Protein profile of halotolerant bacteria under salt-stress

A Dissertation Submitted in partial fulfilment
of the requirements for the degree of
Masters of Science in Marine Biotechnology

by

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APRIL 2023

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12/05/2023

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Protein profile of halotolerant bacteria under salt stress" is a bonafide work carried out by Mr. Manas Upadhyay under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Masters of Science in Marine Biotechnology in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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Signature and Name of Supervising Teacher: Ms. Dviti Mapari
Name of Discipline: Marine Biotechnology

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Place: Goa University

DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, "Protein profile of halotolerant bacteria under salt stress" is based on the results of investigations carried out by me in the Discipline of Marine Biotechnology at the School of Biological sciences and Biotechnology, Goa University under the Supervision/Mentorship of Ms. Dviti Mapari and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.
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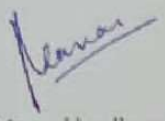

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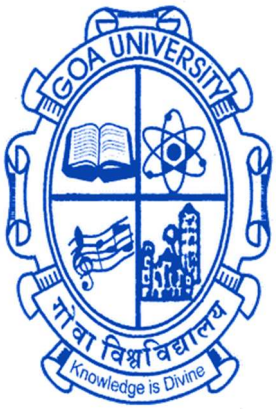

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Acknowledgement

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I also extend a big thank you to all my friends and classmates for creating a fun and supportive environment, making it a memorable experience for me.
Once again, thank you to all!

Abbreviations

1. µL	Microlitres
2. mL	Millilitres
3. SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
4. kDa	Kilo Daltons
5. 2D-GE	Two-dimensional gel electrophoresis
6. LAF	Laminar air flow
7. Rpm	Revolutions per minute
8. OD	Optical density
9. TCA	Trichloroacetic acid
10. BSA	Bovine serum albumin
11.FCP	Folin-Ciocalteu reagent
12. g/L	Grams per litre
13. M	Molar concentration

1. INTRODUCTION

1.1 Overview of halotolerant bacteria

Halotolerant bacteria have been described as being "capable of growing in the absence as well as in the presence of relatively high salt concentrations", and the ones showing growth in salt concentration higher than 2.5 M are classified as extreme halotolerant (Remonsellez et al., 2018). The halophilic organisms are found in all three domains of classification i.e., Bacteria, Archaea and Eukarya (Shivanand & Mugeraya, 2011) and are known to inhabit coastal dunes, salt pans, saline lakes and springs. Also, halotolerants have been identified from salted food, such as brined cucumber, as early as 1918 (LeFevre & Round, 1919). Similarly, halotolerant cocci were isolated from dried, cured cod fish (Vilhelmsson et al., 1997).

1.2 Characteristics of halotolerant bacteria

One of the most important characteristics of halophilic bacteria is their ability to maintain cell integrity in high-salt conditions. These bacteria have evolved a number of strategies to prevent the loss of water due to osmosis, including the synthesis of compatible solutes such as trehalose and glycine betaine, which help to maintain cell turgor and prevent cell damage (Oren, 2011). Additionally, halophilic bacteria have developed unique cell membrane structures that allow them to maintain ion gradients and prevent ion toxicity, which can be a major problem in high-salt environments ("Book Review: Brock Biology of Microorganisms – 14th Edition," 2016).

Apart from being able to thrive in high salt conditions, the ability to produce bright pigments-ranging from yellow to red to purple, is their characteristic feature. Striking changes is caused in the landscape because these bacteria impart various shades of red to natural salterns, spoiled foods and discoloured hides. Carotenoids, similar to one found in tomatoes and peppers, along with Rhodopsin are two common pigments produced by these bacteria (Khanafari et al., 2010).

Another important characteristic of halophilic bacteria is their ability to synthesize enzymes and other proteins that are stable in high-salt conditions. Many of these enzymes are highly valuable for biotechnology applications, as they can be used in processes that require high-salt conditions, such as the production of cheese and other dairy products (Ventosa et al., 1998).

1.3 Classification

Based on their genetic and morphological characteristics, halotolerant bacteria have been divided into different taxonomic groups. Presence of halotolerance in such different groups indicate that adaptation to salt stress evolved in a 'convergent' manner, and not from a direct common ancestor (Gunde-Cimerman et al., 2018). The major taxonomic groups having halotolerant representatives are as follows:

1. Halophilic Archaea: Archaea are a group of prokaryotic microorganisms that are phylogenetically distinct from bacteria and eukaryotes. Halophilic archaea are found in extreme saline environments such as salt lakes and salt flats. They are classified into two major groups, Halobacteria and Haloarchaea. Some common genera of halophilic archaea include *Haloferax, Halorubrum*, *and Natronomonas*.

2. Halophilic Bacteria: Bacteria are a group of prokaryotic microorganisms that are found in diverse environments. Halophilic bacteria are adapted to high salt concentrations and are

found in saline habitats such as salt marshes, salt pans, and saline soils. Some common genera of halophilic bacteria include Halomonas, Salinibacter, and Bacillus.

3. Halophilic Eukaryotes: Eukaryotes are microorganisms with a true nucleus and other organelles. Halophilic eukaryotes are found in saline environments such as salt lakes and salt flats. They are classified into different groups, including algae, fungi, and protozoa. Some common genera of halophilic eukaryotes include Dunaliella, Thraustochytrium, and Halocynthia.

4. Halophilic Viruses: Viruses are microscopic entities that can infect different types of cells. Halophilic viruses are viruses that infect halophilic microorganisms. They are found in saline environments such as salt flats and salt pans. Some common types of halophilic viruses include haloviruses and saliniviruses.

2. Literature Review

2.1 Salt stress in halotolerant bacteria

Halotolerant bacteria have the ability to survive in high salt concentrations but high salinity in the surrounding is not an exclusive parameter for their optimal growth. This unique property is a result of physiological adaptation, along with expression of biomolecules that increase their tolerance to elevated salt levels. Naturally, it makes these taxa a topic oof interest for biotechnologist which are interested in exploiting their potential. They are already being studies for use as bioremediating agents (Singha & Kumarb, 2017). This makes understanding of their sub-cellular processes, which grants them 'protection to salt-stress, a necessity in order to expand the scope of their use in biological as well as allied disciplines.

2.2 Molecular mechanisms of salt tolerance

Halotolerants have developed fundamentally different and specialized mechanisms that are responsible for their tolerance to high salinity. For example, either the cell uptakes certain ions to counter-act the effect of increased ambient salt levels, or there is increase in proteins that 'heal' the cellular damage, and there could be increased expression of molecules that increase the cell's osmolarity (Vaidya et al., 2018).

- Salt-in strategy

  This strategy has been adapted by different halophilic archaea, bacteria and fungal groups. But, among the bacterial kingdom this not the common strategy and is seen predominantly in archaeal families (Gunde-Cimerman et al., 2018).
  Such microorganisms tolerate high salinity levels by influxing high concentrations of Cl^- and K^+ ions in their cytoplasm. This decreases the osmolarity balance between intracellular region and the surroundings, helping them to avoid water loss.
  Additionally, they house modified enzymatic machinery and proteins that are rich in acidic-amino acids, such as aspartate and glutamate. Microorganisms utilizing this strategy tend to be extreme halophiles and often require high osmolarity as a pre-requisite for their growth (Vreeland, 1987).

- Accumulation of compatible solutes

  This is also known as 'salt-out' strategy and is a more commonly-utilized adaptation strategy involving expulsion of salt from the cell, while also synthesizing/accumulating 'compatible' solutes. Compatible solutes are small molar mass molecules having the ability to protect the cellular machinery against elevated salinity levels as a result of contributing by increasing intracellular osmolarity (Vreeland, 1987). Proline, Betaine, Ectoine are such examples of compatible solutes. They usually have a net zero charge on them, and do not interfere with the regular function of enzymes and other macromolecules, hence the term given to them. The biomolecules, for example enzymes, in this case are not 'specialized' to work at high salinity levels, rather they are being protected by the toxix effects of high salinity by compatible solutes. Due to this reason, organisms having the 'salt-out' strategy are not dependent on increased salonity levels for growth and can survive in both the absence and presence of salt ions (Weinisch et al., 2018).

- Increased expression of repair proteins

  Heat shock proteins (Hsps) are a class of conserved intracellular proteins involved in preventing protein denaturation and aggregate formation, and are usually synthesised by the cells as a result of external stress. They were first identified because of their role in heat-shock to cells, hence, the name. Despite being 'shock' proteins, they're still expressed in a cell under normal condition too but with lower levels of expressions.

  They are classified into six major conserved families according to their molecular weight:

  a) Hsp100,
  b) Hsp90,
  c) Hsp70,
  d) Hsp60,
  e) Hsp40, and
  f) sHsp (small heat shock proteins).

The two most studied families are Hsp70 and Hsp60, which consists of 70 kDa and 60 kDa long polypeptide, respectively. These have mostly been studied in many bacterial and eukaryotic species. The Hsp60 family is also called chaperonins and is included within the molecular chaperones. Hsp70 (DnaK) carries out its chaperone functions by teaming up with Hsp40 (DnaJ) and GrpE in the cytosol (Hartl, 1996). This archaeal machinery is much closer to bacterial equivalents due to previous events of lateral transfer from bacteria (Zmijewski et al., 2004), whereas, chaperonins are closely related to eukaryotic counterparts. In addition, the chaperone machinery (DnaK, DnaJ, GrpE) is usually present in bacteria and eukaryotes, while some species of archaea lack it or do not have all the components of this machinery (Petitjean et al., 2012). The function and activity of Hsps is similar in case of both heat-shock and osmotic shock. The difference lies in the signalling pathways that initiates their induction (Callahan et al., 2002).

Unlike the previously two mentioned strategies, expression of Hsps is not a unique stress-response. It works in conjugation with both 'salt-in' and 'salt-out' strategy to provide protection to toxicity by elevated osmotic pressure.

2.3 Comparison with other salt-tolerant microorganisms

There are several other salt-tolerant microorganisms that share similar adaptations to halophiles.

One of the most well-known salt-tolerant microorganisms is the bacterium *Staphylococcus aureus*. It is commonly found in human skin and mucous membranes and can tolerate high salt concentrations of up to 10% NaCl. Unlike halophiles*, S. aureu*s does not require high salt concentrations to grow, but it can survive in such environments. The primary mechanism by which S. aureus can tolerate salt stress is through the synthesis of compatible solutes such as proline and glycine betaine. These molecules help to maintain cellular osmotic balance and prevent the influx of water from the surrounding environment.

Another group of salt-tolerant microorganisms are the cyanobacteria. These photosynthetic microorganisms can grow in a wide range of salt concentrations and are essential contributors to primary production in hypersaline environments. Cyanobacteria are capable of adapting to

high salt concentrations by changing their photosynthetic pigments and adjusting their photosynthetic rates. They also synthesize compatible solutes and accumulate them in their cells to maintain osmotic balance (Cleland et al., 2004).

In contrast to halophilic prokaryotes, halophilic eukaryotes have developed more complex mechanisms to cope with high salt concentrations. For example, some halophilic eukaryotes, such as the green algae *Dunaliella*, accumulate high concentrations of glycerol to balance the osmotic pressure between the cytoplasm and the external environment (Feng et al., 2012). Other halophilic eukaryotes, such as the yeast *Candida*, have evolved a unique protein, called *Hal1*, which helps to maintain intracellular pH and ion balance in high salt concentrations (Loeto et al., 2021).

Furthermore, halophilic bacteria and halophilic eukaryotes differ in their genetic makeup. Halophilic bacteria are typically characterized by their small genomes and high levels of horizontal gene transfer, which allows them to quickly acquire new genetic traits that enhance their survival in high salt concentrations. In contrast, halophilic eukaryotes have larger genomes and a lower frequency of horizontal gene transfer. However, they have developed complex genetic mechanisms, such as alternative splicing and gene duplication, to regulate gene expression and adapt to high salt concentrations.

2.5 Experimental methods used to study salt stress

Salt stress is a significant environmental factor that can affect the growth, metabolism, and survival of halophilic microorganisms and to understand the mechanisms underlying salt stress response in halophiles, various experimental methods have been developed.

2.5.1. Growth curve analysis:

Growth curve analysis is a widely used method to evaluate the effects of salt stress on halophiles. In this method, halophilic microorganisms are grown in media with varying salt concentrations, and their growth is monitored over time. The growth curve provides information about the lag phase, exponential phase, and stationary phase of the microbial growth. This method has been used to study the salt tolerance of various halophiles, including bacteria, archaea, and eukaryotes (Kishimoto et al., 1991).

2.5.2. Enzyme assays:

Enzyme assays are another useful tool to study salt stress in halophiles. Salt stress can alter the activity of various enzymes, including those involved in osmoregulation, energy metabolism, and stress response. Enzyme assays allow researchers to measure the activity of specific enzymes under different salt concentrations. For example, the activity of enzymes involved in glycolysis, such as hexokinase and pyruvate kinase, has been shown to decrease in response to salt stress in halophilic bacteria (Cánovas et al., 1996).

2.5.3. Transcriptomics:

Transcriptomics is a high-throughput method that allows researchers to study the gene expression patterns in response to salt stress. In this method, RNA is extracted from halophilic microorganisms grown under different salt concentrations, and the expression of genes is analyzed using microarrays or RNA sequencing. Transcriptomics has been used to identify genes involved in salt stress response in various halophiles, such as the halophilic archaea Haloferax volcanii (Maurer et al., 2005).

2.5.4. Proteomics:

Proteomics is a method that allows researchers to study the changes in protein expression in response to salt stress. In this method, proteins are extracted from halophilic microorganisms

grown under different salt concentrations, and their expression is analyzed using techniques such as two-dimensional gel electrophoresis and mass spectrometry. Proteomics has been used to identify salt stress-responsive proteins in halophilic bacteria, such as Salinibacter ruber (Santos et al., 2011).

2.5.5. Metabolomics:

Metabolomics is a method that allows researchers to study the changes in metabolite levels in response to salt stress. In this method, metabolites are extracted from halophilic microorganisms grown under different salt concentrations, and their levels are analyzed using techniques such as nuclear magnetic resonance spectroscopy and gas chromatography-mass spectrometry. Metabolomics has been used to identify metabolites involved in osmoregulation and energy metabolism in halophilic bacteria, such as Halomonas elongata (Kjelleberg et al., 1991).

3. Aim & Objective

3.1 AIM

To check the protein profile of halotolerant bacteria under salt-stress.

3.2 OBJECTIVES

1. To extract protein from whole cell lysate.
2. To quantify the amount of protein
3. Find the molecular weight of each protein using SDS-PAGE

4. Materials & Methods

4.1. Subculturing

The sample PSDM 20 was subcultured from a master plate, by streaking an isolated colony on a Nutrient Agar (NA) plate having 8% salt concentration. Before the preparation of media plates, the glass plates were thoroughly cleaned, kept in acid-wash, rinsed with water, and autoclaved at 121◦C at 15 psi for 20 minutes. Streaking was done in a LAF and near the burner's flame to avoid contamination.

4.2. Inoculum preparation

50 mL Nutrient broth of salt concentration 8% was prepared and later autoclaved. This was inoculated with a loopful of isolated colonies from NA 8%. The broth was kept on Shaker incubator, with 28◦C temperature, for 24-48 hrs and used for secondary inoculation.

4.3. Secondary inoculation

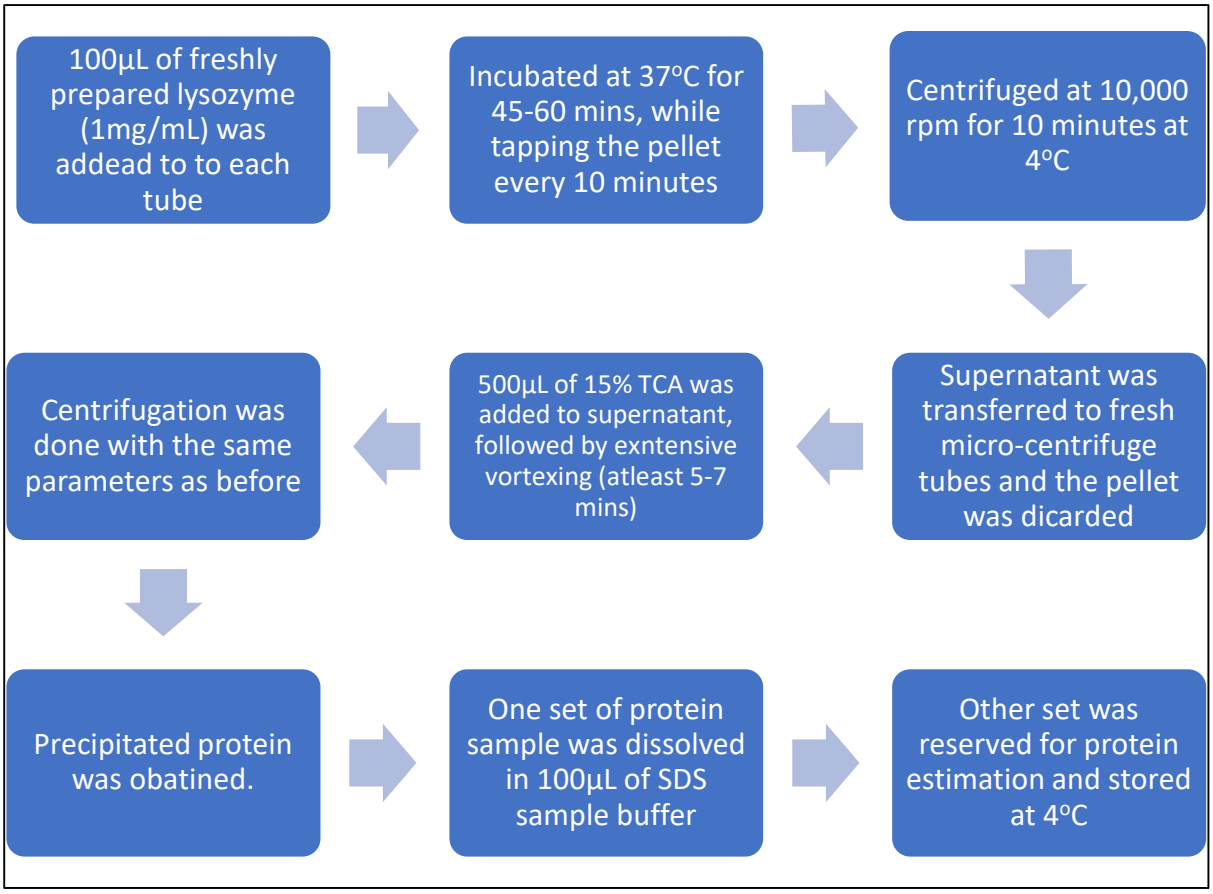
50 mL Nutrient broth (NB) of salt concentrations (0%, 0.5%, 2%, 8%, 12% and 16%) were prepared, by mixing appropriate proportions of NB, NaCl and distilled water in 100mL conical flasks. The broths prepared were then autoclaved and inoculated with 1mL of the primary inoculum. Later, it was kept in the shaker incubator at 28ºC for 24-48hrs.

4.4. Cell harvesting

In sterile microcentrifuge tubes, 1.5mL of culture broth was taken from each broth of different concentrations. 2 such sets were made; one was used for SDS-PAGE and the other for protein estimation.

They were centrifuged at 10,000 rpm for 10 mins at 4ºC. Supernatant was discarded carefully, ensuring that the pellet isn't disturbed. Pellet obtained was washed with a salt solution of concentration same as that of the sample's salt concentration to avoid any osmolarity changes. It was centrifuged again at 10,000 rpm for 10 minutes at 4◦C. Supernatant was discarded. Washing and subsequent centrifugation step was carried out one more time and the microcentrifuge tubes, having the pellet, were kept on ice.

4.5. Protein Isolation



4.6. Protein Estimation

Lowry's assay was used to determine the protein concentration in the given samples (Khalighi et al., 2022).

Reagents:

- BSA stock (1mg/mL)
- Reagent A- 2% of sodium carbonate dissolved in 0.1N of sodium hydroxide
- Reagent B- 1% of sodium potassium tartrate in distilled water
- Reagent C- 0.5% of copper sulphate pentahydrate in distilled water
- Chemical II- Folin & Ciocalteus Phenol (FCP) Reagent

Procedure:

(A)Standard curve preparation

a) 10 mL of BSA stock having concentration 1mg/mL was prepared.
b) To 10 clean test tubes, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mL of BSA stock solution was added
c) Final volume of each test tube was made up to 1 mL by adding an appropriate amount of distilled water.
d) 48mL, 1mL and 1mL of Reagent A, B and C respectively were mixed together. This solution was labelled as Chemical I.
e) 4.5 mL of Chemical I was added to each test tube, the solutions were mixed and then allowed to incubate for 10 mins at room temperature.
f) Later, 0.5mL of Folin's reagent was added to each test tube and incubated in dark for 30 minutes.
g) Absorbance was recorded at 660nm, and a standard curve was plotted.

(B) Sample's protein estimation

a) The pellets of all 6 samples were resuspended by adding 1mL of distilled water in each micro-centrifuge tube. If they were insoluble, 50µL of 0.1M NaOH was added to solubilize them.
b) From 'Standard curve preparation', steps d) to f) were repeated
c) Absorbance was measured at 660 nm and using the standard curve the concentration of unknown protein samples was calculated.

4.7. SDS- PAGE analysis

Requirements:

Acrylamide-bis acrylamide mix (29:1), 1.5M Tris-HCl and 0.5M Tris-HCl buffer, running buffer, 10% and 20% SDS solution, Staining and Destaining solution, Milli-Q water, voltage regulator, SDS-PAGE assembly

Procedure:

(A) Making the gel

a) The glass plates were fitted with spacers on both sides, and were secured with cello tape to keep the spacers in place and to close the sides and bottom end of the plates.
b) Molten 1% agar was poured along the sides of the plates to seal the sides and the bottom portion. The thickness of agar on the bottom shouldn't be more than 4-5mm.
c) Mixed all the components of resolving gel and carefully poured it in the gel cassette till desired height. Air bubbles' presence in gel should be avoided.

d) Poured a few hundred microlitres of milli-q water or iso-butanol on top of resolving gel, in order to avoid contact with air.
e) After the gel had solidified, the water/isobutanol was decanted and any residual amount was absorbed with blotting paper.
f) Next, mixed the component of stacking gel and the mixture was carefully poured into the cassette avoiding air bubble formation. Immediately, the required comb-well was placed on.
g) After ensuring the gel had fully solidified, the comb was removed slowly.

(B) Loading samples & initiation of electrophoresis

a) Removed cello tape from all the ends of gel and fixed it on the SDS-PAGE assembly. The notched glass plate should face the centre of assembly.
b) Before loading, the protein sample (suspended in sample buffer) was heated in a watered bath at 95-100^{o}C for 5mins.
c) 20 ul of protein ladder was loaded in the first well.
d) Similarly, 20µl of each sample was loaded in the next 6 wells.
e) Turn on the powerpack and set the starting voltage to 100 volts.
f) Run was started at 100 volts initially, and increased to 150 volts, once the dye front had migrated ahead of the stacking and resolving gel interface.
g) Power supply was turned off once the dye front was a few millimetres above the agar layer.

(C.) Visualizing the bands

a) Gel was removed from the cassette, rinsed with water and placed in a tray. Staining solution was added to it.
b) The tray was placed on a gel rocker overnight.
c) Staining solution was discarded and the tray was filled with Destaining solution, and kept back on the rocker.
d) Every 40 minutes the solution was replaced with a new one, until the gel was transparent and bands were visible.
e) Gel was carefully placed on a light table and bleu-coloured bands were visualized. An image was captured of the bands for further analysis.
f) Using GelAnalyser software (version 19.1), standard curve was plotted and subsequently the molecular weight corresponding to each band was calculated with the help of this software.

5. RESULTS

5.1. PROTEIN ESTIMATION

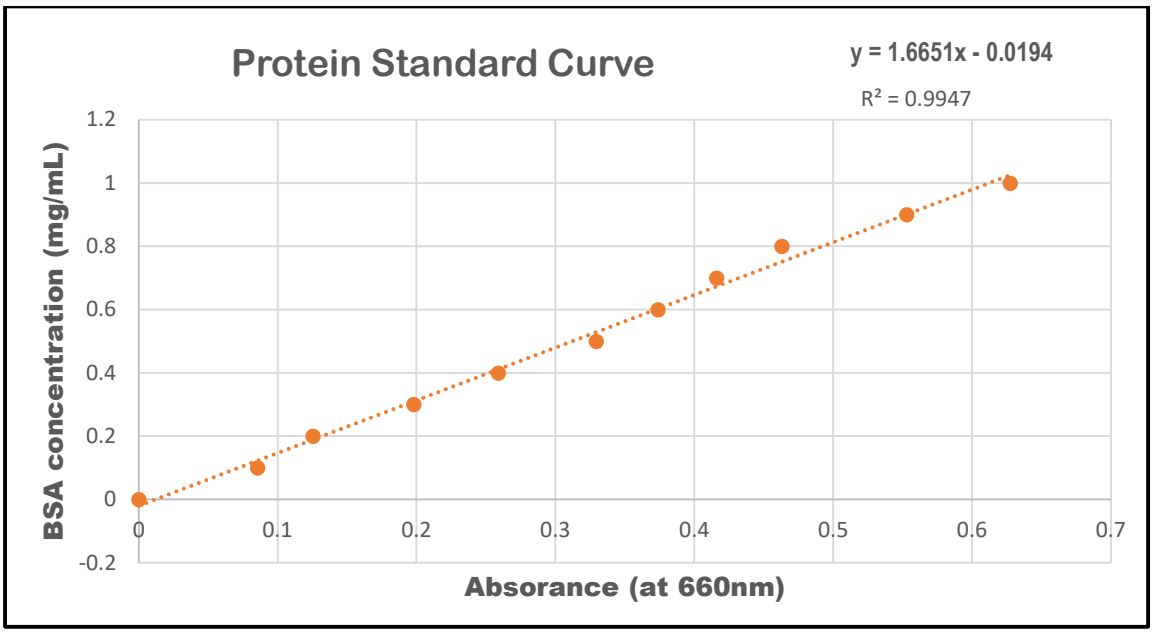


Fig.5.1.1. Standard curve used to determine the concentration of protein content in unknown samples.

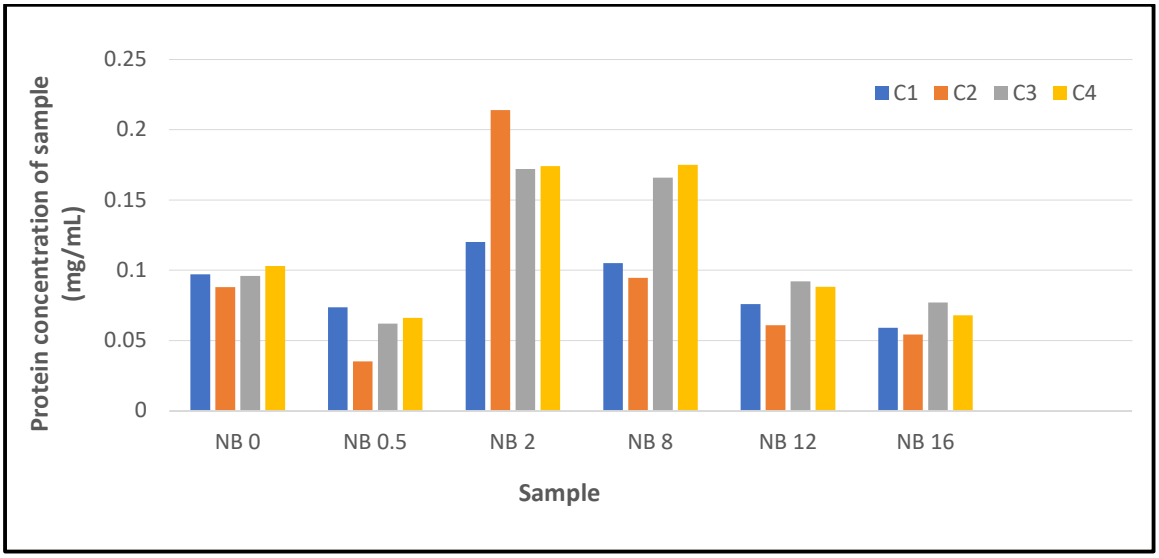


Fig. 5.1.2. Avg. protein concentration in each sample. C1, C2, C3 and C4 represent the different sets of samples.

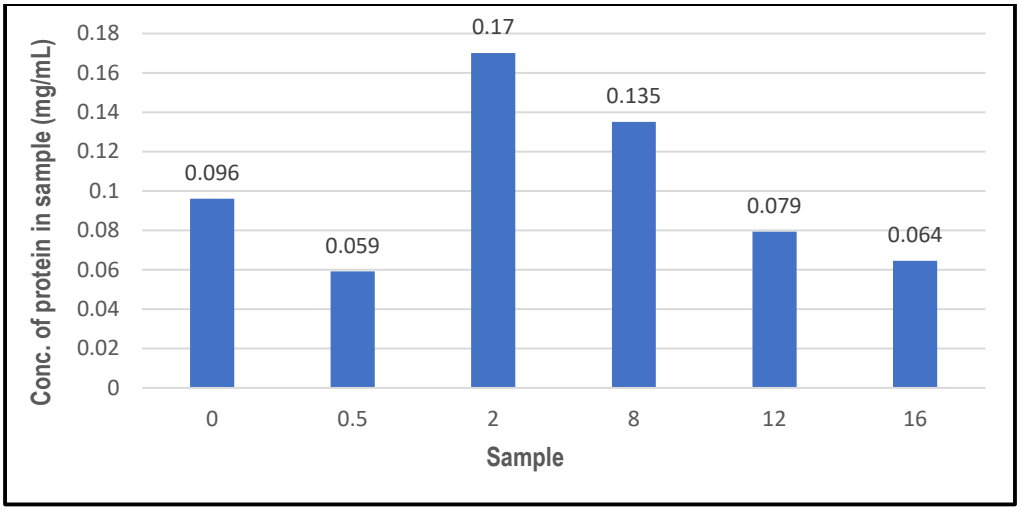


Fig. 5.1.3. Average protein content in samples of all the 4 sets.

5.2. SDS-PAGE GEL

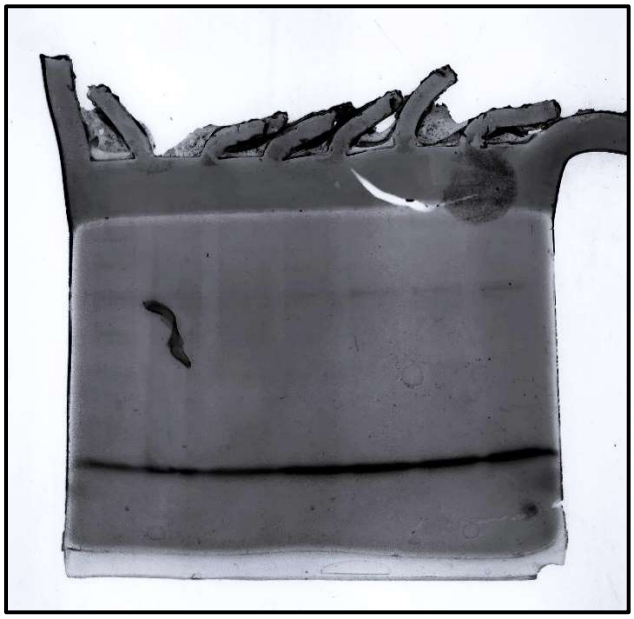


Fig. 5.2.1 12% acrylamide gel. From left to right, lane 1 loaded with protein marker followed by samples (0%,0.5%, 2%, 8%, 12% and 16%) in subsequent wells. SRL's following protein ladder was used= 220, 116, 95, 66, 45, 25 and

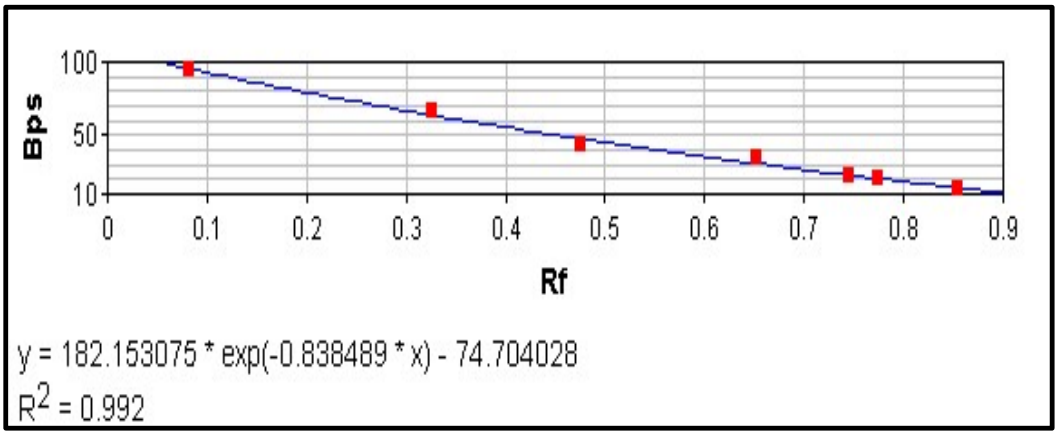


Fig. 5.2.2 Protein marker standard curve, plotted size of polypeptide (in KDa) v/s Log_{10} of Rf value.

(a)

Lane	Band number	Rf	MW (KDa)
2	1	0.068	97
2	2	0.323	64
2	3	0.765	21
2	4	0.871	13

(b)

Lane	Band Number	Rf	MW (KDa)
3	1	0.052	100
3	2	0.21	78
3	3	0.326	64
3	4	0.754	22

(c)

Lane	Band number	Rf	MW (KDa)
4	1	0.132	88
4	2	0.321	65
4	3	0.642	32

(d)

Lane	Band number	Rf	MW (KDa)
5	1	0.277	70
5	2	0.584	37
5	3	0.62	34
5	4	0.858	14

(e)

Lane	Band number	Rf	MW (KDa)
6	1	0.289	68
6	2	0.368	59
6	3	0.91	10

(f)

Lane	Band number	Rf	MW (KDa)
7	1	0.312	65

Table 5.2.1 Bands obtained on the gel, along with their respective molecular weights. Sample 0%,0.5%, 2%, 8%, 12% and 16% are represented in tables (a), (b), (c), (d), (e) and (f), respectively.

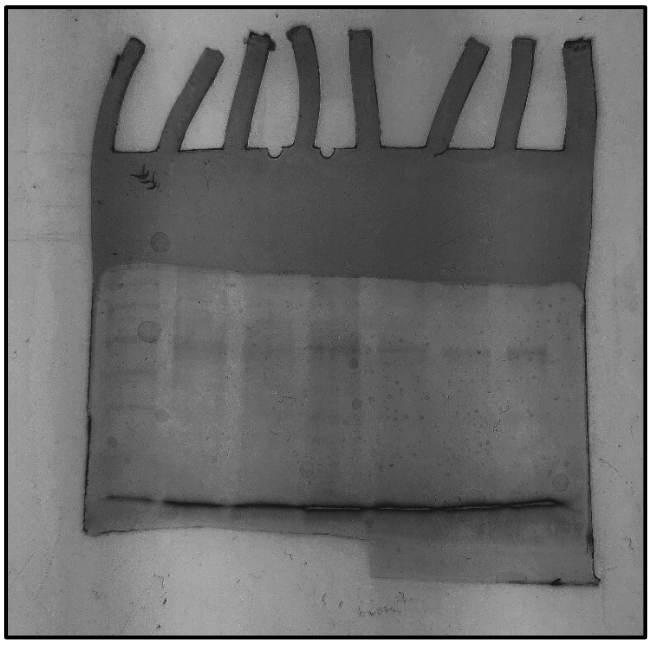


Fig. 5.2.3. 2.1 12% acrylamide gel. From left to right, lane 1 loaded with protein marker followed by samples (0%,0.5%, 2%, 8%, 12% and 16%) in subsequent wells. SRL's following protein ladder was used= 95, 66, 43, 35, 22, 20 and 14 kDa.

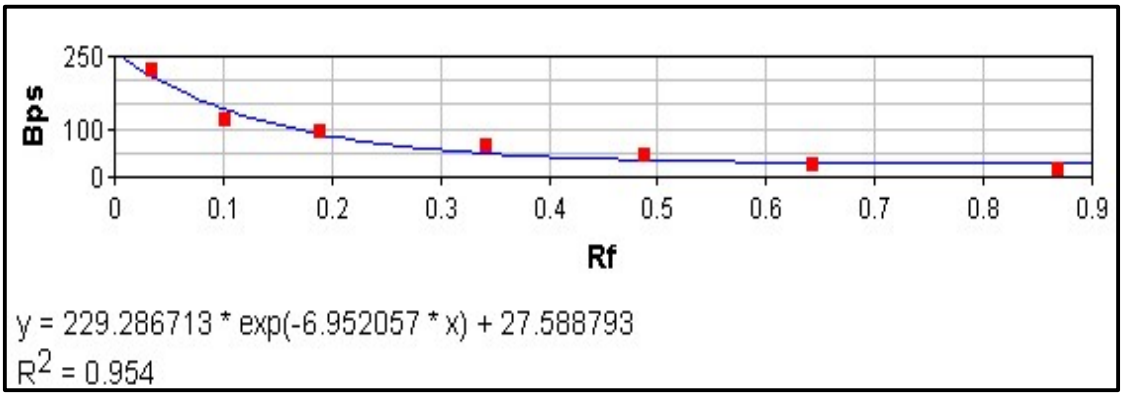


Fig. 5.2.4. Protein marker standard curve, plotted size of polypeptide (in KDa) v/s Log_{10} of Rf value.

(a)

Lane	Band number	Rf	MW (KDa)
2	1	0.069	170
2	2	0.157	105
2	3	0.195	87
2	4	0.355	47
2	5	0.642	30
2	6	0.724	29

(b)

Lane	Band number	Rf	MW (KDa)
3	1	0.048	192
3	2	0.126	123
3	3	0.21	81
3	4	0.355	47
3	5	0.653	30
3	6	0.732	29

(c)

Lane	Band number	Rf	MW (KDa)
4	1	0.119	128
4	2	0.318	53
4	3	0.505	34
4	4	0.633	30
4	5	0.731	29

(d)

Lane	Band number	Rf	MW (KDa)
5	1	0.306	55
5	2	0.622	31
5	3	0.795	28

(e)

Lane	Band number	Rf	MW (KDa)
6	1	0.318	53

(f)

Lane	Band number	Rf	MW (KDa)
7	1	0.318	53
7	2	0.609	31

Table 5.2.2 Bands obtained on the gel, along with their respective molecular weights. Sample 0%,0.5%, 2%, 8%, 12% and 16% are represented in tables (a), (b), (c), (d), (e) and (f), respectively.

6. DISCUSSION

- Protein estimation yielded almost consistent results when compared with different sets of data. Culture grown in NB supplemented with 2% NaCl showed highest avg. protein content. While, the ones growing in 0.5% and 16% salt-supplemented NB had the lowest protein concentration among all 6 samples. Higher protein content is an indicator of high biomass, therefore, greater growth too (Zubkov et al., 1999).
- Presence of 2% salt seems to be the optimum growth condition for the bacterium and presence of growth at 16% salt level indicate that the bacterium is a slight halophile species, with an ability to tolerate greater salt concentrations than its optimum level.
- Moreover, presence of diffused bands in 16% and 12% lanes further confirms the low-protein content and retarded growth at higher salinity levels.
- Lowry's assay accuracy is affected by the presence of few interfering compounds. Tris-HCl and EDTA, used during the protein extraction, are two such compounds (Shen, 2019). Remnants of these compounds in the extracted protein can skew the readings calculated for the sample's protein content.
- Certain bands were present across all three lanes, for example the 65 kDa band. Similarly, a band of molecular weight 53-55 kDa was consistently observed across different lanes too. Presence of these bands across different samples indicates that the said protein is constitutively expressed, and may have role in salt-stress response. Moreover, the 53-55 kDa protein band was observed in high salinity lanes (12% and 16%), this further adds merit to its role in salt stress response and the latter could have triggered its elevated expression in cells.
- Certain bands obtained were faint and they were probably a result of incomplete protein precipitation by TCA, and/or low concentration of protein after resuspending in sample buffer.

7. FUTURE PROSPECTS

1. Standardization of protein extraction protocol required to further optimize the yield of protein. Certain proteins don't precipitate by TCA method, an alternative method such as TCA/acetone precipitation can be employed.

2. SDS-PAGE can only point-out to the probable identity of the protein band. 2D-gel electrophoresis and Mass spectroscopy can be used for proper characterization of the protein bands obtained.

8. APPENDIX

9.1 Reagents

Reagents	Grams/ml
1. 1N HCl	150
Concentrated HCl	12.5
Deionized water	150
2. 1N NaOH	150
Sodium hydroxide pellets	6.0
Deionized water	150
3. Coomassie brilliant blue dye	100
Coomassie brilliant blue R-250	0.25
Methanol	45
Glacial acetic acid	10
Deionized water	45
4. TE butter (pH 8.0)	100
Tris chloride	1.57
EDTA	0.372
Deionized water	100
5. 10% Glycerol	10
Glycerol	1.0
Deionized water	9
6. Lysozyme	10
Lysozyme powder	0.01
1X TE buffer (pH 8.0)	10
7. Protein extraction buffer	100
50 mM Tris HCl (pH 8.0)	10
5mM EDTA (pH 8.0)	1.0
0.1% triton-X 100	0.1
0.01% Lysozyme	0.01
8. Bovine serum albumin	10
BSA	0.1

TE buffer	10
9. 0.5% Sodium chloride solution	100
Sodium chloride powder	0.5
Deionized water	100
10. 2% Sodium chloride solution	100
Sodium chloride powder	2.0
Deionized water	100
11. 5% Sodium chloride solution	100
Sodium chloride powder	5.0
Deionized water	100
12. 8% Sodium chloride solution	100
Sodium chloride powder	8.0
Deionized water	100
13. 12% Sodium chloride solution	100
Sodium chloride powder	12.0
Deionized water	100
14. 16% Sodium chloride solution	100
Sodium chloride powder	16
Deionized water	100
15. Saline	100
Sodium chloride powder	8.0
Deionized water	100

9.2 Reagents for SDS-page

Reagents	Grams/ml
1) Acrylamide-bisacrylamide stock solution 30%	100
N, N- bisacrylamide	29.0
Deionized water	100
2) Resolving gel buffer (pH 8.8)	100
1.5M tris	18.75
Deionized water	100
3) Stacking gel buffer (pH 6.8)	100
1M tris	12.114
Deionized water	100
4) 10% Ammonium persulfate solution	100
APS	10
Deionized water	100
5) 10% Sodium dodecyl sulfate (SDS)	100
SDS	10
Deionized water	100
6) 20% Sodium dodecyl sulfate (SDS)	100
SDS	20
Deionized water	100
7) 1% Bromophenol blue	100
Bromophenol blue	1
Deionized water	100

8) 1X Tris-glycine buffer (pH 8.3)	100
25 mM tris base	0.302
250 mM glycine	1.876
0.1% SDS	1
9) 2X Sample gel/loading buffer (pH 6.8)	10
50 mM tris-chloride	1 ml of 1M tris stock solution
200 mM β-mercaptoethanol	280 µl
2% SDS	4 ml of 10% SDS stock solution
0.1% bromophenol blue	2 ml of 1% stock solution
10% glycerol	2
10) Staining solution	100
Coomassie brilliant blue	0.25
Methanol	45
Glacial acetic acid	10
Deionized water	45
1) Destaining solution	100
Methanol	45
Glacial acetic acid	10
Deionized water	45
2) 1X SDS running buffer	1L
Tris base	3.03
Glycine	14.44
SDS	1.0

9.3 Gel Preparation

Components	**12% Resolving gel (10ml)**	**5% Stacking gel (5ml)**
Milli Q water	3.1	3.4
30% Acrylamide mix	4.3	830 ul
1.5 M tris (pH 8.8)	2.5	-
1 M tris (pH 6.8)	-	630 ul
10% SDS	100 ul	-
20% SDS	-	100 ul
10% APS	150 ul	50 ul
TEMED	10 ul	8 ul

9.4 Reagents for Folin-Lowry Assay

Reagents	**Grams/ml**
0.1 N NaOH	100
Sodium hydroxide pellets	0.4
Deionized water	100
Lowry A	100
2% sodium carbonate	2.0
0.1N NaOH	100
Lowry B	10
0.5% copper sulfate pentahydrate	0.05
Deionized water	10
Lowry C	10
1% sodium potassium tartrate	0.1
Deionized water	10
Final reagent	100
Lowry A	98
Lowry B	1.0
Lowry C	1.0
Reagents	**Grams/ml**
0.1 N NaOH	100
Sodium hydroxide pellets	0.4
Deionized water	100
Lowry A	100
2% sodium carbonate	2.0
0.1N NaOH	100
Lowry B	10
0.5% copper sulfate pentahydrate	0.05

9.5 Media preparation

Media	**Grams/ml**
Nutrient broth	100
Nutrient broth powder	1.3
Deionized water	100
0% Nutrient broth	100
Peptone	0.05
Beef extract	0.15
Yeast extract	0.15
Deionized water	100
0.5% Nutrient broth	100
Nutrient broth powder	1.3
Deionized water	100
2% Nutrient broth	100
Nutrient broth powder	1.3
Sodium chloride	1.5
Deionized water	100
8% Nutrient broth	100
Nutrient broth powder	1.3
Sodium chloride	7.5
Deionized water	100
8% Nutrient agar	100
Nutrient broth powder	1.3
Sodium chloride	7.5
Agar	2.0
Deionized water	100
12% Nutrient broth	100
Nutrient broth powder	1.3
Sodium chloride	11.5
Deionized water	100
16% Nutrient broth	100
Nutrient broth powder	1.3
Sodium chloride	15.5
Deionized water	100

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