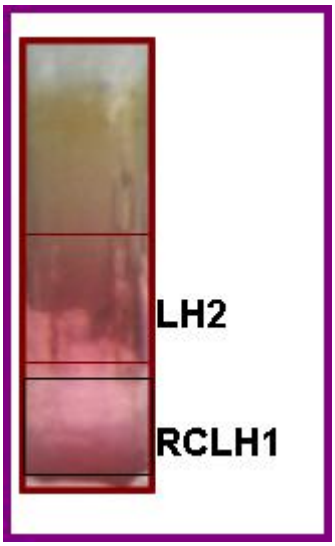

Fig.19 The isolated fractions of the core photosynthetic complexes (LH2, RCLH1) of *Rhodovibrio salinarum* JA137 grown at 5%, NaCl concentration. LH2; Light Harvesting complex II, RCLH1; Reaction Centre Light Harvesting Complex I.

Isolates of *R.salinarum* JA137 grown at varied salt concentrations were obtained as described in methodology section (2.21).

Pigment-protein complexes were solubilised from intra-cytoplasmic membranes and fractionated on sucrose density gradients by ultracentrifugation for the purpose of investigating the impact of salt stress on the photosynthetic unit and its integrity. The culture of strain JA137 grown at 5% and 30% NaCl concentrations till the log phase was harvested. The reaction centers (RCs) from *R. salinarum* JA137 were isolated, and the results of this analysis are summarized (Fig. 19). The gradients for all the NaCl concentrations and those supplemented with the osmolytes exhibited an upper intense pigmented band corresponding to LH II (Fig. 20) and a lower band corresponding to monomeric RC–LH I complexes (Fig. 21).

The spectral peaks 460, 490, 510 and 550 nm are ascribed to carotenoid absorption. The absorption spectra of the isolated light harvesting complexes are shown in (Fig. 20,). The absorption bands at 801 and 849 nm are due to the presence of the LH II antenna. The absence of LH I is demonstrated by a loss of the broadening on the spectra A1, A2 (Fig. 20). The BChl 850 absorption band has shifted further to 820 nm and the presence of LH I is demonstrated by a much more clearly resolved band at 875 nm (Fig. 20B1). The shoulder peak at 875 nm is ascribed to the presence of the LH I complex.

Both NaCl-grown and NaCl and glycine betaine grown cells had a band corresponding to monomeric RC–LH I complexes (Fig. 20). The spectra of monomers was similar to that of the glycine betaine monomers (Fig. 20B1, B2), but with a ~2 nm blue shift of the LH I absorbance band.

3.4.1 Influence of salinity on the Light Harvesting complex 2 (LH2)

The observations of phenotypic variations through a change in the colour of the culture at high salt concentrations, indicated the probable influence of salinity on the photosynthetic units. The photosystems being influenced at 30% NaCl was more because of a bleached phenotype observed compared to cultures grown 5% NaCl concentration (Fig. 20). The integrity of the photosynthetic unit complexes was analysed by taking the absorption spectra of the isolted LH and RC fractions. Absorption spectral analysis revealed the decrease in the relative absorbance of LH-II,I in 30% NaCl indicating that the photosynthetic complexes i.e LH-II,Iwere disturbed by high salt concentration 30% compared to 5% salt concentration (Fig. 20A1. A2). However, when supplemented with glycine-betaine in the media it regains the integrity of LH complex which was

indicated by the regain of color from the bleached phenotype (Fig. 20B). This represents that the glycine-betaine has a protective effect on the growth as well as the photosynthetic pigments biosynthesis, which is line with previous reports in *Synechocystis* (Allakhverdieva et al., 2007) suggesting the effect of salinity on the photosystem integrity. However there is slight shift in the spectra at 850 nm region towards 875 nm in high salt concentration (30%) supplemented with glycine-betaine (Fig. 20B1). Therefore, the integrity of photosystems examined at different salt concentrations and also its integrity in the presence of osmolyte, glycine-betaine (Fig. 20,21) indicates the protective influence of glycine betaine and thus might be resulting in enhanced growth.

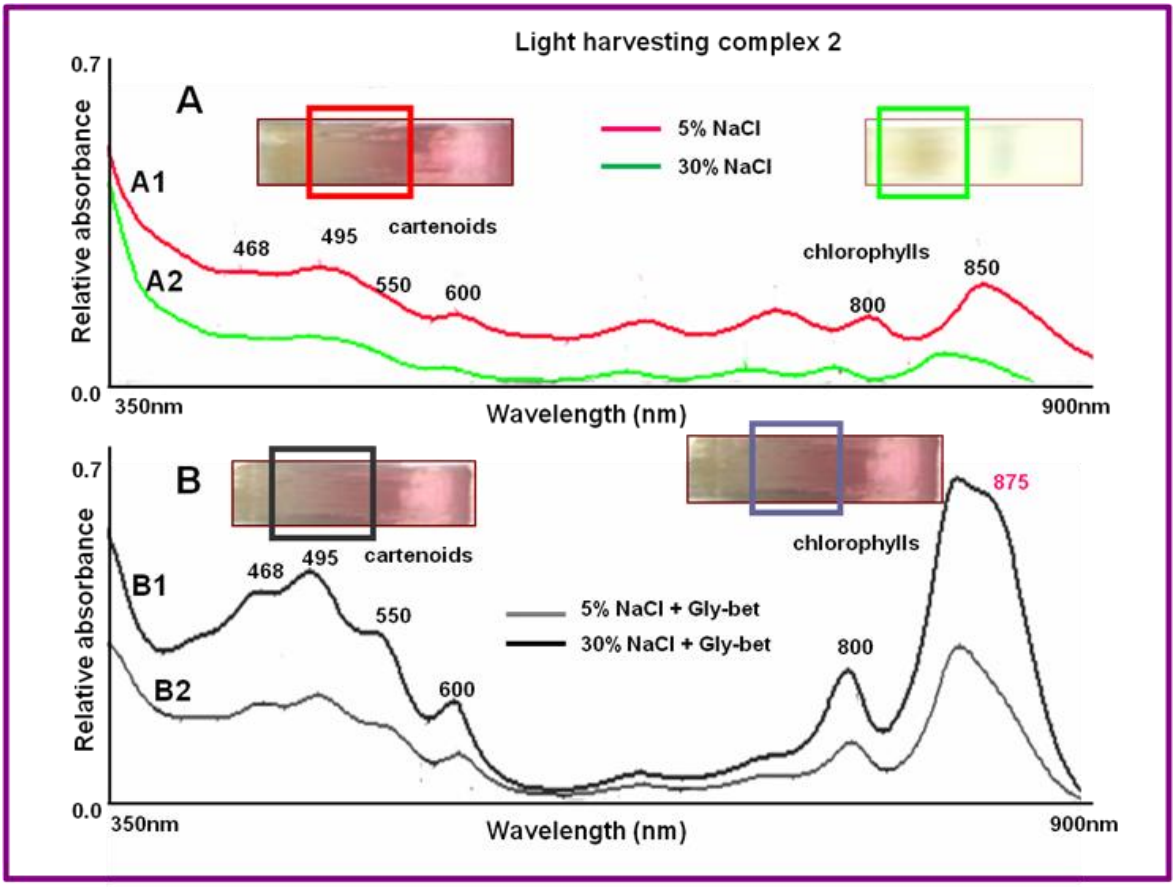

Fig. 20: Absorption spectra of the fractionated membrane proteins-pigment of the Light Harvesting II (LHII) complex of the photosynthetic apparatus, from *Rhodovibrio salinarum* JA137 grown at NaCl concentrations. (A) 5%(0.8M) and 30%(5M)NaCl (without osmolytes); (B) salt 5%(0.8M) and 30%(5M) along with osmolyte Glycine-betaine. (A1) red color represents 5% (0.8 M) NaCl; (A2) Green colored spectra represent 30 %(5 M) NaCl; (B1) black represents 30% (5M) + Gly-Bet; (B2) Grey color represents 5%(0.8M) NaCl + Gly-Bet.

Light harvesting complexes were separated from the strain JA137 and the spectra were recorded as mentioned according to the protocol mentioned in the materials and methods section (2.18).

The presence of the 800-850 nm complex is demonstrated by the absorption bands at 801 nm and 850 nm (Fig. 20). The 875 complex is manifested as a broadening of the red side of the dominant 850 nm band (Fig. 20B1). The bands near 468, 495, 550 and 600 nm are ascribed to carotenoid absorption pertaining to this spectra. The absorption bands at 801 and 849 nm are due to the presence of the LH II antenna. The absence of LHI is demonstrated by a loss of the broadening on the red side of the BChl 850 absorption band observed in spectra of the 30% NaCl grown cells (Fig. 20A2). In comparison, the absorption spectrum shows a blue shift of the BChl 850 absorption band of approximately 20 nm, may be with a peak at 830nm (Fig. 20A2). There were no clear bands which can be ascribed to carotenoids, neither at 801 nm, which is in complete contrast to that observed (Fig. 20A1). B1 shows peaks ascribed to carotenoids and bands at 800 and 850 nm which may be due to the presence of LH II comparable to 5% NaCl spectra but the intriguing aspect observed in B1 is the shoulder at 875 nm (glycine betaine supplemented). B2 shows the normal cartenoid bands and bands at 800 and 850 for LH II.

3.4.2 Influence of salinity on the Reaction Centre Light Harvesting complex I-RCLH I

The fraction of Reaction Center Light Harvesting complexI- A1 andC2 shows bands at 468, 495, 550 and 600 nm ascribed to the carotenoids pertaining to this spectra. However A2 (Fig. 21) spectra altogether shows an absolutely different picture with a shift in the carotenoid bands as well as 850 nm band with a shoulder and band widening towards the red side. Only compatible observation of A2 with A1 (Fig. 21) is the band ascribed to LHII at 801. The absorption spectra of the RCLH I, D1 and B2 show contrasting results. B1 showed well resolved bands which correspond to carotenoids and the bands at 801 and 850 nm correspond to LH II and LHI but B2 showed no such clear bands and no band corresponding to LHI and LHII levels are prominent. The absence of bands corresponding to carotenoids in B2 (Fig. 21) and their appearance at A2 indicates that halo stress probably effects the carotenoid synthesis thereby affecting the photosynthetic efficiency which is evident by the fact that became evident in the case of the culture supplemented with glycine betaine showing the carotenoids. Therefore we can conclude that glycine betaine might be playing a vital role in maintaining the photosystem integrity by its probable role in carotenoid biosythesis, thereby promote growth at high salt which can be clearly seen when we observe the A2 spectra which showed peaks that may be ascribed to carotenoids with slight shift from the normal peaks observed in B1 and also peak with a shoulder of 850 broadening towards 875 nm (Fig. 21).

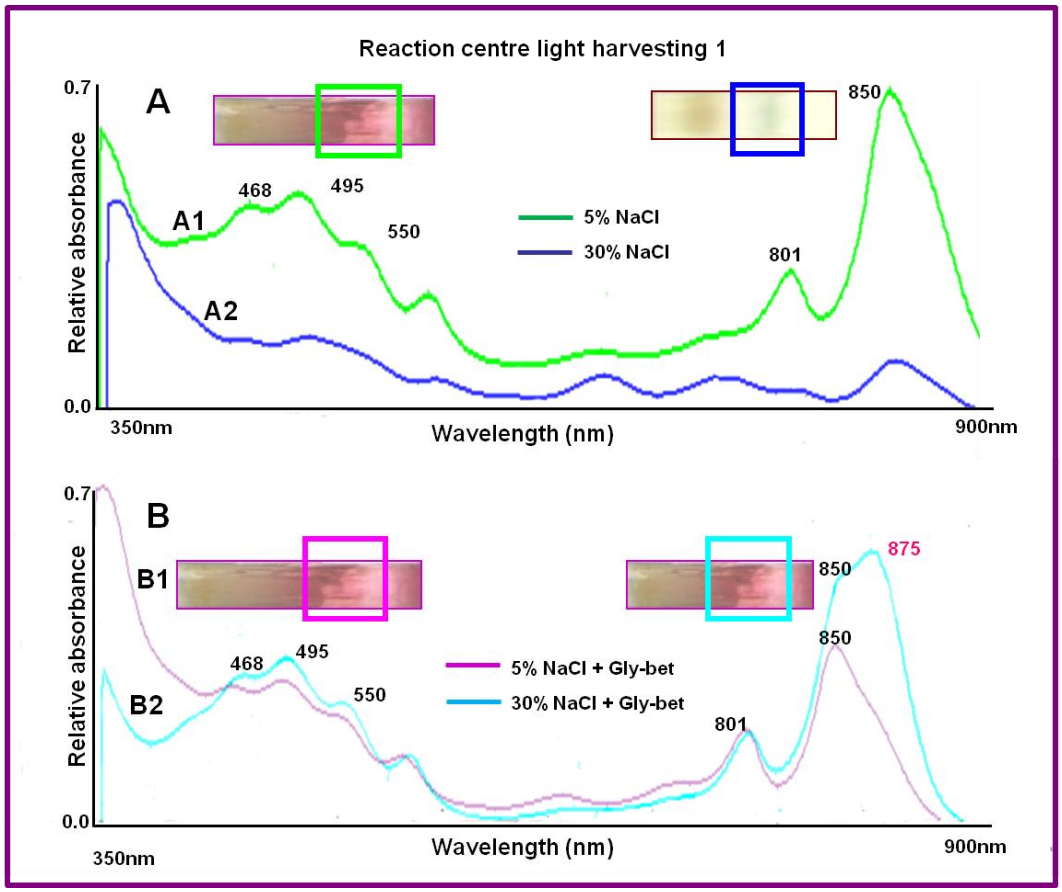

Fig. 21: Absorption spectra of the fractionated membrane pigment-protiens of the Light harvesting complex-I (LH I) of the photosynthetic unit from *Rhodovibrio salinarum* JA137. (A) 5%(0.8M) and 30%(5M)NaCl (without osmolytes); (B) salt 5%(0.8M) and 30%(5M) along with osmolyte Glycine-betaine. (A1) Green colored spectra represent 5%(0.8M) NaCl; (A2) blue color represents 30%(5M) NaCl; (B1) purple color represents 5%(0.8M) NaCl + Gly-Bet; (B2) light blue represents 30%(5M) + Gly-Bet.

Light harvesting complexes were separated from the strain JA137 and the spectra were recorded as mentioned according to the protocol mentioned in the materials and methods (2.18).

Table 5. Average values of ALH1/ARC of *R. salinarum* JA137 cells grown at 0.8 M and 5 M NaCl and of cells grown with glycine betaine supplementation

SALINE CONCENTRATION (%)	NORMAL A LH1/A RC	GLYCINE BETAINE A LH1/A RC
5	2.3	2
30	1.6	3

The absorption spectra of a range of the RC–LH1 complexes, presenting ALH1/ARC ratios obtained by dividing the absorbance maxima of the LH1 band (which was between 850 and 875 nm) by the absorbance at the maximum of the RC band at around 805 nm. Average values of ALH1/ARC were slightly lower than those of the equivalent glycine betaine complexes, but this difference was not significant for only NaCl-grown cells and had probable physiological implication for NaCl-grown cells in presence of glycine betaine.

For the RCLH I and LH II from salt grown and osmolyte supplemented cultures, this ratio had average values between 2.5-3 (Table 5). This might be due to aggregates of LHI around RC. This ratio was calculated as a simple indicator of core complex composition, and to give a convenient way of quantifying typical variability in the absorbance spectra obtained for a particular complex. As such it did not take into account variations in the width of the LH1 absorbance band or differences in its overlap with the underlying absorbance band of the RC. The only clear trend that can be pointed to was the significant increase in ALH1/ARC ratio seen in high NaCl monomers, where values between 1.6-3 (Table 5) obtained might be due to the additional BChls and associated proteins that close the ring of LH1 around the RC in the absence of osmolytes as far as the literature suggests (Vreeland, 2012) which needs further insights to have a clear understanding of this aspect of the effect of glycine betaine's counter effect on photosystems from salt stress.

3.4.3 The whole cell absorption spectra of *Rhodovibrio salinarum* JA137 at different salt concentrations and with osmolytes

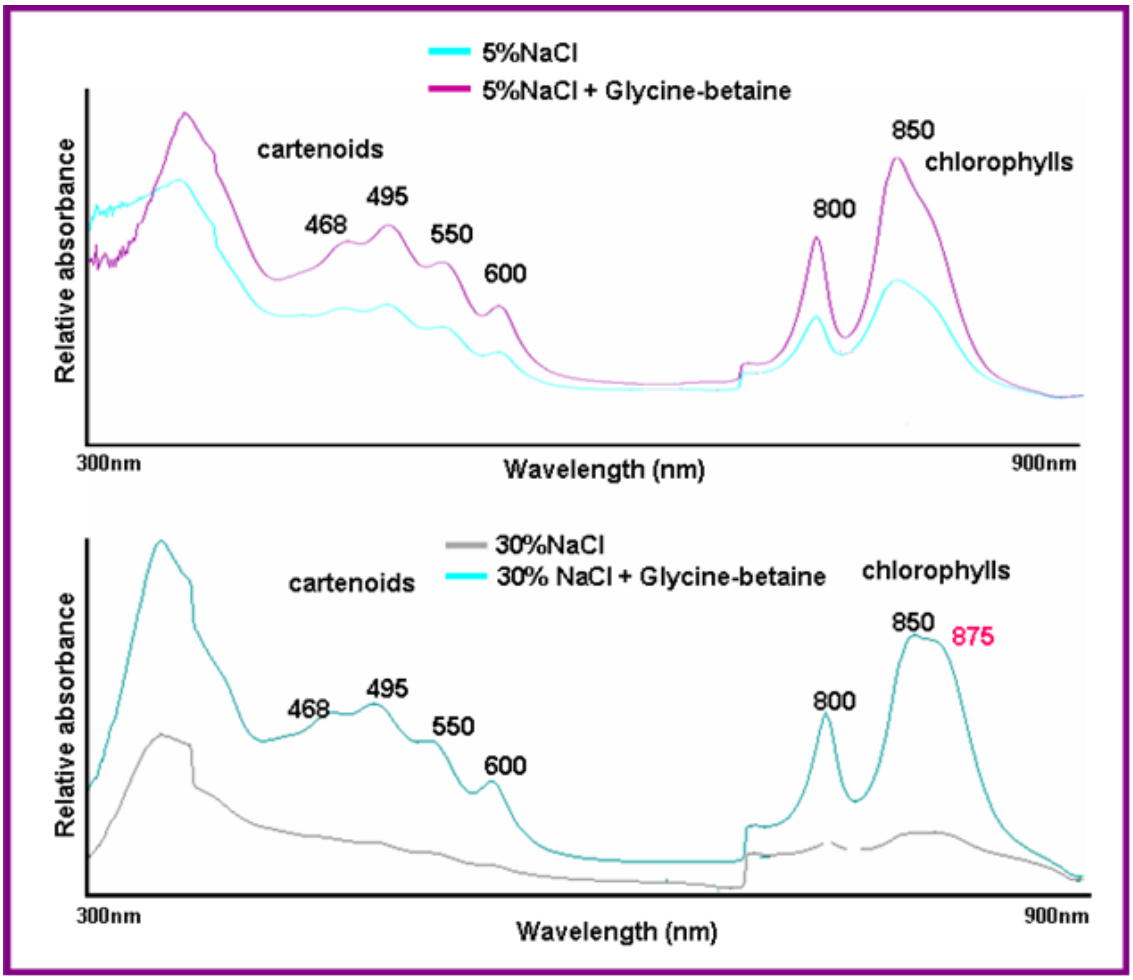

Fig. 22 The whole cell absorption spectra of *Rhodovibrio salinarum* JA137 cultured at upper panel blue color represents 5% (0.8 M), and pink color 5 % (0.8 M) + glycine-betaine; Down panel grey color spectra represents 30 % NaCl; blue color represents 30% NaCl + glycine-betaine.

The pigments were extracted into acetone and the spectra were recorded as described in the materials and methods section (2.18).

The whole cell absorption spectrum of *R.salinarum* pigments extracted with acetone gave absorption maxima at 468, 495, 550, 600, 800 and 850 nm, confirming the presence of bacteriochlorophyll a and carotenoids. The absorption spectrum gave an absorption maxima at 468, 495 and 550, 600 nm indicating the presence of carotenoids spirolloxanthin. The cells grown in the presence of 5% NaCl, showed bands at 468, 495, 550, and 600 nm which can be ascribed to the carotenoids and 801 and 850 bands can be ascribed to LH II and LHI. However the absorption spectra of 30% NaCl grown cells showed no such bands at all corresponding to either carotenoids or light harvesting complex I and II (Fig. 22). But interestingly the absorption spectra of 30% NaCl cells supplemented with glycine betaine showed quite contrasting results with well resolved peaks at 468, 495, 550, and 600 nm. We could also observe a peak at 800nm and the band at 850 had a broad shoulder towards 875 nm towards the red side which can be ascribed to LHI. These observations are in accordance with previous literature of glycine-betaine reversing the high salt stress and there by promoting growth at high salinity which is a clear evidence that this halophile is well equipped with the repair machinery to encounter the adverse conditions of salt. However, all these are operating in concert to maintain the cell and its components at various cellular and molecular levels for the ultimate purpose of survival still remains elusive and the depths of dynamics are still to be explored.

3.5 The influence of salinity on the carotenoid profile of *Rhodovibrio salinarum* JA137

The observations from the absorption spectra clearly indicated that salt stress greatly influenced the carotenoid biosynthesis. The osmolyte glycine betaine is significantly influencing growth probably by playing a role in carotenoid biosynthesis. Therefore performed carotenoid profiling by thin layer chromatography of *R. salinarum* JA137 grown at optimum and hyper saline concentrations of NaCl (Fig. 23). The significant observation was that the carotenoid profile at optimum salt concentrations was different from the hyper saline concentrations, where we observed absence of some carotenoids. However the interesting aspect is that when the high salt concentration (30%) was also supplemented with glycine betaine we could observe the recovery of those carotenoids and could view the corresponding carotenoids bands popping up on the TLC plate. This was observed particularly with 20 and 30% NaCl grown cells (Fig. 23). These observations clearly indicate that in high salt conditions the carotenoids are effected, thereby probably the difference in the photosynthetic efficiency and in the growth physiology.

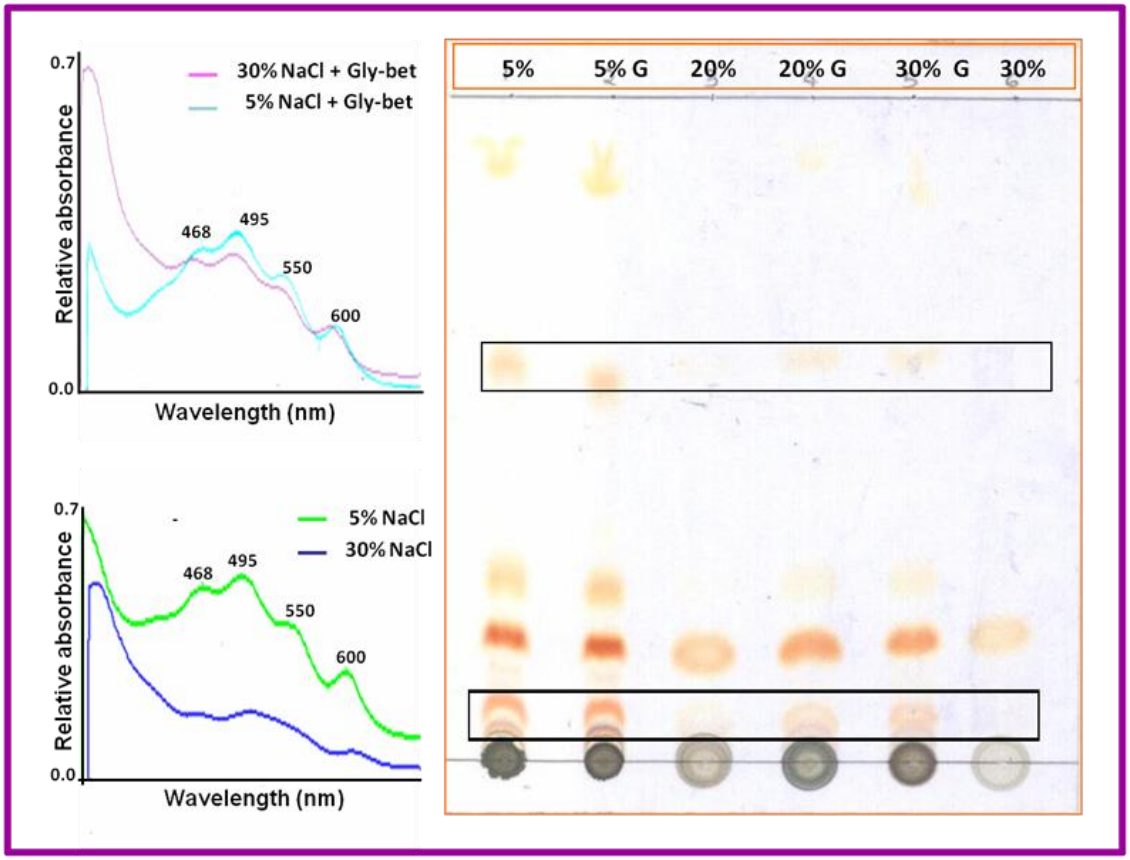

Fig. 23 Thin layer chromatography of the carotenoid profile of *Rhodovibrio salinarum* JA137 grown at different NaCl concentrations of (5%, 20%, and 30%), and also supplemented with the osmolyte glycine betaine. The corresponding absorption spectra of the carotenoid extracts are also represented.

Carotenoid extraction, carotenoid profiling by TLC and spectral analysis was done as described in the methods section (2.17.3).

We observed differences in the absorption spectra of the carotenoids grown at different NaCl concentrations and also significant changes in the spectra of those cultures supplemented with glycine betaine.

3.6 Lipidome of *Rhodovibrio salinarum* JA 137at different NaCl concentrations

3.6.1 Polar lipid profiling of *Rhodovibrio salinarum* JA137 at different salt concentrations and supplemented with glycine betaine.

Information on the influence of medium salinity on the lipid profile has also been obtained in the course of our study by the conclusions based on TLC analysis of total lipid extract of *R. salinarum* JA137.

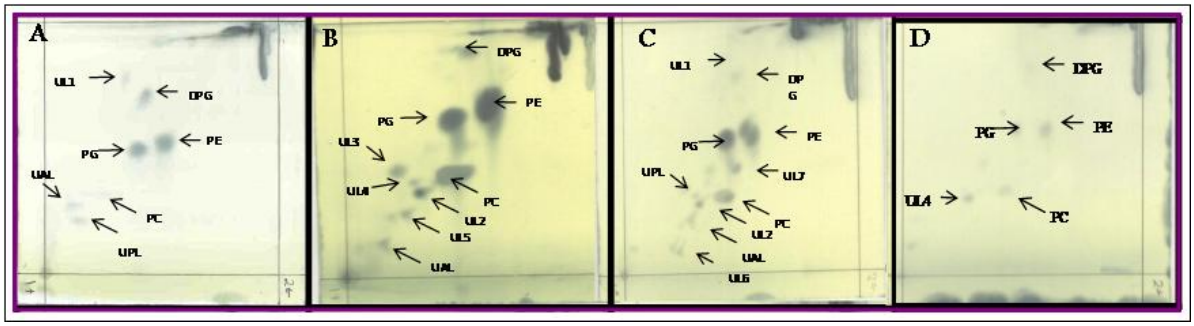

Fig. 24 Thin Layer Chromatography profile of the lipid extracts of *Rhodovibrio salinarum* JA137 grown in different salt concentrations 0.35 M (A), 0.8 M (B), 3.5 M (C) and 5 M (D).

DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; UL, unidentified lipid; UAL, unidentified amino lipid. Lipid extraction and lipid profiling was done as described in the materials and methods section (2.17.2).

We present the characterization of the polar lipids found in the lipid extracts of *R. salinarum* JA137 cells grown in 0.35 M, 0.8 M, 3.5 M and 5 M NaCl by thin-layer chromatography (TLC) to gain information on the effects of increased salinity in the culture medium on cell lipid composition as well as to see the overall lipid profile at all concentrations both optimum and hyper salt. We also observed the effect of low salt on the polar lipids of *R. salinarum* JA137 (Fig. 24). We observed difference in the retention factors of the major and minor lipids and their intensities. Here we have analysed *R. salinarum* JA137 lipidome and gained information on changes in the membrane polar lipid profile under high salt stress conditions. To our knowledge, literature studies on membrane polar lipids, and the impact of changing NaCl concentrations on polar lipids in *R. salinarum* JA137 have not yet been elucidated. Individual polar lipid components were identified

by comparison of their retention factors (Rf) values with those of authentic standard markers. Molybdophosphoric acid was used to detect lipids.

It is evident that there are three major lipid classes in the lipid extracts of cells (Fig. 24, 25). Although it was observed that the main polar lipid components in cells grown at 0.35, 0.8, 3.5, 5 M NaCl were the DPG, PG, PE, and PC, there were differences in the Rf (retention factors) of the TLC spots of the lipids, which might be because of the possibility of differences in the branching, length and unsaturations of hydrophobic chains of the different lipids (Fig. 25). There were no clear differences seen in the lipid profiles of 3.5 M and 5 M cell lipid extract when the minor lipids were considered. The limited comparative analysis of the present study of lipids, has revealed a decrease in the intensity of the major and minor lipids in the membranes of cells grown in 3.5 M, 5M NaCl, the physiological implication of this difference is not yet clear. The polar lipid profiling revealed the presence of many unidentified lipids at high salt concentrations in strain JA137 (Table.).

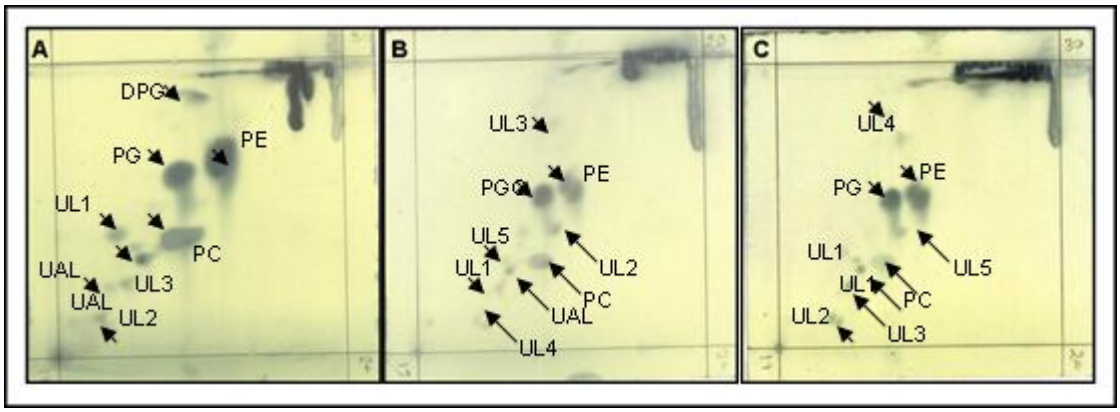

Fig. 25 TLC profiles of lipid extracts of *Rhodovibrio salinarum* JA137cells grown in different salt concentrations supplemented with Glycine-betaine 0.8 M (A), 3.5 M (B) and 5 M (C).

DPG, diphosphatidylglycerol; PE, phosphatidylethanloamine; PC, phosphatidyl choline; UL, unidentified lipid; UAL, unidentified amino lipid. Lipid extraction and lipid profiling was done as described in the materials and methods section (2.17.2).

This is in line with previous reports. In the case of halotolerant or moderate halophilic bacteria from high NaCl environments, a huge spectrum of adaptive responses like growth inhibition, metabolic diversions as is the case of intracellular compounds used as osmolytes were reported (Ventosa et al., 1998; Catucci et al., 2004). There are many reports regarding the alterations in the membrane lipid compositions under stress. In this context it is worth mentioning the example of *Planococcus* when grown in high NaCl media, the (cardiolipin) CL mole per cent content increased and those of PG (phosphatidylglycerol) and phosphatidylethanolamine (PE) decreased. It was reported that in bacteria the CL (cardiolipin) increases at the expense of PG (Lattanzio et al., 2009; Vreeland, 2012). A similar observation of an increase in the abundance of CL at the expense of PG had been noticed as a protective measure taken by Gram positive bacteria consequent to hyperosmotic stress conditions (De Leo et al., 2009). Previously it was shown that bacteria adjust their membrane lipid composition by modifying the types of fatty acids and even by changes of pre-existing phospholipids (Lobasso et al., 2012). In particular, the relative abundance of shorter fatty acids increased and changes in the unsaturation levels of lipid chains were observed (Lopalco et al., 2013). It is well known that the length and the degree of unsaturation of fatty acid chains have a profound effect on membrane fluidity. The unsaturated lipids and chain shortening determine a lowering of the melting temperature and an increase in the membrane integrity as a consequence. The changes in the lipid core of the membrane matrix could represent a compensation for the increase of packing of the phospholipid head groups in high salt therefore contributing to the homeostasis of membranes (Vreeland, 2012). In accordance to earlier reports devoted to lipid involvement in adaptation processes proposed that there are significant changes in the composition and quantity of sulphoquinovosyldiacylglycerol by the impact of abiotic and biotic factors.

However there were some unidentified lipids also apart from the major lipids at all concentrations of NaCl but their intensity decreased with the concentration of salt and also observed that the unidentified lipids differed in the retention factors. Adjustments in the chain structures of main membrane lipid components of *R. salinarum* JA137 are in line with the patterns of other moderate halophiles. On increasing the salinity, an increase in the shorter chains and the presence of chain unsaturations and branching were observed. In order to contribute to the membrane homeostasis in the adverse salt conditions, these changes in the lipid core might be for serving the pupose of packing and to establish the integrity of membranes.

Table 6. Polar lipid profile of *R. salinarum* JA137 grown at different(0.35M,0.8M,3.5M, and 5M) NaCl concentrations with the osmolyte supplementation. G-glycine betaine,P-proline. DPG-diphosphatidylglycerol, PG-phosphatidylglycerol, PC-phosphatidylcholine, PE-phosphatidylethanolamine, UN-unidentified lipid, ND-not determined. Those marked in red box are the novel lipids, with anticipated role in salt tolerance at high salinity.

Sample	DPG	PG	PC	PE	UNL1	UNL2	UNL3	UNL4	UNL5	UNL6	UNL7	UNL8
2	+	+	+	+	+	+	+	-	-	-	-	-
5	+	+	+	+	-	+	+	+	+	+	-	-
20	+	+	+	+	+	+	+	-	+	+	-	-
30	+	+	+	+	+	+	+	+	+	+	+	+
2+G	+	+	+	+	-	-	+	-	+	-	-	-
5+G	+	+	+	+	+	+	-	+	+	+	+	-
20+G	+	+	+	+	+	+	-	+	+	+	+	-
30+G	+	+	+	+	-	-	-	+	-	-	-	-
2+P	ND	ND	ND	+	ND	ND	+	ND	ND	ND	ND	+9
5+P	ND	ND	ND	+	-	+	-	-	-	-	+	+
20+P	ND	ND	ND	+	-	+	-	-	-	-	+	+
30+P	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

3.7 The fatty acid profile of *Rhodovibrio salinarum* JA137 at different salt concentrations

3.7.1 Saturated and unsaturated fatty acids at hyper and hypo salinity

Fatty acids play an important role in the integrity of membrane under stress conditions in bacteria. Bacteria to overcome the stress by modifying the composition of its membrane fattyacids accordingly and protect itself from stress by maintaining the membrane integrity. salinity stress has been reported to have more impact on membrane fatty acids .Hence an attempt was made to know the effect of salinity stress on the membrane fatty acids.

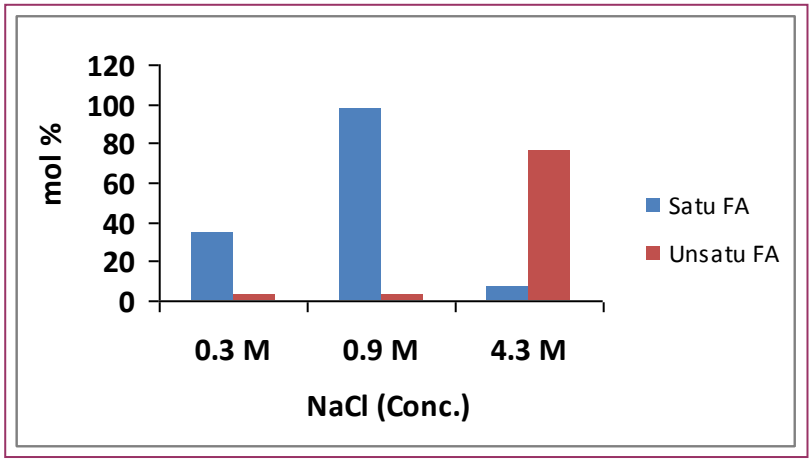

Fig. 26 Saturated and unsaturated fatty acids profiling at optimum NaCl and high saline concentrations of *Rhodovibrio salinarum* JA137 (0.3 M, 0.8 M, and 4.3 M) NaCl concentrations.

Fatty acid methyl esters analysis was done as mentioned in the materials and methods section (2.17.1).

Influence of salinity on the total membrane fatty acids was revealed by Fatty acid methyl esters analysis revealed the total fatty acid profiling at different salt concentrations. The saturated and unsaturated fatty acids profile was carried out with the cells of *R. salinarum* JA137 grown at different salt concentrations. This depicts the saturation and unsaturation fatty acids at optimum and hyper saline conditions. At optimum salt concentrations there are more of saturated fatty acids and in significant contrast with more of unsaturated fatty acids with cells grown at hyper saline concentrations. The saturated and unsaturated fatty acids at the hypo, optimum and hyper NaCl concentrations represented by a bar graph shows that in high salt concentrations the unsaturation of

the fatty acids has increased. This was not reported so far in *R. salinarum* JA137. Saturated to unsaturated fatty acid ratio increased double fold (20%) in NaCl exposed cells compared to those at optimum and low salt (Fig. 26). These observations are in line to the previous reports which suggest that this is a survival strategy exhibited by halophiles to maintain membrane integrity and thus establish cellular homeo stasis. High saturated fatty acids content in NaCl exposed cultures indicate increased membrane fluidity and rigidification of membranes. Reports propose that the unsaturation of fatty acids counteract water or salt stress (Vreeland, 2012).

3.7.2 Branched and unbranched fatty acids at different salt concentrations in *Rhodovibrio salinarum* JA137

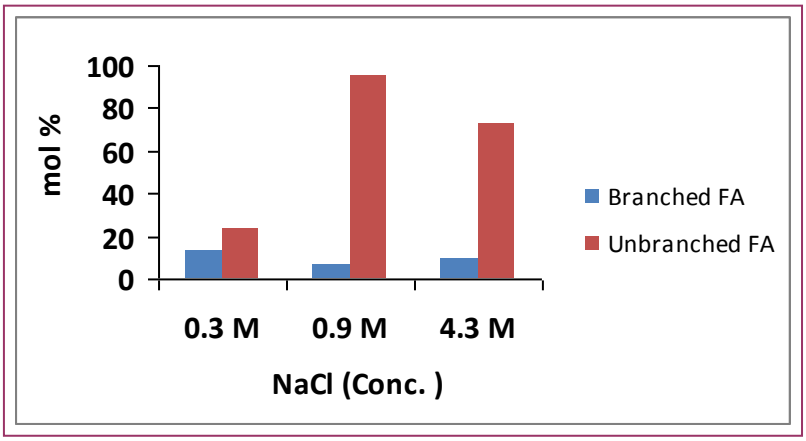

Fig. 27 Branched and Unbranched fatty acids profile of the cells of *Rhodovibrio salinarum* JA137 grown at different NaCl concentrations (0.3 M, 0.8 M, 4.3 M).

Fatty acid methyl esters analysis was done as mentioned in the materials and methods section (2.17.1).

The fatty acid profile analysis was performed to examine the influence of increasing medium salinity on the fatty acid composition of *R. salinarum* JA137. At optimum salt concentrations, there are more of unbranched fatty acids and in contrast to that observed with cells grown at hyper- and hypo-saline concentrations with increase in branched chain fatty acids. The unbranching is observed to be more in low salt concentration as compared to the optimum NaCl

concentration. The exact reasons need further study to confirm particularly about this aspect of more unbranched fatty acids in low salt and high salt (Fig. 27).

3.7.3 Long and short chain fatty acids at hyper and hypo salinity

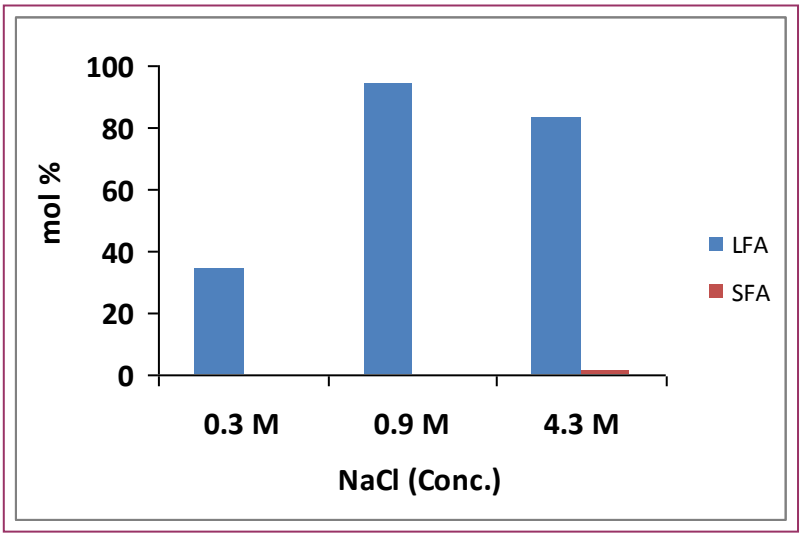

Fig. 28 Long chain and short chain fatty acids of the cells of *R. salinarum* JA137 grown at different salt concentrations of NaCl (0.35 M, 0.8 M, 4.3 M)

LFA, Long chain fatty acids; SFA, Short chain fatty acids. Fatty acid methyl esters analysis was done as mentioned in the materials and methods section (2.17.1).

Effect of NaCl on fatty acid composition of *R. salinarum* JA137. At optimum salt concentrations there are more of long chain fatty acids and in significant contrast to that, observed more of short chain fatty acids with cells grown at hyper saline concentrations. Fatty acid composition analysis of salt exposed and unexposed cells was done to monitor any change or impact on fatty acid content or composition due to NaCl stress. C16:0(2%), and C17:0(2.4%) were two major fatty acids identified in NaCl exposed cells of *R. salinarum* JA137 along with other minor fatty acids (Fig. 28). The fatty acid profile analysis of *R. salinarum* JA137 grown at different salt concentrations revealed that though long chain fatty acids were observed at all the salt concentrations range, remarkably there were short chain fatty acids in cells grown at high salt concentration of 25%.

3.8 Salinity influence on the reactive oxygen species (ROS) in *Rhodovibrio salinarum* JA137

The homeostatsis of cells is usually disturbed by an imbalance in the production of ROS which is generally due to the stress factors like drought and salinity, or in combination with other stresses. Reports have revealed that the ROS might be playing a dual role in the mechanisms of response towards stresses, functioning as toxic by-products of stress metabolism, as well as important signal transduction molecules of stress. Thus the focus is towards the understanding of ROS contributions in stress and regulation of adaptive responses (Kwak et al., 2006; Miller et al., 2009, 2010).To study the osmoprotective role of the compatible solutes on the management of ROS under salt stress. ROS levels were estimated with H_2DCFDA and the flourescence intensity is taken as a measure of the ROS levels during salt stress and the impact of osmolytes on the levels of ROS in high salt conditions was also observed.

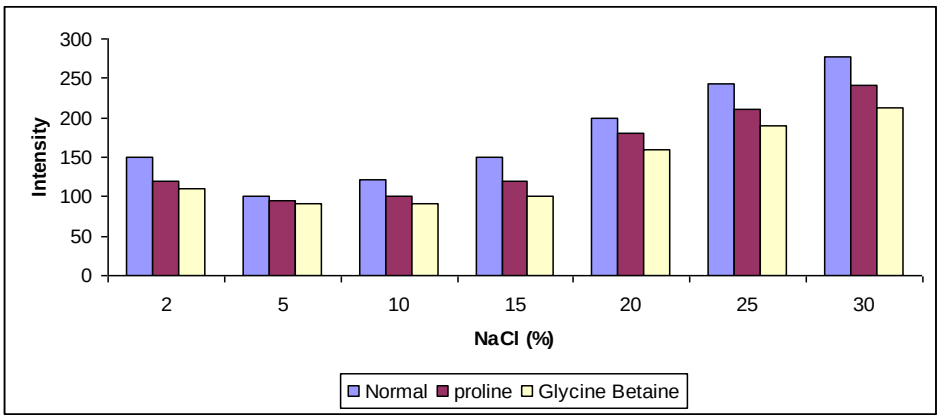

Fig. 29 ROS (reactive oxygen species) levels estimated in the cells of *Rhodovibrio salinarum* JA137 grown at different NaCl concentrations and supplemented with the osmolytes glycine betaine and proline. Y-axis-flouresence intensity, X-axis-percent NaCl concentration.

Reactive oxygen species levels were values were estimated according to the protocol in methods section (2.22).

It is a well known fact that ROS is generated always during the normal metabolism of cell and during stress, there is an imbalance of the ROS generated and that scavenged. Therefore this leads to damage of cells at all levels. However each system has its own unique way of managing

ROS under different conditions of stress as there are many ways of ROS getting generated and getting effected by it which remains unclear. How there is a coordination among all the compartments of cellular metabolism for a common cause of surviving the stress. This cross talk is not yet understood or reported. Therefore when salinity causes stress and its obvious consequence is the production of ROS and we observed that it seems to be increasing with medium salinity, but how about the levels of ROS when the medium is supplemented with osmolytes which are acknowledged to be protectants (Fig. 29). Therefore this Endeavour to examine what is the influence of salinity on ROS and whether it is decreased or increased in the presence of osmolytes and how both osmolytes together show their influence whatsoever positive or negative on the regulation of ROS generation. Our observations are, that the ROS levels seem to decrease by 20% with osmolyte supplementation in the medium as reflected in the intensity. In the limited comparative analysis of the present study, we have observed a decrease in the intensity of the ROS levels (Fig. 29). The observation through the present investigation is that glycine betaine seems to reduce the ROS levels more, in comparison to proline. However, both play the same role as osmoprotectants. However needs further in depth studies to shed light on this aspect of ROS in salt stress in halophiles.

3.9 Cloning of *groESL* of *Rhodovibrio salinarum* JA 137

Genome walking was performed with idea of cloning out the chaperonins GroEL and GroES from this halophile *Rhodovibrio salinarum* JA137 to investigate the role of these chaperones in this organism as biological chaperones, although halophiles are well known to be inherent with the chemical chaperones, the osmolytes. Therefore we wanted to elucidate how they act in concert in halophiles and make a comparative study of the chaperones from a halophile with that of non halophiles and gain information on their molecular evolution in halotolerance. However, the objective of *groESL* cloning is to express and characterize them in vitro to better understand the salt adaptation at molecular level. The illustration of the cloning of *groESL* in strain JA137 as represented in figure 30

3.9.1 PCR amplification of *groESL* of *Rhodovibrio salinarum* JA137

The PCR amplification of *groESL* gene from *R. salinarum* JA137 had been carried out with the specific degenerate primers designed, GroES forward primer, GroELR reverse primer by the protocol described in methodology(Fig. 30). *Rhodovibrio salinarum* JA137 genome is not yet fully sequenced, therefore, using NCBI blast search, retrieved the fully sequenced genomes of the phylogenetically closely related organisms of *R. salinarum* JA137 (*Rhodospirillum rubrum* which is very closely related to *Rhodovibrio salinarum* JA137). Performed the blast analysis to get the best hits of the sequences of *groESL* all the closely related organisms of *R. salinarum* JA137 and performed clustal alignment of the *groESL* gene sequences to get the conserved regions. Taking the conserved regions from the clustal aligned sequences, designed degenerate primers for *groES* and *groEL*(Fig. 30).

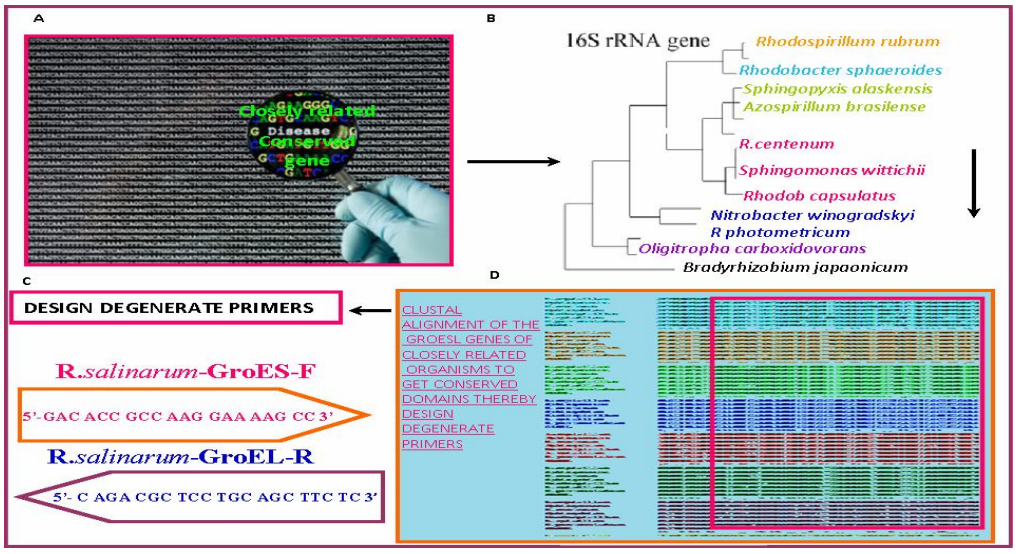

Fig. 30 Schematic Illustration of the *groESL* gene amplification from *Rhodovibrio salinarum* JA137. A) Blast search for the conserved sequences of *groESL* genes from phylogenetic close relatives of *R. salinarum,* JA137. B) Best hits of the closely related organisms *of R. salinarum* JA137, *C*) Designed degenerate primers, D) clustal alignment of the conserved genes of *groESL*. BLAST analysis was done using NCBI website. (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Clustal aligned all the sequences of *groESL* of the best ten hits and then designed the degenerate primers based on the conserved sequences of *groESL* from all the closely related organism best hits (Fig. 30). The degenerate primers were used for the PCR amplification of *groESL* from *R. salinarum* JA137 (Fig. 32). The thick band (*groESL*) obtained is gel extracted and purified and sequenced (Fig. 32). The sequencing results were confirmed by the blast analysis and clustal alignment with the *groESL* of the closely related organism *Rhodospirillum rubrum*(Fig. 30). The gene specific primers are designed based on the sequence of the *groESL* PCR amplified product of *R. salinarum* JA137 for the purpose of genomic walking. (Fig. 31). As the sequence obtained from the PCR amplification of *groESL* gene from *R. salinarum* JA137 was only approximately 1500 bp but the actual expected length of the *groESL* gene of *R. salinarum* JA137 is around 2.5kb in comparison to the closely related organisms like *Rhodospirillum rubrum*. Therefore performed genome walking to get the flanking sequences of *groESL* sequence of *R. salinarum* JA137 (Fig. 31)

Partial *groESL* of *R. salinarum* JA137 sequence aligned from 140 bp to 1402 bp (expected PCR product size is 1.5kb) with that of *groESL* of *Rhodospirillum rubrum*. To obtain a full length *groESL* gene, we designed gene specific primers at 715 bp (GSPUW) and 1135 bp (GSPDW) on the obtained partial *groESL* DNA sequence and used for genome walking (Fig. 33). The primary and secondary PCR for downstream walking resulted in 900, 2000 bp DNA fragments probably covering the stop codon of *groEL* sequence (Fig. 33, 34). Thus, the primary PCR products obtained were expected to cover the entire *groESL* genes of *R. salinarum* JA137. Based on the sequencing results, we designed a set of gene specific primers covering the full length *groESL* genes and amplified to obtain complete *groESL* gene from *Rhodovibrio salinarum* JA137.

3.9.2 The *groESL* gene cloning of *Rhodovibrio salinarum* JA137

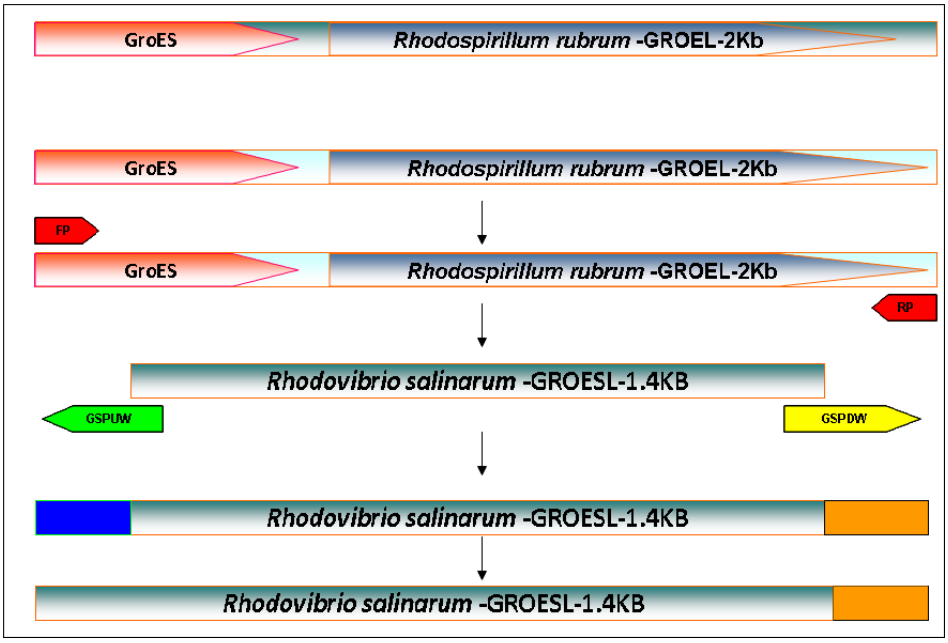

Fig. 31 Schematic illustration of the cloning of the *groESL* genes of *Rhodovibrio salinarum* JA137 by genome walking. *groES* is shown in pink and *groEL* in grey colour. *groESL* of *Rhodospirillum rubrum* is 2kb. Degenerate primers designed based on the conserved sequences of *groESL* best hits of the close relatives of *R. salinarum* JA137 are shown in red. The PCR product of *groESL* of *R.salinarum* JA137 obtained is approximately 1.4kb in size and shown in grey colour. The gene specific primers designed based on the sequence of *R. salinarum* JA137 *groESL* are shown in green (genespecific primer upstream walk) and the yellow (genespecific primer downstream walk). The part of the *groEL* which is not showing complete alignment with *Rhodospirillum rubrum* is shown in orange colour.

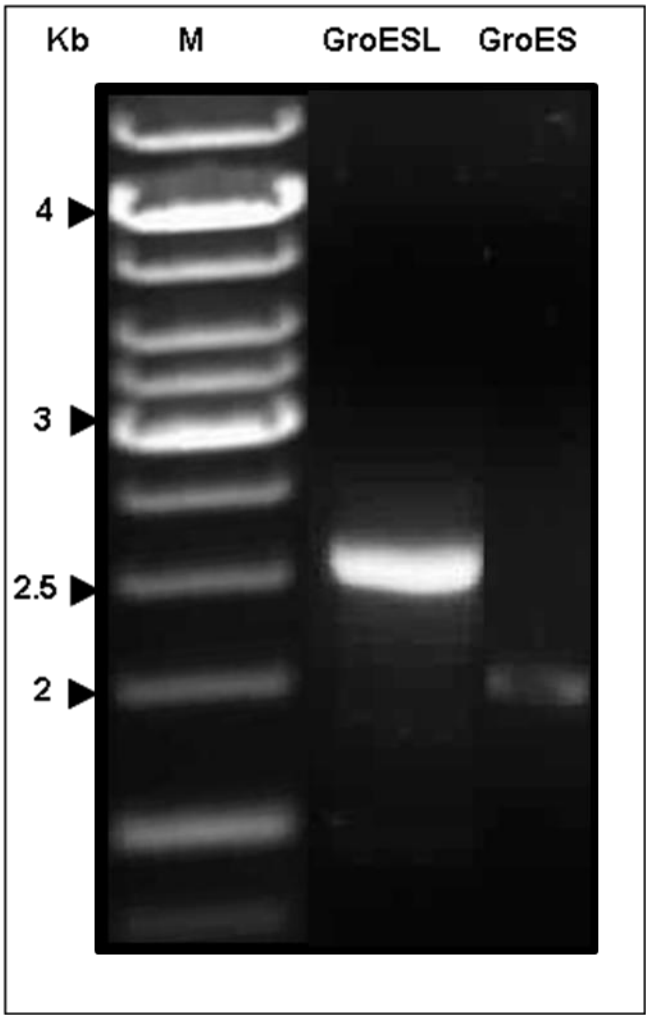

Fig. 32 The PCR amplified product of *groESL* of *Rhodovibrio salinarum* JA137

The thick band is of *groESL* of approximately 2.5kb, *groES* is a faint band of 1.5kb with reference to the ladder. The degenerate primers used were GroES forward primer and GroEL reverse primer. The experiment was done as mentioned in the methods section (2.24.8)

Therefore to obtain the flanking sequences of the unknown sequences of *groESL* sequence of *R. salinarum* JA137, we opted for genome walking. Thereby designed gene specific primers based on the PCR amplified products for the primary PCR of the upward and downward walk. As per the protocol of genome walking(refer methods section-2.30), the primary PCR product is used as a template for the secondary PCR for upward and downward walk and thereby get the full length of *groESL* gene sequence from *R. salinarum* JA137.

Genomic DNA was isolated according to protocol mentioned in the methodology (section). The genomic DNA isolated is ensured for its adequate quality, through agarose gel electrophoresis to check its purity. Digestion of the isolated genomic DNA was carried out as per the genome walking protocol mentioned in the methodology (section2.25). These digested products are used for performing primary PCR of the upward and downward walk of the *groESL* gene.

3.9.3 Primary PCR for the downward walk of *groEL* flanking Sequence of *R. salinarum* JA137

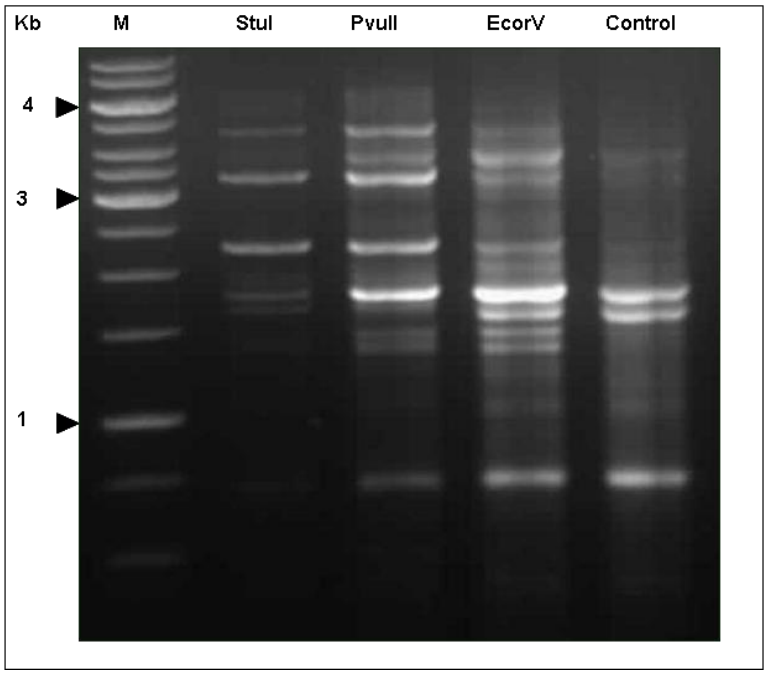

Fig. 33 Schematic Illustration of the amplified Primary PCR product for the downstream *groEL* flanking sequence of *Rhodovibrio salinarum* JA137.

The primary PCR is carried out with adaptor primers and gene specific primers designed for the downward walk. This is carried out with the digested products with the digestive enzymes PvuII, EcorV, StuI and with a control. The PCR amplifications of the digested products resulted in multiple bands and these PCR products are used as templates for the secondary PCR amplification for the downward walk as per the protocol mentioned in methodology (2.27).

3.9.4 Secondary PCR for the downward walk of *groEL* flanking sequence of *Rhodovibrio salinarum* JA137

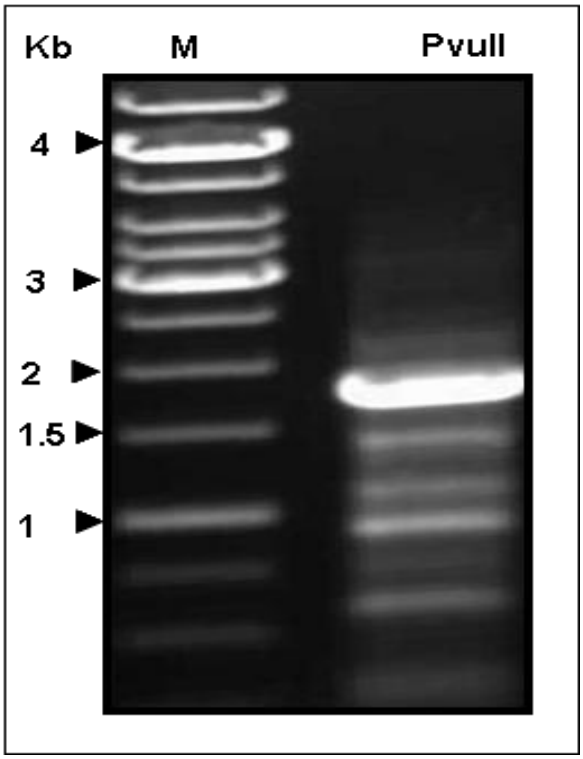

Fig. 34 Schematic Illustration of the amplified secondary PCR product for the downstream walk of *groEL* flanking sequence of *Rhodovibrio salinarum* JA137

This is the gel picture of the secondary PCR product of the downward walk carried out with the gene specific primer and adaptor primer 2 with the primary PCR product as the template. PCR amplification for the downward walk as per the protocol mentioned in methodology (2.27).

3.9.5 Primary PCR for the upward walk of *groEL* flanking sequence of *Rhodovibrio salinarum* JA137

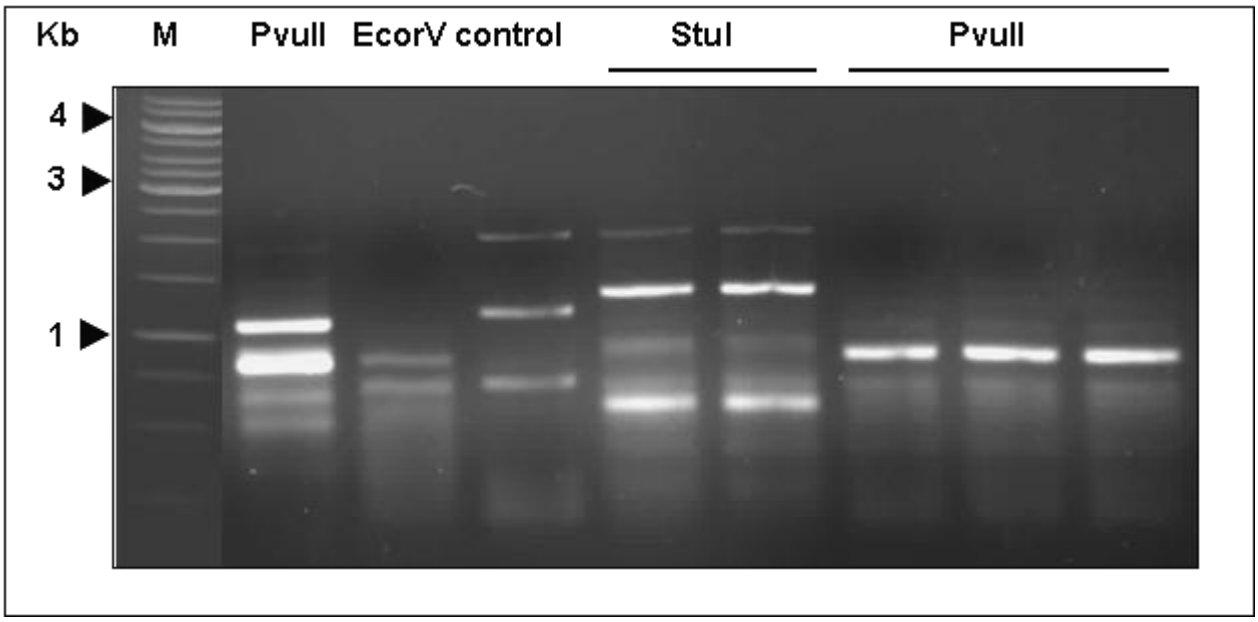

Fig. 35 Schematic Illustration of the amplified primary PCR product for the upstream flanking sequences of *groES* of *Rhodovibrio salinarum* JA137

The primary PCR for the upward walk is performed as per the protocol mentioned in the methodology (2.27). In order to obtain the upstream flanking sequence of *groESL* from *R. salinarum* JA137, the primary PCR is performed with the respective gene specific primers designed and adaptor primers. The primary PCR amplification carried out with the digested products of EcorV, PvuII, StuI resulted in many multiple bands.

The primary PCR products with single bands were gel extracted and purified and are used as templates for the secondary PCR for the upstream walk of the *groESL* sequence of *R. salinarum* JA137.

3.9.6 Secondary PCR for the upward walk of *groEL* flanking sequence of *Rhodovibrio salinarum* JA137

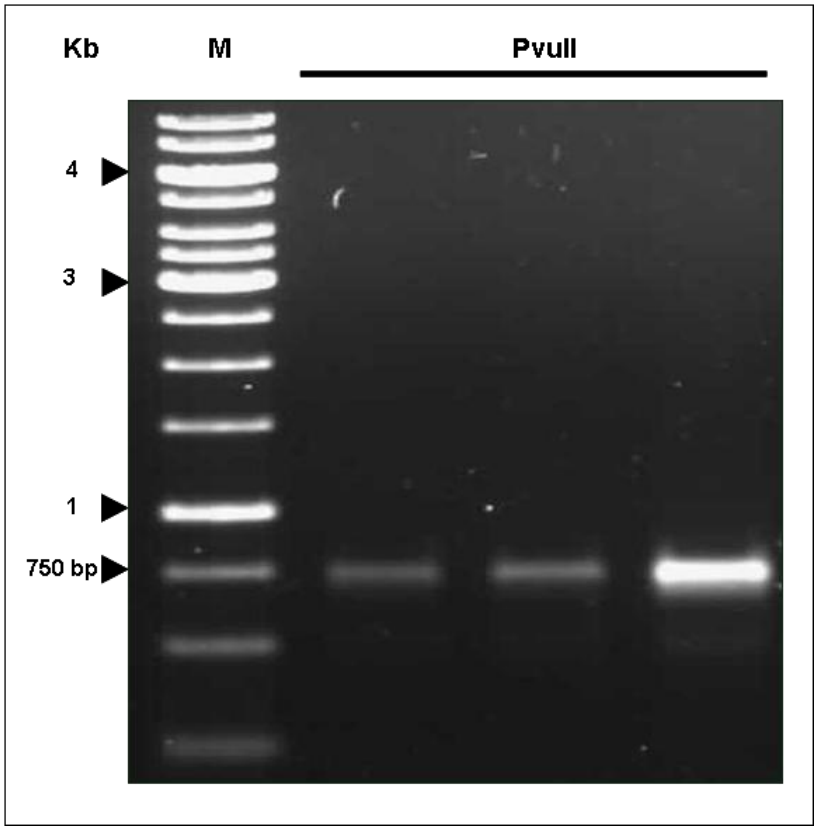

Fig. 36 Schematic illustration of the amplified secondary PCR products of the upstream flanking sequences of *groES* of *Rhodovibrio salinarum* JA137

The secondary PCR reaction is carried out with the primary PCR product as template with the adaptor primer and gene specific primer. We obtained a thick band at 750 bp.

The PCR products were purified and were sequenced. The sequencing results were analysed by Blast and clustal aligned with *groESL* of *Rhodospirillum rubrum* for the similarity match. The PCR product of 750 bp showed almost 60% alignment with *Rhodospirillum rubrum groESL* sequence as seen in (Fig. 37). The primary and secondary PCR for upstream walking resulted in 750 bp DNA fragment (Fig. 35, 36) probably covering the start codon and promoter of the *groESL* operon.

3.9.7 Sequencing of the PCR amplified product of *groESL* of *Rhodovibrio salinarum* JA137

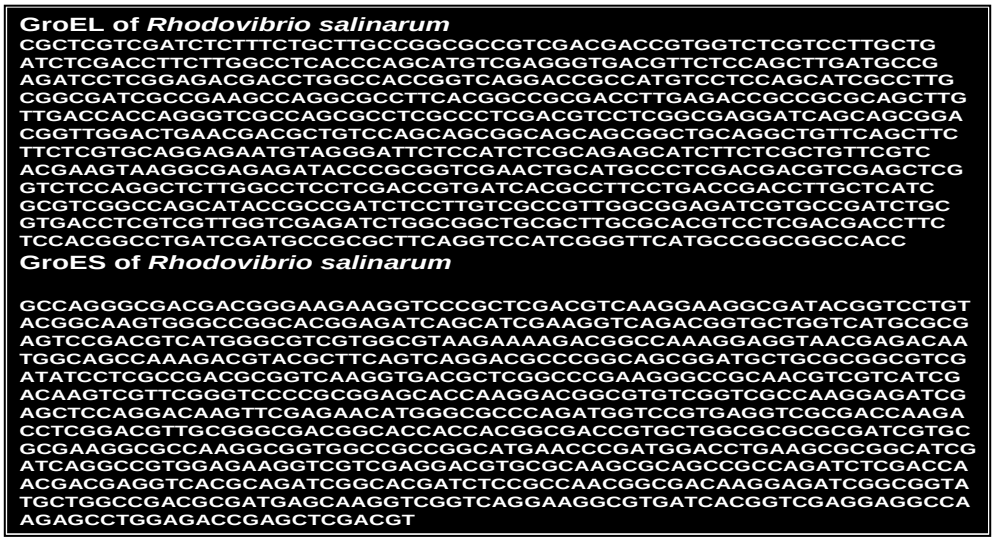

Fig. 37 The sequence of the PCR amplified product of *groESL* of *Rhodovibrio salinarum* JA137

Using the degenerate primers (GROESF-84nt-104nt, GROELR-1454-1475nt) and genomic DNA of *R. salinarum* JA137 as template, approximately 1.5 kb PCR product was obtained.

Sequencing and blast analysis indicated that the amplified DNA fragment corresponds to the partial gene sequence of *groESL* of strain JA137 (Fig. 37, 38). The sequencing results were clustal aligned for finding the high similarity match and selected the high similarity sequence 79% (percentage of similarity with *Rhodospirillum rubrum*) and 75% with *groEL* and *groES* respectively for designing gene specific primers for performing primary and secondary PCR's in order to walk the upstream and downstream flanking sequences to get the full length gene of *groESL* of *R. salinarum* JA137 by genome walking method.

We obtained thick band of approximately 750 bp (Fig. 36). This PCR product is gel extracted and purified and sequenced. The sequenced product is analysed by blast analysis and clustal aligned with the *groESL* of *Rhodospirillum rubrum* (Fig. 37, 38). This analysis resulted in almost 60% similarity with the *groESL* sequence of *Rhodospirillum rubrum*. However we probably did not get the complete length of the this gene because when compared to the close relative *Rhodospirillum rubrum*, the complete length of *groESL* was around 2.2 kb but what we obtained from *R. salinarum* JA137 is (approximately 1.9 kb) not completely matching when only the downstream part of around 300 bp is considered (Fig. 40). From these observations we could only say that may be the *groESL* gene of *R. salinarum* JA137 is a shorter fragment or its downstream base pairs are missing. Needs further insights to confirm on this aspect of its short length or missing of certain base pairs with sequencing of the complete genome of *R. salinarum* JA137.

3.9.8 Blast and clustal alignment of the PCR amplified products of *groESL* of *Rhodovibrio salinarum* JA137

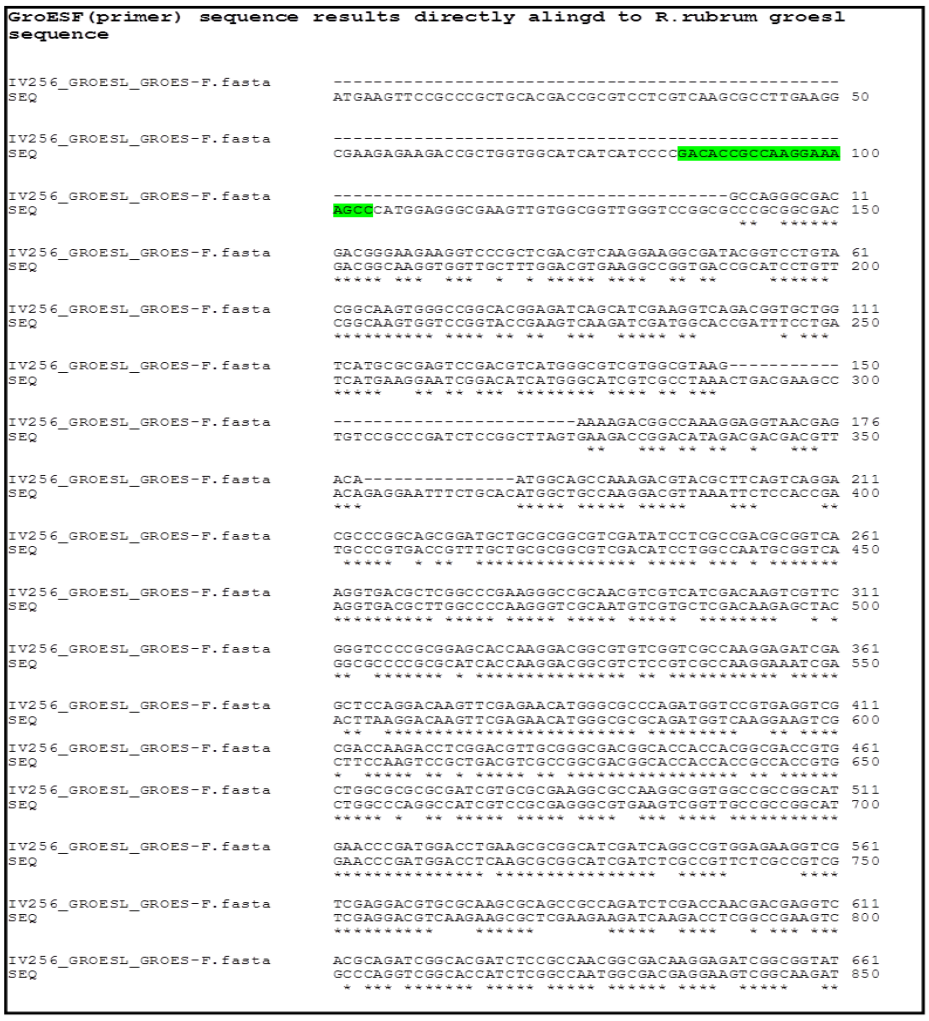

Fig. 38 The schematic representation of the alignment of *groESL* gene sequences obtained from *R. salinarum* JA137 with that of *Rhodospirillum rubrum groESL* nucleotide sequence (accession number-is CP000230.1). The gene specific primers designed for the upstream walk are highlighted in green.

The above illustrated shows the clustal alignment of *groES* of *R. salinarum* JA137 to *R. rubrum* which is the closely related organism. The alignment starts from 140 bp of *R. rubrum* and 11 bp of *R. salinarum* JA137 and it continues upto 745 and 950 bp respectively.

Fig. 39 The Schematic illustration of the alignment of the sequenced PCR products of *groESL* of *Rhodovibrio salinarum* JA137 to the *groESL* sequence of *Rhodospirillum rubrum* and the gene specific primers designed for the downstream walk are highlighted in green.

The above illustration shows the clustal alignment of *groES* of *R. salinarum* JA137 to *groES* of *Rhodospirillum rubrum* which is the closely related organism. The alignment starts from140bp and 717bp of *Rhodospirillum rubrum* and 15bp of *R. salinarum* JA137 and it continues upto 1402 bp respectively.

3.9.9 Clustal alignment of *groESL* flanking sequences of *Rhodovibrio salinarum* JA137 with *groESL* of *Rhodospirillum rubrum* by genome walking

>GROES
TAATATGACATTTCCCCCCCCTCTGCATGACCGCGTTGTGGTCA
AGCGTCTTGAGCAGGATGAGAAGACCACCGGCGGAATCATCAT
TCCGGATACGGCGAAGGAAAAGCCGATGCAGGGCGAAGTGCT
CGCCGTCGGCCCCGGCGCCAAGGACGAGCAGGGCGCCGTCCA
GCCGCTCGACGTCAAGGTCGGCGACACCGTGCTGTTCGGCAAG
TGGTCGGGCACCGAGGTGACGGTCGACGGTCAGGACGTCCTG
ATCATGAAGGAATCCGACATCATGGGCGTCCTCGAAGGCCAGG
CCGCGGGCAAGAAGGCCGCGTAACGCGCCGCTTGCCCGATCC
GCTTCACGAGAACAAAACGTCAATATATTTCCGAAAAGTAGGA
AACACAGACGATGGCTGCCAAAGACGTCAAATTCGGCACCGAC
GCGCGCGACCGCATGCTGCGCGGCGTCGATATCCTGGCCAACG
CCGTGAAGGTCACGCTCGGCCCGAAAGGTCGCAACGTGGTGAT
CGACAAGTCGTTCGGCTCCCCGCGCACGACCAAGGACGGCGTC
ACCGTCGCCAAGGAGATCGAGCTTAAGGACAAGTTCGAGAACA
TGGGCGCTCAGATGGTCCGCGAGGTTGCGTCCAAGACCGATCT
GGCCGGTGACGGCACCACCACCGCGACCGTGCTGGCCCAGGC
GCTGGTGCGCGAAGCGCCCCCATC

>GroEL
CGCTCGTCGATCTCTTTCTGCTTGCCGGCGCCGTCGACGACC
GTGGTCTCGTCCTTGCTGATCTCGACCTTCTTGGCCTCACCC
AGCATGTCGAGGGTGACGTTCTCCAGCTTGATGCCGAGATC
CTCGGAGACGACCTGGCCACCGGTCAGGACCGCCATGTCCT
CCAGCATCGCCTTGCGGCGATCGCCGAAGCCAGGCGCCTTC
ACGGCCGCGACCTTGAGACCGCCGCGCAGCTTGTTGACCAC
CAGGGTCGCCAGCGCCTCGCCCTCGACGTCCTCGGCGAGGA
TCAGCAGCGGACGGTTGGACTGAACGACGCTGTCCAGCAG
CGGCAGCAGCGGCTGCAGGCTGTTCAGCTTCTTCTCGTGCA
GGAGAATGTAGGGATTCTCCATCTCGCAGAGCATCTTCTCG
CTGTTCGTCACGAAGTAAGGCGAGAGATACCCGCGGTCGA
ACTGCATGCCCTCGACGACGTCGAGCTCGGTCTCCAGGCTC
TTGGCCTCCTATACCGCCGATCTCCTTGTCGCCGTTGGCGGA
GATCGTGCCGATCTGCGTGACCTCGTCGTTGGTCGAGATCT
GGCGGCTGCGCTTGCGCACGTCCTCGACGACCTTCTCCACG
GCCTGATCGATGCCGCGCTTCAGGTCCATCGGGTTCATGCC
GGCGGCCACC

Fig. 40 Schematic Illustration of the alignments of the sequenced PCR products of the upstream and downstream flanking sequences of *groES* and *groEL* of *R. salinarum* JA137. Blue coloured is the sequence of *groES* of *R.salinarum* JA137 and its alignment with *Rhodospirillum rubrum*, orange coloured is the gene sequence of *groEL* of *R. salinarum* JA137 and its alignment (pink,blue) with *groEL* of *Rhodospirillum rubrum*.

The above illustrations are the flanking sequences of *groES* and *groEL* of *R. salinarum* JA137 and their alignments with *groESL* of *Rhodospirillum rubrum* respectively. The upward sequence of *groES* was showing (65%) alignment to *Rhodospirillum rubrum.* However the downward stream sequence of *groEL* was not showing complete alignment with *groEl* of *Rhodospirillum rubrum* (Fig. 33,34). This probably needs further study. Might be the *groESL* of strain JA137 is a shorter fragment or its orientation may be different when compared to *Rhodospirillum rubrum*. Complete Genome sequencing of strain JA137 might help us get the information about the complete *groEL* sequence.

CONCLUSIONS

4.0 Conclusions

This Endeavour to study the salt adaptive strategies of *Rhodovibrio salinarum* JA137 was to gain insights into their distinct patterns of genetic makeup evolved to survive high salt. Through the investigations performed on the growth patterns at varied NaCl concentrations and the influence of osmolytes on the growth profiles at varied salt concentrations revealed the organisms abilities to grow over a range of NaCl concentrations and also its unique capability to grow at high salt. The examination of the influence of NaCl on the photosystems integrity and carotenoids, fatty acid and polar lipid profile, cell morphology revealed the inherent molecular mechanisms and thereby the capability of this organism to tune itself to the impact of changing salinity and thus adapt accordingly. The investigations made it evident that the inherent genetic make up, molecular mechanisms all tuned to contribute for halo adaptations in high salinity conditions.

Although the observations were in line with previous reports, it was clear that all these mechanisms involved, operate in synchrony making the organism survive the salty environment and thus adapt. But what remains unexplored still are the different ways and styles of adaptations. Our investigations have evidenced the diverse ways the organism responded to salinity and the means of responses are quiet distinct and reported in strain JA137. The underlying synchrony which operates across the different compartments of the cell to sense, and respond facilitating adaptation to salinity is quiet interesting and that was our prime focus and therefore this endeavour to examine its response towards salinity. The sequence of dynamics that operates during the stress episode still needs further in depth analysis.

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5.0 References

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