

EXTRACTION AND CHARACTERIZATION OF PURPLE MEMBRANE PIGMENT FROM HALOARCHEAL BACTERIA

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

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**INSTITUTE OF SCIENCE AND TECHNOLOGY
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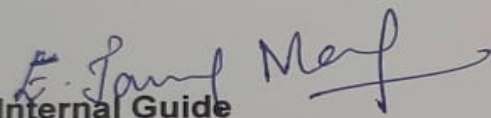
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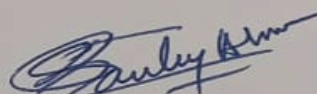
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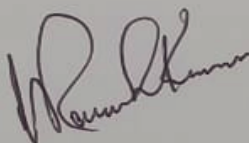
This is to certify that this Project Report is the bonafide work of and **Akula Charishma** (41300002) and **Guduru Naga Vinuthna** (41300011), who carried out the project entitled "**EXTRACTION AND CHARACTERIZATION OF PURPLE MEMBRANE PIGMENT FROM HALOARCHEAL BACTERIA**" under our supervision from January 2024 to April 2024.


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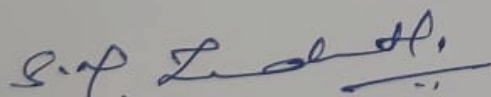
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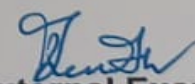


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We Akula Charishma (41300002) and Ms. Guduru Naga Vinuthna (41300011) hereby declare that the project report entitled "EXTRACTION AND CHARACTERIZATION OF PURPLE MEMBRANE PIGMENT FROM HALOARCHEAL BACTERIA" done by me under the guidance of internal guide Ms. E. Jancy Mary, Assistant Professor, Department of Biotechnology, Sathyabama Institute of Science and Technology and external guide Dr.L.Stanley Abraham, Scientist F / Professor (Research), Centre for Ocean Research, Sathyabama Research Park is submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in Microbiology.

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ABSTRACT

Bacterial pigments are of higher interest in industrial sectors as they are highly soluble and have greater stability with potent unique applications. Haloarchaea species which survives in extreme conditions, consists of a transmembrane protein bacteriorhodopsin which possess various advantages. These species found in extreme hypersaline regions like Marakanam, Vedaranyam and Tuticorin of Tamil Nadu, samples from these regions were collected and cultured in modified Mineral salt (MS) media. The present study was aimed to carry out to extract and purify bR by comparing three different methods to estimate the highest yield which was successfully carried out by Aqueous Two-Phase, Freeze Thaw and Bead Mix. Absorption Spectroscopy analysis showed maximum peak at 560nm which results in the presence of bacteriorhodopsin protein. The purified bacteriorhodopsin protein was visualized by SDS-PAGE with molecular mass being seen between 25kDa - 27kDa. The identity of the protein was confirmed by Fourier Transform Infrared Spectroscopy.

KEYWORDS: Haloarchaea, Bacteriorhodopsin, Freeze Thaw Method, Salt pans and retinal protein

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LIST OF ABBREVIATIONS

S.NO	ACRONYM	ABBREVIATION
1.	BR	Bacteriorhodopsin
2.	K ⁺	Potassium
3.	Na ⁺	Sodium
4.	Cl ⁻	Chloride
5.	M	Molar
6.	MS media	Mineral salt media
7.	ATP	Adenosine triphosphate
8.	Cys	Cysteine
9.	DNA	Deoxyribonucleic acid
10.	GPCR	G protein - coupled receptor
11.	HTR	Halo transducer
12.	Ile	Isoleucine
13.	Leu	Leucine
14.	Met	Methionine
15.	NaCl	Sodium chloride
16.	pH	Potential of Hydrogen
17.	Arg	Arginine
18.	UV	Ultraviolet
19.	Val	Valine
20.	FTIR	Fourier Transform Infrared
21.	SM 1	Sample isolated from Marakkanam
22.	TMR 1	Sample isolated from Tuticorin
23.	TMR 2	Sample isolated from Tuticorin
24.	SDS PAGE	Sodium dodecyl – sulfate polyacrylamide gel electrophoresis
25.	PEG	Polyethylene glycol
26.	RPM	Revolution per minute

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CHAPTER 1

INTRODUCTION

1.1 HALOPHILIC BACTERIA

Extreme circumstances (salinity, pH, temperature, pressure, light intensity, oxygen, and nutritional conditions) are all capable of supporting microbial life. Because of their high salinity, exposure to both high and low temperatures, low oxygen levels and in certain situations, high pH values, hypersaline environments are common examples of settings with severe conditions. In these conditions, the most widely spread species are bacteria and archaea (Ventosa *et al.*, 2006). Kushner and Kamekura distinguished four groups of halophilic microorganisms according to the ideal salt concentration at which they grow most efficiently: Microorganisms classified as halotolerant are those that can with stand high concentrations of salt, while non-halophilic organisms are those that need less than 1% NaCl. When it comes to halophilic microorganisms, the classification makes a distinction between moderate halophiles (marine bacteria), which grow best in media containing 3% to 15% NaCl, extreme halophiles (which grow best in media containing 15% to 30% NaCl), and slight halophiles (marine bacteria), which grow best in media with 1% to 3% NaCl (Kushner and kamekura *et al.*,1988). Because the high concentration of NaCl in their typical surroundings causes osmotic pressure, halophiles have evolved two distinct adaptation techniques to deal with this situation (Madigan *et al.*,1999). To maintain the medium's osmotic pressure, halo bacteria and certain very halophilic bacteria accumulate inorganic ions (K^+ , Na^+ , and Cl^-) in their cytoplasm. As a result, these bacteria have evolved certain proteins that remain stable and functional in the presence of salts. On the other hand, moderate halophiles cause large numbers of particular organic osmolytes to collect in the cytoplasm. These osmoprotectants maintain osmotic balance without interfering with the cell's regular metabolism (Neito and Vargas.,2002). Halophilic bacteria have been investigated recently for their potential in a variety of biotechnological sectors (Mellado and Ventosa.,2003).

Applications include the employment of these microbes in environmental bioremediation procedures as well as the utilization of various products, such as

suitable solutes, biopolymers, or carotenoids, in a range of businesses. Halophilic enzymes present significant prospects in biotechnological applications such as food processing, environmental bioremediation, and biosynthetic processes, in addition to being inherently stable and active at high salt concentrations. Thus, it is crucial to discover new enzymes that exhibit optimal activity across a variety of pH, temperature, and salt concentrations (Gómez and Steiner.,2004). It is important to highlight that the use of enzymes from halophiles in industrial applications are not limited to their stability at high salt concentrations, since these extremozymes usually are also tolerant to high temperatures and they are stable in presence of organic solvents (Oren.,2010). Because of the decreased hydration, low water activity (a_w) generally causes conformational changes in the enzymes that impact the catalytic activity. Nonetheless, even with low a_w , enough water is present to maintain an appropriate charge distribution at the active site, retaining the conformation of the enzyme, which is why halophilic enzymes are stable and active in conditions with low a_w (Zaccai.,2004). Organic solvents with a log P lower than 2, generally are considered to cause enzyme denaturation, producing the distortion of the water biocatalyst interactions. However, several studies have been reported on hydrolases produced by extremophiles isolated from saline environments that are very stable in solutions containing organic solvents. This will be focused on the diversity and biotechnological applications of the novel described enzymes from two groups of bacteria: the moderate and the extreme halophiles. The adaptation to live in hypersaline environments give rise to these extremophiles advantages to be exploited from a biotechnological point of view (Salameh and Wiegel.,2007).

1.2 PROTEIN IN HALOPHILIC BACTERIA

Halophilic archaea and bacteria can adapt to three different salinity concentrations: low, moderate, and excessive. These kinds of adaptations could be brought about by structural alterations in proteins and other modifications in various cell organelles. As a result, proteins might be crucial in helping halophilic bacteria and archaea adapt to salinity. Proteins can be fibrous or globular, depending on the purpose of the protein. A polypeptide is a single linear polymer chain made up of neighbouring amino acids bound together by peptide bonds. It is well known that halophilic proteins

are extremely stable. Acidic amino acids, which are mostly found on the protein surface, are abundant in these proteins. The majority of the acidic residues are found on the surface of these, according to the three-dimensional structural analysis. The majority of these proteins contains acidic residues which are present on their surfaces, which makes excessive protein hydration easier, according to the three-dimensional structural analysis. As a result, the surface becomes more flexible and hydrophilic. Thus, nonspecific electrostatic interactions with salts in solution are encouraged (Kennedy *et al.*, 2001) (Paul *et al.*, 2008).

On the surface of dihydrofolate reductase, acidic amino acids form clusters that multiply the cell nuclear antigen (PCNA) from *Haloferax volcanii* (Pace., 1990) (Horovitz and Fersht., 1992) and the glucose dehydrogenase from *Haloferax mediterranii*. In addition to preventing protein aggregation (Dill., 1990), interactions between hydrated salt ions and acidic residues on the surface preserve the protein's functioning. Making protein surfaces lacking in lysine residues is another tactic to promote hydration on protein surfaces (Morroll *et al.*, 2009) (Mevarech *et al.*, 2000) (Marqusee., 1994). The secret of halophilic adaption is electrostatic stabilisation. Ion pair or salt bridge is an important determinant of stability of proteins (Pfeil., 1986) (Stickle *et al.*, 1992). This is more so in the case of proteins adapted to extreme environmental conditions such as high salt or temperature. Interaction energy of salt-bridge could impart stability (Jencks., 1969) (Von Hippel *et al.*, 1969) (Abram and Gibbons., 1961) or be destabilising for the protein (Brown., 1963, 1964) as shown by both theoretical and experimental studies. Mostly, halophilic enzymes function at 1–4 M salt concentration. This range is required for the stability and activity of halophilic enzymes (Mevarecha., 2000).

1.3 AMINO ACID COMPOSITION OF PROTEIN IN HALOPHILIC BACTERIA

Halophiles exhibit biased amino acid composition, similar to proteins from thermophiles. This bias is characterized by an abundance of acidic residues on the protein surface relative to the inside. This universal characteristic seems to be independent of specific protein architectures and to be present for all proteins encoded across the genome. Protein surfaces with distinct compositions are frequently seen in thermophiles and halophiles. According to statistical studies, there are notable

differences in the surface composition of thermophiles, halophiles, and non-halophiles, but there are no significant differences in the internal content of any of the three types of organisms. While both acidic and basic residues are about equally abundant in thermophilic proteins, halophilic proteins appear to have a significant excess of acidic residues offset by a deficiency of basic residues. The compositional variations between mesophiles, thermophiles and halophiles were largely attributed to aspartic acid, lysine, asparagine, alanine, and threonine. Only aspartic acid, however, differed significantly from the predicted quantity based on the dinucleotide makeup of the halophile's genomic DNA sequence, which has a very high G+C content (68%). As a result, the other residues (Lys, Ala, etc.) that had significant deviations from their non-halophilic frequency might have developed only as "dragging effects" due to the DNA's altered composition, which would have increased the aspartic acid proportion exclusively (Eisenberg and C Ebel *et al.*, 1992;F Bonnete *et al.*, 1994;C Ebel *et al.*, 1995).

1.4 HALOBACTERIUM SALINARIUM

A halophilic archaeon, *Halobacterium salinarium* thrives in salinity levels close to or at saturation. Despite being identified from salted fish a century ago, *H. salinarium* gained attention in a variety of fields, including biophysics, chemistry, molecular evolution, and biotechnology, in 1971 when bacteriorhodopsin, the light-driven proton pump, was discovered. Since then, *H. salinarium* has been involved in many discoveries, including the first instance of a non-eukaryotic glycoprotein and advancements in the determination of membrane protein structures. The third domain's molecular mechanisms have also been clarified by research on *H. salinarium*, one of the species used to define Archaea. *H. salinarium*, finally, offers inventive answers to the problems associated with living in high salinity (C Ebel *et al.*, 1995).

1.5 TAXONOMY

Domain: Archaea;

Class: Halobacteria;

Order: Halobacteriales;

Family: Halobacteriaceae;

Genus: Halobacterium;

Species: *Halobacterium salinarium*;

Phylum: Euryarchaeota;

Clade: Stenosarchaea group.

There are now six strains of *H. salinarium*, with strain 91-R6 serving as the type strain (Pfeiffer *et al.*, 2020).

1.6 PROPERTIES OF *Halobacterium salinarium*

High cytosolic K⁺ levels (~4 M) balance the external Na⁺ concentration, allowing *Halobacterium salinarium* to grow at NaCl values close to saturation (Christian *et al.*, 1962). Even though *Halobacterium salinarium* is an aerobic chemoorganotrophic, it may also grow anaerobically by depending on bacteriorhodopsin, which is a light-driven proton pump present in their purple membranes. Because of its high acidity (average pI ~5), the *H. salinarium* proteome is adapted to help halo archaeal proteins fold in their hypersaline environment (Lanyi.,1974). Furthermore, *Halobacterium salinarium* exhibits colour-sensitive photo taxis, chemotaxis, and aero taxis because it thrives in situations with intense UV radiation and variable nutritional availability (Dassarma *et al.*, 2001).

1.7 GENOME

The first genome from a halophilic archaeon to be sequenced was that of *H. salinarium* strain NRC-1, which was released in 2000. The three closed mini-chromosomes that make up the Halobacterium NRC-1 genome are 200 kbp, 350 kbp, and 2 Mbp in size, and they are all home to vital genes. The GC level of the mini-chromosomes is lower (59.2 and 57.9%) than that of the main chromosome, which has a high GC content of 67.9 percent. The genome contains almost 100 transposable insertion sequence elements, which accounts for the haloarchaeon's observed genetic flexibility. The genome of the *H. salinarium* strain NRC-1 was reported to encode 2360 proteins at the time of its publication; 36% of these were thought to be unrelated to any other known proteins at that time. Lastly, *H. salinarium* is a polyploid, similar to other halo archaea, with about 25 copies of the genome in the exponential phase and only

15 copies in the stationary phase. Mini-chromosome copy numbers are likewise controlled by the growth phase (Ng *et al.*, 2000).

1.8 PHYLOGENY

H. salinarium was originally isolated from salted fish in 1922, but since the original strain was destroyed, the type strain (strain 91-R6) was changed to a neotype that was obtained from salted cowhide in 1934. In the end, isolates that were first identified as *H. salinarium*, *H. cutirubrum*, or *H. halobium* were found to be so similar that they were all classified as *H. salinarium* species. Thus far, the type strain's genome and the genomes of two lab strains (*H. salinarium* species NRC-1 and R1) have been sequenced. Only 12 changes were found between *H. salinarium* strains NRC-1 and R1, which included variations linked to the abundance of insertion sequence elements in *H. salinarium* genomes, five single-base frameshifts, four point mutations, and three insertion/deletion events. Only 38 strain-specific sections, or more than 99 percent of the genomes of the laboratory strains, are shared by the *H. salinarium* strain 91-R6 (Pfeiffer.,2020).

1.9 HALOBACTERIUM SALINARIUM

H. salinarium is one of the microbes that helped to first define the Archaea, and it has shed new light on a number of topics related to this unique realm of life. *H. salinarium* is the species that first revealed the distinct ether-linked isoprenoid-based phospholipids that make up the archaeal plasma membrane. The first instance of protein N- and O-glycosylation outside of the Eukarya was found in the surface (S)-layer protein that makes up the protein shell around *H. salinarium* cells (Mescher.,1976). The earliest transformation and gene deletion mechanisms created for Archaea originated from *H. salinarium* (Cline *et al.*,1987). Furthermore, *H. salinarium* offered Archaea's first instance of a two-component regulatory mechanism. It is now established that these systems are necessary for *H. salinarium*'s phototactic responses in reaction to orange and blue light, respectively, sensory rhodopsin's I and II and the transducers Htr I and Htr II mediate positive and negative phototactic responses. Halorhodopsin also promotes Cl⁻ uptake in yellow light concurrently (Spudich.,1998). Nevertheless, the discovery of bacteriorhodopsin in 1971 served to

bring *H. salinarium* to the attention of scientists across all disciplines, leading to a number of ground breaking and even paradigm-shifting discoveries. When exposed to UV light while under oxygen shortage, patches made up of bacteriorhodopsin trimer clusters arranged in a two-dimensional lattice structure are known as purple membranes. Under these circumstances, the cell expresses about 300,000 copies of bacteriorhodopsin, or 75% of the membrane's dry mass. Because of this configuration, bacteriorhodopsin was among the first membrane proteins for which structural data was available. The creation of optogenetics, an experimental technique in which light regulates the activity of neurons or other cell types, was finally prompted by the introduction of bacteriorhodopsin into non-native cells. It has long been known that bacteriorhodopsin has biotechnological potential for use in bioelectronic applications (Oesterhelt.,1971).

Research on *H. salinarium* has also improved our knowledge of life at high salinities. Aspartate and glutamate are more prevalent on *H. salinarium* protein surfaces than positively charged residues like lysine and arginine. This adaptation is likely to help the proteins stay soluble and flexible in hypersaline environments. Intracellular gas vesicles were initially discovered in *H. salinarium* and are believed to enhance buoyancy in low oxygen hypersaline settings. Last but not least, *H. salinarium* is remarkably resistant to UV radiation- and desiccation-induced DNA damage, even in the presence of high amounts of sunshine. This may be because the bacterium is polyploid (Henderson.,1975).

1.10 BEHAVIOUR OF ARCHAEON *HALOBACTERIUM SALINARIUM*

Light-dependent sensory responses in motile eubacteria were first discovered by Engelmann (Engelmann *et al.*, 1883). He observed that motile cells of *Bacterium photometricum* reversed the direction of movement when entering a dark zone. Photo stimuli control swimming behaviour in the Archaeon *Halobacterium salinarium* by modulating the frequency of reversals. An increase in the intensity of blue light increases the probability of reversal, whereas decreases in the intensity suppress reversal probability. Changes in green-red light intensity have the opposite effect. There are now two known photo sensory receptors. Separate from the retinal-based, light-

driven ion pumps bacteriorhodopsin (BR) and halorhodopsin (HR) are both proteins that contain retinals (Hildebrand *et al.*, 1975, Spudich *et al.*, 1982, Takahashi *et al.*, 1985). The photoreceptor for green-red and UV light stimuli is sensory rhodopsin I (SRI) (Bogomolni *et al.*, 1982), and the photoreceptor for blue light is sensory rhodopsin II (Takahashi *et al.*, 1985, Spudich *et al.*, 1986, Wolff *et al.*, 1986, Marwan *et al.*, 1987). Proton pumping by BR produces photo taxis responses even in the absence of specific sensory rhodopsin's, most likely as a result of impacts on membrane potential or proton motive force (Bibikov *et al.*, 1993, Yan., 1992). Isolating *H. salinarium* mutants devoid of BR and HR (Spudich *et al.*, 1982) has made studying the sensory rhodopsin's much easier. There are two spectrally different forms of the SRI protein (SR587 and S373). These forms can be photo activated to produce sensory signals (Bogomolni *et al.*, 1982, Takahashi *et al.*, 1985, Spudich 1984), which are then transferred by a 57-kDa methyl accepting membrane transducer (Yao *et al.*, 1992). A considerable degree of sequence similarity exists between the C-terminal region of this first Archaeal photo transducer Htr I and the signalling and methylation domains of eubacterial chemotaxis transducers. Regarding SRII, also known as phoborhodopsin, less is known (Bourret *et al.*, 1991).

1.11 BACTERIORHODOPSIN

It has been discovered that bacteriorhodopsin, the only protein of the purple membrane of halophilic bacteria that can survive in harsh environments and efficiently use light energy to carry out their essential activity (Oesterhelt., 1976), also acts as a light-driven proton pump, creating a significant pH gradient in the membrane that is needed for ATP synthesis (Racker *et al.*, 1974). Bacteriorhodopsin becomes lodged in the lipid phase when it regularly crosses the purple membrane. This explains the protein's strong lipid affinity and its relative "hydrophobicity," which are traits shared by most integral membrane proteins. Bacteriorhodopsin is a light-driven proton pump. This 248-residue integral membrane protein from *Halobacterium salinarium* provides an excellent model (mainly in the sense of being the best understood model and relatively simple in its mechanism) for how cells use available energy to actively transport protons (H⁺) across a membrane, thereby converting the original energy source into the form of a transmembrane proton gradient. Although the energy to drive the proton pumping

by the membrane protein complexes of the electron transport chain in eukaryotic mitochondria is provided by redox reactions, not light, similar principles appear to apply to the proton translocation mechanism. The structure of bacteriorhodopsin consists for the most part of seven transmembrane helices. This seven-helix tertiary structure is a common protein fold for integral membrane proteins. Bacteriorhodopsin in fact serves as a paradigm for the family of integral membrane proteins that includes G-protein-coupled receptors (GPCRs; examples of GPCRs are rhodopsin and adrenergic receptors). There is a polar channel through the centre of the structure that also contains a retinal prosthetic group. The retinal is covalently attached to Lys216, via a Schiff base linkage (Khorana *et al.*, 1979) (Dunn *et al.*, 1981).

1.12 APPLICATIONS OF BACTERIORHODOPSIN

- BR-based hybrid electrodes are efficient alternate for sensors and solar/fuel cells.
- It can be used in the light, biological, chemical, optical, gas, and electrical sensors, immunosensors, nano sensors, and various other sensors that are the subject of current research programs.
- The ability of BR (Bacteriorhodopsin) to convert light energy into chemical energy which is known as photocurrent activity has taken as application in the field of optical electronic devices, chemical sensing and drug delivery.
- It acts as a light sensor which can effectively be used as a solar collector to collect energy from (sun) light. This energy could either be consumed directly or stored in batteries.
- Applications include protein films, used in artificial retinal implants, light modulators, three-dimensional optical memories, colour photochromic sensors, photochromic and electrochromic papers and ink, biological camouflage and photo detectors for biodefense and non-defense purposes (Saeedi *et al.*, 2021).

1.13 BIODIVERSITY OF *H. SALINARIUM* IN TAMILNADU

- *H. salinarium* often found from multipond solar salterns in Kelambakkam, Vedaranyam, Tuticorin, Tamil Nadu, India.
- This has been found in Marakanam, Tamil Nadu (12°18'69.52" N, 79°92'78.95" E) during two seasons namely pre-monsoon and post-monsoon.
- This bacterium is also seen at salt pan of Kovalam at four intervals for one year in Kanyakumari district ((Burns *et al.* 2004).

1.14 GLOBAL STATUS OF *H. SALINARIUM* IN INDIA

- Other than Tamil Nadu, this is identified at 'Rock Garden' situated on sea shore of Malvan, Sindhudurga Dist., Maharashtra, India.
- The Lake Magadi, the Great Salt Lake, and any other body of water with a high salinity content are home to halobacterium species.
- *H. salinarium* are often found in places with high salt concentration like San Francisco Bay, the Great Salt Lake, Yellowstone National Park, and many other places with saline levels around 4M+(Oren., 1990).

CHAPTER 2

LITERATURE SURVEY

2.1 CELL STRUCTURE AND METABOLISM

The rod-shaped *halobacterium* species are encased in an S-layer composed of the glycoprotein found on the cell surface, which is encircled by a single lipid bilayer membrane. Under aerobic conditions, they thrive on amino acids. Despite having genes for both the enzymes involved in the fatty acid oxidation pathway and the breakdown of glucose, *Halobacterium* NRC-1 does not appear to be able to utilise both as energy sources. The cell uses numerous active transporters to maintain a high potassium content even if the cytoplasm maintains an osmotic balance with the hypersaline environment. Gas vesicles are proteinaceous organelles found in many species of *Halobacterium* (Sayer *et al.*, 2022).

2.2 ECOLOGY

The light-detecting pigment bacteriorhodopsin, which gives halobacterium its reddish colour and gives it chemical energy, is used to identify the archaeon in water bodies. A temperature of 37 °C has been found to be ideal for growth. One possible indicator of a life form on Mars is halobacterium. The harmful ultraviolet radiation on Mars is one of the issues with survival. A thin layer of salt that these microbes form can help to mitigate some UV radiation. The most prevalent salt is sodium chloride, and chloride salts block short-wave UV light. Because of its red hue, the longer-wavelength UV light is actually blocked out by the photosynthetic pigment called bacteriorhodopsin. Furthermore, bacterioruberin, which are pigments produced by Halobacterium, are believed to shield cells from UV light damage. Being able to develop at a low temperature for a potentially brief period of time, when a pool of water may be liquid, is the challenge they must overcome (Gontia-Mishra *et al.*, 2017).

2.3 SIGNIFICANCE

Eukaryotic-like RNA polymerase II and translational machinery that are related to *Escherichia coli* and other Gram-negative bacteria are used by the *Halobacterium* spp. NRC-1, according to genome sequencing of the species. Furthermore, they have genes that are comparable to those found in bacteria, yeasts, and bacteriophages for DNA replication, repair, and recombination. This type of *Halobacterium* is useful as a model organism in biological research because of its ease of culture and genetic modification (Kennedy *et al.*, 2000). Moreover, *Halobacterium* NRC-1 has also been used as a possible vaccine delivery vector. They specifically create gas vesicles that can be genetically modified to exhibit particular epitopes. The gas vesicles also show that they can act as organic adjuvants to support the induction of more robust immune responses. Since halobacteria need a high salt environment to thrive, making these gas vesicles is a cheap and effective process that simply requires tap water to isolate the bacteria (Stuart *et al.*, 2001). Additionally, the cell membranes of halobacteria include a protein called Bacteriorhodopsins, which is a light-driven proton pump. Because of its stability even in these harsh conditions, this protein has demonstrated potential for use in biotechnology, even though the majority of halophile proteins require high salt concentrations for correct structure and function. Because of their potential use in electronics and optics, bacteriorhodopsins that have been isolated from *Halobacterium salinarum* have received particular attention. Particularly, bacteriorhodopsins have been used in holographic storage, optical switching, motion detection, and nanotechnology. Despite the fact that this protein has been shown to have many functions, no large-scale commercial applications have been established as of yet (Oren *et al.*, 2010).

2.4 STRUCTURE

The solitary lipid bilayer membrane of each rod-shaped, about 2–5 μm long cell is encased in a glycoprotein cell wall. Phospholipids in the cell membranes of archaea are different from those in bacteria and eukaryotes. The flagellated cells of *Halobacterium* are able to move in the direction of light, particularly light in the yellow-

green region at a wavelength of around 560 nm, which is the maximal absorbance of the photosynthetic pigment (George.,2022).

2.5 MATTER AND ENERGY

Photoheterotrophs are halobacteria. They require the consumption of organic substances to develop, just like other heterotrophs, but they also require light energy to produce ATP. Photosynthetic creatures, on the other hand, are autotrophic; they produce food, or carbohydrates, to produce ATP, and use the remaining portion as building blocks. Regular heterotrophs consume in order to get resources for growth as well as energy, which is often obtained by cellular respiration. In the event that halobacteria are deprived of light, they will perform like "normal" heterotrophs, utilizing the food they absorb meet their energy requirements in addition to growing larger.

Bacteriorhodopsin, a kind of rhodopsin—the same pigment that our eyes use to see is the pigment that reacts to light. In both cases, the protein undergoes a conformational change due to light. This results in the transmission of a nerve impulse in our eyes; in *Halobacterium*, it causes protons to build up on one side of a membrane, and proton flow down the gradient that is formed permits the synthesis of ATP. Another protein pigment found in halobacterium, called halorhodopsin, is capable of pumping chloride ions into the cell using light energy, raising the concentration of the solute and limiting excessive water loss (George.,2022).

2.6 RETINAL PROTEINS OF *HALOBACTERIUM SALINARIUM*

Four photosynthetic pigments called retinal proteins which include a retinal chromophore and are involved in signal transduction and light energy conversion are found in *Halobacterium salinarium*. The four proteins found in the retina are:

- Bacteriorhodopsin (Haupts *et al.*,1999,Oesterhelt.,1998)
- a) The pigment used in photosynthetic reactions, which enables *Halobacterium* to grow only on light, functions as a light-driven proton pump, transforming light energy into a gradient of protons.

- b) There are various applications for the energy contained in the proton gradient, including as the production of ATP-by-ATP synthase.
- Halorhodopsin (Kolbe *et al.*, 2000)
- a) A light-driven chloride pump that enables *Halobacterium* to continue growing with a high concentration of salt within.
- Sensory rhodopsin I
- a) Participate in photo taxis, promotes the reaction of the photophile to orange light and the reaction of the photophobic to UV light, and forms a complex with the transducer protein Htr I.
- Sensory rhodopsin II (Oesterhelt.,1998)
- a) Participates in photo taxis, promotes the blue light-induced photophobic reaction, and forms a complex with the transducer protein Htr II.

2.7 ENERGY METABOLISM

Unlike the methanogens, which are their closest cousins, halophiles may grow in both aerobic and anaerobic environments. There are three different ways for halobacterium to obtain energy.

1. Different metabolites oxidation in aerobic environments

- a) Oxidizes pyruvate, which pyruvate-ferredoxin oxidoreductase channels into the acid cycle
- b) All five of the respiratory chain's main complexes are found in halobacterium.

2. Photosynthesis

- a) A proton gradient is produced by the light-driven proton pump bacteriorhodopsin. The energy of the proton gradient can be used by ATP synthase to propel ATP production.

3. Arginine fermentation (*Plaga et al.*, 1992).

- a) Through the fermentation of arginine, halobacterium produces ATP.

2.8 RESPONSE TO EXTERNAL STIMULI

A flagellated organism with (chemo)tactic behaviour is called halobacterium. It has the capacity to perceive oxygen (aero taxis), react to light (photo taxis), and identify necessary amino acids (chemotaxis) as well as osmotically active substances (osmo taxis). The receptor/transducer, which can consist of two different proteins or just one, is the first step in the signal transduction cascade. A two-component regulatory system made up of the response regulator cheY and the histidine kinase cheA forwards the signal to the flagellar motor switch. The signal is amplified and combined with other signals during relay (according to the principles of signal transduction).

Methyl transferase (cheR) and regulated methyl esterase (cheB) methylate and demethylate transducer proteins during adaptation. According to genome research, Halobacterium has 18 different transducers, which suggests that it can detect a wide variety of different stimuli. There are now seven identified transducers for these stimuli.

- Photo taxis involves two transducers:
 - a) Transducer protein Htr I in conjunction with its photoreceptor sensorhodopsin I, produces a photophilic reaction.
 - b) Transducer protein htrII in conjunction with its photoreceptor sensor hodopsin II, produces a photophobic response.
- In aero taxis, two transducers are involved:
 - a) Transducer protein hem AT in the aerophilic response and transducer protein htr VIII in the aero phobic response (Hou *et al.*, 2001).
- Chemotaxis toward amino acids involves two transducers:
 - a) Arg-specific chemotactic response: soluble transducer protein car (Storch *et al.*, 1999).
 - b) Periplasmic substrate binding protein bas B in conjunction with transducer protein bas T mediates chemotactic reaction towards Leu, Ile, Val, Met, and Cys (Kokoeva *et al.*, 2000, 2002)
- The chemotaxis of one transducer toward osmolytes:
 - a) Transducer protein cosT combined with periplasmic substrate binding protein cos B in the chemotactic response towards suitable osmolytes (Kokoeva *et al.*, 2000).

- Transducer mpcT is a sensor for membrane potential (Koch *et al.*, 2005)
 - a) This contributes to phototaxis mediated by BR.

2.9 GENOME ANALYSIS

Twice has the genome of *Halobacterium salinarium* been deciphered have sequenced the genome of strain R1 of *Halobacterium salinarium*. Ng *et al.*, have deciphered the genome of the *Halobacterium salinarium* strain NRC-1. The halobacterium possesses a 2 Mb chromosome with 68% GC and many mega plasmids with an average GC content of 58%. Mega plasmid counts for strain R1 are four, whereas strain NRC-1 is said to contain two. The whole genome of strain R1 encodes about 2,837 proteins (Oesterhelt *et al.*, 1998).

2.10 ADAPTATION OF H. SALINARIUM TO EXTREME CONDITIONS

2.10.1 High salt

This archaeon, like other halophilic Archaeal species, uses suitable solutes (potassium chloride, in particular) to lessen osmotic stress in order to thrive in excessively salted conditions (Pérez-Fillol *et al.*, 1986). Because the environment's potassium levels are out of balance, *H. salinarium* expresses a number of active transporters that bring potassium into the cell (Kennedy *et al.*, 2000). There will be protein precipitation at very high salinity levels. *H. salinarium* mostly encodes acidic proteins, which help to avoid the salting out of proteins. *H. salinarium* proteins have an average isoelectric point of 5.03 (Kozłowski, 2016). Even at high concentrations of salt, these extremely acidic proteins may stay in solution because they have a strong negative charge (Dassarma *et al.*, 2007).

2.10.2 Low oxygen and phototrophy

In salt ponds, *H. salinarium* can develop to such densities that oxygen is rapidly consumed. Despite being an obligatory aerobe, it uses light energy to thrive in low oxygen environments. Bacteriorhodopsin is a membrane protein that functions as a light-driven proton pump and is expressed by *H. salinarium* (Oesterhelt and Stoeckleins 1973). It is composed of two components: the light-sensitive cofactor, retinal, and the

7-transmembrane protein, bacterioopsin Retinal undergoes a conformational shift upon photon absorption, which also affects the bacterioopsin protein and promotes proton transport (Andersson *et al.*, 2009). ATP synthase can then employ the proton gradient that was created as a result to produce chemical energy. *H. salinarium* creates gas vesicles in order to get more oxygen, which enables them to float to the surface where there is more light and a higher oxygen content. These vesicles are intricate structures composed of proteins that are at least 14 genes' worth of encoding (Oren *et al.*, 1993). In *H. salinarium*, gas vesicles were first identified in 1967 (Larsen *et al.*, 1967).

2.10.3 UV protection and colour

Salt ponds offer minimal shade from the sun, excessive UV radiation exposure is commonplace for *H. salinarium*. They have developed an advanced DNA repair system in order to make up for it. DNA repair enzymes that are identical to those found in both bacteria and eukaryotes are encoded in the genome. Because of this, *H. salinarium* is far more UV-tolerant than other organisms and is able to repair DNA damage more quickly and effectively (Dassarma *et al.*, 2007).

Bacterioruberin, a 50-carbon carotenoid Alcohol (polyol) pigment found in the membrane of *Haemophilus salinarium*, is the main cause of its red hue. Bacterioruberin's main function in the cell is to shield DNA from UV radiation damage (Shahmohammadi *et al.*, 1998). However, the capacity of bacterioruberin to absorb UV light is not the cause of this protection. Bacterioruberin functions as an antioxidant to shield DNA instead of directly obstructing UV rays. By serving as a target, it can shield the cell from reactive oxygen species brought on by UV radiation. Since the generated bacterioruberin radical is less reactive than the original radical, it is likely to react with another radical to end the radical chain reaction (Saito *et al.*, 1997).

2.10.4 Protection against radiation and desiccation

Because of its polyploid nature (Soppa, 2011), *H. salinarium* is extremely resilient to desiccation and ionizing radiation, two factors that can cause double-strand breaks in DNA (Kottemann *et al.*, 2005). Complete chromosomes can be recreated by utilizing over-lapping fragments, despite the fact that they are first broken up into several pieces.

Regeneration is probably a type of homologous recombinational repair that is mediated by a DNA single-stranded binding protein (DeVeaux LC *et al.*, 2007).

2.11 APPLICATIONS IN VARIOUS SECTORS

2.11.1 Food Industry

Certain *Halobacterium* species may find application in the food sector (*Paterson et al.*, 2007). The synthesis of beta-carotene, a pigment that gives halophilic bacteria their red hue, is one example of an application. It is also utilized as a natural food colouring in the food business. Additionally, halophiles generate degradative enzymes that are employed in a variety of food processing techniques, including lipases, amylases, proteases, and xylanases. These enzymes have several notable uses, such as promoting the fermentation of salty foods, boosting bread dough quality, and aiding in the creation of coffee (*Paterson et al.*, 2007) (*Gontia-Mishra.,2017*).

2.11.2 Bioremediation

Exopolysaccharides (EPS), which are produced by a variety of halophilic bacterial species, are employed in the bioremediation industry. Numerous halophilic bacteria also emit bio surfactants, which are amphiphilic substances that have been applied to soil remediation. Due to their high tolerance for heavy metals, many halophiles have the potential to be helpful in the bioremediation of xenobiotics and heavy metals released into the environment as a result of metal plating and mining. Halophiles transform xenobiotics into less hazardous substances, which helps in the bioremediation of pollutants. It has been demonstrated that certain *Halobacterium* species are useful in the bioremediation of contaminants, including as aromatic hydrocarbons like 4-hydroxybenzoic acid, which is a contaminant in certain high salinity industrial runoffs, and aliphatic hydrocarbons like those present in crude oil (*Dassarma et al.*, 2007).

2.11.3 Pharmaceuticals

The potential medical uses of certain strains of Halobacterium, such as *Halobacterium salinarium*, are being investigated due to their radiation-resistance mechanisms. A carotenoid pigment called bacterioruberin is present in Halobacterium and reduces the sensitivity of the bacteria to UV and γ -radiation (Jung *et al.*, 2017). Studies on knockouts have demonstrated that the lack of bacterioruberin makes the bacteria more vulnerable to oxidative chemicals that damage DNA. For instance, hydrogen peroxide and bacterioruberin combine to reduce the oxidative capability of the DNA-damaging substance, protecting the bacterium by preventing the generation of reactive oxygen species (Shahmohammadi *et al.*, 1998). High intracellular concentrations of potassium chloride, which has previously been demonstrated to confer radiation resistance, are also present in *H. salinarium*. The medicinal uses of the bioactive molecules that halobacterium produce—such as anticancer drugs, antimicrobial bio surfactants, and antimicrobial metabolites—are also being investigated (Jung *et al.*, 2017).

In this project, objective were formed to isolate and characterize haloarcheal bacteria from Tuticorin and Marakkanam salt pans.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

The main aim of the project is to extract the protein bacteriorhodopsin and to characterize it.

3.2 OBJECTIVES

- To scale up to increase the yield of bacteriorhodopsin.
- To isolate and characterize the protein.
- To confirm the protein by using SDS PAGE.

CHAPTER 4

MATERIALS AND METHODS

4.1 PREPARATION OF MEDIA

4.1.1 Selection of Strains

The halophilic bacterial samples that were collected from various sites were isolated and inoculated in the MSM. The culture that was inoculated has been kept under continuous light condition in room temperature for 20 days. Absorbance is checked at 560nm and 660nm. Where, the presence of BR at 560nm and bacterial growth at 660nm can be identified (Ispirli *et al.*,2019).

4.1.2 Ms (Mineral Salt) Media (For 1000 ml)

Minimal saline medium is used for the inoculation of isolated strains. MSM is a cost effective medium than conventional Zobell marine medium. It was found to have nutrients required for the growth of bacteria than Marine broth medium.

Composition of MSM media is (for 1000ml):

Amino acid of 7.5 g, Yeast extract of 1g, Sodium citrate of 3g, Sodium chloride of 250g, Magnesium sulphate 20g, Potassium chloride 2g, Ferrous sulphate of 0.05g and Manganese sulphate of 0.2g are added and made up to 1000ml.

The pH of the media was checked and maintained to 7.2 to 7.4 (Zeng.,2006).

4.2 HARVESTING

- Well grown broth was harvested by centrifugation at 10,000 RPM for 15mins at 4°C.
- Then the pellet was washed twice with 5M NaCl and Distilled Water to remove the lipids.
- The pellet was stored at 4°C for further analysis (Calo *et al.*,1995).

4.3 EXTRACTION OF PROTEIN

The protein was isolated by following three different methods to compare the better yield:

Freeze-thaw method

Bead-mixing method

Aqueous-two phase

4.3.1 Freeze-thaw method

Harvested pellet was freezed at -20 °C for 60 mins. Then, thawed it by resuspending the pellet in Tris-HCl buffer, mixed it by shaking it in a horizontal shaker for 1hr to get suspension. Then the suspension was freezed at - 20°C for 30 min. Suspension was thawed at room temperature and centrifuged at 10, 000 RPM for 10 min at 10 °C. Pellet was resuspended in PBS buffer & stored for further use (*Anthony C. Grabski 2009*).

4.3.2 Bead-mixing method

Harvested pellet was freezed at - 20 °C for 30 minutes. After 30 minutes, the pellet was thawed at room temperature and 1g of glass beads was added and mixed well by vortexing for 10 minutes, then the supernatant was removed. Then the suspension was centrifuged at 12,000RPM for 10 min, and the supernatant was removed. Then the pellet was dissolved in 1ml deionized water and 60µl of CHAPS was added, well mixed and stored at 4 °C for 1 hr. Then the suspension was thawed and centrifuged at 12,000RPM for 10 minutes. The supernatant was collected and centrifuged at 24,000RPM for 30 minutes and the pellet was collected and suspended in PBS buffer and stored at 4 °C for further use.

4.3.3 Aqueous-two phase

Pellet was suspended in 1.5ml of distilled water, then 68.7µl of DNase solution was added and mixed thoroughly for 30 minutes at orbital shaker. Then 10ml of 24% potassium phosphate solution and 10ml of 24% PEG solution was added and mixed for 1 hour to get suspension. Then the suspension was centrifuged at 8000 rpm at 4°C for 15 min, then the pellet was collected and suspended in deionized water. Suspension

was centrifuged at 4000RPM, and the supernatant was collected. Then the supernatant was centrifuged at 24,000RPM for 30 minutes at 4°C. The pellet was collected and washed with distilled water several times and stored in PBS buffer (Szundi and Stoeckenius.,1987).

4.4 CALCULATION OF TOTAL BIOMASS

Total cell weight is measured to check the yield of biomass present in the sample (Ispirli *et al.*,2019).

$$\text{Biomass cell weight} = \frac{\text{Weight of the tube with pellet} - \text{Weight of empty tube}}{\text{Volume of the sample}}$$

4.5 ESTIMATION OF PROTEIN

Bradford assay was carried out to estimate the protein present in the sample. Standard with seven concentrations of BSA S10, S20, S40, S80, S160, S320, S640 was prepared and absorbance was checked at 595nm by using UV spectrophotometer. (UV-1800, Shimadzu,Japan).

Linear graph was plotted and Coefficient of Determination R² value was found to check how much deviation has occurred. Using the slope equation, $y = mx + b$, the amount of protein present in a sample was found.

4.5.1 Preparation of Bradford reagent

Coomassie brilliant blue	-	10mg
Orthophosphoric acid	-	10ml
Ethanol (95%)	-	5ml

Left for overnight stirring.

4.5.2 Preparation of Bradford assay

Standard for Bradford assay was prepared by using bovine serum albumin (BSA), distilled water and Bradford reagent in the given concentrations.

TABLE 4.1 Standard for Bradford assay

	BSA (μl)	DW(μl)	Bradford reagent (ml)
S10	10	990	5
S20	20	980	5
S40	40	960	5
S80	80	920	5
S160	160	840	5
S320	320	680	5
S640	640	360	5
Blank	-	1	5

4.6 CHARACTERIZATION OF PROTEIN

Samples of 500 μ l were taken for characterization purpose by using FTIR (Cary 630, Agilent).

Protein is characterized using FTIR (Fourier Transform Infrared Spectroscopy). Analysing the peak position will help us to know the presence of bacteriorhodopsin.

4.7 QUANTIFICATION OF PROTEIN

- The hydroxylamine method was employed to quantify the bR content in the samples.
- To a 0.6mL sample, 60 μ l pH 7, 2 M hydroxylamine solution was added and the absorbance's at 568nm was immediately measured for bR, respectively.

The bleaching reaction of bR with hydroxylamine was carried out for 24h under illumination (40W) and the absorbance difference was employed to calculate the bR concentration based on molar extinction coefficient of 63,000 M⁻¹CM⁻¹. (Shand and Betlach., 1991).

$$\text{Bacteriorhodopsin concentration (mg/mL)} = \frac{26,800 \times A^{0\ 568} - A^{24\ 568}}{63,000}$$

where 26,800 is the molecular weight of bR.

4.8 CONFIRMATION OF THE PRESENCE OF PROTEIN

Presence of a certain desired protein can be conformed based on their size, charge, affinity towards a particular substrate, molecular weight. Bacteriorhodopsin is said to have a molecular weight of 27kDa. With this as a crutch to conform the presence of bacteriorhodopsin, the process is done by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as per the all-inclusive description mentioned below.

- In a clean beaker , 30g of acrylamide and 0.8g of methylene bisacrylamide were dissolved in 70ml of distilled water to make 100ml of acrylamide (30% stock). kept it at 4°C in amber bottles.
- In order to make the resolving gel, dissolved 18.17 g of tris base in 70ml of distilled water, set pH to 8.8, and then make up to 100ml.
- To prepare the stacking gel buffer (0.5 M tris HCL), dissolved 6.05g of tris base in 70ml of distilled water, adjust the pH to 6.8, and then blend the mixture to a volume of 100ml.
- Dissolved 3g tris base, 14.4g glycine and 1g SDS in 1000ml of distilled water (pH should be 8.3) to prepare electrophoresis tank buffer.
- APS (AMMONIUM PERSULFATE)- Dissolved 100mg of APS in 1ml of double distilled water.
- SDS (SODIUM DODECYL SULPHATE) 10% - Dissolved 2.5g of SDS in 25ml of distilled water.

For SDS sample buffer ,

5mg of bromophenol blue,15mg SDS,1ml of glycerol,1.25ml of stacking gel buffer and 0.5ml of 2 mercaptoethanol in glass beaker was added and mixed well and made up to 10ml.

TABLE 4.2 Preparation of Stacking gel

STACKING GEL	15%
Distilled water	2.93 μ L
Acrylamide	0.69 μ L
0.5M Tris HCL	1.3 μ L
10% SDS	50 μ L
10% APS	50 μ L
TEMED	10 μ L

TABLE 4.3 Preparation of Resolving gel

RESOLVING GEL	12%
Distilled water	3.2ml
Acrylamide	4.0ml
1.5M Tris HCL	2.6ml
10% SDS	100 μ l
10% APS	100 μ l
TEMED	10 μ l

Staining solution

Coomassie Brilliant Blue [R-250]	-	250mg
Methanol	-	50ml
Acetic acid	-	7ml
Distilled water	-	43ml

Destaining solution

Methanol	-	30ml
Acetic acid	-	7ml
Distilled water	-	63ml

Storing buffer

Acetic acid	-	7ml
Distilled water	-	93ml

CHAPTER 5

RESULTS AND DISCUSSION

5.1 SCALE UP



Fig 5.1: Sub-Culturing using MS media

Strains were taken from Marakanam and Tuticorin .MS (Mineral salt) media is used to cultivate halophilic bacteria which has a higher salt content that is conducive to their growth. The bacterium is transferred into the freshly prepared media under sterile conditions. Incubation was carried out for 15 days for its growth under 1500 Lux. The growth of the bacteria is good at 25% NaCl, so it is similar to the related publication (Zeng.,2006).

5.2 HARVESTING THE BROTH

Well, grown bacterial cells were taken and harvested by using centrifugation process and later it was washed twice with NaCl and the pellet was collected which is used for further protein isolation process. Harvest is done as mentioned in this publication (Calo *et al.*,1995) and the pellet was collected.



Fig 5.2: Harvesting grown bacterial cells

5.3 ISOLATION OF PROTEIN



***Fig 5.3: Isolated protein using
(i) freeze-thaw method,
(ii) bead mix method and
iii) aqueous two phase method***

Protein was isolated using three methods in which out of three methods, freeze-thaw had shown the best result by following this methodology (*Anthony C. Grabski 2009*). This method entails freezing a cell suspension in a freezer, and then thawing the material at 37°C or room temperature. As ice crystals form during the freezing process and then contract during the thawing, this lysis approach causes cells to expand and finally rupture.

5.4 ABSORBANCE at 600 & 660 nm

Table 5.1: Absorbance for SM1 strain at both 600 and 660nm.

Purpose	Wavelength	Absorbance
Bacterial growth	600 nm	1.628
Bacteriorhodopsin	660 nm	1.51

Table 5.2: Absorbance for TMR2 strain at both 600 and 660nm.

Purpose	Wavelength	Absorbance
Bacterial growth	600 nm	0.241
Bacteriorhodopsin	660 nm	0.243

Table 5.3: Absorbance for TMR1 strain at both 600 and 660nm.

Purpose	Wavelength	Absorbance
Bacterial growth	600 nm	0.169
Bacteriorhodopsin	660 nm	0.204

To identify the bacterial growth, absorbance was taken at both 600 and 660 nm as mentioned in this article (*Ispirli et al.,2019*). Measuring at 600 nm (or 660 nm) minimizes the absorbance of the media and corresponds to the reflected and dispersed light interactions of the bacteria because the bacteria are suspended in the media rather

than dissolved in it. Determines which portion of the light passes through a sample, more specifically through a suspension of microorganisms. SM1 has shown the best absorbance at 600 and 660nm which indicates a good bacterial growth.

5.5 CALCULATION OF TOTAL BIOMASS

Total cell weight is measured to check the yield of biomass present in the sample

Table 5.4: Biomass results for SM1, TMR1 and TMR2 strains

Samples	Biomass
SM 1	0.015g/ml
TMR 1	0.005g/ml
TMR 2	0.012g/ml

Calculated by measuring the weight of the pellet along with the tube which gets subtracted by the weight of an empty tube divided by its volume. Out of the three strains, SM1 shown a better biomass.

5.6 ESTIMATION OF PROTEIN

Table 5.5: Reading at 595nm for Bradford assay Standard

Concentration	Absorbance
0	0.178
10	0.238
20	0.333
40	0.385
80	0.411
160	0.5
320	0.59
640	0.686

Protein estimation is done by using Bradford assay where the standard is first prepared for that by using different concentrations (Dr. Mahesha).

5.6.1 Standard graph for Bradford assay

Standard graph is used to quantify the protein in an unknown sample by comparing its measurement to the standard curve and we got the coefficient of determination as 0.9849.

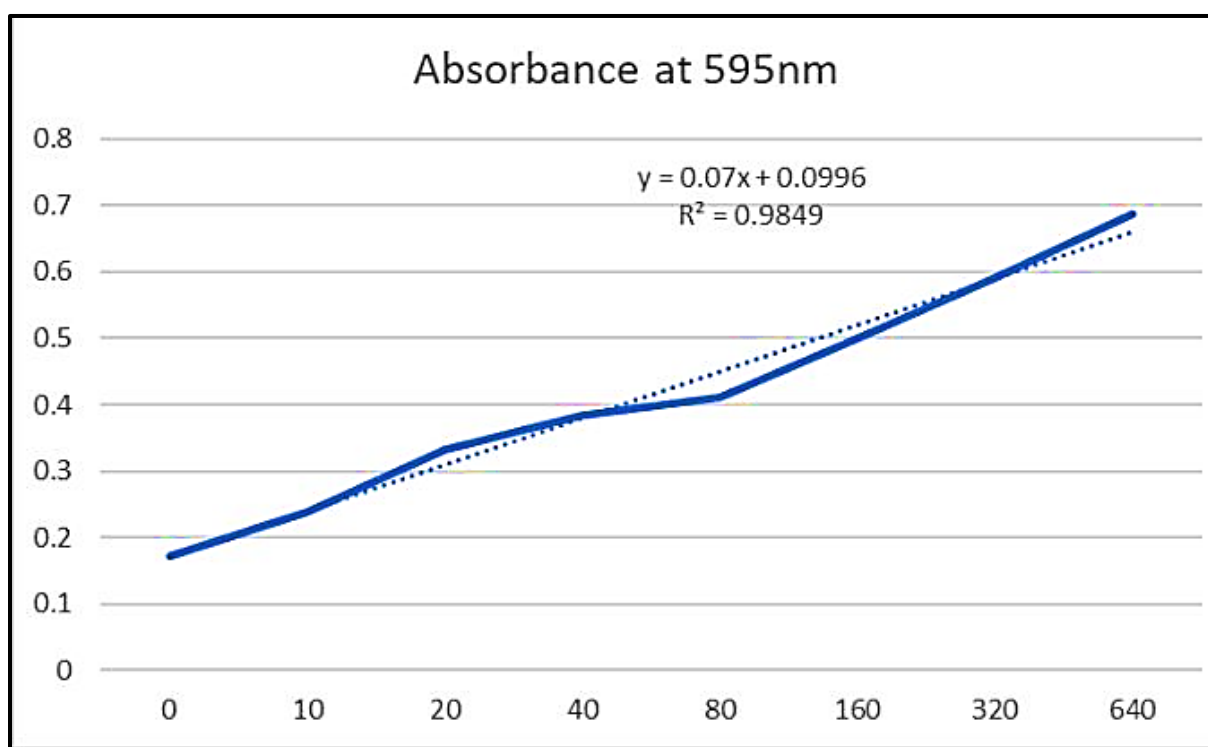


Fig 5.4: Standard graph curve

5.6.2 Sample calculation

Protein of 50µl was taken and added 150µl of reagent to it, later it was placed in 96 well plate and took reading at 595nm. This method is used to estimate the protein in the sample.

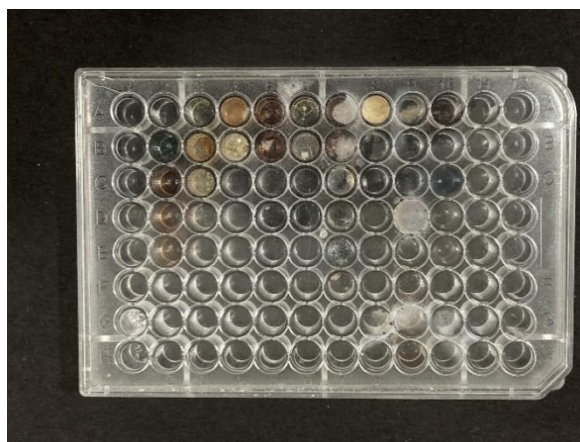


Fig 5.5: Estimation of protein

Table 5.6: Estimation of protein results for SM1, TMR1, TMR2 strains

Strains	Freeze thaw method	Bead mix method	Aqueous - two phase method
SM 1	17.86 $\mu\text{g/ml}$	9.72 $\mu\text{g/ml}$	2.705 $\mu\text{g/ml}$
TMR 1	12.7 $\mu\text{g/m}$	8.24 $\mu\text{g/ml}$	2.477 $\mu\text{g/ml}$
TMR 2	10.22 $\mu\text{g/ml}$	9.67 $\mu\text{g/ml}$	7.14 $\mu\text{g/ml}$

The protein samples isolated by using all the three protein extraction methods namely, freeze thaw, bead mix and aqueous two phase were estimated using Bradford assay and the values were mentioned. Among SM1, TMR1 and TMR2, best result is shown in SM1 strain and out of three methods used, freeze thaw gave the better yield.

5.7 QUANTIFICATION OF PROTEIN

5.7.1 Sample calculation

For quantification, hydroxylamine method is used in which 2M hydroxylamine is used, it was mixed with the sample and absorbance was measured at 568. Bleaching of the mixture was carried under illumination (40W) for 24 hours. Absorbance was checked at 568nm after bleaching reaction. The absorbance difference was employed to

calculate the concentration of BR. Later the values were substituted in the formula which quantifies the total protein in the given sample (Shand and Betlach.,1991).

Table 5.7: Quantification of protein results for SM1, TMR1, TMR2 strains

Strains	Freeze thaw method	Bead mix
SM 1	1.23µg/ml	0.131 µg/ml
TMR 1	0.116 µg/ml	0.0029 µg/ml
TMR 2	0.049 µg/ml	0.009 µg/ml

5.8 CHARACTERIZATION OF PROTEIN BY FTIR

5.8.1 Strain-SM1

FTIR spectra of bacteriorhodopsin from SM1 strain:

SM1 strain has first peak picking at position 3340.1 with the intensity of 48.9941 which indicates stretching vibrations of O-H bonds. Next peak picking at 2364.3, 2118.42 with the intensity of 98.2319, 97.2542 respectively which shows proteins of bacteriorhodopsin. Next picking at 1637.27 with intensity of 72.327 lack of strong O-H bond. Position at 1076.08, with the intensity of 92.2964 lack of strong O-H bond.

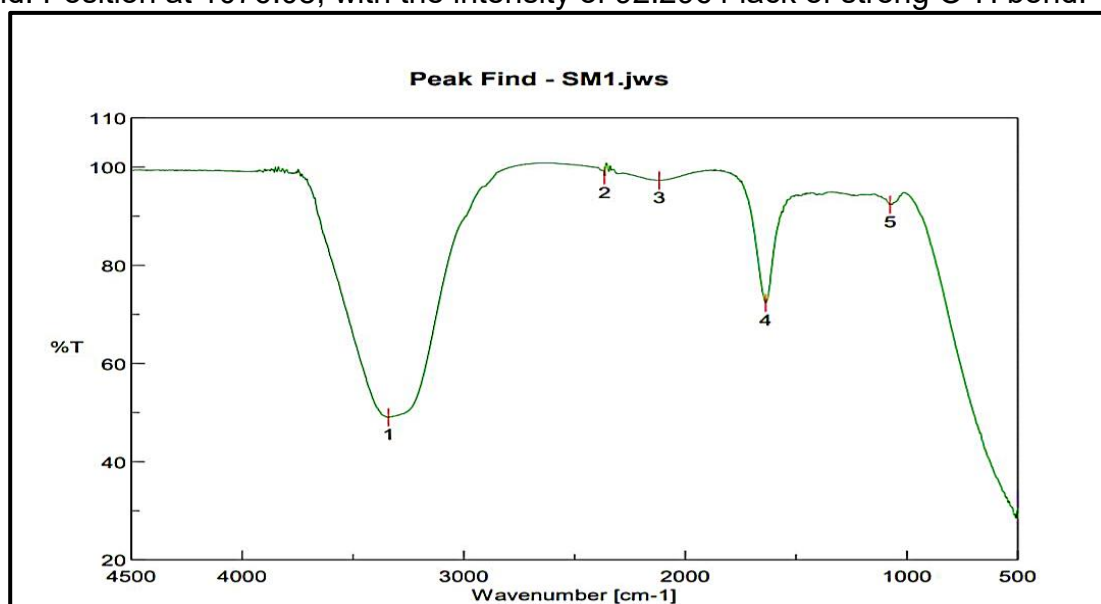


Fig 5.6: FTIR characterization results for SM1 strain

5.8.2 Strain- TMR1

FTIR spectra of bacteriorhodopsin from TMR1 strain:

Position at 3338.18 with the intensity of 49.4004 indicates stretching vibrations of O-H bonds. Peak position at 2364.3 shows intensity of 98.2607 indicates the presence of mutant proteins of bacteriorhodopsin. Next peak picking at 2118.42 with intensity of 97.2733 shows mutant proteins of bacteriorhodopsin. Peak at 1637.27 shows 72.6777 range of intensity lack of strong O-H bond. 1076.08 peak position shows 92.9621 range of frequency lack of strong O-H bond. Final peak at 504.294 shows intensity of range 30.4235 which is a kind of error raised due to impurity.

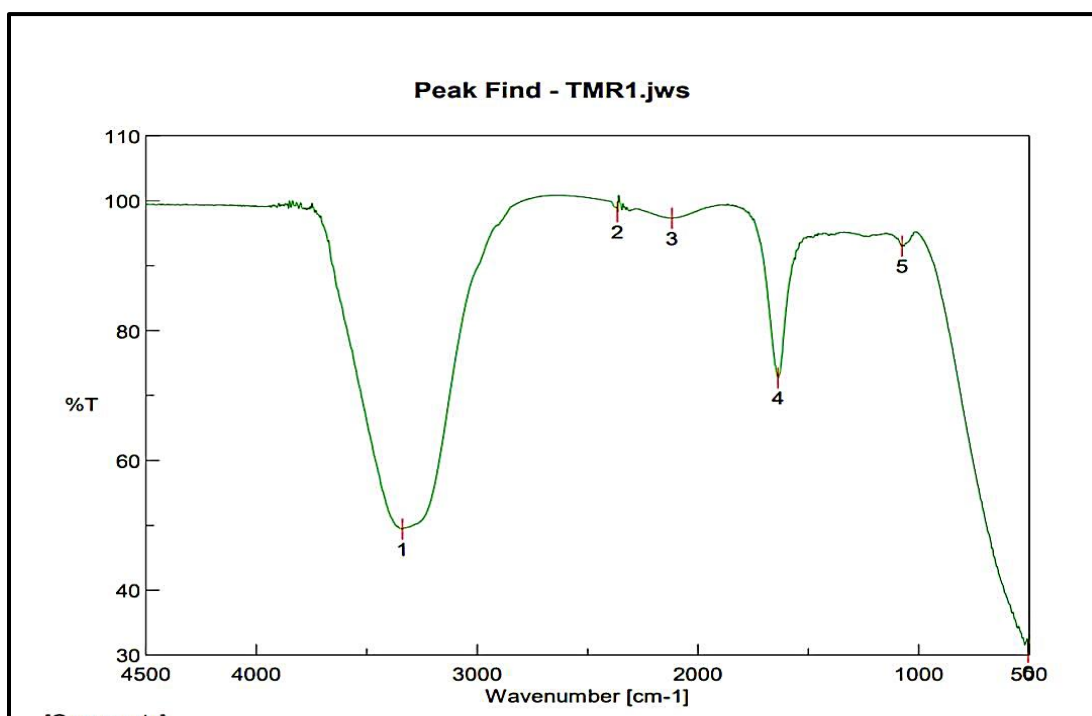


Fig 5.7: FTIR characterization results for SM1 strain

5.8.3 Strain- TMR2

FTIR spectra of bacteriorhodopsin from TMR2 strain:

Position at 3340.1 with an intensity of 48.5557 indicates stretching vibrations of O-H bonds. Next peak at 2120.35 of intensity range 97.0363 shows mutant proteins of bacteriorhodopsin. Peak at 1637.27, 1066.44 shows intensity of 71.9829, 91.9097 respectively lack of strong O-H bond.

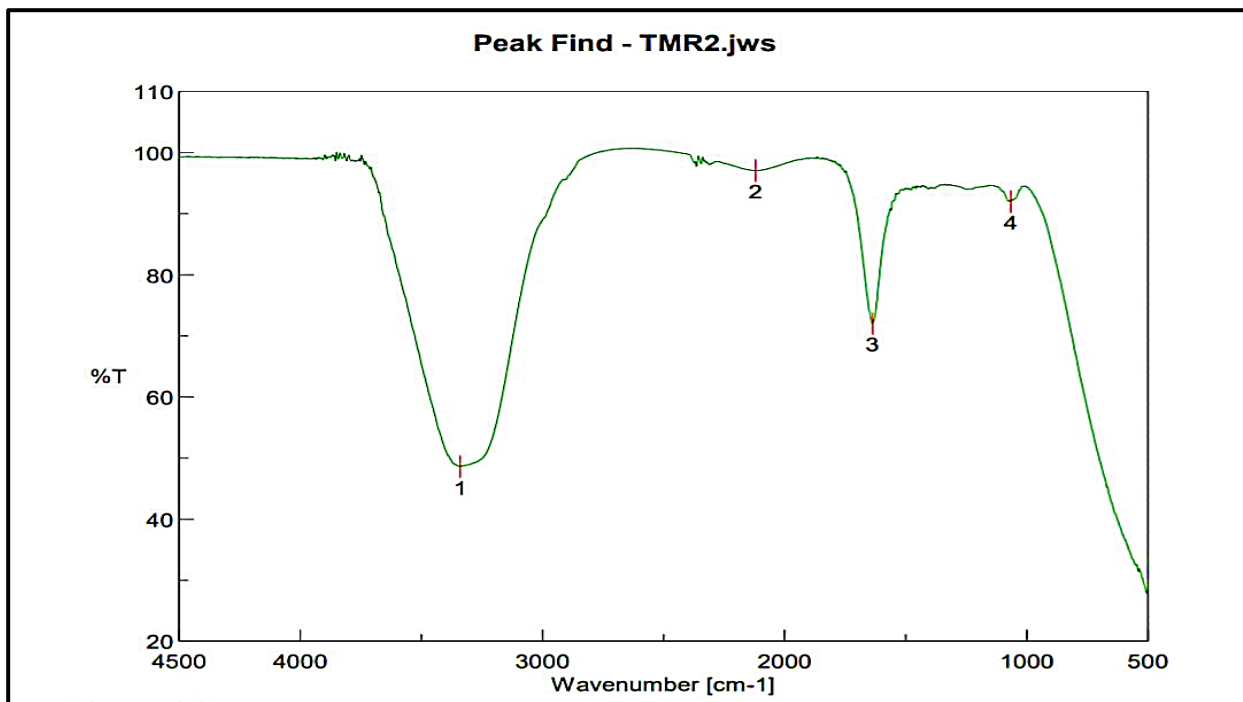


Fig 5.8: FTIR characterization results for TMR2 strain

Using spectroscopy, the Fourier transform infrared (FTIR) approach can identify changes in functional groups, which in turn can reveal changes in the overall composition of biomolecules. FTIR is used to quantify the vibration and rotation of molecules that are affected by infrared radiation at a specific wavelength.

Using FTIR characterisation we can detect the presence of bacteriorhodopsin in our sample. Peak at certain wavelengths signify the presence of bacteriorhodopsin. A water molecule has two O-H groups, and their frequencies are distributed in the wide 3700-2700 cm^{-1} region dependent on their coupling and hydrogen-bonding strength. The QM/MM calculations resulted in the O-D stretches at 2257 and 2423 cm^{-1} for BR and K, respectively. We found that BR and pharaonis phoborhodopsin, both of which pump protons, possess such water molecules (O-D stretch at $<2400 \text{ cm}^{-1}$) (Eisenberg, et al 1969).

5.9 CONFIRMATION OF PROTEIN by SDS-PAGE SDS PAGE

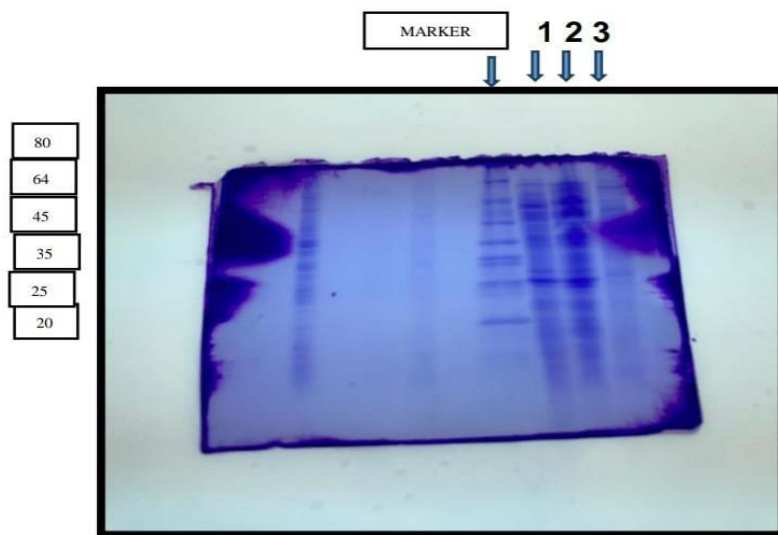


Fig 5.9.1: Confirmation test to check Bacteriorhodopsin by SDS-PAGE SDS PAGE

Well1: TMR2

Well2: TMR 1

Well3: SM1

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli. (After heating the sample at 90° for 5 min, the molecular mass of the sample was determined in a 12% cross-linked polyacrylamide gel containing 10% SDS. The presence of certain desired protein can be confirmed based on their size, charge, molecular weight so the samples should obtain a band between 25kDa - 35kDa as this molecular weight depicts the presence of bacteriorhodopsin. In our sample study, most of the strains such as TMR2, TMR1 and SM1 strains have obtained sharp band between the range of 25kDa - 35kDa which confirm the presence of bacteriorhodopsin.

CHAPTER 6

SUMMARY AND CONCLUSION

Halobacterium salinarium which belongs to domain Archaea has shown a wide range of applications. The Tuticorin and Marakanam strains were selected and grown in MS (Mineral Saline) media, in which 25% percentage of sodium chloride was used as it is a halophilic organism. *Halobacterium* genus were isolated from areas like Marakanam, Vedaranyam, Tuticorin. The isolates shown the best growth at pH 7.2.

Extractions process like freeze thaw, bead mix and aqueous two phase were used. Freeze thaw has shown the better yield. Later protein estimation and quantification was done by using the protein which we extracted from various extraction methods. Protein estimation is done by using Bradford assay and taking absorbance at 595 nm which showed the yield of 17.86µg/ml for SM1 strain and 12.7µg/ml for TMR1 and for TMR2 10.227µg/ml Hydroxylamine is used to quantify the protein by taking absorbance at 568nm and bleaching reaction is allowed to happen by leaving it in illumination chamber for about 24 hours and again reading was taken at same wavelength out of which SM1 has shown the best result by showing 1.23µg/ml followed by TMR2 which showed 0.116 µg/ml and the least TMR2 of 0.049µg/ml.

Meanwhile the growth is monitored using UV spectrophotometer by taking absorbance at 600 and 660nm. Biomass is also calculated for all the three strains used SM1, TMR1 gave yield of 0.015g/ml, 0.005g/ml respectively and TMR2 showed 0.012g/ml yield. FTIR characterisation is done to observe the peak which indicates the presence of bacteriorhodopsin between 2380 cm⁻¹ to 2000 cm⁻¹. SM1 strain showed peak at 2364.3 cm⁻¹ and 2118.42 cm⁻¹, TMR 1 at 2118.42 and 2364 cm⁻¹ and TMR 2 at 2120.35 cm⁻¹ which showed the presence of bacteriorhodopsin SDS PAGE was performed to confirm the presence of bacteriorhodopsin. Band between 25KDa-37KDa confirms the presence of bacteriorhodopsin.

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