

PIGMENT EXTRACTION AND CHARACTERIZATION FROM HALOPHILIC BACTERIA

Submitted in partial fulfillment of the requirements for the award of Bachelor of
Technology degree in Biotechnology

By

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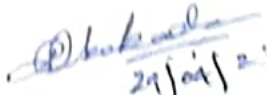
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This is to certify that this Project Report is the bonafide work of **NANDA KUMAR R (39230028)**, **VALLABA M (39230055)**, who carried out the project entitled "**SCREENING OF MARINE ACTINOMYCETES FOR NATURAL PRODUCTS AND ITS POTENTIAL APPLICATION IN MARINE ANTI-BIOFILM ACTIVITY**" under my supervision from September 2022 to April 2023.


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ABSTRACT

Haloarchaea from high saline habitats are known to contain industrially important value-added bioactive compounds such as bacteriorhodopsin, enzymes, carotenoids, lipids, and proteins. The diversity of Haloarchaea from geographically less explored saline habitats has received less attention. In this study isolation and characterization of the biotechnological potential of novel haloarchaea strains from solar saltern, a hypersaline environment in Marakanam, Tamilnadu was carried out. Bacteriorhodopsin (BR) is an integral purple membrane protein found in "cellular membrane" (the Archaea cell membrane) mainly in Halobacteria. This protein absorbs green light (wavelength 500-650 nm, with the absorption maximum at 568 nm) and converts it into an electrochemical gradient. This gradient in turn is used for ATP production.

The isolates of SM1 and M4 strain were taken. Extraction and characterization of bacteriorhodopsin from this strain has been done use FTIR and Raman spectroscopy. Molecular characterization of these two strains that is SM1 and M4 has been done individually. The ability of BR to convert light energy into chemical energy or sunlight into electricity has been used in different applications mainly optical appliances but also for therapeutic/medical applications and research.

Keywords – Halophilic bacteria, pigment extraction, salt-rich, salinity, purple membrane, characterization

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LIST OF ABBREVIATIONS

1. ATP - Adenosine triphosphate
2. ADP - Adenosine di-phosphate
3. NMR - Nuclear Magnetic Resonance
4. K - Potassium
5. Na - Sodium
6. Cl - Calcium
7. BR - Bacteriorhodopsin
8. CHAPS - 3-((3-cholamidopropyl) dimethylammonio)
-1- propanesulfonate
9. EDTA - Ethylenediaminetetraacetic acid
10. FTIR - Fourier Transform Infrared
11. g - Grams
12. HCl - Hydrochloric acid
13. ml - Millilitres
14. mM - Millimolar
15. NaCl - Sodium chloride
16. nm - Nano meter
17. PBS - Phosphate buffer saline
18. RNA - Ribo nucleic acid
19. R^2 - Coefficient of determination
20. UV - Ultraviolet

- 21. μl - microlitre
- 22. μg - Micrograms
- 23. $^{\circ}\text{C}$ - Celsius
- 24. % - Percentage

CHAPTER 1

1. INTRODUCTION

The most prevalent natural pigments are lycopene (red), beta carotene (orange), and chlorophyll (green) (the red color). The essential elements of human life, pigments are linked to nearly every item utilised in society today. The majority of synthetic pigments are non-biodegradable and harmful (Sahoo *et al.*). Due to the drawbacks of synthetic pigments, natural pigments—found in plants, insects, and microbes—need to be looked as an alternative

The second-largest (Ryu *et al.*,1998, Mizukami *et al.*,1978) source of natural colour, behind plants, is microbes. These pigments work as antioxidants in humans and exhibit pro-vitamin A activity that guards against lifestyle-related illnesses like cardiovascular disease and age-related muscle degeneration (Young and Lowe, 2001). Proteins are specifically linked to translocation ions and molecules, regardless of whether they are such proteins act as channels and carriers in the membrane either receptors. Such a membrane one of the most interesting and extensively explored, undoubtedly is bacteriorhodopsin (Fegina *et al.*,1979).

Bacteriorhodopsin, the sole protein of the purple membrane of halophilic bacteria that can survive in harsh environments and efficiently use light energy to carry out their essential functions (Esterhelt, 1976) has been discovered to act as a light-driven proton pump, creating a significant pH gradient in the membrane that is used in ATP generation (acker and Stoeckeniiis,1974).

Halophilic bacteria are inherently unlikely, organisms that can grow in high or saturated salt concentrations or require them fascinate biologists. Bacteria are a real problem since they grow slowly and produce salt. Their diets are complicated and most likely ill-defined (Kushner, 1968). Applications for BR's capacity to transform light energy into chemical energy include optical electronic devices, chemical sensors, and medication delivery (Puthenveetil and Vinogradova, 2013). Extreme halophiles' environments are typically dominated by archaea with considerable adaptability (Luque *et al.*, 2012)

Bacteriorhodopsin is one of the most useful by products of halophilic archaea. It has a special ability to absorb light, which makes it useful for biosensor applications. *Halobacterium salinarum* is the archaea most thoroughly studied for generating BR. For this microbe to survive, high light, high salt, and greater temperature are optimum (Lee *et al.*, 1998). Because *Halobacterium salinarum* is difficult to culture, a lot of research has been done in recent years to reduce production costs and boost BR production output (Kalenov *et al.*, 2016 and Shiu *et al.*, 2014). Even while these programmes have contributed to the manufacture of BR to some level, reducing costs is still a primary concern (Lu *et al.*, (2015) Peck *et al.*, (2001)

In the early stages of bR research, researchers were more interested in the structure and biochemical reaction process of bR than its application (Frydrych *et al.*, 2000). Following that, bR-related artificial retinal prostheses were initially proposed due to the retina's and bR's similar optical characteristics. Then, bR was utilised in the development of sensors and computer systems, resulting in the creation of optical volumetric memory (Fisher *et al.*, 2003, Imhof *et al.*, 2004 and Yu *et al.*,) processors with holographic associations (Hillebrecht *et al.*, 2005) early in the new millennium, and motion biosensors (Okada *et al.*, 2016, Wang *et al.*, 2005 and Ahmadi *et al.*, 2011). The spectral range of detectors related to bR was expanded in 2011. Radiation detection is now possible with the development of an X-ray sensor based on bR by Ahmadi *et al.*, with a detecting region on a flexible substrate with a radius of $r = 3$ mm (Oren, 2001)

In this case, bacteriorhodopsin-producing haloarchaea from Marakanam, Tamil Nadu were isolated and grown to examine the cultivable diversity of haloarchaea species and the yield of bR production. Another potential application of bR is in the development of bioelectronic devices. Bacteriorhodopsin can be incorporated into lipid bilayer membranes and used as a photoactive component in bioelectronic devices. These devices could be used for a range of sensing and energy harvesting applications, including solar cells, light sensors, and pumps for microfluidic systems.

In summary, bacteriorhodopsin is a photoactive protein that has potential applications in optogenetics and bioelectronic devices. Its ability to convert light energy into electrical energy makes it a promising component for a range of sensing and energy harvesting applications. Ongoing research in this area is likely to lead to new innovations and applications for this versatile protein

CHAPTER 2

2. LITERATURE SURVEY

2.1 HALOPHILES

Hypersaline conditions are home to species known as halophiles, which love salt. Prokaryotic and eukaryotic microorganisms with the ability to manage environmental osmotic pressure and withstand the denaturing effects of high salt concentrations make up the majority of the group. Typically, osmosis causes organisms that live in salty environments to lose water and perish. Halophiles need their cytoplasm to be isotonic with their surroundings in order to thrive in salt-rich environments (Santos, 2002). They employ two distinct strategies to get there. To endure osmotic stress, the first type of organism (mostly bacteria, some archaea, yeasts, algae, and fungi) stores organic molecules in its cytoplasm (Fraser *et al.*, 2010).

Neutral amino acids and sugars are the solutes that are most frequently utilised in this process (Stolp, 1998). This approach has a significant drawback in that it makes the organism expend a lot of energy. The second, less frequent adaptation to salt, involves the cytoplasmic uptake of potassium (K^+) ions on a selective basis. In exchange, the organism uses the sodium-potassium pump to expel sodium (Na^+) ions (Cavicchioli *et al.*, 2007). However, sodium ions are employed less frequently than potassium ions (Oren, 2002).

Only one family of Archaea and one order of bacteria exploit this adaption (Kato, 1977). This method has the benefit of using less energy than the adaptation stated above. The fundamental drawback of this strategy is that it requires more work than using suitable solutes because it requires adapting all of the cell's machinery (enzymes, structural proteins, etc.) to high amounts of non-organic ions and salt. Though a few halophiles are capable of doing both, the majority of halophiles only employ one of these two strategies.

2.2 HABITATS OF HALOPHILES

2.2.1 SALTURNS

Saltern systems around the world have evolved into favoured model systems for the explanation of the microbial diversity at various salt concentrations due to their high community densities and the (at least relatively) simple community structure of their biota. Both cultivation -

independent approaches have been used in such research. The bacterial community associated with brine shrimps (*Artemia spp.*) and the community composition within the gypsum crust have both been studied using 16s Rrna gene library- based cultivation – independent approaches (Lee *et al.*,1998)

2.2.2 SALT LAKES

Extremophilic halophilic microbes supply the system with energy and nutrients. Communities comprising bacteria, archaea, phytoplankton, protists and fungi are all part of the microbial diversity; the latter is the subject of new information provided here. We examine the biogeochemistry of microbialites as an illustration of complex microbial communities cooperating in the lake. The Great Salt Lake serves as a metaphor for both the limitations of life on earth and the possibilities for life outside in space. Halophilic cells and their molecules in brine fluid inclusions are protected by the lake's minerals, haite and the gypsum which are present on the shoreline in the sediment and in the nearby evaporite deposits (Li and Jin, 2010).

2.3 PIGMENTS OF HALOPHILES

The pigment of bacteriorhodopsin acts as a green light driven photon pump of wavelength 500 – 650 nm that converts light energy into chemical energy. This converted light driven energy is utilised by halobacterium for the production of ATP, amino acid uptake and locomotion (Oesterhelt and Hess 1973; Oesterhelt 1976; Stoeckenius and Bogomolni 1982).

Bacterioruberin is a carotenoid pigment produced by halophilic archaea such as *Halobacterium salinarum* strains NRC-1 and R1, *Halorubrum sodomense*, *Haloarcula vallismortis*, *Haloarcula japonica*, *Halococcus morrhuae*, *Haloferax volcanii* etc. (Mandelli *et al.*, 2012; Jehlicka 2013; Yatsunami *et al.*, 2014).

The bacterioruberin is synthesized with geranyl geranyl phosphate as the initial precursor and the pathway deviates from lycopene to monoanhydrobacterioruberin, bisanhydrobacterioruberin finally giving the product bacterioruberin. The pigment bacterioruberin acts as antioxidant and during the recent years it has found to be an integral part of ion channel protein of archaea halorhodopsin and archaeorhodopsin. The synthesis of bacterioruberin by *Halobacterium salinarum* was found to be initiated from Geranyl geranyl phosphate and during the formation of lycopene the pathway gets divided into two where lycopene gets converted to retinal,

bacterioopsin and bacteriorhodopsin or it gets converted to monohydrobacterioruberin, bisnahydrobacterioruberin finally producing bacterioruberin. The pigment bacterioruberin is now been explored for its application in photovoltaics and optoelectronic (Ronnekleiv *et al.*, 1995; Bidle *et al.*, 2007).

2.4 ISOLATION OF HALOPHILES

Halophiles are microorganisms that are able to grow and thrive in high salt concentrations. They have unique adaptations that enable them to survive and function under these extreme conditions, making them of significant scientific and industrial interest. The extraction of halophiles from various environments is crucial for the study of their physiology, genetics, and potential application

There are several methods for extracting halophiles from different sources, including salt lakes, salt pans, salted fish, and pickled vegetables, among others. Here are a few key reference papers on the extraction of halophiles:

1. A simple and effective method for the isolation of halophilic bacteria from salted fish and pickled vegetables. The authors used a selective enrichment culture technique and were able to isolate a range of halophilic bacteria, including species of *Halomonas* and *Marinococcus* (Oyaizu and Muramatsu, (1982)).
2. A method for the enrichment and isolation of halophilic bacteria from solar salt works. The authors used a salt gradient to selectively enrich for halophilic bacteria and were able to isolate a range of halophilic species, including *Halomonas* and *Deleya* (Ventosa, Nieto, and Oren (1998)).
3. A method for the extraction of halophilic microorganisms from a salt mine. The authors used a combination of enrichment cultures and direct plating to isolate a range of halophilic bacteria, including species of *Halomonas* and *Halobacterium* (Gallego-Gomez , Ventosa and Oren (1999)

In conclusion, the isolation of halophiles is a crucial step in the study of these microorganisms and their potential applications. The methods described in these reference papers provide a foundation for the isolation of halophiles from a variety of sources, including salt lakes, salt pans, salted fish, and pickled vegetables.

2.5 CHARACTERIZATION OF BACTERIORHODOPSIN

Bacteriorhodopsin is a type of membrane protein found in archaea, particularly in the membrane of Haloarchaea. It is a light-driven proton pump that converts light energy into chemical energy by transporting protons from the cytoplasm to the extracellular space. Bacteriorhodopsin has a characteristic structure composed of seven transmembrane helices, with a retinal molecule embedded in the middle of the protein. This retinal molecule undergoes structural changes upon exposure to light, leading to conformational changes in the protein and the initiation of proton transport. Bacteriorhodopsin is also involved in maintaining the ionic balance of the cell by regulating the proton concentration gradient across the membrane. Overall, bacteriorhodopsin is a remarkable protein with unique properties that make it a useful tool in various applications, including optogenetics and biophysics research.

The results of this study demonstrated the diversity of bacteriorhodopsin present in solar salterns and highlighted the importance of these microorganisms in the cycling of carbon and other nutrients in high salt environments (Rodolfo *et al.*, 1999)

2.6 APPLICATION OF BACTERIORHODOPSIN

Bacteriorhodopsin (bR) is a membrane protein found in the plasma membrane of the archaea *Halobacterium salinarum*. Bacteriorhodopsin absorbs light energy and converts it into a proton motive force, which is used to generate ATP.

Bacteriorhodopsin has many applications, including:

1. Optical storage – Bacteriorhodopsin has been used as a recording medium for optical storage devices. When exposed to light, it changes its state and can retain that state for a long time.
2. Sensors – Bacteriorhodopsin can be used to detect signals from various biological and chemical molecules. It can also detect changes in pH and temperature.
3. Medical imaging – Bacteriorhodopsin has been used as an imaging agent in medical imaging. It has been shown to be effective in imaging the brain and other organs
4. Energy production – Bacteriorhodopsin can be used in bioenergy systems to generate energy from light.

Overall, bacteriorhodopsin has a lot of potential in a variety of applications due to its unique light – absorbing energy from light.

The study of bacteriorhodopsin, which are microorganisms that are able to grow and thrive in high salt concentrations. The authors discuss the various applications of these bacteria in industrial processes, such as the production of enzymes, biopolymers, and other biologically-derived products. They also examine the potential for using halophilic bacteria in bioremediation, or the use of living organisms to clean up environmental pollutants (Reverchon and Riggio, 2010) PHAs are a class of biodegradable and biocompatible polymers that can be used as an alternative to petroleum-based plastics. The authors of the paper investigated the production of PHAs by various haloarchaeal species and found that these microorganisms are capable of synthesizing a wide range of PHAs with different physical and chemical properties (Beliaev and Saffarini, 1998)

The results of this study demonstrate the potential of haloarchaea as a source of sustainable, renewable PHAs, which could be used to replace petroleum-based plastics in a wide range of applications. The findings of this research contribute to the development of alternative and more sustainable methods for the production of bioplastics.

The diversity of halophilic bacteria, their potential for biotechnology applications, and their significance in biogeochemical processes. Halophilic bacteria are microorganisms that are able to grow and thrive in environments with high salt concentrations, making them unique and valuable in a variety of fields

The authors provide an overview of the different types of halophilic bacteria, their ecological niches, and their metabolic pathways. They also discuss the biotechnological applications of halophilic bacteria, including their potential use in the production of enzymes, organic acids, and other bioproducts. Additionally, the authors examine the role of halophilic bacteria in biogeochemical cycles and their impact on the global carbon and nitrogen cycles

Overall, the paper provides a comprehensive overview of the diversity, biotechnology, and biogeochemical significance of halophilic bacteria and highlights their importance in various fields (Qiu *et al.*, 2015)

2.7 GENERAL STRUCTURE AND OPERATION PRINCIPLES OF *bR*

Three membrane proteins containing retinaldehyde, including phobo-rhodopsin, slow-rhodopsin, and halor-rhodopsin, were later discovered in halophilic bacteria in addition to the discovery of BR from *H. salinarium* (sensory-rhodopsin II). These four membrane proteins, collectively known as rhodopsin in *H. salinarum*, share a great deal of structural and functional similarities. (Lanyi and Kato, 2000)

It highlights the parallels and discrepancies between these four proteins. However, compared to BR, each bacterium only has molecules, which are rarely extractable. These three proteins were later discovered due to the low content, and the associated investigation was far less thorough. Consequently, the review of BR-based applications is important and useful (Spudich and Bogomolni, 1988)

All-trans and 13-cis retinal chromophores are the two types that naturally exist in bR. The retinal chromophore in the BR population is all-trans in the dark and is attached to the protein by a protonated Schiff base bond to lysine-216. All-trans retinal is transformed to 13-cis retinal when light is stimulated, and photoisomerization of the retinal chromophores causes a photochemical response in BR proteins (Maeda *et al.*, 1977)

The photocycle of BR, a complicated process that includes several intermediate states with distinct spectral absorption maxima, typically takes about 15 ms to move a proton from the cytoplasmic side to the extracellular side of the membrane, completing the photoelectric energy conversion (Birge, 1990). Not all of the BR photo-intermediates can return to the ground bR state through illumination during the photocycle (Trissl and Montal, 1977) Only a few intermediates can either transition instantly back to the BR state when triggered by photons of the right wavelength or thermally relax to the next intermediate. The branching photocycle and the irreversible change the two distinctive characteristics of the BR photocycle. (Birge,1994)

Table.1 Classification of microorganisms according to their tolerance to salinity

S.NO	CATEGORY	SALT TOLERANCE	EXAMPLES	REFERENCE
1.	Non halophilic	<0.2	• <i>Pseudomonas aeruginosa</i> • <i>Bacillus sp.</i>	Sigee (2005) Saiz-Jimenez and Laiz (2000)
2.	Slight halophile	0.2 -0.5	• <i>Alkalibacterium thalassium sp.</i> • <i>Erwinia sp</i> • <i>Dethiosulfovibrio salsuginis</i> • <i>Bacillus xiaoxiensis</i> • <i>Bacillus hunanensis</i> • <i>Halomonas zhaodongensis</i> • <i>Alkalibacterium gilvum</i> • <i>Roseovarius aquimarinus</i>	Ishikawa <i>et al.</i>, (2009) Li <i>et al.</i>, (2010) Di´azCa´rdenas <i>et al.</i> (2010) Chen <i>et al.</i>, (2011) Chen <i>et al.</i>, (2010) Jiang <i>et al.</i>, (2013) Kang <i>et al.</i>, (2015)

3.	Moderate halophile	0.5-2.5	• <i>Halobacteroides halobius</i> • <i>Halanaerobaculum tunisiense</i> • <i>Halorhodospira halophila</i> • <i>Desulfovibrio retbaensea</i>	Oren (2002c) Hedi et al., (2008) Mehrshad et al., (2015b) Oren (2002a)
4.	Extreme halophile	4 - 5.9	• <i>Halococcus salifodinae</i> • <i>Salinibacter ruber</i> • <i>Limimonas halophila</i> • <i>Halanaerobium sehlinense</i> • <i>Sporohalobacter salinus</i> • <i>Lentibacillus kimchii</i>	Denner et al., (1994) Oren (2002c) Anton et al., (2002) Amoozegar et al., (2013b) Abdeljabbar et al., (2013) Abdallah et al., (2015)
5.	Halo - tolerant	non-halophilic tolerates salt; if it is viable 2.5M salt, it is considered extremely halotolerant	• <i>Staphylococcus aureus</i> • <i>Anaerosalibacter bizertensis</i> • <i>Methylobacillus halotolerans</i> • <i>Solikamskensis</i> • <i>Geodermatophilus africanus</i>	Sheehan et al., (1992) Rezgui et al., (2012) Doronina et al., (2013) Mc et al., (2013) Zhu et al., (2014)

			• <i>Bacillus ligniniphilus</i> • <i>Bacillus pakistanensis</i> • <i>Tamilnaduibacter salinus</i> • <i>Salinicoccus sediminis</i> • <i>Halomonas salicampi</i>	Roohi <i>et al.</i>, (2014) Verma <i>et al.</i>, (2015) Kumar <i>et al.</i>, (2015) Lee <i>et al.</i>, (2015a) Jayaraman (2016)
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CHAPTER 3

3. AIM AND SCOPE

3.1 AIM OF THE PROJECT

The aim of the project is that:

- To extract and characterize the bacteriorhodopsin from isolated halophilic bacteria
- To optimize the bacteriorhodopsin production using cheaply available carbon sources
- To scale up the bacteriorhodopsin pigment production from the optimized halophilic bacteria

3.2 SCOPE OF THE PROJECT

The scope of the project is ,

To develop a low-cost strategy by finding a suitable alternate cheap carbon sources for the production of bacteriorhodopsin.

CHAPTER 4

4. MATERIALS AND METHODOLOGY

4.1 SAMPLE COLLECTION

The soil samples were collected from several salterns in the region of Marakkanam (12° 11' 23.7948" N, 79° 55' 29.5212" E) which is located in Vilupurapm district, Tamil Nadu - India during pre-monsoon season and post monsoon season. The samples were transferred to laboratory and stored under refrigeration before isolation of halophilic strains.

4.2 ISOLATION AND SELECTION OF BACTERIORHODOPSIN PRODUCING HALOARCHAE

4.2.1 SERIAL DILUTION:

- Soil sample of 1g was weighed
- 9ml of water was added in five tubes.
- For serial dilution, 1 ml of the measured sample was added in the first tube.
- From the first tube 1 ml of sample was added to the second tube.
- Then the same method was followed for five tubes until dilution factor of 10^{-5} was obtained.
- Then 1 ml of sample was discarded from 10^{-5}
- Three concentration 10^{-3} , 10^{-4} , 10^{-5} was taken to inoculate in spread plate.

4.3 CULTIVATION OF HALOBACTERIAL STRAINS

4.3.1 PREPARATION OF MEDIA

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

Composition of MSM media is (for 1000ml):

- Casamino acid - 7.5 g
- Yeast extract - 1 g
- Sodium citrate - 3 g
- Sodium chloride [25%] - 250 g
- Magnesium sulphate - 20 g
- Potassium chloride - 2 g
- Ferrous sulphate - 0.05 g
- Manganese sulphate - 0.2 g

Then finally the pH was checked for the medium. The pH should range from 7.2 to 8

4.4 INOCULATION OF STRAIN

The isolates of various bacterial strains were plated, those were TMR2, TSP2, TSP4, TSP10, TP5, M1, M3, M4, SM1, SM2, SM3, and SM4. These 12 strains were serial diluted and isolated.

4.5 INCUBATION CONDITION

The culture was inoculated and has been kept under continuous light condition in room temperature for 20 days.



Fig.1 Light set up for incubation

4.6 SELECTION OF MAXIMUM bR PRODUCING STRAINS

Out of those 12 strains that were collected before the following strains that is SM1 and M4 were selected after the screening process that is used for identifying the maximum bR producing strains. SM1 and M4 strain was found to be the maximum bR producing strains and then they were isolated and inoculated in the MSM. Their growth was checked after every two days of incubation using cheap carbon sources and growth curve has been plotted. Absorbance was checked at 560nm and 660nm. Where, we can find the presence of BR at 560nm and bacterial growth at 660nm.

Table 2: Isolated strains inoculated in MSM

SM1	M4
-----	----

These two strains have been inoculated in MSM along with cheap carbon sources.



Fig.2 Maintenance of Br producing strains in MSM media

4.7 MOLECULAR CHARACTERIZATION

1. Protein sequencing: Once the bacteriorhodopsin is identified, the next step is to determine the sequence of amino acids which make up the protein. This can be done using various sequencing techniques such as Edman degradation, peptide mapping, and MS/MS sequencing.
2. Structural analysis: Bacteriorhodopsin is a transmembrane protein, meaning it resides in the cell membrane. Determining the structure of the protein requires techniques such as X-ray crystallography or NMR spectroscopy.
3. Functional analysis: Bacteriorhodopsin functions as a light-driven proton pump, which generates a transmembrane proton gradient used by the cell to generate energy. The functional analysis involves measuring the protein's ability to pump protons when irradiated with light of different wavelengths, determining the rate of proton pumping and pH change using fluorescence-based assays, and studying its stability and activity under different conditions.
4. Bioinformatics analysis: Finally, the amino acid sequence and structure of bacteriorhodopsin can be compared to other known proteins, and phylogenetic analysis can be used to trace the evolutionary history of the bacteriorhodopsin protein family.

4.8 BIOCHEMICAL TEST OF THE STRAINS

- This is a comprehensive test system that can be used for differentiation of gram-negative species. Inoculation of each well with 50 micro liter of the above inoculum by surface inoculation method.
- Alternatively the kit was inoculated by stabbing each individual well with a loopful of inoculum
- Incubation: Incubation temperature was 35 - 37°C. Duration of incubation: 14 days
- Interpret results as per the standards given in the Result Interpretation Chart. Addition of reagents in well number 1,2, and 3 should be done at the end period, that is after 18 - 24 hours. Following reagents was added to the respective wells

Indole Test: Well No. 1

• 1-2 drops of Kovac's reagent (R008) was added. Development of reddish pink colour within 10 seconds indicates positive reaction.

Methyl Red Test: Well NO. 2

- 1-2 drops of Methyl Red reagent (1007) was added.
- Reagent remains red in colour , the test was positive.

Voges Proskaeur's Test: Well NO. 3

- 1-2 drops of Baritt reagent A (ROzg) and 1-2 drops of baritt reagent B(RO30) was added.
- Pinkish red colour development within 5-10 minutes indicated a positive test.
- No change in colour or a slight copper colour (due to reaction of Baritt reagent A with Baritt reagent B) denoted a negative reaction.

4.9 QUANTIFICATION OF BACTERIORHODOPSIN

Samples (400 µl) from each time point were harvested as described above, quickly frozen and stored at -650°C. Pellets were resuspended and lysed in 100 µl) of DNase A (40 µl/ml; Sigma) in H₂O. The lysate was mixed well until homogeneous and no longer viscous. For 11-7 cultures, BR content was quantified spectrophotometrically by scanning the lysed samples from 400 to 700 nm on a Beckman DU50 spectrophotometer. Bacteriorhodopsin concentrations were determined according to the molar extinction coefficient for purple membrane ($6568 = 63,000M - 1 \text{ cm}; 24$). For NRC817 cultures, the presence of bacterio-uberins interferes with the spectrometric quantitation of BR. A freshly prepared 2M stock solution of NH₂OH was made by mixing equal volumes of 4M NH₂OH and 4M NaOH. One tenth volume of the 2M NH₂OH solution was added in the dark to the lysed NRC817 sample, and an absorption spectrum was taken on a Shimadzu UV160U spectrometer. Spectra were taken until there was no further decrease in absorption and the amount of BR determined from the difference between the bleached

and unbleached spectra at 568nm. Total protein was determined by Bradford assay. The BR concentration was calculated as follows:

$$\text{Bacteriorhodopsin (g/l)} = 26,000 \times [(A1 - A2) \div 63,000]$$

Where,

A1 - Absorbance (560nm) before bleaching

A2 - Absorbance (560nm) after 48 hours of bleaching

26,000 - Molecular weight of BR

63,000/M cm – Molar extinction co efficient

4.10 SELECTION OF CHEAP CARBON SOURCES

Cheap carbon sources are used in MSM at three different concentrations that is 0.5%, 1.0% and 1.5% for increasing the productivity of bacteriorhodopsin. The two cheap carbon sources that we used here was Diary effluent and Distillery spent wash. Out of these two, the Diary effluent showed maximum production of bacteriorhodopsin. So only the Diary effluent was used for further production of bacteriorhodopsin.

4.11 PHYSICO-CHEMICAL CHARACTERIZATION OF CHEAP CARBON SOURCES

1.Sample preparation: The selected cheap carbon sources were cleaned, dried, and grounded into a fine powder to ensure uniformity in the samples.

2. Proximate analysis: The proximate analysis involves determination of moisture content, ash content, volatile matter, and fixed carbon content of the carbon sources. This analysis provided an idea about the availability of carbon and the impurities present in the samples.

3.Ultimate analysis: The ultimate analysis involves determination of the elemental composition of the carbon sources, including carbon, hydrogen, nitrogen, sulfur, and oxygen. This analysis provided information on the potential energy content and the possible formation of undesirable products during combustion.

4.Thermogravimetric analysis: The thermogravimetric analysis involves studying the behavior of the carbon sources when subjected to heating under controlled conditions.

This analysis provided information on the thermal stability of the carbon sources and the pyrolysis characteristics.

5. Fourier-transform infrared spectroscopy: The Fourier-transform infrared spectroscopy involves studying the functional groups present in the carbon sources. This analysis provided information on the chemical composition and the possible interactions with other compounds.

6. Surface area analysis: The surface area analysis involves studying the surface area of the carbon sources using techniques such as nitrogen adsorption-desorption isotherms. This analysis provided information on the pore size distribution and the surface area available for adsorption.

By following these steps, the physico-chemical properties of the cheap carbon sources can be characterized, and they can be used for various applications such as adsorption, catalysis, and fuel production

4.12 ESTIMATION OF BIOMASS DRY WEIGHT

At the end of each optimization study, the dry weight (g/ml) of the biomass was obtained by centrifuging 2ml of the culture samples at 10,000 rpm for 10 minutes at 4 degree celcius. After centrifugation, the supernatant was discarded and the pellet in the centrifuge tube was air dried for 2 days. Then, the dried pellet was weighed and dry weight of the biomass was calculated according to the below mentioned formula.

$$\text{Dry cell weight (g/ml)} = (W3 - W2) \div W1$$

Where,

W3 = Final weight of the tube along with biomass

W2 = Final weight of the control tube

W1 = Volume of culture samples

4.13 EXTRACTION AND PURIFICATION OF BACTERIORHODOPSIN

Bacteriorhodopsin was isolated from the purple membrane of the isolated strain using the modified aqueous two-phase extraction system reported by (Alsafadi *et al.*, 2018), which was originally developed by Shiu *et al.*, 2014. The culture sample was collected and centrifuged at 10,000 rpm for 15 minutes. The obtained pellet was resuspended in 100 mM of Tris – HCl buffer, (pH 7.5) having 2M of NaCl and 2mM of EDTA. The cell lysis was performed by using sonicator at 6W at 4°C. The sonication was performed at an interval of 30 seconds till the lysate becomes transparent. Dialysis was performed by adding the cell lysate to the dialysis bag provided with 100 µl of DNase I allowing the digestion of genomic DNA.

Then, the dialysis bag was kept immersed in a water under a stirring condition at 4 °C for 24 h. Then, the obtained cell lysate was stored at 20 °C. From which, 5ml of the cell lysate was mixed with 10ml of 50% Polythene glycol and 10ml of 24% Potassium phosphate buffer containing 22g of monobasic potassium phosphate, 2g of dibasic potassium phosphate (pH 8). The mixture was added with 100 µl of 60mM CHAPS and kept in shaking condition at about 170 rpm for 1 hour at 25°C. The CHAPS is an ionic detergent used to remove the lipids that are associated with the BR layer. The two phases were separated by 1,960 x g for 15 minutes. The red band formed at the interface of the two phases was collected and was again centrifuged at 13,835 x g for 15 minutes . The total amount of protein in the obtained cell lysate was estimated using the standard procedure of Bradford assay. Bovine albumin serum (BSA) was used a standard. The presence of BR was then confirmed by SDS PAGE.

4.14 REAGENTS USED FOR EXTRACTION

4.14.1 100mM Tris-HCl buffer

To prepare the stock solution of Tris-HCl, the following concentrations were dissolved in 50ml of water separately.

- 0.5M Tris - HCl – 6.055g
- 0.1M EDTA – 1.461g
- 4M NaCl – 11.68g

To prepare the working solution of Tris-HCl, we require the following concentrations.

- 5ml of Tris-HCl
- 1ml of EDTA
- 25ml of NaCl

4.14.2 50% Polyethylene glycol

Dissolve 50g of PEG in 100ml of DW.

4.14.3 60mM CHAPS

Dissolve 0.9225g of CHAPS in 25ml of DW.

4.14.4 24% potassium phosphate

Dissolve 22g of dibasic potassium phosphate, 2g of monobasic potassium phosphate and 100µl of 60mM CHAPS and make up to 100ml with DW

4.15 STANDARDISATION OF BR PROTEIN EXTRACTION FROM HALOPHILIC PROTEIN

The intracellular membrane protein extraction from halophilic bacteria was carried out using three different approaches to obtain maximum yield of protein. Modified two aqueous method was used for extracting BR membrane protein. The method mentioned above.

Freeze thaw method (Sinha *et al.*, 2019) for the extraction of membrane protein. The 15 ml of halobacterium culture was centrifuged at 10000 rpm, 15 minutes after the centrifugation pellet were collected and freeze completely at -20°C for 1 h and resuspended the cell pellet in 1ml of lysis buffer by pipetting. Mix the reaction on horizontal shaking platform 1 h at RT. Then Freeze the suspension again at -20 for 30 minutes after that the pellet suspension was Thaw at RT, mix and remove 60 µl of the mixture 10000 rpm, 5 minutes and remove the supernatant for analysis for soluble proteins. The pellet is resuspended in PBS buffer and stored in -20 degree celcius. The other method for protein extraction from halophilic bacteria was carry out using glass beads. Following steps was under taken, Pelletizing the 100ml of halophilic strain 10000 rpm for 10 minute. The supernatant was discarded and the pellet was freezed for 30 minutes at -20°C.

Resuspended the pellet in Tris HCl buffer and dissolve the pellet with pipetting, then added 1 g of glass beads. Vortex the mixture for 10 minutes transferred the supernatant solution into new 2ml vials. Centrifuge the tube at 12000 rpm, 10 minutes. Then the supernatant removed for further analysis. Dissolve the pellet completely with the proper mixing. 60 µl of CHAPS added into the tube, then mixed and stored at 4 °C for 1 hour. Thawed in RT, then centrifuge under 12000 rpm, 10minutes. The red colored supernatant collected by pipetting and added into 2ml tubes. Then centrifuged under 24000 rpm.

For each extraction method we used step yields instead of overall yields. A step yield was the yield from a single step in the purification procedure, that is the amount of target protein or activity after that steps compared to that in the previous step of the procedure. Calculate the yield of total protein; yield is always recovery of total target protein or activity. SDS PAGE analysis was carried out to confirm the presence of 27KD protein of the isolated samples.

4.16 PROTEIN ESTIMATION BY BRADFORD ASSAY

4.16.1 BRADFORD REAGENT PRINCIPLE

Bradford assay was done for **Protein estimation**. Standard with seven concentrations of BSA S10, S20, S40, S80, S160, S320, S640 is added in each well and absorbance is checked at 595nm. Linear plot R^2 value is found to check how much deviation has occurred. Using the slope equation, y we find the amount of protein present in a sample.

4.16.2 PREPARATION OF BRADFORD REAGENT

- Coomassie brilliant blue(G-250) – 5mg
- Ortho phosphoric acid (85%) – 5ml
- Ethanol (95%) – 2.5ml



Fig 3. Bradford reagent preparation

4.16.3 PROTEIN ESTIMATION STANDARD

- Seven standards are prepared of different concentrations of BSA and DW
S10, S20, S40, S80, S160, S320, S640, blank

Table 3. Standard preparation for Bradford assay

	BSA[μ l]	DW[μ l]	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

- The standard was added in 96 well plate. 50µl of extracted protein sample and 150µl of Bradford reagent was added in other wells and absorbance is checked at 595nm

Subtract the obtained value for the blank with the other standards and plot linear graph and get R^2 value.

Using y equation from the plot, we calculate x value for all the samples were added in the plate.

4.17 SCALE UP OF BACTERIORHODOPSIN

The scale up of these two strains that is SM1 and M4 were inoculated in MSM. The scale up was done for 100ml, 1000L, 7.5L and 10L to increase the productivity of bacteriorhodopsin.



Fig 4. Scale up of 10L

4.18 LYOPHILIZATION

- The extracted sample and their supernatant were transferred into a labelled 6 well plates.
- The samples were then frozen at -80°C for three days.
- The well plates were taken to another cryo-preserve where the lids of the plates are replaced with aluminium foil.

- When the temperature was reached to -80°C , the plates were transferred into freeze-dryer.
- The freeze-dryer was run overnight.
- The dried sample was then scraped from the well plate and transferred to autoclaved vials for further analysis.
- Protein characterization like ATR/FTIR, Raman Spectroscopy was done.



Fig. 5 Freeze- dryer set up for lyophilization of extracted protein samples for further protein analysis such as FTIR/ATR and Raman

4.19 BACTERIORHODOPSIN CHARACTERIZATION

4.19.1 FTIR SPECTROSCOPY

Fourier transform infrared spectroscopy (FTIR) is commonly used to analyze the structural characteristics of proteins such as bacteriorhodopsin. The following is a standard procedure for FTIR methodology for protein characterization of bacteriorhodopsin:

1. **Sample Preparation:** Pure bacteriorhodopsin was isolated from the strains SM1 and M4 and extracted from the microbial membrane. The sample was washed several times with distilled water, centrifuged, and freeze-dried.
2. **FTIR Spectroscopy:** The FTIR spectrum was recorded using a Fourier transform infrared spectrophotometer by averaging 128 scans with a resolution of 4 cm⁻¹ in the mid-IR range (4000-400 cm⁻¹). A high-quality diamond crystal has been typically used for taking measurements.
3. **Baseline Correction:** The baseline was corrected for the interference using a standard correction method.
4. **Deconvolution:** The FTIR spectrum was obtained in step 2 was deconvoluted using a Gaussian curve-fitting method for identifying and quantify the different functional groups of the protein.
5. **Analyzing the FTIR Spectrum:** Using software like Fourier Transform Infrared Spectroscopy (FTIR) software, the characteristic absorption bands of biomolecules, such as amide I and II, has been identified. The C=O and C-N stretching modes of the peptide bonds and the C-H stretching modes of the amino acid side chains was also analyzed.
6. **Comparison of the spectra:** The FTIR spectra of a fully denatured bacteriorhodopsin solution in urea solution and the native protein should be compared. This will provide insight into the structural characteristics of the protein.
7. **Interpretation of Results:** The spectroscopic data was then analyzed to calculate important parameters such as the number of helices, β -sheets, and their secondary structure, which will give detailed information about the protein backbone structure. The results was obtained from FTIR can help researchers characterize the protein, predict drug interactions with it, and ultimately improve our understanding of how to modulate their physiological processes.

4.19.2 RAMAN SPECTROSCOPY

Raman spectroscopy is a powerful analytical technique used for the structural and functional characterization of biomolecules in different biological systems. It is a non-destructive, label-free method that provides information about the molecular vibrations

and rotations of the sample. Bacteriorhodopsin (bR) is a light-driven proton pump that is commonly used as a model protein for Raman spectroscopic analysis.

The Raman spectroscopy methodology for protein characterization of bacteriorhodopsin involves the following steps:

1. Sample preparation: The bR protein was extracted from the cells of *Halobacterium salinarum* and purified using standard protein purification techniques. The purified bR sample was then dissolved in a buffer solution to obtain a homogeneous sample. The buffer solution used for the sample preparation should be pH stable and should not interfere with the Raman signal.
2. Raman spectroscopic analysis: The Raman spectra of the bR sample were obtained using a high-resolution Raman spectrometer equipped with a laser source. The laser source was selected based on the excitation wavelength that maximizes the Raman scattering of the sample. The laser power used for the analysis was kept low to prevent the sample from photodegradation.
3. Data analysis: The Raman spectra obtained from the bR sample were analyzed using software tools. The Raman peaks in the spectra correspond to the distinct vibrational modes of the sample. The peak positions, intensities, and widths were used to extract relevant information about the protein structure and dynamics.
4. Interpretation of results: The Raman spectra obtained from the bR sample were interpreted based on the known spectroscopic features of the protein. The Raman peaks corresponding to the vibrational modes of the protein's C-C, C-N, N-H, and C-O groups provide information about the protein's secondary structure, folding, and dynamical behavior, respectively

CHAPTER – 5

5. RESULTS AND DISCUSSION

5.1 ISOLATION AND SELECTION OF BACTERIRHODOPSIN PRODUCING *HALOARCHAE*

The goal of the current study is to gather sand samples from various salt pans in Tamil Nadu, including Marakkanam which is located in Vilupuram district. The serial dilution procedure, with a dilution factor of up to 10^{-5} , was used to isolate the strains. Three dilution factors of 10^{-3} , 10^{-4} , 10^{-5} were further obtained from these diluted samples for the spread plating procedure in an agar medium, as indicated in (Fig. 5a and 5b)



Fig. 6a & 6b. Serial dilution and isolation of bacterial strain by spread plate method

5.2 PHYSICO-CHEMICAL ANALYSIS OF CHEAP CARBON SOURCE (DIARY EFFLUENT & DISTILLERY SPENT WASH)

Table. 4 Test report of the cheap carbon source (diary effluent)

S.NO	PARAMETERS	METHOD	UNITS	RESULTS
1	Biochemical Oxygen Demand (BOD) 3 days at 27°C	5210-B APHA 23rd Ed.2017	mg/l	1490
2	Chemical Oxygen Demand (COD)	5220-B- APHA 23rd Ed.2017	mg/l	12014
3	Chloride (as Cl ⁻)	IS 3025 (Part 32)-1988 (R.2014)	mg/l	471
4	Sulphate (as SO ₄)	IS 3025 (Part 24)-1986 (R.2014)	mg/l	194
5	Nitrate (as NO ₃)	IS 3025 (Part 34)-1988 (R.2014)	mg/l	129
6	Ammonia (as Total Ammonia-N)	IS 3025 (Part 34)-1988 (R.2014)	mg/l	79
7	Total Hardness as CaCO ₃	IS 3025 (Part 21)-1983 (R.2014)	mg/l	567
8	Volatile Solids	2540-E- APHA 23rd Ed.2017	mg/l	316

Table. 5 Test report of the cheap carbon source (distillery spent wash)

S.NO	PARAMETERS	METHOD	UNITS	RESULTS
1	Biochemical Oxygen Demand (BOD) 3 days at 27°C	5210-B APHA 23rd Ed.2017	mg/l	13300
2	Chemical Oxygen Demand (COD)	5220-B- APHA 23rd Ed.2017	mg/l	159660
3	Chloride (as Cl ⁻)	IS 3025 (Part 32)-1988 (R.2014)	mg/l	11316
4	Sulphate (as SO ₄)	IS 3025 (Part 24)-1986 (R.2014)	mg/l	4419

5	Nitrate (as NO ₃)	IS 3025 (Part 34)-1988 (R.2014)	mg/l	56
6	Ammonia (as Total Ammonia-N)	IS 3025 (Part 34)-1988 (R.2014)	mg/l	376
7	Total Hardness as CaCO ₃	IS 3025 (Part 21)-1983 (R.2014)	mg/l	15200
8	Volatile Solids	2540-E- APHA 23rd Ed.2017	mg/l	5270

5.3 INOCULATION OF BACTERIAL STRAIN

The isolates of two bacterial strains that were plated are SM1 and M4 that showed growth of colonies in about 5-7 days. These strains were then inoculated in MSM media which contained Casamino acid- 7.5 g; Yeast extract- 1 g; Sodium citrate- 3 g; Sodium chloride [25%]- 250 g; Magnesium sulphate- 20 g; Potassium chloride- 2 g; Ferrous sulphate- 0.05 g; Manganese sulphate- 0.2 g. pH of the medium was 7.2. The culture was then incubated at room temperature with proper monitoring. The inoculated cultures were investigated for growth by taking absorbance at 560nm where a peak is obtained around 500nm – 550nm which determines the presence of bacteriorhodopsin as shown in (Fig. 6a and 6b) and at 660nm we have obtained the growth curve as shown in (Fig. 7a and 7b) using spectrophotometer. Cheap carbon source – Dairy effluent was also added for the growth of bacteria at three different concentrations that is 0.5%, 1% and 1.5%

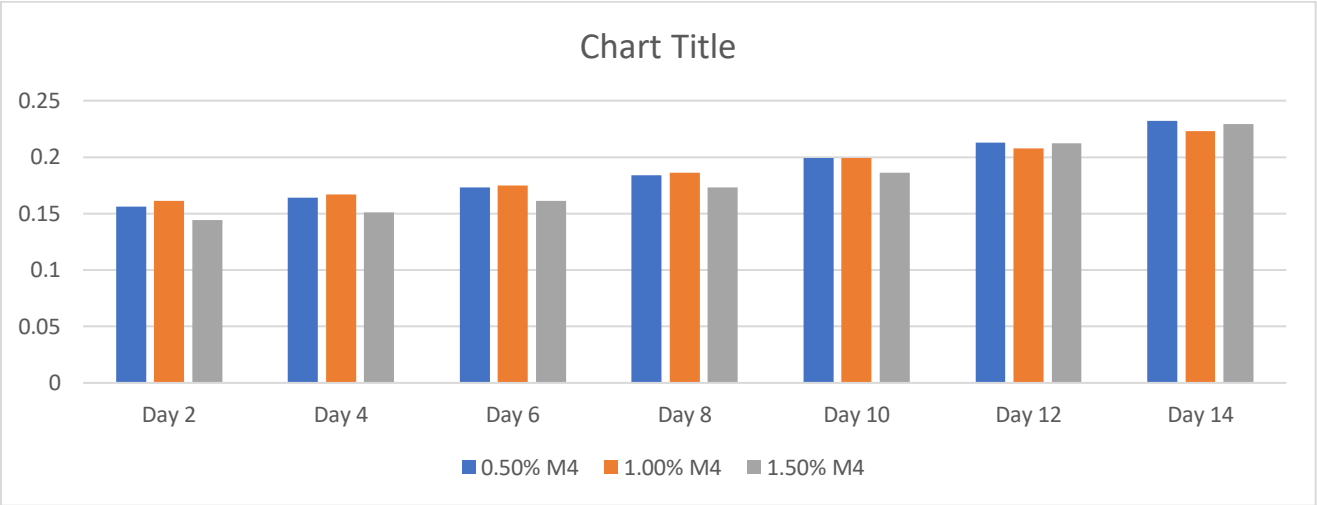
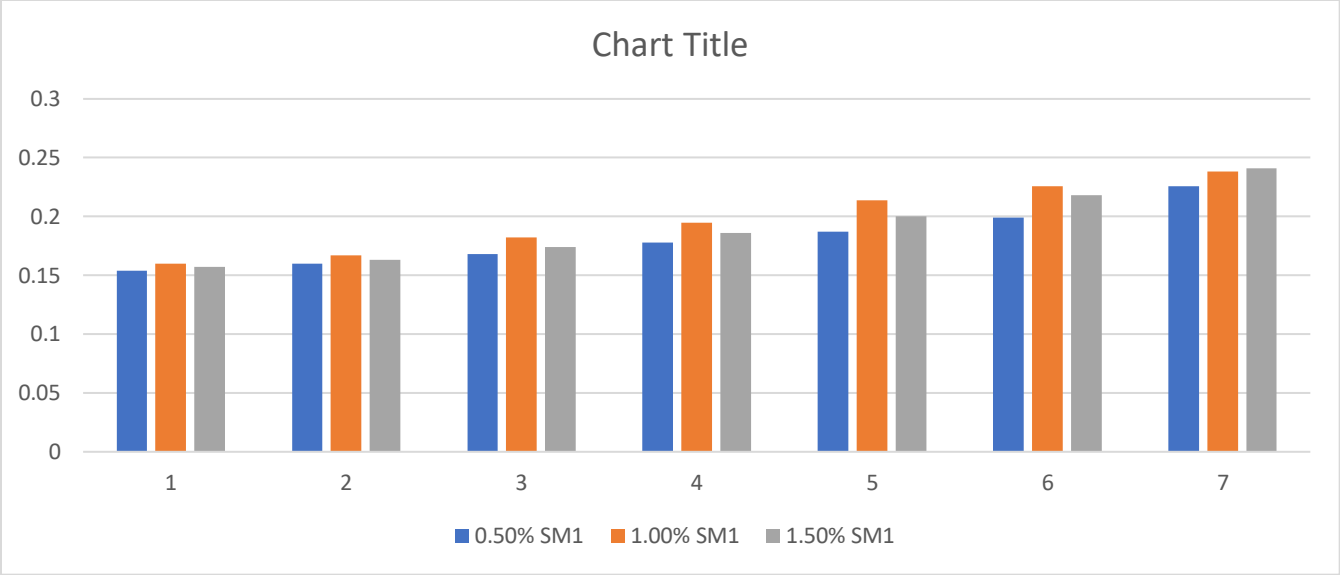


Fig 7. 7a and 7b Growth curve of the bacteria measured at 560 nm for SM1 and M4 strain

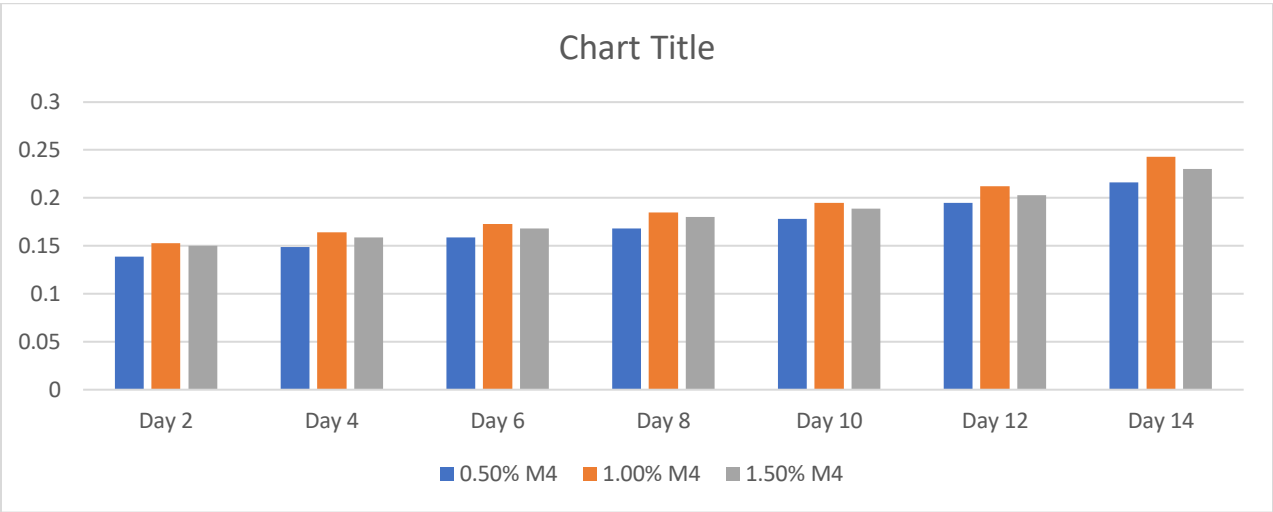
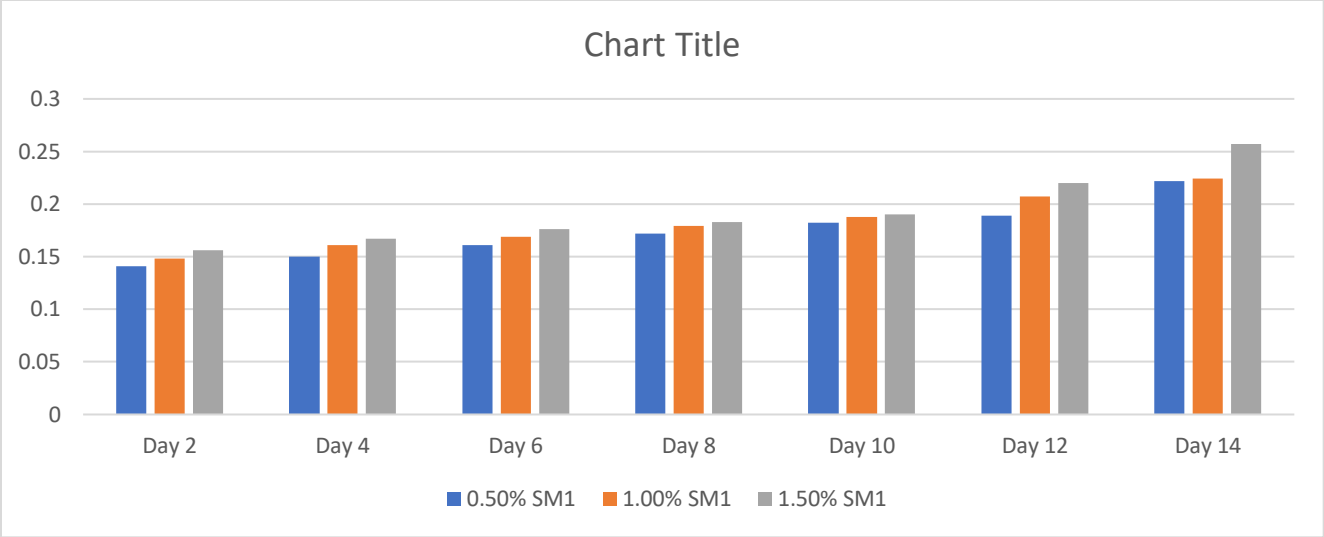


Fig 8. 8a and 8b Growth curve of the bacteria measured at 660 nm for SM1 an M4 strain

5.4 16srRNA IDENTIFICATION OF ISOLATED STRAIN

M4 - Haloferax sp. 16S ribosomal RNA gene, partial sequence.

SOURCE - Haloferax sp.

ORGANISM - [Haloferax sp.](#)

ORIGIN - Archaea; Euryarchaeota; Stenosarchaea group; Halobacteria;
Haloferacales; Haloferacaceae; Haloferax

SEQUENCE

```
1 cgaatttagc catgctagtt gcacgagttg agactcgtgg caaatagctc agtaacacgt
61 ggccaaacta ccctatcgag aacgatactc tcgggaaact gaggctaata gtgcata
121 gactcatgtc tgggagtgca cgcactcccc agaaacgctc aggcgctgat aggatg
181 tgcggccgat taggtagacg gtggggtaac ggcccaccgt gccgataatc ggtacg
241 gtgagagcaa gagcccggag acggaatctg agacaagatt ccggggcccta cggg
301 caggcgcgaa acctttacac tgcacgcaag tgcgataagg ggactccaag tgcgag
361 tatagtctc gcttttcccg actgtaagggt ggtcggggaa taagagctgg gcaagacch
421 tgccagccgc cgcggttaata ccggcagctc aagtgatgac cgatattatt gggcctaah
481 cgtccgtagc tggccacgaa ggttcacggt gaaatctctc cgctcaactg acaggcgctc
541 ggtgaaaact gtgtggcttg ggaccggaag attcgaaggg tacgtctggg gtaggagtg
601 aatcccgtaa tcctggacgg accaccggtg gcgaaagcgc tctggaaaga cggatcc
661 ggtgaggggac gaaagctcgg gtctcgaacc ggattagata cccgggtagt cctagctgt
721 aacgatgctc gctaggtgtg acacaggcta cgagcctgtg ttgtgccgta gggaagccga
781 gaagcgaacc gcctgggaag tacgtctgca aggatgaaac ttaaaggaat tggcgggg
841 gcactacaac cggaggagcc tgcggtttaa ttggactcaa cgccggacat ctcaccagc
901 tcgactacag tgatgacaaa tccgttgatt acctatctg gaccctctga agaggaggtghg
961 catggccgcc gtcagctcgt accgtgaggc gtctgttaa gtcaggcaac gaccggtghh
//
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SM 1 - Haloferax sp. 16S ribosomal RNA gene, partial sequence.

SOURCE - Haloferax sp.

ORGANISM - Haloferax sp.

ORIGIN - Archaea; Euryarchaeota; Stenosarchaea group; Halobacteria; Haloferacales; Haloferacaceae; Haloferax.

SEQUENCE

```

1 gctagttgca cgagttcata ctcgtggcga aaagctcagt aacacgtggc caaactaccc
61 tacagagaac gataacctcg ggaaactgag gctaatagtt catacgggag tcatgtctgg
121 atgccgactc cccgaaacgc tcaggcgctg taggatgtgg ctgcggccga ttaggttag
181 ggtggggtaa cggcccaccg tgccgataat cgggtacgggt tgtgagagca agagccc
241 gacggaatct gagacaagat tccggggcct acggggcgca gcaggcgcca aaccttt
301 ctgcacgcaa gtgcgataag gggaccccaa gtgcgagggc atatagtcct cgcttttca
361 gactgtaagg cggtcgtgga ataagagctg ggcaagaccg gtgccagccg ccgcgggt
421 accggcagct caagtgatga ccgatattat tgggcctaaa gcgtccgtag ccggccacg
481 aggttcacg ggaaatccgc cagctcaact ggcgggcgtc cgtgaaaac cacgtggc
541 gggaccggaa ggctcgaggg gtacgtccgg ggtaggagtg aaatcccgt atcctgga
601 gaccaccgat ggcgaaagca cctcgagaag acggatccga cggtagggga cgaaah
661 ggtctcgaac cggattagat acccgggtag tcctagctgt aaacgatgct cgctaggtgtah
721 gacacaggct acgagcctgt gctgtgccgt agggaagccg agaagcgagc cgctggg
781 gtacgtccgc aaggatgaaa cttaaaggaa ttggcggggg agcactacaa ccggagga
841 ctgcggttta attggactca acgccggaca ttcaccagc tccgactaca gtgatgacgah
901 tcaggttgat gacctatca cgacgtgta gagaggaggt gcatggccgc cgtcagctcgh
961 taccgtgagg cgtcctgtta agtcaggcaa cgagcgagac ccgcacttct aattgccagch
1021 agcagtttcg actggctggg tacattagaa ggactgccgc tgctaaagcg gaagaagga
1081 cgggcaacgg ta

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5.5 TAXONOMY OF HALOPHILIC STRAIN

5.5.1 PHYLOGENETIC TREE OF M4 STRAIN

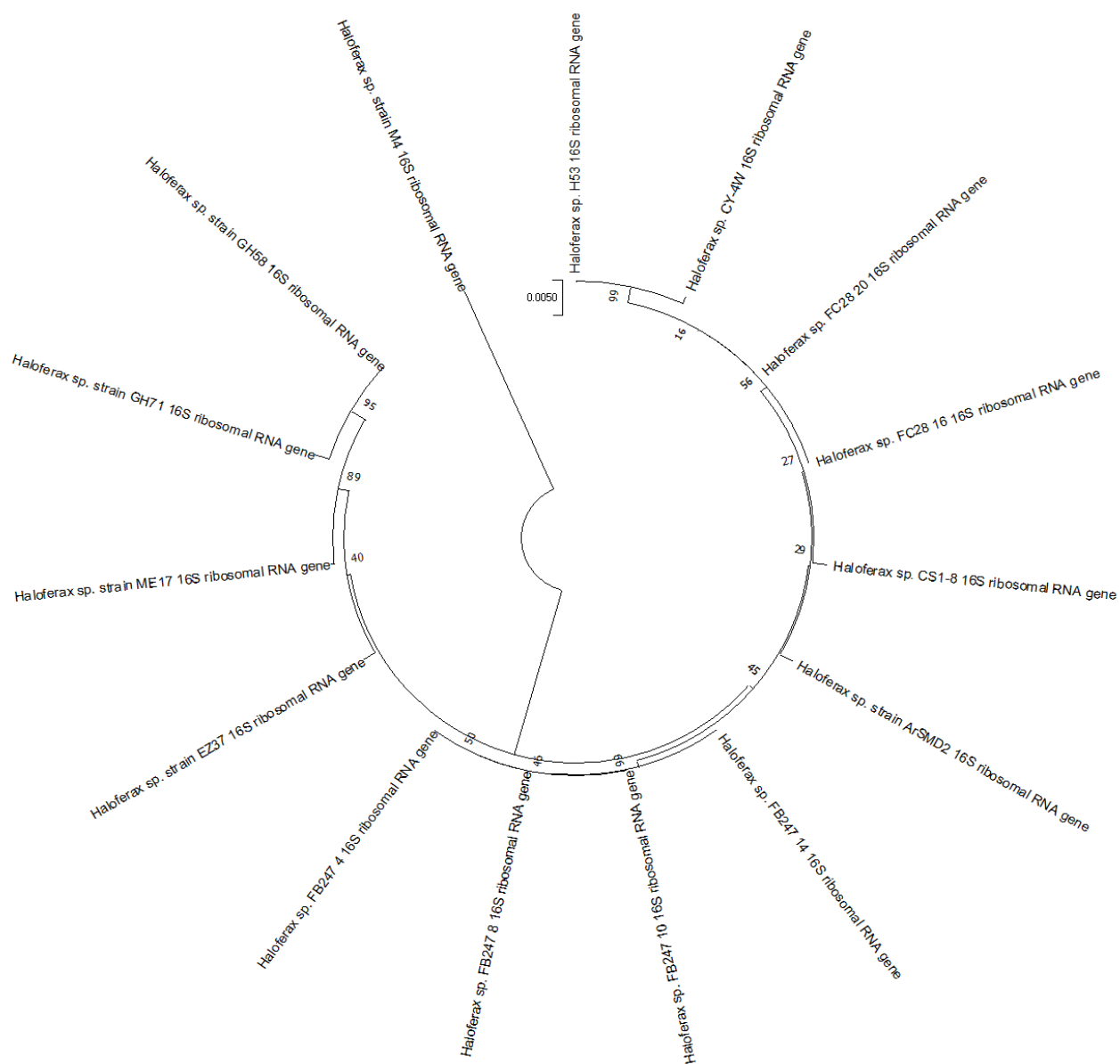


Fig.9 Phylogenetic tree of M4 strain

5.6 EXTRACTION AND PURIFICATION OF PROTEIN FROM THE BACTERIAL STRAIN

The cultured samples of SM1 and M4 strains were subjected to various processes such as deep freezing, thawing, and vortexing with glass beads which were methods of physical and chemical methods of disintegration of bacterial cells (Muriana,1987). Among the different methods that are followed, we have done a comparative study between the Freeze Thaw method and Glass Bead Mix method to check which method produces the highest amount of biomass from each of the different cultured samples. Photo-active membrane protein bacteriorhodopsin (Br) from different inoculated cultures of halophilic bacteria were extracted using the Freeze Thaw method as described in the method part. A good amount of protein biomass of each bacterial strain was obtained in this extraction method. The same inoculated cultures of halophilic bacteria were extracted using Bead Mix method was described in the method part where a good amount of protein biomass of each bacterial strain was obtained in the extraction method. The extracted protein biomass from both Freeze thaw and Bead mix methods were collected and stored in Phosphate Buffer Saline as shown in Fig.8 for both Freeze thawed samples and Bead mixed samples and used for further protein analysis. From this study, it was found that the biomass that was obtained from freeze thawed samples had a higher amount of biomass compared to the biomass that was obtained from bead mix samples as shown in (Table. 5)

5.6.1 PROTEIN EXTRACTION BY FREEZE – THAW METHOD

By following freeze-thaw method of protein extraction from inoculated culture, we obtain the biomass of the bacterial strain. As seen in Fig. 8 the extracted protein samples of bacterial strains are stored in PBS buffer and taken for further protein analysis.

5.6.2 PROTEIN EXTRACTION BY BEAD MIX METHOD

By following bead mix method of protein extraction from inoculated culture, we obtain the biomass of the bacterial strain. As seen in Fig. 8 the extracted protein samples of bacterial strains are stored in PBS buffer and taken for further protein analysis.

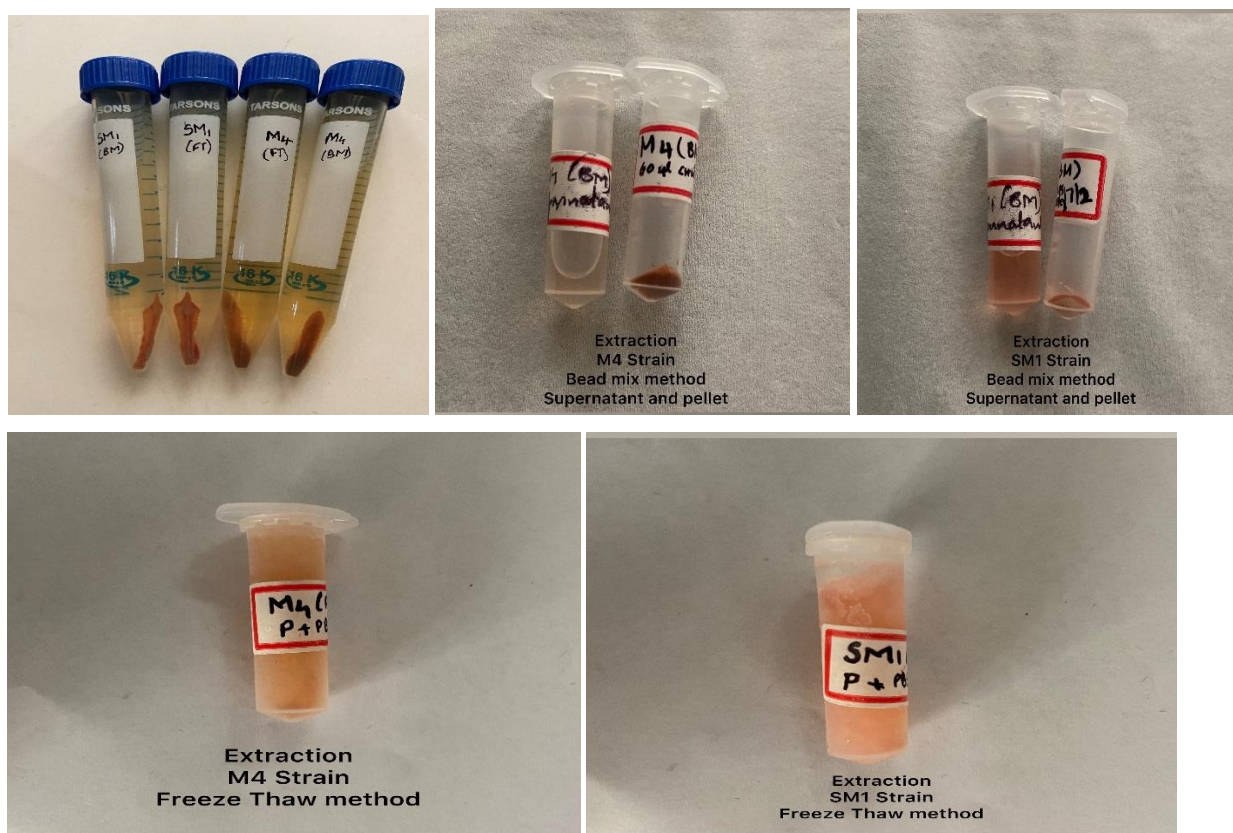


Fig. 10. Extraction of bacteriorhodopsin by bead mix method and freeze thaw method

5.7 ESTIMATION OF TOTAL BIOMASS

SAMPLE CALCULATION

FOR M4 STRAIN –

Weight of the centrifuge tube with obtained pellet = 13.2036 g

Weight of empty tube = 12.9542 g

Volume taken = 25 ml

Biomass cell weight = 13.2036 – 12.9542

$$\frac{\text{-----}}{25} \text{ mg/ml}$$
$$= 0.0099 \text{ g/ml}$$

BIOMASS = 9.96 mg/ml

Table 6. Estimation of Biomass of the halophilic strains

STRAINS	BIOMASS CELL WEIGHT BY BEAD MIX METHOD	BIOMASS CELL WEIGHT BY FREEZE THAW METHOD
SM1	8.63 mg/ml	8.18 mg/ml

5.8 PROTEIN ESTIMATION BY BRADFORD ASSAY

To quantify the protein content in the inoculated SM1 and M4 strain, procedures are available where we have used the most widely utilized method for the analysis of protein contents by Bradford Assay method (Bonjoch *et al.*, 2001). Nonetheless, many authors recommend the spectrophotometric assay of Bradford (1976) because of its multiple advantages if compared with other methods (Snyder and Desborough, 1978; Berges *et al.*, 1993). Bradford reagent was prepared and standardized by making seven different standards (Kruger, 2009) as given in (Table 3) which was checked under absorbance of 595nm where the coefficient of determination, $R^2 = 0.9776$ was plotted and the slope equation of $y = mx + b$ is obtained, as seen in (Graph 1). The Bradford assay method

was carried out as described in the method part using BSA as standard. The absorbance at 595nm for the freeze thawed samples using Bradford assay method was done in triplicates and the average values of the pellet obtained from freeze thaw method are shown in (Table 7) where the maximum absorbance was found in SM1 isolate. The average triplicate values of the supernatant which contains soluble proteins that are obtained from freeze thaw method are shown in (Table 8) where maximum absorbance was found in and SM1 isolates and the absorbance at 595nm for the bead mix samples using Bradford assay method was done in triplicates and the average values of the pellet obtained from bead mix method are shown in (Table 9) where the maximum absorbance was found to be in SM1.

Table 7. Absorbance value of pellet samples obtained by freeze-thaw

STRAINS	ABSORBANCE AT 595nm
SM1	1.049
M4	0.423

Table 8. Absorbance value of supernatant obtained by freeze-thaw method

STRAINS	ABSORBANCE AT 595nm
SM1	0.635
M4	0.517

Table 9. Absorbance value of pellet samples obtained by bead mix method

STRAINS	ABSORBANCE AT 595nm
SM1	1.613
M4	0.388

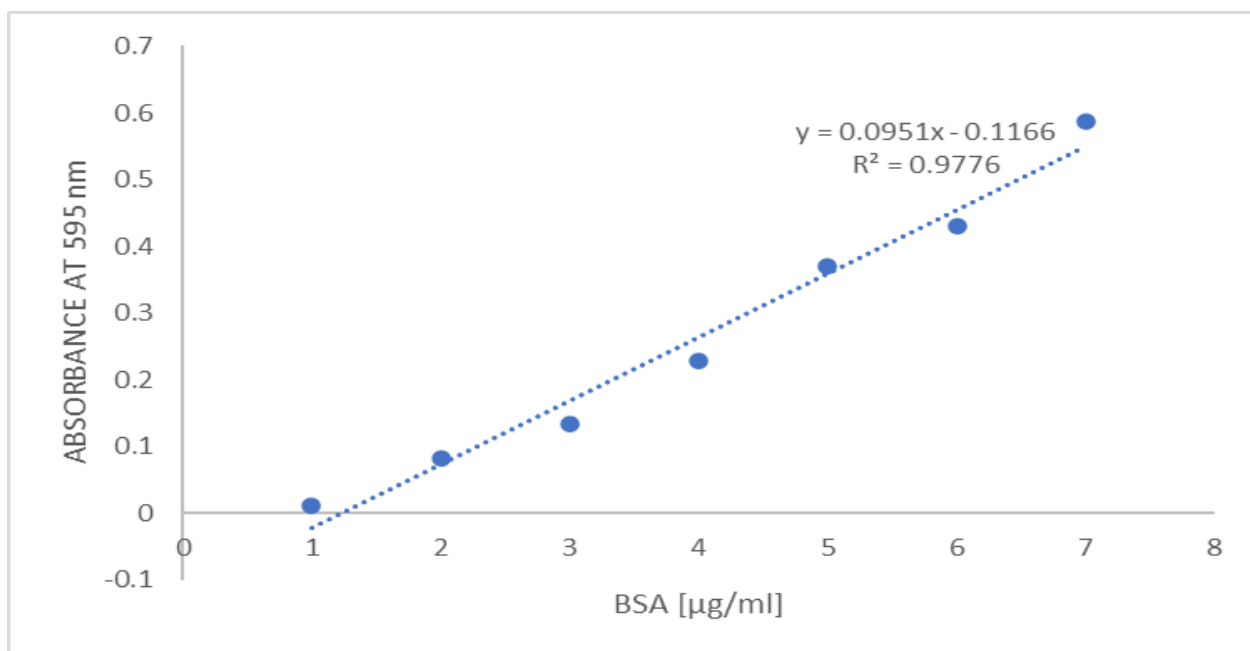


Fig 11. Linear graph that determines Coefficient of determination and slope equation for protein estimation calculation

The amount of BR protein that are present in SM1 and M4 strain sample is calculated by applying the absorbance values that are given in (Table 7, Table 8 and Table 9) to the above mentioned formula. The calculated values of the amount of bacteriorhodopsin protein in each strain are given in (Table 10) where the SM1 strains show a high amount of BR protein content in it.

Table. 10. Bacteriorhodopsin estimation using Bradford assay

STRAINS	BR in FREEZE THAW SAMPLES (μg/ml)	BR in BEAD MIX SAMPLES (μg/ml)
SM1	12.25	18.18
M4	5.67	5.30

5.9 QUANTIFICATION OF BACTERIORHODOPSIN BY HYDROXYLAMINE METHOD

The BR content in different strains was quantified spectrophotometrically based on light induced bleaching reaction in which the added hydroxylamine reaction takes place (Pei-Jing-Shiu.2013). Initial absorbance of the sample was checked at 568nm and hydroxylamine solution was added and the absorbance was checked to indicate the initiation of the bleaching process. The samples were exposed to constant light conditions for 24 hours and the absorbance was noted and the concentration of the bacteriorhodopsin was calculated using the given formula and are depicted in (Table 9).

The concentration of bacteriorhodopsin protein was quantified by Hydroxylamine method. Initially, absorbance of the samples was checked at 568nm which was taken as 0th hour. Immediately after the addition of hydroxylamine solution, the absorbance is checked at 568nm indicating the bleaching process of the protein has been started. The well plate was kept under constant light condition for 24 hours and after 24 hours the absorbance is checked once again which is noted as 24th hour.

The values were calculated by using the formula given. The concentration of BR after bleaching process is given in the (table 10) below.

Table 11. Concentration of bacteriorhodopsin was quantified using hydroxylamine method

STRAIN	BACTERIORHODOPSIN CONCENTRATION FT (mg/ml)	BACTERIORHODOPSIN CONCENTRATION BM (mg/ml)
SM1	0.359	0.553
M4	0.003	0.027

5.10 LYOPHILIZATION

Extracted protein samples are then transferred to 6 well plate and cryo-preserved for three days after transferring them to freeze-dryer. The freeze-dryer is left to run overnight. The dried samples of SM1 and M4 strains were then collected in Eppendorf tubes and further protein characterization processes like ATR/FTIR and Raman Spectroscopy was done.



Fig 12. Extraction of bacteriorhodopsin of different halophilic strains after lyophilization for protein characterization

5.11 BACTERIORHODOPSIN CHARACTERIZATION

5.11.1 FOURIER TRANSFORM INFRA RED SPECTROSCOPY

The FTIR spectroscopy results for the protein characterization of bacteriorhodopsin showed several distinct peaks that correspond to the different functional groups in the protein. The spectrum reveals the presence of several amide bands, indicating the presence of peptide bonds in the protein structure.

The amide I band observed at around 1650 cm^{-1} is primarily due to the $\text{C}=\text{O}$ stretching vibrations in the protein backbone, indicating the presence of alpha-helix and beta-sheet structures. The band at 1540 cm^{-1} is due to the N-H bending vibrations, while the band at 1240 cm^{-1} is due to the C-N stretching vibrations, indicating the presence of amide II and III bands.

The FTIR spectrum also shows the presence of several other peaks that correspond to the various functional groups in the protein. The bands at around 2930 and 2850 cm^{-1}

are due to the C-H stretching vibrations, while the peaks at 1460 and 1375 cm^{-1} indicate the presence of CH_2 and CH_3 groups.

Overall, the FTIR spectroscopy results provide a comprehensive analysis of the structural characteristics of bacteriorhodopsin. The study confirms the presence of several functional groups and supports the alpha-helix and beta-sheet structure of the protein. The FTIR data can also be used to compare the protein's structure to other proteins and to monitor structural changes during activity or under different conditions.

Table 12. FTIR Spectroscopy result for SM1 strain

Peak Number	Wavenumber (cm^{-1})	Intensity
1	859.15137	66.94326
2	982.15352	57.17798
3	1069.74596	46.03095
4	1399.61536	80.86250
5	1474.16211	82.99110
6	1543.11786	71.25536
7	1561.75455	73.04372
8	1638.16498	60.79353
9	1997.85308	97.19617
10	2122.71890	94.66731

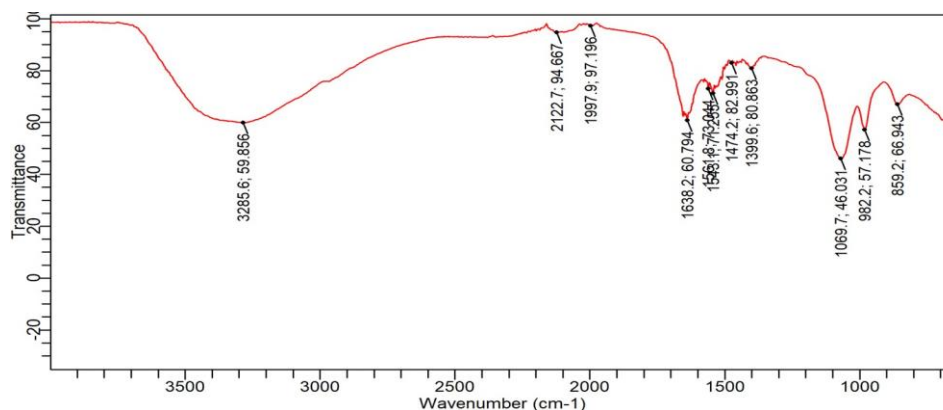


Fig 13. FTIR peak results for SM1 strain

Table 13. FTIR Spectroscopy result for M4 strain

Peak Number	Wavenumber (cm ⁻¹)	Intensity
1	698.87584	63.89576
2	859.15137	70.96465
3	985.88086	61.13495
4	1064.15495	47.65823
5	1407.07003	84.87953
6	1438.75241	85.42900
7	1474.16211	84.50113
8	1544.98153	77.86918
9	1559.89088	79.00983
10	1638.16498	68.22167

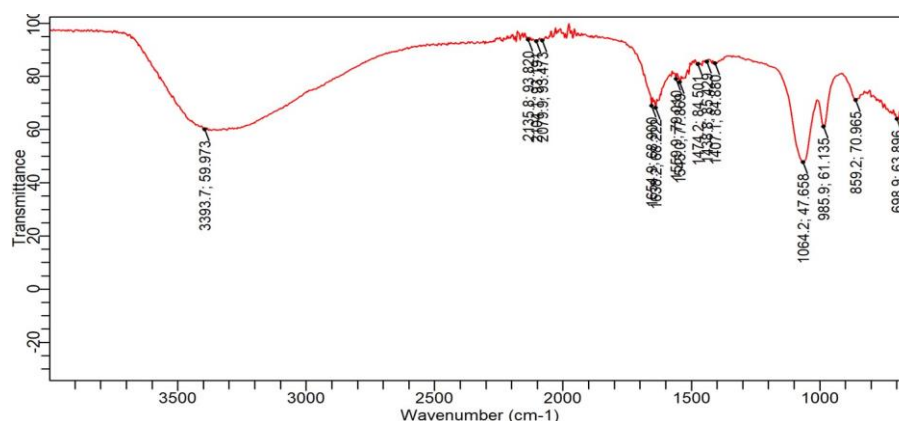


Fig 14. FTIR peak results for M4 strain

5.11.2 RAMAN SPECTROSCOPY

One scientific paper that focuses on the use of Raman Spectroscopy in studying bacteriorhodopsin is by Yoshini Shichida et al., (2001). The authors report that their Raman spectroscopy of bacteriorhodopsin showed characteristic peaks corresponding to various vibrational modes of the protein. Raman spectroscopy is a powerful tool for the characterization of proteins, including bacteriorhodopsin. Bacteriorhodopsin is a transmembrane protein found in the plasma membrane of Halobacteria, which belongs to the family of retinal-binding proteins. Bacteriorhodopsin has a long alpha-helix structure containing 248 amino acids and a prosthetic retinal chromophore. The retinal chromophore in bacteriorhodopsin undergoes a conformational change upon absorbing light, leading to the pumping of protons across the membrane and generating a proton motive force for ATP synthesis.

Raman spectroscopy is based on the inelastic scattering of light, which provides information on the vibrational modes of molecular bonds. The amide group is a key component of proteins, consisting of the carbonyl and amino groups linked by a peptide bond. The Raman spectrum of the amide group contains characteristic peaks corresponding to the stretching and bending vibrations of the peptide bond, providing valuable information on the protein conformation and secondary structure. For example, α -helices exhibit a strong amide I band at ~ 1650 cm⁻¹, while β -sheets have a strong amide I band at ~ 1630 cm⁻¹.

Using Raman spectroscopy, the vibrational modes of the amide group of bacteriorhodopsin can be monitored in real-time, providing insight into the protein structure and dynamics. The retinal chromophore absorbs light at ~ 575 nm, inducing a conformational change in the protein. Raman spectroscopy can detect these conformational changes in real-time, providing valuable insight into the protein mechanism of action. In addition, the Raman spectrum of bacteriorhodopsin can be used to distinguish between different retinal conformations or mutants, as well as to monitor protein stability under different conditions.

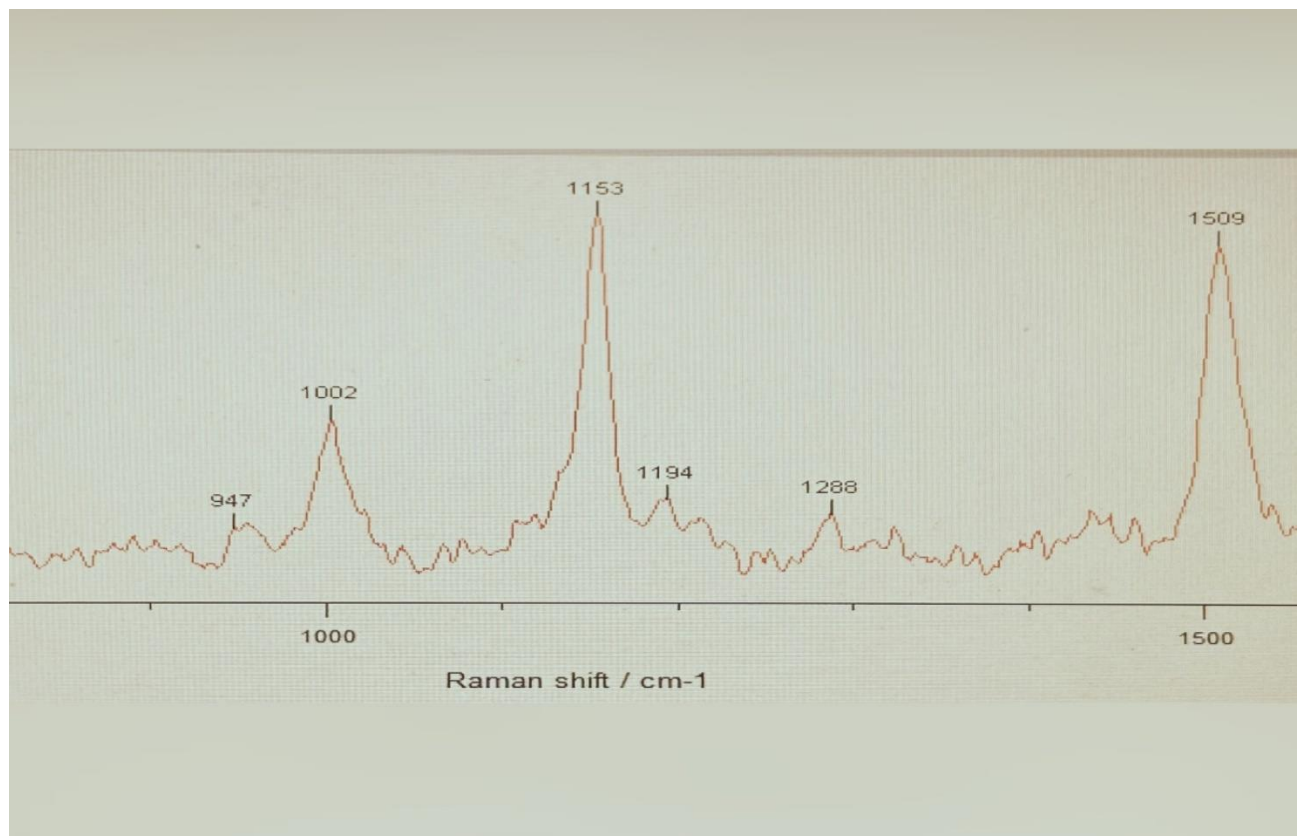


Fig 15. Graph of protein characterization from bacteriorhodopsin using raman spectroscopy

CHAPTER 6

6.CONCLUSION

The samples are collected from various sites such as Marakkanam. The halophilic bacteria are isolated by serial dilution and spread plating. Fresh Minimal Saline Media is prepared and the strains are inoculated. The growth was higher using dairy effluent as a cheap carbon source. SM1 strain showed the maximum production of bacteriorhodopsin. The culture is given proper incubation and growth is monitored. After certain growth is attained, the protein biomass is extracted by Freeze-Thaw and Bead Mix method. The amount of protein present in each sample is estimated by Bradford Assay. The BR protein was quantified by Hydroxylamine method and BR concentration was obtained. The samples are freeze dried or Lyophilized for further protein characterization like ATR/FTIR, Raman spectroscopy. 16sr RNA gene sequencing was done to find bacteriorhodopsin-producing isolates.

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